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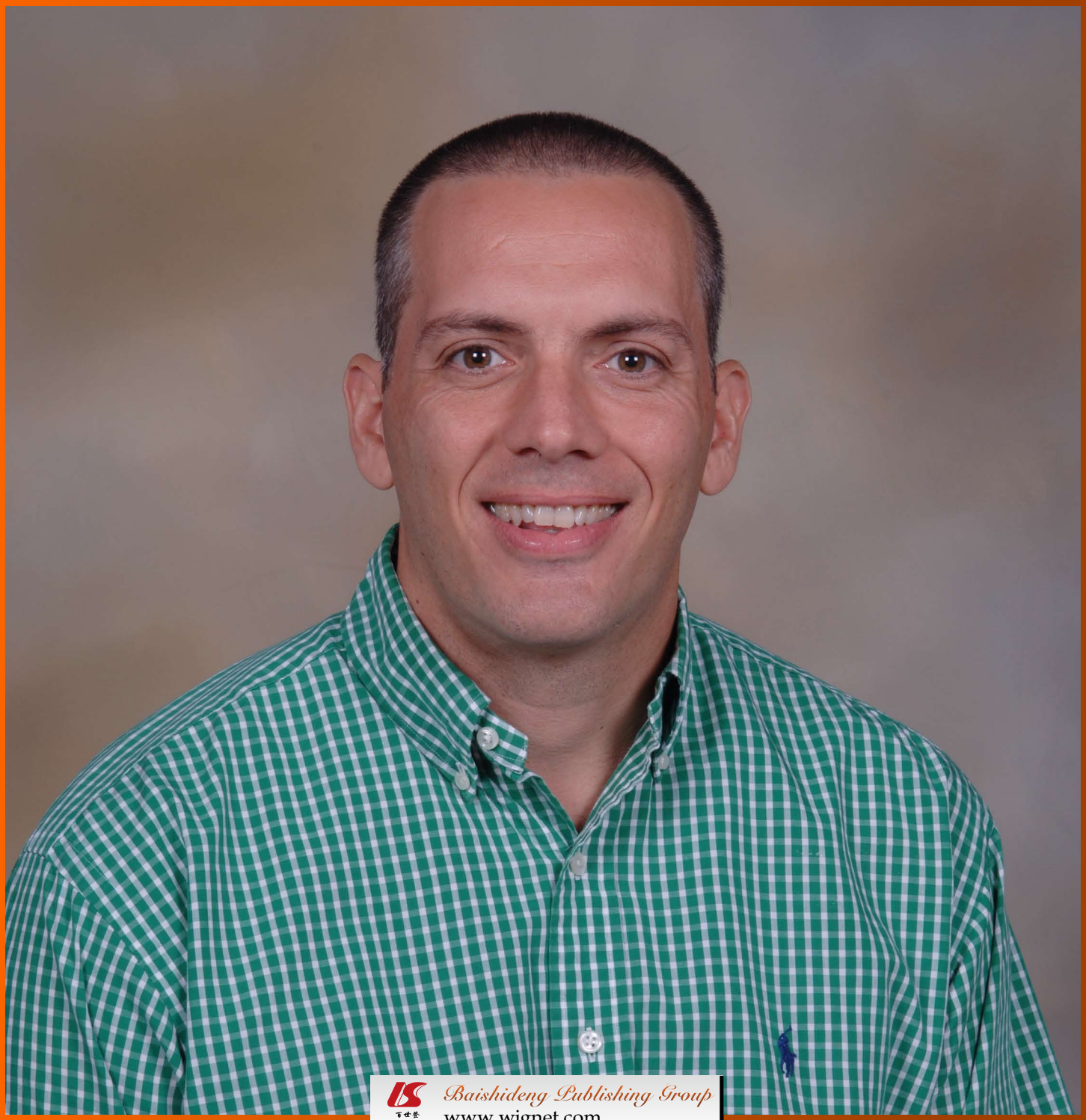

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REVIEW

Sonoporation: Gene transfer using ultrasound

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Abstract

Genes can be transferred using viral or non-viral vectors. Non-viral methods that use plasmid DNA and short interference RNA (siRNA) have advantages, such as low immunogenicity and low likelihood of genomic integration in the host, when compared to viral methods. Non-viral methods have potential merit, but their gene transfer efficiency is not satisfactory. Therefore, new methods should be developed. Low-frequency ultrasound irradiation causes mechanical perturbation of the cell membrane, allowing the uptake of large molecules in the vicinity of the cavitation bubbles. The collapse of these bubbles generates small transient holes in the cell membrane and induces transient membrane permeabilization. This formation of small pores in the cell membrane using ultrasound allows the transfer of DNA/RNA into the cell. This phenomenon is known as sonoporation and is a gene delivery method that shows great promise as a potential new approach in gene therapy. Microbubbles lower the threshold of cavity formation. Complexes of therapeutic genes and microbubbles improve the transfer efficiency of genes. Diagnostic ultrasound is potentially a suitable sonoporator because it allows the real-time monitoring of irradiated fields.

Key words: Gene therapy; Cavity; Microbubbles; Contrast agent; Diagnostic ultrasound

Core tip: Ultrasound causes cavitation bubbles to form cell membrane pores through which DNA/RNA are transferred. This phenomenon is known as sonoporation. Microbubbles lower the threshold of cavity formation. Sonoporation is less toxic and not associated with tumorigenicity as compared with retroviral and adenoviral vectors. Sonoporation does not require surgical procedure and enhances gene transfer with lipofection. Current limitations of sonoporation are low efficiency of gene transfer and damage of target cells are The use of complexes with chemicals and diagnostic ultrasound are promising approaches to overcome these limitations.

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INTRODUCTION

Gene therapy is a promising approach to treat diseases

and is applicable to tissue engineering controlling differentiation of cells to form tissues[1,2]. Therapeutic genes are transferred using viral or non-viral vectors. Viral vectors are mainly retroviral vectors, adenoviral vectors and adeno-associated vectors. Retroviral vectors are the cause of tumorigenicity[3]. Adenoviral vectors provoke a severe systemic immune response[4]. Adeno-associated vectors do not cause major immune response, but they are not permissive to some types of cells[5]. On the other hand, plasmid DNA does not induce host immune responses because exogenous proteins such as viral capsid proteins are not produced[6]. Moreover, plasmid DNA rarely integrates with the host genome upon introduction into target cells and not associated with tumorigenicity[3,7]. Plasmid DNA is safe for gene transfer therapy, but its transfer efficiency is low. Methods should therefore be developed to improve transfection efficiency of plasmid DNA. Irradiation with low-output intensity ultrasound causes mechanical perturbation in the vicinity of cavitation bubbles. Collapse of the bubbles generates small transient holes in the cell membrane and induces cell membrane permeabilization. The membrane poration (cavitation) increases the efficiency of drug and gene delivery. This phenomenon, sonoporation, is a gene delivery technique that could potentially be used for gene therapy. Sonoporation, a method for targeted drug delivery and non-viral gene transfection, has new and advantageous possibilities. Sonoporation stimulates endocytosis of adno-associated virus and enhances efficiency of gene transfer[8,9]. However, no clinical trials using sonoporation have been reported to date because it is not yet a satisfactory technique for efficient and reliable gene transfer. In this chapter, we will review the potential application of sonoporation in gene therapy, with a focus on mircobubbles as a drug delivery agent. We will also discuss other non-viral delivery methods. Finally, we will outline the future direction of ultrasound-assisted gene delivery aiming at improvement of sonoporation and enhancement of gene expression for clinical applications.

NON-VIRAL GENE DELIVERY

In this section, we review non-viral gene delivery methods. Gene therapy requires the delivery of nucleic acid material to target tissues and its entry into target cells. Given that DNA and the cell membrane are both negatively charged, electrostatic forces result in the repulsion of DNA by the cell membrane. To overcome this limitation, a physical or chemical approach needs to be applied. Another restriction of gene transfer is the rapid degradation of DNA by nucleases in the plasma after systemic administration.

Electroporation

Electroporation is useful for gene transfer to primary cells. Primary cells are not introduced with genes with lipofection. Electroporation is the only method to introduce genes to cells, auch as normal human dermal fibroblasts[10]. Electroporation, in which high-voltage electrical currents create transient pores on the cell membranes, allows the transport of nucleic acid material into the cell[11]. Therapeutic genes are successfully transferred *in vivo*[12]. The electroporation method, however, has limitations such as short range of gene transfer, necessity of a surgical procedure, and tissue damage. Distance between electrodes normally requires 1 cm and is not suitable for a large area. Placement of internal electrodes requires a surgical procedure, and high voltage can damage tissue when applied. Therefore, less invasive methods are desirable.

Lipofection

Chemical non-viral vectors have been studied because they are generally considered safer than viral vectors. In addition to safety, liposomes can transfer larger genes with less toxicity and are relatively easy to prepare. Cationic lipids and polymers form complexes with negatively charged DNA. The complexes protect the DNA from nucleases and increase the effectiveness of transfection through the cell membrane. One major problem with lipofection is low efficiency of gene transfer. Transfection efficiency of lipofection is improved by condensing DNA with a double chain monovalent quaternary ammonium lipid[13]. Still genes are not introduced to target cells efficiently. More efficient methods have been waited.

Sonoporation

Sonoporation refers to the formation of small pores in cell membranes by using ultrasound for the transfer of nucleic acid materials (Figure 1). The biological effects of ultrasound are categorized as thermal and non-thermal. Non-thermal effects are composed of mechanical perturbation in the vicinity of bubbles. Cavitation bubbles cause membrane poration[14]. High speed camera images reveal that the cell membrane is fractionated, and cavitation bubbles are formed[15]. The cavitation bubbles induce cell death or permeability to allow the entry of a drug or genes into the cells. Sonoporation is similar to electroporation, wherein DNA is driven by an electrical force along the electric field. Sonoporation is mediated by passive diffusion. The transfer efficiency depends on ultrasound frequency and intensity[16]. The major advantages of sonoporation are its non-invasiveness and ability to transfer genes to internal organs without a surgical procedure[17]. Targeted gene transfer can be facilitated by ultrasound irradiation of selected tissues after systemic administration[18].

SONOPORATION

Emergence of sonoporation

Drug delivery with ultrasound was first reported by Tachibana *et al*[16]. The delivery of insulin on the skin surface when exposed to ultrasound energy in the range of 3000-5000 Pa or 5000-8000 Pa at 48 kHz for 5 min decreased blood glucose levels to 22.4% of the control

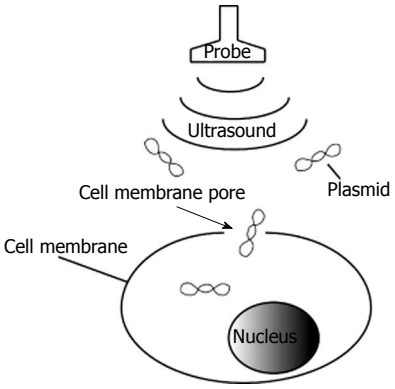

Figure 1 Formation of cell membrane pores after ultrasound irradiation. Nucleic acid such as plasmids enters the cells through the membrane pores that are formed with ultrasound.

in 120 min. It was postulated that insulin absorption increased with ultrasound vibration after intradermal injection. This report is interesting because it demonstrates the potential of ultrasound as a method to improve absorption of therapeutic materials. With ultrasound, chemotherapeutic agents are more efficiently absorbed in the mouse xenograft model of cancer[19].

In vitro experiments are simplified models to investigate the mechanisms of sonoporation *in vivo*. Fechheimer *et al*[20] transferred plasmid DNA encoding the G418 resistance gene to cultured mouse fibroblasts by sonoporation. Colonies were observed after G418 was added to the media. This was the first report demonstrating that plasmid DNA can be transferred to cells *in vitro* by using ultrasound. Kim *et al*[21] also reported that rat joint cells can be successfully transfected with plasmid DNA. Plasmid DNA encoding green fluorescent protein (GFP) was injected as a reporter of expression into the left ventricle of mice percutaneously[22]. Mice were irradiated with transthoracic ultrasound at 1 MHz for 1 min. Histological examination showed GFP expression in the subendocardial myocardium. Intraventricular co-injection of siRNA and GFP exhibited reduced expression of GFP in the coronary artery. These data indicate that plasmid DNA and siRNA can be introduced into cells *in vivo* by sonoporation.

Mechanism of sonoporation

Biophysical effects of ultrasound include cavitation, radiation pressure, and microstreaming[23]. Cavitation refers to the growth and collapse of microbubbles. Radiation pressure is the force in the irradiation field. Microstreaming is the shear forces that exist near the microbubbles. Formation of cavitation increases with a rising ultrasound intensity while the frequency decreases[24]. Mechanical index (MI) is defined as PRP/$\sqrt{F0}$ (PRP: peak rarefactional pressure; $\sqrt{F0}$: transmission center frequency)[25]. MI should be lower than 1.9 for clinical use of ultrasound. Forsberg *et al*[26] reported that cavitation is unlikely to occur at an MI of less than 0.7. Cavitation has been well investigated with ultrasound contrast agents because microbubbles lower the threshold of cavitation[27]. Zhou *et al*[28] developed a device to observe the behavior of a single bubble near the cell membrane. The motion of a single bubble was monitored with a high-speed camera. Cell membrane disruption was assessed by monitoring the transmembrane current. This study showed that a single microbubble expands and contracts with ultrasound irradiation. When the bubble collapses, the cell membrane is ruptured and a pore is generated. Changes in membrane permeability are directly correlated with the formation of pores. Carugo *et al*[29] reported that a standing wave is involved in sonoporation in the absence of microbubbles.

Microbubbles

The presence of microbubbles reduces the threshold of cavitation. Microbubble contrast agents are spheres filled with gas and stabilized with shells. Their size ranges between 1 and 10 μm[30]. They are small enough to circulate in blood vessels, but do not exit from the vessels. The contrast agents scatter ultrasound stronger than the surrounding blood and tissue. Thus, they are used as contrast agents for daily clinical practice. Tachibana *et al*[31] reported that albumin microbubbles (Albumex) accelerate thrombolysis by ultrasound. This was the first report that microbubbles improve the effects of ultrasound in tissues for purposes other than diagnostic imaging. Transfection of plasmid DNA into rat joint cells was improved in the presence of Albumex[21]. This report paved the way for the utilization of ultrasound contrast agents in sonoporation. Microbubbles expand and contract in response to compression and rarefaction of ultrasound (Figure 2). The microbubbles collapse at the high-pressure phase, emitting shock waves that perturb the cell membrane and increase permeability. Qiu *et al*[32] reported that pores on the cell membrane, generated by sonoporation with microbubbles, ranged from 100 nm to 1.25 μm in size. Their experiments used 1 MHz ultrasound at low acoustic pressures from 0.05 to 0.3 MPa. The pores generated with sonoporation were examined by scanning electron microscopy. The size of the pores enlarged with increased acoustic pressure or longer treatment. They concluded that the pores formed with shear stress. Liquid microjet, visualized with a high-speed camera in cultured cells exposed to single-shot short-pulsed ultrasound has been demonstrated[33]. Contrast agents are shells containing gas. Ultrasound scatters on the surface of contrast agents, and are visible as high echo on the display of diagnostic ultrasound. Physical and biological characteristics of contrast agents are basically the same as those of microbubbles. When a contrast agent (Levovist) was added to the media, the jet caused cell membrane damage. Interestingly, the cell membrane repair process suggests that the Ca^{2+}-independent and Ca^{2+}-trigger mechanisms are involved in rapid resealing. Positively charged microbubbles are more efficient in gene transfer than neutral ones[34]. It is hypothesized that positively charged microbubbles are more close to the cell membrane to enable more efficient gene transfer. Plasmids are

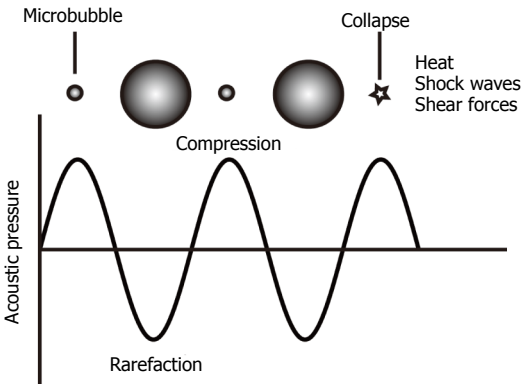

Figure 2 Microbubble response to an ultrasonic pressure wave. Microbubbles expand and contract when exposed to ultrasound at rarefaction and compression, respectively. At high pressure, microbubbles collapse and a shock wave is emitted.

introduced into cells *in vitro* and *in vivo* with sonoporation with microbubbles more efficiently than liposome that is commonly used for transfection[35,36].

Clinical trials

To date, no clinical trials have been reported using sonoporation. There are several reasons for this, including low transfer efficiency and difficulty in monitoring irradiated fields using sonoporators.

Limitations

Sonoporation harbors complicated aspects. Gene transfer efficiency is low with sonoporation. 37.5-50 μg of plasmid should be applied to a single rat with sonoporation *in vivo*[34]. This limitation is one of the reasons that sonoporation has not been applied clinically as mentioned above. Another limitation is cell damage caused with sonoporation. Miller *et al*[37] irradiated cells with 2.25 MHz continuous ultrasound for 1 min. When an ultrasound contrast agent (Definity, a perflutren lipid microsphere injectable suspension; Bristol Myers Squibb Medical Imaging, N. Billerica, MA) was added to the culture media, the irradiated cells underwent apoptosis. Enzymatic activity and mitochondrial membrane are changed after sonoporation[38]. Stresses to endoplasmic reticulum and mitochondria trigger apoptosis[39,40]. Sonoporation delays DNA synthesis to arrest cell cycle[41]. It should be noted that sonoporation itself may cause apoptosis when it is applied to cancer with therapeutic genes[37].

FUTURE PROSPECTS

Microbubbles are expected to improve gene transfer at lower MI for the safety of patients; therefore, microbubbles may be suitable for sonoporation in human trials. Microbubbles are destroyed more efficiently with decreased pulse frequency and increased acoustic pressure and pulse length[42]. Care should be taken when applying *in vitro* data to *in vivo* studies because the effects of MI on microbubble destruction might not be similar[43]. Standing wave may be another option to improve sonoporation. Real-time monitoring of the irradiated field is desirable to introduce therapeutic genes to target tissues. Diagnostic ultrasound could therefore be used as a sonoporator.

Cationic microbubbles

Wang *et al*[27] analyzed the protection of plasmid DNA xenograft tumors in mouse hind limb after intravenous administration of a complex of plasmid DNA (luciferase) with cationic or neutral microbubbles. Luciferase activity was 3.8-fold stronger in complexes with cationic microbubbles than in those with neutral microbubbles. Factors influencing the transfection efficiency were analyzed, and the superiority of cationic microbubbles was more evident at lower doses of microbubbles and plasmid DNA[34]. This strategy appears suitable for this application because nucleic acids are negatively charged. It is expected that plasmid DNA or siRNA will interact and form better complexes with cationic microbubbles than with neutral microbubbles.

Liposomal bubbles

It is difficult to modify surfaces of microbubbles with functional molecules for targeting. On the other hand, liposomes are easy to modify for targeting. Suzuki *et al*[44] developed polyethylene glycol-modified liposomes containing perfluoropropane, a contrast agent for ultrasound imaging. These so-called "bubble liposomes", significantly improved the transfection efficiency of plasmid DNA encoding luciferase into cultured cells. The luciferase reporter plasmid was injected into the femoral artery of mice. Two days after ultrasound irradiation, a signal was detected along the artery. These data suggest that the bubble liposome is a candidate for gene delivery both *in vitro* and *in vivo*. Bubble liposomes were transferred with the interleukin-12 gene in xenograft ovarian cancer mice, and the tumor sizes were reduced[45]. These animal experiments demonstrate that bubble liposomes may be applicable to clinical studies.

Diagnostic ultrasound

Sonoporators are typically used for sonoporation. One of the problems with sonoporators is that the irradiation field cannot be monitored. Diagnostic ultrasound is widely used in daily clinical practice. Diagnostic ultrasound is equipped with a display for image diagnosis. If diagnostic ultrasound could be utilized for sonoporation, therapeutic genes can be accurately introduced into the target fields. Miller and Quddus transfected cultured cells with plasmid DNA using diagnostic ultrasound[46]. They used a 3.5 MHz curved linear transducer of diagnostic ultrasound. A contrast agent of ultrasound (Optison) was added to the media. Although Optison may improve transfection efficiency, even without contrast agents, plasmid DNA was successfully introduced into cultured cells by using diagnostic ultrasound[47]. SiRNA of frizzled (Fz)-9, a receptor of the Wnt signaling pathway, transferred into cultured cells using diagnostic ultrasound sup-

presses cell proliferation[48]. SiRNA of Fz-9 transferred into cultured cells using lipofection also suppresses cell proliferation[49]. These 2 papers indicate that transfection efficiency of siRNA with diagnostic ultrasound is comparable with that of lipofection. Wang *et al*[27] investigated cell permeability of Evans Blue *in vivo* with diagnostic ultrasound. They observed the dye in xenograft hepatoma. Interestingly, they reported that efficiency of Evans Blue transfer was affected by MI sonication duration and dye dose. These data clearly demonstrate that ultrasound causes sonoporation, a biological process that is a promising new approach for the delivery of DNA/RNA for gene therapy.

CONCLUSION

Sonoporation is able to introduce plasmids to cells. Sonoporation is less toxic method of gene transfer as compared with retro viral vectors and adenoviral vectors because plasmids hardly causes immune response and are not associated with tumorigenicity. Sonoporation does not require surgical procedure and enhances gene transfer with lipofection. Current limitations of sonoporation are low efficiency of gene transfer and damage of target cells are the use of complexes with chemicals and diagnostic ultrasound are promising approaches to overcome these limitations.

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An overview of translational (radio)pharmaceutical research related to certain oncological and non-oncological applications

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Abstract

Translational medicine pursues the conversion of scientific discovery into human health improvement. It aims to establish strategies for diagnosis and treatment of diseases. Cancer treatment is difficult. Radiopharmaceutical research has played an important role in multiple disciplines, particularly in translational oncology. Based on the natural phenomenon of necrosis avidity, OncoCiDia has emerged as a novel generic approach for treating solid malignancies. Under this systemic dual targeting strategy, a vascular disrupting agent first selectively causes massive tumor necrosis that is followed by iodine-131 labeled-hypericin (^{123}I-Hyp), a necrosis-avid compound that kills the residual cancer cells by crossfire effect of beta radiation. In this review, by emphasizing the potential clinical applicability of OncoCiDia, we summarize our research activities including optimization of radioiodinated hypericin Hyp preparations and recent studies on the biodistribution, dosimetry, pharmacokinetic and, chemical and radiochemical toxicities of the preparations. Myocardial infarction is a global health problem. Although cardiac scintigraphy using radioactive perfusion tracers is used in the assessment of myocardial viability, searching for diagnostic imaging agents with authentic necrosis avidity is pursued. Therefore, a comparative study on the biological profiles of the necrosis avid ^{123}I-Hyp and the commercially available ^{99m}Tc-Sestamibi was conducted and the results are demonstrated. Cholelithiasis or gallstone disease may cause gallbladder inflammation, infection and other severe complications. While studying the mechanisms underlying the necrosis avidity of Hyp and derivatives, their naturally occurring fluorophore property was exploited for targeting cholesterol as a main component of gallstones. The usefulness of Hyp as an optical imaging agent for cholelithiasis was studied and the results are presented. Multiple uses of automatic contrast injectors may reduce costs and save resources. However, cross-contaminations with blood-borne pathogens of infectious diseases may occur. We developed a radioactive method for safety evaluation of a new replaceable patient-delivery system. By mimicking pathogens with a radiotracer, we assessed the feasibility of using the system repeatedly without septic risks. This overview is deemed to be interesting to those involved

in the related fields for translational research.

Key words: Translational medical research; Cancer treatment; OncoCiDia; Vascular disrupting agent; Hypericin; Myocardial infarction; Gallstone; Transflux

Core tip: Translational medicine converts scientific discovery into clinical applications. Radiopharmacy has played a multidisciplinary role. Based on unique necrosis avidity, OncoCiDia presents a generic approach for management of cancers, on which recent results on its optimization are summarized. Myocardial infarction is a clinical problem. A comparative study between infarct avid iodine-131 labeled-hypericin and commercial ^{99m}Tc-Sestamibi is presented. Cholelithiasis may cause biliary complications. The usefulness of Hyp as an optical imaging agent for cholelithiasis is demonstrated. Multiple uses of automatic contrast injectors may reduce costs but can cause cross-contaminations. We developed a radioactive method for safety evaluation of a new replaceable patient-delivery system.

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INTRODUCTION

Translational medicine refers to the creativity of joining knowledge from "bench to bedside" or from laboratory experiments to clinical trials for producing new drugs, devices, diagnostic and therapeutic options for patients. Hence, translational research is identified as a crucial interface between basic science and clinical medicine. It intends to discover better ways to solve real practical problems enhancing human health and well-being. Another current trend in translational medical research is to hybrid diagnosis and therapy into a combined approach as newly termed a "theragnostic" modality.

In the area of cancer therapeutics, transforming basic research results into clinical practice is becoming increasingly important. Cancer is one of the leading causes of mortality worldwide and little progress has been achieved in treating most of the solid tumors, which could be resistant to common therapies. Based on necrosis-avidity, OncoCiDia is a generic and unconventional theragnostic strategy recently introduced as a complementary modality to improve cancer treatability[1,2]. Unlike other cancer therapies directly attacking multimutant and refractory cancer cells, OncoCiDia may selectively treat solid malignancies by massively necrotizing the tumors plus radioactively cleansing their microenvironments, meanwhile the tumors under treatment can be visualized by nuclear scintigraphy. Thus, a dedicated acronym OncoCiDia is created to portray this cancer (Onco) management approach with both tumoricidal (Ci) and diagnostic (Dia) effects. It consists of two sequential complementary treatments involving the intravenous application of a vascular disrupting agent (VDA) followed by systemic targeted radiotherapy (STR) using a potent necrosis-avid compound iodine-131 labeled-monoiodohypericin (^{131}I-Hyp). Furthermore, to make this novel anticancer strategy clinically applicable, optimizations of the procedures for labeling, purification and formulation of the radioiodinated hypericin (Hyp) have been performed and the outcomes are summarized and commented in this overview article. Results on biodistribution, dosimetry, pharmacokinetic and, chemical and radiochemical toxicities of OncoCiDia have been also presented, which hopefully may boost the advance of this strategy into the clinic.

Myocardial infarction constitutes a health problem with large morbidity, mortality and economic burden[3]. Nuclear imaging based on myocardial perfusion tracers, which distribute in proportion to the regional myocardial blood flow, has played an important role in the assessment of tissue viability[4,5]. However, the development of diagnostic imaging agents with authentic specific avidity for necrosis has been a desired goal due to the numerous utilities they may offer. In earlier studies, the necrosis avid ^{123}I-Hyp has shown its potential usefulness as a diagnostic cardiac agent due to its notable uptake in necrotic tissues[6,7]. In a more recent experiment, its biodistribution and targetability have been compared to those of the commercially available myocardial perfusion tracer ^{99m}Tc-Sestamibi and the results have been herein summarized.

Cholelithiasis is the medical term for gallstone disease. Gallstones are hard, rock-like collections, mainly from cholesterol and bile salts that build up in the gallbladder or bile duct. Eventually, they can cause gallbladder inflammation resulting in pain, jaundice, infection and other serious complications. Because the high affinity between cholesterol and the naturally occurring fluorophore hypericin has been reported[8,9], a preliminary *in vitro* study for assessing the potential suitability of Hyp as an optical diagnostic imaging agent in patients with gallstones was performed and the results are presented in this work.

Multiple uses of automatic contrast injection systems during imaging procedures can reduce costs and save resources. However potential outbreak associated with cross-contaminations with blood-borne pathogens of infectious diseases through the contrast medium may occur. The Transflux contrast delivery system is a simple tube delimited by two one-way valves intended to deliver contrast media from a reservoir to the patient and with the need to only change the tubing in direct contact with the patient blood. It incorporates a safety zone and a one-way valve in the patient line that allow the delivery system and the vein to be flushed and the blood reflux to be prevented. By mimicking microbial pathogens

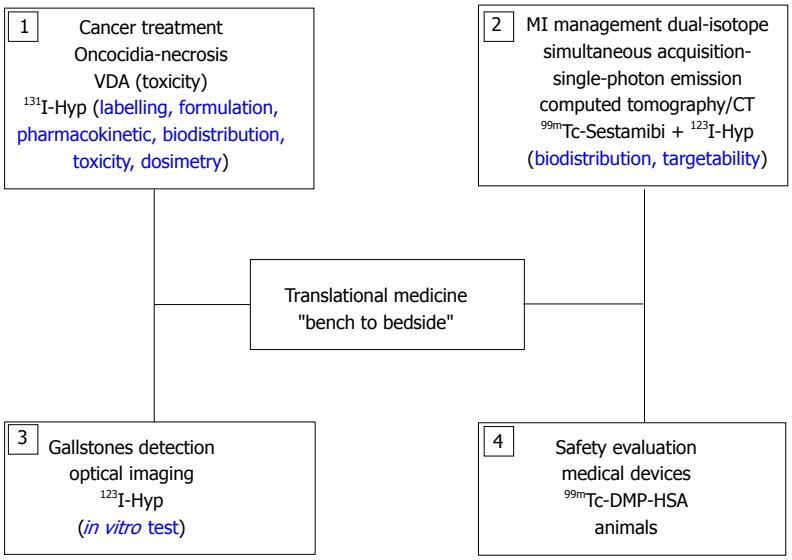

Figure 1 Schematic structure of the translational research activities covered by the overview article. ^{99m}Tc-sestamibi: Technetium-99m labeled-hexakis (2-methoxyisobutylisonitrile); ^{99m}TcDMP-HSA: Technetium-99m dimercaptopropionyl human serum albumin; $^{123/131}$I-Hyp: Iodine-123/iodine-131 labeled monoiodohypericin; MI: Myocardial infarction; VDA: Vascular disrupting agent.

with a particulate radiotracer, we developed a radioactive method for quantitative safety evaluation of this new replaceable patient-delivery system and the main findings are reported here.

This overview paper is in the framework of the full 4-year doctoral training, in which translational research has been attempted as a key component for resolving important problems in diverse medical fields (Figure 1).

APPLICATION OF TRANSLATIONAL RESEARCH IN THERAGNOSTIC ONCOLOGY

Background

Cancer is a complex group of malignant diseases influenced by genetic and environmental factors. Over the past decades, the incidence and prevalence of cancer have raised with an overall estimation of about 20 million new cases by 2030[10]. The costs associated with cancer diagnosis, therapy, and follow-up have drastically soared. Conventional therapies including surgery, chemotherapy and external beam radiotherapy are often ineffective for treating resistant and disseminated solid malignancies. Novel and cost-effective approaches, once available, are essential to improve cancer treatability and curability.

STR is a radiotherapy that makes use of systemically administered radioactive compounds for delivering lethal radiation doses to the tumor while preserving normal tissues. Several radioactive agents have been clinically used, for instance, radioiodine for thyroid cancer owing to its specific uptake by thyroid glandular tissues[11]; iodine-131 metaiodobenzylguanidine for treating pheochromocytoma[12] and neuroblastoma[13]; Metastron (strontium-89 chloride) as a palliative treatment in patients with bone metastases[14]; and radioactive microspheres for radioembolization of liver cancer[15]. Anti-CD20 monoclonal antibody (MoAb) conjugated to I-131 (tositumomab, Bexxar®)[16] or yttrium-90 (ibritumomab tiuxetan, Zevalin®)[17] for treating non-Hodgkins lymphoma, and somatostatin derivatives labeled with bound indium-111, lutetium-177 or yttrium-90 for neuroendocrine tumors (NETs)[18,19] are lately introduced, constituting a step forward in tumor specific targeting.

However, most of the above-mentioned cancer types represent a small proportion among the overall cancer cases. Malignant solid tumors, which represent the major cancer incidence worldwide, have been difficult to treat due to their histological diversity, disorganized angiogenesis and unpredictable mutations. Once carcinogenesis is established, tumor cells become resistant to therapies due to the multiple escape mechanisms facilitated by intrinsic mutations and/or overlapping molecular pathways. Even if a proper radioactive MoAb is chosen, in most of the cases, only small amounts of injected dose (0.001%-0.1% /g) could accumulate in the tumor[20]. Low absorbed doses (1500 cGy) are subsequently reached in cancer cells that are much lower than the usually required doses (5000 cGy) for getting therapeutic responses[21,22]. With somatostatin derivatives-based radiopharmaceuticals characterized by high affinity for distinct receptors overexpressed in the tumor, short-term accumulation in the tumor and retention in normal tissues have also been reported[23]. Therefore, necrosis as a generic alternative target has been utilized for potential theragnostic applications (Figure 2).

Tumor necrosis treatment

Rather than hitting cancer cells undergoing numerous mutations[24,25] that cause uncontrollable growth and escape from annihilation, leading to post-therapeutic cancer resistant clones[24], an innovative anticancer approach called tumor necrosis treatment (TNT) was introduced[26,27]. Since the proportion of dead tissue in fast-growing tumors can be more than 50% of the total cancer volume due to tumor vascular deformation or insufficient blood supply[28,29], necrosis could become a generic target in almost all solid tumors. TNT approach uses radiolabeled MoAbs that spare normal tissue and target naturally occurring intracellular antigens (a complex of double-stranded DNA and histone H1-antigens)

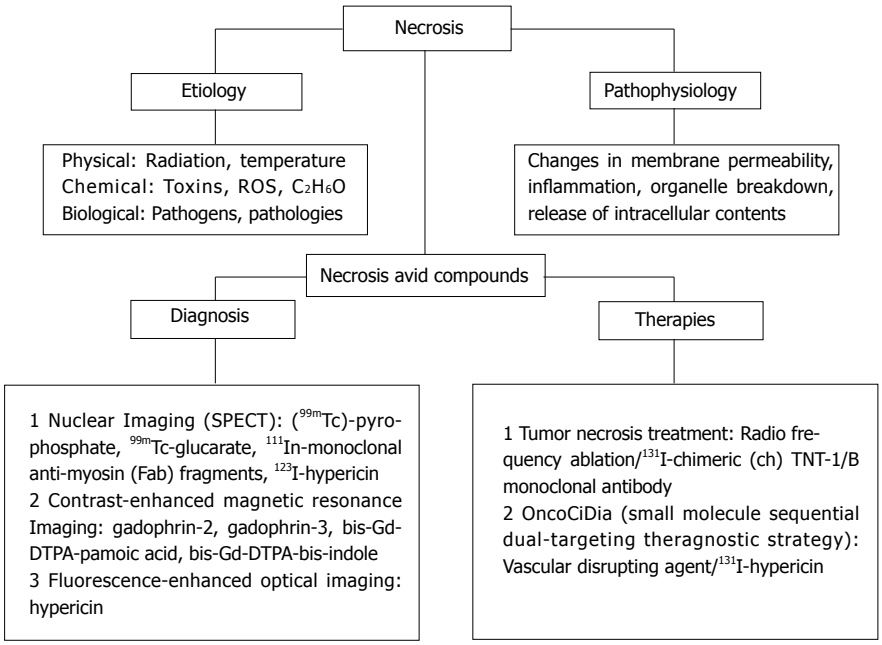

Figure 2 Flow diagram of the included research topics. ^{99m}Tc: Technetium-99m; ^{111}In: indium-111; ^{123}I: Iodine-123; ^{131}I: Iodine-131; C_2H_6O: Ethanol; DTPA: Diethylene triamine pentaacetic acid; Fab: Antigen-binding fragment; Gd: Gadolinium; ROS: Reactive oxygen species; SPECT: Single photon emission computed tomography; TNT: Tumor necrosis treatment.

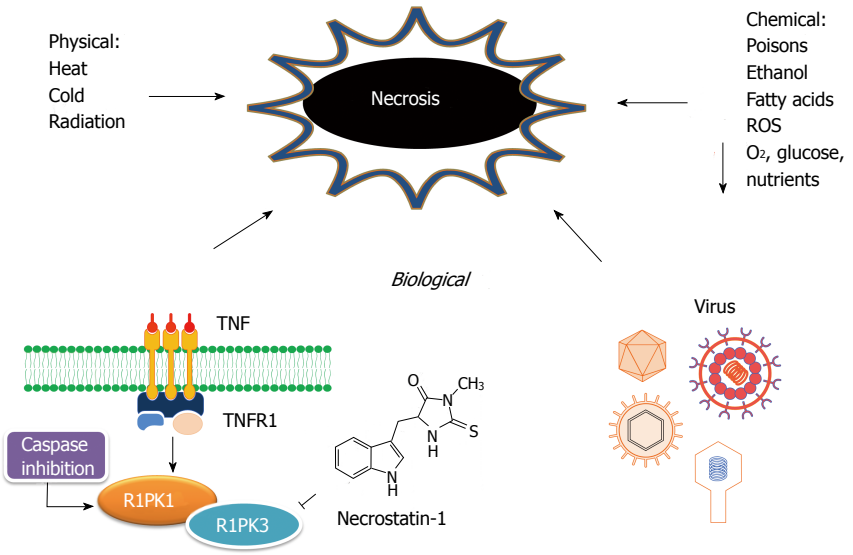

Figure 3 Schematic representation of the etiology of necrosis. ROS: Reactive oxygen species; TNT: Tumor necrosis treatment; RIPK1/3: Receptor-interacting protein kinase 1 and 3; TNFR1: Tumor-necrosis factor receptors 1.

present throughout tumor necrosis[30,31]. Unlike conventional STR, it is a crossfire-dose therapeutic modality, in which the radiation dose deposited to cancer cells only comes from radionuclides on surrounding necrosis.

Definition, etiology and pathophysiology of necrosis: The term of necrosis is originated from the Greek prefix "necros", meaning "dead". It constitutes an irreversible process or "no return" status in the cell life[32]. Cell death by necrosis has been historically stereotyped as an unregulated process[33]. Any severe lesions caused by physical stresses, toxins, infections or genetically programmed injuries if reaching a certain degree and receiving no intervention may alter physiological homeostasis, eventually leading to tissue or organ necrosis[34]. However, it turns increasingly evident that the multi-pathway cell-death program apoptosis may not be the only cellular mechanism involved in regulating cell death[35]. Programmed necrosis or necroptosis has emerged as a specialized biochemical mechanism that can be induced by different stimuli such as tumor-necrosis factor receptors (TNFR1, TNFR2)[36], inactivation of cysteine-aspartic acid proteases (caspase)[37] and caspase-8 mutations[38]. It becomes clear that necroptosis could be regulated by the kinase receptor-interacting protein 1 (RIPK1), which constitutes the molecular target of necrostatins, an emerging class of cytoprotective drugs inhibiting specifically necroptotic cells[37]. Recently, the kinase activity of RIPK3, a family member of RIPK1, has also been related to programmed necrosis[39] (Figure 3).

Necrosis is commonly believed to be a passive process since it involves no protein production, is not restrained by any homeostatic mechanisms, and includes almost negligible energy requirements. The necrotic cells can no longer retain the integrity of the cell or cytoorganelle membranes and perform inherent functions. Af-

ter losing the ability to maintain homeostasis, biological fluids from the blood cross the damaged cell membrane and enter the intracellular space, leading to organelles enlargement, production of toxins and activating enzymes associated to the degradation of cellular life molecules. The swollen organelles become nonfunctional, ceasing the synthesis of proteins and ATP. The mitochondrial swelling causes cytolysis and the debris is discharged into the surroundings, which triggers tissue inflammation regulated by small proteins-cytokines, reactive oxygen species and certain immune system cells[40]. The organism interprets the presence of the debris as signal of tissue injury and reacts to defend itself. In response, immune system cells migrate into the site of damage and combat the supposed invading microorganisms[40]. Dissociation of ribosomes from the endoplasmic reticulum and nucleus disintegration with chromatin condensation take place in turn[41]. The dead cells eventually fade away because of the combination of enzymatic denaturation and fragmentation process, followed by polymorphonuclear leukocyte phagocytosis of solid particles[42].

Preclinical studies and clinical trials on TNT: In a pioneer pre-clinical study conducted on a ME-180 human cervical carcinoma model with ^{131}I-labeled TNT-1 MoAb, Chen *et al*[31] proved the effective and preferential targeting of this radiotherapeutics within the tumor, which established the potential clinical usefulness of TNT. To date, about 200 patients have been treated with TNT worldwide. A phase I study of ^{131}I-chimeric(ch) TNT-1/B MoAb for the treatment of advanced colon cancer was performed. The infusion of ^{131}I-chTNT-1/B MoAb was well tolerated and showed no significant non-hematologic effects. Based on tumor cross-product response criteria, however, none of the patients exhibited complete or partial response[43]. Phase I and Ⅱ trials of convection-enhanced delivery of ^{131}I-chTNT-1/B MoAb were conducted on patients with high-grade adult gliomas, showing promising therapeutic outcomes[44]. Similar results were found in a pivotal study in patients with advanced lung cancer treated with ^{131}I-chTNT-1/B MoAB[45]. More recently, genetically engineered Fab' and F(ab') 2 constructs of chimeric TNT (chTNT)-3 antibody labeled with indium-111 were prepared and preclinically evaluated. The conjugates showed faster body clearance, better biodistribution but lower tumor uptake than the parental ^{111}In-labeled chTNT-3 in tumor-bearing mice[46].

To increase the amount of MoAb binding-necrotic sites in the tumor, necrosis-inducing treatments (NITs) such as radiofrequency ablation were also used as starting complementary techniques[47].

However, myelosuppression due to unfavorable pharmacokinetic properties of MoAbs constitutes an important dose limiting factor that prevents substantial improvement of TNT-based modality[43,44].

OncoCiDia

OncoCiDia, also known as small molecule sequential dual-targeting theragnostic strategy[1], is a novel anticancer approach with great potential for treating solid tumors. Relying on a soil-to-seeds concept, it offers a one-stop-shop for diagnostic imaging, treatment and follow-up[2]. Similar to the TNT approach, it is based on the natural phenomenon of necrosis. However, instead of using radioactive MoAb with large molecular size (150 kDa) and complex pharmacokinetics, it involves two small compounds (< 1 kDa) with pre-identified high and divert but complementary targetability. The intravenously (IV) administered VDA triggers selective tumor vascular shutdown and subsequent central necrosis. However, a viable rim of tumor cells in the periphery always exists as seeds for repopulation of cancer cells[48]. ^{131}I-Hyp is then IV injected, which preferentially localizes at the newly generated necrotic sites and acts as a cleansing shot to lethally irradiate residual tumor cells through a crossfire effect[2]. The small molecular size of ^{131}I-Hyp makes it possible to permeate fast through tissues and target less accessible sites throughout the solid tumor. This may overcome the initial barriers faced by the systemic delivery of MoAb, which limits diffusion from blood vessels and inhibits drug tumor penetration[21,49].

Vascular disrupting agents: Vascular disrupting agents (VDAs) are a novel category of potential anticancer drugs that induce tumor vascular shutdown by destroying the endothelium of tumor vasculature. It has been reported that blood vessels in tumors proliferate more rapidly than those in normal tissues[49]. Newly formed endothelial cells are more sensitive than mature ones that own a well-developed actin cytoskeleton and may retain the cell shape in spite of depolymerization of the tubulin cytoskeleton caused by the VDA[50]. After VDA administration, the occlusion of blood-supplying vessels and capillary sprouts obstructs oxygen and nutrient supply to the tumor cells, compromising cellular integrity and eventually leading to hemorrhagic tumor necrosis[51]. Different groups of VDAs have been developed, *e.g.,* tubulin-binding agents cause microtubule depolymerization by binding either the colchicine or vinblastine sites, whereas flavonoid derivatives selectively obstruct tumor-related vessels due to their indirect pharmacodynamic effects[51]. VDAs can be obtained from nature such as combretastatins (CA4P, OXi-4503, and AVE-8062), colchicines (ZD6126) and phenylahistin (NPI-2358), whilst others are synthetic compounds (DMXAA, MN-029 and EPC2407)[51].

Hyp: Hyp is a red-colored anthraquinone derivative (naphthodianthrone), which is one of the principal active compounds of the genus Hyp (Clusiaceae) comprising roughly 450 species worldwide[52]. Hyp was initially found in the dark glands of the flowering parts from Hyp perforatum L (St. John's Wort)[53], an aromatic, perennial plant. Hyp can be also obtained from fungi Dermocybe[54] or from endophytic fungi growing in different plant species[52]. However, the most commercially available Hyp

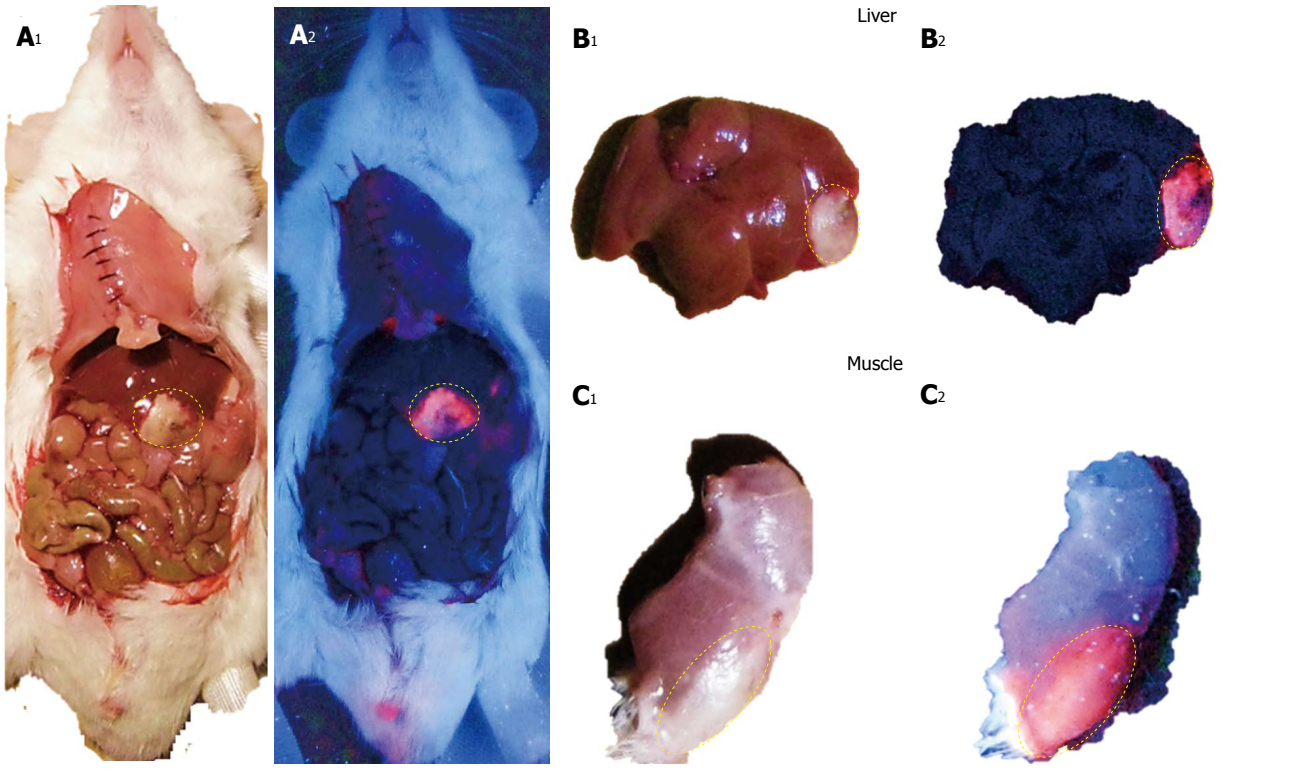

Figure 4 Macroscopic digital imaging of a mouse with acute ethanol-induced necrosis in the liver and muscle having received 5.0 mg/kg hypericin in DMSO/PEG400/water (25:60:15, v/v/v). Under normal tungsten light, viable liver, intestines and muscle show normal appearances whereas hepatic infarction and muscle necrosis appear as white cheesy tissue (A_1, B_1, C_1). With a UV light of 254 nm, a bright red fluorescence from liver (A_2, B_2) and muscle necrosis (C_2) but a lack of fluorescent signal from the liver (A_2, B_2), viable muscle (C_2) and other abdominal structures were observed (A_2). DMSO: Dimethyl sulfoxide; PEG400: Polyethylene glycol 400; UV: Ultraviolet.

compounds are synthesized.

Hyp has been considered a vinylogous carboxylic acid. Its deprotonations are likely at the phenolic hydroxyl groups at the peri- and bay-regions having different acidities. In aqueous system the bay- and peri-regions show estimated pKa values of 1.7 and 12.5[55], respectively. Hyp showed a non-planar conformation owing to the repelling interactions among the side chains of the aromatic skeleton[56]. The proximity of acidic and basic functional groups allows the formation of intramolecular hydrogen bonds, which influence the tautomeric equilibria and acid-base properties[55]. Hyp has 16 conceivable tautomers[57]. Among them, the most stable is the 7, 14-dioxoisomer[58]. Hyp dissolves in polar solvents over concentrations of 10^{-3} mol/L, producing red fluorescent solutions. It is soluble in Dimethyl sulfoxide (DMSO), ethanol, pyridine, methanol, acetone, butanone, ethyl acetate and aqueous alkaline solutions[59]. It has been found in soluble form under physiological conditions due to the complex formation with biological macromolecules, mainly low-density lipoprotein (LDL)[60].

Hyp is a natural product of pharmaceutical interest due to its ever-expanding anti-inflammatory[61], antiretroviral[62], antimicrobial[63], antitumor[64] and antidepressive[65] activities. Recently, it has been found with a highly selective affinity for necrosis[6,7] (Figure 4).

The mechanisms associated with the necrosis avidity of Hyp remain unknown and a number of hypotheses have been proposed. Hyp specifically accumulates in exposed sites of degraded life molecules in the necrotic cell debris[66]. Binding to LDL[60] and serum or interstitial albumins[67] have been put forward as potential interaction pathways. Hyp has also been found to show highly selective avidity for lipid components including cholesterol[8], phosphatidylserine and phosphatidylethanolamine[68] present in the cell membrane bilayer.

Iodine isotopes: Iodine-123 (^{123}I) is a halogen with a physical half-life of 13.1 h. It decays by electron capture to tellurium-123, emitting gamma radiation with a main energy of 159 kEV, which is exploitable for nuclear scintigraphy, biodistribution and radiodosimetry studies.

Iodine-131 (^{131}I) with a decay half-life of 8.02 d is the most common iodine radioisotope utilized in medical applications owing to its relatively easy availability and low cost. It decays by emission of beta minus electrons with a maximal energy of 606 kEV (89% abundance) and a tissue penetration of 0.6-2.0 mm[69] as well as 364 kEV gamma rays of 81% abundance. ^{131}I destroys tissue by short-range beta radiation, causing DNA damage and cell death to the cell that takes up the tracer by self-dose effect and to other cells up to several micrometers away by cross-fire effect.

Due to radioprotection reasons, ^{123}I is frequently used

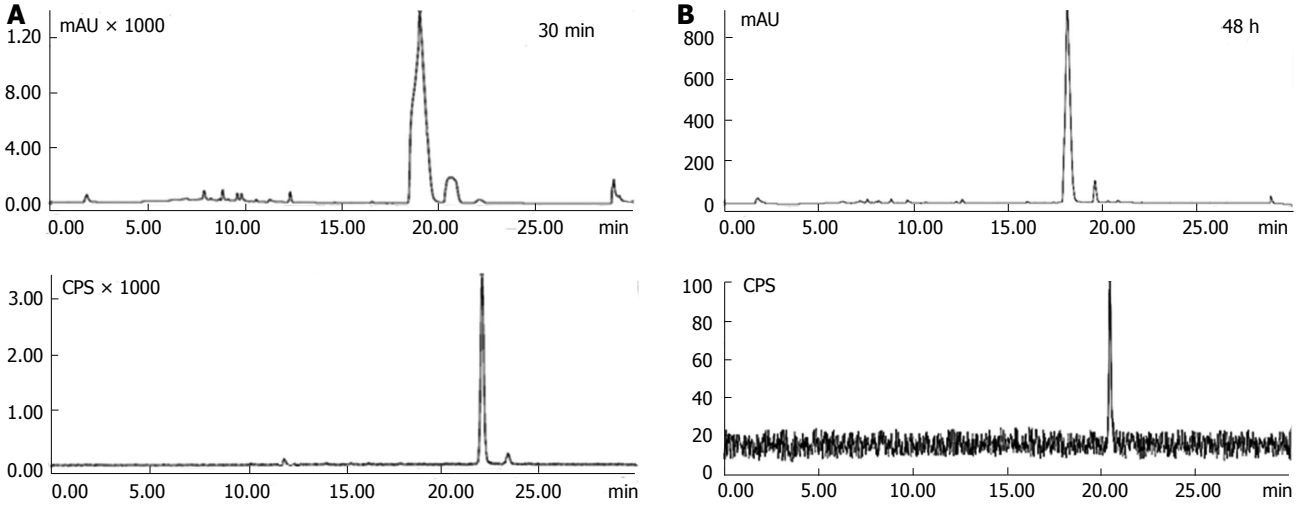

Figure 5 High performance liquid chromatographic analysis of the non-purified iodine-123 labeled hypericin with Ultraviolet (254 nm) and radiometric detection. A: Ultraviolet-chromatogram of the starting reagent hypericin (Hyp) with a retention time of 19.18 min (upper part) and radiochromatogram (lower part) of ^{123}I-Hyp eluting at 22.13 min with a mean radiochemical yield of 95.4%; B: High performance liquid chromatographic analysis of the non-purified ^{123}I-Hyp at 48 h after labeling. A single narrow peak coming out at 21.90 min (lower part) suggests the *in vitro* stability of ^{123}I-Hyp over time. CPS: Count per second.

as surrogate of ^{131}I for labeling optimization, biodistribution and dosimetry studies with the mutually interpretable outcomes.

Preparation of $^{123/131}$I -labeled-monoiodohypericin: Radioiodination *via* direct electrophilic substitution is a simple method based on *in situ* formation of positively charged iodine (I^+) using mild oxidants such as *N*-chloro para-toluenesulfonylamide (chloramine T), peracetic acid and 1,3,4,6-tetrachloro-3α, 6α-diphenyl glycoluril (Iodogen). The radioactive iodine atom in an oxidized form replaces a hydrogen atom of an activated aromatic ring. Since Hyp is a polycyclic aromatic quinone having hydroxyl substituents, it can be efficiently radioiodinated.

Two main methods for direct radiolabelling of Hyp with iodine isotopes have been described. Bormans *et al*[70] reported a radioiodination procedure of Hyp in ethanol using phosphoric acid and peracetic acid as oxidant for 30 min. On high performance liquid chromatography (HPLC), radiochemical yields ranging between 70%-97% were achieved[70]. Sun *et al*[71] described a simple method, in which Hyp in DMSO is labeled with $^{131/123}$I using iodogen as oxidant (either in a pre-coated tube or in powder form), at pH value between 6.5-7.5 for 2 to 10 min. Labeling yields higher than 99% were attained as indicated by paper chromatography (PC).

However, although PC is useful for the purpose of identification due to its convenience and simplicity, we investigated the method developed by Sun and Ni using HPLC. This technique provides high resolution and allows identifying and quantifying small amounts of substances. Labeling conditions were screened for varying reaction parameters such as Hyp mass, Hyp/iodogen molar ratio and reaction time. Stability over time of the radioactive Hyp was also checked. For radiochemical yield determination and purification, the effect of different mobile phases either in gradient or isocratic modes was studied on a quaternary HPLC system equipped with a Ultraviolet (UV) absorbance (254 nm) and radiometric detector. An XTerra® C18 column (4.6 mm × 150 mm, 5.0 μm) and a flow rate of 1.0 mL/min were used for radiochemical yield analysis. On the other hand, an XTerra® C18 semi-preparative column (10 mm × 250 mm, 10 μm) and a flow rate of 3.0 mL/min were set for purification. The peak areas of Hyp and radioiodinated Hyp were considered as response variables in this optimization test.

The preferred conditions for Hyp radioiodination were 2.0 mg Hyp in a molar ratio (Hyp: iodogen) of (3.4:1); 90/10, mL/L DMSO/50 mmol/L sodium phosphate buffer at pH 7.4 for 20 min. For radiochemical yield determination, a mobile phase consisting of acetonitrile/5 mmol/L ammonium acetate buffer pH 7.0 in gradient mode (0 min: 5:95 v/v, 25 min: 95:5 v/v, 30 min: 5:95 v/v) provided the best resolution between adjacent peaks. UV/radio-chromatograms showed unlabeled Hyp and iodine-123-labeled monoiodohypericin (^{123}I-Hyp) with t_R of 19.18 ± 0.15 min and 22.13 ± 0.05 min, respectively. Free iodide was not observed (Figure 5A). ^{123}I-Hyp was prepared with specific activity above 50 GBq/μmol in a radiochemical yield of 95.4% and remained stable over 48 h at room temperature (Figure 5B). As confirmed by mass spectrometry, the small differences between the labeling yield obtained by PC and HPLC were due to the concurrent formation of di-[(^{123}I) iodohypericin in low percentage (approximately 3%), which was detected together with mono-(^{123}I)] iodohypericin by PC.

After labeling, excessive reagents (unlabeled Hyp and iodogen) were removed by HPLC using acetonitrile/5 mmol/L ammonium acetate buffer at pH 7.0 as mobile phase in a gradient mode (0 min: 75:25, v/v, 5 min: 75:25, v/v, 30 min: 90:10, v/v). Good separation between Hyp

and radioiodinated Hyp peaks was achieved. ^{123}I-Hyp was obtained with a radiochemical purity above 99.0%. However, broad peaks with long retention times for Hyp and radioiodinated Hyp were typically observed during the purification process. It seems once radioiodinated Hyp is mixed with unlabeled Hyp in a total mass of about 2 mg, they might undergo partial retention in the HPLC system due to aggregate formation. Under physiological conditions, such aggregates may show reduced necrosis affinity and increased uptake in organs of the mononuclear phagocyte system (MPS), hampering the potential clinical usefulness of ^{131}I-Hyp for OncoCiDia.

Confronted problems: OncoCiDia has shown the best results using a mixture of radioiodinated Hyp/unlabeled Hyp[1]. To overcome the limitations related to the purification process, we recommended the clinical use of the non-purified radioiodinated Hyp, which is attained with high labeling yields (> 95%). However, conditions for Hyp radioiodination require excess of starting material at high concentrations of DMSO, which also dissolved the oxidizing agent iodogen. As a result, both reagents remain in the formulation of the non-HPLC purified radioiodinated Hyp, giving toxicity concerns. Moreover, the unlabeled Hyp present in the mixture is in a concentration range of 10^{-3} mol/L, in which it may aggregate in biocompatible aqueous formulations. Regarding $^{123/131}$I-Hyp, the incorporation of an iodine atom into a molecule can also result in a more lipophilic and less water soluble derivative[72]. Under these circumstances, a proper delivery system is essential for preventing aggregate formation and subsequently ensuring efficient targeting to necrotic tumor. Another potential issue arises with the co-injection of unlabeled Hyp which could influence $^{123/131}$I-Hyp on the biodistribution and targetability over time. Since the treatment of solid tumors requires the preferential delivery of a radiotherapeutic dose to the tumor while preventing normal tissues from undesired side effects[73], the dosimetry of this co-injection approach has to be estimated, as well. These above-mentioned problems have been assessed or addressed below.

Formulation: For $^{123/131}$I-Hyp/Hyp, the co-solvency approach seems to be a good alternative due to its rapidness and simplicity. In preclinical investigations, a formulation consisting of water/ polyethylene glycol (PEG 400) (80/20, v/v) has been reported[1]. Pure DMSO as solvent for the poorly water soluble $^{123/131}$I-Hyp/Hyp has also been used[74]. However, further optimizations are needed.

In a recent study, we tested several delivery systems for $^{123/131}$I-Hyp/Hyp using macroscopic and microscopic techniques and the results are summarized in Table 1[75]. Overall, formulations with a water content below 40% showed red fluorescent solutions without aggregate formation. In contrast, formulations containing around 70% water appeared as cloudy brownish solutions with reduced fluorescent properties. Animal studies confirmed the previous *in vitro* observations. For instance, when DMSO/PEG 400/water (25:60:15, v/v/v) was used as a vehicle, $^{123/131}$I-Hyp/Hyp showed low uptake in MPS organs, high necrosis affinity and striking tumoricidal effects days after OncoCiDia application. With $^{123/131}$I-Hyp/Hyp in DMSO/saline (20:80, v/v), instead, radioactivity accumulation in MPS organs but low uptake in necrotic tumor were found. Consequently, poor radiation dose was deposited in the tumor, leading to disease progression because of rapid repopulation of residual cancer cells at the tumor periphery after VDA attack[75].

However, earlier studies have reported that the common pharmaceutical solvents may have biological and pharmacological activity mainly when given undiluted[76-78]. Alternatively, the water-soluble sodium cholate (NaCh), a naturally occurring liver-produced surfactant with low toxicity, was assessed as a potential solubilizing agent for ^{123}I-Hyp/Hyp in an animal model of acute myocardial infarction (MI) (Cona *et al*[79]). The amphiphilic NaCh molecule with hydrophilic and hydrophobic sides of different solubility properties forms micelles, which act as emulsifier above the critical micellar concentration. Necrosis avidity of ^{123}I-Hyp/Hyp dissolved in a NaCh solution and its favorable biodistribution were demonstrated (Figure 6). The suitability of NaCh as a solubilizing agent of ^{123}I-Hyp for hotspot imaging of acute MI could be demonstrated (Cona *et al*[79]).

Biodistribution and dosimetry studies: Tissue distribution of ^{123}I-Hyp/Hyp was studied on animal models either of reperfused partial liver infarction (RPLI)[80] or ethanol-induced muscle necrosis. Dosimetric extrapolations of ^{131}I-Hyp from animals to humans were attempted using biodistribution data of ^{123}I-Hyp in RPLI animals in combination with Organ Level Internal Dose Assessment/Exponential Modeling software, microsphere model and human phantoms of both genders.

^{123}I-Hyp was accumulated at high concentrations in hepatic infarction and muscle necrosis but low uptake either in viable liver or muscle was detected (Figure 7), as previously reported[1,7,74,75]. Dosimetry studies revealed much higher (> 100 times) absorbed doses of ^{131}I-Hyp in hepatic infarction than in normal liver (Cona *et al*[79]). Based on this finding, such doses seem to be much higher than those estimated with other radiotherapeutics under investigation or currently used in clinic (Table 2)[31,81-88]. This corroborates the high affinity for tumor necrosis as well as the tumor shrinkage and growth delay previously observed in animals bearing different engrafts tumors after a single treatment with OncoCiDia (Figure 8)[1,75,89].

In biodistribution studies, ^{123}I-Hyp was cleared within 24 h with reduced blood pool radioactivity. Thyroid, the dose limiting organ for ^{131}I-labeled products, showed almost no radioactivity concentration due to the absence free iodide at earlier time points, suggesting *in vivo* stability of $^{123/131}$I-Hyp. However, an increased uptake in the gland was detected, starting at the second day after tracer administration. In the lungs, a persistent ^{123}I-Hyp uptake was found, leading to a moderately absorbed radiation

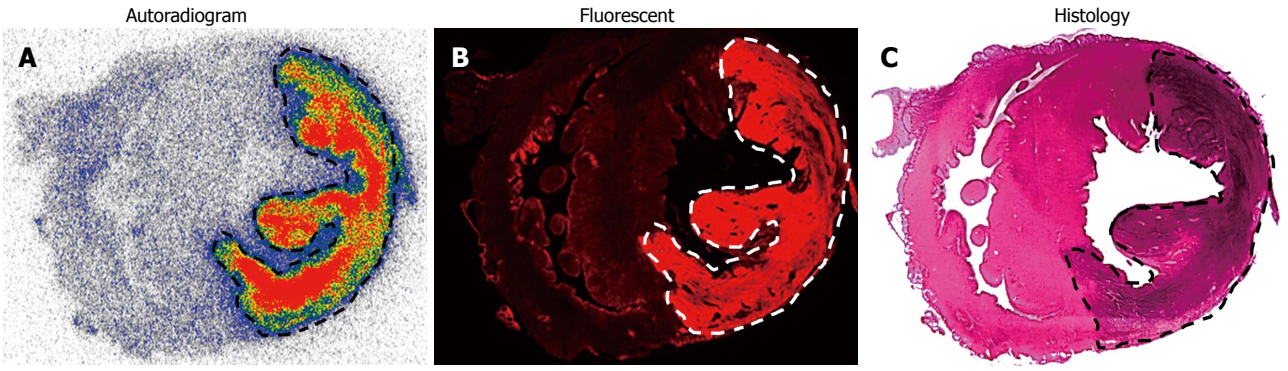

Figure 6 Post-mortem analysis of infarcts and viable heart tissues from rabbits with acute reperfused myocardial infarction having intravenously received iodine-123 labeled hypericin/hypericin dissolved in a 0.07 mol/L solution of the liver-produced surfactant sodium cholate. A: A typical autoradiogram of 50-μm-thick sections reveals higher tracer uptake in infarction than in viable myocardium. The color code bar indicates the coding scheme for the radioactivity; B: Microscopic images of 50-μm-thick sections confirm the selective affinity of the highly fluorescent iodine-123-labeled-hypericin/hypericin for acute myocardial infarction in contrast to the low fluorescent signal found in viable myocardium; C: The H and E-stained section corroborates the location of the viable myocardium tissue and the presence of myocardial necrosis characterized by scattered hemorrhage.

Table 1 Evaluation of physical properties of different formulations of ^{123}Hyp/Hyp examined by macroscopic digital imaging under white light and ultraviolet light (254 nm) and by microscopy over a corresponding drop in bright field and fluorescence illumination mode

Formulation	Macroscopic digital imaging	Microscopy
DMSO/saline (20/80, v/v)	Cloudy brownish solution, no fluorescence	Massive formation of aggregates reduced fluorescent intensity
DMSO/water (25/75, v/v)	Cloudy brownish solution, no fluorescence	Massive formation of aggregate reduced fluorescent intensity
DMSO/D10W (25/75, v/v)	Cloudy brownish solution, no fluorescence	Massive formation of aggregates reduced fluorescent intensity
DMSO/D20W (25/75, v/v)	Cloudy brownish solution, no fluorescence	Massive formation of aggregates reduced fluorescent intensity
DMSO/serum (25/75, v/v)	Cloudy brownish solution, moderate fluorescence signal	Massive formation of aggregates moderate fluorescent intensity
DMSO/PEG 400/water (25/60/15, v/v/v)	Bright red solution, highly fluorescent	No aggregates formation; strong, homogeneous fluorescence
EtOH/PEG 400/water (10/50/40, v/v/v)	Dark red solution, moderate fluorescence	Aggregate formation; moderate, heterogeneous fluorescence,
EtOH/PEG 400/water (10/60/30, v/v/v)	Red solution, minimum aggregation, high fluorescence	Some aggregates; strong, homogeneous fluorescence
PEG 400/water (60/40, v/v)	Red solution, high fluorescence	Some aggregates; strong, homogeneous fluorescence
PEG 400/water (70/30, v/v)	Bright red solution, highly fluorescent	Some aggregates; strong, homogeneous fluorescence
PVP-10000	Dark red solution, moderate fluorescence	Aggregate formation; reduced fluorescence intensity
PVP-29000	Dark red solution, moderate fluorescence	Aggregate formation; reduced fluorescence intensity
β-Cyclodextrins	Cloudy brownish solution, no fluorescence	Massive formation of aggregates reduced fluorescent intensity

DMSO: Dimethyl sulfoxide; D10W: 10% dextrose; D20W: 20% dextrose; EtOH: Ethanol; PEG 400: Polyethylene glycol 400; PVP: Polyvinylpyrrolidone.

dose of ^{131}I-Hyp. The highest levels of radioactivity were found in the intestines, which constitute the major elimination pathway of this radioactive compound. As a consequence, bowel structures received a high radiation dose, being identified as one of the dose limiting organs for OncoCiDia.

Effect of added Hyp on biodistribution and targetability of $^{123/131}$I-Hyp: In STR, it is known that the mass of the unlabelled (carrier) compound present in the final radioactive solution can be critical for high specific activities that are required for maximal radioactivity accumulation in the disease site. In a recent investigation for OncoCiDia, we proved that the co-injection of unlabelled Hyp positively affected the necrosis uptake of the radioiodinated Hyp in RPLI rats[90]. Although both preparations of ^{123}I-Hyp with micro- or Hyp-added dosing showed similar tissue distributions and major hepatobiliary excretion, it was found that the carrier-added ^{123}I-Hyp accumulated at higher concentrations in necrosis. Similarly, long retention into tumor necrosis for several weeks could characterize the carrier-added ^{131}I-Hyp (Figure 8 case 2), which explains the striking therapeutic effects observed in the previous experiments[1,75,89].

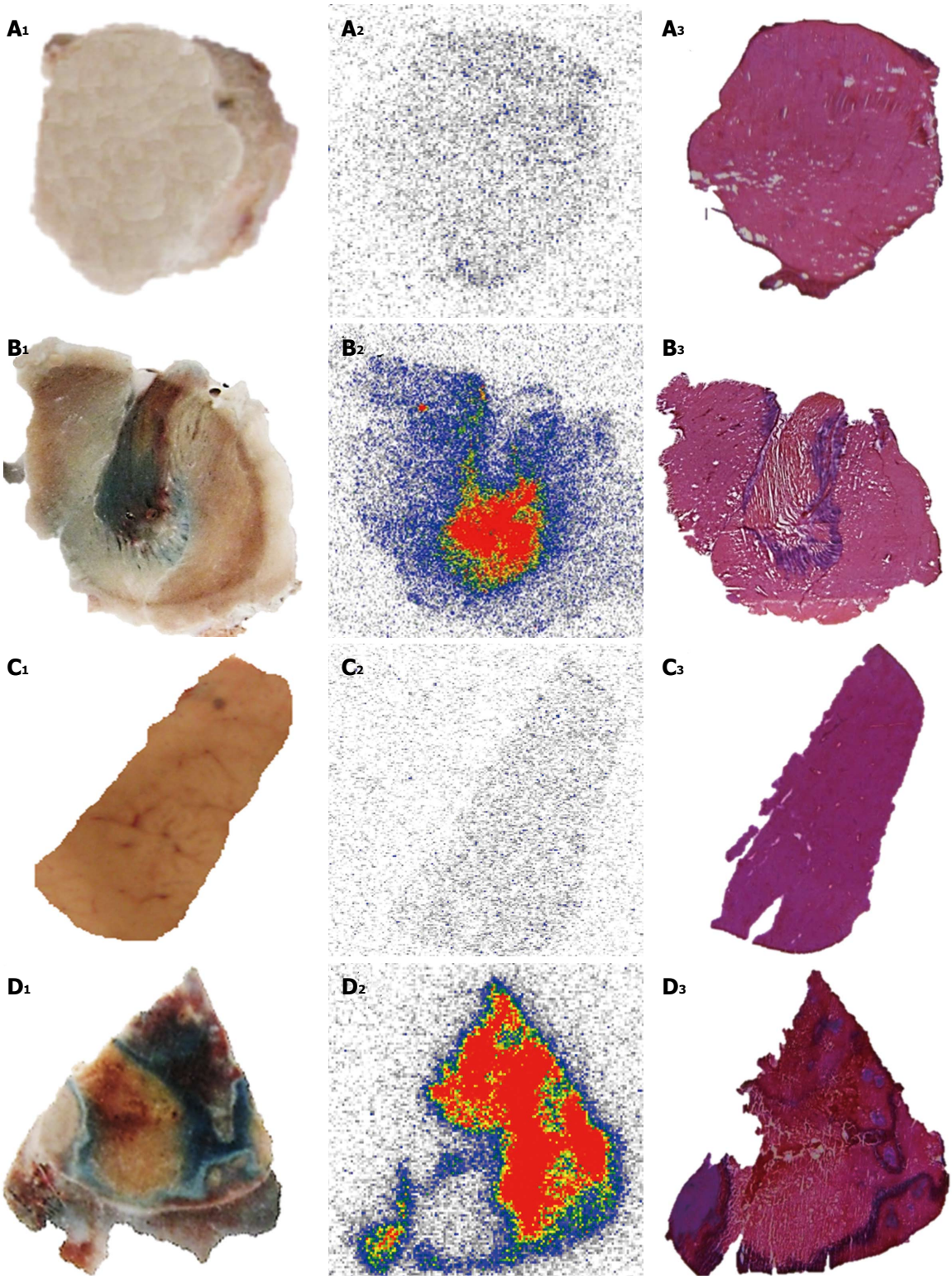

Figure 7 Post-mortem study of necrotic and viable tissues in the liver and muscle from animal models either of reperfused partial liver infarction or ethanol-induced muscle necrosis pre-injected with iodine-123 labeled hypericin/hypericin followed by 1% Evans blue solution. A, B: Muscle; C, D: Liver. The hepatic infarction (A_1) and necrotic muscle (B_1) retain Evans blue as blue hyper intense areas, with viable liver (C_1) and normal muscle (D_1) without staining. Autoradiograms of 50-μm-thick sections show high tracer uptake in hepatic infarction (A_2) and muscle necrosis (B_2) but low accumulation either in viable liver (C_2) or muscle (D_2). The color code bar represents the code for the radioactivity concentration. By histology, the presence of hepatic infarction (A_3) and muscle necrosis (B_3) and the location of the viable liver (C_3) and muscle (D_3) tissues are verified.

Toxicity studies: OncoCiDia is a two-step anticancer strategy involving different compounds with potential chemical and/or radiochemical toxicities, which have been investigated, and possible solutions are herein proposed.

Toxicity from VDAs can happen as a result of the ef-

Table 2 Dosimetry aspects of different anticancer therapeutic agents under pre-clinical and clinical investigation

Therapeutics	Dosimetry calculations	Species	Targeting tissue	Pathology	Dose to tumor (mGy/MBq)	Ref.
^{131}I-Hyp	OLINDA/EXM software	RPLI rats	Necrotic tissue	Solid tumors	276–93600	[79]
^{131}I-Labeled TNT-1 monoclonal antibody	Organ uptake-time integration by trapezoid method Whole body image analysis	Nude mice bearing ME-180 human cervical tumors	Histone fraction H1 in necrotic tissues	Cervical carcinoma cell	366–3610	[31]
^{131}I-m-iodobenzylguanidine (MIBG)	MicroPET/CT 124I-MIBG OLINDA/EXM software	Mice bearing A431 human epithelial carcinoma xenografts	Norepinephrine transporter	Neuroblastoma	97–380	[80]
^{131}I- labeled monoclonal antibody MN-14	MIRDOSE3 software	Nude mice with intraperitoneal LS174T tumors	Carcinoembryonic antigen	Peritoneal metastases of colorectal origin	-16200	[89]
^{131}I-tositumomab	SPECT/CT Imaging DPM Monte Carlo electron and photon transport program	Humans	CD20-positive B-cells	Refractory B-cell NHL	2.81 (mean)	[81]
^{177}Lu-DOTA-AE105	Organ uptake-time integration by trapezoid method Sphere model	Nude mice bearing colorectal HT-29 tumor	uPAR-positive HT-29 xenograft	Colorectal cancer	5.8	[82]
^{177}Lu-pertuzumab	Organ uptake-time integration by trapezoid method Sphere model	BALB/c (nu/nu) Mice with HER-2–overexpressing xenografts	HER-2 tyrosine kinase receptor	Breast cancer	-6900	[83]
^{186}Re-1-hydroxy- ethylidene-1,1 diphosphonic acid	MIRDOSE 3.1 software	Humans	Bone mineral metabolite	Skeletal metastases	23–34	[84]
^{90}Y-ibritumomab tiuxetan	PET/CT Imaging DPM ^{89}Zr-ibritumomab tiuxetan OLINDA/EXM software	Humans	CD20-positive B-cells	Relapsing NHL	8.6–28.6	[85]
^{90}Y- DOTA0-DPhe1-Tyr3-octreotide	SPECT/CT Imaging DPM ^{111}In-DOTA-TOC	Humans	Somatostatin receptor subtype 2	NETs	4-31 (mean 10)	[86]

CT: Computed tomography; DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; ^{131}I: Iodine-131; ^{177}Lu: Lutetium-177; NETs: Neuroendocrine tumors; OLINDA/EXM: Organ level internal dose assessment/exponential modeling; PET: Positron emission tomography; ^{186}Re: Rhenium-186; RPLI: Reperfused partial liver infarction; SPECT: Single-photon emission computed tomography; TNT: Tumor necrosis treatment; DPM: Dose planning method; NHL: Non-Hodgkin lymphoma; DOTA-TOC: ^{90}Y- DOTA0-DPhe1-Tyr3-octreotide; ^{90}Y: Yttrium-90.

fect of VDAs either on tumor blood vessels or normal tissues. Lack of complete specificity for tumor-related vasculature or downstream effects induced by cytokines and other released factors can contribute to the toxic signs or effects. A distinctive transient and acute toxicity pattern with minor cumulative side effects such as tumor pain, nausea and vomiting, headaches, vision changes, symptoms associated to serotonin release, neuromotor abnormalities and cerebellar ataxia, acute hemodynamic disturbances, abdominal pain, hypertension, and tachycardia have been reported after VDA administration in clinical trials[91]. The degree and types of the side effects might differ among flavonoids and tubulin-binding agents[91].

So far, combretastatin A-4 phosphate (CA4P), a synthetic phosphorylated derivative of the natural product combretastatin A-4, is a tubulin-binding agent that has been most extensively used in OncoCiDia experiments. In preclinical studies, intravenous CA4P at a dose of 10 mg/kg has shown a complete and rapid vascular shutdown of the tumor with minimal effects at the well-perfused periphery[1,89]. However, some discrepancies have been noted in animal studies and clinical trials concerning the anticancer effect of VDAs[92]. According to the Food and Drug Administration-approved rules for the correct dose calculation[93], such a dose of 10 mg/kg in rodents would be equivalent to 60 mg/m^2 in humans. However, phase I clinical trials of CA4P have demonstrated a minimal objective tumor response by using similar doses of 52 to 68 mg/m^2[94]. The reason for this difference is not yet clear but could be caused by either miscalculations of the dose based on body surface area in mg/m^2 or due to inaccurate dose translation from animal (mg/kg) to humans (mg/m^2) in addition to the less likely interspecies differences. Based on our experiences, we believe that it may hamper the actual potentialities of VDAs for getting desired anticancer effects in human patients if the dose issues are not properly settled. To overcome the problem, an alternative option could be to increase the total injected dose of CA4P but in an approach of multiple small doses for preventing acute side effects[95]. Moreover, since cardiovascular toxicity seems to be with the most toxicity concerns for CA4P[96], pretreatments with high doses of intravenous diltiazem for preventing hypertension accompanied with amlodipine for secondary prophylaxis or nifedipine and atenolol for blocking tachycardia can be also applied[97,98]. Patients taking QT prolonging drugs, or with a history of significant cardiovascular disease, hypokalemia or hypomagnesemia should be handled with great caution[99].

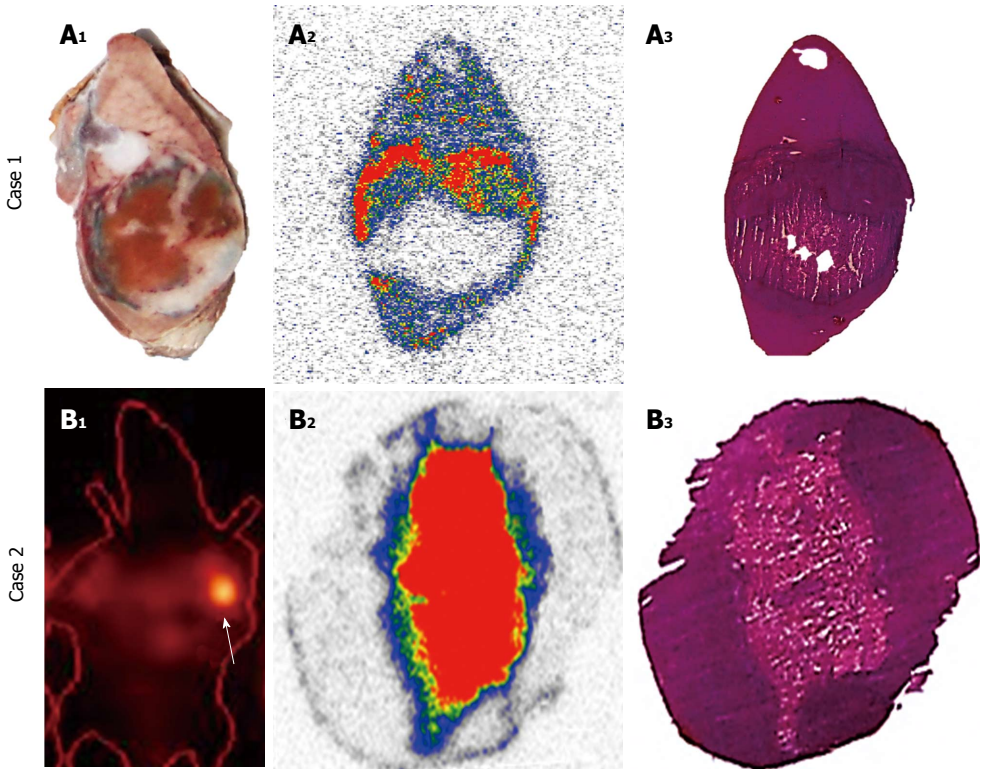

Figure 8 Difference in accumulation patterns of iodine-123/131 labeled hypericin/hypericin in tumor necrosis over time. Case 1: *Ex-vivo* analysis of necrotic tumors from rats with hepatic rhabdomyosarcoma (R1) in the first 24 h after the administration of iodine-123 labeled hypericin/hypericin (^{123}I-Hyp/Hyp) in DMSO/PEG 400/propylene glycol/water (25:25:25:25, v/v/v/v) followed by 1% Evans blue solution. At 24 h after ^{123}I-Hyp/Hyp injection, the tumor necrosis is perfectly outlined by the Evans blue as a blue rim, with viable tumor residues and normal liver with almost no staining (A_1). On the autoradiogram, a perfect match was seen between the high levels of ^{123}I-Hyp/Hyp accumulated in the ring (A_2) and the rim of Evans blue previously observed (A_1). Histology analysis confirms the regions of the liver, necrotic and viable tumors (A_3). Case 2: Single-photon emission computed tomography (SPECT), autoradiograms and histology in severe combined immunodeficiency mice bearing bilateral radiation-induced fibrosarcoma-1 subcutaneously having received combretastatin A-4 phosphate (CA4P) to induce tumor necrosis followed 24 h later by ^{131}I-Hyp/Hyp in DMSO/PEG 400/water (25:60:15, v/v/v). Twelve days after ^{131}I-Hyp/Hyp administration, SPECT detects a persistent intense high radioactivity mainly inside the tumor necrotic core (arrow). Autoradiography (B_2)[1] and histology (B_3) of the tumor after 30 d of tracer injection correspond well with the hotspot imaging on tumor (B_1). [1]The color code bar indicates the coding scheme for the radioactivity. DMSO: Dimethyl sulfoxide; PEG 400: Polyethylene glycol 400.

A study on plasma pharmacokinetics and cerebrospinal fluid penetration of Hyp in rhesus monkeys was conducted. Intravenous administration of 2 mg/kg Hyp was well tolerated. At a dose of 5 mg/kg, a transient severe photosensitivity rash was seen at 12 h that resolved within 12 d[100]. In a phase I study to evaluate the safety and antiretroviral activity of Hyp in thirty HIV-infected patients, weekly repeated IV doses of 0.25 or 0.5 mg/kg were tested. Eleven out of twenty-three patients developed severe cutaneous phototoxicity[101]. In patients with recurrent malignant glioma, a newly developed water soluble formulation of Hyp was IV given (0.1 mg/kg) for tumor visualization. Hyp application proved to be safe with no side effects[102]. Therefore, a low single dose of Hyp at < 0.2 mg/kg for OncoCiDia should be free of noticeable side effects.

I-131 has been used successfully for over 70 years to treat hyperthyroidism and papillary or follicular thyroid cancer and its proper therapeutic use is almost without side effects[103].

However, since accumulation of radioactive iodine was observed in the thyroid, potential damage to the gland could occur due to unnecessary radiation exposure. Therefore, patients undergoing OncoCiDia treatment should take a thyroid-blocking agent on a daily basis before, during and after radiopharmaceutical administration. Either Lugol's solution consisting of elemental iodine and potassium iodide in water or supersaturated potassium iodide solution or oral potassium iodide can be used for this purpose[104].

In a toxicity study with Hyp labeled with non-radioactive iodine (^{127}I), the animals tolerated well IV injections of 0.1 and 10 mg/kg without any signs of clinical toxicity or obvious side effects. The median lethal dose (LD_{50}) of 20.26 mg/kg for ^{127}I-Hyp was above 1000 times of the experimental chemical dose of ^{131}I-Hyp used in OncoCidia, suggesting a wide safety margin with insignificant chemotoxicity[105].

On the other hand, radiation toxic effects could be an issue for ^{131}I-Hyp. Experimental evidence indicated that persistent radioactivity retention mainly occurred in the intestines within few days after injection due to its hepatobiliary excretion[70,75,90]. The acute gastrointestinal syndrome is a main concern following irradiation of the intestinal tract[106]. In a recent study, we tested the suitability of using a newly designed catheter to reduce intestinal

retention of radioactivity after IV administration of ^{131}I-Hyp. Biodistribution and pharmacokinetics of radioiodinated Hyp were also investigated in animals with and without catheterization. In general, the total radioactivity accumulated in the intestines was dramatically reduced by ten times in those animals with catheter placement. Improved tissue biodistribution and kinetic parameters of ^{123}I-Hyp were also seen in cannulated animals. By using this approach, radiation overexposure because of prolonged excretion of ^{131}I-Hyp can be prevented in the future clinical practice[107].

A safety study of IV administered iodogen in DMSO was conducted in mice of both sexes. LD_{50} was determined with iodogen/DMSO doses ranging from 40.0 to 70.0 mg/kg. Toxicity at 30.0 mg/kg was tested for changes in behavior, body weight, and serum biochemistry over 14 d. Due to the toxicity concerns associated with the use of DMSO, a high dose of the solvent was also concurrently tested.

Good safety profile was demonstrated with iodogen in DMSO with LD_{50} values above 50.0 mg/kg and pure DMSO. No animal deaths, pathologies or clinical toxicities were recorded after 30.0 mg/kg iodogen in DMSO, which is 3000 times the dose intended for possible human applications[108].

Overall these data put forward a solid indication for the manageable and tolerable safety of OncoCiDia and potential upcoming clinically applicable formulations in terms of both radiotoxicity and chemotoxicity.

TRANSLATIONAL RESEARCH ON CARDIAC APPLICATIONS OF IODINATED HYPERICIN

Nuclear imaging for diagnosis of myocardial ischemia/infarction

Coronary heart disease, in which MI is a major component, represents the most common cause of death in the Western world. To assess myocardial viability, nuclear imaging uses radiolabelled compounds recognizing specific structures, receptors or antigens to scrutinize the molecular process under physiological conditions in a noninvasive manner. Several myocardial perfusion tracers for single photon emission computed tomography (SPECT) such as thallium-201 (^{201}Tl) and technetium-99m (^{99m}Tc) labeled agents (*e.g.*, sestamibi and tetrofosmin) are currently available in the clinic. They evenly distribute throughout the normal myocardium in proportion to the blood flow, depicting the dead/ischemic tissues as "black spot". However, the development of specific targeting agents with genuine necrosis affinity constitutes an important goal in the management of cardiac pathologies. They may allow early detection, delineation of the infarcted or ischemic area, patient follow-up over-time and evaluation of the response to revascularization therapies[109]. Other cardiovascular diseases related to cardiac cell death could be identified including diverse cardiomyopathy[110], myocardial inflammation, acute myocarditis[111]. Acute or chronic diffuse myocardial damage due to cardiac transplant rejection could also be detected[112].

Various "hot spot" imaging tracers have been exploited for the visualization of MI. Technetium (^{99m}Tc)-pyrophosphate accumulates in necrotic myocardium by targeting the calcium phosphate present in the mitochondria of infarcted or harshly damaged myocardium[113]. ^{99m}Tc-glucarate complex preferentially localizes into basic protein histones within denatured nuclei and subcellular organelles in the dead cardiomyocytes[114]. ^{111}In-labelled monoclonal anti-myosin Fab specifically recognizes the intracellular heavy chain of the exposed cardiac myosin of severely damaged cells[115]. Unfortunately, overestimation of the infarct size due to poor specificity for distinguishing ischemic and necrotic tissues[116,117], and reduced diagnostic accuracy and low target to background ratio on scintigraphic images because of the prompt dissociation of the tracer *in vivo* and short-term accumulation at the damage site[7] have been noticed.

^{123}I-Hyp as a complementary necrosis avid cardiac scintigraphic agent

By micro-single photon emission computed tomography (μSPECT), the potential usefulness of the necrosis avid ^{123}I-Hyp for detection and quantification of acute MI has been reported[118], which is essential for clinical management of ischemic heart disease. In a more recent study[119], ^{123}I-Hyp was compared with the commercial myocardial perfusion agent technetium-99m-labeled-hexakis (2-methoxyisobutylisonitrile) (^{99m}Tc-Sestamibi) in organ distribution and targetability in rabbits with acute MI using dual-isotope simultaneous acquisition-μSPECT/computed tomography and postmortem methods. ^{123}I-Hyp underwent hepatobiliary excretion whereas ^{99m}Tc-Sestamibi distribution was characterized by more rapid hepatorenal elimination. ^{99m}Tc-Sestamibi preferentially accumulated in the normal myocardium, whereas ^{123}I-Hyp confirmed to be a necrosis specific agent that allowed hot spot imaging of irreversibly damaged myocardium or acute MI. Therefore, ^{99m}Tc-Sestamibi and ^{123}I-Hyp can be considered as a pair of complementary tracers for DISA-SPECT/CT in nuclear cardiology[119].

A TRANSLATIONAL APPLICATION ELICITED FROM MECHANISM STUDY ON HYPERICIN

Gallstone basics and pathologies

Cholelithiasis refers to the presence of gallstones in the gallbladder. Although these supersaturated deposits of bile are initially formed within the gallbladder, they may distantly pass into other parts of the biliary tract, reaching the common bile duct, the cystic duct and the pancreatic duct. They can broadly vary in size and appear

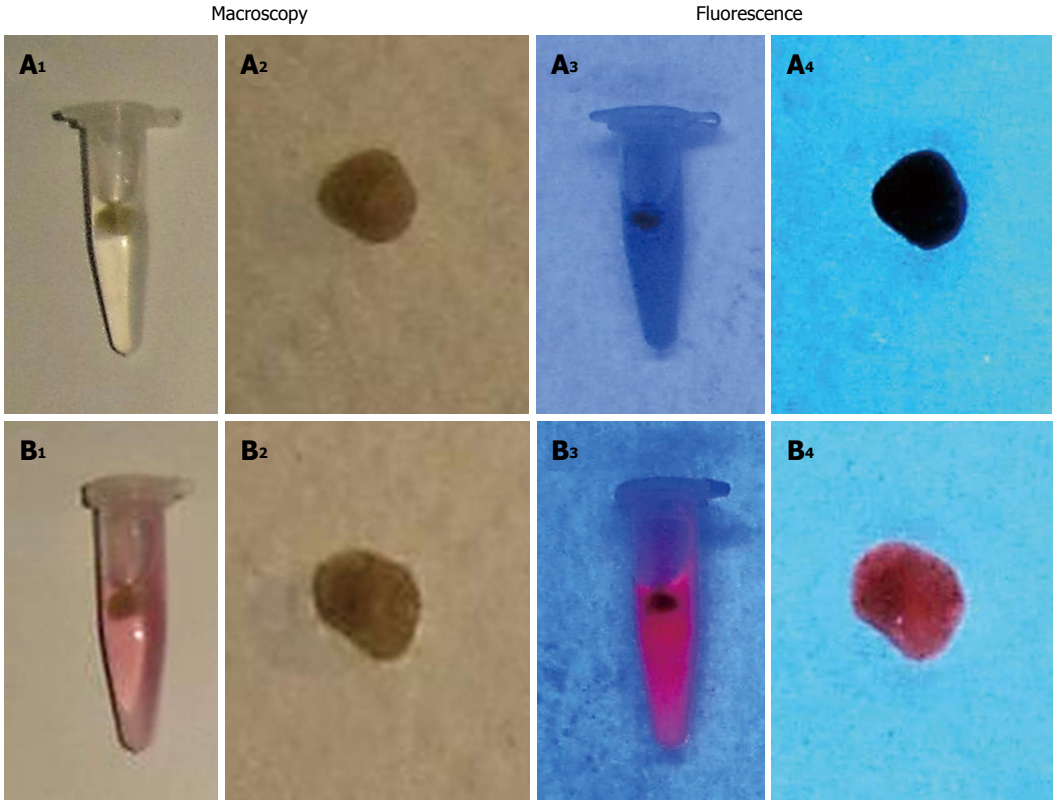

Figure 9 Macroscopic digital imaging of gallbladder stones which were extracted from patients. Stones previously incubated in DMSO/PEG400/water (25:60:15, v/v/v) for 72 h and set as control (A_1, A_2) lack of fluorescent properties (A_3, A_4). Stones treated for 72 h with a solution of Hyp in DMSO/PEG400/water (25:60:15, v/v/v) (B_1, B_2) reveals fluorescence (B_3, B_4) under the UV light of 254 nm. DMSO: Dimethyl sulfoxide; PEG 400: Polyethylene glycol 400; UV: Ultraviolet.

as a single stone or as an assortment of stones with different sizes. Gallstones generally come in three different types including cholesterol stones that represent about 80%, pigment stones composed of bilirubin, the yellow breakdown product of normal heme catabolism found in bile, and mixed stones. Gallstones in the gallbladder may cause acute cholecystitis[120], an inflammatory condition distinguished by bile retention leading to secondary infection by intestinal microorganisms, mainly Escherichia coli, Klebsiella, Enterobacter, and Bacteroides species[121]. Presence of gallstones in the biliary tract can produce obstruction of the bile ducts, leading to severe ascending cholangitis or pancreatitis, which can be life-threatening. Eventually, they can be very painful and may require surgical intervention to remove the gallbladder and/or stones.

Hypericin as an optical imaging agent for fluorescent detection of gallstones

Since Hyp is primarily excreted *via* bile and its interaction with cholesterols has been proved[8,9], the potential use of Hyp as an optical imaging agent for fluorescent detection of gallstones in the clinic was explored. Cholesterol, pigment and mixed gallstones were derived from cholecystectomy patients. *In vitro* studies were conducted by incubating the gallstones with Hyp solutions at increasing concentrations (0-0.01 mg/mL) either in solvent or bile. Under UV light at 254 nm wavelength, red fluorescence was seen on stones previously incubated with Hyp, but this was not observed on only solvent or bile-treated gallstones (Figure 9). The intensity of stone fluorescence depended on Hyp concentration. Although other techniques like ultrasonography or cholescintigraphy scan with ^{99m}Tc-hepatobiliary iminodiacetic acid can usually detect gallstones, the use of Hyp may aid fluorescent detection and removal of gallstones during open and/or endoscopic cholecystectomy and cholangiotomy. *In vivo* studies are needed to prove whether it is a vital and applicable approach.

SAFETY EVALUATION OF MEDICAL DEVICES USING RADIOPHARMACEUTICAL APPROACH

Potential contamination risk from multiple uses of a contrast injection pump

Multiple uses of automatic contrast injection systems for automatic delivery of contrast media during enhanced imaging procedures can reduce costs and save resources. However, cross-contaminations with blood-borne pathogens of infectious diseases may occur[122,123]. To avoid possible nosocomial outbreaks, the injection system including the power syringes, filling and injecting set and

the patient line has to be completely changed for each patient. However, this proves expensive and time consuming due to the wasted surplus contrast materials in the setup from each exam, the consumptions of disposable devices, and the long pauses for changing the entire setup per patient. To reduce material and costs, several institutions worldwide have been applying multiple usages of the syringes with automatic injectors for serial patients. Generally, these commercially available injection systems contain a special one-way-valve tube device. However, nosocomial outbreaks between patients are still a problem because of contamination of the injection system with blood-borne pathogen[124].

A radioactive method for assessing microbial safety of an infusion set

The purpose of this experiment was to develop a radioactive method for quantitative safety evaluation of a new replaceable patient-delivery system[125]. This system (Transflux™ Diepenbeek, Belgium) contains a safety zone composed by a tube and two one-way valves. It permits to flush the whole injector system and the vein but prevents blood reflux during contrast-enhanced imaging. This system is replaced for each new patient, whereas the power syringes need to be changed only once a day after multiple uses for a series of patients. It has been applied for years in many radiology units without any contaminative infections reported, which though has to be experimentally justified.

By mimicking pathogens with a diffusible radiotracer, we evaluated the feasibility of using this system repeatedly without septic risks. The experiment was performed by intravenous injection of ^{99m}Tc-dimercaptopropionyl-human serum albumin in rabbits previously connected *via* an endovenous catheter to an automatic contrast injection system. Protocols with normal saline and contrast agent plus saline loaded in the injection system were compared. By sampling and analyzing aliquots from the filling and injecting set, patient line and blood, it was checked if the radiotracer from the patient line in contact with animal blood was able to cross the safety zone and reach the power syringes.

Overall, with both protocols, radioactivity was found in blood and in patient line but in none of the samples from the filling-injecting set. This radioactive method appears accurate and reliable. The patient-delivery system proves safe and convenient, which is in line with the clinical experiences collected to date. By replacing the patient delivery system, cross-contamination risks can be avoided without changing the main part of injection system. This method can be applied for evaluation of similar devices before human use.

CONCLUSION

Translational medicine aims at identifying solutions to specific health problems. Different but important difficulties faced in cancer treatment, identification of cardiac infarction, detection of cholelithiasis and safety evaluation of medical devices might be considerably tackled by well-designed laboratory experiments.

OncoCiDia presents an unconventional but general approach based on the necrosis avidity for treating multifocal and multitype malignant tumors. It uses a combined sequence of a vascular disrupting agent for triggering massive tumor necrosis followed by the necrosis avid ^{131}I-Hyp to destroy remaining tumor cells. Some technical optimizations have been performed and herein demonstrated to assist in introducing OncoCiDia to the possible clinical practice. The feasible Hyp radioiodination with good radiochemical yields and a proper formulation for *in vivo* applications have been investigated and discussed. The favorable biodistribution, dosimetry and pharmacokinetic patterns as well as good *in vivo* tolerance and low toxicity of radioiodinated Hyp have been exhibited in animal experiments. In general, the genuine benefits of ^{131}I-Hyp distinguished by high and unprecedented long-term accumulation in tumor necrosis in the vicinity of cancer cells and its convenient clearance mechanism through bile without renal retention could noticeably impact on cancer theragnostic management or open doors for handling a wide diversity of cancers in future clinical practice.

Targeting necrosis may offer new opportunities for the management of cardiac pathologies. The clinical introduction of radioactive necrosis-specific agents like ^{123}I-Hyp might play a complementary role in detection and quantification of acute myocardial infarction. The combination of the genuine ^{123}I-Hyp necrosis avidity with the preferential uptake of the currently used commercial myocardial perfusion agent by normal myocardium might offer additional information in the clinical management of this life-threatening pathology.

On the other hand, the use of the fluorophore hypericin as an optical imaging agent with low *in vivo* toxicity could be an excellent diagnostic tool for the detection and removal of gallstones in patients suffering from such common clinical conditions.

Finally, the risk of accidental cross-contamination in medical devices can be minimized through safety evaluation studies based on the inherent sensitivity of radioactive methods in preclinical animal experiments.

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MINIREVIEWS

Bone marrow cell-based regenerative therapy for liver cirrhosis

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Abstract

Bone marrow cells are capable of differentiation into liver cells. Therefore, transplantation of bone marrow cells has considerable potential as a future therapy for regeneration of damaged liver tissue. Autologous bone marrow infusion therapy has been applied to patients with liver cirrhosis, and improvement of liver function parameters has been demonstrated. In this review, we summarize clinical trials of regenerative therapy using bone marrow cells for advanced liver diseases including cirrhosis, as well as topics pertaining to basic *in vitro* or *in vivo* approaches in order to outline the essentials of this novel treatment modality.

Key words: Bone marrow; Liver regeneration; Cirrhosis; Stem cell; Transplantation

Core tip: Bone marrow cells, which include multipotent progenitor cells, are capable of differentiation into liver cells. Autologous bone marrow infusion therapy has been applied to cirrhotic patients, and improvement of liver function parameters has been demonstrated. Although the efficacy of this treatment modality needs to be evaluated in more detail in a large number of patients, regenerative therapy using bone marrow cells for advanced liver diseases has considerable potential.

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INTRODUCTION

Bone marrow cells (BMCs) are capable of differentiating into liver cells[1-4] because they include stem cells known as multipotent adult progenitor cells[5,6]. These cells have been shown to produce albumin when cultured with hepatocyte growth factor (HGF)[7] and various liver-specific proteins, including albumin, when cultured with mature hepatocytes[8]. Using cells obtained with a negatively selective magnetic cell separation system for efficient sorting of rat BMCs enriched with stem cells, we have shown that BMCs differentiate into cells expressing liver-specific genes when cultured with mature hepatocytes or HGF[9]. As there is now much evidence indicating that BMCs can differentiate into cells resembling liver cells *in vitro*[6-11], the characteristics of such BMCs are of great interest in the context of liver-regenerative medicine[12-14].

Liver cirrhosis is the end stage of chronic liver disease, and is associated with many serious systemic complications resulting from both liver failure and portal hypertension. This condition has a poor prognosis and is difficult to treat. Therefore, development of an effective liver-regenerative therapy for liver cirrhosis is an urgent priority. Liver transplantation is the only curative remedy for cirrhotic patients, but is associated with many problems such as donor shortage, surgical complications,

rejection and high cost. As an alternative approach, regenerative cell therapy using stem cells is now attracting attention. Multipotent stem cells present in bone marrow are a particularly promising candidate for this purpose. In this review, we summarize clinical trials of liver-regenerative therapy using BMCs for advanced liver diseases including cirrhosis, as well as topics pertaining to basic *in vitro* or *in vivo* approaches in order to outline the essentials of this novel treatment modality.

MIGRATION AND ENGRAFTMENT OF TRANSPLANTED BMCs TO THE INJURED LIVER IN STUDIES USING ANIMAL MODELS

Although BMCs can show liver cell lineage differentiation *in vitro*, an understanding of the dynamics of transplanted BMCs *in vivo* is essential for the development of BMC-based regenerative therapy. In this context, two important issues need to be clarified: (1) How do transplanted BMCs migrate to and engraft in the liver? and (2) Is there a relationship between the degree of liver damage and the extent of migration of transplanted cells? A previous study using model rats with carbon tetrachloride (CCl_4)-induced liver injury has demonstrated that transplanted BMCs derived from transgenic rats expressing green fluorescent protein[15] in the spleen migrated to and remained in the periportal area of the recipient's damaged liver[16]. These transplanted cells expressed liver cell markers such as alpha-fetoprotein as well as Notch signaling markers for stem cells, suggesting that the BMCs retained in the recipient liver possess the potential to differentiate into liver cells.

Migration of transplanted BMCs to the liver after injection into the spleen has been compared in two models of liver injury induced by administration of CCl_4 and 2-acetylaminofluorene (2-AAF)[17], respectively, focusing particularly on differences in levels of liver mRNA for growth factors such as HGF and fibroblast growth factor (FGF), which have been shown to be responsible for efficient liver cell lineage differentiation of BMCs[9,18,19]. Interestingly, transplanted BMCs were found to engraft into CCl_4-induced injured liver characterized by submassive hepatic necrosis and induction of high levels of HGF and FGF, but not into liver damaged by 2-AAF[20]. A higher degree of HGF induction is characteristic of more severe liver damage[21,22]. These findings suggest that transplanted BMCs migrate more effectively to a liver with greater damage, and that this transplantation approach would be clinically promising for treatment of advanced liver diseases. However, further studies are needed to clarify the factors produced by both BMCs and hepatocytes that contribute to better differentiation of BMCs into liver cells *in vivo*, thus improving the effectiveness of BMC transplantation.

HUMORAL FACTORS BENEFICIAL FOR LIVER REGENERATION AFTER BMC TRANSPLANTATION

The degree of liver function and fibrosis, as well as survival rate, have been shown to improve significantly after BMC transplantation in animal models of severe liver injury[23,24]. With regard to the mechanisms of liver regeneration resulting from BMC transplantation, many of the physiological and regenerative roles of transplanted BMCs remain unclear. However, it can be said with certainty that humoral factors produced in the liver during the regenerative process after BMC transplantation have a crucial role in both improvement of liver fibrosis and liver cell lineage differentiation of stem cells originating from BMCs and hepatic epithelial stem cells.

Improvement of liver fibrosis results from fibrolysis through the proteolytic action of BMC-induced factors. In this context, matrix metalloproteinase (MMP) activity is particularly noteworthy[25]. Sakaida *et al*[23] showed that BMC transplantation ameliorated liver fibrosis in the CCl_4-induced liver-injury model, and that the fibrolytic change was attributable to MMP-9 secreted by BMCs that had migrated to fibrotic areas of the liver.

The liver cell lineage differentiation of BMCs occurs through the cooperative action of a variety of growth factors such as HGF or FGF induced in the injured liver[11,20,26]. Such differentiation may be accompanied by early elevation of the apolipoprotein A1 level in serum and liver[27]. Administration of FGF2 in combination with BMC transplantation synergistically ameliorates liver fibrosis in models of liver injury induced by CCl_4[28]. In addition, in severe liver injury where hepatocyte proliferation is strongly inhibited, hepatic stem cells such as oval cells are induced and show differentiation toward a liver cell lineage, thus leading to liver regeneration[29,30].

As BMC transplantation is successfully adaptable to cases of severe liver injury, it has been hypothesized that transplanted BMCs interact with hepatic epithelial stem cells and influence the subsequent proliferation and differentiation of stem cells. Studies of the interaction between BMCs and hepatic stem cells can provide new insight into the mechanisms of recovery from severe liver damage through liver regeneration after BMC transplantation. In this context, *in vitro* analysis using a system for co-culture of BMCs and an established epithelial hepatic stem cell line has been conducted. Haga *et al*[31] demonstrated that the expression of *FGF2* mRNA was upregulated in BMCs co-cultured with hepatic stem cells, and that expression of mRNAs for both albumin and tyrosine aminotransferase, representative of mature hepatic cells, became detectable in hepatic stem cells after culture with FGF2 protein. Thus, BMCs stimulate both proliferation and differentiation of hepatic stem cells into the hepatocyte lineage, and FGF2 is one of the factors produced by interaction with BMCs, which stimulates

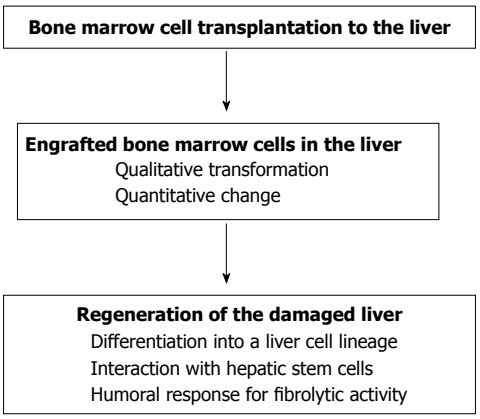

Figure 1 Putative action of transplanted bone marrow cells that include multipotent stem cells for regeneration of damaged liver.

such differentiation. Cross-talk between bone marrow stem cells and hepatic epithelial stem cells may underlie the process of liver regeneration, and this is an area of interest for future investigation. Figure 1 shows an overall representation of the putative action of transplanted BMCs in the regeneration of damaged liver.

CLINICAL TRIALS OF BMC TRANSPLANTATION FOR ADVANCED LIVER DISEASES

BMC transplantation has received increasing attention as a promising therapy for advanced and severe liver diseases such as cirrhosis. Clinical trials of BMC administration to patients with advanced liver diseases have been performed, and improvement of liver function parameters such as the serum level of albumin, Child-Pugh score or Model for Endstage Liver Disease score have been reported[32-40]. Another study has shown that intraportal administration of autologous $CD133^+$ BMCs and subsequent portal venous embolization of right liver segments resulted in a 2.5-fold increase in the mean proliferation rate of the left lateral segment, in comparison with controls not receiving BM transfusion[41]. These findings suggest that transplanted BMCs have a potential role in liver regeneration and proliferate in the recipient liver. Recently, autologous BMC transplantation - a technique named autologous BMC infusion (ABMi) therapy - has been applied to multi-center patients with liver cirrhosis due to hepatitis C[42], hepatitis B[43] and excess alcohol intake[44] using almost the same protocol, and a series of studies have demonstrated improvement of the serum albumin level, leading to improvement of the Child-Pugh score.

Although BMC administration for advanced liver diseases including cirrhosis is an attractive strategy in the field of cell therapy for liver regeneration, many concerns need to be addressed[45-47]. As *in vitro* and *in vivo* experiments have clearly shown, BMCs induce fibrolysis and show hepatocyte differentiation, and they may interact with hepatic epithelial stem cells to aid their differentiation into the hepatocyte lineage. However, it is still unclear how infused BMCs work to improve liver function in humans. A clinical trial of ABMi for patients with cirrhosis demonstrated that the number of AFP-positive cells increased significantly in the liver relative to the situation before ABMi[42]. In addition, ABMi appeared to induce hepatocyte proliferation in the liver, as expression of proliferating cell nuclear antigen, a marker of hepatocyte proliferation, was significantly increased after ABMi in comparison with the pretreatment situation. Although these findings suggest that transplanted BMCs have a potential role in liver regeneration and proliferate in the recipient liver, it remains unknown whether fully functional hepatocytes are induced by ABMi. The characteristics of stem cells present among BMCs that show hepatocyte differentiation require further elucidation.

The factors that determine the difference between effectiveness and non-effectiveness of ABMi are unclear. Collateral circulation resulting from the portal vein disorganization that characterizes liver cirrhosis may affect the flow and effective migration of infused BMCs to the liver, and thus migration of infused cells to the liver may partly depend on the portal venous pressure. In addition, the expression levels of cellular adhesion molecules associated with the attachment of infused cells to liver tissue may vary a great deal among patients. The long-term effectiveness of this therapy in terms of survival rate has not been demonstrated. These issues should be evaluated by a randomized controlled trial involving a large number of patients. Additionally, other issues that impact the efficacy of this therapy, *i.e.*, the long-term culture conditions optimal for stocking BMCs for repeated infusion, the optimal cell population to employ, the optimal number of cells to infuse, the effectiveness of repeated infusion and the optimal route for cell delivery need to be investigated further.

In conclusion, regenerative therapy using BMCs for advanced liver diseases including cirrhosis has considerable potential. Further studies are needed to develop a better method of BMC transplantation that can contribute to improvement of liver function and to clarify the long-term effectiveness of this therapy.

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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