Conclusions—Early administration of these agents to patients having STEMI is associated with improved outcomes, but this strategy has not been adopted widely because of the difficulty of administering the drugs in the prehospital period by Emergency Medical Service personnel. Attempts to develop oral αIIbβ3 antagonists that might be more easily administered failed in trials of chronic therapy because of lack of efficacy, an increased risk of death with some agents, an increased risk of bleeding, and infrequent thrombocytopenia. It has been proposed that the thrombocytopenia associated with these agents is caused, in part, by their inducing the receptor to undergo a major conformational change that exposes neoepitopes to which some patients have preformed antibodies. In fact, 2 of the oral agents associated with increased mortality, xemilofiban and orbofiban, were reported to expose a ligand-induced binding site epitope on the β3 subunit, but variable results have been reported with other ligand-induced binding site antibodies and other αIIbβ3 antagonists. Similarly, the paradoxical increase in mortality has been proposed to result from their inducing the receptor to adopt the high-affinity ligand-binding conformation, thereby priming the receptor to bind ligand when the drug dissociates.
from the receptor.\textsuperscript{14,15,17,20–22} However, priming by αIIbβ3 antagonists has only been reported with purified receptor or when platelets are fixed in the presence of the αIIbβ3 antagonist and then the antagonist is washed away.\textsuperscript{23–26} An alternative explanation for the paradoxical increase in mortality with the oral agents is the increased bleeding associated with these drugs,\textsuperscript{26,27} which likely reflects their narrow therapeutic window, because such events commonly lead to cessation of antiplatelet therapy. Moreover, oral agents are problematic when administered early to patients with STEMI because absorption is poor and erratic. In fact, there are data with all of the approved oral P2Y\textsubscript{12} antagonists demonstrating marked delays in the onset of action, even with high loading doses.\textsuperscript{28–30} Thus, intramuscular administration is preferable because it assures absorption without the technical challenges associated with intravenous administration under emergency conditions in the field.\textsuperscript{31}

We recently described a novel αIIbβ3 antagonist termed RUC-2, a derivative of a smaller compound (RUC-1) identified in a high throughput screen.\textsuperscript{19,32} RUC-1 and RUC-2 lack a carboxyl group analogous to the carboxyl group in the ligand Asp and in the αIIbβ3 antagonists that coordinates the Mg\textsuperscript{2+} ion in the β3 subunit’s metal ion-dependent adhesion site.\textsuperscript{32,33} Interactions between the ligand (or antagonist) carboxyl group and the backbone nitrogens in the β1-α1 loop of β3 result in the movement of that loop toward the metal ion-dependent adhesion site, initiating the dramatic swing-out motion of the β3 hybrid domain that leads to the receptor adopting a high-affinity ligand-binding conformation.\textsuperscript{34} In support of this hypothesis, neither RUC-1 nor RUC-2 induced the reorganization of divalent cations in the β3 ligand-binding pocket nor did they induce conformational changes in β3 detectable by a conformation-specific monoclonal antibody or by electron microscopy.\textsuperscript{19,32,33} Moreover, unlike eptifibatide and tirofiban, neither RUC-1 nor RUC-2 primed the receptor to bind the ligand fibrinogen.\textsuperscript{19,32,33} RUC-2 is ≈100-fold more potent than RUC-1 (IC\textsubscript{50}\textsuperscript{3} of ≈90 nmol/L and 13 μmol/L, respectively) and has a unique mechanism of action, with x-ray crystallography demonstrating that its amine group competes with the metal ion-dependent adhesion site Mg\textsuperscript{2+} for binding to the carboxyl of β3 Glu220, thus displacing the Mg\textsuperscript{2+} and locking the receptor in the inactive conformation.\textsuperscript{32} As detailed in this article, RUC-2 has potent antithrombotic effects in an animal model and favorable pharmacokinetics and pharmacodynamics for use in the prehospital setting, but it has limited solubility, essentially precluding it from being able to be delivered intramuscularly by autoinjector under emergency conditions. As a result, we synthesized congeners of RUC-2 and identified RUC-4, which is slightly more potent than RUC-2 and >500-fold more soluble.\textsuperscript{34} We now report on RUC-4’s properties with regard to specificity, priming, and ability to induce conformational changes in the β3 subunit. We also provide data on its mechanism of action, pharmacokinetics, and pharmacodynamics in mice and nonhuman primates and antithrombotic properties in mouse models that use both mouse and human platelets. We conclude that RUC-4 has favorable properties for further development for prehospital intramuscular therapy of STEMI by autoinjector.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Comparison of the Properties of RUC-2 and RUC-4

The structures and properties of RUC-2 and RUC-4 are presented in Figure 1 and Table. RUC-4 differs from RUC-2 in being ≈20% more potent as judged by its IC\textsubscript{50} for 5 μmol/L ADP-induced platelet aggregation using citrated platelet-rich plasma and, most notably, being >500-fold more soluble in aqueous buffer at neutral pH. Because some αIIbβ3 antagonists are more potent when assayed in citrated platelet-rich plasma compared with anticoagulants that do not chelate divalent cations,\textsuperscript{35,36} we compared the inhibition of ADP-induced platelet aggregation in blood anticoagulated with citrate and blood anticoagulated with bivalirudin. The IC\textsubscript{50} were 85±22 nmol/L and 100±23 nmol/L (n=4; \(P=0.24\)), respectively, indicating a small effect of citrate.

Given their similarity in IC\textsubscript{50}, we went on to assess RUC-4’s specificity, ability to induce conformational changes in the β3 subunit, and ability to prime αIIbβ3 to bind the ligand fibrinogen. We used concentrations of each agent expected to inhibit completely platelet aggregation induced by ADP. RUC-4, like RUC-2, demonstrated specificity for αIIbβ3 relative to αVβ3 as shown by its inability to inhibit the binding of cells expressing αVβ3 to vitronectin while inhibiting the binding of cells expressing αIIbβ3 to fibrinogen (Figure 2). Similarly, like RUC-2, but unlike eptifibatide, RUC-4 did not induce increased binding of mAb AP5, which recognizes a calcium sensitive ligand-induced binding site epitope in the β3 PSI domain (Figure 3). Moreover, RUC-4, like RUC-2, but unlike eptifibatide and an RGD-containing peptide (RGDS), did not prime αIIbβ3 to bind fibrinogen (Figure 4). In the latter studies, we could not directly test that RUC-4 was removed from the receptor by the washing procedures, but we inferred that it was removed because eptifibatide, which has >4-fold higher affinity for αIIbβ3 than RUC-4 as judged by its IC\textsubscript{50} for platelet aggregation,\textsuperscript{32} did prime the receptor to bind fibrinogen under the same experimental conditions.

Molecular Dynamics Simulation Studies of RUC-4’s Mechanism of Action

The structural basis of RUC-4’s higher potency compared with RUC-2 was evaluated by molecular dynamics simulations.
Detailed analysis of representative snapshots from the 50 ns simulation trajectories of RUC-2-αIIb3 (Figure 5A) and RUC-4-αIIb3 (Figure 5B) showed that RUC-4 binds to the protein the way RUC-2 does. Stable interactions that were identified during the simulations of both the RUC-2 and RUC-4 complexes (Figure I in the online-only Data Supplement) included (1) a direct hydrogen bond between the piperazine nitrogen and one of the oxygens of the side chain carboxyl group of αIIb D224, (2) a direct hydrogen bond between the primary amine and one of the oxygens of the side chain carboxyl group of β3 E220, as well as with the backbone carboxyl oxygen of β3 A218, (3) a direct hydrogen bond between the phenylacetamide nitrogen with the backbone carbonyl oxygen of β3 N215, (4) a π-π stacking interaction between RUC-4’s fused ring and the αIIb Y190 aromatic ring, and (5)

Table. Comparison of RUC-2 and RUC-4

<table>
<thead>
<tr>
<th></th>
<th>RUC-2</th>
<th>RUC-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>385</td>
<td>386</td>
</tr>
<tr>
<td>IC_{50} ADP-induced platelet aggregation, nmol/L</td>
<td>95±20</td>
<td>57±10*</td>
</tr>
<tr>
<td>Solubility in aqueous buffer at pH 7.4, mg/mL</td>
<td>0.068–0.092</td>
<td>60–80</td>
</tr>
<tr>
<td>Reactivity with murine platelets</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Specificity for αIIb3 vs αV3</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Ability to prime αIIb3 to bind fibrinogen</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Ability to induce conformational changes in αIIb3 identifiable by LIBS mAb</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

LIBS indicates ligand-induced binding site.

* n=8; P<0.001.

Figure 3. The effects of RUC-2 and RUC-4 on the binding of the ligand-induced binding site mAb AP5. Platelets from blood collected into acid-citrate dextrose were washed with HEPES-modified Tyrode’s (HMBT) buffer containing 1 μmol/L PGE1 and resuspended to 2.5×10^9 platelets/μL in HMBT containing 1 mmol/L MgCl2 and 2 mmol/L CaCl2. After adding buffer (control; C), EDTA (10 mmol/L), eptifibatide (Epti; 10 μmol/L), RUC-2 (100 μmol/L), or RUC-4 (100 μmol/L), the platelets were incubated for 15 minutes. mAb AP5, labeled with Alexa488, was added and incubated for another 30 minutes. Samples were then diluted, washed, and analyzed by flow cytometry. Data shown are mean±SD for 4 separate analyses.

Figure 4. The effects of RUC-2 and RUC-4 on the binding of αIIb3 to fibrinogen. Platelets were washed as per the studies on AP5 binding and resuspended at 1×10^9/μL in HEPES-modified Tyrode’s (HMBT) buffer containing 1 mmol/L MgCl2 and 2 mmol/L CaCl2. After adding buffer (control; Con), EDTA (10 mmol/L), eptifibatide (1 μmol/L), RGDS peptide (100 μmol/L), RUC-2 (100 μmol/L), or RUC-4 (300 μmol/L), the platelets were incubated for 20 minutes at room temperature and then fixed with equal volume of 2% paraformaldehyde in PBS, pH 7.4, for 40 minutes. The paraformaldehyde was quenched with glycine (5 mmol/L) in PBS and then washed X3 with HMBT buffer and resuspended in HMBT with 1 mmol/L MgCl2 and 2 mmol/L CaCl2. Fibrinogen (200 μg/mL) labeled with Alexa488 was then added and after 30 minutes of incubation at room temperature, the platelets were washed and analyzed by flow cytometry. Data shown are mean±SD for 3 separate analyses.

Figure 2. The effects of RUC-2 and RUC-4 on the adhesion of cells expressing αIIbβ3 to fibrinogen and cells expressing αVβ3 to vitronectin. HEK293 cells expressing αIIbβ3 were incubated for 15 minutes at room temperature with buffer (control; Con), mAb 10E5 (anti-αIIbβ3; 40 μg/mL), EDTA (10 mmol/L), mAb LM609 (20 μg/mL), RUC-2 (100 μmol/L), or RUC-4 (300 μmol/L) and then 1000000 were added to microtiter wells precoated with fibrinogen at 50 μg/mL in HEPES-modified Tyrode’s buffer containing 1 mmol/L MgCl2 and 2 mmol/L CaCl2. After 1 hour at 37°C, the wells were washed and the adherent platelets quantified by detecting their acid phosphatase activity. Similarly, HEK293 cells expressing αVβ3 were added to wells precoated with vitronectin (5 μg/mL) in buffer containing 1 mmol/L MgCl2 and adhesion quantified as indicated for cells expressing αIIbβ3. Data shown are mean±SD for 4 separate analyses.

Figure 1. The effects of RUC-2 and RUC-4 on the binding of fibrinogen complexes (Figure I in the online-only Data Supplement) included (1) a direct hydrogen bond between the piperazine nitrogen and one of the oxygens of the side chain carboxyl group of αIIb D224, (2) a direct hydrogen bond between the primary amine and one of the oxygens of the side chain carboxyl group of β3 E220, as well as with the backbone carboxyl oxygen of β3 A218, (3) a direct hydrogen bond between the phenylacetamide nitrogen with the backbone carbonyl oxygen of β3 N215, (4) a π-π stacking interaction between RUC-4’s fused ring and the αIIb Y190 aromatic ring, and (5)
We previously reported that RUC-2 at 1 μmol/L does not inhibit murine αIIbβ3 (32) and RUC-4 shares this property (data not shown). To assess the antiplatelet effects of RUC-2 and RUC-4, we therefore used the mice developed by Ponzc’s group that express human αIIbβ3 in combination with murine β3 (hαIIb/mβ3) because the αIIb subunit primarily determines the binding specificity of RUC-2 and RUC-4. These mice express 58±8% (mean±SD) of the amount of platelet αIIbβ3 expressed by wild-type mice (n=6; data not shown) and have mild to moderate thrombocytopenia (635±112×10^3 platelet/μL in the mice we studied in the reported experiments [n=26] compared with 1257±179×10^3 platelet/μL in a group of wild-type C57Bl/6 mice [n=21]). Our goal was to identify a dose of each agent that could completely inhibit platelet aggregation induced by 20 μmol/L ADP within 15 minutes of administration while allowing for at least partial return of platelet aggregation within 2 to 4 hours. RUC-2 administered at 0.39 mg/kg (0.1 mL) IP produced complete inhibition of platelet aggregation induced by 20 μmol/L ADP within 15 minutes, with return of the aggregation response beginning at 45 minutes (Figure 6A). Because the duration of inhibition was <2 to 4 hours we hoped to achieve, we treated another group of mice with RUC-2 at 3.85 mg/kg (0.3 mL) IP. Platelet aggregation in these mice was completely inhibited within 15 minutes, and the high-grade inhibition lasted for ≈2 hours, at which time the platelet aggregation response returned toward normal (Figure 6B). Because RUC-4 was more soluble than RUC-2, it could be administered intramuscularly in a smaller volume (0.05 mL). At 1.2 mg/kg, RUC-4 produced complete inhibition of platelet aggregation at 5 minutes, with partial return of aggregation at 4 hours (Figure 6C). A series of 7 mice that received saline instead of RUC-2 or RUC-4 showed variable partial reductions in the initial slope of platelet aggregation (47±26%) at different time points, but there was no temporal pattern, and none of them showed the complete inhibition of platelet aggregation consistently observed after receiving RUC-2 or RUC-4.

The plasma concentrations of RUC-2 in the same samples used for the platelet aggregation studies, along with the primary slopes of platelet aggregation, are provided in Table I in the online-only Data Supplement. With the exception of an outlier value in each series, the time to maximum plasma concentration was 15 minutes with RUC-2 and 5 minutes with RUC-4, perhaps reflecting more rapid absorption after intramuscular than intraperitoneal administration. The plasma levels of each agent dropped rapidly thereafter. The correlations between platelet aggregation and plasma concentrations comport well with RUC-4’s IC50 for hαIIb/mβ3 platelets (=0.01 μmol/L; data not shown, n=3).

### Pharmacokinetic and Pharmacodynamic Studies of RUC-2 and RUC-4 in Mice

We previously reported that RUC-2 at 1 μmol/L does not inhibit murine αIIbβ3, and RUC-4 shares this property (data not shown). To assess the antiplatelet effects of RUC-2 and RUC-4, we therefore used the mice developed by Ponzc’s group that express human αIIbβ3 in combination with murine β3 (hαIIb/mβ3) because the αIIb subunit primarily determines the binding specificity of RUC-2 and RUC-4. These mice express 58±8% (mean±SD) of the amount of platelet αIIbβ3 expressed by wild-type mice (n=6; data not shown) and have mild to moderate thrombocytopenia (635±112×10^3 platelet/μL in the mice we studied in the reported experiments [n=26] compared with 1257±179×10^3 platelet/μL in a group of wild-type C57Bl/6 mice [n=21]). Our goal was to identify a dose of each agent that could completely inhibit platelet aggregation induced by 20 μmol/L ADP within 15 minutes of administration while allowing for at least partial return of platelet aggregation within 2 to 4 hours. RUC-2 administered at 0.39 mg/kg (0.1 mL) IP produced complete inhibition of platelet aggregation induced by 20 μmol/L ADP within 15 minutes, with return of the aggregation response beginning at 45 minutes (Figure 6A). Because the duration of inhibition was <2 to 4 hours we hoped to achieve, we treated another group of mice with RUC-2 at 3.85 mg/kg (0.3 mL) IP. Platelet aggregation in these mice was completely inhibited within 15 minutes, and the high-grade inhibition lasted for ≈2 hours, at which time the platelet aggregation response returned toward normal (Figure 6B). Because RUC-4 was more soluble than RUC-2, it could be administered intramuscularly in a smaller volume (0.05 mL). At 1.2 mg/kg, RUC-4 produced complete inhibition of platelet aggregation at 5 minutes, with partial return of aggregation at 4 hours (Figure 6C). A series of 7 mice that received saline instead of RUC-2 or RUC-4 showed variable partial reductions in the initial slope of platelet aggregation (47±26%) at different time points, but there was no temporal pattern, and none of them showed the complete inhibition of platelet aggregation consistently observed after receiving RUC-2 or RUC-4.

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### Pharmacokinetic and Pharmacodynamic Studies of RUC-4 in Macaca Fascicularis

RUC-4 was administered intramuscularly in volumes ranging from 0.26 to 0.47 mL to 3 cynomolgus monkeys at doses of 3.86 mg/kg, 1.93 mg/kg, and 1.0 mg/kg (Figure 6D; Table II in the online-only Data Supplement). At the highest dose, aggregation was completely inhibited within 15 minutes and the high-grade inhibition lasted for >4.5 hours, but <24 hours; at the intermediate dose, inhibition was complete within 15 minutes and the aggregation response began to return to normal by 4.5 hours; at the lowest dose, inhibition of aggregation was partial at 15 minutes and complete at 30 minutes, with return toward normal aggregation evident at 2 hours. The injection of the vehicle control (0.45% NaCl) did not inhibit platelet aggregation of samples obtained at multiple time points (Figure 6D). The platelet counts in all 3 animals remained stable throughout the 24-hour period (Table III in the online-only Data Supplement).

Clinical evaluation of the animals revealed that RUC-4 was well tolerated, with little or no purpura at the sites of administration or blood drawing. Transient gum bleeding was noted in the animal receiving the 1.93 mg/kg dose on one occasion. All animals were judged by the veterinary staff to be clinically normal before being released back to the test facility.
Antithrombotic Effects of RUC-2 and RUC-4: FeCl₃ Murine Carotid Artery Model
The antithrombotic effects of RUC-2 and RUC-4 were assessed in hαIIbβ3 mice using the FeCl₃ carotid artery model. Mice treated with saline had platelet counts similar to those of mice treated with either RUC-2 (587±82 versus 677±132x10³ platelets per μL, respectively) or RUC-4 (707±204 versus 674±176x10³ platelets per μL, respectively). αIIbβ3 surface
expression, judged by the binding of the mAb 10E5, was also similar on saline-treated mice compared with those treated with RUC-2 (231±53 versus 249±10 arbitrary fluorescence units, respectively) or RUC-4 (141±43 versus 137±35 arbitrary units, respectively). RUC-2 at 3.85 mg/kg IP and RUC-4 at 1.2 mg/kg IM protected mice from vaso-occlusion; the protection was complete with RUC-2 (8/8) and incomplete with RUC-4, with 2 of 11 mice developing occlusion during the experiment (Figure 7).

Antithrombotic Effects of RUC-2 and RUC-4: Transgenic vWF Mouse With Infused Human Platelets

To assess the antithrombotic effect of RUC-4 on human platelets in a physiological relevant setting, we used a genetically modified murine model in which substituting His for Arg at position 1326 in the vWF A1 domain results in a decrease in the ability of murine platelets to form thrombi in response to laser injury in the cremasteric circulation while dramatically increasing in the ability of transfused human platelets to form thrombi.38 Intravital microscopy demonstrated that intravenous RUC-4 at 1.5 mg/kg resulted in a marked decrease in thrombus formation (>80%), comparable to the decrease found with the αIIbβ3 antagonist abciximab (P=0.15; Figure 8; Movie I and Table IV in the online-only Data Supplement).

Discussion

Despite universal agreement on the benefits of early treatment of myocardial infarction,39,40 administering an effective agent in the prehospital setting poses several challenges. The first is the ability of emergency medical service personnel to diagnose STEMI in the field based on clinical and electrocardiographic criteria. In the IMMEDIATE trial, improvements in training and the algorithms used to assess the field ECGs resulted in a relatively low rate of misdiagnosis, with 88.7% of the patients with ST segment elevation in the field ECG later demonstrating evidence of myocardial infarction.41

The second is the impact of cardiovascular instability on the patient’s ability to absorb oral medications. Studies of P2Y12 antagonists provide strong evidence that impaired gastrointestinal absorption during acute coronary syndromes results in marked, and variable, delays in the onset of the antiplatelet effect.28–30

The third is the need for a rapid and convenient method of administration. The current αIIbβ3 antagonists all require intravenous administration with ongoing infusion, which can be difficult to achieve under emergency conditions. For example, in a study comparing intravenous lorazepam with an intramuscular midazolam administered by autoinjector by emergency service personnel for the treatment of status epilepticus, 42 of 445 (9.4%) of patients did not receive the lorazepam because the personnel could not obtain intravenous access. In contrast, only 5 of 448 patients (1.1%) did not receive the intramuscular midazolam, and in each case it was because of autoinjector failure. The median time to drug administration was also shorter for the midazolam group (∼1 versus ∼5 minutes). In the IMMEDIATE trial of patients with acute coronary syndromes, the emergency medical service personnel could not obtain intravenous access in 51 of 1483 (3.4%) and in those with intravenous access, subsequent infusion pump failure occurred in 16 of 1087 (1.5%) patients.41
receiving hospital can introduce the therapy they think best, including surgery. Thus, an ideal antiplatelet agent for prehospital therapy of STEMI should (1) have the potency of an αIIbβ3 antagonist, the most potent of currently available agents; (2) be rapidly absorbed and achieve high-grade inhibition of platelet aggregation within minutes when administered intramuscularly; (3) have its antiplatelet effects begin to work off within several hours; and (4) possess sufficient solubility so that it can be administered in \( \approx 1.5 \) mL, the practical limit for autoinjector.

Our data demonstrate that RUC-4 fulfills these criteria, being a potent inhibitor of platelet aggregation and thrombus formation in several different models that correlate with the efficacy of known antiplatelet agents, including a nonprimate primate model and a transgenic mouse model that uses human platelets. Moreover, RUC-4, like RUC-2, has a unique mechanism of action that does not trigger the conformational changes in the receptor induced by other αIIbβ3 antagonists based on the R(K)GD sequence that prime the receptor to bind fibrinogen and perhaps expose neoepitopes that lead to thrombocytopenia.

Prehospital therapy with a potent antiplatelet agent raises important safety concerns, especially because the use of αIIbβ3 antagonists used during percutaneous coronary artery interventions is associated with increased risk of bleeding and bleeding is associated with adverse outcomes. Some of the bleeding associated with these agents can be ameliorated by proper dosing, especially in women and by using radial rather than femoral access. Fortunately, αIIbβ3 antagonists have been associated with a much lower frequency of intracranial hemorrhage relative to thrombolytic therapy, with data from 307,294 patients treated with αIIbβ3 antagonists in the United States between 2000 and 2002 showing a rate of 0.13%, some of which may have been unrelated to the therapy. Moreover, the reported bleeding complications associated with αIIbβ3 antagonists comes from studies in which patients received aspirin and an anticoagulant, whereas in the prehospital setting, patients will likely only receive aspirin and the αIIbβ3 antagonist. In addition, a sizable fraction of the bleeding associated with αIIbβ3 antagonists occurs at the percutaneous coronary interventions arterial access site, and because arterial access only occurs after hospitalization, it will not contribute to prehospital hemorrhagic risk. None-the-less, the possibility of increased bleeding associated with therapeutic doses of RUC-4 remains to be evaluated.

Finally, in addition to the potential short-term benefits of early potent antiplatelet therapy, there is emerging evidence that it may also decrease the longer term risk of congestive heart failure. This is important because in 2010, 14.2% of Medicare patients with myocardial infarction were hospitalized for congestive heart failure within 1 year, and among those hospitalized, 45.5% died within the next year. In a recent review, Goel et al concluded that every 1-hour delay in time to reperfusion is associated with an \( \approx 4\% \) to 12% increase in risk of new-onset congestive heart failure. It is important, therefore, to assess whether the rapid increase in cardiac blood flow associated with αIIbβ3 antagonist treatment of STEMI will translate into decreased morbidity and mortality from congestive heart failure.

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Disclosures

In accord with Federal law and the policies of the Research Foundation of the State University of New York, Mount Sinai School of Medicine, and Rockefeller University, respectively, B.S. Coller has royalty interests in abciximab (Centocor) and the VerifyNow assays (Accumetrics). B.S. Coller, M. Filizola, and C.J. Thomas have royalty interests in RUC compounds.

References

RUC-4: A Novel αIIbβ3 Antagonist for Prehospital Therapy of Myocardial Infarction
Jihong Li, Spandana Vootukuri, Yi Shang, Ana Negri, Jian-kang Jiang, Mark Nedelman, Thomas G. Diacovo, Marta Filizola, Craig J. Thomas and Barry S. Collier

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Materials and Methods

Synthesis of RUC-2 and RUC-4: RUC-2 was synthesized and purified as previously described. Their solubility, plasma stability, microsomal stability, and pharmacokinetics in mice have also been previously described.

Pharmacokinetic and pharmacodynamics studies in mice: These studies were approved by the Rockefeller University Institutional Animal Care and Use Committee. RUC-2 or RUC-4 was administered to mice expressing human αIIb in complex with mouse β3 (hαIIb/mβ3; a kind gift of Dr. Mortimer Poncz, Children’s Hospital of Philadelphia) at the indicated concentrations IV, intraperitonealy (IP), or intramuscularly (IM). The IM injections were delivered to the caudal thigh muscles (semitendinosus-semimembranosus) with a 27½ g needle, with the needle directed away from the femur and the sciatic nerve. RUC-2’s lower solubility than RUC-4 resulted in the need to administer it in a larger volume (0.3 vs 0.05 ml), and thus it was administered IP rather than IM. At timed intervals, 600 µl of blood was drawn percutaneously from the left ventricle under ultrasound guidance as previously described and anticoagulated with bivalirudin (0.2 mg/ml). Platelet-rich plasma was prepared and the platelet count was adjusted to 3.6 X 10^9/µl with platelet-poor plasma. Platelet aggregation was initiated with ADP (final concentration 20 µM) and monitored in a PAP-8E aggregometer (BioData Corporation) at 37°C with stirring. Residual plasma was spiked with an internal standard, extracted with methanol:acetone:acetonitrile (1:1:1; 4: vol/vol) for RUC-2 or methanol for RUC-4, and analyzed for RUC-2 or RUC-4 by LC/MS/MS using a C-18 column and a Thermo-Finnagan TSQ Vantage quad mass spectrometer.

Pharmacokinetic and pharmacodynamics studies in cynomolgus monkeys (Macaca fascicularis): These studies were conducted at the Charles River Lab (Montreal, Canada) and approved by its Institutional Animal Care and Use Committee. Three male monkeys weighing 4.0, 4.2, and 7.3 kg, respectively, were pre-anesthetized IM (23 g needle, right semitendinosus muscle) with glycopyrrolate (0.01 mg/kg)/ketamine (10 mg/kg)/xylazine (10 mg/kg), intubated, and anesthetized with isoflurane (1-2 %) and oxygen. Thereafter, RUC-4 was administered at doses of 3.86, 1.93, and 1.0 mg/kg IM by injecting 0.064 ml/kg of RUC-2 (stock solutions in 0.45% saline of 60, 30, and 15.6 mg/kg) into the left semitendinosus muscle using a 23 g needle. Blood was obtained prior to dosing and at the indicated times after RUC-4 administration by syringe with a 21 g needle from the femoral vein and anticoagulated with 0.1 volume 3.2% citrate for ADP (20µM)-induced platelet aggregation or EDTA for blood cell counts. After the final blood sample was obtained, animals were released to the test facility.

Thrombosis assays

Mouse carotid artery injury: These studies were approved by the Rockefeller University Institutional Animal Care and Use Committee. hαIIb/mβ3 mice were anesthetized with isoflurane, the carotid artery was exposed, and then the mice were injected with RUC-2 IP at a dose of 3.85 mg/kg, RUC-4 IM at 1.2 mg/kg, or saline (IP and IM, respectively). 20 minutes following administration of test article, filter paper soaked in 20% FeCl3 was applied to the artery for 3 minutes and then blood flow was monitored with an ultrasound flow probe (Transonics MA 0.5PSB). The time to total occlusion was monitored. All of the RUC-2 mice and their controls, and the first 5 RUC-4 mice and 4 RUC-4 controls were studied without blinding; the last 7 control and RUC-4-treated mice were studied with the operator blinded and the sequence of administration randomized (randomization.com).
Inhibition of human platelet thrombus formation after laser injury of mouse cremasteric arterioles: These studies were approved by the Columbia University Institutional Animal Care and Use Committee and performed in transgenic mice with a mutation in von Willebrand factor (vWF) designed to increase its interaction with human platelet GPIb and decrease its interaction with murine GPIb as previously described. Human platelets (700,000/µl) were continuously infused at 25 µl/min through a catheter placed in the ipsilateral femoral artery 2 min prior to throughout laser-induced injury (~15 min) to ensure a constant level of circulating human platelets at counts comparable to those in normal humans. A minimum of 5 arteriole segments were studied per mouse and 5 mice were studied with control or experimental agent.

For the αIIbβ3 inhibition studies, abciximab was first given as an IV bolus (0.025 mg/kg) and then as a continuous infusion (0.0125 µg/kg/min) as recommended for treatment of human MI. RUC-4 (1.5 mg/kg) was administered as an IV bolus. To compare mean thrombus areas between different treatment groups, we fit linear mixed models with random intercepts for each study animal. Linear mixed models permit comparison of mean differences between treatment groups while also considering the effect of the clustering of the data on the standard errors resulting from multiple measurements obtained from each mouse.

Molecular Dynamics (MD) simulations: We extended our recent 20 ns MD simulations of the integrin αIIbβ3 headpiece (specifically: αIIb residues 1-452 and β3 residues 108-352) bound to RUC-2 or RUC-4 in equivalent conformations to the recent crystal structure of the αIIbβ3 complex with RUC-2 (PDB code: 3T3M) to elaborate on the mechanism of action of these drugs at the atomic level of detail. To confirm thorough sampling of the configuration of water molecules within the binding pocket during these relatively short MD runs, independent grand canonical Monte Carlo (MC) simulations using the cavity biased MC method were carried out, and the results compared to those obtained with MD. The details of these computations are given in the Data Supplement.

Other assays: Platelet aggregation in response to adenosine diphosphate (ADP; 5 µM) using human blood anticoagulated with 0.1 volume 3.8% sodium citrate or 0.5 mg/ml bivalirudin, studies of RUC-4’s specificity for αIIbβ3 compared to αVβ3, receptor priming, and induction of the conformational change in β3 detectable by monoclonal antibody AP5 were conducted as previously described. These studies were approved by the Rockefeller University Institutional Review Board and all blood donors gave informed consent.
References


Molecular Dynamics (MD) simulations: The two αIIbβ3 headpiece systems in complex with RUC-2 or RUC-4, and including the SYMBS and ADMIDAS Ca^{2+} metal ions, as well as the crystallographic water molecules within the RUC-2 binding sites, were immersed in truncated octahedral boxes of ~22,000 TIP3P water molecules, and neutralized by the addition of 7 Na^{+} counter-ions at random locations. Each system was simulated by all-atom, standard MD for a total of 50 ns, using the ff12SB and general AMBER (GAFF)^1 force fields for protein and ligands, respectively, within the AMBER12.0 suite of programs (http://ambermd.org/).

The crystal structure corresponding to PDB code 3T3M, and without the crystallographic waters, was used as a reference conformation for independent grand canonical Monte Carlo (MC) simulations carried out to confirm thorough sampling of the configuration of water molecules within the binding pocket using the cavity biased MC method. Convergence of the configuration of the water molecules was inferred from converged values of the water density (0.997 g/ml) in a thermodynamic reservoir that is formed between a 97x70x95 Å ('inner') box containing the protein and its directly surrounding environment, and a 106x81x104 Å ('outer') box composed of bulk solvent in periodic boundary conditions, as well as converged values of the total energy for the full system. Convergence in the thermodynamic reservoir implies that equilibrium is reached between the water molecules that are in contact with the protein and those in the bulk. Using force field parameters that are consistent with the MD simulations presented herein, and a chemical potential parameter tuned to the target density using the procedure described by Speidel et al. 300 million MC steps were carried out in the grand canonical ensemble at 300 K to sample water molecule configurations while the protein structure was kept rigid. Generic solvation sites were calculated from the last 40 million MC steps, and representative configurations extracted for visualization. The PyMOL and VMD
packages were used for visualization of all structures and trajectories, while the AMBER ptraj module was used to monitor selected inter-atomic distances.

Supplementary figure SI. Distance measurements of protein-ligand interactions during simulations of RUC-2 (A) and RUC-4 (B) bound to the αIIbβ3 headpiece.
Supplementary figure SII. Distribution of water molecules (within 4 Å of the ligand heavy atoms) achieved by independent MD and MC simulations and mapped onto the αIIbβ3 crystal structure corresponding to PDB 3T3M. (A) Binding pocket of RUC-2 bound to the αIIbβ3 headpiece shown as cartoon. RUC-2 is shown as yellow sticks, crystal water as red spheres, water molecules from the MC simulation of the RUC-2-αIIbβ3 complex as pink spheres, and
water density map (0.4 particle/Å³ isosurface) from MD simulation of the RUC-2-αIIbβ3 complex as a grey mesh. The positions where crystal and MC water molecules overlap are indicated with black arrows. (B) Binding pocket of RUC-4 bound to the αIIbβ3 headpiece shown as cartoon. RUC-4 is shown as green sticks, water molecules from the MC simulation of the RUC-4- αIIbβ3 complex as smudge spheres, and water density map from MD simulation of the RUC-4- αIIbβ3 complex as a cyan mesh. (C) Overlap of the RUC-2 and RUC-4 binding pockets (only RUC-2 is shown). The water bridging RUC-4 and Y166 as a result of the MD simulations is indicated with a magenta arrow.

Supplementary table SI. Plasma concentrations and corresponding primary slopes of platelet aggregation in mice treated with 0.3.85 and 1.2 mg/kg of RUC-2 and RUC-4, respectively.

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>RUC-2</th>
<th></th>
<th>RUC-4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (µM)</td>
<td>Primary Slope</td>
<td>Concentration (µM)</td>
<td>Primary Slope</td>
</tr>
<tr>
<td>Prebleed</td>
<td>0.00</td>
<td>71</td>
<td>0.00</td>
<td>50</td>
</tr>
<tr>
<td>Prebleed</td>
<td>0.00</td>
<td>74</td>
<td>0.00</td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>3.23</td>
<td>0</td>
<td>2.58</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0.07</td>
<td>32</td>
<td>1.12</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>12.13</td>
<td>0</td>
<td>0.19</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>4.42</td>
<td>0</td>
<td>0.42</td>
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</tr>
<tr>
<td>30</td>
<td>7.31</td>
<td>0</td>
<td>0.61</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>23.37</td>
<td>0</td>
<td>13.36</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>2.50</td>
<td>0</td>
<td>N/P</td>
<td>–</td>
</tr>
<tr>
<td>45</td>
<td>2.78</td>
<td>0</td>
<td>N/P</td>
<td>–</td>
</tr>
<tr>
<td>60</td>
<td>0.28</td>
<td>0</td>
<td>0.14</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>0.36</td>
<td>0</td>
<td>&lt;0.08</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>0.06</td>
<td>17</td>
<td>&lt;0.08</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>0.07</td>
<td>0</td>
<td>&lt;0.08</td>
<td>0</td>
</tr>
<tr>
<td>240</td>
<td>0.04</td>
<td>26</td>
<td>&lt;0.08</td>
<td>32</td>
</tr>
<tr>
<td>240</td>
<td>N/P</td>
<td>–</td>
<td>&lt;0.08</td>
<td>22</td>
</tr>
</tbody>
</table>

*Values are below lower limit of detection.
**Supplementary table SII.** Initial slope of platelet aggregation induced by 20 µM ADP in *M. fascicularis* treated IM with RUC-4 at the indicated dose.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>RUC-4 Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.85 mg/kg</td>
</tr>
<tr>
<td></td>
<td>132*</td>
</tr>
<tr>
<td>15 min</td>
<td>0</td>
</tr>
<tr>
<td>30 min</td>
<td>6</td>
</tr>
<tr>
<td>60 min</td>
<td>0</td>
</tr>
<tr>
<td>120 min</td>
<td>0</td>
</tr>
<tr>
<td>270 min</td>
<td>0</td>
</tr>
<tr>
<td>23-24 hours</td>
<td>109</td>
</tr>
</tbody>
</table>

Values are the means of duplicate determinations except for those indicated with an asterisk, in which only single values were obtained.

**Supplementary table SIII.** Platelet counts (X 10⁵/µl) in *M. fascicularis* before and after RUC-4 administration*.

<table>
<thead>
<tr>
<th>Animal #1</th>
<th>Animal #2</th>
<th>Animal #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3.86 mg/kg)</td>
<td>(1.93 mg/kg)</td>
<td>(1.0 mg/kg)</td>
</tr>
<tr>
<td>Predose</td>
<td>384</td>
<td>408</td>
</tr>
<tr>
<td>15 min</td>
<td>438</td>
<td>357</td>
</tr>
<tr>
<td>30 min</td>
<td>418</td>
<td>395</td>
</tr>
<tr>
<td>1 hr</td>
<td>428</td>
<td>356</td>
</tr>
<tr>
<td>2 hr</td>
<td>422</td>
<td>377</td>
</tr>
<tr>
<td>4-5 hr</td>
<td>439</td>
<td>435</td>
</tr>
<tr>
<td>Day 2</td>
<td>459</td>
<td>410</td>
</tr>
</tbody>
</table>

*Normal range 350 ± 95 X 10³/µl⁴

**Supplementary table SIV.** Linear mixed model comparing average human platelet-mediated thrombus formation (max. thrombus size in µm²) in VWF^{R1326H} mutant mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>Regression coefficient</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7,211</td>
<td>172</td>
</tr>
<tr>
<td>RUC-4</td>
<td>-6,293*</td>
<td>249</td>
</tr>
<tr>
<td>Abciximab</td>
<td>-6,684*</td>
<td>259</td>
</tr>
</tbody>
</table>

The column values are estimates of differences compared to control group (no drug). The RUC-4 treated group has, on average, 6,293 lower thrombus measurement values compared to control. The abciximab-treated group has, on average, 6,684 lower thrombus measurement values compared to control.

*p<0.01 comparing treatment group to control group. The difference between treatment groups was ns (p=0.15).
Supplementary video SI. The effect of RUC-4 on human platelet thrombus formation in laser-injured arterioles contained within the cremaster muscle of a VWF<sup>R1326H</sup> mutant mouse.

Left panel demonstrates the ability of fluorescently labeled human platelets to form a large thrombus in the absence of drug. Middle panel shows that intravenous administration of RUC-4 can prevent the formation of an occlusive thrombi formed of human platelets. Right panel demonstrates that abciximab administered per recommendations of ACC/AHA guidelines also prevents human platelet-mediated thrombus formation in a VWF<sup>R1326H</sup> mutant mouse.

References


