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Pharmacodynamic interplay of the $P2Y_1$, $P2Y_{12}$, and TxA_2 pathways in platelets: The potential of triple antiplatelet therapy with $P2Y_1$ receptor antagonism

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ABSTRACT

Introduction: Previous work suggests that the extent of platelet inhibition by $P2Y_1$ receptor antagonism may be underappreciated, particularly in the context of dual antiplatelet therapy with aspirin and clopidogrel.
Materials and Methods: Using $P2Y_1$, $P2Y_{12}$, and TxA_2 receptor antagonists individually and in combination, we assessed the incremental changes from baseline platelet reactivity in blood collected from healthy volunteers.
Results: The $P2Y_1$ receptor antagonist further inhibited platelet activation and aggregation in several assay conditions *ex vivo* when combined with $P2Y_{12}$ and/or TxA_2 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 $P2Y_1$, $P2Y_{12}$, and TxA_2 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 $P2Y_1$, $P2Y_{12}$, and TxA_2 receptor antagonists and support further testing of $P2Y_1$ receptor antagonists as an option for alternative, synergistic, or triple antiplatelet therapy.

Introduction

$P2Y_1$ and $P2Y_{12}$ are two adenosine diphosphate (ADP) receptors important for platelet activation and aggregation. As these two receptors were identified and characterized in the late 1990s and early 2000s [1], priority was given to the $P2Y_{12}$ pathway for clinical drug development because of the documented antithrombotic success of ticlopidine and clopidogrel. Nonetheless, the $P2Y_1$ 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 $P2Y_{12}$ pathway explains a higher proportion of this later effect [3].

Many of the studies evaluating the pharmacodynamic effects of the platelet $P2Y_1$ receptor have tested specific inhibitors in isolation. Without blockade of the $P2Y_{12}$ pathway, $P2Y_1$ antagonists might shunt ADP response to the $P2Y_{12}$ pathway, potentially masking some of the impact of the $P2Y_1$ receptor. Indeed, studies of both ADP receptor blockers indicate synergy between $P2Y_1$ and $P2Y_{12}$ 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 $P2Y_{12}$ 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 $P2Y_{12}$ 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, $P2Y_1$ and $P2Y_{12}$ 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 $P2Y_1$ 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 TxA_2 receptor blockade on platelet activation and aggregation.

Abbreviations: P2Y, purinergic receptor subtype Y; TxA_2, thromboxane A2; LTA, light transmittance aggregometry; ADP, adenosine diphosphate; TRAP, thrombin receptor activating peptide; MFI, mean fluorescence intensity.

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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 μM) 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 μM) 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 μL [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 $P2Y_1$ and $P2Y_{12}$ 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 TxA_2 pathway was assessed with the TxA_2 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 $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 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 TxA_2 antagonists was demonstrated by collagen in LTA experiments (Fig. 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 μg/mL collagen in 36 healthy volunteers (median = 88%, range = 70 to 102%). Inhibition with the $P2Y_1$ receptor antagonist MRS2179 did not significantly affect platelet aggregation (median = 86%, range = 77 to 108%; p = NS). However, the $P2Y_{12}$ receptor antagonist cangrelor did significantly reduce collagen-stimulated platelet aggregation (median = 79%, range = 45