Life Sciences 238 (2019) 116967

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Pharmacokinetics of PEGylated recombinant human endostatin in rhesus monkeys

Lifang Guo[a,1,***], Zuogang Li[c,1], Benshan Xu[a], Min Yu[c], Yan Fu[b], Lihong Liu[a,****], Junzhi Wang[c,**], Yongzhang Luo[b,*]

[a] Pharmacy Department of Beijing Chao-Yang Hospital, Capital Medical University, Beijing, 100020, China
[b] National Engineering Laboratory for Anti-tumor Protein Therapeutics, School of Life Sciences, Tsinghua University, Beijing, 100084, China
[c] National Center for Safety Evaluation of Drugs, National Institute for Food and Drug Control, Beijing, 100176, China

ARTICLE INFO

Keywords:
M_2ES
Rhesus monkeys
Pharmacokinetics

ABSTRACT

To investigate the pharmacokinetics of PEGylated recombinant human endostatin (M_2ES) in rhesus monkey. M_2ES was administered to rhesus monkeys by intravenous bolus injection, and serum M_2ES concentration was determined by a self-developed ELISA assay. Pharmacokinetic parameters were calculated using a non-compartmental model of WinNonlin V2.1A software. The standard curve of self-developed ELISA assay was fitted by four-parameter method. The limit of detection (LOD) and LOQ were 0.3050 ng/mL and 0.9140 ng/mL, respectively. Following IV infusions of M_2ES at 0.3, 1, and 3 mg/kg in rhesus monkeys, the serum M_2ES concentration-time curve was fitted with a non-compartment model. The pharmacokinetic parameters were evaluated as follows: Terminal elimination half-life ($T_{1/2}$) of M_2ES were 3.30 ± 0.70, 29.50 ± 18.80 and 24.60 ± 8.90 h. Systemic clearance (CLsys) of M_2ES were 339.60 ± 66.30, 161.40 ± 18.20 and 260.10 ± 43.70 mL/h/kg. $AUC_{(0-\infty)}$ values of M_2ES were 909.30 ± 199.60, 6251.00 ± 739.60 and 11758.00 ± 2010.10 ng·h/mL. The dosage was positively correlated with AUC, and the correlation coefficient r^2 = 0.9327. Self-developed ELISA assay could meet the requirements of serum M_2ES concentration detection. PEGylation modification substantially expands the circulation time of recombinant human endostatin and effectively improves its pharmacokinetic behavior.

1. Introduction

Endostatin, a 20-kDa C-terminal fragment of collagen XVIII, is a potent endogenous angiogenesis inhibitor [1]. Several studies have demonstrated that endostatin exhibits broad spectrum anti-tumor activity via retarding endothelial cell proliferation, migration, and tube formation [2–6]. Both nucleolin and integrins have been reported to mediate the anti-tumor effects of endostatin [7]. In 2005, a N-terminus (MGGSHHHHH) modified human endostatin, expressed by *E. coli*, was approved by the China Food and Drug Administration (CFDA) for the treatment of non-small-cell lung cancer (NSCLC) [8]. In clinical trials, endostatin monotherapy exerted significant antitumor activity, and showed a synergic effect with no apparent harmful side-effects in combination with chemotherapy. However, endostatin exhibits a short half-life in vivo, and was easily degraded by proteolytic enzymes, and rapidly cleared by the kidneys [9–12].

Polyethylene glycol (PEG), a biologically safe polyether compound, has been widely applied for the modifications of peptides and proteins [13–16]. PEGylation could improve the pharmacokinetic behavior of the therapeutic proteins through increasing their molecular mass, shielding them from proteolytic enzymes, and extending their circulating half-lives in vivo [14,17]. Nowadays, several therapeutic drugs, such as insulin, interferon, granulocyte-colony stimulating, has been PEGylated [18–20]. To reduce the dosing frequency and prolong the half-life of endostatin, recombinant human endostatin was modified at the N-terminus through conjugation with a methoxy polyethylene glycol aldehyde. Indeed, PEG modification improved the anti-tumor activity, and prolonged the circulation time of endostatin [21,22].

* Corresponding author.
** Corresponding author.
*** Corresponding author.
**** Corresponding author.
E-mail addresses: glf03@163.com (L. Guo), liulihong@bjcyh.com (L. Liu), wangjz@nicpbp.org.cn (J. Wang), yluo@mail.tsinghua.edu.cn (Y. Luo).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.lfs.2019.116967
Received 9 July 2019; Received in revised form 5 October 2019; Accepted 13 October 2019
Available online 15 October 2019

However, there were no reports on the evaluation of pharmacokinetics of M_2ES in rhesus monkeys.

The present study attempted to reveal the pharmacokinetics of M_2ES following intravenous injection of M_2ES at a dose level of 0.3, 1, and 3 mg/kg in rhesus monkeys, and provide scientific basis for rational drug use in clinical trials.

2. Materials and methods

2.1. Drugs and reagents

The competitive ELISA Kit used in this study was developed by Tsinghua University and NCSED, MES Injection (purity: 99.50%; specification: 2 mg/mL) and M_2ES Injection (purity: 98.77%; specification: 6 mg/mL) were obtained from Protgen Ltd (Beijing, China). All other reagents were purchased from commercial sources and were of analytical grade.

2.2. Animals

Rhesus monkeys (7 females, 8 males; aging 2–3 years; weighing 3.25 ± 0.31 kg) were provided by the Pingan Animal Breeding Research Base Co., Ltd. (Sichuan, China). The animals were housed in an environmentally controlled breeding room and provided with full price pellet feed, fruit (full price pellet feed and fruit ratio 1:1, daily 150 ± 10 g) and water was available adlibitum.

2.3. Experimental design

Rhesus monkeys (7 females, 8 males) were quarantined and domesticated, and were divided into single dose groups (high, medium and low dose groups; 3 mg/kg, 1 mg/kg, 0.3 mg/kg), multiple dose groups and control groups according to their body weight by stratified randomization. The single dose group was given M_2ES injection. In the multiple dose group, M_2ES was intravenously injected at the same time for 4 consecutive days. The control group received single intravenous injection of MES.

2.4. Sample collection and assay

Whole blood samples were collected from the forearm veins of the animals using a puncture needle. In the single dose group, the samples were collected at time of 10min before administration and at time intervals of 10, 30min, 1, 2, 4, 8, 12, 24, 48, 72, 96 and 120 h after administration. In the multiple dose group, the samples were collected at time of 10min before the first dose and at time intervals of 10, 30min, 1, 2, 4, 8, 12, and 24 h after the first dose, at time of 10min before the last dose and at time intervals of 10, 30min, 1, 2, 4, 8, 12, 24, 48, 72 and 96 h after the last dose. In the control group, the samples were collected at time of 10min before administration and at time intervals of 5, 10, 30min, 1, 2, 4, 8, 12, 24, 48 h after administration. The unanticoagulant blood samples were centrifuged at 8000 rpm/min for 5 min to separate the serum after placed in an ice box for 30 min (the process should be completed within 2 h). Then take 40 μL serum repackaging into eppendorf tubes, serum samples were stored at - 80 °C until analysis.

The competition ELISA kit was utilized to determine the M_2ES in the serum samples from the animals. Briefly, ELISA plates (Costar, Cambridge, MA, USA) were coated with 100μL/well of a 2 mg/L solution of anti-MES polyclonal antibody in buffer, at 4 °C overnights. After washing the coated plates three-times with 150μL/well PBST and one-time with 150μL/well PBS, blocking was realized with 100μL/well 0.5%(w/v) PBS-BSA at 37 °C during 2 h. After washing the plates, 100μL/well of series calibrators, QCs and samples were added in duplicates. The plates were incubated at 37 °C during 1.5 h and were washed five-times with PBST. 50μL/well of HRP - Avidin (1:1000 dilution in PBST) was added and incubated at room temperature during 1 h. After washing the plate five-times with PBST, 100 μl TMB was added to each well and the plates were stored for 15 min in the dark at room temperature. In each well, 50 μl of 2 M sulfuric acid were added to stop the reaction, the plates were measured at 450 nm with an ELISA reader (Spectra Max Plus, Molecular Devices, Sunnyvale, CA, USA).

2.5. Data analysis

The absorbance values at 450 nm and the known concentration of M_2ES (log values) were plotted using SoftMax Pro (V4.8) software (Molecular Devices, Sunnyvale, CA) by a sigmoid curve defined by a 4-parameter logistic equation, Y = Bottom + (Top-Bottom)/(1 + 10^((LogIC$_{50}$-X) ^Hill Slope). The quantitative range was the highest and lowest values that can be determined by this equation, and the unknown concentration of M_2ES serum samples that were on the same plate could be measured was calculated in the range.

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.

All data are represented as mean ± SD, except where otherwise mentioned. Statistical analysis was performed using GraphPad Prism 5 statistical software (GraphPad Software, Inc. La Jolla, CA). Statistical significance was set as $P < 0.05$.

3. Results

3.1. ELISA assay validation for evaluation of serum M_2ES in rhesus monkeys

ELISA assay developed jointly by Tsinghua university and National institutes for food and drug control was used to detect serum samples collected from rhesus monkeys prior to administration or limit of quantification (LOQ) serum sample with 0.914 ng/mL MES standard protein. Compared with the OD value of LOQ serum sample with 0.914 ng/mL MES standard protein (0.296 ± 0.001, n = 3) and the upper limit OD value (0.3) of blank serum sample was calculated by the correction equation obtained from the fitting curve, endogenous MES background and other non-target proteins in serum did not interfere with this method for the quantification of serum samples.

The standard curve was fitted by four-parameter method, and the regression correction equation was y = {(A-D)/[1 + (x/C)^B]} 3.2 + D,A = 0.370,B = 0.903,C = 57.159,D = 0.066,R2 = 0.9998. Desired working range had been determined as 2000 ~ 0.914 ng/mL. The limit of detection (LOD) and LOQ were 0.305 ng/mL and 0.914 ng/mL, respectively.

As shown in Table 1, inter-day and intra-day recovery ranged between 100.00%-106.44% and 100.91%–117.30%. Intra-day and inter-day assay CVs were between 2.70%-11.7% and 1.50%–5.50%. MES stability in rhesus monkey serum was evaluated, as shown in Table 2, the accuracy and precision of the measured data could meet the experimental requirements.

Table 1
Accuracy, precision and recovery results from MES serum drug concentration determination method.

	Reference Conc.(ng/mL)	Mean ± SD(ng/mL)	Recovery%	CV%
Inter-day accuracy and precision (n = 4)	666.67	666.65 ± 50.59	100.00	7.60
	74.07	77.38 ± 2.12	104.46	2.70
	8.23	8.76 ± 1.02	106.44	11.70
Intra-day accuracy and precision (n = 6)	666.67	676.73 ± 37.22	101.51	5.50
	74.07	74.75 ± 1.50	100.91	2.00
	8.23	9.65 ± 0.15	117.30	1.50

Table 2
Stability and recoveries of MES in serum of rhesus monkey.

Tested conditions	Reference Conc.(ng/mL)	Mean ± SD(ng/mL)	Recovery%	CV %
Freeze/Thaw stability at -80 °C (n = 6)	82.31	96.21 ± 1.92	116.89	2.00
	740.74	825.22 ± 39.61	111.40	4.80
	6666.67	7571.71 ± 310.44	113.58	4.10
Short-term stability at room temperature (6.5Hours, n = 8)	82.31	79.40 ± 2.30	96.47	2.90
	740.74	698.65 ± 21.66	94.32	3.10
	6666.67	6584.06 ± 342.37	98.76	5.20
Short-term stability at 4 °C (20Hours, n = 8)	82.31	74.16 ± 2.00	90.11	2.70
	740.74	697.30 ± 19.52	94.13	2.80
	6666.67	7645.69 ± 145.27	114.69	1.90
Long-term stability at -80 °C (50Days, n = 5)	82.31	86.07 ± 1.98	104.57	2.30
	740.74	764.53 ± 9.94	103.21	1.30
	6666.67	7070.24 ± 537.34	106.05	7.60

3.2. Serum concentration-time curves of M_2ES and MES in rhesus monkeys

After a single iv infusion of M_2ES at 0.3 mg/kg, 1 mg/kg and 3 mg/kg in rhesus monkeys, the concentration of M_2ES at most time points (0.17–24 h) showed statistically significant differences between the high and medium dose groups, the high and low dose groups, and the medium and low dose groups ($P < 0.05$). The three dose groups reached the highest blood concentration at the first time point of 10min after iv injection, and then gradually decreased, and the trend was consistent (Fig. 1).

After a single iv injection MES at a dose of 1 mg/kg in rhesus monkeys, the maximum blood concentration was reached after 5 min, and then gradually decreased. Compared with the drug concentration at each time point (M_2ES, 1 mg/kg), there was statistically significant difference between the two groups in blood collection points between 10 min and 12 h ($P < 0.05$) (Fig. 2).

After multiple intravenous injection of M_2ES (1 mg/kg) in rhesus monkeys, the blood concentration at each time point in last administration group was higher than that in first administration group, and the difference was statistically significant after 4 h ($P < 0.05$) (Fig. 3).

3.3. Serum pharmacokinetic of M_2ES and MES in rhesus monkey

The pharmacokinetic parameters were evaluated as follows: Terminal elimination half-life ($T_{1/2}$) of M_2ES were 3.30 ± 0.70, 29.50 ± 18.80 and 24.60 ± 8.90 h. Systemic clearance (CLsys) of M_2ES were 339.60 ± 66.30, 161.40 ± 18.20 and 260.10 ± 43.70 mL/h/kg. $AUC_{(0-\infty)}$ values of M_2ES were 909.30 ± 199.60, 6251.00 ± 739.60 and 11758.00 ± 2010.10 ng·h/mL.

After a single intravenous infusion of M_2ES at 0.3 mg/kg, 1 mg/kg and 3 mg/kg in rhesus monkeys, the terminal elimination half-life ($T_{1/2}$) of M_2ES was 3.30 ± 0.70, 29.50 ± 18.80 and 24.60 ± 8.90 h. The

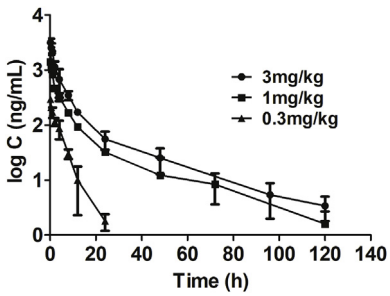

Fig. 1. Concentration-time curves of M_2ES after a single intravenous injection at different dosages in rhesus monkey.

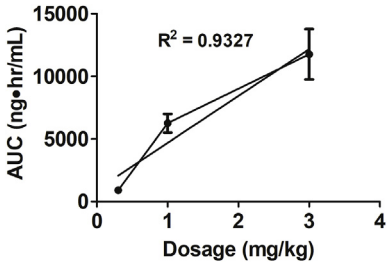

Fig. 2. AUC-Dosage curve of M_2ES after a single intravenous injection at three dosages in rhesus monkey.

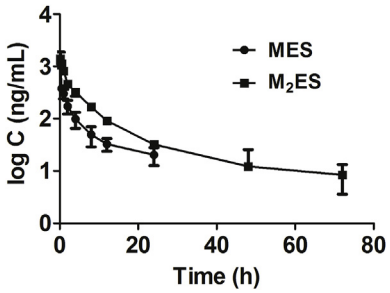

Fig. 3. Concentration-time curves of MES and M_2ES after a single intravenous dose of 1 mg/kg in rhesus monkey.

systemic clearance (CLsys) of M_2ES was 339.60 ± 66.30, 161.40 ± 18.20 and 260.10 ± 43.70 mL/h/kg. The $AUC_{(0-\infty)}$ values of M_2ES was 909.30 ± 199.60, 6251.00 ± 739.60 and 11758.00 ± 2010.10 ng·h/mL (Table 3). The AUC-dose relationship of the three doses was shown in Fig. 4.

After a single intravenous infusion of 1 mg/kg MES in rhesus monkeys, the $T_{1/2}$ of MES was 2.50 ± 0.30 h, the CLsys of MES was 440.90 ± 69.00, and the $AUC_{(0-\infty)}$ of MES was 2306.90 ± 374.90. Compared with the kinetic parameters of M_2ES (1 mg/kg), PEG modification substantially prolonged the half-life of MES and improves its pharmacokinetic behavior (Table 3).

4. Discussion

The evaluation of the pharmacokinetics profile are the requirement of new drug registration and clinical application. A competitive ELISA kit was developed to evaluate the serum concentration of M_2ES. In the GLP-compliant study, for the first time we reported the pharmacokinetics of M_2ES in rhesus monkey.

Endostatin, a well-documented endogenous inhibitor of angiogenesis, was firstly described by Folkman et al. [23]. Although the function, and molecular mechanisms of endostatin have been extensively documented, its clinical effects were reported different [24,25]. *P. pastoris*-expressed endostatin developed by Entered were failed during phase II clinical testing [26], whereas the N-terminus modified endostatin expressed by *E. coli*, was approved by the CFDA for its good clinical responses [27,28]. To explore the reason for the difference, both *P. pastoris*-expressed and *E. coli*-expressed endostatin were analyzed. We found that almost 93% of *P. pastoris*-expressed endostatin was truncated, and further study confirmed that this truncated form leads to reduce stability and lower anti-tumor capacity of endostatin [9]. However, endostatin expressed by *E. coli* was proved to be an intact molecule, and showed full anti-angiogenic activity [29].

The competitive ELISA assay is a common method in pharmacokinetic studies of protein drugs. We developed a competitive ELISA kit to determine the M_2ES concentration in the serum. Since endogenous MES background and other non-target proteins in serum was excluded, the results of ELISA assay could reflect the concentration of profile of M_2ES. PEGylation could protect protein drugs against enzymatic degradation,

Table 3
Pharmacokinetic parameters of M_2ES and MES after a single intravenous administration of rhesus monkey.

Parameter	Unit	M_2ES (3 mg/kg)	M_2ES (1 mg/kg)	M_2ES (0.3 mg/kg)	MES (1 mg/kg)	P_1	P_2	P_3	P_4
C_{max}	ng/mL	3627.30 ± 1150.10	1941.70 ± 284.0	347.80 ± 37.40	1806.50 ± 499.40	0.705	8.301	4.034	4.266
$AUC_{(0-48h)}$	ng hr/mL	11552.10 ± 1841.30	5844.10 ± 355.80	891.70 ± 204.90	2232.50 ± 343.80	0.001	16.950	7.875	9.077
$AUC_{(0-\infty)}$	ng hr/mL	11758.00 ± 2010.10	6251.00 ± 739.60	909.30 ± 199.60	2306.90 ± 374.90	0.001	15.130	7.450	7.680
CL_{sys}	mL/h/kg	260.10 ± 43.70	161.40 ± 18.20	339.60 ± 66.30	440.90 ± 69.00	0.002	6.563	3.635	2.928
V_{dss}	L/kg	3.20 ± 0.90	3.30 ± 1.40	1.30 ± 0.10	4.90 ± 1.20	0.221	3.723	—	—
$T_{1/2}$	hr	24.60 ± 8.90	29.50 ± 18.80	3.30 ± 0.70	2.50 ± 0.30	0.068	3.773	—	—

***P*$_1$**:M_2ES (1 mg/kg) *vs* MES (1 mg/kg).
***P*$_2$**:M_2ES (0.3 mg/kg) *vs* M_2ES (3 mg/kg).
***P*$_3$**:M_2ES (0.3 mg/kg) *vs* M_2ES (1 mg/kg).
***P*$_4$**:M_2ES (1 mg/kg) *vs* M_2ES (3 mg/kg).

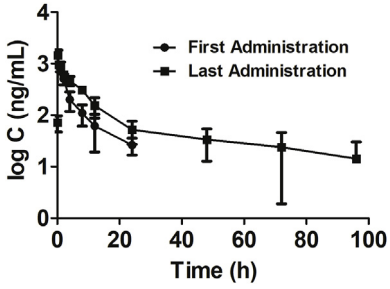

Fig. 4. Concentration-time curves of M_2ES after a multiple intravenous dose of 1 mg/kg at the first and last administration in rhesus monkey.

slow their glomerular filtration, therefore increasing the retention of the drugs in the blood [10,14,17]. Previous study reported that the half-lives of rh-endostatins (4.5 mg/kg) in rats and in rhesus monkeys were 3.91 and 3.10 h, respectively [11,30]. PEGylation significantly extended the half-life of rh-endostatin to 24.60 h at a dose of 3 mg/kg (Table 3). In our study, statistically significant differences were also observed in Cl and $AUC_{(0-\infty)}$ among pharmacokinetics parameters between M_2ES and rh-endostatin in rhesus monkeys (Table 3). The CLsys of rh-endostatin (1 mg/kg) was 440.90 $mL{\cdot}h^{-1}{\cdot}kg^{-1}$, while the CLsys of M_2ES (1 mg/kg) was 161.40 $mL{\cdot}h^{-1}{\cdot}kg^{-1}$, which was much slower than those of rh-endostatin in rhesus monkeys. The slower degradation of M_2ES in the blood resulted in greater values for the area under the serum concentration-time curve, with an $AUC_{(0-\infty)}$ value of 6251.00 $ng{\cdot}equ{\cdot}h{\cdot}mL^{-1}$ at the dose of 1 mg/kg, compared to those of rh-endostatin (1 mg/kg) in rhesus monkeys, which was 2306.90 ng·hr/mL (Table 3). Above mentioned results demonstrated that PEGylation indeed improved rh-endostatin pharmacokinetics profiles and prolonged its retention time in bodies.

Based on our results, we compared the main pharmacokinetic parameters of M_2ES with those of YH-16 in rhesus monkeys. After single dose of M_2ES or YH-16, the half-life ($T_{1/2}$) of M_2ES (3 mg/kg) was significantly longer than that of YH-16 (4.5 mg/kg) (24.60 ± 8.90 h vs. 0.04 ± 0.03 h) [11]. Previous reported that both M_2ES and Yh-16 had a tendency to accumulate in the animal body after successive IV administrations [9]. Of note, endostatin may cause side effects in the cardiovascular system and kidney, especially a dose-dependent toxicity in the heart [21,31]. It is best to avoid the excessive accumulation of endostatin in the body by adjusting the intervals between each IV infusions.

5. Conclusions

We developed and validated a sensitive ELISA assay to evaluated the serum concentration-time curves of M_2ES. According to our pharmacokinetic parameters, PEGylation significantly extended the circulation retention of rh-endostatin in rhesus monkeys, manifesting as a longer $T_{1/2}$, greater $AUC_{(0-\infty)}$, and slower CLsys. The present pharmacokinetics in rhesus monkeys study provides reference for the application of M_2ES in clinical practice.

Author contribution to study

Lifang Guo searched the literature and wrote the manuscript, Zuogang Li conceived, designed and performed the experiments, Benshan Xu prepared the figures and tables, Min Yu and Yan Fu analyzed the data, Lihong Liu, Junzhi Wang and Yongzhang Luo designed and supervised the study, revised the manuscript and gave final approval for publication.

Declaration of competing InterestCOI

All authors declare no conflicts of interest.

Acknowledgments

This work was supported by grants from the National Science & Technology Major Project (Grant No. 2009ZX09306-002); Major scientific and technological special project for “significant new drugs creation” (Grant No. 2009ZX09102-243); and Protgen Ltd. (Beijing, China).

References

[1] M.S. O'Reilly, T. Boehm, Y. Shing, N. Fukai, G. Vasios, W.S. Lane, et al., Endostatin: an endogenous inhibitor of angiogenesis and tumor growth, Cell 88 (1997) 277–285.
[2] A. Abdollahi, L. Hlatky, P.E. Huber, Endostatin: the logic of antiangiogenic therapy, Drug Resist. Updates : Rev. Comment. Antimicrob. Anticancer Chemother. 8 (2005) 59–74.
[3] D. Ribatti, Judah Folkman, a pioneer in the study of angiogenesis, Angiogenesis 11 (2008) 3–10.
[4] H. Shi, Y. Huang, H. Zhou, X. Song, S. Yuan, Y. Fu, et al., Nucleolin is a receptor that mediates antiangiogenic and antitumor activity of endostatin, Blood 110 (2007) 2899–2906.
[5] A. Sudhakar, H. Sugimoto, C. Yang, J. Lively, M. Zeisberg, R. Kalluri, Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alpha v beta 3 and alpha 5 beta 1 integrins, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 4766–4771.
[6] W. Zhuo, C. Luo, X. Wang, X. Song, Y. Fu, Y. Luo, Endostatin inhibits tumour lymphangiogenesis and lymphatic metastasis via cell surface nucleolin on lymphangiogenic endothelial cells, J. Pathol. 222 (2010) 249–260.
[7] L. Guo, N. Song, T. He, F. Qi, S. Zheng, X.G. Xu, et al., Endostatin inhibits the tumorigenesis of hemangioendothelioma via downregulation of CXCL1, Mol. Carcinog. 54 (2015) 1340–1353.
[8] B. Rong, S. Yang, W. Li, W. Zhang, Z. Ming, Systematic review and meta-analysis of Endostar (rh-endostatin) combined with chemotherapy versus chemotherapy alone for treating advanced non-small cell lung cancer, World J. Surg. Oncol. 10 (2012) 170.
[9] B. Hu, H.W. Zhu, L.P. Zhu, C. Li, Z.G. Rong, J.M. Xu, et al., Bioactivity, pharmacokinetics, and immunogenicity assays in preclinical and clinical trials for recombinant human endostatin, Acta Pharmacol. Sin. 29 (2008) 1357–1369.
[10] Y. Nie, X. Zhang, X. Wang, J. Chen, Preparation and stability of N-terminal mono-PEGylated recombinant human endostatin, Bioconjug. Chem. 17 (2006) 995–999.
[11] H.F. Song, X.W. Liu, H.N. Zhang, B.Z. Zhu, S.J. Yuan, S.Y. Liu, et al., Pharmacokinetics of His-tag recombinant human endostatin in Rhesus monkeys, Acta Pharmacol. Sin. 26 (2005) 124–128.

[12] L. Yang, J.W. Wang, Y. Sun, Y.Z. Zhu, X.Q. Liu, W.L. Li, et al., Randomized phase II trial on escalated doses of Rh-endostatin (YH-16) for advanced non-small cell lung cancer. Zhonghua zhong liu za zhi, Chin. J. Oncol. 28 (2006) 138–141.
[13] M.M. Fiume, B. Heldreth, W.F. Bergfeld, D.V. Belsito, R.A. Hill, C.D. Klaassen, et al., Safety assessment of alkyl PEG ethers as used in cosmetics, Int. J. Toxicol. 31 (2012) 169S–244S.
[14] J.M. Harris, R.B. Chess, Effect of pegylation on pharmaceuticals, Nat. Rev. Drug Discov. 2 (2003) 214–221.
[15] S. Jevsevar, M. Kunstelj, V.G. Porekar, PEGylation of therapeutic proteins, Biotechnol. J. 5 (2010) 113–128.
[16] M.J. Roberts, M.D. Bentley, J.M. Harris, Chemistry for peptide and protein PEGylation, Adv. Drug Deliv. Rev. 54 (2002) 459–476.
[17] F.M. Veronese, G. Pasut, PEGylation, successful approach to drug delivery, Drug Discov. Today 10 (2005) 1451–1458.
[18] R. Becker, C. Dembek, L.A. White, L.P. Garrison, The cost offsets and cost-effectiveness associated with pegylated drugs: a review of the literature, Expert Rev. Pharmacoecon. Outcomes Res. 12 (2012) 775–793.
[19] T.M. Caparrotta, M. Evans, PEGylated insulin Lispro, (LY2605541)–a new basal insulin analogue, Diabetes Obes. Metab. 16 (2014) 388–395.
[20] J.S. Kang, K.C. Lee, In vivo pharmacokinetics and pharmacodynamics of positional isomers of mono-PEGylated recombinant human granulocyte colony stimulating factor in rats, Biol. Pharm. Bull. 36 (2013) 1146–1151.
[21] L. Guo, X. Geng, Y. Chen, F. Qi, L. Liu, Y. Miao, et al., Pre-clinical toxicokinetics and safety study of M2ES, a PEGylated recombinant human endostatin, in rhesus monkeys, Regul. Toxicol. Pharmacol. : RTP (Regul. Toxicol. Pharmacol.) 69 (2014) 512–523.
[22] Z.G. Li, L. Jia, L.F. Guo, M. Yu, X. Sun, W. Nie, et al., Pharmacokinetics of PEGylated recombinant human endostatin (M2ES) in rats, Acta Pharmacol. Sin. 36 (2015) 847–854.
[23] A. Sibilly, C. Chemorin, B. Mathey, F. Jung, [Colonic perforation of neoplasic origin (author's transl)], Ann. Chir. 31 (1977) 201–205.
[24] Y. Fu, H. Tang, Y. Huang, N. Song, Y. Luo, Unraveling the mysteries of endostatin, IUBMB Life 61 (2009) 613–626.
[25] Z.A. Khan, J.M. Melero-Martin, X. Wu, S. Paruchuri, E. Boscolo, J.B. Mulliken, et al., Endothelial progenitor cells from infantile hemangioma and umbilical cord blood display unique cellular responses to endostatin, Blood 108 (2006) 915–921.
[26] A. Whitworth, Endostatin: are we waiting for Godot? J. Natl. Cancer Inst. 98 (2006) 731–733.
[27] C. Cui, L. Mao, Z. Chi, L. Si, X. Sheng, Y. Kong, et al., A phase II, randomized, double-blind, placebo-controlled multicenter trial of Endostar in patients with metastatic melanoma, Mol. Ther. : J. Am. Soc. Gene Ther. 21 (2013) 1456–1463.
[28] B. Han, Q. Xiu, H. Wang, J. Shen, A. Gu, Y. Luo, et al., A multicenter, randomized, double-blind, placebo-controlled study to evaluate the efficacy of paclitaxel-carboplatin alone or with endostar for advanced non-small cell lung cancer, J. Thorac. Oncol. 6 (2011) 1104–1109 official publication of the International Association for the Study of Lung Cancer.
[29] Y. Fu, Y. Luo, The N-terminal integrity is critical for the stability and biological functions of endostatin, Biochemistry 49 (2010) 6420–6429.
[30] X.X. Yang, Z.P. Hu, E. Chan, W. Duan, S. Zhou, Pharmacokinetics of recombinant human endostatin in rats, Curr. Drug Metabol. 7 (2006) 565–576.
[31] Q. Jing, Q. Xinyu, L. Rougcheng, Recombinant human endostatin-associated acute left heart failure, Clin. Oncol. 20 (2008) 268.