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# Preclinical studies of RUC-4, a novel platelet $\alpha$ IIb $\beta$ 3 antagonist, in non-human primates and with human platelets

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## Abstract

**Introduction:** We are developing the novel  $\alpha$ IIb $\beta$ 3 antagonist, RUC-4, for subcutaneously (SC)-administered first-point-of-medical-contact treatment for ST segment elevation myocardial infarction (STEMI). **Methods:** We studied the (1) pharmacokinetics (PK) of RUC-4 at 1.0, 1.93, and 3.86 mg/kg intravenous (IV), intramuscular (IM), and SC in non-human primates (NHPs); (2) impact of aspirin on RUC-4 IC<sub>50</sub> in human platelet-rich plasma (PRP); (3) effect of different anticoagulants on the RUC-4 IC<sub>50</sub> in human PRP; and (4) relationship between  $\alpha$ IIb $\beta$ 3 receptor blockade by RUC-4 and inhibition of ADP-induced platelet aggregation. **Results:** (1) All doses of RUC-4 were well tolerated, but animals demonstrated variable temporary bruising. IM and SC RUC-4 reached dose-dependent peak levels within 5–15 minutes, with T<sub>1/2</sub> s between 0.28 and 0.56 hours. Platelet aggregation studies in NHPs receiving IM RUC-4 demonstrated >80% inhibition of the initial slope of ADP-induced aggregation with all three doses 30 minutes post-dosing, with subsequent dose-dependent loss of inhibition over 4–5 hours. (2) The RUC-4 IC<sub>50</sub> for ADP-induced platelet aggregation was unaffected by aspirin treatment (40±9 nM vs 37±5 nM; *p* = 0.39). (3) The RUC-4 IC<sub>50</sub> was significantly higher in PRP prepared from D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK)-anticoagulated blood compared to citrate-anticoagulated blood using either thrombin receptor activating peptide (TRAP) (122±17 vs 66±25 nM; *p* = 0.05; *n* = 4) or ADP (102±22 vs 54±13; *p* < 0.001; *n* = 5). (4) There was a close correspondence between receptor blockade and inhibition of ADP-induced platelet aggregation, with aggregation inhibition beginning with ~40% receptor blockade and becoming nearly complete at >80% receptor blockade. **Discussion:** Based on these results and others, RUC-4 has now progressed to formal preclinical toxicology studies.

## Introduction

$\alpha$ IIb $\beta$ 3 (GPIIb/IIIa) antagonists are effective in decreasing mortality and morbidity when administered to patients undergoing percutaneous coronary artery interventions (PCI), with significant reductions in the odds of both all-cause mortality at 30 days [odds ratio (OR) 0.79, 95% confidence interval (CI) 0.64–0.97] and death or myocardial infarction at 30 days (OR 0.66, 95% CI 0.60–0.72) in more than 40 studies conducted on more than 30,000 patients [1]. Whereas thrombi causing ST segment elevation myocardial infarction (STEMI) have in general, but not uniformly, been found to be platelet rich early after symptom onset and less platelet rich over time [2–4], there is reason to expect that potent antiplatelet agents would be more effective when administered early after the onset of symptoms. Consistent with this hypothesis, meta-analyses of studies comparing administration of  $\alpha$ IIb $\beta$ 3 antagonists relatively early after symptom onset to administration at the time of the PCI demonstrated significant benefits from early therapy in pre-PCI blood flow in the target vessel, time to ST segment resolution, left-ventricular ejection fraction, distal embolization, and mortality [5–7]. Similar benefits of early versus late abciximab were observed in studies of patients with STEMI transferred for primary PCI who received abciximab before transfer rather than during PCI, with a higher percentage of patent arteries pre-PCI and lower rates of death and a composite clinical outcome at 30-day follow-up [8]. Most importantly, in the randomized studies, the mortality benefit from early  $\alpha$ IIb $\beta$ 3 antagonist therapy increased over an 11-year period, with those treated within 65 minutes manifesting less than 5% mortality at 11 years compared to greater than 30% mortality for those treated >178 minutes after the onset of symptoms [6].

Despite these data, early treatment of STEMI with an  $\alpha$ IIb $\beta$ 3 antagonist is not in common use, in part because all of the agents require both an intravenous (IV) bolus injection followed by a

continuous IV infusion controlled by a pump. We previously reported on a novel small molecule  $\alpha$ IIB $\beta$ 3 antagonist, RUC-4 [9], which is a derivative of a compound (RUC-1) we identified in a high throughput screen for inhibitors of platelet adhesion to fibrinogen [10–12] and RUC-2, which was modified from RUC-1 [13]. RUC-2 and RUC-4 exclude the divalent cation required for ligand binding from the  $\beta$ 3 subunit metal ion-dependent adhesion site (MIDAS) domain and thus prevent ligand binding,  $\alpha$ IIB $\beta$ 3-mediated platelet aggregation, and in vivo thrombus formation [9,13]. Moreover, since the interaction between the ligand's carboxyl oxygens with the metal ion in the MIDAS triggers the receptor to undergo a major conformational change that places it in a high-affinity ligand binding state [reviewed in [14]], both RUC-2 and RUC-4 lock the receptor in its inactive conformation. This contrasts with other small molecule  $\alpha$ IIB $\beta$ 3 antagonists based on the Arg-Gly-Asp (RGD) sequence, since they act as pseudo-ligands, binding to the MIDAS metal ion through their carboxyl oxygens and inducing the conformational change. RUC-4's unique mechanism of action may offer clinical advantages since the conformational change produced by the other agents may contribute to the thrombocytopenia observed in a small percentage of treated patients who have performed antibodies to the altered conformation [15–17], and induction of the high affinity state may limit efficacy. The latter may even have contributed to the increased risk of death observed with some of the oral  $\alpha$ IIB $\beta$ 3 antagonists, all of which have carboxyl oxygens that are presumed to bind to the MIDAS metal ion [18–20].

We previously reported that intramuscular administration of RUC-4-free base to male cynomolgus monkeys (*Macaca fascicularis*) non-human primates (NHPs) at doses between 1 and 3.86 mg/kg led to the onset of high-grade inhibition of platelet aggregation within 15–30 minutes that lasted from ~2 to >4.5 hours in a dose-dependent manner [9]. To achieve our goal of making it as easy as possible for first-point-of-medical-contact emergency medical service and emergency room medical personnel to administer RUC-4 rapidly to patients with STEMI, and knowing that it is challenging to reproducibly administer drugs intramuscularly by autoinjector in the emergency setting with a single needle size [21,22], we have now compared the pharmacokinetics (PK) of RUC-4 administered subcutaneously (SC) with the PK and pharmacodynamics (PD) of RUC-4 administered intramuscularly (IM).

We also conducted additional preclinical studies of RUC-4 using human blood in anticipation of starting human studies. Since immediate aspirin treatment is approved for treatment of myocardial infarction and is considered the current standard of care for STEMI, [23,24] we plan to administer RUC-4 in combination with aspirin to STEMI patients. As a result, we also studied the effect of aspirin on the antiplatelet effect of RUC-4. In addition, since the anticoagulant utilized to prepare platelets for platelet aggregation studies can influence the response to different antiplatelet agents, [25–28] we also studied the impact of different anticoagulants on the sensitivity of platelets to the inhibitory effects of RUC-4. Finally, to obtain a better understanding of the correlation between inhibition of platelet aggregation and the percentage of receptors blocked by RUC-4, we studied this relationship directly.

## Materials and Methods

### RUC-4

RUC-4 was synthesized at the National Center for Advancing Translational Science at the National Institutes of Health as previously described or with minor modifications [29].

### Study Comparing the Effects of IM and SC RUC-4 in Cynomolgus Monkey (*M. fascicularis*) NHPs

**Platelet aggregation and Determination of the RUC-4 IC<sub>50</sub> in NHPs.** Light transmission platelet aggregation studies were performed with a PAP-8E aggregometer (Bio/Data Corporation, Horsham, PA) at 37°C with stirring using 5  $\mu$ M ADP to induce aggregation. Blood (5–7.5 ml) was obtained from four NHPs with a 21-gauge needle and anticoagulated with 1/10<sup>th</sup> volume of 3.8% sodium citrate to determine the RUC-4 IC<sub>50</sub> for platelet aggregation. Platelet-rich plasma (PRP) was prepared by centrifuging whole blood at 600  $\times$  g for 1–2.5 minutes at room temperature. The remaining blood was centrifuged at 1200  $\times$  g for 8 minutes at room temperature to prepare platelet-poor plasma (PPP). Platelet counts on PRP were performed with an automated platelet counter (IDEXX ProCyt Dx Hematology Analyzer, Westbrook, ME) and were adjusted down to a count of 3.0  $\times$  10<sup>5</sup>/ $\mu$ l with PPP when the PRP platelet counts were higher. PRP (0.25 ml) was incubated with increasing concentrations of RUC-4 for 20 minutes before initiating aggregation with 5  $\mu$ M ADP. Platelet aggregation was quantified based on the instrument's measurement of the primary slope of aggregation and the percentage inhibition produced by RUC-4 calculated by reference to the slope of the untreated sample. To determine the RUC-4 IC<sub>50</sub>, PRP from four animals was either untreated (control) or incubated with RUC-4 at final concentrations of 0.01, 0.03, 0.1, 0.3, 0.5, 1.0, 3.0, or 5.0  $\mu$ M before adding ADP at 5  $\mu$ M. The 0.01 and 5.0  $\mu$ M doses were only studied in two animals. In one animal, aggregation was not assessed at 1  $\mu$ M because of technical problems. The percentage inhibition of the initial slope of aggregation relative to the control value was calculated for each concentration for each animal, averaging duplicate values when they were available (11/32 samples), and the RUC-4 IC<sub>50</sub> was calculated using the Levenberg–Marquardt algorithm to perform the linear regression fit using the Hill equation.

### Animal Handling

This study was performed at Biomere, Worcester, MA, on six adult male animals aged 3.6–4.8 years obtained from Charles River Laboratories. Animals used on study followed the test facility's standard operating procedures for receipt of NHPs and underwent a physical examination, tuberculosis and measles testing, and evaluation of clinical pathology. All animals were considered in good health by the attending veterinarian before being released for study. The veterinary care of the animals was in accordance with the protocol, Biomere's standard operating procedures, and regulations outlined in the applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR), the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the Guide for the Care and Use of Laboratory Animals, and the Biomedical Research Models, Inc., Policy on Humane Care. The protocol and any amendments or procedures involving the care or use of animals in this study were reviewed and approved by Biomere's Institutional Animal Care and Use Committee (IACUC) before the initiation of such procedures.

The six NHPs used in these studies were single or socially housed, when possible, in stainless steel cages equipped with a stainless steel mesh floor and an automatic watering valve in a room that was well ventilated and maintained at a temperature of 18–29°C and a relative humidity of 30%–70%. Fluorescent lighting provided illumination approximately 12 hours per day. Animals were fed Monkey Diet 5038 (Lab Diet) daily and filtered tap water was provided *ad libitum* via an automatic watering

**Table 1.** NHP study experimental design

Group No.	No. of animals per group	Treatment	Dose level (mg/kg)	Dose volume (mL/kg)	Dose concentration (mg/mL)	ROA	Blood collection
<b>Arm 1 (IM)</b>							
1	2	RUC-4	3.86	0.064	60	IM	At pre-dose and at 30, 120, and 270 minutes, and ~27 hours post-dose
2	2		1.93	0.064	30		
3	2		1.00	0.064	15		
<b>Arm 2 (IV)</b>							
4	2	RUC-4	3.86	0.064	60	IV	At pre-dose and at 30, 120, and 270 minutes post-dose
5	2		1.93	0.064	30		
6	2		1.00	0.064	15		
<b>Arm 3 (IV)</b>							
4	2	RUC-4	3.86	0.064	60	IV	At pre-dose and at 1, 5, and 15 minutes post-dose
5	2		1.93	0.064	30		
6	2		1.00	0.064	15		
<b>Arm 4 (IM)</b>							
1	2	RUC-4	3.86	0.064	60	IM	At pre-dose and at 5, 15, and 45 minutes post-dose
2	2		1.93	0.064	30		
3	2		1.00	0.064	15		
<b>Arm 5 (SC)</b>							
1	2	RUC-4	3.86	0.064	60	SC	At pre-dose and at 5, 15, 30, and 60, and 120 minutes
2	2		1.93	0.064	30		
3	2		1.00	0.064	15		

No., number; ROA, route of administration; IV, intravenous; IM, intramuscular; SC, subcutaneous.

The same animals were studied in each arm of the study and so each animal received five doses via different ROAs over a period of 6 months.

system. All animals received environmental enrichment including manipulable devices, foraging opportunities, food items, structural and environmental enhancements, and positive human interaction. Enrichment devices were rotated on a weekly basis and include toys, mirrors, radios, TV, foraging boards, and a variety of complex foraging devices. After the completion of sample collection, the animals were released back to the Biomere colony. Manual and/or mechanical (chair) restraint was used as necessary to facilitate animal handling and performance of the technical procedures. Blood was collected either from a vein or artery in the femoral triangle from animals acclimated to restraint.

Prior to dose administration, hair was shaved and trimmed at the injection site in order to improve the visualization of any injection site observations. RUC-4 was administered as a single IM or IV injection at a constant dose volume of 0.064 mL/kg under manual restraint without any sedation. All IM injections were administered directly into the thigh semitendinous muscle, which was marked for later evaluation. Direct pressure to the injection site was maintained for at least 5 minutes to minimize the risk of bleeding. IV injections were made via an angiocatheter placed into the saphenous vein. The dose volume for each animal was based on the most recent body weight measured on the day before or the day of dosing. SC injections were made on a shaved portion of the abdomen at a constant volume of 0.064 mL/kg using a 23-gauge, 3/4 inch needle. The site of injection was compressed manually for 5 minutes after the injection.

## Dosing

The six animals were divided into three dose groups of two animals/group and they received RUC-4 at doses of 3.86 mg/kg, 1.93 mg/kg, and 1 mg/kg IM, IV, and SC as indicated in Table 1. Since there were five arms to the study, and each animal was studied in each arm, each animal received repeated doses a total of five times over the course of approximately 6 months.

## Blood Sampling for PK, PD, and Blood Counts

For PK studies, 0.5 mL of whole blood was immediately added to a chilled tube containing 2 mL of a mixture of water and acetonitrile (70:30 vol/vol), and then the tube was immediately vortexed and stored at  $-80^{\circ}\text{C}$  until analyzed. In the first four arms, samples were collected and evaluated ( $n = 2/\text{dose}/\text{time point}$ ) for PK analysis at pre-dose and at 5, 15, 30, 45, 120 and 270 minutes after IM and IV injection; additional samples were obtained at 1 minute after IV administration and at ~27 hours after administering 3.86 mg/kg IM in arm 1, the latter because the platelet aggregation responses had not returned to baseline levels at 270 minutes. In arm 5, animals ( $n = 2/\text{dose}/\text{time point}$ ) received RUC-4 at the same three doses and blood was obtained pre-dose and at 5, 15, 30, 60, and 120 minutes after dosing for PK analysis.

Blood (2.5 mL) for light transmission platelet aggregation studies was collected into 1/10<sup>th</sup> volume of 3.8% sodium citrate from the animals receiving RUC-4 IM pre-dose and at 30, 120, and

270 minutes after RUC-4; an additional sample was obtained at ~27 hours from animals receiving 3.86 mg/ml RUC-4 in arm 1 because the platelet aggregation responses were still inhibited at 270 minutes post-dose. Blood was centrifuged at  $650 \times g$  for 1–2.5 minutes at room temperature to prepare PRP and the remaining blood was centrifuged at  $1200 \times g$  for 8 minutes at room temperature to prepare PPP. Platelet counts were performed on PRP as above and samples were adjusted to a count of  $3 \times 10^5/\mu\text{l}$  with PPP if the PRP count was higher.

Blood (0.5 ml) was collected into EDTA for complete blood counts (CBC) in both animals in each dose group in arms 1 and 2 pre-dose and at 30, 120, and 270 minutes post-dose. In arm 3, both animals had blood drawn for a CBC pre-dose and 15 minutes post-dose; in arm 4, both animals had blood drawn for CBC pre-dose and at 45 minutes post-dose; in arm 5, both animals had blood drawn for CBC pre-dose and at 120 minutes post-dose.

### Analysis of RUC-4 Whole Blood Concentrations

Whole blood samples collected in water-acetonitrile solution were analyzed for RUC-4 by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) with an Acquity UPLC BEH HILIC column (130 Å, 1.7  $\mu\text{m}$ ; 50 x 2.1 mm) and an API-6500 mass spectrometer in positive ion mode using electrospray and a temperature of 550°C. Mobile phase A was 10 mM ammonium acetate in 100:0.1 (vol/vol) water: $\text{NH}_4\text{OH}$  and mobile phase B was 10 mM ammonium acetate in 100:0.1 (vol/vol) acetonitrile: $\text{NH}_4\text{OH}$ . Purified RUC-4 (387.1/330.1 amu) was used as a calibration standard and deuterated RUC-4 (D8; 395.1/338.1 amu) served as an internal control. The standard curve was based on quadratic regression with acceptance criteria of  $\pm 20\%$  and an accepted curve range of 5–5000 ng/ml. The blood concentrations of RUC-4 after IM and SC administrations were evaluated by non-compartmental analyses (NCA) with Phoenix WinNonlin software (version 6.3, Pharsight, St. Louis, MO, USA).

### In Vitro Platelet Aggregation Studies in Healthy Human Volunteers

**Blood sampling and preparation of PRP.** All studies were approved by the Rockefeller University Institutional Review Board (IRB) and all blood donors gave informed consent. Blood from healthy volunteers was obtained via venipuncture with a 19-gauge needle and anticoagulated with 1/10<sup>th</sup> volume 3.8% sodium citrate (final concentration 0.38%), ~1/15<sup>th</sup> volume D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK; final concentration 0.1 or 0.3 mM), or dried sodium heparin in a vacuum tube (Vacutainer, Becton Dickinson) designed to achieve a final concentration of 15.8 USP U/ml). Samples were gently inverted and kept at room temperature until processed. PRP was prepared by centrifuging the whole blood at  $650 \times g$  for 4 minutes at room temperature. The remaining blood was centrifuged for  $1200 \times g$  for 8 minutes at room temperature to collect PPP. Platelet counts in the PRP were adjusted to  $3.0 \times 10^5/\mu\text{l}$  with PPP. Platelet aggregation studies were conducted by incubating 225  $\mu\text{l}$  of PRP with 2  $\mu\text{l}$  of test compound for 20 minutes at room temperature and then adding 25  $\mu\text{l}$  of agonist with stirring to initiate aggregation.

**Preparation of RUC-4 stock solutions for platelet aggregation studies.** Working stock dilutions of RUC-4 in arms 1, 2, and 5 were made up in 0.9% sterile saline at concentrations of 375  $\mu\text{M}$ , 125  $\mu\text{M}$ , 62.5  $\mu\text{M}$ , 37.5  $\mu\text{M}$ , 25  $\mu\text{M}$ , 12.5  $\mu\text{M}$ , 3.75  $\mu\text{M}$ , and 1.25  $\mu\text{M}$  to achieve final concentrations of 3  $\mu\text{M}$ , 1  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 0.3  $\mu\text{M}$ , 0.1  $\mu\text{M}$ , 0.03  $\mu\text{M}$ , and 0.01  $\mu\text{M}$  when 2  $\mu\text{l}$  was added to 250  $\mu\text{l}$  of PRP.

**Preparation of agonist stock solutions for platelet aggregation studies.** Working stock solutions of a thrombin receptor activating peptide (TRAP; SFLLRN) of 200  $\mu\text{M}$  and ADP of 200  $\mu\text{M}$  and 50  $\mu\text{M}$  were prepared with 0.9% sterile saline and final concentrations 20 and 5  $\mu\text{M}$  were achieved by adding 25  $\mu\text{l}$  to 225  $\mu\text{l}$  PRP. The arachidonic acid stock was made by dissolving 5 mg in 1 ml of water.

**Preparation of aspirin-treated PRP.** An initial 1 M aspirin stock solution was made by dissolving acetyl salicylic acid in water and a 75 mM working stock solution was made by diluting the initial stock solution with water. The final concentration of aspirin used in the study (0.83 mM) was achieved by adding 2.5  $\mu\text{l}$  of 75 mM aspirin to 225  $\mu\text{l}$  of PRP for 10 minutes, followed by incubation with RUC-4 at the indicated concentrations for 20 minutes before initiating platelet aggregation with either arachidonic acid (1.5 mM) or ADP (5  $\mu\text{M}$ ).

**Flow cytometry studies with whole blood utilizing monoclonal antibody (mAb) PAC-1 to assess the percentage of receptors occupied by RUC-4.** PAC-1 is an IgM mouse mAb that selectively binds to activated  $\alpha\text{IIb}\beta_3$  in the same region occupied by RUC-4 [30]. As a result, PAC-1 binding to activated platelets identifies the receptors that are not already bound by RUC-4, providing information on the percentage of receptors occupied by RUC-4. In the assay, 25  $\mu\text{l}$  of whole blood from five separate donors (three female and two male) was incubated for 5 minutes at room temperature with 12.5  $\mu\text{l}$  of FITC-labeled 25  $\mu\text{g}/\text{ml}$  PAC-1 (final concentration: 6.25  $\mu\text{g}/\text{ml}$ ), either 0.4  $\mu\text{l}$  of 1 mM PGE-1 (negative control) or 0.4  $\mu\text{l}$  of varying concentrations of RUC-4 (final concentrations 3  $\mu\text{M}$ , 1  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 0.3  $\mu\text{M}$ , 0.1  $\mu\text{M}$ , 0.03  $\mu\text{M}$  and 0.01  $\mu\text{M}$ ), and 27  $\mu\text{l}$  of 1X HBMT with 1 mM  $\text{Mg}^{2+}$ . Five microliters of 200  $\mu\text{M}$  ADP (final concentration 20  $\mu\text{M}$ ) was added to the above mixture and incubated for 30 minutes at room temperature. Four hundred and fifty microliters of 1X HBMT with 1 mM  $\text{Mg}^{2+}$  was added to make up the final volume to 500  $\mu\text{l}$  and flow cytometry was conducted immediately at room temperature using the FACSCalibur (Becton Dickinson, BD).

Flow cytometry data were analyzed using BD CellQuest™ Pro Analysis software. Data were expressed as both the geometric mean fluorescence and the percentage of the mean geometric fluorescence obtained in the absence of RUC-4.

**Data analysis.** Platelet aggregation responses and RUC-4  $\text{IC}_{50}$  determinations were quantified as described previously for the NHP studies.

## Results

### Studies in NHPs

**Determination of RUC-4  $\text{IC}_{50}$  for platelet aggregation in NHP.** The RUC-4  $\text{IC}_{50}$  values for the 4 NHPs tested ranged from 499 to 1060 nM, with a mean  $\pm$  SD of  $807 \pm 231$  nM.

**Study comparing the effects of IM and SC RUC-4 in NHPs.**

**Clinical assessments.** IM administration: All animals exhibited some minor bruising and red, raised areas limited to site of injection (thigh) or venipuncture (left and right inguinal areas) shortly after treatment that resolved over time. In addition, minor bruising was seen at sites on the animal where manual restraint was performed (i.e., shoulders and arms). The clinical observations were similar at all doses of RUC-4 tested. On Day 2, clinical observations were for the most part limited to areas of venipuncture with blood sampling, and all animals were considered healthy at the end of the observation period.

**Table 2.** Whole blood levels ( $\mu\text{M}$ ), half-lives, and bioavailability of RUC-4 in non-human primates after IM and SC administration

Time (minutes)	Dose 3.86 mg/kg		Dose 1.93 mg/kg		Dose 1.00 mg/kg	
	IM	SC	IM	SC	IM	SC
5	16.009	6.036	3.796	3.445	3.415	0.746
15	8.783	5.310	3.692	3.056	2.118	0.819
30 <sup>a</sup>	0.837 <sup>b</sup>	3.523	0.297 <sup>b</sup>	1.295	0.164 <sup>b</sup>	0.497
45	0.952	1.098	0.333	0.551	0.236	0.241
120 <sup>a</sup>	0.016 <sup>b</sup>	0.225	0.008 <sup>b</sup>	0.104	<0.008 <sup>c</sup>	0.077
270 <sup>a</sup>	<0.007 <sup>c</sup>		<0.007 <sup>c</sup>		<0.007 <sup>c</sup>	
T <sub>1/2</sub> (hour)	0.53	0.39	0.28	0.41	0.28	0.56
Bioavailability	0.88	0.60	0.80	0.78	1.2	0.55

IM, intramuscular; SC, subcutaneous.

<sup>a</sup>Time points in which platelet aggregation studies were performed on animals treated with RUC-4 IM.

<sup>b</sup>Values of whole blood RUC-4 levels corresponding to an average of >80% inhibition of the initial slope of platelet aggregation in the two animals tested.

<sup>c</sup>Values of whole blood RUC-4 levels corresponding to an average of 0%–80% inhibition of the initial slope of platelet aggregation in the two animals tested.

**IV administration.** Animals exhibited similar clinical observations as those seen after IM administration, but one animal in arm 2 and both animals in arm 3 of the study vomited immediately after the IV injection of 3.86 mg/kg. In addition, one animal given 1.93 mg/kg in arm 2 had minor nose and gingival bleeding. On Day 2, clinical observations of bruising were noted as minor or moderate, and limited for the most part to sites of venipuncture and manual restraint.

**SC administration.** Clinical findings included red discoloration of the skin at the venipuncture and administration sites and bruising at sites of injection and inguinal areas. These clinical signs resolved by ~24 hours post-dose.

All animals were found to be healthy by the examining veterinarian prior to return of the animals to the colony at the end of all arms of the study.

**PK and PD data.** The whole blood levels ( $\mu\text{M}$ ), T<sub>1/2</sub> values, bioavailability, and impact on platelet aggregation of RUC-4 in NHPs after IM and SC administration are given in Table 2 and Fig. 1. Both IM and SC RUC-4 reached dose-dependent peak levels within 5 minutes, with the exception of the 1.0 mg/kg SC dose, which peaked at 15 minutes. The C<sub>max</sub> after IM dosing was higher than after SC dosing, but all of the SC values between 30 and 120 minutes were higher than the IM values. The calculated T<sub>1/2</sub> values were between 0.28 and 0.56 hours. The bioavailability of RUC-4 IM and SC compared to RUC-4 administered IV ranged between 0.60 and 0.88 at doses of 1.93 and 3.86 mg/kg, but there was greater variability in bioavailability at 1 mg/kg (1.2 IM and 0.55 SC). Platelet aggregation studies demonstrated >80% inhibition of the initial slope of aggregation in response to ADP in all samples in which the whole blood RUC-4 concentration was  $\geq$  ~8 nM, with partial inhibition at lower concentrations.

**Clinical pathology.** The hematocrits and platelet counts of the animals before and after treatment with RUC-4 in arms 1, 2, and 5 are shown in Table 3. The reductions in hematocrit were considered consistent with the blood drawing from the animals. The platelet counts varied, but did not demonstrate obvious drug-related trends by dose or time after administration with the possible exception of a decrease in the count in response to the 1.93 mg/kg IM RUC-4 dose in animal 4. Repeat RUC-4 doses of 1.93 mg/kg in this animal IV and SC in subsequent studies did not, however, elicit a similar response.

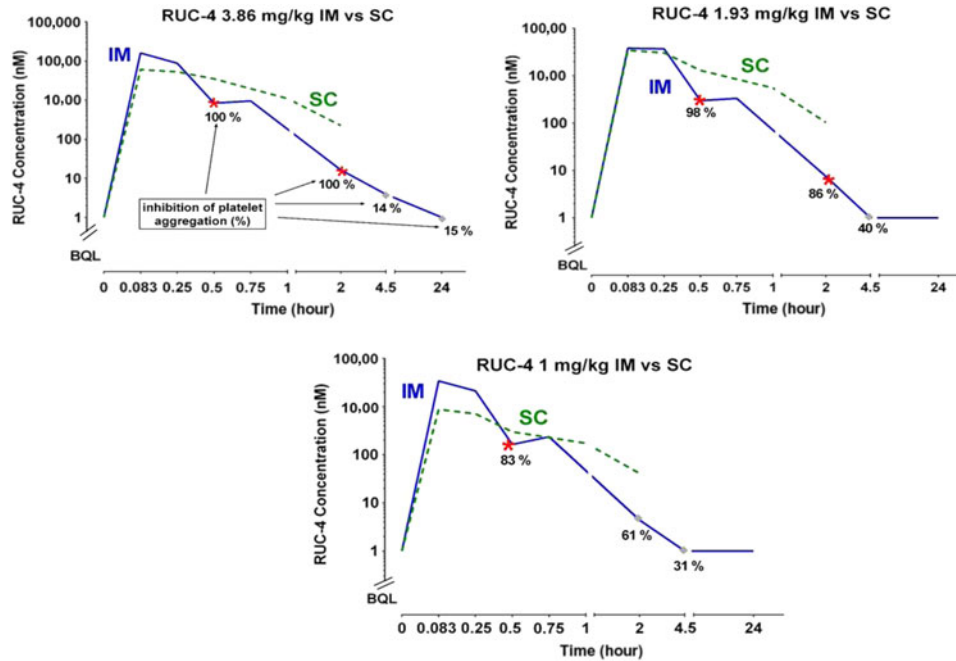
### In Vitro Platelet Aggregation Studies in Healthy Human Volunteers.

**Effect of aspirin on the IC<sub>50</sub> of RUC-4.** The IC<sub>50</sub> of untreated citrated PRP using 1.5 mM arachidonic acid was 61 ± 1 nM (Table 4). Aspirin eliminated arachidonic-acid-induced platelet aggregation, confirming its inhibition of thromboxane production.

The IC<sub>50</sub> of RUC-4 for ADP-induced aggregation of untreated platelets was 40 ± 9 nM, which was similar to the IC<sub>50</sub> for aspirin-treated platelets (37 ± 5 nM; *p* = 0.39).

**Effects of different anticoagulants on RUC-4 IC<sub>50</sub>.** The IC<sub>50</sub>s for RUC-4 in response to 20  $\mu\text{M}$  TRAP activation were similar for PRP prepared from whole blood anticoagulated with heparin (15.8 U/ml) or PPACK (0.3 mM), 111 ± 7 and 122 ± 17 nM, respectively (*p* = 0.16; *n* = 4). In contrast, with PRP prepared from blood anticoagulated with sodium citrate (final concentration 0.38%), the IC<sub>50</sub> was significantly lower, 66 ± 25 nM (*p* = 0.04 vs heparin and 0.05 vs PPACK; *n* = 4). Another series of experiments compared the RUC-4 IC<sub>50</sub>s in response to ADP activation (20  $\mu\text{M}$ ) of PRP prepared from blood anticoagulated with either sodium citrate (0.38%) or PPACK (0.1 mM). Once again, the IC<sub>50</sub> was significantly higher in the PRP prepared from the PPACK-anticoagulated blood (102 ± 22 nM) compared to PRP from the citrate-anticoagulated blood (54 ± 13 nM; *p* < 0.001; *n* = 5).

**Experiments to assess the percentage of unoccupied  $\alpha\text{IIb}\beta\text{3}$  receptors when platelets are treated with different doses of RUC-4.** To assess the percentage of  $\alpha\text{IIb}\beta\text{3}$  receptors that are not occupied by RUC-4 at different doses, we: (1) added FITC-labeled mAb PAC-1 (which selectively reacts with activated  $\alpha\text{IIb}\beta\text{3}$  receptors) to PRP samples prepared from blood anticoagulated with PPACK (0.1 mM); (2) incubated the PRP with varying doses of RUC-4; (3) activated the receptor with ADP (20  $\mu\text{M}$ ); and (4) analyzed PAC-1 binding by flow cytometry. In parallel studies with the same PRP samples, we assessed the effect of the same concentrations of RUC-4 on platelet aggregation in response to ADP (20  $\mu\text{M}$ ) activation. The correlation between the inhibition of ADP-induced platelet aggregation and inhibition of PAC-1 binding for the five separate donors is shown in Fig. 2. With each donor, inhibition of platelet aggregation was discernible when there was ~40% or more inhibition of PAC-1 binding, presumably reflecting occupancy by RUC-4 of ~40% of the  $\alpha\text{IIb}\beta\text{3}$  receptors. The inhibition



**Fig. 1.** RUC-4 whole blood concentrations at various times after IM and SC injections, along with inhibition of ADP-induced platelet aggregation data after IM injection in NHPs. A total of six NHPs were studied. At each dose, the two animals in each group received the RUC-4 by the IM route, and after a washout period, the same dose by the SC route. For PK studies, blood was obtained pre-dose and at the indicated time points. The RUC-4 blood levels reported are the averages of the blood levels of the two animals obtained at that time point. Platelet aggregation in response to 5  $\mu$ M ADP was studied in animals receiving RUC-4 IM in duplicate at the indicated time points and the duplicate values averaged. In some cases, duplicate samples were not able to be obtained (pre-treatment of one animal receiving 3.86 mg/kg and one receiving 1.93 mg/kg, one animal at 2 hours receiving 1.93 mg/kg, and one at 4.5 hours receiving 1.93 mg/kg) and so the single value was used. The values for the average percentage inhibition of the initial slope of aggregation compared to the control value in the two animals studied are included on the graphs at the indicated time points. Platelet aggregation was only performed 24 hours after receiving RUC-4 in animals receiving 3.86 mg/kg. The time points when platelet aggregation inhibition equaled or exceeded 80% are indicated with a red asterisk.

of platelet aggregation was then an approximately linear function of inhibition of PAC-1 binding, reaching nearly complete inhibition of platelet aggregation when the inhibition of PAC-1 binding reached between 80% and 90%. The average correlation between inhibition of platelet aggregation and inhibition of PAC-1 binding was 0.96 ( $p < 0.0001$ ).

## Discussion

Early administration of  $\alpha$ IIB $\beta$  antagonists, the most potent antiplatelet agents approved for human use, after STEMI symptom onset as a prelude to PCI improves both mortality and surrogate indicators of cardiac damage, including pre-PCI blood flow in the target vessel, time to ST segment resolution, left-ventricular ejection fraction, and distal embolization [5–7,31]. The time to treatment is especially important as indicated by De Luca *et al.*'s finding that the cumulative mortality at approximately 11 years after STEMI in 814 patients was ~30% for those treated with an  $\alpha$ IIB $\beta$  antagonist more than 178 minutes after symptom onset, ~16% for those treated between 101 and 178 minutes, ~10% in those treated between 65 and 110 minutes, and ~3% for those treated in less than 65 minutes (HR 1.41; 95% CI 1.02–2.21,  $p = 0.042$  after correction for baseline confounding factors) [6]. Further support for the importance of early therapy comes from a study of 10,730 patients with STEMI transported between 2015 and 2017 by emergency medical service personnel directly to a PCI-capable hospital in which 13% of the patients suffered a cardiac arrest or required intubation before the PCI was performed [32]. It is challenging, however, to administer the currently

available  $\alpha$ IIB $\beta$  antagonists at the first point of medical contact because they all require IV administration as a bolus and a continuous infusion, the latter controlled by an electric pump. In addition, the current small molecule agents eptifibatid and tirofiban induce the receptor to undergo a major conformational change that may contribute to the development of thrombocytopenia in a small percentage of patients [15]. The conformational change also results in the receptor developing high affinity for its ligands, which may limit the efficacy of the agents as their blood concentrations drop and they elute from the receptors [18]. The oral P2Y<sub>12</sub> antiplatelet agents, which are less potent than the  $\alpha$ IIB $\beta$  antagonists, require hours to achieve their full antiplatelet effects, and studies in which they have been administered to STEMI patients as soon as possible have been disappointing when compared to the studies of the  $\alpha$ IIB $\beta$  antagonists. [33,34] As a result, to achieve the antiplatelet benefits of the  $\alpha$ IIB $\beta$  antagonists without the potentially negative effects of inducing the receptor to undergo the conformational change and without the need for IV administration by bolus and infusion, we developed RUC-4. We initially considered IM administration, but because SC administration is easier to deliver in the emergency setting, we now have assessed the feasibility of SC administration of RUC-4.

In accord with our previous studies with RUC-4-free base [9], IM RUC-4 achieves peak blood levels in NHP within ~5 minutes, resulting in >80% inhibition of platelet aggregation that persists for several hours at a dose of 3.86 mg/kg. SC RUC-4 also achieves peak blood levels within ~5 minutes; while the peak concentrations are lower than those with IM RUC-4 at the same doses, the blood levels are more sustained than with IM administration. Thus, SC

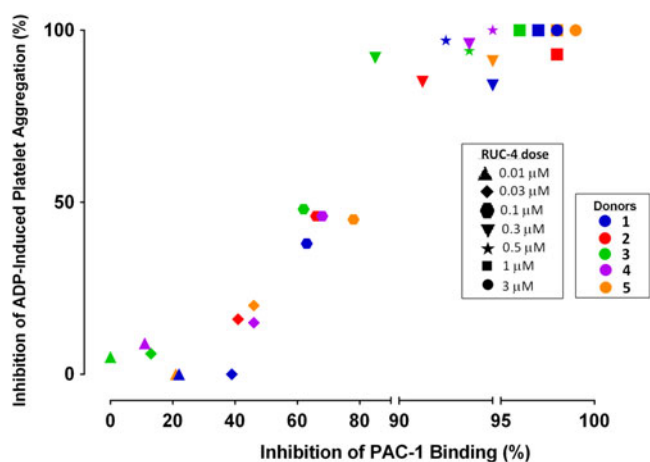
**Table 3.** Hematocrit and platelet count values before and at timed intervals after administering RUC-4 IM (A), IV (B), and SC (C)

A. IM administration						
Platelet counts ( $\times 10^{-3}/\mu\text{l}$ )						
Dose (mg/kg)	3.86		1.93		1.0	
	Animal					
Time (hours)	1	2	3	4	5	6
0	319	345	424	287	104	426
0.5	337	351	409	271	428	401
2	331	330	416	166	378	387
4.5	306	329	373	120	427	381
~27	275	264				
Hematocrits (%)						
Dose (mg/kg)	3.86		1.93		1.0	
	Animal					
Time (hours)	1	2	3	4	5	6
0	44.0	42.9	38.3	42.7	41.5	44.1
0.5	42.8	41.3	37.2	40.8	40.9	43.9
2	41.7	40.7	35.6	40.7	42.1	41.6
4.5	39.1	39.6	33.6	38.7	41.6	40.2
~27	37.5	36.2				
B. IV administration						
Platelet counts ( $\times 10^{-3}/\mu\text{l}$ )						
Dose (mg/kg)	3.86		1.93		1.0	
	Animal					
Time (hours)	1	2	3	4	5	6
0	358	329	452	439	490	118
0.5	237	422	224	240	305	511
2	338	322	461	426	345	434
4.5	435	257	244	163	553	524
Hematocrits (%)						
Dose (mg/kg)	3.86		1.93		1.0	
	Animal					
Time (hours)	1	2	3	4	5	6
0	41.7	39.4	36.1	33.6	42.9	39.5
0.5	43.8	41.2	40.7	40.9	45.6	39.6
2	40.1	38.4	35.1	33.4	40.1	40.3
4.5	41.8	39.1	40.4	39.5	42.3	37.8
C. SC Administration						
Platelet counts ( $\times 10^{-3}/\mu\text{l}$ )						
Dose (mg/kg)	3.86		1.93		1.0	
	Animal					
Time (hours)	1	2	3	4	5	6
0	411	276	390	251	411	362
2	326	410	388	268	451	406
Hematocrits (%)						
Dose (mg/kg)	3.86		1.93		1.0	
	Animal					
Time (hours)	1	2	3	4	5	6
0	41.8	43.6	39.2	41.3	42.6	50.3
2	41.2	38.8	34.5	39.3	42.3	45.4

IM, intramuscular; SC, subcutaneous; IV, intravenous

**Table 4.** RUC-4  $IC_{50}$  values ( $\mu M$ ) for untreated platelets activated with arachidonic acid (1.5 mM) and untreated or aspirin-treated platelets activated with ADP (5  $\mu M$ )

Patient	Arachidonic acid (1.5 mM)		ADP (5 $\mu M$ )	
	Vehicle	Aspirin (0.83 mM)	Vehicle	Aspirin (0.83 mM)
1	0.067	0.041	0.052	0.041
2	0.052	0.033	0.038	0.033
3	0.041	0.032	0.034	0.032
4	0.072	0.043	0.047	0.043
5	0.071	0.037	0.031	0.037
Mean	0.061	0.037	0.040	0.037
SD	0.014	0.005	0.009	0.005



**Fig. 2.** Correlation between inhibition of platelet aggregation (%) and inhibition of PAC-1 binding (%) at different RUC-4 doses in samples from five volunteers.

Samples were collected from three female and two male donors. Blood was anticoagulated with PPACK (0.1 mM) and platelet aggregation was performed on platelet-rich plasma activated with 20  $\mu M$  ADP; binding of fluorescently labeled mAb PAC-1, specific for activated  $\alpha IIb\beta 3$ , to platelets was measured in parallel on the same samples by flow cytometry of whole blood after activation with 20  $\mu M$  ADP. In both cases, RUC-4 at doses between 0 and 3  $\mu M$  ADP was added before activation. All of the individual data points are reported and coded by donor and dose of RUC-4.

administration at 1 mg/kg produced for at least 2 hours whole blood levels exceeding those required for  $\geq 80\%$  inhibition of the initial slope of ADP-induced platelet aggregation after IM administration. These PK and PD data of SC RUC-4 are consistent with our goal of achieving rapid onset, short-term, high-potency inhibition of platelet aggregation as part of first point of care treatment of STEMI.

The inhibition of platelet aggregation correlated in general with the whole blood concentrations of RUC-4, but based on the NHP  $IC_{50}$  value, the extent of inhibition of aggregation was greater than expected at the lower levels of RUC-4 at the later time points. Potential explanations for this finding include incomplete measurement of RUC-4 by the bioanalytical assay, incomplete dissociation of RUC-4 from platelets by the lysing solution, or the generation of an RUC-4 metabolite that retains antiplatelet activity. Additional studies will be required to assess the contribution of each of these possibilities. In addition, since the  $IC_{50}$  value was obtained by adding RUC-4 to PRP, whereas the PK analysis is of whole blood, assuming a hematocrit of  $\sim 40\%$ , the effective

plasma concentration is 67% higher than the measured whole blood concentration.

Whereas aspirin is considered the standard of care for myocardial infarction, we plan to treat patients with STEMI with RUC-4 in combination with aspirin. To assess any pharmacologic drug–drug interactions between these agents, we assessed whether pretreating human platelets with aspirin would have an impact on RUC-4's  $IC_{50}$  for inhibition of platelet aggregation. We found that treating platelets with aspirin at 0.83 mM, which was sufficient to eliminate platelet aggregation induced by arachidonic acid, did not materially affect the RUC-4  $IC_{50}$ . Future studies will need to address the potential for drug–drug PK and bleeding tendency and severity interactions between aspirin and RUC-4.

Previous studies demonstrated that the platelet inhibitory effects of  $\alpha IIb\beta 3$  antagonists differed when assessed with blood anticoagulated with citrate, which chelates divalent cations, including calcium and magnesium, and anticoagulants that do not chelate divalent cations, including heparin and PPACK [25–28]. In each case, the observed  $IC_{50}$  was lower with citrate-anticoagulated blood than with blood anticoagulated with non-chelating anticoagulants, although the agents varied considerably in the effect of citrate. As a result, we compared the RUC-4  $IC_{50}$  in PRP prepared from blood anticoagulated with citrate, heparin, and PPACK. We also observed a lower  $IC_{50}$  in PRP prepared from blood anticoagulated with citrate, with the difference being approximately twofold. Previous investigators emphasized the importance of calcium chelation by citrate, but magnesium is also chelated by citrate [35] and magnesium occupies the MIDAS in  $\beta 3$  that is vital for ligand binding [36]. Moreover, RUC-4, like RUC-2, displaces the magnesium ion from the MIDAS [9,13]. Thus, it is possible that the mechanism by which citrate sensitizes platelets to RUC-4 involved chelation of magnesium. Selecting the proper anticoagulant for studies of RUC-4's antiplatelet effect in humans is very important since early studies of eptifibatide's antiplatelet effects using citrate anticoagulant led to the selection of dosing regimens later found to be inadequate to sustain its antiplatelet effect when the studies were performed using PPACK as the anticoagulant [37].

We also studied the effect of RUC-4 on mAb PAC-1 binding as a surrogate indicator of receptor occupancy by RUC-4. The results were similar to those we previously obtained with the mAb 7E3, the parent mAb of abciximab, and mAb 10E5. [38–40] We were able to measure receptor occupancy directly with those antibodies by measuring the inhibition of the binding of radiolabeled antibody. The data are also similar to those observed with eptifibatide, where receptor occupancy was measured indirectly by the binding of an activation-dependent mAb [37]. Since we do not have radiolabeled RUC-4, we analyzed the ability of PAC-1 to bind to unoccupied receptors after activation by ADP. In particular, we found that inhibition of platelet aggregation begins when more than 40% of the receptors are blocked and that nearly complete inhibition of platelet aggregation requires  $\sim 80\%$  or greater inhibition. The antithrombotic effect of mAb 7E3 was apparent at  $\sim 70\%$  receptor blockade with less stringent thrombotic models and greater than  $\sim 80\%$  inhibition with more stringent models [39–41].

We conclude that RUC-4's PK and PD in NHPs after SC administration support its potential as a potent, rapidly-acting, short-term antiplatelet therapy for first-point-of-medical-contact therapy of STEMI. In addition, aspirin does not affect RUC-4's  $IC_{50}$ , so combined use of the two agents for STEMI does not have to take into consideration a functional drug–drug interaction. Inhibition of platelet aggregation by RUC-4 is mildly enhanced by citrate anticoagulation and so monitoring of RUC-4's



antiplatelet effect should be conducted with blood anticoagulated with PPACK or another non-chelating anticoagulant. Finally, the relationship between the percentage of receptors blocked by RUC-4 and inhibition of platelet aggregation is very similar to that observed with other antiplatelet agents that target  $\alpha$ IIb $\beta$ 3. Based on these results, RUC-4 has now progressed to evaluation in separate toxicology and PK studies in mice and similar studies in NHPs in the presence and absence of aspirin [42,43].

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