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Pharmacodynamic interplay of the P2Y₁, P2Y₁₂, and TxA₂ pathways in platelets: the potential of triple antiplatelet therapy with P2Y₁ receptor antagonism

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Abstract

Introduction—Previous work suggests that the extent of platelet inhibition by P2Y₁ receptor antagonism may be underappreciated, particularly in the context of dual antiplatelet therapy with aspirin and clopidogrel.

Materials and Methods—Using P2Y₁, P2Y₁₂, and TxA₂ receptor antagonists individually and in combination, we assessed the incremental changes from baseline platelet reactivity in blood collected from healthy volunteers.

Results—The P2Y₁ receptor antagonist further inhibited platelet activation and aggregation in several assay conditions *ex vivo* when combined with P2Y₁₂ and/or TxA₂ receptor blockers. Collagen and TRAP-induced platelet aggregation measured by light transmittance aggregometry was inhibited to a greater extent with the triple combination relative to each of the antagonists alone. The triple combination of P2Y₁, P2Y₁₂, and TxA₂ receptor antagonists also significantly shifted adenosine diphosphate (ADP)-stimulated platelet glycoprotein IIb/IIIa receptor and P-selectin expression compared to individual or dual antagonists.

Conclusions—These results substantiate that additional platelet inhibition occurs with the triple combination of P2Y₁, P2Y₁₂, and TxA₂ receptor antagonists and support further testing of P2Y₁ receptor antagonists as an option for alternative, synergistic, or triple antiplatelet therapy.

Keywords

MRS2179; P2Y₁ receptor; ADP receptors; platelet pharmacology; platelet function test

P2Y₁ and P2Y₁₂ are two adenosine diphosphate (ADP) receptors important for platelet activation and aggregation. As these two receptors were identified and characterized in the

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late 1990s and early 2000s [1], priority was given to the P2Y₁₂ pathway for clinical drug development because of the documented antithrombotic success of ticlopidine and clopidogrel. Nonetheless, the P2Y₁ receptor is important for intracellular calcium release and platelet shape change [2], and both receptors are required for initiation and complete ADP-mediated platelet aggregation even though the P2Y₁₂ pathway explains a higher proportion of this later effect [3].

Many of the studies evaluating the pharmacodynamic effects of the platelet P2Y₁ receptor have tested specific inhibitors in isolation. Without blockade of the P2Y₁₂ pathway, P2Y₁ antagonists might shunt ADP response to the P2Y₁₂ pathway, potentially masking some of the impact of the P2Y₁ receptor. Indeed, studies of both ADP receptor blockers indicate synergy between P2Y₁ and P2Y₁₂ receptor antagonism for ADP-induced platelet signaling and aggregation, suggesting that the combination may have expanded efficacy [4, 5].

In today's environment of standard dual antiplatelet therapy with aspirin and a P2Y₁₂ antagonist, alternatives may be required for some patients with high platelet reactivity, genetic risk factors, or adverse cardiovascular events despite adherence to therapy. Although one solution may be more potent P2Y₁₂ receptor antagonists, triple therapy in addition to aspirin and clopidogrel with a third antiplatelet agent or even an anticoagulant continues to be explored as the clinical need remains for effective long-term antithrombotic protection with minimal bleeding risk [6-10].

Previous work measuring calcium and single platelet counts suggests that triple antiplatelet therapy with aspirin, P2Y₁ and P2Y₁₂ antagonism may also have value [11, 12]. Labarthe and colleagues demonstrated potent inhibition of platelet reactivity with MRS2179 in coronary artery disease patients administered aspirin and clopidogrel indicating that P2Y₁ inhibition may be more important than previously thought [13]. Here, we expand upon that work by using multiple pharmacodynamic assays for testing various measures of platelet function in the setting of single, double and triple platelet inhibition. The purpose of these experiments was to evaluate the combined influence of ADP and TxA₂ receptor blockade on platelet activation and aggregation.

Materials and Methods

Healthy volunteers with no significant medical history of abnormal clotting or bleeding were recruited for the study and were not permitted to take any medications affecting platelet function seven days prior to blood collection. All eligible subjects completed a consent form to demonstrate willingness to participate, and methods followed a protocol approved by the Institutional Review Board at the University of Kentucky. LTA data (except for ADP 2 μ M) were collected throughout a screening period for a separate study (n = 14 to 43 per testing condition), and 29 of these subjects were recruited for additional platelet testing (flow cytometry curves and LTA-ADP 2 μ M) approximately one year later.

Venous blood was collected in sodium citrate 3.2% Vacutainer® collection tubes by the University of Kentucky General Clinical Research Center. Following determination of red blood cell count, hemoglobin, hematocrit, and platelet count using a Beckman Coulter Counter (Model ACT 10, Beckman Coulter Inc., Brea, CA, USA), whole blood samples were immediately utilized for flow cytometry or processed by differential centrifugation for use in LTA. Platelet-rich plasma (100 g for 15 minutes) was diluted by platelet-poor plasma (2400 g for 20 minutes) to obtain a concentration of 200,000 to 300,000 platelets per μ L [14].

Adenosine diphosphate (ADP), collagen (both from Chrono-log, Havertown, PA, USA), and thrombin receptor activating peptide-6 amide (TRAP-6; Bachem Americas, Inc., Torrance,

CA, USA) were utilized as platelet agonists for all *ex vivo* platelet assays. Collagen and TRAP were used at 5 µg/mL and 10 µM, respectively for LTA. In flow cytometry, the TRAP concentration was set at 5 µM. Contributions of the P2Y₁ and P2Y₁₂ receptors were determined with the specific and reversible antagonists MRS2179 (10 µM; 2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt; Tocris Bioscience, Bristol, UK) and cangrelor (455 nM=250 ng/mL for LTA and 182 nM=100 ng/mL for flow cytometry; The Medicines Company, Parsippany, NJ, USA), respectively. The impact of the TxA₂ pathway was assessed with the TxA₂ receptor antagonist SQ29548 (1 µM; [1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; Cayman Chemical Company, Ann Arbor, MI, USA) alone and in combination with the ADP receptor antagonists.

Light transmittance aggregometry [14]

Final samples (400 µL) were incubated for 3 minutes in pre-warmed glass cuvettes in a 570VS 4-channel aggregometer (Chrono-log). Platelet poor plasma (400 µL) served as a control for maximal transmittance. Aggregometry samples were incubated for one minute at 1200 rpm in the presence of platelet receptor antagonists as applicable. Following the addition of antagonists, samples were stimulated with a platelet agonist and evaluated for platelet shape change and aggregation for a minimum of 6 minutes with AGGRO/LINK software (version 5.2.1, Chrono-Log). Results were analyzed as maximal (highest percent aggregation recorded) and residual (actual percent aggregation at 6 minutes) platelet aggregation as measured by percent increase in light transmission.

Flow cytometry of PAC1 and CD62P [15, 16]

Whole blood was diluted 1:7 (v/v) in a modified Tyrode's buffer (137 mM NaCl, 2.8 mM KCL, 1 mM MgCl₂, 12 mM NaHCO₃, 0.4 mM Na₂HPO₄, 10 mM HEPES, 0.35% bovine serum albumin, and 5.5 mM glucose; filtered, pH 7.4) and analyzed by flow cytometry as previously described. Aliquots of diluted whole blood (45 µL) were spiked with appropriate platelet antagonists, and then incubated with 5 µL of a platelet agonist (test condition) or buffer (for resting control) for 10 min at 37°C. The antibodies CD42b-phycoerythrin (PE), PAC1-fluorescein (FITC), and CD62P-allophycocyanin (APC) were all purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA) and added for a 20-minute incubation period at room temperature and protected from light. PAC1 and CD62P antibodies bind the activated glycoprotein IIb/IIIa receptor and P-selectin which are selectively expressed on the platelet surface after platelet activation and alpha granule release, respectively. IgM-FITC and IgG-APC were added to a separate group of samples and used as negative antibody controls. Samples were subsequently fixed with 1% paraformaldehyde for 45 min at room temperature, diluted with buffer, and analyzed the same day at the Flow Cytometry Core Research Facility at the University of Kentucky. A FACSCalibur flow cytometer (Becton, Dickinson and Company) and CellQuest Pro software (Version 5.2.1) were utilized to identify, acquire and analyze platelet events in each sample. Platelets were identified by forward and side scatter along with the CD42b-PE antibody, a platelet-specific surface marker. Data were captured for 10,000 platelet events, and platelet activation was determined by calculating the mean fluorescence intensity (MFI) for PAC1-FITC and CD62P-APC.

Statistical analyses

For repeated measures data, we generated a mixed effect ANOVA model using subject as the random variable and group as a fixed effect. Post hoc analysis for multiple comparisons was made with the Tukey HSD test or the Sidak test when the overall effect was significant. The Pearson product-moment coefficient was used to assess the strength of the relationship between maximal and residual platelet aggregation. Half-maximal effective concentrations

(EC50s) and related parameters were determined by nonlinear regression—sigmoidal dose response with variable slope function—for 12 concentrations of ADP with and without individual and combinations of the platelet receptor antagonists (GraphPad Prism 5.01, La Jolla, CA, USA). We used the EC50 shift function for comparison of multiple concentration response curves. In addition, the EC50 ratio for each antagonist group was determined by global nonlinear regression (EC50 shift equation) with the least squares fit. Individual points on a curve were omitted if calculated as outliers by a separate nonlinear regression analysis (sigmoidal dose response) on individual curves by group. The logEC50 control (ADP) was held constant for all groups, and the top value was constrained specifically for each subject, typically to the highest MFI value obtained with ADP alone. EC50s, EC50 ratios, and hill slopes were compared with the nonparametric Friedman test. When significant, post-hoc analysis was performed by pairwise Wilcoxon rank sum tests with Bonferroni correction for multiple comparisons. P-values less than 0.0033 were considered statistically significant for EC50s and hill slopes, and p-values less than 0.005 were deemed significant for EC50 ratios. Statistical analyses were performed in JMP 7.0 (SAS Institute Inc., Cary, NC, USA) and SPSS 19.0 (IBM, Armonk, NY, USA).

Results

Subject characteristics and laboratory values for the healthy volunteers are depicted in Table 1.

Collagen-Stimulated Platelet Aggregation

Evidence of a synergistic effect between ADP and TxA₂ antagonists was demonstrated by collagen in LTA experiments (Figure 1). Maximal and residual aggregation values were essentially identical for all groups tested ($r = 0.986$ to 0.998). Complete and consistent platelet aggregation was obtained with 5 $\mu\text{g/mL}$ collagen in 36 healthy volunteers (median = 88%, range = 70 to 102%). Inhibition with the P2Y₁ receptor antagonist MRS2179 did not significantly affect platelet aggregation (median = 86%, range = 77 to 108%; $p = \text{NS}$). However, the P2Y₁₂ receptor antagonist cangrelor did significantly reduce collagen-stimulated platelet aggregation (median = 79%, range = 45 to 103%; $p < 0.05$). The combination of P2Y₁ and P2Y₁₂ inhibition (median = 72%, range = 9 to 89%; $p < 0.05$) was statistically different from both MRS2179 and cangrelor alone and similar to platelet function in the presence of the TxA₂ inhibitor SQ29548 (median = 66%, range = 31 to 85%; $p = \text{NS}$). Antagonizing both ADP and TxA₂ pathways had a dramatic and potentially synergistic effect on collagen-induced platelet aggregation (median = 20%, range = 9 to 26%; $p < 0.05$).

TRAP-Mediated Platelet Aggregation

Evidence of a synergistic effect was also observed with TRAP using LTA (Table 2). As previously demonstrated with collagen, maximal and residual aggregation were highly correlated ($r = 0.88$ to 0.99). Maximal platelet aggregation was achieved when the thrombin receptor was activated by 10 μM TRAP (median = 90%, range = 71 to 100%). However, TRAP-induced platelet aggregation depended upon P2Y₁₂ receptor signaling as there was a small, but statistically significant decrease in maximal aggregation with cangrelor compared to TRAP alone (~6%). P2Y₁ receptor antagonism with MRS2179 alone did not inhibit TRAP-induced platelet aggregation, and the combination of MRS2179 and cangrelor reduced activity to the same extent as cangrelor alone ($p = \text{NS}$). Inhibiting the TxA₂ receptor with SQ29548 did not affect aggregation, but in combination with the ADP antagonists cangrelor and MRS2179, the triple combination resulted in a ~20% reduction in maximal aggregation ($p < 0.05$ versus all other groups).

TRAP-Mediated Platelet Activation

The impact of the 3 antagonists on platelet activation differed from their influence on platelet aggregation (Table 2). Maximal inhibition of TRAP-induced PAC1 expression was achieved with cangrelor alone, so combination effects were unable to be evaluated. MRS2179 significantly inhibited TRAP-stimulated PAC1 expression, but SQ29548 did not affect response. Cangrelor and all other groups with cangrelor maximally blocked activation to levels indistinguishable from each other and from the resting samples (all p values > 0.05). For P-selectin (CD62P) expression, no significant effect was identified for MRS2179 alone or SQ29548. Cangrelor significantly inhibited CD62P expression ($p < 0.05$), and this effect was not statistically different from the combination of cangrelor with MRS2179 or the triple combination. Unlike the PAC1 results, cangrelor did not maximally inhibit P-selectin expression to resting levels ($p < 0.05$ versus all other groups).

ADP-Induced Platelet Aggregation

The influence of the ADP and TxA_2 receptor pathways was evaluated with ADP $2 \mu\text{M}$, a concentration within the linear portion of the concentration-response curve for about half of the subjects (Figure 2; median = 54%, range = 17 to 93%). There was a significant effect of each antagonist, and the combination of P2Y_1 and TxA_2 receptor antagonism (median = 19%, range = 8 to 33%) was statistically lower in value than MRS2179 alone (median = 25%, range = 6 to 81%; $p = 0.004$) and SQ29548 alone (median = 35%, range = 18 to 54%; $p < 0.001$). Combination with P2Y_{12} antagonism was unable to be assessed since cangrelor achieved maximal inhibition (median = 2%, range = 0 to 12%). Similar trends were identified with residual aggregation (data not shown), but the MRS2179 effect was not as pronounced. Interestingly, the TxA_2 receptor inhibitor decreased variability of platelet aggregation with ADP $2 \mu\text{M}$, but P2Y_1 receptor blockade did not.

At a maximum ADP concentration, the effect of P2Y_1 receptor antagonism was much different. Aggregation with $20 \mu\text{M}$ ADP was complete and consistent with a median value of 88% as demonstrated in Figure 3 (range = 72 to 111%). Inhibiting the P2Y_1 receptor with MRS2179 had no statistically significant effect on aggregation at maximal concentrations of ADP (median = 85%, range = 65 to 99%, $p = \text{NS}$ vs. ADP alone). In combination with cangrelor, MRS2179 significantly inhibited platelet response (median = 6%, range = 0 to 20%) compared to cangrelor alone (median = 21%, range = 3 to 41%; $p < 0.05$). TxA_2 generation contributed to the overall ADP response as treatment with SQ29548 decreased maximal aggregation to a median of 70% (range = 56 to 96%; $p < 0.05$ vs. ADP alone). Inhibiting both the P2Y_1 and TxA_2 pathways reduced aggregation to a median value of 68% (range = 47 to 76%), but this was not statistically different from ADP plus SQ29548. Similar results were obtained for residual aggregation ($r = 0.931$ to 0.998 ; $p < 0.0001$ for all groups), although there was no significant difference between cangrelor and MRS2179 versus cangrelor since almost complete inhibition occurred with cangrelor alone (data not shown).

ADP-Induced Platelet Activation

For glycoprotein IIb/IIIa receptor activation (PAC1 expression), ADP concentration-response curves were generated for all antagonist groups tested (Figure 4). MRS2179 increased the median ADP EC_{50} from $3.1 \mu\text{M}$ (range = 1.1 to $8.6 \mu\text{M}$) to $37 \mu\text{M}$ (range = 8.7 to $132 \mu\text{M}$; $p < 0.001$). In combination with SQ29548, MRS2179 further reduced platelet activation to a median EC_{50} of $97 \mu\text{M}$ (range = 4.6 to $361 \mu\text{M}$; $p < 0.001$ versus MRS alone) even though SQ29548 alone had a negligible effect (median $\text{EC}_{50} = 3.3 \mu\text{M}$, range = 1.2 to $41 \mu\text{M}$; $p = 0.002$ versus ADP). As expected, P2Y_{12} receptor antagonism with cangrelor had the greatest effect as ADP EC_{50} s reached a median of $607 \mu\text{M}$ (range = 236 to $1164 \mu\text{M}$; $p < 0.001$ versus ADP). MRS2179 was able to shift the ADP plus

cangrelor curve as the combination of cangrelor with MRS2179 and SQ29548 resulted in a median EC₅₀ of 706 μ M (range = 297 to 1294 μ M; $p = 0.002$ versus cangrelor alone). EC₅₀ ratios demonstrated similar results although there was no significant difference between cangrelor and the triple combination after adjusting for multiple comparisons ($p = 0.005$).

In addition to the differences in EC₅₀s, the calculated hill slopes were also significantly different. All hill slopes were statistically significantly different from each other except for ADP (median = 0.78, range = 0.47 to 1.17) and ADP plus SQ (median = 0.75, range = 0.55 to 1.70; $p = 0.25$). The hill slope was significantly decreased with MRS2179 (median = 0.61, range = 0.41 to 1.44; $p = 0.001$ versus ADP) and further decreased with the combination of MRS plus SQ (median = 0.51, range = 0.38 to 1.05; $p < 0.001$ versus MRS). With cangrelor, the hill slope was significantly increased (median = 1.17, range = 0.78 to 1.51; $p < 0.001$ versus ADP) and further increased with the triple combination (median = 1.44, range = 1.13 to 2.93; $p < 0.001$ versus cangrelor). All nonlinear regression curves fit the data extremely well as the median R^2 value was 0.99 (range = 0.95 to 1.0).

The extent of ADP and TxA₂ receptor involvement in ADP-stimulated platelet aggregation was also assessed with the flow cytometric marker for P-selectin expression (CD62P; Figure 5). The relative changes in EC₅₀s between groups remained consistent with the PAC1 results as all EC₅₀s were significantly different from each other (all p -values < 0.003). Similarly, all pairwise comparisons of EC₅₀ ratios were statistically significant except MRS versus MRS with SQ ($p = 0.006$). The relative trends for calculated hill slopes were also consistent for the P-selectin data. The hill slope for the triple combination of antagonists—MRS, cangrelor, and SQ (median = 1.60, range = 1.08 to 3.46)—was significantly different than ADP alone (median = 0.97, range = 0.67 to 1.69; $p < 0.001$), cangrelor (median = 1.29, range = 0.86 to 2.25; $p = 0.001$), MRS2179 (median = 0.80, range = 0.36 to 5.40; $p < 0.001$), and MRS2179 with SQ29548 (median = 0.73, range = 0.44 to 3.08; $p < 0.001$). The R^2 values were particularly exceptional with a median of 0.99 (range = 0.94 to 1.0).

Discussion

We assessed the interplay of three separate platelet signaling pathways with three platelet antagonists—MRS2179 (P2Y₁), cangrelor (P2Y₁₂), and SQ29348 (TxA₂)—to inhibit different pathways involved in platelet activation and aggregation *ex vivo*. Several assays demonstrated increased inhibition when the P2Y₁ receptor antagonist was added to the P2Y₁₂ and/or TxA₂ receptor antagonists.

In LTA with collagen or TRAP, potential synergism was suspected as the increased inhibition with the triple combination was greater than expected for the additive effects of each antagonist alone. The suspected synergism was most evident with the agonist collagen, as MRS2179 potentiated the cangrelor response and the triple combination markedly reduced platelet aggregation by 77%. These results represent a more potent effect than previously demonstrated with a single-platelet counting technique and different P2Y₁ and TxA₂ antagonists [11]. A similar effect for the triple combination was also demonstrated for LTA-TRAP, albeit not nearly as profound. These effects were apparent in LTA even though maximal concentrations of agonist were used—collagen 5 μ g/mL and TRAP 10 μ M.

Platelet sensitivity to the individual receptor antagonists and combinations was analyzed in detail with 12 separate concentrations of ADP to generate concentration-response curves. The triple combination of ADP and TxA₂ receptor antagonists was able to shift the EC₅₀ compared to the cangrelor curve. Combined with a previous report documenting a synergistic effect between MRS2179 and cangrelor [4], these findings suggest that further

platelet inhibition can be achieved by adding MRS2179 to P2Y₁₂ and TxA₂ inhibitors. The dual combination of TxA₂ and P2Y₁ antagonists was also significantly different than either antagonist alone even though the TxA₂ antagonist demonstrated a negligible effect, suggesting that potential synergism may also occur without P2Y₁₂ receptor antagonism. Interestingly, the triple combination increased the hill slope value compared to cangrelor, even though MRS2179 and SQ29548 decreased the hill slope value from the ADP curve. If, and how these differences relate to the pharmacodynamic interplay between pathways has yet to be determined.

One limitation of this study is that we used potent receptor antagonists *ex vivo*. In many assays and assay conditions, cangrelor achieved complete or near complete platelet inhibition which prevented assessment of combination therapy. Nonetheless, increased inhibition with MRS2179 as a combination agent was a consistent theme in our study even if the results were agonist and assay dependent. Furthermore, because cangrelor is a potent and consistent P2Y₁₂ receptor antagonist, we expect that the P2Y₁ receptor effect on platelet inhibition may be even greater with clopidogrel—which is less potent and more variable [17]. Similarly, any variable effect of aspirin responsiveness was also evaded in this study by use of a direct TxA₂ receptor antagonist.

Since adverse cardiovascular events still occur in patients treated with dual antiplatelet therapy, the pursuit is ongoing for new P2Y₁₂ receptor antagonists and additional antithrombotic agents for use in combination. Cilostazol [6], thrombin receptor (PAR-1) antagonists [7, 10], and factor Xa inhibitors [8, 9] have all been evaluated in addition to dual antiplatelet therapy for acute coronary syndrome patients. Previous work has identified synergy between P2Y₁ and P2Y₁₂ receptor antagonism [4], and a study of blood from aspirin and clopidogrel-treated coronary artery disease patients also suggested that P2Y₁ receptor blockade can further inhibit thrombotic potential [13]. In our study, we were able to assess incremental changes from baseline platelet reactivity for P2Y₁, P2Y₁₂, and TxA₂ receptor antagonism, using detailed EC50s in some cases. Our *ex vivo* results in blood collected from healthy volunteers substantiate that further platelet inhibition occurs with the triple combination of ADP and TxA₂ receptor antagonists. As it remains unclear what measure of platelet inhibition, with what agonist, at what concentration is the appropriate surrogate for antiplatelet efficacy, perhaps the discussion and future study of options for triple antiplatelet therapy should also include P2Y₁ receptor antagonism as a candidate.

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Abbreviations

P2Y	purinergic receptor subtype Y
TxA₂	thromboxane A ₂
LTA	light transmittance aggregometry
ADP	adenosine diphosphate
TRAP	thrombin receptor activating peptide
MFI	mean fluorescence intensity

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Figure 1. Inhibition of ADP and TxA₂ signaling demonstrated potential synergistic inhibition on collagen-induced platelet aggregation as measured by LTA

Platelet-rich plasma was activated with the endogenous agonist collagen in the presence and absence of MRS2179 (10 μ M), cangrelor (455 nM), and SQ29548 (1 μ M) alone or in combination in healthy volunteers. Lines indicate median values. Symbols indicate $p < 0.05$ versus all other groups. $n = 36, 19, 36, 34, 21$, and 15 for Co, Co+M, Co+C, Co+M+C, Co+S, and Co+M+C+S, respectively. Co = collagen; M = MRS2179; C = cangrelor; S = SQ29548.

Figure 2. ADP and TxA₂ receptor blockade significantly affected aggregation induced by 2 μ M of ADP

There was a significant effect of the combination of P2Y₁ and TxA₂ receptor antagonism compared to either inhibitor alone. Cangrelor maximally inhibited platelet aggregation. A = ADP; M = MRS2179; C = cangrelor; S = SQ29548. Lines indicate median values. Symbols indicate $p < 0.05$ versus other groups. $n = 26$ for A+C+S and A+M+C+S, and $n = 28$ for all other groups.

Figure 3. P2Y₁ receptor antagonism impacted maximal ADP-induced platelet aggregation in combination with P2Y₁₂ receptor inhibition

Antagonizing the P2Y₁ receptor pathway further inhibited aggregation in the presence of cangrelor for maximal aggregation. ADP (A) = adenosine diphosphate; MRS2179 (M) = P2Y₁ receptor antagonist; cangrelor (C) = P2Y₁₂ receptor antagonist; SQ29548 (S) = thromboxane A₂ receptor antagonist. Lines indicate median values. Symbols indicate $p < 0.05$ versus all other groups. $n = 43, 39, 41, 10, 43$, and 42 for ADP, A+M, A+S, A+M+S, A+C, and A+M+C, respectively.

Group	EC50 (μM)	EC50 Ratio	Hill Slope	R ²
ADP	3.1 (1.1 - 8.6)	—	0.78 (0.47 - 1.2)	0.99 (0.96 - 1.0)
ADP + SQ	3.3* (1.2 - 41)	1.1 (0.48 - 10)	0.75 (0.55 - 1.7)	0.99 (0.95 - 1.0)
ADP + MRS	37 (8.7 - 132)**	12 (2.4 - 44)*	0.61 (0.41 - 1.4)*	0.99 (0.97 - 1.0)
ADP + MRS + SQ	97 (4.6 - 361) [#]	32 (2.5 - 260) [#]	0.51 (0.38 - 1.1)**	0.99 (0.96 - 1.0)
ADP + Cangrelor	607 (236 - 1164) ^{##}	174 (36 - 686) [†]	1.2 (0.78 - 1.5) [‡]	0.99 (0.97 - 1.0)
ADP + MRS + Cangrelor + SQ	706 (297 - 1294) [‡]	226 (40 - 932) [‡]	1.4 (1.1 - 2.9) [‡]	0.99 (0.98 - 1.0)

Figure 4. P2Y₁ receptor antagonism significantly impacted ADP EC50s for PAC1 activation
MRS2179 shifted the EC50 curve versus ADP alone and in combination with SQ29548 versus each antagonist alone. The triple combination of P2Y₁, P2Y₁₂, and TxA₂ receptor antagonism was also statistically significant from all other groups and achieved the highest hill slope. Although not statistically significant, the p-value for comparing EC50 ratio between cangrelor and the triple combination was 0.005, and the corrected level of significance was 0.005. All values indicate the median and range. Symbols indicate $p < 0.0033$ (EC50 and hill slope) or $p < 0.005$ (EC50 ratio) versus all other groups. ADP = adenosine diphosphate; MRS = MRS2179; SQ = SQ29548; Can = cangrelor; EC50 = half-maximal effective concentration.

Group	EC50 (μM)	EC50 Ratio	Hill Slope	R ²
ADP	1.9 (0.54 - 3.9)	—	0.97 (0.67 - 1.7)	0.98 (0.95 - 1.0)
ADP + SQ	2.3 ^a (0.77 - 57)	1.2 (0.82 - 43)	0.99 ^a (0.73 - 5.7)	0.99 (0.95 - 1.0)
ADP + MRS	18 ^{**} (0.85 - 100)	11 [*] (1.6 - 49)	0.80 [*] (0.36 - 5.4)	0.98 (0.96 - 1.0)
ADP + MRS + SQ	33 [#] (1.0 - 130)	16 [*] (1.7 - 67)	0.73 [*] (0.44 - 3.1)	0.99 (0.94 - 1.0)
ADP + Cangrelor	272 ^{##} (91 - 723)	135 [#] (47 - 526)	1.3 ^b (0.86 - 2.3)	0.99 (0.95 - 1.0)
ADP + MRS + Cangrelor + SQ	377 [†] (211 - 1055)	198 [†] (59 - 943)	1.6 [#] (1.1 - 3.5)	0.99 (0.96 - 1.0)

Figure 5. MRS2179 in combination with other antagonists significantly shifted the ADP EC50s for P-selectin expression

P2Y₁ antagonism further inhibited platelet activation (increased EC50) compared to ADP alone, ADP plus SQ, and ADP plus cangrelor (all $p < 0.0033$). All values are represented as median and range. Symbols denote $p < 0.0033$ (EC50 and hill slope) or $p < 0.005$ (EC50 ratio) versus all other groups. a = $p < 0.0033$ versus all groups except ADP and ADP + cangrelor; b = $p < 0.0033$ versus all groups except ADP+SQ. ADP = adenosine diphosphate; MRS = MRS2179; SQ = SQ29548; EC50 = half-maximal effective concentration.

Table 1
Subject characteristics and laboratory values for healthy volunteers

Laboratory values depict the mean $\pm$ SD. RBC = red blood cells; n = 75.

Gender (% male)	29%
Race (% caucasian)	81%
RBC ($\times 10^6/\mu\text{L}$)	4.4 ± 0.5
Hemoglobin (g/dL)	12.4 ± 1.3
Hematocrit (%)	39 ± 4
Platelets ($\times 10^3/\mu\text{L}$)	262 ± 56

Table 2
Combined inhibition of ADP and TxA2 pathways significantly decreased TRAP-mediated platelet aggregation, but not platelet activation

Although MRS2179 or SQ29548 alone did not affect aggregation ($p = \text{NS}$), the combination of ADP and TxA2 receptor antagonists further reduced maximal and residual aggregation compared to cangrelor and all other groups ($p < 0.05$). For the conditions evaluated by flow cytometry, maximal inhibition of PAC1 expression was obtained with cangrelor, and no combination effects were observed for either activation marker (PAC1 or CD62P). Values indicate the median and range. MFI = mean fluorescence intensity; MRS = MRS2179; SQ = SQ29548. # = $p < 0.05$ versus TRAP, TRAP+SQ, and TRAP+MRS+Cangrelor+SQ. ** = $p < 0.05$ versus all groups except TRAP+Cangrelor. Other symbols indicate $p < 0.05$ versus other groups. For aggregation, $n = 35, 34, 18, 31, 20$, and 14 for each group, respectively. For flow cytometry, $n = 29$.

Group	Maximal Aggregation (%)	Residual Aggregation (%)	PAC1 expression (MFI)	CD62P expression (MFI)
TRAP	90 (71-100)	89 (66-98)	111 (48-206)	115 (69-184)
TRAP + Cangrelor	85 (57-98)#	82 (55-98)*	46 (36-70)*	87 (54-127)†
TRAP + MRS	87 (80-104)	86 (77-104)	83 (41-144)†	111 (67-181)
TRAP + Cangrelor + MRS	82 (59-101)**	80 (54-98)*	44 (35-111)*	86 (52-124)†
TRAP + SQ	86 (73-110)	84 (72-108)	108 (52-183)	117 (67-189)
TRAP + SQ + Cangrelor + MRS	74 (50-84)†	62 (40-76)†	44 (34-61)*	82 (56-118)†
Resting (no agonist)			42 (24-66)*	32 (30-54)*