Full-length article

Bioactivity, pharmacokinetics, and immunogenicity assays in preclinical and clinical trials for recombinant human endostatin¹Bi HU², Hao-wen ZHU⁴, Li-ping ZHU², Chen LI², Zhi-gang RONG⁴, Jia-ming XU⁴, Zhi-wei WU³, Jian-jun WANG^{2,5}, Gen-xing XU^{3,4,5}²Department of Biological Science and Technology and State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210093, China; ³Center for Public Health Research, Nanjing University, Nanjing 210093, China; ⁴Jiangsu Research Center for Gene Pharmaceutical Engineering and Technology, Suzhou 215128, China**Key words**

recombinant human endostatin; bioactivity; pharmacokinetics; immunogenicity

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Abstract

Aim: To determine the *in vitro* and *in vivo* bioactivity of recombinant human endostatin (rhEndostatin) and to analyze its pharmacokinetics and immunogenicity in rhesus monkeys and patients. **Methods:** The physical chemical characteristics of rhEndostatin were detected according to *Pharmacopoeia of the People's Republic of China* (2005 edition, part III). Its *in vitro* and *in vivo* bioactivities were assayed via proliferation–inhibition on human umbilical vein endothelial cells and their inhibitory effect on tumor-bearing mice models. Serum concentrations of rhEndostatin in monkeys and patients were determined by an enzyme immunoassay method. **Results:** The corresponding specific *in vitro* activities of rhEndostatin obtained from the cell counting method, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and lactate dehydrogenase assay, respectively, were 6.4×10^7 , 6.7×10^7 , and 3.8×10^8 U/mg, and the *in vivo* antitumoral potency was 4.04×10^7 U/mg. In rhesus monkeys, there were no gender differences in all pharmacokinetic parameters. Serum anti-rhEndostatin immunoglobulin (Ig)G antibodies were generated quickly after intravenous (iv) administration and decreased rapidly when therapy was stopped. In phase I clinical trials, linearity in the pharmacokinetics of rhEndostatin was indicated by dose-proportionate increases in the area under the curve and the maximum serum concentration. Serum rhEndostatin reached a steady-state level after 7 d of successive administration with the average concentration at a steady state of 272.44 ± 91.98 ng/mL. Neither IgG nor IgM antibodies against rhEndostatin were observed in patients. **Conclusion:** RhEndostatin exhibited a definite proliferation–inhibition effect on HUVEC, and significant antitumoral activity in mice. The immunoreactivity of rhesus monkeys to rhEndostatin is common, and rhEndostatin showed no immunogenicity in patients in this trial. The results provide a basis for further clinical trials.

Introduction

Endostatin has attracted wide attention due to its specific inhibition of new vessel generation since its discovery by O'Reilly *et al* in 1997^[1]. Human endostatin is the NC1 domain of the $\alpha 1$ chain of type XVIII collagen with 183 amino acids^[2], and has been shown to have an inhibitory effect on new vessel formation in many experimental

models, including the chick embryo allantochorion, rat cornea, and arterial circle cultured *in vitro*^[1,3,4]. Several studies have also proven that endostatin has an inhibitory effect on vessel generation in tumors and in pathological vascular inflammation in a definite dose-dependent manner without any apparent side-effects, toxicity, or developmental drug resistance^[1,5–7]. In addition, endostatin directly targets vascular endothelial cells whose hereditary feature

is stable. Therefore, the chance of inducing drug resistance is markedly reduced. Due to the complexity of the therapeutic efficacy, recent studies have focused on the bioactivity of recombinant proteins of human endostatin^[8–11]. With the development of recombinant DNA technology, many highly-effective expression and purification systems have been established to obtain recombinant human endostatin (rhEndostatin) with high purity^[1,4,12].

Several reports have indicated that no toxic effects or resistance to therapy was observed even after prolonged therapy with endostatin in experimental mouse models^[6,13,14]. RhEndostatin has also been proven to be biologically active and non-toxic in preclinical pharmacology and toxicology studies^[15,16]. The results provide the impetus to initiate clinical trials of endostatin in patients with cancer. Some clinical trials have been performed during the past decade with the results demonstrating that rhEndostatin ranges from no apparent clinical activity (eg advanced neuroendocrine) to significant tumor inhibition though all clinical trials, showing high safety, rare untoward reactions or anaphylactic responses, and no drug resistance^[17–25]. The differences of the expression system, protein structure, purification means, and tumor types may be responsible for the discrepancy in the bioactivity of rhEndostatin.

In our previous work, we expressed, purified, and assayed the bioactivity of rhEndostatin^[26]. In the present study, we report on further tests of purified rhEndostatin expressed from *Escherichia coli* according to *Pharmacopoeia of the People's Republic of China* (2005 edition, part III)^[27]. We measured the *in vitro* bioactivity and *in vivo* antitumoral activity of rhEndostatin, and then analyzed the pharmacokinetics and immunogenicity of rhEndostatin in rhesus monkeys and patients. The results of these studies are described in this report.

Materials and methods

Samples The rhEndostatin samples were supplied by ZhongKai Bio-Pharma Co, Ltd (Suzhou, China).

Analysis of rhEndostatin preparations

Molecular weight test and purity determination^[27]

A mass spectrographic analysis was performed by the National Biomedicine Analysis Center (Beijing, China). SDS-PAGE was prepared and performed according to Appendix IVC of *Pharmacopoeia of the People's Republic of China* (2005 edition, part III). The samples were analyzed for the molecular weight of the rhEndostatin protein by 12.5% reduced SDS-PAGE. Proteins were stained with Coomassie brilliant blue R250.

Chromatographic conditions for reverse phase HPLC were performed according to Appendix IIIB of *Pharmacopoeia of the People's Republic of China* (2005 edition, part III). The fixed phase involved water symmetry C4, and the mobile phases involved 0.1% trifluoroacetic acid (TFA)-water (1:1000, v/v) and TFA-acetonitrile (ACN)-water (1:950:50, v/v/v), respectively. After balance, the sample (10 μ L) was added to the column, and the flow rate was set at 1.0 mL/min. The separation and the analysis were conducted at 30 °C, and the detection wave length was set at 280 nm.

N- and C-terminal sequence assay and amino acid composition assay The N- and C-terminal sequence of rhEndostatin was analyzed by an amino acid sequenator, and the analysis was performed by the National Biomedicine Analysis Center. The amino acid composition was determined by an amino acid composition analyzer by the State Key Laboratory of Pharmaceutical Biotechnology of Nanjing University (Nanjing, China).

Peptide mapping and mass peptide spectrum analysis^[27] Peptide mapping analysis was done based on the Appendix VIIE of *Pharmacopoeia of the People's Republic of China* (2005 edition, part III). Briefly, the rhEndostatin samples were analyzed by reverse-phase HPLC after trypsin digestion. The chromatographic conditions for reverse-phase HPLC were the same as those mentioned earlier. The mass spectrum analysis of the peptides was determined by mass spectrometer, and the analysis was carried out by the Drug and Biological Product Standardization Research Center of China (Beijing, China).

Measurement of other parameters^[27] Other parameters, in accordance with the requirement and methods of the Appendix of *Pharmacopoeia of the People's Republic of China* (2005 edition, part III) were also measured. They included pH values (Appendix VA), residual antibiotics (Appendix IXA), exogenous DNA content (Appendix IXB), host cell protein content (Appendix IXC), sterility test (Appendix XIA), pyrogen test (Appendix XIID), residual endotoxin content (Appendix XIIE), and undue toxicity test (Appendix XIIF). In addition, a disulfide bond allocation analysis was carried out by the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

In vitro activity study

Preparation and culture of human umbilical vein endothelial cells Human umbilical vein endothelial cells (HUVEC) were separated from the cord blood of neonates. Neonate cord blood was obtained and kept in preservation solution (supplemented with 140 mmol/L NaCl, 4 mmol/L

KCl, 5.2 mmol/L Na₂HPO₄, 1.5 mmol/L KH₂PO₄, 110 mmol/L glucose, and 10⁵ U/mL penicillin-streptomycin). After repeated rinsing with phosphate-buffered saline (PBS) containing penicillin (100 U/mL) and streptomycin (100 µg/mL), the umbilical vein was perfused with collagenase II prepared in Hanks' solution for 20 min and rinsed with culture medium. The wash was pooled and centrifuged to recover HUVEC. The recovered HUVEC were cultivated in complete RPMI-1640 medium (supplemented with 20% fetal bovine serum [FBS], 2 ng/mL basic fibroblast growth factor (bFGF), 1% bovine serum albumin [BSA], 90 µg/mL heparin, 100 U/mL penicillin, and 100 µg/mL streptomycin) at 37 °C in a humidified 5% CO₂ atmosphere.

Detection of proliferation-inhibition effect of rhEndostatin on HUVEC The rhEndostatin solutions were prepared with NaAc/HAc buffer (pH 7.0; 10 mmol/L) to various concentrations of 31.25, 62.5, 125, 250, 500, 1000, 2000, and 4000 ng/mL before use. According to a previously-established protocol^[26], the plasmid pcDNA3.0-vascular endothelial growth factor (VEGF) was transfected using Lipofectamine 2000 as instructed by the manufacturer. Stable transfectants (HUVEC carrying plasmid pcDNA3.0-VEGF) were selected and preserved for future use after routine identification. The transfectants were seeded onto 96-well plate at 1×10⁴/pool and maintained in complete RPMI-1640 medium with 10% FBS at 37 °C in a humidified 5% CO₂ atmosphere for 24 h. RhEndostatin (150 µL/well) was then added at serial concentrations, while an equal amount of buffer was added to the control group. Each concentration of rhEndostatin had 9 repeat wells (3 wells per method). Seventy-two hours later, the number of cells was counted under a microscope after treatment with 0.05% trypsin (0.5 mL) or assayed using colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or lactate dehydrogenase (LDH) as the substrate following the manufacture's instructions^[28]. The proliferation-inhibition rate of rhEndostatin on HUVEC at each concentration was calculated using the following formula:

$$\frac{\text{Cell number or OD value of (control group - treatment group)}}{\text{Cell number or OD value of control group}} \times 100\% \quad (1)$$

where OD is the optical density.

Dosage linear regression analysis and the effective concentration for 50% inhibition of cell proliferation potency assay The proliferation-inhibition rate of HUVEC was performed as the ordinate, and the according logarithm of each concentration of rhEndostatin as the abscissa to make the linear regression. The regression

equation $y=a+bx$ was obtained, where a and b represent the intercept and slope rate of each regression curve, respectively. The effective concentration for 50% inhibition of cell proliferation (IC₅₀) was calculated from the regression equation. The dose of IC₅₀ representing 1×10⁵ units was obtained according to the amount and concentration of the samples, and the specific activity was deduced from IC₅₀.

***In vivo* activity study^[29]**

Animals and tumor cells Six-to-eight-week-old male Kunming mice (grade SPF; 20±2 g), were purchased from the Animal Center of Nanjing Medical University (Nanjing, China). The animals were supplied with laboratory chow and water *ad libitum* and kept in an animal facility maintained at 22 °C on a 12 h light/dark cycle. H22 murine hepatoma cells were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The experiments were carried out by the Animal Welfare Committee of the institutions.

Tumor growth inhibition of rhEndostatin A liver tumor model was established by a subcutaneous injection of H22 tumor cells resuspended in PBS (1×10⁶ cells/0.2 mL) into the flank of each mouse^[30]. Forty mice were divided into 4 groups ($n=10$ in each group). The control group received diluents (10 mmol/L NaAc/HAc buffer, pH 7.0); the other 3 groups received rhEndostatin reference preparation at doses of 5, 10, and 20 mg/kg, respectively. Each mouse was intravenously injected with the diluents of rhEndostatin via the tail vein daily for 7 continuous days. The animal experiment was repeated 12 times. The tumor growth inhibition rate (IR) was determined by the following formula:

$$\frac{\text{Tumor weight of control group} - \text{tumor weight of treatment group}}{\text{Tumor weight of control group}} \times 100\% \quad (2)$$

In order to quantify the antitumoral activity of rhEndostatin and compare the *in vivo* potency with the *in vitro* activity, we assumed that the estimated effective dose for 50% inhibition of tumor growth (ED₅₀) represented 1×10⁷ units, and the potency of each rhEndostatin preparation was calculated using the following formula:

$$\text{Potency (U)} = \frac{\text{rhEndostatin (1 mg)}}{\text{ED}_{50} \text{ value (mg/kg)} \times \text{mouse weight (0.02 kg)}} \times 10^7 \quad (3)$$

Pharmacokinetics and immunogenicity of rhEndostatin in rhesus monkeys

Animals Rhesus monkeys (weighing 5.5±1.2 kg, approximately 5 years old, grade I, certificate SCXK [Su] 2002-0007) were provided by the Suzhou Xishan Laboratory (Suzhou, China). The animals were individually housed in stainless steel cages and fed a standard monkey

diet; water was available *ad libitum*. The Tianjin Drug Academy (Tianjin, China) conducted the pharmacokinetic study, and Shanghai Medicine Industry Institute (Shanghai, China) made the immunogenicity assays for the preclinical trial, respectively. The experiments were carried out by the Animal Welfare Committee of the institutions.

Pharmacokinetics Six rhesus monkeys were administered rhEndostatin at the dose of 3 mg/kg by ulnar vein over a 30 min period. Whole blood samples (1.5 mL) were drawn from the saphenous veins of the animals at the following time points: before dose administration (0 h), during first infusion (0.083, 0.25, and 0.5 h), and after the first dose (0.583, 0.75, 1, 1.25, 1.5, 2.5, 4.5, 8.5, 12.5, 24.5, and 48.5 h) of rhEndostatin. The blood was centrifuged at $3000\times g$ for 10 min, and the separated serum was stored at -20°C until assayed.

The human endostatin protein accucyte enzyme immunoassay kit (Cytimmune Sciences, Germany) was used to determine the rhEndostatin levels in the serum samples. The assay was performed according to the operating instructions specified by the manufacturer. A series of calibration standards were set in each microplate and the concentration range on the approaching straight line segment was utilized to assess the relative recovery and precision of rhEndostatin. The rhEndostatin levels in the unknown samples were obtained by calculation from the calibration standard curves on the same microplate. The serum drug concentration-time data were analyzed by a compartmental analysis using the practical pharmacokinetic program 3p97 (Chinese Pharmacological Society and the Mathematic and Pharmacology-Professional Committee, Beijing, China).

Serum antibodies to rhEndostatin in rhesus monkeys Twenty-four rhesus monkeys were divided into 4 groups ($n=6$ per group). One negative control group was administered with physiological saline (10 mL/kg) and 3 treated groups were administered rhEndostatin at doses of 6, 20, and $60\text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for 90 d using an iv drip, respectively. The serum samples were obtained at the following time points: before dose administration, 1, 2, 4, 11, and 13 weeks after the first dose, and 4 weeks after treatment.

ELISA was used to assess the immunogenicity of rhEndostatin during therapy. The assay was performed according to the guidelines of the procedure specified by the manufacturer. The serum samples were tested at dilutions of 1:40, 1:80, 1:160, and 1:320. In total, 200 μL horseradish peroxidase (HRP)-conjugated Staphylococcal protein A (SPA) at a dilution of 1:40 was added as a second antibody, and tetramethylbenzidine (TMB) was used as a substrate. Each sample was measured in duplicate. The OD_{450} was

determined after 30 min of incubation. The data were analyzed to determine an endpoint titer of immunoreactivity.

Pharmacokinetics and immunogenicity of rhEndostatin in patients

Healthy volunteers, patients, and administration^[31] The eligibility criteria excluded those who were pregnant or breast feeding or had a history of cardiac, hepatic, nephritic, or hematological diseases, those aged between 18 and 45 years, and those weighing above 50 kg. Twenty-four volunteers were divided into 3 groups ($n=8$ per group) and received rhEndostatin intravenously over a 2 h period at a single administration of 60, 120, and $180\text{ mg}/\text{m}^2$, respectively.

The eligibility criteria included patients aged between 18 and 65 years with progressive advanced tumors, for whom no standard treatment was available. The patients had to have a life expectancy of at least 3 months and an Eastern Cooperative Oncology Group performance status of 0 or 1. Patients were excluded if they were pregnant or breast feeding, had a history of a primary or metastatic brain tumor, or had a history of hematological diseases. The patients received a 28 d continuous iv infusion of rhEndostatin at dose of 10 ($n=10$), 20 ($n=3$), and $30\text{ mg}/\text{m}^2$ ($n=3$), respectively.

All volunteers and patients were informed about the experimental nature of this program and signed an approved informed consent form before therapy with rhEndostatin, as required by the institutional review boards of the respective institutions. The phase I clinical trial (lot 2005L02614) was licensed by the State Food and Drug Administration of China and carried out in Tianjin Tumor Hospital (Tianjin, China).

Pharmacokinetics of rhEndostatin in healthy volunteers and patients^[31] In the single-dose group, whole blood samples (3 mL) were obtained before dose administration at 1, 5, 15, and 30 min, and 1, 2, 4, 8, 12, 24, 48, and 72 h after the first injection of rhEndostatin. In the successive administration of rhEndostatin ($10\text{ mg}/\text{m}^2$) group, whole blood samples (3 mL) were obtained on d 1 and 7 before and at 15, 30, and 45 min, and 1, 1.5, 2, 4, 8, 12, and 24 h after administration. They were also obtained 2 and 24 h before administration on d 3, 5, 14, and 28, and at 48 and 96 h after administration on d 28. The blood samples were centrifuged at $3000\times g$ for 10 min and the resulting serum was stored at -20°C until assayed.

The concentration of rhEndostatin in the serum specimens was measured using an enzyme immunoassay kit (R&D Systems, Minneapolis, USA). Pharmacokinetic parameters were estimated by compartmental modeling of

the serum concentration-time data obtained from each patient. Parameters such as time-to-peak concentration (T_{max}), the maximum serum concentration (C_{max}), half-lives, and clearance were calculated from the primary parameters. The area under the curve (AUC) was calculated from the start until the end of the iv infusion by the linear trapezoid method.

Serum antibodies to rhEndostatin in patients Serum samples were obtained from patients in the successive administration of rhEndostatin ($10 \text{ mg} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$) group on d 7, 14, and 28 during therapy. Serum was removed for storage at -70°C until endpoint titers of anti-rhEndostatin immunoglobulin (Ig)G and IgM antibodies were determined by ELISA. Briefly, rhEndostatin ($300 \mu\text{g/mL}$), resolved in carbonate-bicarbonate buffer (0.05 mol/L , pH 9.6) was coated in Immulon 96 plates (Costar, Corning, NY, USA) as the antigen. After blocking for 45 min at 37°C with 0.2 mL PBS containing 5% BSA and 1‰ Tween-80, 0.1 mL serum samples non-diluted and diluted at 1:2, 1:4 with PBS plus 2% BSA, 2‰ Tween-20, 1% glycerol, and 1‰ sodium azide were added in duplicate. After 2 h incubation at 37°C , the wells were aspirated and washed 3 times with washing solution (4 g NaCl , 0.1 g KCl , $1.445 \text{ g Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, $0.1 \text{ g KH}_2\text{PO}_4$, and 0.05 mL Tween-20, pH 7.2). The plates were tamped dry, and the wells were incubated for 40 min at 37°C with 0.1 mL of either a 1:300 dilution of protein A-peroxidase (Boster Biological Technology Company, Wuhan, China) or rabbit antihuman IgM (Beijing Zhongshan Jinqiao Biotechnology Co, Beijing, China). In the anti-rhEndostatin IgM antibody assay, the wells were incubated for another 40 min at 37°C with 0.1 mL of a 1:300 dilution of HRP-goat antirabbit IgG (ZyMax; ZYMED Laboratories, San Francisco, California, USA). All of the wells were then washed 3 times with the above-mentioned washing solution, and 0.1 mL TMB substrate was added. The OD_{450} was determined after 40 min of incubation. Positive results was defined as the OD_{450} value resulting in a 2-fold increase over baseline absorbance.

Statistical methods The Student's *t*-test and one-way ANOVA were employed for the statistical analysis of difference in 2 groups and multiple groups, respectively. All values were expressed as mean $\pm$ SD. and $P < 0.05$ was considered to be significant.

Results

Analysis of rhEndostatin The mass spectrographic analysis showed that the ion peak of the molecular weight (MW) of rhEndostatin was 20.227 kDa (data not shown). The MW of rhEndostatin detected by SDS-PAGE was

20.873 kDa and 20.564 kDa for 2 repeats, respectively (data not shown), thus the average value of 20.718 kDa was chosen and conformed to the theoretical value. The mass spectrum analysis of peptides showed that the MW of 11 main peptide fragments in 19 enzymolysis fragments conformed to theoretical value. The purity of rhEndostatin as measured by reversed phase (RP)-HPLC and non-reduced SDS-PAGE both exceeded 98% (data not shown). The disulfide bridges were determined to locate at Cys-33/Cys-173 and Cys-135/Cys-165, and N- and C-terminal analyses showed that the frontal 16 amino acids of the N-terminus were M-H-S-H-R-D-F-Q-P-V-L-H-L-V-A-L and the last 3 amino acids of the C-terminus were A-S-K. These results were in accordance with previous studies^[32]. Furthermore, the amino acid composition analysis revealed that the amino acid composition was consistent with previously-published results with the exception of asparagines and glutamine being replaced by aspartate and glutamate, respectively. Rypthophane was destroyed by acid hydrolysis. The amino acid sequence of rhEndostatin was consistent with the sequence deduced by the DNA sequence. Peptide mapping of 3 batches of rhEndostatin digested by L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (TPCK)-TRYPSIN was completely identical (Figure 1). All parameters measured in this study conformed to the relevant regulations as shown in Table 1.

Proliferation-inhibition of rhEndostatin on HUVEC

In the current study, the proliferation of HUVEC was significantly suppressed when concentrations of rhEndostatin reached 1000 ng/mL in any method used. A dose-dependent inhibition of proliferation was observed in the treated groups, while no proliferation-inhibition occurred in the

Figure 1. Peptide mapping result of rhEndostatin.

Table 1. Analytic results of rhEndostatin preparations.

Test	Requirement	Result
Peptide mapping	Identical for batches	Qualified
Purity (SDS-PAGE)	≥96.0%	>98%
Purity (HPLC)	≥97.0%	>98%
Molecular weight (MS)	20.0 kDa±10%	20.227 kDa
Molecular weight (SDS-PAGE)	20.0 kDa±10%	20.718 kDa
Isoelectrical point	9.3±0.5	9.6
Maximal UV absorbance wave length	282±3	283
Residual DNA content (ng/15mg)	<0	<10
Residual host cell protein content	<0.1%	0.0056%
Residual antibiotic activity	Negative	Negative
Endotoxin (EU/mg)	<10	<10
N-terminal acid sequence	Conform to theoretics	Qualified
C-terminal acid sequence	Conform to theoretics	Qualified
<i>In vitro</i> specific activity(U/mg)	≥5.0×10 ⁵	Qualified

control group. For the proliferation-inhibition assessment, the MTT colorimetric method was shown to have lower sensitivity and efficiency than the LDH colorimetric method ($P<0.05$ and $P<0.01$, respectively) because of its lower OD values under specific absorbance wavelengths (Figure 2). These results were consistent with those of our previous observation^[26].

Although the stability for passages of HUVEC transfected with VEGF was improved and the deviation induced by the operation was reduced without stimulation by the addition of bFGF or VEGF in the culture, the cell proliferation-inhibition rate of rhEndostatin measured by different methods were shown to be highly variable (Table 2). Thus the *in vitro*-specific activity of rhEndostatin determined by the cell counting method, MTT assay, and LDH assay were 6.4×10^7 , 6.7×10^7 , and 3.8×10^8 U/mg, respectively, and the corresponding regression coefficient was 0.95, 0.93, and 0.98, respectively, as shown in Table 2. Therefore, we could conclude from the regression coefficient that comparatively, the LDH assay was the most sensitive and effective method.

Owing to the significant differences among the *in vitro* activity assays, it is necessary to develop *in vivo* activity assays for the quantification of bioactivity for rhEndostatin.

***In vivo* antitumoral potency of rhEndostatin** After treatment with rhEndostatin at doses of 5, 10, and 20 mg/kg or 10 mmol/L NaAc/HAc buffer daily for 7 continuous days, the tumor-bearing mice were killed. The tumors were then excised and weighed after an additional 3 d following treatment. The analysis was repeated 12 times. The values of the y intersection (a), the slope (b), and correlation coefficient (r) of each regression line

Figure 2. Proliferation-inhibition of different concentrations of rhEndostatin on HUVEC measured by the cell counting method (A), MTT assay (B), and LDH assay (C). Every concentration had 6 duplicate wells of cells, and the same volume of 10 mmol/L NaAc/HAc buffer was added as the control. (■), control group; (▲) treated group. Significant differences between the control and treated groups were evaluated using Student's *t*-test, ^b $P<0.05$, ^c $P<0.01$.

were analyzed using the least-squares linear regression analysis. The averaged values were 20.16, 2.42, and 0.98 and the standard deviations were 3.79, 0.39, and 0.02, respectively, suggesting that the potencies determined by the ED₅₀ potency assay were reproducible. The results demonstrated that rhEndostatin significantly inhibited the growth of the H22 tumor and its inhibitory effect was dose-dependent. The tumor inhibition rate and the *in vivo* potency of rhEndostatin calculated from the corresponding formula are shown in Table 3.

Pharmacokinetics of rhEndostatin in rhesus monkeys and patients In preclinical trials, the concentration-time curves of rhEndostatin were best fitted to a 2-compartment open model. Following the repeated iv administration of rhEndostatin over a 90 d period (once daily) at the dose of 3 mg/kg, the serum concentration of rhEndostatin

Table 2. Mean cell proliferation-inhibition rate (MCIR) and *in vitro* activity of rhEndostatin.

Items of <i>in vitro</i> assay	Cell counting method*	MTT assay*	LDH assay*
MICR at 8 concentration (ng/mL)			
31.25	5.0%	5.1%	5.4%
62.5	2.3%	8.8%	4.8%
125	8.6%	10.7%	9.4%
250	12.6%	12.3%	17.4%
500	20.1%	15.0%	32.8%
1000	23.5%	28.4%	46.4%
2000	30.7%	35.3%	52.5%
4000	53.4%	50.0%	62.3%
IC ₅₀ (ng/mL)	10444	9914	1774
Specific activity (U/mg)	6.4×10 ⁷	6.7×10 ⁷	3.8×10 ⁸
Regression coefficient	0.95	0.93	0.98

* Replication=3

in the rhesus monkeys significantly decreased after the injection, with an initial half-life ($t_{1/2\alpha}$) of 0.293 ± 0.096 h, and a terminal elimination phase ($t_{1/2\beta}$) half-life of 6.0 ± 1.7 h. A serum concentration-time profile is shown in Figure 3A. The mean area under the serum concentration-time curve ($AUC_{(0-\infty)}$) was 10.31 ± 1.98 h·μg/mL, C_{max} was 5.14 ± 0.88 μg/mL, and the systemic clearance (Cl_s) was 325 ± 53 mL·kg⁻¹·h⁻¹. No gender difference was found in the pharmacokinetic studies of rhesus monkeys as illustrated in Table 4. The main pharmacokinetic data are shown in Table 5.

In clinical trials^[31], patients treated with $10\text{ mg}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ rhEndostatin, given as a 2 h iv infusion for 28 d, were included in the pharmacokinetic analysis. A serum concentration-time curve from 0 to 24 h was determined after the administration of the first dose of rhEndostatin, as shown in Figure 3B. The serum concentration of rhEndostatin significantly decreased after the iv injection and returned to baseline level 12 h after the injection. No drug accumulation was found, and the serum concentration of rhEndostatin reached a steady-state level after 7 d of successive administration with the C_{av}^{ss} of 272.44 ± 91.98 ng/mL.

Figure 3. Concentration-time curves of rhEndostatin in preclinical and clinical studies. (A) concentration-time curves of rhEndostatin ($3\text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, iv, $n=6$) from 0 to 24.5 h after the first injection in rhesus monkeys. (B) concentration-time curves of rhEndostatin ($10\text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, iv, $n=10$) from 0 to 24 h after the first injection in patients. (C) concentration-time curves of rhEndostatin for single administration at different doses in volunteers. (■), iv $60\text{ mg}/\text{m}^2$, $n=8$; (+), iv $120\text{ mg}/\text{m}^2$, $n=8$; (▲), iv $180\text{ mg}/\text{m}^2$, $n=8$.

Table 3. Mean tumor inhibition rate and *in vivo* activity of rhEndostatin.

Drug	Replication	MIR ^a at 3 dose (mg/kg)			ED ₅₀ ^b (mg/kg)	Potency (U/mg)	SD
		5	10	20			
rhEndostatin	12	30%	48%	67%	12.41	4.04×10^7	3.73

^a MIR, mean tumor inhibition rate.

^b ED₅₀, the effective dose for 50% inhibition of tumor growth.

Table 4. Comparison of main PK parameters of different gender in Rhesus monkey.

Parameters	Male (n=3)	Female (n=3)	P value
$t_{1/2\beta}$ (h)	6.8±1.3	5.3±2.0	>0.05
$C_{\max}$ (ng/mL)	5188±377	5085±1337	>0.05
V_d (mL/kg)	359±18	314±82.8	>0.05
$AUC_{(0-24\text{ h})}$ (h·μg/mL)	9.521±1.005	10.837±2.103	>0.05
$AUC_{(0-\infty)}$ (h·μg/mL)	10.172±1.221	10.456±2.866	>0.05
Cl_s (mL·kg ⁻¹ ·h ⁻¹)	331±48	320±69	>0.05

Table 5. Main pharmacokinetic parameters of rhEndostatin and YH-16 in rhesus monkeys.

Parameters	rhEndostatin (3 mg·kg ⁻¹ ·g ⁻¹) for 90 days	YH-16* (mg·kg ⁻¹ ·d ⁻¹) for 7 days		
		1.5	4.5	13.5
$t_{1/2\beta}$ (h)	6.0±1.7	8±8	3.1±1.4	20±14
$C_{\max}$ (μg/mL)	5.14±0.9	NA	NA	NA
V_d (mL/kg)	337±59	390±220	120±40	148±13
$AUC_{(0-24\text{ h})}$ (h·μg/mL)	10.18±1.6	NA	NA	NA
$AUC_{(0-\infty)}$ (h·μg/mL)	10.31±1.98	15±5	33±7	93±25
Cl_s (mL·kg ⁻¹ ·h ⁻¹)	325±53	110±30	46±10	17±4

* The data was from ref. 16

NA, not available

The $C_{\max}^{ss}$ and the $C_{\min}^{ss}$ were 861.29±160.25 ng/mL and 166.31±53.36 ng/mL, respectively. The AUC_{ss} ranged from 31.76 to 48.88 h·μg/mL. The V^s , mean residence time (MRT), Cl_s , and $t_{1/2z}$ were 5.96±2.07 L/m², 105.68±17.87 h, 0.23±0.07 L/h·m², and 19.56±10.07 h, respectively. The main pharmacokinetic data are shown in Table 6.

In the single-administration groups of the clinical trials, baseline endostatin serum concentrations were measured in 24 volunteers. The mean baseline (before rhEndostatin therapy) circulating endostatin concentrations were 126.54±25.04, 110.44±22.82, and 114.41±29.18 ng/mL in the 60, 120, and 180 mg/m² dose groups, respectively, and showed little interclass difference. Serum concentration-time profiles of rhEndostatin following iv injections in the single-dose group were best fitted to a 2-compartment open model. The results are shown in Figure 3C. After injection, rhEndostatin was rapidly absorbed with the $T_{\max}$ of 1.79±0.35, 1.75±0.46, and 1.75±0.46 h, respectively. The terminal half-lives ($t_{1/2\beta}$) differed dramatically with values of 8.76±19.34, 6.59±9.33, and 26.84±27.19 h, respectively, while the biological half-lives ($t_{1/2z}$) were 22.87±12.01, 25.11±15.73, and 24.93±17.10 h, respectively, with no significant difference. The Cl_s were 3.48±1.03, 4.92±2.28,

Table 6. Serum pharmacokinetics of a 28-day continuous intravenous administration of rhEndostatin from *E coli* and *P pastoris* strain respectively.

Parameters	A ^a (10 mg·m ⁻² ·d ⁻¹)	B ^b (15 mg·m ⁻² ·d ⁻¹)	C ^c (mg·m ⁻² ·d ⁻¹)	
			7.5	15
C_0 (ng/mL)	166.31±53.36	25±17	159±119	224±60
$C_{\min}^{ss}$ (ng/mL)	166.31±53.36	48±25	220±120	240±110
$C_{\max}^{ss}$ (ng/mL)	861.29±160.25	394±55	870±470	1610±180
$AUC_{0-24\text{ h}}$ (h·μg/mL)	40.32±8.56	31±15	2.66±1.76	6.32±1.28
V^s (L/m ²)	5.96±2.07	NA	12.3±7.7	8.2±0.8
MRT (h)	105.68±17.87	NA	3.2±0.2	4.6±0.7
Cl_s (L/h·m ²)	0.23±0.07	32.0±15.5	NA	NA
$t_{1/2z}$ (h)	19.56±10.07	NA	10.2±1.0	14.4±6.4

^a, Mean values of rhEndostatin from *E coli* during 28-day therapy;

^b, Mean values of rh-Endo from *P pastoris* during 28-day therapy, data was from ref.22;

^c, Values of YH-16 from *E coli* at day 7 during 28-day therapy, data was from ref.25;

NA, not available

and 5.31±1.73 L/h·m², respectively, in the 60, 120, and 180 mg/m² dose groups. The main pharmacokinetic parameters are shown in Table 7. As illustrated in Figure 4, the fact that the $C_{\max}$ and AUC with different rhEndostatin dosages increased as the dosage increased suggested that the $C_{\max}$ and AUC were dose-dependent in the single-administration clinical studies.

Immunogenicity of rhEndostatin in rhesus monkeys and patients In the preclinical trials, studies to assess the immunogenicity of rhEndostatin were performed in rhesus monkeys. IgG antibodies to rhEndostatin were not detectable in the sera obtained both from rhesus monkeys in the treated groups before treatment and in the control group during administration. After receiving rhEndostatin for a week, 1 animal in the group received a low-dose rhEndostatin injection (6 mg/kg, n=6), which had positive IgG immunoreactivity with the titer of 1:160. Two animals in the high-dose group (60 mg/kg, n=6) were positive with titers of 1:320. All of the animals in the middle-dose group (20 mg/kg, n=6) showed negative results. The number of animals that showed an immunoreaction to rhEndostatin rapidly increased after 2 weeks of administration. Nearly all of treated groups were positive for IgG immunoreactivity to rhEndostatin during the next 10 weeks with little difference in titers. The administration of rhEndostatin was stopped at d 91 (13 weeks), and the animals showing immunoreactivity and titers in each group began to decrease. Thirteen weeks later, 3 of 6 (50%), 2 of 6 (33.3%), and 1 of 6 (16.7%) animals in the low-, middle-, and high-dose

Table 7. Comparison of main pharmacokinetic parameters of rhEndostatin and YH-16 according to dose levels during single-administration therapy.

Parameters	rhEndostatin (mg/m ²)			YH-16* (mg/m ²)	
	60 (n=8)	120 (n=8)	180 (n=8)	60 (n=3)	120 (n=3)
C ₀ (ng/mL)	126.54±25.04	110.44±22.82	114.41±29.18	256±69	222±94
t _{1/2β} (h)	8.76±19.34	6.59±9.33	26.84±27.19	8.2±1.9	9.8±0.1
C _{max} (μg/mL)	4.89±1.27	6.54±2.21	7.88±4.01	13.87±0.61	11.49±3.29
MRT (h)	17.74±10.56	19.29±6.80	29.78±18.79	2.9±0.9	3.8±0.8
AUC ₍₀₋₄₎ (h·μg/mL)	18.22±5.36	27.83±9.94	35.78±10.18	18.67±4.98	58.94±22.52
AUC _(0-∞) (h·μg/mL)	19.07±7.38	28.16±10.14	37.03±11.66	20.30±6.83	62.83±24.93
Cl/s (L/h·m ²)	3.48±1.03	4.92±2.28	5.31±1.73	3.2±0.9	2.1±0.7

* Data of YH-16 was from ref.25

Figure 4. AUC (A) and C_{max} (B) of serum endostatin versus dose level are shown for volunteers. Three different dose levels of rhEndostatin were intravenously administered as a 2 h infusion in the single-administration group. AUC and C_{max} both showed a linear relationship as the dosage of rhEndostatin increased (A, R²=1; B, R²=0.9962).

groups showed negative results. Two of 6 (33.3%) and 3 of 6 (50%) in each corresponding group were positive with titers of 1:320; other titers ranged from 1:40 to 1:160. No positive results were obtained 4 weeks after the cessation of administration, with the exception of 1 in the high-dose group which had a low titer of 1:40. The number of positive results in the treated groups at different times is shown in Figure 5A. The data strongly suggest that immunoreactivity of rhesus monkeys to rhEndostatin used in this trial is common.

In the clinical trials, IgG and IgM antibodies specific for rhEndostatin were not detectable in the sera obtained from patients before treatment. Sera from 10 patients at d 7, 14, and 28 were assessed for IgG and IgM antibodies to rhEndostatin. As shown in Figure 5B and 5C, the OD₄₅₀ value in all of the serum samples did not exceed critical value, which was defined as a 2-fold increase of the initial value. The results demonstrated that neither IgG nor IgM antibodies to rhEndostatin were detected, and rhEndostatin had no immunogenicity in this trial.

Discussion

Anti-angiogenic therapy targeting the internal vas capillary network of tumors may become predominant tumor biotherapy in this century. It is considered to be another effective means for tumor treatment besides conventional surgery, chemotherapy, and radiotherapy^[33]. As an angiogenic inhibitor, endostatin can inhibit the growth or metastasis of tumors by promoting apoptosis of malignant cells and has a definite antitumoral effect without toxicity and drug resistance after prolonged use^[6,34]. Therefore, the advantages of endostatin make it a promising candidate for antitumor therapy. It is known that quality assurance is of great important in the biological product preparation process. In the present study, authorized institutions analyzed main parameters of rhEndostatin to make sure that the samples met set standards. For genetically-engineered products, peptide mapping is an important index for evaluating the integrity of the protein structure and the stability of production technology. In addition, we determined other relevant parameters according to *Pharmacopoeia of the People's Republic of China* (2005 edition, part III) and ensured the safety and availability for the application of rhEndostatin preparations in animals and patients.

At the beginning of the experiments, the bioactivity of rhEndostatin was measured by *in vitro* inhibition of

Figure 5. (A) number of positive results for IgG antibodies to rhEndostatin in rhesus monkeys at different times. RhEndostatin was administered daily for 90 d at dose of $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$. (B,C) OD_{450} value of serum specimen in IgG and IgM antibodies to the rhEndostatin assay, respectively. Patients received rhEndostatin daily for 28 d at the dose of $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$. Critical value was defined as a 2-fold increase over baseline absorbance. Practical value exceed critical value was defined as positive.

proliferation or migration of endothelial cells after stimulation by bFGF or VEGF, and the instability of bFGF or VEGF in the culture medium contributed to low sensitivity and reproducibility^[35,36]. The stability of cells was improved by transfecting the endothelial cells with VEGF or bFGF, which made the *in vitro* activity assay more convenient, efficient, and rigorous^[26]. To gain a more accurate insight into the biological activity of rhEndostatin,

animal models, including chick embryo chorioallantoic membrane (CAM) and tumor-bearing models, have been used to assess the therapeutic effectiveness of rhEndostatin^[1,14]. However, it was found that CAM has a number of disadvantages, including its limited availability and quantification difficulty since no dose-dependent relationship was observed^[37]. Alternatively, many results have revealed that rhEndostatin acts in a significant dose-dependent manner in tumor-bearing mouse models^[26,38]. In the present study, the application of HUVEC transfected with VEGF resulted in highly reproducible results and reduced deviations. Three methods were used the *in vitro* activity assay for comparison. The cell counting method proved to be inconvenient and time costly. The results suggested that the LDH assay was more sensitive with low deviations and high reproducibility. Based on these results, we believe that the LDH assay is a practical method for quantifying the *in vitro* activity of rhEndostatin. We used H22-tumor-bearing mice to assay the *in vivo* activity of rhEndostatin, which was repeated 12 times in order to diminish the deviation from individual differences of the animals, concentration of the drugs, and potency of the samples so that the authenticity of the experiments and fidelity of potency was ensured. Due to the fact that the *in vitro* activity was not always a good predictor for *in vivo* therapeutic effectiveness, we created a different definition of units. We used the ED_{50} from our previous study in the present study to ensure the concordance of the *in vitro* and *in vivo* bioactivities^[29]. The results revealed that the *in vitro* specific bioactivity (6.4×10^7 , 6.7×10^7 , and $3.8 \times 10^8 \text{ U/mg}$) was 1.6–9.4 times that of the *in vivo* antitumoral potency ($4.04 \times 10^7 \text{ U/mg}$). Thus we reached the conclusion that the *in vivo* and *in vitro* bioactivities were on the whole concordant, and this method serves as a candidate pharmacological method for the potency assessment of the bioactivities of rhEndostatin *in vivo* and *in vitro*.

Based on above results, preclinical trials in rhesus monkeys and a phase I clinical trial were carried out to assess the safety and pharmacokinetic profile of rhEndostatin. We compared main pharmacokinetic parameters of rhEndostatin (Met-endostatin) with that of YH-16 (MGSSH-HH-HH-endostatin) in both preclinical and clinical trials. The results were dramatically different, although 2 protein products were derived from *Escherichia coli*. In rhesus monkeys, the results of the pharmacokinetic studies of YH-16 suggested that endostatin tended to accumulate in the body, and that an exorbitant dose (13.5 mg/kg) of endostatin might affect its clearance^[16]. The main pharmacokinetic parameters of rhEndostatin at a dose of 3 mg/kg were

similar to the results of YH-16 at a dose of 1.5 mg/kg, with the exception that the Cl_s was much higher (325 ± 53 vs 110 ± 30 mL·kg⁻¹·h⁻¹), as shown in Table 5. The difference of the Cl_s implied that rhEndostatin may not contribute to drug accumulation. Furthermore, the YH-16 studies show great individual differences while there was little difference between the individuals in our study. In the successive-administration clinical studies, the main pharmacokinetic parameters of rhEndostatin (10 mg·m⁻²·d⁻¹, 28 d, o.d iv) was compared to *P pastoris*-derived rhEndostatin (15 mg·m⁻²·d⁻¹, 28 d, o.d iv) and YH-16 (7.5, 15 mg·m⁻²·d⁻¹, 28 d, o.d iv), as illustrated in Table 6^[22,25]. Interestingly, the difference of the basal endogenous endostatin levels in Chinese (100–300 ng/mL) and American (<50 ng/mL) patients suggest that race disparity exist^[22,25,31]. The discrepancy between rh-Endostatin and rhEndostatin was extremely great, many pharmacokinetic parameters such as the MRT, Cl_s , and V^{ss} resulted in tens, even hundreds times' differences, except that the $AUC_{0-24\text{ h}}$ was close (40.32 ± 8.56 vs 31 ± 15 h·μg/mL). The greatest difference of baseline endostatin levels between Chinese and American patients may be as a result of the significant differences of pharmacokinetic parameters between rhEndostatin and rh-Endostatin. The difference between YH-16 and rhEndostatin was much less: the $C_{\min}^{ss}$ (166.31 ± 53.36 vs 220 ± 120 ng/mL) and $C_{\max}^{ss}$ (861.29 ± 160.25 vs 870 ± 470 ng/mL) of rhEndostatin were very close to those of YH-16 at a dose of 7.5 mg·m⁻²·d⁻¹. RhEndostatin showed a little lower V^{ss} (5.96 ± 2.07 vs 12.3 ± 7.7 and 8.2 ± 0.8 L/m²) and a little longer $t_{1/2z}$ (19.56 ± 10.07 vs 10.2 ± 1.0 and 14.4 ± 6.4 h), but the $AUC_{0-24\text{ h}}$ and MRT of rhEndostatin were much higher than those of YH-16, as shown in Table 6. In the single-administration groups of the clinical trials, different dosages of rhEndostatin had a similar $T_{\max}$ because the same delivery route was applied. Despite the different basic endogenous endostatin levels (C_0), the $t_{1/2\beta}$, $AUC_{(0-t)}$, $AUC_{(0-\infty)}$, and Cl_s were highly similar at the dose of 60 mg/m², as shown in Table 7. The significant differences in $C_{\max}$ (4.89 ± 1.27 vs 13.87 ± 0.61 μg/mL) and MRT (17.74 ± 10.56 vs 2.9 ± 0.9 h) suggests that rhEndostatin is released slower than YH-16 and may exert a prolonged action^[25]. Moreover, the $C_{\max}$ and AUC of both rhEndostatin and YH-16 were dose-dependent and showed a much rise in the YH-16 studies as the dose increased.

As for serum antibodies to rhEndostatin, we found that anti-rhEndostatin IgG antibodies generated quickly during administration and decreased rapidly after treatment in rhesus monkeys. We consider the result reasonable because rhEndostatin is a heterogeneous protein. In our clinical

studies, neither IgG nor IgM antibodies for rhEndostatin were detected in the patients ($n=10$). The clinical trial of YH-16 showed that 2 of 20 (10%) patients were positive for IgA immunoreactivity to the rhEndostatin formulation, 1 of 20 (5%) patients was positive for IgA immunoreactivity to His-tag, and 2 of 11 (18%) healthy volunteers were positive for IgG immunoreactivity to rhEndostatin. The immunogenicity of rh-Endostatin was found to be more common with 9 of 13 (69%) patients positive for IgG immunoreactivity to the rh-Endostatin formulation after the first 28 d of treatment. Eight of 11 (73%) patients had positive results after the second 28 d treatment; the data were similar for IgM immunoreactivity^[23]. A recent study showed that a natural immune reaction against endostatin can occur in breast cancer patients, and the presence of antibodies to endostatin is not associated with circulating endostatin levels^[19].

The difference between rhEndostatin, YH-16, and rh-Endostatin in pharmacokinetic parameters and immunogenicity were significant, as described earlier. First, the difference between rhEndostatin and YH-16 was minimal. The difference between rhEndostatin and rh-Endostatin was much greater. RhEndostatin and YH-16 are derived from *Escherichia coli* while rh-Endostatin is derived from *P pastoris*, therefore, different sources may be responsible for the great disparity. Second, no IgG antibodies for endostatin were detected in phase I clinical studies of rhEndostatin, but 18% of volunteers had positive results for IgG immunoreactivity and 5% of patients had positive results for His-tag immunoreactivity in YH-16 clinical trials. The positive rates of IgG and IgM antibodies for rh-Endostatin in patients were both above 60%. Generally, the genecity of antibodies for certain drugs may contribute to drug resistance or pharmacodynamic action attenuation which is closely associated with clinical outcome. The last point refers to the amino sequence. Compared with the amino sequence of rh-Endostatin reported in previous literature^[22], Met was added at the N-terminal of the protein in rhEndostatin, an additional 9 amino acid sequence (MGGSH-HHHH) was added at the N-terminal of the protein in YH-16^[16]. A recent report revealed that approximately 50% of the clinical grade endostatin (rh-Endostatin) molecules lack 4 amino acids at their NH₂ termini. The lack of these amino acids gives rise to a molecule that is devoid of zinc, resulting in no antitumoral activity^[39]. In contrast, no lack of amino acids in rhEndostatin was found in our trials. This result indicates that our rhEndostatin preparation was more stable and available. Based on the above analysis, we conclude that the expression system, immunoreactivity,

and structure of the rhEndostatin protein may be the main reasons contributing to different outcomes of endostatin in clinical trials. Other factors, such as race disparity of basal endogenous endostatin levels, viable therapeutic regimens, and the application of different compartment models, were also included. In conclusion, we have established a method determining the bioactivity of rhEndostatin. We also analyzed the main differences between preclinical and clinical outcomes of different recombinant human endostatin proteins, including rhEndostatin, YH-16 and especially rh-Endo and the pharmacokinetic parameters and immunogenicity. The ongoing phase II trial of rhEndostatin (lot 2007L01486) will help to address some of the questions raised during the current study.

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Author contribution

Bi HU, Zhi-wei WU, Jian-jun WANG and Gen-xing XU designed research; Bi HU, Hao-wen ZHU, Li-ping ZHU, Chen LI and Jia-ming XU performed research; Zhigang RONG provided the research materials; Bi HU, Zhi-wei WU and Jian-jun WANG wrote the paper.

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