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Modified diadenosine tetraphosphates with dual specificity for $P2Y_1$ and $P2Y_{12}$ are potent antagonists of ADP-induced platelet activation

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Summary

Background—Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A), a natural compound stored in platelet dense granules, inhibits ADP-induced platelet aggregation. Ap_4A inhibits the platelet ADP receptors $P2Y_1$ and $P2Y_{12}$, is a partial agonist of $P2Y_{12}$, and is a full agonist of the platelet ATP-gated ion channel P2X1. Modification of the Ap_4A tetraphosphate backbone enhances inhibition of ADP-induced platelet aggregation. However, the effects of these Ap_4A analogs on human platelet $P2Y_1$, $P2Y_{12}$ and P2X1 are unclear.

Objective—To determine the agonist and antagonist activities of diadenosine tetraphosphate analogs towards $P2Y_1$, $P2Y_{12}$, and P2X1.

Methods—We synthesized the following Ap_4A analogs: P^1,P^4-dithiotetraphosphate; P^2,P^3-chloromethylenetetraphosphate; P^1-thio-P^2,P^3-chloromethylenetetraphosphate; and P^1,P^4-dithio-P^2,P^3-chloromethylenetetraphosphate. We then measured the effects of these analogs on: (i) ADP-induced platelet aggregation; (ii) $P2Y_1$-mediated changes in cytosolic Ca^{2+}; (iii) $P2Y_{12}$-mediated changes in vasodilator-stimulated phosphoprotein phosphorylation; and (iv) P2X1-mediated entry of extracellular Ca^{2+}.

Results—Ap_4A analogs with modifications in the phosphate backbone inhibited both $P2Y_1$ and $P2Y_{12}$, and showed no agonist activity towards these receptors. The dithio modification increased inhibition of $P2Y_1$, $P2Y_{12}$, and platelet aggregation, whereas the chloromethylene modification increased inhibition of $P2Y_{12}$ and platelet aggregation, but decreased $P2Y_1$ inhibition. Combining the dithio and chloromethylene modifications increased $P2Y_1$ and $P2Y_{12}$ inhibition. As compared

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with Ap_4A, each modification decreased agonist activity towards P2X1, and the dual modification completely eliminated P2X1 agonist activity.

Conclusions—As compared with Ap_4A, tetraphosphate backbone analogs of Ap_4A have diminished activity towards P2X1 but inhibit both $P2Y_1$ and $P2Y_{12}$ and, with greater potency, inhibit ADP-induced platelet aggregation. Thus, diadenosine tetraphosphate analogs with dual receptor selectivity may have potential as antiplatelet drugs.

Keywords

diadenosine; platelet; receptor

Introduction

Combined antiplatelet therapy with aspirin and an inhibitor of the platelet ADP receptor $P2Y_{12}$ significantly reduces the risk of ischemic events in patients with acute coronary syndromes and those undergoing percutaneous coronary intervention [1]. However, ischemic events still occur, and these agents do not inhibit platelet activation mediated through other receptors, thereby raising the question of whether inhibition of other pathways of platelet activation would be clinically beneficial.

Platelets possess three purinergic receptors: $P2Y_{12}$, a G_i-linked ADP receptor that mediates the propagation of stable platelet aggregation and is the target of the Food and Drug Administration (FDA)-approved antiplatelet agents clopidogrel, prasugrel, and ticagrelor; $P2Y_1$, a G_q-linked ADP receptor whose activation results in Ca^{2+} mobilization from intracellular pools, platelet shape change, and rapidly reversible platelet aggregation; and P2X1, an ATP-stimulated ligand-gated ion channel whose activation results in entry of extracellular Ca^{2+} and platelet shape change [2].

Diadenosine $5',5'''$-P^1,P^4-tetraphosphate (Ap_4A), a natural compound stored in platelet dense granules, is released along with ADP, ATP and other diadenosine polyphosphates ($Ap_{3–7}A$) upon platelet activation [3]. Ap_4A inhibits ADP-induced platelet activation [4] but, until recently, its specific activity towards P2X1, $P2Y_1$ and $P2Y_{12}$ was unclear. We have demonstrated that Ap_4A is an antagonist of platelet $P2Y_1$ and $P2Y_{12}$, a partial agonist of $P2Y_{12}$, and an agonist of P2X1 [5]. Other previous studies also showed that the antiplatelet effect of Ap_4A is enhanced by modification of the polyphosphate chain, yielding derivatives that resist degradation in plasma and may be clinically useful antithrombotic agents [4,6]. However, the relative antagonist potencies of these analogs against $P2Y_1$ and $P2Y_{12}$ have not been characterized, and their effects on platelet P2X1 have not been studied. Platelet P2X1 is involved in clot formation in high shear force conditions, such as arterial stenosis [7], and unmodified diadenosine polyphosphates are agonists of P2X1 expressed on a variety of human and rat cell types [8,9]. If Ap_4A analogs are also P2X1 agonists, their potential as therapeutic antiplatelet agents may be limited. Therefore, the goal of the present study was to investigate the antiplatelet potency of Ap_4A modified tetraphosphate backbone analogs, and their structure–activity relationships with regard to signaling through $P2Y_1$, $P2Y_{12}$, and P2X1.

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Methods

Chemicals and reagents

Ap_4A and its P^1,P^4-dithio derivative (subsequently referred to in this article as compound 1), P^2,P^3-chloromethylene derivative (subsequently referred to as compound 2) and P^1,P^4-dithio-P^2,P^3-chloromethylene derivative (subsequently referred to as compound 4) were synthesized as previously described [10]. The unsymmetrical P^1-thio-P^2,P^3-chloromethylene derivative (subsequently referred to as compound 3) was prepared by extension of a method for the synthesis of unsymmetrical bis-nucleoside tetraphosphates [11]. The purity of compounds 1–4 was > 95%, as determined by reverse-phase HPLC, LCMS, and proton and phosphorus NMR (data not shown). The structures of Ap_4A and its analogs are shown in Fig. 1. MRS2179, probenecid, adenosine 5′-(β,γ-methylene)triphosphate (β,γ-CH_2-ATP), EGTA and apyrase (grade VII) were from Sigma-Aldrich (St Louis, MO, USA). D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was from Calbiochem (EMD Biosciences, La Jolla, CA, USA). FLUO-4 was from Invitrogen (Carlsbad, CA, USA). ADP was from Bio/Data (Horsham, PA, USA). CD41–phycoerythrin–Cy5 was from Beckman Coulter (Fullerton, CA, USA).

Blood collection and sample preparation

After Instutional Review Board-approved written informed consent had been obtained, blood was collected into tubes containing 3.2% sodium citrate from healthy aspirin-free (7 days) and non-steroidal anti-inflammatory drug-free (3 days) volunteers. Anticoagulated whole blood was used in vasodilator-stimulated phosphoprotein (VASP) phosphoryla tion and cytosolic Ca^{2+} assays. Platelet-rich plasma (PRP) and platelet-poor plasma for platelet aggregation assays were prepared as previously described [5]. For assays of platelet P2X1 function, blood was drawn into tubes containing PPACK (0.3 mM final concentration), apyrase (1.8 μM final concentration), and PRP prepared as previously described [5].

ADP-induced platelet shape change and platelet aggregation

ADP-stimulated (3 μM) platelet shape change in PRP with 10 mM EDTA was evaluated as previously described [5]. Light transmission platelet aggregation in response to ADP (3 μM) was measured with the 96-well microplate reader method, as previously described [5].

$P2Y_{12}$-mediated VASP phosphorylation assay

$P2Y_{12}$-mediated changes in VASP phosphorylation were measured by flow cytometry with a kit from BioCytex (Marseilles, France).

$P2Y_1$-mediated cytosolic Ca^{2+} increase

The ADP-dependent, $P2Y_1$-mediated increase in platelet cytosolic Ca^{2+} was measured by detection of changes in FLUO-4 fluorescence, as previously described [5]. The cytosolic Ca^{2+} increase was calculated as the ratio of the maximal post-stimulation FLUO-4 fluorescence to the baseline FLUO-4 fluorescence. The percentage inhibition of the ADP-induced Ca^{2+} increase caused by the addition of Ap_4A analogs was calculated relative to ADP (3 μM) plus vehicle (Hepes–saline).

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To test Ap_4A analogs for their potential $P2Y_1$ agonist properties, a high concentration of each compound (100 μM for compound 1, 250 μM for compound 2, 200 μM for compound 3, and 80 μM for compound 4), was added as a stimulant to whole blood incubated with FLUO-4 in the absence of additional ADP.

P2X1-mediated entry of extracellular Ca^{2+}

Measurement of P2X1-mediated entry of extracellular Ca^{2+} based on changes in FLUO-4 fluorescence was performed as previously described [5]. The non-hydrolyzable ATP analog β,γ-CH_2-ATP (20 μM) was used as a positive control. To confirm that any increases in intracellular Ca^{2+} observed were unrelated to $P2Y_1$ activation, experiments were repeated with 100 μM MRS2179, a selective $P2Y_1$ inhibitor, in the ambient buffer. The ability of high concentrations of Ap_4A analogs to antagonize P2X1 activation by 20 μM β,γ-CH_2-ATP was also tested.

Statistical analysis

The activation or inhibition parameters were analyzed with GRAPHPAD PRISM software, version 4.00 for Windows (Graph-Pad Software, San Diego, CA, USA). All data are expressed as mean ± standard error of the mean. Student's *t*-test was used to determine statistical significance when two groups of data were compared. One-way ANOVA and Bonferroni's multiple comparison test were used when three or more groups of data were compared.

Results

Inhibition of ADP-induced platelet shape change and platelet aggregation

All four Ap_4A analogs inhibited ADP-stimulated (3 μM) platelet shape change (data not shown) and both primary and secondary platelet aggregation (Fig. 2). Figure 2A shows representative aggregation tracings for compound 4. Figure 2B shows the concentration-dependent inhibition of 3 μM ADP-induced aggregation of human platelets. Corresponding IC_{50}s are shown in Table 1. As previously reported [4,6], both the P^1,P^4-dithio modification (compound 1) and the P^2,P^3-chloromethylene modification (compound 2) enhanced the inhibition of platelet aggregation: approximately two-fold for compound 1, and approximately six-fold for compound 2. The effect of these modifications was additive; that is, the greatest inhibition was observed with the dually modified P^1,P^4-dithio-P^2,P^3-chloromethylene derivative (compound 4), which was an approximately 60-fold more potent inhibitor than Ap_4A. The previously unreported non-symmetrical P^1-thio-P^2,P^3-chloromethylene Ap_4A analog (compound 3) showed an inhibitory potency intermediate between those of compound 2 and compound 4.

Agonist and antagonist effects of Ap_4A derivatives on the $P2Y_{12}$-mediated decrease in VASP phosphorylation

VASP phosphorylation at Ser239, measured by flow cytometry with a specific mAb, was elevated in prostaglandin E_1-treated platelets (Fig. 3, solid gray bars) and, as expected, was reduced by addition of the $P2Y_{12}$ agonist ADP (Fig. 3, open bars). We previously reported that Ap_4A is a partial $P2Y_{12}$ agonist, and also produces a dose-dependent reduction in VASP

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phosphorylation [5]. In contrast, none of the four Ap_4A analogs reduced VASP phosphorylation (Fig. 3A–D, striped bars), indicating that, at the concentrations tested, the compounds are not $P2Y_{12}$ agonists. Compounds 1–4 each dose-dependently antagonized the ADP-induced reduction of VASP phosphorylation (Fig. 3A–D, hash-marked open bars). The concentrations of compounds 1–4 required for 50% inhibition of ADP-induced reduction of VASP phosphorylation were ~ 15–50-fold greater than that needed to inhibit ADP-induced platelet aggregation (Table 1). Nevertheless, the Ap_4A and compound 1–4 IC_{50}s for the ADP-induced reduction of VASP phosphorylation correlated with the IC_{50}s for ADP-induced platelet aggregation (Pearson $r^2 = 0.98$, $P = 0.005$).

Agonist and antagonist effects of Ap_4A derivatives on $P2Y_1$-mediated cytosolic Ca^{2+} increase

Addition of 100 μM compound 1, 250 μM compound 2, 200 μM compound 3 or 80 μM compound 4 to FLUO-4-loaded platelets in the absence of ADP did not result in increased fluorescence (data not shown), indicating that, at these concentrations, these analogs lack $P2Y_1$ agonist activity.

The dose-dependent inhibition of the ADP-induced $P2Y_1$-mediated cytosolic Ca^{2+} increase by the four Ap_4A analogs is shown in Fig. 4, and the corresponding IC_{50}s are shown in Table 1. Relative to Ap_4A, compound 1 had ~ 10-fold increased $P2Y_1$ antagonistic potency, but compound 2 and compound 3 had < 50% of the potency of Ap_4A. Compound 4 had a $P2Y_1$ antagonist effect that was approximately three-fold greater than that of Ap_4A, but less than that of compound 1. The concentrations of compounds 2–4 required for 50% inhibition of the ADP-stimulated Ca^{2+} increase were 85–150-fold greater than that needed for inhibition of ADP-induced platelet aggregation (Table 1). In contrast, compound 1 inhibited ADP-stimulated Ca^{2+} flux and ADP-stimulated platelet aggregation with nearly identical IC_{50}s (Table 1). The Ap_4A and compound 1–4 IC_{50}s for ADP-stimulated Ca^{2+} flux and for ADP-stimulated platelet aggregation were not significantly correlated ($P = 0.77$).

Agonist effects of Ap_4A derivatives on P2X1-mediated cytosolic Ca^{2+} influx

We previously reported that Ap_4A is a potent (maximal response at 1 μM) agonist of P2X1 on human platelets, as shown by the influx of extracellular Ca^{2+} [5]. Figure 5 shows the increase in FLUO-4 fluorescence caused by entry of extracellular Ca^{2+} with the Ap_4A derivatives. Relative to Ap_4A, compound 1-stimulated Ca^{2+} entry was reduced (maximal extracellular Ca^{2+} influx at 10 μM), and compound 2-stimulated Ca^{2+} entry was strongly reduced (sub-maximal extracellular Ca^{2+} influx at 200 μM), and the effect of compound 3 was intermediate between those of compound 1 and compound 2, inducing strong Ca^{2+} influx at 100 μM (Fig. 5). Compound 4 did not induce influx of extracellular Ca^{2+} at concentrations up to 200 μM. To determine whether the observed Ap_4A derivative-stimulated increase in cytosolic Ca^{2+} was attributable to $P2Y_1$-mediated release of Ca^{2+} from intracellular stores, rather than P2X1-mediated influx of extracellular Ca^{2+}, we evaluated FLUO-4 changes stimulated by compound 1 in the presence of the specific $P2Y_1$ inhibitor MRS2179 (100 μM) and in samples where extracellular Ca^{2+} was chelated by EGTA (1 mM). MRS2179 did not alter the compound 1-stimulated increase in cytosolic

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Ca^{2+}, whereas EGTA at 1 mM completely eliminated changes in cytosolic Ca^{2+} at all concentrations of compound 1 (data not shown).

Maximal P2X1-mediated Ca^{2+} influx occurred at 10 μM compound 1; a higher concentration (100 μM compound 1) not only failed to generate a greater response, but caused significantly lower Ca^{2+} influx (Fig. 5). A similar biphasic dose response has been observed with the non-hydrolyzable ATP analog β,γ-CH_2-ATP (data not shown) and with Ap_4A [5].

None of the four Ap_4A analogs, at any concentration tested, reduced the FLUO-4 fluorescence increase caused by 20 μM β,γ-CH_2-ATP, indicating that these compounds are not platelet P2X1 antagonists (data not shown).

Discussion

The main findings of this study are as follows. (i) All four Ap_4A analogs inhibit both platelet $P2Y_1$ and $P2Y_{12}$ function, but have no agonist activity towards either receptor. Thus, inhibition of both receptors contributes to the previously reported inhibition of ADP-induced platelet aggregation by compounds 1, 2, and 4 [4,6], and to the currently reported inhibition of platelet aggregation by compound 3. (ii) Dithio modification at P^1 and P^4 increased the inhibition of $P2Y_1$, $P2Y_{12}$, and platelet aggregation, and the P^2,P^3-chloromethylene modification increased the inhibition of $P2Y_{12}$ and platelet aggregation, but decreased the inhibition of $P2Y_1$. The simultaneous effect of both modifications was increased inhibition of both $P2Y_1$ and $P2Y_{12}$. (iii) Inhibition of platelet aggregation by Ap_4A and compounds 1–4 correlates with their inhibition of the $P2Y_{12}$-mediated VASP phosphorylation decrease, but not with their inhibition of $P2Y_1$-mediated Ca^{2+} flux. However, the concentrations of compounds 2–4 required to inhibit platelet aggregation are many times lower than that required to inhibit either $P2Y_1$ or $P2Y_{12}$ activity, suggesting a synergistic effect, as previously suggested by the combined use of individual selective antagonists of $P2Y_1$ and $P2Y_{12}$ [12,13]. (iv) The P2X1 agonist property indigenous to Ap_4A was diminished by the polyphosphate chain modifications, and completely abolished in the dithiochloromethylene derivative, compound 4, the most potent inhibitor of platelet aggregation. In sum, the potency and novel dual receptor antagonism mechanism of Ap_4A analogs suggest that they have potential as antiplatelet drugs.

Platelet purinergic receptors are important targets for the development of antiplatelet agents. Thus far, only platelet $P2Y_{12}$ inhibitors have been successfully applied in clinical practice [14]. $P2Y_1$-selective antagonists have been developed and proposed as antiplatelet agents, but none has advanced to clinical development [15–22]. Likewise, Ap_4A analogs with a modified polyphosphate chain have been proposed as potential antiplatelet drugs [4,6,23], but their development has not been pursued. Furthermore, the specific mechanism(s) for their enhanced potency and the structure–activity relationship with respect to platelet purinergic $P2Y_1$ and $P2Y_{12}$, and the effect on platelet P2X1, have not previously been described. Our recent study [5] showed that Ap_4A can inhibit both platelet $P2Y_1$ and $P2Y_{12}$, but that the IC_{50} for inhibition of each of these receptors was much greater than the IC_{50} for

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inhibition of ADP-stimulated platelet aggregation, suggesting a possible synergistic effect of such dual inhibition.

Structure–activity relationships of Ap_4A derivatives with regard to human platelet $P2Y_1$ and $P2Y_{12}$

The present study demonstrates that, like Ap_4A, all four Ap_4A derivatives possess antagonist activities towards both $P2Y_1$ and $P2Y_{12}$ receptors. However, the thio modification and chloromethylene modification have distinctly different effects on the ability of the corresponding Ap_4A analogs to inhibit platelet $P2Y_1$ and $P2Y_{12}$. The dithio modification (compound 1) strongly increased (~ 10-fold relative to Ap_4A) the inhibitory effect on $P2Y_1$, whereas the chloromethylene modification (compound 2) decreased it (approximately three-fold relative to Ap_4A). In agreement with the relative sizes of these two opposing effects, the dually modified compound 4 was apprximately three-fold stronger as an inhibitor of $P2Y_1$ than Ap_4A. Interestingly, the monothio derivative (compound 3) was not significantly different from compound 2, which has no thio substitution, in its $P2Y_1$ inhibitory properties, suggesting that both P^1 and P^4 need to be thio-modified for the inhibition-enhancing effect of this thio modification to take place, and that perhaps both P^1 and P^4 are involved in a direct interaction with $P2Y_1$.

In the case of $P2Y_{12}$, and similar to the effect on $P2Y_1$, the dithio modification increased (more than three-fold relative to Ap_4A) the inhibitory properties of the Ap_4A scaffold. However, in contrast to the negative effect that the chloromethylene modification has on the inhibition of $P2Y_1$, the chloromethylene modification strongly increased the inhibition of $P2Y_{12}$ (> 10-fold relative to Ap_4A). Addition of a single thio group in the presence of the chloromethylene modification (i.e. compound 3) did not further enhance the inhibition of $P2Y_{12}$, whereas the dithio modification did (approximately three-fold, compound 4 relative to compound 2). These results suggests that, as for $P2Y_1$, both P^1 and P^4 need to be thio-modified for the $P2Y_{12}$ inhibition-enhancing effect, and that perhaps both P^1 and P^4 are involved in a direct interaction with $P2Y_{12}$.

The finding that Ap_4A and its analogs antagonize both platelet $P2Y_1$ and $P2Y_{12}$ is remarkable when contrasted with distinctively different interactions of ATP and its analogs with these receptors. ATP is an inhibitor, albeit a weak one, of $P2Y_{12}$ [24], and its modification led to the development of the highly active and selective $P2Y_{12}$ inhibitor cangrelor (ARC69931MX) [25]. On the other hand, ATP is an agonist of $P2Y_1$ [26], and ATP analogs have been developed as highly potent $P2Y_1$ agonists. For example, 2-methylthio-ATP activates human $P2Y_1$ ($hP2Y_1$) in transfected HEK cells with an EC_{50} of 1 nM [27].

Structure–activity relationships of Ap_4A derivatives with regard to human platelet P2X1

The emerging role of P2X1 in the activation of platelets has been documented [7]. Platelet P2X1 is activated by ATP, and is believed to be involved in platelet activation under high shear stress conditions, e.g. in partially occluded blood vessels. In addition, platelet P2X1 synergizes with other platelet purinergic receptors to enhance downstream signal transduction, such as the Ca^{2+} increase mediated by the $P2Y_1$ pathway and the cAMP

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decrease mediated by the $P2Y_{12}$ pathway. Ap_4A is an agonist of rat and human P2X receptors from various tissues [28–30]. In platelets, Sage *et al.* [31] showed that Ap_4A may induce a rise in cytosolic Ca^{2+}, and suggested that this was mediated by P2X1. We have previously shown [5] that Ap_4A increases platelet cytosolic Ca^{2+} levels by P2X1-mediated extracellular Ca^{2+} influx, and that this effect has an unusual dose-dependence, with a maximum at 1 μM Ap_4A. In the present study, we have found that the P^1,P^4-thio and the P^2,P^3-chloromethylene modifications reduce the P2X1 agonist properties of the Ap_4A scaffold. The dose–response curve of compound 1 was similar to that of Ap_4A (Fig. 5), with a maximum effect ~ 150% of that of 20 μM β,γ-CH_2-ATP, but with an approximately one order of magnitude rightward shift. The agonist properties were even more significantly reduced by the P^2,P^3-chloromethylene modifications, with compound 2 achieving only 50% of the 20 μM β,γ-CH_2-ATP response at the highest studied concentration (200 μM). The effect of these modifications is additive, because compound 4, even at 200 μM, appears to be devoid of P2X1 agonist effect (Fig. 5). Elimination of P2X1 agonist activity is important in the development of antiplatelet agents, not only to avoid P2X1-mediated platelet activation, but also to avoid activation of P2X1 on cells in other tissues [32].

Like Ap_4A, most analogs in the present study have IC_{50}s for inhibition of ADP-induced platelet aggregation that are much lower than their IC_{50}s for antagonism of $P2Y_1$ or $P2Y_{12}$ (Table 1), thus reaffirming the possibility of synergism in the simultaneous inhibition of $P2Y_1$ and $P2Y_{12}$. Both $P2Y_1$ and $P2Y_{12}$ are necessary for full-scale ADP-induced platelet aggregation [33,34]. Other derivatives of ADP, coenzyme A and acetyl-coenzyme A, also inhibit ADP-induced platelet aggregation [35], and inhibit both $P2Y_1$ and $P2Y_{12}$, albeit with micromolar activities [36], leading to the proposal that agents that act at both of these receptors may provide more protection against the effects of ADP than agents acting at only one of these receptors [36]. Synergism from inhibition of both platelet ADP receptors was previously suggested by the combined use of antagonists with individual selectivity for either $P2Y_1$ or $P2Y_{12}$: MRS2179 or A3P5P for $P2Y_1$, and AR-C69931MX for $P2Y_{12}$ [12,13]. This effect may be explained by the complex interplay between platelet ADP receptors [37,38]. However, in the case of Ap_4A and its analogs, we cannot exclude the possibility that diadenosine polyphosphate compounds as a class inhibit platelet aggregation through an additional separate, as yet unknown, mechanism.

Conclusions

Ap_4A analogs with modifications in the phosphate backbone inhibit both $P2Y_1$ and $P2Y_{12}$, and show no agonist activity towards these receptors. The dithio modifications at P^1 and P^4 increase the inhibitory activity towards $P2Y_1$, $P2Y_{12}$, and platelet aggregation, and the P^2,P^3-chloromethylene modification increases $P2Y_{12}$ and platelet aggregation inhibition, but decreases $P2Y_1$ inhibition. The simultaneous effect of both modifications is an increase in $P2Y_1$ and $P2Y_{12}$ inhibition. Both modifications decrease the agonist activity of Ap_4A towards P2X1, and the dual modification completely eliminates P2X1 agonist activity.

Current FDA-approved (ticlopidine, clopidogrel, prasugrel, and ticagrelor) or experimental (cangrelor and elinogrel) ADP receptor antagonist antiplatelet therapy is solely directed against $P2Y_{12}$. The present results identify derivatives of Ap_4A as prototypical members of a

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class of antiplatelet agents that inhibit platelet function by targeting both $P2Y_1$ and $P2Y_{12}$, with an apparently synergistic effect on inhibition of platelet aggregation. Previous studies [4,6] and the present results suggest that such compounds would cause a rapid onset of platelet inhibition and, although the modifications allow them to resist degradation in plasma [4,6], they would probably be rapidly cleared from the bloodstream. These properties make Ap_4A analogs potentially appropriate for clinical situations such as urgent percutaneous coronary intervention (where rapid platelet inhibition would be beneficial) and the need for emergency surgery, such as coronary artery bypass grafting (where rapid reversibility of platelet inhibition would be beneficial). Thus, Ap_4A derivatives represent a unique class of antiplatelet agents with potential clinical benefits.

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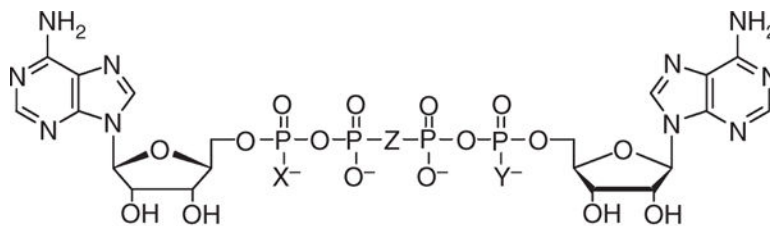

Compound	Modification X	Y	Z
Ap_4A	O	O	O
1	S	S	O
2	O	O	CHCl
3	S	O	CHCl
4	S	S	CHCl

Fig. 1.

Chemical structure of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) and its analogs. Compounds: 1, diadenosine 5′,5″-P^1,P^4-dithiotetraphosphate; 2, diadenosine 5′,5‴-P^2,P^3-chloromethylenetetraphosphate; 3, diadenosine-5′,5‴-P^1-thio-P^2,P^3-chloromethylenetetraphosphate; 4, diadenosine-5′,5‴-P^1,P^4-dithio-P^2,P^3-chloromethylenetetraphosphate.

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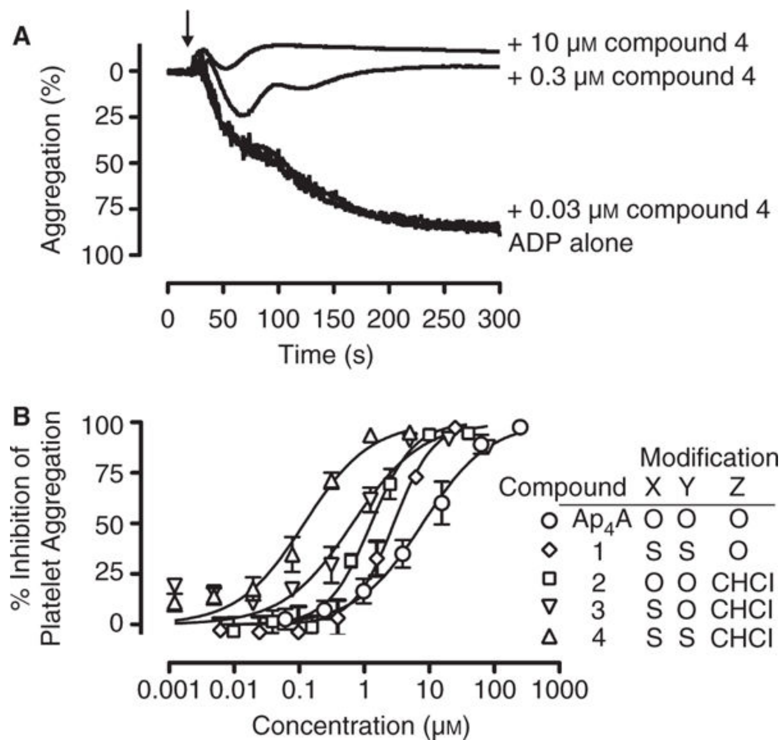

Fig. 2.

Inhibition of ADP-induced platelet aggregation by diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) analogs. (A) Representative turbidometric aggregation tracings for platelet-rich plasma stimulated with ADP alone (final concentration 3 μM) and in combination with 0.03, 0.3 or 10 μM compound 4. (B) Percentage inhibition of maximal platelet aggregation. The results shown are mean ± standard error of the mean. Data are from three independent experiments. Compounds: 1, diadenosine 5′,5‴-P^1,P^4-dithiotetraphosphate; 2, diadenosine 5′,5‴-P^2,P^3-chloromethylenetetraphosphate; 3, diadenosine-5′,5‴-P^1-thio-P^2,P^3-chloromethylenetetraphosphate; 4, diadenosine-5′,5‴-P^1,P^4-dithio-P^2,P^3-chloromethylenetetraphosphate.

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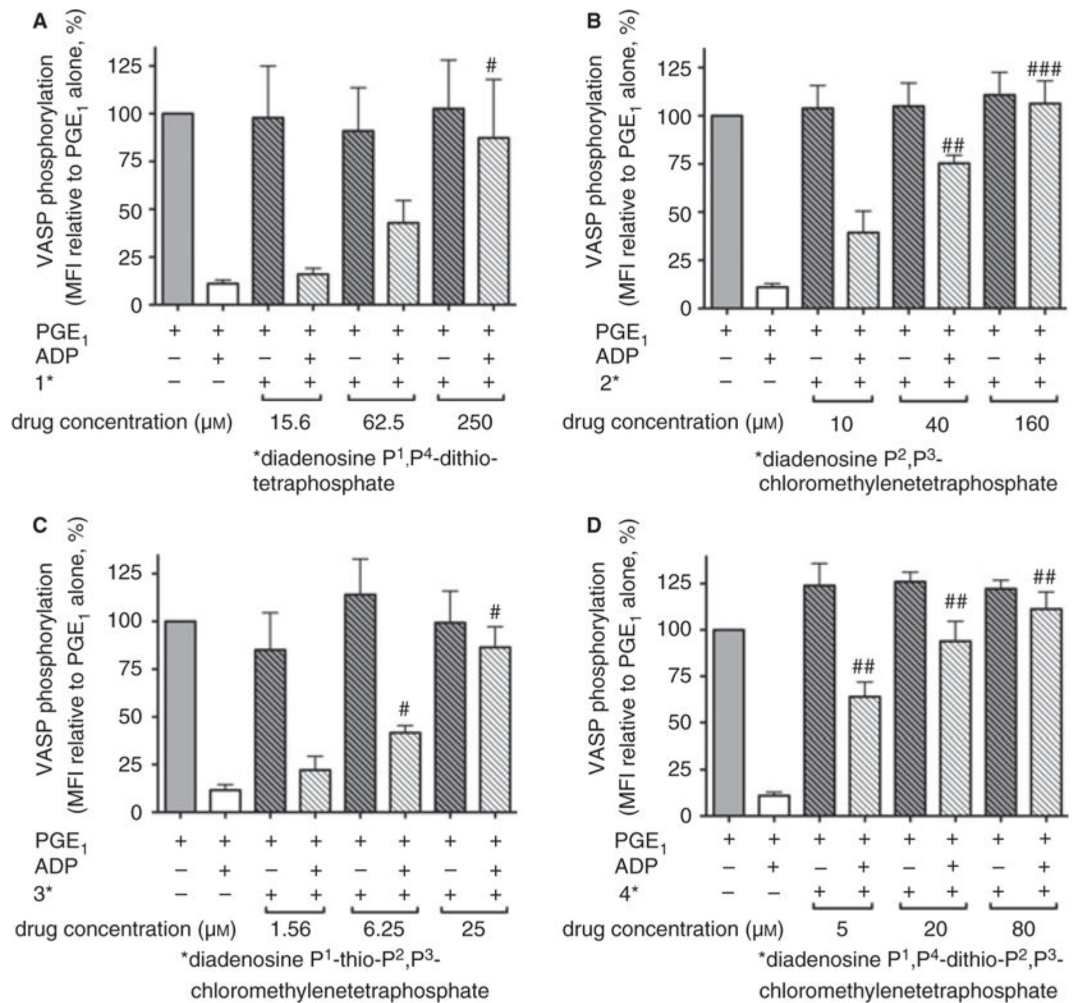

Fig. 3.

Inhibition of ADP-induced, $P2Y_{12}$-mediated decrease in vasodilator-stimulated phosphoprotein (VASP) phosphorylation by diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) analogs. Prostaglandin E_1 (PGE_1)-stimulated VASP phosphorylation and its attenuation by ADP in the presence and absence of Ap_4A analogs were measured by flow cytometry. (A) Compound 1. (B) Compound 2. (C) Compound 3. (D) Compound 4. The results shown are mean ± standard error of the mean. Data are from three independent experiments ($P < 0.05$, ##$P < 0.01$, and ### $P < 0.001$, as compared with PGE_1 plus ADP). MFI, mean fluorescence intensity. Compounds: 1, diadenosine 5′,5‴-P^1,P^4-dithiotetraphosphate; 2, diadenosine 5′,5‴-P^2,P^3-chloromethylenetetraphosphate; 3, diadenosine-5′,5‴-P^1-thio-P^2,P^3-chloromethylenetetraphosphate; 4, diadenosine-5′,5‴-P^1,P^4-dithio-P^2,P^3-chloromethylenetetraphosphate.

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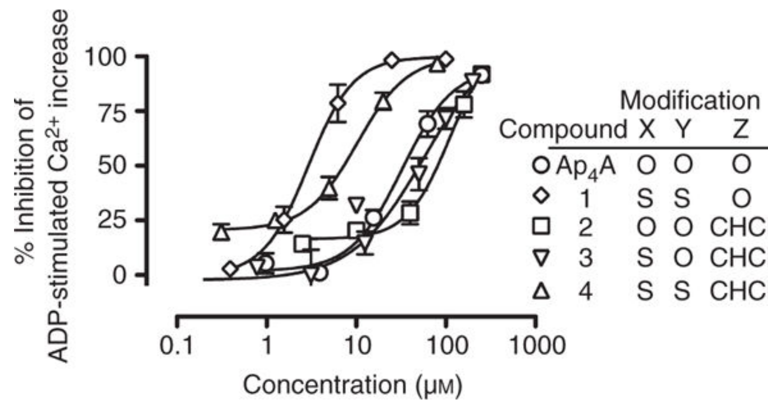

Fig. 4.

Inhibition of ADP-induced, $P2Y_1$-mediated platelet Ca^{2+} increase by diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) analogs. The increase in platelet cytosolic Ca^{2+} in response to 3 μM ADP, with and without addition of Ap_4A analogs, was measured by whole blood flow cytometry with the Ca^{2+} indicator FLUO-4. The percentage inhibition was calculated relative to ADP + vehicle (0% inhibition) and vehicle alone (100% inhibition). The results shown are mean ± standard error of the mean. Data are from three or four independent experiments. Compounds: 1, diadenosine 5′,5‴-P^1,P^4-dithiotetraphosphate; 2, diadenosine 5′,5‴-P^2,P^3-chloromethylenetetraphosphate; 3, diadenosine-5′,5‴-P^1-thio-P^2,P^3-chloromethylenetetraphosphate; 4, diadenosine-5′,5‴-P^1,P^4-dithio-P^2,P^3-chloromethylenetetraphosphate.

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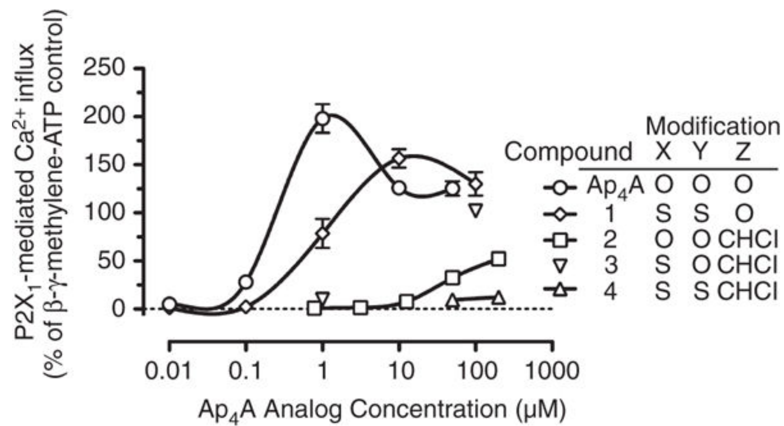

Fig. 5.

Agonist effects of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) analogs on P2X1-mediated entry of extracellular Ca^{2+}. Increasing concentrations of Ap_4A analogs were added to FLUO-4-loaded platelets, and the change in FLUO-4 fluorescence was measured by flow cytometry. The results shown are mean ± standard error of the mean normalized to the response to adenosine 5′-(β,γ-methylene)triphosphate (β,γ-CH_2-ATP) (20 μM) alone; compound 3 was evaluated at only 1 and 100 μM. The results for Ap_4A and compounds 1, 2 and 4 are from four to eight independent experiments. Compounds: 1, diadenosine 5′,5‴-P^1,P^4-dithiotetraphosphate; 2, diadenosine 5′,5‴-P^2,P^3-chloromethylenetetraphosphate; 3, diadenosine-5′,5‴-P^1-thio-P^2,P^3-chloromethylenetetraphosphate; 4, diadenosine-5′,5‴-P^1,P^4-dithio-P^2,P^3-chloromethylenetetraphosphate.

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Table 1

Inhibition of platelet aggregation and P2 receptor selectivity by diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) and its analogs

	Platelet aggregation	$P2Y_{12}$ VASP	$P2Y_1$ Ca^{2+} flux
	IC_{50} (µM) (95% CI)	**IC_{50} (µM) (95% CI)**	**IC_{50} (µM) (95% CI)**
Ap4A	7.9 (5.5–11.4)	> 250	32.5 (22.1–47.8)
Compound 1	2.8 (2.0–4.0)	88.6 (59.8–131.3)	2.9 (2.0–4.2)
Compound 2	1.3 (1.0–1.7)	19.3 (10.2–36.5)	111.5 (51.8–239.9)
Compound 3	0.66 (0.43–1.01)	23.9 (12.8–44.3)	57.2 (15.5–211.5)
Compound 4	0.12 (0.08–0.17)	6.4 (3.7–11.4)	10.2 (6.7–15.7)

CI, confidence interval; VASP, vasodilator-stimulated phosphoprotein. Compounds: 1, diadenosine 5′,5‴-P^1,P^4-dithiotetraphosphate; 2, diadenosine 5′,5‴-P^2,P^3-chloromethylenetetraphosphate; 3, diadenosine 5′,5‴-P^1-thio-P^2,P^3-chloromethylenetetraphosphate; 4, diadenosine 5′,5‴-P^1,P^4-dithio-P^2,P^3-chloromethylenetetraphosphate.