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REVIEW

Updates on therapies for chronic prostatitis/chronic pelvic pain syndrome

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Abstract

Prostatitis comprises of a group of syndromes that affect almost 50% of men at least once in their lifetime and makeup the majority of visits to the Urology Clinics. After much debate, it has been divided into four distinct categories by National Institutes of Health namely (1) acute bacterial prostatitis; (2) chronic bacterial prostatitis; (3) chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) which is further divided into inflammatory and non-inflammatory CP/CPPS; and (4) asymptomatic inflammatory prostatitis. CP/CPPS has been a cause of great concern for both patients and physicians because of the lack of presence of thorough information about the etiological factors along with the difficult-to-treat nature of the syndrome. For the presented manuscript an extensive search on PubMed was conducted for CP/CPPS aimed to present an updated review on the evaluation and treatment options available for patients with CP/CPPS. Several diagnostic criteria's have been established to diagnose CP/CPPS, with prostatic/pelvic pain for at least 3 mo being the major classifying symptom along with the presence of lower urinary tract symptoms and/or ejaculatory pain. Diagnostic tests can help differentiate CP/CPPS from other syndromes that come under the heading of prostatitis by ruling out active urinary tract infection and/or prostatic infection with uropathogen by performing urine cultures, Meares-Stamey Four Glass Test, Pre- and Post-Massage Two Glass Test. Asymptomatic inflammatory prostatitis is confirmed through prostate biopsy done for elevated serum prostate-specific antigen levels or abnormal digital rectal examination. Researchers have been unable to link a single etiological factor to the pathogenesis of CP/CPPS, instead a cluster of potential etiologies including atypical bacterial or nanobacterial infection, autoimmunity, neurological dysfunction and pelvic floor muscle dysfunction are most commonly implicated. Initially monotherapy with anti-biotics and alpha adrenergic-blockers can be tried, but its success has only been observed in treatment naïve population. Other pharmacotherapies including phytotherapy, neuromodulatory drugs and anti-inflammatories achieved limited success in trials. Complementary and interventional therapies including acupuncture, myofascial trigger point release and pelvic floor biofeedback have been employed. This review points towards the fact that treatment should be tailored individually for patients based on their symptoms. Patients can be stratified phenotypically based on the UPOINT system constituting of Urinary, Psychosocial, Organ-specific, Infectious, Neurologic/Systemic and symptoms of muscular Tenderness and the treatment algorithm should be proposed accordingly. Treatment of CP/CPPS should be aimed towards treating local as

well as central factors causing the symptoms. Surgical intervention can cause significant morbidity and should only be reserved for treatment-refractory patients that have previously failed to respond to multiple drug therapies.

Key words: Chronic prostatitis; Antibiotics; Myofascial trigger point; Pelvic biofeedback; Chronic pelvic pain syndrome; Phytotherapy; Nanobacteria; Dysfunctional voiding; Acupuncture

Core tip: Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is difficult-to-treat because of the multitude of potential etiologies that are not easily observed and delayed diagnosis. Pharmacological monotherapy with antibiotics, alpha-blockers and anti-inflammatories provide significant symptomatic improvement in a limited number of patients. Multidrug therapies are recommended for monotherapy refractory patients. Complementary and interventional therapies such as acupuncture, myofascial trigger point release and pelvic floor biofeedback can provide additional symptomatic relief. Current recommendations involve a treatment algorithm based on UPOINT phenotypic presentation for CP/CPPS patients. Keeping in mind the high prevalence of CP/CPPS, development of novel therapies and an effective vaccine for prevention of CP/CPPS is crucial.

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INTRODUCTION

Prostatitis is a term that identifies a number of syndromes ranging from acute or chronic pain to bacterial infection of the prostate gland[1]. Prostatitis makes up the majority of Urology clinic visits with as high as 2 million office visits annually by men suffering from prostatitis in the United States[2] and yet it is one of the least understood diseases in the field. Almost 50% of all men are symptomatic for prostatitis at some point in their lives[3]. Over time diagnostic and therapeutic modalities for prostatitis have evolved significantly and patients are now given specific treatments in accordance with the set of determined subtypes formulated by physicians based on clinical presentations[4].

The national institute of health (NIH) has classified chronic prostatitis into four different categories: Category Ⅰ: Acute bacterial prostatitis is an infection caused by an underlying uropathogen, which presents with signs of systemic infection including fever and chills. Category Ⅱ: Chronic bacterial prostatitis is caused by chronic bacterial infection of the prostatitis secondary to recurrent urinary tract infections. Category Ⅲ: Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) primarily presents with pain and sometimes presents with voiding symptoms in absence of any urinary tract infection (UTI). It is sub-grouped into two more categories: Category Ⅲa: Inflammatory CP/CPPS; Category Ⅲb: Non-Inflammatory CP/CPPS. Category Ⅳ: Asymptomatic inflammatory prostatitis is diagnosed incidentally on prostate biopsy or other pathologic specimen by presence of prostatic inflammation without the evidence of any genitourinary symptoms[5].

The majority of patients with CP/CPPS present with pain symptoms ranging from lower abdominal to ejaculatory pain but the pain is not necessarily associated with lower urinary tract symptoms (LUTS) or sexual dysfunction[6]. Apart from being one of the least understood diseases, CP/CPPS comprises almost 90% of the prostatitis syndromes in patients[7].

Given the high prevalence of the disease and the lack of good diagnostic and treatment strategies, NIH formulated a classification system to stratify the types of prostatitis in order to devise treatment modalities accordingly[8]. The guidelines provided by the NIH in the form of NIH chronic prostatitis symptom index (NIH-CPSI) now serve as the international standard for non-diagnostic symptom evaluation of prostatitis in clinical practice as well as in research protocols (Table 1)[9]. An extensive search on PubMed was conducted for CP/CPPS with an aim to present an updated review on the evaluation and treatment options available for patients with CP/CPPS and to provide an in-depth review of the possible causative factors of CP/CPPS.

UNDERSTANDING THE BASES OF CP/CPPS

CP/CPPS is a syndrome without evident pathophysiology. For long it has been postulated that pathogens are the inciting factors and the early treatments focused on microbial eradication[10]. Recently researchers identified that uropathogens are present for only a few cases, which suggests that other unidentified causative factors play a role in the disease[10] including atypical microbes or pathogens that are difficult to culture[11]. The lack of readily identifiable bacteria rationalized the initial term Chronic Non-Bacterial Prostatitis, which was later changed to chronic prostatitis or CPPS[12]. CPPS is further divided into two types based on the number of leukocytes/high power field on microscopic examination of the expressed prostatic secretion (EPS) or semen. The prostatic fluid leukocyte count is used to differentiate between CPPS type Ⅲa (5-10 leukocytes/hpf) and type Ⅲb (< 5 leukocytes/hpf)[13]. Type Ⅲa CP/CPPS patient samples also have higher levels of pro-inflammatory markers, which are absent in type Ⅲb[12]. Despite the absence of bacteria in patients with CP/CPPS, it is hypothesized that bacterial infection acts as the initiating factor for the development of the disease.

Table 1 National institute of health prostatitis classification system

Categories
Acute bacterial prostatitis
Chronic bacterial prostatitis
Chronic prostatitis/chronic pelvic pain syndrome
Inflammatory
Non-inflammatory
Asymptomatic inflammatory prostatitis

CP/CPPS has also been linked to depression, and similar conditions with chronic pain symptoms such as fibromyalgia, chronic fatigue syndrome and irritable bowel syndrome[14,15].

"Difficult to culture or atypical bacteria" the main culprits

Efforts have been put into finding the role of sexually transmitted pathogens such as Chlamydia trachomatis, trichomonas vaginalis, Ureaplasma urealyticum or mycoplasma hominis in causing CP/CPPS but no researcher yet been able to link the two[16]. A notion passed on by the researchers is that Bacterial involvement in CP/CPPS is only in 10% of the patients made evident by their response to antibiotics[17].

Atypical bacteria's haven been implicated as one of the potential precipitants of the pathogenic process in CP/CPPS. Atypical bacteria's are difficult to cultivate and require amplification at molecular level hence there's an important role of polymerase chain reaction (PCR) in the investigation[18]. Using PCR Hochreiter *et al*[19] were able to identify the presence of 16 s sub-unit of ribosomal RNA in CP/CPPS patients who had previously been cultured without evidence of bacterial growth in culture media.

Ever since *Helicobacter pylori* (*H. pylori*) has been established as causative for diseases other than gastric ulcers, researchers have been investigating its contribution in development of CP/CPPS. One such study by Karatas *et al*[20] looked for *H. pylori* seropositivity in CP/CPPS patients. They found that 76% of the cases were seropositive for *H. pylori* as compared to 62% controls. They posit a possible role of *H. pylori* in CP/CPPS, but large multicenter studies are necessary to establish a firm link between the two.

An atypical *Escherichia Coli* strain known as CP1 has been associated with CP/CPPS. As this strain was isolated from a patient suffering from CP/CPPS, non-obese diabetic (NOD) mice were inoculated with CP1 to assess its role in the disease. CP1 was found to be invasive into the urothelium and could chronically colonize the urinary tract of the mice and initiate pain as seen in the patients of CP/CPPS[21].

Function of mast cells, interleukins and cytokines in the development of the disease

Much work has gone into elucidating the role of the immune system in the pathophysiology of CP/CPPS[22]. One of the cells thought to play a major role are mast cells, which are derived from $CD34^+$ hematopoietic precursor cells. Mast cells carry and release potent pro-inflammatory and vasoactive substances such as histamine, serotonin, proteases, leukotriene's and nerve growth factor (NGF)[23,24]. Seminal NGF levels are a reliable predictor of mast cell activity and increased levels are linked to increased pain symptoms, suggesting that NGF is a possible pain inducer[25]. A study was conducted in which EPS from patients diagnosed with CPPS ⅢB was compared to controls. Samples were assayed for mast cell tryptase and NGF levels. It was shown that patients diagnosed with CPPS ⅢB had significantly increased levels of tryptase and NGF in their prostatic fluid[25]. Interestingly increased tryptase-PAR2 axis activity is linked to pain in animal models through the activation of the dorsal root ganglion[26].

It has been demonstrated that T cells can provoke prostatic and pelvic pain in the absence of any ongoing bacterial infection. In a study, interleukin-17a (IL-17a), which is secreted primarily by Th1 helper cells, was shown to induce pain symptoms in murine models without interferon γ (IFNγ) playing any major role in the process indicating that T cells are primary mediators of pain in the mouse CPPS model[27].

Autoimmunity has been considered as a possible cause of CP/CPPS since autoreactive T-cells have been found within the prostate, which can trigger IFNγ release[22]. Recently a study was conducted to assess the role of autoimmunity in the development of CP/CPPS in an experimental autoimmune prostatitis model in NOD mice. The study demonstrated that Th1/Th17 cells expressing CXCR3 receptors were able to infiltrate and damage the prostate gland through the induction of pro-inflammatory chemokines[28]. Several pro-inflammatory cytokines including IL-1b, -6 and -8, tumor necrosis factors α and IgA have been linked to the development of CP/CPPS following an event that triggers initiation of the inflammatory process[29].

Chemokines appear to play a significant role in the development of the CP/CPPS. Chemokines are a subgroup of cytokines responsible for regulating and recruiting inflammatory cells; of them, two chemokines, monocyte chemotactic protein 1 (MCP-1) also known as C-C motif ligand 2 and macrophage inflammation protein 1α also called C-C motif ligand 3 can enhance the pain symptoms of CPPS moreover both chemokines are elevated in CP/CPPS type ⅢA and type ⅢB patients[28,29].

Despite the presence of inflammatory markers in the EPS, Thumbikat *et al*[30] observed in their study that MCP-1 has no chemoattractant potential in CP/CPPS patients; the underlying mechanism can be caused by extracellular proteases and induced chemoattractant signal loss. In other words, the normal inflammatory pathway is altered within the patient's prostate and this aberrant inflammatory pathway is playing part in

pathogenesis of the disease.

Neurological dysfunction as the primary cause of pain

Neurological dysfunction has been a major focal point for the etiology of the pain in CP/CPPS. A group of men diagnosed with CP/CPPS found to have high amounts of prostaglandin E2 (PGE2) present in their prostatic fluid were treated with antibiotics (Quinolones or Macrolides) and anti-oxidants (Prosta-Q). Subsequently, the level of PGE2 decreased by 50% and β-endorphin levels concurrently increased 2.75 fold, which coincided with reported improvement in patient's pain using the NIH-CPSI[31]. This provided quantifiable effects presumedly due to local neurological effects of prostaglandins along with its role in inhibition of β-endorphins[32].

NGF is a known neurotrophic agent that has a direct role in pain induction in CP/CPPS patients[33]. Prostate inflammation leads to the release of cytokines such as IL-10, which may induce the expression of NGF[33] or direct neuronal damage may bring about NGF release, prompting excitation of C-fibers and mast cell degranulation leading to further release of NGF[34]. Though NGF is released peripherally, it can sensitize central neurons once its concentration exceeds a threshold causing constant depolarization of those neurons. This, in turn, leads to central hyper-sensitization and chronic pain[32].

Furthermore, autonomic dysfunction has been implicated in patients with CP/CPPS as a key contributor in the network of processes promoting symptom development. Abnormal postural blood pressure response has been noted in CP/CPPS patients, along with elevated peripheral blood pressure readings[35]. Cho *et al*[36] also reported that CP/CPPS patients had lower heart rate variability as compared to the controls, which suggests that autonomic dysfunction could be a causative as well as an aggravating factor in CP/CPPS.

Role of nanobacteria

Recently, compelling results have been obtained highlighting the role of nanobacteria in CP/CPPS. Nanobacteria are newly discovered cell-walled organisms that have an annular structure, display slow growth in cell culture, are able to induce cell death in fibroblasts and require electron microscopy for visualization[37]. They do not require host cells in order to replicate and are believed to form the apatite core of prostatic calculi, as under physiological conditions they are present in mineralized apatite crystal form[36-38]. A study was performed to establish the treatment modalities that can be effective against nanobacteria-causing CP/CPPS in the presence of prostatic calculi; in this study patients refractory to multiple prior therapies were given tetracycline, multivitamin (Nanobac OTC supplement) and an ethylenediaminetetraacetic acid rectal suppository for 3-4 mo. Ultrasound and nanobacterial antigen testing was done before the treatment process was initiated. Patients reported marked improvement in their mean NIH-CPSI score ($P < 0.0001$), after 3 mo consequently few patients who underwent transrectal ultrasound displayed decreased or absent prostatic stones once the treatment was completed[38].

Pelvic injuries, chronic pelvic floor muscle tension and dysfunctional voiding as possible etiologies

The process of CP/CPPS appears to begin after an initiator causes inflammatory or neurogenic damage within or outside the prostate[39]. An important consideration in CP/CPPS patients is that similar symptomatology can also be seen in patients with bladder neck obstruction and external sphincter dyssynergia[40]. These patients often complain of dysfunctional voiding. It is hypothesized that psychological stress causes aberrant pelvic floor muscle function which triggers dysfunctional voiding and eventually full-blown CP/CPPS[41-43]. There is evidence that increased intraprostatic pressure due to enhanced sympathetic activity and can lead to urine reflux from the urethra into prostate, which subsequently causes prostatic inflammation[44]. The prostatic inflammation is thought to cause edema that disrupts the microvasculature, causing tissue hypoxia and pain initiation[45].

A study by Shoskes *et al*[46] demonstrated myofascial pain as an etiology of CP/CPPS. They deduced that most of the patients with CP/CPPS had point myofascial tenderness with the prostate being the tenderest point. Although the cause of myofascial spasm can be infectious, inflammatory or traumatic like CP/CPPS, there was no association between the myofascial spasm and prostatic inflammation. Neuromuscular trigger points have been identified in CP/CPPS patients and it is hypothesized that painful myofascial tissue plays a major role in the syndrome[47].

A racially and ethnically diverse study known as the Boston Area Community Health survey comprised of 2301 men was conducted by Daniels *et al*[48] between April 2002 through June 2005, demonstrating men with frequent UTI's had higher odds of having symptoms of CP/CPPS ($P < 0.01$). Similarly a study conducted to investigate the symptoms of prostatitis amongst male health professionals concluded that individuals with a history of sexually transmitted disease had 1.8 times increased odds of having the disease[49].

DIAGNOSING THE DISEASE

Patients with CP/CPPS primarily complain of pain in the pelvic region including perineum, rectum, prostate, penis, testicles and abdomen. Along with the pain occasionally patients also complain of LUTS or obstructive symptoms[50]. Pain lasting longer than 3 mo[51] is an imperative symptom and is the most consistent finding in CP/CPPS patients[6]. Patients also experience a wide array of sexual dysfunctions including erectile dysfunction, painful ejaculation and premature ejaculation[52].

Diagnosing CP/CPPS can be difficult as there are

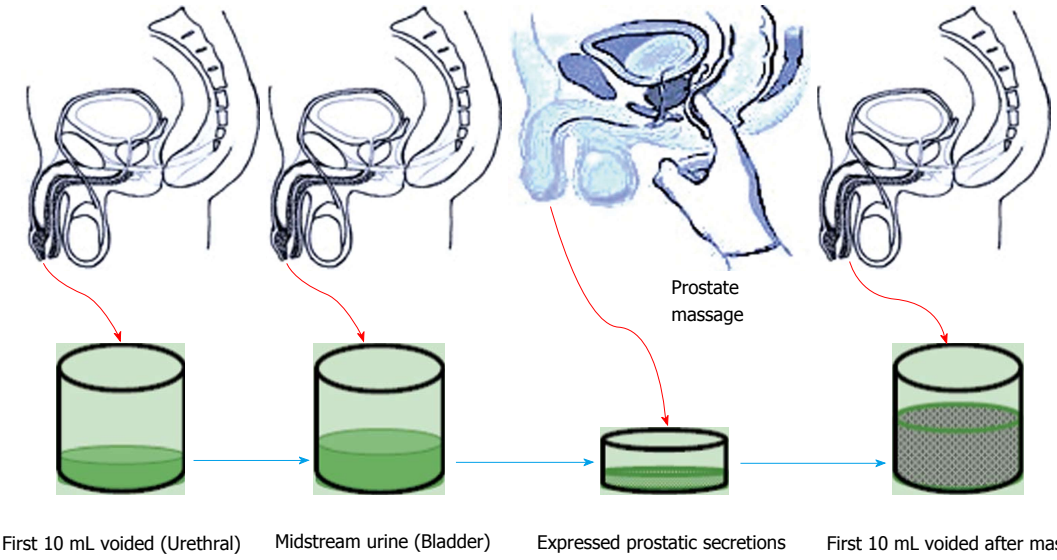

Figure 1 Meares-Stamey four glass test.

Table 2 Diagnostic evaluation of chronic prostatitis/chronic pelvic pain syndrome

Primary evaluation
History: Should include complete background and symptom evaluation
Physical exam: Complete physical exam including a digital rectal exam and check for myofascial tenderness
Pain evaluation: Use the National Institute of Health-Chronic Prostatitis Symptom index for quantification of the symptoms
Urinalysis and culture
Specialized evaluation
Meares-stamey four glass test
Pre and post-massage two glass test
Urodynamic studies (only if lower urinary tract symptoms or outflow obstruction present)
New/optional evaluation
Serum prostate-specific antigen
Prostatic fluid nerve growth factor levels
Cystoscopy
Transrectal ultrasound or computerized tomography scan
Intra-anal electromyography
Pelvic floor ultrasound

no validated tests for the disease and it is largely a diagnosis of exclusion[53] once other disease such benign prostatic hyperplasia (BPH), interstitial cystitis, Genitourinary stones or cancer and prostatic abscess have been ruled out[54]. Adequate history, a physical examination including a digital rectal examination and urinalysis are mandatory in every patient[55]. Physicians are advised to adopt a step-by-step approach (Table 2) including subjective evaluation and quantification of pain symptoms using the NIH-CPSI followed by physical evaluation and laboratory tests while investigating a patient for CP/CPPS[56]. Meares-Stamey-four glass[57] and the modified two glass tests[13] are commonly used laboratory investigations.

Meares-Stamey four-glass test

This test involves performing a prostatic massage followed by collection of four samples of Urine and expressed prostatic fluid. Each sample collection takes place after voided bladder 2 (VB2) has been collected. Initial 10 mL of voided urine constitutes urethral flora. Subsequently 200 mL of urine is voided and midstream urine is collected (VB2) this constitutes the bladder flora. Collection of VB2 is followed by a prostatic massage to collect EPS afterwards EPS and the first voided 10 mL are collected (VB3) (Figure 1). These samples are cultured and evaluated microscopically to look for bacterial presence and confirm prostatic inflammation[58]. Meares-stamey test can help differentiate between type Ⅱ, ⅢA and ⅢB on the bases of presence of leukocytes (> 5 in ⅢA and < 5 in ⅢB)[50].

Pre-, post-massage two-glass test

Meares-Stamey four-glass test was modified to pre and post-massage two-glass test (Figure 2), which utilizes only VB2 and VB3 and provides fairly accurate results. Along with being cost effective it is easier to perform[59].

Role of serum prostate-specific antigen

Studies have been conducted to find a link between CP/CPPS and total or free prostate-specific antigen (PSA) but a statistically significant connection is yet to be established between the two[60,61]. PSA levels should be checked even without the presence of pain in patients older than 50 years.

Nerve growth factor levels

As discussed earlier a positive correlation between nerve growth factor levels and NIH-CPSI score have been found, presence of higher levels of NGF in CP/CPPS than control group further strengthens the use of NGF as a biomarker[62]. Higher levels of NGF in seminal fluid of CPPS patient heralds prostatic inflammation[33] hence it can be used to assess treatment response in

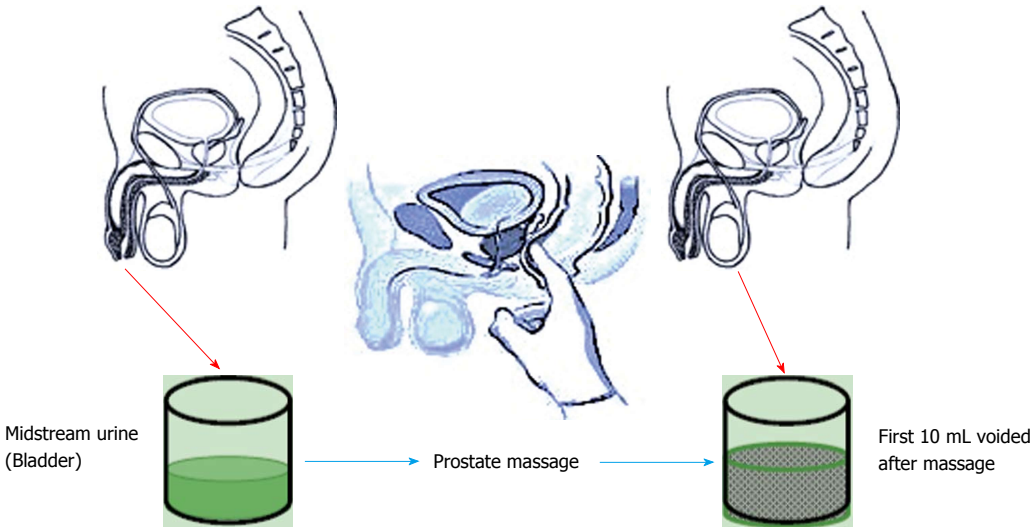

Figure 2 Pre- and post-massage two glass test.

the patients.

Imaging studies

CP/CPPS patients are not required to undergo unnecessary imaging or endoscopic studies until and unless there is evidence of LUTS or bladder outflow obstruction in which case urodynamic evaluation helps guide treatment[56]. Pelvic imaging such as transrectal ultrasound, computed tomography scan or magnetic resonance imaging can be helpful in ruling out bladder, prostate, seminal vesicular or ejaculatory duct pathology[55]. As pelvic floor muscle dysfunction has been contemplated as one of the possible causes of CPPS, intra-anal electromyography and pelvic floor ultrasound has successfully been used to assess higher pelvic resting muscle tone[63].

TREATMENT MODALITIES

Pharmacological

Antibiotics: Despite the fact that bacterial involvement is one of the least likely causes of CP/CPPS and there are apprehensions surrounding the etiology of CP/CPPS, the treatment primarily constitutes of empirical therapy and in it antibiotics are amongst the most prescribed medications. In a some patients with bacteria identified as the primary etiological factor in causing the disease the antibiotic therapy helps improve symptoms by eradicating the pathogen, however the conducive effects of antibiotics have largely been linked to their anti-inflammatory activity[17].

A randomized multicenter trial by Alexander *et al*[64] conducted to analyze the effect of ciprofloxacin over the period of 6 wk in 49 patients, did not result in any statistically significant improvement in the NIH-CPSI score. Another study conducted to infer efficacy assessment of levofloxacin[65] proved to be fruitless in exhibiting significant improvements in patients NIH-CPSI score on the other hand a course of tetracycline over the period of 12 wk did prove efficacious but the study had lacking in several fronts including small patient population and use of combination therapy[38]. Despite the negative outcomes of the above studies, a study undertaken by Canadian Prostatitis Research Group did show promising results with likelihood of symptom improvement being almost 65% after a 12-wk course of ofloxacin was administered to CP/CPPS patients[66].

Recently Choe *et al*[67] performed a multicenter randomized pilot trial to compare the effect of roxithromycin with ciprofloxacin and aclofenac in 75 patients divided into three groups. Patients were treated for 4 wk and subsequently followed for 12 wk. Results obtained showed decrease in NIH-CPSI score in roxithromycin group comparable to results of the other two groups for type ⅢA patients and even lower scores in type ⅢB patients. This outcome supports the notion that though CP/CPPS is considered primarily to be a non-infectious condition, post-antibiotic improvement in symptoms attests their definitive role in disease treatment.

Use of trimethoprim-sulfamethoxizole has also been found to improve symptoms in CP/CPPS[68] and a single 4-6 wk course of trimethoprim-sulfamethoxizole can be tried as a first line treatment therapy[58]. In a small study, Zhou *et al*[69] found out that tetracycline was able to improve the mean NIH-CPSI scores from 35.6 ± 5.2 to 17.1 ± 2.8 in the treatment group ($P < 0.01$). This result warrants performing further investigations to evaluate the effects of tetracycline therapy in CP/CPPS. Antibiotic use is recommended only in antibiotic naive population and should not be considered as a monotherapy in patients who had prior antibiotic treatment. (Evidence level: 1, recommendation grade: A).

Anti-inflammatories: As prostate inflammation is one of the possible etiologies of CP/CPPS, the use of anti-inflammatories to curb the inflammation has been a mainstay of treatment and plays a discrete role in

disease management. Mast cells are considered to play an important part in pathological process of CP/CPPS for this reason a small-randomized single center study conducted to identify the effect of a leukotriene inhibitor zafirlukast did not exhibit promising results[70]. Cycloxygenase inhibitors are an important class of drugs with important anti-inflammatory effects. Rofecoxib, a COX-2 inhibitor, was given to 161 patients. Efficacious effects were seen in the treatment group against the placebo group but only at high doses given to the patients over a span of 6 wk[71]. Non-steroidal anti-inflammatory drugs have been commonly used in the treatment of CP/CPPS as a first line therapy[72]. Few trials are available, one of which was conducted by Tuğcu *et al*[73] who treated CP/CPPS patients with Ibuprofen together with doxazosin and thiocolchicoside (muscle relaxant) as a triple therapy and compared the results with alpha blocker (Doxazosin) monotherapy and placebo groups for six months, which displayed mean improvement of NIH-CPSI score from 23.1 to 10.7 in the triple therapy group and 21.9 to 9.2 in the the monotherapy group with a stable score in the placebo group indicating there is no advantage of triple therapy over mono therapy ($P < 0.05$) in CP/CPPS. Similarly, a placebo controlled study to assess the role of celecoxib in CP/CPPS by Zhao *et al*[74] determined that 6 wk of celecoxib therapy reduced total NIH-CPSI score from 23.91 ± 5.27 to 15.88 ± 2.51, a statistically significant decrease in score ($P < 0.006$).

Pentosan polysulfate sodium is a FDA approved glycosaminoglycan used in interstitial cystitis chiefly because of its anti-inflammatory effect on bladder mucosa[75]. A 16-wk randomized double-blinded multicenter study failed to show its substantial effect in improving NIH-CPSI scores in CP/CPPS patients[76]. There is an ample room for research and large multicenter studies would certainly reveal more information regarding the potential effects of pentosan in CP/CPPS patients. Keeping in mind the pathogenic role of neurotrophin Nerve growth factor in CP/CPPS, a randomized double-blinded multicenter study was conducted with 62 patients divided in two groups to evaluate the efficacy of tanezumab a humanized monoclonal antibody. Tanezumab was given as a single 20 mg intravenous dose per day to the treatment group for 6 wk but only modest response was observed as compared to the placebo group[77]. These results warrant further trials on a larger scale to broaden the scope of the available treatment options.

Anti-inflammatories play an important role in CP/CPPS therapy when given as a part of the multimodal regimen. (Evidence level: 2, recommendation grade: C).

Alpha-adrenergic blockers: Often, men suffering from CP/CPPS also present with LUTS such as urgency, frequency and incomplete voiding. These manifestations along with the fundamental CP/CPPS symptom of genitourinary pain give way to the use of α-blockers. Alpha blockade is thought to exert its pacifying effects on prostate, urethra and bladder neck and in turn improve the LUTS along with improvement in NIH-CPSI scores especially in alpha-blocker-naive patients[78]. Various randomized placebo controlled trials have been performed to ascertain effects of alpha-blockers in CP/CPPS. Alpha-blockers commonly used to minimizing LUTS in CP/CPPS are alfuzosin[79,80], tamsulosin[64,81], terazosin[82], doxazosin[73] and silodosin[83]. Despite the fact that two NIH-sponsored studies failed to demonstrate any usefulness of alfuzosin or tamsulosin in CP/CPPS[84], beneficial effects of alpha blockers are pertinent to their long term use as illustrated in a randomized placebo control study by Nickel *et al*[80] performed to evaluate the role of alfuzosin against placebo in recently diagnosed alpha-blocker naive CP/CPPS patients over a period of 12 wk. The study failed to demonstrate any significant improvement in the NIH-CPSI score of the group treated over the placebo group and postulated that long-term therapy might be warranted to observe treatment effects. Taken together, alpha-blocker monotherapy is not recommended, especially in patients previously treated with alpha-blockers. (Evidence level: 1, recommendation grade: A).

Combination therapy trials: Due to the complex nature of the disease, lack of effectiveness of monotherapies and the postulated role of multiple patho\genic factors for CP/CPPS, various combination therapies have been devised by researchers to address specific etiologic factors. The three A's of CP/CPPS including alpha-blockers, anti-inflammatories and antibiotics are an effective combination therapy for CP/CPPS[85]. A recent meta-analysis of all the available treatments for CP/CPPS and their subsequent effects on patient NIH-CPSI scores reported multimodal therapy with alpha-blockers, anti-inflammatories and anti-biotics was superior to monotherapies for optimal disease management[72]. Combination therapies with alpha-blockers and anti-biotics have revealed positive outcomes[64,86], but current recommendations are to tailor the treatment regimen to the patient's symptoms. (Evidence level: 2, recommendation grade: A).

Phytotherapy: Phytotherapy is one of the alternative pharmacotherapies believed to abate the inflammatory process that occurs in the prostate, but the exact mechanism is still unknown[87]. Phytotherapy includes pollen extracts, quercetin and saw palmetto. Various pollen extract preparations are available. In one randomized double-blinded trial, 60 patients were divided into treatment groups that received prostat/poltit (a pollen extract) or placebo. After 6 mo, the pollen treatment group reported marked improvement in symptom scores compared to placebo group[88]. A larger randomized, multicenter, placebo-controlled trial by Wagenlehner *et al*[89] enrolled 139 participants, randomly allotted to treatment and placebo groups. The 12-wk study resulted in improvement in pain

(P = 0.0086), quality of life (P = 0.0250) and NIH-CPSI score (P = 0.0126) in the Pollen extract group. These studies highlight the fact that pollen extract to some extent is effective in the treatment of CP/CPPS. (Evidence level: 1, recommendation grade: B).

Quercetin is a bioflavonoid found in plants, as well as in green tea, onions and red wine and is a known anti-oxidant and anti-inflammatory agent[90]. A prospective placebo controlled, double-blind trial by Shoskes *et al*[91] demonstrated mean improvement of NIH-CPSI score from 21.0 to 13.1 (P = 0.003) in the group taking quercetin. Apart from the quercetin and placebo group, a third group received a quercetin formulation mixed with digestive enzymes bromelain and papain. This group demonstrated an 82% improvement in mean NIH symptom scores from 25.1 to 14.6. (Evidence level: 2, recommendation grade: C)

Saw Palmetto (Serenao repens), a herbal lipid extract, is one of the most frequently used phytotherapies in symptomatic benign prostatic hyperplasia (BPH) patients[92,93], but its use in CP/CPPS still remains controversial because of limited number of trials. A prospective, randomized open-label study by Kaplan *et al*[94] compared saw palmetto with finasteride, 5-alpha reductase inhibitor, in 64 participants. After 1 year, mean NIH-CPSI score in the finasteride group decreased from 23.9 to 18.1 (P = 0.003) and from 24.7 to 24.6 (P = 0.41) in saw palmetto group. These authors concluded that the use of saw palmetto does not improve the symptoms in CP/CPPS patients significantly. (Evidence level: 3, recommendation grade: C).

Neuromodulatory drugs: Neurological dysfunction has been implicated as a prime culprit in men with CP/CPPS. Depression and psycho-emotional changes usually accompany neurogenic pain in CP/CPPS[95,96]. Thus, anxiolytics and anti-depressants might have a therapeutic role in disease management. Several uncontrolled trials have been performed to evaluate different pharmacological interventions for neuropathic pain symptoms. Recently, Giannantoni *et al*[97] conducted a small study to analyze the effectiveness of duloxetine being given as part of a multidrug regimen for CP/CPPS. After 16 wk of treatment in 38 men, randomly divided into two groups, one of which received an alpha-blocker (Tamsulosin) and saw palmetto, while the other group received triple therapy with an alpha-blocker (Tamsulosin), saw palmetto and duloxetine. Significant improvement in total NIH-CPSI score (25.1-14.17, P < 0.01) was observed in the group receiving the triple therapy. The anticonvulsants pregabalin and gabapentin play a major role in chronic pain syndromes treatment and also have been used in CP/CPPS[98]. A multi-center randomized double-blinded placebo controlled trial failed to show significant NIH-CPSI score improvement in participants receiving pregabalin[99]. Nonetheless well-controlled studies are required to investigate possible benefits of these drugs in the management of CP/CPPS. Neuromodulatory drugs should not be recommended as a primary treatment modality for CP/CPPS (Evidence level: 2, recommendation grade: B).

Hormonal therapy: Finasteride is a 5-α reductase inhibitor used as a treatment to alleviate symptoms and prevent surgical intervention in men with BPH[100]. It blocks the conversion of testosterone to the more potent dihydrotestosterone. The use of finasteride in CP/CPPS needs further study because of the lack of data that supports its positive role in improving patient symptoms. Nickel *et al*[101] conducted a placebo-controlled, randomized trial to determine the effectiveness of finasteride in reducing CP/CPPS symptoms. Only 75% of participants receiving finasteride had > 25% improvement in the subjective oral assessment and a similar trend was observed in the NIH-CPSI scores. Use of mepartricin, an estrogen-lowering drug[102], has been tested in a small placebo controlled trial. It was found to be effective in decreasing the NIH-CPSI score from 25.0 to 10.0 in the treatment group along with a statistically significant decrease in scores of pain (11.0-4.0) and quality of life (10.0-5.0)[103]. Despite the fact that this study displayed some benefit of mepartricin in CP/CPPS patients, larger multicenter center studies are still required to confirm the results. Hormonal therapy is not considered as a first line treatment in CP/CPPS and should be reserved in patients with symptoms of BPH (Evidence level: 2, recommendation grade: C).

Others: Recently a 12-mo randomized placebo controlled double-blinded study was conducted to observe the effects of immunostimulation in CP/CPPS by giving an oral immunostimulatory agent known as OM-89, which is a lysed pathogenic *E. coli* extract[104]. The study did not demonstrate a significant difference in improvement of NIH-CPSI scores between the treatment and the placebo groups despite the fact that the long term therapy was well tolerated by the patients[105]. Hormonal therapies as yet are only advisable to patients with CP/CPPS with prior symptoms of prostatic hyperplasia. Use of allopurinol[106,107] and oral corticosteroids[108] in the management of CP/CPPS remains uncertain and their effects have not comprehensively studied and more detailed and well planned trials are necessary to ascertain the therapeutic role these drugs. (Evidence level: 3, recommendation grade: C).

Complementary and interventional therapies

Physical therapy, myofascial trigger point release and pelvic floor biofeedback: Large number of patients with CP/CPPS also have pelvic floor muscle dysfunction[109] and myofascial pain. In order to relax these pelvic muscle and decrease the pain associated with hypersensitive regions in muscles or fascia, pelvic floor physical therapy and myofascial trigger point release has been devised. Researchers from stanford have identified various myofascial trigger points[47] and have devised a protocol accordingly employing myofascial trigger point release physical therapy along

with paradoxical relaxation technique in CP/CPPS patients. The beneficial effect of this treatment can be assessed by one of their trials which resulted in a 72% rate of moderate to marked improvement of symptoms along with improvement in NIH-CPSI scores with a median decrease of 10.5 ($P < 0.001$) in markedly and 6.5 ($P = 0.008$) in moderately improved groups of CP/CPPS patients[110].

A randomized, multi-center, feasibility trial performed by FitzGerald *et al*[111] evaluated the effectiveness of physical therapy in urological chronic pelvic pain syndromes with global therapeutic massage (GTM) *vs* myofascial physical therapy (MPT). In the study, 45% participants had CP/CPPS and 42% received GTM and 48% received MPT. MPT resulted in improved symptom scores ($P = 0.0003$) in CP/CPPS patients but was only significantly better than GTM on the urinary symptom scale of the NIH-CPSI (-3.9 *vs* -0.3, $P = 0.007$).

Along with myofascial trigger point release, the rationale behind physical therapy is to restore proper use of pelvic floor muscles. Pelvic floor biofeedback and pelvic floor re-education were investigated by Cornel *et al*[112] in a study using rectal Electromyogram probes to monitor therapy response. Pelvic floor biofeedback helped improve the NIH-CPSI score from 23.6 to 11.4 ($P < 0.001$) along with a decreased pelvic muscle tone ($P < 0.001$). Similarly He *et al*[41] used pelvic floor biofeedback in 21 patients and attained comparable results with improvement in NIH-CPSI score ($P < 0.05$) along with improvement urodynamics ($P < 0.05$). Nadler[113] had previously demonstrated the beneficial effects of biofeedback and bladder training in CP/CPPS patients in a small pilot study. Overall, these results point towards therapeutic benefit when targeting pelvic floor muscle dysfunction for CP/CPPS patients.

Acupuncture: Acupuncture has been put forward as a safe and beneficial procedure for CP/CPPS patients. Recently several publications have evaluated the role of acupuncture[114-117], though the exact mechanism of pain relief is unknown. But, its utility in other neuropathic pain entities has already been established[114]. A recent review found acupuncture effective in ameliorating CP/CPPS pain symptoms and the authors endorsed acupuncture as part of a standard CP/CPPS treatment[118].

A pilot study to determine the validity of acupuncture use to improve CP/CPPS symptoms was performed. Chen *et al*[115] provided acupuncture therapy for 6 wk in men refractory to standard therapy. Total NIH-CPSI score decreased from 28.2 to 8.5. The NIH-CPSI pain (14.1-4.8), urinary (5.2-1.3), and NIH-CPSI quality-of-life (8.8-2.3) scores all decreased after a median follow-up of 33 wk. Although this had significant limitations including small study population and the lack of control and placebo groups, acupuncture achieved impressive results and highlighted a need for randomized placebo controlled studies. Lee *et al*[116] compared acupuncture with sham acupuncture and found out that 32 (73%) of 44 participants responded with 4.5 points decrease in NIH-CPSI score on average in the acupuncture group compared to the score of 21 (47%) participants in the sham group ($P = 0.03$). After 24 wk 32% participants in the acupuncture group demonstrated long-term response as compared to 13% participants ($P = 0.04$) in sham acupuncture group without any additional treatment.

Electroacupuncture was noted to significantly reduce NIH-CPSI scores (details) at 6 wk compared to sham electroacupuncture and to medical advise and exercise regimens[119].

In a recent randomized double-blinded trial, results of acupuncture and sham acupuncture in CP/CPPS were compared over the period of 10 wk. In sham acupuncture, participant's short needles were placed 0.5 cm away from the true acupuncture points. Clinical response criterion was achieved in 73% of the acupuncture participants compared with 47% of sham acupuncture participants ($P = 0.017$). Higher levels of β-endorphins and leucine-enkephalin levels were noted in the acupuncture group ($P < 0.01$)[117]. A recent systemic review analyzed 27 clinical trials including 890 patients and concluded that acupuncture can be contemplated as an efficacious treatment modality in CP/CPPS[120]. Nevertheless, high quality randomized, placebo-controlled trials are needed to investigate acupuncture as a first-line treatment modality for CP/CPPS.

Posterior tibial nerve stimulation and sacral neuromodulation: United States Food and Drug Administration has approved sacral nerve stimulation and posterior tibial nerve stimulation for use in CP/CPPS patients with urinary symptoms. Despite the fact that these therapies have been approved, they are still not considered first line treatments[121]. Both therapies have improved NIH CPSI scores, but larger and better-designed multicenter trials are still required in order to include them into the treatment algorithm[122-124].

Botulinum toxin injection: Botulinum toxin is a potent neurotoxin already being used in different muscular and neurological disorders. Likewise its use has been advocated in the treatment of CP/CPPS patients when multi-therapy is employed[125].

Others: Several other techniques including prostatic massage, sitz bath and frequent ejaculations are prescribed, but they only have a supportive role in the treatment of CP/CPPS. Nickel *et al*[126] devised a comprehensive 8-wk program consisting of cognitive behavioral therapy (CBT) in patients with CP/CPPS in order to help the patients manage their condition and in turn improve their quality of life (QoL). This technique was again tested by Tripp *et al*[127] and obtained satisfactory results. Patients displayed improved scores

in the categories of pain, disability and catastrophizing. Follow-up CPSI scores were significantly decreased (P = 0.007) in particular, significant decrease was noted in CPSI pain (P = 0.015) and QoL domains (P = 0.013). These results support the use of CBT, but additional randomized controlled trials are required to assess its long-term effects in CP/CPPS patients.

These interventions are helpful in CP/CPPS patients with pelvic floor muscle dysfunction and help relieve the symptoms significantly (Evidence level: 4, recommendation grade: C).

Surgical treatment

Surgical treatments have been found to help patients suffering from CP/CPPS refractory to other types of treatments. Various types of surgical interventions are available but their level of benefit is still questionable.

Transurethral needle ablation: Transurethral needle ablation (TUNA) is already one of the primary treatment methods with clinical efficacy BPH/LUTS[128]. Its therapeutic effect in CP/CPPS is yet to be determined because of the limited number of studies available. A Pilot study performed by Aaltomaa *et al*[129] along with the studies conducted by Lee *et al*[130] and Chiang *et al*[131] have shown favorable outcomes in the patients, but further prospective trials are still required[130].

Transurethral balloon dilation: Transurethral balloon dilation (TUBT) is another treatment modality offered to CP/CPPS patients, though, as in the case of TUNA, limited number of studies only allows TUBT to be offered as a secondary treatment choice. A small study by Lopatin *et al*[132] performed TUBT on 7 patients presenting with bladder neck or prostatic urethra obstruction which improved the overall symptoms of CP/CPPS in all the participants.

Transurethral microwave therapy: Transurethral microwave therapy (TUMT) is one of the primary treatments BPH. In CP/CPPS patients, TUMT have been used and results obtained are promising though its use as a first line therapy is not recommended[5].

Extracorporeal shockwave therapy and Extracorporeal magnetic stimulation: Most recently, extracorporeal shock wave therapy has been shown to have potential benefit in CP/CPPS patients. Zimmermann *et al*[133] followed 34 patients for 12 wk and found significant improvement in patients pain and QoL. They followed their initial trial with a double-blinded, placebo-controlled, randomized trial with 60 patients. Extracorporeal shockwave therapy was significantly effective in improving pain, QoL and voiding symptoms[134].

Kim *et al*[135] used a recent technique known as extracorporeal magnetic stimulation to treat CP/CPPS. In their study 46 patients were enrolled and the treatment was provided for 6 wk. The patients were followed 24 wk post treatment. More than 70% of the patients registered a positive outcome of the treatment with an improvement in NIH-CPSI (P < 0.05) and pain scores (P < 0.05). Results for this study are promising but it would be too early to say if it can be included as a primary mode of treatment until and unless more multicenter trials are performed and validate its effectiveness.

The above interventions are reserved for patients with refractory symptoms and should not be offered as first line treatment modality (Evidence level: 2, recommendation grade: A).

Transurethral resection of prostate and Prostatectomy: Transurethral resection of prostate and prostatectomy have been reserved only for CP/CPPS patients with intractable pain and is not routinely recommended because it involves significant morbidity and persistence of symptoms post-surgery[39,96] (Evidence level: 4, recommendation grade: D).

Multimodal therapy

CP/CPPS is a heterogeneous syndrome, which makes it difficult to treat. Monotherapies have worked in some of the patients, but there is no data to support the use of a single therapy in most patients. Monotherapy failed to find major success in treating the disease because it acts against a single target but there are multiple etiologies that likely lead to the development of the disease[136].

A more tailored treatment approach was required to treat this condition with varied symptomatology and consequently Shoskes *et al*[137] and Nickel *et al*[138] came up with a phenotypic approach for the management of CP/CPPS. The UPOINT phenotypic approach comprises of six clinical domains including Urinary symptoms, Psychosocial dysfunction, Organ specific findings, Infection, Neurologic dysfunction and Tenderness of muscles[136]. Recently sexual dysfunction was included in this phenotypic approach, which led to the modified UPOINTS system after a study showed sexual dysfunction domain correlated with the NIH-CPSI score significantly[139,140].

To test the treatment efficacy of the UPOINT system, a prospective study was conducted, including 100 patients treated with multimodal therapy. Men were re-evaluated after 26 wk. The treatment proved to be effective and almost 86% patients experienced at least a 6-point decrease in NIH-CPSI score and total improvement in NIH-CPSI scores were from 25.2 ± 6.1 to 13.2 ± 7.2 (P < 0.0001). These results are comparable to some of the large monotherapy trials[141].

An algorithm has been constructed to provide individualized therapy based on the phenotypes within the UPOINT system (Figure 3). Because of the fact that monotherapies give modest therapeutic outcomes, a multimodal treatment approach would be considered more rational and should be considered as a primary

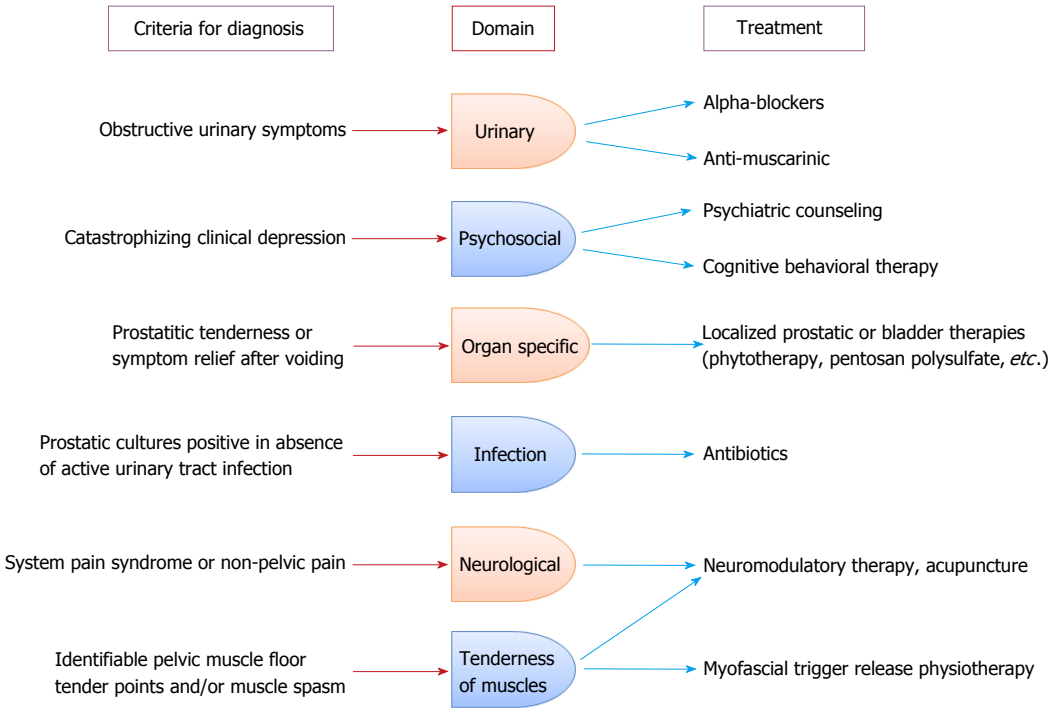

Figure 3 Multimodal therapy based on clinical phenotype (urinary, psychosocial, organ specific, infection, neurological, tenderness of muscle) approach for chronic prostatitis/chronic pelvic pain syndrome.

recommendation based on patients phenotype.

CONCLUSION

CP/CPPS has been baffling the physicians since long because of its complex etiology and difficult-to-treat nature. No single etiological factor has been linked strongly to CP/CPPS, instead a cluster of factors cause symptoms in CP/CPPS patients. This relates to the fact that monotherapy does not always proves effective in treating the symptoms. Primary role of the Urologist is to rule out differentials that present with similar symptoms as CP/CPPS and utilize diagnostic modalities that cause minimum physical and psychological distress to the patients and provide most accurate results. Multimodal therapy such as UPOINT has been beneficial because of its role in influencing multiple constituents that lead to CP/CPPS. Overall CP/CPPS should not be considered as a localized pathology and a centralized approach is recommended to reverse or halt the progression of symptoms. There is still space for development of novel therapeutic regimens including the development of a vaccine that can be offered to CP/CPPS patients. Psychological stress should always be ruled out and psychiatric counseling needs to be offered along with pharmacological treatment. Pelvic floor biofeedback along with acupuncture proved to help patients but larger multicenter studies are still required to prove its effectiveness in CP/CPPS patients. It is recommended that physicians keep in touch with new researches and treatments coming out for CP/CPPS, in order to provide their patients a chance for an up to date and improved treatment opportunities. For now the best available strategy would be to individually tailor treatment plan for each patient.

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REVIEW

Levomilnacipran and vortioxetine: Review of new pharmacotherapies for major depressive disorder

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Abstract

Major depressive disorder (MDD) is a common psychiatric disorder with an estimated lifetime prevalence rate in the range of 13% to 16% in the United States population. Patients with MDD often have symptoms such as depressed mood, loss of interest or pleasure in usual activities, changes in eating or sleeping patterns, fatigue, difficulty concentrating and thoughts of suicide. Although many pharmacotherapy treatment options are available for MDD, antidepressants can often cause adverse effects that could affect adherence to the medication. Additionally, it is estimated that MDD is unremitting in 15% of patients and 35% can have recurrent episodes. Given the high rate of recurrence and the adverse effects associated with existing medications, new treatment options for depression are needed. Both levomilnacipran and vortioxetine are new antidepressants that were approved by the food and drug administration in 2013 for the treatment of MDD in adults. Levomilnacipran is a serotonin norepinephrine reuptake inhibitor that was effective in several short term studies and sustained efficacy and tolerability was demonstrated in a 48-wk extension study. Vortioxetine is a multi-modal antidepressant and it is thought to work *via* inhibition of the serotonin (5-HT) transporter, 5-HT$_{3A}$, 5-HT$_{7}$ and 5-HT$_{1D}$ antagonist, a 5-HT$_{1B}$ partial agonist, and a 5-HT$_{1A}$ agonist. Vortioxetine was effective in the treatment of MDD in both short-term trials as well as in the prevention of relapse in a 24-36 wk trial. Sustained efficacy and tolerability was demonstrated in several long-term open-label trials. Further studies comparing levomilnacipran and vortioxetine to other currently available antidepressants are needed to establish its place in therapy.

Key words: Levomilnacipran; Vortioxetine; Adult; Major depressive disorder; Antidepressive agents

Core tip: Levomilnacipran and vortioxetine are the two newest antidepressant medications to join the armamentarium of treatment choices for major depressive disorder. Levomilnacipran, a serotonin norepinephrine reuptake inhibitor, is an enantiomer of the previously approved fibromyalgia agent milnacipran. Vortioxetine is a multimodal antidepressant with a unique mechanism of action, affecting several serotonin receptors as well as inhibiting serotonin reuptake. This review summarizes the clinical trial data as well as pharmacokinetic, dosing and

safety concerns with these two new agents.

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INTRODUCTION

Major depressive disorder (MDD) is a common psychiatric disorder with an estimated lifetime prevalence rate in the range of 13% to 16% in the United States population[1]. Patients with MDD often have symptoms such as depressed mood, loss of interest or pleasure in usual activities, changes in eating or sleeping patterns, fatigue, difficulty concentrating and thoughts of suicide. These symptoms persist over a period of at least 2 wk and are not attributable to other disorders such as substance-induced or general medical conditions[1].

Many pharmacotherapy treatment options are available for MDD. They include selective serotonin reuptake inhibitors (SSRIs), serotonin norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants, monoamine oxidase inhibitors (MAOIs), miscellaneous antidepressants such as bupropion and mirtazapine and antipsychotics such as quetiapine and aripiprazole. Despite the wide variety of antidepressants available, antidepressants can often cause adverse effects such as weight gain, sexual dysfunction, anxiety, headache, nausea, and sleep disturbances which could affect adherence to the medication. Additionally, it is estimated that MDD is unremitting in 15% of patients and 35% can have recurrent episodes[1]. If no prophylactic maintenance treatment is provided, the risk of recurrence approaches 100% in patients with three or more lifetime depressive episodes[1]. Given the high rate of recurrence and the adverse effects associated with existing medications, new treatment options for depression are needed.

The objective of this article is to provide an overview of the two newest antidepressants, levomilnacipran and vortioxetine, and review the safety and efficacy data on these two medications.

LEVOMILNACIPRAN

Levomilnacipran (1S, 2R-milnacipran) was initially approved by the Food and Drug Administration (FDA) on July 26, 2013 for the treatment of MDD in adults (Table 1). Although levomilnacipran is the L-isomer in racemate milnacipran (Savella®) and it is reported to be the more active form of the two isomers, levomilnacipran is not approved for fibromyalgia and efficacy and safety of levomilnacipran for the management of fibromyalgia have not been established[2]. Interconversion between levomilnacipran and its stereoisomer does not occur in humans[3].

Pharmacology

Levomilnacipran is categorized as a SNRI antidepressant because it is thought to increase serotonin and norepinephrine (NE) in the central nervous system through inhibition of reuptake at serotonin (5-HT) and norepinephrine transporters. Levomilnacipran lacks significant affinity to other receptors such as adrenergic, muscarinic, or histaminergic receptors[3]. Levomilnacipran has 2-fold greater potency for inhibition of NE relative to serotonin reuptake[2]. Compared to other SNRIs such as duloxetine and venlafaxine, which preferentially inhibit 5-HT relative to NE reuptake, levomilnacipran has more than 10-fold higher selectivity for NE relative to serotonin reuptake inhibition[2]. Antidepressants with noradrenergic mechanisms of action may be effective in improving depression symptoms related to social functioning such as decreased concentration and loss of energy[4].

Pharmacokinetics

The concentration of levomilnacipran at steady state is proportional to the dose when administered within the range of 25 to 300 mg once daily. It reaches maximum plasma concentration within 6 to 8 h and terminal elimination half-life is approximately 12 h. The relative bioavailability of levomilnacipran is 92% compared to oral solution and its concentration is not significantly affected when administered with food. Levomilnacipran is metabolized primarily by cytochrome P450 (CYP) 3A4 with minor contribution by CYP2C8, 2C19, 2D6, and 2J2. Levomilnacipran and its inactive metabolites are eliminated primarily by renal excretion (Table 2)[3].

Clinical trials

Levomilnacipran has been studied in over 2600 patients with MDD in 7 clinical trials including: 5 short-term (10-11 wk) acute MDD trials, one long-term (≥ 24 wk) relapse prevention trial and one long-term (48 wk) extension study. Four of the short-term trials with results published to date have demonstrated positive efficacy for levomilnacipran for the acute treatment of MDD[5,6], the remaining study is not yet published. One of the studies was a 10-wk Phase Ⅱ study conducted outside of the United States and the other three studies were 10-11 wk Phase Ⅲ studies that included United States sites[7-11]. Two of the studies were fixed dose and two were flexible dose studies (Table 3).

Short-term trials: In the three published Phase Ⅲ short-term studies, patients were included if they were adult out-patients who met the Diagnostic and Statistical Manual of Mental Disorders, Fourth edition, Text Revision (DSM-Ⅳ-TR) criteria for MDD with an ongoing episode of at least 4 to 8 wk duration and a minimum Montgomery-Asberg Depression Rating Scale (MADRS) score ranging from 26-30 at baseline indicating depression symptoms of moderate severity[12,13]. Patients

Table 1 Basic drug information for levomilnacipran and vortioxetine[3,20]

	Levomilnacipran	Vortioxetine
Brand names	Fetzima	Brintellix
Mechanism of action	Serotonin-norepinephrine reuptake inhibitor	Serotonin receptor reuptake inhibitor, serotonin-3 receptor antagonist and serotonin-1A receptor agonist
FDA approval date	July 26, 2013	September 30, 2013
Recommended dosing range	40 mg to 120 mg once daily with or without food	10 mg to 20 mg once daily
Dosage form	Extended-release capsules in 20 mg, 40 mg, 80 mg and 120 mg	Immediate release tablets in 5 mg, 10 mg, 15 mg and 20 mg

FDA: Food and Drug Administration.

Table 2 Pharmacokinetic parameters for levomilnacipran and vortioxetine[3,20,21,24]

	Levomilnacipran	Vortioxetine
Bioavailability	92%	75% ± 9%
T_{max}	6-8 h	3-16 h
Volume of distribution	387-473 L	2400 L
Metabolism	Oxidation (primarily through CYP3A4), glucuronidation	Oxidation (primarily through CYP2D6), glucuronidation
Elimination	58% urine	50% urine, 26% feces
Clearance	21-29 L/h	38 L/h
Half-life	12 h	57-66 h
Protein binding	22%	98%-99%

CYP: Cytochrome P450; T_{max}: Time to maximum plasma concentration.

Table 3 Randomized controlled trials of levomilnacipran for major depressive disorder[3,5,7-11]

Ref.	*n*	Duration	Key inclusion criteria	Doses	Primary outcome
Montgomery *et al*[7] Phase Ⅱ	563	10 wk	Age 18-70 yr HAMD-17 > 22	Levomilnacipran 75-100 mg/d Placebo	Positive Change from baseline in MADRS Placebo: -14.5 Levomilnacipran: -18.7 ($P < 0.001$)
Greenberg[5] Phase Ⅲ	362	11 wk	Age 18-80 yr Clinician-rated MADRS ≥ 30 Self-rated MADRS ≥ 26	Levomilnacipran 40-120 mg/d Placebo	Negative Change from baseline in MADRS Placebo: -14.2 Levomilnacipran: -15.7 ($P = 0.249$)
Asnis *et al*[8] Phase Ⅲ	724	11 wk	Age 18-65 yr MADRS ≥ 30	Levomilnacipran 40 mg/d Levomilnacipran 80 mg/d Levomilnacipran 120 mg/d Placebo	Positive Change from baseline in MADRS Placebo: -11.6 40 mg: -14.8 ($P < 0.05$) 80 mg: -15.6 ($P < 0.01$) 120 mg: -16.5 ($P < 0.001$)
Bakish *et al*[9] Phase Ⅲ	568	10 wk	Age 18-75 yr MADRS ≥ 26	Levomilnacipran 40 mg/d Levomilnacipran 80 mg/d Placebo	Positive Change from baseline in MADRS Placebo: -11.3 40 mg: -14.6 ($P < 0.003$) 80 mg: -14.4 ($P < 0.004$)
Sambunaris *et al*[10] Phase Ⅲ	442	11 wk	Age 18-80 yr Clinician-rated MADRS ≥ 30 Self-rated MADRS ≥ 26	Levomilnacipran 40-120 mg/d Placebo	Positive Change from baseline in MADRS Placebo: -12.2 Levomilnacipran: -15.3 ($P < 0.01$)
Shiovitz *et al*[11] Phase Ⅲ	734	24 wk	Age 18-65 yr MADRS ≥ 22	Levomilnacipran 40 mg/d Levomilnacipran 80 mg/d Levomilnacipran 120 mg/d Placebo	Failed Percent of patient relapse Placebo: 13.91% Levomilnacipran: 20.54% ($P = 0.1651$)

HAMD: Hamilton Rating Scale for Depression; MADRS: Montgomery-Asberg Depression Rating Scale.

were required to have normal physical examination and clinical laboratory results and electrocardiogram findings. Patients were excluded if they had other comorbid DSM-Ⅳ-TR Axis Ⅰ disorders within the last 6 mo, history of nonresponse to adequate treatment with 2 or more antidepressants, medical conditions that may have interfered with the study, if they were pregnant or had significant risk of suicide. Patients

taking concomitant psychotropic medications (with the exception of eszopiclone, zolpidem, or zaleplon) were also excluded[8-10].

In the fixed-dose study by Bakish *et al*[9], the average age of participants ranged from 42.3-43.1 years, 62.2%-66% were female, and 72.6%-77.5% were Caucasian. Patients had an average of 3.5 depressive episodes and the duration of illness ranged from 12.8-14.7 years. The mean MADRS score at baseline ranged from 30.8-31.2 indicating moderate depression. Baseline demographics were similar between the treatment groups. The primary outcome for this study was change from baseline to end of study on the MADRS total score. Secondary outcomes included improvement in the Sheehan Disability Scale (SDS), response (≥ 50% improvement in MADRS score) and remission (MADRS score ≤ 10 at study endpoint). This study found statistically significant differences in change of MADRS total score between both levomilnacipran groups (40 mg and 80 mg) compared to placebo [40 mg: -3.3 (95%CI: -5.5 to -1.1), P = 0.003; and 80 mg: -3.1 (95%CI: -5.3 to -1.0), P = 0.004)][9]. A treatment effect is considered clinically significant if there is at least a 2-point difference between the active drug and the placebo group[7]. Levomilnacipran also demonstrated superiority over placebo in change in SDS total score [40 mg: -1.8 (95%CI: -3.6 to 0) P = 0.046; 80 mg: -2.7 (95%CI: -4.5 to -0.9) P = 0.003]. Response rates were found to be statistically significantly higher than placebo in both 40 mg (49%) and 80 mg (47%) groups (placebo 34%, P < 0.01). Remission rates were statistically significantly higher than placebo in the 40 mg and 80 mg groups, at 18% *vs* 30% and 32%, respectively (P < 0.01). Significantly more patients in the levomilnacipran 40 mg/d (P = 0.032) and 80 mg/d (P < 0.001) groups than the placebo group prematurely discontinued the study due to adverse events. Adverse effects were generally considered mild to moderate, with the most frequent and statistically significant adverse effects being nausea, dry mouth, constipation, increase heart rate, dizziness, hyperhidrosis, urinary hesitation and erectile dysfunction[9].

In another fixed-dose study of levomilnacipran 40 mg, 80 mg or 120 mg *vs* placebo, the average age of participants in ranged from 40.3-41.3 years, 58.9%-68.5% were female and 72.2%-76.1% were Caucasian. The average number of depressive episodes experienced per patient ranged from 5.3-9.7 and the duration of illness ranged from 10.2-12.6 years. The mean MADRS score at baseline ranged from 35.6-36.1, indicating severe depression. Baseline demographics were similar between the treatment groups. The primary outcome for this study was change from baseline to end of study on the MADRS total score. Secondary outcomes included improvement in SDS total score, response and remission rates as defined above. This study found statistically significant differences in change of MADRS total score between all 3 levomilnacipran groups compared to placebo [40 mg: -3.23 (P = 0.0186), 80 mg: -3.99 (P = 0.0038), and 120 mg: -4.86 (P = 0.0005)]. Levomilnacipran demonstrated superiority over placebo in change in SDS total score in the 80 mg and 120 mg groups only (-2.51 and -2.57, respectively, P < 0.05). Response rates were found to be statistically significantly higher than placebo only in the 120 mg group with a rate of 41.5% compared to 29.1% with placebo (P = 0.0107). Remission rates were found to be similar between the groups. Significantly more levomilnacipran than placebo patients discontinued due to adverse events. The most common adverse effects that led to discontinuation were nausea, vomiting and palpitations[8].

In the flexible-dose study by Sambunaris *et al*[10] (with a dose range 40 mg to 120 mg/d of levomilnacipran) the average age of participants was 45 years and 65% were female. The mean MADRS score at baseline was 35 indicating that the patients had at least moderate to severe depression. Baseline demographics were similar between the treatment groups. The primary and secondary outcomes for this study were the same as the above described trials. This study found statistically significant differences in change of MADRS total score and SDS total score between the levomilnacipran group compared to placebo [-3.095 (-5.256 to -0.935); P = 0.0051 and -2.632 (-4.193 to -1.070); P = 0.0010, respectively]. Response rates were found to be statistically significantly higher than placebo with a rate of 41.9% in the levomilnacipran compared to 29.4% in the placebo group (P = 0.0083). Remission rates were found to similar between the groups. The incidence of premature discontinuation due to adverse events was higher for levomilnacipran (7.8%) than placebo (3.2%) but the difference was not statistically significant (P = 0.0567)[10].

Long-term trials: One randomized, double-blind, placebo-controlled, relapse prevention study over ≥ 24 wk was conducted in 348 patients age 18 to 65 years with MDD in 36 sites in the United States and Canada[11]. Patients were included in the study if they met the DSM-Ⅳ-TR criteria for MDD with an ongoing episode of at least 4 wk duration, the diagnosis was confirmed by the Mini International Neuropsychiatric Interview and if they had a MADRS score ≥ 22 indicating moderate severity[12,13]. Patients were required to have normal physical examination and clinical laboratory results and electrocardiogram findings. Patients were excluded if they had other comorbid DSM-Ⅳ-TR Axis Ⅰ disorder within the last 6 mo, history of nonresponse to 2 or more adequate trials with antidepressants, medical conditions that may interfere with the study, if they were pregnant or if they had significant risk of suicide. Eligible patients were treated first with 12 wk of open-label, flexible-dose levomilnacipran 40-120 mg/d. Patients who met criteria for response (MADRS score ≤ 12) and had

a Clinical Global Impressions-Improvement (CGI-I) score ≤ 2 at both weeks 10 and 12, were randomized to receive either levomilnacipran or placebo once daily for 24 wk. Relapse was defined as MADRS score ≥ 22, CGI-I increase by ≥ 2 points or insufficient clinical response as judged by the investigators. The primary efficacy endpoint was the time to relapse within the first 24 wk of the double-blind period. Time to relapse was longer in the levomilnacipran group than the placebo group, but the difference was not statistically significant [HR = 0.68 (95%CI: 0.4-1.17, P = 0.165)]. The relapse rates observed in this study (placebo = 20.5%, levomilnacipran = 13.9%) were lower than the relapse rates anticipated in the statistical analysis (placebo = 38%, levomilnacipran = 20%), which compromised the projected statistical power. This study was underpowered and therefore it can be considered a failed study rather than a negative study[11].

One open-label extension study has been published so far[14]. It was a multicenter, open-label, flexible-dose study (40-120 mg/d) in adult patients with MDD who completed 1 of the 3 short-term studies. The study was 52 wk in duration and consisted of a 48-wk open-label period followed by a down-taper period of up to 4 wk. The median duration of treatment was 280 d and the final daily dose was 40 mg/d for 27%, 80 mg/d for 26% and 120 mg/d for 47% of patients. A mean decrease in MADRS total score of -23.6 was seen from baseline to the end of the 48-wk period. Rates of withdrawal due to adverse effects or insufficient therapeutic response were 13% and 8.1%, respectively The most common that were considered severe were nausea and headache[14].

Dosage recommendations

The recommended dose range of levomilnacipran is 40 mg to 120 mg once daily. The dose should be starting at 20 mg once daily for 2 d, and then increased to 40 mg once daily. The dose can be further increased by 40 mg at intervals of every 2 or more days. The maximum recommended dose is 120 mg once daily. The dose of levomilnacipran should not exceed 80 mg once daily when use concomitantly with strong CYP3A4 inhibitors. Levomilnacipran should be swallowed whole due to the extended-release formulation and it can be taken with or without food[3].

No dose adjustment is required in patients with mild renal impairment [creatinine clearance (CrCl) of 60-89 mL/min]. The maximum recommended dose is 80 mg once daily or 40 mg once daily for patients with moderate renal impairment (CrCl of 30-59 mL/min) or patients with severe renal impairment (CrCl of 15-29 mL/min), respectively. Levomilnacipran is not recommended for patients with end stage renal disease[3].

Drug interactions

Levomilnacipran appears to have a low potential to cause any clinically relevant inhibitory or inducing effects on the CYP450 system. Dose adjustment is recommended when co-administered with strong CYP3A4 inhibitors such as ketoconazole. MAOIs should be discontinued for at least 14 d prior to starting levomilnacipran. Levomilnacipran should be discontinued for at least 7 d prior to starting MAOI. Alcohol can cause a more rapid release of drug into the blood stream and it is not recommended to take levomilnacipran with alcohol[3].

Adverse effects

The most common adverse effects (≥ 5%) seen in both short and long-term trials of levomilnacipran were headache, nausea, dizziness, constipation, dry mouth, increased heart rate, tachycardia, erectile dysfunction, urinary hesitation, insomnia, vomiting, and hyperhidrosis[7-11,14]. Most adverse reactions were considered mild or moderate in intensity and 9% of patients in the short-term placebo-controlled trials discontinued treatment due to an adverse event. The most common adverse reaction leading to discontinuation in at least 1% of patients was nausea. Only urinary hesitation and erectile dysfunction appear to be dose-related[3,6].

Levomilnacipran had a mean increase in systolic blood pressure (SBP) of 3 mmHg and diastolic blood pressure (DBP) of 3.2 mmHg in the short-term, placebo-controlled studies. Mean increase in SBP was 3.9 mmHg and DBP was 3.3 mmHg in the long-term, 48-wk extension study[3,14]. Levomilnacipran had a mean increase in heart rate of 7.4 beats per minute (bpm) compared to a mean decrease of 0.3 bpm in placebo group. Orthostatic hypotension was observed in 11.6% of patients in the levomilnacipran group compared to 9.7% in the placebo group[3]. Mean increase in corrected QT interval (QTc) by Bazett's formula (QTcB) was 9.5 ms in levomilnacipran compared to 0.1 ms in the placebo group in the short-term studies. However, changes in QTc by Fridericia's method (QTcF) were small between groups (-2.5 and -1.4 ms for levomilnacipran and placebo, respectively). No patients had QTcF interval greater than 500 ms in short-term or extension studies[6]. One patient has QTcB greater than 500 ms in the extension study[14]. Since patients with significant medical conditions such as cardiovascular disease were excluded from the clinical trials, clinicians should use caution in these patients, and should monitor blood pressure and heart rate periodically.

All clinical trials had a down-taper period range from 1 to 4 wk[7-11,14]. Patients should be monitored for symptoms of discontinuation syndrome when discontinuing levomilnacipran and reduce the dose gradually[3]. No clinically significant effects on body weight or laboratory tests were reported in the short-term studies but five patients experienced potentially clinically significant high transaminase values (≥ 3x upper limits of normal of aspartate aminotransferase and alanine aminotransferase levels) in the 48-wk extension study[6,14].

Levomilnacipran is contraindicated in patients with hypersensitivity to levomilnacipran, milnacipran, or any excipients in the formulation. Patients with uncontrolled narrow-angle glaucoma should not use levomilnacipran since it is associated with an increased risk of mydriasis. Similar to other antidepressants, levomilnacipran is contraindicated to use with MAOI, methylene blue, or linezolid concurrently due to the increased risk of serotonin syndrome. Levomilnacipran should be used with caution in patients with controlled narrow-angle glaucoma and patients with raised intraocular pressure or those at risk of acute narrow-angle glaucoma should be monitored. Patients being started on any antidepressant medication should be screened for bipolar disorder and monitored closely for clinical worsening, suicidality and behavior changes. Serotonin syndrome, abnormal bleeding, activation of mania/hypomania and hyponatremia are all class warnings of the SSRI and SNRI antidepressants that also apply to levomilnacipran[3].

Levomilnacipran is listed as pregnancy category C. No teratogenic effects have been seen in the offspring of rats and rabbits exposed to levomilnacipran at doses up to 8 to 16 times the maximum recommended human dose (MRHD). An increase in early post natal rat pup mortality was seen at a dose equivalent to 5 times the MRHD given during pregnancy and lactation[3]. Pregnancy was reported in three patients during the 48-wk open-label extension study conducted by Mago *et al*[14]. Two of the three patients prematurely discontinued from the study and all three pregnancies resulted in live births with no complications. Levomilnacipran has been detected in the milk of lactating rats, but no data is available for nursing women[3].

VORTIOXETINE

Vortioxetine was initially approved by the FDA on September 30, 2013 and became available on the United States market in early 2014[15,16]. Like levomilnacipran it is currently approved for use in MDD. Trials supporting its use in generalized anxiety disorder have also been completed; however, they are beyond the scope of this review.

Pharmacology

Vortioxetine is categorized as a "multi-modal" antidepressant because it is thought to work *via* two complementary mechanisms of action[17,18]. In addition to being an inhibitor of the 5-HT transporter [serotonin transporter (SERT)], vortioxetine also functions as a 5-HT receptor modulator, acting as a 5-HT_{3A}, 5-HT_{7} and 5-HT_{1D} antagonist, a 5-HT_{1B} partial agonist, and a 5-HT_{1A} agonist[17,19,20]. It is unclear exactly which of these activities contributes to its antidepressant efficacy though it is likely a combination of these receptor modulatory effects coupled with the SERT blockade[19,20].

Most selective SSRI antidepressants, which are so named because they are thought to work exclusively through their inhibition of SERT, require approximately 80% SERT occupancy to exert therapeutic efficacy. Vortioxetine exhibits approximately 50% SERT occupancy at 5 mg/d, 65% at 10 mg/d and 80% at 20 mg/d[20]. Given its demonstrated efficacy in clinical trials at doses as low as 5 mg/d, it can be postulated that other mechanisms are actively involved[19,21-23].

Pharmacokinetics

Vortioxetine exhibits linear and dose-dependent pharmacokinetics with a terminal half-life of approximately 57-66 h[20,24]. The absolute bioavailability has been established to be approximately 75% after a single administration of 20 mg, and its absorption does not appear to be effected by the presence or absence of food. It is a lipophilic compound with a high affinity for peripheral tissue, causing it to have a large volume of distribution. It is also highly protein bound (98%-99%)[24]. Vortioxetine is extensively metabolized *via* oxidation *via* the cytochrome P450 system, followed by glucuronidation, primarily to a metabolite that is likely pharmacologically inactive due to its inability to cross the blood brain barrier[20,24]. Vortioxetine is metabolized primarily by CYP2D6 and poor metabolizers of this enzyme have approximately twice the vortioxetine plasma concentration of extensive metabolizers[20]. Approximately 59% of vortioxetine metabolites are eliminated through the urine and 26% through the feces. Negligible amounts of unchanged vortioxetine are excreted in the urine[20]. The presence of renal impairment or mild-moderate hepatic impairment does not seem to affect the clearance of vortioxetine[20].

Clinical trials

Vortioxetine has been studied in over 9000 patients with MDD in 25 clinical trials including: 14 short-term (6-12 wk) acute MDD trials, one long-term ($\geqslant$ 24 wk) maintenance of remission study, 6 long-term (52 wk) extension studies, 3 short-term (2-8 wk) studies focused on cognition and one short-term (8 wk) trial focused on sexual dysfunction[25,26]. Seven of the 12 short-term trials with results published to date have demonstrated positive efficacy for at least one dose of vortioxetine for the acute treatment of MDD (Table 4)[25-27]. One of these trials demonstrated efficacy in the elderly and another in patients who had an inadequate response to previous antidepressant treatment[25]. The only dose to show clinical efficacy in a United States population is the 20 mg/d.

Short-term trials: The first of the short-term trials was a multinational (excluding the United States) phase Ⅱ trial conducted by Alvarez and colleagues in 429 patients with moderate to severe MDD over 6 wk[21]. Patients were randomized equally to a fixed dose of vortioxetine 5 or 10 mg, venlafaxine XR 225 mg, or placebo for 6 wk[21,25]. Patients who had failed

Table 4 Randomized controlled trials of vortioxetine for major depressive disorder[21-23,27,29,31-36]

Ref.	*n*	Duration	Key inclusion criteria	Doses	Primary outcome
Alvarez *et al*[21] Phase Ⅱ	429	6 wk	Age 18-65 yr MADRS ⩾ 30	Vortioxetine 5 mg/d Vortioxetine 10 mg/d Placebo Venlafaxine XR 225 mg/d	Positive Change from baseline in MADRS Placebo: -14.5 5 mg: -20.4 (P < 0.001) 10 mg: -20.2 (P < 0.001) Venlafaxine: -20.9 (P < 0.001)
Baldwin *et al*[29] Phase Ⅲ	776	8 wk	Age 18-75 yr MADRS ⩾ 26	Vortioxetine 2.5 mg/d Vortioxetine 5 mg/d Vortioxetine 10 mg/d Placebo Duloxetine 60 mg/d	Failed Change from baseline in MADRS Placebo: -14.8 2.5 mg: -16.2 (P = 0.219) 5 mg: -16.5 (P = 0.132) 10 mg: -16.3 (P = 0.185) Duloxetine: -16.8 (P = 0.074)
Jain *et al*[32] Phase Ⅲ	600	6 wk	Age 18-75 yr MADRS ⩾ 30	Vortioxetine 5 mg/d Placebo	Negative/failed Change from baseline in HAM-D$_{24}$ Placebo: -13.87 5 mg: -14.61 (P = 0.407)
Mahableshwarkar *et al*[33] Phase Ⅲ	611	8 wk	Age 18-75 yr MADRS ⩾ 22	Vortioxetine 2.5 mg/d Vortioxetine 5 mg/d Placebo Duloxetine 60 mg/d	Negative Change from baseline in HAM-D$_{24}$ Placebo: -10.5 2.5 mg: -12.05 (P = 0.138) 5 mg: -11.08 (P = 0.577) Duloxetine: -13.47 (P = 0.005)
Henigsberg[23] Phase Ⅲ	560	8 wk	Age 18-75 yr MADRS ⩾ 26	Vortioxetine 1 mg/d Vortioxetine 5 mg/d Vortioxetine 10 mg/d Placebo	Positive Change from baseline in HAM-D$_{24}$ Placebo: -11.3 1 mg: -14.82 (P < 0.001) 5 mg: -15.42 (P < 0.001) 10 mg: -16.23 (P < 0.001)
Boulenger *et al*[31] Phase Ⅲ	608	8 wk	Age 18-75 yr MADRS ⩾ 26	Vortioxetine 15 mg/d Vortioxetine 20 mg/d Placebo Duloxetine 60 mg/d	Positive Change from baseline in MADRS Placebo: -11.7 15 mg: -17.2 (P < 0.0001) 20 mg: -18.8 (P < 0.0001) Duloxetine: -21.2 (P < 0.0001)
NCT01153009[34] Phase Ⅲ	614	8 wk	Age 18-75 yr MADRS ⩾ 26	Vortioxetine 15 mg/d Vortioxetine 20 mg/d Placebo Duloxetine 60 mg/d	Positive Change from baseline in MADRS Placebo: -12.83 15 mg: -14.3 (NS) 20 mg: -15.57 (P < 0.05) Duloxetine: -16.9 (P < 0.001)
NCT01163266[36] Phase Ⅲ	462	8 wk	Age 18-75 yr MADRS ⩾ 26	Vortioxetine 10 mg/d Vortioxetine 20 mg/d Placebo	Positive Change from baseline in MADRS Placebo: -10.8 10 mg: -13.0 (NS) 20 mg: -14.4 (P < 0.01)
NCT01179516[35] Phase Ⅲ	469	8 wk	Age 18-75 yr MADRS ⩾ 26	Vortioxetine 10 mg/d Vortioxetine 15 mg/d Placebo	Negative/failed Change from baseline in MADRS Placebo: -12.87 10 mg: -13.66 (P = 0.597) 15 mg: -13.36 (P = 0.745)
NCT01255787[27] (unpublished) Phase Ⅲ	600	8 wk	Age 20-64 yr MADRS ⩾ 26	Vortioxetine 5 mg/d Vortioxetine 10 mg/d Vortioxetine 20 mg/d Placebo	Negative Change from baseline in MADRS Placebo: -13.99 5 mg: -14.61 (P = 0.907) 10 mg: -15.68 (P = 0.301) 20 mg: -15.82 (P = 0.240)
Boulenger *et al*[22] Phase Ⅲ	400	24-64 wk	Age 18-75 yr MADRS ⩾ 26	Vortioxetine 5-10 mg/d Placebo	Positive Time to relapse-vortioxetine *vs* placebo HR = 2.01 (95%CI: 1.26-3.21) (P = 0.0035)

NCT: National clinical trial; MADRS: Montgomery-Asberg Depression Rating Scale; HAM-D$_{24}$: Hamilton Rating Scale for Depression (24-items).

adequate trials of two or more antidepressants, who were receiving formal behavioral or psychotherapy, or who had significant suicidal thoughts were excluded[21]. Of the 426 patients who were treated in the trial, the average age was 43.3, 62.7% were women and 92% were Caucasian. The mean baseline MADRS score was 34, indicating a severe level of depression[12,13,25]. There were no statistically significant differences between treatment groups at baseline[21]. Both doses of vortioxetine were statistically significantly superior to placebo on the primary endpoint of mean change from baseline in MADRS total score at week 6. The mean difference from placebo was 5.9 points for 5 mg, 5.7 points for 10 mg, and 6.4 points for venlafaxine, translating to medium effect sizes of 0.56 for 5 mg and 0.54 for 10 mg of vortioxetine ($P < 0.001$ for each)[28]. A statistically significant difference compared to placebo was seen from week 2 onwards in the venlafaxine and vortioxetine 5 mg group and week 3 onwards in the vortioxetine 10 mg group. Response and remission rates (as defined in the description of the levomilnacipran trials above) were also statistically significantly higher for all of the active treatment arms when compared to placebo, ranging from 67%-73% response and 45%-49% remission in the vortioxetine 5 mg arm, 68%-76% response and 45%-50% remission in the vortioxetine 10 mg arm, and 72%-77% response and 46%-55% remission in the venlafaxine arm as compared to 40%-49% response rates and 26%-28% remission rates in the placebo arm depending on how response or remission was defined. The study was not powered to be able to detect statistical differences between vortioxetine and venlafaxine. Adverse effects were generally considered mild to moderate, with the most frequent and statistically significant adverse effects being nausea, hyperhidrosis and vomiting. Venlafaxine patients also experienced significantly more dry mouth, constipation and sexual dysfunction, particularly anorgasmia, than placebo-treated patients. Only the venlafaxine group had more patients withdraw from the trial due to adverse effects (14%) than placebo group (4%) ($P = 0.009$)[21].

A similar study conducted by Baldwin *et al*[29] in 776 patients with moderate to severe MDD, which was also conducted in several countries outside of the United States. Patients also had to be in a current major depressive episode (MDE) for at least 3 mo. Other inclusion and exclusion criteria were similar to the Alvarez trial. Patients were randomized to either a fixed dose of vortioxetine 2.5, 5 or 10 mg, duloxetine 60 mg or placebo for 8 wk[29]. The mean baseline MADRS score was 31.9 indicating moderate to severe MDD and the mean Hamilton Rating Scale for Anxiety (HAM-A) score was 23.0, indicating at least a mild to moderate level of anxiety[30]. The average age of trial participants was 45 years, approximately two-thirds were women, and the majority (78%) were Caucasian, with a significant minority being Asian (21%). As none of the active treatment arms separated from placebo on the primary endpoint, mean change in MADRS score at week 8, using a last observation carried forward analysis approach, this was considered a failed trial. However, when using the mixed model method of analysis with repeated measures, the vortioxetine 5 and 10 mg and duloxetine groups were statistically significant when compared to placebo on the primary and most secondary outcomes, including mean change from baseline in the Hamilton Rating Scale for Depression (24-items) (HAM-D_{24}), HAM-A, and CGI-I scores. The clinical significance of these outcomes, however, is questionable, as the difference from placebo on the MADRS scores were only 1.6, 2.5, 2.6 and 3.0 points for vortioxetine 2.5, 5, 10 mg and duloxetine, respectively. This study was also not powered to be able to detect a difference between vortioxetine and the active control, duloxetine. In terms of adverse effects of vortioxetine, only the incidence of nausea was statistically significantly greater than placebo, while patients in the duloxetine arm experienced significantly more nausea, dizziness, hyperhidrosis and decreased appetite than placebo-treated patients. The rates of withdrawal from the study were comparable between each of the treatment arms[29].

Two similar trials demonstrated efficacy for vortioxetine in a multinational population, excluding the United States. In these trials, patients were treated for 8 wk with vortioxetine 1, 5, 10 mg or placebo once daily in the trial conducted by Henigsberg *et al*[23] and vortioxetine 15 or 20 mg, duloxetine 60 mg or placebo in the study by Henigsberg *et al*[23] and Boulenger *et al*[31]. Other inclusion and exclusion criteria were similar to the above described trials. The average age of patients in these trials was approximately 47 years, the great majority were Caucasian and roughly two-thirds were female[23,31]. All doses of vortioxetine, as well as duloxetine, showed a statistically significant improvement on the primary outcome, change from baseline HAM-D_{24} in the Henigsberg trial, and change in MADRS in the Boulenger trial (Table 4). In the Henigsberg trial, these differences translated to moderate effect sizes of 0.37, 0.41 and 0.54 for vortioxetine 1, 5 and 10 mg, respectively[23]. MADRS and HAM-D_{24} scores began to separate from placebo at week 2 for most doses, and week 4 for the vortioxetine 15 mg/d arm. Statistically significant differences were seen for many of the secondary outcomes as well, with the exception of the change in the SDS, which did not show a difference from placebo with any dose of vortioxetine in the Henigsberg trial, but did show improvement in the Boulenger trial[23,31]. Such positive secondary outcomes included change in MADRS score, change in HAM-D_{24} in patients with a baseline HAM-A score $\geqslant$ 20, and response and remission rates based on both the HAM-D_{24} and the MADRS[23,31]. Response rates ranged from 46.8%-61.6% and remission rates from 20.9%-38.4% for vortioxetine as compared to 74.0% response and 54.1% remission

in the duloxetine group and 23.0%-32.3% response and 11.5%-19.0% remission for placebo. Adverse effects occurring in ⩾ 5% of subjects in any of the vortioxetine treatment groups included nausea, headache, nasopharyngitis, and dizziness. One incidence of pancreatitis with vortioxetine 10 mg/d was judged by the investigator to be possibly related to the medication[23]. No statistically significant changes in the Arizona Sexual Experiences (ASEX) scale were seen in any of the treatment arms in the Boulenger trial, even when stratified according to sex or baseline sexual function status. However, the most frequent primary reason for withdrawing from the study was adverse events and the percentage of patients in the vortioxetine 20 mg arm that discontinued due to adverse events was significantly higher than placebo [11.3% *vs* 4.4%, respectively (*P* value not reported)]. Nausea and dry mouth occurred statistically more often with duloxetine than with placebo ($P < 0.001$)[31].

In patients being switched from vortioxetine 15 or 20 mg to placebo at the end of 8 wk, only the 20 mg group experienced a statistically significantly higher score on the Discontinuation Emergent Signs and Symptoms Scale (DESS). This difference was seen in week 9 ($P = 0.0297$), but was not statistically significant at week 10 ($P = 0.1690$), indicating short-lived discontinuation symptoms. Increased dreaming or nightmares was the most reported new or worsening symptom in the vortioxetine-treated patients. Patients in the duloxetine arm were tapered down to 30 mg/d for one week and then switched to placebo. Duloxetine-treated patients also scored higher on the DESS during the taper period with patients reporting new or worsening dizziness/lightheadedness, trouble sleeping, insomnia, irritability, fatigue/tiredness, anxiety, tearfulness, headache, agitation, and mood swings[31].

The first United States short-term trial was published by Jain and colleagues and enrolled 600 patients age 18-75 years with moderate to severe MDD. Patients had to have a MADRS score ⩾ 30 at baseline and were excluded if they had significant risk of suicide or history of non-response to 2 or more antidepressants[32]. Patients were required to be in a current MDE for at least 3 mo. Patients were randomized to vortioxetine 5 mg once daily or placebo for 6 wk. Vortioxetine did not separate from placebo in the primary outcome, change in HAM-D_{24} total score at week 6 (Table 4), nor most of the secondary outcomes such as response and remission. Vortioxetine did, however, statistically significantly decrease HAM-D_{24} scores (-13.4 points) in individuals with a baseline HAM-A score > 19 (indicating moderate anxiety) ($P = 0.049$). The effect size in this subgroup was small (0.21), indicating a low level of clinical significance[28,32]. The most common adverse effects seen in the vortioxetine-treated group were nausea (19%), headache (17.1%), diarrhea (11.4%), dry mouth (8.4%) and dizziness (6.4%). The rates of nausea and diarrhea appeared much higher than with placebo (9.4% and 7.0%, respectively). There were no differences between the groups in the number of patients who withdrew from the study due to adverse effects. Additionally, no significant discontinuation effects were seen after stopping vortioxetine during the 2-wk follow up period[32].

A series of three 8-wk trials were conducted in the United States by Mahableshwarkar *et al*[33]. Each of these trials included patients aged 18-75 years with similar inclusion and exclusion criteria as the above described trials, with the exception of the cutoff requirements on the MADRS at baseline. One trial had a relatively modest cutoff of ⩾ 22 and the other two trials required a score of ⩾ 26. Two of the trials used duloxetine 60 mg/d as an active reference. The first trial included vortioxetine 2.5 mg, 5 mg, placebo or duloxetine, the other trial to include a duloxetine arm also studied vortioxetine 15 or 20 mg/d. The trial without an active reference included vortioxetine 10 or 15 mg/d. The average age of participants in these trials ranged from 42.7-45.1 years, 63.5%-73.8% were female, 74.2%-76.5% were Caucasian, and a significant minority (17.3%-22.7%) were Black. The mean MADRS score at baseline ranged from 29.8-33.7[33-35]. Baseline demographics were similar between the treatment groups, however there was a significant difference in baseline body mass index (BMI) in one trial, with patients in the vortioxetine 5 mg group having a slightly higher mean BMI (31.38 kg/m^2) than the vortioxetine 2.5 mg, duloxetine or placebo treatment arms (29.48-30.14 kg/m^2) (*P* value not reported)[25,33]. Only the 20 mg/d arm in any of the vortioxetine arms showed statistically significant improvement in the primary outcome, in this case change in MADRS from baseline (Table 4). Duloxetine, however, did show a significantly greater improvement in the primary outcome in both trials to include it, and was significant in many of the secondary outcomes in these trials as well[33,34].

Nausea, dry mouth and headache were the most frequently reported adverse effects with vortioxetine in these trials and nausea was also the most common adverse events leading to discontinuation. Sexual side effect rates, as measured by the ASEX scale, were similar to that of duloxetine in one trial (51.0% with vortioxetine 2.5 mg, 37.5% with 5 mg, 46.9% with duloxetine, as compared to 33.3% with placebo)[18,33]. In the trials that examined higher doses of vortioxetine, rates of sexual dysfunction as measured by the ASEX were generally similar to what was seen in placebo-treated patients[25,26]. No significant differences in DESS scores were seen in the vortioxetine groups as compared to placebo in the higher dose vortioxetine trial, despite abrupt discontinuation from 15 or 20 mg/d[25].

The second study to show efficacy for vortioxetine in a United States population was conducted by Jacobsen and colleagues in 462 patients randomized

to vortioxetine 10 mg, vortioxetine 20 mg or placebo once daily. Inclusion and exclusion criteria where the same as the above studies by Mahableshwarkar *et al*[33] and Boulenger *et al*[22,31]. The average age of patients in this trial was 42.8 years, 69.9% were Caucasian, 27.9% were Black, and 72.5% were female[25]. The mean MADRS score at baseline was 32.2[36]. Vortioxetine 20 mg/d separated from placebo on the primary outcome, change from baseline in MADRS total score, with a mean difference of 14.41 points (P = 0.002), while vortioxetine 10 mg/d did not, with a mean difference of 12.96 as compared to placebo, with a mean difference of 10.77 (P = 0.058)[36].

The most recently completed short-term placebo-controlled trial was a combined phase Ⅱ and Ⅲ trial conducted in 600 patients, age 20 to 64 years, in Europe and Asia. Patients in this trial were treated with vortioxetine 5, 10, or 20 mg or placebo. Average age of included participants was 44.4 years, 62.5% were female and 69% were Caucasian, with the remaining 31% being Asian. The mean MADRS score at baseline was 31.7. None of the treatment arms separated from placebo on the primary endpoint, change in MADRS (Table 4). Statistical analysis was not performed for any of the secondary outcomes. Nausea was the most commonly reported adverse effect and the rate was the highest in the 20 mg vortioxetine group (24.7%)[27].

The only short-term trial to include an active comparator was a trial of 501 patients with moderate to severe MDD who had a documented inadequate response to SSRI or SNRI monotherapy. This trial was conducted in Europe and compared flexibly-dosed vortioxetine 10-20 mg/d to flexibly dosed agomelatine 25-50 mg/d. Agomelatine works as a NE and dopamine disinhibitor and is marketed in Europe as an antidepressant. Average age of participants in this trial was 46.3 years, 74.7% were female, and almost 100% of patients were Caucasian. The mean baseline MADRS score at baseline was 28.9[25,37]. At weeks 8 and 12, vortioxetine provided significantly greater improvement in the MADRS as compared to agomelatine, with a between group difference of 2.2 points at week 8 (P = 0.018) and 2.0 points at week 12 (P = 0.0054)[25,37]. Change from baseline in HAM-A score, response and remission rates, and all other secondary outcomes were also significantly superior in vortioxetine-treated patients as compared to agomelatine-treated patients[37]. Fewer patients in the vortioxetine group discontinued due to adverse effects than the agomelatine group and the most commonly reported adverse effects with both treatments were nausea, headache, dizziness and somnolence. Numerically more patients in the agomelatine group reported headache, dizziness and somnolence and more patients in the vortioxetine group reported nausea[25].

The efficacy of vortioxetine in an elderly population (≥ 65 years) was established in an 8-wk trial conducted by Katona *et al*[38] in 453 patients randomized to vortioxetine 5 mg/d, duloxetine 60 mg/d or placebo. The mean age of patients in this trial was 71 years, approximately two-thirds were women, and 95% were Caucasian. Mean baseline MADRS score was approximately 30. Approximately 91% of patients had concurrent medical, psychiatric or neurologic disorders and the mean baseline HAM-A score was 19. Both vortioxetine and duloxetine were statistically significant when compared to placebo on the primary outcome, change in HAM-D_{24} at week 8. Vortioxetine had a 3.3 point difference from placebo (P = 0.0011) and duloxetine had a 5.5 point difference (P < 0.0001). Response and remission rates were also significant for both drugs with rates of 53.2%-61.7% response and 29.2%-33.8% remission for vortioxetine, depending on the definition, 63.3%-72.1% response and 34.7%-46.9% remission with duloxetine and 35.2%-38.0% response and 19.3%-20.7% remission with placebo[38].

Secondary exploratory outcomes focused on cognition, which were measured with the Digit Symbol Substitution Test (DSST) and the Rey Auditory Verbal Learning Test (RAVLT) were statistically significantly superior to placebo for vortioxetine. Duloxetine had statistically significant improvement on the RAVLT but not the DSST. Effect sizes on these outcomes were generally small for vortioxetine (0.24-0.27) which calls into question the clinical significance of these findings[38]. The only adverse effect that occurred significantly more frequently in the vortioxetine group than placebo was nausea. Significantly more patients in the duloxetine group experienced nausea, fatigue, constipation, dry mouth, hyperhidrosis and somnolence[38] .

The final short-term trial in adult MDD patients examined sexual functioning in adults experiencing SSRI-related sexual dysfunction[39]. Preliminary results have recently been presented at a meeting of the American Society of Clinical Pharmacology and are currently available from the manufacturer's website[40]. In this trial, 447 patients with recent major depressive episodes that were currently being treated with an SSRI were discontinued from their current treatment and randomized to vortioxetine titrated to 20 mg once daily or escitalopram 20 mg once daily for 8 wk. Patients treated with vortioxetine experienced statistically significant improvement in sexual functioning as measured by the changes in sexual functioning questionnaire short-form-14 (P = 0.013).

Long-term trials: The only long-term trial to employ a double-blind, randomized, placebo-controlled design was a 24-64 wk relapse prevention study conducted in 404 patients, age 18-75 years with MDD in 17 countries in Europe, Asia and Africa. Inclusion and exclusion criteria were similar to the short-term phase Ⅱ trial conducted by Alvarez *et al*[21]. Eligible patients were treated first with 12 wk of open-label, flexible-

dose vortioxetine 5-10 mg/d. The study design was nearly identical to the relapse prevention study conducted for levomilnacipran. Patients who achieved remission (MADRS score ⩽ 10) at both weeks 10 and 12, were randomized to receive vortioxetine or placebo once daily for 24-64 wk. Patients were withdrawn from the study if at any time they suffered a relapse (MADRS score ⩾ 22) or an insufficient clinical response as judged by the investigator. The primary efficacy endpoint was the time to relapse within the first 24 wk of the double-blind period. There were no significant differences between the treatment groups at time of randomization. The mean MADRS score at the time of randomization was 4.8% and 65% of the vortioxetine-treated patients were on 10 mg/d, with the remaining 35% on 5 mg/d. Patients treated with vortioxetine were statistically significantly less likely to relapse with a hazard ratio of 2.01 (95%CI: 1.26-3.21, P = 0.035)[22].

The proportion of patients that relapsed on placebo was also statistically significantly higher in the placebo group (26%) than in the vortioxetine group (13%) (P = 0.013)[22]. Interestingly, there was a lower risk of relapse for Caucasian patients than for Asian patients, however the number of Asian patients enrolled in the study was small (17.7% of the placebo group and 15.7% of the vortioxetine group)[22]. Nausea and headache were the most common adverse effects in the open-label period (25.7% and 18.3% respectively). The only adverse effect that was statistically significantly higher in the vortioxetine group during the double-blind phase was nausea (8.8% *vs* 3.1% with placebo)[22].

Out of the six open-label extension trials that have been conducted for vortioxetine for the treatment of MDD, five of them currently have results available. Doses in these trials ranged from 2.5-20 mg/d. Three of these trials were performed outside of the United States, one exclusively in the United States and one in multiple countries including the United States. Depression scale scores continued to improve in all of the open-label extension studies, as did rates of response and remission in trials that measured this information. The most common adverse effects seen in the long-term trials were nausea and headache. Other commonly reported adverse effects reported in one or more of the trials included dizziness, nasopharyngitis and weight increase. Rates of withdrawal due to adverse effects were generally low and ranged from 5.9%-10.9%[25,41-43].

Dosage recommendations

Vortioxetine is available in dosage strengths of 5, 10, 15 and 20 mg immediate release tablets. The recommended starting dose of vortioxetine is 10 mg once daily with or without food. It is recommended that this be titrated to 20 mg once daily, as tolerated. No specific titration recommendations are outlined in the package insert, however, most clinical trials increased to 15 or 20 mg/d after one week at a dose of 10 mg/d. Patients who do not tolerate 10-20 mg/d can be decreased down to 5 mg/d[20].

The maximum recommended dose is 10 mg/d in patients who are known CYP2D6 poor metabolizers or who are on a strong CYP2D6 inhibitor (such as bupropion, paroxetine or fluoxetine). Patients who are taking concomitant CYP strong inducers (such as carbamazepine, phenytoin or phenobarbital) for more than 14 d may need a dose increase to maintain adequate plasma levels; however, it is not recommended to go above three times the original dose[20]. No dose adjustment is recommended based on age, race, gender, ethnicity or renal function. Patients with mild-end stage renal impairment or mild-moderate hepatic impairment can be administered vortioxetine safely without dose adjustment. Vortioxetine is not recommended in patients with severe hepatic impairment since it has not been studied in this population[20].

Drug interactions

Vortioxetine does not appear to have any clinically relevant inhibitory or inducing effects on the CYP450 system[24]. It is a substrate of several of the CYP450 enzymes and dose adjustments are recommended when used concomitantly with strong CYP2D6 inhibitors or strong CYP450 inducers. Because vortioxetine is highly protein bound, it is possible that coadministration with other highly protein bound drugs may increase the free concentrations of the other drug. Yet, in a study with coadministration of warfarin, a highly protein-bound drug, no significant change in international normalized ratio was seen[20].

Adverse effects

The most common adverse effects seen in both short and long-term trials of vortioxetine were nausea, constipation and vomiting. Nausea appears to be dose-related, with almost one-third of patients on 15 or 20 mg/d experiencing nausea. It also seems to be transient for most patients, with an average duration of 2 wk, however, as many as 10% of patients continued to experience nausea at the end of the 6- to 8-wk trials[20].

Spontaneously reported sexual adverse effects were low in the clinical trials for vortioxetine. In order to more accurately capture a side effect that is generally underreported, the ASEX was used in several of the clinical trials for vortioxetine. For patients without sexual dysfunction at baseline, rates of treatment emergent sexual dysfunction according to the ASEX were somewhat higher than placebo, with rates of 22%-34% in females and 16%-29% in males as compared to 20% and 14% with placebo, respectively. As with nausea, this adverse effect appears to be dose-dependent, with the highest rates occurring at 20 mg/d[20].

Most of the clinical trials employed a rapid dis-

continuation upon cessation of vortioxetine. Rates of discontinuation symptoms were low overall, with some patients reporting headache and muscle tension[20]. One of two trials that measured such symptoms *via* the DESS showed a statistically significant difference[25,31]. It is recommended that patients taking 15 or 20 mg/d of vortioxetine be tapered down to 10 mg/d for one week before completely discontinuing the medication[20].

Contraindications and warnings related to vortioxetine include hypersensitivity to vortioxetine or any excipients in the formulation as well as concomitant use of MAOIs, linezolid or methylene blue. Vortioxetine should be discontinued at least 21 d before starting one of these medications and usage of vortioxetine within 14 d of use of an MAOI is also contraindicated. Patients being started on any antidepressant medication should be screened for bipolar disorder and monitored closely for clinical worsening, suicidality and behavior changes. Serotonin syndrome, abnormal bleeding, activation of mania/hypomania and hyponatremia are all class warnings of the SSRI and SNRI antidepressants that also apply to vortioxetine. No abnormal laboratory (except sodium), weight or vital sign changes have been noted in clinical trials with vortioxetine. According to the product information, a clinical study has demonstrated that a single dose of vortioxetine 20 or 40 mg does not appear to increase the impairment of mental or motor skills due to alcohol consumption[20].

Vortioxetine is listed as pregnancy category C. Developmental delays, but no teratogenic effects have been seen in the offspring of rats and rabbits exposed to vortioxetine. The medication has been seen to enter the milk of lactating rats, but no data is available for nursing or pregnant women[20].

CONCLUSION

Both levomilnacipran and vortioxetine are new antidepressants that were approved by the FDA in 2013 for the treatment of MDD in adults. Levomilnacipran is a SNRI that was effective in several short term studies and sustained efficacy and tolerability was demonstrated in 1 long-term (48 wk) extension study. The most commonly observed adverse effects are headache, nausea, dizziness, constipation, dry mouth, increased heart rate, tachycardia, erectile dysfunction, urinary hesitation, insomnia, vomiting, and hyperhidrosis. Levomilnacipran seems to have a weight-neutral profile that was demonstrated in both short and long-term studies. Although most of the adverse effects are considered mild to moderate, this may limit its clinical utility, especially in patients with baseline cardiovascular disease. Additional long-term studies and studies comparing levomilnacipran to other antidepressants are needed to establish its place in therapy.

Vortioxetine was effective in the treatment of MDD in both short-term (6-8 wk) trials as well as in the prevention of relapse in a 24-36 wk trial. Sustained efficacy and tolerability was demonstrated in several long-term open-label trials. Its efficacy was also established in a trial of patients who had unsatisfactory response to an SSRI or SNRI. Some unique benefits of vortioxetine may include its ability to improve depressive symptoms in patients with high levels of baseline anxiety, its low-moderate risk for causing sexual dysfunction and its relatively low risk for sedation, weight gain, and discontinuation symptoms upon rapid withdrawal. Vortioxetine's drug interaction potential, high rates of nausea and cost may limit its clinical utility. Currently the only dose of vortioxetine that has been proven to be effective in a United States population is 20 mg/d, which is also the dose associated with the most adverse effects.

Several generic antidepressants are currently available at a modest cost. Compared to these medications, these brand-name products may be cost prohibitive. Further studies comparing levomilnacipran and vortioxetine to other currently available antidepressants may be needed to establish its place in therapy.

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REVIEW

Antimicrobial resistance in clinically important biofilms

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Abstract

A biofilm contains a consortium of cohesive bacterial cells forming a complex structure that is a sedentary, but dynamic, community. Biofilms adhere on biotic and abiotic surfaces, including the surfaces of practically all medical devices. Biofilms are reported to be responsible for approximately 60% of nosocomial infections due to implanted medical devices, such as intravenous catheters, and they also cause other foreign-body infections and chronic infections. The presence of biofilm on a medical device may result in the infection of surrounding tissues and failure of the device, necessitating the removal and replacement of the device. Bacteria from biofilms formed on medical devices may be released and disperse, with the potential for the formation of new biofilms in other locations and the development of a systemic infection. Regardless of their location, bacteria in biofilms are tolerant of the activities of the immune system, antimicrobial agents, and antiseptics. Concentrations of antimicrobial agents sufficient to eradicate planktonic cells have no effect on the same microorganism in a biofilm. Depending on the microbial consortium or component of the biofilm that is involved, various combinations of factors have been suggested to explain the recalcitrant nature of biofilms toward killing by antibiotics. In this mini-review, some of the factors contributing to antimicrobial resistance in biofilms are discussed.

Key words: Biofilm; Bacteria; Antimicrobial agent; Medical devices; Nosocomial infections; Resistance

Core tip: Biofilm formation on host tissues and medically implanted devices is a major health problem, and the infections caused by bacteria in biofilms are hard to treat with antimicrobial agents. They are the cause of frequent and recurrent infections after the termination of antimicrobial treatments. The reasons for the recalcitrant nature of biofilms to antimicrobial treatment are varied and have been attributed to different factors, including impermeability of biofilms, slow rates of growth and metabolic activity, and the presence of small colonies and persisters. They have been the subject of many investigations that will be discussed in this minireview.

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INTRODUCTION

Over the past three decades, it has become increasingly clear that microbial biofilms represent the norm and not the exception for microbial life. Most microorganisms reside under diverse environmental stresses, with less than optimal levels of such essentials as nutrients and oxygen, and under a constant threat of physical removal. To combat such a hostile environment, microorganisms form surface-associated communities, embedded in a self-produced extracellular matrix. This is evident in the clinical setting, where the biofilm survival lifestyle affords resistance to high concentrations of antimicrobials and to the host defense system[1,2]. It has been estimated that 60% to 80% of infections in the developed world involve biofilms[1,3]. By definition, biofilms are microbially derived sessile communities characterized by cells that are irreversibly attached to a substratum or interface or each other, are embedded in a matrix of extracellular polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription[4]. The bacterial population in biofilms may be comprised of one or more bacterial species that have formed a cohesive matrix, consisting of live bacteria embedded in polysaccharides, proteins, and extracellular DNA, all of which are bacterial byproducts, and may also include fungi and host-related materials[5]. This matrix, which is produced over time, is important for the structural stability of biofilms and the protection of living microorganisms in the biofilm from antimicrobial agents and the immune system[6]. Clinically important microbial biofilm growth may occur on various host tissues and medically-implanted foreign bodies, resulting in a variety of infections[7-9]. Bacteria in the biofilm matrix have a survival advantage, including protection from the host' s immune defense system of antibodies and phagocytic leukocytes, host-derived inhibitory substances, and antimicrobial agents that encompass antibiotics, disinfectants, and germicides[4,10]. Consequently, these biofilm infections tend to be chronic or recurring, even when formed by opportunistic bacterial pathogens[11]. For further reading, we suggest these excellent reviews[12-14].

MEDICALLY IMPORTANT BIOFILMS

Biofilm formation occurs on a variety of surfaces and can be either indwelling medical device-associated[15] or formed on native host tissues[16]. In most cases, formation of a biofilm on a medical device results in failure of the device, requiring removal of the device and/or debridement, which leads to significant morbidity and economic loss[17-19]. These devices include intravenous catheters, biliary and urinary stents, prosthetic heart valves, joint prostheses, peritoneal dialysis catheters, cardiac pacemakers, cerebrospinal fluid shunts, endotracheal tubes, breast implants and urethral catheters[20-24]. It has been estimated that 1.8 billion dollars per year are spent on treating orthopedic implant-related infections in the United States alone[25,26]. Most, if not all, implanted medical devices are susceptible to biofilm formation, as devices are coated by host matrix proteins, such as fibronectin and collagen, which serve as sites for adherence by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)[17,27-31].

In addition to being the cause of local infection, a biofilm on an implanted device may shed bacteria that produce abscesses in other locations or cause systemic infections[32]. The presence of a biofilm may result in blockage of an indwelling medical device, resulting in complication of treatment. Implanted tube devices may be clogged by biofilms, which necessitate their removal[24,33]. In addition to nosocomial and other infections associated with insertion of tubes, catheters and other devices, biofilms also are involved in causing atherosclerosis, sinusitis, otitis media, chronic wound infections, endocarditis, bronchopneumonia, urinary tract infection, cystic fibrosis, osteomyelitis, colitis, dental plaque and gingivitis[6].

Although a variety of microorganisms are involved in biofilm formation, the bacteria most frequently associated with biofilms are *Staphylococcus epidermidis* (*S. epidermidis*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), and *Enterobacteria*, such as *Escherichia coli* (*E. coli*)[24]. Other more frequently isolated bacteria, depending on the site of infection, are *Haemophilus influenzae*, *Burkholderia cepacia* (*B. cepacia*), *Enterococcus* spp., *Klebsiella* spp., *Proteus* spp., and *Helicobacter pylori*[24,34,35]. Other bacterial genera, including *Bacteroides*, *Clostridium*, *Finegoldia* and *Fusobacterium*, have been shown to form biofilms *in vitro*, and some have been isolated from clinical biofilms[6,36]. Regardless of their location and bacterial makeup, the bacterial populations in biofilms coexist and form a cohesive matrix, which allows them to survive and be protected from the detrimental effects of antibiotics and the immune system[37].

BACTERIAL BIOFILM DEVELOPMENT

The formation of a biofilm occurs in three sequential phases, each involving specific factors - irreversible attachment to the surface, growth and production of an extracellular matrix leading to a mature biofilm, and finally, detachment or dispersal[38]. Abiotic biofilm formation starts with planktonic bacterial cells that attach to the foreign body surface by reversible, specific or nonspecific adhesion[39]. The initial attachment is primarily governed by physicochemical forces, such as hydrophobicity and electrostatic forces, between the surface of attachment and the attaching microorganism[20,21,36]. In addition, bacterial appendages, such as flagella, pili, have been shown to be associated with attachment in the case of *P. aeruginosa*[40,41].

More than likely, the production of different bacterial cell wall-associated proteins that adhere to many of the host matrix proteins, either on tissue surfaces or on the surfaces of medically-implanted devices, plays a more important role in attachment[42]. Curli fibers which are proteinaceous extracellular compounds produced by many *Enterobacteriaceae* and belong to class of fiber called amyloids have been shown to be involved in the bacterial attachment and biofilm formation[43]. In *S. aureus*, the MSCRAMMs have been identified not only as important components of staphylococcal infection but also as adhesins for attachment to host tissues and/or foreign bodies covered with host matrix proteins[44,45]. Human plasma has been shown to enhance the expression of genes encoding these proteins in *S. aureus*[46,47]. These proteins include fibronectin-binding proteins[48,49], fibrinogen-binding proteins[50-52], extracellular matrix binding protein[53], protein A[54], and accumulation-associated protein[55-57].

Once attached, bacteria proliferate and produce an extracellular polymeric substance (EPS) matrix consisting, in the staphylococci, of polysaccharides[58], proteins[59], and extracellular DNA[60]. This EPS, also known as glycocalyx or slime[7,61], surrounds the cells and functions as an intercellular adhesin that leads to the formation of a microcolony, which is irreversibly bound to the surface. In the staphylococci, polysaccharide intercellular adhesin (PIA) or poly-N-acetyl glucosamine (PNAG) is usually the main component of the EPS[62-64], but recent studies indicate that the PIA/PNAG may be less important in methicillin-resistant *S. aureus* than in *S. epidermidis* and methicillin-susceptible *S. aureus*[65-67]. Other microbial components have recently been determined to be involved in biofilm maturation. These include extracellular DNA, which is hypothesized to be released from either small vesicles secreted from the outer membranes[68,69] or released by prophage-mediated cell death[70], in *P. aeruginosa*, or programmed cell death, in *S. aureus*[60,71]. However, the exact role is not known, as this has only been demonstrated under *in vitro* conditions. Other staphylococcal polymers that have been implicated in biofilm formation are the cell wall-associated teichoic acids[72,73]. In the Gram-negative bacterium *P. aeruginosa*, three different polysaccharides are produced[74-77].These are the glucose-rich Pel polysaccharide[78], the mannose-rich PSl polysaccharide[78], and alginate[79-81]. Alginate is a key component in the mucoid phenotype of *P. aeruginosa* and a contributing factor to chronic cystic fibrosis pathology[79,82]. Over time, as bacteria are surrounded by a much larger amount of EPS matrix, the biofilm continues to grow in thickness, and mushroom-like or column-like structures up to 10-100 μm thick have been observed *in vitro*[75]. Detailed analyses of mature biofilms show a heterogeneous structure, in which bacterial biomass exists in a polymeric matrix surrounded with water-filled spaces, thought to be essential for providing nutrients to the deeper layer of sessile bacteria but constituting only 15% of the total volume of the biofilm community[8,83]. It is at this stage of biofilm maturation that the cells are recalcitrant to antimicrobial agents[6].

Bacteria from a biofilm may be released and dispersed, which results in the spread of bacteria and the potential for the formation of new biofilms in other locations. Detached bacteria may produce other types of infections[84]. Dispersion could be either by the release of individual cells or aggregates of cells into the fluid or surrounding substances or by surface dispersal and movement of biofilm structures across a surface as in the case of motile bacteria[7,85]. The biofilm matrix formed in the laboratory may differ from those formed in the body, in which adherent bacteria in biofilms may be associated with molecules of host origin or with fungi[7].

Development of a biofilm may also be influenced by cell-to-cell communication and quorum sensing (QS), in which certain genes are activated when the concentration of bacteria in a given space in the environment reaches a critical level[86-89]. In the staphylococci, the primary QS system that has been most studied, with respect to biofilms, is the accessory gene regulator (Agr) system[90]. The Agr QS system is comprised of two divergent transcripts, one containing a four-gene operon (*agrA*, *agrB*, *agrC*, and *agrD*) that functions as a sensor/response regulator sensing a secreted self-made autoinducing peptide (the product of the *AgrD* gene). The autoinducing peptide is then sensed by the AgrAC two-component, trans-membrane transduction system that, in turn, generates the effector molecule, RNAⅢ. This effector molecule then regulates the expression of numerous cell wall-associated and secreted proteins in a temporal fashion[91]. In biofilms, activation of the Agr system negatively affects biofilm formation, as the Agr system downregulates many of the MSCRAMMs shown to be involved with adhesion and activates proteases, which are most likely responsible for biofilm maturation by degrading many of the proteins known to be involved with adhesion in staphylococcal strains independent of PIA for the formation of biofilms[92-94]. Also, in the staphylococci, the DNA-binding protein SarA is an important regulator of not only virulence gene expression but also biofilm formation[92,95-97]. SarA does so by Agr-dependent and independent mechanisms that contribute collectively to switching between planktonic and sessile lifestyles[92,95-97].

In Gram-negative bacteria, the QS molecules are N-acyl-L-homoserine lactones[86,98]. For example, in *P. aeruginosa*, there are three QS systems. The Pqs system senses a specific quinolone, referred to as the *Pseudomonas* quinolone signal, the Las system senses 3-oxododecanoyl-L-homoserine lactone, and the Rhl system senses N-butanoyl-L-homoserine lactone[99-101]. The activation of these systems may result in the production of extracellular polysaccharides,and a variety of enzymes, including virulence factors[99,100,102-104]. Collectively, these QS systems regulate the expression

of various genes in a coordinated fashion[105]. Natural and synthetic QS inhibitors are being considered for the treatment of infections caused by bacteria in biofilms[87-89,106].

In *P. aeruginosa*, the cyclic AMP signaling, in addition to regulating other genes, controls biofilm formation, alters cell surface hydrophobicity and signals irreversible attachment[107]. *In vitro* experiments with *E. coli* have shown crosstalk between QS and hormones from the host. Yang *et al*[108] showed that in *E. coli* the *qseC* gene regulates the histidine kinase gene, which senses epinephrine/norepinephrine hormone and stimulates biofilm formation, and found that the addition of these hormones increases the thickness of biofilms. The biofilm thickness and ureolytic activity of *Proteus mirabilis* were shown to increase with the QS signal molecule N-butanoyl-L-homoserine lactone[109].

Other factors that have been shown to be involved with biofilm maturation and dispersal include surfactants. For example, a surfactin is found in *Bacillus subtilis*[110,111] and, in *P. aeruginosa,* a rhamnolipid has been shown to contribute to biofilm maturation[112,113]. In the staphylococci, a group of amphipathic, alpha-helical peptides with surfactant activity, known as the phenol-soluble modulins, have been shown to be involved in biofilm maturation[114,115].

CHARACTERISTICS OF BACTERIA IN BIOFILMS

Biofilm-grown bacteria have different properties from those of free-living bacteria, which affects the diagnosis and treatment of infections caused by biofilms. The gene expression profile in a staphylococcal biofilm is considerably different from the profile of a planktonic culture[96,116,117]. Genes for arginine deaminase and urease are upregulated, probably to maintain a pH homeostatic environment, due to anaerobic growth that results in the formation of acidic by-products. In addition, Scherr *et al*[118] recently reported a significant reduction in gene expression when an *S. aureus* biofilm was exposed to macrophages, but very little change in the biofilm transcriptome when exposed to neutrophils; these are two important cellular components of the innate immune response. An increase in pyrimidine nucleotide biosynthesis is also involved in staphylococcal, *E. coli* and *Streptococcus pneumoniae* biofilm formation[119,120].

Biofilms are formed by heterogeneous bacterial aggregates surrounded by a self-produced matrix, which also may contain host constituents[6]. The physiological heterogeneity of bacteria in biofilms enables the subpopulation of bacteria with specialized activities to tolerate the hostile environment and survive[13,121]. Study of *in vitro* biofilms has shown that the concentration of oxygen may be higher at the surface of a biofilm and lower in the center[122,123]. In *P. aeruginosa*, the oxygen is depleted in mucoid macrocolonies, and in cystic fibrosis, this disease-causing bacterium functions in an anaerobic environment[123,124]. Similarly, there are differences in the concentrations of nutrients and chemicals at the surface and in the center of microcolonies[125,126]. As a result, growth, metabolic activities and protein synthesis are reduced at the center of a biofilm and are higher at the surface[125,126]. There is population diversity within the biofilms; some cells have slow or no growth and other cells are in stationary phase[123,126-129]. There may be also phenotypic variants of regular cells, called persisters, which have reduced cellular activity, are non-growing or dormant, and are tolerant to antimicrobial agents[130-134]. This state of cell growth, which reduces its susceptibility to antimicrobial agents and contributes to relapsing and chronic infections[135], will be discussed later. Also, there is a higher rate of mutation in growing biofilm bacteria, in comparison with free-living bacteria, including mutations in the DNA repair genes[121,136]. Low nutrients, including the lack of some amino acids, and stress responses result in tolerance of *E. coli* biofilms to ofloxacin[121]. The dense population of cells in a biofilm facilitates plasmid transfer, and the frequency of gene transfer is increased in the bacterial population in a biofilm[137]. *In vitro* study of *P. aeruginosa* has shown genetic differences between the planktonic and surface-attached bacteria, which results in differences in biochemical and phenotypic properties, and specific mechanisms to avoid the bactericidal action of antibiotics[138].

FACTORS AFFECTING ANTIMICROBIAL RESISTANCE IN BACTERIAL BIOFILMS

The colonization of natural surfaces or medically implanted devices with biofilm-forming bacteria results in the infection of local and surrounding tissues, which if not treated, may result in systemic infection, require prolonged treatment with antimicrobial agents, and may require the removal of infected devices[6,7,12,13]. The bacteria forming a biofilm could be 10-1000 times more resistant to antimicrobial agents than planktonic cells, even when they are formed by *E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae*, *S. epidermidis*, *S. aureus*, and *Enterococcus faecalis*, which are commensal bacteria[6,139]. Various factors contribute to the recalcitrant nature of these bacteria. Vancomycin used for the treatment of methicillin-resistant *S. aureus* was shown to be effective only against planktonic cells, not biofilm cells[135,140].

The mechanisms of resistance to antimicrobials, such as antibiotic-modifying enzymes and efflux pumps, that have long been established for planktonic bacteria, are only marginally involved in resistance of bacteria in a biofilm[141,142]. The role of exopolysaccharides in restricting antimicrobial penetration, which was originally thought to be the key to biofilm resistance, remains uncertain. Additional factors contributing to antimicrobial resistance in biofilms include specific

genetic determinants, such as the *ndvB* gene, which codes for a glycosyltransferase required for the synthesis of a cyclic-β-(1,3)-glucan, which is found in the periplasmic space of *P. aeruginosa* and is thought to be involved with antibiotic sequestration[143]. Given the dynamics of a biofilm lifestyle with cells at various stages of growth, it is not surprising that antimicrobial resistance of a biofilm is multifactorial.

PENETRATION RESTRICTION

Insufficient exposure of bacteria to antimicrobial agents, because of the limitations of transport of antimicrobial agents to the bacteria in a biofilm, contributes to the lack of antimicrobial effectiveness in eradicating bacteria in the biofilm[144]. The exopolysaccharides, proteins and extracellular materials that form the biofilm matrix may prevent the penetration of antimicrobial agents to various layers of the biofilm where the bacteria are vulnerable[144]. The exopolymer matrix of a biofilm may also restrict penetration of antimicrobial agents by binding to the compounds and preventing their diffusion[6]. Reversible or irreversible binding of antimicrobial agents to biofilm components retards antibiotic transport to cells within the biofilm[145]. Gentamicin was shown to penetrate *E. coli* biofilms but not the biofilm with *P. aeruginosa*, which has negatively charged polysaccharides that can bind to gentamicin[146]. Bacterial species and the age of the biofilm affect the retardation of antimicrobial agents[146]. Also, exopolysaccharides and other components of the matrix may present a barrier preventing certain compounds from entering the biofilm and not others[147].

The charge of the matrix also affects diffusion of some antimicrobial agents, and this diffusion barrier is specifically effective against large molecules[6,148]. Aminoglycosides are positively charged and may bind to the negatively charged exopolysaccharide matrix of a biofilm, restricting access to the live cells in the biofilm[149]. Tseng *et al*[150] showed that in *P. aeruginosa* the penetration of tobramycin is limited by the presence of an extracellular matrix.

Some antimicrobial agents, including fluoroquinolones, readily equilibrate across a biofilm and have been shown to be effective. A *P. aeruginosa* biofilm was shown to prevent the penetration and diffusion of piperacillin[151]. However, a biofilm formed by *S. epidermidis* did not prevent the diffusion of vancomycin and rifampicin across the biofilm, indicating that biofilm resistance to antimicrobial agents is not solely because of lack of penetration[152].

Restricted diffusion also may result in the enzymatic degradation of some antimicrobial agents by enzymes produced by the bacteria in the biofilm. The combination of retarded diffusion and enzymatic degradation of antibiotics has an additive effect in rendering antimicrobial agents ineffective for the treatment of bacteria in a biofilm[131,153]. It appears from these reports that whether or not an EPS can serve as a suitable permeability barrier to antibiotics and disinfectants[145,154] depends upon the antimicrobial agent in question and certainly also on the chemical composition of the EPS that surrounds the microorganisms.

INACTIVATION OF THE ANTIMICROBIAL AGENTS

Inactivation of antimicrobial agents by extracellular enzymes also has been suggested to be a contributing factor in the inefficiency of antimicrobial agents in eradicating the cells in biofilms. Extracellular enzymes, like β-lactamases, have been suggested to be involved in the ineffectiveness of β-lactam antibiotics[153]. However, the extent of their involvement in rendering antibiotics ineffective for clinically important biofilms is not known. Vrany *et al*[155] showed that ciprofloxacin and levofloxacin are transported into the *P. aeruginosa* biofilm. Anderl *et al*[147], using ampicillin and ciprofloxacin in biofilms formed by a strain of *Klebsiella pneumoniae* that produced β-lactamase and its mutant lacking β-lactamase, showed that resistance to these antibiotics is not the result of slow diffusion or antibiotic inactivation, and suggested other mechanisms for the resistance of biofilms. In clinical isolates, recurrent infections by *P. aeruginosa* result from persisters, which develop tolerance to a host of antibiotics under a variety of growth conditions and will be discussed further below[156].

GROWTH RATE AND PRESENCE OF PERSISTERS

All antimicrobial agents are more effective in killing rapidly growing cells; decreased growth rates reduce the efficacy of antimicrobial agent killing[131,132]. Several subpopulations of *P. aeruginosa* found in biofilms have different susceptibilities to antimicrobial agents. During treatment, antimicrobial agents preferentially kill the metabolically active cells in biofilms. However, the less active dormant cells that are covered with various substances within the biofilm layers are protected[148]. Considering the heterogeneous nature of microbial subpopulations in a biofilm, multiple resistance mechanisms may be involved in the protection of the different subpopulations[157]. Treatment with a single antimicrobial agent, while bactericidal for part of the population, is not enough to eradicate the infection. In addition to slow growth, induction of stress response genes also could contribute to resistance of bacteria in biofilms[132]. The physicochemical structure of the biofilm components also may eliminate the biocide from the microbial community. Gilbert *et al*[158] showed that sensitivities of *P. aeruginosa*, *E. coli* and *S. epidermidis* in planktonic cultures to tobramycin and ciprofloxacin increased with increasing rates of growth, but the

slow rate of growth of cells in a biofilm protected the cells from antimicrobial action. However, although both planktonic and biofilm cells of *P. aeruginosa* are resistant to ciprofloxacin at slower growth rates, only the planktonic cells of *P. aeruginosa* become more susceptible to ciprofloxacin when the growth rate increases[159]. Other factors in the biofilm, in addition to the slower rate of growth, must contribute to antimicrobial resistance. Desai *et al*[160] found that *B. cepacia* cells in biofilms were 15 times more resistant to antibiotics than planktonic bacteria, and that the growth phase and mode of growth affect the susceptibility of *B. cepacia* to antimicrobial agents. Factors affecting resistance may differ for different antibiotics[161]. Low nutrients, including lack of some amino acids, and stress responses result in tolerance of biofilms to ofloxacin[121].

The majority of cells in a biofilm are killed within the clinically achievable concentration range of antimicrobial agents; however, after the initial 3-4 log drop in the bacterial number, further addition of antimicrobial agents has no effect on bacterial killing[131]. This indicates that a small fraction of the cells persisting in the biofilm are the source of antimicrobial resistance and account for the failure of antimicrobial agents to eradicate bacteria in the biofilm[135].

It is now thought that the main contributor to increased antimicrobial resistance of biofilms is a subset of cells known as persisters[131,162]. By definition, persisters are "small subpopulations of bacteria that survive lethal concentrations of antibiotics without any specific resistance mechanisms"[162]. These bacteria represent a small percentage (0.1%-10%) of the entire population and appear to be the product of a non-heritable phenotypic switch rather than a result of antimicrobial pressure[130,163].

Biofilm exopolymers shield bacteria from the assault of immune factors. During therapy with antimicrobial agents, most of the planktonic cells shed from a biofilm are eliminated and only a small fraction of persisters remains after the symptoms disappear and treatment is discontinued[131,132]. The persisters eventually start shedding new planktonic cells, resulting in the relapse of symptoms. This dynamic cycle of decrease in planktonic bacteria and bacterial shedding from existing biofilms explains the need for lengthy antibiotic therapy and continuous recurrent infection following termination of the use of antimicrobial agents[131]. Although the persisters are not necessarily resistant to antimicrobial agents, being shielded from the effect of the immune system enables them to survive and initiate recurring infections. The persisters cause recurrent meningitis by *S. pneumoniae* and *Helicobacter pylori,* both of which are shielded from the immune system, and cause relapse of infections following therapy[132]. Persisters in a biofilm can be assayed using antimicrobial agents. If the biofilm restricts penetration of antimicrobial agents across the exopolymers, the bulk of bacteria in the biofilm should survive drugs like aminoglycosides, which have restricted penetration and diffusion, and be killed by fluoroquinolones, which can diffuse and penetrate into a biofilm[131].

Both *in vitro* and *in vivo* studies of Gram-positive and Gram-negative bacteria support the theory of persisters in biofilms[37,164-166]. The difficulties in the eradication of persisters in biofilms have been shown for different bacteria and antimicrobial agents[133,135,167,168]. After exposure to a high concentration of ciprofloxacin, a small number of *E. coli* cells insensitive to this drug remain in a biofilm[131,169]. Similarly, the effects of amoxicillin and clindamycin on *Lactobacillus acidophilus*, and those of erythromycin and metronidazole on *Gardnerella vaginalis,* reach a plateau in a biofilm after initial bacterial killing[170].

From cystic fibrosis patients, a high-persister mutant of *P. aeruginosa* has been isolated. Its presence has been speculated to be the main reason for the recalcitrant nature of this disease to antimicrobial therapy[132]. In *E. coli*, the stress response results in the formation of persisters. The stress response activates the overexpression of TisB, which is a membrane-acting dipeptide, and decreases the ATP level and proton motive force in the cell, leading to cell dormancy and persister formation[132]. Persisters also occur in planktonic cells; most of what is known of persisters has been done with planktonic cells[132]. Whether biofilm bacteria produce more persisters than planktonic cells is not known; however, planktonic cells are subject to elimination by the immune system[6,132].

Clearly, the generation of persisters is multifactorial and most likely involves environmental cues and the expression of genes in response to those cues. It is beyond the scope of this review to discuss all of these factors. For an in-depth discussion, the reader is encouraged to peruse the recent review by Lebeaux *et al*[14]. Many factors have been shown to be involved in the generation of persisters, such as nutrient limitations and the induction of the stress response and stringent responses. However, what is known of persisters from planktonic growth conditions does not necessarily explain the occurrence of persisters under biofilm conditions[121]. Because the environmental cues are numerous, the response to such cues involves several molecular mechanisms and pathways that are overlapping and may actually "cross-talk". Given the dynamic community of a biofilm, the generation of subsets of persisters is essentially endless. While, in general, the phenotypic switch that results in the formation of persisters is transient, exposure of these subsets of cells to continuous antibiotic treatments may lead to a greater genetic diversity with time due to mutational changes. In the staphylococci mutability in the biofilm lifestyle is significantly higher than that in the planktonic lifestyle[171].

In addition to persisters and the ability to hyper-mutate, many Gram-positive and Gram-negative bacteria form small-colony variants (SCV)[172]. These include *S. aureus*[173], methicillin-resistant *S. aureus*[174],

S. epidermidis[175], and *P. aeruginosa*[176]. All of these bacteria are also known to be involved in biofilm-associated infections, and they have been the most studied. This is rightfully so, as *P. aeruginosa* is notorious for causing severe chronic infections in patients with cystic fibrosis[177,178]. The staphylococci represent the leading cause of hospital-acquired infections on indwelling medical devices[179,180]. Both *P. aeruginosa* and *S. aureus* also coexist in the lungs of cystic fibrosis patients and in chronic wounds[181,182]. These same bacteria have also been isolated exhibiting the SCV phenotype in clinical samples that include blood, abscesses, skin and soft tissues, bones and joints, and the respiratory tract[172].

Whereas many alterations in metabolic activity can yield small, slow-growing colonies[183], thus far only a limited number of defects have been associated with clinical strains[172]. In clinical isolates of the staphylococci, the primary determinants of SCV are a reduction in electron transport and thymidine biosynthesis[172,184]. These determinants have been linked to defects in menadione, hemin, and thymidine biosynthesis by using staphylococcal laboratory strains and generating mutations within the *men, hem,* and *thy* operons, respectively[172]. However, a recent study examining the whole genomes of five different clinical isolates of *S. aureus* (four were either hospital-acquired or community-associated United States 300 MRSA strains) demonstrated that all five contained a single-nucleotide polymorphism in one of the menadione biosynthesis genes, specifically *menC*, *menE*, or *menF*[185], thereby identifying the genetic basis for the staphylococcal SCV phenotype.

In addition to exhibiting a small colonial, slow-growing morphology, SCV cells of *S. aureus* produce less pigmentation and exhibit a reduction of hemolytic and coagulase activity as well as other virulence factors. The SCV are slow-growing and tend to have increased resistance to antibiotics, which is strikingly similar to bacteria growing in a biofilm, especially when one considers that the diseases with which SCV and biofilms are most closely associated (endocarditis, pneumonia, soft-tissue infection and osteomyelitis) are persistent, recurrent, and tend to be resistant to most classes of antibiotics[186-188]. The SCVs of *S. epidermidis* and *S. aureus* have an increased ability to form biofilms, which is partly due to increased expression of PIA[189,190]. Similarly, SCVs of two different strains of *S. pneumoniae* were shown to emerge in the initial stages of biofilm formation[191,192]. These SCVs had increased attachment capabilities to solid surfaces and formed mature, three-dimensional[191,192] biofilm structures but had decreased capsules[191,192]. Likewise, in *P. aeruginosa*, both an increase in hydrophobicity[193,194] and EPS production[195,196] have been observed in SCV.

The clinical and laboratory standards institute (CLSI) performance standards for antimicrobial susceptibility testing have recommendations (Fifteenth informational supplement. CSLI/NCCLS document M100-S15.2005) for measuring the susceptibility of bacteria in a biofilm, quantified as the minimal biofilm eradication concentration (MBEC). After exposure to antibiotics, samples are transferred to fresh medium. The MBEC value is the lowest concentration of an antibiotic that prevents regrowth. The recommended MBEC for an antibiotic is defined as the concentration of antibiotic that causes a > 99.9% drop in cell number.

In a clinical setting, increased production of persister cells in biofilms is one reason for the recurrence of infection following discontinuation of treatment[132,135]. Although the small population of persister planktonic cells will be eliminated by the immune system following antimicrobial treatment, antibiotics are not effective for eliminating the persisters in a biofilm because they are protected by the biofilm matrix. The remaining persisters are involved in regrowth after cessation of antimicrobial treatment[135].

Bacteria with serious defects do not survive but undergo programmed cell death (PCD). It is hypothesized[131,197] that persisters may have disabled their PCD to allow survival of a few cells if the antimicrobial agent reaches the whole population. Lewis[131] hypothesized that production of persisters by bacteria is a lifestyle for bacterial perseverance. Persisters survive challenge by factors that kill planktonic bacteria; the rate of development of persisters is 10-10000 fold higher than the rate of development of mutants[197]. This process of variation in lifestyle insures cell survival.

OXIDATIVE STRESS

Differences in the expression of phenotypes between planktonic cells and cells in biofilms may also include differences in sensitivity to antimicrobial agents. In a mature biofilm, bacteria have slower growth than those in the planktonic state[4]. Changes in growth rate in the biofilm, which may be accompanied by limitation in nutrients, may affect the components of the bacterial cell envelope. Both growth rate and low nutrients affect antibiotic efficacy and tolerance[121,198-200]. The constituent of proteins, polysaccharides, extracellular enzymes, fatty acids, phospholipids and metal cations in the bacterial cell envelope are affected by low nutrients, which in turn affect the bacterial susceptibility to antimicrobial agents[121,200,201]. By growing in an environment with lower nutrients, the bacteria also avoid oxidative stress, which results from the effect of bactericidal antibiotics, the generation of harmful reactive oxygen species (ROS) and the production of cytotoxic hydroxyl radicals (•HO), which damage cellular macromolecules[202].

The interactions of β-lactams, fluoroquinolones and aminoglycosides with the target cells induces •HO formation in bacteria. It is suggested that the production of •HO depends on the activity of the tricarboxylic acid cycle (TCA)[12]. Drug-target interaction results in the oxidation of NADH that is produced during the TCA

cycle. Conversion of NADH to NAD^+, which generates ATP, results in the production of superoxide (O_2^-). The superoxide damages the iron-sulfur cluster in proteins and releases Fe^{2+}. The released Fe^{2+}, in combination with H_2O_2, produces highly reactive •HO, which is damaging to macromolecules. The production of •HO is prevented in the bacteria in biofilms that have low metabolic activities[12]. Roles of oxidative stress and •HO in cell death have mainly been established in planktonic cells. However, Battán *et al*[203] showed that for the induction of ROS production in *Pseudomonas* in biofilms, a higher concentration of piperacillin and ceftazidime is required than for planktonic cells. Also, although ciprofloxacin induces ROS production in *P. aeruginosa* biofilms, in the areas of biofilms with low metabolic activities, •HO production is prevented[202]. A study of the effect of the aminoglycoside tobramycin on a *Burkholderia cenocepacia* biofilm showed that although the production of ROS increased in treated cells, 0.1% of the cells survived the treatment[204]. The genes for the glyoxylate cycle, which allows the cells to utilize simple carbon compounds as carbon sources rather than glucose, were upregulated in the surviving cells in comparison with the untreated cells of a biofilm[204]. However, the genes for the TCA cycle and electron transport were downregulated, avoiding the production of reactive oxygen intermediates. Similarly, low number of persisting cells were produced in catalase mutants[204]. Khakimova *et al*[205] showed that the stress response in *P. aeruginosa* regulates catalase, which is important in protecting biofilm bacteria from antibiotic-mediated killing.

Biofilm bacteria are also exposed to ROS from activated polymorphonuclear leukocytes. If there is a deficiency in the antioxidant system, the production of ROS increases in a biofilm[206]. The increase in oxidative burden and decrease in antioxidant defense results in oxidative stress in biofilms[6,178]. The oxidative stress affects the bacterial DNA break repair mechanism and is a contributing factor in the increased mutability of bacteria. This may lead to the diversity and adaptability of a biofilm community[127]. Boles *et al*[127] showed that in *P. aeruginosa*, endogenous oxidative stress in biofilms promotes antimicrobial resistance and that the addition of antioxidants reduces the occurrence of diversity.

EFFLUX PUMP

Induction of the biofilm phenotype is another suggested reason for the resistance of bacteria in biofilms to antimicrobial agents. It is hypothesized that a sub-population of bacteria in the biofilm expresses an active mechanism to avoid the bactericidal effect of antimicrobial agents[157,207,208]. The multidrug-resistant efflux pump MexAB-OprM may be involved in the resistance of *P. aeruginosa* to ofloxacin, and biofilms lacking this pump are more susceptible to ofloxacin[103,161], but resistance of *P. aeruginosa* to ciprofloxacin is not attributed to this pump[161]. Another efflux pump reported in *P. aeruginosa* is PA1874-1877, which confers resistance to ciprofloxacin, gentamicin and tobramycin[209]. Other efflux pumps in biofilms that contribute to drug resistance are *MacABCsm*, an ABC-type tripartite efflux pump in *Stenotrophomonas maltophilia,* which also is involved in oxidative stress response and biofilm formation[210].

In *E. coli*, acrAB (AG100-B) protects biofilms from 0.004 mg/L, but not 0.1 mg/L, of ciprofloxacin, indicating that biofilm resistance to ciprofloxacin in *E. coli* is not the result of multiple antibiotic resistance operons (*mar*) and the multidrug efflux pump *acrAB*[10]. In *E. coli*, a putative multidrug resistance pump, yhcQ, may be responsible for resistance to penicillin[211]. In *E. coli*, the *emrD*, *emrE*, *emrK*, *acrD*, *acre* and *mtd* genes, which encode the proton motive force multidrug efflux pump, also contribute to the formation of biofilms[212]. In *Salmonella enterica*, serovar Typhimurium there is a link between the multidrug resistance efflux pump and biofilm formation[213]. In the mutants that lack a functional multidrug resistance efflux pump AcrB and TolC the transcription of proteinacous materials of amyloid class (curli) biosynthesis is repressed[213].

Inactivation of the NfxB negative regulator of the MexAB-OprM efflux pump affects the resistance mechanism of *P. aeruginosa* in a biofilm[214].

OTHER FACTORS

In vitro study of *P. aeruginosa*, *E. coli* and *S. epidermidis* has shown genetically based differences between planktonic and surface-attached bacteria, which results in differences in biochemical and phenotypic properties. The bacteria in a biofilm use a specific mechanism to avoid the bactericidal action of antibiotics[138,215]. Mah *et al*[138] detected a periplasmic glucan produced from the *ndvB* locus in the biofilm-forming, antimicrobial-resistant strains that interacted with tobramycin. They suggested that the interaction of the glucose polymer with tobramycin may prevent the antibiotic from reacting with the target.

A biofilm-specific *BrlR* gene product, contributes to a high level of drug tolerance in *P. aeruginosa*[216]. BrlR has similarity to the MerR family of transcription regulators, which function as multidrug transporter activators. MerR transcription regulators activate the expression of multidrug transporters in *B. subtilis* and *Streptomyces lividans*[216]. In *P. aeruginosa*, BrlR activates the multidrug efflux pump operons *mexAB-oprM* and *mexEF-oprN*, which are involved in resistance to a variety of antibiotics[216].

Other genes are also implicated in conferring resistance to *P. aeruginosa* in biofilms. Gupta *et al*[217] found that in *P. aeruginosa*, biofilm development and tolerance to antimicrobial agents are linked. They found a two-component hybrid, SagS that regulates the transition attachment of *P. aeruginosa* during biofilm development and also regulates the tolerance to

antimicrobials. The inactivation of SagS resulted in an increase in susceptibility of biofilm cells to bactericidal compounds, more than that of the planktonic cells, and also contributed indirectly to *BrlR* activation. *SagS* functions upstream of *BrlR*, so its inactivation correlates with reduction of the level of BrlR in biofilms[217]. The activities of the QS system also appear to affect antimicrobial resistance in some bacteria. Unlike their wild types, QS deficient mutants of *P. aeruginosa* are susceptible to kanamycin[218]. By *in vitro* quantitation of biofilm formation in wild type and three QS deficient mutants of *P. aeruginosa*, they showed that the quorum sensing Las system plays an important role, not only in biofilm formation thickness, but also in the production of *ampC* lactamase[219]. A further complication of biofilm infections is the potential for horizontal gene transfer[220]. Both conjugation and plasmid transformation have been shown to occur at a higher frequency in *E. coli in vitro* biofilms[221-223]. Clinically speaking the serious implications of such transfer are the generation of either more virulent bacterial strains and/or bacterial strains having acquired antibiotic resistance determinants. In addition, biofilms and horizontal gene transfer may very well promote and maintain an environment for bacterial heterogeneity. In addition, biofilm may also provide a communal environment where transformation of mobile genetic elements is more conducive not only between cells of the same species but between cells of different species, thus providing a mechanism of evolutionary change[220].

CONCLUSION

Clinically important microbial biofilms are formed on host tissues and medically-implanted devices, resulting in a variety of hard-to-treat infections. Biofilm formation is a major health problem, as microorganisms within biofilms are difficult to eradicate with conventional therapeutic treatments. Bacteria in biofilms persist under less than the optimal conditions that would be required for growth and survival of planktonic bacteria. The sessile bacteria in a biofilm are embedded in a self-produced extracellular matrix that shields them from the host's immune system. The population of bacteria in a biofilm exhibits a heterogeneous physiology that enables the sessile community to survive environmental stresses and allows them to escape the bactericidal activities of antimicrobial agents. After termination of treatment with antimicrobial agents, biofilms may shed bacteria and cause recurrent infections. A variety of mechanisms have been investigated in the last several decades to elucidate the reason for the recalcitrant nature of bacterial biofilms; in this literature review, we have attempted to highlight some of these mechanisms. Various studies have implicated as possible causes the inaccessibility of antibiotics to bacteria because of the protective matrix, the production of antimicrobial-degrading enzymes and efflux pumps, and the lack of oxidative stress. Some of these mechanisms are known to be involved in resistance in planktonic cells. Other studies have shown hypermutability of the cells, as well as the existence of persisters and small colony variants that are characteristic of biofilms of several clinically important bacteria, as contributors to the recalcitrant nature of biofilms to high concentrations of antimicrobials. The reason for the recalcitrance appears to be multifactorial, which challenges the development of strategies for the prevention and treatment of biofilm related diseases.

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REVIEW

Importance of microenvironment in preclinical models of breast and prostate cancer

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Abstract

The majority of cancer drugs entering clinical trials fail to reach the market due to poor efficacy. Preclinical efficacy has been traditionally tested using subcutaneous xenograft models that are cheap, fast and easy to perform. However, these models lack the correct tumor microenvironment, leading to poor clinical predictivity. Selecting compounds for clinical trials based on efficacy results obtained from subcutaneous xenograft models may therefore be one important reason for the high failure rates. In this review we concentrate in describing the role and importance of the tumor microenvironment in progression of breast and prostate cancer, and describe some breast and prostate cancer cell lines that are widely used in preclinical studies. We go through different preclinical efficacy models that incorporate the tissue microenvironment and should therefore be clinically more predictive than subcutaneous xenografts. These include three-dimensional cell culture models, orthotopic and metastasis models, humanized and transgenic mouse models, and patient-derived xenografts. Different endpoint measurements and applicable imaging techniques are also discussed. We conclude that models that incorporate the tissue microenvironment should be increasingly used in preclinical efficacy studies to reduce the current high attrition rates of cancer drugs in clinical trials.

Key words: Tumor microenvironment; Breast cancer; Prostate cancer; Preclinical; Efficacy

Core tip: It is today a recognized major problem in cancer drug development that the vast majority of drugs entering clinical trials fail to reach the market due to poor efficacy. One important reason for this is the wide use of subcutaneous xenograft models that are cheap, fast and easy to perform, but lack tumor microenvironment. Concentrating on breast and prostate cancer, we explain why the presence of tumor microenvironment is important, and describe different types of preclinical efficacy models that incorporate

tumor microenvironment. We state the importance of using these models to reduce the high failure rates in clinical trials.

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INTRODUCTION

During the course of multistep tumorigenesis of breast and prostate carcinomas, neoplastic epithelial cells are in a continuous interplay with mesenchymal cells that form the tumor-associated stroma. This tumor microenvironment is constituted by endothelial cells, pericytes, myoepithelial cells, osteoblasts, immune cells, fibroblasts, cancer stem cells, and many other cells that secrete growth factors and cytokines[1]. While complex interactions between these different cell types reshape the surrounding extracellular matrix (ECM) as cancer progresses, also neoplastic and stromal cells undergo constant changes. Endpoint of this extreme plasticity is that a tumor almost never contains two completely identical cells[2]. While tumor heterogeneity remains a major obstacle to effective cancer treatment and personalized medicine, it can also be used as a biomarker to predict the risk of progression and therapeutic resistance[3].

An optimal preclinical model mimics these plastic genetic and phenotypic changes that occur within human disease, is heterogenic, and results in appropriate tumor growth and spread[4]. Mouse (Mus musculus) has emerged as the main species of *in vivo* tumor biology due to its basic physiology and genome size that are similar to human[5]. Other advantages for using mice include the ease of genetic manipulation, low maintenance cost, and short gestation period[6]. Here we rationalize how mouse models of breast and prostate cancer can help us to understand the interaction between microenvironment and cancer cells in neoplastic progression. Major differences between human and mouse tissue architecture and different research models will be discussed.

MOUSE *VS* HUMAN BREAST AND PROSTATE TISSUE

In mammals, the morphology of mammary gland changes throughout the entire reproductive life. Ductal morphogenesis, as well as carcinogenesis, are regulated by steroid and polypeptide hormones and growth factors that act as local epithelial-mesenchymal inductive signals. The glandular part of the human and murine mammary tissue is composed of major lactiferous ducts that arise inside the nipple, branch into terminal ducts, and end up in acini that are embedded in the intralobular stroma[7,8]. The acini are composed of a bilayer of inner milk producing luminal cells and outer myoepithelial cells[9]. The human acini with the surrounding intralobular stroma are termed terminal ductal lobular unit. It is comprised of a small group of lobules, resembling a cluster of grapes at the end of a stem[10]. The murine mammary tissue is organized differently. The corresponding functional units are termed lobuloalveolar units. Unlike in human, the individual ducts branch minimally and end in single bulbous terminal end-buds (Figure 1)[11].

Breast cancer usually originates from the epithelium, but the stroma has a profound effect on tumor growth, invasion, metastasis, and drug resistance[12]. The mouse mammary stroma is histologically different from the human stroma[13,14]. Human mammary epithelium is surrounded by fibrous connective tissue, whereas mouse tissue consists of larger number of adipose cells and smaller proportion of connective tissue (Figure 1). Also, the human breast contains fat, but it is not in contact with the epithelium[11].

Both human and murine prostates are muscular glands that surround urethra. The prostate is covered with a capsule, and it is in close contact with accessory sexual glands such as coagulating gland in mice, bulbourethral gland in humans, and seminal vesicles in both. The obvious difference in gross anatomy between human and murine prostates is that murine prostate is composed of separate ventral, dorsal, and lateral lobes, whereas human prostate is a single nut-shaped gland that is divided to lobes or zones according to their location and function. In humans, there are two lateral lobes in the anterior end of the gland. The anterior lobe is located behind the lateral lobe, anterior to urethra. It is constructed of fibromuscular tissue, and activates during ejaculation. On the posterior to the urethra there is an area called median lobe, and on the posterior to the median lobe a very thin area called posterior lobe. The human prostate can also be divided into an anteriorly located central zone, an urethra surrounding transition zone, and a peripheral zone, which is the largest zone and the most common location of a tumor[4,15].

The prostatic tissue is composed of exocrine glands, ducts, and fibromuscular stroma. The human and mouse prostates contain similar cell types, but the proportion of stroma is larger in the human prostate (Figure 1). Of the mouse prostatic lobes, the dorsolateral lobe resembles most the human prostate histologically and biochemically[4,16]. Therefore, the dorsolateral prostate is an appropriate inoculation or implantation site in xenograft models.

During carcinogenesis, the stroma undergoes extensive changes in gene expression, and often proliferates actively[17]. The stroma co-evolves with its tumor and adapts to the needs of the tumor[18]. For

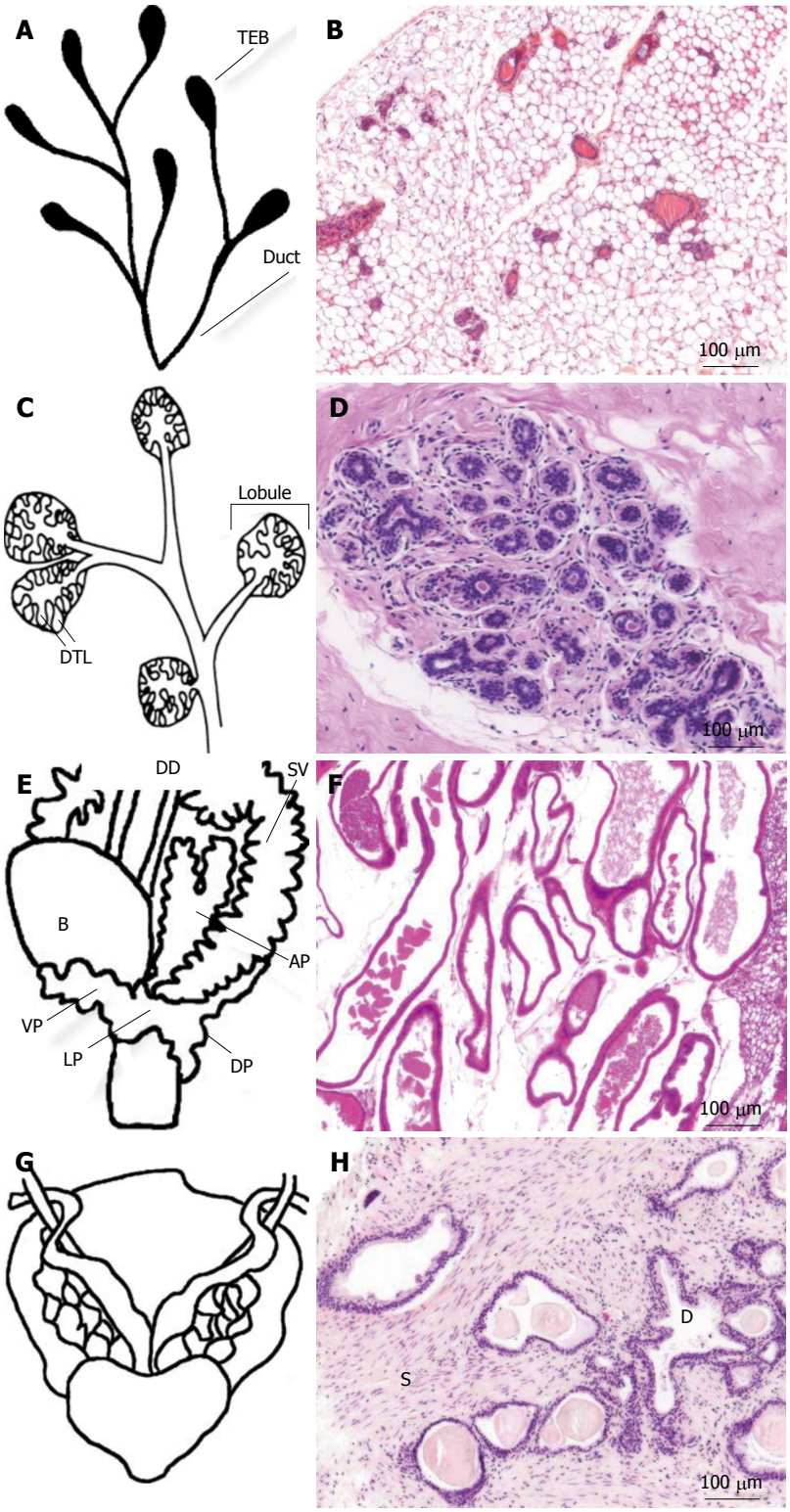

Figure 1 Anatomical and histological comparison of mouse and human mammary gland (A-D) or prostate (E-H). A: Schematic representation of pubertal mouse mammary tree ducts, which end in club shaped terminal end buds (TEBs); B: Hematoxylin & eosin (H&E) stained section of mouse breast tissue, showing ducts imbedded in a stroma composed of adipose tissue; C: Human mature nulliparous terminal ductal lobular unit, 30-50 ductules (DTL) are present in each lobule; D: H&E stained section of human mammary gland showing a terminal ductal lobular unit comprised of ducts and acini in a fibrous connective tissue stroma; E: Mouse prostate surrounds urethra and has distinct lobes: ventral lobe (VP), dorsal lobe (DP) and lateral lobe (LP); F: H&E stained section of mouse ventral prostate; G: Human prostate is a nut shaped glad which also surrounds the urethra; H: The proportion of stroma in human prostate is larger compared with mouse prostate, H&E staining shows secreting ducts (D) and stroma (S). B: Bladder; DD: Ductus deferens; SV: Seminal vesicle; AP: Anterior prostate.

example the amount of collagens increases in tumor ECM, which makes it thicker and may act as a physical or cell attachment - based barrier to drugs. Despite the differences in organization of the stroma between humans and mice, similar gene activation as in patients is seen in the stroma of transgenic and xenograft-

Table 1 Classification of the most commonly used human breast cancer cell lines

Name	Histopathological classification	Immunohistochemical classification
MCF-7	Luminal A	ER^+, PR^+, $Her2^-$
SUM185	Luminal A	ER^+, PR^-, $Her2^-$
T47D	Luminal A	ER^+, PR^+, $Her2^-$
BT-474	Luminal B	ER^+, PR^+, $Her2^+$
ZR-75	Luminal B	ER^+, PR^-, $Her2^+$
SKBR3	Her2-positive	ER^-, PR^-, $Her2^+$
MDA-MB-453	Her2-positive	ER^-, PR^-, $Her2^+$
MDA-MB-468	Basal	ER^-, PR^-, $Her2^-$
SUM190	Basal	ER^-, PR^-, $Her2^+$
BT-20	Basal	ER^-, PR^-, $Her2^-$
MDA-MB-231	Claudin-low	ER^-, PR^-, $Her2^-$
HS-578T	Claudin-low	ER^-, PR^-, $Her2^-$
Cal-51	Claudin-low	ER^-, PR^-, $Her2^-$

Adapted from a review of Holliday and Speirs 2011[24]. $ER^{+/-}$: Estrogen receptor-positive/negative; $PR^{+/-}$: Progesterone receptor-positive/negative; $Her2^{+/-}$: Human epidermal growth factor-positive/negative.

Table 2 Classification of the most commonly used human prostate cancer cell lines

Name	Site of origin	Hormonal status	PSA expression
PC-3	Bone	AR^-	No
DU-145	Brain	AR^-	No
LNCaP	Lymph node	AS	Yes
C4-2B	Subline of LNCaP	AI	Yes
VCaP	Bone	AS	Yes
CWR22	Prostate	AS	Yes
22Rv1	Subline of 22Rv1	AI	Yes
PC-346	Prostate	AS	Yes

AR^-: Androgen receptor negative; AS: Androgen sensitive; AI: Androgen independent.

bearing mice[19,20].

HUMAN BREAST AND PROSTATE CANCER CELL LINES

BT-20 was the first commercial breast cancer cell line. It was established in 1958, followed by the still very popular MD Anderson series (MDA), and MCF-7 cell lines 20 years later[21-23]. Breast cancer, as well as prostate cancer, is a very heterogenous disease, and until today there are no comprehensive models available to study them. However, human breast cancer cell lines (summarized in Table 1) are available that represent the main categories of breast cancer[24].

Table 2 summarizes the most commonly used human prostate cancer cell lines. PC-3 and DU-145 cells were originally cloned from bone and brain metastases of prostate cancer, respectively[25,26]. Their tumorigenicity is high and they form metastases when inoculated into immunodeficient mice[27], and they can thus be considered as models of advanced disease. However, these very popularly used cell lines lack expression of androgen receptor (AR) and prostate specific antigen (PSA), which are both characteristic for hormone-responsive prostate cancer. LNCaP cells express AR and secrete PSA, but they have limited tumorigenicity and respond aberrantly to androgen therapy because of a mutated AR, and they are also sensitive to other sex steroids[28]. Some newer prostate cancer cell lines respond to androgens and secrete PSA, including VCaP cells[29-31], 22Rv1 cells[32] and PC-346 cells[33]. A panel of transplantable human-derived xenografts (CWR, MDA Pca, LuCaP, and LAPC series) have interesting characteristics that mimic human disease[26]. Their benefit is the relevant tissue architecture with stromal support, which improves tumor growth and metastasis.

THREE-DIMENSIONAL CELL CULTURE MODELS FOR STUDYING THE IMPACT OF MICROENVIRONMENT

Currently, *in vitro* drug testing is mostly based on traditional two-dimensional (2D) monoculture models that utilize immortalized cancer cell lines in systems that cannot incorporate the tissue microenvironment. However, 3D cell cultures have raised considerable attention in recent years because of their potential to deliver higher quality and more accurate information that is more representative and predictive of drug responses *in vivo*. Currently, the main applications of 3D cell cultures include cancer therapy and studies of cell-to-cell and cell-to-matrix interactions. It is known that both cancer cells and normal cells cultured in 3D in the presence of ECM components show differences in gene expression, differentiation and proliferation when compared to cells cultured as monolayer in 2D. The importance of the microenvironment was highlighted by Mina Bissell's research group, who were the first to recognize that normal mammary epithelial cells grown in monolayers divided exponentially through several passages, but when the cells were grown in 3D Matrigel culture, they responded to microenvironmental signals by reducing proliferation and differentiating into nearly normal-sized mammary acinar structures[34]. An interesting finding was also that when cultured in the presence of a matrix that contained a combination of reconstituted basement membrane proteins, including type Ⅰ collagen and normal breast fibroblasts, MCF-7 cancer cells were induced to near-complete tumor phenotype reversion[35].

The most widely used 3D culture structures are spheroids that can be formed by multiple different approaches, including scaffolds such as hydrogels, and as floating structures formed either by hanging drop method or by low attachment coatings. The spheroid systems allow co-culturing of different cell populations for studying the role of cell-to-cell or cell-to-ECM interactions, and therefore provide an improved approximate of the *in vivo* tissue architecture. Multiple cell types, such as stromal fibroblasts, nerve

ganglia or endothelial cells, have been seeded within a matrix gel to influence spheroid growth and define specific roles or interactions with prostate cancer cells, including DU-145, LNCaP and PC-3 cells[36]. Also, co-culture of bone stromal derived HS5 cells and PC-3 cells in Matrigel scaffold displays up-regulated invasion and proliferation, along with altered expression of epithelial-to-mesenchymal and chemokine protein constituents involved in metastatic progression[37]. Additionally, multiple cells, including PC-3, osteoblasts and endothelial cells, have been seeded into hanging drops to form heterogeneous aggregates recapitulating the *in vivo* growth behavior of cancer cells within the bone metastatic prostate cancer microenvironment[38]. In breast cancer, the surrounding microenvironment, including stromal fibroblasts, is believed to promote the progression of ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma[39-43]. Indeed, human mammary fibroblasts cultured in a 3D matrix have been shown to secrete more paracrine signaling molecules than in 2D monolayer cultures, increasing the invasive progression in MCF10-DCIS.com cells[44]. Even though the role of the matrix in regulating fibroblast behavior has been studied, the consequences of modified fibroblast behavior with cancer cells remains poorly understood.

XENOGRAFT AND SYNGENEIC ANIMAL MODELS

The term xenograft implies transplantation of material between species. Most commonly, human cells or tissue implants are grafted into immunodeficient mice. If the transplanted material is from genetically nearly identical individuals, it can be transplanted into immunocompetent mice to produce syngeneic tumors. Syngeneic models allow to study the role of adaptive immunity in tumor progression, which is a benefit compared with xenografts. However, the fact that the cells are from murine origin and very rarely respond to hormonal therapy may hamper the results. There are several good syngeneic models for breast cancer, such as Balb/cC3H-originated 4T1 subline grafted into Balb/c mice[45], S115 cells grafted into DD/Sio mice[46], and Py8119 cells grafted into C57BL mice[47]. Until now, there are only few syngeneic models for prostate cancer such as RM1 cells or TRAMP-C2 cells in C57BL mice[48,49].

SUBCUTANEOUS, ORTHOTOPIC AND METASTASIS MODELS

Subcutaneous inoculation of tumor cells is a popular and inexpensive way to perform xenograft models. However, these models can be used only in studies of primary tumor growth because of restricted spread and formation of metastases due to incomplete blood and lymphatic vasculature[50,51]. This, and the fact that these models lack the correct microenvironment for the tumor cells, leads to poor clinical predictivity. The correct tumor microenvironment is important not only for the processes of tumorigenesis, invasion and metastasis, but also for its potential effects on efficacy of tested drug candidates. The correct microenvironment can either improve the efficacy of tissue-specific targeted therapies, or protect the cancer cells from the therapy[12]. The wide use of subcutaneous xenografts and relying on the obtained results is probably one important reason why a very high number of cancer drug candidates fail in clinical trials due to poor efficacy[52]. However, many other reasons such as non-enhanced patient groups, tumor heterogeneity, and low number of clinically relevant events also contribute to the high failure rates.

Clinically much more relevant xenograft models are orthotopic models, where breast cancer cells are inoculated into the mammary fat pad, and prostate cancer cells into the prostate. In these models the cancer cells form primary tumors in the relevant tumor microenvironment and interact with the mouse stromal cells[53-55]. Orthotopic models can also include formation of metastases, depending on the characteristics of the used cell line[56,57]. Typically, orthotopic breast and prostate tumors metastasize into local (inguinal or iliac and sacral, respectively) lymph nodes, liver and lungs[58,59]. Bone metastasis is a common and deadly complication of both breast and prostate cancer. Some breast and prostate cancer models produce bone metastases, but macroscopic bone tumors are rarely, if ever, observed using orthotopic models[56]. By inoculating tumor cells into the bone marrow cavity of the mouse tibia, tumor cell-bone interactions can be studied. Although several steps of the metastasis cascade remain unstudied in this model, the intratibial tumors provide valuable information about the tumor-bone interaction.

Tumor cells can also be inoculated directly into the tail vein or the left cardiac ventricle in order to mimic metastatic disease[60,61]. These models are clinically highly relevant, since at the time of diagnosis of breast and prostate cancer, dormant tumor cells can be found in bone marrow cavity[62]. The models are based on Paget's seed and soil-hypothesis, where a small number of tumor cells have evolved towards metastatic phenotype after a series of somatic mutations[63]. Some laboratories have succeeded in enrichment of bone- or lung-seeking tumor cell populations, and created sublines of some commonly used cell lines. Examples of such breast cancer sublines are bone-seeking MDA-MB-231(SA) and MDA-MB-231(B02) cells[64,65], and MDA-MB-231(LM) cells that form tumors in lungs when inoculated into the blood stream[59].

HUMANIZED MICE

The major limitation of using xenograft models with immunocompromised mice is the lack of immune cells

in the tumor microenvironment. The use of human stroma may be a solution to this problem. Kuperwasser *et al*[66] injected human mammary stromal and epithelial cells into cleared murine mammary fat pads. This chimeric mouse "humanized mammary fat pad" was found to be similar to that of humans and allowed genetic manipulation of the human stroma. Currently, there are no xenograft models where bone metastases are formed from orthotopic tumors with a relevant rate. Several laboratories have introduced a humanized mouse, where human bone tissue is first grafted into immunodeficient mice and after inoculation of the human breast or prostate tumor cells, metastases have been formed into human bone instead of mouse bone[67-69], underlining the importance of species-specificity of the microenvironment in metastasis formation. However, the effect of possible differences in bone metabolism of the transplant *vs* normal bone cannot be ruled out, since there is clear evidence of higher bone metabolism connected to higher metastasis rate[70]. Challenges of the model include the availability of human bone, donor-related variance, immune reactions, and difficulties in implant functionality and viability[71,72].

TRANSGENIC MOUSE MODELS

Genetically engineered mouse models are physiologically relevant models to study tumor progression, because they include natural microenvironment and immune competence. However, most transgenic breast and prostate cancer models are hormone-independent and do not respond to hormone therapy[73,74]. Also, mouse tumors are often mesenchymal instead of epithelial origin[75], and none of the transgenic models include the entire heterogeneity and plasticity of human carcinogenesis.

When an oncogene is overexpressed in mammary gland or prostate epithelium, the most commonly used promoter elements are the mouse mammary tumor virus (MMTV) long terminal repeat, human cytomegalovirus and ubiquitin promoters, the rat probasin gene, the rat C3 prostate steroid-binding protein gene, the human *PSA* gene, and the mouse cryptic gene[76-79]. Hruska *et al*[80] created an estrogen receptor overexpressing conditional mouse line that developed mammary adenocarcinomas, which responded to estrogen and had similarities to human breast cancer histology. The transgenic adenocarcinoma mouse prostate (TRAMP) model was established in 1995, and TRAMP mice have been widely used in oncology[78,81]. In the TRAMP model, SV40 small and large T-antigens inactivate tumor-suppressor proteins and enhance the development of neoplasia[78,82]. TRAMP mice develop prostate adenocarcinoma and metastasize into para-aortic lymph nodes and lungs, and occasionally to distant sites[78]. Disadvantage of the model is that metastases develop at a relatively low frequency[4]. In addition, Chiaverotti *et al*[83] have shown that the background of TRAMP mice (FVB instead of C57/BL) influenced the tumor type. FVB mice frequently developed neuroendocrine-type prostate tumors, while C57/BL mice developed adenocarcinomas. In addition to TRAMP mice, a popular transgenic model is c-Myc overexpression[84]. A structural variation of the c-Myc gene is common in cancer, and accordingly the increased copy number of c-Myc results in a homologous gene-expression profile with human c-Myc-overexpressing cancer, such as disappearance of NKX3.1 during tumorigenesis[85].

Alternatively, the role of specific genes in breast and prostate tumorigenesis can be studied using knockout mice. Since ablation of important genes often leads to embryonic or early fatality, genetically modified mice with conditional knockouts have been developed. Germ-line mutations in oncogenes BRCA1 and BRCA2, in which DNA repair function is interrupted, account for the majority of familial breast cancers. In order to study the role of BRCA1 in breast cancer, MMTV-cre mice have been created, and used to produce conditional mammary BRCA1 knockout mice[86].

Inactivation of the tumor suppressor gene *PTEN* is associated in approximately 70% of advanced human prostate cancers[87]. PTEN$^{+/-}$, PTEN hypomorph, and PTEN conditional knock-out models have been established to study prostate cancer progression[87-89]. Conditional PTEN knock-out leads to prostate cancer with lymph node and lung metastases[88,89]. In addition to the cre-loxP system, tissue-specific, conditional knock-out models have been created using the tetracycline promoter system under the regulation of tet operator promoter. In this model, the specific gene is expressed only under doxycycline supplementation[80].

PATIENT-DERIVED XENOGRAFTS

While cell line based models have provided invaluable knowledge of cancer progression, the utility of these systems is diminished in the light of the findings that patient derived tumor cell lines have significantly different gene expression patterns when compared to the original cell lines or the xenografted tumors[90-92]. Patient-derived xenografts (PDXs) are recent advances in personalized medicine. These models use mouse avatars, where fresh tumor tissue from the patient is grafted in order to study which therapies are most effective for an individual cancer patient. A large number of drugs or drug combinations can then be screened in the mice, which increases the likelihood that a given treatment will benefit the patient. In addition to clinics, PDX models are used increasingly as tumor models in drug development. An obvious benefit of PDX models *vs* traditional cell line - based subcutaneous xenografts is that they possess the natural tissue architecture and composition[93].

However, PDX models have many challenges. The success rate for implanting human tumors in mice is low and depending on the tumor type, engraftment efficiencies vary a lot. In clinical use, it takes more

Table 3 Comparison of different types of breast and prostate cancer xenograft models

Type	Relevant ME	Metastases	Costs	Ease	Ref.
Subcutaneous	No	No	Low	Easy	[51]
Orthotopic BrCa	Yes	Yes	Low	Easy	[45]
Orthotopic PCa	Yes	Yes	Medium	Difficult	[55]
Intratibial	Yes	Yes	Medium	Difficult	[56]
Intravenous/cardiac	Yes	Yes	Medium	Medium	[60]
Humanized	Yes	Yes	High	Difficult	[67]
PDX	Yes	No	High	Difficult	[94]

ME: Microenvironment; BrCa: Breast cancer; PCa: Prostate cancer; PDX: Patient-derived xenograft.

than six months to generate PDXs and screen potential therapies, and many patients die before they can benefit from the results. Although the patient tumor is engrafted along with human stromal components and is sustained during several passages[94], murine stroma may gradually replace the human stroma and lead to confounding results. High cost of PDX technology also limits their use. However, increased use of PDX systems with modern molecular biology techniques will continue to improve the methodology and may help more patients in the future.

There are several companies that offer breast cancer PDX models, but none that offer prostate cancer PDX models. Human prostate cancer xenografts have been implanted in immunodeficient mice subcutaneously or under the renal capsule to study, maintain, or even expand the tumor tissue[95]. This technique has been particularly tested for the propagation of the tumor tissue from castration resistant prostate cancer, which is available for research only in very limited amounts from biopsy samples.

ENDPOINTS AND IMAGING

Experimental tumors are evaluated using immuno-histochemical markers and histomorphometry that are already established in clinic. The major obstacle of comparing experimental tumors with clinical specimens is the mouse background, which may hamper immuno-histochemical stainings. Also the need for an experienced disease model pathologist may be an obstacle.

The classical endpoint in subcutaneous xenograft models is tumor dimension measurement by caliper, where tumor volume can be calculated using the formula $V = a \times b^2/2$, "a" being the biggest dimension of the tumor and "b" the perpendicular dimension[96]. If the tumors are dissected the formula of three dimensions can be used, where $V = \pi/6\ (a \times b \times c)$[97]. Naturally, caliper measurements can only be used if the tumors are palpable. The rapid evaluation of novel drugs in animal models requires developing clinically translatable noninvasive imaging strategies, which are discussed below.

Optical imaging is based on a signal produced by a reporter protein. The signal can be produced by constitutive expression of a fluorescent protein[98], or by enzymatic activation of an inactive substrate[99]. In both options, tumor-producing cell lines need to be transfected with a reporter molecule. A popular method of transfection is the use of genome-integrated viruses. However, they contain a risk of genotoxicity and unpredicted effects due to random integration, which may directly affect the expression levels of not only surrounding but also distant genes. Also, both plasmid and virus based methods can modify the cell behavior indirectly because they typically contain unmethylated or hypomethylated CpG sequences that act as ligands for Toll-like receptor 9, and therefore activate the immune system[100,101]. The third obstacle is that cells may spit out the redundant reporter material during the course of the experiment[102]. In a recent study, these problems were avoided by transfecting cells using non-integrated, episomal CpG-depleted lentivector with a scaffold/matrix-attachment region that acts as an initiation point of replication during mitosis, and enables efficient and stable production of labelled cell lines[103,104].

In addition to optical imaging, bone metastases can be imaged and quantitatively analysed using radiography, micro-computed tomography (CT)[105,106], or micro-magnetic resonance imaging (MRI)[107]. Multimodality functional imaging approach effectively combines the advantages of optical imaging, CT and MRI to analyze breast or prostate cancer bone lesions. Soft tissue metastases can be detected using ultrasound imaging[108], MRI[107], or *ex vivo* by histology and quantitative polymerase chain reaction[56]. Micro-ultrasound imaging can be used to image the surrounding tissue at 3 cm depth, which is usually sufficient for detecting tumors in mice, but difficult for detecting metastases due to their small size. Micro-MRI combined with a contrast agent that specifically attaches to prostate specific membrane antigen receptor, a marker implicated in prostate tumor progression and metastasis, may prove to be a sensitive technique[109].

Today, popular methods of functional imaging are single-photon emission CT and positron emission tomography combined either with CT or MRI. Although clinical use of these techniques is increasing in oncology for diagnosis and image guided radiotherapy planning, their use in preclinical studies is still limited due to their poor resolution and because they are very expensive[110].

CONCLUSION

There are several types of xenograft models available for breast and prostate cancer research (summarized in Table 3). Subcutaneous models are most widely used because they are cheap, fast and easy to perform, but they lack the correct tumor microenvironment. The presence of tumor microenvironment is very

important and necessary for obtaining results that are clinically predictive. It would be important to use preclinical efficacy models that incorporate tumor microenvironment instead of or in addition to subcutaneous models to decrease the very high number of cancer drugs that fail in clinical trials due to poor efficacy.

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REVIEW

Improving cancer therapy by targeting cancer stem cells: Directions, challenges, and clinical results

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Abstract

Cancer stem cells (CSC) are a rare cell population within a tumor characterized by the ability to form tumors following injection into an immunocompromised host. While the role of CSC has been clearly established in animal models, evidence of their clinical relevance has been harder to demonstrate. A number of markers, or combination thereof, have been used to detect and measure, although non-specifically, CSC in almost all human tumors. Several pathways have been identified as crucial for, but not necessarily unique to, CSC survival and proliferation, and novel agents have been designed to target such pathways. A number of such agents have entered early phase development. Further, drugs that have long been marketed for non-oncological indications have been redirected to oncology as they appear to affect one or more of such pathways. This article aims to review the available evidence on the clinical relevance of CSC from a drug development standpoint and the results of early phase clinical trials of agents interfering with the above pathways. It also discusses limitations of current clinical trial design and endpoints to demonstrate anti-CSC activity as well as possible strategies to overcome these limitations.

Key words: Cancer stem cells; Cancer; Time to new metastasis; Tumor heterogeneity; Drug development

Core tip: Cancer stem cells are a rare cell population with two embedded fundamental properties: self-renewal and differentiation into the heterogeneous lineages of cancer cells that comprise the tumor. While the identification and role of such cells has been clearly established *in vitro* and in animal models, uncertainty remains as to the role they play in human tumors and their relevance as therapeutic targets. The article summarizes and discusses the latest findings and challenges on cancer stem cell research, the significance of these cells beyond experimental models and suggests avenues for development of cancer stem cell targeting agents.

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INTRODUCTION

In order to explain tumor heterogeneity, the clonal theory of cancer initiation and progression was proposed[1], stating that each cell within a tumor has equal potential in acquiring genetic and/or epigenetic changes, conferring growth advantages and generating new tumors. This model presumes that all cells within a given tumor have the same tumorigenic potential, and it is called classical or stochastic.

In the cancer stem cell paradigm of tumors, heterogeneous neoplastic cells are organized in a hierarchy where only a small subset of cancer cells, the cancer stem cells (CSC), is responsible for tumor initiation and growth maintenance. According to a consensus definition[2], a CSC is a cell within a tumor that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor. CSC could be tissue stem cells or a more differentiated progeny, which acquired self-renewal capacity[3-5]. There are 2 mechanisms that could mediate the transformation of normal stem cells to CSC[6,7]: (1) Early progenitor cells can gain mutations, which gives them self-renewal capacity; and (2) Fully differentiated cells or cells in the late progenitor stage can become de-differentiated to acquire the properties of stem cells.

Activation of oncogenes or inactivation of tumor suppressor genes are the major types of mutations involved in these processes. CSC may indeed arise from normal stem cells by mutation of genes that make the stem cells cancerous, but this may not be the case in all tumors. For example, in blast crisis chronic myelogenous leukemia (CML), a committed granulocyte-macrophage progenitor may acquire self-renewal capacity and thus "reacquire" stem-like properties due to the effects of later mutations[2]. It is conceivable that more differentiated cells can, through multiple mutagenic events, acquire the self-renewal capacity and immortality that typify CSC. In these examples, a differentiated cell, not the tissue stem cell, eventually evolves to become a full-blown CSC.

The first compelling evidence on the existence of CSC was presented by Bonnet *et al*[8] through their seminal work on leukemic stem cells, where they showed that these cells possess characteristic functional properties of stem cells. They demonstrated that the cell capable of initiating human acute myeloid leukemia in non-obese diabetic mice with severe combined immunodeficiency (SCID)-termed the SCID leukemia-initiating cell-possesses the differentiative and proliferative capacities and the potential for self-renewal expected of a leukemic stem cell. The SCID leukemia-initiating cells were able to differentiate *in vivo* into leukemic blasts, indicating that the leukemic clone is organized as a hierarchy.

CSC have been identified also in solid tumors, including breast, lung, colon, prostate and pancreatic cancers, all supporting the model that cancer derives from a subpopulation of CSC capable of self-renewal to initiate and sustain tumor growth[9,10].

Functional assays were performed in all of these studies, in which cell suspensions of cancer cells were inoculated into immunodeficient mice. This assay is currently considered the gold standard for studying the degree of stemness in a subpopulation of human cancer cells that drives tumorigenicity. In fact, CSC are not necessarily derived from stem cells although they share functional similarities to normal stem cells.

Therefore, the definition of CSC remains mainly operational, *i.e.*, the ability to form tumors in an animal model, which is, although imperfect[11], regarded as the best functional assay to meet the two critical criteria of the consensus CSC definition[2]. The implementation of this approach explains the use of alternative terms in the literature, such as "tumor-initiating cell" and "tumorigenic cell" to describe putative CSC.

However, the results of this assay have been questioned, as it may measure the ability of human tumor cells to grow in immunodeficient mice and not necessarily the actual frequency of CSC in the tumor. It has been demonstrated that only rare human cancer cells (0.1%-0.0001%) form tumors when transplanted into immunodeficient mice. However, modifications to xenotransplantation assays (*i.e.*, the degree of immunodeficiency of mice) can dramatically increase the detectable frequency of tumorigenic cells, demonstrating that they are common in some human cancers[12]. Furthermore, introducing human tumor cells in Matrigel containing a cocktail of growth factors to a foreign species such as a mouse may compromise the growth of human cancer cells[13], indicating the importance of the tumor microenvironment in tumorigenesis. Last, the nature of the proteolytic enzymes used, the duration of incubation, and the temperature at which the tumor cells are dissociated into single cells in order to perform xenotransplantation might further result in the under-estimation of the percentage of CSC, even with the same type of tumor. It follows that if the cancer initiating cells are not rare, the hierarchical model of CSC may be questioned[12]. It should be stressed that the aforementioned findings do not disprove the CSC paradigm. The CSC paradigm is directly related to tumor heterogeneity and tumor's hierarchical organization, and not to the absolute number of these cells[14]. Thus, evidence of CSC in many human tumors has been described using the xenotransplantation model by highlighting that a fraction of tumor cells, and not all cancer cells, are tumorigenic and may thus represent a distinct therapeutic target[15].

From a therapeutic perspective, it is important to acknowledge that other targets exist beyond the CSC themselves to affect this cell population. In fact, the tumor microenvironment, the CSC niche, and cytokine loops play essential roles in the maintenance of CSC and in tumor growth and development[16,17].

Table 1 Commonly used Cancer Stem Cells Markers

Marker	Association with chemoresistance	Reported malignancies
CD34		Hematological malignancies (in combination with CD38)[8,36]
CD38		Hematological malignancies (in combination with CD34)[8,36]
CD24	Yes (in combination with CD44)[63]	Breast, prostate, pancreas (in combination with CD44)[15,23]
CD44	Yes (in combination with CD24)[63]	Breast, pancreas, stomach, prostate, head and neck, ovary, colon (in combination with CD24)[15,23]
CD133	Yes[24,25]	Brain, pancreas, lung, liver, prostate, stomach, colon, head and neck[15,23]
ALDH	Yes[60,61]	Breast, lung, head and neck, colon, liver, ovary[15,26]

ALDH: Aldehyde dehydrogenase.

For example, Feng *et al*[18] demonstrated that tumor microenvironment stromal cells create a growth advantage for CSC in a model of multiple myeloma. In that study, the proliferative capacity of multiple myeloma stem cells was stronger when grown in the presence of stromal cells from patients with myeloma than it was in the presence of stromal cells from normal control bone marrow.

If CSC are relatively refractory to therapies (*e.g.*, chemotherapy, radiotherapy) that have been developed to eradicate the rapidly dividing cells that constitute the majority of the non-stem cell component of tumors, then such therapies are unlikely to be curative and relapses would be expected. If correct, the cancer stem cell hypothesis would require adjustments to the way tumors are diagnosed and treated. The objective would have to be not only to eliminate the bulk of rapidly dividing but terminally differentiated components of the tumor responsible for tumor-associated symptoms, but also the minority stem cell population that fuels tumor growth and is responsible for disease relapse and metastases.

CANCER STEM CELL SURFACE MARKERS

Besides discussion about the pitfalls of the functional assay used to identify CSC, it is intuitive that such an assay is unfit for clinical development of any novel drug as it would require ready availability of immunodeficient mice to be injected with single cell suspensions of patient-derived cancer cells before and after treatment, if one is to demonstrate presence of CSC and their reduction following treatment.

Therefore, more practical assays have been developed to be implemented in clinical research. An *in vitro* tumorsphere assay was used to evidence the self-renewal properties of CSC in human brain tumors[19,20]. This assay is now widely used in clinical research inasmuch as it requires live cells, and this requirement may not be always satisfied in large, multicenter clinical trials. The easiest way to identify and measure CSC within tumors or in the bloodstream would be to use CSC-specific or - associated cell surface marker proteins. By staining cells with antibodies against these markers, populations of interest could be easily identified and measured by either flow cytometry (which requires live cells) or immunohistochemistry (which does not require live cells).

Several markers are commonly used to isolate CSC in normal and tumor tissue, but it is not clear if they are universal markers for characterizing CSC from all tumor types. Furthermore, expression of markers may not be restricted to the CSC population and may be present in normal stem cells, early progenitor cells and normal tissues.

Although many markers have been proposed to define CSC populations, here we focus on the ones most widely used in the literature (Table 1).

The pentaspan transmembrane glycoprotein CD133, also known as prominin-1, was discovered as the target of the AC133 monoclonal antibody, specific for the $CD34^+$ population of hematopoietic stem cells. Subsequently, it was shown to be expressed by a number of normal stem and progenitor cells including those of the epithelium, where it is expressed on the apical surface[21]. The biological role of CD133 has yet to be clarified.

Best known for being expressed on the tumor-initiating population of brain neoplasms[22] the $CD133^+$ phenotype has recently been used to define the CSC populations in lung, pancreatic, liver, prostate, gastric, colorectal, as well as head and neck cancers, and $CD133^+$ cells clearly generated tumors in immunocompromised mice more efficiently than their CD133-counterpart[23]. Also, $CD133^+$ CSC display resistance to traditional chemotherapies[24,25].

A valid stem cell marker among several malignant and non-malignant tissues is aldehyde dehydrogenase (ALDH). ALDH is an intracellular enzyme that oxidizes aldehydes. It holds the attractive distinction of being not only a potential marker of "stemness", but it potentially plays a role in the biology of tumor initiating cells as well[26].

Traditional methods such as immunohistochemistry and blotting are used to detect levels of ALDH in tissues and cells, but the method of choice to measure ALDH activity is the flow cytometry-based Aldefluor assay[27]. In the past few years, Aldefluor has been used to characterize CSC in breast, lung, head and neck, colon, and liver tumors and cell lines. Although CSC are enriched in $ALDH^+$ populations in several tissues,

it is important to acknowledge possible limitations of this marker, especially when used as a single marker. Enzymatic activity measured by Aldefluor is much more transient than the expression of traditional surface markers and may be altered by treatment of tumors or cells[23]. ALDH activity has been used as a marker for CSC in malignancies characterized by the presence and/or absence of cell-surface markers like the $CD44^+/CD24^{-/low}$ and $CD133^+$ populations such as breast cancer and ovarian cancer, respectively[9,28] and early reports suggest that the use of ALDH may "refine" the CSC population because the CSC populations defined above and the $ALDH^+$ populations rarely overlap completely[23].

CD44 is a transmembrane glycoprotein which mediates cell adhesion and migration by binding extracellular matrix components such as hyaluronic acid and osteopontin, or by activating receptor tyrosine kinases related to tumor progression, invasion and metastasis. CD44 is reported as a CSC marker across several tissues, including breast, pancreas, gastric, prostate, head and neck, ovarian, and colon[15]. In both preclinical models and clinical research, however, CD44 is most commonly used in combination with other markers, *e.g.*, CD24.

CD24 is a glycosylphosphatidylinositol-linked cell surface protein expressed in various solid tumors[29]. Expression of CD24 in adult non-malignant tissue is limited to B cells, granulocytes, and epithelial cells[30,31]. The normal function of CD24 outside of B cells is poorly understood. The presence or absence of CD24 on the cell surface has been used as a marker for putative CSC. Breast and prostate CSC ($CD44^+/CD24^-$) were found to have increased adhesion, invasion and migration characteristics when compared with CD24-expressing cells[32], and prostate cells that were able to migrate through Matrigel had suppressed CD24 expression along with decreased levels of E-cadherin[33].

The presence or absence of CD24 as a CSC marker seems to be tissue specific, *i.e.*, breast ($CD44^+/CD24^{-/low}$)[9], prostate ($CD44^+/CD24^-$)[34] and pancreatic ($CD44^+/CD24^+/ESA^+$)[35] cancers.

All the above markers have been used alone or in combination to define CSC populations in solid tumors. The most commonly used markers to define CSC in hematological malignancies are CD34 and CD38, which are used in combination[8,36]. Also for these markers, expression is not restricted to CSC, as they are expressed also by normal hematopoietic and non-hematopoietic cells as well as hematopoietic stem and progenitor cells (CD34).

CLINICAL EVIDENCE FOR A ROLE OF CSC IN CANCER INITIATION, RELAPSE AND METASTASES

Following the demonstration that only CSC identified in patients' tumor cells by the previously described markers can form tumors following injection into immunodeficient mice, a number of studies has been conducted to measure CSC in human tumors and to explore their association with prognosis. These studies are important as they would contribute to validate CSC as a therapeutic target and to confirm the correlation between the presence of such cells in tumor samples with unfavorable outcome, independent of any other prognostic factor. Methodologically, most of these studies are retrospective, which in itself carries limitations due to change over time in diagnostic and therapeutic procedures. Breast cancer is by far the most thoroughly investigated solid tumor. Two meta-analyses have been published. The first reported on 9 studies including a total of 2121 patients and demonstrated that the presence of $ALDH^+$ CSC and $CD44^+/CD24^{-/low}$ CSC was significantly associated with poor overall survival[37]. All of the studies included in the meta-analysis used either ALDH or CD44/CD24 as CSC marker. More recently, a second meta-analysis on 3274 patients using ALDH as the sole CSC marker (sharing 7 out of 16 studies and more than 2000 patients with the first meta-analysis) confirmed the association between the presence of $ALDH^+$ CSC and reduced patient survival irrespective of the different cutoff values used in the different studies to define ALDH positivity[38]. However, breast cancer can be divided into several subtypes, either into histopathological categories based upon expression or lack of hormone receptors and HER2 amplification, or into molecularly identifiable categories using gene expression signatures[39]. The presence and clinical significance of CSC have been investigated in most subtypes[40,41]. In this respect, it is interesting to note that CSC have been shown to be represented at an increased percentage in some breast cancer subtypes. For example, Triple Negative Breast Cancer (TNBC) has been described as the most CSC enriched breast cancer subtype[42-44] Further molecular dissection of TNBC identified mesenchymal stem cell-Like[45] and claudin-low[46] as the most CSC enriched subgroups. It has been shown *in vitro* that breast cancer cell lines of the luminal subtype do not always contain a sizeable $ALDH^+$ cell population, whereas 16/16 basal-like cell lines displayed a CSC population identified by the same marker[47]. Indeed, with a cutoff for positivity set at $\geqslant$ 5% in most studies, it would appear that a minority of tumors display a sizeable CSC population identified by a single marker or combination of markers (*i.e.*, $CD44^+/CD24^-$, $ALDH^+$)[37,38]. It remains to be demonstrated whether this is due to suboptimal markers to identify CSC and/or the limited overlap between CSC populations identified by either marker alone[41,48,49].

In other solid tumors similar results have been reported, although the number of patients studied is in general far lower. In ovarian cancer, the presence of $ALDH^+/CD133^+$ cells in debulked primary tumor specimens correlated with reduced disease-free and overall survival in 56 ovarian cancer patients[28]. These findings were confirmed by a meta-analysis of 1258

patients from 7 studies, in which ALDH was used as a single CSC marker[50]. In prostate cancer, detection of ALDH$^+$ CSC correlated with shorter median survival in hormone naïve, but not in castration resistant, tumors. The two groups consisted of 100 and 107 patients, respectively[51]. In gastric cancer, presence of ALDH$^+$ CSC was found to be an independent prognostic factor for both overall survival and recurrence-free survival[52] in a population of 216 patients. In pancreatic cancer, expression of the CSC markers CD133, CD44 and CD24 was found to correlate with poor prognosis in a sample of 65 patients[53]. In hematological malignancies, it has been found that CD34$^+$/ALDH$^+$ CSC in the bone marrow of 68 patients with acute myeloid leukemia at diagnosis correlated with reduced survival probability[54]. In conclusion, retrospective studies have found a negative prognostic role of pretreatment CSC, in keeping with their alleged resistance to chemo- and radiotherapy.

EVIDENCE OF CSC RESISTANCE TO CHEMOTHERAPY

Breast cancer is the solid tumor in which this issue has been explored most thoroughly. Breast cancer CSC have been shown to display resistance to commonly used chemotherapeutic agents (*e.g.*, paclitaxel) *in vitro*[55] and in mice[56,57]. In patients, neoadjuvant (*i.e.*, administered before surgery) chemotherapy represents the ideal setting for assessing the efficacy of chemotherapy against CSC, as the primary tumor is readily available to serial biopsies before, during and at the completion of treatment. In the majority of clinical trials of neoadjuvant chemotherapy, the proportion of CSC identified by marker expression and/or mammosphere forming efficiency was found to be increased following treatment[58-60] despite reduction of tumor size, highlighting that chemosensitivity of the bulk tumor cells is not shared by CSC.

In these studies, 56% or fewer patients had ALDH$^+$ CSC in their tumors at diagnosis, using a cutoff of $\geqslant$ 5% to $\geqslant$ 20% (reviewed in[61]). In keeping with preclinical findings[41,62], it was reported that the ALDH$^+$ phenotype[60,61], or the CD44$^+$/CD24$^-$ phenotype[63], is associated with chemoresistance However, the studies by Lee *et al*[59] and Gong *et al*[62] came to the opposite conclusion, *i.e.*, ALDH$^+$ CSC correlated with increased pathologic or clinical responses, respectively. The reasons for this discrepancy could be different chemotherapy regimens administered to patients, different patient populations, or different antibody and cutoff values used to evaluate ALDH positivity in breast cancer samples.

The study by Alamgeer *et al*[61] is the only prospective evaluation of ALDH$^+$ cells in serial biopsies of breast cancer. Biopsies were obtained before neoadjuvant chemotherapy, following 4 cycles of either one of two chemotherapy regimens, and at completion of treatment in 119 patients. Dynamic changes of ALDH were recorded, with patients switching from ALDH$^-$ to ALDH$^+$ and vice versa in 27% and 19% of cases, respectively, at the biopsy after 4 cycles of chemotherapy. There are several possible explanations for these findings. First, there may be interconversion between the stem cell like and non-stem cell like phenotype, as previously described in preclinical models[64]. However, conversion from ALDH$^+$ to ALDH$^-$ with chemotherapy alone (*i.e.*, without a CSC targeting agent) is not in keeping with the alleged chemoresistance of CSC. Second, ALDH$^+$ cells have been described to be localized at the center of the tumor mass, whereas CD44$^+$/CD24$^-$ cells are localized at the edges[49]. Thus, serial biopsy of a tumor mass may lead to increase or decrease of either cell population depending on the area sampled. Third, for biopsies negative at baseline, which convert to positive after 4 wk of treatment, there might have been ALDH positivity below the 5% cutoff value of an otherwise positive baseline sample. However, the most intriguing finding of the study is that the disease-free survival of those patients who did not achieve pathologic complete response (pCR) but displayed elimination of ALDH$^+$ cells in the primary tumor, is similar to that of patients experiencing pCR[61].

Another prospective study, the AVASTEM trial, was designed to evaluate whether the addition of bevacizumab to conventional chemotherapy alters the proportion of CSC in breast cancer patients receiving neoadjuvant treatment. It is an open label, randomized phase II trial in which tumor biopsies are required at baseline and after the 4 initial cycles of treatment to evaluate variations in the ALDH$^+$ CSC population[65]. Preclinical work demonstrated that anti-angiogenic agents (*e.g.*, bevacizumab) may increase breast cancer CSC *via* the generation of tumor hypoxia[66]. Indeed, CSC have been described to reside near hypoxic regions in solid tumors[67-70]. Results of AVASTEM will confirm or disprove these preclinical findings.

MOLECULAR SIGNALING PATHWAYS AS THERAPEUTIC TARGETS IN CSC

Signaling pathways are essential for normal stem cells with respect to self-renewal, proliferation and differentiation. Accumulating evidence suggests that maintenance of the CSC population in different human tumors employs the same signaling pathways as in normal stem cells, although an alteration of these pathways during the development of cancer has occurred, and this event has led to dysregulation of stem cell self-renewal and contributes to tumor proliferation[71,72]. Therefore, targeting these aberrant signaling pathways that are important for the formation of CSC seems to offer a new strategy for cancer therapy.

Most of these pathways have been described in dedicated reviews[15]. In this section, we will present

Table 2 Pathways and drug candidates targeting cancer stem cells

Pathway	Targets	Compounds	Clinical development
Hedgehog	Smoothened	[1]Vismodegib[80-82]	Medulloblastoma Pancreatic cancer Hematological malignancies
Notch	γ-secretase DLL4	MK-0752[94] Demcizumab[88-90]	Breast cancer Pancreatic cancer NSCLC Ovarian cancer
Wnt	β-catenin	MEDI0639 Vantictumab[101]	Solid tumors Breast cancer Pancreatic cancer NSCLC
		OMP-54F28[102]	Ovarian cancer Pancreatic cancer Hepatic cancer
CXCR1/2-CXCL8	CXCR1	Reparixin[113,114] SCH563705	Breast cancer
IL-6/JAK/STAT3	CXCR1/2 IL-6 JAK1, JAK2	[2]Tocilizumab [3]Tofacitinib [4]Chloroquine	Breast cancer[57]

[1]Erivedge™, FDA and EMA approved for metastatic or locally advanced basal cell carcinoma; [2]RoActemra™, FDA and EMA approved for rheumatoid arthritis; [3]Xeljanz™, FDA approved for rheumatoid arthritis; [4]FDA approved for malaria and extraintestinal amebiasis. DLL4: Delta-like ligand 4; FDA: Food and Drug Administration; NSCLC: Non-small-cell lung carcinoma; CXCR1/2-CXCL8: CXC chemokine receptor 1/2-interleukin 8; IL-6/JAK/STAT3: Interleukin 6/Janus kinase 2/Signal transducer and activator of transcription 3.

and discuss clinical data concerning anti-tumor activity and side effects related to targeting of each pathway.

The hedgehog signaling pathway

The hedgehog (Hh) pathway plays a crucial role in development and patterning during mammalian embryogenesis, and it is essential for the maintenance of stem cells[73]. The Hh pathway is activated when Hh protein binds to and inhibits the transmembrane protein called Patched, leading through activation of the Smoothened function to the regulation of target genes involved in many cellular functions including metastasis, proliferation, survival, and pathway auto-regulation[74,75]. Deregulation of the Hh pathway is associated with numerous human malignancies and is a critical factor affecting the outcome of treating the disease[76]. Recently, it has been suggested that the Hh pathway is essential for the maintenance of CSC in various human cancers, including colorectal, pancreatic and gastric cancer[77,78], and it is also responsible for treatment resistance of cancer cells[79]. Thus, inhibitors that block any step of the Hh signaling pathway may result in reduction of CSC and overcoming treatment resistance.

One Hh inhibitor, vismodegib, is approved for treatment of metastatic or locally advanced, unresectable basal cell carcinoma. Vismodegib 150 mg/d administered orally produced a sizeable and lasting response rate, including complete responses, in patients with basal cell carcinoma, suggesting activity also on bulk tumor cells[80,81]. In a randomized trial of vismodegib *vs* placebo in patients with basal cell nevus syndrome, an autosomal dominant disorder causing a markedly increased incidence of basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma, new basal cell carcinoma formation rate was decreased by vismodegib from 29/year to 2/year[82]. This tumor prevention capability would suggest suppression of CSC activity, however, no formal investigation of anti-CSC activity has been reported. Vismodegib administration may result in adverse reactions, so that in the latter trial, 54% of patients had to discontinue treatment due to adverse reactions[82], with 25% of patients experiencing serious adverse events in the phase Ⅱ trial regarding basal cell carcinoma[81]. Ongoing and completed clinical trials using Smoothened antagonists were recently reviewed by Amakye *et al*[83] (Table 2).

Notch signaling pathway

The Notch signaling pathway plays several crucial roles in the communication between cells[84,85]. The Notch pathway is activated through ligand-receptor interactions of four receptors (Notch-1-Notch-4) and five Notch ligands (Delta-like1, 3, 4 and Jagged1, 2)[85], resulting in the expression of multiple target genes. The Notch pathway is involved in stem cell proliferation, differentiation, and apoptosis, but its role in tumorigenesis can be either oncogenic or oncosuppressive[85]. In fact, Notch functions as an oncogenic protein in most human cancers including cervical, lung, colon, head and neck, prostate and pancreatic cancer, while it may act as a tumor suppressor in hepatocellular carcinoma, skin and small cell lung cancer[86,87].

The delta-like ligand 4 (DLL4) contributes to CSC self-renewal and vascular development and can bind to any of the 4 receptors. Two anti-DLL4 monoclonal antibodies are in development (Table 2). Demcizumab (OMP-21M18, Oncomed) is a humanized IgG2 antibody specific for DLL4 with as dual mechanism of action: targeting of cancer stem cells and inhibition of angiogenesis. It has been evaluated as a single agent in patients with advanced malignancies[88] and in two phase Ib dose escalation clinical trials for frontline treatment of metastatic pancreatic adenocarcinoma[89] and non-squamous non-small cell lung cancer[90] in combination with gemcitabine and carboplatin + pemetrexed, respectively. In pancreatic adenocarcinoma, median Progression-Free Survival observed did not differ significantly from what would be expected from single agent treatment with gemcitabine in the same patient population[89]. In order to comply with the new standard of care in this disease[91], patients are now being treated with demcizumab in combination with gemcitabine + nab-paclitaxel[89]. In non-squamous non-small cell lung

cancer, median Progression-Free Survival was around 6 mo[90]. In both studies, the objective response rate was significant (25% and 46%, respectively), but the dual mechanism of action makes it difficult to disentangle effects on CSC from anti-angiogenic activity. The most common adverse reactions included nausea, fatigue and hypertension, the latter of which was managed with anti-hypertensives[88-90]. Also, late cardiopulmonary toxicity was recorded in a few patients treated for over 5 mo, leading to a shorter course of treatment and monitoring for early signs of toxicity by serial measurements of B-type natriuretic peptide levels[89,90].

MEDI0639 (medimmune) is an IgG1 lambda (IgG1λ) antibody that selectively binds to DLL4. Clinical results are undisclosed at the time of writing (Table 2).

Notch signaling affects lineage-specific differentiation and self-renewal of normal human breast stem cells[92]. Moreover, Notch-4 activity is elevated in breast CSC, and inhibiting Notch4 activity can reduce the breast CSC population, thereby suppressing tumor initiation[93]. Cleavage of the Notch receptor by gamma-secretase is required to release the Notch intracellular domain, which then translocates to the nucleus, turning on genes involved in cell differentiation and proliferation. Thus, gamma secretase inhibition results in a loss of Notch function in cells. In breast cancer xenografts, the gamma-secretase inhibitor MK-0752 reduced CSC and enhanced the efficacy of docetaxel[94]. Subsequently, 30 patients with locally advanced or metastatic breast cancer were treated with escalating doses of oral MK-0752[95] plus docetaxel every 3 wk. All patients could be evaluated for toxicity. Overall, 12/30 patients experienced G3 adverse reactions including G3 diarrhea (2 patients) and G3 liver function tests elevation at the selected dose level. Further, 24 patients could be evaluated for efficacy. In 11/24 patients, partial remissions were recorded, and in 9/24 patients, stable diseases were recorded. Since docetaxel has an established activity against breast cancer[96], the response rate should be interpreted with caution. Tumor biopsies were optional for participants in the study, and were performed more frequently in patients with locally advanced disease for whom surgery following initial medical treatment was planned. Serial biopsies were taken from 6 patients, at baseline, after cycle 1 and 3 and at progression or completion of treatment. All patients but one were treated at the selected dose level. Four patients experienced Partial Response, and 2 patients achieved stable disease. CSC were evaluated by flow cytometry examining both $ALDH^+$ and $CD44^+/CD24^-$ cell populations and by mammosphere forming efficiency. $CD44^+/CD24^-$ cells decreased from baseline to surgery in 3/5 patients, while $ALDH^+$ cells decreased from baseline to surgery in 3/6 patients. Mammosphere forming efficiency decreased over the course of treatment[94]. Despite these results, later phase clinical trials with MK-0752 are not listed on http://clinicaltrials.gov, neither for breast cancer nor for any other tumor type.

Wnt signaling pathway

The Wnt signaling pathway is another developmental pathway involved in multiple biological processes including embryogenesis, development, cell proliferation, survival and differentiation[97]. The Wnt/β-catenin signaling pathway is the best characterized one of the Wnt pathways[74]. Wnt/β-catenin signaling is activated when secreted glycoprotein, Wnt ligand, binds to the cell membrane Frizzled receptors and LRP5/6 co-receptors, which results in triggering of target genes[97]. Alteration in Wnt/β-catenin signaling has been identified in various malignancies, such as leukemia, colon, breast and cutaneous carcinoma. Wnt signaling in many human tumors arises from mutations in pathway components, resulting in aberrantly high levels of activation. For example, defective mutations in the adenomatous polyposis coli gene, a key downstream regulator of Wnt signaling, result in the inappropriate stabilization of β-catenin, thus activating the Wnt cascade and inducing epithelial cell transformation in patients with familial adenomatous polyposis[98,99]. The Wnt signaling pathway is associated with the maintenance of CSC stem cell properties, but it also regulates important stem cell niches within the body, including normal hematopoietic stem cells and adult stem cells within various tissues.

OMP-18R5 (vantictumab) (Table 2), a fully human IgG2 monoclonal antibody targeting the Frizzled receptors 1, 2, 5, 7 and 8, displays strong anti-tumor activity in patient-derived xenografts in association with standard of care chemotherapy[100]. It also induces cancer cell differentiation. A phase Ib study of the single agent OMP-18R5 in 25 patients demonstrated pharmacodynamic activity on hair follicles and bone turnover. Prolonged stabilization of disease was recorded in 3 patients with neuroendocrine tumors. Analysis of pre- and post-treatment tumor biopsies showed inhibition of Wnt and CSC genes, as well as upregulation of differentiation genes[101].

OMP-54F28 is a fusion protein based on a truncated form of the Frizzled8 receptor, or Fzd8, and the Fc fragment of human immunoglobulin IgG1. It is designed to bind Wnt ligands thus blocking Wnt signaling. In patient-derived xenograft models, OMP-54F28 acts synergetically with chemotherapy in a broad spectrum of malignancies. This fusion protein was tested as a single agent in a phase Ⅰ study in patients with advanced solid tumors[102]. Intravenous injections every 3 wk did not result in any grade ≥ 3 adverse reactions at doses up to 20 mg/kg, double the target efficacious dose established by animal data and pharmacokinetic modeling. Most common (> 20% of patients) grade 1-2 adverse reactions were dysgeusia, decreased appetite, fatigue, muscle spasms, nausea and vomiting. No objective responses were recorded in 25 patients treated in 7 dose escalation cohorts, whereas several patients experienced prolonged stabilization of the disease. Pharmacodynamic modulation of Wnt pathway genes was shown in hair follicles. Five patients experienced doubling of the bone turnover marker

β-C-terminal telopeptide, consistent with Wnt pathway inhibition in bone. This adverse reaction was easily managed with a single dose of zoledronic acid. Phase Ⅰb studies of OMP-54F28 in combination with gemcitabine and nab-paclitaxel (pancreatic cancer), carboplatin and paclitaxel (ovarian cancer) and sorafenib (hepatocellular carcinoma) are currently in progress (Table 2).

CXCR1/2-CXCL8 signaling pathway

CXCL8 [formerly interleukin (IL-8)] is a small soluble protein belonging to the chemokine family[103]. It was originally identified as a potent neutrophil activator and chemotactic factor mainly secreted by activated monocytes and macrophages[104,105]. The biological effects of CXCL8 are mediated by two class A, rhodopsin-like guanine-protein-coupled receptors: CXC chemokine receptor 1 (CXCR1) (IL-8RA) and CXCR2 (IL-8RB)[106,107]. CXCL8 is upregulated in a wide variety of solid cancers, such as prostate, gastric, bladder, ovarian, lung cancer and melanoma. It has also been reported to contribute to multiple hallmarks of cancer, such as increased proliferation, angiogenesis, invasion, and metastases[108,109]. CXCL8 is overexpressed in breast cancer, compared with normal breast tissue, and although there is substantial evidence that CXCL8 may promote breast cancer initiation and progression, more recent evidence indicates that this cytokine is a key regulator of CSC activity[110]. A search for actionable molecules on $ALDH^+$ breast cancer CSC identified CXCR1 as a target almost exclusively expressed by CSC as compared with bulk tumor cells[47]. It was shown *in vitro* that breast cancer CSC proliferate in response to exogenous CXCL8 and that addition of a small molecular weight antagonist of CXCR1/2 (reparixin, formerly repertaxin)[111] or a blocking anti-CXCR1 (but not anti-CXCR2) monoclonal antibody depleted CSC *in vitro*[56]. Interestingly, a FAS-FASL mediated bystander effect killed the vast majority of bulk tumor cells *in vitro*, suggesting the possibility of synergistic effects with chemotherapy[56]. In breast cancer patient-derived xenografts or human breast cancer cell lines, the combination of docetaxel and reparixin was more effective than chemotherapy alone in reducing tumor size[56]. As expected, administration of the single agent reparixin did not result in tumor shrinkage over a 4 wk treatment period. However, tumors recovered from mice that had been treated with reparixin alone or in combination with chemotherapy displayed a far lower abundance of CSC than tumors recovered from mice receiving chemotherapy alone[56]. In addition, reparixin reduced metastasis formation in mice following injection of luciferase-transfected human breast cancer cells into the bloodstream[56]. These results were integrated into a model in which following administration of chemotherapy, dying bulk tumor cells release CXCL8 and FASL. However, $CXCR1^+$ CSC are sheltered from apoptotic signals from FASL unless CXCR1 is blocked by reparixin. Reparixin is a clinical grade molecule already used in patients with non-oncological conditions[112]. Therefore, clinical trials of oral reparixin were started in combination with weekly paclitaxel in HER2-negative pretreated metastatic breast cancer (NCT02001974)[113], and as a single agent in a window of opportunity trial in HER2-negative operable breast cancer (NCT01861054)[114]. Indirect support for this model comes from two lines of evidence. First, Bolha *et al*[55] reported release of CXCL8 from tumor cells exposed to taxane *in vitro*. Also, they observed a marked and dose-dependent increase in mammosphere forming efficiency in TNBC tumor cells recovered from immunocompromised mice treated with 2 doses of paclitaxel[55]. Second, Singh and colleagues[115] demonstrated measurable IL-8 levels in pleural effusions and ascites from breast cancer patients When tumor cells from pleural effusions and ascites were cultured *in vitro*, a direct correlation between IL-8 levels and CSC activity could be measured by mammosphere formation[115]. Surface CXCR1 was detected on the majority of mammosphere cells, and a CXCR1/2 inhibitor, SCH563705, blocked the effects of exogenous CXCL8 on mammosphere formation[115]. In partial contrast to findings by Ginestier pointing to a role for CXCR1 but not CXCR2 in anti-CSC activity[56], CXCR2 inhibition was shown to lead to a significant growth inhibition of basal like breast cancer cell lines *in vitro*[116], further highlighting the potential of CXCR1/2 inhibition. The activity was far less pronounced on non-basal like breast cancer cell lines. Also, in the report by Ginestier, most of the cell lines and patient-derived xenografts were triple negative breast cancers[56]. Although basal like and triple negative breast cancers do not fully overlap[44], these findings suggest that basal like and/or triple negative breast cancer could be most sensitive to CXCR1/2 inhibition, possibly due to an enrichment in CSC in these breast cancer types[42-44].

A possible role for CXCL8 on CSC has been recently highlighted also in pancreatic cancer[53]. The authors found a positive correlation between CXCR1 and both CD44 and CD133 expression in human pancreatic cancer samples. Furthermore, addition of exogenous CXCL8 *in vitro* increased sphere formation, CSC population, and cell invasion of pancreatic cancer cells, all these effects being reversible upon addition of a CXCR1-blocking monoclonal antibody[53].

CXCR1/2 blockade may lead to anti-CSC effects also by an indirect mechanism. In fact, mesenchymal stem cells, a stromal cell type secreting factors that sustain CSC, are often recruited to tumors by tumor cell-derived CXCL8[117]. Thus, inhibition of CXCL8 activity and its homing signal may prevent localization of mesenchymal stem cells to the tumor stroma, hindering the development of a supportive CSC niche[15].

Finally, considering the multiple roles played by CXCL8 in cancer (reviewed in[118], it should be borne in mind that inhibition of CXCL8 biological activity may lead to anti-tumor activity also by other mechanisms not directly related to CSC, such as inhibition of angiogenesis and modification of the leukocyte infiltration into tumors.

IL-6/Janus kinase 2/Signal transducer and activator of transcription 3

The IL-6/Janus kinase 2 (JAK2)/Signal transducer and activator of transcription 3 (Stat3) pathway was found to be preferentially activated in $CD44^+/CD24^-$ breast CSC[116]. This opens the possibility of targeting CSC by compounds interfering with any of the 3 pathway components. Approved anti-IL-6 compounds (tocilizumab, anti-IL-6R mAb) and JAK inhibitors (*i.e.*, tofacitinib) are available for the treatment of rheumatoid arthritis, thus making this pathway a readily available clinical target. Furthermore, it has been shown in preclinical studies that breast cancer cell resistance to trastuzumab, an approved monoclonal antibody for treatment of $HER2^+$ breast cancer, is mediated by an IL-6 feedback loop, leading to an expansion of the CSC pool[119]. Indirect support for a role played by JAK2 comes also from preclinical studies using chloroquine (another approved drug for treating malaria), which suppressed $CD44^+/CD24^-$ breast CSC *via* inhibition of the JAK2-Stat3 pathway[57].

HER2/AKT

HER2 has been reported to be an intrinsic regulator of breast cancer CSC[120,121]. This regulation occurs through activation of the Wnt/β-catenin pathway *via* Akt mediated phosphorylation of GSK3B and nuclear translocation of β-catenin[122]. In patients with HER2-positive breast cancer undergoing neoadjuvant treatment, lapatinib administration led to a decrease, although not a statistically significant one, in both the proportion of $CD44^+/CD24^-$ CSC and mammosphere forming efficiency[58]. However, HER2 regulation of CSC possibly extends to HER2-negative breast cancer (reviewed in[123], as suggested by the clinical benefit observed in patients with HER2-negative breast cancer receiving adjuvant anti-HER2 treatment[124]. It has been shown that in HER2-negative breast cancer, CSC selectively express, and are regulated by, HER2[125]. This is in line with the observation that *in vitro*, lapatinib inhibits CXCL8-stimulated mammosphere formation from both HER2-positive and HER2-negative patient breast cancer samples, highlighting a key role of HER2 in regulating CXCR1/2 mediated activation of breast cancer CSC[115]. This data was put into context in a model in which CSC activity is increased following CXCL8 binding to CXCR1/2 *via* both HER2-independent and HER2-dependent (through CXCR1/2 mediated transactivation) mechanisms[115]. From a clinical standpoint, this data suggests that combination therapy with anti-HER2 and anti-CXCR1/2 compounds may affect breast cancer CSC to a greater extent than either compound alone[115,126].

REPURPOSED DRUGS

A number of approved drugs that have been used for many years in non-oncological indications have been shown to have anti-tumor activity *in vitro* and/or *in vivo*. The obvious advantage of such drugs is the extensive knowledge of their safety profile (although not in association with standard of care chemotherapy), which may open the possibility for single agent window of opportunity trials in a preoperative setting for selected cancers, or possibly neoadjuvant trials if overlapping toxicities are not to be expected[127]. In other instances, high throughput screening programs have identified known compounds as potential CSC targeting agents[128,129]. Several such drugs are being tested as anti-CSC.

Chloroquine is an antimalarial drug. It is capable of inhibiting autophagy, which is a mechanism exploited by cancer cells to cope with stress[130]. In particular, autophagy has emerged as a mechanism by which CSC maintain their capability of self-renewal[131]. *In silico* gene expression analysis of the $CD44^+/CD24^-$ treatment resistant breast cancer cell population identified chloroquine as a candidate anti-CSC compound[57]. *In vitro* chloroquine reduced, in a dose-dependent manner, mammosphere forming efficiency in TNBC cell lines as well as $CD44^+/CD24^-$ cells when used alone or in combination with paclitaxel[57]. *In vivo*, growth of orthotopically implanted tumors as well as lung metastases were reduced, and so were CSC when chloroquine was administered in combination with paclitaxel[57]. A clinical trial (NCT01446016) is currently in progress, in which chloroquine is administered in combination with a taxane or taxane-like drug (*i.e.*, nab-paclitaxel and ixabepilone) to women with advanced or metastatic breast cancer, who have not responded to anthracycline therapy. An interim report showed that in two out of three patients, from whom pre- and post-treatment biopsies were taken, a clear reduction in $CD44^+/CD24^-$ cells was recorded[57]. Chloroquine has been investigated as a CSC targeting agent not only in breast cancer but also in CML[132].

A significant number of studies has been performed with the anti-diabetic drug metformin[133]. Its activity against CSC has been described in a number of preclinical reports[134,135], whereas the issue of its clinical activity against CSC has remained unexplored despite a number of window of opportunity studies in patients with operable breast cancer that established safety of the drug in this setting[136].

Itraconazole and arsenic trioxide, two drugs approved for fungal infections and acute promyelocytic leukemia, respectively, have been shown to be Hedgehog pathway inhibitors[137,138]. In an open label phase 2 clinical trial in patients with basal cell carcinoma, a 24% decrease in tumor area, a 65% reduction in Hedgehog pathway activity and a 45% reduction in the proliferative marker Ki-67 were observed following treatment with itraconazole in comparison with untreated patients[139]. The potential for all-trans retinoic acid as an anti-CSC agent has been recently and thoroughly reviewed[140].

Salinomycin, which is used in chicken fodder as a coccidiostat, has been shown to reduce the proportion of CSC by > 100-fold relative to paclitaxel, a commonly

used breast cancer chemotherapeutic drug[128]. Treatment of mice with salinomycin inhibits mammary tumor growth *in vivo* and induces increased epithelial differentiation of tumor cells[128]. In addition, global gene expression analyses show that salinomycin treatment results in loss of expression of breast CSC genes previously identified by analyses of breast tissues isolated from patients[128]. However, no clinical trials testing salinomycin are registered on http://clinicaltrials.gov.

Thioridazine, an antipsychotic drug, selectively targets leukemic stem cells while sparing normal hematopoietic stem cells[129]. The drug antagonizes dopamine receptors that are expressed on CSC[129]. It is currently being tested in a phase I trial in combination with cytarabine in patients with acute myeloid leukemia (NCT02096289).

ISSUES IN DESIGNING CLINICAL TRIALS FOR CSC-TARGETING AGENTS

Early drug development in cancer usually requires signs of anti-tumor activity as measured by shrinkage of metastatic lesions. Once this is demonstrated, a novel agent is then moved to the adjuvant setting, *i.e.*, after removal of the primary tumor, to reduce recurrence risk. Based on the CSC model, and considering that CSC represent only a tiny percentage of the total tumor cells (*i.e.*, < 5%) of any given tumor, single agent CSC-specific agents would not be expected to display antitumor activity measurable by RECIST criteria[141]. As a consequence, in metastatic patients such agents would not be expected to affect tumor growth and tumor-related symptoms in the short term. Thus, in this setting, CSC-specific agents likely need be tested in combination with standard of care chemotherapy that will address the proliferating, non-CSC portion of the tumor. When a CSC-targeting agent is used in combination with standard of care chemotherapy, the objective response rate is still an obvious endpoint, but its relevance is mostly to ensure that the CSC-targeting agent does not hinder chemotherapy activity. In fact, it is unlikely that a specific CSC-targeting agent augments chemotherapy activity as measured by the objective response rate unless indirectly mediated through a bystander effect on bulk tumor cells[56].

The same consideration applies to neoadjuvant chemotherapy, the purpose of which is to downsize the primary tumor and to eliminate micrometastases. Also, in this setting, it is not intuitive that a CSC-specific agent can significantly increase the rate of pCR as compared to chemotherapy alone, unless a bystander effect is postulated. In this specific setting, one may hypothesize that in a randomized clinical trial, the rate of pCR as well as complete and partial clinical responses between the two treatment groups could be very similar, but disease-free survival may differ, favoring the regimen including a CSC-targeting agent. Partial support for this hypothesis comes from the study by Alamgeer *et al*[61], in which patients who experienced less than pCR but had eliminated $ALDH^+$ CSC in their primary tumor following neoadjuvant chemotherapy had the same disease-free survival as patients achieving a pCR.

Therefore, the objective response rate *per se* is not necessarily indicative of anti-CSC activity, whereas the duration of response would be more informative. These considerations are relevant for the identification of effective compounds for adjuvant treatment, in which activity on CSC is expected to be most relevant, according to the CSC paradigm[123]. Evaluation of pre- and post-treatment biopsies is conceivably the most compelling evidence for anti-CSC activity. However, post-treatment biopsy can be difficult to obtain from metastatic patients[94]. Other challenges also may hinder the value of repeated biopsies even in the neoadjuvant setting, in which tumor tissue is readily available. In breast cancer, the most widely used CSC markers are $ALDH^+$ and $CD44^+/CD24^-$. However, it has been shown that cells displaying either phenotype are largely not overlapping, with each marker(s) identifying a distinct CSC population[41,48,49]. Furthermore, breast cancer CSC can transition from one phenotype to the other. Little is known about the role, and the therapeutic relevance, of each CSC population in primary breast cancer. However, recent evidence shows that $ALDH^+$ cells are proliferative, epithelial cell-like CSC, whereas $CD44^+/CD24^-$ CSC are quiescent, mesenchymal-like cells poised to invade and metastasize[49]. In keeping with their different putative role in cancer, $ALDH^+$ and $CD44^+/CD24^-$ cells tend to be localized in different areas of the tumor, *i.e.*, center and edges, respectively[49]. The location of these CSC populations in anatomically distinct areas of a tumor, coupled with their plasticity, poses theoretical challenges to comparative measurement of CSC in serial biopsies taken before, during and after treatment.

In drug development, in order to treat patients with potentially curable diseases with a novel agent, a robust safety database from the metastatic setting or from other indications for repurposed drugs must be available. Thus, most novel agents designed to target CSC will be tested first in patients with metastatic disease due to safety reasons. One may argue that the metastatic setting is less than ideal to test CSC-targeting agents, since they are conceivably best suited to prevent or reduce metastatic spread and thus should be applied to the (neo)adjuvant setting. In order to circumvent these limitations, surrogate clinical endpoints have been devised to evaluate the potential of a novel agent to reduce the metastatic spread of tumors[142]. This concept fits into the CSC model and can be applied to the development of a CSC-targeting agent which, as a novel chemical entity or biologic,

cannot be tested in settings of earlier disease stages due to safety concerns. Time to new metastasis (TTM) is an exploratory endpoint that measures the time elapsing from study entry or randomization to detection of metastatic lesions in a new organ system as compared to baseline[143]. Examples of application of this endpoint can be found in lung cancer[144,145]. In a less stringent fashion, this endpoint can be measured also until appearance of new lesion(s) in an organ already metastatic at baseline. In breast cancer, considering the proportion of patients developing either new metastatic sites or new lesions at their first tumor progression[146], this endpoint can certainly be read with the possibility of demonstrating the potential for a novel agent to reduce metastatic spread. It should be noted that TTM has also immediate clinical relevance since the development of new metastases in the brain, bones or viscera can lead to significant morbidity. The data with denosumab in the prevention of bone metastases in patients with castration-resistant, non-metastatic prostate cancer[147], as well as the reduction of skeletal-related events in patients with metastatic breast cancer[148], is reminiscent of this endpoint. However, TTM is a challenging endpoint to measure, as in several metastatic tumors, patients with progression will be put on another treatment so that TTM can be read only until RECIST 1.1 progression[143]. The most thorough way to assess TTM would be to design trials allowing treatment beyond progression[143]. One possible way to measure anti-CSC activity circumventing the need for serial biopsies would be to analyze cancer cells and/or tumor DNA in patients' bloodstream. The prognostic role of circulating tumor cells (CTCs) has been established since 2004[149,150]. However, the relationship between the number of CTC and tumor-localized or circulating CSC has not been established yet. The potential, but also the current pitfalls, of CTC and circulating DNA have been recently reviewed elsewhere[151,152].

Targeted anti-cancer therapies are best developed in conjunction with biomarkers (*e.g.*, crizotinib, vemurafenib) that can measure treatment efficacy and/or can identify patients with a higher chance to benefit from the treatment. As discussed earlier, not all tumors display a sizeable CSC population, although this finding may be related to the sensitivity of the assay used and the use of a single marker. The ideal biomarker for pharmacodynamic evaluation of cancer stem cell therapies would be able to accurately measure the proportion of stem cells within the tumor, so as to select patients with a high proportion of stem cells for treatment, to identify presence of the target on CSC for targeted therapies, and to determine on pre- and post-therapy specimens (*i.e.*, tumor or blood) whether the proportion of stem cells within the tumor decreased under the therapy. Unfortunately, the tiny percentage of CSC in a tumor mass makes it difficult to develop a companion diagnostic for any CSC targeting agent with a molecular target.

CONCLUSION

The existence of multiple subpopulations of cells within a tumor with distinct tumor initiating capacity has been clearly demonstrated[14,15]. The clinical relevance of CSC is being highlighted by both retrospective analyses and prospective studies, challenging clinical paradigms[61]. There is still controversy about the most suitable markers to identify CSC in order to understand their role and therapeutic relevance in different stages of human cancers, as well as about monitoring effects of CSC targeting agents. There is a requirement to identify cell surface marker and gene activity profiles that can be readily and reliably used to identify CSC in human tumors. However, as these genes frequently operate in other cell types, they cannot be called "stemness" genes. In order to identify true signatures and pure populations, microarray and genome-wide techniques can be applied to detect trends in genetic and epigenetic models for cancer stem cells. This is especially true for cells expected to be rare, such as cancer stem cells, the expression signature of which would be saturated by the majority of non-stem cells in a whole tumor sample. Even after a cancer stem cell signature from a particular type of tumor is identified, one cannot assume that a given signature is useful for identifying cancer stem cells in a different tumor type, unless it is validated by a functional assay. The use of gene inactivation to eliminate "stemness" or gene activation to engender "stemness" would be required to functionally link any marker to stem cell identity. This can be applied to clinical trials as exemplified by the study that identified RPL39 and MFL2 as potential targets in CSC[153].

A number of novel or repurposed agents interfering with pathways considered relevant for CSC are being evaluated in patients with advanced tumors following demonstration of activity in patient-derived xenografts. A recommended phase 2 dose has been identified for most of such agents either alone or in combination with chemotherapy. For some agents, pharmacodynamic demonstration of target pathway modulation of non-cancer targets (*e.g.*, hair follicles) has been obtained, and some long term responders or at least non-progressors have been described. Formal demonstration of an anti-CSC effect is still lacking in most cases, due to a number of factors including the challenge to obtain serial biopsies in metastatic patients, and the interference of several anti-CSC agents with multiple pathways makes it difficult to disentangle the relative contribution of the anti-CSC *vs* non-anti-CSC activity. Clinical surrogate endpoints may prove useful in suggesting an anti-CSC activity, such as reduction of metastatic spread measured as time to new metastasis[143]. However, in order to seek marketing authorization, a CSC-targeting agent should demonstrate an advantage at least in terms of Progression-Free Survival and Disease-Free Survival as compared with standard of care in the metastatic and

(neo)adjuvant setting, respectively. In this respect, the goals recently put forward by the American Society of Clinical Oncology Cancer Research Committee[154] may be within reach if the CSC model holds true for a given malignancy. Achievement of these goals raises the issue of patient selection, a rather unexplored issue thus far, since trials to date have been conducted on unselected patient populations. First, it is not known whether the CSC paradigm is universal to all human cancers[14]. Second, it has been clearly established that administration of chemotherapy in the adjuvant setting (*i.e.*, following surgery of the primary tumor) increases 30-year disease-free survival as compared with surgery alone in node-positive breast cancer patients[155]. This suggests that a fraction of patients does not seem to require a CSC targeting agent, whereas the opposite is entirely possible in another set of patients or a different breast cancer subtype. Third, aberrant activation of any of the pathways discussed here due to mutations, as seen in a proportion of tumors, may represent one selection step. Lastly, it is possible that CSC may eventually show resistance to CSC targeting agents. At least two drug resistance pathways have been identified for the Hedgehog inhibitor vismodegib in patients[156,157].

In conclusion, given the extraordinary complexity of CSC clinical targeting, a bench to bedside to bench circle[94] is the most sensible approach to develop anti-CSC agents.

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REVIEW

Carbapenemases: A worldwide threat to antimicrobial therapy

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Abstract

Carbapenems are potent β-lactams with activity against extended-spectrum cephalosporinases and β-lactamases. These antibiotics, derived from thienamycn, a carbapenem produced by the environmental bacterium *Streptomyces cattleya*, were initially used as last-resort treatments for severe Gram-negative bacterial infections presenting resistance to most β-lactams but have become an empirical option in countries with high prevalence of Extended Spectrum β-lactamase-producing bacterial infections. Imipenem, the first commercially available carbapenem, was approved for clinical use in 1985. Since then, a wide variety of carbapenem-resistant bacteria has appeared, primarily *Enterobacteriaceae* such as *Escherichia coli* or *Klebsiella pneumoniae* (*K. pneumoniae*), *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, presenting different resistance mechanisms. The most relevant mechanism is the production of carbapenem-hydrolyzing β-lactamases, also known as carbapenemases. These enzymes also inactivate all known β-lactams, and some of these enzymes can be acquired through horizontal gene transfer. Moreover, plasmids, transposons and integrons harboring these genes typically carry other resistance determinants, rendering the recipient bacteria resistant to almost all currently used antimicrobials, as is the case for *K. pneumoniae* carbapenemase - or New Delhi metallo-β-lactamases-type enzymes. The recent advent of these enzymes in the health landscape presents a serious challenge. First, the emergence of carbapenemases limits the currently available treatment options; second, these enzymes pose a risk to patients, as some studies have demonstrated high mortality associated with carbapenemase-producing bacterial infections; and third, these circumstances require an extra cost to sanitary systems, which are particularly cumbersome in developing countries. Therefore, emphasis should be placed on the early detection of these enzymes, the prevention of the spread of carbapenemase-producing bacteria and the development of new drugs resistant to carbapenemase hydrolysis.

Key words: Carbapenemases; Origin; Epidemiology; Evolution; Multi-Drug-resistant bacteria

Core tip: Carbapenemase-producing bacteria were a rare curiosity 15 years ago, as these bacteria were primarily detected in hospital settings. However, now carbapenemase-producing bacteria are observed in farms, companion or wild animals and even in distant glaciers, becoming an epidemic. The relevance this subject has acquired can be easily demonstrated through a search in any medical database; more than 1500 articles have been published depicting the exponential isolation of these bacteria since 1990, with an alarming acceleration in the last seven years.

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INTRODUCTION

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Imipenem, the first commercially available carbapenem, was approved for clinical use in 1985. Since then, a wide variety of carbapenem-resistant bacteria has appeared, primarily *Enterobacteriaceae* such as *Escherichia coli* (*E. coli*) or *Klebsiella pneumonia*, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumannii* (*A. baumannii*), presenting different resistance mechanisms.

The most relevant mechanism is the production of carbapenem-hydrolyzing β-lactamases, also known as carbapenemases. These enzymes also inactivate all known β-lactams, and some of these enzymes can be acquired through horizontal gene transfer *via* conjugation, transduction or transformation through outer membrane vesicles. Moreover, plasmids, transposons and integrons harboring these genes typically carry other resistance determinants, rendering the recipient bacteria resistant to almost all currently used antimicrobials, as is the case for *Klebsiella pneumoniae* (*K. pneumoniae*) carbapenemase (KPC) - or New Delhi metallo-β-lactamase (NDM)-type enzymes.

The recent advent of these enzymes in the health landscape presents a serious challenge. First, the emergence of carbapenemases limits the currently available treatment options; second, these enzymes pose a risk to patients, as some studies have demonstrated high mortality associated with carbapenemase-producing bacterial infections; and third, these circumstances require an extra cost to sanitary systems, which are particularly cumbersome in developing countries. Therefore, emphasis should be placed on the early detection of these enzymes, the prevention of the spread of carbapenemase-producing bacteria and the development of new drugs resistant to carbapenemase hydrolysis.

Carbapenemase-producing bacteria were a rare curiosity 15 years ago, as these bacteria were primarily detected in hospital settings. However, now carbapenemase-producing bacteria are observed in farms, companion or wild animals and even in distant glaciers, becoming an epidemic. The relevance this subject has acquired can be easily demonstrated through a search in any medical database; more than 1500 articles have been published depicting the exponential isolation of these bacteria since 1990, with an alarming acceleration in the last seven years.

The objective of this paper is to summarize the current understanding of the epidemiological, biochemical and diagnostic characteristics of carbapenemases.

CLASIFICATION

Carbapenem-hydrolyzing β-lactamases are classified according to Bush, Jacoby and Medeiros[1], updated in 2010[2], proposing a functional classification dividing β-lactamases into 4 groups (1-4) according to the target substrate and inhibition profile of these enzymes and the phenotypic expression of the clinical isolates (Table 1).

This functional classification correlates with the Ambler molecular classification[3], which divides β-lactamases into 4 classes (A-D) according to the amino acid sequence and spatial structure of these molecules. Two large families have been determined based on the hydrolysis active site: serine-β-lactamases (classes A, C and D), possessing a serine residue at the active center, and metallo-β-lactamases (class B), requiring the presence of one or two divalent Zn^{2+} cations[4].

Serine carbapenemases

Class A carbapenemases (functional group 2f): Class A carbapenemases hydrolyze carbapenems, cephalosporins, penicillins and aztreonam and are typically inhibited by clavulanic acid and tazobactam. Characteristically, imipenem susceptibility widely varies from actual resistance to a slight susceptibility reduction that is typically undetected in routine microbiological tests.

Class A carbapenemases have been detected in *Enterobacter cloacae*, *Serratia marcescens* (*S. marcescens*), *Klebsiella* spp. and *E. coli* in the bacterial chromosome or in mobile elements[5-8].

Among these class A enzymes, KPC, *S. marcescens*

Table 1 Carbapenemase classification

Bush-Jacoby (2010)	Bush-Jacoby-Medeiros (1995)	Molecular class	Distinctive substrate	Inhibited by		Characteristics	Representative enzyme
				CA or TZB	EDTA		
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyamino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
2df		D	Carbapenems	Variable	No	Hydrolizes oxacillin and carbapenems	OXA-23, OXA-48
1e		C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	CMY-10
3a	3	B1	Carbapenems	No	Yes	Broad.spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
3a	3	B3	Cephalosporins	No	Yes	Broad.spectrum hydrolysis including carbapenems but not monobactams	L1, CAU-1, GOB-1, FEZ-1
3b	3	B2	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, SFh-1

CA: Clavulanic acid; TZB: Tazobactam; KPC: *Klebsiella pneumoniae* carbapenemase; IMI: Imipenem hydrolyzing B-lactamase; SME: *Serratia marcescens* enzyme; VIM: Verona integron-encoded metallo-β-lactamase; EDTA: Ethylenediaminetetraacetic acid.

enzyme (SME), imipenem hydrolyzing B-lactamase (IMI) and non metallo-carbapenemase of class A (NMC-A) are the most similar in structure, with the largest deviations observed in areas distant from the active site.

SME and the NMC-A/IMI group are the most important chromosomally encoded enzymes, sharing 97% amino acid sequence identity.

Class A carbapenemases enzymes occur rarely and spread depends on the clonal expansion of the bacteria harboring these enzymes. Class A carbapenemases are susceptible to broad-spectrum cephalosporins, but resistant to carbapenems, penicillins, aztreonam and first generation cephalosporins. The production of these enzymes is induced through imipenem and cefoxitin *via* an AmpC type LysR-dependent regulation system involving the proteins SmeR, ImiR and NmcR[9,10].

SMEs have been detected in a small number of *S. marcescens* isolates, whereas IMI/NMC group enzymes have been detected in *Enterobacter* spp[10,11].

KPCs are clinically the most relevant among plasmid-encoded class A carbapenemases. Not only do these enzymes hydrolyze almost all known β-lactams[12], but KPCs are also inserted in mobile genetic elements bearing resistance genes for antimicrobials other than β-lactams. Remarkably, *K. pneumoniae* accumulates and transfers virulence factors, such as plasmids, to other genera[13-17].

To date, 20 different types of KPCs, differing by 1-3 amino acid substitutions and presenting different hydrolysis profiles (www.lahey.org/studies), have been described. Interestingly, these enzymes share 61% homology with SFC-1 from *Serratia fonticola*, another class A carbapenem[18].

KPCs efficiently hydrolyze nitrocefin, cefalotin, cephaloridine, benzylpenicillin, amoxicillin and piperacillin and also imipenem, meropenem, cefotaxime and aztreonam, but with 10-fold lower activity. Remarkably, KPCs present weak hydrolyzing activity on cefoxitin and ceftazidime[4].

Guiana extended spectrum (GES) enzymes were initially classified as ESBL, but subsequent studies have demonstrated that GESs hydrolyze imipenem, although less efficiently. These enzymes have been detected in *P. aeruginosa* and *K. pneumoniae*, and to date, 24 GES types have been described (www.lahey.org/studies).

Class D (functional group 2df): Class D β-lactamases, also known as oxacillinases (OXAs) due to their ability to hydrolyze oxacillin, are classified according to their hydrolysis spectrum. Broad-spectrum OXA enzymes hydrolyze carbapenems and represent a heterogeneous group including more than 100 genetically different enzymes subdivided into 9 clusters based on amino acid sequence (Table 2)[4]. Carbapenems are weakly hydrolyzed through OXA carbapenemases, rendering high minimum inhibitory concentrations (MICs) below the resistance cut-off value. Nevertheless, OXA-producing bacteria are typically resistant to carbapenems, reflecting concurrent resistance mechanisms, such as permeability defects or the production of other β-lactamases[19,20]. OXA carbapenemases are variably inhibited by clavulanic acid, sulbactam and tazobactam.

The vast majority of OXA carbapenemases, except the OXA-48 cluster, have been detected in *Acinetobacter* spp. isolates, primarily *A. baumannii*, presenting high resistance to carbapenems[21,22].

However, the OXA-48 cluster is the most important among class D carbapenemases. The activity of these enzymes is 10-fold higher than that of other OXA enzymes, and the OXA-48 cluster has been identified in *K. pneumoniae*, from which it has spread to other *Enterobacteriaceae*[23-27].

OXA enzymes hydrolyze penicillins and first generation cephalosporins, but have a weak activity on oxyimino-cephalosporins. OXA-163, an OXA-48-like enzyme with a single amino acid substitution and a four-amino-acid deletion, exhibits lower affinity for carbapenems, but is active on extended-spectrum cephalosporins and partially inhibited by clavulanic acid, mimicking an ESBL phenotypic profile.

Class C (functional group 1e): CMY-10 is the only carbapenem-hydrolyzing enzyme within class C β-

Table 2 Oxacillinase-type clusters

Cluster	Subfamily	Other members	Microorganisms associated
1	OXA-23	OXA-27, OXA-29	*Acinetobacter* spp.
2	OXA-24/40	OXA-25, OXA-26, OXA-72	*Acinetobacter* spp. and *P. aeruginosa*
3	OXA-51	OXA-64-71, OXA-75-78,OXA-83, OXA-84, OXA-86-89, OXA-91, OXA-92, OXA-94, OXA-95	*Acinetobacter* spp.
4	OXA-58		*Acinetobacter* spp.
5	OXA-55	OXA-SHE	*Shewanella* spp.
6	OXA-48	OXA-54, OXA-181, OXA-163, OXA-204, OXA-232, OXA-247, OXA-SAR2	*Enterobacteriaceae*
7	OXA-50	OXA-50a-d, POXB	*P. aeruginosa.*
8	OXA-60	OXA-60a-d	*Ralstonia pickettii*
9	OXA-62		*Pandoraea pnomenusa*

P. aeruginosa: *Pseudomonas aeruginosa*.

lactamases. This enzyme is an extended-spectrum cephalosporinase, reflecting a three-amino-acid deletion in the R2-loop, and has been detected in *Enterobacter cloacae* (*E. cloacae*) in South Korea[28].

Class B or metallo-β-lactamases (functional group 3): Class B or metallo-β-lactamases (MBL) vary greatly in amino acid sequence and molecular structure, but share some features: a divalent cation, primarily Zn^{2+}, for substrate hydrolysis; the hydrolysis of carbapenems, penicillins and cephalosporins, but not monobactams, such as aztreonam, reflecting low affinity interactions; and inhibition through metal chelators, such as ethylene-diaminetetraacetic acid (EDTA), which inhibit the activity of these enzymes through Zn^{2+} sequestration.

In *Enterobacteriaceae*, the expected phenotype includes resistance to amino-, carboxy-, and ureido-penicillins, penicillin-clavulanate combinations, and cefoxitin; decreased susceptibility to piperacillin-tazobactam and oxyimino cephalosporins and elevated MICs compared with the epidemiological cut-off values[19].

MBLs are subdivided into three subclasses, B1, B2 and B3, depending on the primary amino acid sequence and the structural characteristics of the active site, presenting low homology[29].

B1 and B3 subclasses use two Zn^{2+} ions in the active site and have a broad hydrolysis spectrum, whereas subclass B2 employs a single Zn^{2+} ion in the active site and only shows activity on carbapenems[30].

MBLs have been detected in the bacterial chromosome and mobile genetic elements. MBLs in the bacterial chromosome were the first type studied, as these enzymes have been detected in environmental organisms or opportunistic pathogens, such as *Bacillus cereus*, *Aeromonas hydrophila*, *Chryseobacterium* spp. or *Stenotrophomonas maltophilia*[31,32].

Acquired MBLs are easily transferred among different species, as these enzymes can be inserted into mobile genetic elements. These MBLs are primarily classified as B1 subclass enzymes, likely reflecting increased predisposition for insertion into gene cassettes, integrons, transposons or plasmids[33,34].

NDMs have been recently described as MBLs with high dissemination tendencies and have become the most relevant enzymes within this family[35]. NDM-1 has been identified in *Enterobacteriaceae*, and Gram-negative non-fermenters[36,37]. Some variants, such as NDM-4, -5 and -7, present higher efficiencies of carbapenem hydrolysis[38]. The systematical association of NMDs with other resistance determinants and spread rapidity to other bacterial strains poses a serious challenge to sanitary systems worldwide.

IMP (active on imipenem) was first detected in Japan during the 80's[39]. Since then, 48 different subtypes have been described in *Enterobacteriaceae* and Gram-negative non-fermenting rods, particularly *Pseudomonas* and *Acinetobacter*, worldwide (www.lahey.org/studies). These enzymes exhibit a high specificity for hydrolyzing cephalosporins and carbapenems and characteristically, exhibit low activity on temocillin[40]. Enzymatic kinetic differences have been identified among different subtypes, but with low clinical relevance.

Verona integron-encoded metallo-β-lactamase (VIMs), first identified during the 90's, are currently detected worldwide[41], and 41 VIM subtypes have been described (www.lahey.org/studies). VIM-1 and VIM-2 are distributed worldwide and spread easily. Typically, these enzymes exhibit a high specificity for hydrolyzing carbapenems, and different from IMPs, these enzymes also hydrolyze temocillin. The hydrolysis profile varies among different VIM subtypes with no clinical relevance[30].

SPM-1s (São Paulo metallo-β-lactamases) were first identify in Brazil in 2001[42]. These enzymes have a broad hydrolyzing profile, including penicillins, cephalosporins and carbapenems[43]; but due to the associated mobile genetic elements, these enzymes exhibit a low tendency toward spreading.

GIM-1s (German imipenemases) have only been identified in Germany, originally associated with *P. aeruginosa*; however, these enzymes have recently been identified in *Enterobacteriaceae*[4,38,44].

Other acquired MBLs, including SIM, AIM, DIM and the recently described KHM-1 in a Japanese isolate from 1997, exhibit a low dissemination range and are clinically less relevant.

CARBAPENEMASES: MECHANISMS OF ACTION

β-lactamases catalyze the hydrolysis of the β-lactam ring, using water in nucleophilic ring opening, generating an innocuous β-amino acid[45].

Classes A, C and D possess a serine amino acid in the active site, while class B carbapenemases are metallo-enzymes possessing a zinc in the active site[4].

The catalytic mechanism of serine β-lactamases involves acylation, which is common to serine-β-lactamases and penicillin-binding proteins (PBP), and deacylation, which is absent in PBPs and is the essence of antibiotic resistance through serine-β-lactamases[46].

Class A carbapenemases

Class A β-lactamases possess four catalytic residues (Ser70, Lys73, Ser130 and Glu166) and use water as a nucleophile.

First, Lys73 is deprotonated through a concerted double proton transfer from Lys73Nζ to Ser130Oγ and from Ser130Oγ to C3-carboxylate in the substrate; Lys73Nζ-Ser130Oγ-C3-carboxylate in the acyl-enzyme intermediate forms a hydrogen bond network. Second, the acyl-enzyme tetrahedral intermediate is formed, with Glu166 acting as a general base catalyst. This step is the rate-determining process. Third, Lys73 is protonated through the concerted double proton transfer from C3-carboxylate to Ser130Oγ and from Ser130Oγ to Lys73Nζ. Finally, the degraded substrate is detached from the enzyme in concert with the transfer of a single proton from Lys73Nζ to Ser70Oγ to cleave the C7-Ser70Oγ bond[47].

Plasmidic class A carbapenemases: KPCs, GESs

The active site of KPC-2 contains the catalytic Ser70 residue and harbors the deacylation water molecule that is primed through an interaction with Glu166, Asn170 and Ser70. The oxyanion hole formed by the nitrogen backbones of S70 and Thr237 is partially occluded by the side chain of Ser70, which is somewhat unusual for class A β-lactamases. Cys69, adjacent to Ser70, is involved in a disulfide bond formation with Cys238. This disulfide bond formation is characteristic of class A carbapenemases.

A comparison of the KPC-2 structure with that of non-carbapenemases and previously determined NMC-A and SME-1 carbapenemase structures revealed several active-site alterations unique to carbapenemases. An outward shift of the catalytic Ser70 residue renders the active sites of carbapenemases more shallow, likely facilitating easier access for bulkier substrates. Further space for the α-substituents is likely provided through shifts in Asn132 and Asn170 and concerted movements in the postulated carboxyl-binding pocket to facilitate substrate binding at a slightly different angle to accommodate these α-substituents. Thus, the structure of KPC-2 provides key insights into the carbapenemase activity of emerging Class A β-lactamases[48].

The importance of Asn170, a ligand for the hydrolytic water, is further supported by the lack of this residue in GES-1, an enzyme exhibiting characteristic active-site disulfide bond formation and possessing a structure similar to other known carbapenemases, but lacking carbapenem-hydrolyzing activity. Although Asn170 is replaced with a Gly in GES-1, this residue is present in GES-2 and other class A carbapenemases[49-52].

Cromosoma class A carbapenemases: NMCs, SMEs, and IMIs

Cromosoma class A carbapenemases exhibit sequence differences compared with traditional class A β-lactamases, such as TEM-1 and SHV-1, likely contributing to the unique substrate specificity of these enzymes.

The activity of SME-1 reflects a highly distributed set of interactions that subtly alter the structure of the active-site pocket, although no single position uniquely contributes to carbapenem hydrolysis[53]. Nonetheless, Ser-237 is significant to the carbapenemase activity of SME-1[54]. SME-3 differs from SME-1 by a single amino acid substitution of Tyr for His at position 105 at the entrance of the active site, a position that plays a subtle role in the binding affinities of some substrates[6,10,11,55].

Class D carbapenemases

OXA enzymes present markedly different amino acid sequences, however the most characteristic feature is the presence of an unusual N-carboxylated lysine post-translational modification, a general base for the activation of the serine nucleophile in the acylation reaction, and deacylating water[56-58]. In the first step of the acylation reaction, nucleophilic Ser attacks the carbonyl carbon of the β-lactam ring, leading to ring cleavage and the formation of a stable carbapenem-enzyme acylate. In the second step of the acylation reaction, a water molecule attacks the same carbon, the Ser-carbapenem bond breaks and the inactivated ligand is released from the active site[59].

In addition, some OXAs form dimers, and a dimer-monomer equilibrium has been implicated in the kinetic behavior of these enzymes[60].

The strictly conserved residues that participate in the acylation and deacylation enzymatic reactions of class D β-lactamases include the Ser67 nucleophile[59,61,62], the carboxylated Lys70, which activates Ser67 and is essential for the deacylation reaction[63], and Lys212, which plays a role in the protonation of the antibiotic substrate in concert with the acylation reaction.

The active site of OXA-24 has two main elements. Catalytic Ser-81, Thr-82, Phe-83 and Lys-84 form the first element[62]. As well as establishing contacts with water molecules in the cleft, the hydroxyl group of Ser-81 establishes a weaker hydrogen bond with the Nζ of Lys-84 (3.35 Å) than that observed in other oxacillinases. The relative weakness of this interaction is compensated by the slight movement of Lys-84 within the crevice, forming a hydrogen bond with the side chain nitrogen of the conserved Trp-167.

The conserved motif Ser-128/X/Val-Ile represents the second active site element of OXA-24. The lateral hydroxyl chain of Ser-128 is accommodated in the direction of active serine Ser-81, forming hydrogen bonds with the neighboring residues. Another characteristic feature within this second element is the presence of a non-polar residue (Val-130) unique to class D enzymes and contributing to the broad substrate specificity of

OXA-24.

A hydrophobic barrier established through the specific arrangement of Tyr-112 and Met-223 side chains, which define a tunnel-like entrance to the active site, determines carbapenem substrate specificity. This hydrophobic environment over the active site is stabilized through other hydrophobic contacts involving Thr-111, Trp-115, and Trp-221. The tunnel diameter regulates the shape and chemical nature of the antibiotics accessing the active site[64].

Unexpectedly, the structure of OXA-48 was similar to that of OXA-10, an enzyme without carbapenemase activity, indicating that the hydrolysis of these compounds depends on subtle changes in the active site region. Moreover, the active site groove of OXA-48 is different in shape, dimensions, and charge distribution from that of OXA-24[65].

In OXA-58, the deacylating water molecule approaches the acyl-enzyme species, anchored at Ser-83, from the alpha-face. Additional studies comparing OXA-24, OXA-48 and OXA-58 carbapenemases with OXA-10 support the theory that OXA carbapenemases retain the catalytic machinery associated with class D β-lactamases, and residues located close to the β5-β6 loop might play a role in the mechanism of carbapenem hydrolysis[66,67].

Class B carbapenemases

MBLs require zinc ions for β-lactam ring hydrolysis. The active site has two potential zinc ion binding sites, often referred to as sites 1 and 2[68-70]. His, Asn, Gln, Asp and Cys residues typically form the zinc ligands in the two binding sites, and these residues are not fully conserved between different MBLs.

It has been suggested that metal ions act as Lewis acids based on coordination to the peptide carbonyl oxygen, providing a more electron-deficient carbonyl carbon to facilitate nucleophilic attack. Thus, metal ions stabilize the negative charge developed on the carbonyl oxygen of the tetrahedral intermediate anion. In many other metallo-proteases, metal ions lower the pKa of the coordinated water, thereby increasing the concentration of the metal-bound hydroxide ion. Although different in nature from simple solvated ions, coordinated hydroxide ions are better nucleophiles than water[71].

C-N bond fission is the most energetically difficult process in peptide hydrolysis. This fission reaction could be facilitated through the direct coordination of the departing amine nitrogen to the metal ion[72]. Alternatively, a metal-bound water could act as a general acid catalyst, protonating the amine-nitrogen-leaving group to facilitate C-N bond fission.

The catalytic mechanism suggested for BCII from *Bacteroides fragilis* was based on the crystal structure of this enzyme at a resolution of 2.5 Å, showing only one zinc-ion bound to the enzyme at the His site. Following substrate binding, the zinc-bound water molecule, deprotonated by the Asp120 residue, attacks the carbonyl, forming a negatively charged tetrahedral intermediate, which is stabilized through interactions with the metal ion. The Asp120 residue donates a proton to the nitrogen, and C-N bond cleavage occurs, followed by product dissociation from the enzyme active site[73,74].

In this mechanism, the bridging hydroxide ion is responsible for the nucleophilic attack, generating a negatively charged intermediate, stabilized through the oxyanion hole of the enzyme. The apical water molecule bound to zinc is optimally positioned to donate a proton to the leaving nitrogen, and the newly formed hydroxide ion moves to occupy the vacated Wat1 site, followed by product dissociation from the enzyme active site. This mechanism was initially proposed to involve zinc coordination to the β-lactam nitrogen, which is unlikely because of the relatively low electron density of zinc due to amide-resonance. However, the increased basicity of this ion, suggests that nitrogen binding to zinc likely occurs once the tetrahedral intermediate is formed[72].

An unusual mechanism has been proposed for subclass B2 CphA from *Aeromonas hydrophila* based on the crystal structures of the free enzyme and a reaction intermediate between the enzyme and the substrate biapenem[75]. Nucleophilic attack is performed through a water molecule, which is not coordinated to zinc, but activated through His118 catalysis, followed by C-N bond fission, which occurs prior to nitrogen protonation. This proposed mechanism uses zinc as a Lewis acid to facilitate C-N bond fission and stabilize the leaving group[72,73], whereas most mechanisms use the zinc ion as a Lewis acid to stabilize oxyanion formation from the β-lactam carbonyl oxygen. Rearrangement of the proposed intermediate amine anion, whose negatively charged nitrogen is stabilized through an interaction with the zinc ion, generates a bicyclic intermediate, which is observed in the crystal structure, although this rearrangement might not occur in the active site of the enzyme. A solvent molecule replaces the water molecule used in the nucleophilic attack, protonates the nitrogen and releases the final product[71].

ORIGIN OF CARBAPENEMASES

Bacteria appeared on Earth approximately 3.5 billion years ago. Subsequently, these evolved, differentiated and adapted to new environments, initiating competition among different species for the many resources needed for survival.

One of the survival strategies of early microorganisms involved the production and secretion of antimicrobial molecules that would eliminate any competitor but not affect the producer. To this end, bacteria synthesize both antimicrobials and antimicrobial neutralizers from the molecules already present in the metabolic networks of these organisms. The next step in this biochemical warfare involved the development of resistance mechanisms in non-antimicrobial-producing bacteria.

It has been suggested that antimicrobials developed from signaling molecules, such as those used in *quorum* sensing, while β-lactamases are primarily derived from PBPs, enzymes for the production and maintenance of the bacterial wall[76,77].

Currently, many different biochemical products with antimicrobial or resistance properties are found ubiquitously, as this biochemical warfare has been ongoing for billions of years. Thus, it is only now that we are beginning to understand antimicrobial resistance. β-lactams and β-lactamases regularly occur in nature, therefore to understand the origin and evolution of the carbapenemase enzymes, it is necessary to introduce and explain the term "resistome", *i.e.*, the global set of molecules that confer antimicrobial resistance to microorganisms[78]. According to this theory, antimicrobials act as selectors and mutation accelerators of the resistance mechanisms present in nature; then, these resistance mechanisms are selected and transferred from one bacteria to another, as horizontal gene transfer is advantageous in dynamic environments[79-81]. Moreover, the level of antimicrobial pressure will determine the evolutionary rate[78].

Origin of serine β-lactamases

PBPs are a group of transpeptidases, transglycosylases and carboxypeptidases belonging to the active-site serine family of enzymes, except for one Zn^{2+}-dependent PBP. These enzymes have been divided into low- and high-molecular-weight molecules responsible for building and maintaining the peptidoglycan structure of the bacterial cell wall. To this end, PBPs are fixed to the inner bacterial membrane with the active site facing the periplasmic space[77]. Interestingly, these enzymes cluster into six different groups closely related to different β-lactamases, suggesting that these enzymes developed independently from PBPs under different selection pressures in a two-step evolution to acquire acylation/deacylation properties[77,82-84]. Hall *et al*[85,86] showed that serine β-lactamases are ancient enzymes, over two billion old, which form three different clusters: classes A and B are sister taxa, while class C separated earlier from a common ancestor of classes A and B[85,86]. Recently, a new family of PBPs, PBP-A, has been described in *Cyanobacteria*, and these enzymes represent an intermediate step between class A β-lactamases and PBPs[84].

Phylogenetic analysis revealed that class A carbapenemases generate six related branches sharing 32%-70% amino acid sequence identity and group together with other β-lactamases and low-molecular-weight class C PBPs[77]. This observation suggests that these clusters evolved from different ancestors[18].

Class A carbapenemases existed before carbapenems were clinically used, as chromosomally encoded SME-1 and IMI-1 were detected in *S. marcescens* and *E. cloacae*, respectively, a few years before imipenem was launched[7,10]. Thus, carbapenem use acted as a selector. Indeed, Walther-Rasmussen *et al*[18] conducted a phylogenetic analysis, and the resulting phylogram showed that NMC-A, SME, KPC and SFC-1 cluster together and could share a common ancestor with carbapenemase activity.

In contrast, GES and SHV-38 have different origins, and only SHV-38 has a known ancestor[87]. Both enzymes cluster with other ESBLs, indicating that the carbapenemase activity of these enzymes evolved through various mutations in response to carbapenem use[18].

Class D β-carbapenemases might have also been derived from low-molecular-weight class C PBPs, and similar to class A, these enzymes predated carbapenem clinical use, as the first member of this family, ARI-1 (subsequently referred to as OXA-23), was identified in 1985 from an *A. baumannii* isolate[88]. Interestingly, BlaR and MecA transducers are included in branches belonging to these β-lactamases, potentially representing reverse evolution from β-lactamases[77,83]. Most OXA carbapenemases are primarily chromosomally encoded in *Acinetobacter* spp. and might represent a natural component of some subgroups[89]. Other OXA carbapenemases have been identified in bacteria, such as *P. aeruginosa* or *Shewanella* spp. Notably, the most clinically relevant enzyme in this family is the OXA-48 cluster, which exhibits the highest carbapenem hydrolysis rate, is plasmid encoded and has been detected in *Enterobacteriaceae*. Interestingly, OXA-48 might have originated from *Shewanella xiamensis*, an environmental bacterium isolated from seepage water that harbors an OXA-48-like chromosomal gene, namely OXA-181. However, the genetic structure of $bla_{OXA-181}$ is different from that of bla_{OXA-48}, indicating an unrelated origin. Thus, *Shewanella* spp. might represent a reservoir for OXA-type carbapenemases[90-93].

Origin of MBL

MBLs belong to a superfamily whose members exhibit a four-layered αβ/αβ structure and share five conserved motifs. Many enzymes of this superfamily are involved in hydrolysis and redox processes, mRNA maturation and DNA reparation, suggesting that MBLs could represent divergent evolution from an early ancestor[94].

Bayesian phylogenic studies indicate that subclasses B1 and B2 form one cluster that split approximately 2.2 billion years ago and developed β-lactamase activity approximately 1 billion years ago, whereas B3 is older, splitting earlier from a common ancestor and developing β-lactamase activity approximately 2.2 billion years ago[94,95]. Therefore, although the 3-dimensional structure of these enzymes is similar, with structural features consistent with the enzymatic activity profiles, the amino acid sequences of MBLs significantly diverge[94,95].

MBLs are chromosomally encoded in bacteria from different phyla, including δ-*Proteobacteria*, which are primarily soil inhabitants and bacterial predators, harboring

as many as 30 gene copies, *Firmicutes*, *Bacteroidetes*, and α-, β- and γ-*Proteobacteria*[96,97].

Environmental bacteria exposed to antimicrobial pressure might be a plausible source of MBLs. For example, the sequence analysis of the blaNDM-1-positive IncA/C plasmid revealed that the plant pathogens *Xanthomonas* spp. or *Pseudoxanthomonas* spp. might be the origin of the NDM-1 β-lactamase[98], the pLD209 plasmid containing the *Pseudomonas putida* blaVIM-2 is a mosaic of both clinical and environmental bacteria[99], or the *blaIMP* gene, found in bacteria isolated from glaciers[100].

GENETIC CONTEXT

The expansion of chromosomally encoded carbapenemases, such as SME, IMI or NMC, depends on clonal replication; therefore, although common pathogens can easily spread among humans, the clinical impact of these microorganisms is typically limited to sporadic and local outbreaks that can be controlled.

However, mobile genetic elements carrying carbapenemases are a greater threat and challenge. These elements can be interchanged between different species and rapidly spread beyond clinical control. Indeed, horizontal gene transfer can occur in natural environments and remain unknown.

Among class A, KPCs are the most successful enzymes[101]. blaKPC has been identified in *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* species and is typically inserted in TN4401, a TN-3 based transposon that evolves and mobilizes genes at a high frequency[102-105]. Interestingly, blaKPC success has been associated with a single *K. pneumoniae* sequence type, ST-258, which harbors the TN4401 transposon. Naas et al suggested that the transposition of these elements into various-sized plasmids is a recent event[95].

GES has also been identified in different species worldwide, but this enzyme is associated with single outbreaks[4], although a new type of mobile genetic element, namely the integron mobilization unit, has been described for blaGES-5[106]. IMI-2 has been identified in plasmids in *Enterobacter asburiae* and *E. cloacae*, but the clinical import of this enzyme is minimal[107,108].

Class D carbapenemases are transferred from plasmids to bacterial chromosomes and vice versa[109]. blaOXA-23 has been identified in certain *A. baumannii* clones and is generally located in a Tn2006 transposon, bracketed by the ISAba1 insertion sequence and associated with other antimicrobial resistance islands[110]. blaOXA-48 is associated with TN1999 in *K. pneumoniae* and Tn1999.2 in *E. coli*, where an ISR1 insertion sequence transfers this enzyme from an IncL/M plasmid to the bacterial chromosome. The high conjugative frequency of this plasmid among *Enterobacteriaceae* accounts for its overall success[109,111,112].

Until the emergence of blaNDM type MBLs, blaVIM and blaIPM were the most successful enzymes in this family. These enzymes are associated with IncA/C, IncF, IncHI1, IncL/M, IncN, and IncK incompatibility group plasmids and intercountry, interhospital, intrahospital, interspecies, and intraclonal spread[113]. These genes have primarily been identified on class I integrons as gene cassette arrays with other antimicrobial and antiseptic resistance genes[114,115]. In contrast, in a surprisingly short period of time, the *blaNDM* gene has become the most disseminated MBL among countries and species[35,116]. However, blaNDM is harbored in highly transferable plasmids containing resistance genes to almost all known antimicrobials[116,117]. Indeed, blaNDM-1 is considered a chimera constructed in *A. baumannii* through the initial capture of a precursor gene by ISCR27 and subsequent fusion to the aphA6/ISAba125 promoter, which was incorporated into the existing large multi-resistance regions of different *Enterobacteriaceae* plasmids. The gene *blaNDM-1* has been associated with the remnants of the Tn125 transposon, which was identified in the chromosomes or plasmids of *Acinetobacter* spp. and is positioned adjacent to a complete or truncated *bleMBL* gene[118-121]. The high genetic mobilization of blaNDM-1 has been demonstrated through reports of a new chromosomally located class I Tn402-like structure in *P. aeruginosa*[122].

CMY-10, the only class C carbapenemase, has recently been associated with a new complex class 1 integron within a conjugative plasmid[123].

EPIDEMIOLOGY

blaSME has been identified in some subpopulations of *S. marcescens* located primarily in the United Kingdom and sporadically in North America. This gene has been associated with single cases or small outbreaks, but the spread is most likely hindered through fitness costs associated with the expression of SME protein. However, recently, blaSME has been detected on a genomic island, SmarGI1-1, which can be excised and circularized, likely contributing to the dissemination of this gene among *S. marcescens*[4,18,124,125].

blaIMI/NMC-A has been identified in *Enterobacter* spp. in sporadic cases in the United States, France, Finland, Croatia, Argentina and China[4,38,126].

blaKPC was first described in *K. pneumoniae* in cases in the east coast of the United States, but has now been identified worldwide in other *Enterobacteriaceae* and Gram-negative non-fermenters[12,18,127]. The spread of blaKPC has become an endemic problem in the east coast of the United States, Puerto Rico, Colombia, Greece, Italy, Israel and the east coast of China, and regional or local outbreaks have been reported worldwide[101,128,129]. The high adaptability and inter-genera spread of KPC has been demonstrated by the first KPC-3 outbreak in Canada, involving five different *Enterobacteriaceae* in eight patients[130].

blaGES genes have been detected worldwide in both

Enterobacteriaceae and Gram-negative non-fermenters, but these genes pose a minor challenge compared with *bla*$_{KPC}$. Nevertheless, bla$_{GES}$ has been identified in plasmids recovered from environmental samples easily replicated in *E. coli* and *P. aeruginosa*[4,131].

OXA-48-like carbapenemases, a source of frequent nosocomial outbreaks, were first detected in Turkey, but has recently spread primarily across North Africa[132,133]. In France, The Netherlands and Morocco, OXA-48-like carbapenemases have been associated with *K. pneumoniae* ST395, suggesting geographical dispersion through immigration. OXA-181 is a growing concern in the Indian subcontinent, and sporadic cases have been reported worldwide, although most cases have been associated with this geographical area[38]. OXA-163 has also been identified in Argentina and Egypt [38].

Chromosomally encoded MBLs are ubiquitous in environmental bacteria, but acquired MBLs have recently become a global concern. IMP type carbapenemases were first reported in Japan in the late 1980s in *P. aeruginosa*, subsequently these enzymes were identified in *S. marcescens* and are currently primarily detected in *Enterobacteriaceae* and *Pseudomonas* across Eastern Asia[38,39,134-136].

Similarly, VIM-type enzymes have primarily been detected in *Enterobacteriaceae* and *Pseudomonas*. These enzymes were first detected in the 1990s in Italy and France and are now endemic in Greece, Italy, Spain, South Korea and Taiwan[38,41,137,138].

However, the most challenging MBLs are undoubtedly, the NDM-type carbapenemases. These enzymes were first detected in *K. pneumoniae* and *E. coli* in a Swedish patient returning from India, showing the propensity for interspecies spread[117,139]. The Indian subcontinent has been identified as both the origin and reservoir, where the prevalence of these enzymes is approximately 5%-18%[140,141], but the Balkans has also been recently identified a reservoir for NDM-type carbapenemases[142]. NDM producers have been reported worldwide, associated with intercontinental travel[38]. Disturbingly, NDM producers are involved in nosocomial infections and community-acquired infections.

CARBAPENEMASE COSTS

The emergence of carbapenemase-resistant bacteria is a global health threat and a medical challenge, implicating an extremely high cost for hospitals. This cost is associated with prolonged hospital stays, higher health care expenses, and increased mortality, particularly when a second antibiotic is needed for better coverage of the causative pathogen[143]. Significantly, the length of stay in hospital, the hours of mechanical ventilation and the time spent on an intensive care unit also contribute to increased costs[144].

The actual cost of controlling infection has been associated with several factors, and some of these factors, such as new antimicrobial development (estimated at U$ 1 billion per drug), the need for increased surveillance within each hospital to determine problematic pathogens, enforced isolation procedures to control spread, and education (primarily interns, residents, and medical students who have not yet developed specific treatment habits), are unsuspected.

Other costs involve the aging population and the delivery of quality health care at reasonable prices[143]. For optimal economic understanding, considering that high-cost drugs can also be cost-effective, the clinicians, pharmacists and economists at each hospital should work together to achieve cost-savings with effective antibiotic therapy, rather than making choices based on drug-acquisition costs[144].

With regard to the host, particularly in multi pathological patients, there is a synergy between the severity of the underlying disease and infection with a resistant microorganism. The consequences are higher mortality rates, extended hospitalization and increased costs[145]. A common problem in endemic settings or during outbreaks is that therapeutic options become limited and are not always optimal[146]. Consequently, the appropriate selection of the initial antibiotic therapy before susceptibility tests are available is difficult. Thus, patients under these conditions have poorer clinical outcomes. Indeed, the death rates associated with KPC producers are higher than 50%, while rates associated with MBL producers range from 18%-67%[101].

Other challenges include clinical laboratory testing and the obtained results. Ideally, rapid and optimal susceptibility tests are in demand, but each pathogen varies, even within the same family, reflecting delayed resistant pathogen identification, treatment errors and higher costs[143].

The ability to efficiently treat carbapenemase-producing bacterial infections requires a multifactorial approach, including continuous investigation, the development of new antibiotics and the adequate use currently available treatments, considering that the first approach starts with an appropriate means of infection control[147].

CARBAPENEMASE DETECTION

A series of non-molecular-based tests have been proposed for the detection and identification of carbapenemases, but none of these analyses have 100% specificity or sensitivity. Therefore, the use of routine susceptibility tests to detect carbapenem resistance should be followed by genotypic and phenotypic confirmation.

Accordingly, Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for carbapenems have been significantly lowered to improve the detection of carbapenem-resistant isolates[148,149].

Both organizations recommend reporting carbapenem MICs, regardless of carbapenemase production, and suggest that special tests for carbapenemase detection

Table 3 Breakpoint and screening cut-off for carbapenemase-producing *Enterobacteriaceae*

	MIC (mg/L)				Disk-diffusion zone diameter (mm) using 10 μg disks			
	S breakpoint		Screening cut-off		S breakpoint		Screening cut-off	
	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI
Meropenem	≤ 2	≤ 1	> 0.12	2-4	≥ 22	≥ 23	< 25	16-21
Imipenem	≤ 2	≤ 1	> 1	2-4	≥ 22	≥ 23	< 23	-
Ertapenem	≤ 0.5	≤ 0.5	> 0.12	2-4	≥ 25	≥ 22	< 25	19-21

S: Susceptibility; MIC: Minimum inhibitory concentration; CLSI: Clinical Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing.

be performed only for epidemiology purposes and infection control issues. However, partial or total susceptibility to carbapenems has been observed in carbapenemase producers, and carbapenem elevated MICs have been associated with decreased outer membrane permeability or the overexpression of other β-lactamases or chromosomally encoded AmpC cephalosporinases[101,150].

The detection of carbapenemase producers in clinical specimens is based on a careful analysis of the susceptibility results obtained from automated systems, liquid media or disc diffusion tests according to standardized guidelines. The CLSI and EUCAST have defined cut-off values for carbapenemase detection in *Enterobacteriaceae* because carbapenem MICs for carbapenemase-producing bacteria might be below the clinical breakpoints. However, while the CLSI values lie within the intermediate category, the EUCAST values lies within the susceptible range. According to EUCAST epidemiological cut-off values, meropenem offers the best sensitivity and specificity balance (Table 3). Imipenem shows a narrow range between the wild-type bacteria and carbapenemase producers, and ertapenem, although it presents excellent sensitivity, has a variable positive predictive value, depending on the carbapenemase prevalence and presence of different resistance mechanisms[151,152].

Day *et al*[153] recently evaluated the effectiveness of disc diffusion using faropenem to predict carbapenemase activity in *Enterobacteriaceae.* These authors concluded that a zone diameter of 6 mm for a 10 μg faropenem disc predicted carbapenemase activity with a sensitivity (99%) and superior specificity (94%) equivalent to any recommended screening indicator of carbapenemase activity. Moreover, most carbapenemase-producing isolates grow up to the disc, and consequently these bacteria are easily detected. Interestingly, a double inhibition zone with colonies growing up to the disc was observed for isolates carrying OXA-48 carbapenemase.

Automated systems

Automated systems present poor specificity and cannot distinguish carbapenemase producers from isolates with porin loss and ESBL or AmpC overproduction[152]. These systems might not reliably detect all carbapenemase producers[154], particularly when a single carbapenem is tested[153]. Additionally, discrepancies arise with automated systems, such as Vitek2, which reported meropenem MICs for KPCs that were significantly lower than those obtained through broth microdilution[155]. OXA-48 producers are also poorly detected.

The modification of antibiotic testing panels for the inclusion of synergy tests between carbapenems and β-lactamase inhibitors, such as boronic acid for KPCs, cloxacillin for AmpCs and dipicolinic acid or EDTA for MBLs, would facilitate more stringent interpretive criteria and improve specificity[156].

Modified hodge test

The cloverleaf or modified hodge test (MHT) is based on the inactivation of a carbapenem using carbapenemase-producing organisms. Briefly, an inoculum of the indicator organism *E. coli* ATCC 25922, adjusted to 0.5 McFarland turbidity standards, is used to inoculate the surface of a Mueller-Hinton agar plate. After incubation for 10 min at room temperature, a disc containing 10 μg of meropenem is placed onto the agar plate. Subsequently, 3-5 colonies of the test organisms are inoculated in a straight line from the edge of the disc to the periphery of the plate. The growth of the indicator strain toward the meropenem disc is interpreted as a positive result for carbapenem hydrolysis.

CLSI recommends this test for screening purposes, except for *P. aeruginosa*. An optimization of MHT (PAE-MHT) using *K. pneumoniae* ATCC 700603 as an indicator has demonstrated 100% sensitivity and 98% specificity for detecting carbapenemase activity without indeterminate results, including KPC and carbapenem-susceptible MBL-producing *P. aeruginosa*[157].

The MHT works well for the detection of KPC and OXA-48 producers, but is time consuming, cannot distinguish the type of carbapenemase involved and, most importantly, false positive results have been observed with isolates producing CTX-M-type ESBL or AmpC β-lactamases at high level. Moreover, false negative results may occur, primarily among NDM producers. Adding zinc to the culture medium significantly improves the sensitivity in detecting NDM producers by increasing the stability of the enzyme or modifying porin expression[158]. The replacement of Mueller-Hinton agar with MacConkey agar also increases the sensitivity for MBL or OXA producers. The

enhanced detection reflects the presence of bile salts in the medium, which improve the release of periplasmic enzymes[159].

A modification of the MHT using discs containing carbapenem supplemented with boronic acid, which inhibits both class A carbapenemases and AmpC β-lactamases, can be used to reduce the number of false positives compared with the MHT results obtained using discs containing carbapenem alone or carbapenem supplemented with cloxacillin, which only inhibits AmpC enzymes. The proposed procedure results in high sensitivity and specificity for class A carbapenemase detection, regardless of the carbapenem tested. A low performance level is observed only among mucoid phenotype strains[160].

Overall, MHT, although remaining a convenient screening assay, cannot be used as the sole method for the detection of carbapenemase-positive isolates in the clinical laboratory.

Biochemical tests

Biochemical tests based on the detection of β-lactam ring hydrolysis have been developed for the early identification of carbapenemases in *Enterobacteriaceae* and *P. aeruginosa*.

The Carba NP test is a colorimetric test based on the detection of acidification resulting from imipenem hydrolysis coupled with inhibitors that reliably identify carbapenemase producers and discriminate between the three main types of carbapenemases (Ambler classes A, B and D). Ambler classes A and B are inhibited through tazobactam and EDTA, respectively, while class D production is deduced from the lack of inhibition.

This test is highly sensitive and specific, easy to perform and interpret, cost-effective, adaptable to any laboratory, and the results are obtained within 2 h. Additionally, the Carba NP test is cheaper and faster than molecular techniques and can be used to select isolates for subsequent analyses using polymerase chain reaction (PCR) methods[161,162]. False negative results have been observed using this test, particularly for OXA-48 and GES[163].

The Carba NP test has been evaluated to directly detect carbapenemase-producing *Enterobacteriaceae* from positive blood cultures, yielding high sensitivity and specificity, which might be useful for the rapid detection of carbapenemase producers in endemic countries and outbreak situations and for the early implementation of appropriate antimicrobial therapies[164,165].

The Rapid CARB Screen Kit (Rosco Diagnostica A/S, Taastrup, Denmark) is another method based on the identification of β-lactam ring hydrolysis in the presence of an indicator. This kit is technically easier to use, but the results are more difficult to interpret, and this assay does not discriminate carbapenemase types. Furthermore, the number of false positives and uninterpretable results present another major disadvantage of this assay. Thus, this kit can be used for the direct detection of carbapenemases in positive urine and blood samples.

Both tests should be used with caution in areas with high OXA-48 prevalence and should be evaluated in epidemiological settings where carbapenemases with lower hydrolytic activity might be detected, *i.e.*, IMP, GES or OXA-198[166].

The Blue-Carba test is a variation of the Carba NP test, validated for the direct detection of carbapenemase-producing strains from bacterial cultures. This test uses tienam instead of the more expensive imipenem monohydrate. The sensitivity and specificity of the Blue-Carba test are similar to those of the Carba NP test, but the former has an additional advantage of detecting *Acinetobacter* OXA-type carbapenemases[167].

Detection of MBLs based on chelating agents

The phenotypic detection of MBL producers in the clinical laboratory is based on the specific inhibition of MBLs using zinc-depriving compounds, such as EDTA, dipicolinic acid, and 1,10-phenanthroline or thiol compounds, such as 2-mercaptopropionic and mercaptoacetic acid.

The double-disc synergy test (DDST) employs a disc containing hydrolyzable β-lactam (typically a carbapenem, although ceftazidime has also been widely used) placed near a disc containing an MBL inhibitor. The formation of a synergistic inhibitory zone indicates MBL production[168].

Alternatively, in the combined disc test (CDT), the inhibition zone of a disc containing β-lactam combined with an inhibitor is compared with that of a disc containing β-lactam alone. An increase in the inhibition zone diameter above a predefined cut-off value indicates MBL activity[169].

Based on similar principles, gradient diffusion methods (*e.g.*, IP/IPI Etest, bioMérieux, France) use strips containing imipenem and imipenem plus EDTA. A MIC reduction greater than or equal to eight-fold in the presence of EDTA indicates MBL activity[170,171]. New E-test strips containing other inhibitors or carbapenem molecules would facilitate MBL detection, but these strips are not yet commercially available.

Another strategy to facilitate easy interpretation, regardless of the carbapenem MIC, is the use of an inhibitor-impregnated agar. EDTA-impregnated agar is prepared by spreading 2 mL of a 5 mmol/L EDTA solution on the surface of a mueller Hinton agar plate (MH). Differences in the inhibition zone of the carbapenem discs are compared between inhibitor-impregnated and a regular MH agar, searching for differences $\geq$ 10 mm. This technique facilitates the easy detection of simultaneously associated mechanisms of β-lactam resistance and carbapenemase production in clinical isolates[172].

When using methods based on β-lactam-chelator combinations, it is important to consider the potentially detrimental effects of chelating agents on bacterial

growth, which might yield false positive results.

Detection of class A carbapenemases based on boronates

The phenotypic detection of class A carbapenemase production is based on the susceptibility of these enzymes to boronic acid and boronic acid derivatives, *i.e.*, phenylboronic and 3-aminophenylboronic acid (PBA). For MBL detection, DDST, CDT and carbapenemase PBA-impregnated agar techniques are used.

PBA and PBA-combined discs are prepared as previously described[173]. To prepare PBA-impregnated agar, 750 μL of PBA at 10 mg/mL is spread onto a Mueller Hinton agar plate[172].

Because boronic acid derivatives also inhibit AmpC-type β-lactamases, the simultaneous use of cloxacillin, which only inhibits AmpC but not class A enzymes, enhances the specificity of these tests. MH agar containing cloxacillin and discs containing boronic acid alone or meropenem in combination with boronic acid or cloxacillin are also commercially available (MAST, United Kingdom; Rosco, Denmark).

Notably, isolates with both KPC and metallo-beta-lactamase enzymes might not show synergy in inhibitor-based assays and appear highly resistant to carbapenems.

Detection of OXA-48 based on temocillin-resistance

CLSI and EUCAST interpretative criteria exhibit poor sensitivity in the detection of OXA-48 producers. The zone diameters for meropenem in OXA-48-producers can be up to 26 mm, thus < 27 mm must be used as a screening cut-off in countries where OXA-48 is endemic, at the expense of lower specificity[149].

Because OXA-48 carbapenemases confer high-level temocillin resistance, a disc diffusion assay using discs containing 30 μg of temocillin provides a reliable phenotypic test for the detection of OXA-48 in *Enterobacteriaceae*[174].

The combination of both temocillin and piperacillin/tazobactam diameters, < 12 mm and < 16 mm, respectively, is used to identify OXA-48 *Enterobacteriaceae* producers with high sensitivity and specificity[175].

Detection using chromogenic media

Several selective chromogenic media, in which species are distinguished according to colony color, are commercially available.

CHROMagar-KPC medium lacks sensitivity, as this medium only detects carbapenemase producers resistant to high levels of carbapenems. Thus, the detection of low-level resistance, as observed with KPC, IMP, VIM, NDM, and OXA-48, might not be achieved using this medium.

Supercarba medium is a Drigalski agar-based culture medium containing a low concentration of ertapenem, cloxacillin to prevent the growth of non-carbapenemase-producing carbapenem-resistant isolates, and zinc sulfate to improve the expression of MBLs. This medium might be used for the detection of not only KPC and MBL producers but also OXA-48 producers that do not co-express ESBLs. Supercarba medium exhibits higher sensitivity and specificity than other media and is useful for the specific selection of carbapenemase-producers in stools, as this medium inhibits the growth of ESBL producers.

CHROMagar-KPC and Supercarba medium are only suitable for *Enterobacteriaceae* testing because non-enterobacterial non-carbapenemase-producing Gram-negative rods grow on these media[176].

ChromID CARBA is also designed for the specific detection of carbapenemase-producing *Enterobacteriaceae*, and the sensitivity and specificity of this medium is high. ChromID CARBA inhibits all ESBL producers and has demonstrated the excellent detection of CPE, with MICs from 2 to > 32 mg/L, however OXA-48 was only detected at a high inoculum (10^7 CFU/mL)[177].

Oxoid Brilliance CRE Agar exhibits high sensitivity for the detection of KPC and MBL-producing *Enterobacteriaceae*, but lower sensitivity if observed for the detection of OXA-48 producers. The specificity of this agar is relatively low, reflecting the growth of AmpC and ESBL producers that require further confirmation[178].

ChromID CARBA SMART is a new bi-plate combining two chromogenic media to rapidly and reliably identify all carbapenemase-producing *Enterobacteriaceae*, particularly KPC, NDM-1 and OXA-48; however, this medium has not yet been evaluated[179].

Molecular detection of carbapenemase genes

Molecular techniques, primarily based on PCR, are considered the reference standard for the identification and differentiation of carbapenem resistance genes based on the excellent specificity, sensitivity, accuracy and rapidity of these methods. If identification of a carbapenemase is required for epidemiological purposes, then the PCR products are subjected to additional sequencing. These techniques generate results within 4-6 h, or even less when real-time PCR techniques are used. The main disadvantages to the use of these techniques are high costs, requirement of trained technicians, and inability to detect novel carbapenemase genes.

Simplex PCR assays, using various primers and several in-house and commercial multiplex PCR tests, facilitate the simultaneous identification of multiple carbapenemase types[4,180,181], but the development of methods that further shorten the detection time, such as real-time systems, is needed[182,183].

The Xpert MDRO assay (Cepheid, Sunnyvale, CA) uses GeneXpert cartridges for the detection of carbapenemase-producing Gram-negative bacterial colonization directly from rectal swab samples in 47 min[184].

For the detection of the main carbapenemase gene types, PCR and hybridization-based kits, such as Hyplex MBL ID and Hyplex CarbOxa ID kits (BAG Health Care,

Lich, Germany), are commercially available.

Microarray technology facilitates the rapid and reliable identification of multiple resistance determinants. Check-KPC ESBL and Check-MDR CT102 microarray analyses (Check-Points Health BV, Wageningen, Netherlands) have been successfully used for the detection of most clinically relevant carbapenemases within a single reaction[185].

Spectrometry

Spectrometry is based on a reduction in imipenem absorbance in the presence of carbapenemase-containing extracts of bacterial enzymes at a wavelength of 297 nm. This extract is obtained from an overnight culture of the isolate through mechanical lysis using sonication. Spectrometry accurately differentiates between carbapenemase producers and carbapenem-resistant bacteria with non-carbapenemase-mediated resistance mechanisms. Moreover, this method is cheap compared with molecular techniques and has excellent sensitivity and specificity (100% and 98.5%, respectively). However, spectrometry does not discriminate between different types of carbapenemases, is time-consuming and requires trained microbiologists[186]. Therefore, this technique is considered a reference method for the confirmation of carbapenemase activity: however, spectrometry is laborious and technically demanding, which limits the use of this method in reference laboratories.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) is based on the detection of enzyme activity through the direct detection of hydrolysis degradation products, which show a different molecular mass from that of the native molecules[187].

Antibiotics and their degradation products are typically small molecules (< 1000 Da), making the analysis of these compounds difficult because the matrix is also visible in mass spectra and produces interference with high background levels. Thus, for the detection of these molecules, different matrices and modified approaches for sample preparation have been described[188,189].

The MALDI-TOF methodology involves the suspension of a fresh bacterial culture in a buffer, followed by centrifugation. The pellet is subsequently re-suspended in a reaction buffer containing carbapenem. After 1-3 h of incubation at 35 ℃, the reaction mixture is centrifuged, and the supernatant containing carbapenem and carbapenem metabolites is assessed through MALDI-TOF. Carbapenem activity is determined based on the disappearance of the peak corresponding to native carbapenem and the appearance of peaks corresponding to metabolites as a result of carbapenem hydrolysis[190]. All reported studies have presented similar results, demonstrating the excellent sensitivity and specificity for all carbapenemases, including OXA-type carbapenemases in *Enterobacteriaceae* and *A. baumannii*[191,192].

Manual measurements and analyses of raw spectra, however, can be difficult and require trained microbiologists. Therefore, the availability of software for the automatic acquisition and interpretation of results is desirable in diagnostic laboratories.

The direct detection of carbapenemase activity from positive blood culture is currently being evaluated, having achieved adequate sensitivity[193].

FIGHTING CARBAPENEMASES

The fight against carbapenemase-producing bacteria should be based on three pillars: preventing transmission, avoiding antimicrobial selective pressure and developing new treatments.

Preventing transmission is presently the starting point to win this war. Therapeutic options are limited, particularly for NDM and KPC producers, making prevention the best strategy to avoid life-threatening infections, prolonged hospital stays and increased health care costs[146].

The first step in the prevention of transmission is to emphasize adherence to hand hygiene and standard precautions in daily clinical practice. In addition, the early identification of carriers is needed to apply control measures. Early detection can be achieved passively, selecting potential carriers through risk factor evaluation, or actively, through microbiological screening techniques.

The risk factors for carbapenem-producers are similar to those other multi-drug-resistant bacteria (MDR), including hospitalization, critical illness, surgery, ICU stays, the use of invasive devices and prior exposure to antimicrobial treatments. Recent travel to endemic countries is also important, particularly for medical tourism and military conflicts[194-196].

The active screening of patients is the gold standard to identify carriers. The number and type of patients investigated, the use of cultures, biochemical or molecular tests, and the duration of the surveillance program will depend on the economical characteristics of the country and whether the infection represents an outbreak event or an endemic situation[146].

Once the carriers have been identified, contact precautions and pre-emptive contact isolation must follow. Additionally, environmental decontamination and patient decolonization are recommended[129,197].

Antimicrobial stewardship to avoid antimicrobial selective pressure should be mandatory in sanitary institutions. Antimicrobials act as selectors and accelerators of diversity in resistance mechanisms, promoting MDR and selecting new resistance enzymes[78,198]. Thus, the high prevalence of ESBL-carriers in India encouraged the use of carbapenems as empirical treatment and selected carbapenemase-producers[199]. Nevertheless, this issue is controversial and more research is needed[146].

Finally, the third and most important pillar for

clinical use against carbapenemase producers involves the development of new molecules that are either active against these pathogens or act as inhibitors of carbapenemases, which might extend the life of existing antimicrobials.

New antimicrobials are being developed to fight MDR. Sulfactams are monocyclic β-lactams structurally related to monobactams, such as BAL30072, which exhibit potent activity against MDRs, particularly when combined with colistin or meropenem[200]. Plazomicin is a new-generation aminoglycoside with bactericidal effects on carbapenem-resistant bacteria, although more studies are needed to assess the activity of this antimicrobial[201,202]. TP-434 and GSK2251052 are antimicrobials that target protein synthesis and exhibit a spectrum encompassing multidrug-resistant Gram-negative pathogens[203].

Moreover, carbapenemase inhibitors have also been developed, including Penem derivatives, such as BRL 42715, which inhibit serine β-lactamases, and BLI-489, which inhibits KPC; 1-β-methylcarbapenem compounds, such as J-110,411 and J-11,225, which inhibit classes A and C and IMP-type enzymes; Thiols, such as thiomandelic acids, which inhibit MBLs; and avibactam, which inhibits serine β-lactamases. These compounds will likely extend the life and increase the activity of existing antimicrobials and bring hope in the carbapenemase era[19,204].

CONCLUSION

Carbapenem-resistant bacteria, particularly *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter*, have become a challenging health issue worldwide. Fourteen years have passed since Livermore *et al*[205] published the article entitled "Carbapenemases: a problem in waiting?". These authors discussed the emergence of new carbapenem-hydrolyzing enzymes and the possibility that these molecules could pose a threat to infection treatment in the near future. Five years later, Walsh *et al*[206] warned about the perils of MBLs, remarking on the spread of these enzymes through horizontal gene transfer and resistance mechanisms to other antimicrobial families. These authors suggested the development of both surveillance programs and new inhibitors.

Moreover, in 2012, Nordmann *et al*[207] published an article entitled "Carbapenem resistance in *Enterobacteriaceae*: here is the storm!", stating that carbapenemase-producing *Enterobacteriaceae* constituted a growing threat to public health.

To paraphrase John Donne, "no man is an island", in this case "no country is an island". Thus, now that the storm has arrived, international organizations, such as the World Health Organization, Centers for Disease Control and Prevention and European Centre for Disease Prevention and Control, have called for a worldwide collaboration to contain this epidemic[146]. Migration fluxes, vacations and medical travels or distant military conflicts likely contribute to the spread of resistance genes across the globe, and only through a collaborative global effort, will we successfully address this problem.

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REVIEW

Hepatocellular carcinoma and multidrug resistance: Past, present and new challenges for therapy improvement

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Abstract

Hepatocellular carcinoma (HCC) is the most frequent form of liver cancer and the third most common cause of cancer-related death in the world. The main risk factor worldwide for this type of malignancy is chronic hepatitis caused by hepatitis B virus and hepatitis C virus infections. Advances in early detection and treatment have improved life expectancy of patients with HCC. However, this disorder remains as a disease with poor prognosis. In fact, epidemiological studies have revealed that there is an 8-mo median survival rate in patients, approximately 20% of whom survive one year while only 5% remain alive after three years. Additionally, HCC is particularly difficult to treat because of its high recurrence rate, and its resistance to conventional chemotherapy is due, among other mechanisms, to several members of the ATP-Binding Cassette protein family involved in drug transport being overexpressed. Fortunately, there is evidence that these patients may benefit from alternative molecular-targeted therapies. This manuscript intends to provide further insight into the etiology and molecular mechanisms related to HCC development and the latest therapeutic approaches to treat this malignancy. The development of effective delivery systems of antitumor drugs able to target the liver parenchyma is also assessed. Finally, the prospects in the development of more efficient drug therapies to overcome multidrug resistance are also examined.

Key words: Hepatocellular carcinoma; Therapy; Multidrug resistance; Drug delivery systems; Liver targeting

Core tip: Hepatocellular carcinoma (HCC) is the most frequent malignancy of the liver. Despite the advances in early detection and treatment, this disorder still has a poor prognosis. This manuscript reviews the ongoing knowledge regarding the etiology and molecular mechanisms implicated in HCC development and the therapeutic strategies for the management of this malignancy. Finally, the development of effective delivery systems of antitumor drugs able to target the liver parenchyma as well as the prospects in the development of a more efficient drug therapy to overcome multidrug resistance are also examined.

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INTRODUCTION

Liver cancer, which is ranked fifth in frequency of occurrence worldwide and third in cancer mortality, is one of the most frequent types of cancer[1]. Hepatocellular carcinoma (HCC) represents 85%-90% of primary liver cancers and is the main subtype in terms of histologic origin. Its clinical course is aggressive, while frequent recurrence and metastasis are often associated with this malignancy. It is characterized by late presentation, fast progression, limited response to therapy and a very poor survival rate (6%)[2]. Asia and Africa are the countries where HCC is more prevalent; however, there has been a rising trend of HCC in Western countries[3]. Chronic liver diseases, such as chronic hepatitis B (CHB) and CHC[4] are among the major risk factors for HCC development. Other common causes leading to the development of this malignancy are: hemochromatosis, fatty liver diseases unrelated to alcohol consumption (non-alcoholic fatty liver disease), primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis, excessive alcohol use, ingestion of food contaminated with aflatoxin, vinyl chloride, and use of radioactive agents such as thorotrast[5,6].

The development of HCC involves several steps of a complex process characterized by both genetic and epigenetic changes that may activate cellular oncogenes, inactivate tumor suppressor genes and/or dysregulate multiple cell signal transduction pathways, such as the Wnt/β-catenin, the Ras-Raf-mitogen-activated protein kinase (MAPK), the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and the transforming growth factor-β (TGF-β) pathways[7-9].

Several potentially curative or palliative approaches to the treatment of HCC are available. The surgical approaches that are most commonly chosen are: surgical resection and orthotopic liver transplantation. However, preserved or adequate liver function is an essential criterion for surgical resection. In this regard, this surgical approach is not a feasible option for HCC patients[10] when the tumor is at an advanced stage, or is located in close proximity to important hepatic vessels within the liver preventing a negative-margin resection, or when there are tumors at multiple sites or there is inadequate remaining hepatic function. Furthermore, about 17%-69% of patients suffer from recurrence, thus limiting their long-term survival at 5 years postoperatively[11]. Orthotopic liver transplantation is considered to be the only curative solution for HCC that cannot be surgically removed. Candidates for this procedure are those patients having solitary HCCs of less than 5 cm in size or up to three nodules, each smaller than 3 cm[12,13]. Nevertheless, this procedure has limited availability due to the great difficulty in finding organ donors[10].

Non-surgical therapeutic approaches for HCC such as radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), transarterial embolization (TAE) and transarterial chemoembolization (TACE) are other therapeutic tools used to substitute first-line procedures; however, the probable course of the disease for the patients undergoing such procedures is still bleak. The annual recurrence rate is approximately 15%-20%, reaching 80%-90% within the 5-year post-treatment period[14,15]. A research study confirms that so far there are no adjuvant therapeutic postoperative regimens to successfully treat HCC.

A clinical investigation indicates that none of the adjuvant therapies is particularly effective in the treatment of HCC after surgery[16]. Systemic chemotherapy with doxorubicin, immunotherapy using interferon and hormonal therapy with tamoxifen, on the other hand, yielded poor results, with no significant survival benefits compared with symptomatic management[17-19]. One important limitation in the chemotherapy for HCC is the emergence of multidrug resistance (MDR) to conventional anti-tumoral agents[20]. This phenotype is commonly related to cancer cells that are able to overexpress drug transporter proteins belonging to the ATP-binding cassette (ABC) superfamily of proteins that move drugs out of cells, such as P-glycoprotein (P-gp), the multidrug resistance-associated proteins (MRPs) and the breast cancer resistance protein (BCRP)[21]. Additionally, current anti-tumoral drugs used in HCC treatment, also promote significant toxicities in other non-target organs affecting patient compliance and adherence to these therapeutic regimens[22]. Enhanced delivery[23] of these commercially available anti-cancer agents to liver parenchyma may provide an opportunity to selectively improve the efficacy of the current therapies and simultaneously reduce the adverse effects that often lead to treatment failure.

Up to now, no succesful systemic chemotherapy for patients with advanced and unresectable HCC is available. However, on November 16, 2007, the Federal and Drug Administration (FDA; United States) had approved sorafenib tosylate (Nexavar® tablets, made by Bayer Pharmaceuticals Corp.), as "a small molecule Raf kinase and VEGF receptor kinase inhibitor, for the treatment of patients with unresectable hepatocellular carcinoma (HCC)". Unfortunately, this promising treatment has demonstrated limited survival benefits with very low response rates[24,25]. Therefore, new approaches are urgently needed for: (1) improving the activity of prevailing antichemotherapeutic agents by targeting them to the liver using drug delivery systems designed with targeting moieties; (2) overcoming MDR by sensitizing tumor cells to conventional chemotherapeutics; and (3) improving the prognosis of HCC by

further development of the latest molecular targeting agents, such as sorafenib and rapamycin which - although limited - at present are deemed to be the most effective agents for managing unresectable HCC.

In this analysis we review the available information regarding the latest pharmacotherapy options for the treatment of patients suffering from advanced HCC, including molecular targeting agents. Prospects regarding a more effective pharmacotherapy for liver targeting and avoiding/preventing multidrug resistance in cancer cells are also addressed.

CURRENT THERAPIES FOR HCC

Unfortunately, owing to the asymptomatic nature of early HCC, in a majority of patients, HCC is usually diagnosed at an advanced stage, when most potentially curative therapies such as surgical resection, orthotopic liver transplantation and local ablation display a low efficacy. Moreover, in 60% to 80% of patients with liver cancer, the treatment is complicated by underlying liver cirrhosis and hepatic dysfunction[26]. In these advanced stages, systemic treatments are commonly used; however, they are also minimally effective, have severe side effects, develop high drug resistance, and most importantly, patient survival is not improved. HCC is rarely amenable to radiotherapy, leaving this disease with no effective therapeutic options and a very poor prognosis[27]. Through better understanding the molecular basis of hepatocarcinogenesis (*e.g.*, signal pathways and molecular alterations that promotes tumor growth and cell survival), new treatment modalities have recently emerged including molecular targeted therapy and gene therapy, such as antisense gene targeting.

Surgical therapies

At present, surgical resection and orthotopic liver transplantation offer the only chance for long-term cure of patients suffering from HCC. Surgical resection is an effective treatment for those patients with HCC that is not associated with liver cirrhosis or in patients whose hepatic function is well compensated. This means, that surgical resection is an option for only a small proportion of patients, less than 18%, since underlying chronic liver disease or cirrhosis accounts for about 85%-90% of HCC patients[28]. Thus, both tumor extent and hepatic function must be evaluated pre-operatively to avoid hepatic failure following resection, which is usually a fatal condition possibly requiring urgent liver transplantation. There is a 5-year post-operative survival rate of 40%-70% of duly selected candidates for liver resection; however, relapse takes place in near 70%, especially in patients with cirrhosis[29]. For this reason, orthotopic liver transplantation is considered to be the best choice for those patients suffering from HCC and cirrhosis, showing 5-year post-transplant survival rates of 65%-80% among well-selected candidates. Nevertheless, there is a limitation as to the use of this procedure, since the shortage of human donors is an unfortunate event these days[10]. Due to the strictness of Milan criteria regarding transplantation and the restrictions in finding available donors, scientists are now devoted to exploring other therapies for managing the disease in order to provide a solution for the disadvantages arising from transplantation or surgical resection[30].

Non-surgical therapies

Locoregional therapies: Percutaneous treatments: Percutaneous ablation (PA) is now the first alternative treatment when resection or orthotopic liver transplantation has been ruled out in patients suffering from early-stage HCC. PA can be thermal or chemical. The thermal ablation procedure destroys cancer cells by cryoablation or by heat using lasers, high intensity focused ultrasound, microwaves or radiotherapy. Chemical ablation consists on cancer cells destruction by injecting chemicals - *e.g.,* ethanol/acetic acid - introduced into the tumor mass by means of a very fine needle[10]. The ablative method as a treatment of choice will be based on the size of the tumor.

PEI was introduced in the 1980's, nowadays being the most prevalent kind of PA for treating HCC. Cohort studies and retrospective series analysis have shown that a five - year survival might be possible in 50%-80% of patients having a single tumor smaller than 5 cm in size or up to three nodules, lesser than 3 cm in size[31]. The main drawback of PEI is the high local recurrence rate (33%-43% at three years)[32]. RFA (radiofrequency ablation) is another ablative procedure initially outlined by Rossi *et al*[33], in 1993, and since then, it has become the favorite form of ablation for small tumors. When comparing RFA with PEI, the former showed to be better in relapse prevention and in improving tumor necrosis[30]. However, surgical resection is so far very superior to PA techniques.

Chemoembolization: TAE is another locoregional palliative treatment option in cases where surgical resection or other forms of treatment with curative potential are not advised for specific HCC tumors. The hepatic artery is responsible for the supply of blood to the tumor; therefore, the obstruction caused by TAE produces extended tumor necrosis as a result of ischemia, thus providing the rationale for its wide use in patients with HCC[34]. When this procedure is performed in combination with chemotherapeutic agents such as doxorubicin and cisplatin, usually mixed with lipiodol, it is termed TACE. The addition of chemotherapy aims to enhance the anti-tumoral action of ischemia. Usually, in TACE, anti-neoplasic drugs are mixed with lipiodol. By injecting the patient with a combination of anti-tumoral agents with the radio-opaque contrast agent lipiodol into the hepatic artery, drug delivery to tumor cells is expected to increase. Likewise, the chances of systemic side-effects related to chemotherapy are expected to decrease. Unfortunately, the use of

either TAE alone or TACE, remains a controversial treatment approach for patients with HCC, because some randomized controlled trials have failed to disclose a significant benefit in terms of survival of treated patients as compared with untreated patients[34,35]. Moreover, several studies demonstrated disappointing results, showing that TACE enhances intrahepatic and extrahepatic metastases, and even reduce survival[36]. Accordingly, anti-angiogenic therapy enhances the efficacy of transcatheter arterial embolization for HCC hepatocellular carcinomas[37].

Furthermore, severe side-effects produced by the arterial obstruction and by the toxicity of the injected anti-tumoral agents during the TACE procedure, counteract the anti-tumoral action resulting from arterial obstruction. It should be highlighted that the absence of effects due to chemotherapy is not the result of ineffective drug delivery but of the presence of MDR due to the over-expression of efflux pumps that belongs to the ABC superfamily of protein transporters, as well as to an abnormal p53 function that leads to an inhibition of apoptosis making tumor cells resistant to anti-tumoral treatment[38-40]. Dysfunctional p53 makes tumoral cells also less sensitive to hypoxia.

Radiation therapy

External beam radiation therapy: Before the 1990s, external beam radiation therapy (EBRT) has played a minor role in the primary treatment of HCC. However, EBRT was mainly used in the palliative setting for metastatic disease because of an intolerance of the adjacent normal liver to tolerate radiation that precluded a more intense use of radiation[41].

In 1987, the radiation therapy oncology group outlined the outcomes of a randomized clinical trial including radiotherapy of the whole-liver with a dose of 21 Gy in seven fractions or combined with the radiosensitizer misonidazole[42]. Although a whole-liver EBRT provided a significant palliative effect, the addition of misonidazole did not significantly improve the outcomes[43].

The dose-limiting complication of delivering EBRT to the liver is radiation-induced liver disease (RILD) a clinical entity characterized by the presence of anicteric hepatomegaly and ascites (associated with high levels in sera of hepatic enzymes) that may lead to liver failure and death[44]. Due to this reason, several approaches were designed by researchers at the University of Michigan to administer higher radiation doses to smaller liver portions, in order to produce greater tumor control rates without an increase in the damage to the liver parenchyma that is likely to be caused by radiation[45].

Based on the above, with the advent of intensity-modulated radiation therapy, image-guided radiation therapy and stereotactic body radiation therapy (SBRT; as described below, separately), higher doses could be delivered safely since the radiation dose can be distributed tightly into the tumor while preserving normal tissue in the liver from the effects of high doses of radiation[41].

Selective internal radiotherapy: Intrahepatic radiotherapy, better known as radioembolization or selective internal radiation therapy (SIRT), is a therapy based on the intrahepatic delivery of Yttrium-90 (Y-90)-labeled microspheres into the arteries that supply blood to the tumor, where the microspheres come into contact with tumor cells which are hit by radiation emitted by the radioisotope[46].

The microspheres are an implantable medical device consisting of resin-based or glass-based biocompatible microspheres loaded with Y-90[47,48].

The process of release of the microspheres occurs by using a flexible catheter inserted into the femoral artery which is moved forward by the radiologist until the hepatic artery is reached[47,49].

SIRT demonstrated an 89% treatment response with resin microspheres and 78% with glass microspheres, respectively, in patients suffering from HCC[49]. The median overall survival ranged 16.4-18 mo[50,51].

SIRT is a minimally invasive technique and a well-tolerated therapy. It is a new therapy for treating liver cancer and liver metastases originated from colorectal cancer.

Finally, SIRT represents a new therapeutic option for patients with unresectable HCC. Clinical studies showed an increase in terms of survival when this technique is used in combination with chemotherapy. Noteworthy, SIRT tends to reduce the size of the tumor and allows some patients to become eligible for surgical resection[52].

SBRT: As a means to ablate primary or metastatic liver tumors, technical advances in tumor localization and motion management were achieved.

SBRT has become an optimistic approach for the treatment of liver cancer as a result of the complex character of liver tumor motion along with the priority of decreasing the volume irradiated to the minimum to reduce the probability of RILD.SBRT[45].

Focal, high dose SBRT delivers ablative doses in fewer fractions and highly conformational radiotherapy volumes[43].

To avoid damaging nearby critical structures and organs, doses are minimized using tight margins. A robust immobilization device is thus crucial to achieve a reproducible and accurate setup. Image guidance can be accomplished by using a megavoltage/kilovoltage cone beam computed tomography (CT) or stereoscopic X-rays[43]. The local tumor control of SBRT turns out to exceed that of conventional fraction EBRT[42].

A retrospective analysis carried out by Choi *et al*[53], demonstrated that a dose of 50 Gy of SBRT in 5 or 10 fractions for primary liver tumor produced a median survival of 20 mo**.** Another study carried out by Tse *et al*[54] using SBRT at a dose of 24-54 Gy in 6 fractions, demonstrated that the median survival rate turned out

to be 13.4 mo.

Although liver metastasis is not the subject of the present review, it is noteworthy to point out that survival outcomes are better in patients with liver metastasis than with HCC. In both groups, there appears to be a dose-response for local control. For HCC, the dose of SBRT should be based on the cirrhotic status. For patients with Child-Pugh A cirrhosis, 48 Gy or higher distributed in 3 fractions is recommended. For patients with Child-Pugh B cirrhosis, more fractionated schemes are suggested (5 fractions of 40 Gy, for example). For liver metastases, doses greater than 48 Gy divided into 3 fractions or 14-26 Gy in one fraction is recommended[43].

Finally, with the use of innovative tools combined with radiotherapy such as advanced imaging and immunotherapy, further advances in liver cancer could be achieved. Research is under way to analyze the way of optimizing radiation delivery by using other procedures such as TACE and sorafenib administration[45].

Systemic treatments

Hormonal therapy: Since 15%-39% of HCC express estrogen receptor (ER), and overexpression of the progesterone receptors was detected in up to 39% of tumors, in the last decades there have been clinical trials with tamoxifen for patients with HCC[55,56]. However, later studies have shown that patients suffering from HCC and receiving tamoxifen did not have a survival benefit, reaching the conclusion that this anti-estrogen drug, either alone or in combination with other chemotherapy agents to treat advanced HCC is ineffective. According to Di Maio *et al*[57], a possible explanation for these unfavorable results resides on the selection of the patients in the clinical trials, since none of them had selected patients based on the expression-status of the hormonal receptor. Therefore, this constitutes a significant problem. It should be pointed out that in breast cancer, for example, it is well known that the adequacy of hormonal treatment is pertinent; however, it is only restricted to those patients having tumors with expressed hormone receptors. Moreover, in some HCC patients, a variant form of the ER alpha (vER) transcript derived from an exon 5-deleted transcript lacking the hormone-binding domain of the receptor, yet having an intact DNA-binding domain keeps constitutive transcriptional activity. These tumors with vER, which account for an important percentage of HCCs, have a bleaker prognosis characterized by faster doubling time and shorter survival[57]. Tamoxifen is ineffective in the treatment of tumors with vER because tamoxifen is not able to bind to the receptor. Thus, by choosing anti-hormonal treatment according to the presence of wild-type or variant ERs in the tumor, a significant improvement to the response rate to tamoxifen is observed[58]. Efficacy of megestrol acetate has been tested in HCC tumors expressing vER in a randomized study of 45 patients with advanced HCC. Although in this study it was observed that megestrol notoriously increases survival in this reduced group of patients (untreated patients: 7 mo; patients treated with megestrol: 18 mo)[59], an adequately powered randomized trial should be carried out to confirm these results.

As in the case of estrogens, it has been proved that androgens positively influence HCC growth; thus, androgens or luteinizing hormone-releasing hormone agonists (nilutamide, goserelin acetate, triptorelin, flutamide, leuprorelin) will possibly play a part in treating HCC. However, no benefit in terms of survival was found with anti-androgenic treatment in male patients with advanced HCC[60,61].

Finally, hormonal compounds have proved to be totally ineffective as regards patient survival. Although tamoxifen and anti-androgen drugs failed to prolong survival in advanced HCC cases, somatostatin -whose receptor is expressed in HCC - and its synthetic analogs like octreotide may play a role in prolonging survival in patients with advanced disease[57]. However, the results obtained so far are conflicting; therefore - as in the case of megestrol - further studies are required.

Systemic chemotherapy: Many patients seek systemic chemotherapy and for more than 50 years, conventional systemic cancer chemotherapy has been developed with the so-called anti-tumoral agents. However, in patients with HCC, the role of chemotherapy is quite limited due to inefficacy and toxicity of these antineoplastic drugs[62]. Single chemotherapy with cytotoxic agents such as cisplatin or 5-fluorouracil showed a low response rate (< 10%) without a clear benefit in overall survival[63]. In a recent clinical trial involving a large number of HCC patients, systemic administration of doxorubicin has provided a very low response rate (4%)[64].

Combination therapy is broadly regarded as a treatment option and used in oncology practice to enhance the efficacy of systemic chemotherapy. Moreover, it is the only treatment choice for those patients in whom unresectable HCC is not feasible for intra-arterial treatment. Although many regimens have not proved to be efficient for HCC patients, the combination of doxorubicin with paclitaxel (a microtubule stabilizer deemed to be one of the leading anti-tumoral agents in the past 10 years) showed a synergistic anti-tumor activity *in vitro* and *in vivo*[65].

A randomized phase Ⅲ trial assessing doxorubicin combination chemotherapy (cisplatin, interferon, doxorubicin and 5-fluorouracil, PIAF) revealed a higher overall response rate and better survival rates than those of patients receiving doxorubicin; unfortunately, these differences were not statistically significant. Moreover, increased toxicity was also related to PIAF[66].

The result in a double-blind phase Ⅱ multinational study assessing the treatment using sorafenib plus doxorubicin was greater median time to progression, overall survival and progression-free survival than doxorubicin monotherapy with treatment using sora-

fenib[67]. However, the combination therapy of sorafenib and doxorubicin is not yet indicated for routine clinical use.

The poor response nature of HCC to systemic chemotherapy is mainly due to its extreme chemo-resistance. Overexpression of several members belonging to the ABC-transporters superfamily leads to its MDR phenotype. At present, there is an intense search of agents for overcoming MDR, as it is discussed in the last section.

Immunotherapy: Immunotherapy is considered to be a possible treatment choice for those suffering from HCC, mainly as a second-line treatment to prevent relapse. In accordance with previous studies, there is direct correlation between patient survival and the the type and number of immune cells infiltrating the tumor, which indicates that there is a direct effect of immune responses on the disease evolution[68].

Immunotherapy represents an attractive alternative tool based on sensitivity, specificity against tumor cells, on the immune system capacity to renew itself, and its potential to eradicate residual tumors after conventional treatment. Therefore, results from several clinical trials have shown that immune-based therapy can improve outcomes in patients with HCC[69].

A randomized clinical trial demonstrated that there were statistically significant improvements in relapse time and relapse-free survival with the administration of interleukin 2 (IL-2) and anti-CD3 activated peripheral blood mononuclear cell in HCC patients that underwent surgical resection[70].

Interferons (IFNs) have immunomodulatory and anti-proliferative activities on tumor cells, and are widely used as therapy for neoplasias and viral diseases[71]. A randomized study carried out by Lai *et al*[63], reported that recombinant IFN-α turned out to be superior to doxorubicin in terms of survival, tumor response and toxicity in patients with unresectable HCC, both in prolonging survival and in inducing tumor regression.

One area of active research is immunotherapy with cytokine-induced killer cells (CIK)[71]; unfortunately, its efficiency is limited because of its low specificity to cancer cells. Another approach is the tumor-associated antigen (TAA)-pulsed dendritic cells (DC) therapy, but the outcomes remain unsatisfactory due to the poor immunogenicity of TAA that make tumor cells to fail to adequately stimulate DCs for effective presentation to immune cells[72]. A possible method for increasing the uptake of TAAs by DCs is to complex them with an IgG antibody, so that the resulting immune complexes may bind to Fcγ receptors (Fcγ-Rs) on DCs and induce phagocytosis of TAAs, leading to an effective immune response against the tumor cells[73]. Such targeting strategy was achieved by complexing the tumor cell membranes expressing α-Gal epitopes (Gal-α1, 3Gal-β1, 4-GlcNAc-R, α-Gal) with the anti-Gal IgG antibody (a natural antibody comprising 1% of IgG in humans)[74]. This opsonized binding complex may be phagocyted by DC and then enhance TAA presentation to naïve T or CIK cells, which are then activated and attack the remaining tumor cells *in vivo*[75]. In this study, the authors demonstrated that this anti-tumor vaccine could significantly increase the tumor-specific immune responders in circulation and the survival of advanced HCC patients (17.1 mo *vs* 10.1 mo in control groups) with no serious side effects.

In addition, results from a larger trial testing infusion of antigen-presenting cells that included 31 HCC patients receiving autologous tumor lysate-pulsed DC, showed an important 1 year survival (63% *vs* 10%), which supports the idea of immunotherapy for HCC based on DC[76].

Immunotherapy was also supported by rat models, since it was shown that there was a reduction in HCC relapse when administering DC in combination with IL-12 activated T and NK cells[77].

Antigen-specific immunotherapy and Treg ($CD25^+$ T-cells) depletion are worth mentioning as promising plans of action in physiologically important HCC preclinical models[68]. For example, immunization with a DNA-based synthetic vector (DNAmAFP/704) as an antigen-specific approach for targeting α-fetoprotein (AFP) proved to considerable reduce (65%) the tumor burden in an autochthonous model of a chemically produced hepatocarcinoma. Similarly, $CD25^+$ T-cell depletion by injecting the PC61 antibody significantly protected against tumor growth in an orthotopic HCC model[68]. Treg-depleting reagent Denileukin diftitox (Ontak) targets the constitutively expressed molecule CD25, thus producing the elimination of circulating Tregs without coordinating depletion of activated CD25-expressing T effector cells[78].

Another research work reported that the *ex vivo* treatment of $CD8^+$ T cells isolated from HCC patients with CTLA-4 blocking antibodies (ipilimumab) produced an expanded antigen-specific T cell repertoire, suggesting that this monoclonal antibody is likely to be highly effective in the treatment of HCC[79].

Direct reactivation of hyporesponsive tumor-specific T cells by providing T cell growth factors (IL-15, IL-7) or costimulatory agonists (anti-4-1BB, anti-OX40)[80,81] is another possible approach to successfully deal with tumor-mediated immunosuppression.

Furthermore, the use of therapeutic reagents inducing chemokine and adhesion molecule expression through blood vessel activation is also an interesting strategy for HCC treated with immunotherapy, since this kind of strategy may help restore T cell infiltration of the tumor[82].

Finally, it is expected that chemoimmunotherapy, that is, immunotherapy in combination with conventional therapy or other types of immunotherapies will elicit synergistic anti-tumor activity.

It has been earlier suggested that during or immediately following ablative therapy, immunotherapy will have its highest observed efficacy when tumor cells are about to die and the immune response has begun

its activity. In HCC, combined therapy of TAE with intratumoral DC infusion produced higher frequencies of AFP-specific T cells in comparison with TAE alone[83,84].

Advantageous therapeutic approaches in HCC will probably include combinations of immunotherapy involving several immune effector mechanisms, such as vaccines and T cell immune-modulators, along with immunotherapy supplemented with molecularly targeted inhibitors of tumor signaling pathways[84].

MOLECULAR TARGETED THERAPY

In the last decades, research on the molecular pathology of HCC has uncovered a plethora of molecules that are critical in the onset and progression of this human disease. With regard to cancer investigation, in order to target key molecules involved in cancer genesis and growth, several compounds for disease treatment were developed. The present section summarizes the status of the different therapeutic compounds developed for the targeting members of different signaling pathways that are crucial in the pathogenesis of HCC, *e.g.*, inhibitors of the epidermal growth factor receptors (EGFR) and the vascular EGFR (VEGFR), families, as well as inhibitors of the TGF-β and the mTOR signaling pathways (Table 1 and Figure 1).

Anti-angiogenic therapy

HCC is one of the most vascularized solid tumors, having high vascular endothelial growth factors (VEGF) and microvessel density levels. In addition, other relevant angiogenic factors involved in HCC pathogenesis are: VEGFs, fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs).

VEGF seems to be primary a mediator of angiogenesis in HCC. Moreover, a higher level of VEGF is associated with a more aggressive disease evolution and possible poor treatment response[85]. Therefore, VEGF/VEGFRs and PDGF/PDGFRs signaling pathways are prime targets for the development of anti-angiogenic treatments for cancer. The anti-VEGF antibody bevacizumab and the multi-targeted tyrosine kinase inhibitors (TKI) sunitinib, sorafenib and pazopanib, which inhibit VEGFRs and other receptor tyrosine kinases are agents approved by the FDA to directly aim at the VEGF pathway[86]. So far, the only agent that has been proven to be effective in terms of survival of patients with HCC is sorafenib, which has become the current standard for palliative treatment[86].

Unfortunately, resistance to anti-angiogenic therapy was described (Figure 2). Hypoxia-Inducible Factor-1α and -2α (HIF-1α and HIF-2α) may be caused by the use of anti-angiogenic agents due to constriction of tumor blood vessels, decrease in blood flow and intratumoral hypoxia[87]. HIF-1α and HIF-2α transactivate genes causing tumor angiogenesis, tumor cell growth and energy metabolism, therefore causing anti-angiogenic drugs to become resistant and leading to poor prognosis[88]. It was reported that HCC overexpress HIF-1α and that this overexpression is triggered by tissue hypoxia, aberrant growth factor receptor signaling and mutations in oncogenes and tumor supressor genes[87].

Furthermore, previous cancer experiences have shown that the expression of other angiogenic factors such as the FGF are up-regulated upon anti-VEGF treatment as an alternate escape mechanism. Thus, inhibitors of the FGF pathway such as brivanib were recently investigated for the treatment of advanced HCC as an option for patients with HCC following failure of sorafenib.

Sorafenib: Sorafenib is a multitargeting small molecule that exerts its anti-angiogenic effect through inhibition of VEGFR-1, VEGFR-2, VEGFR-3, CD135 or Fms-like tyrosine kinase-3 (Flt-3), PDGFR-β, and FGF receptor-1 (FGFR-1) promoting the formation of new blood vessels[89]. Sorafenib also acts blocking cellular proliferation mediated by the Raf/MAPK/ERK signaling pathway[89] and inducing both apoptosis and autophagy in human hepatoma cells[90,91]. As previously mentioned, the FDA has approved sorafenib for treating both HCC and renal cell cancer in 2007, and is the first systemic therapy to show some survival advantage. In 2008, a promising prospect for sorafenib monotherapy in the treatment of advanced HCC had been provided by a multicenter double-blind Phase Ⅲ trial (the Sorafenib HCC Assessment Randomized Protocol) which demonstrated a 44% increase in the median overall survival (10.7 mo in the sorafenib group and 7.9 mo in the placebo group)[24]. In the following year, an Asia-Pacific trial corroborated sorafenib efficacy reporting a median overall survival of 6.5 mo, whereas in the placebo group the reported median overall survival was 4.2 mo[92]. However, problems of drug-toxicity have been reported; among the most frequently observed drug-related adverse events, fatigue, anorexia, diarrhea, rash/desquamation, and hand - foot skin reactions were described[93]. Furthermore, other studies have shown that patients with severe liver dysfunction had a limited life expectancy after treatment with sorafenib (1.5 mo)[94]. In a meta-analysis of five randomized controlled trials encompassing 1462 patients with unresectable HCC, Shen *et al*[95], have recently shown that sorafenib use - as compared with placebo - improved the disease control rate (RR = 1.85, 95%CI: 1.55-2.20, $P < 0.001$), decreased tumor progression (HR = 0.61, 95%CI: 0,51-073, $P < 0.001$) and reduced mortality (HR = 0.71, 95%CI: 0.56-0.89, $P < 0.001$). Interestingly, further subgroup analyses demonstrated that results obtained were not modified by HCC etiology, performance status nor Barcelona Clinic Liver Cancer-stage[95] (Figure 3). Sorafenib has also shown benefit when combined with doxorubicin. In a phase I study combining sorafenib/doxorubicin, all four patients with metastatic HCC maintained stable disease state for more than 1 year of treatment[96]. In a randomized, double-blind, phase Ⅱ trial, the sorafenib/doxorubicin combination prolonged the median overall survival and progression-free survival when compared with doxorubicin alone[97].

Table 1 Molecular targeted therapy

Type of drug	Drug	Target	Stage of use (for HCC)
Inhibitors of angiogenesis	Sorafenib[1]	VEGFR members	Approved
		PDGFR-β	
		Flt-3	
		FGFR-1	
		Raf/MAPK/ERK signaling pathway	
	Bevacizumab	VEGFR members	Phase Ⅱ
	Sunitinib	VEGFR members	Phase Ⅱ
		PDGFR-α	
		PDGFR-β	
		Flt-3	
		c-Kit	
		RET kinases	
	Pazopanib	VEGFR members	Phase Ⅰ
		PDGFR-α	
		PDGFR-β	
		c-Kit	
	Brivanib	VEGF signaling pathway	Phase Ⅱ
		FGF signaling pathway	
	Axitinib	VEGFR members	Phase Ⅱ/Ⅲ
		PDGFR-α	
		PDGFR-β	
		c-Kit	
	Linifanib	VEGF	Phase Ⅱ
		PDGFR-α	
		PDGFR-β	
	TSU-68	VEGFR-2	Phase Ⅱ
		PDGFR-α	
		PDGFR-β	
		FGFR-1	
		c-Kit	
		Flk-1	
	Foretinib	VEGFR-2	Phase Ⅰ/Ⅱ
		c-Met	
	Dovitinib	VEGFR members	Phase Ⅰ/Ⅱ
		PDGFR-β	
		FGFR members	
		Flt-3	
		c-Kit	
	Ramucirumab	VEGFR-2	Phase Ⅱ
Inhibitors of EGFR	Erlotinib	EGFR/HER-1	Phase Ⅱ
	Lapatinib	EGFR/HER-1	Phase Ⅱ
		HER-2/NEU	
	Gefitinb	EGFR/HER-1	Phase Ⅰ
	Cetuximab	EGFR/HER-1	Phase Ⅱ
Inhibitors of the mTOR pathway	Rapamycin	PI3K/Akt/mTOR pathway	Phase Ⅰ/Ⅱ
	Everolimus	PI3K/Akt/mTOR pathway	Phase Ⅰ/Ⅱ

[1]Sorafenib also induces apoptosis and autophagy. HCC: Hepatocellular carcinoma; VEGFR: Vascular endothelial growth factor receptors; Flt-3: Fms-like tyrosine kinase-3; FGFR-1: FGF receptor-1; MAPK: Ras-Raf-mitogen-activated protein kinase; EGFR: Epidermal growth factor receptors; FGF: Fibroblast growth factor; PDGFR: Platelet-derived growth factor receptors; RET: Rearranged during transfection; HER-1: Human epidermal growth factor receptor-1; mTOR: Mammalian target of rapamycin.

A phase Ⅱ multicenter study of combined sorafenib/octreotide showed a higher disease control rate than sorafenib monotherapy (76% *vs* 43%, respectively) achieving an overall survival of 12 mo[98] sorafenib combined with TACE is currently under clinical investigation[99].

Inhibition of autophagy with specific pharmacological inhibitors such as chloroquine, produced more pronounced tumor suppression in HCC *in vivo* and *in vitro*[24]. Thus, the combination of sorafenib and autophagy modulation is a promising therapeutic option in unresectable HCC[91]. Moreover, up-regulation of HIF-2α induced by sorafenib contributes to drug resistance by activating the TGF-α/EGFR pathway in HCC cells[100], overcoming the negative modulation exerted by HIF-1α (Figure 2).

Bevacizumab: The FDA also approved a recombinant monoclonal anti-VEGF antibody to be used in advanced breast, non-squamous non-small cell lung and colorectal cancers in combination with chemotherapy. In Siegel's Phase Ⅱ study it was shown that bevacizumab as a single agent was effective, showing a 13% rate of objective tumor response and a median overall survival of 12.4 mo in patients suffering from non-metastasized HCC unable to be resected[101]. However, its use was

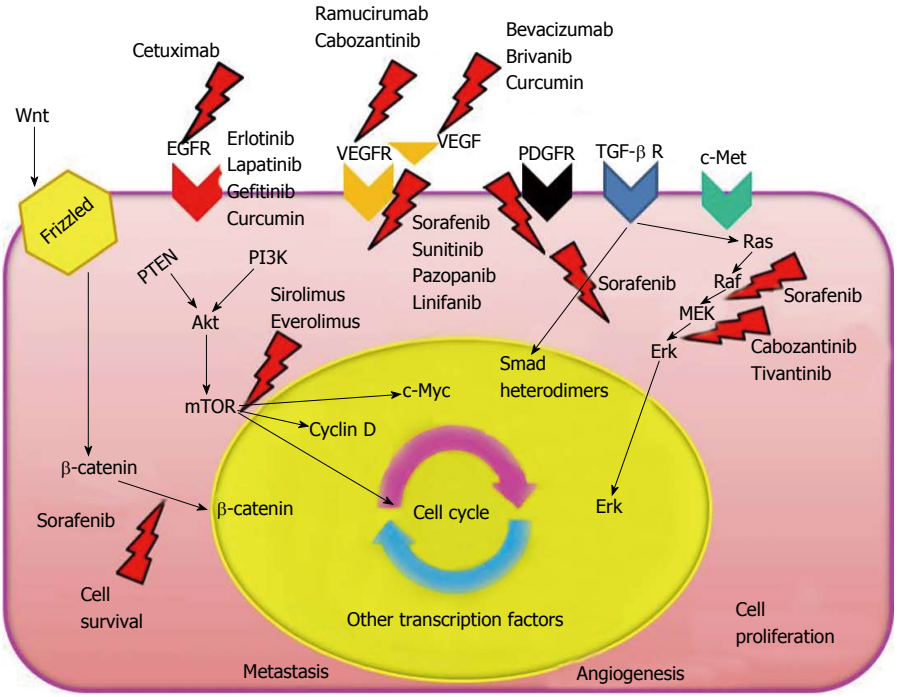

Figure 1 Hepatocellular carcinoma pathogenetic pathways. Main molecular targets of the major anti-tumoral drugs are indicated. VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptors; TGF-β: Transforming growth factor-β; Erk: Extracellular signal-regulated kinase; EGFR: Epidermal growth factor receptors; PDGFR: Platelet-derived growth factor receptors; TGF-β R: TGF-β receptor; RAS: Rat Sarcoma; RAF: Rapidly accelerated fibrosarcoma; MEK: Mitogen-activated protein kinase kinase; c-Myc: Myelocytomatosis cellular oncogene; PTEN: Phosphatase and tensin homology; PI3K: Phosphoinositide 3-kinase.

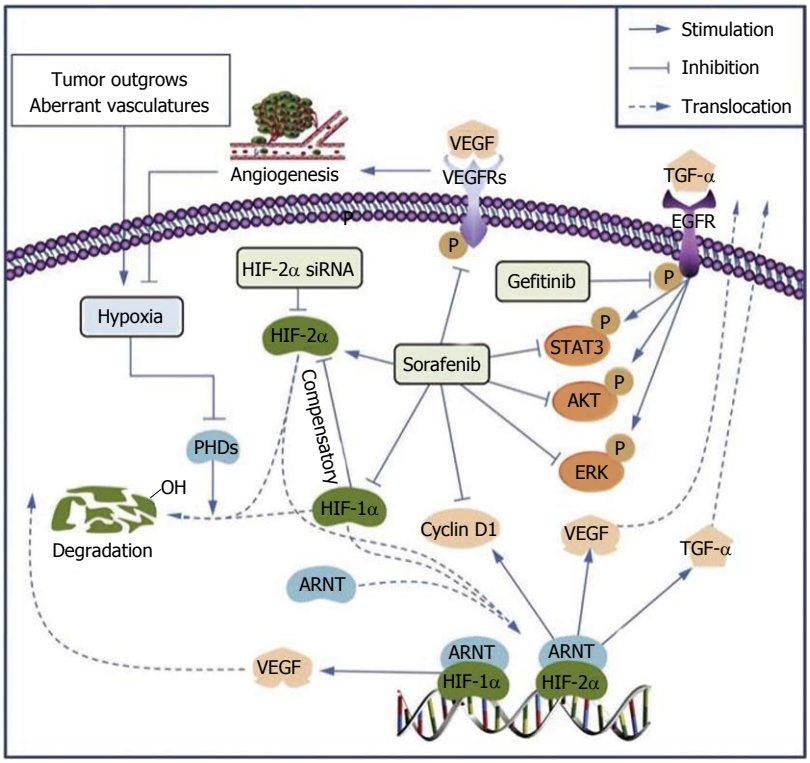

Figure 2 Proposed mechanisms by which upregulation of hypoxia-inducible factor-2α induced by sorafenib contributes to the resistance by activating the transforming growth factor-α/epidermal growth factor receptors pathway in hepatocellular carcinoma cells. ARNT: Aryl hydrocarbon receptor nuclear translocator; EGFR: Epidermal growth factor receptor; ERK: Extracellular signal-regulated kinase; HIF-1α: Hypoxia-inducible factor-1α; HIF-2α: Hypoxia-inducible factor-2α; PHD: Prolyl hydroxylase; STAT3: Signal transducer and activator of transcription 3; TGF-α: Transforming growth factor-α; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor. Reprinted from ref. [100] with permission from Elsevier.

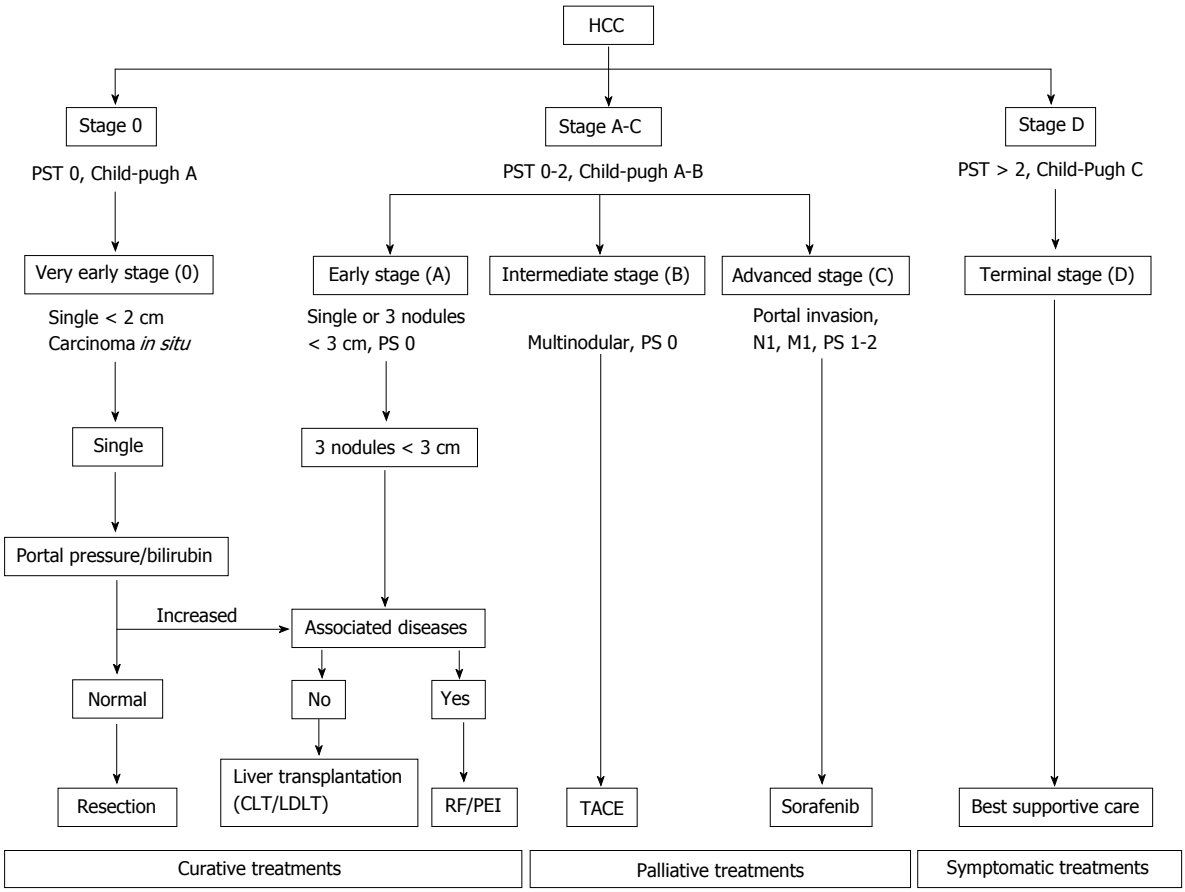

Figure 3 Barcelona Clinic Liver Cancer staging system and treatment strategy (2011). HCC: Hepatocellular carcinoma; CLT: Cadaveric liver tansplantation; LDLT: Living donor transplantation; RF: Radiofrequency; PEI: Percutaneous ethanol injection; TACE: Transarterial chemoembolization; PST: Performance status test; PS: Performance status.

associated with considerable bleeding in 11% of cases and thrombosis in 6% of the patients, therefore it is prone to drug-related complications. Patients receiving the combination of bevacizumab with gemcitabine-oxaliplatin (GEMOX)[102] or capecitabine-oxaliplatin[103] responded in up to 20% of cases, however the overall survival rate was 9.6 mo. The administration of these drugs also resulted in considerable toxicity associated with the treatment, causing leukopenia, transaminitis, hypertension and fatigue. To summarize, it has been proved in previous clinical studies that bevacizumab was relatively effective in HCC; therefore, since some severe drug-related complications such as thrombosis, hemorrhage and even death have been reported, further studies are needed to clarify its efficacy and safety.

Sunitinib: Sunitinib is an oral multi-targeted TKI that inhibits VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α, PDGFR-β, c-kit, Flt3, and rearranged during transfection (RET) kinases. Sunitinib has been approved by the FDA to treat renal adenocarcinoma and gastrointestinal stromal tumors. In a phase Ⅱ clinical trial to analize the efficacy of sunitinib as monotherapeutic agent in advanced stages of HCC, it was reported that this drug shows modest antitumor activity with a very low rate response and a median overall survival between 8 and 9.8 mo[104]. It is worth mentioning that sunitinib had been negative for its primary overall survival endpoint and proved to have greater toxicity than sorafenib. Thus, based on these results, the use of sunitinib as first line treatment in advanced HCC was not supported, being sorafenib monotherapy the standard of care in these cases. However, when sorafenib fails, sunitinib might be chosen as second-line treatment[105].

Pazopanib: Pazopanib, a synthetic indazolyl-pyrimidine is an oral angiogenesis inhibitor, recently approved by FDA for the treatment of patients with renal cell cancer[106]. This novel multitargeted TKI acts inhibiting VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α, PDGFR-β and c-Kit. It is still being assessed as potential treatment for HCC[107]. Phase Ⅰ clinical studies established that since the toxicity level of pazopanib is acceptable, it might be a possible option for advanced HCC treatment[108].

Brivanib: Brivanib, a TKI, is the first oral selective dual inhibitor of VEGF and FGF signaling pathways, that has showed encouraging antitumor activity in preclinical and phase Ⅰ studies. In a phase Ⅱ open-label study of brivanib as first-line therapy in patients with unresectable, locally advanced, or metastatic HCC,

brivanib demonstrated promising antitumor activity with a median overall survival of 10 mo. Furthermore, this anti-angiogenic drug showed a manageable safety profile, being fatigue, diarrhea, anorexia, vomiting, hypertension, constipation and nausea the most frequent reported adverse events[109]. In another phase Ⅱ, open label study of brivanib, which this time was assessed as a second-line drug treatment for HCC patients not responding to the administration of anti-angiogenic therapy, showed encouraging results in this group of patients for whom no approved treatment is currently available[110]. Recently, a multinational, randomized, double-blind, phase Ⅲ trial compared brivanib with sorafenib as first-line treatment for HCC. Results demonstrated that both drugs displayed a similar anti-tumor activity, based on secondary efficacy end points, although brivanib was less well-tolerated than sorafenib[111]. Finally, brivanib, as an adjuvant therapy to TACE in patients with HCC, failed to improve overall survival[112].

Axitinib: Axitinib is another multi-targeted TKI with activity against VEGFR-1, VEGFR-2, VEGFR-3, VEGFR-4, PDGFR and c-Kit. This drug has shown promising results for renal cell cancer and thyroid cancer. Phase Ⅱ/Ⅲ trials assessing this medication for HCC are still being planned[113,114].

Linifanib: Linifanib is an innovative and potent selective inhibitor aimed at inhibiting angiogenesis, tumor growth and metastasis. A phase Ⅱ clinical trial in patients undergoing the advanced stages of HCC showed that linifanib is clinically active for unresectable HCC with an acceptable safety profile. The median overall survival was 9.7 mo. A phase Ⅲ trial for comparing linifanib with sorafenib is currently under way[115].

TSU-68: TSU-68 is an oral compound which inhibits VEGFR, PDGFR and FGFR. A phase Ⅰ/Ⅱ clinical trial in patients with advanced HCC has shown promising efficacy with a median overall survival of 13.1 mo and a high safety profile even in patients who had been heavily pre-treated[116].

Foretinib: Foretinib is a novel receptor TKI that targets VEGFR-2 and c-Met that demonstrated significant anti-tumor activities in preclinical models of HCC. At present, phase Ⅰ and Ⅱ clinical trials are under way[117].

Dovitinib

Dovitinib potently inhibits receptor TKs, showing specificity for VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-β, FGFR-1, FGFR-2, FGFR-3, Flt-3 and c-Kit. Several phase Ⅰ/Ⅱ studies have been carried out to assess the efficacy, pharmacokinetics, pharmacodynamics and safety profile of this drug. In xenografts models of human HCC it was reported that this compound reduced angiogenesis and cell proliferation, inducing apoptosis of tumor cells[118].

Ramucirumab: Ramucirumab is a recombinant human monoclonal antibody that binds to the extracellular domain of VEGFR-2. A phase Ⅱ study of ramucirumab as first-line monotherapy in patients with advanced HCC showed that this monoclonal antibody has been well tolerated and has conferred a moderate disease control[119].

Inhibitors of the human EGFR

It has been broadly accepted that the role of growth factors and their receptors is crucial for several cancers to develop and progress, HCC among them[120]. In fact, inhibitors of the human EGFR-1 (HER-1) is usually overexpressed in chronic hepatitis, fibrosis, cirrhosis and HCC cases[121]. EGFR/HER-1 ligands such as epidermal growth factor (EGF), hepatocyte growth factor, TGF-α, TGF-β, and insulin-like growth factors (IGF) were shown to be mitogenic for hepatocytes, therefore contributing significantly to hepatocarcinogenesis[122]. Furthermore, hypomethylation of the EGFR/HER-1 gene was also described to be associated with the development of HCC[123]. Since drugs targeting EGFR have proved to increase survival rates in patients in whom cancer has metastized to the lungs and pancreas[124,125], there is a logic for analyzing the effectiveness of this novel class of compounds in patients with unresectable HCC. Regarding HER-2/NEU and its significance in HCC, the international literature shows conflicting data. Some studies have demonstrated that HER-2/NEU is rarely overexpressed in HCC and might not play a role in this kind of cancer[126], whereas the opposite has been shown by other studies[127].

Erlotinib: Erlotinib is an orally active selective inhibitor of the EGFR/HER-1-related thymidine kinase (TK) enzyme that inhibits its autophosphorylation process[128]. Erlotinib blocks the EGF-dependent growth of tumoral cells at submicromolar concentrations and arrests cell-cycle progression in the G_1 phase[129]. FDA has approved this selective inhibitor of the EGFR/HER-1-related TK enzyme for treating advanced lung and pancreatic cancers. A phase Ⅱ study of the single-agent erlotinib in patients with unresectable HCC reported that tolerance to this drug was good but had a modest benefit in controlling HCC, which was evidenced as a 13-mo discrete prolonged overall survival[130]. However, another phase Ⅱ study demonstrated a median overall survival of 10.75 mo[129]. Interestingly, it was also demonstrated in this study that overall survival between the group of patients that showed high EGFR/HER-1 expression and those with low EGFR/HER-1 expression was not significantly different[129]. This means that there was no correlation with EGFR/HER-1 expression and overall survival. The toxicity to erlotinib was mainly cutaneous and similar in profile to other drugs that target the EGFR/HER-1-related TK activity. A phase Ⅱ, single-arm, open-label trial of erlotinib in combination with bevacizumab obtained encouraging results and a favorable toxicity profile. The

best response showed minor tumor shrinkage, decreased tumor vascularity or increased necrosis. Adverse effects consisted on transaminases elevation, hyperkalemia, diarrhea, proteinuria, gastrointestinal bleed, fatigue and hypertension[130]. Further studies with erlotinib as a single agent or in combination with other agents are needed.

Lapatinib: Lapatinib is a dual inhibitor of EGFR/HER-1 and HER-2/NEU by docking into the ATP-binding site of the two receptors, thus inhibiting their autophosphorylation and the corresponding downstream signaling with consequent down-regulation of MAPK, AKT and p70S6 kinase, inhibiting tumor growth[131]. Clinical studies using lapatinib demonstrated that this drug was well-tolerated and displayed anti-tumor activity in heavily pretreated patients with several solid tumors. The most common adverse effects reported were rash and diarrhea. Lapatinib was recently approved by the FDA for use in metastatic breast cancer[132]. A phase II study of single agent lapatinib in patients with advanced HCC demonstrated that this drug was well-tolerated but revealed a minimal anti-tumoral activity based on the lack of objective responses and an overall survival of 12.6 mo[133]. The use of single-agent lapatinib in advanced HCC was tested in another phase Ⅱ study which revealed a lower median overall survival of 6.2 mo. Authors reported that this low median survival might be due to the small sample size. Anyway, they concluded that treatment with lapatinib failed to meet predefined efficacy standards and did not have significant activity on HCC[134].

Gefitinib: Gefitinib, an adenosine triphosphate mimetic anilinoquinazoline is an orally active EGFR-TKI that reduces EGF-stimulated tumor cell growth[135]. Results from the Eastern Cooperative Oncology Group's Study E1203 had shown modest activity in advanced HCC with a median overall survival of 6.5 mo[136]. Interestingly, combination of gefitinib and sorafenib has demonstrated synergistic effects to inhibit cell proliferation by promoting apoptosis *in vitro* and tumor growth suppression *in vivo*[99].

Cetuximab: Cetuximab is a chimeric (human and mouse) monoclonal antibody directed against EGFR, approved by the FDA for the treatment of squamous cell carcinoma of the head and neck and metastatic colorectal cancer. In phase Ⅱ clinical studies in patients with advanced and unresectable HCC, the use of cetuximab - as single agent therapy, as well as in combination therapy with GEMOX - demonstrated modest activity[137,138].

Inhibitors of the mTOR pathway

The PI3K/Akt/mTOR signal pathway is crucial in promoting protein synthesis and is implicated in various cellular functions such as proliferation, differentiation, tumorigenesis and apoptosis. In approximately 15%-41% of HCC patients, activation of this signaling pathway has been reported[139]. This event is implicated in metastasis, invasion and poor prognosis[140]. Blocking the mTOR pathway confers anti-cancer, anti-angiogenic and immunosuppressive properties. Preclinical data have shown that mTOR inhibitors were effective in both cell growth and tumor vascularity suppression in HCC cell lines and HCC tumor models[141]. According to this, rapamycin - the naturally occurring inhibitor of mTOR - and a number of recently developed rapamycin-analogues inhibit the growth of cell lines derived from multiple tumor types *in vitro* and tumor models *in vivo*. LY294002 is a PI3-kinase inhibitor that decreased the viability of HCC cells by inhibition of Akt activation. Other Akt inhibitors include wortmannin and inhibitor Ⅷ[142].

In addition, cyclooxygenase-2 (COX-2) has been recently implicated in the pathogenesis of HCC through Akt activation. According to this, the level of COX-2 expression and Akt phosphorylation is positively correlated in cultured HCC cells and human liver cancer tissues[143]. In this regard, Leng *et al*[143] demonstrated that HCC cells treated with the COX-2 inhibitor celecoxib showed significant reduction of Akt phosphorylation and induced apoptosis.

Sirolimus: Sirolimus (Rapamycin) is a macrolide antibiotic and antifungal drug isolated from *Streptomyces hygroscopicus.* Since it has been proved to have both immunosuppressive and antiproliferative effects, it has been regarded as an adjuvant therapy designed to treat cancer[88]. This specific mTOR inhibitor exerts its action in association with its intracellular receptor FKBP-12. Sirolimus may both inhibit rejection in liver transplantation patients and prevent the recurrence of HCC[144].

Everolimus: Everolimus is an oral inhibitor of mTOR. A phase Ⅰ/Ⅱ study carried out in patients with un-resectable or metastatic HCC showed modest anti-tumor activity with a median overall survival of 8.4 mo and a disease control rate of 44%. Everolimus was well tolerated in patients with advanced HCC. The most frequent adverse effects reported were fatigue, hyperglycemia, diarrhea, anemia, leukopenia and lymphopenia, thrombocytopenia, hyponatremia, anorexia, stomatitis and rash[145].

Curcumin

Curcumin is a naturally occurring and biologically active compound extracted from the rhizomes of Curcuma longa. *In vitro*, it was shown that this natural compound was able to induce apoptosis of HCC cell lines. In this regard, Cao *et al*[146] reported that curcumin induced apoptosis in human HepG2 cells through mitochondrial hyperpolarization and damage.

Wang *et al*[147] also demonstrated that in HCC J5 cells, curcumin induced apoptosis *via* Ca^{2+}-regulated mitochondria-dependent pathway.

Furthermore, curcumin has also been shown to

inhibit several angiogenic biomarkers, including VEGF and COX-2 expression[148]. This means that curcumin could be used as a candidate for the combined drug therapy for HCC in the future.

Other drugs

Cediranib blocks VEGFR, PDGFR and c-KIT. Similarly, BIBF-1120 targets VEGFR, PDGFR and FGFR; E-7080 inhibits VEGFR, FGFR, PDGFR and c-KIT; XL-184 targets VEGFR-2, MET and RET; vandetanib targets VEGFR and EGFR; BIIB-022, AVE1642 and cixutumumab inhibits IGF-1R; CT-011 inhibits PD-1/2; MEDI-575 inhibits PDGFR; BAY73-4506 inhibits VEGFR, PDGFR, FGFR-1, Raf, RET, and c-KIT; GC33 inhibits Glypican-3, which is highly expressed in HCC; salirasib blocks ras and mTOR activation, and finally, PI-88, which targets heparanases as well as sulfatases is now in Phase Ⅲ clinical trials for the treatment of HCC[149].

DRUG DELIVERY SYSTEMS AND TARGETING STRATEGIES TO THE LIVER PARENCHYMA OF ANTI-TUMORAL COMPOUNDS

As described above, since HCC is asymptomatic at an early stage, most cases are often diagnosed when the disease has advanced and most of the potentially effective treatments such as surgical resection, orthotopic liver transplantation and local ablation demonstrate poor efficacy. In these advanced stages, systemic treatments are commonly used. However, the efficacy of the current anti-tumoral drugs used in advanced HCC treatment cause significant toxicity in other non-target organs, therefore influencing on the patients' willingness to comply with and adhere to these treatments. Therefore, the effectiveness of treatments using anti-tumoral drugs for advanced HCC significantly depends on their pharmacokinetics, particularly in, their distribution and accumulation in the liver. An interesting approach to enhance anti-HCC drug action is to direct them into the liver by drug delivery systems (DDS) that recognize hepatocyte surface receptors. Thus, those strategies targeting the drug to its site of action, - in this case, the liver - may cause an increase in drug efficacy and a decrease in possible collateral effects in other non-target organs[150]. Indeed, several studies discussed below, have attempted to target anti-tumoral drugs to the liver for the treatment of advanced HCC, using novel formulations including liposomes, polymeric micelles, polymeric nanoparticles, dendrimers, nanocapsules and microspheres.

As mentioned above, since HCC originates from liver parenchyma cells, it is desirable to deliver drugs selectively to hepatocytes. To this end, asialoglycoprotein receptors (ASGPRs) are usually used as liver target due to their high expression on the surface of hepatocytes and in HCC-derived cell lines. ASGPRs specifically recognize ligands with terminal galactose or N-acetylgalactosamine residues, and endocytose them through an intracellular degradation process. The use of their natural ligand (asialofetuin) or synthetic ligands with galactosylated or lactosylated residues, has achieved significant targeting efficacy to the liver[22,151]. In this regard, Xu *et al*[152] synthesized a lactobionic acid conjugate of dioleoylphosphatidyl ethanolamine (Lac-DOPE) for targeting of solid lipid docetaxel-loaded nanoparticles. Following this approach, other works used the synthesis of lactosylated liposomes for targeted delivery of doxorubicin to HCC as a possible strategy to treat the disease[153]. Other groups used a cleavable poly(ethylene glycol) (PEG)-lipid [methoxypolyethyleneglycol 2000-cholesteryl hemisuccinate, PEG (2000)-CHEMS] linked *via* an ester bond and a galactosylated lipid {(5-cholesten-3 beta-yl) 4-oxo-4-[2-(lactobionyl amido) ethylamido] butanoate, CHS-ED-LA} to modify doxorubicin. Results demonstrated that modification of liposomes with PEG (2000)-CHEMS and CHS-ED-LA turned out to be a potentially advantageous strategy for HCC therapy[154].

Polymeric micelles also constitute a safe and effective delivery system. Bei *et al*[155] designed three novel polymers named palmitoyl-trimethyl-chitosan (TPCS)-1, TPCS-2 and lac-TPCS-2, that hold a great potential in the development of nanomedicine for the therapy of liver tumors, especially lac-TPCS-2. On the other hand, polymeric micelles self-assembled from amphiphilic block copolymers of PEGs and poly(D,L-lactide) (PDLLA) with folate as a targeting ligand attached to the distal ends of the PEG (Folate-PEG-PDLLA) were prepared. Such Folate polymeric micelle was demonstrated to selectively deliver the anti-tumoral drug doxorubicin to HCC cells, since they also overexpress surface receptors for folate[156]. Cuestas *et al*[157] reported the synthesis of galactosylated poly(ethylene oxide)-poly(propylene oxide) block copolymers, proposed for potential targeting to the liver.

The nanoparticle DDS, which uses polymeric material from natural or synthetic sources as a carrier in drug delivery to targeted tissues, has remarkable targeting, slow-release and biodegradable properties that also makes it a promising therapeutical option. Regarding this, Cheng *et al*[158] reported the use of chitosan and the hepatoma cell-specific binding molecule glycyrrhetinic acid to synthesize glycyrrhetinic acid-modified chitosan (GA-CTS). The anti-tumoral drug 5-fluorouracil (5-FU) was conjugated onto this newly synthesized nano-material, thus forming the corresponding GA-CTS/5-FU nanoparticles. Results demonstrated that these nanoparticles accumulated selectively in the liver blocking tumor growth in an orthotopic liver cancer mouse model[158]. Another group reported the preparation of nanoparticles composed of galactosylated chitosan oligosaccharide and adenosine triphosphate for HCC cell-specific uptake[159].

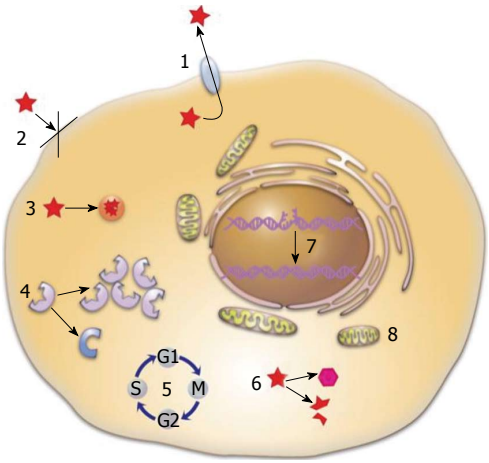

Figure 4 Mechanisms of multidrug resistance in cancer cells. (1) Active drug efflux by drug transporters, such as Pgp, multidrug resistance-associated protein, and breast cancer resistance protein; (2) Loss of cell surface receptors and/or drug transporters or alterations in membrane lipid composition; (3) Compartmentalization of the drug in cellular vesicles; (4) Altered/increased drug targets; (5) Alterations in cell cycle; (6) Increased drug metabolism/enzymatic inactivation; (7) Active damage repair; and (8) Inhibition of apoptosis. Reprinted with permission from ref. [181].

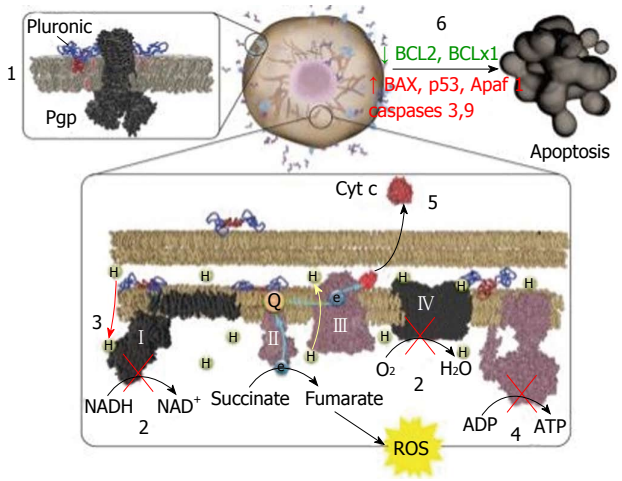

Figure 5 Summary of Pluronic effects in cancer cells. Pluronic binding with plasma membrane of multidrug resistance (MDR) cancer cells (1) induces membrane fluidization, disruption of membrane microdomains, and inhibition of drug efflux transporters' activity (Pgp shown as an example). Pluronic also reaches mitochondria where it (2, 3) inhibits complexes I and IV of mitochondria respiratory chain and (3) induces inner mitochondrial membrane depolarization. This (4) results in ATP depletion and (5) promotes cytochrome c release and ROS generation in MDR cells. Altogether, the MDR cells respond to a Dox/Pluronic combination by (6) an increased proapoptotic signaling and decreased antiapoptotic defense. Reprinted with permission from ref. [181]. ROS: Reactive oxygen species.

Poly(amidoamine) dendrimers are branched water-soluble polymers defined by consecutive generation numbers (Gn) indicating a parallel increase in size, molecular weight, and number of surface groups available for conjugation of bioactive agents. In this connection, Medina *et al*[160] targeted hepatic cancer cells with pegylated dendrimers displaying N-acetylglucosamine and SP94 peptide ligands. Lactosylated dendrimers were also used as a liver-targeting DDS[161].

An alternative strategy is to use microspheres[162] and nanocapsules[163]. For example, Kang *et al*[163] reported an innovative hepatoma-targeted gene delivery system which was prepared with a combination of a human liver cell-specific bionanocapsule and a tumor cell-specific gene regulation polymer that responded to hyperactivated protein kinase C in liver cells.

OVERCOMING MDR DUE TO OVEREXPRESSION OF ABC PROTEINS

As mentioned above, HCC is a molecular complex tumor with high intrinsic MDR (Figure 4). An increased cellular extrusion of chemotherapeutic drugs due to over-expression of MDR mediating ABC transmembrane proteins leads to a reduced effectiveness with response rates below 10%[164]. Actually, there are 49 known ABC transporters divided into 7 distinct subfamilies of proteins[165]. The most studied proteins were P-gp, MRP1 and BCRP.

A classic approach for overcoming MDR involves the use of low molecular mass ABC inhibitors co-administered with the pharmacotherapeutic agent, such as verapamil and valspodar. However, limited success has been achieved so far with these chemosensitizing agents that inhibit these efflux proteins. New advances to overcome MDR consists on the use of block copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) also known as poloxamers or Pluronics® and poloxamines or Tetronics® (Figure 5). Poloxamers consist of a central hydrophobic PPO molecule flanked on both sides by two hydrophilic chains of PEO. A slightly different structure is exhibited by poloxamines, which are tetrafunctional block co-polymers with four PEO-PPO blocks joined together by a central ethylene diamine bridge[166]. These surfactants have found a wide range of pharmaceutical, biomedical, clinical and nanotechnological applications. Some of them, such as Pluronic P85 were shown to sensitize MDR tumors refractory to many chemotherapeutic agents[167]. In addition, Cuestas *et al*[168] explored the prospective capacity of PEO-PPOs to overcome MDR in HCC-derived cell lines. Results demonstrated that there is multiple-inhibitory activity of poloxamines on P-gp, MRP1 and BCRP in two human hepatoma cell lines, Huh7 and HepG2[168]. Copolymers of intermediate to high hydrophobicity (*e.g.*, Tetronic® 304, 904 and 1301) inhibited P-gp and BCRP but not MRP1 in both cell lines[168]. This activity was related to both copolymer concentration and hydrophobicity. Conversely, there was no evidence of an inhibitory effect in Tetronic® 1107, a more hydrophilic counterpart[168]. Furthermore, the work by Cuestas *et al*[169], also analyzed for the first time the effect of branched PEO-PPOs on the expression of mRNA encoding for the main ABCs in a human hepatoma cell line and gave evidence of the down-regulation of mRNA levels corresponding to *p-gp* and *bcrp*[169].

All these technological strategies constitute a

positive starting point that will require further research to evaluate their potential efficacy in treating HCC.

FUTURE PERSPECTIVES

HCC remains a disease with poor prognosis despite recent advances in the knowledge of both its patho-physiology and therapy.

Since aberrant epigenetic deregulation events such as hyper-methylation (silencing) of tumor suppressor genes, hypo-methylation (activating) of proto-oncogenes, as well as abnormal expression of histone modifying enzymes and non-coding RNAs (microRNAs and long non-coding RNAs) have been associated with genetic instability and altered gene expression, this landscape should be analyzed as a complex network of crosstalk and cooperation (synergism) leading to HCC. Bearing in mind the potential reversibility of epigenetic changes, plausible next generation treatments might also consider the use of drugs that modify DNA methylation and/or those that promote histone modifications (such as DNA methyl transferases - or histone deacetylases - inhibitors to activate tumor suppressors), either as mono- or combined-treatment, together with conventional chemotherapeutic agents. Moreover, encouraging results obtained with the up-regulation of some anti-tumoral miRNAs (such as adenoviral vectored-miR-122[170], and adeno-associated-miRNA-26a[171], respectively) allow to consider this strategy as a candidate for the treatment of HCC[172].

Although only modest results have been thus far obtained with immunotherapy[173], a plausible use of immune-stimulating monoclonal antibodies (such as anti-CTLA-4/anti-programmed death ligand-1) together with inhibitors of the immune regulatory (suppressor) mechanisms exerted by Tregs and/or - as already demonstrated - locoregional conventional treatments intended to increase immunity and unmask TAA-specific T cell responses[174] might be envisaged as a next approach for HCC treatment. Moreover, the recent development of the calixarene compound OTX008[175] as an inhibitor of galectin 1[176] - a key regulator of extracellular matrix interactions, cell proliferation, invasion, angiogenesis and escape from the immune response by favoring the expansion of Tregs and the differentiation of tolerogenic dendritic cells, as well as by limiting T cell viability, and maintaining T cell anergy - promises a future view of tumor halting by selectively counteracting tumor immune escape[177]. A phase Ⅰ, first-in-man - study of OTX008 treatment to patients with advanced solid tumors is ongoing (Clinical trial NCT01724320). Treating patients suffering from advanced HCC and overcoming MDR still remain an important challenge. Since an association between miR-122 down-regulation and MDR has been established, and an *in vitro* therapeutic effect on MDR of HCC cell lines with adenovirus-vector miR-122 has been reported[178], it seems plausible that miR-122 treatment in human HCC might be worth to evaluate.

In this regard, a very recent report using cabozantinib (a VEGFR and MET inhibitor) demonstrated that patients with HCC with high level expression of phosphorylated-MET (activated by the hepatocyte growth factor) are associated with resistance to adjuvant sorafenib treatment. The dual blockade of VEGFR2 and MET by cabozantinib leads to significant anti-tumor activities in HCC by suppressing both tumor growth and metastasis[179]. Therefore, the use of this drug might help to overcome to some extent the resistance to sorafenib. Likewise, the oral use of tivantinib in a Phase Ⅱ placebo-control study demonstrated promising results in patients with HCC with high level of MET, which might be a second choice therapeutic in treating patients suffering from advanced HCC[180].

The challenge of the heterogeneous nature of HCC - and the corresponding biomarkers - needs the expedited discovery of novel chemotherapeutic and immunotherapeutic agents, in order to have multiple choices for therapy which can then be used alone, in combination and/or sequentially, as wellas the design of technological or pharmaceutical strategies for chemosensitizing HCC cells. Furthermore, despite the availability of several drugs for the treatment of advanced HCC, implementing liver-targeting DDS strategies in general and nanotechnologies in particular may result in future tools to: (1) enhance the efficacy and application of approved drugs to overcome and delay cellular resistance development; (2) limit systemic side effects by promoting selective accumulation in the liver; and (3) increase patient adherence to treatment by reducing administration frequency.

Finally, there are still many unknown technological drawbacks to be faced in the discovery and assessment of new drug candidates, which will demand the design of more suitable drug carriers to deal with their preliminary preclinical assessment.

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REVIEW

Drug therapy for Parkinson's disease: An update

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Abstract

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, affecting about 1% of the population above the age of 65. PD is characterized by a selective degeneration of the dopaminergic neurons of the substantia nigra pars compacta. This results in a marked loss of striatal dopamine and the development of the characteristic features of the disease, *i.e.*, bradykinesia, rest tremor, rigidity, gait abnormalities and postural instability. Other types of neurons/neurotransmitters are also involved in PD, including cholinergic, serotonergic, glutamatergic, adenosine, and GABAergic neurotransmission which might have relevance to the motor, non-motor, neuropsychiatric and cognitive disturbances that occur in the course of the disease. The treatment of PD relies on replacement therapy with levodopa (L-dopa), the precursor of dopamine, in combination with a peripheral decarboxylase inhibitor (carbidopa or benserazide). The effect of L-dopa, however, declines over time together with the development of motor complications especially dyskinesia in a significant proportion of patients within 5 years of therapy. Other drugs include dopamine-receptor-agonists, catechol-O-methyltransferase inhibitors, monoamine oxidase type B (MAO-B) inhibitors, anticholinergics and adjuvant therapy with the antiviral drug and the N-methyl-D-aspartate glutamate receptor antagonist amantadine. Although, these medications can result in substantial improvements in parkinsonian symptoms, especially during the early stages of the disease, they are often not successful in advanced disease. Moreover, dopaminergic cell death continues over time, emphasizing the need for neuroprotective or neuroregenerative therapies. In recent years, research has focused on non-dopaminergic approach such as the use of A_{2A} receptor antagonists: istradefylline and preladenant or the calcium channel antagonist isradipine. Safinamide is a selective and reversible inhibitor of MAO-B, a glutamate receptor inhibitor as well as sodium and calcium channel blocker. Minocycline and pioglitazone are other agents which have been shown to prevent dopaminergic nigral cell loss in animal models of PD. There is also an evidence to suggest a benefit from iron chelation therapy with deferiprone and from the use of antioxidants or mitochondrial function enhancers such as creatine, alpha-lipoic acid, l-carnitine, and coenzyme Q10.

Key words: Parkinson disease; Levodopa; Dopaminergic drugs; Neuroprotective agents; Non-dopaminergic drugs; Mitochondrial enhancers

Core tip: Parkinson's disease (PD) is a neurodegenerative disorder for which currently there is no cure. The advent

of many therapies such as levodopa (L-dopa), dopamine-receptor-agonists, monoamine oxidase type B inhibitors, and catechol-O-methyltransferase inhibitors helped much to ease the life and to improve health-related quality of life of PD patients. Among these drugs, L-dopa remains the most effective agent for treatment of motor symptoms in PD. These agents provide symptomatic relief for motor symptoms but there is no evidence that these could alter the natural course of the disease and prevent the progressive dopaminergic neuronal loss. There is, however, encouraging data that suggest a benefit from iron chelation therapy with deferiprone and from the use of antioxidants or mitochondrial function enhancers in preventing or delaying the progression of PD.

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INTRODUCTION

Idiopathic Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by bradykinesia, tremor, rigidity and impaired postural reflexes. It is the 2nd most common neurodegenerative disorder after Alzheimer's disease. It is estimated to affect approximately 1% of the population over 65 years of age[1,2]. The main neuropathology in PD is the progressive loss of nigrostriatal dopaminergic neurons and consequent striatal dopamine depletion[3]. When there is a loss of about 60%-70% of neurons of the substantia nigra pars compacta (SNc) and the striatal dopamine content is reduced by 70%-80%, symptoms start to appear[4,5]. The definitive diagnosis of PD is based on post-mortem histopathological findings of degeneration and loss of pigmented neurons of the SNc and the presence of intracytoplasmic eosinophilic inclusions bodies (Lewy bodies) and dystrophic neurites (Lewy neurites) present in the remaining dopaminergic neurons of the substantia nigra. The major compound of Lewy bodies is aggregated forms of the normally presynaptically located protein α-synuclein[6]. Abnormal signaling in PD is not confined to nigrostriatal dopaminergic pathwaysrestricted. Other types of neurons/neurotransmitters including cholinergic, serotonergic, glutamatergic, adenosine, and GABAergic neurotransmission are also involved in PD. Alterations in these neurotransmitter systems contributes to the development motor, non-motor, neuropsychiatric and cognitive disturbances that occur in the course of the disease and are possible targets for drug therapy [7,8].

PD is essentially a sporadic disorder, commonly referred to as idiopathic PD, while a minority of cases is familial (approximately 5%)[9]. These rare familial forms of PD are usually of early onset in contrast to the late-onset idiopathic PD. The past few years have witnessed the identification of distinct genetic loci responsible for rare Mendelian forms of PD and both autosomal dominant and recessive patterns of inheritance have been described. The rare genetic forms have helped in understanding the molecular mechanisms involved in PD including protein misfolding and aggregation, mitochondrial defects, and oxidative stress[10]. The cause of idiopathic PD is not yet fully understood, but there is accumulating evidence to support a role for environmental toxin(s) and a genetic background[11,12]. PD can also be caused by drugs especially neuroleptic agents and dopamine-blocking drugs, toxins (manganese, carbon dioxide), head trauma, tumours of basal ganglia. This is termed secondary parkinsonism[13-15].

CURRENT PHARMACOLOGIC THERAPY FOR PARKINSON DISEASE

Classic drugs

Levodopa: The main neurochemical deficit in PD is the progressive loss of dopamine producing neurons in the SNc and subsequent striatal dopamine depletion[16]. This deficit can be partially compensated for by the administration of levodopa (L-dopa) (L-3,4-dihydroxyphenylalanine), the precursor of dopamine (Figure 1). Following oral ingestion, L-dopa is actively transported from the upper small intestine into the circulation by a mechanism specific for large, neutral L-amino acids[17]. L-dopa is subject to high presystemic metabolism to dopamine in the gut by the enzyme L-amino acid decarboxylase, with only approximately 30% of an L-dopa dose reaching the systemic circulation[18]. The small fraction of the drug that reaches the brain after active transport across the blood brain barrier is rapidly converted to dopamine by aromatic L-amino acid decarboxylase. In order to inhibit conversion of L-dopa to dopamine outside the central nervous system, thus limiting systemic side effects, it is combined with a peripherally acting decarboxylase inhibitor (carbidopa or benserazide)[17]. The combined administration of L-dopa with either carbidopa (L-dopa/carbidopa at the dose ratio of 10/1 and 4/1) or benserazide (L-dopa/benserazide 4/1), have nearly tripled L-dopa oral bioavailability, markedly reducing both the required L-dopa therapeutic dose and severity of dopamine-mediated gastrointestinal and cardiovascular side-effects[18]. Ever since its introduction in 1960s, L-dopa has remained the most effective treatment in controlling the symptoms of PD and is considered the gold standard regarding the symptomatic treatment of patients with PD[19]. Compared with other available dopaminergic therapies, dopamine replacement with L-dopa is associated with the greatest improvement in motor function, as assessed by reduced scores in the Unified PD Rating Scale (UPDRS). L-dopa is the preferred therapy in patients above 65 years of age, while those below 65 are better treated with dopamine agonists[20].

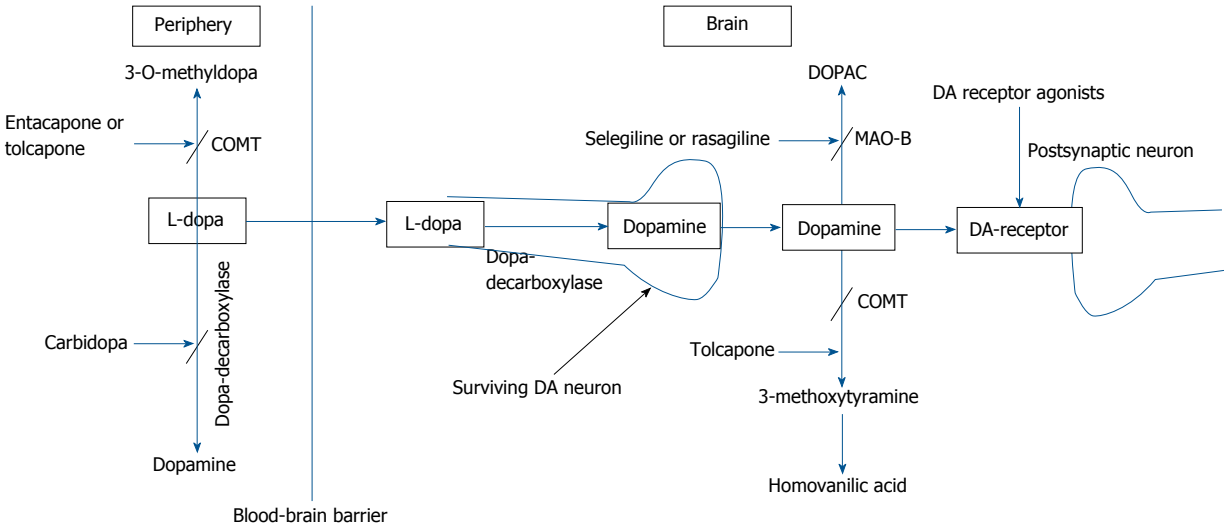

Figure 1 Site of action of antiparkinsonian drugs. DA: Dopamine; DOPAC: Dihydroxyphenylacetic acid; MAO-B: Monoamine oxidase-B; L-dopa: Levodopa; COMT: Catechol-O-methyltransferase.

When used as an initial monotherapy, L-dopa delayed the need for supplementary treatment and was well tolerated compared with dopamine agonists; 50% of the patients starting L-dopa received supplementary therapy with-in 3.6 years compared with 2.3 years in case of dopamine agonist monotherapy[21].

However, as the disease progresses, long-term use of L-dopa leads to the development of motor response complications, particularly "wearing-off", "on-off", dyskinesias and dystonias. These L-dopa-associated motor problems result in marked disability and decreased quality of life. End-of-dose or wearing-of phenomenon is the reappearance of motor symptoms before the next scheduled dose of L-dopa. Wearing-of usually emerges within 1-3 years of initiation of levodopa treatment[20-25]. Patients may also report a "delayed-on" that is unusual delay between a given dose of levodopa and the start of its effects. The phenomenon of "no-on" is the failure of a given dose of levodopa to elicit any response. These manifestations are related to variations in gastrointestinal transport and absorption of levodopa. On-off motor oscillations on the other hand are characterized by sudden and unpredictable shifts between functioning "on" and non-functioning "off" states[26].

Under physiological conditions, stimulation of dopamine receptors occurs in a continuous fashion. This contrasts with the effect of L-dopa which because of its short half-life of 90 min, results fluctuations in plasma levodopa levels and in pulsatile, rather than continuous stimulation of striatal dopamine receptors. It is thought that the ability of striatal neurons to take up L-dopa and store dopamine for continuous release is lost in advanced disease because of the diminished number of available neurons. It is suggested that this non-physiological pulsatile stimulation of dopamine receptors, might trigger a dysregulation of many neuro-transmitter systems within the basal ganglia and is the cause for the motor fluctuations and dyskinesias[22,25,27].

Reducing "off" time can be achieved by increasing the dosage of L-dopa. Increase in L-dopa dosage, however, can be associated with hyperkinetic movements or dyskinesias. The latter are involuntary movements other than tremor and most commonly consists of chorea which represent a peak-dose effect when L-dopa brain concentration is highest and is caused by excessive levels of dopamine ("peak-dose dyskinesia")[28]. Dyskinesia can be alleviated through reducing the dose of L-dopa, but only at the expense of worsening parkinsonism and an increase in the number of "off" episodes[22-24]. The risk of developing dyskinesia or wearing-off was found to be closely linked to L-dopa dose. Young age at onset, higher L-dopa dose, low body weight, female gender, and more severe UPDRS Part Ⅱ were among factors predictive of dyskinesia[29]. The approach to manage wearing-off and dyskinesia involves the addition of drugs which would permit more continuous dopaminergic stimulation, such as dopamine agonists, catechol-O-methyl transferase inhibitors, and monoamine oxidase type B (MAO-B) inhibitors, controlled-release formulation of L-dopa, transdermal delivery (rotigotine), infusion therapies (intravenous L-dopa, subcutaneous application of apomorphine and lisuride, duodenal infusion of L-dopa)[23,24,30] (Figure 2).

One therapeutic modality involves the use of L-dopa-carbidopa intestinal gel delivered continuously through an intrajejunal percutaneous gastrostomy tube. This form of therapy has been proved successful in controlling motor symptoms in advanced disease with fluctuating symptoms unresponsive to conventional oral treatment[31,32]. This treatment reduced "off" time and increased "on" time without troublesome dyskinesia at week 12 compared to baseline[31]. Improved motor complications and improvement in quality of life, autonomy and clinical global status have been reported after long-term treatment (over 7 years) with L-dopa/

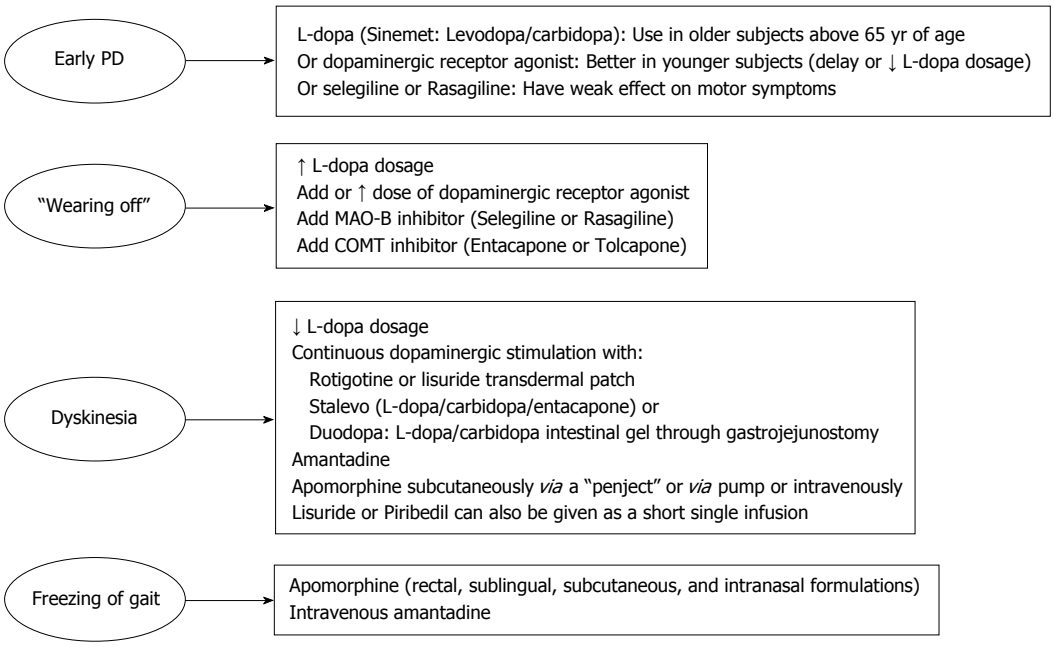

Figure 2 Drugs used to treat Parkinson's disease. L-dopa: Levodopa; PD: Parkinson's disease; MAO-B: Monoamine oxidase type B; COMT: Catechol-O-methyltransferase.

carbidopa intestinal gel[32]. It has also been shown that in advanced PD patients, continuous intrajejunal L-dopa-carbidopa intestinal gel infusion maintained stable plasma L-dopa levels with minimal degree of fluctuation in L-dopa, 3-OMD, and carbidopa plasma concentrations during 2-16 h of infusion, thereby, providing continuous dopaminergic stimulation[33]. In a randomized, double-blind trial in adults aged ⩾ 30 years with advanced PD and motor complications, the intestinal gel was more effective in decreasing "off" time and in increasing "on" time without troublesome dyskinesia when compared with immediate-release oral L-dopa-carbidopa[34]. L-dopa-carbidopa intestinal gel is not without complications. The technique involves an invasive procedure with attendant surgical and postsurgical complications. There are also problems related to the pump and tube, *e.g.*, dislocation and kinking of the intestinal tube, abdominal pain. Other complications are vitamin deficiency and polyneuropathies[31,32,35]. Subacute axonal neuropathy[36] and refractory seizures and a complex partial status epilepticus[37] likely to be due to vitamin B6 and/or vitamin B12 deficiency have been described under L-dopa-carbidopa intestinal gel infusion. Studies reported increased prevalence of neuropathy in chronic L-dopa treated patients and that cumulative levodopa exposure was significantly and positively associated with the severity of peripheral neuropathy. The cause of neuropathy is not clear but elevated plasma homocysteine level methylmalonic acid and reduced vitamin B12 levels that occurs under high doses of orally administered or continuously infused levodopa/carbidopa might be involved[38,39]. Monitoring vitamin B12/B6 status before and after starting L-dopa-carbidopa intestinal gel infusion and careful observation for signs of peripheral neuropathy have been advised[40].

Dopamine-receptor-agonists: These agents exert their antiparkinsonian effects by acting directly on dopamine receptors, thereby, mimicking the endogenous neurotransmitter. Several dopamine agonists are available for the treatment of PD. These include the ergot derivatives bromocriptine, cabergoline, alpha-dihydroergocryptine, lisuride, pergolide, the non-ergot agonists pramipexole, ropinirole, rotigotine, piribedil as well as apomorphine[41]. The non-ergot group of drugs is currently the most frequently prescribed oral dopamine receptor agonists. These agents are indicated both as an initial monotherapy in early PD to delay the need for L-dopa and in combination with L-dopa in advanced disease, enabling patients to take lower doses of L-dopa, thereby reducing the frequency of L-dopa induced motor complications[42]. The addition of dopamine agonists allows around a 20%-30% reduction in the dose of L-dopa[41]. When used as an initial monotherapy, these agents are effective in controlling motor symptoms. Overtime, however, dopamine receptor agonists lose efficacy and after 3 years of treatment with agonist monotherapy, the number of patients remaining decreases to less than 50%. These patients will then require the addition of L-dopa, so as to achieve better control of their motor symptoms[43]. The rate of discontinuation of dopamine receptor agonists also appears to higher compared with L-dopa monotherapy (20% *vs* 1% over four years) (due to impulse control disorders, somnolence and light-headedness)[21].

The use of dopamine receptor agonists might have several benefits. First, their use early in the disease is likely to delay or reduce the incidence of dyskinesia,

Table 1 Currently available dopaminergic receptor agonists

Drug	D-receptor specificity	Dose range	Side effects
Ergot derivatives			
Bromocryptine	D2 receptor agonist with partial dopamine D1 antagonist activity	7.5-15 mg/d orally	Risk of developing valvular regurgitation (cumulative and dose-dependent)
Lisuride	D2 receptor agonist (also has a weak dopamine D1 antagonistic, serotonin 5-HT1A agonistic, and 5-HT2B receptor antagonist properties)	0.2-3 mg/d orally	Dry mouth, nausea, weakness, postural hypotension, and headache
Cabergoline	D1 and D2 receptor agonist	0.5-4 mg/d orally	Risk of developing moderate to severe valvular regurgitation
Non-ergot derivatives			
Piribedil	D2/D3 receptor agonist with alpha(2) antagonist properties	150-300 mg/d orally	
Rotigotine transdermal patch	D1, D2, and D3 receptor agonist	2-16 mg patch/d	Patch site reactions, nausea, vomiting, dry mouth, somnolence , peripheral edema, and dyskinesia
Ropinirole (immediate and extended release)	D2/D3 receptor agonist	4-24 mg/d orally	Nausea, dyspepsia, dizziness, back pain, headache, uncontrollable sleep attacks, orthostatic hypotension, leg oedema
Pramipexole (immediate and extended release)	D2/D3 receptor agonist	0.25 - 4.5/d orally (Extended release pramipexole is approved as monotherapy in early PD, as well as an adjunct therapy to levodopa in advanced PD	Somnolence, cognitive adverse events, fatigue, nausea, constipation, and peripheral oedema
Apomorphine	D1 and D2 receptor agonist (also stimulates serotonin and α-adrenergic receptors)	4 mg subcutaneously 20 mg continuously daily *via* a pump system	Local inflammation and granuloma at the subcutaneously infusion site Intravascular thrombotic complications secondary to apomorphine crystal accumulation after *iv* administration

iv: Intravenous.

dystonia, and motor fluctuations resulting from long-term L-dopa therapy, possibly due to better continued rather than pulsatile stimulation of postsynaptic dopamine receptors[44,45]. One recent study showed that an initial treatment with dopamine agonists resulted in 87% lower risk for dyskinesia compared with treatment with L-dopa[46]. Second, dopamine-receptor-agonists are not metabolized to active chemicals, do not produce toxic metabolites or lead to the cytotoxic free radical formation that might be associated with metabolism of dopamine[41,47]. Third, there is also evidence that dopamine-receptor-agonists might slow the progression of disease. Clinical imaging studies targeting dopamine function with by [^{123}I]β-CIT or [^{18}F]Dopa imaging suggested that the rate of loss of [^{123}I]β-CIT or [^{18}F]Dopa uptake in early PD patients treated with dopamine agonists is slower as compared with L-dopa[48]. Table 1 lists the currently available dopaminergic receptor agonists and their side effects.

Ergot derivatives

Bromocryptine: This is the earliest dopamine agonist in use which was introduced in the 1970s as adjunct therapy to L-dopa for PD patients with motor complications[49]. It is structurally related to dopamine and activates postsynaptic dopamine D2 receptors with partial dopamine D1 antagonist activity[50]. Compared with low-dose L-dopa, dyskinesia and dystonia were delayed by early use of bromocriptine, but end-of-dose failure appeared at a similar time once L-dopa was added. The rate of disease progression, however, was similar whether treatment was initiated with L-dopa or bromocryptine[51]. There was no evidence of a long-term benefit or clinically relevant disease-modifying effect on initiating treatment with bromocryptine. The initially reduced frequency in motor complications was not sustained and motor disability or mortality was not reduced over the long term. Moreover, disability scores and physical functioning were better in the L-dopa than in the bromocriptine treatment group[52]. Patients on bromocriptine have the risk of developing valvular regurgitation which occurs in a cumulative dose-dependent manner[53].

Lisuride: The drug exerts its activity primarily at postsynaptic dopamine D2 receptor sites. It has also a weak dopamine D1 antagonistic and serotonin 5-HT1A agonistic effects[50] as well as 5-HT2B receptor antagonist properties[54]. Lisuride is short-acting with a plasma half-life of 1-3 h. In contrast to bromocriptine, cabergoline, and pergolide, the drug is not associated with fibrotic cardiac valvulopathy[54,55]. Transdermal delivery using lisuride patches has been shown to improve the motor changing rate in advanced PD patients with unpredictable on-off phenomena[56].

Cabergoline: Cabergoline has selective affinity for D2-like dopamine receptors. It has a long plasma

half-life of 65 h[41]. In early PD, initiating therapy with cabergoline improved motor disability and delayed the development of motor complications compared with L-dopa-treated patients[57]. A reduction in daytime sleepiness by 70% was reported following a fast switch-over from the currently used dopamine agonist to a single equivalent dose of cabergoline, administered at bedtime[58]. In patients with motor fluctuations and dyskinesia, cabergoline improved "off" or "on" hours, or both[59], improved "on" with dyskinesia', mean dystonia intensity, time spent in severe "off' condition, severity of "off" periods and allowed the reduction of L-dopa requirements[60]. Side effects of cabergoline include gastric upset, orthostatic hypotension, and ankle edema[59]. There were also reports of constrictive pericarditis, cardiac valvular regurgitation and pleuropulmonary disease (pleural effusion/pulmonary fibrosis)[59,61-63]. The ability of cabergoline (and bromocriptine) to cause fibrotic cardiac valvulopathies and consequent valvular regurgitation appears to be mediated through stimulation of valvular 5-HT(2B) serotonin receptors that might mediate mitogenesis and, in turn, the proliferation of fibroblasts[64,65]. Lisuride and non-ergot dopamine agonists are devoid of 5-HT(2B) agonistic activity and hence might not induce heart valve fibrosis[54,55].

Non-ergolinic dopamine receptor agonists

Piribedil: Piribedil [1-(3,4-methylenedioxybenzyl)]-4-[(2-pyrimidinyl)]piperazine is a D2/D3 receptor agonist with alpha(2) antagonist properties. This last property of piribedil might favorably influence motor function, cognition, mood and the integrity of dopaminergic neurons[66]. In early PD, the administration of piribedil (150-300 mg/d) improved UPDRS Ⅲ over a 7-mo period; the proportion of responders (UPDRS Ⅲ improvement > 30%) was significantly higher for piribedil (42%) than for placebo (14%)[67]. In PD patients insufficiently controlled by L-dopa alone, early combination of piribedil (150 mg) or bromocriptine (25 mg) with L-dopa resulted in similar long-term improvement of motor symptoms (UPDRS Ⅲ) over 12 mo. Piribedil-treated patients, however, required less L-dopa dose increase than those on bromocriptine[68]. In PD patients with motor fluctuations, piribedil improved motor UPDRS scores, and activities of daily living, increased the duration of effect of L-dopa and permitted the mean daily L-dopa dose to be decreased by 17%[69]. Piribedil can also be given as a short single infusion of at 2 to 16 mg to improve motor symptoms, including akinesia in PD patients with fluctuations[70]. The new sublingual formulation of piribedil at a single dose of 60 mg was superior to placebo in improving UPDRS Ⅲ and aborting "off" in patients with advanced PD[71]. In PD presenting with apathy following subthalamic nucleus stimulation, piribedil improved apathy by 46.6% as well as depression[72]. Side effects of piribedil include hallucinations, dyskinesias, dizziness[69], gastrointestinal complaints[67] and sleep attacks[73].

Rotigotine: This dopamine D1, D2, and D3 receptor agonist is administered through a silicone-based transdermal patch designed for once-daily application. Steady-state plasma levels of rotigotine can be reached between 8 and 12 h, and a stable drug release is maintained throughout the 24-h patch application[74]. Rotigotine patch thus allows constant delivery of the drug and possible continuous dopaminergic stimulation. Rotigotine improved motor scores, and the activities of daily living[75]. The drug is indicated both as monotherapy for the treatment of early PD, and as adjunctive therapy to L-dopa in advanced PD with motor fluctuations, significantly reducing "off" time[76,77]. In patients with early-stage PD, rotigotine was generally well tolerated for up to approximately 6 years[76]. The majority of patients experiencing dyskinesia reported first appearance after starting L-dopa[76]. Most common adverse events reported were application site reactions, nausea, vomiting, dry mouth, somnolence, peripheral edema, and dyskinesia[75-77]. Rotigotine transdermal patch was shown to be of similar efficacy to oral pramipexole in patients with fluctuating PD over 6 mo of treatment. The absolute change in "off" time from baseline compared with placebo was -1.58 h for rotigotine and -1.94 h for pramipexole and responder rates were 67% for pramipexole, 59.7% for rotigotine[78].

Ropinirole: Immediate- and extended-release once-daily formulations of ropinirole are available. Ropinirole prolonged-release formulations has also been shown to delay the onset of dyskinesia in early PD, compared with increasing doses of L-dopa. This was achieved without significant change in UPDR Scale activities of daily living or motor scores[79]. In advanced PD patients not optimally controlled with L-dopa and who suffered troublesome nocturnal disturbance, the extended-release formulation provided 24-h symptom control and improved nocturnal symptoms. In these patients, the drug resulted in reduction in awakenings and in an increase in awake time "on"/"on" without troublesome dyskinesia during night-time and early morning[80-82]. In moderate-to-advanced PD, symptom control could be achieved 2 wk after treatment initiation[83]. Patients on pramipexole could be switched overnight to extended release ropinirole without serious adverse events[84]. Ropinirole most commonly causes nausea and sleepiness, less commonly uncontrollable sleep attacks, vertigo, dyspepsia, orthostatic hypotension, leg oedema, back pain, and headache[81,85]. Gastrointestinal complaints and sleep/fatigue were significantly higher for ropinirole than for pramipexole[86].

Pramipexole: Pramipexole shows high affinity for the D2 subfamily of dopamine receptors. The drug is effective as a symptomatic treatment in early PD,

reducing UPDRS by 4-5 points relative to placebo[87]. When used as a monotherapy in early idiopathic PD, pramipexole was of comparable clinical efficacy to rasagiline[88]. It alleviated L-dopa dyskinesia when used as an "add on" therapy or in place of ergot dopamine agonist[89]. Switching patients with PD from ergot dopamine agonist, *e.g.*, cabergoline to pramipexole, appeared to be well tolerated and effective, but adjustment of pramipexole dose is required in some patients to reduce side effects[90,91]. Significant improvement in the UPDRS was evident after 2 wk of initiating therapy with pramipexole and maintained up to 12 wk of treatment[91]. Pramipexole was associated with significantly low rates of fatigue[92] and improved depressive symptoms[93] in PD patients. There appear, however, to be no significant difference between early and delayed pramipexole initiation on UPDRS total score or striatal dopamine-transporter binding in patients with early PD[94].

Extended release pramipexole is approved as monotherapy in early PD, as well as an adjunct therapy to L-dopa in advanced PD. It has the advantage over the immediate release formulation of improved compliance because of once-daily dosing regimen and steadier plasma levels over 24 h[95]. In patients with early PD not receiving L-dopa or dopamine receptor agonists, once-daily extended-release pramipexole was of similar efficacy to the immediate release preparation (taken 3 times daily) in controlling motor symptoms, and in safety and tolerability[96,97]. In those with motor fluctuations on L-dopa therapy, the addition of either the extended-release and immediate-release preparations was of similar efficacy in improving UPDRS score and off-time compared with placebo, with similar tolerability, and safety[98]. In advanced PD, switching to one daily pramipexole formulation from thrice daily immediate-release tablet formulation was also effective in controlling motor symptoms[99].

Somnolence, fatigue, nausea, constipation, and peripheral oedema are common side effects of pramipexole[87,96]. The drug administered in a single oral dose to healthy young subjects, reduced mean sleep latency and increased total duration of sleep. These effects were not observed with L-dopa and bromocryptine[100]. In early PD, pramipexole monotherapy resulted in higher incidence of cognitive adverse events compared with ropinirole[86]. Other dopamine-receptor-agonists, *e.g.*, rotigotine and cabergoline did not affect cognitive function in patients with early-mild disease. It has been suggested that their combined stimulation of both dopamine (D1 and D2) receptor families might account for preserving cognitive functions compared with pure D2 family stimulation that occurs with pramipexole[101].

Apomorphine: This synthetic morphine derivative exerts antiparkinsonian effects by non-selective stimulation of dopamine receptors. The drug also stimulates serotonin and α-adrenergic receptors. It is currently used in patients with advanced PD for the treatment of persistent and disabling motor fluctuations unresponsive to conventional therapy with L-dopa or dopamine receptor agonists, with or without deep brain stimulation[102]. In late stage PD, apomorphine administered *via* subcutaneous, intravenous routes or by inhalation, has been shown to result in long term symptomatic improvement, effectively abort "off" episodes and significantly decrease L-dopa equivalent dose[103-106]. Non-motor symptoms as hyperhidrosis, nocturia, urgency of micturition, and fatigue improved as well[106]. Switching patients with refractory motor fluctuations from subcutaneous to intravenous therapy with apomorphine resulted in 59% decrease in their additional oral anti-parkinsonian medication. Dyskinesias also significantly decreased and "off" time was virtually eliminated[104]. Continuous subcutaneous apomorphine infusion proved of symptomatic benefit in those with untreatable motor fluctuations but in whom subthalamic nucleus deep brain stimulation was contraindicated (because of L-dopa-resistant axial motor symptoms and/or cognitive decline). Daily "off" time decreased while "on" time improved together with a significant reduction in mean oral L-dopa equivalent dose[107]. In PD patients undergoing deep brain stimulation, subcutaneous apomorphine reduced the risk of neurologic and respiratory deterioration caused by perioperative withdrawal of dopaminergic medication[108].

Apomorphine causes severe nausea and vomiting. It has been suggested that the activation of human sensory transient receptor potential A1 channels by apomorphine, might contribute to adverse side effects such as nausea and painful injections[109]. The most common side effect to subcutaneous apomorphine is local inflammation at the infusion site[103,107]. Moreover, intravascular thrombotic complications, secondary to apomorphine crystal accumulation, necessitating cardiothoracic surgery, complicate intravenous therapy with apomorphine[104].

Monoamine oxidase inhibitors

Selegiline and rasagiline: Both MAO-A and MAO-B contribute to dopamine metabolism. MAO-A is the main enzyme responsible for the metabolism of the monoamines, noradrenaline, serotonin and dopamine. MAO-B is more specific to dopamine metabolism[110]. MAO-B inhibitors are clinically being used to treat PD by blocking the degradation of dopamine and thereby providing a symptomatic relief in these patients. Selegiline (Deprenyl/Eldepryl) and rasagiline (Azilect) are irreversible selective inhibitors of the enzyme MAO-B. Selegiline, the R-optical enantiomer of deprenyl (phenyl-isopropyl-methyl-propargylamine) was approved by the Food and Drug Administration (FDA) in 1996. Selegiline is a propargyl amphetamine derivative that undergoes extensive first-pass metabolism to L-methamphetamine, L-amphetamine, and desmethyl-deprenyl. Rasagiline [N-propargyl-1-(R)-aminoindan]

is a novel, highly potent irreversible MAO-B inhibitor, recently introduced in the treatment of PD. Rasagiline has received FDA approval in 2006. Rasagiline's major metabolite is aminoindan, which has no amphetamine like properties[111,112] and thus is not likely to cause sleep disturbances compared with selegiline. In patients treated with selective MAO-B inhibitors, the risk of serotonin toxicity due to a concomitant serotonergic agent or hypertensive crisis due to dietary tyramine or sympathomimetic amines appears to be minimal and should not preclude the use of MAO-B inhibitors in treating PD[113]. There is evidence, however, that daily treatment with MAO-B inhibitor may also influence MAO-A activity. Thus in plasma samples from patients with MAO-B inhibitor therapy, there was 70% reduction of MAO-A activity compared with patients without MAO-B inhibitor treatment or healthy controls[114].

Selegiline and rasagiline are effective as initial monotherapy in early PD and as adjunctive therapy in advanced PD[115-117]. MAO-B inhibitors provide mild symptomatic benefit, compared with L-dopa and dopamine agonists. These drugs are indicated for the treatment of akinesia and motor fluctuations associated with L-dopa therapy. Both agents are safe and well tolerated at the recommended daily doses. They might delay the need start L-dopa therapy, reduce disability and reduce the rate of motor fluctuations compared with initial L-dopa therapy[96,118,119]. Rasagiline inhibits MAO-B more potently than selegiline and has the advantage of once-daily dosing and favorable tolerability[116,120]. Rasagiline was effective both as monotherapy in early PD and as adjunctive treatment in advanced PD and motor fluctuations. As monotherapy, however, rasagiline provided modest yet clinically meaningful benefit on motor symptoms (compared to other drugs)[121,122]. Early in the disease, rasagiline monotherapy at 1 mg/d improved symptoms. In advanced PD, rasagiline adjunct therapy (0.5 or 1 mg/d) to L-dopa significantly reduced the total daily "off" time[123]. Rasagiline (1 mg/d), in L-dopa-treated PD patients with motor fluctuations produced a significant improvement over placebo in UPDRS motor "off" score. Rasagiline significantly improved bradykinesia and showed trends for improvements in facial expression, speech, and axial impairment during OFF time[124]. Rasagiline has a rapid beneficial effect on PD symptoms from the first week of therapy. Objective and subjective measures of symptom severity improved at 1 wk (change from baseline in bradykinesia scores and physicians' and patients' global impression). The magnitude of benefit was similar in patients treated with once-daily rasagiline either as monotherapy (1.0 mg) or as adjunct therapy (0.5 mg)[125]. Rasagiline might also possess antidepressant effect. In patients with newly diagnosed PD with comorbid untreated depression, rasagiline monotherapy 1 or 2 mg/d for 8 wk, improved the activity of daily living and motor function as well as symptoms of depression. The latter effect was observed at the higher dose of 2 mg/d and appeared not to be related to the motor improvement[126]. Motor behavior, motor complications, mood and sleep improved when patients on selegiline were switched to 1 mg rasagiline[127]. Rasagiline monotherapy in early untreated disease also demonstrated better adverse events profile in the incidence of gastrointestinal symptoms and sleep disorders and less incidence of dropout rates compared with pramipexole[88].

The use of selegiline and rasagiline in the early stage of the disease might also improve long-term outcome. L-dopa-treated patients who received selegiline within 5 years from the onset of the disease exhibited significantly lower UPDRS motor scores over 7 years compared with those who received selegiline 9 to 11 years after the onset of the disease[117]. One study suggested that selegiline use (≥ 3 years) in early PD patients who were of younger age, shorter PD duration, lower UPDRS motor scores was associated with a slower progression of PD[128]. Early-start treatment with rasagiline at a dose of 1 mg/d (though not 2 mg/d) caused a smaller increase in rate of worsening in the UPDRS score between weeks 12 and 36, less worsening in the score between baseline and week 72 compared with the placebo group. The study suggested a disease-modifying effect for rasagiline[129]. In the ADAGIO study, Rascol *et al*[130], assessed the ability of rasagiline to modify need for additional antiparkinsonian therapy and changes in non-motor and motor changes in patients with untreated early PD. Patients received rasagiline 1 mg/d or 2 mg/d for 72 wk (early-start groups) or placebo for 36 wk followed by rasagiline 1 mg/d or 2 mg/d for 36 wk (delayed-start groups). The findings of the study suggested that rasagiline delayed the need for symptomatic antiparkinsonian drugs.

Recent interest in selegiline and rasagiline has focused on their possible neuroprotective effects that have been delineated in preclinical models of PD[131-133].

Catechol-O-methyltransferase inhibitors

In the presence of aromatic amino acid decarboxylase inhibitors, L-dopa metabolism is predominantly shifted to the formation of 3-O-methyldopa by the enzyme catechol-O-methyltransferase (COMT), which has the highest activity in the liver and kidney[18]. The reversible COMT inhibitors tolcapone and entacapone, are being used as an adjunct to L-dopa for the symptomatic treatment of PD patients with motor fluctuations. These agents extend the elimination half-life of L-dopa by inhibiting the peripheral breakdown of L-dopa, thereby increasing L-dopa bioavailability, which will decrease "of" time and increase "on" time in fluctuating PD patients and allow the dosage of L-dopa/carbidopa to be reduced. Moreover, by stabilizing plasma L-dopa concentrations, tolcapone and entacapone permit a more continuous stimulation of dopamine receptors which in theory would reduce the risk of motor complications[25,134].

Entacapone

When used as an adjunct to concomitant treatment with L-dopa and a dopa decarboxylase inhibitor (DDCI), entacapone showed benefits in the quality of life and activities of daily living and was efficacious in increasing "on" time and decreasing "of" time in PD patients with wearing-off fluctuations[135-140]. Moreover, compared with L-dopa/carbidopa or L-dopa/benserazide, treatment with L-dopa/carbidopa/entacapone resulted in significantly greater improvements in non-motor domains such as depression, personal relationships, and communication[139]. In randomized, open-label study, entacapone was as effective as cabergoline in conjunction with L-dopa in decreasing the daily "off"-time and in improving the quality of life (a decrease of approximately 20% was detected in UPDRS Ⅱ and Ⅲ motor scores, with no differences between the groups). The effect of entacapone, however, was more quickly apparent compared with that of cabergoline[141]. In patients receiving L-dopa and a DDCI, the addition of entacapone improved UPDRS Ⅲ motor scores during the first 6 mo of combined therapy, increased daily "on" time and the response duration to a single morning dose of L-dopa. The mean daily dose of L-dopa did not increase over the 5-year follow-up period, suggesting the long-term efficacy of L-dopa/DDCI and entacapone[142].

Studies suggested that early rather than delayed addition of entacapone to L-dopa/DDCI in PD patients with wearing-off provides a modest clinical benefit over L-dopa/DDCI that is maintained for up to 5 years, with an improvement in UPDR motor scale[143]. In patients with early PD, compared with L-dopa/carbidopa (Sinemet), L-dopa/carbidopa/entacapone (Stalevo) resulted in significantly greater improvement in activities of daily living and subject-reported clinical global impression without increasing motor complications[144]. Studies also suggested that switching from L-dopa/DDCI and entacapone and L-dopa/DDCI provides a significant benefit in PD patients with wearing-off[145]. Comparing immediate and delayed switch to L-dopa/carbidopa/entacapone was in favor of immediate switch in terms of greater motor improvement and quality of life[146].

The most common adverse effect of adding entacapone is the increase in dyskinesia[135,141] which would necessitate reducing the dose of L-dopa. In one study, patients with PD and with mild-to-moderate wearing-off without or with mild dyskinesias were randomly assigned to either receiving the same L-dopa-carbidopa dosage or 15%-25% less total L-dopa-carbidopa amount. The findings showed that either regimen resulted in increase in daily "on" time and a reduction in the daily time spent in "off" 4 wk after the change[147]. In a randomized, open-label trial in patients with wearing-off with conventional L-dopa/DDCI therapy, adjunct therapy with entacapone or increasing dose frequency of L-dopa without an increased total daily dose (dose fractionation) reduced the mean "off" time, and the rate of motor complications[148].

On the other hand, entacapone did not improve motor scores on the UPDR Scale when used as an adjunct to L-dopa in PD patients who do not experience motor fluctuations[149]. Moreover, initiating L-dopa therapy with L-dopa/carbidopa/entacapone was associated with a shorter time to onset and increased frequency of dyskinesia compared to L-dopa/carbidopa[150]. Entacapone was also non-efficacious in the prevention/delay of motor complications (reviewed by Fox *et al*[151]). In another study, entacapone (200 mg with each L-dopa dose) was ineffective in reducing the severity of motor symptoms in the "off" state in L-dopa-treated PD patients with motor fluctuations[124]. Dyskinesia is the most common adverse event of entacapone[152,153]. Entacapone was not associated with an increased risk of acute myocardial infarction, stroke, or death in elderly patients with PD[154].

Tolcapone

Tolcapone is a longer acting and more potent COMT inhibitor compared with entacapone[155]. The agent is used in patients with severe motor fluctuations inadequately controlled with entacapone[153]. In patients with advanced PD who were switched to tolcapone because of persisting "off" periods despite treatment with entacapone, there were significant reductions in mean daily off-time duration and L-dopa dose at follow up[156]. The daytime sleepiness, global clinical impression of change, activities of daily living, and quality of life were also significantly improved after adjunctive tolcapone treatment to L-dopa/carbidopa in fluctuating PD patients[157,158]. A randomized, open-label, trial of 150 patients on a stable regimen of L-dopa/DDCI in combination with bromocriptine, lisuride, or pergolide, conducted to assess the efficacy of switching from a dopamine agonist to tolcapone, found the drug to be effective in decreasing daily "off" time, increasing "on" time (as well as other efficacy variables, *e.g.*, UPDR scale Ⅱ, Ⅲ, and Ⅳb and investigator's global assessment scores)[159].

The most common adverse event with tolcapone is dyskinesia which might require decreasing the dose of L-dopa. Elevations of the serum liver enzymes aspartate aminotransferase and alanine aminotransferase have been reported in patients on tolcapone treatment. Therapy with tolcapone thus requires monitoring for of liver function every 2 to 4 wk for 6 mo for hepatotoxicity[156,160,161]. Severe liver injury due to tolcapone, however, appears to be a rare event[160]. In addition, studies on the safety and efficacy of the long-term use of tolcapone concluded that significant liver transaminase elevations were rare and these returned to normal in most patients[162,163]. Tolcapone causes severe diarrhea more often than entacapone[155].

Nebicapone

Nebicapone is a new COMT inhibitor which has been found efficacious for the treatment of motor fluctuations

in PD patients[164]. In randomized, double-blind, placebo-controlled study, nebicapone 75 mg and 150 mg showed greater effect in increasing "on" time and decreasing "off" time compared with entacapone 200 mg. The drug produced more sustained COMT inhibition compared with entacapone 200 mg. Nebicapone 150 mg increased L-dopa area under the plasma concentration time curve by 48.4% compared to a value of 33.3% after entacapone 200 mg[165]. Nebicapone has the risk of increasing liver transaminases[164].

N-methyl-D-aspartate glutamate receptor antagonism

Amantadine: Amantadine is an antiviral drug which was found to exert beneficial antiparkinsonian effects[166]. As an alternative to L-dopa in early PD, amantadine is associated with improvement in functional disability, and in a subset of PD patients, there is a robust symptomatic improvement[167]. The main current indication of amantadine is, however, as an adjunctive treatment for L-dopa-induced dyskinesia in late-stage PD. Amantadine, is an N-methyl-D-aspartate glutamate receptor antagonist. Increased glutamate transmission contributes to the motor symptoms in PD, and also to the progression of neurodegeneration through excitotoxic mechanisms[168]. Amantadine might also improve apathy and fatigue in PD patients[169]. The drug is well absorbed and widely distributed, little drug being present in the circulation, and is primarily eliminated through the kidneys both by glomerular filtration and tubular secretion. The dose of amantadine, therefore, requires adjustment in in patients with renal dysfunction[170]. In patients with PD on amantadine therapy, plasma amantadine concentration increased according to increasing renal dysfunction[171].

Dyskinesia can improve with amantadine and in a multi-center, double-blind, randomized, placebo-controlled trial, dyskinesia rating scale improved in 64% patients treated with amantadine compared to 16% on placebo[172]. In a randomized placebo-controlled study of 32 patients who have been on stable amantadine therapy for L-dopa-induced dyskinesia over at least one year, dyskinesia duration and intensity (assessed by UPDRS Ⅳ items) significantly increased at three-week follow-up after being switched to placebo[173]. Wash-out of amantadine in dyskinetic patients with PD significantly worsened L-dopa induced dyskinesia (with greater worsening of abnormal involuntary movement scale score)[169].

Amantadine is also effective in the treatment of freezing of gait in patients with advanced PD. In one study, freezing of gait improved by treatment with amantadine in 11 patients with advanced PD. The effect, however, decreased in a proportion (approximately 36%) of patients after 4 mo[174]. In a randomized, double-blind, placebo-controlled, multicenter trial of 42 subjects with freezing of gait, 5 d intravenous amantadine attenuated freezing severity and improved patients' mobility[175]. Intravenous administration of amantadine has also been effective in improving parkinsonian symptoms after surgery[176]. In PD patients with subthalamic nucleus deep brain stimulation and incomplete axial benefit, gait scores significantly improved with amantadine treatment. Patients also reported subjective improvement in speech, gait or balance[177]. Side effects of amantadine includes blurred vision, visual hallucinations, peripheral edema (Malkani *et al*[174], 2012), reversible corneal edema after long term use[178], auditory hallucinations[179]. Myoclonus, hallucination, or delirium might develop when the plasma concentration of amantadine exceeds 3000 ng/mL[171]. Cardiac arrest, ventricular tachycardia and prolonged QTc interval have been reported following amantadine[180,181].

Studies have shown that amantadine might possess neuroprotective properties. The dug protected rat midbrain cultures from either MPP(+) or lipopolysaccharide. Amantadine possibly exerts its neuroprotective effects through the inhibition of the release of microglial pro-inflammatory factors, and/or an increase in expression of neurotrophic factors such as glial cell line-derived neurotrophic factor released from astroglia[182].

Anticholinergic drugs

The anticholinergic drugs were the first agents to be used in the pharmacological management of PD[183]. Nowadays, however, they have limited place in the treatment of the disease. These drugs are also prescribed to ameliorate extrapyramidal symptoms caused by antipsychotic medications[184], but this also appears to be declining owing to an increase in the use of atypical anti-psychotic agents[185]. When used as monotherapy in early disease or as an adjunct to other antiparkinsonian drugs, anticholinergics are more effective than placebo in control of symptoms. Because of the high risk of cognitive, neuropsychiatric and autonomic adverse events, these agents are best avoided in the elderly[186-188].

The current place of anticholinergic agents in treatment of PD is limited to early cases and in younger patients (*i.e.*, 60 years of age) with troublesome resting tremor because of the evidence that these agents are better than levodopa for tremor[189]. Studies, however, have shown that dopaminergic agents are as effective as anticholinergics in reducing tremor in idiopathic PD. Single-dose challenges with biperiden or apomorphine significantly reduced the amplitude of resting, postural, and action tremor. UPDRS scores for rigidity and akinesia, however, were only reduced by apomorphine[190]. Moreover, the effect of biperiden on the amplitude of the resting tremor was weaker than that of L-dopa had a good effect on the amplitude of the resting tremor[191].

Anticholinergic drugs are of little value in the treatment of rigidity, akinesia, gait dysfunction, or impaired postural reflexes[189]. It has been shown however that trihexyphenidyl might be of benefit in

patients whose axial symptoms worsened after deep brain stimulation of the subthalamic nucleus. In this study UPDRS Ⅱ and Ⅲ decreased in response to the anticholinergic agent[192]. Studies also suggested that the use of anticholinergic drugs early in the disease progression might be of potential benefit in delaying the need for L-dopa treatment[183]. Side effects due to anticholinergic agent include dry mouth, blurred vision, tachycardia, urinary retention, constipation, impaired sweating, and central nervous system effects, *e.g.*, memory impairment, confusion, and hallucinations, especially in older individuals[183,189].

NEWER DRUGS

Adenosine A_{2A} receptor antagonism

Istradefylline: Istradefylline (KW-6002) is a selective adenosine A2A receptor antagonist which exhibit antiparkinsonian activity without worsening L-dopa induced dyskinesia. Istradefylline is not yet an FDA-approved drug. Istradefylline has been licensed as an anti-parkinsonian drug this year in Japan[193]. Istradefylline exhibits high affinity for A2A receptors, but lower affinities for the other subtypes of adenosine receptors (A1, A2B, and A3) in humans, marmosets, dogs, rats, and mice. The agent does not influence other neurotransmitter receptors, inhibit monoamine oxidases, or catechol-O-methyl transferase[194]. Unlike L-dopa, the chronic administration of istradefylline (and also of other A2A receptor antagonists: SCH 412348, vipadenant and caffeine) to rats did not result in dyskinetic activity or worsen dyskinesias when co-administered with L-dopa[195]. In non-human primates with haloperidol-induced extrapyramidal symptoms (EPS) and catalepsy, A2A receptor antagonists, SCH 412348 and KW-6002 and the A1/A2A receptor antagonist, caffeine significantly increased the time to the onset of EPS. Moreover, SCH 412348 and caffeine significantly reduced haloperidol-induced catalepsy[196]. In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated marmosets with L-dopa dyskinesia, single dose acute oral administration of istradefylline enhanced and prolonged the anti-parkinsonian effects of a sub-optimal dose of L-dopa, while its chronic administration did not worsen dyskinesia[197].

When evaluated as monotherapy in patients with early PD, istradefylline 40 mg/d for 12-wk was safe and well tolerated, but failed to significantly improve motor symptoms[198]. In PD patients on L-dopa therapy with motor complications, istradefylline 10, 20 or 40 mg/d, administered as adjunctive treatment to L-dopa for 12 wk in a double-blind study did not affect "off" time duration, though at 40 mg/d it significantly improved the motor score[199]. Other studies, however, showed that istradefylline 20, 40 or 60 mg/d, given once daily for 12 wk to L-dopa-treated patients with motor complications was well tolerated and reduced daily "off" time[200-204]. The most common adverse event was dyskinesia[200-205]. Other side effects reported with istradefylline were lightheadedness, tremor, constipation, weight decrease[201], nausea, dizziness, and hallucinations[200].

Preladenant: Preladenant (SCH 420814) is an orally bioavailable selective adenosine A_{2A} receptor antagonist in phase Ⅲ development for PD treatment. In MPTP-treated primates, preladenant improved motor ability without causing any dopaminergic-mediated dyskinetic or motor complications. The drug also delayed the onset of EPS symptoms evoked by an acute haloperidol challenge in primates with previous chronic haloperidol treatment[206]. Two randomized, double-blind, placebo-controlled, ascending-dose studies, showed that the drug was generally well tolerated up to 200 mg/d. Peak plasma concentrations were reached in approximately 1 h and then declined rapidly. Preladenant caused transient mild increases in blood pressure within a few hours of administration[207]. In patients with PD and motor fluctuations who were receiving L-dopa, preladenant at 5 and 10 mg given twice daily for 12 wk reduced the mean daily "off" time relative to baseline. The most common adverse events in the L-dopa/preladenant group *vs* placebo were worsening of PD (11% *vs* 9%), somnolence (10% *vs* 6%), dyskinesia (9% *vs* 13%), nausea (9% *vs* 11%), constipation (8% *vs* 2%), and insomnia (8% *vs* 9%) [208]. In another study, preladenant treatment (5 mg twice a day) for 36 wk as a L-dopa adjunct in subjects with fluctuating PD, provided sustained "off" time reductions (1.4-1.9 h/d) and "on" time increases (1.2-1.5 h/d) relative to the baseline. The main side effects were dyskinesia (33%) and constipation (19%)[209].

Safinamide: Safinamide is a novel anti-parkinsonian drug currently in phase 3 clinical trials, as add-on therapy to L-dopa or a dopamine agonist in early and advanced stage PD. It is an oral alpha-aminoamide derivative, with dopaminergic and non-dopaminergic mechanisms of action involving inhibition of dopamine and noradrenaline reuptake, a selective and reversible inhibition of MAO-B, blockage of voltage-dependent sodium channels, modulation of calcium channels as well as an inhibitor of glutamate release[210-212]. Safinamide is administered once daily at doses of 50 and 100 mg[211]. In an open pilot study, safinamide (100, 150, and 200 mg once a day) improved motor performance when added to a stable dose of dopamine agonist and also decreased motor fluctuations in those treated with L-dopa[213]. Two randomized double-blind studies suggested that safinamide 100 mg/d may be effective as an "add-on" treatment to a dopaminergic agonist in early PD[214,215]. In 24-wk, double-blind study, safinamide 100 mg added to a dopamine agonist improved motor symptoms (UPDRS motor total score)[214]. Safinamide 100 mg/d for 12-mo resulted a lower rate of intervention (increase in

dopamine agonist dose; addition of another dopamine agonist, L-dopa or other PD treatment) and a delay in median time to intervention of 9 d compared with placebo[215]. In both studies, there was no benefit from safinamide 200 mg. A more recent study showed that oral safinamide 50 or 100 mg/d added to L-dopa in patients with PD and motor fluctuations for 24 wk, increased time with no or non-troublesome dyskinesia, decreased "off" time, and improved UPDRS motor scores as well as clinical global impression-change[216]. In MPTP- lesioned dyskinetic macaque monkey made dyskinetic by treatment with L-dopa, pre-treatment with safinamide (3, 10, 20 and 30 mg/kg) dose-dependently reduced dyskinesia scores and prolonged the duration of the antiparkinsonian effect of L-dopa. Moreover, combined amantadine (5 mg/kg) and safinamide (20 mg/kg) exerted additional beneficial effects on L-dopa-induced dyskinesia[217].

Safinamide, also appear to exert neuroprotective effects by blocking the voltage-dependent Na^+ and Ca^{2+} channels and the Ca^{2+}-mediated glutamate release processes. Safinamide provided significant protection against neurological deficit and axonal degeneration in experimental autoimmune encephalomyelitis, possibly *via* reduction in the activation of microglia/macrophages, resulting in suppressed microglial superoxide production [218].

Monoamine oxidase inhibition

Zonisamide: Zonisamide (1,2-benzisoxazole-3-methanesulfonamide) is a new antiepileptic drug for treating refractory epilepsy. It is licensed in Europe and the United States for the adjunctive treatment of partial seizures (with or without secondary generalization) in adults. It is also licensed in Europe as monotherapy for adults with newly diagnosed partial epilepsy[219]. The drug inhibits voltage-gated Na^+ channel, T-type voltage-sensitive Ca^{2+} channel, Ca^{2+}-induced Ca^{2+} releasing system, and neuronal depolarization-induced glutamate release; and enhance the release of inhibitory neurotransmitters. The drug has been found by chance to exert beneficial anti-parkinsonian effects. Early studies on patients with PD demonstrated lessening of symptoms, especially wearing-off when using zonisamide (50-200 mg/d) as an "add-on" treatment[220]. When used as an adjunctive therapy in patients with insufficient response to L-dopa treatment, zonisamide (25, or 50 mg/d) resulted in significant motor improvement and reduced the duration of "off" time compared with placebo. Dyskinesia was not increased in zonisamide-treated groups[221]. In two 12-wk, randomized, double-blind trials in PD patients inadequately controlled with L-dopa, zonisamide (25 mg once daily) significantly improved motor function (UPDRS Part Ⅲ total score), compared with placebo[222]. The drug appears to be generally well tolerated at doses of 25-50 mg/d[221,222]. Zonisamide also led to marked reduction in the severity of impulsive behaviors and global impulsiveness in PD patients with impulse control disorders who did not improve following a reduction of either L-dopa or dopamine agonists[223].

In experimental models of PD, zonisamide displayed antiparkinsonian and neuroprotective effects[224-228]. Several mechanisms have been proposed including (1) increased expression of astrocyte-mediated neurotrophic and anti-oxidative factors, *e.g.*, astrocyte-derived neurotrophic factor, vascular endothelial growth factor, copper/zinc superoxide dismutase, and manganese superoxide dismutase[227]; (2) upregulating levels of manganese superoxide dismutase[225]; (3) anti-apoptotic effect[225,227-229]; (4) antioxidant effect[227-229]; (5) increased S100β-positive and glial fibrillary acidic protein-positive astrocytes and dopamine turnover[226]; (6) potent and reversible inhibition of MAO-B activity[224]; (7) delta (1) receptor mediated inhibition of the indirect pathway[230]; (8) dopamine release[231]; and (9) prevention of dopamine quinone formation[232]. Table 2 summarizes the findings of randomized double blind studies on novel antiparkinsonian drugs.

Mitochondrial enhancement therapy

Coenzyme Q10: Coenzyme Q10 (CoQ10) or ubiquinone is a lipid-soluble molecule present in all membranes throughout the cell. It acts as an electron carrier in the mitochondrial electron transport chain, located within the inner mitochondrial membrane (transfers an electron between complexes Ⅰ/Ⅱ and Ⅲ). CoQ10 also functions as an antioxidant, thereby protecting cellular membranes and macromolecules (*e.g.*, proteins, lipids, DNA). CoQ10 also regenerates the pool of tocopherol[233-235]. In brains from PD patients postmortem, CoQ10 decreased in the cortex[236]. In a study involving 33 patients with PD, Jiménez-Jiménez *et al*[237] found no difference in serum levels of CoQ10 between patients with PD and controls. In contrast, CoQ10/cholesterol ratio inversely correlated with duration of the disease, total UPDRS score and motor examination of the UPDRS. Treatment with L-dopa or dopamine agonists had no significant effect on CoQ10/cholesterol ratio. Other studies, however, have shown elevation in oxidized form of CoQ10 in plasma[238] or decreased CoQ10 in peripheral blood lymphocytes from patients with PD[239]. Moreover, increased percentage of oxidized to total CoQ10 was detected in the cerebrospinal fluid (CSF) of patients with PD. The concentration of 8-OHdG in the CSF also increased and correlated with concentrations of oxidized to total CoQ10, thereby linking both mitochondrial oxidative damage and oxidative DNA damage in the disease process[240].

In primate model of PD induced by the nigrostriatal toxin MPTP, dopamine cell loss was prevented by treatment with coenzyme Q[241]. In the MPTP rat model of PD, both CoQ10, reduced CoQ10 (ubiquinol) exerted neuroprotective effects against MPTP induced dopamine depletion, loss of tyrosine hydroxylase neurons and the development of alpha-synuclein inclusions in SNc[242]. Orally administered CoQ10 also halted the progression of nigrostriatal degeneration

Table 2 Results of randomized, double-blind, placebo-controlled trials involving new antiparkinsonian drugs

Drug	Study objective	Outcomes	Adverse events	Ref.
Istradefylline	Evaluated the efficacy and safety of istradefylline, 20 and 40 mg once daily as adjunctive to L-dopa in patients with motor complications (12 wk)	↓ daily change in "off" time *vs* placebo	↑ dyskinesia	[203]
Istradefylline	Evaluated the efficacy and safety of istradefylline, 10, 20 and 40 mg once daily as adjunctive to L-dopa in patients with motor complications (12 wk)	No effect on "off" time duration Improved motor scores at 40 mg	-	[199]
Istradefylline	Evaluated the efficacy of istradefylline at an oral dose of 20 and 40 mg once daily for 12 wk in PD patients with motor complications on levodopa therapy	↓ "off" time *vs* placebo	↑ dyskinesia	[204]
Istradefylline	Evaluated the safety and efficacy of istradefylline 40 mg, as monotherapy in patients with PD	No significant effect in improving motor symptoms	-	[198]
Istradefylline	To evaluate efficacy, safety, and tolerability of istradefylline 20 mg once daily *vs* placebo as an adjunct to levodopa in PD subjects with motor fluctuations	↓ "off" time	Dyskinesia, lightheadedness, tremor, constipation, and weight decrease	[201]
Istradefylline	To evaluate safety and efficacy of istradefylline 20 or 60 mg/d in L-dopa-treated PD subjects with motor complications	↓ "off" time without an increase in "on" time	Dyskinesia, nausea, dizziness, and hallucinations	[200]
Istradefylline	To evaluate safety and efficacy of istradefylline 40 mg/d in L-dopa-treated PD subjects with prominent wearing-off motor fluctuations	↓ "off" time without increased troublesome dyskinesia	-	[202]
Istradefylline	To evaluate safety and efficacy of istradefylline 20 or 40 mg/d in patients with L-dopa-motor fluctuations and peak-dose dyskinesias	↓ "off" time	Severity of dyskinesia was unchanged, but "on" time with dyskinesia increased	[205]
Preladenant	To evaluate efficacy of using preladenant 5 mg twice a day as a levodopa adjunct in subjects with fluctuating PD	↓ "off" time ↑ "on" time throughout the 36-wk treatment relative to the baseline	Dyskinesia and constipation	[209]
Preladenant	To evaluate safety of single and multiple rising preladenant doses compared with placebo	Preladenant was generally well tolerated up to the maximum dose tested (200 mg/d)	Transient mild increases in blood pressure within a few hours after preladenant administration	[207]
Preladenant	To evaluate efficacy and safety of 1, 2, 5, or 10 mg oral preladenant twice daily in patients with PD and motor fluctuations on L-dopa	5 and 10 mg preladenant ↓ "off" time	Worsening of PD, dyskinesia, nausea, constipation, and insomnia	[208]
Safinamide	To evaluate efficacy and safety of safinamide 50 or 100 mg/d, as add-on to L-dopa in the treatment of PD patients with motor fluctuations	↑ total on time with no or nontroublesome dyskinesia, ↓ decreased off time, without worsening dyskinesia	-	[216]
Safinamide	To evaluate efficacy of safinamide 100 or 200 mg/d as add-on treatment to single dopaminergic receptor agonist single in early PD	Safinamide 100 mg/d may be effective as add-on treatment	-	[215]
Safinamide	To evaluate efficacy and safety of once-daily 100 or 200 mg safinamide in patients with early PD receiving a stable dose of a single dopaminergic receptor agonist	Safinamide 100 mg/d improved motor symptoms (UPDRS part Ⅲ total score)	-	[214]
Zonisamide	To evaluate the efficacy, safety and tolerability of daily doses of 25, 50, and 100 mg of zonisamide as an adjunctive treatment in PD	Zonisamide 25 and 50 mg/d improved motor symptoms (UPDRS part Ⅲ total score) Zonisamide 50 and 100 mg ↓ "off" time without ↑ dyskinesia	-	[221]
Isradipine	To establish a tolerable and efficacious dosage of isradipine controlled-release in subjects with early PD not requiring dopaminergic therapy	The tolerability of 5, 10, or 20 mg of isradipine was dose dependent No difference in change in UPDRS among dosages	Peripheral oedema and dizziness	[295]
Isradipine	To evaluate safety and tolerability of isradipine controlled release in patients with early PD	Tolerability of isradipine CR 5, 10, 15, or 20 mg was dose dependent Isradipine had no significant effect on blood pressure or PD motor disability	Leg oedema and dizziness	[294]

L-dopa: Levodopa; PD: Parkinson's disease; UPDRS: Unified Parkinson's disease rating scale; CR: Controlled release.

induced in rats by paraquat[243] and in the MPTP mouse model of PD[244].

In patients with PD without motor fluctuations and on stable antiparkinsonian treatment, nanoparticular CoQ10 (100 mg 3 times a day) for 3 mo failed to demonstrate clinical benefit. The formulation used was associated with CoQ10 plasma levels similar to 1200 mg/d of standard formulations[245]. In another randomized, double-blind, placebo-controlled trial, treatment of PD patients with CoQ10 (300-1200 mg/d) increased plasma level in a dose-dependent manner. CoQ10 was well-tolerated and at 1200 mg/d there were significant slowing the progression of PD as measured by the total UPDRS score [246]. Improvements in the total UPDRS were also observed following 2 wk treatment with CoQ10 in 16 subjects with early idiopathic PD. Moreover, F_2-isoprostanes in plasma were significantly reduced in the 400-1200 mg/d dose range (but increased at 2400 mg/d dosage). Symptomatic benefit from CoQ10 appeared to depend on initial plasma ubiquinol and F_2-isoprostanes[247]. More recent phase Ⅲ randomized, placebo-controlled, double-blind clinical trial, however, reported no evidence of clinical benefit (total UPDRS) from treatment with 1200 mg/d or 2400 mg/d in patients who received a diagnosis of PD within 5 years[248,249].

Creatine: Creatine is a naturally occurring amino acid consumed in meat and fish. It is also synthesized in liver, kidneys, and pancreas from glycine, arginine and methionine. The highest concentration of creatine is found in skeletal muscles (95% of body stores) with most of the remaining stores found in the heart, brain and testes. After its synthesis, creatine is released into the blood stream, from where it is taken up by cells against a concentration gradient *via* the creatine transporter. The active form of creatine is phosphorylcreatine (PCr). Creatine kinase is the enzyme catalyzing the reaction of the phosphorylation of creatine to PCr through the transfer of the γ-phosphate group of adenosine triphosphate to the guanidino group of creatine to yield adenosine diphosphate and high-energy PCr. In brain, the active form of creatine or phosphocreatine serves as an energy reserve being a donor of high energy phosphate molecules to adenosine diphosphate to form adenosine triphosphate (ATP). One key function of phosphocreatine is to increase ATP levels in tissue so as to stabilize neuronal membranes. Creatine and phosphocreatine are involved in the shuttle of ATP from the mitochondria, site of synthesis to the site of use in the cytosol[252,253]. Creatine is widely used as a nutritional supplement and ergogenic aid for athletes[254].

In PD patients analysis of whole brain metabolite changes using proton magnetic resonance spectroscopy, indicated higher creatine values, which might reflect greater neuronal energy expenditure early in the disease process that is compensatory[255]. Other researchers detected significantly decreased creatine (as well as N-acetylaspartate, choline, myo-inositol, glutathione and dopamine concentrations) in patients with PD. This decrease in creatine levels possibly reflected impaired energy metabolism due to mitochondrial dysfunction[256]. In the MPTP model of PD in mice, creatine protected against striatal dopamine depletions and loss of substantia nigra tyrosine hydroxylase immunoreactive neurons[257].

Creatine supplementation has been proposed as an adjunct to medication for the treatment of brain-related disorders associated with bioenergetic deficits like PD[258]. Creatine intake seems to be safe in healthy individuals and in patients with PD. in healthy males undergoing aerobic training, creatine supplementation (approximately 10 g/d) over 3 mo does not provoke renal dysfunction. Serum creatinine serum and urinary sodium and potassium were unchanged, while cystatin C levels decreased over time, suggesting an increase in glomerular filtration rate[259]. In patients with PD, creatine 10 g/d was well tolerated[260] and creatine (4 g/d) for 2 years was well tolerated, apart from gastrointestinal complaints. Despite increased serum creatinine levels, other markers of tubular or glomerular renal function, especially cystatin C, remained normal, indicating unaltered kidney function[261]. In patients with PD, a 2-year placebo-controlled randomized clinical trial showed that creatinine improved patient mood and led to a smaller dose increase of dopaminergic therapy. Creatinine, however, had no effect on overall UPDR scale scores or dopamine transporter SPECT[262]. Creatine supplementation (20 g/d for the first 5 d and 5 g/d thereafter) has been shown to enhance the benefits of resistance training in patients with PD[263].

L-Carnitine: Carnitine (3-hydroxy-4-N-trimethy-lammoniobutanoate) is a quaternary amine synthesized in the body from lysine and methionine mainly in liver, kidney, and muscle. Dietary carnitine from meat and dairy products provides 75% of body carnitines. Carnitine or acylcarnitines including acetyl-L-carnitine are important in the oxidation of fatty acids in mitochondria. Acetyl-L-carnitine is a constituent of the inner mitochondrial membrane[264]. In humans, plasma acetyl-L-carnitine and L-carnitine short chain esters increases following oral treatment with acetyl-L-carnitine. CSF concentrations also increases, suggesting that the agent easily crosses the blood-brain barrier[265]. The administration of acetyl L-carnitine in elderly subjects (2 g twice-a-day) improves physical and mental fatigue as well as functional status and cognitive functions[266], Preclinical studies have shown that L-carnitine and its acetyl ester, acetyl-L-carnitine exert neuroprotective effects. Acetyl-L-carnitine act to preserve mitochondrial respiratory chain complex activity in face of inflammatory cytokine insult[267], prevent age-related oxidative mitochondrial decay[268] and maintain mitochondrial respiration and enzyme

activities (NADH dehydrogenase, cytochrome C oxidase and pyruvate dehydrogenase) following contusion spinal cord injury[269]. The neuroprotective effects of acetyl-L-carnitine involves induction of heme oxygenase-1, up-regulation of heat shock protein 60, increased expression of the redox-sensitive transcription factor Nrf2[267], reduction of carbonyl formation and decreased mtDNA deletion[270]. Acetyl-L-carnitine has been reported to protect against MPTP-induced toxicity in the nonhuman primate[271].

N-acetyl cysteine: The brain in PD is exposed to inappropriately high levels of oxygen and nitrogen-derived free radicals. Post-mortem studies of PD brains have detected increased lipid peroxidation products[272] and increased protein carbonyls indicative of protein oxidation[273] as well as reduced glutathione levels[274]. One of the most important intracellular redox buffers and free radical scavengers in brain is glutathione, a tripeptide thiol that consists of glutamate, cysteine and glycine. It exists mainly in a reduced form (GSH) and the ratio of GSH to the oxidized form (GSSG) determines the oxidative status of the cell[275,276]. Therefore a decrease in glutathione bioavailability would have serious consequences on the ability of cells to withstand oxidative burden. Glutathione concentrations can be increased by N-acetyl cysteine, the N-acetyl derivative of the amino acid L-cysteine, which is rapidly hydrolyzed intracellularly to cysteine, the rate limiting substrate for glutathione synthesis[277,278].

There is accumulating evidence to suggest the usefulness of supplementation with N-acetylcysteine in neurodegenerative disorders including PD[277-279]. In transgenic mice overexpressing wild-type human alpha-synuclein, N-acetylcysteine attenuated the loss of dopaminergic terminals at 1 year and also significantly decreased the levels of human alpha-synuclein[280]. Mice lacking the excitatory amino acid transporter EAAC1 have impaired neuronal cysteine uptake and consequent reduced neuronal glutathione content. These mice exhibited age-dependent loss of dopaminergic neurons in the SNPc, nitrosative stress and neuroinflammation, which were alleviated by N-acetylcysteine treatment[281]. In rats in which GSH was depleted by treatment with 2-cyclohexene-1-one, treatment with 1.6 g/kg of N-acetylcysteine rescued the depleted levels of GSH in the brain and restored cognitive deficits[282].

In a recent clinical study, single N-acetylcysteine infusion (150 mg/kg) was able to increase blood GSH redox ratios and increase brain GSH concentrations (measured using 7-T magnetic resonance spectroscopy) in those with PD and Gaucher disease and healthy controls[283].

Alpha lipoic acid: Lipoic acid (thioctic acid, 1,2-dithiolane -3-pentanoic acid) functions as a cofactor in multienzyme complexes that catalyze the oxidative decarboxylation of pyruvate, a-ketoglutarate, and branched-chain α-keto acids. Lipoic acid and its reduced form dihydrolipoic acid are also potent antioxidants capable of scavenging a number of reactive oxygen and nitrogen species. Lipoic acid is rapidly absorbed in the gut and passed to various tissues for catabolism[284]. Alpha lipoic acid is likely to be of benefit in several brain pathologies and neurodegenerative disorders. Chronic treatment with alpha lipoic acid (and also N-acetylcysteine) was found to improve cognition In SAMP8 mice that overexpress amyloid precursor protein[285]. In rat brain glial cultures, alpha-lipoic acid decreased viral double-stranded RNA-stimulated inflammatory signaling by down-regulating interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase transcripts. It also prevented cultured glial cytotoxicity[286]. Alpha lipoic acid has been shown to protect dopaminergic neurons in vitro against apoptosis induced by the nigrostriatal toxin 1-methyl-4-phenylpyridinium (MPP+). This effect of was associated with decreased intercellular levels of reactive oxygen species and the mitochondrial transmembrane permeability[287]. It also protected dopaminergic neurons in the animal model of PD induced by stereotaxic injection of 6-hydroxydopamine in rat striatum[288]. It has been suggested that a combination of mitochondrial antioxidants/nutrients could improve mitochondrial function and/or attenuate oxidative damage implicated in PD[289]. The authors found that the combined treatment with alpha-lipoic acid and acetyl-L-carnitine was more effective than either agent alone in protecting SK-N-MC human neuroblastoma cells against rotenone-induced mitochondrial dysfunction and oxidative damage.

Neuroprotective agents

Isradipine: Isradipine is a dihydropyridine calcium channel antagonist, with high affinity for Cav1.3 L-type channels. L-type Ca^{2+} channels with a pore-forming Cav1.3 subunit underlie autonomous pacemaking in adult dopaminergic neurons in the SNPc. This poses a sustained stress on mitochondrial ATP generating oxidative phosphorylation, accelerating cellular aging and death, and rendering dopaminergic more susceptible to the effect of nigrostriatal toxins[290]. Antagonism of these channels with isradipine has been shown to exert neuroprotective effects in animal models of PD[291,292]. Isradipine afforded neuroprotection against the nigrostriatal toxin 6-OHDA injected intrastriatally, sparing dopaminergic fibers and cell bodies[291]. In human neuroblastoma SH-SY5Y cells, isradipine antagonized many effects of rotenone including production of reactive oxygen species, G1/G0 cell cycle arrest, and activation of p53/p21 signaling proteins as well as the decreased expression of the signaling proteins for cell proliferation and survival, Cyclin-dependent kinase 2, cyclin D1, and Akt[292]. Isradipine also reduced L-dopa-induced rotational behavior and abnormal involuntary movements in animal model of L-dopa-induced dyskinesia[293].

Isradipine studies are thus being conducted in

humans to establish the dosage, safety and tolerability of the drug. In these studies, controlled release isradipine 10 mg daily was tolerated by 73% and 87% of patients with early PD, respectively. Peripheral edema and dizziness were most common adverse events encountered[294,295]. Isradipine displayed no significant effect on blood pressure or PD motor disability[294].

Pioglitazone: Pioglitazone is a peroxisome proliferator-activated receptor-gamma agonist of the thiazolidinedione class. The administration of this antidiabetic agent has been shown to protect dopaminergic neurons preclinical rodent models of PD[296-299]. The drug also exhibited neuroprotective properties in the non-human primate model of MPTP-induced PD. Significant improvements in clinical rating score was associated with preservation of nigrostriatal dopaminergic markers, *e.g.*, cell counts of tyrosine hydroxylase immunoreactive- and vesicular monoamine transporter-2immunoreactive-nigral neurons[300]. Several mechanisms have been postulated to account for pioglitazone-induced neuroprotection. This included attenuation of toxin-induced glial activation and consequent suppression of pro-inflammatory cytokine (TNF-α, IL-1β, interferon-gamma) release[296,301,302]. Pioglitazone also attenuated oxidative stress[302], interfered with phosphorylation of jun N-terminal kinase and nuclear factor kappa-B, and suppressed cyclooxygenase 2 expression and the subsequent prostaglandin E(2) synthesis[298] and showed dose-dependent modulation of CD68-ir inflammatory cells[286]. Other researchers provided data that pioglitazone is effective in the MPTP mouse model through inhibition of MAO-B[299]. It has been shown that pioglitazone is a specific and reversible inhibitor of human MAO B. Other members of the glitazone class, rosiglitazone and troglitazone are weaker inhibitors of both MAO-A and MAO-B[303].

Minocycline: Minocycline is a second-generation, semi-synthetic tetracycline that received much interest for its dopaminergic neuroprotective effects observed in experimental models of PD. Minocycline inhibits microglial activation[304], exerts antioxidant and anti-inflammatory effects[305], and prevents apoptotic cell death, possibly due to attenuating endoplasmic reticulum stress and mitochondrial dysfunction[306]. Ongoing clinical trials for evaluating the effect of minocycline on disease progression, however, failed to demonstrate clinical benefit for minocycline. An 18-mo phase Ⅱ trial of minocycline 200 mg/d in subjects with early PD, found no effect for the drug in slowing down the progression of disability. Symptomatic treatment of PD symptoms was required in 62% of minocycline, and 60% of placebo-treated subjects[307]. In a randomized, double-blind in patients with Multiple-System-Atrophy Parkinson-type, the progression rate over 48 wk of minocycline 200 mg/d (the change in motor function) did not differ from that of placebo. A significant deterioration in motor scores occurred in both groups. Positron emission tomography-data in two patients in the minocycline group, however, suggested that minocycline might interfere with microglial activation[308].

Non-steroidal anti-inflammatory drugs

Neuroinflammation is a major contributing factor in the pathogenesis of PD[309]. *In vitro*, several non-steroidal anti-inflammatory drugs (NSAIDs) including ibuprofen, aspirin, acetaminophen, meclofenamic acid sodium salt, sulindac sulfide, ketoprofen (but not naproxen and indomethacin) inhibited the formation and stabilization of alpha-synuclein fibrils[310]. In this context, it is to be noted that the use of NSAIDs is frequent among PD patients. The chronic prescription of analgesic drugs was more prevalent in PD patients than in the general population and similar to that in osteoarthritis patients[311]. Several studies have assessed the potential for NSAIDs in lessening the progression of PD. The results of these studies were, however, inconclusive. In one study, the regular use of non-aspirin NSAIDs was associated with a lower risk of PD compared with non-regular users. A lower risk of PD (though of no statistical significance) was also observed among men and women who took 2 or more tablets of aspirin per day[312]. In their study, Wahner *et al*[313] found that regular use of aspirin ($\geqslant$ 2 pills/wk for at least 1 mo) was associated with a decreased risk of PD; an effect seen only in women. Regular non-aspirin NSAID usage was even associated with a stronger protective effect, particularly those who reported 2 or more years of use. Other researchers provided data that ibuprofen users had a significantly lower PD risk than non-users with this effect of the drug being a dose-dependent one. The same studies found no effect for aspirin, other NSAIDs or acetaminophen in reducing the risk for PD[314,315]. Hernán *et al*[316], however, found that non-aspirin NSAID use was associated with a higher risk in women and a lower risk in men. Other researchers, found no evidence that NSAID use reduces the risk of PD[317-321], although one study suggested that long-term use of NSAIDs was associated with a slightly lower PD risk[321].

Deferiprone

Studies in humans suggest the accumulation of iron in the substantia nigra of PD patients. Consequently excess iron has been implicated in the pathogenesis of PD[322-325]. One approach to halt nigrostriatal degeneration in PD might therefore involve the reduction of iron-mediated oxidative stress through the use of iron chelators. In a clinical trial in early PD patients, one such iron chelator that is deferiprone slightly improved motor signs at 6 mo and decreased motor handicap progression and iron overload at one year[326]. In a randomized clinical study on 23 patients with early stage PD (< 5 years from diagnosis), deferiprone for 6 mo elicited a non-significant small improvement in the motor UPRDS scores compared to placebo. Magnetic resonance

imaging indicated significantly reduced iron content in the dentate nucleus and caudate nucleus though not in substantia nigra following deferiprone therapy[327]. In these two studies, deferiprone was well tolerated. Side effects were neutropenia or agranulocytosis[326,327]. In one study, treatment with deferiprone for 4 years in 6 patients with neurodegeneration with brain iron accumulation was associated with stabilization in motor symptoms in 5/6 patients[328].

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REVIEW

Role of opioid receptor heterodimerization in pain modulation and tolerance development

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Abstract

Protein to protein interactions leading to homo/heteromerization of receptor is well documented in literature. These interactions leading to dimeric/oligomers formation of receptors are known to modulate their function, particularly in case of G-protein coupled receptors. The opioid receptor heteromers having changed pharmacological properties than the constituent protomers provides preferences for novel drug targets that could lead to potential analgesic activity devoid of tolerance and physical dependence. Heterodimerization of opioid receptors appears to generate novel binding properties with improved specificity and lack of side effects. Further the molecules which can interact simultaneously to both the protomers of the heteromer, or to both the binding sites (orthosteric and allosteric) of a receptor protein could be potential therapeutic molecules. This review highlights the recent advancements in exploring the plausible role of heteromerization of opioid receptors in induction of tolerance free antinociception.

Key words: Opioid receptors; Receptor heterodimers; G-protein coupled receptors oligomerization; G protein coupled receptors; Opioid tolerance; G-protein coupled receptors; Allosteric regulation; Antinociception

Core tip: Endogenous opioid peptides are known for their analgesic effects. However their analgesic effect is downplayed by the side-effect of tolerance development. To maintain homeostasis to their effect, other endogenous anti-opioid peptides works parallel to it. The present work highlights the role of anti-opiates in development of tolerance to opiate drugs.

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OPIOID RECEPTOR HETEROMERIZATION

Introduction

Opioid system comprising endogenous opioid peptides and receptors is mainly expressed in the central nervous

system[1]. A prominent role is played by the opioid system in modulation of nociception, neuroendocrine physiology, autonomic functions and reward processing[2]. Opioid receptors are a part of the G-protein coupled receptors (GPCRs) family. There exist three types of homologous opioid GPCRs *viz.* mu opioid receptor (MOR), delta opioid receptor (DOR) and Kappa opioid receptor (KOR)[3]. Extensive studies have been done to enunciate their implications in pain control, mood disorders and drug abuse[4,5]. GPCRs are seven transmembrane spanning proteins, which represent powerful targets to modulate both, the physiological and pathological states as they are involved in many biological processes. Regulation of GPCR activity is through various mechanisms *viz.* phosphorylation, endocytosis, desensitization, *etc.* is substantially evident. Among these, the interactions leading to heteromerization in which one protein receptor interacts with other protein receptor particularly modulates the GPCR function[6]. Decades of research over opioid pharmacology has discovered the complexity underlying the physiology of opioid system[7]. Various homotypic and heterotypic interactions have been revealed among the three types of opioid receptors (μ, KOR and DOR), when they are expressed in heterologous cells[8-10], which were considered as single units functionally. As a result the ligand binding and signaling properties get altered for these receptors. However occurrences of such interactions in live cells and *ex vivo* have not been reported until now and even no clue was there whether they were physiologically relevant. Protein complexes give rise to functional interactions within intracellular partners, resulting from shared association or downstream effectors competition. However, these interactions are highly debated whether they occur at neuronal level through signaling pathways or at molecular level exhibiting physical association of receptors by direct contact and with existing tools it is very difficult to claim any specific answer for them.

Recent reports shed some light over the functional interactions across receptors, by analysing the opioid drug effects for MOR and DOR receptors *in vivo*[11]. Well known example is of DORs to have implication in opioid tolerance development to morphine[12]. To further explore on this growing notion a "two-state dimer receptor model" has been recently proposed to understand and interpret heteromer operation by binding to lead molecules[13]. A receptor is a cellular macromolecular assembly which specifically transduces chemical signals inside and between cells. Whereas a receptor heterodimer is composed of two units and its biochemical properties may be different from its individual protomers[14]. This could be either due to some sort of intermolecular interaction or allosteric interaction which leads to the changes in binding properties of other protomer upon ligand binding to the first protomer. Thus generating novel pharmacological and signalling properties[15] which when targeted by specific ligands leads to improved efficacy with reduced undesirable effects[16]. Such specific ligands could be dualsteric compounds and may interact simultaneously to both the protomers or to the orthosteric site of one and then allosterically modulating the other protomer. In case of the allosteric interactions receptor subtype-selectivity is achieved and it may also modulate the efficacy as well as intracellular signaling pathway activation. The occurrence of opioid receptor heteromers uncovers a new side of novel drug targets which could be capable of combating a variety of diseases with potentially fewer side effects.

Since all the aspects of receptor physiology, pharmacology, trafficking, signaling, ligand affinities, etc. are affected due to the heteromer formation, it offers a very useful handle to obtain reliable macroscopic dissociation constant (K_D) values from binding data for biphasic kinetics. A new parameter, degree of cooperativity (Dc) could quantitatively define the intramolecular communication within the dimer. This new parameter has enabled vision of the occurrence of receptor heterodimers unfolding the new functional and pharmacological perspectives for GPCRs[17].

Initially the opioid receptors heteromerization was studied in artificial cell systems but now the focus has been shifted to its *in vivo* relevance. Many compounds have been identified that could selectively target the opioid heteromers of DOR with KOR and MOR influencing the opioid analgesic effect and modulating its ethanol consumption side effect. In some cases the differences in receptor trafficking properties have been attributed to the specific physiological response produced by the heteromers in comparison to their homomeric counterparts. For opioid receptor heteromers the easier detection of pharmacological profile modification has been achieved which has enabled the consideration of making opioid drugs like morphine more effective while restricting its side effects[18]. One of the examples is of DOR ligands which have shown potentiation of morphine's efficiency. Although, the molecular mechanism underlying such observations is still not clear, they have been attributed to the modulation of receptor function due to physical association between them. This hypothesis has been supported by the existence of mu-delta receptors complexes in live cells and the enhancement of their binding and signaling activity by antagonist occupancy of receptors. Thereby suggesting that heterodimeric association of opioid receptors could be used as a model to develop novel drug compounds for pain modulation[19].

An important aspect of opioid pharmacology is the establishment of the side effect, tolerance and dependence[20]. Tolerance may be defined as a phenomenon in which an increased amount of drug is required to produce the same level of drug effect after repeated use of the drug. Development of tolerance involves complex biochemical procedures at the cellular as well as sub-cellular level. Though many mechanisms have been proposed for the same but due to conflicting reports, no mechanism is yet universally accepted.

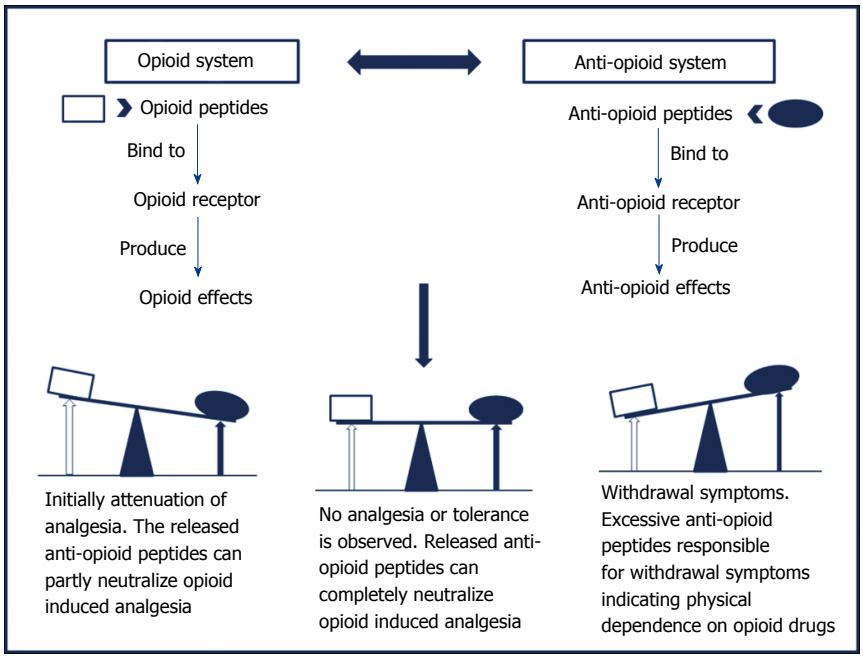

Figure 1 Anti-opioid model for tolerance and physical dependence.

Several lines of evidence propose that development of opioid tolerance may have multiple causes involving complex physiological adaptations, but majorly includes molecular level receptor trafficking.

The classical approach attributes opioid tolerance development and physical dependence in the body to the changes that occur at the receptor level *viz*. desensitization, down-regulation or internalization of opioid receptors. But it could not completely explain all the aspects of tolerance and physical dependence which gave rise to an alternative model, the "anti-opioid model"[21], shown in Figure 1. This model postulates the existence of an anti-opioid system within the body which works parallel to the endogenous opioid system, to neutralize the antinociceptive opioid effects upon chronic treatment. Certain endogenous neuropeptides such as Phe-Met-Arg-Phe-NH_2 (FMRFa), Phe-Leu-Phe-Gln-Pro-Gln-Phe-NH_2 (NPFF), Tyr-Pro-Leu-Gly-NH_2 (Tyr-MIF-1) and orphanin FQ/nociceptin, exhibits anti-opioid effects. These peptides mediate their biological effects through a different set of receptors, distinct from opioid receptors[22] which are also G protein-coupled receptors[23,24]. Together these peptides and their receptors are known to have role in the development of tolerance and physical dependence upon chronic administration of opioids.

Taking for example, morphine chronic treatment should increase the release of anti-opioid peptides which thereby will lead to attenuation of the morphine antinociceptive effects. Further opioid treatment will cause more release of anti-opioid which will induce tolerance to the opioid effects. The residual excess of anti-opioid left after termination of opioid administration, is partially responsible for the withdrawal syndrome. This model suggest the participation of NPFF in modulation of opioid effects and therefore present them as important targets for novel pharmacological agents which can intensify the opioid antinociceptive effects by modulating endogenous opioid function. In view of this point, the agonists of the anti-opioid system would block the opioid effect whereas their antagonists are expected to potentiate it. Such hypothesis broadens the vision to describe the role of NPFF system in the control of opioid function mainly in supraspinal regions. In particular, NPFF exhibiting anti-opioid effects emerges as a modulator of the opioid effects along with having significant role in the development of opioid tolerance and physical dependence.

This story has another side to be viewed, where experimental evidences have shown that NPFF exhibits opioid-like effects and it also potentiates in both the spinal as well as supraspinal regions the analgesic effect of morphine. Earlier NPFF has been placed in the anti-opioid category inspite of demonstration of both pro- and anti-opioid effects because the underlying mechanisms were not clear. However, recent studies are revealing the complexities of NPFF effects on opioid analgesic activity and thereby limiting the use of anti-opioid term to describe the physiological role of NPFF[25].

Evidences for opioid receptor heteromerization

Opioid GPCRs appear to form homo/heteromers having altered ligand binding and activation of G-protein[26,27]. The opioid receptor heteromerization was believed to occur intracellularly, where the two receptors may have physical association, thereby operating as homo/heteromers with distinct signaling and trafficking properties as compared to their monomeric counterparts[11]. The concept of homo/heteromers prevails from the reports on heteromerization of many GPCRs including

MOR and DOR[8,9]. The first example of role of heteromerization in pharmacological diversity was observed in studies on DOR and KOR receptors[28]. These studies exhibited the ligand binding, functional and trafficking properties of DOR/KOR complex that are distinct from those of each individual receptor[8]. *In vivo* operation of these mechanisms indeed is still unresolved in GPCR research[11]. Until now, there is very little evidence supporting the *in vivo* co-expression of MOR/DOR receptors. Few examples have been reported in dorsal root ganglia[29], spinal cord[19], rostroventral medulla[11] and in a limited number of brain areas which are tested by specific antibodies raised particularly against these heteromers[30]. Various studies *viz.* coimmunoprecipitation and bioluminescence resonance energy transfer studies suggest physical association between MOR and DOR receptors upon co-expression[19,31], although their distinct localization and activation have been recently reported in studies on mice[32]. It is revealed from one of the study that chronic administration of morphine upregulates the MOR/DOR dimers[30] and during this the activation of the DOR subunit in the dimer results in degradation of MOR and cellular response[33].

A study supporting MOR/DOR heteromer formation demonstrating oligomerization of MOR receptor dimers into heteromer complex formation with DOR receptor following prolonged morphine treatment, and the influence on these interactions of DOR receptors. The changes in association were measured by förster resonance energy transfer (FRET), the mobility of receptors by fluorescence correlation spectroscopy and the degree of oligomerization by number analysis. Moreover the structural interpretation was done *via* computational modelling. With morphine there was little effect on the diffusion properties and interaction with G proteins of MOR. On the other hand, presence of DOR increases the oligomerization along with association of MOR receptors with G-proteins upon morphine treatment, indicating correlation of functional properties of MOR with its oligomerization[34].

Amongst the GPCR heteromers studied so far, opioid receptors were the first to be exhaustively studied *via* functional (changes in pharmacology), biochemical (immunoprecipitation, cross linking) and biophysical (FRET, BRET) methods. The affinity for heteromer formation is same for all the opioid receptors[35]. For example DOR can heteromerize with either MOR[36] or/ and KOR[37]. Taking a deep insight into the heteromers formed could reveal the presence of opioid receptor subtypes that have been pharmacologically defined, but not ascribed as splice variants. This could be exemplified by delta opioid receptor subtype 1 (DOR1) [preferred (D-Pen2,D-Pen5)enkephalin (DPDPE) and 7-Benzylidenenaltrexone (BNTX)] and DOR2 (preferred deltorphin Ⅱ and naltriben), as DOR1 forms DOR/KOR heteromer and DOR2 forms DOR/MOR heteromer. Conversely in other study more pronounced affinity was observed for DOR/MOR heteromer by DOR1 (BNTX) ligand[10]. Supporting the previous observations, some behavioural effects *viz*. ethanol consumption, of DOR1 ligands are affected by disruption of MOR, partially confirming involvement of DOR1 in DOR/MOR heteromer[38]. Similarly exists the subtypes of MOR and KOR receptors. MOR antagonist naloxonazine inhibits MOR1 only out of the two MOR1 and MOR2. In case of KOR three subtypes have been pharmacologically identified out of which KOR1 binds arylacetamides, KOR2 does not, while KOR3[39] is insensitive to KOR (U50,488) ligand. Interestingly the subtypes being classified for various opioid receptors may be same, *e.g.*, MOR2 may be DOR2[40], whereas DOR1 and KOR2 may be the same DOR/KOR heteromer[41].

Another example includes the co-immunoprecipitation of MOR1 and DOR1 in the central nervous system tissue[19] which have been selectively recognized by the antibodies for DOR/MOR heteromer *in vitro* and *in vivo* as well[42]. Furthermore, only a single ligand, 6'-guanidinonaltrindole (6'-GNTI), has been identified that selectively activates a heteromer *in vitro* and produces a biological effect *in vivo*[43]. Although there is ongoing debate over the *in vivo* role of DOR/MOR or DOR/KOR heteromers, some evidences do exist for their existence *in vivo*.

Taking together the synergism between DOR and KOR receptor agonists and the reports on co-localization of opioid receptors from spinal region, DOR receptor agonist DPDPE was proposed to interact with DOR recognition site in an allosteric DOR/KOR heteromer. This heteromer is said to be allosteric because this model considers that upon norBNI binding to KOR recognition site induces conformational changes in the other protomer of the heteromer, DOR which results in antagonism of DPDPE antinociception. Another support for this observation came from a study on porcine ileum where DOR and KOR receptors were found co-localized and norBNI significantly antagonized the DOR selective agonists[44]. Thus reinforcing the existence of DOR1 receptor as DOR/ KOR heteromer in the mouse spinal cord and that the two subunits are allosterically coupled[45].

In another study the DOR agonist deltorphin-Ⅱ has been reported as functional agonist of the MOR/DOR heteromer, which not only induced desensitization but also inhibited the adenylyl cyclase through a pertussis toxin-insensitive G-protein. Stimulation of the heteromer MOR/DOR lead to the activation of $G\alpha_z$, which was demonstrated by the incorporation of $GTP\gamma^{35}S$, whereas, individual activation of separate MOR and DOR receptors activated $G\alpha_i$ preferably. This specific behaviour has been attributed to the shared involvement of both receptors distal carboxyl tails, so that truncation of distal carboxyl tail of one of the receptor modified the selective ligand-binding pocket of the other. The significance of the role of distal carboxyl tails in the receptor interaction could be viewed from the reduction in their co-immunoprecipitation upon truncation of the carboxyl tails of both the receptors.

Thereby suggesting the occurrence of interaction upon co-expression only, indicating the generation of MOR/DOR heteromer by a co-translational mechanism[46].

One of the studies further provides new understandings of the MOR/DOR receptor heteromer trafficking *via* clathrin and dynamin endocytic machinery. The insight of this mechanism was that MOR/DOR receptor heteromers internalization by DOR agonists needed the modification of MOR agonist binding to MOR/DOR heteromer caused by the DOR agonists which occupied both the receptors binding pockets and remained intact in a morphine tolerance model[47]. Another study suggested the existence of DOR/KOR heteromers in the sensory neurons of rat and the modulation of DOR agonist responses *via* KOR antagonists through the allosteric interaction of the protomers[48].

Opioid receptors are known for modulation of all levels of brain function including autonomic, sensory, emotional and cognitive processing. A very recent study has provided a proof-of-principle brain atlas using the MOR/DOR interacting model for the co-expression of GPCRs *in vivo*[11]. And now it has become an established fact that MOR and DOR receptors have functional interactions *in vivo* but still the underlying mechanism is still unresolved. To gain an insight of the existence of MOR/DOR heteromer in brain a double knock-in, redMOR/greenDOR (red protein mcherry tagged and green fluorescent protein tagged) mice model was generated. The data has been reported for the mapping of both receptors throughout the nervous system and is accessible online offering subcellular level visualization of opioid receptor atlas with associated MOR/DOR. Further where co-immunoprecipitation revealed heteromerization of receptors, high-order processing of forebrain suggested system-level interactions between MOR and DOR receptors as they were detected in separate neurons. On the contrary, subcortical networks highlighted co-localization which is crucial for eating, perception, sexual behaviours and response to aversive stimuli[11].

Although in literature all the three possible receptor heterodimer formations have been reported for the three known opioid receptors *viz.* KOR/MOR, MOR/DOR and KORDOR, the most importantly known and proven till date has been the heteromer MOR/DOR. Apart from this there is another interesting possibility of opioid receptors forming heteromers with other receptors outside their family having distinct pharmacological properties, different affinities for ligands as well as different signaling and receptor trafficking.

Astonishingly recent reports have identified and proposed the presence of some sort of association within the cell membrane between opioid and anti-opioid NPFF receptors. A study performed on rats revealed strong and long-lasting antinociceptive effects induced by NPFF when administered intrathecally along with potentiation of the morphine-induced analgesia[49]. This model proposed NPFF as a functional DOR/opioid autoreceptor antagonist causing an increase in the release of endogenous opioid peptides in spinal cord[50].

Another *in vitro* study done on SH-SY5Y cells showed a physical interaction between NPFF2 and MOR receptors, explaining the anti-opioid activity of NPFF2 receptors. By promoting a heteromeric association with MOR receptors, NPFF agonists cause changes in the diffusion properties of MOR receptors, as if they are moving MOR receptors away from their signaling associates. As a consequence, the response to opioids is reduced. The modulation of the delivery and trafficking of DOR/opioid receptors at the cell surface is assumed to be a means for regulating MOR receptor function, hence opioid analgesia and tolerance[51]. Likewise, the molecular mechanism described here for NPFF, comparable to the diffusion-trap system of the synapse, could represent another way to modulate the opioid response.

NPFF2 receptors exert a nonreciprocal antagonism on opioid receptors. Although, many other peptides including the opioid peptides, have been known to modulate the opioid receptors activity but it is only NPFF which exerts the antiopioid activity. To study this, a model SH2-D9 has been provided which enables the characterization of the molecular mechanisms involved in NPFF and opioid receptors interaction[52].

It is clearly evident at various, behavioural and receptor levels that NPFF and opioid systems interact. Though the most extensively studied physiological function is nociception, others like reward, locomotion, feeding, and intestinal motility are also affected. The two reciprocate each other as endogenous opioids are implied in analgesia upon spinal injection of NPFF and upon chronic opioid treatment, the endogenous NPFF peptides results in analgesic tolerance/hyperalgesia. These pharmacological modifications could be explained by the cellular anti-opioid effect of NPFF which has been attributed to the heteromerization mediated direct cross-talk between the two types of receptors studied in model cell lines. The validation of this hypothesis on endogenous receptors in neurons is a great challenge which will unveil some new perspectives for pain modulation and tolerance development[53]. This review focuses on the latest developments in the field of opioid receptor heteromerization and role in tolerance development.

PHARMACOLOGY AND THERAPEUTIC POTENTIAL OF OPIOID RECEPTOR HETEROMERS

Previously opioid receptors were considered as monomers and the selection of ligands binding to these receptors was based on observed pharmacological parameters. However, recent evidence from converging methodologies suggests that opioid receptors are expressed as homo/heterodimers. Opioid receptors homo/heteromerization

Table 1 Alteration of receptor properties by heteromerization over the years

Year of study	Receptors involved	Mode of study	Possible mechanism for interaction	Changes in binding properties	Heteromer specific ligands	Changes in G-protein activation and coupling	Possible therapeutic implications	Ref.
2000	MOR/DOR	*In vivo*	Direct interaction (heterodimers are preferred)	Decrease in affinity for selective agonists			Pain relief	George *et al*[9]
2000	MOR/DOR	*In vivo*	Direct interaction (heterodimers are preferred)	Allosteric effect of DOR ligands on MOR binding			Pain relief	Gomes *et al*[10]
2004	MOR/DOR	*In vivo*	Direct interaction (heterodimers are preferred)			Decrease in G protein coupling	Pain relief	Gomes *et al*[19]
2000	MOR/DOR	*In vivo*	Direct interaction (heterodimers are preferred)			Allosteric effect of DOR ligands on MOR coupling; signaling through a β-arrestin2-mediated pathway	Pain relief	Rozenfeld *et al*[6]
2007	MOR/DOR					Shift in coupling from Gαi to Gαz	Pain relief	Hasbi *et al*[84]
2005	MOR/DOR	COS-7 and CHO-K1 cells	Direct interaction (heterodimers are preferred)				Pain relief, tolerance development	Fan *et al*[46]
2003	KOR/DOR	Mouse spinal cord	Direct interaction (heterodimers are preferred)				Pain relief	Portoghese *et al*[45]
2001	KOR/DOR	Porcine ileum	Direct interaction (heterodimers are preferred)				Pain relief	Poonyachoti *et al*[44]
2010	MOR/DOR	HEK-293 cells	Direct interaction (heterodimers are preferred)				Pain relief	Yekkirala *et al*[57]
2005	MOR/DOR	*In vivo*	Direct interaction (heterodimers are preferred)		Specific heteromer activation by the agonist 6-GNTI		Pain relief	Waldhoer *et al*[43]
2014	DOR/KOR	*In vivo* (rat trigeminal ganglia)	Allosteric interactions				Thermal allodynia	Erbs *et al*[11]

MOR: Mu homologous opioid G-protein coupled receptors; DOR: Delta homologous opioid G-protein coupled receptors; KOR: Kappa homologous opioid G-protein coupled receptors.

is assumed to play a role in the activation of receptors and their internalization. This review focuses on advancements in the field of opioid receptor heteromerization and its impact on their pharmacological behaviour, and is summarised in Table 1. Since the theory behind heteromerization is still unknown, various models have been devised to identify the neurotransmitters binding to opioid receptors and their activation mediated by the allosteric interactions taking place between the protomers. Although there is a lack of a model predicting heteromerization signalling, functional data supports their occurrence as well as effects produced by them in response to a single neurotransmitter[54].

It has been very recently known that heterodimerization of opioid receptors modulates their pharmacology. The variation in the opioid receptor number, their distribution and the post-translational modifications may have great impact on the various adaptive changes following the acute (*e.g.*, desensitization) and chronic (*e.g.*, tolerance and down-regulation) opioid administration. Although each protomer of the heteromer is distinct, the heteromer too on the whole represents a distinct entity. Therefore depending on the heteromer different affinities and efficacies are expected for compounds. Thus the existence of opioid receptors heteromers open up a new field for the identification of compounds with improved specificity and reduced undesirable effects as they generate novel pharmacological and signaling properties.

Several laboratories have indeed started using this interesting therapeutic approach to design such compounds which may act on two receptors of the heteromer. In doing so, there arise two possibilities for the development of "dual" compounds. In first the dual compounds have moderate affinity for the two receptors whereas in second case the "dimeric" compounds would activate simultaneously both the receptors of the heterodimer. Out of these the second would serve to be an excellent tool for detection

of heteromerization in natural tissues. Many such heteromer specific compounds do exist which have been identified serendipitously. These rationally designed dualsteric GPCR agonists have allowed the simultaneous exploitation of favourable characteristics of orthosteric and allosteric receptors and prove to be a promising new approach for the achievement of fine-tuned GPCR modulation[55]. Following this approach it is expected that more rational (perhaps modelling based) approaches will emerge in the future.

The dualsteric ligands would enable both *in vivo* heteromer localization as well as their dynamics. Moreover, tissue-selective expression of opioid receptors would also enable prevention of the opioid induced side-effects *viz.* constipation and respiratory depression. In addition, the alteration of heteromers expression during morphine tolerance development could represent unexplored and selective targets for the pain modulation and reversing the development of tolerance and physical dependence[56].

LIGANDS TARGETING OPIOID HETEROMERS

Out of the ligands identified so far that are selective for the opioid receptor types, some may exhibit selectivity towards the heteromer in a differential manner in comparison to the individual receptors. In this context, various studies have been initiated to evaluate the classical MOR and DOR ligands selectivity towards MOR/DOR heteromer and also some MOR/DOR heteromer selective compounds have been identified and synthesized.

Given below are some examples of ligands targeting opioid receptor heteromers having analgesic effects *in vivo* and their possible role in side-effects, *e.g.*, tolerance.

Classical MOR agonists

Morphine has long been known as one of the choice analgesic used to treat chronic pain, but its usage is restricted due to the development of tolerance and physical dependence. To overcome this shortcoming, various strategies have been considered to enhance the potency of morphine while limiting its abuse. The most important out of them is using a combination of drugs, to increase the effectiveness of morphine.

Measurement of intracellular calcium release *via* chimeric G proteins or GTPγS binding has enabled the examination of the signaling properties of both the classical and clinically used MOR agonists (DAMGO, morphine, fentanyl and methadone) which is done in the cells stably expressing homo/heteromeric opioid receptors[57]. The potencies of these agonists increased to almost 7-12 folds higher in MOR/DOR heteromers than in MOR homomers and showing no significant results in DOR homomers. Whereas the DOR selective antagonist, naltrindole, antagonized the morphine, fentanyl and methadone mediated signaling exclusively in MOR/DOR heteromers expressing cells and also the antinociceptive effects of these drugs in monkeys[58]. Thus, suggesting MOR/DOR heteromers as prime targets for exhibiting antinociception and in tolerance development of these drugs.

Studies have shown that selective ligands activate distinct signaling pathways in cells expressing MOR/DOR heteromers in comparison to the cells expressing MOR homomers. As for example, DAMGO activates $G\alpha_{i/o}$-mediated signaling in MOR alone expressing cells while β-arrestin mediated signaling is activated in MOR/DOR heteromers expressing cells[59]. Since the β-arrestin mediated signaling is known for involvement in tolerance development, the MOR/DOR heteromers are suggested to play a role in the same[60].

Various *in vivo* studies have revealed that modulation of morphine-mediated antinociception involves MOR/DOR heteromers like morphine antinociception is enhanced by endogenous DOR agonist, Leu-enkephalin, by synthetic analog of enkephalin, FK33824 or by DOR opioid antagonist, TIPPΨ[19].

Further studies have promoted the DOR involvement in morphine tolerance development. Like in one study DOR receptor antagonist, naltrindole have been shown to block the morphine tolerance development[61] and in other study the DOR knockout mice did not developed the antinociceptive tolerance to morphine[62,63].

Recent reports have shown increased levels of MOR/DOR heteromers in brain and spinal regions following the morphine chronic treatment, using heteromer selective antibodies[30]. Another study using (transactivator of transcription) peptide (GRKKRRQRRRPQ) (TAT) peptide which targets the transmembrane 1 of MOR, preventing its heteromerization, demonstrated prevention of morphine tolerance development upon pretreatment with TAT[33]. Thus clearly indicating the crucial role of MOR/DOR heteromers in morphine induced anti-nociceptive tolerance.

Further studies have shown that MOR subunits interactions or interactions with other G-proteins are not disturbed by the morphine treatment rather it destabilizes the MOR/DOR heteromers. This could be seen in a recent study which shows that activation of the DOR protomer in MOR/DOR heteromer causes degradation of MOR protomer rather than recycling and thus decreasing its cellular response[33]. This is explained on the basis of sizes where MOR/DOR heteromers can be easily transferred to the lysosomal pathway as compared to MOR homomers which are larger in size, through more accessible sites for proteolysis or modifications, such as ubiquitination. Finally, proposing MOR/DOR heteromer as a suitable example to demonstrate the role of GPCR heteromerization causing differences in the cellular responses[34].

Classical DOR agonists

The antinociceptive effects of DOR selective agonist SNC80 have clearly depicted the involvement of

MOR/DOR heteromers. It was also shown in cells co-expressing a chimeric G-protein with either opioid receptor heteromers or individual receptor homomers that SNC80 induced intracellular calcium release only in cells expressing MOR/DOR heteromers[64]. Additionally MOR knockout animals did not showed the antinociceptive effect of SNC80 which was right shifted by almost 3-fold and with DOR knockouts the right shift was almost 6-fold[64]. Combining these results emphasized on the necessary presence of both MOR and DOR for the antinociceptive activity of SNC80. Further in a study combination of highly selective MOR agonists with DOR antagonists (and vice versa) were used to explore MOR/DOR heteromer mediated signaling.

As for example reversal of the MOR mediated signaling from β-arrestin-mediated into Gαi/o-mediated, in cells co-expressing MOR/DOR heteromers by blocking of the DOR by its selective antagonist which also resulted to enhancement of morphine-mediated antinociception[19,59].

These results highlight the functioning of DOR ligands in allosteric modulation of MOR (protomer) activity among the MOR/DOR heteromer. Moreover, in a report loss of the antidepressant and anxiolytic effects of the DOR agonist, UFP-512, were shown using a fusion of TAT peptide to the peptide corresponding to the distal C-tail of DOR to disrupt MOR/DOR heteromers signifying their potential role in anxiety and depression[65].

Heteromer specific ligands

Many studies have reported that receptor heteromerization leads to new binding properties[8-10], suggesting that heteromerization induces an alteration in the conformation of the ligand-binding site. The identification of an agonist, 6′-GNTI [6′-guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5a-epoxy-3,14-dihydroxy-6,7-2′,3′ indolomorphinan], an analgesic showing relative selectivity for DOR/KOR heteromers, supports the notion that receptors within a heteromer are capable of adopting active conformations that are absent in their homomeric counterparts[43]. 6′-guanidinonaltrindole precisely activates only the opioid receptor heteromers and not homomers[43]. Occurrence of heterodimers is tissue-specific as can be seen from the example of 6-GNTI which upon administration to spinal region induces analgesia but not in the brain. This study has given a proof-in-principle for the compounds targeting opioid heterodimers that they could be a better probe with increased analgesia and lesser side effects.

Another example is of a DOR1 selective agonist, 2-methyl-4 alpha alpha-(3-hydroxyphenyl)-1,2,3,4,-4a,5,12,12 alpha alpha-octahydro-quinolino[2,3,3-g]isoquinoline (TAN-67), which is known for reducing ethanol consumption in mice. The study reveals that its activity depends on presence of both the DOR and MOR receptors suggesting DOR1 to be a DOR/MOR heteromer which exhibited reduced ethanol consumption without dysphoria production. One more such example is of CYM51010 which is a MOR/DOR heteromer selective agonist[66]. The biasing of CYM51010 towards the MOR/DOR heteromer was determined by the tail-flick antinociception assay which demonstrated analgesic activity comparable to morphine and during chronic administration it resulted in lesser tolerance development in comparison to morphine[66]. Further the MOR/DOR heteromer selective antibody treatment although partially but significantly blocked CYM51010-induced β-arrestin recruitment, GTPγS binding and intrathecal antinociception[66] reinforcing the result that CYM51010 exhibited its effect mainly through activation of MOR/DOR heteromer. Thereby, proposing CYM51010 as potent analgesic with lesser tolerance development as compared to morphine.

In addition, drug "cocktails" targeting homo/heteromers could be therapeutically valuable. As for example an opioid cocktail comprising of morphine and either methadone or DAMGO enhances the morphine induced endocytosis. It is benefitted by the homomeric MOR resulting into reduced development of tolerance and dependence[67].

Dualsteric/dual ligands (allosteric ligands)

Dualsteric ligands represent a novel mode of targeting GPCRs as they can bind simultaneously to both, the orthosteric and allosteric sites of a receptor protein or two receptors present in the near vicinity or heterodimers[68], as shown in Figure 2. This approach facilitates the exploitation by a single compound, of both the orthosteric and the allosteric sites. The orthosteric interaction on one hand provides high affinity for binding and activation of receptors, the allosteric interaction on the other hand results in receptor subtype-selectivity thereby modulating both the efficacy and the activation of intracellular signaling pathway. With the increase in reports on allosteric interactions for GPCRs and the insight of spatial geometry of ligand/GPCR/complexes, the rational design of dualsteric ligands promises the achievement of finely tuned GPCR modulation.

GPCRs physical interaction is becoming progressively more recognized. The mechanism behind allosteric modulation of receptor function may possibly be provided through these interactions. Such allosteric interactions are expected to occur in a way that when a ligand binds to one protomer, it behaves as an allosteric enhancer of the other protomer[69]. Apart from allosteric modulations, heteromerization also affects the ligand recognition, G protein-coupling and trafficking. One such example is of opioid receptor heteromers having difference in ligand recognition at the receptor heteromer. According to recent models only one G protein binds to two receptor units meaning that in case of a heteromer it will have to select a particular G protein it should bind to as these receptors are usually coupled to different G proteins[70].

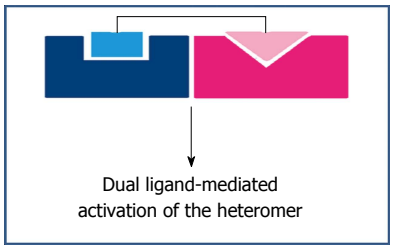

Figure 2 Dualsteric ligand mediated activation of the heteromer.

Following this rational the attempts of synthesizing heteromer selective ligands has given rise to the generation of dual ligands *viz.* the mu-delta agonist-antagonist (MDAN) series of ligands linked by a spacer of variable length. Out of these the ligand MDAN21 consists of two pharmacophores, MOR agonist MA19 and DOR antagonist DN20, which are separated by a 21-atom spacer[71]. In comparison to morphine, MDAN21 exhibited 100-times greater potency without significant development of tolerance and dependence[71]. Furthermore, this ligand also immobilized the heteromer at the cell surface possibly by bridging both the protomers and thereby inhibiting the MOR receptor internalization in cells expressing MOR/DOR heteromers[72]. More examples of such ligands are ENTI, which comprises of oxymorphone (high affinity μ-agonist) linked by a spacer arm to a low affinity DOR antagonist and DM-SNC80, containing naltrexone (high affinity μ-antagonist) joined by a spacer arm to a low affinity DOR agonist[73]. Though, the analgesic activity and the side effects of these ligands have not yet been adequately evaluated. These studies altogether, projects dual ligands crucial role in the examination of both the *in vitro* and *in vivo* properties of MOR/DOR heteromers.

Certain endogenously occurring amphiactive peptides as MERF, containing overlapping opioid and anti-opioid peptide sequences represent important molecules to study these protein-protein interactions that modulate endogenous opioid function[74]. Also anti-opioid peptides which bind to specific anti-opioid receptors attenuate opioid analgesia and are also involved in tolerance development and physical dependence. Thus in accordance to anti-opioid hypothesis, if the anti-opioid receptors can be blocked by a suitably designed peptide which can block or act as an antagonist at anti-opioid receptors, then the tolerance development and physical dependence might be reduced or attenuated.

In this connection, based on MERF and well known modulation of opioid system by NPFF/FMRFa peptides a chimeric peptide, of Met-enkephalin (YGGFM) and FMRFa, YFa (YGGFMKKKFMRFamide) was previously designed by our group[75]. The tetrapeptide (YGGF) sequence is a common endogenous opioid peptides allosteric (message) sequence that activates the receptor and cascades the intracellular signaling pathways. Whereas, the (FMRFamide) sequence is a positive allosteric modulator of anti-opioid receptors and here is orthosteric (address) sequence having role in opioid effect modulation and development of tolerance. YFa can bind to opioid receptors through its N-terminus Tyr-Gly-Gly-Phe (YGGF), (allosteric, message) sequence and to anti-opioid receptors through its C-terminus Phe-Met-Arg-Phe (FMRFamide), (orthosteric, address) sequence, which are separated by 3-lysine residues based on Schwyzer compartment theory[76].

YFa induced naloxone-reversible antinociception suggesting opioid receptors mediated analgesia it potentiated morphine induced antinociception and attenuated tolerance development to morphine analgesia, upon intraperitoneal administration[77]. Antagonist and protein expression studies revealed that YFa produces tolerance free KOR specific antinociception[77] may be due to its ability to adopt a helical conformation. In addition, it induced after 4 d pretreatment, cross tolerance to 20 mg/kg morphine analgesia with 80 mg/kg YFa[78] and these results have been further substantiated by forskolin-stimulated cAMP inhibition and Eu-GTP-γS binding studies[79]. Moreover, smooth muscle contraction study performed on ileum of guinea pig and vas deferens of mouse revealed role of anti-opioid receptors in normalizing the effects mediated by opioid receptors[80]. These observations proposed the role of this amphiactive peptide in pain modulation.

OPIOID RECEPTOR HETEROMER TRAFFICKING

Opioid receptor heteromerization has lead to the alteration of opioid ligand properties and it also affects the trafficking of receptors in the cell culture model systems[19,81-83]. A number of studies have reported the heteromers trafficking from an intracellular compartment to the cell surface. On the other hand a few studies have examined the MOR/DOR heteromers trafficking from the cell surface to an intracellular compartment (endocytosis) among which some conflicts about their presence only at the cell surface while others presume their pre-assembling in the endoplasmic reticulum prior being trafficked to the cell surface. To exemplify this one of study was done with MOR expressing cells in which DOR expression could be induced and it was revealed that the two receptors form heteromers only when present at the cell surface as this required the interactions with G proteins. Another study reported MOR/DOR heteromers presence in the endoplasmic reticulum where they were associated with $G\alpha_z$ protein, using BRET in combination with cell fractionation[84]. These two conflicting results having differences in the detection of heteromerization site is due to the disparity of the experimental conditions. The first study used induction of DOR expression in MOR expressing cells which is staggered receptor expression while the other study specifically picked up co-expressing MOR/luciferase and DOR/GFP.

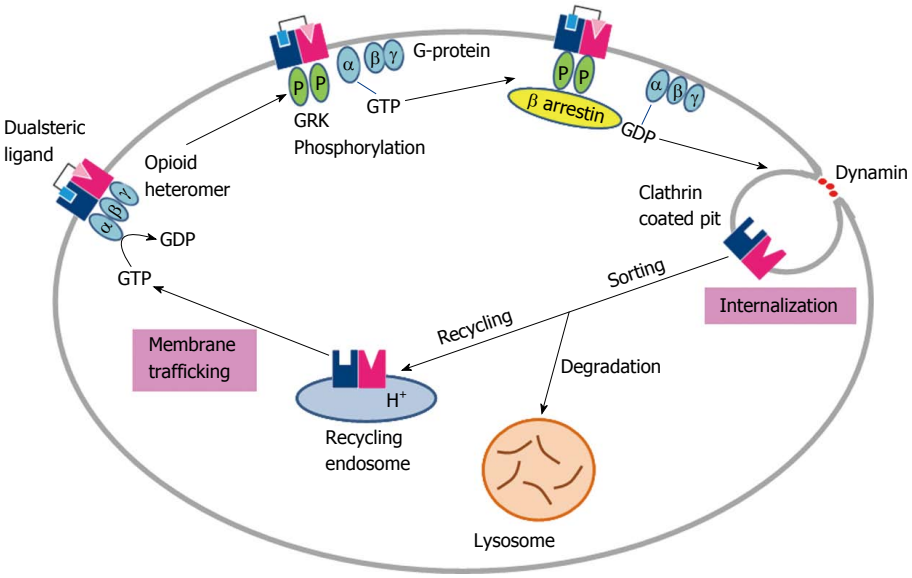

Figure 3 Dualsteric ligand induced regulation of opioid receptors heteromer. Without agonist treatment, about 15% of the receptors are intracellular and G protein trimers are associated with each other with GDP bound to Gα subunits. Upon exposure to agonist, the receptor is activated and coupled to G proteins, triggering the exchange of GDP with GTP and dissociation of Gα from Gβγ subunits, which in turn activate down-stream effectors. Activation of the receptor also causes translocation and activation of GRKs, which phosphorylated the receptor in intracellular domains. Receptor phosphorylation enhances binding of β-arrestins, leading to uncoupling of receptors from G proteins. β-arrestins bind to clathrin and initiate movement of receptors into clathrin-coated pits, which are pinched by dynamin to become endosomes. Low pH in endosomes facilitates dissociation of agonists from the receptor and dephosphorylation of the receptor is believed to occur here. Internalized receptors are routed to two different pathways. Some are sorted to recycling endosomes and returned to plasma membranes. Alternatively, the receptors lead to degradation forming lysosomes. GRK: G-protein coupled receptor kinase; GDP: Guanosine-5'-diphosphate; GTP: Guanosine-5'-triphosphate.

Another twist arises with the studies claiming heteromer endocytosis to be probe selective, by showing that some of the agonists (damgo, Deltorphin Ⅱ, SNC80, methadone) induces the MOR/DOR heteromers endocytosis but others (DPDPE, DSLET) do not[42]. Furthermore, studies report blocking of MOR selective agonist induced endocytosis by the DOR selective antagonists[81]. Additionally, endocytosed MOR/DOR heteromers were found to be targeted for degradation whereas the MOR homomers were found to be recycled back to the cell surface[81]. Overall these studies proposed that changes in receptor trafficking properties are due to heteromerization, as shown in Figure 3.

Further studies using a MOR/DOR heteromer selective dual ligand, MDAN21, revealed blocking of heteromer endocytosis, it was successfully induced by the combination of individual monovalent pharmacophores (DN20 and MA19). This observation highlighted the importance of the 21-atom spacer in MDAN21 joining the two pharmacophores, which has effectively bridged the protomers, thereby immobilizing the MOR/DOR heteromer and preventing its internalization. Further studies are still required for the characterization of the underlying mechanisms involved in the differential trafficking of MOR/DOR heteromers.

In one of the study it was shown that chaperone proteins are required for the efficient cell surface expression of MOR/DOR heteromers while examination of their biosynthesis and maturation. Therefore in MOR and DOR co-expressing cells the heteromer was found to localize in the Golgi apparatus significantly, clearly indicating the requirement of presence of receptor transport protein 4 for cell surface expression of the heteromer[42]. The heteromer was protected from ubiquitination and proteasomal degradation by this chaperone during folding and maturation[42]. Although the role of this chaperone in the unique binding and signaling properties of the MOR/DOR heteromer is not clear.

Taking the other side of the story and looking at the antibodies which have been classically used as analytical tools for the identification, localization and quantification of different antigens including hormones and pathogens. Last decade has seen their usage as therapeutic agents in experimental and clinical medicine[85]. They have significant advantage of electivity, potency and efficacy over the conventional chemical drug-based therapies which makes them more effective in the treatment of various conditions *viz.* cancer and immune disorders. Additionally they have shown direct impact on signaling pathways within the targeted cells either by binding to the cell surface proteins or acting on their intracellular targets. A study on generation of heteromer specific antibody reported a MOR-DOR heteromer selective antibody that enabled examination of up-regulation of the heteromer in endogenous tissue upon chronic morphine treatment. This subtractive immunization strategy could be used for the generation of MOR-DOR heteromer selective antibodies and also to generate other antibodies selective for other GPCR heteromers.

This would then help in studying the physiological and pathophysiological conditions and the role of GPCR heteromers in them[30].

Screening for ligands targeting the MOR/DOR heteromer and their pharmacology in pain-regulation

The above mentioned studies put forward the MOR/DOR heteromers as potential targets for the development of novel therapeutics in treatment of pain with lesser side-effects due to their unique pharmacological and signaling properties. Since this would require high-throughput screening (HTS) of a large number of compounds leading to the identification of MOR/DOR heteromer selective ligands, suitable screening assays have to be determined[86].

All the observations of this study points towards the anti-analgesic role of the DOR/MOR heteromer in case of thermal nociception. The anti-analgesic effect exhibited by the combined treatment of methadone and naltriben was dependent DOR dependent, as it was not present in the DOR knockout mice. Moreover, reversal of the anti-analgesic effect was demonstrated which may be either due to the increase in degradation of DORs or by selective blocking of signaling only from the DOR/MOR heteromers but not MORs[87]. Thus below we describe a few of the HTS assays suitable for the heteromer selective ligand screening.

HTS using calcium signaling

Out of the several assays *viz*. adenylyl cyclase/cAMP, phospholipase C/Ca^{2+}, or Rho, that are used to measure the G-protein mediated signaling which could be used for HTS, the one measuring the intracellular Ca^{2+} release is commonly used for the screening of ligands to Gαq coupled receptors. Furthermore, measuring the intracellular Ca^{2+} release could also be used in the screening of ligands for $G\alpha_i$ or $G\alpha_s$ coupled receptors, because of the development of chimeric G-proteins ($G\alpha q_s$ or $G\alpha q_i$) which provide for these receptors the calcium readout, that do not normally signal *via* the Gαq pathway[88]. Therefore by monitoring the release of intracellular Ca^{2+}, the activation of opioid receptors co-expressed with chimeric $G\alpha q_i$ protein can be detected.

Recently, a screening assay has been reported which uses the terminal-carboxyl truncated GPCRs fused to chimeric Gαqi proteins for the detection of heteromer-mediated signaling. These fused receptors do not induce the intracellular Ca^{2+} release upon agonist binding; it is only observed upon their co-expression with the wild-type receptors[88]. The co-expression of wild-type MOR and $G\alpha q_i$-fused DOR receptor allowed the detection of Ca^{2+} release for MOR/DOR heteromers[88]. The most important advantage of this method is it detects only the heteromer mediated signaling in heteromers expressing cells. To exemplify, this assay has been used with a compound ADL5859 which exhibited weak signals in case of MOR/DOR heteromers as compared to DOR homomers[88]. Thus proving that Ca^{2+} signaling based assays could be very useful in the identification of heteromer-selective compounds.

HTS using β-arrestin signaling

The two subtypes of β-arrestin (1 and 2) exhibit 78% homology and contain binding sites for clathrin and the β2-adaptin subunit in their C-terminal tail which allows them to function as adaptor proteins and target GPCRs to clathrin coated pits for endocytosis[89,90]. The different opioid receptor subtypes exhibit different requirements for binding β-arrestin (type 1 and 2)[91] that could lead to different signaling outcomes[92,93], as shown in Figure 4. Glutathione S-transferase pull down assays show that the third intracellular loop and the C-terminal tail of DOR and only the C-terminal tail of KOR can interact with β-arrestin 1 or β-arrestin 2[91]. These studies did not observe any interaction between β-arrestin 1/2 and MOR. However, studies in HEK-293 cells using β-arrestin 2 tagged to GFP or in striatal neurons using dominant negative β-arrestin 2 show that agonist activated MOR can recruit β-arrestin 2[94,95] although the efficacy of recruitment is agonist dependent[96]. Interestingly, mice lacking β-arrestin 2 potentiated and increased duration of the analgesic effect induced by morphine underscoring the importance of β-arrestin 2 in mediating MOR function[97]. In the case of DOR, a BRET assay suggested that receptor phosphorylation promoted receptor selectivity for β-arrestin 2 over β-arrestin 1 without affecting the stability of the receptor-β-arrestin complex[98]. However, another study used fluorescence and co-immunoprecipitation to show that agonist treated DOR bound and recruited β-arrestin (1 and 2) to the plasma membrane[99]. In addition, it has been shown that over expression of type 1 β-arrestin leads to an attenuation of DOR and KOR but not MOR mediated activation of G-proteins and inhibition of cAMP levels[100].

In addition to being involved in the attenuation of G protein mediated signaling, studies have shown that β-arrestins can induce a sustained extracellular signal-regulated kinases (ERK) phosphorylation that is distinct from the transient G-protein mediated ERK phosphorylation[101]. A recent study showed that MOR ligands such as etorphine and fentanyl, but not morphine or methadone, induced phosphorylation of ERK through a β-arrestin dependent pathway. This led to the translocation of phosphorylated ERK to the nucleus leading to an increase in the activity of Elk-1 and in the transcription of G-protein coupled receptor kinases 2 and β-arrestin 2[102]. More recently, a study showed that heterodimerization between MOR and DOR promotes the recruitment of type 2 β-arrestin onto the plasma membrane thereby changing the spatio-temporal dynamics of ERK mediated signaling that are quite distinct from those observed with the

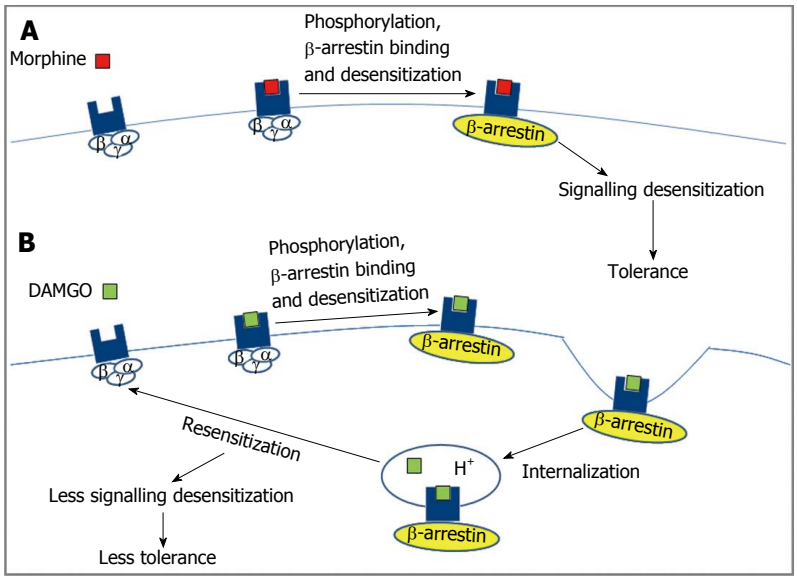

Figure 4 Hypothesis about the role of receptor internalization in the development of opioid tolerance. A: In the presence of "non-internalizing" opioids, exemplified by morphine, receptor signaling is rapidly terminated by receptor phosphorylation and β-arrestin binding. The desensitized receptors remain inactivated in the plasma membrane leading to signaling desensitization and opioid tolerance; B: In contrast, "internalizing" opioids such as DAMGO [D-Ala2, N-MePhe4, Gly-ol]-enkephalin lead to receptor desensitization by phosphorylation and β-arrestin binding but also to rapid receptor internalization and resensitization counteracting signaling desensitization and opioid tolerance.

MOR homodimer. Altogether, these studies show that β-arrestins plays crucial role in mediating opioid receptor signaling by serving as a switch between G protein dependent and independent signaling mechanisms[103].

Role of anti-opioid compounds in tolerance development

Recent researches have though made somewhat clear the mechanism underlying pain, but still the opioids are the most powerful analgesics, inspite of their limitation of tolerance development and dependence following chronic exposure. To overcome this, a hypothesis has been proposed according to which the chronic administration of opioids is counteracted by anti-opioid system that produces opposite effects[21]. This hypothesis has been supported by a number of experimental and clinical data which clearly shows that opiates activate anti-opioid systems, producing opposite and long-lasting effects which results in opioid induced hyperalgesia (OIH) thereby reducing the analgesic effects (tolerance)[104]. Since the molecular mechanisms that underlie OIH are not clear, this phenomenon is attributed to the sensitization of pronociceptive pathways in response to opioid treatment. Some other adverse effects *viz*. dependence and abstinence syndrome, may also involve the activation of anti-opioid systems[21]. Therefore it is assumed that compounds that can block the stimulation of anti-opioid systems could lead to the prevention of opioid tolerance development and thus limit the side effects developing due to chronic opioid exposure. This may be due to some sort of physical association between the opioid and anti-opioid receptors[105].

Looking at the other side of the story the discovery of a potent NPFF receptors (anti-opioid) selective antagonist, upon systemic administration prevents the hyperalgesia development which consequently opposes the associated decrease in analgesic effect induced by heroin. This observation reinforces the hypothesis that opioid tolerance development is not only due to a reduction in cellular responsiveness but could also be arising from the secondary up-regulation of the antiopioid systems having pronociceptive properties which renders long-lasting enhancement in pain sensitivity[106].

Looking at the other side of the story suggests that NPFF plays an important role in pain modulation, opioid tolerance development and several other physiological processes[106]. Recent reports proposed the discovery of a potent NPFF receptors (anti-opioid) selective antagonist, RF9 which upon systemic administration prevents the OIH development which consequently opposes the associated decrease in analgesic effect induced by heroin. These observations clearly mark NPFF receptors as part of anti-opioid system and put forward their antagonists as useful therapeutic agents for the improvement of opioids efficacy during chronic pain treatment[23].

Based on certain endogenous amphiactive peptides as MERF, which represent important molecules to study presence of homo/heteromer as well as their interactions that modulate endogenous opioid function[74] a chimeric peptide YFa (YGGFMKKKFMRFamide-YFa) was designed by our group. YFa can bind to opioid receptors through its N-terminus Tyr-Gly-Gly-Phe (YGGF), (allosteric, message) sequence and to anti-opioid receptors through its C-terminus Phe-Met-Arg-Phe (FMRFamide), (orthosteric, address) sequence, separated by 3-lysine

residues based on Schwyzer compartment theory.

Following the same lines of scrutiny presently in our group another dualsteric ligand NPYFa (YGGFM-KKKPQRFamide) based on YFa, having mammalian anti-opioid sequence in order to achieve a more potent and tolerance free chimeric peptide. NPYFa contains both endogenous opioid (YGGFM; Met-enkephalin) at N-terminus and anti-opioid (NPFF-endogenous mammalian peptide)[107] at C-terminus (C-terminal modified analogue of YFa), separated by 3 lysine residues. Pharmacological profiling of dualsteric ligand NPYFa is in progress.

CONCLUSION

Decades of research over opioid receptors has recently discovered heteromers existence, opening new ways of investigating the functioning of opioid receptors. This has also given insight to how heteromerization affects the receptor trafficking and vice versa. This review highlighted the dualsteric ligand approach targeting opioid heteromers with improved analgesic efficacy and less tolerance development. Rationally designed dualsteric GPCR agonists targeting opioid receptors heteromers allows exploitation of binding, orthosteric or orthosteric and allosteric sites of two distinct receptors simultaneously. This could be due to orthosteric receptor activation which may be followed by the allosteric subtype-selectivity further leading to intracellular signaling pathway selectivity. Further understanding of such mechanisms enables the selection of ligands with enhanced intrinsic efficacy and lesser side effects.

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Chemoprevention of gastrointestinal cancers by natural honey

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Abstract

Gastrointestinal cancers are the most common human cancers in both men and women worldwide. Several epidemiological and experimental studies suggest a relationship between gastrointestinal cancers risk and dietary factors. Natural honey has been widely used in traditional medicine for many centuries to treat a wide range of ailments and complaints. Honey contains various components that exhibit wide activities including antibacterial, anti-inflammatory, antioxidant and anticancer properties. The anticancer effects of honey are mediated *via* diverse mechanisms, including inhibition of proliferation, induction of apoptosis, suppression of free radicals and modulation of inflammatory signalling pathways. The present review assesses the chemopreventive effects of natural honey and its components in the modulation of gastrointestinal cancers and its modes of action in the prevention of the development of gastrointestinal tumors. Honey can be an approach as a cancer-preventive strategy which merits further experimental and clinical research in the near future.

Key words: Honey; Gastrointestinal; Cancer; Chemoprevention; Anticancer

Core tip: Natural honey has been widely used in traditional medicine to treat a wide range of ailments and complaints. Honey contains various components that exhibit wide activities including antibacterial, anti-inflammatory, antioxidant and anticancer properties. The present review assesses the chemopreventive effects of natural honey and its components in the modulation and prevention of gastrointestinal cancers.

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GASTROINTESTINAL CANCERS

Cancer is a leading cause of death worldwide. It strikes more than one third of the world population and it's the cause of more than 20% of all deaths[1]. Gastrointestinal cancers (cancers of the digestive

system) include cancers of the esophagus, gallbladder, liver, pancreas, stomach, small intestine, large intestine (colon) and rectum. The risk of gastrointestinal cancers varies greatly by individual ethnic group, lifestyles and risk factors[2]. Considering the magnitude of the problem of cancer and the failure of conventional therapy of affects greatly the mortality rates for many types of cancers, new approaches to control of cancer and discovery of new agents is of great importance.

In recent years, with rising prevalence of gastrointestinal cancers by several factors such as changing lifestyle and improved screening and diagnosis, besides the obstacles of the current cancer therapy (chemotherapy and radiotherapy), there has been a great trend towards the use of dietary factors and natural products among cancer patients[3,4]. The incidence of gastrointestinal cancers varies considerably from place to place and from time to time. It is clear that environmental factors play an important part in the development of these cancers and that many of these factors may be preventible. Oesophageal adenocarcinoma has been dramatically rising over the past decades[5], and this rise in this cancer has been associated with an increased prevalence of gastroesophageal reflux disease and Barrett's esophagus. Fruit and vegetable consumption has been reported to have a protective effect and associated with a lower risk of oesophageal cancer. Gastric adenocarcinoma is the second leading cause of cancer worldwide[5]. Foods that are smoked, dried, or pickled have been associated with an increased risk of gastric cancer[6,7]. Vegetable and fruit intake has consistently been associated with a decreased incidence of gastric cancer[8]. Pancreatic cancer is also one of the most devastating cancers, with a 5-year survival of only 6%[9]. High dietary fiber intake, vegetable and fruit intake reduces pancreatic cancer risk[10]. Colorectal cancer is also one of the leading cancers in both men and women in several countries[11], and several epidemiological and experimental studies suggest a link between colon cancer risk and dietary factors[12,13].

CANCER CHEMPREVENTION

Carcinogenesis is a multistep process induced by molecular and genetic changes that disrupt signaling pathways regulating proliferation, apoptosis and differentiation[14]. The search for anticancer agents from natural sources for prevention and treatment of cancer is of a considerable interest in recent years. Several approaches are explored for the prevention and treatment of cancer including chemoprevention in animal models and clinical trials[15]. Dietary supplements and natural compounds are one approach is used to reverse or prevent the development of cancer by modulating the molecular processes of initiation, promotion, and progression stages. It has been reported that diet components such as turmeric, garlic, ginger, cruciferous vegetables and green tea play an important role in cancer prevention[16,17]. The role of diet and prevention of gastrointestinal cancers is evolving and much data from basic science and animal models. Natural honey has been recently the focus of basic research and clinical studies for its several therapeutic benefits including cancer.

Figure 1 Variety of natural honey.

NATURAL HONEY

Since ancient times, natural honey has been widely used as a conventional medicine, and is extensively used for its therapeutic effects in recent years. Ancient Egyptians, Chinese, Greeks and Romans employed honey for wounds and diseases of the intestine[18]. In the Holy Quran, Almighty Allah mentioned the special ability of honey to heal and cure disease. Scientific research has proven the therapeutic benefits of honey in treating several human diseases. The physical properties of natural honey depend on water content, temperature, the type of flora and the proportion of its sugars. The color of the honey varies according to the floral source and its mineral content, which usually ranges from water white to dark amber (Figure 1).

TYPES OF NATURAL HONEY

A large number of natural honeys are available worldwide and are either locally produced or imported from other countries. The type of natural honey depends on which species of plants were flowering when the bees were producing the honey. Honey from a single floral source greatly varies from honey of the same floral source that obtained from different locations or seasons. The percentage of fructose, glucose, amount and type of amino acids and the organic acids vary by floral source. The floral source affects not only the physicochemical properties of the honey, but also the antimicrobial activity of honey[19-21]. Different types of honey differ in their color, flavor and density. The antibacterial quality of honey varies among different types of honey depending on geographical locations, seasonal source, harvesting,

Table 1 Composition of natural honey[18,31]

Component	Average (value per 100 g)
Carbohydrates	82.4 g
Fructose	38.5 g
Glucose	31 g
Sucrose	1 g
Other sugars	11.7 g
Dietary fiber	0.2 g
Fat	0 g
Protein	0.3 g
Water	17.1 g
Riboflavin (Vit. B2)	0.038 mg
Niacin (Vit. B3)	0.121 mg
Pantothenic acid (Vit. B5)	0.068 mg
Pyridoxine (Vit. B6)	0.024 mg
Folate (Vit. B9)	0.002 mg
Vitamin C	0.5 mg
Calcium	6 mg
Iron	0.42 mg
Magnesium	2 mg
Phosphorus	4 mg
Potassium	52 mg
Sodium	4 mg
Zinc	0.22 mg

purity and storage conditions[22-24]. A survey of the antibacterial activity of 52 samples of 24 types of honey of Saudi and some international honeys revealed that the antibacterial activity of the majority of the investigated honeys did not show large variations[25]. The equivalent phenol percent concentrations for the majority of honeys ranged between 5.5% and 7.9%. It was also noticed in this study that there was no relationship between color and antibacterial activity of honey. The antimicrobial activity of honey could be attributed to several factors like the osmotic effect of honey, acidity, the presence of hydrogen peroxide, the presence of antibacterial phytochemical components and the *in-vivo* antibacterial activity of honey[22,26-29]. *In-vitro* antioxidant activity of Saudi Sidr honey "monofloral type of honey" revealed a strong antioxidant activity[30]. Furthermore, pretreatment with Sidr honey prior to the administration of CCl_4 significantly prevented the increase of the serum levels of enzyme markers and reduced oxidative stress in rats.

COMPOSITION OF NATURAL HONEY

The composition of natural honey varies, depending on many factors such as the geographical areas, source of honeybee food, climate, environmental conditions and the processing it undergoes[31-35]. Honey contains about 200 substances including fructose, glucose, amino acids, vitamins, minerals, water and enzymes[18,31], as shown in Table 1. Natural honey uses a combination of components, including hydrogen peroxide, acidity, osmotic effect, high sugar concentration and polyphenols to prevent diseases and fight infection. All natural honey contains flavonoides, phenolic acids,

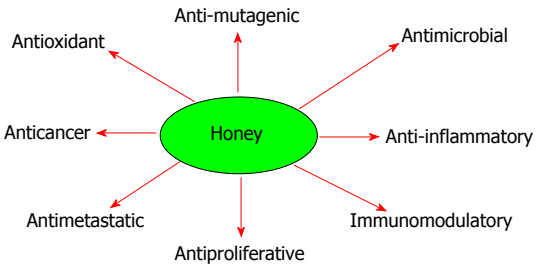

Figure 2 Actions of natural honey.

ascorbic acid, tocopherols, catalase, superoxide dismutase, reduced glutathione[18,36]. Some of these flavonoids and phenolic compounds include chrysin, kaempferol, quercetin, pinobanksin, pinocembrin, luteolin, apigenin, genistein, naringenin, hesperetin, *P*-coumaric acid, gallic acid, ellagic acid, ferulic acid, syringic acid, caffeic acid and vanillic acid. Natural honey and its components have been shown to possess a wide range of medicinal properties such as anti-inflammatory, gastroprotective, antioxidant, antitumor and anticancer effects[37-41]. Figure 2 depicts some of the actions of natural honey.

Potential mechanisms of honey actions were found to include regulation of cell cycle, induction of apoptosis, activation of mitochondrial pathway, inhibition of angiogenesis and modulation of oxidative stress. Honey can inhibit the development of cancer by blocking the three stages of carcinogenesis (initiation, promotion and progression). The inhibitory effect of honey on carcinogenesis can be attributed to the presence of its active components, especially flavonoids and phenolic constituents. Honey plays an important role in preventing inflammatory tissues from producing free radicals[42]. Caffeic acid phenethyl ester (CAPE), an active component of propolis, has many biological and pharmacological activities including antioxidant, antiinflammatory, antiviral action, anti-proliferative effect, apoptosis-inducing effect and anticancer effect[43-46]. Caffeic acid esters have been shown to have an inhibitory effect on tumor cell proliferation and transformation by the down regulation of many cellular enzymatic pathways including protein tyrosine kinase, cycloxygenase and ornithine decarboxylase pathways[47-50].

NATURAL HONEY AND GASTROINTESTINAL INFLAMMATION

Inflammation plays an important role in the development of several diseases including gastrointestinal diseases and cancer[51,52]. Inflammation is recognized as a risk factor for gastric inflammation and *Helicobacter pylori* (*H. pylori*) infection and clinical intervention by natural products such as honey may provide an approach for reducing inflammation and *H. pylori*-associated diseases, particularly gastric cancer.

Honey contains many phenolic compounds such as ellagic acid, gallic acid, caffeic acid, quercetin and chrysin, which correlated to its antioxidant and anti-inflammatory activities[36,52,53]. It was suggested that honey and its components can inhibit inflammation *via* inactivation of nuclear factor kappa-B (NF-κB) and inhibition of transcription of genes for pro-inflammatory mediators such as COX-2, tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and inducible nitric oxide synthase[54,55]. Gelam honey has shown to have anti-inflammatory effects by reducing the edema size and inhibiting the production of the pro-inflammatory mediators nitric oxide, prostaglandin E2, TNF-α, and IL-6 in rats[56]. Honey has been reported to potentiate the gastric protection effects of sucralfate against ammonia-induced gastric lesions in rats[57]. Alagwu has reported that honey intake caused cytoprotection on the gastric mucosa of albino rats[58]. Oral administration of honey has been reported to protect against gastrointestinal infection such as gastritis, duodenitis and gastric ulcer caused by bacteria[59-61]. Nasutia *et al*[62] demonstrated that oral pretreatment of honey prevented indomethacin-induced gastric lesions in rats. Perfusion of the stomach with isotonic honey resulted in a marked reduction of the lesions caused by ethanol and indomethacin in rats[38,63].

NATURAL HONEY AND *H. PYLORI* INFECTION

H. pylori infection represents the most common risk factor underlying chronic inflammation and gastric cancer[64,65]. *H. pylori* can lead to mucosal inflammation and cancer development through several mechanisms including *H. pylori* virulence factors such as CagA and VacA genotypes and inflammatory mediators that induce cellular signalling alterations in gastric cells[65]. The intake of honey also helps treat *H. pylori* infection[66]. Natural honey from New Zealand and Saudi Arabia at concentrations 20% (v/v) inhibited the growth of *H. pylori in vitro*[67,68]. Honey had an inhibitory effect on *H. pylori in vitro* at solutions of both 10% and 20% honey[67]. al Somal *et al*[59] also found that Manuka honey from New Zealand, at concentrations as low as 5% v/v, completely inhibit the growth of *H. pylori,* and that 2.5% v/v partially inhibits the growth of *H. pylori*[68]. Osmotic effects were shown to be the most important parameter for killing *H. pylori* as all carbohydrate solutions > or = 15% (v/v) inhibited 100% of the *H. pylori*. The therapeutic effect of honey was attributed to the antibacterial properties[68,69]. Osato *et al*[70] also reported that commercial honeys and the artificial solution were effective as Manuka honey in inhibiting growth of all *H. pylori* isolates at concentrations 15% v/v. It has also been reported that the use of honey with triple therapy regimen may help shorten the time required to eliminate *H. pylori* from stomach lining of patients with gastritis or duodenal ulcer caused by *H. pylori* infection[71].

H. pylori has been shown to activate mitogen-activated protein kinases and transcription factors such as AP-1 and NF-κB that regulate cell proliferation and differentiation in gastric epithelial cells using several different bacterial components and host signaling pathways[72,73]. NF-κB and activator protein-1 (AP-1) are key regulators of inflammation and signaling cascades that lead to carcinogenesis. There are numerous agents including honey have been reported to suppress NF-κB activation and act as potential chemopreventive agents for inflammation and cancer[74]. CAPE blocked *H. pylori*-induced NF-κB and AP-1 expression in gastric cancer cells, and CAPE also suppressed *H. pylori*-induced cell proliferation and production of the cytokines TNF-α and IL-8 and COX-2 expression[55]. Therefore, the inhibition of these molecules by CAPE could result in suppression of many genes during *H. pylori*-induced inflammation. Wu *et al*[75] demonstrated that the activity of NF-κB and the expression of matrix metalloproteinase-9, IL-1beta, and IL-8 in gastric cancer cells by *H. pylori* significantly reversed by CAPE treatment, which suggested that CAPE could be promising adjuvant agent against gastric cancer. In Monogolian gerbils, CAPE treatment elicited anti-inflammatory effects on *H. pylori*-induced chronic gastritis. CAPE significantly inhibited *H. pylori*-stimulated NF-κB activation and mRNA expression of several inflammatory factors in a dose-dependent manner, and prevented degradation of IκB-alpha and phosphorylation of p65 in gastric cancer cells[76].

NATURAL HONEY AND GASTROINTESTINAL CANCERS

There are many research studies support the use of natural honey for cancer prevention and treatment, especially cancers of the gastrointestinal tract. Nutritional studies have indicated that consumption of honey modulates the risk of the development of gastric cancer, and also honey induced apoptosis in gastric mucosa[77]. It was postulated that CAPE may be a promising adjuvant treatment in gastric cancer[78]. The chemopreventive actions of honey and its components have been also studied in various colon cancer models. Gelam and Nenas honeys suppressed the growth of HT 29 colon cancer cells by inducing DNA damage and apoptosis and suppressing inflammation[79]. Jaganathan also demonstrated the anti-proliferative effect of Caffeic acid, one of the phenolic constituents of honey, inhibited in the colon cancer cells HCT15 and HT29[80]. Honey induced apoptosis by causing the depletion of intracellular non-protein thiols and reduced the mitochondrial membrane potential and increased generation of reactive oxygen species. Furthermore, honey constituents induced apoptosis in colon cancer cells[81]. Orsolić *et al*[82] showed that honey exerted anti-metastatic effect in a murine

tumor model with colon carcinoma. Supplementation of diet with honey and Nigella sativa had a protective effect against methylnitrosourea-induced oxidative stress, inflammatory response and carcinogenesis in Sprague Dawely rats[83]. Caffeic acid esters derivatives inhibited azoxymethane-induced colonic colonie pre-neoplastic lesions, ornithine decarboxylase, tyrosine protein kinase, and lipoxygenase activities and aberrant crypt foci formation, which are relevant to colon carcinogenesis in rat colon[50]. Caffeic acid and its ester are potent inhibitors of human colon adenocarcinoma cell growth[84]. Dietary administration of phenylethyl-3-methylcaffeate significantly inhibited the incidence and multiplicity of invasive, noninvasive adenocarcinomas of the colon, and also suppressed the colon tumor volume by 43% compared to the control diet, and also inhibited the formation in colonic tumors by 15%-30% in the animals[48]. Gribel' *et al*[85] indicated that honey possessed moderate antitumor effect and pronounced antitumor activity of 5-flurouracil and cyclophosphamide against five different strains of rat and mouse tumors. Furthermore, honey potentiated the antitumor activity of the chemotherapeutic drugs 5-fluorouracil and cyclophosphamide in colorectal cancer cells[86,87].

The anticancer effects of natural honey and its components on liver cancer cells have been investigated in a number of studies[88-90]. Treatment of hepatocellular carcinoma HepG2 cells with bee honey and Nigella sativa led to a significant decrease in both the number of viable HepG2 cells and the levels of nitric oxide and improved the total antioxidant status and caspase-3 activity, especially in HepG2 cells treated with higher doses of bee honey Nigella sativa (20% and 5000 μg/mL)[88]. It has been reported that Spanish honeys were most effective in protecting against food mutagen-induced DNA damage in HepG2 cells, which was attributed to its antioxidant and free radical scavenging properties[89]. Gelam honey was selectively cytotoxic to liver cancer cells and found that the IC_{50} value of gelam honey towards HepG2 was 25% whereas it was 70% for normal human hepatocytes (WRL-68)[90]. Abdel Aziz *et al*[91] reported that honey extracts exerted cytotoxic, antimetastatic and anti-angiogenic effects in HepG2 cells. Treatment with diethylnitrosamine induced hepatic cancer in rats and the neoplastic hepatic cells were reduced in the liver of honey-treated DEN-induced rats[92]. These studies indicate that honey has an anticancer effect on various types of cancer cells and exerts its protective effect against the development of cancer by modulating the molecular and cellular mechanisms of carcinogenesis stages. Some of the mechanisms by which honey may exert its anticancer effects are cell cycle arrest, activation of mitochondrial pathway, induction of mitochondrial outer membrane permeabilization, induction of apoptosis, modulation of oxidative stress, amelioration of inflammation, modulation of insulin signaling, and inhibition of angiogenesis[37].

The effect of honey was also investigated in pancreatic cancer. Caffeic acid phenethyl ester induced apoptosis in human pancreatic cancer cells by activation of caspase-3/caspase-7 and mitochondrial dysfunction[93]. Treatment with CAPE slightly restored the expression of E-cadherin and markedly reversed the transforming growth factor-β-induced overexpression of vimentin at 24 h in Human pancreatic cancer cells (PANC-1) cells. CAPE suppressed the expression of Twist 2 and growth of PANC-1 xenografts without significant toxicity in an orthotopic pancreatic cancer model. These data suggest that CAPE could suppress the epithelial-mesenchymal transition in pancreatic cancer[94].

CONCLUSION

Natural honey has many therapeutic benefits and medical uses. The different effects of natural honey including anti-inflammatory antioxidants and anticancer effects highlight its importance in the prevention of gastrointestinal cancers and improvement of cancer therapies. Some evidence of the anticancer effects of honey has been reported from *in-vitro* and *in-vivo* studies in gastrointestinal cancers, however, further investigation of anticancer effects of honey in animal and clinical studies are required to prove its therapeutic efficacy in chemoprevention strategies.

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