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Inhibition of ADP-Triggered Blood Platelet Aggregation by Diadenosine Polyphosphate Analogues

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Abstract—The synthesis and biological evaluation of new diadenosine polyphosphate analogues on blood platelet aggregation are reported. The most active are compounds with a sulfur atom replacing one or both non-bridging oxygens at phosphorus bound to adenosyl residues and hydroxymethyl groups of bis(hydroxymethyl)phosphinic acid.

Blood platelets play an important role in prevention of blood circulation from massive bleeding caused by vascular damage, but on the other hand, they are also responsible for intravascular plaque formation resulting in arteriosclerosis. Thus balanced platelet reactivity is crucial for proper circulation. It is well documented that insufficiently reactive platelets are responsible for bleeding tendency in uremia,[1] although platelet hyperactivity increases the risk of arteriosclerosis and coronary disease.[2] Platelets can be activated by several physiological agonists like adenosine diphosphate (ADP), thrombin, platelet activating factor (PAF), collagen, and others,[3] but the most important seems to be ADP, which can evoke platelet activation and aggregation, and is responsible for proper formation of the second wave of platelet aggregation, followed by ADP release from platelet granules.[4] Attempts for treatment of overactive platelets are numerous, but most frequently aspirin is used for inhibition of platelet cyclooxygenase activity and thromboxanes formation.[5] Also disintegrins and RGD-analogues are used for inhibition of fibrinogen binding,[6] but clinically useful for inhibition of ADP binding to its receptor are ticlopidine and clopidogrel.[7] It is well known that both ATP and AMP nucleotides are very potent competitors and antagonists for ADP action,[8] although both are highly unstable in circulation.

Activation of platelets by ADP is mediated by several purinoceptors showing distinct specificity.[9] For example, P_2Y and P_2T receptors are antagonized by ATP and AMP, whereas P_2X is agonized by ATP but not by ADP.[10] P_2X forms an ionotropic channel which allows sodium and calcium ions to pass the platelet membrane. Though it can trigger platelet activation accompanied by shape change, aggregation of platelets requires involvement of P_2Y and P_2T receptors as well.[11] Diadenosine tetraphosphates (Ap_4A), like other diadenosine polyphosphates, are P_2X purinoceptor agonists,[12] which can evoke platelet activation, but at the same time these agents act as inhibitors for ADP action on P_2Y and P_2T receptors, leading to inhibition of platelet aggregation. Complete and actual information about the meaning of above mentioned receptors can be found in a review article.[13]

Recently, a number of stable analogues of adenosine nucleotides with structure related to diadenosine polyphosphates were described in terms of their antiplatelet activity.[14,15] Among them, diadenosine 5′,5‴′-P^1,P^4-tetraphosphate (Ap_4A) and its chloromethylene thiophosphate analogue ApspCHClppsA appeared to be strong antithrombotic agents that block a very early stage of thrombus formation. Further to these observations, in

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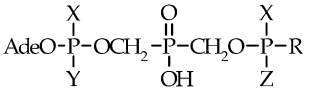
this report we describe new analogues of Ap_nA with antiplatelet activity and focus on their structure–activity relationships. These compounds, synthesized based upon the oxathiaphospholane methodology,[16] show several common structural features. First of all, adenosine 5′-*O*-phosphorothioate units are linked together through polyols, diamines, dimercaptoalkanes or bis(hydroxymethyl)phosphinic acid residues.[17] Interestingly, some of them, like 1,3-bis(adenosine-5′-*O*-phosphorothioyl)-glycerol, have appeared to be superior inhibitors of FHIT protein.[18] Data presented in this report show that these compounds, particularly isosteric analogues of Ap_4A, are also strong inhibitors of ADP-induced platelet aggregation. $N^6,N^6,O^{2'},O^{3'}$-Tetrabenzoyladenosine 5′-*O*-(2-thiono-1,3,2-oxathiaphospholane)[17] was used to synthesize analogues with chemical formulas presented in Figures 1 and 2.

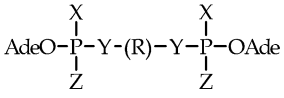
The synthesis of compounds **3**–**5**, **7**–**10** and **13** was described recently.[17] For the synthesis of compounds **1** and **11**, the following procedure has been used: pyridine solution of $N^6,N^6,O^{2'},O^{3'}$-tetrabenzoyladenosine containing 3-fold molar excess of elemental sulfur was treated with two molar equivalents of 2-chloro-1,3,2-dithiaphospholane. Resulting $N^6,N^6,O^{2'},O^{3'}$-tetrabenzoyladenosine 5′-*O*-(2-thiono-1,3,2-oxathiaphospholane) [isolated in 68% yield, ^{31}P NMR ($CDCl_3$) 124.3 ppm; FAB-MS (M−1) *m*/*z* 836.4] in DBU-assisted reaction with half-molar equivalent of bis(hydroxymethyl)phosphinic acid *O*-methyl ester, in acetonitrile solution, provided compound **1** (Scheme 1).

Similarly, $N^6,N^6,O^{2'},O^{3'}$-tetrabenzoyladenosine 5′-*O*-(2-thiono-1,3,2-dithiaphospholane) in reaction with glycerol provided compound **11**. Compound **2** was prepared by reaction of O^1,O^3-(2-thiono-1,3,2-oxathiaphospholanyl)-bis(hydroxymethyl)phosphinic acid *O*-methyl ester with one molar equivalent of $N^6,N^6,O^{2'},O^{3'}$-tetrabenzoyladenosine in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), followed by molar equivalent of β-cyanoethanol. Reaction of two molar equivalents of $N^6,N^6,O^{2'},O^{3'}$-tetrabenzoyladenosine 5′-*O*-(2-thiono-1,3,2-oxathiaphospholane) with *O*,*O*-dimethyl-D,L-tartrate and in the presence of DBU in acetonitrile solution furnished compound **6**. Compound **12** has also been obtained in a two step procedure: treatment of 1,3-diaminopropane with two molar equivalents of 2-chloro-1,3,2-oxathiaphospholane in the presence of triethylamine and elemental sulfur gave N^1,N^3-bis(1,3,2-oxathiaphospholanyl)diaminopropane in 72% yield [^{31}P NMR ($CDCl_3$) 96.7 ppm; FAB-MS (M−1) *m*/*z* 349]. This compound in reaction with two molar equivalents of $N^6,N^6,O^{2'},O^{3'}$-tetrabenzoyladenosine in acetonitrile solution, in the presence of DBU, provided desired compound **12**. All compounds were purified by means of DEAE Sephadex A-25 ion exchange chromatography, using a linear gradient of ammonium bicarbonate buffer (pH 7.5) as an eluent. Their structure and physicochemical characteristics were verified by MS-MALDI and ^{31}P NMR (Table 1).



1, X = Z = Y = S; R = OAde
2, X = S; Z = Y = O; R = OH
3, X = Y = S; Z = O; R = OAde
4, X = S; Z = Y = O; R = OAde
7, X = Z = Y = O; R = OAde

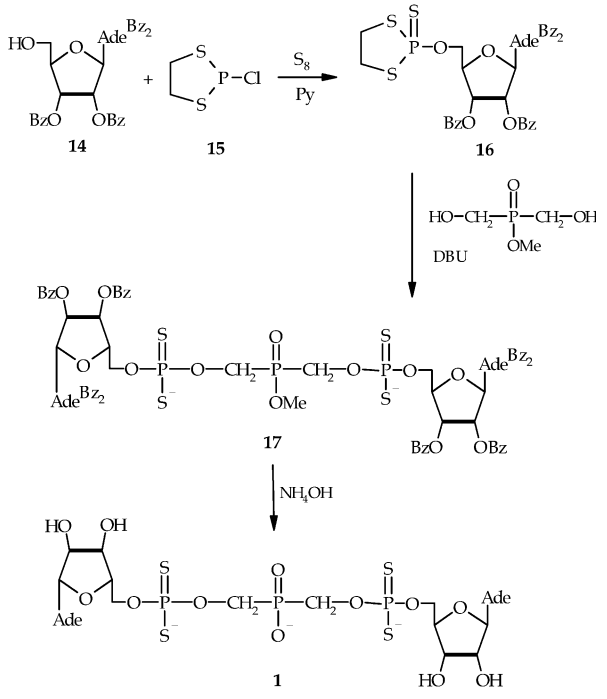
Figure 1.



5, X = S; Z = O; Y = OCH_2; R = CH(OH)CH(OH)
6, X = S; Z = O; Y = OCH(COOH)
8, X = S; Z = O; Y = OCH_2; R = CH(OH)
9, X = S; Z = O; Y = OCH_2; R = $CHOP(S)(OH)_2$
10, X = S; Z = O; Y = OCH_2; R = $C[CH_2OP(S)(OH)OAde]_2$
11, X = Z = S; Y = OCH_2; R = CH(OH)
12, X = S; Z = O; Y = $NHCH_2$; R = CH_2
13, X = S; Z = O; Y = OCH_2; R = CHOP(S)(OH)OAde

Figure 2.

Aggregation test was performed with the recently published method involving microplate reader.[19] Blood was drawn from healthy human volunteers who were not medicated by any drug during at least 2 weeks prior to the examination. Informed consent was obtained from each donor and the study was performed according to the Helsinki Declaration. Blood was anticoagulated with 3.8% sodium citrate added in 1:9 ratio. Freshly collected human citrated blood was subjected to centrifugation (200*g*, 10 min, rt), and platelet-rich plasma



Scheme 1.

(PRP) was used for the aggregation test. Platelet-poor plasma (PPP) was obtained by subsequent centrifugation of PRP at room temperature for 20 min at 1000*g*. The instrument used for aggregation test (EL340 Bio-Tek Instruments) could maintain temperature 37 °C and continuously shake (forth and back) the microplate between readings (20 s). Readings were performed at 595 nm and repeated every 30 s. The results of reading were stored in computer memory for further evaluation. Flat-bottom 96-well Costar microplates were used. The studied analogues and AMP (Sigma Aldrich), dissolved in phosphate buffer (pH 7.4), were prepared as series of dilutions in a microplate (20 μL of each), and to each sample dilution 100 μL of PRP were dispensed. Following tender shaking, samples were preincubated for 30 min at rt, and then 20 μL of ADP (final concentration 5 μM) were injected into each well, shaken and observations of aggregation process were started. For a control, some wells were processed in the presence of 20 μL of phosphate buffer instead of the studied sample, whereas others were filled with PPP alone.

Table 1. The physicochemical characteristics of compounds **1**–**13** and their IC_{50} values

Compd	^{31}P NMR (ppm)	MALDI-MS (M−1) *m/z*	IC_{50} (μM)
1	116.7; 30.7	847	10
2	59.5; 44.9	566	13
3	58.5; 30.3	815	16
4	58.3; 30.3; 2.1	799	40
5	57.1	812	70
6	58.8; 57.7	840	75
7	30.5; 1.7	783	80
8	56.8	782	140
9	56.6; 45.9	877	>160
10	56.3	1516	>160
11	115.9	814	—
12	60.2; 58.5	1125	—
13	57.6	1127	—

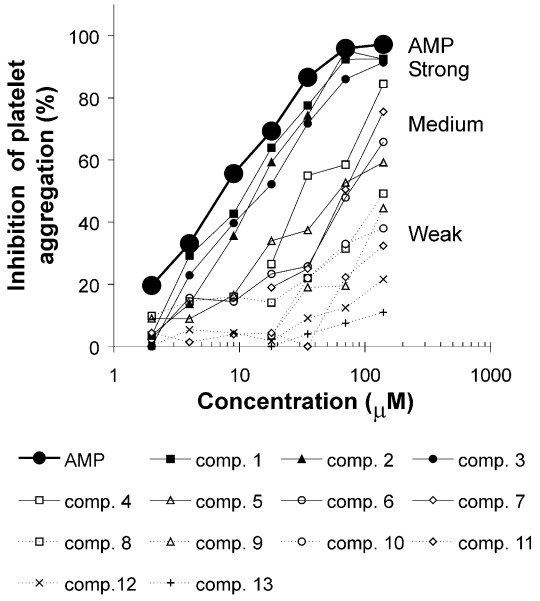


Figure 3. The effect of Ap_4A analogues on platelet aggregation triggered by ADP. Increasing concentrations of the analogues were added to platelet-rich plasma and preincubated for 30 min at room temperature in flat-bottom 96-well Costar microplates. Then, 20 μL of ADP (final concentration 5 μM) were injected into each well, shaken and observation of aggregation process, using EL340 BioTek Instruments, was continued for 10 min with readings performed at 595 nm every 30 s.

In this study, 13 analogues of Ap_4A with chemical structures given in Figures 1 and 2 were synthesized as described above and tested in terms of their inhibitory effects on ADP-induced platelet aggregation. Compounds **2**–**5**, **8**–**10**, **12** and **13** were studied as unseparated mixtures of P-diastereomers. In parallel adenosine 5′-monophosphate (AMP) was used as a positive control. All compounds were used as sodium salts.

We have found three analogues showing very strong potency for inhibition of blood platelet aggregation evoked in PRP by ADP (Fig. 3), comparable with that elicited by AMP. Assigned IC_{50} values for each compound are given in Table 1.

In our tests compounds **1**–**3** were able to inhibit by 50% aggregation process if used at 10–15 μM concentration. In the same experiments 50% inhibition was observed for 7 μM AMP. The next four compounds **4**–**7** exhibited medium inhibitory potency with 50% inhibition observed for concentrations ranging from 40 to 80 μM. The last group consisted of six compounds **8**–**13** with low or no observable inhibitory potency in our test. Compounds **1**, **3** and **4** can be considered as isosteric analogues of Ap_4A; adenosyl moieties are separated by seven atoms, albeit they are not isoelectric, since on the contrary with Ap_4A they possess three formal negative charges. An important feature of their structure is the lack of pyrophosphoryl linkages, an important element of their chemical stabilization and resistance to nucleolytic enzymes, that potentially promotes them as orally administered drugs. Conservation of the distance between adenosyl moieties and three negative charges is salutary for occupation of active site of biological entity they interact with, since compounds **5** and **6** with eight atom spacers are less active. Some years ago it has been reported that adenosine-5′-phosphorothioate also inhibited platelet aggregation.[20] It should be emphasized that key structural motif in the most active compounds is the sulfur atom replacing one or both non-bridging oxygens at phosphorus bound to adenosyl residues and hydroxymethyl groups of bis(hydroxymethyl)phosphinic acid. Our best inhibitor, compound **1**, contains two phosphorodithioate functions within a seven atom scaffold, which additionally stabilize that compound against nucleolytic enzymes. The high avidity of phosphorodithioates incorporated in oligonucleotide constructs towards protein has been reported.[21]

That consistent picture suggesting interaction of our compounds with P_2T receptor is not perturbed by compound **2,** bearing only one adenosyl residue attached to a seven atom scaffold $P(X,Y^-)OCH_2P(O,O^-)CH_2OP(X,Y^-)$ (where the X and Y are both sulfur atoms, or sulfur and oxygen, respectively) and demonstrating high inhibitory effect to platelet aggregation. P_2T purinoceptor,

serving as binding site for ADP, requires the presence of only one adenosyl residue for binding and recognition, and therefore can be competitively occupied by a compound like **2**, albeit that highly speculative explanation requires further studies. In summary, in this communication we present a novel class of compounds with high potency of inhibition of platelet aggregation process. They are easily available due to methodology elaborated in this Laboratory, and of highest activity compound **1**, as a P-achiral species, does not require separation into diastereomers.

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References and Notes

1. Horowith, H. I.; Bronx, N. J. *Arch. Int. Med.* **1970**, *12*, 823.
2. Becker, R. C. *Cardiology* **1999**, *79*, 49.
3. Holmsen, H. *J. Clin. Invest. Eur.* **1994**, *24*, 3.
4. Lages, B.; Weiss, H. J. *Thromb. Haemost.* **1980**, *43*, 147.
5. McAdam, B.; Keimowitz, R. M.; Maher, M.; Fitzgerald, D. J. *J. Pharmacol. Exp. Ther.* **1996**, *277*, 559.
6. McLane, M. A.; Gabbeta, J.; Rao, A. K.; Beviglia, L.; Lazarus, R. A.; Niewiarowski, S. *Thromb. Haemost.* **1995**, *74*, 1316.
7. Topol, E. J.; Easton, J. D.; Amarenco, P.; Califf, R.; Harrington, R.; Graffagnino, C.; Davis, S.; Diener, H. C.; Ferguson, J.; Fitzgerald, D.; Shuaib, A.; Koudstaal, P. J.; Theroux, P.; Van-de-Werf, F.; Willerson, J. T.; Chan, R.; Samuels, R.; Ilson, B.; Granett, J. *Am. Heart J.* **2000**, *139*, 927.
8. Gachet, C.; Cazenave, J. P. *Nouv. Rev. Fr. Hematol.* **1991**, *33*, 347.
9. Kunapuli, S. P. *Trends Pharmacol. Sci.* **1998**, *19*, 391.
10. Mahaut-Smith, M. P.; Eninion, S. J.; Rolf, M. G.; Evans, R. J. *Br. J. Pharmacol.* **2000**, *131*, 108.
11. Rolf, G. M.; Brearley, C. A.; Mahaut-Smith, M. P. *Thromb. Haemost.* **2001**, *85*, 303.
12. Sage, S. O.; MacKenzie, A. B.; Jenner, S.; Mahaut-Smith, M. P. *Prost. Leukot. Essent. Fatty Acids* **1997**, *57*, 435.
13. Gachet, C. *Thromb. Haemost.* **2001**, *86*, 222.
14. Chan, S. W.; Gallo, S. J.; Kim, B. K.; Guo, M. J.; Blackburn, G. M.; Zamecnik, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4034.
15. Zamecnik, P. C.; Kim, B. K.; Guo, M. J.; Taylor, G.; Blackburn, M. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2370.
16. Guga, P.; Koziolkiewicz, M.; Okruszek, A.; Stec, W. J. In *Applied Antisense Oligonucleotide Technology*; Stein, C., Krieg, A., Eds.; John Wiley and Sons: New York, 1998; p. 23.
17. Baraniak, J.; Wasilewska, E.; Korczynski, D.; Stec, W. J. *Tetrahedron Lett.* **1999**, *40*, 8603.
18. Varnum, J. M.; Baraniak, J.; Kaczmarek, R.; Stec, W. J.; Brenner, C. *BMC Chemical Biology* **2001**, *1*, 3 (www.biomedcentral.com/1472-6769/1/3).
19. Walkowiak, B.; Kesy, A.; Michalec, L. *Thromb. Res.* **1997**, *87*, 95.
20. Gough, G. R. *J. Med. Chem.* **1978**, *21*, 520.
21. Yang, X.-B.; Fennewald, S.; Luxon, B. A.; Aronson, J.; Herzog, N. K.; Gorenstein, D. G. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3357.