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

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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·kg⁻¹, respectively) and multiple dosing (3 mg·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 $C_{\text{max}}$ after bolus in three single dose groups were 2.90, 9.78, and 15.68 mg·L⁻¹, respectively. Infusions at rate of 8.35, 25, and 50 μg·kg⁻¹·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·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, Refudan™ (hirudin variant-1, HV-1) was approved in
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-Gly-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°C 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·kg\(^{-1}\), respectively) and 1 multiple dosing group (3 mg·kg\(^{-1}\)·d\(^{-1}\)×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·kg\(^{-1}\)) and the high one (6 mg·kg\(^{-1}\)). Another group was designed to study the pharmacokinetics after multiple dosing at the medium dose of 3 mg·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 μg·kg\(^{-1}\)·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·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°C before analysis.

**Assays of rHV-2 antigen in plasma** IMUBIND\(^\circ\) 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 μg·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.

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 (0-8 h) 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)
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·h·L\(^{-1}\) \((P=0.42)\), respectively. The accumulation factor (AUC\(_{d7}/AUC_{d1}\)) was (1.0±0.4) \((P>0.05)\).

**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·kg\(^{-1}\) group were significantly shorter than that in higher dose groups \((P<0.05\ vs\ 3\ mg\ kg^{-1}\ group\ at\ 40\ min,\ and\ P<0.05\ vs\ 6\ mg\ kg^{-1}\ group\ at\ 1\ min)\).

**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 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\(^{TM}\) (Hoest Marion), Revasc\(^{TM}\) 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 indi-
cations, 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.

REFERENCES


