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ORIGINAL ARTICLE

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Preclinical pharmacology of the novel antitumor agent adaphostin, a tyrphostin analog that inhibits bcr/abl

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Abstract *Purpose*: To define several pharmacological properties for the potential anticancer agent, adaphostin, in order to determine whether the compound is appropriate for clinical evaluation as an anticancer agent. *Methods*: The analytical procedure involved high-performance liquid chromatography and utilized an analytical J'Sphere ODS H-80 column. *Results*: The stability of adaphostin at two different concentrations was determined at temperatures of 37°C, 4°C, and -80°C, in the plasma of mice, rats, dogs, and humans. The compound was most stable at the lower temperatures. At all temperatures, adaphostin was generally most stable in human plasma and least stable in dog plasma. Adaphostin bound strongly (>93%) to proteins in plasma from all four species. Following intravenous (i.v.) administration to mice (50 mg/kg; 150 mg/m^2), plasma concentrations declined rapidly from 50 μM at 2 min to 1 μM at 2 h. Elimination was triexponential, with $t_{1/2}$ values of 1.1, 9.1, and 41.2 min. The Cl_{tb} was 0.411 L/(min·m^2), the V_{dss} was 24.6 L/m^2, and the AUC was 927 μM·min. In a comparison of vehicles for intraperitoneal (i.p.) dosing, PEG 300 allowed the highest plasma concentrations of adaphostin. Bioavailability following an i.p. dose was greater than that following a subcutaneous dose, or that for a dose administered by oral gavage. For rats dosed i.v. with adaphostin (50 mg/kg; 300 mg/m^2), plasma concentrations also decreased triexponentially, with $t_{1/2}$ values of 1.8, 10.6, and 136 min. Other pharmacokinetic values were Cl_{tb} = 0.466 L/(min·m^2), AUC = 1,161 μM·min, and V_{dss} = 8.0 L/m^2. Analysis of samples collected from two dogs dosed i.v. with adaphostin (7.5 mg/kg; 150 mg/m^2) showed that plasma concentrations decreased in a biphasic manner, with individual values for $t_{1/2\alpha}$ of 6.0 and 9.8 min for the distribution phase and $t_{1/2\beta}$ of 40.6 and 66.2 min for the elimination phase. Other pharmacokinetic values were Cl_{tb} = 0.565 and 0.852 L/(min·m^2), AUC = 673 and 446 μM min, and V_{dss} = 29.6 and 56.8 L/m^2. *Conclusions*: The stability of adaphostin in plasma varies with species. In mice and dogs dosed with adaphostin, plasma concentrations of the compound decreased rapidly. The clearance of adaphostin from plasma, on an m^2 basis, was equivalent for mice and rats but more rapid in dogs. These results are relevant for assessing the pharmacologic and toxicologic profiles and the antitumor activity of adaphostin in humans.

Keywords Adaphostin · HPLC · Protein binding · Pharmacokinetics

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Introduction

Transmembrane receptor tyrosine kinases, a family of enzymes involved in signal transduction, share basic structural features: an extracellular domain with ligand binding specificity; a hydrophobic transmembrane domain; and a cytoplasmic domain that contains regulatory regions and catalytic sites for binding both ATP and substrate [1, 2]. Activation of these kinases often requires formation of a hetero- or homodimer with another receptor tyrosine kinase [3]. After dimerization, one receptor molecule is phosphorylated by the other [1]. Ultimately, signals are transmitted through the nuclear membrane, where they induce expression of specific

genes, causing a response in cellular growth, activation, or differentiation. Tyrosine kinases are also regulators of angiogenesis in normal and tumor tissues [4]. Mutations that lead to ligand-independent activation or expanded substrate specificity, altered ATP-binding, or dysregulation of the tyrosine kinase activity can render these genes constitutively active. Aberrant regulation of these proteins is implicated in carcinogenesis, tumor progression, and metastasis [5].

Tyrphostins are small synthetic molecules that specifically inhibit tyrosine kinases by interfering with the binding of ATP or ligands [6]. There is a correlation between tyrphostin-mediated inhibition of $p210^{bcr\text{-}abl}$ kinase, which is involved in growth stimulation, and inhibition of the growth of chronic myelogenous leukemia (CML) cells [7]. A potent tyrphostin is AG957, which blocks DNA synthesis in CML cells at times and concentrations that do not affect RNA or protein synthesis. Such inhibition results in apoptosis [8]. The adamantyl ester of AG957, adaphostin (Fig. 1), down-regulates $p210^{bcr\text{-}abl}$ kinase in CML cells at concentrations lower than those required for the parent compound [8]. Adaphostin, which inhibits the growth of leukemia cell lines in culture at concentrations < 1 μM [9], induces apoptosis in these cells and is active in cells resistant to another inhibitor of bcr/abl kinase [9–11]. An alternative mechanism of action for adaphostin involves production of intracellular peroxide [12]. The formation of peroxide is followed by strand breaks in DNA and, in cells with wild-type p53, a response to DNA damage involving p53 phosphorylation and up-regulation.

The purpose of the present effort was to define several pharmacological properties relevant in determining whether adaphostin is appropriate for clinical evaluation as an anticancer agent.

Materials and methods

Test compounds, chemicals, reagents, and animals

All chemicals and solvents used for sample preparation and high-performance liquid chromatography (HPLC) analysis were of analytical grade. Other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA. Samples of heparinized mouse (non-Swiss albino), rat (Sprague-Dawley), and dog (mixed breed) plasma were purchased from Lampire Biological Laboratories, Pipersville, PA, USA. For human plasma, a blood sample donated from an individual was centrifuged to prepare plasma. The National Cancer Institute supplied adaphostin, male CD2F1 mice (25 g), and male Fischer 344 rats (86–155 g). Beagle dogs (11 months, 9–10 kg, 1 male, 1 female) were obtained from Covance Research Products Inc. The University of Alabama at Birmingham's Institutional Animal Care and Use Committee (IACUC) approved the protocols for care and use of mice and rats, and the Battelle IACUC approved the protocol for care and use of dogs. Studies were accomplished in compliance with appropriate National Institutes of Health guidelines.

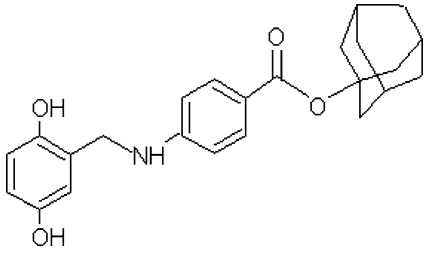

Fig. 1 Structure of adaphostin

Analytical method

Adaphostin in biological samples was analyzed by an analytical procedure involving liquid–liquid extraction and reverse-phase HPLC. For extraction of samples, one portion of plasma was mixed with two portions of cold acetonitrile. These preparations were mixed and then centrifuged to remove the precipitate. The HPLC system consisted of a Hewlett Packard 1050 ChemStation with a UV detector (Agilent 1050 series). Columns used were an analytical J'Sphere ODS H-80 column (4 μm, 150 × 4.6 mm YMC Co., Wilmington, NC, USA) and a LiChroCART 100 RP-18 guard column (EM Sciences, Gibbstown, NJ, USA). The mobile phase was 60:40 acetonitrile/ammonium formate buffer [0.05 M, pH 4.0 (v/v)], and the flow rate was 1 mL/min. The eluate was monitored by UV at 302 nm. The peaks for adaphostin were used to establish standard curves and for quantitative analysis of samples. The retention time for adaphostin was 9 min.

In the investigated concentration range of 0.05–10 μM, the calibration curves for adaphostin in mouse, rat, dog, and human plasma were linear (Fig. 2). The correlation coefficients (r^2) were > 0.998. As determined for mouse plasma, intra-day and inter-day variations were acceptable in that the coefficient of variation (CV) was $< 10.12\%$ (Table 1), and, for concentrations of 0.1, 0.5, 1.0, 2.5, 5, and 10 μM, values for accuracy were within the range of −6.2 to 0.9% (not shown). The specification range was 90% to 110% of the actual concentrations. Data for rat, dog, and human plasma were similar. For plasma from the four species, the lower limit of detection was 0.01 μM and the lower limit for quantitation was 0.05 μM.

Stability of adaphostin in mouse, rat, dog, and human plasma

By use of the validated HPLC method, stability studies of adaphostin (1 and 5 μM) were performed in mouse, rat, dog, and human plasma at temperatures of 37°C, 4°C, and −80°C. Adaphostin was dissolved in methanol

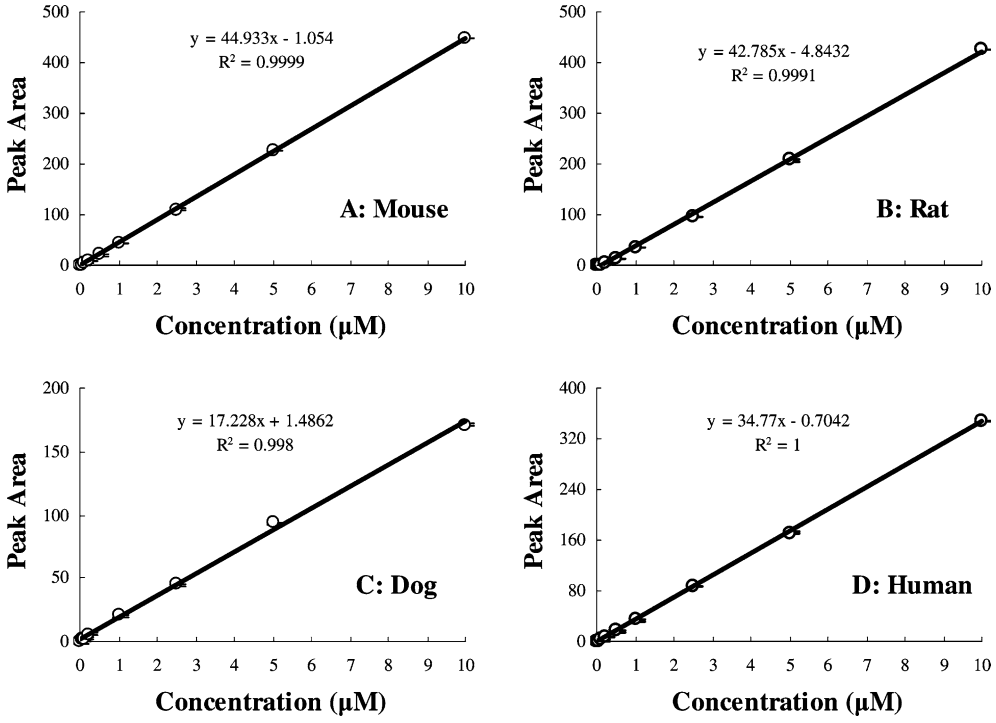

Fig. 2 Standard curves for adaphostin in mouse plasma (**a**), rat plasma (**b**), dog plasma (**c**), and human plasma (**d**)

Table 1 Inter-day and intra-day variation for determination of adaphostin in mouse plasma

Concentration (μM)	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Mean (SD)	CV (%)
Inter-day							
1	0.90	0.95	0.92	1.01	0.92	0.94 (0.04)	4.60
2.5	2.51	2.62	2.64	2.18	2.33	2.45 (0.20)	8.12
5	4.84	4.89	4.94	4.84	4.29	4.76 (0.27)	5.63
Intra-day							
1	0.90	0.98	0.90	0.94	0.96	0.93 (0.03)	3.69
2.5	2.49	2.28	2.64	2.79	2.18	2.48 (0.25)	10.12
5	4.84	5.05	4.54	4.31	4.33	4.61 (0.32)	6.97

and diluted with plasma so that the final concentration of methanol was 0.5%. At selected times, samples were removed and extracted. The adaphostin concentrations were quantified and, to illustrate the in vitro stability, expressed as percentages of the initial concentration.

Binding to plasma proteins

The plasma protein binding of adaphostin was assessed by a previously described procedure involving use of a micro-ultrafiltration system [13]. Adaphostin was dissolved in methanol and added to plasma; the final concentration of methanol was 1%. Samples of mouse, rat, dog, and human plasma containing adaphostin at concentrations of 1 and 5 μM were maintained at 37°C for 20 min. Controls were prepared with water to the same final concentrations. From each of these preparations, a portion was taken and placed in a sample reservoir of an Amicon Centrifree® ultrafiltration system (Millipore Co., Bedford, MA, USA). The filter systems were centrifuged at 2,000 *g* until the reservoirs were dry. From each filtrate, triplicate portions were taken for analysis by HPLC. The amounts present were designated as "free drug" (*F*). The concentrations of the unfiltered solutions were also determined by triplicate analyses. This amount represented the "total drug" concentration (*T*). The amount bound to the filter (*X*) was also considered. The percentage of adaphostin bound to plasma proteins was calculated by the following formula:

$$\% \text{ bound} = [(T - F - X)/T] \times 100$$

Pharmacokinetic studies

Male CD2F1 mice were dosed by i.v. injection of adaphostin via a tail vein (50 mg/kg body weight; 150 mg/m^2). The compound was dissolved in dimethylsulfoxide (DMSO) and administered in a volume of 1 mL/kg body weight. In an experiment involving intraperitoneal (i.p.)

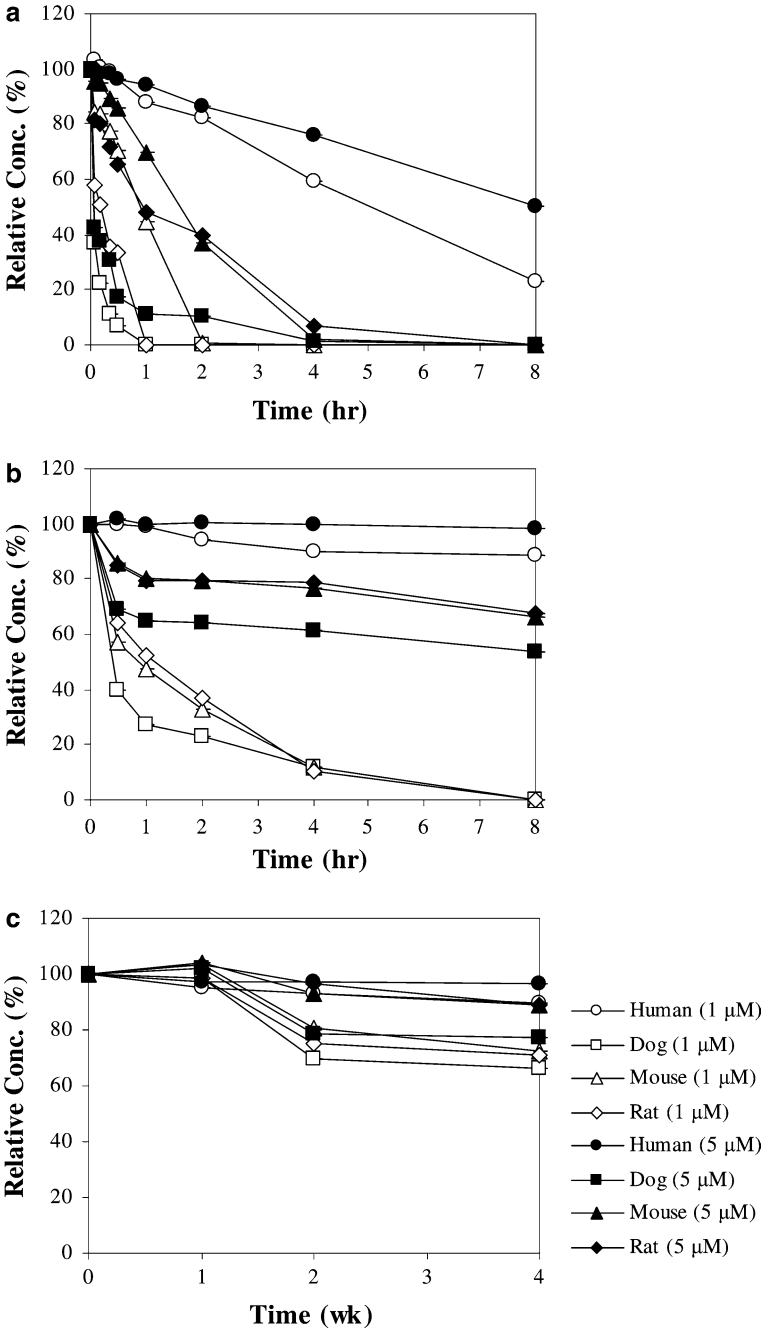

Fig. 3 Stability of adaphostin in mouse, rat, dog, and human plasma at 37°C (**a**), 4°C (**b**), and −80°C (**c**)

Table 2 Binding of adaphostin to proteins present in plasma from various species

Type of plasma	Concentration (μM)	Total bound (%)[a]	Protein binding (%)
Mouse	1	100	98.58
	5	100	93.25
Rat	1	100	98.58
	5	100	93.25
Dog	1	100	98.58
	5	100	93.25
Human	1	100	95.99
	5	100	94.79

[a]% remaining in the reservoir during centrifugation of plasma samples

injection (50 mg/kg), different vehicles were compared. Adaphostin was dissolved either in PEG 300 or DMSO and administered in a volume of 1 mL/kg body weight, or suspended in 0.9% saline containing 0.05% Tween 80 and administered in a volume of 10 mL/kg body weight. To compare different routes of administration, mice (three for each time point) were dosed intraperitoneally, by oral gavage, or by subcutaneous administration (25 mg/kg). For dosing, adaphostin was suspended in PEG 300 and administered in a dose volume of 1 mL/kg for the i.p. dose and 10 mL/kg for the oral and subcutaneous doses. At selected times, groups of three mice were bled from the retro-orbital sinus. Blood was

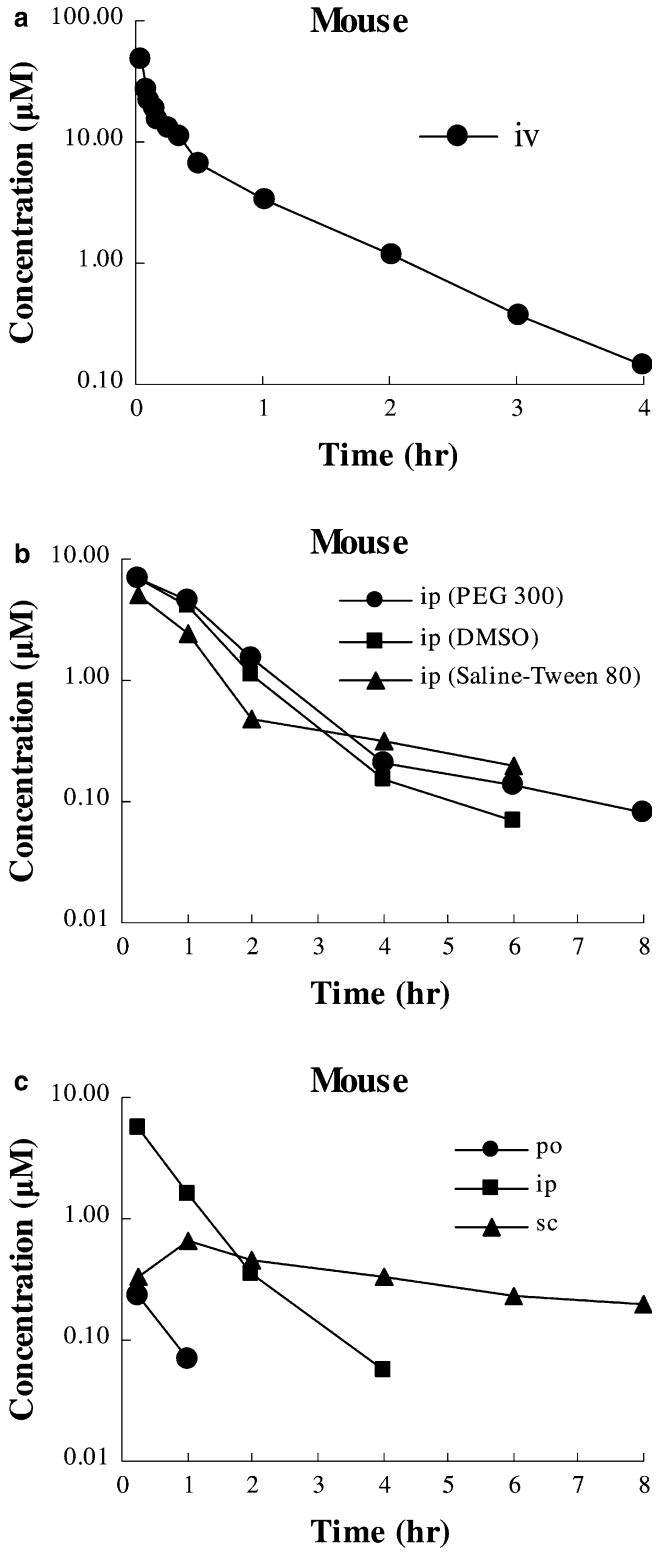

Fig. 4 Plasma concentrations of adaphostin following **a** intravenous administration of adaphostin to mice at a dose of 50 mg/kg, **b** intraperitoneal administration of adaphostin in various vehicles to mice at a dose of 50 mg/kg, **c** intraperitoneal, oral gavage, and subcutaneous administration of adaphostin to mice at a dose of 25 mg/kg. There were three mice for each time point

collected in heparinized tubes, and plasma was prepared by centrifugation. Pharmacokinetic values were derived with the WinNonlin program (Version 4.1, Mountain View, CA, USA).

To determine the pharmacokinetics of adaphostin in male Fischer 344 rats (86–155 g), groups of three were dosed intravenously via a tail vein (50 mg/kg; 300 mg/m^2) in a vehicle of 30% hydroxypropylcyclodextrin (HPCD), pH 4.0. The volume administered was 5 mL/kg. At selected times after dosing, blood samples were collected from the inferior vena cava into heparinized tubes. Plasma was prepared by centrifugation. Urine was also collected from each of the three rats kept in metabolism cages for 4, 8, and 24 h. Pharmacokinetic parameters were derived by use of the WinNonlin program.

Two dogs (dog #1, male; dog #2, female) were dosed by i.v. bolus administration of adaphostin (7.5 mg/kg; 150 mg/m^2) suspended in 0.1 M HPCD, pH 4.0. The volume administered was 0.5 mL/kg and was injected as a bolus via a catheter placed into the cephalic vein of each animal. At selected times after dosing, blood samples were collected from the jugular vein into Vacutainers containing EDTA. Plasma was prepared by centrifugation. Pharmacokinetic values were derived by use of the WinNonlin program.

Results

Stability in plasma

At 37°C and concentrations of 1 or 5 μM, adaphostin was most stable in human plasma and least stable in dog plasma (Fig. 3a). At 1 μM, the half-lives were 60 min, 10 min, <5 min, and 300 min, respectively, for mouse, rat, dog, and human plasma. At 5 μM, the values were about 90 min, 60 min, <5 min, and >480 min, respectively. At 4°C (Fig. 3b), the half-lives for the lower concentration of 1 μM were, respectively, 45 min, 60 min, <30 min, and >24 h. At the higher concentration of 5 μM, the half-life values for mouse, rat, and dog plasma were 24 h; adaphostin was essentially stable in human plasma. For each type of plasma at −80°C (Fig. 3c), the half-lives for adaphostin were all >4 weeks, but the compound tended to be less stable in dog plasma. From these data, we can conclude that adaphostin, in plasma from various species, is more stable at lower temperatures. It is also more stable in human plasma than in plasma from mice, rats, or dogs.

Protein binding

Plasma samples from mice, rats, dogs, and humans were examined for their capacity to bind adaphostin at concentrations of 1 and 5 μM. The results (Table 2) show

Table 3 Pharmacokinetic parameters for adaphostin in mice, rats, and dogs dosed by intravenous injection

Parameter	Description or value			
Species	Mice	Rats	Dog #1	Dog #2
Dose (mg/kg; mg/m^2)	50; 150	50; 300	7.5; 150	7.5; 150
Vehicle	DMSO	30% HPCD	0.1 M HPCD	0.1 M HPCD
Half-life, $t_{1/2\alpha}$ (min)	1.1	1.8	6.0	9.8
Half-life, $t_{1/2\beta}$ (min)	9.1	10.6	40.6	66.2
Half-life, $t_{1/2\gamma}$ (min)	41.2	136	N/A[a]	N/A
Cl_{tb} [L/(min·m^2)]	0.411	0.466	0.565	0.852
AUC (μM·min)	927	1,611	673	446
V_{dss} (L/m^2)	24.6	8.0	29.6	56.8

[a]N/A, not applicable

that >93% of the added adaphostin was bound by plasma proteins from these species.

Pharmacokinetics

Following bolus intravenous (i.v.) administration of adaphostin (50 mg/kg; 150 mg/m^2) to mice, plasma concentrations declined rapidly from 50 μM at 2 min to 1 μM at 2 h (Fig. 4a). Plasma concentrations were >20 μM for about 10 min. At 4 h after dosing, the concentration was <0.2 μM. Regression analysis indicated that plasma concentrations of adaphostin declined triexponentially. The half-lives for the initial, intermediate, and terminal phases were 1.1 min, 9.1 min, and 41.2 min, respectively (Table 3). The Cl_{tb} was 0.411 L/(min·m^2), the V_{dss} was 24.6 L/m^2, and the AUC was 927 μM·min.

Intraperitoneal administration of adaphostin to mice (50 mg/kg; 150 mg/m^2) in PEG 300 or DMSO resulted in plasma concentrations in the range of 5–8 μM for 1 h; at 4 h after dosing, the concentration was 0.2 μM (Fig. 4b). When adaphostin was administered in a saline vehicle containing Tween 80, plasma concentrations were in the range of 2–5 μM for 1 h; at 4 h after dosing, the concentration was ~0.3 μM. Intraperitoneal bioavailability was 77, 66, and 54%, respectively, for the vehicles PEG 300, DMSO, and saline/Tween 80.

In a comparison of the i.p., oral, and subcutaneous routes of administration, adaphostin was given to mice at a dose of 25 mg/kg in a vehicle of PEG 300 (Fig. 4c). Following i.p. dosing, plasma concentrations of adaphostin decreased from 5.6 μM at 15 min to 0.06 μM at 4 h and were not detectable thereafter. For this route of administration, bioavailability was 74%. This value is similar to that derived for a dose of 50 mg/kg in the same vehicle (77%). After subcutaneous dosing, concentrations increased to a maximum of 0.7 μM at 1 h and then declined with a half-life of ~4 h to 0.2 μM at 8 h; bioavailability was 51%. Administration by oral gavage gave low plasma concentrations that fell from 0.2 μM at 15 min to 0.07 μM at 1 h; bioavailability was ~3%.

For rats dosed intravenously with adaphostin (50 mg/kg; 300 mg/m^2), plasma concentrations that were initially 162 μM, decreased rapidly (Fig. 5). Values for the three phases of elimination were: $t_{1/2\alpha}$, 1.8 min; $t_{1/2\beta}$, 10.6 min; and $t_{1/2\gamma}$, 136 min (Table 3). The values derived were from the means for three rats at each time point. After 4 h, adaphostin was barely detectable. Other pharmacokinetic values were Cl_{tb} = 0.466 L/(min·m^2), AUC = 1,161 μM·min, and V_{dss} = 8.0 L/m^2. Over a period of 24 h, the amount of adaphostin excreted in the urine of these rats was <0.01% of the dose administered.

Samples of dog plasma were collected in a pharmacokinetic study of adapahostin administered to two animals at a dose of 7.5 mg/kg; 150 mg/m^2. For dogs #1(male) and #2 (female), the plasma concentrations of adaphostin decreased with $t_{1/2\alpha}$ values of 6.0 min and 9.8 min, and $t_{1/2\beta}$ values of 40.6 and 66.2 min, respectively (Fig. 6a, b; Table 3). Other pharmacokinetic values, for individual dogs, were: Cl_{tb} = 0.565 and 0.852 L/(min·m^2), AUC = 673 and 446 μM·min, and V_{dss} = 29.6 and 56.8 L/m^2.

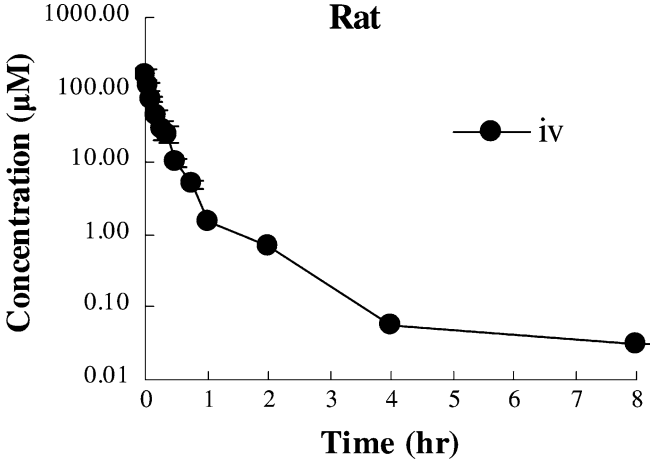

Fig. 5 Plasma concentrations of adaphostin following intravenous administration of adaphostin to rats at a dose of 50 mg/kg. There were three rats for each time point. The standard errors for the values are too small to be readily visible in the figure

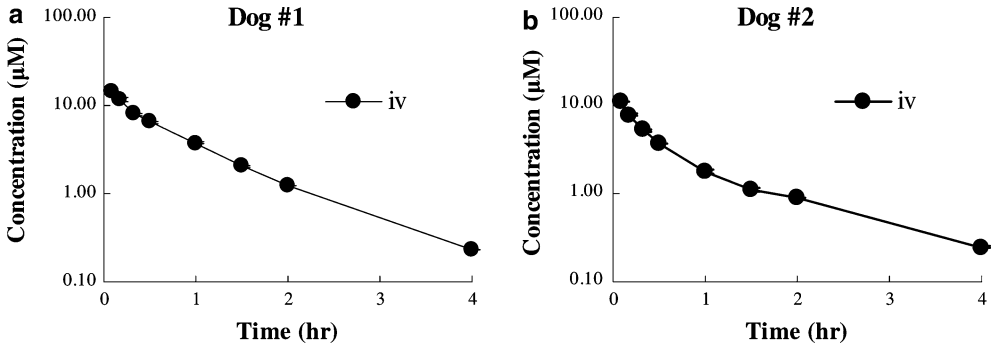

Fig. 6 Plasma concentrations of adaphostin following intravenous administration of adaphostin to each of two dogs at a dose of 7.5 mg/kg

Discussion

The purpose of the present effort was to define several pharmacological properties for adaphostin that may be relevant in determining whether the compound is appropriate for clinical evaluation as an anticancer agent. Evaluations of plasma stability, protein binding, and pharmacokinetics for different species can be important in the selection of an appropriate animal model for preclinical testing of antitumor drugs and for selection of an appropriate dose, route, and schedule of administration to achieve effective plasma concentrations of the test compound. Optimization of a dosing regimen can be achieved through characterization of the pharmacokinetic properties of an agent, in the context of the level and duration of exposure required for activity against cancer cells.

Although it is difficult to extrapolate in vitro results to predict in vivo situations, species differences in biostability of adaphostin may be related to its therapeutic and toxic effects. Since adaphostin is most stable in human and mouse plasma, the mouse model may be better used to predict pharmacologic and toxicologic profiles in humans.

Traditionally, in pharmacokinetic studies, total plasma drug concentrations are used to describe the distribution and elimination of tested drugs and their metabolites. For many drugs, however, the therapeutic and toxic effects correlate better with the concentration of diffusible, unbound drug than with that of the total drug. Thus, in anticipation of future pharmacokinetic and clinical studies, it is important to evaluate the binding to plasma proteins. In the present study, extensive protein binding of adaphostin was found in plasma from mice, rats, dogs, and human plasma. Such binding may play a role in the disposition and therapeutic effects of adaphostin.

Time-course assays designed to determine the adaphostin concentrations and exposure periods required for activity against various human tumor cells revealed that, in the most sensitive cell line (NCI-H522), concentrations as low as 2 μM for a period as brief as 10 min produced total growth inhibition (M. Alley, National Cancer Institute, personal communication). Concentrations of 5 μM caused a 50% reduction in the number of cells over a 10-min period of exposure. When the exposure period was extended to 6 h, the concentration required to reduce growth by 50% was 2 μM. For several other cell lines, concentrations of 10–20 μM were required to inhibit cell growth. Thus, a goal for therapy would be to achieve plasma concentrations of 10–20 μM for 1–3 h or 5–10 μM for 6–12 h.

In the present study, plasma concentrations >10 μM were maintained for 20 min, 30 min, and 5–10 min, respectively, in mice, rats, and dogs dosed intravenously. The lower bioavailability following i.p., oral, and subcutaneous dosing may limit the use of these routes of administration. The extremely low bioavailability following oral dosing is probably due to minimal absorption of precipitated adaphostin. Based on this logic, and considering that plasma concentrations in humans are likely to be considerably lower at comparable mg/kg doses, one would expect that even with i.v. dosing, only tumors remarkably sensitive to this compound would respond in substantial way.

The results, however, can also be viewed differently. Values for AUC and clearance of adaphostin from plasma, on an m^2 basis, were equivalent for mice and rats, and values for AUC were smaller and clearance was more rapid for dogs, perhaps due to greater degradation in plasma. Nevertheless, there is considerably greater in vitro stability of adaphostin in human plasma, and greater stability of this drug would be expected in humans. Such stability may translate into substantial antitumor activity.

Overall, the results provide a basis for further evaluation of adaphostin as a new chemotherapeutic agent for treatment of human cancer.

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