Pharmacokinetics, Pharmacodynamics and Tolerability of a Potent, Non-peptidic, GP IIb/IIIa Receptor Antagonist following Multiple Oral Administrations of a Prodrug Form

Canio J. Refino\(^1\), Nishit B. Modi\(^2\), Sherron Bullens\(^3\), Cheryl Pater\(^1\), Michael T. Lipari\(^1\), Kirk Robarge\(^3\), Brent Blackburn\(^3\), Maureen Beresini\(^4\), Thomas Weller\(^5\), Beat Steiner\(^5\), Stuart Bunting\(^1\)

From the Departments of \(^1\)Cardiovascular Research, \(^2\)Pharmacokinetics, \(^3\)Bio Organic Chemistry, \(^4\)Bio Analytical Technology, Genentech Inc. South San Francisco CA, USA; and the \(^5\)Pharma Division, Preclinical Research, F. Hoffmann-La Roche LTD, Basel, Switzerland

Summary

Ro 44-3888 is a potent and selective antagonist of GP IIb/IIIa. Following IV administration to rhesus monkeys, the (mean ± SD.) clearance, volume of distribution and terminal half-life of Ro 44-3888 were 4.4 ± 1.8 ml/min/kg, 0.8 ± 0.4 l/kg and 2.5 ± 0.8 h respectively. Oral administration of Ro 48-3657 (1 mg/kg), a doubly protected prodrug form, produced peak concentrations of Ro 44-3888 (152 ± 51 ng/ml), 4.2 ± 2.2 h after dosing. Terminal half-life and estimated bioavailability were 5.1 ± 1.6 h and 33 ± 6% respectively. No effect on blood pressure, heart rate or platelet counts were seen. Adenosine diphosphate (ADP) induced platelet aggregation (PA) and cutaneous bleeding times (CBT) were determined prior to and after the last of 8 daily oral administrations of Ro 48-3657 (0.25 or 0.5 mg/kg) to eight rhesus monkeys. Peak and trough plasma concentrations were proportional to dose and steady state was achieved after the second administration. Inhibition of PA and prolongation of CBT were concentration dependent. The ex vivo IC\(_{50}\) (82 nM) for ADP-mediated PA correlated with a value (58 nM) determined in vitro. The CBT response curve was displaced to the right of the PA curve. CBT was prolonged to ≥25 min when levels of Ro 44-3888 exceeded 190 nM and PA was >90% inhibited. Therefore, in rhesus monkeys, Ro 48-3657 is reproducibly absorbed and converted to its active form, is well tolerated, and has a concentration-dependent effect on PA and CBT. These properties make Ro 48-3657 an attractive candidate for evaluation in patients at high risk for arterial thrombosis.

Introduction

Despite advances in the prevention and treatment of atherothrombotic disease, it remains the major cause of death in the industrialized world. Atherothrombosis is a multifactorial process. Lipid deposition in the arterial vascular wall leads to plaque formation and rupture, activation of platelets and coagulation, and ultimately to thrombotic vascular occlusion (1). Platelets are thought to play a central role in this process, particularly in the high shear environment of stenosed arteries (2, 3). Platelet rich thrombi have been observed in coronary arteries obtained from patients shortly after fatal myocardial infarction (MI) (4). More recently, platelet rich thrombi have been observed by coronary angiography in the arteries of patients experiencing unstable angina (UA) or MI (5). Platelet deposition and mural thrombosis also occurs to varying degrees following coronary angioplasty (6) and platelet rich thrombus formation has been implicated in abrupt vessel closure during and immediately following angioplasty (7, 8).

Aspirin, the primary anti-platelet therapy in use today, has been shown to reduce the risk of arterial thrombosis in placebo-controlled clinical trials. Relative risk reductions in composite endpoints (MI, stroke, death) of 25 to 35% have been achieved in patient populations with chronic stable angina, UA, MI, peripheral vascular disease and transient ischemic attacks or stroke (9). Despite aspirin’s proven efficacy there are reasons to believe that substantial improvements in anti-platelet therapy can be made. Primary among these is the fact that aspirin and other currently available, orally administered, anti-platelet agents such as ticlopidine and clopidogrel are selective platelet inhibitors, inhibiting some but not all agonist-induced pathways of platelet activation and recruitment (10-12). Indeed, up to one third of patients do not respond to aspirin therapy as determined by a circulating platelet aggregate assay (13).

Recognition of the role of the platelet glycoprotein IIb/IIIa receptor (GP IIb/IIIa, αIIbβ3) in the final common pathway of platelet aggregation (14-16) has led to the development of GP IIb/IIIa receptor antagonists as potential antithrombotic agents (17). Because of their higher intrinsic potency compared to aspirin, GP IIb/IIIa antagonists are expected to produce better clinical efficacy in preventing arterial thrombosis (18, 19). This has been convincingly demonstrated in a phase III clinical trial in which the intravenous administration of the chimeric Fab fragment of the anti-GP IIb/IIIa monoclonal antibody c7E3 (or Abciximab) proved superior to aspirin as an adjunct to angioplasty in high risk patients (20, 21). In addition to c7E3, a large number of potent GP IIb/IIIa antagonists, including snake venom polypeptides, linear and cyclic peptides, as well as non-peptide inhibitors have been identified (22-24). Some of these, for instance, the cyclic peptide Integrilin (25) and the non-peptide inhibitors Tirofiban (26) and Lamifiban (27), are currently being evaluated in clinical trials as short term intravenous treatments for a variety of thrombotic events (18). However, for the treatment of patients at risk for recurrent vascular events, orally available inhibitors that are effective and safe following long term administration will be required (19).

Aleg et al. have demonstrated that a number of non-peptidic compounds (piperidinoxyacetic acid derivatives) were potent and selective GP IIb/IIIa receptor antagonists. The alanine derivative of this

---

Correspondence to: Dr. Canio J. Refino, Department of Cardiovascular Research, Genentech Inc., 460 PT, San Bruno Blvd., South San Francisco, CA 94080. USA – Tel.: +1 415 225 2200; FAX Number: +1 415 225 6327; E-mail: ken@gene.com

---

Downloaded from www.thrombosis-online.com on 2018-05-06 | ID: 100166444 | IP: 54.70.40.11
For personal or educational use only. No other uses without permission. All rights reserved.
series (37b or Ro 44-3888) inhibited aggregation of human platelet rich plasma induced by 10 μM ADP, 0.2 U/ml collagen, or 0.2 U/ml thrombin with IC₅₀ of 0.05 μM, 0.07 μM and 0.05 μM respectively (28). Further investigation of this series of compounds demonstrated that a hydroxy amidine-ethyl ester prodrug of Ro 44-3888 (18 or Ro 48-3657) had good oral bioavailability in a number of different species. Specifically, following its oral administration to rats, dogs or rhesus monkeys, Ro 48-3657 was metabolized to the pharmacologically active molecule (29). Here we report on the pharmacokinetics, pharmacodynamics and tolerability of Ro 48-3657 following single and multiple oral administrations to rhesus monkeys.

Subjects, Materials and Methods

Materials

Ro 48-3657, Ro 44-3888 (Fig.1) and gelatin were provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Ro 44-3888 was supplied as a lyophilized powder of the trifluoroacetate salt. Prior to its administration, Ro 48-3657 was reconstituted with sterile saline for injection (Baxter, Hayward, CA) and the pH adjusted to 5.0 to 5.5 using sterile filtered 1N NaOH. Ro 48-3657 was supplied as either a lyophilized powder, pressed tablet, or as enteric coated particles in a gelatin capsule. Prior to its administration, the powder was suspended in a 10% gelatin solution and the components mixed as enteric coated particles in a gelatin capsule. Prior to its administration, the powder was suspended in a 10% gelatin solution and the components mixed to homogeneity with a ground glass pestle (Kontes, Vineland, NJ). ADP for platelet aggregations was obtained from Sigma (St. Louis, MO).

Animal Care and Use

All procedures involving the care or use of animals were reviewed and approved by the Institutional Animal Care and Use Committees of the institutions where the experiments were performed (Sierra Biomedical Inc., Sparks, NV and TSI Mason Laboratories, Worcester, MA) and were carried out in accordance with the regulations of the USDA Animal Welfare Act.

Single Dose Pharmacokinetics, Hematology and Hemodynamics

Five rhesus monkeys weighing 3.8 to 4.5 kg were given an intravenous (IV) infusion of Ro 44-3888 (0.2mg/kg, final dose at a constant rate of 2.22 μg/kg/min over 90 min) via an indwelling cephalic vein catheter. Following a 48 h washout period the monkeys were given an oral administration of Ro 48-3657 (1 mg/kg) in either a pressed tablet (n = 2) or enteric coated capsule (n = 3) form. Prior to oral dosing animals were fasted overnight. Administration of oral reagents was accomplished with a gavage tube, followed by 10ml of water.

Prior to both IV and oral dosing the monkeys were briefly anesthetized with Ketamine to facilitate the placement of blood sampling (saphenous vein) and drug delivery (cephalic vein) catheters. Following catheter placement the monkeys were placed in primate restraining chairs. Dosing was initiated after the animals had regained consciousness. Following the start of the Ro 44-3888 IV infusion, blood samples were collected at 0 (pre-dose), 15, 30, 60, 90, 95, 100, 105, 120, 150, 180, 240, 360 min and 24 h. Following oral administration of Ro 48-3657, blood samples were collected at 0 (pre-dose), 1, 2, 3, 4, 6, 8, 10, 12, 14, 20, and 24 h. The pre-dose to 360 min samples were collected from the saphenous vein catheter. Following this sample, catheters were removed, the animals returned to their cages and subsequent samples obtained by direct venipuncture. At each time point, 1.0 ml of blood was collected on 8.5% dipotassium-EDTA (20 μl) and kept on ice until processed and assayed for anti-IIb/IIIa activity as described below. An additional 0.5 ml was collected into dipotassium-EDTA Microtainer® tubes (Becton Dickinson) at pre-dose and at 1, 5, 6, and 24 h post dose. These samples were kept at room temperature until they were analyzed for complete blood counts (CBC) within three hours of collection.

Shortly after the pre-dose, 0.5, 1.5 and 6 h blood samples were collected, the monkeys' blood pressures and heart rates were monitored using a Dinamap® cuff type monitor (Critikon Inc., Miami, FL). Readings were taken from a cuff that was positioned around either the upper thigh or upper arm of the monkey according to the manufacturer’s instructions.

Multiple Oral Dosing: Pharmacokinetics, Pharmacodynamics and Tolerability

Eight rhesus monkeys weighing 4.1 to 6.2 kg were dosed orally once a day for eight consecutive days with a 10% gelatin suspension of Ro 48-3657. Monkeys received a dose of either 0.25 mg/kg (n = 4) or 0.5 mg/kg (n = 4). Blood samples for plasma concentration determinations (1.5 ml/sample) were taken just prior to and 3 h after each dose for the first seven days of dosing and just prior to and 1, 3, 6, 12, 24, 36, and 48 h after the last dose (Day 8). These samples were obtained by peripheral venipuncture and were processed and assayed as described below. Prior to the first, fourth and eighth dose, 0.5 ml of blood was collected for CBC determinations as described above. Additional blood samples (2.7 ml/sample) for determination of ADP-induced ex vivo platelet aggregation were collected on trisodium citrate (3.8%, 9 parts blood to 1 part citrate), prior to dosing on Day 1 and prior to and at 3, 6, 12, 24, and 36 h after the last dose (Day 8). These samples were obtained by peripheral venipuncture while the monkey was in a restraining chair and lightly anesthetized with Ketamine. Blood samples were centrifuged at 12,000 G in a benchtop centrifuge for approximately 4 s and allowed to stand for 5 min before platelet rich plasma (PRP) was decanted. PRP aggregation induced by ADP (17 μM) was measured in a turbidometric aggregometer (Payton Scientific, Inc., Buffalo, NY). For comparison, 15 ml of blood was obtained from an untreated monkey and processed similarly to obtain PRP. Ro 44-3888 was added at various concentrations to this PRP and ADP-induced platelet aggregations were determined. Concomitant with the collection of platelet aggregation samples, CBTs were determined using a Surgicutt Jr® bleeding time device (Baxter Health Care Corp., McGaw Park, IL) according to the manufacturer’s directions. Briefly, the Surgicutt device was used to make an incision on the volar surface of the monkey’s forearm while a pediatric sphygmomanometer, placed on the monkey’s upper arm, was inflated to 30mm of mercury. Following the incision, circular blotting paper was used to wick away the forming blood drop once every 15 s until bleeding had stopped (25 min maximum). The bleeding time was recorded to the nearest quarter minute.

Blood Sample Preparation for anti-IIb/IIIa Activity Assay

Dipotassium EDTA-anticoagulated whole blood was centrifuged at 12,000 × G for 4 min. The resulting platelet poor plasma was transferred to...
fresh tubes and stored at –70°C. Prior to assay, plasma samples were thawed and filtered through Microcon-10™ micro-concentrators (Amicon Inc., Beverly, MA) at 12,000 × g for 15 min. The protein poor filtrate was analyzed for anti-IIb/IIIa activity using a solid phase fibrinogen-soluble human IIb/IIIa receptor binding assay in which plasma sample and IIb/IIIa were added simultaneously to fibrinogen-coated, BSA-blocked, 96-well assay plates. After a one hour incubation, the sample and IIb/IIIa which did not adhere to the plate were washed away. Horseradish peroxidase-labeled mouse monoclonal anti-IIb/IIIa was then added. After another one hour incubation, the unbound labeled antibody was washed away, and the horseradish peroxidase substrate tetramethylbenzidine was added. The enzyme reaction was allowed to develop for approximately ten minutes, after which it was stopped by acidification. The absorbance in the wells was read at 450 nm. A standard curve of response to various concentrations of Ro 44-3888 was constructed, and the samples were quantitated from the standard curve. The lower limit of detection in this assay system was approximately 0.5 ng/ml of plasma. Within-run coefficients of variation (CV) ranged from 11% for the low range control to less than 6% for the high range control. Prior spike recovery studies demonstrated that total (free plus “receptor bound”) Ro 44-3888 could be recovered from plasma of dipotassium EDTA anticoagulated whole blood using these methods (unpublished data). Furthermore, there was a good correlation between results obtained with this assay system and an HPLC method (29).

Data Analysis

Data in the tables and graphs are presented as either a value for a single determination (one animal) or as the mean ± SD of multiple determinations (multiple animals, multiple days, or both). CBC and hemodynamic data from post-dosing time points (each time point treated independently) were compared to pre-dose values using a 2-tailed paired t-test. Between-animal and within-animal contributions to the variability in Ro 44-3888 concentrations following multiple administrations of Ro 48-3657 were estimated separately for each combination of dose and time point in a balanced analysis of variance. Animal identity was treated as a random effect and study day as a fixed effect. In this model, the within-animal contribution to the variability consists of two components, day-to-day and assay variability. The observed mean concentration (Σ, 4 animals by 8 days) was used as the divisor in computing the CV. To determine if there was a trend towards changing plasma concentrations of Ro 44-3888 over the 8 day course of treatment with Ro 48-3657, the mean of day 1 and 2 concentrations for each animal was compared to the mean of day 7 and 8 concentrations using a 2-tail paired t-test. This analysis was performed separately for each combination of dose and time point (3 and 24 h post dose). Regression analysis of platelet aggregation and CBT versus plasma concentrations of Ro44-3888 was performed using KaleidaGraph™ (Synergy Software, Reading PA). Aggregation versus plasma concentration data was fit to a four parameter curve. CBT was best fit to a simple exponential function. The clearance, half-life and steady-state volume of distribution of Ro44-3888 were determined using TOPFIT (30), a pharmacokinetic analysis package. The half life was estimated using the last four data points and the bioavailability of Ro 44-3888, following oral administration of Ro 48-3657, was calculated accounting for the differences in the molecular weights and doses.

Results

Pharmacokinetics of Ro 44-3888 following a Single IV Administration of Ro 44-3888 or a Single Oral Administration of Ro 48-3657

The mean ± SD plasma concentration versus time profile of Ro 44-3888 following intravenous infusion of Ro 44-3888 (0.2 mg/kg over 90 min) to five rhesus monkeys is shown in Fig. 2. During continuous intravenous infusion, plasma concentrations of Ro 44-3888 gradually increased. Following the termination of the infusion there was a biphasic elimination of Ro 44-3888. The terminal half-life (t1/2), determined from a log-linear regression of the last four data points was 2.5 ± 0.8 h. The weight adjusted volume of distribution at steady state was estimated to be 0.8 ± 0.4 l/kg and the total body clearance, estimated by dividing the dose by the trapezoidal AUC(0-∞), was 4.4 ± 1.8 ml/min/kg. The pharmacokinetic parameters for the individual animals following the intravenous regimen are summarized in Table 1. Following a 48 h wash out period the same animals were given an oral administration of Ro 48-3657 (1 mg/kg) in either a pressed tablet form (n = 2) or as enteric coated capsules (n = 3). There were no significant differences in the plasma disposition or pharmacokinetic parameters between the tablet and capsule formulations, therefore, data from both groups were pooled. Prior to oral dosing, the concentration of Ro 44-3888 had decreased to below the detection level of the assay (<0.5 ng/ml). The mean ± SD plasma disposition of Ro44-3888 following the oral administration of Ro 48-3657 is shown in Fig. 2 and the estimated pharmacokinetic parameters for the individual animals are summarized in Table 2. Following oral administration of Ro 48-3657, a peak plasma concentration of 152 ± 51 ng/ml was reached in 4.2 ± 2.2 h. Thereafter,
plasma concentrations decreased gradually in a mono-exponential fashion. The terminal half-life determined from a log-linear regression of the last four data points was 5.1 ± 1.6 h. Oral bioavailability was estimated to be 33 ± 6%.

Tolerability following a Single IV Administration of Ro 44-3888 or a Single Oral Administration of Ro 48-3657

Complete blood counts were determined prior to and at specified intervals after IV and oral dosing. Treating each time point independently, post-dosing values for each parameter were compared to their pre-dose value using a 2-tail paired t-test. IV dosing with Ro44-3888 or orally, post-dosing values for each parameter were compared to their pre-dose values with a 2-tail paired t-test. IV dosing with Ro 48-3657, plasma concentrations of Ro 44-3888 were elevated in a dose dependent fashion and had decreased to low but detectable levels at 24 h (Fig. 3). The mean ± SD concentrations at 24 h post dose were 42 ± 9 and 75 ± 18 ng/ml for the 0.25 mg/kg and 0.5 mg/kg dose respectively. Mean ± SD concentrations at 24 h post dose were 4.1 ± 2.8 and 6.1 ± 2.0 ng/ml respectively (Table 3). Inter- and intra-animal contributions to the variability in Ro 44-3888 concentrations were estimated separately for each combination of dose and time point (3 and 24 h post dose) in a balanced analysis of variance. For drug concentrations 3 h post dose, the inter-animal CV was 7-14% and the intra-animal CV was 17-21%. For the 24 h post dose samples, the inter-animal CV was 0-25% and the intra-animal CV was 34-66% (Table 3). Inter-animal and intra-animal contributions to the variability in Ro 44-3888 concentrations were estimated separately for each combination of dose and time point (3 and 24 h post dose) in a balanced analysis of variance. For drug concentrations 3 h post dose, the inter-animal CV was 7-14% and the intra-animal CV was 17-21%. For the 24 h post dose samples, the inter-animal CV was 0-25% and the intra-animal CV was 34-66% (Table 3). It should be noted that the intra-animal contribution to the variability is made up of not only day to day variability within each animal, but also variability due to the assay. This might explain the larger inter-animal variability observed in the 24 h post dose data since the lower plasma concentrations are read from the portion of the assay standard curve that has a larger variability.

To determine if there was a trend towards changing plasma concentrations over the 8-day course of treatment, the mean of day 1 and 2...
concentrations for each animal was compared to the mean of day 7 and 8 concentrations using a 2-tail paired t-test. This analysis was performed separately for each combination of dose and time point (3 and 24 h post dose). With the exception of a significant difference in the 24 h concentration data of the 0.25 mg/kg dose (p = 0.048), all other comparisons were not significant. For drug concentrations 3 h post dose, P values were 0.73 and 0.63 for the 0.25 mg/kg and 0.5 mg/kg dose groups respectively. At 24 h post dose the P value of the 0.5 mg/kg dose group was 0.99. This analysis suggests that in these animals absorption and conversion mechanisms were neither up- nor down-regulated by repeated administration and that steady-state was achieved in one dosing cycle.

The estimated pharmacokinetic parameters for the individual animals are summarized in Table 4. The mean ± SD estimated terminal half-lives following the last dose were 8.6 ± 2.0 and 12.0 ± 4.8 h for the 0.25 and 0.5 mg/kg dose groups respectively. These means were not significantly different from each other (p >0.05). However they were significantly different from the mean t½ of 5.1 ± 1.6 h following a single administration of Ro 48-3657 (p <0.01).

**Hematological Parameters following Multiple Oral Daily Administration of Ro 48-3657**

Platelet, erythrocyte and leukocyte counts were measured prior to the first, fourth and eighth dose of Ro 48-3657. Day 4 and day 8 values for each animal were expressed as a percentage of the pre-dose value. Since there were no significant differences in daily values between the 0.25 mg/kg and 0.5 mg/kg dose groups, these groups were pooled for further analysis. Day 4 and day 8 values were compared to day 1 values using a 2 tailed paired t-test. These data are summarized in Table 5. There was a trend towards decreasing platelet counts (7% decrease by day 8). However, this change was not statistically significant. In contrast, decreases in red cell counts of 5% and 14% by day 4 and 8 respectively were highly significant (p <0.01). White blood cell counts were elevated to 128% and 127% of day 1 values at day 4 and day 8 respectively. However this increase was not statistically significant. Since these modest changes in blood counts were not dependent on the dose of Ro 48-3657 administered it is unlikely that they were drug related. More likely, as in the single dose experiment, they probably result from blood loss due to repeated blood sampling (RBC and platelets) or from a stress response to handling (WBC).

**Pharmacodynamics following Multiple Oral Administration of Ro 48-3657**

The pharmacodynamic effects of Ro 44-3888 were investigated following the first and last of 8 daily oral administrations of Ro 48-3657. *Ex vivo* platelet aggregation response to ADP (17 μM) and CBT’s were measured prior to the first and last (eighth) dose and at various times between 3 to 36 h after the last dose. Platelet aggregation response and bleeding times were similar at the first and eighth pre-dose time point (data not shown). Subsequent platelet aggregations for each animal were expressed as a percentage of its most recent pre-dose response. These data (mean ± SD) along with mean plasma concentrations of Ro 44-3888 are presented graphically in Fig. 4. From these

---

**Table 4** Pharmacokinetic parameters following multiple oral administrations of Ro 48-3657

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Animal ID.</th>
<th>Conc. (3 hr)a</th>
<th>Conc. (24 hr)b</th>
<th>11/2)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>R05F</td>
<td>35±5</td>
<td>3.2±1.3</td>
<td>9.7</td>
</tr>
<tr>
<td>0.25</td>
<td>R101F</td>
<td>41±8</td>
<td>5.0±4.6</td>
<td>10.0</td>
</tr>
<tr>
<td>0.25</td>
<td>R156F</td>
<td>41±5</td>
<td>2.7±1.2</td>
<td>5.7</td>
</tr>
<tr>
<td>0.25</td>
<td>R758F</td>
<td>49±8</td>
<td>5.4±1.1</td>
<td>9.0</td>
</tr>
<tr>
<td>0.50</td>
<td>R13F</td>
<td>73±22</td>
<td>6.4±2.9</td>
<td>11.0</td>
</tr>
<tr>
<td>0.50</td>
<td>R35F</td>
<td>66±10</td>
<td>6.1±1.3</td>
<td>9.3</td>
</tr>
<tr>
<td>0.50</td>
<td>R60F</td>
<td>84±12</td>
<td>5.0±1.9</td>
<td>19.0</td>
</tr>
<tr>
<td>0.50</td>
<td>R114F</td>
<td>75±8</td>
<td>6.5±0.8</td>
<td>8.7</td>
</tr>
</tbody>
</table>

a Mean±SD of the plasma concentrations over the dosing period.

b Estimated using the data following the last dosing.

**Table 5** Hematological parameters in rhesus monkey following multiple oral administrations of Ro 48-3657

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Day 4</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of day 1</td>
<td>% of day 1</td>
</tr>
<tr>
<td>Platelets</td>
<td>93±11</td>
<td>93±11</td>
</tr>
<tr>
<td>RBC</td>
<td>95±6*</td>
<td>86±4**</td>
</tr>
<tr>
<td>WBC</td>
<td>128±38</td>
<td>127±55</td>
</tr>
</tbody>
</table>

* Mean ± SD are day 4 and day 8 pre-dose values expressed as a percentage of day 1 pre-dose.

*p <0.05, ** p <0.01 (2-tail paired t-test)
The bleeding time-plasma concentration relationship was best described by an exponential fit of the data ($r = 0.82$). Ro 44-3888 and its pharmacodynamic effect. To determine the relationship between plasma concentrations of Ro 44-3888, platelet aggregation, and prolongation of CBT were dependent on the dose of Ro 48-3657 administered. Furthermore, there appeared to be a good correlation between the plasma concentration of Ro 44-3888 and its pharmacodynamic effect.

To determine the relationship between plasma concentrations of Ro 44-3888, platelet aggregation, and bleeding time in more detail, a regression analysis was performed. For the purpose of this analysis, the aggregatory response of each animal’s eighth day predose sample to ADP was considered to be 0% inhibition while lack of a response to ADP was considered to be 100% inhibition. For comparison, the results of a dose-response evaluation of Ro 44-3888 in untreated rhesus monkey PRP were similarly analyzed. These results are summarized in Fig. 5. A four parameter fit of the aggregation versus concentration data produced an IC$_{50}$ of 82 nM (31 ng/ml). This *ex vivo* IC$_{50}$ was similar to the IC$_{50}$ calculated from the *in vitro* titration (58 nM or 22 ng/ml). A comparison of an exponential fit of the CBT versus concentration data and the fitted platelet aggregation versus concentration data, demonstrated that bleeding times equaled or exceeded 25 min when plasma concentrations of Ro 44-3888 exceeded 195 nM (73 ng/ml), a concentration where ADP-induced platelet aggregation was $>90\%$ inhibited.

**Discussion**

GP IIb/IIIa receptor antagonists were first shown to have antithrombotic effects in canine models of arterial thrombosis that are resistant to aspirin (32, 33). Furthermore, the strategy of GP IIb/IIIa receptor blockade continues to show promise in a number of clinical trials in which the intravenous administration of the monoclonal antibody c7E3 or peptidic and non-peptidic small molecule antagonists of GP IIb/IIIa have proven efficacious as an adjunct to PTCA (20, 21, 25) or in the treatment of unstable angina (27, 34, 35). Presumably, as suitable orally active agents become available, the use of GP IIb/IIIa antagonists can be extended to the chronic treatment of patients at risk for recurring thrombotic events (19). Ro 48-3657 is currently being evaluated in patients following an acute coronary syndrome (MI, UA, etc.) (36). In addition to Ro 48-3657, a number of other potent, orally active GP IIb/IIIa antagonists have recently been reported (37-41). For any of these molecules to become viable clinical treatments, they will have to exhibit a number of pharmacological properties. Primary among these will be adequate and reproducible activity and good tolerability following chronic oral administration. Secondarily, the half life of the active compound should be sufficiently long to allow for single or twice daily dosing.

In a previous report we demonstrated that an approximately 20-fold increase in oral activity of a potent GP IIb/IIIa antagonist (Ro44-3888) could be achieved by using a "double pro-drug" strategy to produce Ro 48-3657 (29). In the current report we have extended that work by giving a detailed analysis of the pharmacokinetics, pharmacodynamics and tolerability of Ro 44-3888 following single and multiple oral administrations of Ro 48-3657 to rhesus monkeys. In this study there are a number of notable findings. Single or multiple oral administrations of the inactive prodrug resulted in its absorption and conversion such that the active form was detectable in plasma. As reported previously, bioavailability of Ro 44-3888 following a single oral administration of Ro 48-3657 to rhesus monkeys was greater than 30% (29). Analysis of the peak and trough plasma concentrations of Ro 44-3888 following multiple oral administrations of Ro 48-3657 demonstrate that conversion from the inactive to the active form was predictable (dose proportional) and reproducible (low inter-animal and inter-day variability). While the metabolic processes involved in the conversion of Ro 48-3657 to Ro 44-3888 are still under investigation, preliminary data suggests that reduction of the amidoxime occurs in the liver, predominately in the mitochondria subfraction (42). Therefore, the use of a prodrug approach may have additional benefits for orally administered compounds of this class beyond increasing their bioavailability. Specifically, having an inactive form in the intestinal lumen may reduce the risk of inducing gastrointestinal bleeding.

The terminal half life of Ro 44-3888 following the last of eight daily oral administrations of Ro 48-3657 was estimated to be $10.3 \pm 3.8$ h (mean $\pm$ SD of 0.25 mg/kg and 0.3 mg/kg doses). This was significantly longer than the terminal half life of $5.1 \pm 1.6$ h estimated following a single oral administration of 1 mg/kg. While this difference may reflect a change in clearance rates due to a dose or prolonged exposure effect it is more likely an artifact of the different sampling schedules used in the two experiments. In both experiments, the $t_\beta$ estimate was used the last four data points. In the single dose experiment these samples were collected at 12 to 24 h after dosing while in the multiple dose experiment the sampling schedule was less frequent, encompassing the time frame from 12 to 48h. Since the plasma concentrations in the latter samples were at the low end of the sensitivity range of the assay, where assay variability is greatest, the $t_\beta$ estimate from the multiple dose experiment is probably less reliable than the estimate from the single dose experiment. What is of more interest than a precise measurement of the $t_\beta$ in rhesus monkeys is what this value may be in humans. Interspecies scaling has been used previously to predict the pharmacokinetic profile of various compounds in humans based on their pharmacokinetic profile in lower species (43). If the $t_\beta$s follow-
ing single oral administration to rat, dog and rhesus monkeys (29) are subjected to this type of analysis, the t1/2 of Ro 44-3888 following an oral administration of Ro 48-3657 to a 70 kg human is estimated to be 12.7 h. Depending on the therapeutic window for this compound, a terminal half life of this magnitude would most likely allow for once or twice daily dosing in humans.

In rhesus monkeys, inhibition of platelet aggregation and prolongation of CBT following eight consecutive daily administrations of Ro 48-3657 were dependent on the total plasma concentration of Ro 44-3888. The plasma concentration of Ro 44-3888 that produced a 50% inhibition of ex vivo platelet aggregation was similar to the concentration that produced a 50% inhibition of in vitro platelet aggregation of PRP collected from an untreated donor animal. The similarity of the in vitro and ex vivo IC50 suggests that the platelet inhibitory effect following multiple oral administrations of Ro 48-3657 was the result of its conversion to Ro44-3888 and not an effect of other metabolites or a change in platelet responsiveness. This conclusion is further supported by the results of the single dose study where there was a good correlation in the concentrations of Ro44-3888 determined by either an HPLC method or the activity assay used here (29). The rhesus in vitro IC50 of 58 nM determined in this study is very similar to a human in vitro IC50 of 62 ± 7 nM determined using comparable methodologies (unpublished data). Interestingly, the concentration-effect relationship for CBT was right-shifted relative to ADP-induced aggregation. That is, a higher concentration of drug was required to produce a half-maximal response in CBT relative to a half-maximal response to ADP-induced aggregation. This difference in sensitivity has been previously reported for a number of GP Ib/IIIa inhibitors in both animal models and humans (44-46). More recently, Theroux et al. have demonstrated that in unstable angina patients treated with Lamifiban (another non-peptide GP Ib/IIIa inhibitor) there is a better correlation between prolongation of CBT and inhibition of ex vivo platelet aggregation induced by the synthetic thrombin receptor agonist, TRAP, than with the less potent agonist, ADP (27). This difference in sensitivity to the two agonists could explain the separation in CBT and platelet aggregation responses seen in ours and other studies where ADP was used as the agonist.

Ro 48-3657, like many other GP Ib/IIIa inhibitors, is modeled on the tripeptide motif arginine-glycine-aspartic acid (RGD) found in the Aα chain of fibrinogen and disintegrins like Echistatin and Kistrin (48). Since both monoclonal antibodies against GP Ib/IIIa and certain disintegrins are known to induce thrombocytopenia (20, 49, 50) we felt that it was important to monitor platelet counts in our studies. Single administration of Ro 44-3888 or Ro 48-3657 or repeated administration of Ro 48-3657 had no significant effect on platelet counts in these animals.

In summary, single or multiple administration of the double prod rug Ro 48-3657 to rhesus monkeys resulted in its absorption and conversion to the active form of the drug (Ro44-3888). Furthermore, peak and trough plasma concentrations of Ro 44-3888 following multiple oral administration of Ro 48-3657 were proportional to the dose administered and highly reproducible both between animals and between days. The terminal half life of the active compound was sufficiently long that, when scaled to humans, it would require no more than twice daily administration to maintain platelet inhibitory concentrations. Pharmacodynamic effects (inhibition of ex vivo platelet aggregation and prolongation of CBT) following multiple oral administrations were dose- and concentration-dependent. The concentration-effect relationship of ex vivo aggregation correlated well with an in vitro titration in rhesus PRP. Furthermore, the concentration-effect relationship for prolongation of bleeding times was displaced to the right of the relationship for ADP-induced aggregation. Finally, oral administration of Ro 48-3657 was well tolerated in rhesus monkeys with no significant drug-induced effects on heart rate, blood pressure or CBC.

A number of questions remain to be answered about GP Ib/IIa inhibitors in general, and the chronic administration of orally active inhibitors in particular, before their potential for the prevention and treatment of atherothrombotic disease can be fully realized (19). However, the pharmacokinetic, pharmacodynamic, and tolerability profile demonstrated by Ro 48-3657 in these primate studies make it an excellent candidate for further clinical evaluation.

Acknowledgments

The authors are grateful to Julie Badillo for sample preparation and Ann Wawruckiewicz for performing the anti GP Ib/IIa activity assays. We also appreciate the excellent assistance of James Reiman with the statistical analysis. We would also like to thank the staffs of Sierra Biomedical Research and TSI Mason Laboratories who assisted with the primate studies, especially Belinda Fuller and Christina Gamba-Vitalo.

References

14. Turitto VT, Baumgartner HR. Platelet surface interactions. In: Hemostasis and thrombosis, basic principles and clinical practices. Colman RW,


