



Original Article

Pharmacokinetics of gene recombined angiogenesis inhibitor Kringle 5 in vivo using ^{131}I specific markers and SPECT/CT[☆]Ge Yan^a, Danrong Yang^b, Yan Yu^b, Jianjun Xue^a, Yifan Jia^b, Xuanzi Sun^b, Boyu Wang^b, Zewei Zhao^b, Maode Wang^{a,*}^a The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, China^b Xi'an Jiaotong University Health Science Center, Xi'an 710061, China

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ABSTRACT

The previous pharmacokinetic methods can be only limited to drug analysis in vitro, which provide less information on the distribution and metabolism of drugs, and limit the interpretation and assessment of pharmacokinetics, the determination of metabolic principles, and evaluation of treatment effect. The objective of the study was to investigate the pharmacokinetic characteristics of gene recombination angiogenesis inhibitor Kringle 5 in vivo. The SPECT/CT and specific ^{131}I -Kringle 5 marked by Iodogen method were both applied to explore the pharmacokinetic characteristics of ^{131}I -Kringle 5 in vivo, and to investigate the dynamic distributions of ^{131}I -Kringle 5 in target organs. Labeling recombinant angiogenesis inhibitor Kringle 5 using ^{131}I with longer half-life and imaging in vivo using SPECT instead of PET, could overcome the limitations of previous methods. When the doses of ^{131}I -Kringle 5 were 10.0, 7.5 and 5.0 g/kg, respectively, the two-compartment open models can be determined within all the metabolic process in vivo. There were no significant differences in $t_{1/2\alpha}$, $t_{1/2\beta}$, apparent volume of distribution and CL between those three levels. The ratio of $\text{AUC}_{(0-\infty)}$ among three different groups of 10.0, 7.5 and 5.0 g/kg was 2.56:1.44:1.0, which was close to the ratio (2:1.5:1.0). It could be clear that in the range of 5.0–10.0 g/kg, Kringle 5 was characterized by the first-order pharmacokinetics. Approximately 30 min after ^{131}I -Kringle 5 was injected, ^{131}I -Kringle 5 could be observed to concentrate in the heart, kidneys, liver and other organs by means of planar imaging and tomography. After 1 h of being injected, more radionuclide retained in the bladder, but not in intestinal. It could be concluded that ^{131}I -Kringle 5 is mainly excreted through the kidneys. About 2 h after the injection of ^{131}I -Kringle 5, the radionuclide in the heart, kidneys, liver and other organs was gradually reduced, while more radionuclide was concentrated in the bladder. The radionuclide was completely metabolized within 24 h, and the distribution of radioactivity in rats was similar to normal levels. In our study, the specific marker ^{131}I -Kringle 5 and SPECT/CT were successfully used to explore pharmacokinetic characteristics of Kringle 5 in rats. The study could provide a new evaluation platform of the specific, in vivo and real-time functional imaging and pharmacokinetics for the clinical application of ^{131}I -Kringle 5.

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1. Introduction

Drug metabolism pharmacokinetics was the research on absorption, distribution, metabolism and excretion of the drug in vivo, namely called ADME. At present, ADME had a development trend of diversification and refinement. The commonly research methods included spectrophotometry, chromatography, immunoassays, biological analysis and isotope labeling. However, due to the biological variations, large measurement error, poor

reproducibility and more confounders, bioassay was only the supplement of physical and chemical test. Based on specific antigen-antibody reaction, immunization was characterized by high sensitivity, fast operation and high-throughput assay [1]. Considering the complex sampling process, discontinuity of observation, and large sample size, the methods above mentioned were unsuited to drug metabolism studies in vivo. The previous pharmacokinetic methods could be only confined to drug analysis in vitro, which provided less information on the distribution and metabolism of drugs, and limit the interpretation and assessment of pharmacokinetics, the determination of metabolic principles, and evaluation of treatment effect [2].

Isotope tracer method is the trace analysis in which radionuclides serve as tracers to mark [3]. Compared with the

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conventional pharmacokinetic methods [4], it has specific advantages of high sensitivity, simple operation, more accuracy, less confounders and errors of separation and purification. Radiolabelled tracer technology could be used not only to observe the absorption, distribution, metabolism and excretion of drugs, but also to locate drugs by imaging, and orient cell and organelles using light microscopy and electron microscopy [5–11]. To better investigate the distribution of drugs in vivo, Bergstrom et al. [12] first used positron emission tomography (PET) in pharmacokinetic study. Since the half-life period of markers ^{11}C and ^{18}F of PET is so short (about 20 min and 110 min), it is difficult to analyze drug distribution in the body through special routes [13]. Because of less compatibility and more complexity, PET radioactive labeling is difficult to popularize in a short time [14–16]. In this study, we used ^{131}I with longer half-life to label recombinant angiogenesis inhibitor Kringle 5 and SPECT instead of PET to image in vivo, which could overcome the limitations of previous methods.

2. Materials and methods

2.1. Instruments

SPECT/CT Imaging System was Infinia VC Hawkeye₄ from GE Company Milwaukee (Wisconsin, USA). DFM296 multi-tube radioimmunoassay counter and γ scintillation counter were made in 262 Factory in Xi'an of China. Sephadex G-25 column was from Pharmacia Corporation (Stockholm, Sweden).

2.2. Targeted drugs

Recombinant angiogenesis inhibitor Kringle 5 was white lyophilized powder, split charging 30 mg in vials. The batch number was 20110402; they were provided by Professor Liujiao Bian in National Micro Detection System Engineering Research Center in Northwestern University, China and freshly prepared before testing every time. Iodogen (tetrachloro-diphenyl glycoluril) was from Sigma Company in San Francisco of USA and was used for iodine-labeled-solid-phase oxidant. Fetal bovine serum was obtained from GIBCO Company (New York, USA).

2.3. ^{131}I labeled Kringle 5 using Iodogen method [17–20]

2.3.1. Smear

First of all, 0.5 mg Iodogen was weighed by electronic precision balance and dissolved in 0.25 mL organic solvent of chloroform. Then, 30 μL of solution acquired above (containing approximately 50 μg Iodogen) was taken to pack in the experimental EP tube and normally dry at 4 °C. Finally, it was smeared evenly the bottom of the experimental tube with Iodogen, sealed and cryopreserved at –20 °C for application.

2.3.2. Label

20 μg /20 μL Kringle 5, 500 μCi /10 μL Na^{131}I and 100 μL PBS were mixed. Their action remained approximately 20 min in EP tube, and then the tube was gently shaken. At last, the mixed solution was transferred out of experimental tube and the reaction was stopped. The steps above should be repeated three times.

2.3.3. Purification

In order to avoid albumin iodization in the column of buffer solution, we will keep 5 min to revert unlabeled iodide ions to molecular iodine before chromatography separation. The whole reaction solution was added into Sephadex G-25 gel to filter, and then eluted with 0.05 M (pH7.5) phosphate buffer at a flow rate of 0.5 mL/min and 1 cm above the column side. A total of 16 tubes

were collected at the speed of one/min, and numbered all of them; meanwhile, radiation count per minute (CPM) of each tube was measured and mark rate was calculated. In the end, these tubes were sealed and cryopreserved at –20 °C.

2.3.4. Separation

At the bottom of filter paper, ^{131}I - Kringle 5 solution was dripped rapidly by the capillary, and ammonium hydroxide served as developing solvent. 10 μL eluent in each tube was also taken. The γ scintillation counter was used to count in 10 s. The elution curve was drawn in graph paper, where the first peak was the protein vertex, and the second peak was free iodine vertex or impurity vertex. We further collected protein vertex, diluted it with normal saline, and identified the product. The experiment procedures above should be repeated three times.

2.3.5. Identification

The appropriate amount of eluent of protein peaks was dripped on the paper (What man 1[#]), and chromatographically spread upwards in n-butanol: ethanol: aqueous ammonia system (5:1:2, v/v/v), which was divided into 10 sections. Next, we counted with γ scintillation counter at channel energy 364 keV, and calculated radiochemical purity (radio-chemical purity, RP).

2.4. Pharmacokinetics of specific labeled ^{131}I -Kringle 5 in vivo [21,22]

Eighteen SD rats, with weight 200–220 g, half of who were female, were divided into three groups of different doses (Medicine Laboratory Animal Center of Xi'an Jiaotong University; the production license number: SCXK (Shaanxi) 2012-003, usage license number: SYXK (Shaanxi) 2012-005). ^{131}I -Kringle 5 in 2 μg (4 μCi) was injected into femoral vein of every rat. After 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 24 h respectively of being injected, 0.2 mL blood sample was drawn from rear eye venous plexus of these rats, and EDTA anticoagulant was also required. The blood samples were centrifugalized at 3000 r/min for 10 min at 4 °C. In the next step, we took 100 μL separate plasma and directly measured radioactivity counts with DFM296 multi-tube radioimmunoassay counter. The corresponding plasma concentration was calculated according to the following formula: plasma concentration ($\mu\text{g}/\text{mL}$) = [measured radiation counts (CPM) \times 2 μg] / [injected dose radiation counts (CPM) \times 0.1 mL].

2.5. The imaging distribution of radionuclide-labeled ^{131}I -Kringle 5 in vivo

After 0.5, 1, 2, 6, 24 h of injecting ^{131}I -Kringle 5 into sublingual vein of 5 rats, SPECT system (Infinia Hawkeye 4, GE) with high-energy collimators, energy window 360 keV, and magnification 5 times, the acquisition count 50 counts, was used to obtain planar imaging. Three-dimensional tomography of SPECT/CT combined with image fusion using specific software, was further used to obtain anatomical and functional imaging and fusion imaging in real time.

3. Results

3.1. The labeled rate of ^{131}I -Kringle 5

The labeled rate of ^{131}I -Kringle 5 was approximately 84.6% in this study. Bimodal curve is shown in Fig. 1.

3.2. The concentration of ^{131}I -Kringle 5 in blood within different time points

After 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 24 h of injecting

^{131}I -Kringle 5 $2\ \mu\text{g}$ ($4\ \mu\text{Ci}$) into femoral vein of rats, the concentration of ^{131}I -Kringle 5 in blood is shown in Table 1.

3.3. The pharmacokinetic parameters of ^{131}I -Kringle 5 in vivo

We draw a curve of the relationship between time and the concentration of Kringle 5 in blood of rats using software DAS 2.0. When the doses of Kringle 5 were 10.0, 7.5 and 5.0 g/kg respectively, the results showed that the metabolic process of Kringle 5 in rats was consistent with a two-compartment model. The ratio of $\text{AUC}_{(0-\infty)}$ between the three groups was 2.56:1.44:1.0, which was similar to the ratio (2:1.5:1.0) of the three groups. It is concluded that the first-order kinetics metabolism of Kringle 5 could be found in the study when the doses ranged from 5.0 to 10.0 g/kg (Table 2).

3.4. The imaging distribution of radionuclide-labeled ^{131}I -Kringle 5 in vivo

After 0.5, 1, 2, 6, 24 h of injecting ^{131}I -Kringle 5 into sublingual

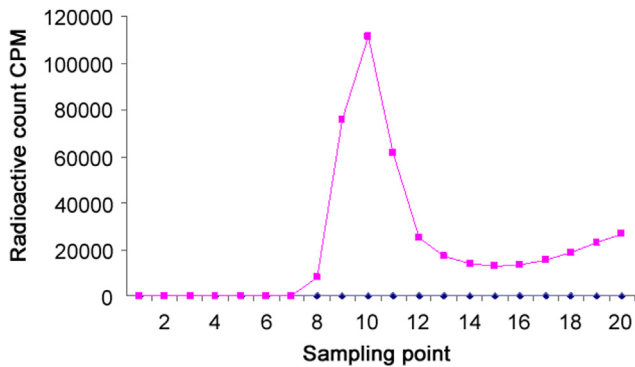


Fig. 1. The curve of labeled rate of ^{131}I -Kringle 5.

Table 1

The concentration of ^{131}I -Kringle 5 in blood within different time periods across different dose groups.

The time of taking blood (h)	Concentration of ^{131}I -Kringle 5 (g/L)		
	10.0 g/kg	7.5 g/kg	5.0 g/kg
0.25	0.1467 ± 0.0124	0.1027 ± 0.0080	0.0563 ± 0.0193
0.5	0.1432 ± 0.0185	0.0907 ± 0.0098	0.0477 ± 0.02096
0.75	0.1330 ± 0.0186	0.0867 ± 0.0152	0.0481 ± 0.0121
1.0	0.1276 ± 0.0367	0.0780 ± 0.0067	0.0469 ± 0.0116
1.5	0.1158 ± 0.0139	0.0684 ± 0.0145	0.0390 ± 0.0099
2.0	0.1061 ± 0.0253	0.0636 ± 0.0125	0.0381 ± 0.01340
4.0	0.1039 ± 0.0218	0.0514 ± 0.0076	0.0340 ± 0.0108
6.0	0.0831 ± 0.0163	0.0486 ± 0.0092	0.0284 ± 0.0048
8.0	0.0809 ± 0.0162	0.0416 ± 0.0119	0.0261 ± 0.0071
12.0	0.0471 ± 0.0071	0.0295 ± 0.0074	0.0190 ± 0.0038
24.0	0.0358 ± 0.0085	0.0206 ± 0.0042	0.01497 ± 0.0021

Table 2

The pharmacokinetic parameters of compartment model of ^{131}I -Kringle 5.

AV parameters	The doses of ^{131}I -Kringle 5 (g/kg)		
	10.0	7.5	5.0
$t_{1/2\alpha}$ (h)	0.484 ± 0.280	0.717 ± 0.324	0.642 ± 0.24
$t_{1/2\beta}$ (h)	14.134 ± 2.426	15.263 ± 4.093	17.612 ± 5.292
VI (L/kg)	60.238 ± 8.896	64.182 ± 4.005	76.504 ± 32.383
CL (L/h/kg)	4.484 ± 1.043	5.866 ± 0.895	5.707 ± 1.176
$\text{AUC}_{(0-t)}$ ($\mu\text{g/L h}$)	1.609 ± 0.190	0.928 ± 0.143	0.573 ± 0.113
$\text{AUC}_{(0-\infty)}$ ($\mu\text{g/L h}$)	2.325 ± 0.513	1.304 ± 0.196	0.905 ± 0.186

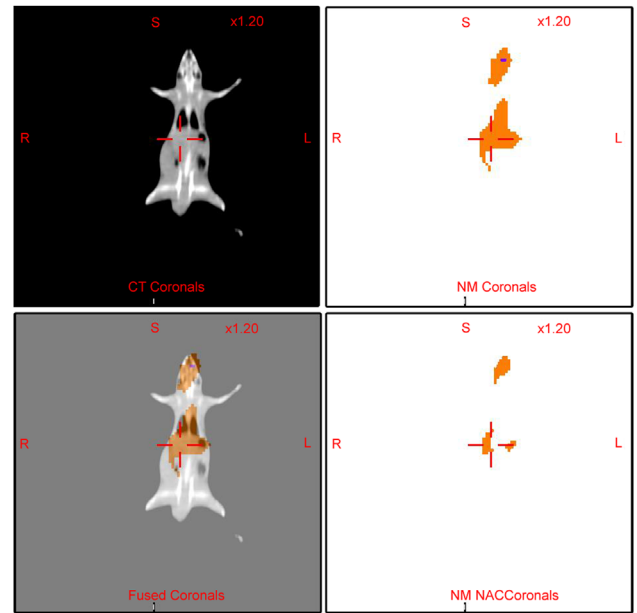


Fig. 2. The imaging of radionuclide distribution at 0.5 h after injection of ^{131}I -Kringle 5.

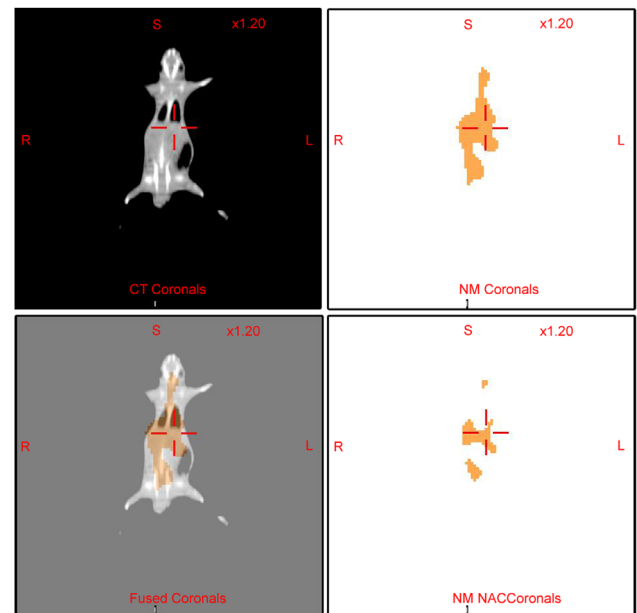


Fig. 3. The imaging of radionuclide distribution at 1 h after injection of ^{131}I -Kringle 5.

vein of rats, functional imaging and real-time fusion imaging using SPECT were obtained. After 30 min, it was clear that ^{131}I -Kringle 5 had concentrated in the heart, kidneys, liver and so on (Fig. 2). After 1 h, there was more radionuclide accumulated in the bladder, but not found in the intestinal (Fig. 3). After 2 h, the concentration of radionuclide in the heart, kidneys and liver were increasingly reduced, while more radionuclide was concentrated in the bladder (Fig. 4). After 6 h, the level of radionuclide in the heart, kidneys, liver and other parts continued to reduce, and no radionuclide could concentrate in the bladder. The distribution of radioactivity in vivo was close to normal background level (Fig. 5). After 24 h, the levels of radioactivity in the heart, kidney and liver had approximated to normal background level. There was not any

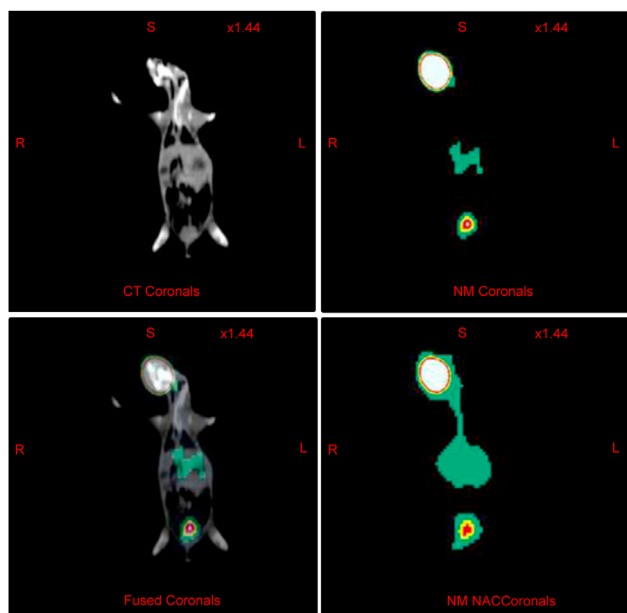


Fig. 4. The imaging of radionuclide distribution at 2 h after injection of ^{131}I -Kringle 5.

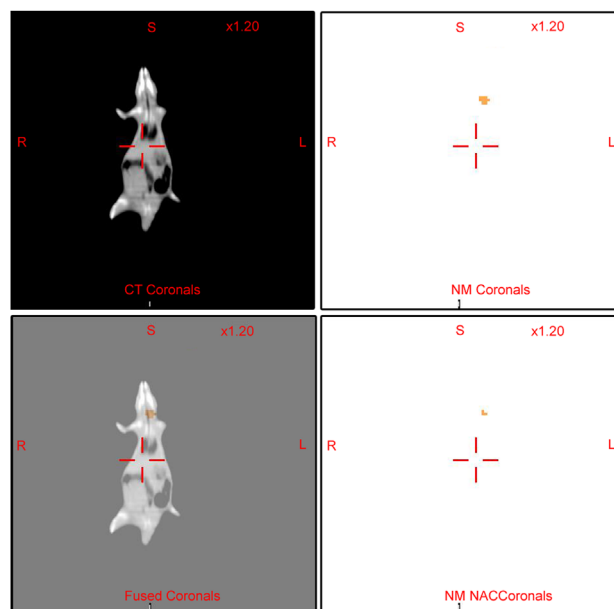


Fig. 6. The imaging of radionuclide distribution at 24 h after injection of ^{131}I -Kringle 5.

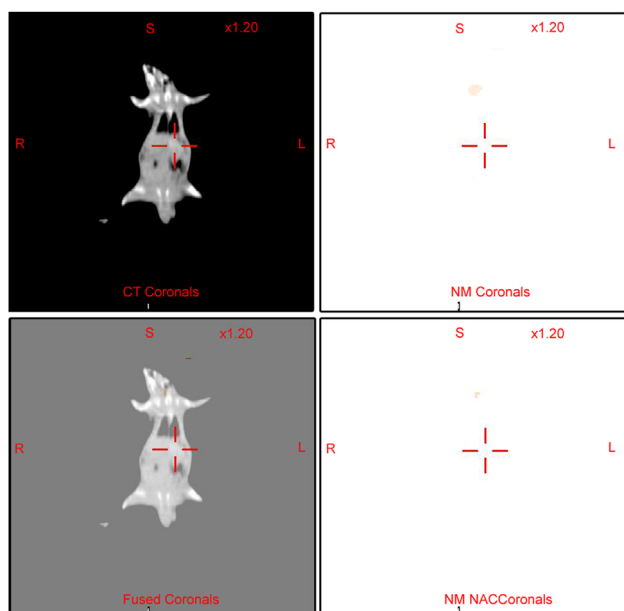


Fig. 5. The imaging of radionuclide distribution at 6 h after injection of ^{131}I -Kringle 5.

radionuclide concentrated in the bladder (Fig. 6). The results suggested that ^{131}I -Kringle 5 was mainly excreted by the kidneys. About 2 h after the injection of ^{131}I -Kringle 5, the level of radionuclide gradually decreased in the heart, kidneys and liver, while an amount of radionuclide was concentrated in the bladder. The radionuclide in vivo was completely metabolic away within 24 h, and reached normal levels.

4. Discussion

In the process of tumor growth and metastasis, recent studies have indicated that there is sustained and uncontrolled angiogenesis, which plays an important role in tumor growth, invasion and metastasis. It is also found that angiogenesis is a complex

process, which includes activation, proliferation and migration of endothelial cell, and damage of vascular basement membrane, and the formation of new blood vessels and vascular network, and connection to the existent vascular. It further proves that angiogenesis is required for further growth of the 1–2 mm tumor; otherwise it could not continue to grow [23]. The latest research shows that Kringle 5 has a strong inhibiting effect on the angiogenesis and growth of tumor [24]. However, the distribution and metabolic characteristics of Kringle 5 in the body have not been found yet so far.

In the study, by using SPECT imaging system and SPECT/CT three dimensional tomographic fusion imaging, we obtained anatomical and functional imaging and real-time fusion imaging, and continuously and dynamically observed the absorption, distribution and excretion of recombinant angiogenesis inhibitor ^{131}I -labeled Kringle 5 in vivo.

^{131}I is one of the most common radioisotopes for labeling, but there must be a persad combined with iodine atom in the interior of marked compound. In other words, it must contain casein or histamine amine residues [17]. As protein lysates of human plasminogen fragment that could specifically prohibit the proliferation of vascular endothelium, Kringle 5 having a strong anti-angiogenic effect is a potential anticancer drug [23]. It can meet the basic requirements for radioactive iodine-labeled, because its amino acid residue contains casein group at site 64, 72, 74, being far from the active site [24]. Yin et al. [25] proved that Indogen labeling method had not any impacts on biological activity and distribution of Kringle 5 in vivo. In the meanwhile, SPECT is considered to a good way of imaging that could present the distribution of labeled drug in vivo. Both comprehensively applications of Indogen labeling and SPECT could not only reflect the biological processes of Kringle 5 in vivo, but also increase the experimental sample size. Moreover, there were higher sensitivity and feasibility compared to other non-isotopic labeling methods. Therefore, a combination of Indogen isotope labeling and SPECT technique is an excellent approach for the pharmacokinetic study of Kringle 5.

In this study, after ^{131}I -Kringle 5 was injected into the sub-lingual vein of rats, the transport rate of the drug in vivo was in accordance with open two-compartment model, that was the

concentration of the organs did not rapidly equilibrium, but delayed to some extent. It showed that Kringle 5 conducts a first-order kinetics metabolism in the dose range of 5.0–10.0 g/kg.

The results of ^{131}I -Kringle 5 pharmaceutical kinetic from SPECT/CT tomographic image showed that ^{131}I -Kringle 5 was absorbed quickly after injection and clearance quickly from the blood pool and finally most of the ^{131}I -Kringle 5 was concentrated in the bladder. This result suggested that ^{131}I -Kringle 5 was mainly excreted through the urinary system, which was difference from the other traditional ^{131}I labeled compounds such as ^{131}I -AFP monoclonal antibody which was mainly excreted through the hepatic system. These metabolic characteristics is more helps to clinic observation of ^{131}I -Kringle 5 concentration in vivo because urinary system clearance is more quickly than the hepatic system, which provides more clear SPECT/CT tomographic image contrast.

The final results in this experiment revealed that it was feasible technologically to label recombinant angiogenesis inhibitor Kringle 5 with radioactive ^{131}I . The pharmacokinetic data on ^{131}I -Kringle 5 in rats provided scientific basis for further clinical application. In the pharmacokinetic study of Kringle 5 in rats, we also used angiogenesis inhibitor Kringle 5 to explore specific target organs and metabolic level using specific markers. The pharmacokinetic study of specifically labeled Kringle 5 in vivo using SPECT imaging system also provided imaging basis of the drug metabolism. The successful application of specific Iodogen labeling method was important to study pharmacokinetics of angiogenesis inhibitor Kringle 5 in vivo, and provided a new approach for pharmacokinetic study.

This study established pharmacokinetic method of Kringle 5 in vivo, based on the specific Iodogen labeling method and SPECT/CT imaging system. The pharmacokinetic status of recombinant angiogenesis inhibitor Kringle 5 in live animals had been clearly shown in this study, which overcome the limitations of previous methods that were subject to changes of blood concentration and organ pathology after administration [23]. According to metabolic level of angiogenesis inhibitor Kringle 5 in specific target organs of live animals, we analyzed the inhibiting effect of Kringle 5 on vascular growth, and then further investigated similar effect on tumor growth [26]. It is expected that the study could provide important basis for inhibiting tumor growth.

The differences between this study and other previous studies are also presented as follows. In our study, the same experimental rats were monitored dynamically in different experimental periods, which ensure the consistency of the experimental conditions, eliminates individual variations, and keeps the accuracy of results. Especially, as optimizing the program, we also made sure “3Rs” welfare of animals [27]. Through dynamically monitoring the same experimental rats in vivo among different administration time in the experiment, which altered traditional program of observation after being killed, it could greatly reduce sample size of laboratory animals. In other words, this is a cost-effective scientific method which uses the relatively small samples to get more experimental data. There were strictly operated and guided programs for the staff during the experiment. When keeping the animal completely under anesthesia during the experiment, we endeavor to alleviate the suffering of rats to the maximum extent and provide humanistic concern. Throughout this study, we continuously improve experiment protocol, to avoid and reduce pain and anxiety of experimental animals, to ensure the reliability of the results. The whole experimental program included “3Rs” welfare of experimental animals, respects for life of them and compliances the ethical principles of experimental animals.

In summary, the applications of specific labeling ^{131}I -Kringle 5 and functional imaging of SPECT/CT establishes pharmacokinetic method of Kringle 5 in rats, and provides the new platform of real-time pharmacokinetic functional imaging in vivo.

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