Effects of skeleton structure on necrosis targeting and clearance properties of radioiodinated dianthrones

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Abstract
Necrosis avid agents (NAAs) can be used for diagnosis of necrosis-related diseases, evaluation of therapeutic responses and targeted therapeutics of tumor. In order to probe into the effects of molecular skeleton structure on necrosis targeting and clearance properties of radioiodinated dianthrones, four dianthrone compounds with the same substituents but different skeletal structures, namely Hypericin (Hyp), protohypericin (ProHyp), emodin dianthrone mesomer (ED-1) and emodin dianthrone raceme (ED-2) were synthesized and radioiodinated. Then radioiodinated dianthrones were evaluated in vitro for their necrosis avidity in A549 lung cancer cells untreated and treated with H2O2. Their biodistribution and pharmacokinetic properties were determined in rat models of induced necrosis. In vitro cell assay revealed that destruction of rigid skeleton structure dramatically reduced their necrosis targeting ability. Animal studies demonstrated that destruction of rigid skeleton structure dramatically reduced the necrotic tissue uptake and speed up the clearance from the most normal tissues for the studied compounds. Among these 131I-dianthrones, 131I-Hyp exhibited the highest uptake and persistent retention in necrotic tissues. Hepatic infarction could be clearly visualized by SPECT/CT using 131I-Hyp as an imaging probe. The results suggest that the skeleton structure of Hyp is the lead structure for further structure optimization of this class of NAAs.

Keywords
 Autoradiography, biodistribution, necrosis targeting, pharmacokinetics, radioiodinated dianthrones, SPECT/CT imaging

Introduction
Necrosis represents a principal feature of numerous pathologies or disorders such as ischemic cardiac disease, atherosclerosis, stroke, sepsis, pancreatitis and malignant tumors [1,2]. It is often classified as an uncontrolled, accidental process of cell or tissue death that results from chemical or physical noxious insults and ischemic or inflammatory injury [3]. Differing from apoptosis, necrosis usually involves groups of cells and is frequently followed by granulation development and fibrosis replacement with distortion of the local tissue’s architecture [4–6]. Thus the presence of necrotic tissue may provide an anchor for the diagnosis, prognosis or treatment of aforementioned diseases. Necrosis avid agents (NAAs) are a class of compounds that selectively concentrate in the necrotic tissue after systemic administration, which can be used for in vivo imaging of necrotic tissue [3,7] and tumor necrosis therapy (TNT) [8–12]. A common strategy for effective molecular imaging and targeted therapeutics is to employ a suitably designed molecular probe that is composed of a targeting group conjugated to a reporter group or therapeutic group. A number of molecular probes with high avidity for necrotic tissue have been explored for diagnosis and treatment of necrosis-related pathologies such as 99mTc-pyrophosphate [13], 99mTc-glucarate [14–16], 111In-antimyosin monoclonal antibody [17,18], 123I-hypericin monocarboxylic acid (123I-HMA) [19], 99mTc(CO)3-bis-DTPA-pamoate [20], 123I/131I-hypericin(123I/131I-Hyp) [21,22], Hoechst-IR [1] and 64Cu-bis-DOTA-Hyp [23], etc. Among them, the most studied is Hyp. 123I-Hyp allowed visualization of necrotic tissue and may serve as a potential necrosis-avid diagnostic agent for assessment of tissue viability [24–27]. 131I-Hyp has been explored for diagnostic and therapeutic utilities in experimental malignancies [8,10,11]. The inherent fluorescence properties of Hyp have also been exploited for
microscopic necrosis imaging [28,29]. However, Hyp is a lipophilic compound that sparingly dissolves and forms aggregates in aqueous solution [30]. It makes systemic administration problematic and, in turn, reduces its biodistribution to necrotic tissue and shows unwanted biodistribution in normal tissue [31,32].

In recent years, we have committed to discover new necrosis avid probes for noninvasive imaging diagnosis of necrosis-related pathologies and dual-targeting theragnostic strategy of tumors. In addition to Hyp, we found that median dianthrone compounds sennidin A (SA) [12] and sennoside B (SB) [33] (Figure 1) also had necrosis targetability. Compared with Hyp, SA and SB both have better water solubility and giant π-conjugation damaged parent nucleus structure. The fewer uptakes of $^{131}$I-SA and $^{131}$I-SB in the lung compared with $^{131}$I-Hyp suggested that SA and SB may be prone to aggregation to a lesser degree than Hyp.

Although dianthrone compounds, Hyp, HMA, SA and SB all reveal necrosis targeting property, the necrosis targeting ability of them is different. This is mainly due to the difference of their parent nucleus structures and their peripheral substituents. To seek for new necrosis targeting compounds and to provide the basis for further structural optimization, we decided to first explore the effects of skeleton structure on necrosis targeting and clearance properties of radioiodinated dianthrones. Therefore, we synthesized four dianthrone compounds with the same substituents but different skeletal structures, namely Hyp, protohypericin (ProHyp), emodin dianthrone mesomer (ED-1) and emodin dianthrone raceme (ED-2) (Figure 2). After radioiodination, necrosis targeting and pharmacokinetic properties of the four $^{131}$I-dianthrones were examined in rat models with reperfused partial liver infarction (RPLI) and absolute ethanol induced muscular necrosis. Thereafter, we further evaluated the ability of $^{131}$I-Hyp to image necrosis in vivo in rats with RPLI.

Materials and methods

Chemistry

All reagents and solvents used, unless stated otherwise, were purchased from Sinopharm Chemical Reagent Co., Ltd or Nanjing Chemical Reagent Co., Ltd (Nanjing, China) and were used without further purification. The starting material emodin was purchased (Chengdu Huaxia Chemical Reagent Co., Ltd, Chengdu, China). Sodium iodide (Na$^{131}$I) was supplied by HTA Co., Ltd (Beijing, China). The radioactivity concentration was 370 MBq/mL and the radionuclidic purity was greater than 99%. Thin layer chromatography (TLC) was performed on Merck alumina-precoated silica gel 60 F$^{254}$ plates or silica gel 60 RP-18 F$^{254}$ plates with spots visualized by UV light (254 nm or 365 nm) and/or by development with magnesium acetate, or aluminum chloride. Column chromatography was carried out with Merck silica gel 60 normal phase (45–75 μm) or Sephadex LH20. $^1$H NMR and $^{13}$C NMR were recorded on a Bruker 300 or 500 MHz spectrometer and $^{13}$C NMR spectra were recorded at 75 MHz. Chemical shifts are reported as parts per million (δ) relative to tetramethylsilane (TMS, 0.00 ppm), which was used as an internal standard. MS spectra were measured by HP1100 mass spectrometer utilizing electrospray ionization (ESI) (Agilent, Santa Clara, CA).

**Synthesis of emodin anthrone**

Emodin anthrone was synthesized following a literature procedure [34] with slight modification. A solution of tin (II) chloride hydrate (29.1 g, 129 mmol) in conc. hydrochloric acid (149 mL) was added to a suspension of emodin (3.49 g, 12.9 mmol) in acetic acid (149 mL) was added to a suspension of emodin (3.49 g, 12.9 mmol) in acetic acid (270 mL) to give the red-colored solution. After the solution was stirred at 120°C for 24 h, it was poured into 1000 mL of ice-water and the precipitate was collected by filtration and dried in vacuo at 60°C to give 1 (3.2 g, 96.9%). The resulting emodin anthrone was directly used in the next reaction without further purification.

**Synthesis of ProHyp**

ProHyp (1.25 g, 63.2%) was synthesized following a literature protocol [35] and purified by preparative reversed phase-high performance liquid chromatography (RP-HPLC) (see Supplementary data). Data for ProHyp: $^1$H NMR (300 MHz, DMSO-d$_6$) δ 14.38 (s, 2H), 12.87 (s, 2H), 7.20 (s, 2H), 6.74 (s, 2H), 6.33 (s, 2H), 2.05 (s, 6H), assignments referencing to the literature [36,37]. $^{13}$C NMR (75 MHz, DMSO-d$_6$) δ 15 pairs of signals are overlapping) 184.4, 173.8, 168.3, 160.0, 142.5, 136.2, 129.7, 127.7, 125.8, 119.6, 118.7, 116.1, 113.3, 104.2, 21.3, assignments referencing to the literature [38], MS (ESI) m/z: 505.2 (M–H)−.

**Synthesis of Hyp**

Hyp (1.19 g, 60.4%) was synthesized following a literature protocol [35]. Data for Hyp: $^1$H NMR (300 MHz, DMSO-d$_6$) δ 14.65 (s, 2H), 14.01 (s, 2H), 7.28 (s, 2H), 6.41 (s, 2H), 2.63 (s, 6H); $^{13}$C NMR (75 MHz, DMSO-d$_6$) δ 15 pairs of signals are overlapping) 183.1, 174.3, 168.0, 161.1, 143.2, 126.7, 125.9, 121.1, 120.5, 119.2, 118.5, 108.3, 105.3, 101.8, 23.5, assignments referencing to the literature [38], MS (ESI) m/z: 503.2 (M–H)−.

**Synthesis of ED-1 and ED-2**

A solution of iron (III) chloride hydrate (1.95 g, 129 mmol) in ethanol (180 mL) was added drop wise to a solution of emodin anthrone (1.58 g, 5.85 mmol) in ethanol (375 mL) over 30 min. After heating for 4 h under reflux, the solution was poured into an aqueous solution of hydrochloric acid (5%, 750 mL). It was cooled to room temperature and the product...
Radiolabelling and in vitro stability

The compounds were radiolabelled with $^{131}$I via a direct oxidation reaction using iodogen as an oxidation agent. Briefly, iodogen (1, 3, 4, 6-tetrachloro-3, 6-di-diphenylglycocol; Pierce Biotechnology, ZI Camp Jouven, France) is first dissolved in dichloromethane and is coated on the wall of the Eppendorf tube according to the required dose (40–60 mg). To an iodogen-coated tube were added 400 mL of 0.5 mg/mL of each compound in DMSO and 100 mL of fresh Na$^{131}$I solution (37 MBq). The method of controlling variables (temperature, reaction time and the amount of iodogen) was used to optimize the radiolabelling yields. Radiochemical yield of each $^{131}$I-dianthrone was determined using RP-HPLC (analysis methods are seen in the Supplementary data).

In vitro stability of $^{131}$I-compounds were incubated by incubation in rat serum at 37°C for 72 h and monitored for degradation by RP-HPLC. Briefly, 100 mL of each reaction solution was added into 900 mL of rat serum and incubated at 37°C for 72 h. After the incubation, 1 mL of a mixture of ethanol and acetonitrile (1:1, v/v) was added to precipitate the serum proteins. The resulting mixture was centrifuged at 12000 rpm for 10 min to collect the supernatant. The supernatant was filtered through 0.22-μm pore-size filters (Membrana, Germany) and then injected into RP-HPLC for analysis using the method described above.

Induction of cell necrosis

An in vitro model of necrosis was set up whereby A549 cells were treated with the H$_2$O$_2$ and analysed for FITC-annexin V and propidium iodide (PI) binding by flow cytometry. The human lung cancer A549 cell line was obtained from American Type Culture Collection (Manassas, VA). The A549 cells were seeded into a six-well cell culture plate at a density of 2.5 × 10$^4$ cells/well and incubated at 37°C for 24 h. After being washed once with phosphate-buffered saline (PBS), cells were incubated at 37°C for 1 h in the presence of increasing concentrations of H$_2$O$_2$ (0, 20, 40, 60, 80, 100, 120 mM) in 1 mL of RPMI-1640 medium. After incubation, the reaction medium was aspirated and the cells were rinsed once with 1 mL of cold pH 7.4 PBS to remove the remaining H$_2$O$_2$. Necrosis and apoptosis were both assessed by using the annexin V/PI kit according to the manufacturer’s instructions (Roche, France). Briefly, cells (5 × 10$^5$) were washed twice with PBS and suspended in annexin V and PI staining solution for 15 min at room temperature. Cells were analysed immediately on a flow cytometer (FACS Calibur; BD Biosciences, Franklin Lakes, NJ).

In vitro necrotic binding assay

The binding of $^{131}$I-Hyp, $^{131}$I-ProHyp, $^{131}$I-ED-1 and $^{131}$I-ED-2 for the cell were determined in A549 cells. The cell binding assay was replicated in triplicate for each $^{131}$I-compound. Control group cells and 100 mM H$_2$O$_2$ treated cells were respectively transferred into 4 mL tubes and coincubated at 37°C for 15 min with 0.037 MBq of $^{131}$I-Hyp, $^{131}$I-ProHyp, $^{131}$I-ED-1 or $^{131}$I-ED-2 in 1 mL of RPMI-1640 medium. After incubation at 37°C for 15 min, the mixture was transferred into 1.5 mL tubes and centrifuged at 12000 rpm for 10 min. The tubes were frozen in liquid nitrogen, the bottom tips containing the cell pellet were cut off, and the cell pellets and supernatants were collected and counted for radioactivity in a WIZARD$^+$ 2470 automated gamma counter (PerkinElmer, Maltham, MA). The data were expressed as activity ratios of the cell pellets to the cell pellets and supernatants (cpm of cell pellets/cpm of cell pellets and supernatants) × 100%.
Animal models of necrosis

Adult male SD rats weighing 250–300 g were provided by Experimental Animal Center, Jiangsu Academy of Traditional Chinese Medicine (Nanjing, China). All animal studies were approved by the Institutional Animal Care and Use Committee.

Reperfused partial liver infarction: Adult male SD rats were anesthetized with intraperitoneal injection of pentobarbital (Nembutal Veterinary; Sanofi Sante Animale, Paris, France) at a dose of 40 mg/kg. Under laparotomy, reperfused hepatic infarction was induced by temporarily clamping the hilum of the right liver lobes for 3 h. After reperfusion by declamping hepatic inflow and by giving massage to the liver lobe, the abdominal cavity was closed with two-layer sutures and the animals were allowed to recover overnight. Moreover, sham operated rats, which were subjected to the same surgery except for not clamping the hilum of the right liver lobes, were also established to be used for control in SPECT/CT imaging study.

Ethanol induced muscle necrosis: Each rat of reperfused hepatic infarction was then intramuscularly injected with 0.2 mL absolute alcohol in the left hind limb to establish the model of chemically induced muscle necrosis. Thus, each rat model had both liver necrosis and muscle necrosis and was allowed to recover overnight after the procedure.

In vivo MRI examination

Magnetic resonance imaging (MRI) was performed to examine whether the model of liver necrosis and muscle necrosis were successfully established. Under anesthesia with 2% isoflurane in a mixture of 20% oxygen and 80% room air, the rat models placed in a wrist coil were scanned using a 1.5 T whole body MRI scanner (Echo speed; GE Co., New York, NY). MRI with T1-, T2- and DWI sequences was performed to acquire T1-weighted imaging (T1WI), T2-weighted imaging (T2WI) and diffusion-weighted imaging (DWI). Moreover, Dotarem (Gd-DOTA, Guerbet, France) was intravenously injected at a dose of 0.2 mmol/kg to obtain contrast enhanced-T1WI (CE-T1WI).

Drug delivery protocol

In order to facilitate intravascular (IV) administration, the solution after radioactive labeling reaction was diluted with polyethylene glycol 400 (PEG400) and propylene glycol (1:1, v/v) to formulate preparation immediately before injection. On day 1 after reperfusion of the partial liver infarction and induction of muscle necrosis, $^{131}$I-Hyp, $^{131}$I-ProHyp, $^{131}$I-ED-1 or $^{131}$I-ED-2 preparation was administered via the tail vein (14.8 MBq/kg according to $^{131}$I dose and 0.2 mg/kg according to compound dose) under anaesthesia for biodistribution and pharmacokinetic studies. For biodistribution study, each rat model was intravenously injected with 0.1 mL of 1% Evans blue solution for intravital staining of necrosis at 8 h before being euthanized.

Biodistribution, autoradiography and histochemical staining

For each $^{131}$I-compound, rat models with RPLI and muscle necrosis were randomly allocated into two groups of four animals. After IV administration of $^{131}$I-Hyp, $^{131}$I-ProHyp, $^{131}$I-ED-1 or $^{131}$I-ED-2, the urine and feces were collected for gamma counting during the whole experiment and then the rats were sacrificed at 24 h and 72 h post injection (p.i.) (n = 4 per time point). Organs of interest were isolated, weighed and counted in a WIZARD$^2$ 2470 automated gamma counter (PerkinElmer, Waltham, MA). The stomach and intestines were emptied of food contents and the bladder was emptied of urine prior to radioactivity measurements. The necrotic liver and muscle tissues that were labeled by Evans blue were carefully sampled for gamma counting. Corrections were made for background radiation and physical decay during counting. Radioactive uptake in the organs was expressed as percentage of the injected dose per gram of tissues (%ID/g) while radioactive content in excretion was expressed as percentage of the injected dose (%ID).

After gamma counting, representative tissues of necrotic liver, normal liver and necrotic muscle were cryosectioned into 30 μm thick slices with a cryotome (Shandon Cryotome FSE; Thermo Fisher Scientific Co., Waltham, MA). Autoradiography were performed by exposing the sections to a high performance phosphor screen (PerkinElmer, Waltham, MA) for 4–48 h. Digital autoradiographic images were obtained by reading out the phosphor screen using a Cyclone Plus Phosphor Imager (PerkinElmer, Waltham, MA). Regions of interest (ROI) from the images were analyzed with OptiQuant$^®$ software (version 5.0; Canberra-Packard, Meriden, CT). Ratios of radioactivity concentration (DLU/mm$^2$) between ROIs on necrotic and viable areas were calculated. Afterwards, the sections were stained with hematoxylin and eosin (H&E) using the conventional procedure and were digitally photographed for macroscopic inspection.

Pharmacokinetics study

Another six rat models with RPLI and muscle necrosis was used for pharmacokinetics study. After IV administration of $^{131}$I-Hyp, $^{131}$I-ProHyp, $^{131}$I-ED-1 or $^{131}$I-ED-2, 10 μL of blood samples were collected via a tail incision at 5 min, 10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 24 h and 48 h p.i. Blood samples were measured for radioactive counts in a WIZARD$^2$ 2470 automated gamma counter (PerkinElmer, Waltham, MA) and corrections were made for its background radiation and physical decay during counting. Radioactivity concentrations in blood samples were calculated and expressed as megabecquerel per liter (MBq/L). The pharmacokinetic parameters including area under the curve (AUC(0–$\infty$), elimination half-life ($t_{1/2z}$), peak time ($T_{max}$), clearance (CL$_z$), peak concentration ($C_{max}$) and apparent volume of distribution ($V_z$) were calculated by Drug and Statistics for windows 2.0 software.

SPECT/CT imaging

$^{131}$I-Hyp was evaluated for its ability to image necrosis in vivo in rats with RPLI or sham surgery. Approximately, 14.8 MBq/kg of $^{131}$I-Hyp was injected into rats with RPLI or sham surgery via the tail vein. SPECT/CT imaging was performed using a variable-angle dual-detector SPECT with 6-slice CT (Symbia T6; Siemens Medical Systems Inc., Chicago, IL). Rats were anaesthetized by intraperitoneal injection of...
sodium pentobarbital at a dose of 30 mg/kg and then secured to the head holder of the patient bed in supine position. SPECT/CT images were acquired at 24 h and 72 h after intravenous injection of $^{131}$I-Hyp using the following acquisition parameters: static image matrix size $128 \times 128$, acquisition count limit 50000, SPECT tomographic image matrix $64 \times 64$, and continuous acquisition $15 \text{s/frame} \times 24 \text{frames}$.

**Statistical analysis**

Numerical data were expressed as mean ± standard deviation (SD), unless otherwise stated. Statistical significance in all experiments was determined by unpaired two-tailed Student’s $t$ test, $p < 0.05$ was considered significant.

**Results**

**Synthesis**

All the target compounds were successfully synthesized starting with the coupling of two molecules of emodin anthrone. The desired intermediate emodin anthrone was prepared from commercially available emodin in high yields. Emodin dianthrone was synthesized as a diastereomeric mixture in the presence of iron (III) chloride hydrate in anhydrous ethanol. The diastereomers are inseparable in silica gel TLC plate and slightly separable in reversed phase TLC plate. They were successfully separated into ED-1 and ED-2 by preparative RP-HPLC.

**Radiolabeling and in vitro stability**

The radiolabeling method [39] used for radioiodination of the target compounds was simple and effective in this work. All target compounds were readily radiolabeled with $^{131}$I with greater than 95% radiolabeling yields. The retention times of $^{131}$I-Hyp, $^{131}$I-ProHyp, $^{131}$I-ED-1 and $^{131}$I-ED-2 were 14.40, 13.52, 12.03 and 12.66 min, respectively. Stabilities of all four $^{131}$I-dianthrones were favorable in rat serum at $37^\circ\text{C}$ for 72 h (Figure 3).

**Induction of cell necrosis**

Necrosis was induced by H$_2$O$_2$ with concentrations of 20, 40, 60, 80, 100, 120 mM for 1 h. As shown in Figure 4(A), the percentage of necrotic cells as evaluated by flow cytometry increased with the concentration of H$_2$O$_2$ from 20 mM to 100 mM while slightly declined with 120 mM. Untreated A549 cells (control cells) showed 4.71% of necrotic cells, whereas cells treated with increasing H$_2$O$_2$ concentrations for 1 h showed gradually increasing necrotic cells from 20.84% to a maximum of 78.13% of necrotic cells with 100 mM H$_2$O$_2$ (Figure 4). The percentage of apoptotic cells reached a maximum of 13.60% with 20 mM H$_2$O$_2$ but there was no proportional change with H$_2$O$_2$ concentration. It is worth noting that there is no significant difference on the percentage of apoptotic cells between controls and treated with 100 mM H$_2$O$_2$ cells (6.22% versus 6.64%, $p > 0.5$).

**In vitro necrotic binding assay**

In order to determine the effect of structure on necrosis targeting of $^{131}$I-dianthrones, in vitro binding studies with H$_2$O$_2$ treated A549 cells were performed. All four tracers were found to have a significantly higher uptake for 100 mM H$_2$O$_2$ treated cells compared to non-treated cells (Figure 5). The ratio of percentage of radioactivity in the pellet for the treated cells compared with non-treated cells were 8.10 for $^{131}$I-Hyp, 5.27 for $^{131}$I-ProHyp, 3.08 for $^{131}$I-ED-1 and 2.22 for $^{131}$I-ED-2. These results demonstrated that the skeleton structure had a profound influence on necrosis targeting ability for the four radiotracers.

**In vivo MRI examination**

All rats survived the anesthesia and surgical procedures. Model rats with RPLI and absolute ethanol induced muscular necrosis were successfully established as proven by MRI. The necrotic liver lobe showed homogeneously isointense on T1WI but hyperintense relative to the adjacent normal liver on T2WI, DWI and contrast enhanced T1WI. The necrotic area in the left leg showed iso- or slightly hyperintense on T1WI but hyperintense on T2WI, DWI and contrast enhanced T1WI compared with the healthy right leg (Figure 6).

**Biodistribution and excretion**

The biodistribution properties of $^{131}$I-Hyp, $^{131}$I-ProHyp, $^{131}$I-ED-1 and $^{131}$I-ED-2 are illustrated in Figure 7 with the results expressed as mean %ID/g ± SD. Among these four $^{131}$I-dianthrones, $^{131}$I-Hyp showed the highest necrotic liver and necrotic muscle uptake both at 24 h and 72 h p.i. Despite higher radioactivity uptake in most normal tissues, $^{131}$I-Hyp had lowest radioactivity retention in normal liver, which results in the highest necrotic to normal liver ratio. Although it had higher radioactivity uptake in normal muscle, $^{131}$I-Hyp still exhibited the highest necrotic/normal muscle ratio. The higher radioactivity uptake and slow clearance in the lung demonstrated that $^{131}$I-Hyp may form coarse aggregates in the physiological environment and were subsequently trapped by pulmonary capillary network. $^{131}$I-ProHyp exhibited similar necrosis uptake pattern to $^{131}$I-Hyp. Although it showed much lower radioactivity uptake and rapid clearance in the lung, $^{131}$I-ProHyp also exhibited much lower radioactivity uptake in necrotic liver and muscle in comparison with $^{131}$I-Hyp. $^{131}$I-ED-1 and $^{131}$I-ED-2 exhibited similar radioactivity uptake pattern in both normal and necrotic tissues. Among these studied $^{131}$I-dianthrones, $^{131}$I-ED-1 and $^{131}$I-ED-2 presented the lowest radioactivity uptake in necrotic tissues and had the lowest necrotic/normal tissue ratio.

As described in Figure 8, $^{131}$I-Hyp was mainly cleared through the hepatobiliary pathway resulting in increasing feces radioactivity with time. More than 61.0%ID of $^{131}$I-Hyp was excreted via feces and only 11.1%ID of $^{131}$I-Hyp were excreted via urine at 72 h p.i. $^{131}$I-ProHyp was also mainly cleared via the hepatobiliary pathway and to a lesser extent via the renal pathway, resulting in 31.3%ID of $^{131}$I-ProHyp in feces and 26.4%ID of $^{131}$I-ProHyp in urine within 72 h. Unlike $^{131}$I-Hyp and $^{131}$I-ProHyp, excretion of $^{131}$I-ED-1 and $^{131}$I-ED-2 was mainly via the renal pathway and to a lesser extent via the hepatobiliary pathway.
Figure 3. Radioactive HPLC profiles of $^{131}$I-Hyp (A), $^{131}$I-ProHyp (B), $^{131}$I-ED-1 (C) and $^{131}$I-ED-2 (D) in rat serum after incubation at 37 °C for 72 h. The arrows denote the peaks corresponding to intact $^{131}$I-Hyp (14.40 min), $^{131}$I-ProHyp (13.52 min), $^{131}$I-ED-1 (12.03 min) and $^{131}$I-ED-2 (12.66 min).
Postmortem autoradiography and histochemical staining

The representative autoradiographs and corresponding H&E staining images of the liver (Figure 9A) and muscle slices (Figure 9B) at 24 h and 72 h p.i. for each 131I-dianthrone are shown in Figure 9. Hepatic infarct and muscle necrosis was identified by H&E staining. Autoradiography showed that radioactivity uptake in necrotic liver was much higher than that in normal liver for each 131I-dianthrone, particularly for 131I-Hyp. Moreover, necrosis targetability of 131I-dianthrones was further verified in muscle tissue as higher radioactivity uptake was seen in the necrotic areas than that in viable areas. By a semiquantitative analysis, ratios of radioactivity in necrotic over normal tissues obtained from the representative autoradiographs are presented in Table 1. At 24 h and 72 h p.i., the necrotic to normal liver uptake ratios for 131I-Hyp were 20.9 and 46.4, respectively; for necrotic to normal muscle, these values were 9.0 and 13.8.

Pharmacokinetics

The mean plasma radioactive concentration–time profiles of 131I-dianthrones from 0 to 48 h in rat models with RPLI and muscle necrosis are shown in Figure 10. The major pharmacokinetic parameters are presented in Table 2. Although there was no significant difference in $t_{1/2}$ for 131I-Hyp and 131I-ProHyp ($p > 0.05$), the $C_{\text{max}}$ value for 131I-Hyp was significantly higher than that for 131I-ProHyp ($p < 0.01$) and the same is true for the AUC$_{0-\infty}$ and AUC$_{1-\infty}$ ($p < 0.01$). The blood clearance of 131I-ED-2 was similar to that of 131I-ED-1, as the $t_{1/2}$, $CL_{\text{f}}$ and $V_{\text{f}}$ with no statistically significant difference ($p > 0.05$). Although the $C_{\text{max}}$ value for 131I-ED-2 was significantly higher than that for 131I-ED-1 ($p < 0.01$), both AUC$_{0-\infty}$ ($p > 0.05$) and AUC$_{1-\infty}$ ($p > 0.05$) were similar to each other.

SPECT/CT imaging

As 131I-Hyp showed higher necrotic liver uptake and lower normal liver uptake than the other three 131I-dianthrones at 24 h and 72 h p.i., we further evaluated its ability for imaging necrosis in vivo in rats with RPLI or sham surgery. Representative SPECT/CT images at 24 h and 72 h p.i. of 131I-Hyp are presented in Figure 11. On the SPECT/CT images, the uptake of radioactivity in the infarcted liver lobe was apparently much higher than that in the non-infarcted liver lobe and that in the liver of the sham-operated rat. Right infarcted liver lobes could be clearly visualized by SPECT/CT using 131I-Hyp as an imaging probe, which suggested that specific high uptake of 131I-Hyp in the infarcted liver was mediated by necrosis and 131I-Hyp can detect necrosis in the liver after a partial liver infarction. The SPECT/CT images of rats with RPLI showed high contrast of infarcted liver lobes to normal liver lobes, which was consistent with the biodistribution results. Moreover, higher radioactivity uptake was seen especially in the intestines, indicating the major excretion pathways of the compound.

Discussion

In our previous reports [33,40,41], we have perceived the important effect of skeleton structure of dianthrones on their necrosis targeting. In order to ascertain in more detail the relationship between molecular skeleton structures of dianthrones and their necrosis targeting and clearance properties, we synthesized and evaluated 131I-Hyp, 131I-ProHyp, 131I-ED and 131I-ED in non-treated (control) and H$_2$O$_2$-treated A549 cells. For all four tracers, the percentage of radioactivity in the pellet is significantly increased for the treated cells in comparison to control cells.
Figure 6. *In vivo* MRI transverse section images of rat model with RPLI and muscle necrosis. The upper row was T1WI, CE-T1WI, T2WI and DWI of the liver with infarcted right lobe (arrow) and the bottom row was that of the leg with muscle necrosis (arrow). Infarcted liver lobe and necrotic muscle appeared hyperintense on CE-T1WI, T2WI and DWI.

Figure 7. Biodistribution of $^{131}$I-dianthrones in rat models with RPLI and muscle necrosis at 24 h (A) and 72 h (B) p.i. ($n=4$ per time point).
The present study showed that the disruption of molecular planarity has a dramatic impact on the necrosis targeting of the $^{131}$I-dianthrones. With the increasing distortion of molecular planarity, the necrosis targeting also continues to reduce. Among the four $^{131}$I-dianthrones, $^{131}$I-Hyp displayed the highest percentage of radioactivity in the pellet for the treated cells followed by $^{131}$I-ProHyp, whereas $^{131}$I-ED-1 and $^{131}$I-ED-2 exhibited significantly lower percentage of radioactivity in the pellet for the treated cells.

In vivo biodistribution study displayed a similar necrotic tissue uptake pattern. Up to now, the mechanism of necrosis avidity behind Hyp and other NAAs has not yet been fully elucidated, although several thoughts or hypotheses were raised to explain the phenomenon, such as cholesterol or phospholipid binding in lipid membranes, or a compound-specific mechanism behind

### Table 1. Ratios of radioactivity uptake between ROIs on necrotic and viable areas from rat models with RPLI and muscle necrosis at 24 h and 72 h after IV injection of $^{131}$I-dianthrones*

| Compounds   | Necrotic liver/normal liver | Necrotic muscle/normal muscle |
|-------------|----------------------------|------------------------------|
|             | 24 h          | 72 h          | 24 h          | 72 h          |
| $^{131}$I-Hyp | 20.9          | 46.4          | 9.0           | 13.8          |
| $^{131}$I-ProHyp | 4.3          | 7.9           | 6.3           | 9.1           |
| $^{131}$I-ED-1 | 2.8           | 4.1           | 5.0           | 7.5           |
| $^{131}$I-ED-2 | 2.4           | 3.2           | 4.5           | 5.4           |

*Regions of interest (ROI) from the autoradiographic images was analyzed with Optiquant software. Radioactivity uptake on ROIs on necrotic and viable areas was expressed as DLU/mm$^2$. Ratios were calculated by dividing DLU/mm$^2$ of ROI on necrotic areas by that on viable areas.

The present study showed that the disruption of molecular planarity has a dramatic impact on the necrosis targeting of the $^{131}$I-dianthrones. With the increasing distortion of molecular planarity, the necrosis targeting also continues to reduce. Among the four $^{131}$I-dianthrones, $^{131}$I-Hyp displayed the highest percentage of radioactivity in the pellet for the treated cells followed by $^{131}$I-ProHyp, whereas $^{131}$I-ED-1 and $^{131}$I-ED-2 exhibited significantly lower percentage of radioactivity in the pellet for the treated cells. In vivo biodistribution study displayed a similar necrotic tissue uptake pattern. Up to now, the mechanism of necrosis avidity behind Hyp and other NAAs has not yet been fully elucidated, although several thoughts or hypotheses were raised to explain the phenomenon, such as cholesterol or phospholipid binding in lipid membranes, or a compound-specific mechanism behind
the necrosis targeting of different NAAs [22]. Although we have demonstrated that the Hyp compounds are indeed more effective than the ED compounds both in vitro and in vivo, we don’t know the exact cause yet. As we know, Hyp has a nearly planar rigid skeleton. The skeleton structure of ProHyp is less planar than that of Hyp as a result of the absence of a single bond. Emodin dianthrone compounds can be free to rotate around a single bond connecting two molecules of emodin anthrone and the overall rigidity was completely broken. Therefore, by analyzing structure–necrosis targeting relationship of the four dianthrone compounds, we can at least draw the conclusion that rigid skeleton structure may play an

**Table 2. Major pharmacokinetic parameters of 131I-dianthrone obtained by non-compartmental modeling after IV injection in rat models with RPLI and muscle necrosis within 48h (n = 6).**

| Parameters                  | Unit       | 131I-Hyp | 131I-ProHyp | 131I-ED-1 | 131I-ED-2 |
|-----------------------------|------------|----------|-------------|-----------|-----------|
| AUC(0-t)                    | MBq/L*h    | 1178.41 ± 62.92 | 864.80 ± 129.92 | 509.71 ± 71.50 | 540.82 ± 104.35 |
| AUC(0-∞)                    | MBq/L*h    | 1268.36 ± 48.63 | 955.14 ± 151.52 | 546.83 ± 79.04 | 557.92 ± 108.16 |
| t1/2z                       | h          | 14.73 ± 2.77 | 15.05 ± 1.28 | 12.71 ± 2.70 | 10.05 ± 1.25 |
| Tmax                        | h          | 0.15 ± 0.04 | 0.13 ± 0.05 | 0.14 ± 0.04 | 0.08 ± 0.01 |
| CLz                         | L/h/kg     | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| Cmax                        | MBq/L      | 200.57 ± 18.38 | 114.86 ± 11.84 | 68.73 ± 12.81 | 96.56 ± 10.84 |
| Vz                          | L/kg       | 0.25 ± 0.05 | 0.34 ± 0.05 | 0.50 ± 0.12 | 0.40 ± 0.09 |

The values are expressed as mean ± SD.
important role in necrosis targeting and molecular morphology of a rigid nearly planar configuration may be more beneficial to its binding to the target site.

The difference in pharmacokinetic behavior suggested that skeleton structures of $^{131}$I-dianthrones have also a profound influence on their pharmacokinetic properties. Fonge et al. [42] confirmed that $^{131}$I-Hyp bind mainly to low-density lipoprotein (LDL) and to a lesser extent to high-density lipoprotein (HDL), which was similar to plasma protein binding profile of Hyp. Considering the similarity in structure that both $^{131}$I-Hyp and $^{131}$I-ProHyp maintain a nearly planar structure, we have reason to believe that $^{131}$I-ProHyp could have a similar plasma lipoprotein binding profile to $^{131}$I-Hyp. This may explain why they showed similar $t_{1/2}$ ($p > 0.05$) despite the fact that lipophilicity of $^{131}$I-ProHyp ($log P = 2.40 \pm 0.18$) [42] is less than that of $^{131}$I-Hyp ($log P = 3.08 \pm 0.03$) [43]. This indicates that not only lipophilicity but also other parameters have a significant impact on the pharmacokinetic properties of the $^{131}$I-dianthrones. Furthermore, the $C_{max}$ value for $^{131}$I-Hyp was nearly two times higher than that for $^{131}$I-ProHyp, which may ensure sufficient hypericin that could be delivered to the target tissues. Although $^{131}$I-ED-1 and $^{131}$I-ED-2 displayed similar plasma radioactive concentration–time profiles, the higher $C_{max}$ ($p < 0.01$) for $^{131}$I-ED-2 relative to $^{131}$I-ED-1 suggested stereoisomerism have an influence on the pharmacokinetic parameters. Moreover, both the AUC$_{(0-\infty)}$ ($p > 0.05$) and AUC$_{(0-\infty)}$ ($p > 0.05$) values were not significantly different, which suggested they may have similar bioavailability after IV injection.

The results of present study showed that the skeleton structure of Hyp was the most attractive for the development of promising NAAs both for necrosis imaging and targeted radionuclide therapy. Further SAR study will involve the effect of various substituent groups on necrosis targeting of Hyp. Owing to the hydrophobic effect of its nearly planar highly conjugated structure and the cofacial $\pi-\pi$ stacking between the molecular skeletons, Hyp is prone to form aggregates in an aqueous environment [30,44]. After intravenous administration, parts of the Hyp might form the aggregates and even coarse pellets in the blood circulation. The formed coarse aggregates are intercepted by mononuclear phagocyte system, especially by pulmonary capillary network, which not only reduces its biodistribution to necrotic tissues but also shows unwanted biodistribution in normal tissues [31,32]. Although distortion and even destruction of $\pi$-conjugated system may reduce the degree of self-aggregation of dianthrones, it also reduced their necrosis targeting. Accordingly, it is desirable to reduce the self-aggregation degree of Hyp without reducing the necrosis targeting as far as possible. A possible strategy is to introduce bulky groups to its rigid skeleton structure to improve the water solubility and hinder the cofacial $\pi-\pi$ stacking [45,46].

The development and application of Hyp necessitate a more complete knowledge of the necrosis-avidity mechanism. Not identifying the specific binding moiety that is responsible for the necrosis avidity of Hyp has seriously hindered our research process. Studies have indicated that Hyp can bind to DNA molecule and 5′AG3′ doublet is a specific DNA target for Hyp binding in vitro [47]. Therefore, the next step, we attempt to study whether DNA binding is responsible for high Hyp targeting to necrosis region.

**Conclusions**

The present study demonstrated that the destruction of rigid skeleton structure dramatically reduced the necrosis targeting ability of $^{131}$I-bianthrones. $^{131}$I-Hyp exhibited sustaining higher necrosis tissue uptake and lower normal liver uptake than those of the other three $^{131}$I-dianthrones. Hepatic infarction could be clearly visualized by SPECT/CT using $^{131}$I-Hyp as an imaging probe. The preliminary SAR study indicated that the skeleton structure of Hyp was the most attractive for further developing NAAs. Future work will focus on reducing the self-aggregation degree of Hyp and understanding the mechanism that yields necrosis targeting in molecular level.

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**Declaration of interest**

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