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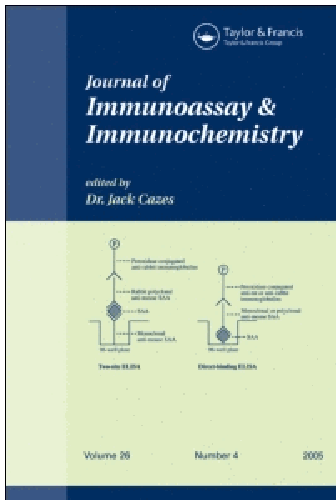

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Development of an Immunoassay for BMS-191352, A Single-Chain Immunotoxin, and Its Application to Toxicokinetic Studies

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DEVELOPMENT OF AN IMMUNOASSAY FOR BMS-191352, A SINGLE-CHAIN IMMUNOTOXIN, AND ITS APPLICATION TO TOXICOKINETIC STUDIES

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ABSTRACT

BMS-191352 is a single-chain fusion protein composed of the variable regions of chimeric BR96 monoclonal antibody and the binding defective form of *Pseudomonas* Exotoxin A (PE40). The immunotoxin exhibits potent cytotoxicity against tumor cells expressing the Lewis^{Y} antigen. A sensitive and specific double antibody sandwich ELISA has been developed and validated for the determination of BMS-191352 in rat and dog EDTA plasma. A monoclonal anti-PE40 antibody (EXA2-1H8) was used to capture BMS-191352 in plasma samples. The captured BMS-191352 was then detected using a biotinylated monoclonal BR96 anti-idiotypic antibody (757-4-1) followed by the addition of streptavidin-horseradish peroxidase conjugate and chromogen 3, 3', 5, 5'-tetramethylbenzidine. The optical density was measured at 450 nm. The standard curve range in rat and dog plasma was 2-32 ng/mL. The RSD for the inter- and intra-assay precision was within 9.2% and the accuracy was greater than 89.0%. The ELISA method was applied to the analysis of BMS-191352 in plasma samples from toxicokinetic studies conducted in rats and dogs. These studies revealed that the systemic exposure of BMS-191352 was dose proportional and the kinetics of BMS-191352 were linear between the dose range of 1.8-7.2 mg/m^2 in the rat and 2.5-15 mg/m^2 in the dog. (KEY WORDS: ELISA, BMS-191352, Immunotoxin, Toxicokinetics)

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INTRODUCTION

Immunotoxins are hybrid proteins consisting mainly of a monoclonal antibody (mAb) linked to a protein toxin (1, 2). Many of these targeting agents are under evaluation in clinical trials for safety and efficacy in the treatment of cancer (3). BMS-191352 (BR96 sFv-PE40) is a 67 kDa single-chain immunotoxin fusion construct of the variable heavy and light chains of a human-murine chimeric mAb (BR96) and the translocation and catalytic domains of *Pseudomonas* exotoxin A (PE40) (4). BR96 recognizes a carbohydrate structure which is composed, at least in part, of the Lewis^{Y} antigen expressed on a wide variety of human carcinomas including that of the lung, breast, colon, ovary, and prostate (5). *Pseudomonas* exotoxin A is a 66 kDa protein produced by the bacterium *Pseudomonas aeruginosa* and contains three structural domains that contribute to the binding, translocation, and catalytic activity (1). PE40, an approximately 40 kDa fraction, is the binding defective form of *Pseudomonas* exotoxin A. Following binding to cell membranes and internalization, PE40 is proteolytically cleaved in the endosome to release the catalytic subunit which accounts for the cytotoxic activity of the toxin (6). The catalytic subunit is responsible for the ADP-ribosylation and inactivation of elongation factor 2, which consequently inhibits protein synthesis and leads to cell death (7, 8). BMS-191352 demonstrates potent cytotoxicity activity, both *in vitro* as well as *in vivo*, against tumor cell lines expressing the Lewis^{Y} antigen (4, 9, 10) and is, therefore, a suitable candidate for development

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as an anticancer agent. To support the preclinical development of BMS-191352, the toxicokinetics of BMS-191352 were evaluated in the rat and the dog. In this paper we report the development and validation of an Enzyme Linked Immunosorbent Assay (ELISA) method, for the quantitation of BMS-191352 in rat and dog plasma and its application to toxicokinetic studies.

MATERIALS AND METHODS

Materials

BMS-191352, anti-*Pseudomonas* exotoxin A mAb (EXA2-1H8), biotinylated monoclonal BR96 anti-idiotypic antibody (757-4-1), PE40 with a lysine residue engineered on the amino terminus (LysPE40), and an irrelevant protein fused with PE40 (Heregulin-PE40) were obtained from Bristol-Myers Squibb (Seattle, WA). BMS-191352 for toxicokinetic studies was supplied by Immunex Corp. (Seattle, WA). Bovine serum albumin (BSA), *Pseudomonas* exotoxin A, and buffers were purchased from Sigma Chemical Co. (St. Louis, MO). Streptavidin/horseradish peroxidase conjugate was purchased from Jackson Immunoresearch Labs (West Grove, PA) and 3, 3', 5, 5'-tetramethylbenzidine (TMB) was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

ELISA Method

The concentration of BMS-191352 was determined by a double antibody sandwich ELISA method. Polystyrene plates (96-well) (Dynatech Laboratories

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Inc., Alexandria, VA) were coated overnight (12-16 hr) at 4°C with 100 μL of anti-PE40 mAb EXA2-1H8 [4 μg/mL in phosphate buffered saline (PBS)]. The coating solution was flicked dry and the plates were blocked for 1-2 hr with 200 μL of blocking buffer [PBS containing 0.05% Tween-20 and 3% BSA]. The plates were rinsed 5 times with wash buffer (PBS containing 0.05% Tween-20) and incubated for 2 hr with 100 μL of standards, quality control samples or study samples; the final amount of plasma on-plate was 10%. After rinsing the plates 5 times with wash buffer, 100 μL of biotinylated monoclonal BR96 anti-idiotypic antibody 757-4-1 (80 ng/mL in PBS containing 0.05% Tween-20 and 0.25% BSA) was added, and the plates were incubated for 1 hr. The plates were then rinsed 5 times with wash buffer, and incubated for 30 min with 100 μL of diluted Streptavidin/HRP conjugate (1:15,000). Excess conjugate was removed by washing 5 times with wash buffer and the color reaction was initiated by adding 100 μL of TMB. The color reaction was stopped after 10 min by addition of 100 μL of 1M H_3PO_4. The optical density (OD) was measured within 1 hr at 450 nm by a Tecan 340 ATTC reader (Tecan Instruments, Research Triangle Park, NC).

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Method Validation

Validation of the ELISA included assessments of the range of reliable response, lower limit of quantitation, precision and accuracy, specificity, and sample stability. The range of reliable response was determined using a seven-point standard curve ranging from 2-32 ng/mL in rat or dog EDTA plasma with each

standard run in triplicate. The sensitivity, expressed as lower limit of quantitation (LLQ), was assessed using 8-10 replicate plasma samples spiked with BMS-191352; blank samples served to determine possible background interference. To be acceptable as LLQ, the concentrations of at least 80% of the samples had to be within 20% of their nominal value. Accuracy and precision was based on predicted concentrations of quality control (QC) concentrations. For all analytical runs in the method validation, the standard curve was run in triplicate with a minimum of three replicates of high, medium, and low QC samples. The predicted QC concentrations, generated from the regression curve on each plate, were then utilized to evaluate accuracy and precision of the assay. To demonstrate the specificity of the assay, chimeric BR96, *Pseudomonas* exotoxin A, LysPE40, or Heregulin-PE40 were spiked into analytical QC samples, prepared in triplicate in dog plasma at a concentration of 2 μg/mL and the samples were assayed for BMS-191352. The concentration of BMS-191352 was determined in plasma samples stored at room temperature, 37°C, and -70°C for varying time periods and in plasma samples subjected to freeze-thaw cycles to evaluate the stability of the immunotoxin.

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Toxicokinetic Studies

Sprague Dawley rats (9-10 weeks old) and purebred Beagle dogs (12-24 months old) were obtained from Harlan Sprague Dawley (Frederick, MD) and Marshall Farms (North Rose, NY), respectively. The animals were housed individually and

were acclimated to in-house conditions prior to the study. At the time of study inception, the rats and dogs weighed between 190-280 gm and 7-12 kg, respectively. Rats had free access to ground certified rodent laboratory chow (#5002, PMI Foods Inc., St. Louis, MO). Each dog was offered 400 (males) and 300 (females) grams/day of Purina certified laboratory canine diet (#5007, PMI Foods Inc.) Animals had free access to water.

For toxicokinetic studies, BMS-191352 was administered as intravenous bolus doses of 1.8, 3.6, and 7.2 mg/m^2 to Sprague Dawley rats (n=4/gender/dose) and as 5-minute intravenous infusion doses of 2.5, 6, and 15 mg/m^2 to Beagle dogs (n=3/gender/dose). Blood samples were collected from jugular vein cannulated rats at predose and at 3 and 30 minutes, and 1, 2, 4, 6, 12, and 24 hr postdose; at each sampling time, approximately 0.05 mL of blood was drawn and discarded and an additional 0.2 mL of blood was collected in microvette tubes containing potassium EDTA. In the dog study, blood samples (approximately 1 mL) were collected via the saphenous vein at predose and at 5 (end of infusion), 15, 30, and 60 minutes, and 3, 6, 9, 12, 16, and 24 hr postdose. Following blood collection, the EDTA tubes were inverted several times to ensure mixing with the anticoagulant. Blood samples were placed on ice and centrifuged for 10 minutes at approximately 1,000xg and 0-5°C within 1 hr of collection. Plasma samples were stored with QC samples (used to assess the stability of toxicokinetic plasma samples during storage) at -70°C until analysis of BMS-191352.

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Data Analysis

The four-parameter logistic model was used to describe the relationship between the OD readings and nominal concentration (CONC) of BMS-191352 for the ELISA method (11):

$$OD = MAX + [(MIN-MAX)/(1+(CONC/EC_{50})^{B})]$$

where, MIN and MAX are the minimum and maximum OD readings, EC_{50} is an estimate of the concentration that yields 50% of the maximum OD, and B is the slope coefficient. Evaluation of potential outliers in the individual plate standard curves was performed according to the procedure described by Dixon and Massey (12). A lack-of-fit parameter, which evaluates the appropriateness of the logistic fit ($p > 0.05$ for adequate fit), was calculated for each standard curve and was used as an additional tool to evaluate model fit (13). Accuracy of the assay was defined as the deviation of the overall mean predicted concentration from its nominal value. To determine assay precision, the predicted QC concentrations were evaluated using an analysis of variance (ANOVA) model (14).

For toxicokinetics, the plasma concentration-time data were analyzed by a non-compartmental method (15). The peak plasma concentration, CMAX was obtained from experimental observations. Using no weighting factor, the terminal log-linear phase of the plasma concentration-time curve was identified by least-square linear regression of at least three data points which yielded a minimum mean square error. The half-life of the terminal log-linear phase, T-HALF, was

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calculated as 0.693/K, where K is the absolute value of the slope of the terminal log-linear phase. The area under the plasma concentration-time curve from zero to infinity, AUC(INF), was determined by summing the areas from time zero to the time of last measured concentration, calculated by using conventional trapezoidal and log-trapezoidal methods, and the extrapolated area. The extrapolated area was determined by dividing the final concentration by the slope of the terminal log-linear phase. The total body clearance (CLT) and the apparent steady-state volume of distribution (VSS) were calculated from the following relationships:

CLT = DOSE/AUC(INF) and VSS = CLT * MRT(INF)

where, MRT(INF) = [AUMC(INF)/AUC(INF)] - [T/2], MRT(INF) is the mean residence time, AUMC(INF) is the area under the first moment curve extrapolated to infinity, and T is the time required for intravenous drug administration (T=0 hr for bolus dose in the rat and T=0.083 hr for the 5-minute infusion dose in the dog).

Statistical analysis of the toxicokinetic parameters was performed using PC SAS (version 6.08) (16). The effect of dose, gender, and period was evaluated by ANOVA. Tukey's unweighted studentized range test was used to make pairwise comparisons for significant effects. Weighted linear regression, with weights of 1/dose, was used to assess dose linearity and proportionality on CMAX and

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AUC(INF) values. All statistical tests were carried out at the 5% significance level.

RESULTS

Standard Curve Characteristics

The assay method resulted in an on-plate range of reliable response of 0.2-3.2 ng/mL; this corresponds to a range of 2-32 ng/mL for the rat or dog plasma assay, since the on-plate concentrations were in 10% plasma. A representative standard curve in rat and dog plasma is given in Figure 1. The standard curve parameters, as determined by the four-parameter logistic model, are shown in Table 1. The R^2 values for the standard curve for the rat and dog plasma assay were ≥0.992 and ≥0.993, respectively.

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Limit of Quantitation

Plasma samples obtained from individual rats (n=10) or dogs (n=8) were spiked with 2 ng/mL of BMS-191352 and assayed for BMS-191352. The predicted concentrations of BMS-191352 ranged between 1.99 to 2.51 ng/mL in rat plasma and 1.73 to 2.40 ng/mL in dog plasma. Eight out of 10 rat plasma samples and 7 out of 8 dog plasma samples deviated from their nominal values by less than 12.5% and 13.5%, respectively. Based on the criterion specified in the Methods section, the LLQ was accepted as 2 ng/mL.

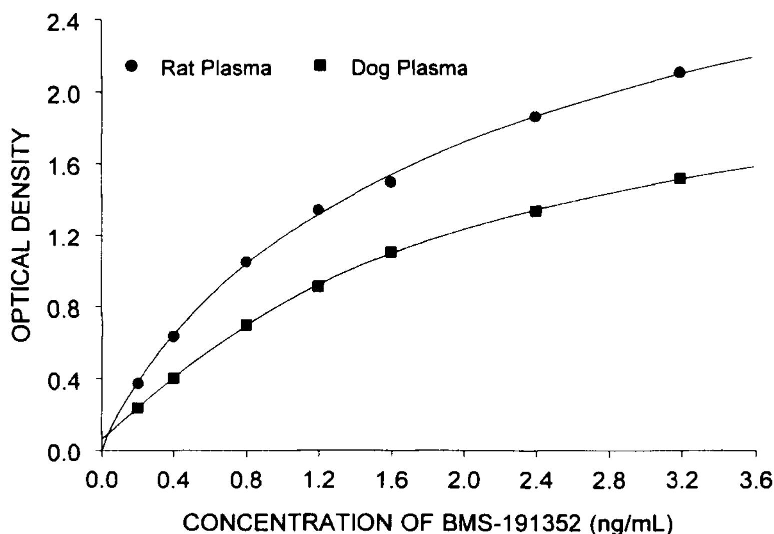

FIGURE 1. Representative standard curve for the ELISA method for quantitation of BMS-191352 in rat (●) and dog (■) plasma.

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TABLE 1

Mean (SD) of the Standard Curve Parameters for the ELISA Method of BMS-191352 in Rat and Dog Plasma.*

Standard Curve Parameters	Rat Plasma Assay	Dog Plasma Assay
MIN	0.03 (0.03)	0.10 (0.03)
MAX	3.7 (0.4)	2.6 (0.4)
ED_{50}	2.2 (0.4)	1.9 (0.1)
B	1.0 (0.1)	1.2 (0.7)
N	9	4

* The four-parameter logistic model used to describe the relationship between optical density and the nominal concentrations of BMS-191352 was as follows:
Optical Density = $MAX + [(MIN-MAX)/(1+(CONC/EC_{50})^{B})]$

Assay Accuracy and Precision

For accuracy and precision determination, QC samples of BMS-191352 were prepared in rat or dog plasma at concentrations of 5, 100, and 10,000 ng/mL; these samples were analyzed for the concentration of BMS-191352 following 10-, 66.6-, and 4,000-fold dilution. The accuracy, as determined by the deviation of the overall mean predicted concentration from its nominal values, was within 5.0 and 11.0% for the rat and dog plasma assay, respectively (Table 2). The precision (%RSD) for the rat and dog plasma assays was within 8.4 and 9.2%, respectively (Table 2).

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Assay Specificity

The specificity of the assay was studied by spiking dog plasma QC samples with structurally similar proteins (chimeric BR96, *Pseudomonas* Exotoxin A, LysPE40, and Heregulin-PE40) each at a concentration of 2 μg/mL. Samples were then analyzed for the concentration of BMS-191352. None of the proteins tested interfered with the quantitation of BMS-191352 (data not shown).

Stability of BMS-191352

To evaluate the stability of BMS-191352 in rat and dog plasma, QC samples at concentrations of 5 (rat plasma only), 1,000, 10,000 ng/mL were assayed after storage at room temperature, 37°C, and -70°C for various time intervals and after three freeze-thaw cycles. The results are presented in Table 3. BMS-191352 was found to be stable in rat and dog plasma for at least 24 hr at room temperature, 2

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TABLE 2

Accuracy and Precision of the ELISA Method for the Determination of BMS-191352 in Rat and Dog Plasma.

Matrix	Concentration of BMS-191352 (ng/mL)		Accuracy (% deviation)	Precision (%RSD)		
	Nominal*	Mean Predicted**		Between-Run	Between-plate Within-run	Within-plate
Rat Plasma	5	5	0.0	8.4	2.1	4.0
	1,000	983	-1.7	6.2	2.4	5.4
	10,000	9,504	-5.0	4.3	0.0**	6.2
Dog Plasma	5	5.5	9.4	2.3	---¶	4.2
	1,000	1,079	7.9	0.0**	---¶	9.2
	10,000	11,100	11.0	3.4	---¶	5.6

* The on-plate concentrations were 0.5, 1.5, and 2.5 ng/mL in rat and dog plasma.

** Represents the mean of n=42 to 69 observations.

*** No significant additional variation was observed as a result of performing the assay in different runs.

¶ Not determined since only one plate per run was used.

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TABLE 3

Stability of BMS-191352 in Rat and Dog Plasma.

Matrix	Storage Conditions	Concentration of BMS-191352 (ng/mL)		Accuracy (% deviation)
		Nominal	Mean Predicted*	
Rat Plasma	Room temperature (24 hr)	5	5.1	2.0
		1,000	1,013	1.3
		10,000	9,956	-0.4
	37°C (2 hr)	5	4.4	-12.0
		1,000	852	-14.8
		10,000	8,830	-11.7
	-70°C (30 days)	5	5.4	8.0
		1,000	951	-4.9
		10,000	9,900	-1.0
	3 freeze-thaw cycles**	5	5.02	0.4
		1,000	1,012	1.2
		10,000	9,934	-0.7
Dog Plasma	Room temperature (24 hr)	1,000	979	-2.1
		10,000	10,092	0.9
	37°C (2 hr)	1,000	901	-9.9
		10,000	8,808	-11.9
	-70°C (30 days)	1,000	953	-4.7
		10,000	8,920	-10.8
	3 freeze-thaw cycles**	1,000	961	-3.9
		10,000	9,572	-4.3

* Represents the mean of triplicate observations.

** QC samples were frozen at -70°C and thawed at room temperature.

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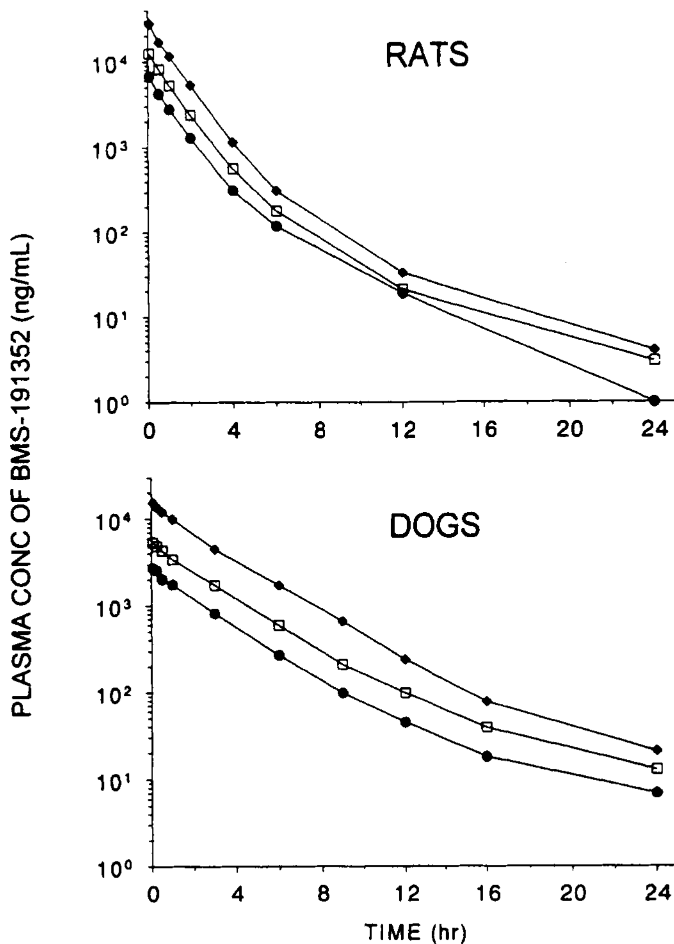

FIGURE 2. Mean plasma concentration-time profiles of BMS-191352 after intravenous doses of 1.8 (●), 3.6 (□), and 7.2 (◆) mg/m^2 in rats (n=8) and 2.5 (●), 6 (□), and 15 (◆) mg/m^2 in dogs (n=6). The data for male and female animals were pooled since the toxicokinetics of BMS-191352 were similar between gender.

TABLE 4

Mean (SD) Toxicokinetic Parameters of BMS-191352 in Sprague Dawley Rats.*

Toxicokinetic Parameter (Units)	Gender	Dose		
		1.8 mg/m^2	3.6 mg/m^2	7.2 mg/m^2
CMAX (ng/mL)	Male	6,279 (1,192)	12,419 (793)	27,756 (3,544)
	Female	7,302 (1,620)	12,523 (1,208)	27,881 (812)
AUC(INF) (hr.ng/mL)	Male	8,948 (1,034)	17,167 (584)	36,069 (2,842)
	Female	8,750 (954)	15,554 (2,454)	3,4091 (2,062)
T-HALF (hr)	Male	2.20 (1.03)	2.85 (0.31)	2.79 (0.16)
	Female	2.20 (0.77)	3.12 (0.14)	3.29 (0.21)
MRT(INF) (hr)	Male	1.70 (0.39)	1.54 (0.10)	1.41 (0.12)
	Female	1.55 (0.54)	1.42 (0.07)	1.37 (0.08)
CLT (mL/min/m^2)	Male	4.02 (0.43)	4.16 (0.11)	4.01 (0.31)
	Female	3.73 (0.41)	4.24 (0.68)	3.81 (0.21)
VSS (L/m^2)	Male	0.42 (0.14)	0.38 (0.03)	0.34 (0.02)
	Female	0.36 (0.17)	0.36 (0.06)	0.31 (0.01)

* Represents mean (SD) of four rats per gender.

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hr at 37°C, and 30 days at -70°C. In addition, plasma samples were found to be stable when subjected to 3 freeze-thaw cycles.

Toxicokinetics of BMS-191352

The plasma concentration-time profiles of BMS-191352 following intravenous administration of BMS-191352 in the rat and the dog are depicted in Figure 2, and

TABLE 5

Mean (SD) Toxicokinetic Parameters of BMS-191352 in Beagle Dogs.*

Toxicokinetic Parameter (Units)	Gender	Dose		
		2.5 mg/m^2	6 mg/m^2	15 mg/m^2
CMAX (ng/mL)	Male	2,957 (608)	5,398 (273)	14,665 (936)
	Female	2,702 (426)	5,494 (550)	16,288 (910)
AUC(INF) (hr.ng/mL)	Male	7,712 (1,734)	15,808 (1,045)	42,603 (4,505)
	Female	6,901 (889)	14,053 (834)	40,704 (3,400)
T-HALF (hr)	Male	4.70 (0.59)	3.42 (0.65)	3.53 (0.19)
	Female	3.96 (0.19)	4.88 (0.26)	3.56 (0.51)
MRT(INF) (hr)	Male	3.02 (0.23)	3.12 (0.33)	2.86 (0.14)
	Female	2.85 (0.08)	3.00 (0.19)	2.82 (0.30)
CLT (mL/min/m^2)	Male	5.55 (1.03)	6.45 (0.54)	6.00 (0.58)
	Female	5.63 (0.83)	6.44 (0.27)	5.53 (0.43)
VSS (L/m^2)	Male	1.00 (0.12)	1.20 (0.14)	1.03 (0.05)
	Female	0.96 (0.14)	1.16 (0.09)	0.93 (0.04)

* Represents mean (SD) of three dogs per gender.

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the key toxicokinetic parameters are summarized in Tables 4 and 5, respectively. Following intravenous administration, BMS-191352 was rapidly eliminated from the plasma in both species in an apparent biexponential manner. In both species, the plasma levels declined to about 95% of the CMAX values within 6 hr after

dosing (Figure 1). Statistical analysis (ANOVA) indicated no significant gender differences in the toxicokinetics of BMS-191352. Weighted linear regression analysis of the relationship between CMAX or AUC(INF) values and the intravenous dose of BMS-191352 indicated that CMAX and AUC(INF) were dose proportional in the rat and the dog ($R^2 \geq 0.96$). ANOVA results indicated that the T-HALF, MRT(INF), CLT, and VSS values were similar across the dose range of 1.8-7.2 mg/m^2 in the rat and 2.5-15 mg/m^2 in the dog. Pooled across gender and dose, the overall mean values of T-HALF, MRT(INF), CLT, and VSS in the rat were 2.74 hr, 1.50 hr, 3.99 mL/min/m^2, and 0.36 L/m^2, respectively; in the dog, the overall mean values were 4.01 hr, 2.94 hr, 5.94 mL/min/m^2, and 1.05 L/m^2, respectively.

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DISCUSSION

This paper reports the development and validation of an ELISA method for the quantitation of BMS-191352, a single-chain construct of BR96 and PE40, in rat and dog plasma and its use in toxicokinetic studies. The four-parameter logistic model adequately described the standard curve data over the range of 2-32 ng/mL of BMS-191352 since the R^2 values exceeded 0.992. The ELISA method gave comparable standard curve parameters in the rat and the dog plasma, suggesting a lack of matrix effect. BMS-191352 was found to be stable in plasma for at least

24 hr at room temperature, 1 month at -70°C, and when subjected to 3 freeze-thaw cycles. Although BMS-191352 appeared to be stable at 37°C for 2 hr based on the percent deviation values to be within the variability of the assay, it should be noted that being a protein, BMS-191352 may be likely to degrade at this temperature if kept for extended time periods.

A notable feature of this assay is the specificity for the quantitation of BMS-191352. This was possible due to the use two monoclonal antibodies, EXA2-1H8, which recognizes an epitope on PE40, and 757-4-1, an anti-idiotypic BR96 mAb. These antibodies provided for the quantitation of intact BMS-191352, and neither chimeric BR96 nor PE40 alone (as LysPE40 which is PE40 with a lysine residue on the amino terminus) could be detected in the assay. Furthermore, both *Pseudomonas* Exotoxin A and Heregulin-PE40, a single-chain construct of an irrelevant protein and PE40, did not cross-react with the assay antibodies or interfered in the immunoassay.

The ELISA method reported here was successful in monitoring plasma levels of BMS-191352 in toxicokinetic studies. The sensitivity of this assay offered the quantitation of BMS-191352 in such studies for at least 6 half-lives following drug administration. The toxicokinetic studies revealed that the systemic exposure of BMS-191352, as determined by the CMAX and AUC(INF) values, was dose proportional; T-HALF, CLT, MRT(INF), and VSS were invariant with respect to dose indicating that the kinetics of BMS-191352 were linear over the dose range

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of 1.8-7.2 mg/m^2 in the rat and 2.5-15 mg/m^2 in the dog. Furthermore, there were no gender differences in the toxicokinetics of BMS-191352. The VSS of BMS-191352 in the rat (0.36 L/m^2) and in the dog (1.05 L/m^2) corresponds to 0.05 L/kg, suggesting that the volume of distribution of BMS-191352 is limited to the blood volume of 0.05 and 0.09 L/kg in the rat and dog, respectively (17).

In conclusion, a specific, sensitive, precise, and accurate assay for the determination of BMS-191352 in rat and dog plasma has been developed. This assay is suitable for the quantitation of BMS-191352 in toxicokinetic studies. BMS-191352 exhibits linear kinetics in the rat and the dog.

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