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P^1,P^4-Dithio-P^2,P^3-monochloromethylene diadenosine 5′,5‴-P^1,P^4-tetraphosphate: A novel antiplatelet agent

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ABSTRACT **We have previously demonstrated in a series of searches for antithrombotic agents that diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA) and its analogues are competitive inhibitors of ADP-induced platelet aggregation. Among various analogues, the P^2,P^3-monochloromethylene analog of AppppA (AppCHClppA) is superior to unmodified AppppA in its antiplatelet and antithrombotic effects. In this communication, we compare the antiplatelet potency of five newly synthesized agents with that of AppCHClppA. The five new agents include four diadenosine polyphosphate analogues [$Ap_spCHClpp_sA$ (p_s indicates a thiophosphate), dAppCHClpdA, $dAp_spCHClpp_sdA$, and AppCHClpCHClppA], and an adenosine tetraphosphate analogue (AppCHClpCHClp). When tested for their inhibitory effects on platelet aggregation by ADP, the most promising agent among them was $Ap_spCHClpp_sA$. Both molecular and functional integrity of this compound proved to be stable in blood at 37°C for at least 3 h. It also showed an excellent heat stability. This agent inhibits a number of aspects of ADP-induced platelet activation—e.g., release reaction, cytoplasmic calcium mobilization, thromboxane production, fibrinogen binding sites, and platelet factor 3 activity. Moreover, platelet aggregation induced by agonists other than ADP—e.g., arachidonic acid, collagen, and epinephrine—was inhibited partially by $Ap_spCHClpp_sA$. It is concluded that (*i*) $Ap_spCHClpp_sA$ is a promising antiplatelet agent; (*ii*) it is resistant to blood phosphodiesterases and stable to heat treatment; (*iii*) platelet aggregation induced by collagen, epinephrine, or arachidonic acid is also inhibited in part by this agent; and (*iv*) specificity of the inhibitory effects is presented by unmodified adenosine moieties of the agent. Resistance to phosphodiesterases raises the possibility of oral administration.**

Thrombosis in arterial blood vessels and prosthetic medical devices is characterized by the adhesion of platelets to the damaged vessel wall or prosthetic surface, the release of their granular content, the synthesis of prostaglandin endoperoxides, and platelet aggregation. The newly formed platelet aggregate is a fragile clump, a so-called "white thrombus." The exposure of procoagulant activity on the surface of aggregated (activated) platelets may enhance the formation of a fibrin network on the white thrombus and stabilize it. Various antiplatelet agents have been studied for many years as potential targets for beneficial clinical inventions with respect to the inhibition of thrombus formation. Some agents such as aspirin and dipyridamole have come into use as prophylactic antithrombotic agents, and others have been the subjects of clinical investigations (1–3).

Recent studies on more potent and selective compounds for inhibition of platelet aggregation suggest greater benefit in their antithrombotic efficacy. A number of studies have suggested that ADP, a common agonist, plays an important role in the initiation and progression of arterial thrombus formation (4–6). A potent inhibitor of ADP-induced platelet aggregation would therefore be of particular interest in searching for antithrombotic agents.

It has been known for some time that diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppppA) is a competitive inhibitor of ADP-induced platelet aggregation (7–10). Previous findings suggested the feasibility of using AppppA or AppCHClppA as an antithrombotic agent in certain clinical situations such as platelet-rich thrombosis (11, 12). We believe that there is substantial clinical need for an antithrombotic agent that is more potent and more selective in action than those now available. Because AppppA and its analogues act at a very early stage of thrombus formation, and encouraged by our data to date, we believe that these compounds may well provide such an agent. The possible analogues are very large in number. Our previous studies have identified analogues that are more suited to this purpose than AppppA itself (7, 12). In the present study, we have identified the chloromethylene thiophosphate analogue $Ap_spCHClpp_sA$ as the most potent agent among the analogues studied in its antiplatelet effects.

MATERIALS AND METHODS

Blood was collected in a 1/10 vol of 3.8% sodium citrate from healthy human volunteers who had abstained from antiplatelet agents for at least 10 days. Platelet-rich plasma was separated by centrifugation at $230 \times g$ for 15 min. Platelet concentration was adjusted to $(3 \pm 0.2) \times 10^8$ cells per ml of plasma, the concentration that has been used in our current study unless otherwise stated.

Biochemicals were purchased as follows: ADP, forskolin, and Sepharose CL-2B from Sigma; arachidonic acid, collagen, epinephrine, luciferin, and luciferase from Chrono-Log (Havertown, PA); anti-fibrinogen antibody linked to fluorescein isothiocyanate (FITC-anti-Fgn) from Accurate (Westbury, NY); and Indo-1 acetoxymethyl ester (Indo-1) from Molecular Probes (Eugene, OR). All other chemicals are analytical grade or HPLC grade.

The synthesis of AppppA analogues was carried out by condensation of AMP, dAMP, adenosine 5′-thiophosphate, and deoxyadenosine 5′-thiophosphate with monochloromethylene bisphosphonate or bismonochloromethylene triphosphonate using diphenyl phosphorochloridate by standard methods (13). The product after chromatography on DEAE-Sepharose was 90% pure by ^{31}P NMR. Analytical purification

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Abbreviations: AppppA, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; ApCHClppA, P^2,P^3-monochloromethylene diadenosine 5′,5‴-P^1,P^4-tetraphosphate; $Ap_spCHClpp_sA$, P^1,P^4-dithio-P^2,P^3-monochloromethylene diadenosine 5′,5‴-P^1,P^4-tetraphosphate; FITC, fluorescein isothiocyanate; PF_3, platelet factor 3; TxB_2, thromboxane B_2.

using reverse-phase HPLC provided material that was at least 99% pure. The concentrations of all stock solutions were determined using the molar absorptivity at 260 nm (ε = 23.3/mM·cm and 20.2/mM·cm, respectively, for AppCHClppA and Ap_spCHClpp$_s$A). The analogues were characterized chemically and quantitated by HPLC using a quaternary amine anion-exchange column (Whatman Partisil 10) and analyzed with Beckman System Gold (San Ramon, CA). Both agents showed their purity with a single peak (Fig. 1) and a linear relationship between the concentration of the agent and peak areas.

Platelet aggregation was measured by the turbidometric method of Born (14) in an aggregometer (model 530VS, Chrono-Log). Platelet release reaction was tested in a lumi-aggregometer (model 560VS, Chrono-Log) that is equipped to detect the luciferin–luciferase reaction of ATP (15).

Fibrinogen binding sites on activated platelets were assayed by means of FITC-conjugated anti-fibrinogen antibody techniques (16) in flow cytometry. Platelets were activated by ADP (5 μM) in the presence or absence of the inhibitors at 22°C for 5 min. A polyclonal antibody, FITC-anti-Fgn, was used to detect bound fibrinogen on the platelets in a flow cytometer (FACS, Becton Dickinson).

Platelet factor 3 (PF_3) activity was measured by preincubating platelets in the presence or absence of the agents (10 μM) at 37°C for 5 min. The platelets were then stimulated by 10 μM ADP and assayed for the Stypven clotting time (17) in a COAG-A-MATE XM (Organon Teknika, Durham, NC).

To assay for cytoplasmic calcium (Ca^{2+}) mobilization, platelets were incubated with Indo-1 at 37°C for 45 min (18). Excess Indo-1 in the medium was removed by gel filtration. The extent of cytoplasmic Ca^{2+} mobilization induced by ADP was measured in the presence or absence of the agents and monitored for an increase of fluorescence in flow cytometry (EPICS, Coulter).

The level of thromboxane B_2 was measured by an enzyme immunoassay kit (Amersham). Cyclic AMP (cAMP) was measured by preincubating platelets with saline (control), agents, or forskolin (Colforsin) at 37°C for 5 min followed by extraction with $Ba(OH)_2$ and $ZnSO_4$. cAMP in the extracts was converted into the etheno-cAMP derivative and the levels were measured by HPLC using a fluorescence detector (19). The level of cAMP (nM) in an unknown extract was determined by extrapolation from a standard graph which showed a linear relationship between the cAMP level and fluorescence detection.

To determine the effect of ADP-induced aggregation on platelets that were exposed previously to the agents, platelets were preincubated with the agents at 22°C for 30 min. Platelets isolated by gel-column filtration (2×10^8 cells per ml) or centrifugation (3×10^8 cells per ml) were resuspended in inhibitor-free plasma and measured for ADP-induced aggregability.

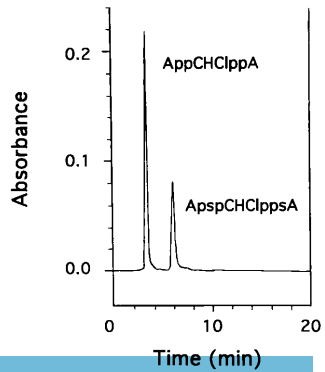

FIG. 1. HPLC Chromatogram of AppppA analogues. The retention times were 3.39 and 5.95 min, respectively, for AppCHClppA and Ap_spCHClpp$_s$A.

Table 1. IC_{50} of the agents for ADP-induced platelet aggregation

Inhibitor	IC_{50}, μM
AppCHClppA	3.28*
Ap_spCHClpp$_s$A	0.81*
dAppCHClppdA	>100
dAp_spCHClpp$_s$dA	9.22
AppCHClpCHClppA	4.0
AppCHCLpCHClp	2.56

*Mean values of three determinations; others are the mean of two determinations.

To test for the stability of the agent in blood, normal human blood was incubated in the presence of agents (250 μM) at 37°C for 4 h. Blood samples were extracted with perchloric acid (3%) at the indicated times and neutralized with K_2CO_3, then kept at −20°C until assay (11).

RESULTS

Inhibition of ADP-Induced Platelet Aggregation. We performed an initial screening for inhibitory effects of the agents on ADP (5 μM)-induced platelet aggregation as demonstrated previously (7). We then extended the study to estimate the IC_{50} values for an individual agent by doing a dose-dependent inhibition. The IC_{50} value of AppCHClppA observed in the present study (Table 1) is consistent with the value we have reported (7). The value of Ap_spCHClpp$_s$A appeared the lowest (0.81 μM) among all the agents tested, indicating the highest inhibitory potency. When both adenosine molecules of Ap_spCHClpp$_s$A were replaced with deoxyadenosine, the inhibitory potency was significantly reduced. Similarly, replacement with deoxyadenosine in the nonthiophosphonate compound resulted in complete loss of inhibitory effects (IC_{50} > 100 μM). It is noted that a monoadenosine tetraphosphate analogue (AppCHClpCHClp), an intermediate product that was left from the synthesis of diadenosine derivatives, shows an inhibitory potency similar to that observed with diadenosine compounds. In addition, we have done an inhibition kinetic study for Ap_spCHClpp$_s$A, as was done previously with ApCHClppA (7). The inhibition constant (K_i) value of Ap_spCHClpp$_s$A was 0.33 μM with a competitive nature indicating the same nature but a higher potency than that of AppCHClppA (1.1 μM). We have, therefore, focused all subsequent studies on Ap_spCHClpp$_s$A and a reference agent, AppCHClppA.

Effect on ADP-Induced Platelet Activation Other Than Aggregation. We have tested the effects of the agent on (*i*) release reaction, (*ii*) fibrinogen receptor sites (GPIIb/IIIa), and (*iii*) PF_3 activation. The values of IC_{50} of the agents on the release reaction are presented in Table 2. The values were estimated from the data of a series of dose-dependent inhibition studies. The inhibition potencies of Ap_spCHClpp$_s$A on the release reaction are far superior to those of AppCHClppA when ADP or collagen was used as agonist. Platelet surface receptors (GPIIb/IIIa) for fibrinogen are known to be increased by activation. Fibrinogen bound on the ADP-activated platelets is inhibited prominently in a dose-dependent manner by Ap_spCHClpp$_s$A as compared with AppCHClppA (Fig. 2 and Table 3). The ADP-stimulated PF_3 activities are presented as a shortening of clotting time (Table 4). The inhibitory effect

Table 2. IC_{50} values of the agents for platelet release reaction

Agent	IC_{50} of agonist, μM	
	ADP (5 μM)	Collagen (1 μg)
AppCHClppA	4.5	35
Ap_spCHClpp$_s$A	0.7	1.7

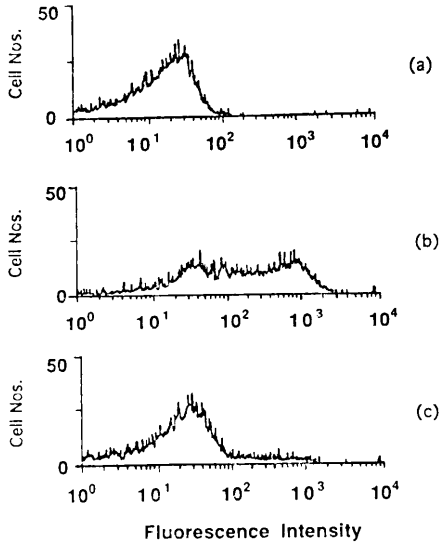

FIG. 2. Effects of $Ap_spCHClpp_sA$ on fibrinogen binding sites of ADP-activated platelets. (*a*) Unstimulated. (*b*) Stimulated with 5 μM ADP. (*c*) Platelets exposed to $Ap_spCHClpp_sA$ for 5 min before ADP stimulation.

of $Ap_spCHClpp_sA$ on PF_3 activation is again better than the nonthio compound (81% vs. 61%).

Effect of the Regulatory Factors in Platelet Activation. Three regulatory factors in the processes of platelet activation are known: cytoplasmic calcium ion mobilization, arachidonic acid metabolic product, and the increase of cAMP levels. The effects of agent on these three regulatory factors are presented as follows.

Cytoplasmic Ca^{2+} mobilization. An increment of cytoplasmic Ca^{2+} in the platelets represented an activated state. Fig. 3 shows a overlay of three histograms generated from a plot of fluorescence intensity (FI, in arbitrary units) against platelet number under various conditions. Unstimulated platelets registered FI between 42 and 250 with a peak at 133 (baseline control). Platelets treated with 2 μM A23187 (a Ca^{2+} ionophore) showed a marked shift to the right (FI between 150 and 1,000 with a peak at 530) representing a positive control. ADP-activated platelets had a mild to moderate shift to the right (FI between 67 and 300 with a peak at 158). The effect of ADP activation on Ca^{2+} mobilization was relatively mild compared with that of the calcium ionophore and was blocked completely in the presence of either agent. The histogram of ADP-activated platelets in the presence of the inhibiting agent showed a pattern identical to that obtained with unstimulated platelets.

Arachidonic acid metabolic products. Thromboxane A_2 is an intermediate product of arachidonic acid metabolism. It is a potent platelet activator and has a short half-life. The metabolic end-product, thromboxane B_2 (TxB_2), is a stable compound and is frequently used to represent thromboxane A_2. The effect of agents on TxB_2 production in ADP- and arachidonic acid-stimulated platelets is summarized in Table 5. Both

Table 3. Effects of agents on fibrinogen binding sites of ADP-activated platelets

Conc. of agent, μM	% binding	
	AppCHClppA	$Ap_spCHClpp_sA$
0	100	100
2.5	71	10
5.0	41	5
7.5	36	Not done
10	24	Not done

Table 4. Effects of agents on ADP-stimulated PF_3 activity

Agent	Clotting time, sec	Net change, sec	% change
Baseline	46	—	—
Control*	39	−7	100
AppCHClppA	43	−3	39
$Ap_spCHClpp_sA$	45	−1	19

*Control represents stimulated PF_3 activity in the absence of agents.

agents showed a dose-dependent inhibition of TxB_2 production on ADP-stimulated platelets. The TxB_2 level was reduced to 53% and 12% of the control, respectively, for AppCHClppA and $Ap_spCHClpp_sA$, when concentrations of the inhibitors were adjusted to the same level as the agonist (5 μM ADP). On the other hand, the agents showed a mild to moderate inhibition (77% and 58%) for arachidonic acid-induced synthesis of thromboxane by platelets.

Level of intracellular cAMP. It is known that cAMP inhibits platelet aggregation. Accordingly, the inhibitory effects of the agents with respect to the cAMP-mediated mechanism was tested. Both agents showed no effect on the cAMP level at 20 μM, but they did show a mild increase at a higher concentration of 100 μM (Table 6). A marked increase of cAMP by 10 μM forskolin, a potent stimulator for adenylate cyclase (20), was demonstrated as the positive control. The results suggest that the inhibitory effect of the analogues on platelet activation by ADP is not mediated by cAMP.

Effect on Aggregation Agents Other Than ADP. We have examined the role of ADP that is released in platelet aggregation induced by other agonists, including arachidonic acid, collagen, epinephrine, thrombin, and ristocetin. None of these agents exhibited an effect on the thrombin- and ristocetin-induced platelet aggregation (data not shown). The IC_{50} values for the other three agonists are summarized in Table 7. Although collagen-induced aggregation appears to be unaffected by AppCHClppA, a noticeable inhibitory effect was detected in the presence of $Ap_spCHClpp_sA$. On the other hand, both agents exhibited moderate inhibitory effects on platelet aggregation induced by arachidonic acid or epinephrine.

Aggregability of the Platelets That Were Exposed Previously to the Agent. To test the stability and inhibitory effects of the bound analogues, the platelets were preincubated with $Ap_spCHClpp_sA$ (25 μM) or AppCHClppA (50 μM). Agent-bound platelets were separated from the plasma by gel filtration or centrifugation and tested for ADP-induced aggregation. Platelets exposed to $Ap_spCHClpp_sA$ showed a moderate inhibition of aggregability (22–33%), whereas those exposed to AppCHClppA exhibited lesser inhibition (8%).

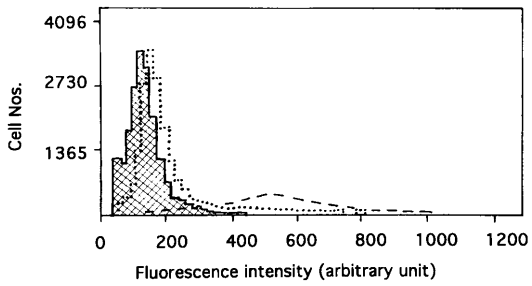

FIG. 3. Effects of agents on cytoplasmic Ca^{2+} mobilization in ADP-activated platelets. Solid line represents unstimulated fluorescence histogram (baseline control). The change in fluorescence after stimulation with 6.7 μM ADP is shown under the three following conditions: in the absence of agents (dotted line); in the presence of 6.7 μM AppCHClppA or 3.3 μM $Ap_spCHClpp_sA$ (solid line, identical to the baseline control); and after the addition of calcium ionophore A23187 (dashed line).

Table 5. Effect of AppCHClppA and $Ap_spCHClpp_sA$ on TxB_2 production in ADP- and arachidonic acid (AA)-stimulated platelets

	TxB_2, pg per 10^9 platelets				
		AppCHClppA		$Ap_spCHClpp_sA$	
Agonist	No inhibitor (saline control)	5 μM	10 μM	2.5 μM	5 μM
ADP (5 μM)	171	91	4	91	21
AA (2 mM)	376	ND	291	ND	219

ND, not done.

Stability of the Agents. The functional stability of the inhibitor was measured by incubating platelets in the presence of the agent at 37°C for 3 h. Samples taken at the times indicated were tested for ADP-induced (10 μM) aggregation (Table 8). The inhibitory effect of AppCHClppA vanished when it was kept in platelet-rich plasma for more than 60 min at 37°C, but $Ap_spCHClpp_sA$ showed no sign of decay in inhibitory effect for at least a 3-h period. $Ap_spCHClpp_sA$ exhibited similar functional stability when incubated in whole blood.

The molecular stability of the agents was determined by incubating whole blood with the agent at 37°C for 4 h. Samples were removed at the indicated times, extracted, neutralized, and assayed by HPLC. We identified two peaks (major and second) from the blood extracts for either agent. The extraction efficiency was maximal at 60 and 120 min for AppCHClppA and $Ap_spCHClpp_sA$, respectively (Table 9). The apparent high value in the extraction efficiency of AppCHClppA suggests that blood-borne adenosine compounds (possible adenosine and AMP) may contribute to the major peak. The major peak, with a retention time of 3.39 min, slowly degrades with time, declining to 70% after 4 h. At least 80% of the degradation product, possibly β,γ-chloromethylene adenosine triphosphate (AppCHClp) is detected in the second peak (X1, retention time = 6.45 min). The extraction efficiency of $Ap_spCHClpp_sA$ was relatively poor (69%) at time 0 but showed an improvement (81–85%) after 60 min. The data indicate that the thio derivative is stable in blood for at least 4 h at 37°C. The second peak (X2, retention time = 4.33 min) in the blood extract of $Ap_spCHClpp_sA$ may be a metabolite—e.g., a monothio compound ($Ap_spCHClppA$). It was barely detected in the pure compound, but constitutes a small portion (10–20%) in the blood extraction.

When both agents were heated to 100°C for 30 min, no changes were detected with respect to the inhibitory effects on platelet aggregability or physical integrity of the agents, as assayed by HPLC.

DISCUSSION

We have previously demonstrated that AppppA and its analogues inhibit ADP-induced platelet activation *in vitro* (7) and arterial thrombosis in a rabbit intracarotid-cannula thrombosis model (11, 12). Analogues having a P—C—P bridge located in the P^2,P^3-position of AppppA are superior to other forms in antiplatelet potency. They are competitive with ADP and resistant to hydrolytic enzymes (7, 13, 21). We have examined in the present study the antiplatelet effects of several newly developed diadenosine tetra- and pentaphosphate derivatives. A dithiophosphonate analogue, $Ap_spCHClpp_sA$, has been found to be the most potent inhibitor among agents so far tested.

Table 6. Effect of agents on platelet cAMP level

Conc. of agent, μM	cAMP, nM		
	AppCHClppA	$Ap_spCHClpp_sA$	Forskolin
0	32	31	32
10	ND	ND	124
20	31	29	ND
100	57	54	ND

ND, not done.

Table 7. IC_{50} values of the agents for platelet aggregation induced by arachidonic acid, collagen, or epinephrine

	IC_{50}, μM	
Agonist	AppCHClppA	$Ap_spCHClpp_sA$
ADP (5 μM)	3	1
Arachidonic acid (1 mM)	19	9
Collagen (1 μg)	>100	9
Epinephrine (10 μM)	24	5

$Ap_spCHClpp_sA$ has been studied in regard to its inhibition mechanism. Platelet responses to agonists are in general complex. Platelets possess a specific purinoceptor (P_{2T}), the binding site for ADP (22–24). Binding of platelet receptor sites by ADP or other agonists stimulate the G proteins, specifically $G_i\alpha 2$, which is responsible for the signal transduction between cell surface receptor and intracellular second messenger generating enzymes (25, 26). The second messengers in the process of platelet activation include inositol 1,4,5-trisphosphate (IP_3), 1,2-diacylglycerol (DAG), and cAMP (27, 28). IP_3 increases the cytoplasmic level of Ca^{2+}, which activates phospholipase A_2 to produce arachidonic acid, the substrate for thromboxane A_2 synthesis. DAG activates protein kinase C, leading to the release reaction. cAMP, on the other hand, mediates the inhibition of the platelet activation at several steps—e.g., thrombin receptor (29), phospholipase C (30), phospholipase A_2 (31), and actin polymerization (32).

A number of studies have suggested that ADP, a common biological agonist, plays an important role in forming arterial thrombi (4–6). The release reaction in normal platelet responses to any agonist is an essential event in which ADP, serotonin, and Ca^{2+} react as the "secondary" agonists in addition to the initial platelet activator (33, 34). Moreover, thromboxane A_2, an endoperoxide of arachidonic acid, is another potent agonist for the platelet activation. These secondary activating factors generated by the platelets in response to exogenous agonists may enhance the activation by additive or synergistic effects on the activity of the initial agonist. Platelet aggregation induced by epinephrine, collagen, or arachidonic acid was partially inhibited by $Ap_spCHClpp_sA$. In this case, the ADP released by other agonists becomes ineffective on platelet activation in the presence of $Ap_spCHClpp_sA$. At least 50% of platelet aggregability induced by arachidonic acid, collagen, or epinephrine is dependent upon ADP being released from the initial platelet activation (Table 7). This finding supports the above-described observations of the inhibitory effects of the agents on the release reaction and aggregation. The mild to moderate inhibition for collagen-induced release reaction is interesting (Table 2). It is conceiv-

Table 8. Time-dependent effects of agents on ADP-induced platelet aggregation

	Aggregability, %		
		Presence of inhibitor	
Incubation time, min	No inhibitor (control)	AppCHClppA (5 μM)	$Ap_spCHClpp_sA$ (2.5 μM)
0	82	13	21
60	69	31	7
120	59	47	5
180	43	42	6

Platelets were incubated with agents at 37°C for 3 h. Aggregability was measured at the time indicated with 10 μM ADP.

Table 9. Stability of AppCHClppA and Ap_spCHClpp$_s$A in whole blood

Incubation time, min	Conc., nmol/ml of blood					
	AppCHClppA			Ap_spCHClpp$_s$A		
	Main peak	X1	Total	Main peak	X2	Total
0	249	Trace	249	158	14	172
60	249	26	275	172	34	206
120	222	37	259	180	31	211
240	173	63	236	173	38	202

Unidentified compounds were designated X1 and X2.

able that initial release of ADP by collagen may amplify the further release of ADP from the activated platelets. In the presence of the inhibitors, especially the Ap_spCHClpp$_s$A analogue, the ADP-mediated release is effectively blocked by the agent.

The inhibition mechanism of Ap_spCHClpp$_s$A in ADP-induced platelet activation appeared to be located at the P_{2T} purinoceptor, the ADP binding site. It is likely that binding of AppppA and its analogues to the P_{2T} receptor somehow uncouples the functional connection between the G protein involved and phospholipase C. This hypothesis is supported by the lack of involvement in signal enhancement at various steps of ADP-induced platelet activation—e.g., intracellular Ca^{2+} mobilization, production of TxB_2 (stable form of thromboxane A_2), release reaction, fibrinogen binding site (activated GPIIb/IIIa complex), and PF3 activation, plus other mechanisms beyond the binding step.

The inhibitory potency is reduced by employing deoxyadenosine, as demonstrated in this study, and also by changing the carbon bridge in the P^1,P^2- and P^3,P^4-positions, as demonstrated previously (7, 20). The results indicate a molecular specificity for the inhibitory potency. The carbon bridge located in the P^2,P^3-position (AppCHClppA) and the two ADP moieties linked symmetrically, provide the specificity for the inhibitory potency. The inhibitory potency appears to be enhanced by the phosphorothioate analogues—e.g., Ap_spCHClpp$_s$A. The enhancement of the inhibitory effect by the phosphorothioate is especially prominent in the deoxyadenosine analogues—e.g., $IC_{50} > 100$ and 9.22 μM for dAppCHClppdA and dAp_spCHClpp$_s$dA, respectively (Table 1). It is interesting to note that a 2-propylthio substitution in ATP, e.g., ARL67085 (2-propylthio-D-β,γ-dichloromethylene-ATP) and ARL66096 (2-propylthio-D-β,γ-difluoromethylene-ATP), other P_{2T} purinoceptor antagonists, also results in a marked increase in potency in washed platelets (21, 35). Similar observations have been reported in inhibition of secretion and aggregation of ADP-stimulated platelets by ticlopidine and its analogue clopidogrel (36, 37). It is also interesting that the phosphorothioate analogues of oligonucleotides showed more potent antiviral effects against influenza viruses (38) and HIV (39–41) than did the phosphodiester forms.

It is known that cAMP inhibits platelet aggregation by means of the counteraction of Ca^{2+} mobilization, inhibition of thrombin receptor (29), actin polymerization (32), and phospholipases (30, 31, 42). We observed a mild increment (≈2-fold) of cAMP in the platelets at a high concentration (100 μM) of the AppppA analogues (Table 6). However, such a mild increment of cAMP may not be a specific reaction. Though we failed to detect adenosine contamination in the tested AppppA analogues, it is possible that either the metabolic products of cellular adenosine nucleotides or contaminated adenosine in the analogues may be responsible for a mild increase of the cAMP level. Nevertheless, cAMP does not appear to be the mediator for the inhibition mechanism of Ap_spCHClpp$_s$A, since no enhancement of cAMP was observed at 20 μM agent, a level that is more than enough to inhibit platelet activation completely.

Both molecular and functional integrity of Ap_spCHClpp$_s$A are stable in blood at 37°C for least 3 h. However, AppCHClppA (5 μM) was shown to be less stable in human blood (Table 8). Previous study with rabbit whole blood showed that when a higher concentration of agent (100 μM) was used, the inhibitory effect of AppCHClppA on 10 μM ADP-induced platelet aggregation was well maintained up to 2 h at 37°C (12). Both agents showed excellent stability to heat treatment. The binding of agent on the platelet surface appeared to be reversible. Platelets that have been exposed previously to the agent for 15–30 min and then washed to remove the agent, showed only mild to moderate inhibition of ADP-induced aggregation. These findings suggest that the inhibitory efficiency *in vivo* may be dependent on clearance of the blood level.

Our results suggest that Ap_spCHClpp$_s$A might prove to be a clinical antithrombotic agent. Toxicity, pharmacokinetics, and the feasibility for oral administration are currently under investigation.

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