A Novel Oligodeoxynucleotide Inhibitor of Thrombin. II. Pharmacokinetics in the Cynomolgus Monkey

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Purpose. To determine the pharmacokinetics of GS-522, an oligodeoxynucleotide (GGTTGGTGTGGTTGG) inhibitor of thrombin, after constant infusion and bolus administration in the cynomolgus monkey.

Methods. Using a stability indicating HPLC method, the GS-522 plasma concentration versus time data were obtained after constant infusion (0.1, 0.3, 0.5 mg/kg/min) and bolus administration (11.25 and 22.5 mg/kg). Plasma data after bolus administration was fit to a three-compartment model.

Results. The half-lives for the α and β phases were 1.4 and 5.4 min, respectively. Steady state GS-522 concentrations were reached within 10 minutes after initiation of constant infusions. Termination of infusions resulted in a rapid elimination of GS-522 with an average elimination half-life equal to 1.5 min. The V_{ss} calculated from both the constant infusion and bolus data approximated the blood volume of the monkey. Substitution of the phosphodiester backbone at the 3′ end of GS-522 with two phosphorothioate linkages did not substantially effect the elimination half-life upon termination of infusion.

Conclusions. These data in conjunction with published biodistribution data suggest that oligodeoxynucleotides are rapidly cleared from plasma by tissue uptake and that little efflux back into blood takes place. Additionally, strategies designed to increase oligodeoxynucleotide resistance to exonucleases will not dramatically increase plasma half-lives.

KEY WORDS: GS-522; oligodeoxynucleotide; thrombin; pharmacokinetics; monkey.

INTRODUCTION

GS-522 is a novel oligodeoxynucleotide, GGTTGGTGTGGTTGG, which inhibits the thrombin catalyzed conversion of fibrinogen to fibrin (1). *In vitro* and *in vivo* studies have demonstrated that GS-522 is a potent inhibitor of thrombin with desirable anticoagulant properties (2). Studies in monkeys and dogs have shown that infusion of GS-522 results in a rapid onset of action and a rapid cessation of anticoagulant activity at the end of infusion. An anticoagulant with a short pharmacological half-life (1–2 min) is highly desirable in a surgical setting. GS-522 is currently in preclinical development as a anticoagulant for use during coronary by-pass surgery.

In the preceding paper (3), we described a stability indicating HPLC method capable of detecting GS-522 in monkey, dog and human plasma. The method was 40-fold more sensitive than the standard prothrombin clotting time (PT) assay used to measure coagulation potential. *In vitro* half-lives of GS-522 in serum and plasma, degradation pathways, and prothrombin binding were also determined. From these studies it was concluded that the *in vitro* half-life of GS-522 in serum or plasma could not explain the short *in vivo* pharmacological half-life observed in the monkey. In order to relate the observed pharmacological effect of GS-522 in monkey to its concentration *in vivo*, we have determined the serum concentrations and pharmacokinetics of GS-522 in cynomolgus monkey after bolus and constant infusion.

The pharmacokinetic literature describing *in vivo* studies of DNA and oligodeoxynucleotides is often contradictory and presents an unclear picture of the effects of backbone modifications on the pharmacokinetic parameters. Some of these discrepancies are due to the lack of specificity of the bioanalytical methods used to measure oligodeoxynucleotide concentrations. The alpha half-lives of unmodified oligodeoxynucleotides or DNA fractions have been reported to be between 0.8–4 minutes (4, 5, 6). Substitution of the phosphodiester backbone with either a phosphorothioate (PS) or methyl phosphonate (MP) backbone has been shown to dramatically stabilize oligodeoxynucleotides towards nuclease degradation (7, 8), while the effects on plasma clearance were less dramatic. In mice, a 28-mer PS sequence was reported to have a plasma half-life of 11.6 min (9) and a 12-mer MP sequence showed a biexponential clearance with an alpha phase $t_{1/2}$ of 6 min and a beta phase $t_{1/2}$ of 17 min (10). In contrast, two additional publications have reported extremely slow plasma clearance of PS sequences. Iversen reported that a 27-mer PS had a distribution half-life of 34 min and an elimination half-life from plasma of 43 hr in the rat (11), while Cossum reported a multi-exponential decrease in plasma concentration for a 20-mer PS with half-lives ranging from 24 min to 51 hr (12). Both studies determined plasma concentrations by total radioactivity (^{35}S and ^{14}C, respectively). The first time point taken in the Cossum report was at 30 min, possibly missing an initial rapid clearance phase reported by other researchers using PS oligodeoxynucleotides (9). In a very interesting study by Sands, et. al., the biodistribution and metabolism of a phosphodiester and a phosphorothioate oligodeoxynucleotide were compared (13). Total blood radioactivity levels declined rapidly ($t_{1/2} < 2$ min) for both compounds. Using total blood radioactivity, elimination half-lives of 15.9 and 66 hr were calculated for the phosphodiester and the phosphorothioate, respectively. In all three reports, no estimate of the percent intact oligodeoxynucleotide actually eliminated in these slower phases was made, therefore it is difficult to evaluate the relevance of the reported long elimination half-lives.

The ability to accurately measure plasma concentrations of intact GS-522 distinct from its metabolites overcomes a major limitation of many of the previous reports of oligodeoxynucleotide pharmacokinetics. The results of our work and the implications for oligodeoxynucleotide therapeutics are discussed.

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MATERIALS AND METHODS

Materials

The ssDNA molecule GGTTGGTGTGGTTGG (GS-522) was prepared by solid-phase phosphoramidite chemistry on a Biosearch 8800 synthesizer and purified as described elsewhere (3). The calculated molecular weight of GS-522 is 5033.7 Daltons. The phosphorothioate analog, GS-1154, was prepared according to published procedures (14).

Formulations

Dosing solutions of GS-522 between 20–32 mg/mL were prepared in 20 mM phosphate buffer, pH 7.4 and filtered through a 0.2 μm filter. The exact concentrations of GS-522 were determined by UV absorbance at 260 nm (6.98 nM/OD). For bolus administration, volumes were adjusted to account for individual monkey weights. In the case of the infusions, after adjusting volumes to body weights, all dosing solutions were diluted to 32.5 ml with normal saline solution.

Animals

Four groups (n = 2 to 4 per group) of adult male cynomolgus monkeys (*Macaca fascicularis*) were used for the study. All procedures were approved by the Institutional Animal Care and Use Committee of the New Mexico Regional Primate Research Center and were performed in compliance with the Animal Welfare Act. The mean ± SD body weight at the time of dosing was 4.9 ± 0.4 kg. Animals were lightly sedated with intramuscular ketamine hydrochloride (10 mg/kg) on the day of the study and were placed in primate restraint chairs. A short (1 inch) catheter was placed in a cephalic vein for drug administration, and a long (12 inch) catheter was placed in a saphenous vein for blood collection.

Bolus Injection

Two animals (Group 1) received a bolus intravenous injection of GS-522 into a cephalic vein over a one minute period, followed by a 1 ml flush of normal saline to rinse the catheter. One animal received a dose of 11.25 mg/kg GS-522 and one animal received a dose of 22.5 mg/kg GS-522. Blood samples (approximately 2.0 ml) were collected at 0 (pre-dose) and 2, 5, 10, 20, 30, 45, 90 and 120 minutes post-dose, transferred immediately to citrated tubes and placed on ice.

Continuous Infusion

All intravenous infusions were performed by continuous administration via a cephalic vein over a 60 minute period. Two animals (Group 2) received a continuous intravenous infusion of GS-522 at a dose of 0.1 mg/kg/min (total dose 6 mg/kg). Four animals (Group 3) received a continuous intravenous infusion of GS-522 at a dose of 0.3 mg/kg/min (total dose 18 mg/kg). Four animals (Group 4) received a continuous intravenous infusion of GS-522 at a dose of 0.5 mg/kg/min (total dose 30 mg/kg). Blood samples (approximately 2.0 ml) were collected from the saphenous vein of each animal into citrated tubes at 0 (pre-dose), 2, 10, 30 and 60 minutes into the infusion, and at 2, 5, 10, 15, 20, 30 and 45 minutes after the end of the infusion.

Sample Processing and Analysis

Blood samples were processed immediately for plasma by centrifugation at 4°C. Plasma samples were frozen and maintained at ≤ -20°C until analyzed. Plasma samples were analyzed using a validated HPLC method for GS-522 in cynomolgus monkey plasma (3). The method was linear over the range 25 ng/ml to 151 μg/ml and the limit of quantitation was 100 ng/ml. The precision determined from 6 injections was 3.2% and the recovery from plasma was 86 ± 7%. Detection was by UV absorbance at 260 nm. PT times were determined as described earlier (2).

Pharmacokinetic Calculations

The pharmacokinetic parameters for intravenous GS-522 were calculated using the nonlinear curve fitting software PCNONLIN. For the bolus intravenous administration, data were analyzed using noncompartmental methods. For the continuous infusions, a one compartment model incorporating continuous input with first order output was employed. The plasma clearance (CL_p) was calculated for bolus and continuous infusion doses as Dose/$AUC_{0-\infty}$, where $AUC_{0-\infty}$ is the area under the plasma concentration time curve projected to infinity. The volume of distribution at steady state (Vd_{ss}) was calculated as MRT × CL_p, where MRT is the mean residence time.

RESULTS AND DISCUSSION

A scatter plot of measured *in vivo* GS-522 concentrations (up to 30 μg/ml) versus the corresponding percent elevations in PT times determined previously for all available plasma samples (2) was linear with a slope of 10.5 and an r^2 value of 0.92. Considering the variability inherent in the PT assay, this correlation of the *in vivo* anticoagulant effect to the *in vivo* concentration is convincing evidence that the effect is due solely to intact GS-522 in plasma. At the higher initial concentrations of GS-522 reached after bolus administration (80 and 150 μg/ml), the concentration vs. effect curve becomes non-linear, consistent with a saturable pharmacological effect. No prolonged anticoagulant activity was observed in the pharmacodynamic study, consistent with a limited distribution and a rapid clearance of GS-522 (2).

Pharmacokinetics

In Figure 1, plasma concentrations detected in the cynomolgus monkey following bolus administration of GS-522 at 11.25 and 22.5 mg/kg are plotted. At both doses, GS-522 concentrations declined in a multiexponential manner. The half-lives of the alpha and beta phases were consistent between doses. The data points associated with a potential third phase were close to or below the limit of quantitation of the analytical method preventing accurate determination of a gamma phase half-life. The non-compartmental pharmacokinetic parameters derived from the data obtained following intravenous bolus administration of GS-522 are summarized in Table I. The AUC and Cmax values indicate apparent dose-proportionality. The volume of distribution at steady

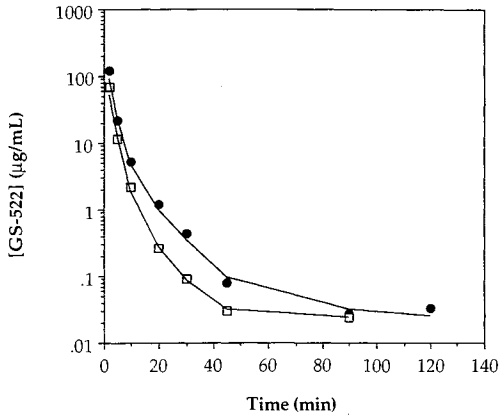

Fig. 1. Plasma concentrations of GS-522 following bolus intravenous injection to cynomolgus monkeys; (□) 11.25 mg/kg; (●) 22.5 mg/kg; (n = 1 per dose level).

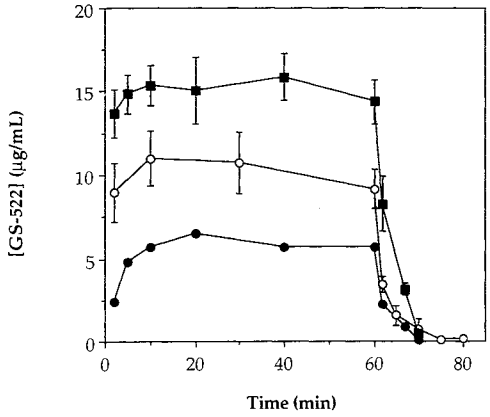

Fig. 2. Plasma concentrations of GS-522 following continuous intravenous infusion in cynomolgus monkeys: (●) 0.1 mg/kg/min (mean ± SEM, n = 2); (○) 0.3 mg/kg/min (mean ± SEM, n = 4); (■) 0.5 mg/kg/min (mean ± SEM, n = 4).

state was 66.5 ml/kg at the low dose and 95.1 ml/kg at the high dose, close to the anticipated blood volume in the monkey of approximately 75 ml/kg (15).

Figure 2 shows the mean ± SEM plasma concentrations of GS-522 following 60 minute continuous intravenous infusion at 0.1, 0.3 and 0.5 mg/kg/min. At all three dose levels, a steady state plateau concentration was achieved within 10 minutes indicating that GS-522 has a very limited distribution equilibrium and a rapid clearance process. Noncompartmental pharmacokinetic data for continuous infusion of GS-522 are summarized in Table II. The mean steady state plasma concentrations observed for the three GS-522 infusion doses deviated slightly from dose proportionality at the lowest dose. Clearance of GS-522 was approximately one third slower at the 0.1 mg/kg/min dose as compared to the higher doses.

Inherent in the assumption of a steady state during continuous infusion is the attainment of a distribution equilibrium. Therefore, the rapid disappearance of GS-522 after cessation of the infusion must represent elimination of the drug. The equivalence of the alpha phase and the elimination rate after continuous infusion suggests that the same process occurred after both dosing regimens and that this process was an elimination and not a distribution phase as has been suggested for other oligos (11). Consistent with a limited distribution, the calculated V_{ss} from both the bolus and the constant infusion data approximate the blood volume of the monkey. By comparison to biodistribution studies in the literature (13,16,17), which demonstrate that radioactivity from labeled phosphodiesters, phosphorothioates and mixed backbone oligodeoxynucleotides was present in most tissues immediately after intravenous administration, rapid tissue uptake for GS-522 is a reasonable conclusion. However, the efflux of GS-522 from these tissues must be limited and therefore the disposition of GS-522 into tissues should be considered a clearance phase. This later conclusion is supported by data presented by Agrawal, et al., showing that plasma radioactivity from a radiolabeled phosphorothioate rapidly drop to levels below those found in liver, intestines, heart, spleen and lungs within 2 hours after intravenous administration (17).

Table I. Pharmacokinetic Parameters for GS-522 Following Bolus Intravenous Administration to Cynomolgus Monkeys

Parameter	Low dose 11.25 mg/kg	High dose 22.5 mg/kg
AUC (μg.min/ml)	373	663
t1/2 alpha (min)	1.4	1.4
t1/2 beta (min)	5.2	5.6
Cmax (μg/ml)	133	212
CL_p (ml/min/kg)	30.1	33.9
AUMC (μg.min²/ml)	824	1878
MRT (min)	2.2	2.8
Vss (ml/kg)	66.5	95.1
AUC/DOSE	33.1	29.5

A second elimination phase was also observed after bolus administration GS-522. The fraction of the dose associated with this phase was less than 10% for the high dose and less than 15% for the low dose. The GS-522 plasma concentration at which the slower beta phase began to appear after bolus administration was close to the predicted prothrombin (P) concentration in the cynomolgus monkey (1–3 μM). This observation suggested that the beta elimination phase was slower than the alpha phase due to the equilibrium binding of GS-522 to prothrombin in the blood. The *in vitro* dissociation constant for GS-522 and human prothrombin is 50 nM (3). A scheme describing this effect is depicted below,

$$\overset{k_{tis}}{\leftarrow} [\text{GS-522}]_f \overset{P}{\rightleftharpoons} [\text{GS-522}]_b$$
$$\downarrow k^s_{obs} \qquad K_p$$

where k_{tis} is the putative elimination rate constant for all tissue uptake, k^s_{obs} is the rate constant for serum metabolism, K_P is the prothrombin dissociation constant and $[GS\text{-}522]_f$ and $[GS\text{-}522]_b$ are free and bound GS-522, respectively. At [GS-522] > 3 μM where prothrombin binding is saturated, the elimination rate constant $k_\alpha = k_{tis} + k^s_{obs}$. As the plasma concentration of GS-522 *in vivo* falls below the prothrombin concentration, a slower beta clearance phase is observed,

$$k_\beta = \left[\frac{K_p}{K_p + [P]_f}\right] k_\alpha \qquad (1)$$

Table II. Pharmacokinetic Parameters for GS-522 Following Continuous Intravenous Infusion at Various Rates in Cynomolgus Monkeys

	0.1 mg/kg/min		0.3 mg/kg/min		0.5 mg/kg/min	
Parameter	Mean (n = 2)	SD	Mean (n = 4)	SD	Mean (n = 4)	SD
AUC (μg.min/ml)	347	20.5	638	181	953	139
t1/2 (min)	2.0	0.2	1.07	0.37	1.5	0.0
Cmax (μg/ml)	5.80	0.33	10.6	3.02	15.8	2.29
CLp (ml/min/kg)	17.3	1.0	30.0	8.9	32.0	4.3
AUMC (μg.min^2/ml)	11400	561	20100	5650	30700	4510
MRT (min)	2.9	0.3	1.54	0.53	2.2	0.0
Vss (mL/kg)	50.7	8.6	46.9	22.2	70.1	9.4
AUC/DOSE	57.8	3.43	35.4	10.0	31.8	4.65

Where k_β is the rate constant for the beta phase and $[P]_f$ is the concentration of free prothrombin. At low GS-522 concentrations (≤0.1 μM), substituting in the values for K_P (50 nM) determined previously for human prothrombin (3), and assuming $[P]_f \sim [P]_{total} = 1.0$ μM, the calculated value for k_β is 0.024 min^{-1}. This value is approximately 5-fold slower than the observed $k_\beta = 0.13$ min^{-1}. A 5-fold difference was also reported between the observed and calculated rate constants for the *in vitro* plasma metabolism of GS-522 (3). Both calculated values assume inhibition due to prothrombin binding. The most likely cause for this 5-fold difference is the use of the K_d for human prothrombin instead of the actual K_d for monkey prothrombin. No sequence has been published for monkey prothrombin, or thrombin, but the percent homology is likely to be high. However, GS-522 was optimized specifically to bind human thrombin from a pool of 10^{13} possible oligodeoxynucleotides and thus a 5-fold decrease in affinity would not be unreasonable.

In the case of the constant infusions, limited data was available after termination of the infusion. As a consequence, the elimination rate was fit to a single exponential and no beta phase was determined. At the 0.1 mg/kg/min infusion rate, the GS-522 steady state plasma concentration reached was approximately the same as the expected prothrombin concentration and this may explain why the elimination rate appears to slow down relative to the 0.3 and 0.5 mg/kg/min infusions.

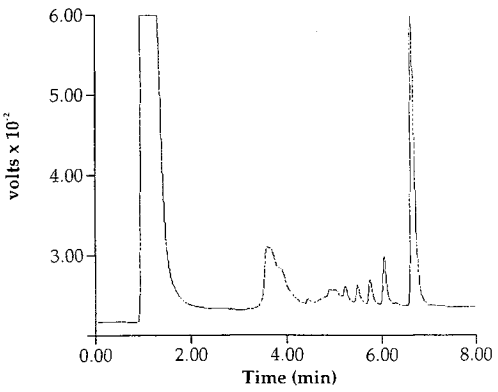

Fig. 3. HPLC Chromatograms of GS-522 in monkey plasma at 60 min after initiation of infusion (0.5 mg/kg/min).

Metabolism

An HPLC chromatogram of a plasma sample taken at 60 minutes after the initiation of the 0.5 mg/kg/min infusion is shown in Figure 3. The relative concentrations of GS-522 and the n-1 through n-4 metabolites remain constant throughout the infusion period at all 3 infusion levels implying a steady state for the initial metabolites. The ladder-like appearance of the initial metabolites was similar to that observed in the *in vitro* experiments and suggests that plasma exonucleases are responsible for the metabolism.

As described above, the plasma elimination rate constant,

$$k_\alpha = k_{tis} + k^s_{obs} \quad (2)$$

Using the k^s_{obs} (0.09 min^{-1}) previously determined in monkey serum (3) and substituting in the observed k_α, $k_{tis} \approx 0.41$ min^{-1}. Thus nuclease degradation originating in the blood should account for approximately 22% of the total clearance of GS-522 *in vivo*.

The above argument implies that efforts to stabilize the GS-522 sequence to exonuclease metabolism will only partially enhance plasma concentrations or plasma half-life. This hypothesis was tested using an analog to GS-522, GS-1154, in which two phosphodiester linkages at the 3′ end were

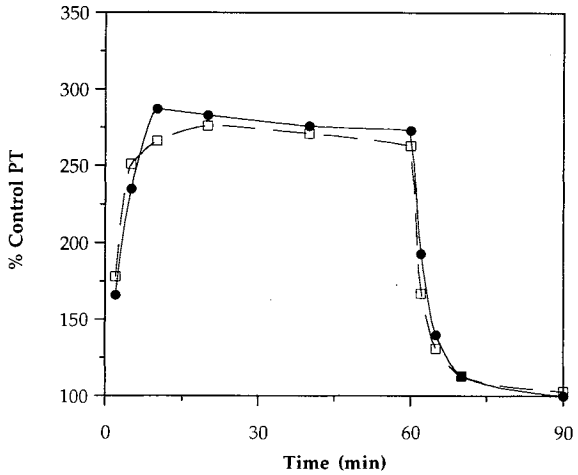

Fig. 4. The percent of control PT versus time for GS-1154 and GS-522 at 0.5 mg/kg/min for 60 min. (●) = GS-1154, n = 2, (□) = GS-522, n = 4.

replaced with phosphorothioate linkages. Double substitution of phosphorothioates at the 3′ end of an phospohodiester oligodeoxynucleotide has been shown to significantly increase the oligodeoxynucleotide stability in human serum and fetal calf serum (18). Additionally, preliminary *in vitro* studies in monkey serum using an HPLC method to analyze the samples showed ≥10-fold increase in stability of GS-1154 as compared to GS-522. In Figure 4, the anticoagulant effect of an infusion of this modified oligodeoxynucleotide is compared to that of GS-522 at the same dose. Upon termination of the infusions, the activities of both oligodeoxynucleotides fall at approximately the same rate ($t_{1/2} \approx 1.5$ min for GS-522 and $t_{1/2} \approx 2.0$ min for GS-1154). Thus plasma clearance of GS-1154 is unaffected by 3′-end substitution of phosphorothioate, supporting the hypothesis that nuclease resistance alone will not dramatically effect the plasma half-lives of modified oligodeoxynucleotides. A similar conclusion was reached by Sands concerning the *in vivo* tissue stability of 3′, 5′-blocked oligodeoxynucleotides (15).

CONCLUSIONS

We have described the pharmacokinetics in monkeys of a novel oligodeoxynucleotide inhibitor of thrombin after constant infusion and bolus administration. The pharmacokinetics of GS-522 are consistent with the pharmacodynamics reported previously. The rapid elimination of this molecule from the plasma compartment makes it an ideal candidate for anticoagulant therapy in coronary bypass surgery. *In vivo,* at therapeutic concentrations, the primary clearance of GS-522 is likely due to extensive tissue uptake, while plasma metabolism makes only a small contribution. From a therapeutic standpoint, rapid and essentially irreversible uptake of antisense oligodeoxynucleotides into tissues could be very advantageous.

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