Synergistic Inhibition of Both P2Y\textsubscript{1} and P2Y\textsubscript{12} Adenosine Diphosphate Receptors As Novel Approach to Rapidly Attenuate Platelet-Mediated Thrombosis

Thomas Gremmel, Ivan B. Yanachkov, Milka I. Yanachkova, George E. Wright, Joseph Wider, Vishnu V.R. Undyala, Alan D. Michelson, Andrew L. Frelinger III, Karin Przyklenk

Objective—Unlike currently approved adenosine diphosphate receptor antagonists, the new diadenosine tetraphosphate derivative GLS-409 targets not only P2Y\textsubscript{12} but also the second human platelet adenosine diphosphate receptor P2Y\textsubscript{1}, and may, therefore, be a promising antiplatelet drug candidate. The current study is the first to investigate the in vivo antithrombotic effects of GLS-409.

Approach and Results—We studied (1) the in vivo effects of GLS-409 on agonist-stimulated platelet aggregation in anesthetized rats, (2) the antithrombotic activity of GLS-409 and the associated effect on the bleeding time in a canine model of platelet-mediated coronary artery thrombosis, and (3) the inhibition of agonist-stimulated platelet aggregation by GLS-409 versus selective P2Y\textsubscript{1} and P2Y\textsubscript{12} inhibition in vitro in samples from healthy human subjects before and 2 hours after aspirin intake. In vivo treatment with GLS-409 significantly inhibited adenosine diphosphate- and collagen-stimulated platelet aggregation in rats. Further, GLS-409 attenuated cyclic flow variation, that is, platelet-mediated thrombosis, in vivo in our canine model of unstable angina. The improvement in coronary patency was accompanied by a nonsignificant 30% increase in bleeding time. Of note, GLS-409 exerted its effects without affecting rat and canine hemodynamics. Finally, in vitro treatment with GLS-409 showed effects similar to that of cangrelor and the combination of cangrelor with the selective P2Y\textsubscript{1} inhibitor MRS 2179 on agonist-stimulated platelet aggregation in human platelet-rich plasma and whole blood before and 2 hours after aspirin intake.

Conclusions—Synergistic inhibition of both P2Y\textsubscript{1} and P2Y\textsubscript{12} adenosine diphosphate receptors by GLS-409 immediately attenuates platelet-mediated thrombosis and effectively blocks agonist-stimulated platelet aggregation irrespective of concomitant aspirin therapy. (Arterioscler Thromb Vasc Biol. 2016;36:501-509. DOI: 10.1161/ATVBAHA.115.306885.)

Key Words: adenosine diphosphate ▪ antiplatelet therapy ▪ aspirin ▪ P2Y\textsubscript{1} ▪ P2Y\textsubscript{12}

Dual antiplatelet therapy with aspirin and an adenosine diphosphate (ADP) receptor inhibitor is a mainstay of pharmacological therapy in acute coronary syndromes (ACS).\textsuperscript{1–3} In aspirin-treated patients with non–ST-segment–elevation ACS, the co-administration of the P2Y\textsubscript{12} ADP receptor antagonist clopidogrel reduced the composite of cardiovascular death, myocardial infarction, and stroke by 20% compared with placebo.\textsuperscript{4} The newer ADP receptor inhibitors prasugrel and ticagrelor yielded an even greater reduction of ischemic outcomes in ACS patients than clopidogrel at the expense of a significantly increased bleeding risk.\textsuperscript{5,\textsuperscript{7}} The latter is particularly problematic in patients who cannot be treated by percutaneous coronary intervention (PCI) but must immediately undergo coronary artery bypass graft surgery. Despite these recent advances, ischemic events like acute stent thrombosis still impair the prognosis of many ACS patients. Consequently, to minimize the risk of thrombotic and bleeding complications in the initial phase of ACS, there is still a need for rapidly acting and reversible but highly potent antiplatelet agents.

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Currently approved ADP receptor inhibitors target only the P2Y\textsubscript{12} receptor, but not the other platelet ADP receptor, P2Y\textsubscript{1}.\textsuperscript{8} P2Y\textsubscript{1} activation initiates ADP-induced platelet aggregation and is responsible for platelet shape change,\textsuperscript{9} whereas P2Y\textsubscript{12} activation results in amplification and stabilization of the aggregation response. There is a complex interplay between P2Y\textsubscript{1} and P2Y\textsubscript{12}\textsuperscript{10} and co-activation of both is necessary for full platelet aggregation.\textsuperscript{11} P2Y\textsubscript{1}-selective antagonists have been identified,\textsuperscript{12,\textsuperscript{13}} but the lack of clinical candidates contrasts with the essential role of P2Y\textsubscript{1} in platelet aggregation.\textsuperscript{14}
Adenosine tetraphosphate (Ap4A) is a naturally occurring compound in mammalian tissues. In human platelets, Ap4A is stored in dense granules and, therefore, released along with ADP and ATP upon platelet activation. Ap4A and its analogs have a very short plasma half-life and are known to inhibit ADP-induced platelet activation. Specifically, they inhibit the ADP-induced platelet release reaction, calcium mobilization, thromboxane production, and platelet factor 3 activities. However, Ap4A also acts as a P2X1 receptor agonist, an undesirable effect for potential antithrombotic drugs. We recently reported that Ap4A and its derivatives synergistically inhibit both human platelet P2Y1 and P2Y12 receptors and efficiently antagonize ADP-induced human platelet aggregation. In a subsequent study, a series of new Ap4A analogs with modifications in the tetraphosphate chain and/or 2- or N6-positions in the base were synthesized and evaluated as platelet aggregation inhibitors and with respect to their effects on platelet P2Y1, P2Y12, and P2X1 receptors. The established structure–activity relations were used to design Ap4A analogs which potently inhibit human platelet aggregation by simultaneously targeting platelet P2Y1 and P2Y12 receptors but, unlike Ap4A, do not activate the platelet P2X1 receptor. The new Ap4A analogs can be administered intravenously and act reversibly, and they show significantly higher plasma stability than Ap4A. Because of their unique and reversible mechanism of action, their route of administration, the fast onset of their antiplatelet effect, and their short plasma half-life, these new compounds may be beneficial in clinical situations where a high atherothrombotic risk and an increased bleeding risk coincide. Accordingly, they are envisioned as a future treatment option in the initial phase of ACS. However, data on the antithrombotic effect of the new Ap4A analogs in vivo, as well as on agonist-induced platelet aggregation, without and with concomitant aspirin treatment are missing, to date. Based on its pharmacokinetic and pharmacodynamic profile, we therefore selected compound GLS-409 from the newly discovered Ap4A derivatives for further studies on antiplatelet efficacy. Specifically, we investigated (1) the effect of GLS-409, administered in vivo to anesthetized rats, on ex vivo agonist-stimulated platelet aggregation, (2) the antithrombotic activity of GLS-409 and the associated effect on the bleeding time in a canine model of recurrent platelet-mediated coronary artery thrombosis mimicking unstable angina, and (3) the inhibition of agonist-stimulated platelet aggregation by GLS-409 versus selective P2Y1 and P2Y12 inhibition in vitro in samples from healthy human subjects before and 2 hours after aspirin intake.

### Materials and Methods

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

## Results

### Protocol 1. Rat Model of In Vivo Treatment–Ex Vivo Platelet Aggregation

There were no significant differences in heart rate or arterial pressures in GLS-409-treated groups versus saline controls (see Figure I in the online-only Data Supplement).

In vivo administration of GLS-409 inhibited ex vivo ADP-stimulated platelet aggregation in a dose-dependent manner, with approximately complete inhibition, assessed at 10 minutes after addition of the agonist, achieved with 0.054 mg/kg bolus+0.0018 mg/kg per min infusion and doses 10×, 100×, and 300× higher than this threshold dose (Table 1). In contrast, the effect of GLS-409 on collagen-induced platelet aggregation was modest and manifest only at 1/4, 1, and 10× the threshold dose: that is, the agent had no effect on collagen-stimulated aggregation at either low or high doses (Table 1).

### Table 1. Rat Model: Ex Vivo Platelet Aggregation After In Vivo Treatment with GLS-409

<table>
<thead>
<tr>
<th>GLS-409: Dose</th>
<th>Platelet Aggregation, ohms, 3 μmol/L ADP</th>
<th>Platelet Aggregation, ohms, 2 μg/mL collagen</th>
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<tbody>
<tr>
<td>Bolus, mg/kg</td>
<td>Infusion, mg/kg per min</td>
<td>Relative to Threshold Dose</td>
</tr>
<tr>
<td>Control (0)</td>
<td>Control (0)</td>
<td>0</td>
</tr>
<tr>
<td>0.000135</td>
<td>0.0000045</td>
<td>1/400</td>
</tr>
<tr>
<td>0.000675</td>
<td>0.000225</td>
<td>1/80</td>
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<tr>
<td>0.0027</td>
<td>0.0009</td>
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<tr>
<td>0.0135</td>
<td>0.0045</td>
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</tr>
<tr>
<td>0.054</td>
<td>0.0018</td>
<td>1</td>
</tr>
<tr>
<td>0.54</td>
<td>0.018</td>
<td>10</td>
</tr>
<tr>
<td>5.4</td>
<td>0.18</td>
<td>100</td>
</tr>
<tr>
<td>16.2</td>
<td>0.54</td>
<td>300</td>
</tr>
</tbody>
</table>

*P<0.01 vs corresponding value in the control group. †P<0.001 vs corresponding value in the control group. ‡P<0.05 vs corresponding value in the control group.
Protocol 2. Canine Model of Recurrent Coronary Thrombosis

As in the rat model, there were no differences in heart rate or arterial pressures in the GLS-409-treated group when compared with saline controls at any time during the 3-hour observation period (all P values for group > 0.22; Figure 1).

As expected, all dogs developed cyclic variations in coronary blood flow (ie, spontaneous episodes of recurrent thrombosis) after coronary injury+stenosis (Figure 2). During the 1 hour pretreatment period, coronary patency was comparable in dogs later assigned to receive GLS-409 versus saline: flow-time area was 30±5% versus 28±4%, and zero flow duration was 22±6% versus 21±5%, respectively (Figure 3). In addition, template bleeding times assessed before the onset of treatment were similar in both groups (mean of 90–93 s; Figure 4).

The saline-control group displayed no change in coronary patency or template bleeding time during the 2 hour treatment period when compared with the pretreatment phase (Figures 3 and 4). In contrast, administration of GLS-409 evoked a significant increase in flow-time area (to 60±10%; P<0.05 versus pretreatment and P<0.05 versus controls) together with a decrease in zero flow duration (to <1%; P value for group =0.14; Figures 2 and 3). The effect of GLS-409 on template bleeding time was variable, averaging 160±48 s at 2 hours post-treatment (P value for group =0.26; Figure 4).

Protocol 3. Effect of GLS-409 Versus Selective P2Y₁ and P2Y₁₂ Inhibition on Agonist-Stimulated Human Platelet Aggregation In Vitro Before and 2 Hours After Aspirin Intake

In the third component of the study, we compared the effects of GLS-409 to those of the selective P2Y₁ inhibitor MRS 2179 and to those of the selective P2Y₁₂ inhibitor cangrelor on ADP-, collagen-, and thrombin receptor-activating peptide (TRAP)-stimulated platelet aggregation in platelet-rich plasma (PRP) from 6 healthy donors before and 2 hours after the intake of 325 mg of uncoated aspirin. Moreover, we compared the effects of GLS-409 to those of the combination of cangrelor with 5 μmol/L of MRS 2179 on ADP-, collagen- and TRAP-stimulated maximal and final platelet aggregation in PRP without and with aspirin treatment (Figures 5 and 6). GLS-409, cangrelor, and the combination of cangrelor with 5 μmol/L of MRS 2179 inhibited maximal and final platelet aggregation in response to all agonists pre- and post-aspirin more strongly than the selective P2Y₁ antagonist MRS2179 (Figures 5 and 6). The IC₅₀ for ADP-, collagen-, and TRAP-stimulated platelet aggregation were lower with GLS-409 compared with cangrelor and MRS 2179, but similar to the combination of cangrelor with 5 μmol/L of MRS 2179 before and 2 hours after the intake of aspirin (Tables 2 and 3). Inhibition of ADP- and collagen-stimulated platelet aggregation by GLS-409 in PRP was not significantly altered by aspirin treatment, whereas the IC₅₀ for TRAP-stimulated aggregation with GLS-409 decreased after the administration of aspirin to healthy donors (Tables 2 and 3). Selective inhibition of P2Y₁ by MRS 2179 at the tested platelet ADP receptors by GLS-409 had similar effects to those of cangrelor and the combination of cangrelor with 5 μmol/L of MRS 2179 on ADP-, collagen- and TRAP-stimulated maximal and final platelet aggregation in PRP without and with aspirin treatment (Figures 5 and 6). GLS-409, cangrelor, and the combination of cangrelor with 5 μmol/L of MRS 2179 inhibited maximal and final platelet aggregation in response to all agonists pre- and post-aspirin more strongly than the selective P2Y₁ antagonist MRS2179 (Figures 5 and 6). The IC₅₀ for ADP-, collagen-, and TRAP-stimulated platelet aggregation were lower with GLS-409 compared with cangrelor and MRS 2179, but similar to the combination of cangrelor with 5 μmol/L of MRS 2179 before and 2 hours after the intake of aspirin (Tables 2 and 3). Inhibition of ADP- and collagen-stimulated platelet aggregation by GLS-409 in PRP was not significantly altered by aspirin treatment, whereas the IC₅₀ for TRAP-stimulated aggregation with GLS-409 decreased after the administration of aspirin to healthy donors (Tables 2 and 3). Selective inhibition of P2Y₁ by MRS 2179 at the tested
concentrations showed no inhibition of collagen- and TRAP-stimulated aggregation in PRP from aspirin-free healthy donors. In contrast, 2 hours after the administration of aspirin, ADP- and collagen-stimulated platelet aggregation was inhibited to a similar extent in PRP from healthy donors concomitantly treated with MRS 2179 (Figures 5 and 6).

Finally, we compared the effects of GLS-409 to those of the selective P2Y1 inhibitor MRS 2179 and to those of the selective P2Y12 inhibitor cangrelor on ADP- and TRAP-stimulated platelet aggregation in whole blood from 6 healthy donors pre- and post-aspirin intake. GLS-409 showed effects similar to those of cangrelor on ADP- and TRAP-stimulated platelet aggregation, and both had more pronounced effects than MRS 2179 on agonist-stimulated platelet aggregation (Figure 7). Inhibition of ADP-stimulated aggregation by GLS-409 in whole blood was not significantly altered by aspirin treatment, whereas the IC₅₀ for TRAP-stimulated aggregation with GLS-409 decreased after the administration of aspirin to healthy donors (Table 4). Similar to the results in PRP, selective inhibition of P2Y1 by MRS 2179 at the tested concentrations showed no inhibition of TRAP-stimulated platelet aggregation pre- and post-aspirin treatment (Figure 7).

Discussion

Our study is the first to investigate the effects of synergistic P2Y₁ and P2Y₁₂ receptor inhibition by a single compound (GLS-409) on agonist-stimulated platelet aggregation in rats and on recurrent coronary artery thrombosis in a canine model of unstable angina. In addition, we studied the antiplatelet effect of GLS-409 versus selective P2Y₁ and P2Y₁₂ receptor antagonists on agonist-stimulated platelet aggregation in PRP and whole blood from healthy human donors in vitro before and 2 hours after aspirin intake. In vivo treatment with GLS-409 significantly inhibited ADP- and collagen-stimulated platelet aggregation in rats. Furthermore, GLS-409, administered intravenously after coronary stenosis and injury, evoked an immediate and significant attenuation of platelet-mediated thrombosis in vivo in our canine model of unstable angina. The improvement in coronary patency was accompanied by a nonsignificant 30% increase in the median bleeding time, an observation that must be interpreted with caution given the small n value of 4 per group and variability in the data. GLS-409 showed a rapid biphasic plasma clearance with a first phase half-life of 9.7 minutes and a second phase half-life of 58 minutes. Of note, GLS-409 exerted its effects in vivo without affecting rat or canine hemodynamics, respectively.

Finally, in vitro treatment with GLS-409 showed effects similar to that of cangrelor and the combination of cangrelor with MRS 2179 on agonist-stimulated platelet aggregation in human PRP and whole blood. The inhibition of ADP- and collagen-stimulated platelet aggregation by GLS-409 in PRP and the inhibition of ADP-stimulated platelet aggregation in whole blood were not altered by aspirin treatment, whereas the IC₅₀ for TRAP-stimulated platelet aggregation in PRP and whole blood with GLS-409 decreased after the administration of aspirin to healthy donors.

P2Y₁ is widely expressed in different tissues, where it is involved in the control of various functions. Therefore, it is of utmost importance for antiplatelet drug candidates inhibiting P2Y₁ to avoid adverse effects associated with non-platelet P2Y₁ targeting. Because GLS-409 inhibited agonist-stimulated human platelet aggregation at levels at which P2Y₁ was not significantly inhibited in our study, such side effects seem unlikely with this agent. Specifically, the IC₅₀ for ADP-stimulated platelet aggregation with the P2Y₁ inhibitor MRS 2179 was significantly higher compared with GLS-409. Moreover, selective blockage of P2Y₁ by MRS 2179 did not affect platelet activation by TRAP and, in line with a previous publication by Mangin et al., inhibited collagen-stimulated platelet aggregation only in samples from aspirin-treated healthy donors. These findings suggest that P2Y₁ inhibition alone may not effectively prevent agonist-stimulated platelet aggregation at acceptable plasma concentrations.
Zhang et al recently investigated the crystal structures of human P2Y$_1$ in complex with a nucleotide antagonist MRS 2500 and with a non-nucleotide antagonist BPTU (1-(2-(2-(tert-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)phenyl)urea), thereby revealing 2 disparate ligand-binding sites of the receptor. Although MRS 2500 and BPTU bind to distinct sites in P2Y$_1$, both ligands stabilize the receptor in similar inactive conformations. Nevertheless, future studies are needed to determine whether nucleotide and non-nucleotide antagonists exert similar antiplatelet effects at P2Y$_1$. Because GLS-409 acts as a nucleotide antagonist at P2Y$_1$, we chose another nucleotide antagonist (MRS 2179) as a comparator for our experiments in aspirin-free and aspirin-treated samples from healthy volunteers.

Activation of P2Y$_{12}$ by ADP inhibits adenylyl cyclase through activation of the $G_{i2}$ G-protein subtype. Thus, P2Y$_{12}$ activation counteracts the antiplatelet effects of prostacyclin, which inhibits platelet function by increasing the levels of cyclic adenosine monophosphate through activation of adenylyl cyclase. Consequently, P2Y$_{12}$ receptor inhibitors exert their antithrombotic effects in part by fostering the antiplatelet potency of prostacyclin. By inhibiting prostacyclin synthesis of endothelial cells, concomitant aspirin therapy may attenuate the antiplatelet effects of P2Y$_{12}$ inhibition. Indeed, it has been speculated that the combination of ticagrelor with high-dose aspirin at many study sites in North America in the PLATO (Platelet Inhibition and Patient Outcomes) trial may have been responsible for the less pronounced effects of ticagrelor on the reduction of adverse ischemic events compared with study sites in the rest of the world. Because ADP receptor antagonists are typically used in conjunction with aspirin in ACS, it is important to show that concomitant aspirin therapy does not reduce the antiplatelet potency of new drugs targeting P2Y$_{12}$. We, therefore, assessed the inhibition of agonist-stimulated human platelet aggregation by GLS-409 in samples drawn before and 2 hours after the intake of 325 mg aspirin.

Table 2. IC$_{50}$s and 95% Confidence Intervals for Adenosine Diphosphate–, Collagen–, and Thrombin Receptor–Activating Peptide–Stimulated Maximal Platelet Aggregation With GLS-409, MRS 2179, Cangrelor, and the Combination of Cangrelor With 5 μmol/L MRS 2179 in Platelet-Rich Plasma From Healthy Donors Before and 2 Hours After the Intake of 325 mg Aspirin

<table>
<thead>
<tr>
<th>Agent</th>
<th>No Aspirin</th>
<th>2 h Post Aspirin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLS-409, nmol/L</td>
<td>5.5</td>
<td>5.9</td>
</tr>
<tr>
<td>MRS 2179, μmol/L</td>
<td>7.5</td>
<td>3.8</td>
</tr>
<tr>
<td>Cangrelor, nmol/L</td>
<td>14.7</td>
<td>15.8</td>
</tr>
<tr>
<td>Cangrelor+MRS 2179, nmol/L</td>
<td>3.6–6.2</td>
<td>4.3–5.6</td>
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</table>

ADP indicates adenosine diphosphate; and TRAP, thrombin receptor–activating peptide.
and inhibition of collagen-stimulated platelet aggregation by GLS-409 was similar in PRP before and 2 hours after aspirin intake. Furthermore, the IC₅₀ values for TRAP-stimulated platelet aggregation in PRP and whole blood with GLS-409 decreased after pretreatment with aspirin. These findings suggest that the strong antiplatelet effects of GLS-409 are retained in aspirin-treated subjects.

Synergistic inhibition of P2Y₁ and P2Y₁₂ by GLS-409 is envisioned as an antiplatelet strategy for the initial phase of ACS. When patients suffer an ACS, they are in a prothrombotic state initiated by the rupture of an atherosclerotic plaque with subsequent coronary artery stenosis and occlusion. In this situation, rapid platelet inhibition is critical to prevent further prothrombotic actions. Moreover, most patients with ACS currently undergo PCI with implantation of a drug-eluting stent. This procedure is associated with a further increased risk of ischemic events, for example, acute stent thrombosis, and, therefore, requires a fast, strong, and consistent antiplatelet therapy. In contrast to the oral antiplatelet agents prasugrel and ticagrelor, GLS-409 is administered intravenously, thereby avoiding the need for intestinal absorption. Consequently, GLS-409 is able to immediately exert its antiplatelet effect, which may be associated with a further reduction of thrombotic events in the initial phase of ACS compared with oral ADP receptor antagonists. Alternatively, GLS-409 could serve as additional intravenous treatment for high-risk patients who are already on prasugrel or ticagrelor.

On the other hand, some patients develop serious bleeding complications while being treated with antithrombotic therapy. Other patients are not eligible for PCI and stent implantation because of their coronary artery anatomy and require emergency coronary artery bypass graft surgery. In these cases, the fast restoration of platelet function would be of great importance. Because GLS-409 has a much shorter plasma half-life than prasugrel and ticagrelor, its use may result in a significant reduction of serious bleeding events compared with the clinically approved, oral P2Y₁₂ inhibitors in the above-mentioned patient populations.

Table 3. IC₅₀ values for adenosine diphosphate (ADP)-, collagen- and thrombin receptor–activating peptide (TRAP)-stimulated final platelet aggregation with GLS-409, MRS 2179, cangrelor, and the combination of cangrelor with 5 μmol/L MRS 2179 in platelet-rich plasma from healthy donors before and 2 hours after the intake of 325 mg aspirin.

<table>
<thead>
<tr>
<th>Agent</th>
<th>No Aspirin</th>
<th>2 h Post Aspirin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP</td>
<td>Collagen</td>
</tr>
<tr>
<td>GLS-409, nmol/L</td>
<td>4.1</td>
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<tr>
<td>MRS 2179, μmol/L</td>
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</tr>
<tr>
<td>Cangrelor, nmol/L</td>
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<td>19</td>
</tr>
<tr>
<td>Cangrelor+MRS 2179, nmol/L</td>
<td>6.3</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>5–7.9</td>
<td>3–9.2</td>
</tr>
</tbody>
</table>

ADP indicates adenosine diphosphate; and TRAP, thrombin receptor–activating peptide.

**Figure 6.** Percent of final human platelet aggregation (mean±SEM) in response to adenosine diphosphate (ADP), collagen, or thrombin receptor–activating peptide (TRAP) as assessed by 96-well optical aggregometry before and 2 h after the ingestion of 325 mg aspirin in platelet-rich plasma preincubated with GLS-409, MRS 2179, cangrelor, or the combination of cangrelor with 5 μmol/L MRS 2179.
In contrast to prasugrel, ticagrelor, and cangrelor, GLS-409 targets not only P2Y\textsubscript{12} but also the second platelet ADP receptor P2Y\textsubscript{1} and may, therefore, provide a more complete inhibition of ADP-induced human platelet aggregation. Our findings indicate that because of the synergistic nature of this inhibition of platelet aggregation, therapeutic inhibition may be achieved at lower concentrations of drug and lower blockade of P2Y\textsubscript{12} and P2Y\textsubscript{1} than with agents that only block P2Y\textsubscript{1} or P2Y\textsubscript{12}. One may speculate that a more comprehensive inhibition of ADP-induced platelet aggregation at lower drug concentrations will be associated with an improved benefit/risk ratio. However, large randomized clinical trials are needed to reveal the potential benefits of the presently described combined inhibition of P2Y\textsubscript{12} and P2Y\textsubscript{1} compared with the currently Food and Drug Administration-approved solely P2Y\textsubscript{12} blockers. Of particular interest would be a clinical study comparing GLS-409 to the intravenous P2Y\textsubscript{12} receptor antagonist cangrelor in the initial phase of ACS because the latter yielded heterogeneous results in large clinical trials. Although cangrelor was not superior to an oral loading dose of 600 mg clopidogrel in reducing the composite of death from any cause, myocardial infarction, or ischemia-driven revascularization at 48 hours in ACS patients undergoing PCI\textsuperscript{34}, it significantly reduced the composite of death, myocardial infarction, ischemia-driven revascularization, or stent thrombosis at 48 hours compared with clopidogrel in patients undergoing urgent or elective PCI\textsuperscript{35}. In both studies, the use of cangrelor was not associated with a significantly increased risk of severe bleeding compared with clopidogrel. Moreover, the use of periprocedural cangrelor during PCI was not superior to placebo in reducing the composite of death from any cause, myocardial infarction, or ischemia-driven revascularization at 48 hours, but significantly reduced the secondary end points of stent thrombosis and death from any cause, albeit at a significantly increased bleeding risk\textsuperscript{36}. It remains to be established whether,

because of its unique mechanism of action, GLS-409 can provide additional reduction of the ischemic risk beyond intravenous P2Y\textsubscript{12} blockade at an acceptable bleeding risk.

Our data demonstrate the antithrombotic efficacy of GLS-409 in vivo and show that it rapidly and potently inhibits agonist-stimulated human platelet aggregation in vitro with and without concomitant aspirin therapy. Moreover, GLS-409 did not affect hemodynamics in our rat and canine model and was only associated with a moderate nonsignificant increase in median bleeding time in our canine model, while showing a fast plasma clearance. These findings, together with the previous data on its high plasma stability and reversibility of its antiplatelet effects\textsuperscript{22}, suggest that GLS-409 could become a meaningful addition to the current pharmacological armamentarium in ACS. Presently, GLS-409 is undergoing late-stage preclinical evaluation with a particular focus on the transition from GLS-409 to clinically approved, oral P2Y\textsubscript{12} inhibitors with a goal of initiating human clinical testing in near future.

Table 4. \( I_{C_{50}} \)s for Adenosine Diphosphate– and Thrombin Receptor–Activating Peptide–Stimulated Maximal Platelet Aggregation With GLS-409, MRS 2179, and Cangrelor in Whole Blood From Healthy Donors Before and 2 Hours After the Intake of 325 mg Aspirin

<table>
<thead>
<tr>
<th>Agent</th>
<th>No Aspirin</th>
<th>2 h Post Aspirin</th>
</tr>
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<tr>
<td>GLS-409, nmol/L</td>
<td>1.6</td>
<td>2</td>
</tr>
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<td></td>
<td>1.1–2.3</td>
<td>1.3–3.1</td>
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<td></td>
<td>1.3–2.3</td>
<td>2.6–6.5</td>
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<td>MRS 2179, ( \mu )mol/L</td>
<td>8</td>
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<tr>
<td></td>
<td>5.5–11.5</td>
<td>3.9–12.2</td>
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<tr>
<td>Cangrelor, nmol/L</td>
<td>7.4</td>
<td>4.8–11.3</td>
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<tr>
<td></td>
<td>3.6–7.7</td>
<td>8.5–51.4</td>
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\( I_{C_{50}} \) indicates adenosine diphosphate; and TRAP, thrombin receptor–activating peptide.
In conclusion, synergistic inhibition of both P2Y1 and P2Y2/12 ADP receptors by GLS-409 immediately attenuates platelet-mediated thrombosis and effectively blocks agonist-stimulated platelet aggregation irrespective of concomitant aspirin therapy. GLS-409 is, therefore, a promising antiplatelet drug candidate, in particular, for the initial phase of ACS.

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**References**


Our study is the first to investigate the effects of synergistic P2Y₁ and P2Y₁₂ adenosine diphosphate receptor inhibition by a single compound (GLS-409) on agonist-stimulated platelet aggregation in rats and on recurrent coronary artery thrombosis in a canine model of unstable angina. In addition, we studied the antiplatelet effect of GLS-409 versus selective P2Y₁ and P2Y₁₂ receptor antagonists on agonist-stimulated platelet aggregation in platelet-rich plasma and whole blood from healthy human donors in vitro pre- and 2 hours post-aspirin intake. Our results show that in vivo treatment with GLS-409 significantly inhibits adenosine diphosphate- and collagen-stimulated platelet aggregation in rats and immediately attenuates platelet-mediated thrombosis in dogs. GLS-409 exerted these effects without affecting rat or canine hemodynamics. Finally, GLS-409 effectively blocks agonist-stimulated platelet aggregation irrespective of concomitant aspirin therapy. GLS-409 is, therefore, a promising antiplatelet drug candidate, in particular, for the initial phase of acute coronary syndrome.
Synergistic Inhibition of Both P2Y₁ and P2Y₁₂ Adenosine Diphosphate Receptors As Novel Approach to Rapidly Attenuate Platelet-Mediated Thrombosis

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Supplemental Figure I: Heart rate and mean arterial pressure (mean ± SEM) in rats randomized to receive GLS-409 or placebo (control). Doses of GLS-409 are expressed relative to the threshold dose of 0.054 mg/kg bolus + 0.0018 mg/kg/min infusion (see Table 1) All p-values for group >0.12 (ns).
Materials and Methods

Ethics Statement

All experiments conducted in preclinical models were approved by the Institutional Care and Use Committee of Wayne State University, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1996, 2011). The human study was approved by the Boston Children’s Hospital Institutional Review Board and written informed consent was obtained from all study participants.

Protocol 1. Rat model of in vivo treatment – ex vivo platelet aggregation

Surgical preparation

Forty-eight female Sprague-Dawley rats (retired breeders: 250-350 grams) were anesthetized with sodium pentobarbital (50-60 mg/kg IP), intubated and mechanically ventilated with room air. Cannulae were placed in the left or right carotid artery for continuous monitoring of heart rate and arterial pressures (DigiMed data acquisition system, Louisville KY), and in the left or right femoral vein for the later administration of GLS-409 or vehicle (saline).

Study design

After stabilization and collection of baseline hemodynamic data, rats were allocated to receive GLS-409 (n=40) or vehicle (saline: n=8), administered as an IV bolus followed by a 30 minute continuous IV infusion. Eight GLS-409-treated groups were enrolled (n=5 rats per group), receiving doses ranging from 0.000135 mg/kg bolus + 0.0000045 mg/kg infusion to 16.2 mg/kg + 0.54 mg/kg (Table 1). At 30 minutes after the onset of treatment, 3 mL of blood was collected from each animal by cardiac puncture into tubes containing hirudin. Rats were then euthanized under deep pentobarbital anesthesia by intracardiac injection of KCl.

Endpoints and analysis

The primary endpoint of Protocol 1 was in vitro platelet aggregation, assessed by whole blood impedance aggregometry. For each sample, blood aliquots (0.5 mL) were diluted with an equal volume of sterile 0.9% saline and maintained at 37°C. Platelet aggregation was initiated by the addition of standard agonists (3 µM ADP; 2 µg collagen) and impedance (in ohms: index of platelet aggregation) monitored for 10 minutes – i.e., a time at which aggregation had reached a stable plateau. For each aliquot, both maximum impedance during the 10-minute observation period and end-impedance at 10 minutes after triggering aggregation were quantified.

Protocol 2. Canine model of recurrent coronary thrombosis

Surgical preparation

Ten adult Class A purpose-bred mongrel dogs (weight: 18-24 kg; 9 females and 1 male)
were anesthetized with sodium pentobarbital (30 mg/kg IV), intubated and mechanically ventilated with room air. Catheters were positioned in the left jugular vein for administration of fluids and supplemental anesthesia, and in the left carotid artery for measurement of heart rate and arterial pressure. The heart was exposed via a left lateral thoracotomy, and two adjacent segments of the left anterior descending coronary artery (LAD) were isolated, usually midway along its course: the distal LAD segment was instrumented with a Doppler flow probe (Transonic Systems Inc., Ithaca NY) for continuous measurement of mean coronary blood flow (CBF), while the proximal segment served as the site of later thrombosis. Arterial pressure and CBF were monitored throughout each experiment using a DigiMed data acquisition system (Louisville KY).

**Study design**

After stabilization, recurrent coronary thrombosis was initiated as described previously by our group. In brief: the proximal LAD segment was compressed with forceps to induce endothelial denudation and medial injury. A micromanometer constrictor was then positioned around the site of injury and tightened such that mean CBF was reduced to 30-35% of its baseline value, triggering the rapid development (within ~5 minutes post-stenosis) of cyclic variations in coronary blood flow (CFVs) caused by platelet activation-aggregation and the resultant spontaneous accumulation/dislodgment of platelet-rich thrombi at the site of injury + stenosis (Figure 1).

At 1 hour after the onset of recurrent thrombosis, five dogs were randomly assigned to receive GLS-409, administered as an IV bolus (0.054 mg/kg) followed by a continuous IV infusion (0.0018 mg/kg/min) for 2 hours. This was the ‘threshold’ in vivo dose of GLS-409 shown in Protocol 1 to achieve near-total ex vivo inhibition of ADP-stimulated platelet aggregation (highlighted in Table 1). The remaining five animals received a volume-matched bolus + infusion of vehicle (saline; n=5). CBF was monitored for an additional 2 hours post-treatment. At the conclusion of the protocol, animals were euthanized under deep pentobarbital anesthesia by intracardiac injection of KCl.

**Endpoints and analysis**

Heart rate and mean arterial pressure were recorded at baseline and throughout the 3 hour observation period. Coronary patency following injury + stenosis was assessed by quantifying two variables: the duration of total thrombotic occlusion (CBF = 0); and flow-time area, defined as [area of the flow-time tracing/baseline coronary flow]. Zero flow duration and flow-time area measured during each phase of the protocol (before versus after randomization and treatment) were normalized and expressed as a % of the respective observation time (60 minutes versus 120 minutes). In addition, in 8 animals (4 treated with GLS-409 and 4 placebo-controls), template bleeding times were assessed immediately before randomization and at the end of the 2-hour treatment period. At each time-point, standardized incisions (1 mm depth and 5 mm length) were made on the anterior surface of the tongue (Surgicutt®; Baxter Healthcare). Blood was wicked onto blotting paper at 10-sec intervals, and bleeding times were calculated as the time from making the incision until transfer of blood to the blotting paper ceased.
Protocol 3. Effect of GLS-409 versus selective P2Y<sub>1</sub> and P2Y<sub>12</sub> inhibition on agonist-stimulated human platelet aggregation in vitro before and 2 hours after aspirin intake

**Human Blood collection and sample preparation**

Human blood samples were taken from 6 healthy volunteer donors (3 male, 3 female, median age [range]: 29 years [24 – 34 years]) free from aspirin and other non-steroidal anti-inflammatory drugs for at least 10 days. The number of healthy volunteers used for the human in vitro studies was based on the variability observed in our previous studies of diadenosine phosphate derivatives<sup>6-8</sup> and that observed by others.<sup>9</sup> Blood was drawn from an antecubital vein into tubes containing 3.2% sodium citrate for light transmission aggregometry and in hirudin tubes for multiple electrode impedance aggregometry. For 96-well light transmission aggregometry the blood was centrifuged at 110 × g for 12 minutes, and platelet-rich plasma (PRP) was immediately removed. Centrifugation at 1650 × g for 10 minutes was applied to obtain platelet-poor plasma (PPP).

**Light transmission aggregometry**

The 96-well microplate method for the detection of agonist-induced platelet aggregation and the concentration dependence of its inhibition by the tested compounds was used as previously described,<sup>10</sup> thereby avoiding the problem of platelet aging. In brief, 90µL of PRP at 37°C were added to a pre-warmed 96-well microplate and incubated with 5µL of GLS-409, cangrelor, MRS 2179 (at various concentrations) or vehicle (10 mM HEPES, 0.15 M NaCl, pH 7.4). After 15 minutes at 37°C, 5µL of ADP (final concentration 3µM), collagen (final concentration 2µg/mL) or thrombin receptor-activating peptide (TRAP; final concentration 5µM) were added. Light transmission at 580 nm was recorded immediately and at 11 second intervals for 8 min at 37°C with intermittent programmed shaking of the plate in a Molecular Devices microplate reader (Sunnyvale, California, USA). Within each experiment samples from 6 healthy donors (3 male, 3 female) were run in duplicate prior to and 2 hours after the intake of 325 mg of uncoated aspirin. In 3 healthy donors we additionally assessed the effects of preincubating the platelets with a combination of cangrelor (at various concentrations) and 5 µM MRS 2179 on platelet aggregation. The results are presented as % of maximal and final aggregation, respectively.

**Multiple electrode aggregometry**

Hirudin-anticoagulated whole blood (247.5µL) was incubated at 37°C with 2.5µL of GLS-409, cangrelor, MRS 2179 (at various concentrations) or vehicle (10 mM Hepes, 0.15 M NaCl, pH 7.4) for 15 minutes. Impedance aggregometry was performed with the Multiplate analyzer (Verum Diagnostica, Munich, Germany). One Multiplate test cell contains two independent sensor units and one unit consists of 2 silver-coated highly conductive copper wires with a length of 3.2 mm. After 1:2 dilution of pre-incubated whole blood with 0.9% NaCl solution and stirring in the test cuvettes for 3 minutes at 37 °C, ADP (6.4 µM) or TRAP (32 µM; both from Verum Diagnostica, Munich, Germany) was added and aggregation was continuously recorded for five minutes. The adhesion of activated platelets to the electrodes led to an increase of impedance, which was detected for each sensor unit separately and transformed to
aggregation units that were plotted against time. The results are presented as % of maximal aggregation.

**Statistical analysis**

The results were analyzed using GraphPAD Prism software, version 4.0 for Windows (GraphPad Software, San Diego, CA). For Protocols 1 and 2, all endpoints (with the exception of the template bleeding time) were normally distributed as determined by the Kolmogorov-Smirnov Test. For the bleeding time, the sample size (n=4 per group) was too small for statistical assessment of normality. Accordingly, all primary endpoints were compared by 2-factor ANOVA (for group and time) with replication, and data are presented as mean ± SEM. For Protocol 3, IC\textsubscript{50}s are expressed as mean followed by 95% confidence interval (95% CI).
References


