Pharmacokinetics of PEGylated recombinant human endostatin (M₂ES) in rats

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Aim: M₂ES is PEGylated recombinant human endostatin. In this study we investigated the pharmacokinetics, tissue distribution, and excretion of M₂ES in rats.

Methods: ¹²⁵I-radiolabeled M₂ES was administered to rats by intravenous bolus injection at 3 mg/kg. The pharmacokinetics, tissue distribution and excretion of M₂ES were investigated using the trichloroacetic acid (TCA) precipitation method.

Results: The serum M₂ES concentration-time curve after a single intravenous dose of 3 mg/kg in rats was fitted with a non-compartment model. The pharmacokinetic parameters were evaluated as follows: $C_{max}=28.3 \mu g\cdot eq/mL$, $t_{1/2}=71.5$ h, $AUC_{(0–∞)}=174.6 \mu g\cdot eq\cdot h/mL$, $Cl=17.2 mL\cdot h^{-1}\cdot kg^{-1}$, $MRT=57.6$ h, and $V_{ss}=989.8 mL/kg$ for the total radioactivity; $C_{max}=30.3 \mu g\cdot eq/mL$, $t_{1/2}=60.1$ h, $AUC_{(0–∞)}=146.2 \mu g\cdot eq\cdot h/mL$, $Cl=20.6 mL\cdot h^{-1}\cdot kg^{-1}$, $MRT=47.4$ h, and $V_{ss}=974.6 mL/kg$ for the TCA precipitate radioactivity. M₂ES was rapidly and widely distributed in various tissues and showed substantial deposition in kidney, adrenal gland, lung, spleen, bladder and liver. The radioactivity recovered in the urine and feces by 432 h post-dose was 71.3% and 8.3%, respectively. Only 0.98% of radioactivity was excreted in the bile in 24 h post-dose.

Conclusion: PEG modification substantially prolongs the circulation time of recombinant human endostatin and effectively improves its pharmacokinetic behavior. M₂ES is extensively distributed in most tissues of rats, including kidney, adrenal gland, lung, spleen, bladder and liver. Urinary excretion was the major elimination route for M₂ES.

Keywords: recombinant human endostatin; PEGylation; trichloroacetic acid precipitation method; pharmacokinetics; drug tissue distribution; drug excretion; rats

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Introduction
Endostatin, a 20-kDa proteolytic fragment of collagen XVIII, is a potent endogenous angiogenesis inhibitor isolated from supernatant of a murine hemangioendothelioma cell line[1]. It has been widely reported that endostatin has potent inhibitory effects on endothelial cell proliferation, migration, and tube formation[2, 3]. Nucleolin, integrins, caveolin and clathrin have been demonstrated to mediate the anti-tumor effects of endostatin[4-6]. Except in endothelial cells, endostatin also has anti-tumor lymphangiogenic and anti-lymphatic metastasis functions[7-9], further supporting the conclusion that endostatin is a potent anti-tumor drug with the potential ability to restrict tumor progression and metastasis[10].

Endostatin shows a broad anti-cancer spectrum and a low toxicity in animal models[11]. Systematic administration of rh-endostatin suppressed the growth of a number of primary tumors in mice, including Lewis lung carcinoma, T241 fibrosarcoma, B16F10 melanoma[12] and renal cell carcinoma[13], without the development of resistance or apparent toxicity. However, the clinical trials of rh-endostatin were terminated at the early phase II stage given production problems and unsatisfactory therapeutic effects[14]. In 2005, ZBP-Endostatin (zinc-binding peptide-Endostatin), a modified recombinant human endostatin, was approved by China Food and Drug Administration (CFDA) for the treatment of non-small-cell lung cancer (NSCLC)[15]. ZBP-Endostatin expressed by Escherichia coli has...
been engineered to contain an additional nine-amino on its N terminus, which has been proved to enhance protein purification, solubility and stability. Because the rh-endostatin produced in the United States suffered from N-terminal truncations during Pichia pastoris expression, we speculate that both the N-terminus integrity and correct folding are critical for the stability and biological functions of endostatin[16, 17].

In clinical trials, endostatin monotherapy showed evident anti-tumor efficacy[18, 19] and exhibited a synergic activity with a favorable toxic profile in combination with chemotherapy[20]. However, the stability and retention of endostatin with a favorable toxic profile in combination with chemotherapeutic agents[21] may potentially increase its drug efficacy.

The tissue distribution and excretion pattern of M2 ES in rats after intravenous injection were evaluated via an isotype-labeled assay. The results confirmed to be accurate, reliable and reproducible.

Preparation of 125I-labeled PEGylated recombinant human endostatin
The M2 ES was radiolabeled according to the IodoGen method as previously described[29]. Briefly, 5.9 mg protein was incubated with 5 mCi Na125I in a calibrated reaction vial coated with 100 μg of iodogen reagent with gentle stirring for 12 min at 20°C. After incubation, the iodinated protein was purified on a Sephacryl S200H gel column at a flow rate of 1 mL/h with 20 mmol/L Tris buffer (pH 7.4) to separate the free 125I from the protein-bound 125I. Column fractions were collected at 3-min intervals. The radioactivity of each eluted fraction was determined by the assembled γ counter. The fractions containing 125I-labeled M2 ES were combined.

The radiochemical purity of the 125I-labeled M2 ES was determined by size-exclusive high performance liquid chromatography (SHPLC) with Superdex™ peptide 10/300GL and 0.1 mol/L NaAc buffer (pH4.0) at a flow rate of 0.5 mL/min. The radio-chromatogram was obtained from a HP1100 system with a detector of Packard Radiomatic™ Flow Scintillation Analyzer 525TR (Meriden, CT, USA).

Determination of 125I-labeled M2 ES activity
Cell migration was assessed using a modified Boyden chamber (8 μm pores, Costar). HUVEC (2.4×10⁵ cells in 100 μL) were seeded in the upper chambers. M2 ES and 125I-labeled M2 ES from 33rd and 31st eluted fractions at indicated concentrations were added in the lower chamber. Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% serum was used as the control. After incubation for 6 h at 37°C, the migrated cells were quantified by counting in randomly selected fields of each chamber. Each experiment was analyzed in triplicate.

Validation of the radioactivity determination by a TCA precipitation assay
The calibration curves were generated by adding a series of concentrations of 125I-labeled M2 ES (0.0257–16.08 ng/μL) into the blank plasma, tissues and excreta samples. After 20% TCA precipitation, the radioactivities of the samples were measured. The results (data not shown) showed good linearity ($r^2>0.999$) and precision (CV%<10%) for all samples. The ratios of radioactivity recovered from the TCA precipitated pellet were more than 90% of the added radioactivity, indicating that the 125I-labeled M2 ES mainly existed in the TCA precipitants. Therefore, the TCA precipitation method was confirmed to be accurate, reliable and reproducible.

Pharmacokinetic study
Six Wistar rats (three males and three females, 250±10 g) were injected with 125I-labeled M2 ES at a single dose of 3 mg/kg (4 MBq/kg) by iv injection via the tail vein. Blood samples were collected from the tail vein into heparinized tubes prior to administration and at 2, 4, 8, 12, 24, 72, 96, 120, 168, 216,
and 264 h after drug administration. Plasma samples in the volume of 50 μL were added with 400 μL 20% TCA to precipitate the proteins. After centrifugation, the radioactivities of precipitants and supernatants were detected to obtain the total radioactivity. The concentrations of 125I-labeled M2ES were expressed as microgram-equivalents per milliliter (μg·equ·mL⁻¹) compared to the injected drug specific activity.

**Tissue distribution study**

Twenty-four Wistar rats (twelve males and twelve females, 250±10 g) were randomly divided into 4 groups (n=6 per group) receiving a single iv bolus injection of 125I-labeled M2ES at 3 mg/kg (4 MBq/kg) via the tail vein. Blood and urine were collected and tissues were excised at 4, 36, 72, and 120 h post-dosing. The tissues included the thyroid, thymus, heart, lungs, liver, spleen, adrenal gland, kidney, bladder, testicle/uterus, intestinal contents, jejunum, adipose, muscle, bone marrow/thighbone and brain. All tissues or organs were individually weighed on an analytical balance and homogenized and then precipitated by adding 400 μL 20% TCA. After centrifugation, the radioactivities of precipitants and supernatants were detected and expressed as cpm/g or cpm/mL and Bq/g or Bq/mL, respectively. Then, the radioactivities of various tissues were expressed in weight, as microgram-equivalents per gram (μg·equ·g⁻¹), or in volume, as microgram-equivalents per milliliter (μg·equ·mL⁻¹), compared to the specific activity of injected drug. The total AUC(0,120 h) and TCA precipitation AUC(0,120 h) in various tissues were calculated in Windows Excel 7.0. The radioactivity distribution and AUC values in different tissues at indicated time points were analyzed.

**Urinary and fecal excretion**

Each of the rats (n=6, three males and three females, 250±10 g) received a single iv injection of 125I-labeled M2ES at 3 mg/kg (4 MBq/kg) and was then individually placed in a metabolic cage. The rats were provided standard food and water throughout the experiment. Total voided urine and excreted feces samples were collected from each rat at intervals of 0–24, 24–48, 48–72, 72–96, 96–120, 120–144, 144–168, 168–192, 192–216, 216–240, 240–264, 264–288, 288–336, 336–360, 360–384, 384–408, and 408–432 h post-dosing. The samples were analyzed for γ-radioactivity, such that the percentage of accumulated radioactivity to total radioactivity could be calculated.

**Biliary excretion**

Briefly, the experimental rats (n=5, two males and three females, 250±10 g) were anesthetized by ip injection with 10% chloral hydrate supplemented with ether. Following a midline abdominal incision, the common bile duct was exposed and the distal end ligated with a silk suture. The bile duct was cannulated with PE tubing for the collection of bile samples. 125I-labeled M2ES was administered as a single iv injection at 3 mg/kg (4 MBq/kg). The bile was collected at intervals of 1 h over 12 h, and the volume of each collected sample was recorded separately. Thereafter, bile was collected over 12-h intervals (thus, the total collection period was 24 h). The 125I radioactivities in the collected bile samples were determined by γ-radioactivity.

**Statistical analysis**

All data are expressed as the mean±standard deviation (SD). The pharmacokinetic analysis of the data was carried out using the non-compartmental model (Model: NCA 201) methods in WINNONLIN Version 2.1 to calculate the PK parameters. Differences in the numeric variables between the groups were assessed with a two-tailed t-test, and differences within the groups were assessed with a self-paired t-test. The data were fitted by linear regression, and the concentration-time curves were drawn with Microcal Origin software.

**Results**

**Biological activity determination of 125I-labeled M2ES**

The motility (the ratio of migrated cells in drug treatment group to control group) of the M2ES group was 64.85%, while the 125I-M2ES with 10.39 kBq/μL radioactivity group showed 63.5% migratory inhibition and the 125I-M2ES with 11.37 kBq/μL radioactivity group showed 48.3% migratory inhibition, with P-values more than 0.05 compared to the motility of the M2ES group. Therefore, 125I-M2ES was found to have a similar biological activity as unlabeled M2ES, and no significant difference was observed, indicating that 125I-labeling did not influence the activity of M2ES.

**Plasma pharmacokinetics of M2ES in rats**

The total plasma radioactivity and TCA precipitant radioactivity versus time after single iv injection at dose of 3 mg/kg are depicted in Figure 1A. The results show that no significant differences (P>0.05) were observed between the total plasma radioactivity concentration and the TCA precipitant radioactivity concentration before 168 h after drug injection. However, the total plasma radioactivity was significantly higher than the TCA precipitant radioactivity at 216 and 264 h following drug injection, indicating that small molecules of radiolabeled degradation products from parent drugs existed in the supernatants after 216 h. More than 80% of the total radioactivity was recovered in the TCA-precipitated pellets in the 2–120 h following drug injection. The ratio of the TCA precipitant radioactivity concentration to the total radioactivity concentration reached 60% at 264 h, suggesting that 125I-labeled M2ES degraded very slowly after iv administration. The corresponding pharmacokinetic parameters were generated by fitting plasma radioactivity concentration profiles to a non-compartmental model as shown in Table 1. The Cmax of the total plasma radioactivity and TCA precipitant radioactivity after iv administration were 28.3±2.4 and 30.3±2.9 μg·eq·mL⁻¹, respectively. The AUC(0,24 h) values of the total plasma radioactivity and TCA precipitant radioactivity were 166.6±7.6 and 142.0±6.5 μg·eq·h·mL⁻¹. The clearance (Cl) values were 17.2±0.9 and 20.6±1.0 mL·h⁻¹·kg⁻¹, the terminal elimination half-life (t1/2) values were 71.5±7.6 and 60.1±7.8 h, the mean residence time (MRT) values were 57.6±5.0 and 47.4±5.2 h, and the Vss values were 989.8±81.6 and 974.6±109.5 mL.
mL/kg for the total plasma radioactivity and TCA precipitant radioactivity, respectively.

**Mass balance study and the biliary excretion of M<sub>2</sub>ES**

After the iv administration of <sup>125</sup>I-M<sub>2</sub>ES to the bile-duct cannulated rats, only 0.98% of the dosed radioactivity was excreted into bile by 24 h post-dosing (Figure 1B), indicating that biliary excretion is a minor pathway for <sup>125</sup>I-M<sub>2</sub>ES in rats.

The urinary and fecal excretions of <sup>125</sup>I-M<sub>2</sub>ES in rats were also investigated following a single iv administration at a dose of 3 mg/kg (4 MBq/kg) to the bile duct intact rats. The results showed that urinary excretion was the dominant route of elimination following iv administration (Figure 1C) because the accumulative urinary excretion of <sup>125</sup>I-M<sub>2</sub>ES reached 59% of the administered radioactivity after 120 h post-dosing, while the cumulative fecal excretion was only 5.0%. Over the 432 h collection period, a mean of 79.6% of the radioactive dose was recovered from the excreta. The bulk (71.3%) was eliminated in the urine, and only a minority (8.3%) was eliminated in the feces.

**Tissue distribution of M<sub>2</sub>ES in rats**

After a single iv injection at a dose of 3 mg/kg in rats, the tissue distributions of <sup>125</sup>I-M<sub>2</sub>ES at 4, 36, 72, and 120 h and the total radioactivity [AUC (0–120 h)] in the tissues were evaluated (Figure 2). The radioactivity levels in order of AUC values from high to low were urine, kidney, adrenal gland, serum, lung, spleen, bladder, jejunum, intestinal contents, heart, liver, testicle/uterus, bone marrow/thighbone, thymus, muscle, adipose and brain. The radioactive <sup>125</sup>I-M<sub>2</sub>ES showed substantial disposition in the urinary excretion system, highly perfused tissues and serum, while the radioactivity levels in the muscle, adipose and brain were the lowest. The radioactivity in the thyroid gland was only 0.0025%±0.0007% at 4 h and 0.0005%±0.0002% at 120 h of the dosed radioactivity after administration, which did not influence the reliability of the conclusions in the <sup>125</sup>I-M<sub>2</sub>ES tissue distribution.

**Table 1. Pharmacokinetic parameters of <sup>125</sup>I-M<sub>2</sub>ES after single iv administration at the dose of 3 mg/kg in rats.** Data are expressed as mean±SD. n=6.

| Parameter | Unit                          | Total radioactivity | TCA precipitant radioactivity | P value |
|-----------|-------------------------------|---------------------|------------------------------|---------|
| C<sub>max</sub> | μg·equ·mL<sup>-1</sup> | 28.3±2.4 | 30.3±2.9 | 0.2301 |
| t<sub>1/2</sub> | h               | 71.5±7.6 | 60.1±7.8 | 0.0277 |
| AUC<sub>(0–264 h)</sub> | μg·equ·h·mL<sup>-1</sup> | 166.6±7.6 | 142.0±6.5 | 0.0001 |
| AUC<sub>(0–∞)</sub> | μg·equ·h·mL<sup>-1</sup> | 174.6±8.7 | 146.2±6.9 | 0.0001 |
| CI | mL·h<sup>-1</sup>·kg<sup>-1</sup> | 17.2±0.9 | 20.6±1.0 | 0.0001 |
| MRT | h               | 57.6±5.0 | 47.4±5.2 | 0.0063 |
| V<sub>ss</sub> | mL/kg            | 989.8±81.6 | 974.6±109.5 | 0.7907 |

C<sub>max</sub>, maximum plasma concentration; t<sub>1/2</sub>, terminal elimination half-life; AUC, area under the plasma concentration versus time curve; CI, systemic clearance; MRT, mean residence time; V<sub>ss</sub>, volume of distribution at steady state. P values are obtained by evaluating the pharmacokinetic parameters between total plasma radioactivity and TCA precipitant radioactivity.
marrow/thighbone, muscle, thymus, adipose tissue and brain. With the exception of the kidney and adrenal gland samples, the highest deposition was found in the serum, which was consistent with the prediction that highly perfused tissues showed greater radioactivity. The radioactivities in adipose and brain were the lowest, and drugs were hardly transported across the blood-brain barrier. The radioactivities in most tissues reached the maximum level by 4 h after administration, decreased rapidly in the 4–36 h after dosing, and decreased slowly in the 72–120 h after dosing.

The ratio of the TCA precipitant radioactivity to the total radioactivity reflected the amounts of $^{125}$I-M$_2$ES parent drug binding to various tissues. The ratios of TCA precipitant radioactivity to total radioactivity at different time points after drug injection are shown in Table 2. The data showed that 70%–90% of the additional radioactivity was recovered in the TCA precipitants in most tissues at 24 h after drug administration and 60%–90% at 120 h after dosing. The ratio of the TCA precipitant radioactivity to the total radioactivity in the serum remained greater than 74% over time, while the ratio of the TCA precipitant radioactivity to the total radioactivity in urine was approximately 7%–11%, indicating that urine mainly contained the soluble degradation products of $^{125}$I-M$_2$ES.

| Tissue                | 24 h (%) | 48 h (%) | 96 h (%) | 120 h (%) |
|-----------------------|----------|----------|----------|-----------|
| Thymus                | 63.8±6.9 | 55.0±4.7 | 67.1±9.3 | 62.3±10.4 |
| Heart                 | 87.6±3.9 | 69.3±2.7 | 79.5±5.4 | 74.6±4.3  |
| Lung                  | 82.7±10.3| 83.0±5.9 | 71.5±23.2| 74.4±4.4  |
| Liver                 | 75.1±6.7 | 80.1±10.1| 88.3±3.1 | 85.8±3.9  |
| Spleen                | 86.3±3.2 | 84.5±7.8 | 84.6±3.1 | 74.6±3.5  |
| Adrenal gland         | 84.8±3.2 | 86.9±10.6| 77.6±23.2| 71.6±2.0  |
| Kidney                | 92.2±1.1 | 94.2±2.4 | 95.2±2.0 | 92.2±3.9  |
| Bladder               | 59.4±3.9 | 77.5±8.0 | 83.3±2.8 | 75.4±3.2  |
| Testicle/uterus       | 79.5±8.3 | 62.8±12.2| 79.6±3.9 | 72.8±6.1  |
| Intestinal contents   | 36.7±12.9| 59.2±18.8| 67.3±8.5 | 60.9±6.2  |
| Jejunum               | 66.7±11.7| 71.2±9.4 | 74.7±6.6 | 65.7±7.1  |
| Adipose               | 73.6±4.4 | 79.7±6.3 | 74.1±3.2 | 70.2±5.8  |
| Muscle                | 70.2±12.1| 74.6±2.4 | 74.4±4.4 | 71.8±3.1  |
| Bone marrow/thighbone | 76.0±6.5 | 64.4±6.3 | 70.6±6.4 | 69.3±5.0  |
| Brain                 | 60.7±4.5 | 58.3±12.0| 70.8±5.7 | 65.8±5.0  |
| Plasma                | 86.7±4.0 | 74.0±4.4 | 87.7±3.2 | 85.1±3.8  |
| Urine                 | 9.7±7.8  | 10.3±4.1 | 7.4±3.3  | 10.7±2.7  |
Discussion

The evaluation of the pharmacokinetics profile, tissue distribution and excretion patterns for a novel drug are essential for its preclinical and clinical application. To the best of our knowledge, this is the first study to report the plasma pharmacokinetics, tissue distribution and excretion patterns for PEGylated rh-endostatin (M\(_2\)ES) in rats.

The TCA precipitation assay is a common method in the pharmacokinetic studies of protein drugs. In this study, the TCA precipitation radioactivity and the total radioactivity were measured to evaluate the M\(_2\)ES concentration in the plasma and in different tissues. Because the interference of free \(^{125}\)I and soluble degradation products of \(^{125}\)I-M\(_2\)ES was excluded, the results of TCA precipitation can better reflect the concentration of parent M\(_2\)ES. The radioactivity concentrations of \(^{125}\)I-M\(_2\)ES in the TCA precipitants were lower than those in the total radioactivity concentrations in both the plasma and other tissues, but they followed approximately the same trend.

It has been reported that PEG conjugation can protect protein drugs against enzymatic digestion, slow their filtration by the kidneys and reduce the generation of neutralizing antibodies, therefore increasing the retention of the drugs in circulation. In previous studies, the pharmacokinetics of rh-endostatin were reported in rats and rhesus monkeys\(^{30,31}\). The half-lives of rh-endostatins (4.5 mg/kg) in rats and in rhesus monkeys were 3.91 and 3.1 h, respectively. PEGylation significantly prolonged the half-life of rh-endostatin to 60.1 h at a dose of 3 mg/kg. Significant differences were also observed in CI and AUC\(_{0–\infty}\) among pharmacokinetics parameters between M\(_2\)ES in rats and rh-endostatin in rats and rhesus monkeys. The clearance of rh-endostatin (1.5 mg/kg) was 140.4 mL h\(^{-1}\) kg\(^{-1}\) in rats and 110 mL h\(^{-1}\) kg\(^{-1}\) in rhesus monkeys, while the clearance of M\(_2\)ES (3 mg/kg) in rats was 20.6 mL h\(^{-1}\) kg\(^{-1}\), which was much slower than those of rh-endostatin in rats and rhesus monkeys. The slower disappearance of M\(_2\)ES resulted in greater values for the area under the serum concentration-time curve, with an AUC\(_{0–\infty}\) value of 146.2±6.9 μg·Equ·h·mL\(^{-1}\) at the dose of 3 mg/kg, compared to those of rh-endostatin (4.5 mg/kg) in rats and rhesus monkeys, which were 28.91 and 33 μg·h/mL, respectively. The comparison of the pharmacokinetic parameters of rh-endostatin and M\(_2\)ES demonstrated that PEGylation indeed improved rh-endostatin properties and prolonged its residence time in bodies. It may thereby improve its therapeutic potential.

The tissue distribution of M\(_2\)ES was investigated following a single iv administration in rats. Widely distributed radioactivities were observed in various tissues and organs within the time course examined. The levels of radioactivity in most tissues decreased rapidly over time. The AUC\(_{0–\infty}\) distribution

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**Figure 3.** M\(_2\)ES tissue distribution. (A–C) The tissue distribution of TCA precipitant radioactivity at 4, 36, 72, and 120 h after single iv administration of \(^{125}\)I-M\(_2\)ES at the dose of 3 mg/kg in rats. The data are expressed as the mean±SD. n=6. (D) Distribution of TCA precipitant radioactivity [AUC(0–120 h)] in tissues after the iv administration of \(^{125}\)I-M\(_2\)ES at the dose of 3 mg/kg in rats. n=6.
of individual tissues in the TCA precipitant peptide showed that except for plasma and urine, the majority of the radioactivity was detected in highly perfused tissues and organs, with the highest deposition levels detected in the kidney, followed by the adrenal gland, lung, spleen, bladder and liver. For the clearance organs, the kidney and liver showed high absorption levels of M2ES, which were also reported in studies of rh-endostatin and other proteins such as angiostatin and porcine fibrinogen. In this study, the radioactivity detected in the kidneys was greater than that in the liver, indicating that the kidney played a more important role in the elimination of M2ES and its metabolites, which was consistent with the results in the excretion studies showing that most M2ES was eliminated in the urine. Therefore, monitoring renal function is necessary when M2ES is used in the clinic, and adjusting the dosage is needed to avoid adverse effects in patients whose kidney functions are impaired. Moreover, high levels of radioactivity were also found in the lungs, and these decreased slowly compared to other tissues, which was similar to previous findings for rh-endostatin. This potentially provided evidence for the application of endostatin in the treatment of lung cancer.

In conclusion, we developed and validated a sensitive 125I-labeled method to investigate the pharmacokinetics, tissue distribution and excretion of M2ES. According to our pharmacokinetic parameters, PEG conjugation significantly enhanced the circulation retention of rh-endostatin, resulting in a longer half-life (t1/2) and slower clearance (Cl). Following intravenous injection, M2ES was widely distributed in most tissues, and urinary excretion was the dominant route for elimination. The present pharmacokinetics study provides helpful information for the application of M2ES in the clinical setting.

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Author contribution
Zuo-gang LI, Lin JIA, Li-fang GUO, Jun-zhi WANG, and Yong-zhang LUO designed the research; Zuo-gang LI, Lin JIA, Li-fang GUO, Min YU, Xu SUN, and Wen NIE performed the research; Zuo-gang LI, Lin JIA, Li-fang GUO, Yan FU and Chun-ming RAO analyzed the data; Zuo-gang LI, Lin JIA, Li-fang GUO, Jun-zhi WANG, and Yong-zhang LUO wrote the paper.

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