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Pharmacokinetics and partial thromboplastin time after intravenous recombinant hirudin variant-2 in rhesus monkeys[1]

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KEY WORDS recombinant proteins; hirudin; pharmacokinetics; enzyme-linked immunosorbent assay; partial thromboplastin time

ABSTRACT

AIM: To study the pharmacokinetics (PK) and changes of kaolin partial thromboplastin time (KPTT) following single or multiple (7 d) dosing of a novel recombinant hirudin variant-2 (rHV-2) *via* the route of iv bolus injection (50 % of the total dose) plus infusion (the remained 50 % of the dose) in rhesus monkeys. **METHODS:** A crossover design was applied to research the PK and KPTT profiles of rHV-2 after single (with total dose at 1, 3, and 6 $mg \cdot kg^{-1}$, respectively) and multiple dosing (3 $mg \cdot kg^{-1}$). An enzyme-linked immunosorbent assay (ELISA) method was utilized to determine the level of rHV-2 in plasma. **RESULTS:** The concentration profiles of rHV-2 during or after administration were dependent both on the loading dose and the infusion rate. Mean C_{max} after bolus in three single dose groups were 2.90, 9.78, and 15.68 $mg \cdot L^{-1}$, respectively. Infusions at rate of 8.35, 25, and 50 $\mu g \cdot kg^{-1} \cdot h^{-1}$ in 1 h resulted in steady-state levels of 0.73 − 0.86, 1.94 −2.04, and 5.41 − 5.59 $mg \cdot L^{-1}$, respectively. The plasma rHV-2 levels during or after administration among doses were significantly different at most of the time points. Area under concentration-time curve (AUC) increased linearly with dose but systemic clearances were similar among different groups. KPTT was significantly prolonged (compared with baseline) at all dose levels, and trended to increase with dose. **CONCLUSION:** Both the loading dose and the infusion rate are very important for controlling the rHV-2 level, and the data may be helpful for optimizing dosage-regimen in clinical trials.

INTRODUCTION

Hirudin, an anticoagulant peptide, which was composed of 65 amino acid residues, was firstly found in the European leech *hirudo medicinalis*. It is believed that the hirudin was one of the most potent natural inhibitors of coagulation. Hirudin rapidly forms a stable 1:1 complex with thrombin *via* the anionic/hydrophobic interaction, which preventing the cleavage of fibrinogen and subsequent fibrin clot formation. In 1998, Refludan™ (hirudin variant-1, HV-1) was approved in

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the United States for the treatment of patients with heparin-induced thrombocytopenia and thromboembolic complications. Studies were carried out for the treatment of other new indications, such as angioplasty, thrombolysis with streptokinase, and acute myocardial ischemia without S and T elevation in electrocardiograph (ECG), *etc*[1–4]. In this study, we investigated the pharmacokinetics (PK) of a novel structural recombinant hirudin variant-2 (rHV-2, Ile-Thr-Tyr-Thr-Asp-Cys-Thr-Glu-Ser-Gly Gln-Asn-Leu-Cys-Leu-Cys-Glu-Gly-Ser-Asn-Gly-Cys-Gly-Lys-Gly-Ser-Arg-Cys-Ile-Leu-Gly-Ser-Asn-Gly-Lys-Gly-Asn-Gln-Lys-Val-Thr-Gly-Glu-Gly-Thr-Pro-Lys-Pro-Glu-Ser His-Asn-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln).

METHODS AND MATERIALS

Test drug rHV-2 was produced by Beijing Institute of Radiation Medicine. It was expressed in *Pichia pastoris* and purified with standard procedure. The amino acid sequence of the peptide was different from that reported in the literatures[3]. The drug was available as white lyophilized powder (lot 20001226, purity >98 %) and was stored at -20 ℃ before use. The drug was dissolved in saline immediately prior to administration.

Animals Rhesus monkeys were supplied by the Animal Raising Center of the Academy of Military Medical Sciences (Grade I, Certificate BDW95002), four females and four males, weighing (5.9±1.0) kg. The animals were individually housed in stainless-steel cages and fed with standard monkey diet. Water was supplied *ad libitum*.

Experimental design and dosage groups The monkeys were randomly divided into 3 single dosing groups (total dose of 1, 3, and 6 $mg \cdot kg^{-1}$, respectively) and 1 multiple dosing group (3 $mg \cdot kg^{-1} \cdot d^{-1} \times 7$ d), each group consisted of two males and two females. Two single dosing groups were cross-overly designed for comparing the PK following administration at the low dose (1 $mg \cdot kg^{-1}$) and the high one (6 $mg \cdot kg^{-1}$). Another group was designed to study the pharmacokinetics after multiple dosing at the medium dose of 3 $mg \cdot kg^{-1}$ and comparing the PK after the first and the last dose. There was a 7-d washing out time between two separated administration periods. The drug was administered through the right posterior tibia vein, and firstly bolus injection 50 % of the total dose, then immediately followed by a constant infusion of another 50 % of dose in 1 h by a peristaltic pump. The infusion rates of low, medium, and high dose groups were 8.35, 25, and 50 $\mu g \cdot kg^{-1} \cdot h^{-1}$, respectively.

Blood sampling Blood samples were collected before and 1, 10, 20, 40, 60 min, 1.5, 2, 3, 4, and 8 h after bolus injection. Freshly drawn whole blood was mixed with sodium citrate (0.109 $mol \cdot L^{-1}$) at the ratio of 9:1. The samples were centrifuged at 3000×*g* for 10 min immediately after sampling. Plasma was collected and kept at -20 ℃ before analysis.

Assays of rHV-2 antigen in plasma IMUBIND® hirudin ELISA kit (lot 853, American Diagnostical Inc) was used to determine the level of hirudin in plasma. The ELISA had been tested to detect native hirudin, hirudin in plasma, the hirudin-thrombin complex, PEG hirudin, a variety of recombinant hirudin variants, and an adenoviral-mediated hirudin expression product. The assay did not detect certain C-terminal hirudin fragments. The assay procedure was according to previously described[5].

Determination of KPTT KPTT was determined by a commercial kit (Shanghai Sun Biotechnology Co, China) and measured by a platelet aggregation and blood coagulation densitometry (Beijing Shidi Scientific Instrument, Model PAPER-1, China).

Data analysis The PK parameters were estimated by non-compartmental analysis. Microcal Origin software was utilized for data fitting or plotting. Student's *t*-test was applied to compare the differences of concentration among dose groups.

RESULTS

Validity of the assay methods Both of the standard curves constructed by the kit and rHV-2 standards appeared as typical linear curves (logarithmic scales of hirudin concentration *vs* absorbance at 405 nm) within the dose range of 0.25–3.0 and 0.5–50 $\mu g \cdot L^{-1}$, respectively. The representative equations of the kit and the rHV-2 standards were as Y=0.020 (±0.013)+

0.64 (±0.03) X (r=0.996) and Y=-0.072 (±0.019)+ 0.320 (±0.022) X (r=0.990).

Standard curves of rHV-2 came from 5 parallel experiments performed on different days revealed that the coefficient of variance (CV %) of intra-assay was less than 7.4 %, and the CV % of inter-assay was less than 9.5 %. The CV % of the slopes and intercepts of standard curve were all less than 11.5 %. The limit of quantitation was 0.5 μ g·L^{-1}.

The results of determination of blank samples spiked with 1, 2, and 4 μ g·L^{-1} tested rHV-2 revealed that the relative standard deviation were -25 % to 5 %.

The validity studies demonstrated that it was reliable to use IMUBIND® hirudin ELISA kit for determination of plasma rHV-2 antigen. All of the specificity, sensitivity, accuracy, and precision met the requirements for PK study.

Concentration-time curves Plasma concentration-time profiles of rHV-2 antigen following iv bolus plus infusion were plotted in Fig 1. The baseline of determination was zero for all animals. Mean peak concentrations (C_{max}) immediately after bolus injections of 0.5, 1.5, and 3 mg·kg^{-1} of rHV-2 were 2.90, 9.78, and 15.68 mg·L^{-1}, respectively. Intravenous infusions at the rate of 8.35, 25, and 50 μg·kg^{-1}·h^{-1} in 1 h resulted in the mean steady-state levels of 0.73–0.86, 1.94–2.04, and 5.42–5.59 mg·L^{-1}, respectively. The rHV-2 concentrations at different doses decreased in parallel and exponential manner, then returned to baseline level at about 8 h after injection. The rHV-2 levels at most of the time point in different dose groups were with significance (P<0.05 or P<0.01). The phenomena indicated the loading dose and the infusion rate strongly affected the levels of the antigens.

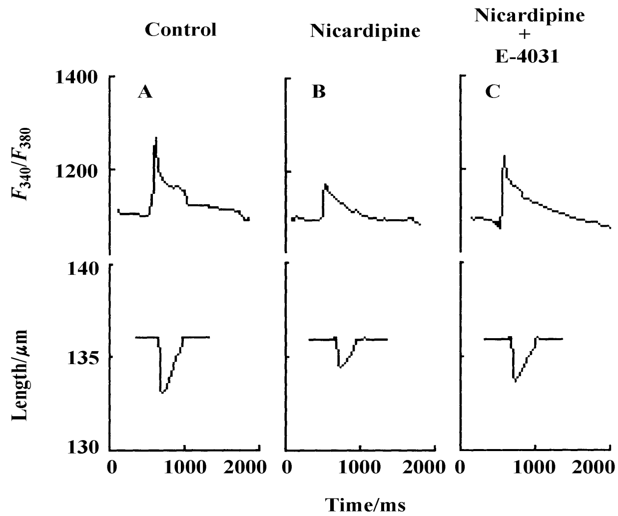

Fig 1. Plasma concentration of rHV-2 antigens following iv bolus injection plus constant infusion in 1 h (bolus:infusion dose ratio=1:1) of 1, 3, and 6 mg·kg^{-1} of recombinant hirudin variant-2 in rhesus monkeys. n=4. Mean±SD. "Start" denotes the beginning of administration and "End" denotes the end of infusion.

Pharmacokinetic parameters The PK parameters were estimated by non-compartmental analysis from the plasma concentration-time data of rHV-2 in Tab 1. After iv bolus plus infusion 1, 3, and 6 mg·kg^{-1} of rHV-2, AUC$_{(0-\infty)}$ were (1.6±0.7) (P<0.05 *vs* 3 and 6 mg·kg^{-1} group), (4.9±1.4) (P<0.05 *vs* 6 mg·kg^{-1} group), and (12±4) mg·h·L^{-1}, respectively. Both of AUC$_{(0-8\ h)}$

Tab 1. Pharmacokinetic parameters of rHV-2 following iv bolus (50 % of dose) plus constant infusion (50 % of dose) for 1 h of rHV-2 in rhesus monkeys. n=4. Mean±SD. ^{b}P<0.05 *vs* 3 mg·kg^{-1} group on d 1. ^{e}P<0.05 *vs* 6 mg·kg^{-1} group.

Dose/mg·kg^{-1}		AUC$_{(0-8\ h)}$ /mg·h·L^{-1}	AUC$_{(0-\infty)}$ /mg·h·L^{-1}	MRT/h	CL$_S$/L·h^{-1}·kg^{-1}	V_{SS}/L·kg^{-1}	$T_{1/2}$/h
1		1.6±0.7^{be}	1.6±0.7^{be}	0.325±0.020	0.7±0.3	0.25±0.11	1.53±0.04
3	d 1	4.9±1.4^{e}	5.0±1.5^{e}	0.300±0.010	0.66±0.26	0.21±0.08	1.6±0.4
3	d 7	4.19±0.27	4.33±0.29	0.35±0.06	0.70±0.00	0.23±0.05	1.48±0.15
6		12±4	12±4	0.32±0.04	0.54±0.22	0.19±0.07	1.85±0.25

AUC: area under concentration-time curve; MRT: mean retention time; CL$_s$: systemic clearance; V_{ss}: apparent distribution volume; $T_{1/2}$: half of the concentration decreased time at the terminal exponential data points.

and $AUC_{(0-\infty)}$ increased linearly with dose. Systemic clearance, terminal $T_{1/2}$, and mean retention time (MRT) were similar among doses.

PK after multiple dosing The rHV-2 concentrations of the same time points following dosing at d 1 and d 7 were with no statistical difference (Fig 2 and Tab 1). $AUC_{(0-8\ h)}$ were (4.9±1.4) and (4.19±0.27) $mg \cdot h \cdot L^{-1}$ (P=0.42), respectively. The accumulation factor ($AUC_{d\ 7}/AUC_{d\ 1}$) was (1.0±0.4) (P>0.05).

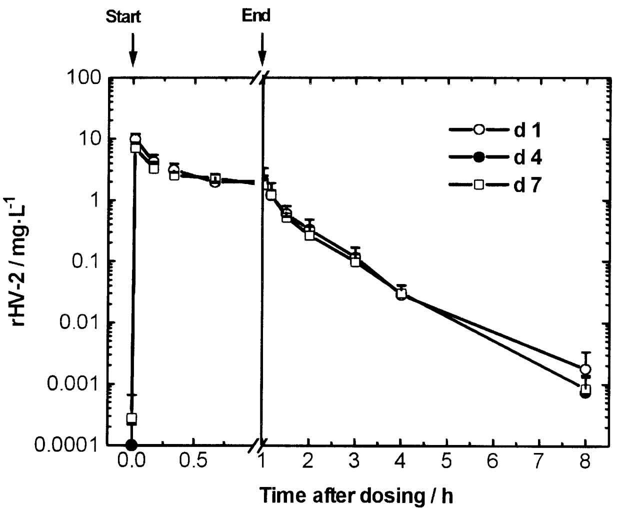

Fig 2. Plasma concentration of rHV-2 antigens following iv bolus injection plus constant infusion in 1 h (bolus:infusion dose ratio=1:1) of 3 $mg \cdot kg^{-1} \cdot d^{-1} \times 7$ d of recombinant hirudin variant-2 in rhesus monkeys. *n*=4. Mean±SD. "Start" denotes the beginning of administration and "End" denotes the end of infusion.

Changes of KPTT Plasma KPTT in all groups were significantly prolonged compared with individual baselines (Fig 3). The prolongation of KPTT at very few time points in 1 $mg \cdot kg^{-1}$ group were significantly shorter than that in higher dose groups (P<0.05 *vs* 3 $mg \cdot kg^{-1}$ group at 40 min, and P<0.05 *vs* 6 $mg \cdot kg^{-1}$ group at 1 min).

DISCUSSION

Studies have demonstrated the important role of C-terminal 10 amino acid residues (Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln)[6] and the thrombin anion-binding exosite-I (ABE-I)[7] in the formation of the thrombin-hirudin inhibitor complex. Experiment showed that

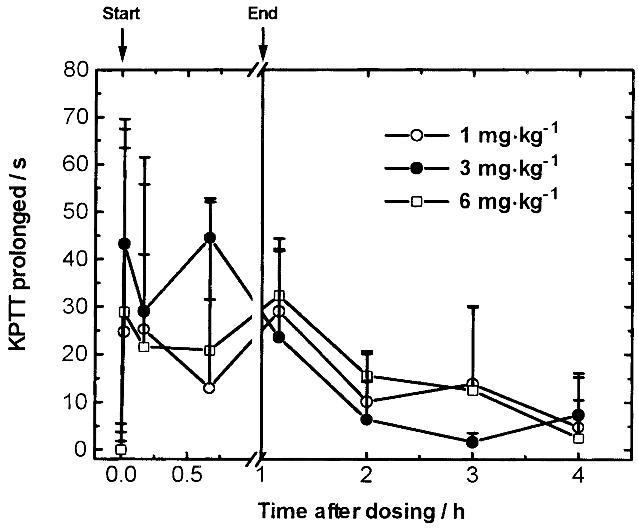

Fig 3. Plasma kaolin partial thromboplastin time (subtracts individual baseline value) following iv bolus injection plus constant infusion in 1 h (bolus:infusion dose ratio=1:1) of 1, 3, and 6 mg kg^{-1} of recombinant hirudin variant-2 in rhesus monkeys. *n*=4. Mean±SD. "Start" denotes the beginning of administration and "End" denotes the end of infusion.

the rHV-2-Lys47 mutation was related to activity[8]. Technology of genetic engineering led to the availability of sufficient quantities of recombinant hirudin for clinical purposes[9]. In this study, we reported a novel variant of rHV-2, which met all necessary requirements but different from Refludan™ (Hoest Marion), Revasc™ and other rHV-2. Preliminary pharmacological profiling of this rHV-2 showed that both of its PK and pharmacodynamic characteristics were similar to those of the hirudin reported, and we expected that it might have some features improved.

The PK study demonstrated that the shapes and plasma concentrations of rHV-2 during and after administration were correlated to the loading dose and the infusion rate. In other words, an optimal target concentration for a particular treatment with the highest efficacy and the minimal adverse reaction could be achieved by modifying the total dose, the loading dose, the infusion rate, and the infusion duration. For example, the protocol in this study using a rather high fraction loading resulted in immediately highest level and then gradually decreased to different level depended on the infusion rate. This protocol meets the requirement of an immediate high rHV-2 level in some special indications, for instance, angioplasty.

An interesting phenomenon observed in this study is that the levels of rHV-2 between different dose pairs were significantly different at most of the time points. With respect to KPTT, although prolongations of KPTT were observed compared with the baseline, the difference of KPTT prolongation among doses was seen at only very few time points. One explanation was probably related to the variation of KPTT among individuals. Another possible reason might be, the rHV-2 antigen determined by ELISA was composed of both free rHV-2 and combined rHV-2 in the thrombin-hirudin complex, so the observed rHV-2 concentration was not concordant with the KPTT prolongation, which was induced only by thrombin-bound hirudin. Further, this phenomenon might result from a saturation of formation of thrombin-hirudin complex.

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猕猴静脉给予重组水蛭素变异体-2的药代动力学和部分促凝血酶原时间[1]

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关键词　重组蛋白质类；水蛭素；药物动力学；酶联免疫吸附测定；部分促凝血酶原时间

目的：研究猕猴单次和多次(7天)静脉推注加滴注(剂量比为1:1)新型重组水蛭素变异体-2 (rHV-2)的药代动力学和部分促凝血酶原时间(KPTT)变化．**方法**：交叉设计比较单次1，3和6 mg·kg^{-1}及多次3 mg·kg^{-1}注射后血浆rHV-2浓度及KPTT．ELISA法测定血浆rHV-2．**结果**：浓度曲线形状和水平与推注量及滴注速率相关．推注后C_{max}分别为2.90，9.78和15.68 mg·L^{-1}，随后分别稳定在0.73-0.86，1.94-2.04和5.41-5.59 mg·L^{-1}，剂量间血浆rHV-2浓度有统计差别．AUC随剂量呈线性增大，剂量间清除率和各时间常数无统计差别．各剂量组KPTT比基线值明显延长，随剂量增加有延长趋势．**结论**：推注剂量和滴注速率对控制rHV-2水平起重要作用，这对临床试验优化给药方案有一定作用．

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