European Journal of Medicinal Chemistry 54 (2012) 202–209

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and *in vitro* stability of nucleoside 5′-phosphonate derivatives

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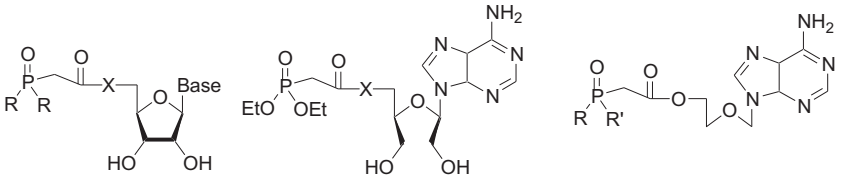
HIGHLIGHTS

- A class of nucleoside derivatives was synthesized.
- Three derivatives were identified as weak inhibitors of platelets aggregation.
- Two derivatives showed high stability in human plasma and in rat liver homogenate.

GRAPHICAL ABSTRACT



Base= Adenine, Guanine, Inosine; X= O, NH; R, R'= OH, OEt

ARTICLE INFO

Article history:
Received 3 February 2012
Received in revised form
27 April 2012
Accepted 30 April 2012
Available online 15 May 2012

Keywords:
Nucleoside 5′-phosphonate derivatives
Pharmacokinetics

ABSTRACT

Nucleoside derivatives are largely synthesized and tested to investigate their influence on platelet aggregation. It's well known that P2Y receptors play an important role in the regulation of platelet function and, as consequence, in controlling atherothrombotic events. The research of compounds that antagonize $P2Y_1$ and, in particular, $P2Y_{12}$ receptors is of great interest in the aim to obtain platelet aggregation inhibitors that are effective in the prevention and treatment of arterial thrombosis. In this study we present the synthesis and *in vitro* metabolic stability in human blood and rat liver homogenate of a new class of nucleoside derivatives, in particular 5′-phosphonate adenosine, inosine, guanosine and thioadenosine analogues also modified at the ribose moiety. On the basis of the results obtained we can hypothesize compounds **4** and **18** to have *in vivo* a relatively high stability.

1. Introduction

Platelets have a crucial role in the maintenance of normal haemostasis, and perturbation of this system can lead to pathological thrombus formation and vascular occlusion, resulting in stroke, myocardial infarction and unstable angina [1] which are the most common cause of morbidity and mortality in Western Word [2]. Intervention in the process of thrombus formation has long been an attractive therapeutic target for the treatment or prophylaxis of such events, and many approaches, centred on the various components of the process, have been investigated [3–7]. It is currently known that ADP released from damaged vessels and red blood cells is a key stimulus inducing platelets activation and aggregation through its action on two G protein-coupled receptor subtypes, $P2Y_1$ and $P2Y_{12}$ [8].

Synergistic activation of both receptors is required to induce ADP-mediated platelet aggregation [3–5]. Modulation of P2Y receptor in platelets appears to be of primary importance in regulating platelet function and, as consequence, in controlling thrombotic disease [9]. Thus, both $P2Y_1$ and $P2Y_{12}$ receptors constitute targets for antithrombotic therapy, antagonizing each subtype separately has antithrombotic effects. $P2Y_{12}$ is the target of platelet aggregation inhibitors that are effective in the prevention and treatment of arterial thrombosis [10]. Indeed many agents currently on the market (such as ticlopidine, clopidogrel, prasugrel, ticagrelor) target the $P2Y_{12}$ receptor. The thienopyridines (ticlopidine,

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doi:10.1016/j.ejmech.2012.04.045

clopidogrel, and prasugrel) inactivate the $P2Y_{12}$ receptor irreversibly via covalent binding of an active metabolite generated in the liver, whereas ticagrelor acts as a competitive antagonist [11].

Oral antiplatelet therapy with platelet $P2Y_{12}$ antagonists, primarily the thienopyridine clopidogrel, is a major strategy for preventing cardiovascular events in patients with ACS and those undergoing percutaneous coronary intervention [12–17]. Limitations of clopidogrel include the requirement for metabolic conversion to its active metabolite, and irreversible binding of the active metabolite to the $P2Y_{12}$ receptor [18–26]. The new thienopyridine prasugrel is metabolized to its active form more efficiently than clopidogrel and produces greater levels of platelet inhibition [27,28]. Unlike thienopyridines, ticagrelor does not require metabolic conversion to an active form; the parent compound and AR-C124910XX (its main active metabolite) have a similar potency in inhibiting the $P2Y_{12}$ receptor [29,30].

Despite recent advances in the treatment of ACS, including dual antiplatelet therapy with aspirin and a thienopyridine during the acute phase and for secondary prevention, this condition remains a leading cause of morbidity and mortality [31]. The limitations of the currently available antiplatelet agents have triggered the development of newer drugs.

Early structure–activity studies on the effects of ADP analogues on human platelets showed that the introduction of substituents, such as alkylthio groups, at the 2-position of the adenine base improved potency, while modifications of ribose sugar or diphosphate chain reduced it [32–34]. On the other hand di- and triphosphates analogues of 2-phenylethynyladenosine appear able to inhibit platelet aggregation; this behaviour may be associated to the lacking of the 2-alkylthio moiety (PEAdo derivatives, Fig. 1) [35].

Xu et al. have focused on a 2-chloro-N^6-methyladenine-9-(2-methylpropyl) scaffold (MRS2395 is the most representative of the series, Fig. 1), characterized by the lack of the sugar portion among the ATP analogues, for the design of P2Y receptors-based inhibitors of platelet aggregation [36].

Douglass et al. have studied the structure–activity relationships of modified mono- and dinucleotides at $P2Y_{12}$, and identified lipophilic modifications to the ribose and base moieties that imparted potent, selective, and reversible antagonist properties at this receptor. This work led to the discovery of INS50589, an adenosine monophosphate derivative with 2′,3′-cyclic acetal and N^6-urea (Fig. 1) [37,38].

Other compounds have been reported to act as antagonist of the $P2Y_{12}$ receptor. In particular, it was reported that 6-amino-2-mercapto-3H-pyrimidin-4-one can be used as lead for the synthesis of ligands for $P2Y_{12}$ receptors on platelets (Fig. 1). This nucleus represents a simplified combination of the active metabolite of the thienopyridines and the ATP derivatives [35].

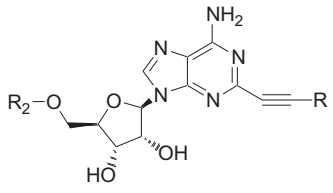
Bases on the above observations about the SAR of the agonist and antagonist of P2Y receptors, we have decided to investigate, in the present study, a series of adenosine, inosine, guanosine and thioadenosine nucleotide derivatives, selected from our previously studies [39–44], and to synthesize new derivatives from these remarks. In particular, we have explored the possibility to obtain a new class of stable nucleotide analogues endowed with isosteric substitution of the diphosphate group by substitution of the diphosphate moiety of natural substrates with phosphonoacetic acid ester and amide moieties. We have also explored the presence of acyclic ribose equivalent and the absence of the ribose moiety.

Pharmacological data on their effect on human platelet aggregation have shown that only compounds **4**, **18** and **33** seem to be able to inhibit the platelet aggregation (see below). We therefore decided to focus our study on the *in vitro* stability of the three compounds in human whole blood and rat liver homogenate to evaluate their stability in physiological environments.

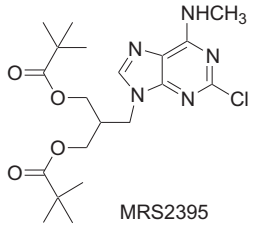
2. Results and discussion

2.1. Chemistry

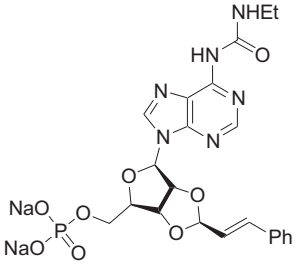
Synthesis of 5′-*O*-phosphonylacetyladenosine (**4**), 5′-*O*-[(diethyl)phosphonoacetyl]-adenosine (**5**), (Diethoxy-phosphoryl)-acetic acid 2-[1-(6-amino-purin-9-yl)-2-hydroxy-ethoxy]-3-hydroxy-propyl ester (**6**), 5′-deoxy-5′-[(phosphonoacetyl)amino]-adenosine (**11**), 5′-deoxy-5′-[[(diethoxyphosphinyl)acetyl]amino]-adenosine (**12**), ({2-[1-(6-Amino-purin-9-yl)-2-hydroxy-ethoxy]-3-hydroxy-propylcarbamoyl}-methyl)-phosphonic acid diethyl ester



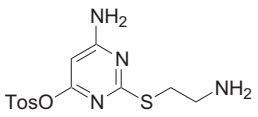
PeAdo derivatives R=C_6H_5
R_2= diphosphate (IC_{50} 60 μM)
triphosphate (IC_{50} 85 μM)



MRS2395
(inhibits ADP-induced aggregation in both rat, K_I 3.6 μM, and human platelets)



INS50589 (IC_{50} 16 nM)



4-Pyrimidinol, 6-amino-2-[(2-aminoethyl)thio]-,
4-(4-methylbenzenesulfonate)
(reduced the response to the agonist 2-methylthio-ADP with an IC_{50} of 8.08 μM)

Fig. 1. Representative examples of $P2Y_{12}$ antagonists.

(**13**), (Diethoxy-phosphoryl)-acetic acid 2-(6-amino-purin-9-ylmethoxy)-ethyl ester (**17**), (Ethoxy-hydroxy-phosphoryl)-acetic acid 2-(6-amino-purin-9-ylmethoxy)-ethyl ester (**18**), and 5′-*O*-(diethyl)phosphonacetyl-8-methyltioadenosine (**21**) is shown in Scheme 1. Adenosine was treated with perchloric acid in dry acetone to obtain an intermediate with the hydroxyl group in 2′ and 3′ position protected. After protection of the N in position 6 with 4,4′-dimethoxytrityl chloride, the reaction with diethylphosphonoacetic acid gave an intermediate that was treated with bromotrimethylsilane to give the expected compound **4** [45], and with trifluoroacetic acid to give compound **5** [43].

Final treatment of **5** with sodium metaperiodate and sodium triacetoxy borohydride gave the desired **6**.

The synthesis of the analogues **11** [42], **12** [42], and **13**, started by tosylation of the 5′-hydroxyl group of Adenosine protected in 2′ and 3′ position and at N6 as described above, with tosyl chloride to give an intermediate subsequently treated with NaN_3 and then reduced with C/Pd under hydrogen atmosphere. The intermediate obtained by reaction with diethylphosphonoacetic acid, was treated with bromotrimethylsilane to give **11** and with trifluoroacetic acid to give **12**, that was treated with sodium metaperiodate and subsequently with sodium triacetoxy borohydride to give the compound **13**.

Adenosine treated with saturated bromine-water gave intermediate **19** [46], subsequently reacted with sodium methanethiolate [47] and finally with diethylphosphonacetic acid gave the desired **21**.

The intermediated **15** [48] obtained by reaction of **14** [49] with 6-chloropurine was treated with saturated methanolic ammonia [50] and then with diethylphosphonacetic acid to give the desired **17**, then treated with bromotrimethylsilane to obtain compound **18**.

Inosine derivatives 5′-*O*-[(diethyl)phosphonoacetyl]-inosine (**24**), 5′-deoxy 5′-*N*-phosphonylmethylinosine (**29**), 5′-deoxy-5′-*N*-[(diethyl)phosphonoacetyl]-inosine (**30**), and Guanosine derivatives 5′-*O*-[(diethyl)phosphonoacetyl]-guanosine (**33**), 5′-(phosphonoacetate)-guanosine (**34**) [51], 2-isobutylamide-5′-*O*-[(diethyl)phosphonoacetyl]-guanosine (**38**), and 2-isobutylamide-5′-*O*-phosphonylacetylguanosine (**39**) are shown in Scheme 2 and were obtained according to the procedures described above.

2.2. Pharmacologicgal data

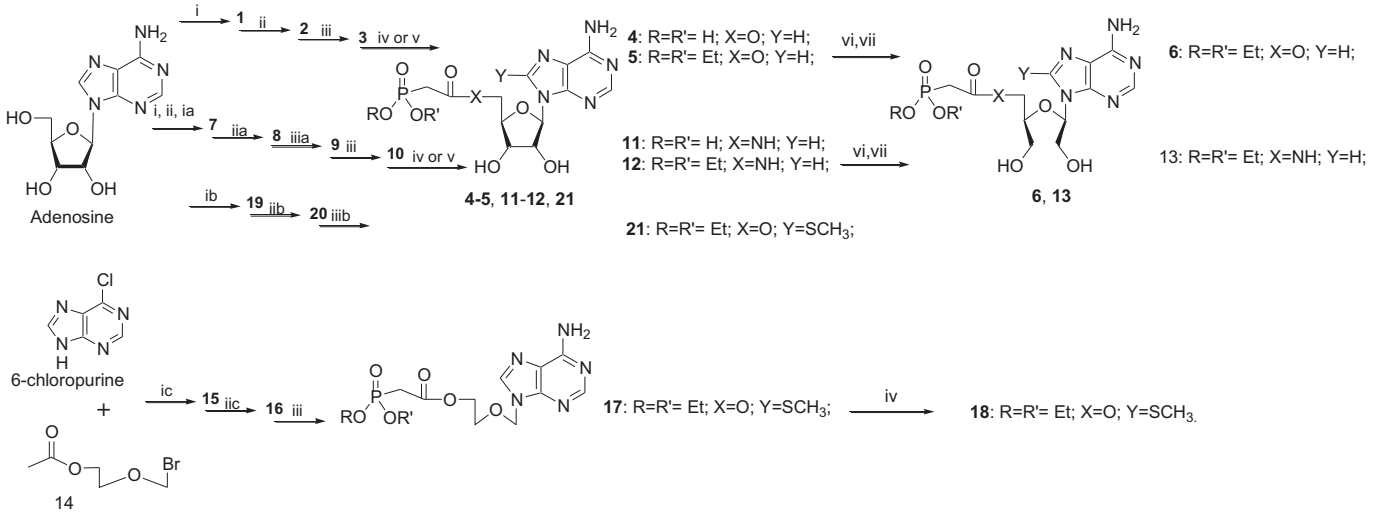
All the newly synthesized compounds were assayed *in vitro* for their activity on the ADP induced aggregation of human platelet-rich plasma (PRP) using light transmission aggregometry. All the compounds were tested at a single concentration (10^{-4} M) throughout the experiments. None of the compounds induced aggregation of human platelets. Among the tested compounds **4**, **18**, and **33** were found able to inhibit the platelet aggregation induced by ADP up to 70% (Fig. 2).

2.3. In vitro metabolism

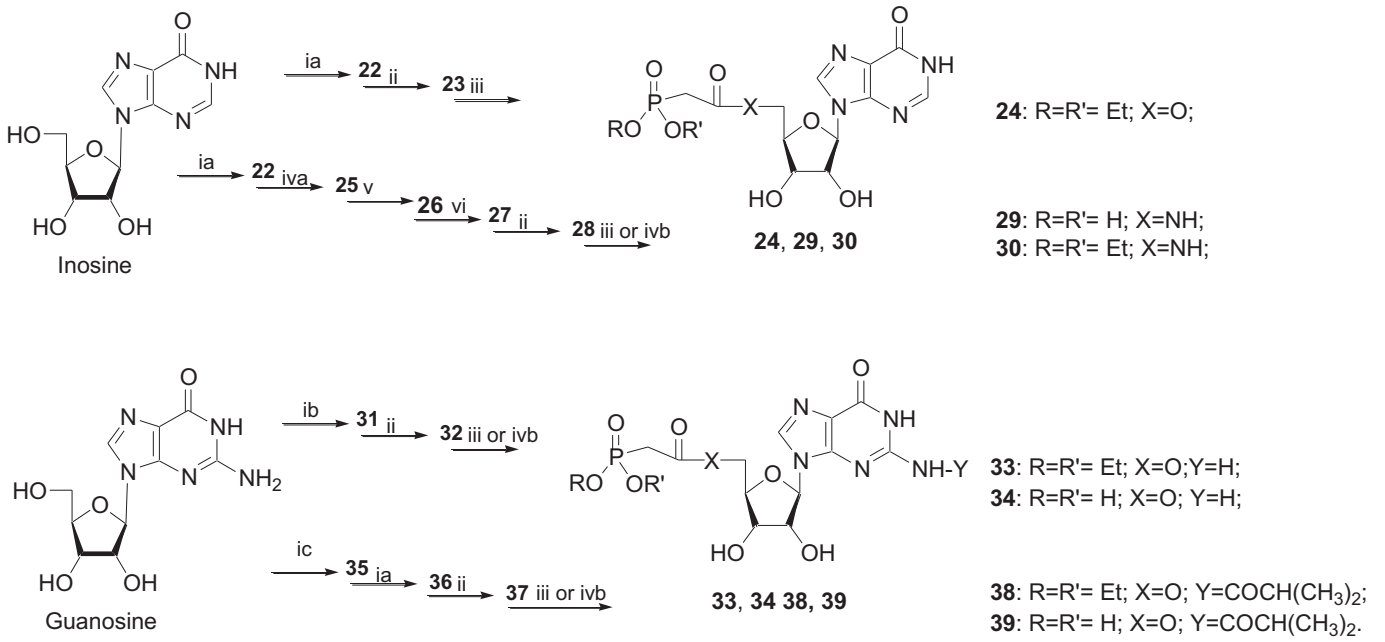
The compounds **4**, **18**, and **33** are characterized by the presence of a 5′-*O*-phosphono acetyl function that can potentially be hydrolysed in physiologic environments [52]. The stability of these compounds has been therefore tested *in vitro*, by evaluating their degradation patterns in human whole blood and rat liver homogenates. As reported in Fig. 3 the inhibitors **4** and **18** were not significantly degraded in human blood during 6 h of incubation at 37 °C, whereas a relatively weak degradation (about 20%), following a first order kinetic, has been registered for compound **33**.

Fig. 4 evidences that this last compound is degraded also in rat liver homogenates, following a first order kinetic, with a half-life value of 2.33 ± 0.25 h. In particular, the degradation of compound **33** was about the 80% after 6 h; on the other hand any or relatively weak degradation was registered for compounds **4** and **18**, respectively.

On the basis of these results we can hypothesize these last compounds to have *in vivo* a relatively high stability. A lower stability can be instead supposed for the derivative **33**, that appears also to be the weaker inhibitor among the three compounds analysed. We have hypothesized that the instability of the derivative **33** could be due to the hydrolysis of the 5′-*O*-phosphono acetyl function, allowing to obtain guanosine as metabolite. As a consequence, we have tested the stability of guanosine in rat liver homogenates and founded that its degradation is very fast, following a first order kinetic with a half-life value of 14.5 ± 1.0 s (Fig. 4). This means that guanosine, if derived by hydrolysis of **33**, cannot be detected in our



Scheme 1. Synthesis of the adenosine derivatives **4**, **5**, **6**, **11**, **12**, **13**, **17**, **18** and **21**. Reagents and conditions: i) $HClO_4$ 60%, dry acetone; ii) TMSCl, 4,4′-dimethoxytrityl chloride, pyridine, r.t.; iii) Diethylphosphonacetic acid, DMAP, DCC, CH_2Cl_2 or DMF; iv) TMSBr, CH_2Cl_2; v) TFA 50%; vi) $NaIO_4$, MeOH, H_2O, r.t.; vii) $Na(AcO)_3BH$, AcOH, r.t; ia) Tosyl chloride, DMAP, CH_2Cl_2, r.t.; iia) NaN_3, DMF; iiia) H_2, C/Pd, MeOH; ib) Saturated bromine-water, NaOAc buffer pH4, 47 h, r.t.; iib) $NaSCH_3$, DMF, 12 h, r.t.; ic) DMF, TEA, 0 °C for 2 h, r.t. for 12 h; iic) NH_3/MeOH, 150 °C.



Scheme 2. Synthesis of the derivatives **24**, **29**, **30**, **33**, **34**, **38**, and **39**. Reagents and conditions: ia) Dimethxypropane, *p*-toluenesulfonic acid monohydrate, DMF; ib) $HClO_4$ 60%, dry acetone; ic) 1) TMSCl, pyridine, 2) Isobutyryl chloride, 3) NH_4OH conc.; ii) Diethylphosphonacetic acid, DMAP, DCC, CH_2Cl_2 or DMF; iii) TFA 50%; iva) Tosyl chloride, DMAP, pyridine, 40 °C; ivb) TMSBr, CH_2Cl_2; v) NaN_3, DMF; vi) H_2, C/Pd, MeOH; ivb) TMSBr, CH_2Cl_2.

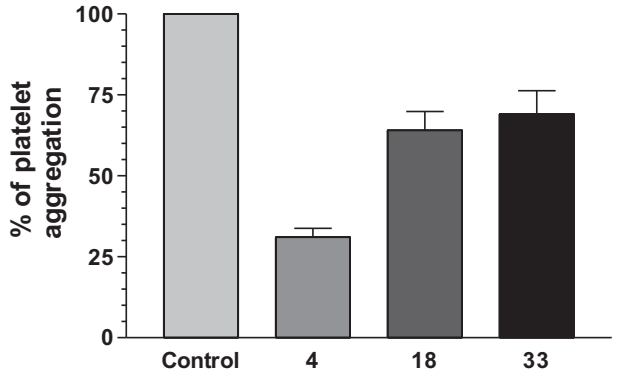


Fig. 2. Effect of compound **4**, **18**, and **33** on platelet aggregation induced by ADP.

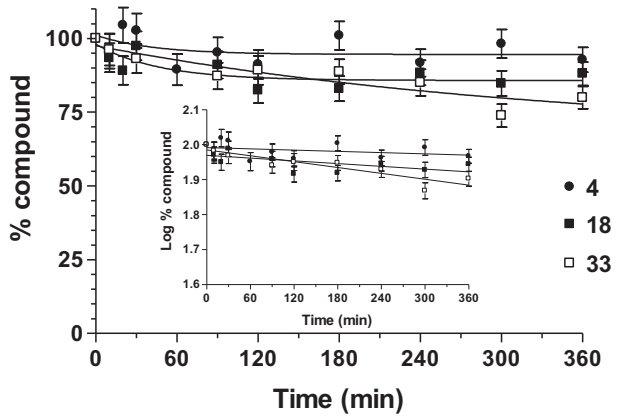


Fig. 3. Degradation time-courses of compounds **4**, **18**, and **33** in human whole blood. Only compound **33** showed a significant degradation (about 20% within 6 h) following a first order kinetic, confirmed by the semilogarithmic plot reported in the inset ($n = 9$, $r = 0.904$, $P < 0.001$). The compounds **4** and **18** were not significantly degraded within 6 h, accordingly the linear regressions of their semilogarithmic plots were not significant ($n = 10$, $r < 0.61$, $P > 0.061$). Data are expressed as the mean ± SD of three independent experiments.

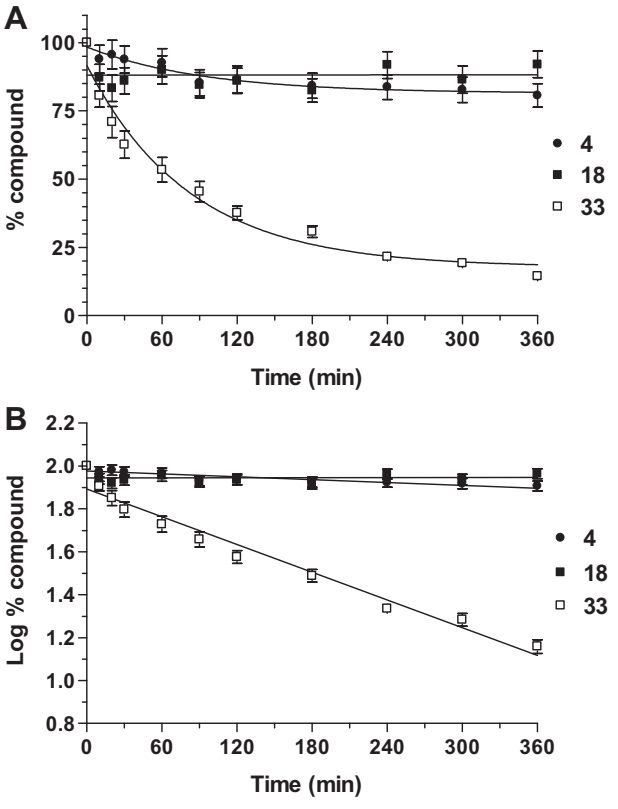


Fig. 4. Degradation time-courses of compounds **4**, **18**, and **33** in rat liver homogenates (A). Compound **33** showed a significant degradation following a first order kinetic, confirmed by the semilogarithmic plot (B) ($n = 11$, $r = 0.983$, $P < 0.0001$) and its half-life was calculated to be 140 ± 15 min. The compound **4** showed a weak but significant degradation (about 20% within 6 h) following a first order kinetic, confirmed by the semilogarithmic plot (B) ($n = 11$, $r = 0.904$, $P < 0.001$). Compound **18** was not significantly degraded within 6 h, accordingly the linear regression of its semilogarithmic plot (B) was not significant ($n = 11$, $r = 0.027$, $P = 0.94$). Data are expressed as the mean ± SD of three independent experiments.

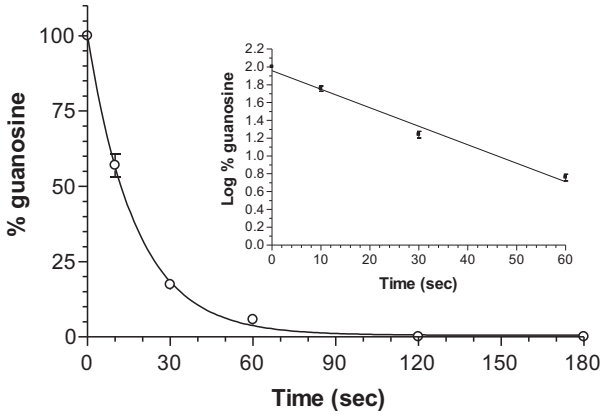


Fig. 5. Degradation time-course of guanosine in rat liver homogenates. This compound showed a relatively fast degradation following a first order kinetic, confirmed by the semilogarithmic plot in the inset ($n = 4$, $r = 0.993$, $P < 0.01$) and its half-life was calculated to be 14.5 ± 1.0 s. Data are expressed as the mean ± SD of three independent experiments.

experimental conditions. We have therefore verified that guanosine was degraded to guanine in rat liver homogenates, being the guanine amounts increased during guanosine degradation (data not shown). Compound **33** was the only one, among those analysed, able to induce the guanine formation in rat liver homogenates during its degradation. To support our data concerning the hydrolysis of **33** in rat liver homogenates, we have observed that this compound differs from **4** and **18** adenosine derivatives by the presence of an ethyl-radical at all the phosphonic hydroxyl groups. Thus, at physiologic pH values the compounds **4** and **18** are negatively charged on the phosphonic group, whereas the derivative **33** is not. It has been reported that some hydrolase activities can be sensibly hindered by the presence of negative charges on the substrates, as demonstrated, for instance, in the case of butyrylcholinesterase [53,54]. Also in our case the hydrolysis of the 5′-*O*-phosphono acetyl function could be possible only in the absence of negative charges. We have recently observed a similar phenomenon also with a novel conjugated molecule between dopamine and an A_{2A} adenosine receptor antagonist, were the presence of negative charges inhibited hydrolysis phenomena in physiologic environments [55] (Fig. 5).

3. Conclusion

The synthesis of a series of adenosine, inosine, guanosine and thioadenosine nucleoside derivatives obtained by substitution of the diphosphate moiety of native compounds with phosphonoacetic acid ester led to the identification of the derivatives **4**, **18**, and **33** identified as weak inhibitors of platelets aggregation induced by ADP. Compounds **4** and **18** showed high stability in human plasma and in rat liver homogenate while compound **33** undergoes degradation in human plasma and rat liver homogenates with different rates. Thus **4** and **18** may represent interesting lead candidate for the development of inhibitors of platelets aggregation.

4. Experimental

4.1. General

Chemicals and solvents were purchased from Sigma–Aldrich and Carlo Erba reagenti (Italia). The molecular weights of the compounds were determined by ESI (MICROMASS ZMD 2000), and the values are expressed as $[MH]^+$. TLC was performed on pre-coated plates of silica gel Macherey–Nagel durasil-25. Nuclear magnetic resonance spectra were determined in DMSO-d_6, and D_2O and solution with a Varian VXR-200 MHz spectrometer or Varian MERCURY*plus* 400 MHz and chemical shifts are presented in ppm from internal tetramethylsilane as a standard. HPLC analysis was performed using an Agilent1100 Series HPLC System equipped with a G1315A DAD and with an Hydro RP18 Sinergi 80A column (4.6 × 150 mm, 4 μm) from Phenomenex. Male Wistar rats were purchased from Harlan SRC (Milan, Italy).

All other reagents were of analytical grade and obtained from commercial sources.

4.2. Compounds synthesis

4.2.1. N^6-(4,4′-Dimethoxytrityl) -2′,3′-isopropylidene-adenosine (**2**)

To a solution of **1** (1.10 g, 3.57 mmol) in pyridine (32 mL) was added trimethylchlorosilane (TMSCl) (0.762 mL, 5.35 mmol). After 1 h, 4,4-dimethoxytrityl chloride (1.68 g, 5.03 mmol) was added and the reaction mixture was stirred for 1.5 h. After this time, 8 mL of NH3 were added and the solution stirred for others 45 min. Then the reaction was evaporated to dryness and the residue obtained was dissolved in CH_2Cl_2 and washed with $NaHCO_3$ at 5%. The organic phase was dried, filtered and evaporated to give a crude residue purified by chromatography over silica gel (eluent: CH_2Cl_2/MeOH, 98:2→95:5, v/v) to afford desiderated compound **2** (yield 62%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.30, 1.52 (6H, s, $(CH_3)_2C$), 3.45–3.50 (2H, m, H-5′), 3.72 (6H, s, $2OCH_3$), 4.20 (1H, m, H-4′), 4.98 (1H, dd, H-3′), 5.12 (1H, t, $J = 2.8$ Hz, OH-5′), 5.38 (1H, dd, $J = 4.0$ Hz, H-2′), 6.10 (1H, d, $J = 2.8$ Hz, H-1′), 6.83–7.30 (15H, m, Ph), 7.94 (1H, s, H-2), 8.42 (1H, s, H-8). ESI MS: m/z 612.8 Da $[M + H]^+$, $C_{34}H_{37}N_5O_6$ Mol. Wt. 611.69.

4.2.2. General synthetic procedures for the preparation of compounds ***3***, ***17***, ***21***, ***23***, ***28***, ***32***, and ***37***

To a solution of **2** (1.00 g, 1.63 mmol), **16** [50] (0.35 g, 1.67 mmol), **20** [47] (1.6 g, 5.1 mmol), **22** [56] (0.2 g, 0.64 mmol), **27** (0.2 g, 0.65 mmol), **31** [57] (0.3 g, 0.92 mmol), or **36** [58] (0.38 g, 0.95 mmol) in CH_2Cl_2 under Ar or dry DMF, were added diethylphosphonoacetic acid (PPA) and 4-(dimethylamino)pyridine (DMAP). At 0 °C N,N′-dicyclohexylcarbodiimide (DCC) was added and then the reaction mixture was stirred at r.t. for 12 h. After filtration of dicyclohexyl urea (DCU), the solvent was evaporated to dryness.

*4.2.2.1. N^6-(4,4′-Dimethoxytrityl)-2′,3′-isopropylidene-5′-O-[(diethyl) phosphonoacetyl]-adenosine (**3**).* The crude residue was purified by chromatography over silica gel (eluent: CH_2Cl_2/MeOH, 98:2→95:5, v/v) to afford the desiderated compound **3** (yield 54%). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.15–1.25 (6H, m, $2CH_3CH_2O$), 1.35, 1.53 (6H, s, $(CH_3)_2C$), 2.72, 2.88 (2H, s, $J = 21.2$ Hz, CH_2P), 3.71 (6H, s, $2OCH_3$), 4.26–4.39 (3H, m, H-4′, 2H-5′), 5.20 (1H, dd, H-3′), 5.40 (1H, dd, $J = 3.8$ Hz, H-2′), 6.10 (1H, d, $J = 6.0$ Hz, H-1′), 6.81–7.27 (15H, m, Ph), 7.95 (1H, s, H-2), 8.39 (1H, s, H-8). ESI MS: m/z 790.7 Da $[M + H]^+$, $C_{40}H_{48}N_5O_{10}P$ Mol. Wt. 789.81.

*4.2.2.2. (Diethoxy-phosphoryl)-acetic acid 2-(6-amino-purin-9-ylmethoxy)-ethyl ester (**17**).* The resulting residue was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH 95/5 v/v) to afford desiderated compound **17** as white foam (yield 75%). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.19 (6H, t, $J = 8.0$ Hz, $2OCH_2CH_3$), 3.08 (2H, d, $J = 20.0$ Hz, CH_2P), 3.75 (2H, m, CH_2), 4.00 (4H, m, $2OCH_2CH_3$), 4.18 (m, 2H, CH_2O), 5.57 (s, 1H, OCH_2N); 7.30 (2H, br s, NH_2); 8.17 (1H, s, H-2); 8.28 (1H, s, H-8). ESI MS: m/z 388.5 Da $[M + H]^+$, $C_{14}H_{22}N_5O_6P$ Mol. Wt. 387.3.

*4.2.2.3. 5′-O-(diethyl)phosphonacetyl-8-methylthioadenosine (**21**).* The resulting residue was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH 95/5, v/v) to afford desiderated compound **21** (yield 10%). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.2 (6H, t, $2OCH_2CH_3$), 2.70 (3H, s, SCH_3), 3.08 (2H, d, J = 21.4 Hz, CH_2P), 3.95–4.08 (2H, m, H-5′, H-5″), 4.2–4.3 (6H, m, $2OCH_2CH_3$, H-3′, H-4′), 5.1 (1H, m, H-2′), 5.4 (1H, br t, OH3′), 5.58 (1H, br t, OH2′), 5.85 (1H, J = 6.8 Hz, H-1′), 7.25 (2H, br s, NH_2), 8.12 (1H, s, H-2). ESI MS: m/z 492.6 Da $[M + H]^+$, $C_{17}H_{26}N_5O_8PS$ Mol. Wt. 491.46.

*4.2.2.4. 2′,3′-Isopropylidene-5′-O-[(diethyl)phosphonoacetyl]-inosine (**23**).* The resulting residue was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH 98/2→95/5, v/v) to afford desiderated compound **23** (yield 70%). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.15–1.22 (6H, m, $2CH_3CH_2O$), 1.32, 1.54 (6H, s, $(CH_3)_2C$), 3.06, 3.17 (2H, s, J = 22 Hz, CH_2P), 4.37–4.39 (4H, m, $2OCH_2CH_3$), 4.96 (1H, m, H-3′), 5.39 (1H, dd, J = 3.7 Hz, H-2′), 6.16 (1H, d, J = 2.0 Hz, H-1′), 8.09 (1H, s, H-2), 8.29 (1H, s, H-8), 12.44 (1H, br s, NH-3). ESI MS: m/z 487.3 Da $[M + H]^+$, $C_{19}H_{27}N_4O_9P$ Mol. Wt. 486.41.

*4.2.2.5. 2′,3′-Isopropylidene-5′-deoxy-5′-N-[(diethyl)phosphonoacetyl]-inosine (**28**).* The resulting residue was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH 98/2→95/5, v/v) to afford desiderated compound **28** (yield 94%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.18–1.22 (6H, m, $2CH_3CH_2O$), 1.23, 1.52 (6H, m, $(CH_3)_2C$), 2.85–2.93 (2H, m, CH_2P), 3.93 (2H, m, H-5′), 3.97–4.04 (4H, m, $2OCH_2CH_3$), 4.16–4.17 (1H, m, H-4′),4.87 (1H, dd, J = 3.2 Hz, H-3′), 5.34 (1H, dd, H-2′), 6.07 (1H, d, J = 2.8 Hz, H-1′), 8.10 (1H, s, H-2), 8.21 (1H, t, NH-5′), 8.35 (1H, s, H-8), 12.47 (1H, br s, NH-3). ESI MS: m/z 486.3 Da $[M + H]^+$, $C_{19}H_{28}N_5O_8P$ Mol. Wt. 485.43.

*4.2.2.6. 2′,3′-Isopropylidene-5′-O-[(diethyl)phosphonoacetyl]-guanosine (**32**).* The resulting residue was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH 95/5, v/v) to afford desiderated compound **32** (yield 63%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.16–1.20 (6H, m, $2CH_3CH_2O$), 1.24–1.51 (6H, m, $(CH_3)_2C$), 3.10–3.16 (2H, s, J = 24 Hz, CH_2P), 3.98–4.02 (4H, m, $2OCH_2CH_3$), 4.03–4.32 (3H, m, H-4′, 2H-5′), 5.11 (1H, dd, J = 3.2 Hz, H-3′), 5.24 (1H, dd, J = 3.8 Hz, H-2′), 6.01 (1H, d, J = 2.0 Hz, H-1′), 6.50 (2H, br s, NH_2), 7.89 (1H, s, H-8), 10.60 (1H, br s, NH-3). ESI MS: m/z 502.3 Da $[M + H]^+$, $C_{19}H_{28}N_5O_9P$ Mol. Wt. 501.43.

*4.2.2.7. 2′,3′-Isopropylidene-2-isobutylamide-5′-O-[(diethyl)phosphonoacetyl]-guanosine (**37**).* The resulting residue was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH 95/5, v/v) to afford desiderated compound **37** (yield 86%) as white foam. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.12–1.22 (6H, m, $2CH_3CH_2O$), 1.26, 1.53 (6H, s, $(CH_3)_2C$), 3.08, 3.18 (2H, s, CH_2P), 3.96–4.07 (4H, m, OCH_2CH_3), 4.28–4.32 (3H, m, H-4′, 2H-5′), 5.03–5.16 (1H, m, H-3′), 5.40 (1H, dd, H-2′), 6.10 (1H, d, J = 2.0 Hz, H-1′), 8.22 (1H, s, H-8), 11.50 (1H, br s, NH–CO), 12.12 (1H, br s, NH-3). ESI MS: m/z 572.4 Da $[M + H]^+$, $C_{23}H_{34}N_5O_{10}P$ Mol. Wt. 571.52.

*4.2.3. General synthetic procedures for the preparation of compounds **6** and **13***

A solution of **5** [43] or **12** [42] (2.2 mmol) and sodium metaperiodate (0.48 g, 2.2 mmol) in a mixture of methanol (14.5 mL) and water (4.4 mL) was stirred at room temperature for 1 h. The iodate salts were removed by filtration, and the obtained filtrate was evaporated *in vacuo*. Sodium triacetoxy borohydride or sodium borohydride was slowly added to a stirred solution of the dialdehyde in acetic acid or ethanol. After stirred for 12 h at room temperature the mixture was evaporated to dryness *in vacuo*.

*4.2.3.1. (Diethoxy-phosphoryl)-acetic acid 2-[1-(6-aminopurin-9-yl)-2-hydroxy-ethoxy]-3-hydroxypropyl ester (**6**).* The crude material was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH 98/2→90/10, v/v) to afford desiderated compound **6** as white foam (yield 60%). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.16 (6H, t, $2OCH_2CH_3$), 2.76 (2H, d, J = 20.6 Hz, CH_2P), 3.63–3.54 (7H, m, $2OCH_2CH_3$, H2′, H3′, H4′), 3.97–3.95 (2H, m, H5′, H5″), 4.94 (1H, br t, OH3′), 5.19 (1H, br t, OH2′), 5.85 (1H, t, J = 6.4 Hz, H1′), 7.25 (2H, br s, NH_2), 8.14 (1H, s, H2), 8.29 (1H, s, H8). ESI MS: m/z 450.3 Da $[M + H]^+$, $C_{16}H_{26}N_5O_8P$ Mol. Wt. 449.40.

*4.2.3.2. ({2-[1-(6-Aminopurin-9-yl)-2-hydroxy-ethoxy]-3-hydroxy-propylcarbamoyl}-methyl)-phosphonic acid diethyl ester (**13**).* The obtained residue, solubilized in water, was applied to a carbon column and the column was eluted with water. The product was then eluted with 3% concentrated NH_4OH in 1/1 water/ethanol. Selected fractions were concentrated to give **13** as brown solid (yield 60%). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.18 (6H, m, $2OCH_2CH_3$), 2.60–2.80 (2H, m, CH_2P), 3.90–3.20 (2H, m, H-5′, H-5″), 3.40–3.60 (3H, m, H-3′, H-4′), 3.80–4.00 (6H, m, $2OCH_2CH_3$, H-2′), 4.80 (1H, br s, OH3′), 5.20 (1H, br s, OH2′), 5.85 (1H, t, J = 5.6 Hz, H-1′), 7.25 (2H, br s, NH2), 7.80 (1H, br t, NH), 8.13 (1H, s, H-2), 8.27 (1H, s, H-8). ESI MS: m/z 447.2 Da $[M + H]^+$, 469.4 Da $[M + Na]^+$, $C_{16}H_{27}N_6O_7P$ Mol. Wt. 446.4.

*4.2.4. General synthetic procedures for the preparation of compounds **18**, **29** and **39***

To a solution of **17** (0.125 g, 0.32 mmol), **28** (0.13 g, 0.26 mmol) or **37** (0.09 g, 0.16 mmol) in CH_2Cl_2 (5 mL) was added TMSBr (10 eq). The reaction mixture was stirred at r.t. for 5 h and the solvent was then evaporated *in vacuo*.

*4.2.4.1. (Ethoxy-hydroxy-phosphoryl)-acetic acid 2-(6-aminopurin-9-ylmethoxy)-ethyl ester (**18**).* The residue obtained was purified by reversed-phase chromatography over silica gel (eluent: H_2O) to afford **18** (yield 60%). ^{1}H NMR (200 MHz, D_2O) δ: 1.02 (3H, t, J = 7.2 Hz, OCH_2CH_3), 2.59 (2H, d, J = 20.6 Hz, CH_2P), 3.74 (4H, m, CH_2, OCH_2CH_3), 4.09(2H, m, CH_2), 5.61 (2H, s, OCH_2N), 8.30 (1H, s, H-2), 8.33 (1H, s, H-8). ESI MS: m/z 360.1 Da $[M + H]^+$, $C_{12}H_{18}N_5O_6P$ Mol. Wt. 359.28.

*4.2.4.2. 5′-Deoxy 5′-N-phosphonylmethylinosine (**29**).* The residue obtained was purified by reversed-phase chromatography over silica gel (eluent: H_2O) to afford **29** (yield 40%). ^{1}H NMR (400 MHz, D_2O) δ: 2.58, 2.63 (2H, s, J = 20 Hz, CH_2P), 3.37 (1H, dd, H-5′a), 3.54 (1H, dd, J = 4.8 Hz, J = 14.8 Hz, H-5′b), 4.12–4.16 (3H, m, H-2′, H-3′, H-4′), 5.88 (1H, d, J = 5.6 Hz, H-1′), 8.1 (1H, s, H-2), 8.3 (1H, s, H-8). ESI MS: m/z 390.2 Da $[M + H]^+$, $C_{12}H_{16}N_5O_8P$ Mol. Wt. 389.26.

*4.2.4.3. 2-Isobutylamide-5′-O-phosphonylacetylguanosine (**39**).* To a solution of **37** (0.09 g, 0.16 mmol) in CH_2Cl_2 (2.5 mL) was added TMSBr (0.210 mL, 1.58 mmol). The reaction mixture was stirred at r.t. for 5 h and the solvent was then evaporated *in vacuo*. The residue obtained was purified by reversed-phase chromatography over silica gel (eluent: H_2O) to afford **39** (yield 40%) as white foam. ^{1}H NMR (400 MHz, D_2O) δ: 1.18–1.20 (6H, s, $(CH_3)_2C$), 2.71–2.77 (1H, m, CH), 2.85, 2.90 (2H, s, J = 24.1 Hz, CH_2P), 4.44–4.47 (4H, m, H-3′, H-4′, 2H-5′), 4.73 (1H, t, H-2′), 6.00 (1H, d, J = 6.0 Hz, H-1′), 8.53 (1H, s, H-8). ESI MS: m/z 476.3 Da $[M + H]^+$, $C_{16}H_{22}N_5O_{10}P$ Mol. Wt. 475.35.

*4.2.5. General synthetic procedures for the preparation of compounds **24**, **30**, **33** and **38***

6 mL of TFA 50% in water were added to **23** (0.22 g, 0.43 mmol), **28** (0.2 g, 0.41 mmol), **32** (0.200 g, 0.40 mmol) or **37** (0.200 g,

0.38 mmol). The reaction mixture was stirred at r.t. for 5 h and then the solvent was evaporated *in vacuo* to give an oil.

*4.2.5.1. 5′-O-[(Diethyl)phosphonoacetyl]-inosine (**24**).* The residue obtained was purified by chromatography over silica gel (eluent: CH_2Cl_2/MeOH, 95:5→90:1, v/v) to afford the desiderated compound **24** (yield 92%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.18 (6H, m, 2CH_3CH$_2$O), 3.11, 3.22 (2H, s, J = 22 Hz, CH_2P), 3.97–4.18 (8H, m, H-3′, H-4′, 2H-5′, 2OCH_2CH$_3$), 4.60 (1H, m, H-2′), 5.40 (1H, d, J = 2.6 Hz, OH-3′), 5.60 (1H, d, J = 3.0 Hz, OH-2′), 5.88 (1H, d, J = 2.8 Hz, H-1′), 8.07 (1H, s, H-2), 8.34 (1H, s, H-8), 12.29 (1H, br s, NH-3). ESI MS: m/z 447.2 Da $[M + H]^+$, $C_{16}H_{23}N_4O_9P$ Mol. Wt. 446.35.

*4.2.5.2. 5′-Deoxy-5′-N-[(diethyl)phosphonoacetyl]-inosine (**30**).* The residue obtained was purified by chromatography over silica gel (eluent: CH_2Cl_2/MeOH, 95:5→90:1, v/v) to afford the desiderated compound **30** (yield 77%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.18–1.22 (6H, m, 2CH_3CH$_2$O), 2.86, 2.92 (2H, s, J = 24 Hz, CH_2P), 3.37–3.41 (2H, m, H-5′), 3.90–3.98 (1H, m, H-4′), 4.00–4.05 (5H, m, H-3′, 2OCH_2CH$_3$), 4.57 (1H, t, J = 5.6 Hz, H-2′), 5.83 (1H, d, J = 6.4 Hz, H-1′), 8.09 (1H, s, H-2), 8.12 (1H, t, NH-5′), 8.37 (1H, s, H-8), 12.42 (1H, br s, NH-3). ESI MS: m/z 446.2 Da $[M + H]^+$, $C_{16}H_{24}N_5O_8P$ Mol. Wt. 445.36.

*4.2.5.3. 5′-O-[(Diethyl)phosphonoacetyl]-guanosine (**33**).* The residue obtained was purified by chromatography over silica gel (eluent: CH_2Cl_2/MeOH, 95:5, v/v) to afford the desiderated compound **33** (yield 76%) as white foam. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.17–1.22 (6H, m, 2CH_3CH$_2$O), 2.86, 2.92 (2H, s, J = 24 Hz, CH_2P), 3.99–4.02 (4H, m, 2OCH_2CH$_3$), 4.11 (1H, dd, H-4′), 4.19 (1H, dd, H-5′a), 5.53 (1H, d, J = 6.0 Hz, OH-2′), 4.27 (1H, dd, J = 3.6 Hz, J = 12.0 Hz, H-5′b), 4.46 (1H, dd, H-3′), 5.32 (1H, d, J = 5.2 Hz, OH-3′), 5.70 (1H, d, J = 5.6 Hz, H-1′), 6.48 (2H, br s, NH_2), 7.95 (1H, s, H-8), 10.64 (1H, br s, NH-3). ESI MS: m/z 462.1 Da $[M + H]^+$, $C_{16}H_{24}N_5O_9P$ Mol. Wt. 461.36.

*4.2.5.4. 2-Isobutylamide-5′-O-[(diethyl)phosphonoacetyl]-guanosine (**38**).* The residue obtained was purified by chromatography over silica gel (eluent: CH_2Cl_2/MeOH, 95:5, v/v) to afford the desiderated compound **38** (yield 90%) as white foam. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.11–1.19 (6H, m, (CH_3)$_2$CH), 2.90–2.96 (1H, m, CH), 3.16, 3.21 (2H, s, J = 20 Hz, CH_2P), 3.97–4.03 (4H, m, 2OCH_2CH$_3$), 4.06–4.20 (4H, m, H-3′, H-4′, 2H-5′), 4.54 (1H, t, H-2′), 5.40 (1H, d, J = 3.9 Hz, OH-3′), 5.60 (1H, d, J = 4.3 Hz, OH-2′), 5.90 (1H, d, J = 5.6 Hz, H-1′), 8.28 (1H, s, H-8),11.67 (1H, br s, NH–CO),12.09 (1H, br s, NH-3). ESI MS: m/z 532.3 Da $[M + H]^+$, $C_{20}H_{30}N_5O_{10}P$ Mol. Wt. 531.45.

*4.2.6. 5′-O-Tosyl-2′,3′-isopropylidene-inosine (**25**)*

To a solution of **22** (0.15 g, 0.49 mmol) in pyridine (10 mL) was added DMAP (0.035 g, 0.2 mmol). At 0 °C tosyl chloride (0.122 g, 0.63 mmol) was added and the reaction mixture was stirred at 40 °C overnight. The reaction mixture was washed with HCl 1 N, sat.d aq. $NaHCO_3$ solution and brine. The organic phase was dried, filtered and evaporated to give a crude residue purified by chromatography over silica gel (eluent: CH_2Cl_2/MeOH 98/2→95/5→90/10, v/v) to afford **25** (yield 53%). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.28, 1.50 (6H, s, (CH$_3$)$_2$C), 2.35 (3H, s, CH$_3$-tos), 4.16–4.35 (3H, m, H-4′, 2H-5′), 4.93 (1H, m, H-3′), 5.25 (1H, dd, J = 3.0 Hz, H-2′), 6.14 (1H, d, J = 2.0 Hz, H-1′), 7.27 (2H, d, Ph), 7.60 (2H, d, J = 8.2 Hz, Ph), 7.96 (1H, s, H-2), 8.15 (1H, s, H-8), 12.43 (1H, br s, NH-3). ESI MS: m/z 463.4 Da $[M + H]^+$, $C_{20}H_{22}N_4O_7S$ Mol. Wt. 462.48.

*4.2.7. 5′-Azido-2′,3′-isopropylidene-inosine (**26**)*

To a solution of **25** (0.487 g, 1.15 mmol) in DMF (20 mL) was added NaN_3 (0.3 g, 4.61 mmol). The reaction mixture was stirred at 80 °C for 3 h. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2 and washed with brine. The organic phase was dried, filtered and evaporated to give a crude residue purified by chromatography over silica gel (eluent: CH_2Cl_2/MeOH 98/2→95/5, v/v) to afford the desired products **26** (yield 45%). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.32, 1.53 (6H, s, (CH$_3$)$_2$C), 3.57 (2H, d, J = 5.8 Hz, H-5′), 4.29–4.31 (1H, m, H-4′), 4.30 (1H, dd, H-3′), 4.95 (1H, dd, J = 3.2 Hz, H-2′), 6.17 (1H, d, J = 2.8 Hz, H-1′), 8.11 (1H, s, H-2), 8.30 (1H, s, H-8), 12.45 (1H, br s, NH-3). ESI MS: m/z 334.1 Da $[M + H]^+$, $C_{13}H_{15}N_7O_4$ Mol. Wt. 333.30.

5. Pharmacological data

5.1. Platelet function studies

The effects of the compounds on platelets were studied using platelet-rich plasma (PRP) obtained from whole blood collected in sodium citrate 10.9 mmol (9:1, v/v) from healthy volunteers, 2 h later the intake of a tablet of acetyl salicylic acid and upon centrifugation at 150g at 21 °C for 10 min. 10^{-6} M ADP, was added to PRP samples, which had been pre-warmed at 37 °C, in the presence of the compounds at study or of their vehicles, in a light transmission aggregometer (Macia Brunelli, Italy). Platelet shape change and aggregation were monitored for 3 min after the addition of ADP [59].

6. Metabolic stability studies

6.1. HPLC methods

The quantification of **4**, **18**, **33**, Guanosine and Guanine in all samples generated from the experimental procedures was performed by HPLC. The detector was set at 259 nm for **4** and **18** analyses and at 254 nm for **33** Guanosine and Guanine analyses.

For **4** and **18** analyses the mobile phase consisted of water (0.01 M H_3PO_4) (solvent A) and acetonitrile (0.01 M H_3PO_4) (solvent B) with a ratio of 98:2 (v/v). The flow rate was of 1 mL/min and the retention times of **4** and **18** were 5.8 and 11.8 min, respectively.

For **33** analyses the mobile phase consisted of water/acetonitrile (95:5, 0.01 M H_3PO_4) (solvent A) and acetonitrile/water (95:5, 0.01 M H_3PO_4) (solvent B) with a ratio of 95:5 (v/v). The flow rate was of 1.2 mL/min and the retention times of **33** were 11.4 min.

For Guanosine analyses the mobile phase consisted of water (0.01 M H_3PO_4) (solvent A) and acetonitrile (0.01 M H_3PO_4) (solvent B) with a ratio of 98:2 (v/v). The flow rate was of 1.2 mL/min and the retention times of Guanosine was 6 min.

For Guanine analyses the mobile phase consisted of water (0.01 M H_3PO_4) (solvent A) and acetonitrile (0.01 M H_3PO_4) (solvent B) with a ratio of 99:1 (v/v). The flow rate was of 0.8 mL/min and the retention times of Guanine was 4.1 min.

Quantification was performed by integration of peak areas using external standardization.

6.2. Preparation of rat liver homogenates

The livers of male Wistar rats were isolated from the rats immediately after their decapitation. The excided tissue was washed in ice-cold saline solution (0.9% sodium chloride), weighted, cut into small pieces and 4 mL of ice-cold incubation buffer (50 mM Tris–HCl, pH 7.4) was added per 1 g of tissue. The tissue was then homogenized with the employment of a Potter–Elvehjem apparatus. The supernatant obtained after centrifugation (2000× g for 10 min, at 4 °C) was

decanted off and stored at −80 °C before its employment for degradation studies. The total protein concentration in the tissue homogenate was determined using the Lowry procedure [60] (29.4 ± 1.1 μg protein/μL).

6.3. Stability studies in human plasma and rat liver homogenates

An aliquot of 15 μL solution of each compounds (10^{-2} M in DMSO) was incubated (at a final concentration of 50 μM) in rat liver homogenate or human blood obtained from healthy subjects (3 mL). Incubation of the aliquots was carried out at 37 °C for various periods up to 360 min. At different incubations times, an aliquot of the solution (100 μL) was removed and compound degradation was blocked by addition of ice water (100 μL). After mixing the solution, 50 μL of SSA 10% and 50 μL of internal standard (50 μM solution) were subsequently added. After centrifugation (10 min at 14,000*g*), the supernatant was evaporated to dryness by N_2 flow. The residue was dissolved in 400 μL of water and after centrifugation (10 min at 19,800*g*) an aliquot (20 μL) of supernatant was injected into RP-HPLC.

Acknowledgements

Authors wish to thank Alberto Casolari and Elisa Durini for careful NMR experiments. This work was supported by Ministry of Education, University and Research (grant FIRB RBNE03YA3L_008).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.04.045.

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