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.1-3 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.\(^1,4,5\) 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].\(^1,4,5\) 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).\(^5\) 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.\(^5\) At each time-point, standardized incisions (1 mm depth and 5 mm length) were made on the anterior surface of the tongue (Surgicutt\(^\circledR\); 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 P2Y1 and P2Y12 inhibition on agonist-stimulated human platelet aggregation \textit{in vitro} before and 2 hours after aspirin intake

\textbf{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 \textit{in vitro} studies was based on the variability observed in our previous studies of diadenosine phosphate derivatives\textsuperscript{6-8} and that observed by others.\textsuperscript{9} 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).

\textbf{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,\textsuperscript{10} 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.

\textbf{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$_{50}$s are expressed as mean followed by 95% confidence interval (95% CI).
References