Department of Pharmacology[1], School of Basic Medicine, Lanzhou University, Key Laboratory of Preclinical Study for New Drugs of Gansu Province; Institute of Integrative Traditional and Western Medicine[2], Lanzhou University, Lanzhou, Gansu; Biotechnology Center of the Fourth Military Medical University[3], Xi'an, China

Pharmacokinetics of rh-IFNα-2a-NGR, a tumor targeted-therapy candidate, following intramuscular administration to mice, rats and monkeys

Li Lu[1], Xuexi Wang[2], Li Wang[1], Baolai Zhang[1], Shuai Yan[1], Xin Zhao[1], Wan Li[1], MingXia Cui[1], MingTang Gao[1], YingQi Zhang[3], YongJie Wu[1]

Received February 13, 2011, accepted March 28, 2011

YongJie Wu, Department of Pharmacology, School of Basic Medicine, Lanzhou University, 199 Western Road of Donggang, Lanzhou 730000, China.
wuyj@lzu.edu.cn

Pharmazie 66: 690–693 (2011) *doi: 10.1691/ph.2011.1521*

The compound rh-IFNα-2a-NGR can inhibit tumor angiogenesis and could be used for targeted therapy. In the present study, double antibody sandwich ELISA analysis was used to determine the concentration of rh-IFNα-2a-NGR in serum after intramuscular administration of various dosages to mice, rats and monkeys. The results showed that the pharmacokinetic properties of rh-IFNα2a-NGR after i.m. administration to mice, rats and monkeys were consistent with a one-compartment open model. The main pharmacokinetic parameters in mice (9.36 μg/kg), rats (4.68 μg/kg) and monkeys (2.34 μg/kg) after i.m. rh-IFNα2a-NGR were as follows: T_{peak} was 0.49, 1.65 and 3.60 h, C_{max} was 3030.20, 654.49 and 268.13 ng/L, $t_{1/2}$ was 0.39, 4.52 and 2.70 h, and $AUC_{(0-\infty)}$ was 4197.65, 5784.58 and 2622.06 ng/L·h, respectively. Also, mice, rats and monkeys had their own distinct metabolic characteristics. These data would provide references for further clinical pharmacokinetic study of rh-IFNα2a-NGR.

1. Introduction

Interferons (IFNs) are groups of cytokines, possessing broad and potent antiviral, antiproliferative and immunoregulatory activity. Since the 1980 s, when IFNs were successfully manufactured by genetic engineering and used clinically on a large scale, they have been regarded as the most promising antitumor and antivirus agents rh-IFNα2a, produced by DNA recombinant technology, is a single strand polypeptide consisting 165 amino acids. Its theoretical molecular weight is 19,219 Dalton, its isoelectric point is 5–6, and it is stable to acid and heat while being sensitive to various proteinases. rh-IFNα2a can promote T lymphocyte differentiation, enhance macrophage activity, regulate NK cell activity and suppress cell division, and has been successfully applied clinically as therapy for chronic active hepatits, renal cell carcinoma, melanoma, lymphoma, breast cancer and so on (Chiarion-Sileni et al. 2002; Maellaro et al. 2003; Koskinas et al. 2009).

The growth and metabasis of primary tumors rely on angiogenesis. Researchers (Curnis et al. 2008) have found that the NGR (aspartic acid-glycine-arginine) motif acts selectively on tumor vessel systems. Drugs combined with the NGR motif have a more potent anti-tumor activity and a lower toxicity. Aminopeptidasse N (APN,CD13) is a receptor of NGR (Pasqualini et al. 2000) that is abundant in tumor vessels in mice and humans but not in normal blood vessels in mice and humans. There is increasing evidence that APN plays an important role the neovascularization process. As a ligand of APN, the NGR motif has properties of tumor vessel selectivity and targeting (Curnis et al. 2000, 2001). Drugs containing the NGR motif can be used for targeted therapy of tumor anti-angiogenesis.

Table 1: Stability of rh-IFNα2a-NGR in rat serum

Concentration (pg/ml) (n = 5)		
Freshly prepared	After three freeze/thaw cycles	Decrease (%)
628.87 ± 35.47	603.98 ± 35.52	3.96
176.28 ± 1.51	175.10 ± 5.75	0.67
39.29 ± 2.79	38.71 ± 1.31	1.48

Storage temperature: −20 °C

Previous studies reported the manufacturing process of rh-IFNα-2a-NGR and its anti-angiogenesis activity *in vivo* and *in vitro* (Meng et al. 2007). In this study, the pharmacokinetic characteristics of rh-IFNα-2a-NGR after i.m. administration to mice, rats and monkeys are reported.

2. Investigations and results

The ELISA method is widely used in pharmacokinetics research due to its high sensitivity, reproducibility and efficiency. In our study, a typical equation of the calibration curve was as follows: $y = 0.0049x + 0.0299$, $r^2 = 0.9976$, while the lower limit of quantification (LLOQ) was 8 pg/ml, as shown in Fig. 1. Serum samples of rh-IFNα2a-NGR stored at −20 °C showed good stability, ranging from 96.14% to 99.37% after three freeze/thaw cycles. The stability data are summarized in Table 1. The recoveries of rh-IFNα2a-NGR ranged from 78.08% to 98.12%, and the intra- and inter-day precisions were less than 11% for concentrations from 32 pg/ml to 500 pg/ml and less than 19% at

Table 2: Recovery, accuracy and precision of rh-IFNα2a-NGR assay in serum ($n = 5$)

Nominal concentration (pg/ml)	Intra-day			Inter-day	
	Measured concentration (pg/ml)	Recovery (%)	CV (%)	Measured concentration (pg/ml)	CV (%)
500	452.39 ± 19.92	90.48 ± 3.98	4.40	482.24 ± 50.41	10.45
125	122.66 ± 4.76	98.12 ± 3.81	3.88	114.46 ± 10.26	8.97
32	27.25 ± 2.10	85.17 ± 6.56	7.71	23.60 ± 2.08	8.82
8	6.25 ± 1.07	78.08 ± 13.33	17.07	4.77 ± 0.90	18.96

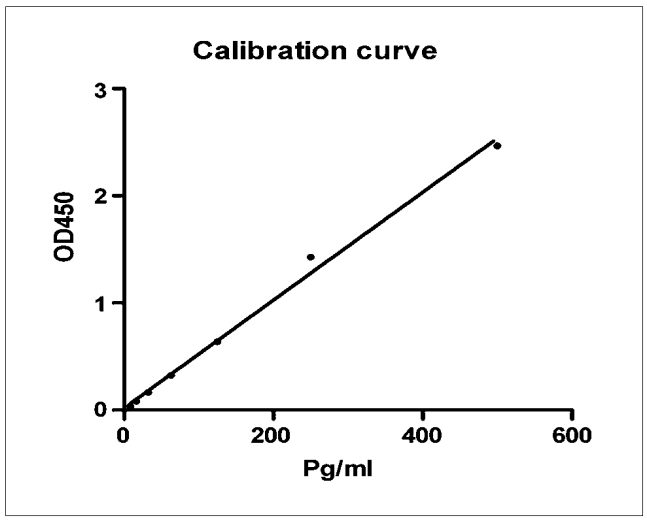

Fig. 1: Calibration curve of rh-IFNa2a-NGR in serum

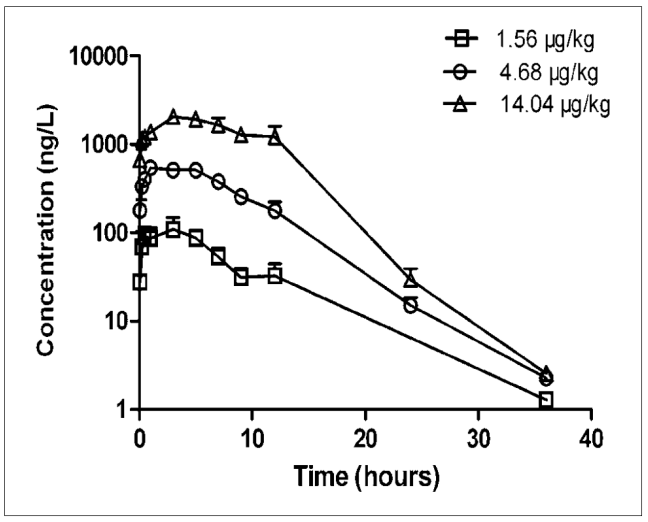

Fig. 3: Pharmacokinetic profile of rh-IFNa2a-NGR following i.m. injection to rats. The observed concentrations were represented by symbols. $n = 3$

8 pg/ml. All the values are shown in Table 2. Therefore, the method was proved to be accurate and validated.

In this study, the pharmacokinetic properties of rh-IFNα2a-NGR administered i.m. were evaluated in mice, rats and monkeys, which are typically used to assess the *in vivo* activity of this drug owing to their ability to respond to IFNα. The pharmacokinetic profiles of rh-IFNα2a-NGR after i.m. administration to mice, rats and monkeys were reasonably well described using a linear one-compartment open model, subject to first-order elimination kinetics, within the given drug dose range. The concentration-time profiles of rh-IFNα2a-NGR are shown in Figs. 2–4, and the mean parameters from the analysis are listed in Tables 3–5. rh-IFNα2a-NGR was rapidly absorbed in mice with peak concentrations occurring around 0.38 to 0.49 h after i.m. administration of 3.12, 9.36 and 28.08 µg/kg of the drug. The maximum serum concentration (C_{max}) was increased proportionally to the dose. A prolonged half life ($t_{1/2}$) was observed at a dose of 28.08 µg/kg. The area under the curve showed a linear correlation to dose ($r = 0.999$, $P < 0.05$). In rats, after i.m. administration of 1.56, 4.68 or 14.04 µg/kg of rh-IFNα2a-NGR, $t_{1/2}$ and T_{peak} values were not statistically significant different among groups ($P > 0.05$). The $AUC_{(0-\infty)}$ linearly correlated to the dose ($r = 0.9993$, $P < 0.05$) as it also did in monkeys.

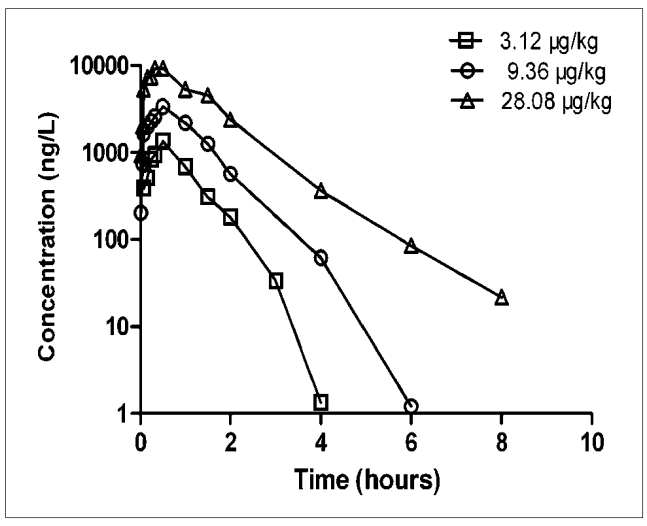

Fig. 2: Pharmacokinetic profile of rh-IFNa2a-NGR following i.m. injection to mice. The observed concentrations were represented by symbols. Every 5 mice at each point as one sample

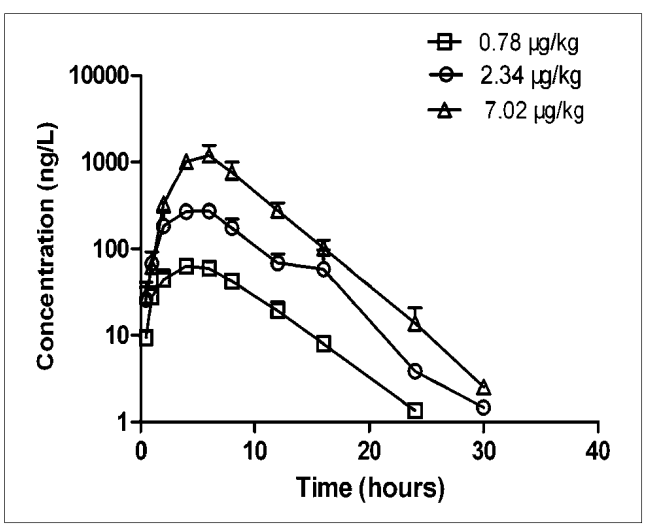

Fig. 4: Pharmacokinetic profile of rh-IFNa2a-NGR following i.m. injection to monkeys. The observed concentrations were represented by symbols. $n = 3$

Table 3: Calculated pharmacokinetic parameters of rh-IFNα 2a-NGR after i.m. administration to mice

Parameter	rh-IFNα2a-NGR		
Dosage	3.12 μg/kg	9.36 μg/kg	28.08 μg/kg
T½ (h)	0.33	0.39	0.79
Vd/F (ml/kg)	5.21	12.63	73.09
CL/F (ml/kg·h)	10.42	22.63	103.09
T_{peak} (h)	0.48	0.49	0.38
C_{max} (ng/L)	1104.36	3030.20	9007.46
$AUC_{(0-\infty)}$ (ng/L·h)	1346.72	4197.65	14271.87

Values were means ± SD. Every 5 mice at one time point taken as one sample. $T^{1/2}$, elimination half-life; Vd/F, volume of distribution/absolutely bioavailability; CL/F, clearance/absolutely bioavailability, Tpeak, time to reach peak concentration; Cmax, peak concentration; AUC, area under serum concentration-time curve.

3. Discussion

As indicated by the data above, the drug was absorbed fastest in mice, its T_{peak} being less than 0.5 h, possibly because of the smaller size of the leg muscle and the relatively abundant blood flow and large absorption area when the volume of drug administered is considered. The absorption rate was slowest in monkeys where T_{peak} was close to that in human (3.8 h) (Motzer et al. 2002). Macromolecule drugs migrate to blood circulation through capillaries or the lymphatic system (Kase et al. 1995), and it may be that the different absorption route in monkeys compared with mice and rats was a reason why T_{peak} was longest in monkeys. Half-life ($t_{1/2)}$, the time required for blood levels of the drug to drop by half, was shortest in mice, whereas in rats it was longest and closest to that in humans (5.28 h), while monkeys showed an intermediate value, implying that the rate of metabolism in the mouse kidney of rh-IFNα2a-NGR was fastest. Simultaneously, the assay results showed that $t_{1/2}$ in mice was prolonged at a dosage of 28.08 μg/kg while rats and monkeys showed no such phenomenon. Jang et al. (1992) reported that pharmacokinetic parameters for i.v. administered IFN altered in a dose-dependent manner and $t_{1/2\ was}$ extended with increased dosage in rabbits. A prolonged $t_{1/2}$ in mice may be an overall consequence of the faster absorption rate and changes in the efficiency or route of metabolism at 28.08 μg/kg. A longer $t_{1/2}$ in rats was possibly correlated to the anatomical characteristics of the rats chosen for the assay. Polypeptide drugs can be readily hydrolysed by proteinase which is widely distributed in every part of the body, so the C_{max} was determined both by the absorption rate and by the elimination rate. The fastest absorption rate in mice gave the highest C_{max}. In contrast, the lowest C_{max} seen in monkeys was partly the result of the slowest absorption and the relatively faster elimination. Though C_{max} in rats was lower than in mice, their AUC values were responding to the rats' slower rate of metabolism and longer $t_{1/2.}$ The AUC of monkeys was also lower because of their slower absorption and relatively faster elimination rate.

As shown above, after i.m. administration of rh-IFNα2a-NGR, mice, rats and monkeys had their own metabolic characteristics. These data would provide references for further clinical pharmacokinetic studies.

4. Experimental

4.1. Method validation

4.1.1. Linearity and lower limit of quantification (LLOQ)

Linearity was assessed by assaying five sets of serum to give a calibration curve, the curves being fitted by the linear weighted least squares regression method. LLOQ was the lowest standard concentration on the curve.

4.1.2. Stability

rh-IFNα-2a-NGR was added to serum at three different concentrations and samples were divided into two parts. One was determined immediately, as the initial concentration of the drug, another was stored at −20 °C. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−20 to 25 °C) on consecutive days.

4.1.3. Recovery experiments

To avoid the interference of serum components with antigen–antibody interaction, a recovery assay was performed by comparing rh-IFNα-2a-NGR concentrations in serum with the corresponding reference standards prepared at the same concentrations.

Table 4: Calculated pharmacokinetic parameters of rh-IFNα2a-NGR after i.m. administration to rats

Parameter	rh-IFNα2a-NGR		
Dosage	1.56 μg/kg	4.68 μg/kg	14.04 μg/kg
T½ (h)	7.08 ± 3.27	4.52 ± 1.40	3.43 ± 0.79
Vd/F (ml/kg)	36.11 ± 17.64	12.54 ± 6.44	1.97 ± 1.36
CL/F (ml/kg·h)	3.49 ± 1.44	2.40 ± 0.59	0.34 ± 0.18
T_{peak} (h)	1.32 ± 0.88	1.65 ± 0.97	2.40 ± 1.79
C_{max} (ng/L)	107.71 ± 52.39	654.49 ± 127.49	2251.70 ± 464.57
AUC(0-∞) (ng/L·h)	1168.71 ± 521.68	5784.68 ± 381.96	25128.87 ± 9734.33

Values were means ± SD. $n = 3$ at each time point

Table 5: Calculated pharmacokinetic parameters of rh-IFNα2a-NGR after i.m. administration to monkeys

Parameter	rh-IFNα2a-NGR		
Dosage	0.78 μg/kg	2.34 μg/kg	7.02 μg/kg
T½ (h)	2.75 ± 0.20	2.35 ± 0.45	2.95 ± 0.27
Vd/F(ml/kg)	0.14 ± 0.07	0.05 ± 0.07	0.04 ± 0.007
CL/F (ml/kg·h)	0.04 ± 0.02	0.02 ± 0.007	0.01 ± 0.00
T_{peak} (h)	3.90 ± 0.71	3.60 ± 0.55	4.59 ± 0.39
C_{max} (ng/L)	61.54 ± 13.67	268.13 ± 108.46	668.82 ± 101.22
AUC(0-∞) (ng/L·h)	573.09 ± 96.12	2348.30 ± 1177.36	6722.09 ± 593.12

Values were means ± SD. $n = 3$ at each time point.

4.1.4. *Intra- and inter-assay coefficients of variation*

To evaluate the precision and accuracy of the method, rh-IFNα-2a-NGR at four concentration levels (500, 125,32 and 8 pg/ml) was analyzed in five replicates on five validation days. The assay accuracy was expressed in terms of coefficient of variation (CV%).

4.2. *Pharmacokinetic studies*

Mice: Three groups of 65 mice received single intramuscular injections of 3.12, 9.36 and 28.08 μg/kg of rh-IFNα-2a-NGR. Following drug administration, 500 μl blood samples were collected from the orbital vein at 0, 0.02, 0.05, 0.083, 0.17, 0.25, 0.5, 1, 1.5, 2, 4, 6, and 8 h.
Rats: Three conscious rats in each group received single intramuscular injections of 1.56, 4.68 or 14.04 μg/kg of rh-IFNα-2a-NGR, and 200 μl blood samples were collected by heart puncture at 0, 0.083, 0.25, 0.5, 1, 3, 5, 7, 9, 12, 24 and 36 h.
Monkeys: Three groups of 9 monkeys received single intramuscular injections of 0.78, 2.34 or 7.07 μg/kg of rh-IFNα-2a-NGR, 1 ml blood samples were collected from the hind limb vein at 0, 0.5, 1, 2, 4, 6, 8, 12, 16, 24, 30 and 36 h.
The serum was isolated by centrifugation (8000 rpm × 10 min, 4 °C) and stored at −20 °C. The concentrations of rh-IFNα-2a-NGR were determined by ELISA analysis.

4.3. *ELISA*

rh-IFNα2a-NGR levels were measured by ELISA according to the manufacturer's instructions. Briefly, 100 μl of the serum sample or standard (0–500 pg rh-IFNα/ml) were added to the wells which had been precoated in duplicate with the capture antibody and incubated for 1 h at room temperature. After washing, 100 μl/well of streptavidin-HRP was added for another 1 h incubation. Following the last washing, 100 μl/well of substrate was added using a 1:1 mixture of stabilized peroxide and stabilized chromogen solutions. The enzymatic reaction was stopped after 15 min by addition of 50 μl/well of 1 M H_2SO_4. The absorbance of the reaction product was measured by a microplate reader over a time span of less than 15 min at 450 nm. The concentration of rh-IFNα2a-NGR in the samples was calculated from the rh-IFNα standard curve by linear regression analysis performed on each microtiter plate, subtracting the non-specific signal from all values obtained.

4.4. *Statistical analysis*

Pharmacokinetic parameters were calculated using DAS professional software (Mathematical Pharmacological Association, Shanghai, China). All values were expressed as mean ± SD. Pharmacokinetic data were analyzed statistically using SPSS 10 software (SPSS Inc. Chicago, IL, USA). A P value less than 0.05 was considered statistically significant

Acknowledgment: This study was supported by the National Science Foundation of China, No. 30572210.

References

Chiarion-Sileni V, Bononi A, Fornasa CV, Soraru M, Alaibac M, Ferrazzi E, Redelotti R, Peserico A, Monfardini S, Salvagno L (2002) Phase II trial of interferon-alpha-2a plus psoralen with ultraviolet light A in patients with cutaneous T-cell lymphoma. Cancer 95: 569–575.

Curnis F, Sacchi A, Borgna L, Magni F, Gasparri A, Corti A (2000) Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). Nat Biotechnol 18:1185–1190.

Curnis F, Sacchi A, Corti A (2002) Improving chemotherapeutic drug penetration in tumors by vascular targeting and barrier alteration. J Clin Invest 110:475–482.

Curnis F, Sacchi A, Gasparri A, Longhi R, Bachi A, Doglioni C, Bordignon C, Traversari C, Rizzardi GP, Corti A (2008) Isoaspartate-glycine-arginine: a new tumor vasculature-targeting motif. Cancer Res 68: 7073–7082.

Jang AH, Lee SH, Ryoo SH, Kim SH, Lee MG (1992) Dose-independent pharmacokinetics of recombinant human interferon-αA in rabbits. Int J Pharm 84: 273–278.

Kase S, Kubota T, Watanabe M, Furukawa T, Tanino H, Kuo TH, Saikawa Y, Teramoto T, Kitajima M (1995) Recombinant human interferon alpha-2a increases the antitumor activity of 5-fluorouracil on human colon carcinoma xenograft Co-4 without any change in 5-FU pharmacokinetics. Anticancer Res 15: 153–155.

Koskinas J, Zacharakis G, Sidiropoulos J, Elefsiniotis J, Savvas S, Kotsiou S, Kountouras D, Schina M, Kostopoulos P, Archimandritis A (2009) Granulocyte colony stimulating factor in HCV genotype-1 patients who develop Peg-IFN-alpha2b related severe neutropenia: a preliminary report on treatment, safety and efficacy. J Med Virol 81: 848–852.

Maellaro E, Pacenti L, Del Bello B, Valentini MA, Mangiavacchi P, De Felice C, Rubegni P, Luzi P, Miracco C (2003) Different effects of interferon-alpha on melanoma cell lines: a study on telomerase reverse transcriptase, telomerase activity and apoptosis. Br J Dermatol 148: 1115–1124.

Meng J, Yan Z, Wu J, Li L, Xue X, Li M, Li W, Hao Q, Wan Y, Qin X, Zhang C, You Y, Han W, Zhang Y (2007) High-yield expression, purification and characterization of tumor-targeted IFN-alpha2a. Cytotherapy 9: 60–68.

Motzer RJ, Rakhit A, Thompson J, Gurney H, Selby P, Figlin R, Negrier S, Ernst S, Siebels M, Ginsberg M, Rittweger K, Hooftman L (2002) Phase II trial of branched peginterferon-alpha 2a (40 kDa) for patients with advanced renal cell carcinoma. Ann Oncol 13: 1799–1805.

Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, Ashmun RA, Shapiro LH, Arap W, Ruoslahti E (2000) Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res 60: 722–727.