Special pharmacokinetics of dipetarudin suggests a potential antitumor activity of this thrombin inhibitor

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Thrombin is a potent mitogen for many tumor cells, suggesting that this enzyme may be involved in tumor genesis and metastasis. Inhibition of thrombin expressed on the surface of tumor cells may improve outcomes in some tumor cases. For this reason, a thrombin inhibitor to be applied in antitumor therapy must have favorable pharmacokinetic attributes to exert its action as long as possible in the extravascular compartment of the extracellular space, with a short action intravascularly, avoiding bleeding and/or other undesirable side-effects. None of the thrombin inhibitors in clinical use has these properties. Here, we report for first time a direct thrombin inhibitor, named dipetarudin that could be very useful in antitumor therapy because of its pharmacokinetic behavior characterized by a rapid distribution in the extravascular space with a slow elimination from this compartment. ***Anti-Cancer Drugs*** **15:145–149 © 2004 Lippincott Williams & Wilkins.**

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Introduction

Thrombin, a trypsin-like serine protease, converts fibrinogen in fibrin and activates enzymatically other blood coagulation factors, such as factor V, VIII, XI, XIII and protein C [1,2]. Thrombin possesses also multiple bioregulatory functions unrelated to hemostasis including fibroblast, macrophages, splenocytes, endothelial cells and smooth muscle cell mitogenesis [3–6].

Most of the biological functions of thrombin are executed through its binding to the cell-surface receptors, the most important being the protease-activated receptors (PARs), which contain a cleavage site in the extracellular extension. The resultant shortened extracellular portion after thrombin cleavage contains a newly exposed N-terminus with a specific amino acid composition that functions as a tethered ligand to activate the receptor [7,8]. Thus, in this cellular process, receptor binding and the enzymatic activity of thrombin are coupled. Three of the four members of this receptor family (PAR-1, PAR-3 and PAR-4) are cleaved and activated by thrombin [9].

Some types of tumors are associated with a coagulation pathway leading to thrombin generation. Moreover, PAR-1 is highly expressed in tumor cells [9–13]. This could stimulate tumor cell growth, adhesion and, possibly, invasion in an autocrine cycle.

At present, the most investigated direct thrombin inhibitor is hirudin, which was purified from the salivary glands of the leech *Hirudo medicinalis* [14]. The recombinant equivalent of hirudin, named lepirudin, is now in clinical use for treatment of heparin-induced thrombocytopenia [15]. Dipetalogastin, another potent thrombin inhibitor, was isolated and cloned in our laboratories from the stomach content of the assassin bug *Dipetalogaster maximus*. Biochemical analysis demonstrated that it has a double head structure with a molecular mass of 12.9 kDa [16]. Furthermore, in order to reduce the molecular mass of dipetalogastin, we designed and cloned an inhibitor which consists of the N-terminal head structure of dipetalogastin II and a fragment of the anion exosite 1 blocking segment of hirudin. This new thrombin inhibitor, named dipetarudin, has a K_i value of 446 ± 85 fM and a molecular mass of 7.5 kDa which is comparable to r-hirudin [17].

On the other hand, synthetic thrombin inhibitors have also been developed, among them, argatroban and bivalirudin are in clinical use, and ximelagatran is in advanced clinical trials for prophylaxis or treatment of thromboembolic disorders.

Although inhibition of thrombin has been considered essential for treatment of thromboembolic disorders [18], its inhibition should also be a useful tool in the treatment of malignancies. Unfortunately, none of the thrombin inhibitors studied until now has a good pharmacokinetic behavior to be applied as an antitumor drug. For this reason, the development of novel inhibitors with

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adequate pharmacokinetic attributes and safety profiles is necessary for effective results in antitumor therapy.

In this study, we report the pharmacokinetics of dipetarudin and propose that it could be useful in antitumor therapy due to its special pharmacokinetic behavior.

Methods

Animal preparation and experimental protocol

Wistar rats were anaesthetized by a parenteral injection of 1.5 g/kg ethylurethane. A catheter was placed in the left jugular vein for blood sampling and another one was placed in the right jugular vein for a continuous infusion to ensure diuresis. For this purpose a 20% mannitol solution was administered at a flow of 12 ml/h for 5 min and then the infusion was continued with 10% mannitol containing 5% bovine serum albumin at 3 ml/h for 10 h.

For bilateral functional nephrectomy, the depilated skin and the muscles were cut by a flank incision. Within the retroperitoneum, surrounding fatty and connective tissue was removed from the kidney. The ureter and vessels were double-ligated and the kidney was left in position. The incision was closed with clips. Then the contralateral kidney was treated accordingly. A 2-h period of recovery from surgery was permitted before the experiment was performed. The body temperature of the animals was maintained at 37°C throughout the experiment by external heating.

Dipetarudin was expressed in *Escherichia coli* and purified as was described previously [17]. It was applied i.v. or s.c. as a single bolus injection of 1 mg/kg body weight.

Blood and urine sampling

The blood samples were drawn into plastic tubes containing one part of 3.13% sodium citrate and filled with nine parts of blood. Blood samples were collected for determination of the thrombin inhibitor concentration, before and at definite time intervals after dosing.

Urine was also collected immediately before dosing and at definite time intervals after dosing, and frozen at –20°C until further analysis.

Determination of thrombin inhibitor concentration

Anti-thrombin activity in urine samples was determined by ecarin clotting time (ECT) [19]. For the measurement of anti-thrombin activity in whole blood, a modification of this method was performed. Briefly, 100 µl of citrated blood was pre-incubated for 2 min at 37°C. The clotting reaction was started by addition of 100 µl ecarin solution (1 EU/ml in 0.05 M Tris–HCl buffer, pH 7.5 containing 0.154 M NaCl, 0.01 M $CaCl_2$ and 10% prionex). Inhibitor concentration in *ex vivo* samples was estimated by means of calibration curves obtained with blood or urine to which defined amounts of dipetarudin were added.

Pharmacokinetic calculations

The pharmacokinetic parameters were calculated from the blood concentration time curve after i.v. administration of inhibitor by means of a two compartment model described by the equation:

$$C_{(t)} - Ae^{-\alpha_t} + Be^{-\beta_t}$$

where $C_{(t)}$ is the dipetarudin concentration in blood at time t, α and β are the slopes of monoexponential distribution and elimination lines, respectively. A and B are the intercepts of these monoexponential lines with the ordinate.

The α-phase half-life ($t_{1/2\alpha}$) was derived from the elimination constant α. The β-phase half-life considered as elimination half-life ($t_{1/2\beta}$) was calculated from the slope of the terminal portion of log blood concentration of inhibitor versus time curve.

The fractional efflux (k_{12}), reflux (k_{21}) and elimination (k_{13}) rate constants were calculated according to the following equations:

$$k_{12} = [AB(\beta - \alpha)^2]/[C_{(0)}(A\beta + B\alpha)]$$

$$k_{21} = (A\beta + B\alpha)/C_{(0)}$$

$$k_{13} = C_{(0)}/(A/\alpha + B/\beta)$$

where $C_{(0)}$ is dipetarudin concentration in blood at time zero.

The area under the blood concentration time curve from zero to the time of the last blood concentration above the limit of detection (AUC_{0-t}) was calculated using the trapezoidal rule, and extrapolated to infinity ($AUC_{0-\infty}$) by adding C_{last}/β where C_{last} is the last measured blood concentration above the detection limit at the time of the last sampling point.

Total clearance from blood (Cl_{tot}) was calculated by dividing the i.v. dose (D) by the AUC value. The quotient $D/C_{(0)}$ represents the apparent volume of distribution (V_c) and the volume in steady state (V_{dss}) was calculated according to the equation:

$$V_{dss} = [(k_{21} + k_{12})/k_{21}]V_c$$

After s.c. administration of the thrombin inhibitor, the peak of its blood concentration (C_{max}) and time to C_{max} (t_{max}) were recorded as observed. The s.c. bioavailability (F) was determined using the formula:

$$F = (AUC_{0-\infty,s.c}/AUC_{0-\infty,i.v})(Dose_{i.v.}/Dose_{s.c.})$$

The urinary recovery of dipetarudin was calculated by multiplication of the inhibitor concentration in the urine

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aliquots (in μg/ml) by the total amount of urine (in ml) collected during the sampling period.

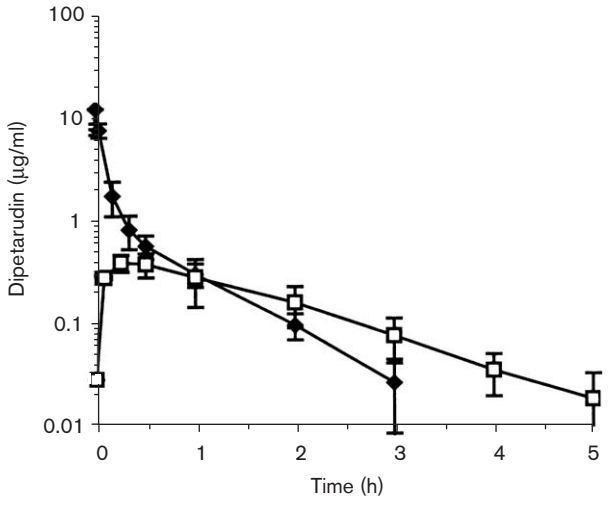
Results

Figure 1 shows the blood concentration time curves of dipetarudin. After i.v. administration, the distribution phenomena are primarily responsible for the decrease of its blood level, this initial distribution was followed by an elimination phase with a half-life ($t_{1/2\beta}$) of 0.5778 ± 0.1625 h. This behavior can be best described by an open two-compartment model with first-order elimination.

The blood concentrations of dipetarudin following s.c. administration are also shown in Figure 1. A maximum blood level (C_{max}) of 0.4037 ± 0.1021 μg/ml was observed 30 min (t_{max}) after inhibitor administration. Mean elimination half-life ($t_{1/2\beta}$) calculated on the 2–6 h interval was approximately 0.9737 ± 0.3387 h, which was markedly prolonged in comparison with the $t_{1/2\beta}$ of the i.v. application. The s.c. bioavailability was calculated as 84.6%.

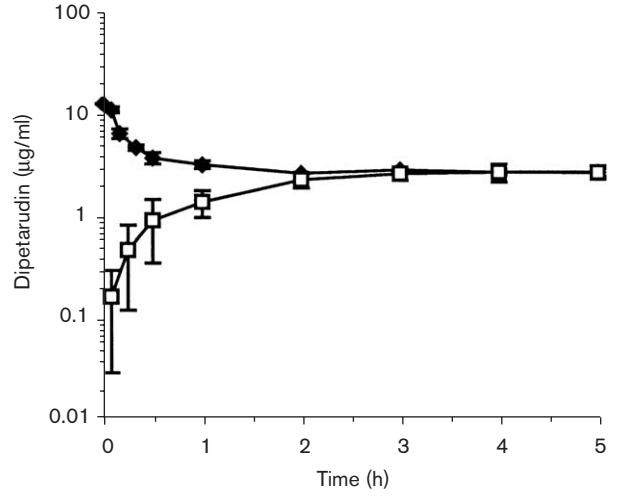
The administration of dipetarudin to nephrectomized rats was always followed by a higher blood level of this substance than found in animals with normal renal function (Fig. 2). After the distribution or absorption phases (for i.v. or s.c. administration, respectively) the blood levels remained nearly constant, which speaks in favor of the exclusive renal elimination of this inhibitor and demonstrates that it does not undergo some metabolism in any other organ of the body. Furthermore,

Fig. 1



Time course of the dipetarudin blood concentration in rats. Dipetarudin was administered as an i.v. (filled diamonds) or s.c. (open squares) bolus of 1 mg/kg. Results are expressed as the mean blood concentration (μg/ml) of $n=4$, showing the standard deviation at each sampling time.

Fig. 2



Time course of the dipetarudin blood concentration in nephrectomized rats. Dipetarudin was administered as an i.v. (filled diamonds) or s.c. (open squares) bolus of 1 mg/kg. Results are expressed as the mean blood concentration (μg/ml ± SD) at each sampling time.

the maximum blood level was reached 4 h after the application of a s.c. bolus, which represents the end of the resorption phase from the injection depot.

The elimination of dipetarudin from the extravascular space was described by a linear kinetics (Fig. 3). About 0.7% of the administered dose was excreted via kidneys per hour. Thus, total urinary excretion of active dipetarudin amounted to approximately 80% of the administered dose within 5 days.

The pharmacokinetic parameters obtained after i.v. dosing of dipetarudin are listed in Table 1. The volume of distribution at steady-state (V_{dss}) amounted to 0.2225 ± 0.0660 l/kg, demonstrating that dipetarudin is distributed in the extracellular space of the organism which is divided into intravascular and extravascular compartments.

The fractional efflux (k_{12}) and reflux (k_{21}) rate constants indicate that there is a special behavior in the transfer of dipetarudin between the vascular and extravascular compartments, which is approximately 2 times faster from the vascular to the extravascular space than vice versa, which could explain why this substance is excreted for a long time (more than 5 days) although its blood level is below the sensitivity limit of the ECT (approximately 10 ng/ml) already a few hours after administration. In fact, at 4 h after application of the inhibitor, the blood concentration of dipetarudin could not be measured by the ECT (Fig. 1), but at this moment only approximately 3% of the administered dose had been excreted (Fig. 3)

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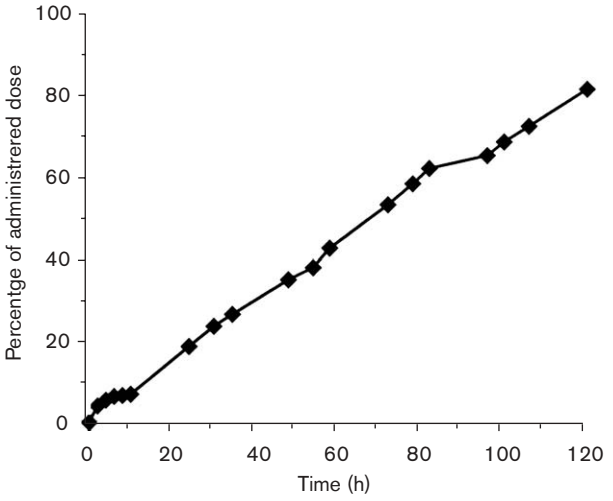
Fig. 3



Cumulative urinary excretion of dipetarudin. The data are expressed as percentage of administered dose, after i.v. injection of 1 mg/kg in a rat. Representative data of one of four experiments.

Table 1 Pharmacokinetic parameters of dipetarudin in rats

Pharmacokinetic parameters	Mean ± SD
A (μg/ml)	12.0280 ± 1.0379
B (μg/ml)	1.2158 ± 0.5720
α (h^{-1})	11.7212 ± 2.6130
β (h^{-1})	1.2739 ± 0.3540
$t_{1/2\alpha}$ (h)	0.0616 ± 0.0148
$t_{1/2\beta}$ (h)	0.5778 ± 0.1625
k_{12} (h^{-1})	3.9255 ± 1.1381
k_{21} (h^{-1})	2.1918 ± 0.7158
k_{13} (h^{-1})	6.8779 ± 1.4805
$AUC_{(0-3)}$ (μg/ml h)	1.8959 ± 0.1382
$AUC_{(0-\infty)}$ (μg/ml h)	1.9217 ± 0.1396
V_c (l/kg)	0.0762 ± 0.0087
V_{dss} (l/kg)	0.2225 ± 0.0660
Cl_{tot} (ml/h/kg)	522.5296 ± 39.6561

Dipetarudin was administered as an i.v. bolus injection of 1.0 m/kg. Values are the mean of $n=4$, showing the standard deviation.

and the remaining 97% had been distributed above all extravascularly; then, a small amount returned continuously to the vascular compartment, but it was quickly excreted in a first-phase manner via the kidneys.

Discussion

Via inhibition of thrombin, it is possible to avoid the activation of PARs expressed on the surface of tumor cells and outcomes in some tumor cases may be improved.

Up to date, some anticoagulant drugs have been used in trial clinical studies and animal models of malignancy. Several studies suggest an improvement in short- to intermediate-term survival in patients with cancer who received low-molecular-weight heparin [20,21], but heparin has serious drawbacks which limit its safe and efficacious use, the most important being heparin-induced thrombocytopenia [22,23]. Moreover, heparin–anti-thrombin III complexes do not inhibit thrombin bound to fibrin clots or to cells [24,25].

Clinical trials were also carried out using another anticoagulant, warfarin, that acts by vitamin K antagonism. However, most of these studies failed to show a clinical benefit [26,27].

Notably, the metastatic potential of transplanted tumors in experimental animals is greatly diminished by hirudin [28], confirming the favorable effect that direct thrombin inhibitors might exert in the antitumor therapy; however, the pharmacokinetic behavior of hirudin is not convenient for a long and safe antitumor therapy. Indeed, a thrombin inhibitor to be used as an antitumor drug should exert its effect as long as possible in the extravascular compartment to block the activation of the thrombin receptors, but a short action intravascularly to avoid hemorrhages and other undesirable side-effects. For this reason, it must be transferred faster from the vascular to extravascular space than vice versa, and thus it can be retained in the extravascular space and block the thrombin generated in this compartment.

In contrast, pharmacokinetic studies of hirudin have demonstrated that it is transferred with the same velocity in both senses. Moreover, it has been reported that 95% of the total amount of hirudin is renally eliminated within the first few hours following its administration [29]. In consequence, for a successful antitumor effect using hirudin, frequent application of this thrombin inhibitor would be necessary, which might increase the risk of hemorrhages or/and other complications in patients.

Similarly, pharmacokinetics of bivalirudin, argatroban and melagatran demonstrated that they are not retained in the extravascular compartment for a long time [30]. In fact, this is the first report of a thrombin inhibitor that is transferred quickly to the extravascular space, but returns very slowly to the vascular space. It is conceivable that a greater and safer degree of antitumor effect might be achieved by treatment with dipetarudin, which possesses an excellent anti-thrombin activity and more favorable pharmacokinetic attributes than the other thrombin inhibitors in clinical use.

Our results permit the hypothesis that administration of dipetarudin in patients with certain types of malignancy might bind and inhibit the thrombin expressed on the surface of the tumor cells, and thus may improve outcomes in some tumor cases. Dipetarudin in combination with other treatment modalities such as chemotherapy, radiation therapy and surgery might constitute an effective cancer therapy.

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