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Synthesis of imidazo[1,2-*c*]pyrazolo[4,3-*e*]pyrimidines, pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines and 1,2,4-triazolo[5,1-*i*]purines: new potent adenosine A_2 receptor antagonists

F Gatta[1], MR Del Giudice[1], A Borioni[1], PA Borea[2], S Dionisotti[3], E Ongini[3]

[1]*Laboratorio di Chimica del Farmaco, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome;*
[2]*Istituto di Farmacologia, Università di Ferrara, Ferrara;*
[3]*Laboratori di Ricerca Schering-Plough SPA, Comazzo, Milan, Italy*

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Summary — A number of 2- or 4-fluorobenzylderivatives of imidazo[1,2-*c*]pyrazolo[4,3-*e*]pyrimidine, pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine and 1,2,4-triazolo[5,1-*i*] purine have been synthesized. The interaction with the adenosine A_2 and A_1 receptors was evaluated using selected biological assays . The highest degree of activity was displayed by the 5-amino-2-(2-furyl)-7-(or 8-)-fluorobenzyl-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine **13e, f** and **18e, f** and -3-fluorobenzyl-1-2-4-triazolo[5,1-*i*] purines **19e, f.** The compound **18f** was found to be the most potent A_2 antagonist in our series with a selectivity similar to that of the reference compound **CGS 15943**, but with 75-fold more activity in the platelet aggregation model.

imidazo[1,2-*c*]pyrazolo[4,3-*e*]pyrimidines / pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines / 1,2,4-triazolo[5,1-*i*]purines / adenosine A_2 receptor antagonists

Introduction

There is evidence that the purine nucleoside, adenosine, specifically modulates neurotransmission *via* interactions with 2 cell surface receptor subtypes designated as A_1 and A_2 adenosine receptors [1]. The therapeutic usefulness of adenosine and its analogues is limited by the ubiquity of A_1 and A_2 receptors and the several pharmacological responses that these receptors mediate in mammalian tissues [1, 2]. Thus a considerable effort has been made to synthesize agonists and antagonists selective for one or the other type of receptor [3–5].

As for adenosine receptor antagonists, several studies on the prototypic compounds, the xanthines theophylline and caffeine, led to the identification of a large number of 8-substituted xanthine derivatives which are potent and selective A_1 antagonists [3, 5]. Recently a series of 8-styryl xanthine derivatives has been described to possess A_2 antagonistic properties [6]. Other classes of non-xanthine heterocyclic compounds have also been found to possess antagonistic properties at either A_1 or A_2 adenosine receptors [3–5]. Of particular interest are 3 classes of related heterocycles which include the triazoloquinazolines [7], triazoloquinoxalines [8] and imidazoquinolines [3]. One reference compound, the 5-amino-9-chloro-2-(2-furyl)1,2,4-triazolo[1,5-*c*]quinazoline (**CGS 15943**, Ciba-Geigy) **1** has been described to have elevated affinity for the A_2 receptor (IC_{50} 3 nM) and 7-fold selectivity for the A_2 *vs* A_1 receptor [7]. However, subsequent studies have shown that the compound predominantly labels A_1 receptors [9] and is also potent at antagonizing functional effects mediated *via* A_1 receptors [4, 10]. Therefore, the need remains to identify within the non-xanthine heterocycle series A_2 receptor antagonists displaying high potency in both binding and functional tests involving selective interaction with A_2 receptors.

On the basis of these reports, and as a further development to our previous works on the preparation of fused-ring systems containing the pyrazole group [11], we extended our investigations in synthesizing some tricyclic compounds **2** related to **CGS 15943** (fig 1).

Our efforts have resulted in the synthesis of some imidazo[1,2-*c*]pyrazolo[4,3-*e*]pyrimidines,pyrazolo-[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines and 1,2,4-triazolo[5,1-*i*]purines, which have been studied for their interactions with either adenosine A_1 or A_2 receptor.

Chemistry

The readily available 5-amino-4-cyano-1-(2- or 4-fluorobenzyl)pyrazoles **3** [1] served as starting materials for the syntheses outlined in scheme 1.

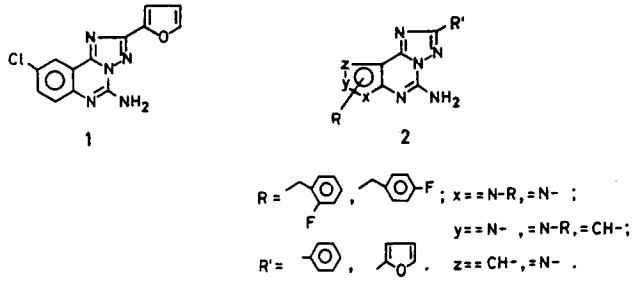
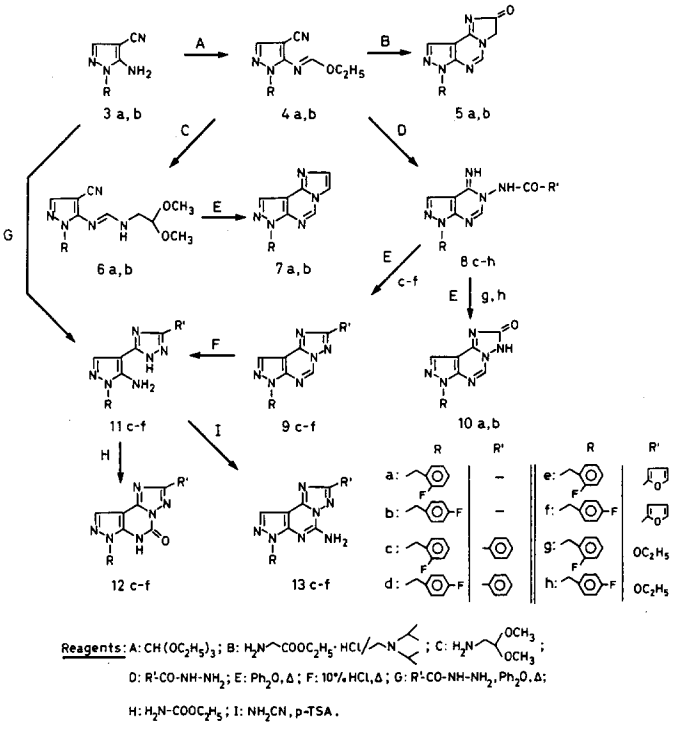


Fig 1. Comparison of structures of our tricyclic compounds **2** and **CGS 15943 1**.

Compounds **3**, by refluxing in triethyl orthoformate gave the imidates **4** which were found to react with glycine ethylester hydrochloride in the presence of *N*-ethyldiisopropilamine (synthetic pathway B), with aminoacetaldehyde dimethylacetal (synthetic pathway C) and, finally, with benzhydrazide or 2-furoic acid hydrazide or ethyl carbazate (synthetic pathway D) to provide compounds **5**, **6** and **8**, respectively.

The chemical structure proof of compounds **8** was substantiated by their IR and ^{1}H-NMR spectral data. The IR spectra showed the absence of any nitrile band in the 2200 cm^{-1} region and the presence of an amide carbonyl band at 1660 cm^{-1}, while the ^{1}H-NMR spectra (DMSO–d_6) showed 2 broad signals between δ 10.7 and 10.1 assigned to the 2 NH groups.

Ring closure to imidazo[1,2-*c*]pyrazolo[4,3-*e*]-pyrimidines **7** and pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]-pyrimidines **9** and **10**, was easily achieved by heating compounds **6** and **8** in diphenyl ether. Treatment of **9** with diluted hydrochloric acid at reflux temperature induced ring opening of the pyrimidine part to furnish the 5-amino-4-(1*H*-1,2,4-triazol-5-yl)-pyrazoles **11** in good to satisfactory yields. An alternative synthetic route to compounds **11** involved the direct condensation of aminopyrazoles **3** with benzhydrazide or 2-furoic acid hydrazide in refluxing diphenyl ether. This



Scheme 1.

Table I. Biological activity of compounds displaying high receptor affinity for both A_1 and A_2 receptors.

Compound [a]	*Binding* [b] *IC_{50} (nM)* A_1	A_2	*Ratio* A_1/A_2	*Adenylate cyclase* [c] *IC_{50} (nM)*	*Platelet aggregation* [d] *ED_{50} (μM)*
13e	550 ± 25	60 ± 10	9.2	7.9	2.0
13f	425 ± 20	18 ± 2	23.6	20.0	10.6
18e	34 ± 4	4.7 ± 0.3	7.2	0.7	0.1
18f	8 ± 1	1.5 ± 0.2	5.3	0.3	0.004
19e	220 ± 10	48.7 ± 3.8	4.5	7.0	ND
19f	550 ± 20	35.3 ± 3.2	15.6	25.0	2.3
CGS 15943	14.5 ± 0.4	1.8 ± 0.2	8.1	0.7	0.3
DPCPX	0.7[e]	502[e]	0.0014[e]	2000	ND
Theophylline	14 900[f]	37 530[f]	0.4[f]	ND	1277

[a]Compounds not reported here displayed an IC_{50} > 10 μM in the binding assay for both A_1 and A_2 receptors; [b]inhibition of [^{3}H]-N^6-cyclohexyladenosine binding in rat brain homogenates (A_1) or [^{3}H]-**GCS 21680** binding in rat striatal homogenates (A_2); [c]dose of antagonist reducing by 50% the inhibitory effect of NECA (1 μM) on stimulation of cAMP levels in washed human platelets; [d]dose of antagonist reducing by 50% the inhibitory effect induced by NECA used at a concentration (0.3 or 1 μM) close to the IC_{50} value on rabbit platelet aggregation; [e]see [15]; [f]see [5]; ND: not determined.

method is more convenient for the preparation of **11**, with yields from 48 to 60%.

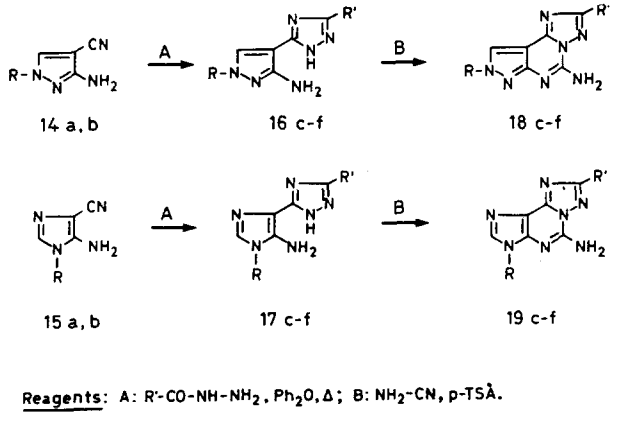
Compounds **11**, on heating in ethyl carbamate were cyclized to pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidin-5(6*H*)-ones **12**, while by reaction with an excess of cyanamide in 1-methyl-2-pyrrolidone at 150–160°C gave the expected 5-amino-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines **13** in 45–63% yields.

Preliminary studies on the affinity at A_1 and A_2 adenosine receptors showed that the most potent ligands in our series were the 5-amino-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines **13e** and **13f** (table I).

These findings prompted us to synthesize the isomers 5-amino-8-(2- or 4-fluorobenzyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines **18** and 5-amino-3-(2- or 4-fluorobenzyl)1,2,4-triazolo[5,1-*i*]purines **19** (scheme 2).

The starting materials **14** were obtained by reaction of 3-amino-4-cyanopyrazole [12] with the appropriate fluorobenzylchloride in *N,N*-dimethylformamide and in the presence of anhydrous potassium carbonate; this pathway provided an approximate 1:2 mixture of the isomers **3** and **14**, from which compounds **14** were easily separated by crystallization. The 5-amino-4-cyanoimidazoles **15** were prepared according to the procedure described for the corresponding 1-benzyl-derivative [13]. Treatment of compounds **14** and **15** with benzhydrazide or 2-furoic acid hydrazide in refluxing diphenyl ether, followed by reaction of the resulting triazolyl derivatives **16** and **17** with cyanamide afforded the desired products **18** and **19**.

In the *Experimental protocols* spectral data on the most significant compounds, with R = 2-fluorobenzyl and R' = phenyl, have been reported.



Scheme 2.

Pharmacology

Compounds displaying high receptor affinity for both A_1 and A_2 receptors have been tested in the 2 functional models for the specific evaluation of agonistic or antagonistic properties at A_2 receptors [1]. Activity on the NECA-stimulated adenylate cyclase was measured in human platelets. Platelet aggregation assay was instead performed on rabbit platelets. This is supported by our previous study showing that the A_2 mediated response in rabbit platelets is highly correlated with that found in human platelets [14]. Pharmacological results are reported in table I and figure 2.

Results and discussion

The results of binding assay have shown that 2-furyl substituent in the position 2 and the amino group in the position 5 in our pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines were essential for the high affinity at both adenosine receptors. This is in agreement with SAR investigation by Francis *et al* [7] showing that the 5-amino-9-chloro-2(2-furyl)1,2,4-triazolo[1,5-*c*]-quinazoline (**CGS 15943**), was the most potent and

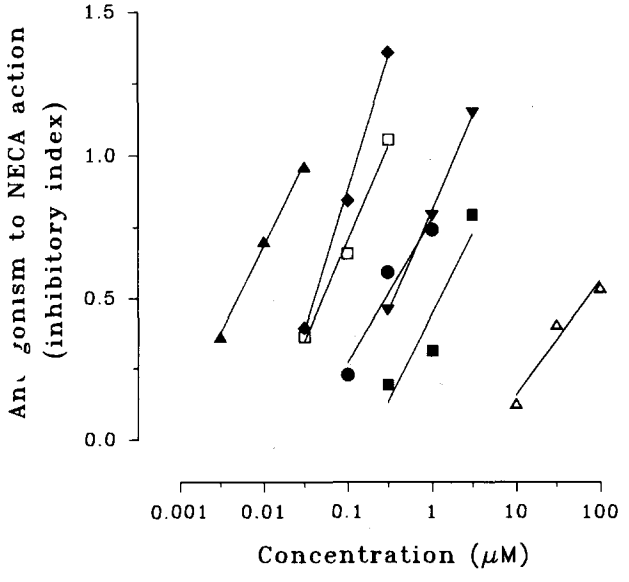


Fig 2. Reversal by selected A_2 adenosine antagonists of the anti-platelet aggregation action of the adenosine agonist NECA (0.3 or 1 μM). Inhibitory index was calculated as (% of aggregation at the selected concentration of antagonist over % of aggregation with NECA) – 1. The compounds were used at the following concentration range: **13e** ▼ and **13f** ■ (0.3–3 μM); **18e** ◆ (0.03–0.3 μM); **18f** ▲ (0.003–0.03 μM); **19f** ● (0.1–1 μM). The reference A_1 (theophylline Δ) and A_2 (CGS 15943 □) antagonists were used at a range of 10–100 and 0.03–0.3 μM, respectively.

selective A_2 antagonist of a series of triazoloquinazolines, in which replacement of either the amino or the 2-furyl group caused a severe loss of activity.

The present study indicates that, compared with this reference compound, replacement of the chlorophenyl group of the quinazoline with the fluorobenzylpyrazole or imidazole retains affinity for A_2 receptors. Marked changes of biological activity were found between the isomers **13** and **18**. In particular, the compound **18f** showed the highest affinity for A_2 receptors, being practically similar to that of **CGS 15943**, and with ≈ 75-fold more activity in the model of platelet aggregation (table I, fig 2). Instead, the replacement of the pyrazole ring by an imidazole moiety afforded slight reduction of potency in all the test systems (table I).

The results obtained in platelets with only A_2 adenosine receptors [14] were consistent, except for the compound **18f**, with the affinity found in the receptor binding assay. This indicates that in addition to having high affinity for A_2 receptors our compounds are also able to antagonize functional A_2-mediated responses in biological models. Although the data demonstrate that the potency at A_2 receptors is very high and even improved in comparison with **CGS 15943**, the A_2 selectivity still remains substantially low. There was a slight increase of separation between affinity at A_2 *vs* A_1 receptors for compounds **13f** and **19f**, at the expense however of potency in the platelet assay. The interesting activity found for **18f** deserves additional comment. This compound showed high potency in functional models compared with the other compounds described in table I. This is in agreement with other data in the literature [3, 10] showing that A_2 agonists, as well as the reference A_2 antagonist **CGS 15943**, are more potent and selective in functional models than in binding assays. Therefore, it could well be that **18f** may display high selectivity in other functional models.

Further experiments in other selected biological models will provide more information on the activity of these compounds in physiological processes modulated by A_2 receptors. In turn, this may provide the basis for the identification of more selective A_2 receptor antagonists.

Experimental protocols

Chemistry

Melting points are uncorrected. ^{1}H-NMR spectra were determined on a T-60 Varian instrument with TMS as internal standard. IR spectra were recorded on a Perkin–Elmer 580 spectrophotometer. Electron ionization mass spectra were obtained on a Finnigan 5100 apparatus. Column chromatographic separations were accomplished on Merck silica gel (70–230 mesh). Sodium sulfate was used to dry organic solutions. Microanalyses, indicated by the symbols of the elements, were performed by the microanalytical section of our Institute and were within ± 0.4% of theoretical values. The synthesis of **3a** and **3b** has been reported elsewhere [11].

*4-Cyano-5-[(ethoxymethylene)amino]pyrazoles **4a, b***
A solution of **3** (4.3 g, 20 mmol) in triethyl orthoformate (40 ml) was heated at reflux under N_2 for 8 h. Excess *ortho*-formate was removed *in vacuo* and the yellow residue crystallized.

*4-Cyano-5-[(ethoxymethylene)amino]-1-(2-fluorobenzyl)pyrazole **4a***. Yield: 4.7 g (87%), mp: 40–42°C (ethyl acetate/hexane). Anal $C_{14}H_{13}FN_4O$ (C, H, N).

*4-Cyano-5-[(ethoxymethylene)amino]-1-(4-fluorobenzyl)pyrazole **4b***. Yield: 5.1 g (94%), mp: 91–93°C (methanol). Anal $C_{14}H_{13}FN_4O$ (C, H, N).

*Imidazo[1,2-c]pyrazolo[4,3-e]pyrimidin-2(3H)ones **5a, b***
A mixture of each compound **4** (2.7 g, 10 mmol), glycine ethylester hydrochloride (2.1 g, 15 mmol) and *N*-ethyldiisopropylamine (1.9 g, 15 mmol) in ethanol (30 ml) was refluxed for 5 h. The solvent was evaporated, and pure compounds were obtained after chromatography on a silica gel column by eluting with ethyl acetate/methanol (95: 5 v/v).

*7-(2-Fluorobenzyl)-imidazo[1,2-c]pyrazolo[4,3-e]pyrimidin-2(3H) one **5a***. Yield: 0.9 g (32%), mp: 219–221°C (methanol). Anal $C_{14}H_{10}FN_5O$ (C, H, N). IR (nujol): ν CO: 1720 cm^{-1}. ^{1}H-NMR (DMSO–d_6), δ: 8.57 (s, 1H, 5-CH), 8.30 (s, 1H, 9-CH), 7.45–6.95 (m, 4H, aromatic protons), 5.57 (s, 2H, benzyl-CH_2), 4.58 (s, 2H, 3-CH_2). MS: *m/z* 283 (M^+).

*7-(4-Fluorobenzyl)-imidazo[1,2-c]pyrazolo[4,3-e]pyrimidin-2(3H) one **5b***. Yield: 1.2 g (42%), mp: 213–215°C (ethanol). Anal $C_{14}H_{10}FN_5O$ (C, H, N).

*4-Cyano-5[(2-dimethoxyethylaminomethylene)amino]pyrazoles **6a, b***
A solution of the appropriate imidate **4** (2.7 g, 10 mmol) and aminoacetaldehyde dimethylacetal (1.1 g, 10 mmol) in ethylene glycol monomethyl ether (30 ml) was refluxed for 4 h. The residue resulting from the solvent evaporation, was chromatographed on a silica gel column and eluted with ethyl acetate/hexane (2:1 v/v).

*4-Cyano-5[(2-dimethoxyethylaminomethylene)amino]-1-(2-fluorobenzyl)pyrazole **6a***. Yield: 2.2 g (66%), mp: 120–122°C (ethyl acetate/hexane). Anal $C_{16}H_{18}FN_5O_2$ (C, H, N).

*4-Cyano-5[(2-dimethoxyethylaminomethylene)amino]-1-(4-fluorobenzyl)pyrazole **6b***. Yield: 2.8 g (85%), mp: 98–100°C (ethyl acetate/hexane). Anal $C_{16}H_{18}FN_5O_2$ (C, H, N).

*Imidazo[1,2-c]pyrazolo[4,3-e]pyrimidines **7a, b***
Each compound **6** (3 g, 9 mmol) and diphenyl ether (30 ml) was placed in a flask fitted with a Dean–Stark trap apparatus. The mixture was heated at reflux temperature and maintained until the starting material had disappeared (≈ 20 min, controlled by TLC with ethyl acetate/hexane 2:1 v/v as eluent). The mixture was allowed to cool to room temperature and hexane (100 ml) was added. The precipitate solid was collected by filtration, washed with additional hexane, then crystallized.

7-(2-Fluorobenzyl)-imidazo[1,2-c]pyrazolo[4,3-e]pyrimidine ***7a***. Yield: 1.8 g (75%), mp: 189–191°C (ethyl acetate). Anal $C_{14}H_{10}FN_5$ (C, H, N). ^{1}H-NMR (DMSO–d_6), δ: 9.23 (s, 1H, 5-CH), 8.30 (s, 1H, 9-CH), 8.00 (d, 1H, J = 2 Hz, 3-CH), 7.47 (d, 1H, J = 2 Hz, 2-CH), 5.70 (s, 2H, benzyl-CH_2). MS: *m/z* 267 (M^+).

7-(4-Fluorobenzyl)-imidazo[1,2-c]pyrazolo[4,3-e]pyrimidine ***7b***. Yield: 2.0 g (83%), mp: 147–149°C (ethyl acetate/hexane). Anal $C_{14}H_{10}FN_5$ (C, H, N).

5-Acylamino- and 5 -ethoxycarbonylamino-4-imino-4,5-dihydropyrazolo[3,4-d]pyrimidines ***8c–h***

A solution of each compound **4** (5.4 g, 20 mmol) and benzhydrazide (2.9 g, 22 mmol) or 2-furoic acid hydrazide (2.5 g, 22 mmol) or ethylcarbazate (2.3 g, 22 mmol) in ethylene glycol monomethyl ether (50 ml) was refluxed for 3 h. On cooling, the crude solid which separated out was recrystallized from the suitable solvent.

5-Benzoylamino-1-(2-fluorobenzyl)-4-imino-4,5-dihydro-pyrazolo[3,4-d]pyrimidine ***8c***. Yield: 3.1 g (43%), mp: 268–271°C (dimethylformamide). Anal $C_{19}H_{15}FN_6O$ (C, H, N). ^{1}H-NMR (DMSO–d_6), δ: 8.33 (s, 1H, 3-CH), 7.95 (m, 2H, aromatic protons), 7.54 (m, 3H, aromatic protons), 7.18–7.00 (m, 3H, aromatic protons), 6.44 (bs, 1H, 4-N=), 6.08 (bs, 1H, NHCO), 5.56 (s, 2H, benzyl-CH_2). MS: *m/z* 362 (M^+).

5-Benzoylamino-1-(4-fluorobenzyl)-4-imino-4,5-dihydro-pyrazolo[3,4-d]pyrimidine ***8d***. Yield: 3.4 g (47%), mp: 260–262°C (methanol). Anal $C_{19}H_{15}FN_6O$ (C, H, N).

5-(2-Furoylamino)-1-(2-fluorobenzyl)-4-imino-4,5-dihydropyrazolo[3,4-d]pyrimidine ***8e***. Yield: 3.4 g (48%), mp: 261–263°C (dimethylformamide/ethanol). Anal $C_{17}H_{13}FN_6O_2$ (C, H, N).

5-(2-Furoylamino)-1-(4-fluorobenzyl)-4-imino-4,5-dihydropyrazolo[3,4-d]pyrimidine ***8f***. Yield: 2.8 g (40%), mp: 257–259°C (methanol). Anal $C_{17}H_{13}FN_6O_2$ (C, H, N).

5-(Ethoxycarbonylamino)-1-(2-fluorobenzyl)-4-imino-4,5-dihydro-pyrazolo[3,4-d]pyrimidine ***8g***. Yield: 4.2 g (64%), mp: 172–175°C (ethyl acetate). Anal $C_{15}H_{15}FN_6O_2$ (C, H, N).

5-(Ethoxycarbonylamino)-1-(4-fluorobenzyl)-4-imino-4,5-dihydro-pyrazolo[3,4-d]pyrimidine ***8h***. Yield: 3.1 g (47%), mp: 193–195°C (methanol). Anal $C_{15}H_{15}FN_6O_2$ (C, H, N).

Pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidines ***9c–f****: pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidin-2(3H) ones* ***10a, b***

Compounds **8c–f** (to obtain **9**) or **8g, h** (to obtain **10**) (10 mmol) were cyclized in refluxing diphenyl ether (40 ml) working up the reaction mixture as described for compounds **7**.

7-(2-Fluorobenzyl)-2-phenyl-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine ***9c***. Yield: 2.8 g (81%), mp: 273–275°C (dimethylformamide). Anal $C_{19}H_{13}FN_6$ (C, H, N). ^{1}H-NMR (DMSO–d_6), δ: 9.11 (s, 1H, 5-CH), 8.76 (s, 1H, 9-CH), 8.20–7.00 (m, 9H, aromatic protons), 5.40 (s, 2H, benzyl-CH_2).

7-(4-Fluorobenzyl)-2-phenyl-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine ***9d***. Yield: 3.1 g (88%), mp: 233–235°C (dimethylformamide). Anal $C_{19}H_{13}FN_6$ (C, H, N).

7-(2-Fluorobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine ***9e***. Yield: 2.6 g (78%), mp: 246–248°C (dimethylformamide). Anal $C_{17}H_{11}FN_6O$ (C, H, N).

7-(4-Fluorobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine ***9f***. Yield: 2.9 g (88%), mp: 243–245°C (dimethylformamide). Anal $C_{17}H_{11}FN_6O$ (C, H, N).

7-(2-Fluorobenzyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidin-2(3H) one ***10a***. Yield: 2.8 g (74%), mp: 271–274°C (methanol). Anal $C_{13}H_9FN_6O$ (C, H, N). ^{1}H-NMR (DMSO–d_6), δ: 12.25 (bs, 1H, exchangeable with D_2O, NH), 8.63 (s, 1H, 5-CH), 8.17 (s, 1H, 9-CH), 7.60–7.00 (m, 4H, aromatic protons) 5.60 (s, 2H, benzyl-CH_2).

7-(4-Fluorobenzyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidin-2(3H)one ***10b***. Yield: 2.6 g (91%), mp: 305–307°C (dimethylformamide). Anal $C_{13}H_9FN_6O$ (C, H, N).

5-Amino-4-(1H-1,2,4-triazol-5-yl)pyrazoles ***11c–f***

These compounds were prepared either by ring opening of the pyrimidine moiety of **9** in diluted hydrochloric acid *(Method A)* or by condensation of compounds **3** with the appropriate hydrazide in refluxing diphenyl ether *(Method B)*.

Method A. A suspension of **9** (10 mmol) in 10% hydrochloric acid (50 ml) was heated at reflux temperature with vigorous stirring for 3 h. After cooling, the mixture was carefully basified with concentrated ammonium hydroxide, and the resulting precipitate was collected by filtration and then crystallized.

Method B. Each compound **3** (4.3 g, 20 mmol) and benzhydrazide (2.9 g, 22 mmol) or 2-furoic acid hydrazide (2.5 g, 22 mmol) in diphenyl ether was stirred and heated at reflux temperature with a Dean–Stark trap until **3** had disappeared, as indicated by TLC using an ethyl acetate/hexane 2:1 mixture as developer (3–4 h). The precipitate separated according to the procedure described for compounds **7**, was purified by chromatography on a silica gel column by eluting with ethyl acetate/hexane (2:1 v/v).

5-Amino-1-(2-fluorobenzyl)-4-(3-phenyl-1H-1,2,4-triazol-5-yl) pyrazole ***11c***. *Method A*, yield: 5.6 g (84%); *Method B*, yield: 4.0 g (60%); mp: 199–201°C (ethyl acetate). Anal $C_{18}H_{15}FN_6$ (C, H, N). ^{1}H-NMR (DMSO–d_6), δ: 13.70 (bs, 1H, exchangeable with D_2O, NH), 8.16–7.80 (m, 2H, aromatic protons), 7.76 (s, 1H, 3-CH), 7.60–6.90 (m, 7H, aromatic protons), 6.40 (bs, 2H, exchangeable with D_2O, NH_2), 5.35 (s, 2H, benzyl-CH_2).

5-Amino-1-(4-fluorobenzyl)-4-(3-phenyl-1H-1,2,4-triazol-5-yl)pyrazole ***11d***. *Method A*, yield: 5.3 g (80%); *Method B*, yield: 4.0 g (60%); mp: 177–179°C (chloroform). Anal $C_{18}H_{15}FN_6$ (C, H, N).

5-Amino-1-(2-fluorobenzyl)-4-[3-(2-furyl)-1H-1,2,4-triazol-5-yl]pyrazole ***11e***. *Method A*, yield: 4.7 g (69%); *Method B*, yield: 3.2 g (47%); mp: 110–112°C (ethyl acetate). Anal $C_{16}H_{13}FN_6O{\cdot}H_2O$ (C, H, N).

5-Amino-1-(4-fluorobenzyl)-4-[3-(2-furyl)-1H-1,2,4-triazol-5-yl]pyrazole ***11f***. *Method A*, yield: 4.9 g (76%); *Method B*, yield: 3.3 g (50%); mp: 170–172°C (chloroform). Anal $C_{16}H_{13}FN_6O$ (C, H, N).

Pyrazolo[4,3-e]-1,2,4-triazolol[1,5-c]pyrimidin-5(6H)ones ***12c–f***

Compound **11** (10 mmol) in ethyl carbamate (15 g, 168 mmol) was heated under nitrogen at 210°C for 3 h. The reaction mixture was allowed to cool and then vigorously stirred in warm water (200 ml) over 1 h to dissolve residual ester. The solid was filtered, washed with water and ethanol, then crystallized.

7-(2-Fluorobenzyl)-2-phenyl-pyrazolo[4,3-e]1,2,4-triazolo-[1,5-c]pyrimidin-5(6H)one ***12c.*** Yield: 2.9 g (81%), mp: > 300°C (dimethylformamide). Anal $C_{19}H_{13}FN_6O$ (C, H, N). IR (nujol): ν CO 1710 cm^{-1}. ^{1}H-NMR (DMSO–d_6), δ: 8.20 (m, 4H, 3H after D_2O exchange, 2 aromatic protons, NH and 9-CH), 7.65–7.00 (m, 7H, aromatic protons), 5.60 (2H, benzyl-CH_2). MS: *m/z* 360 (M^+).

7-(4-Fluorobenzyl)-2-phenyl-pyrazolo[4,3-e]1,2,4-triazolo-[1,5-c]pyrimidin-5(6H)one ***12d.*** Yield: 2.9 g (81%), mp: > 300°C (dimethylformamide). Anal $C_{19}H_{13}FN_6O$ (C, H, N).

7-(2-Fluorobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo-[1,5-c]pyrimidin-5(6H)one ***12e.*** Yield: 2.3 g (68%), mp: > 300°C (dimethylformamide). Anal $C_{17}H_{11}FN_6O_2$ (C, H, N).

7-(4-Fluorobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo-[1,5-c]pyrimidin-5(6H)one ***12f.*** Yield: 2.2 g (65%), mp: > 300°C (dimethylformamide). Anal $C_{17}H_{11}FN_6O_2$ (C, H, N).

5-Amino-7-(2- or 4-fluorobenzyl)-pyrazolo[4,3-e]-1,2,4-triazolo-[1,5-c]pyrimidines ***13***

To a suspension of each compound **11** (10 mmol) in 1-methyl-2-pyrrolidone (40 ml) was added cyanamide (2.5 g, 60 mmol) followed by *p*-toluenesulfonic acid monohydrate (2.9 g, 15 mmol). The mixture was heated at 150–160°C with stirring. After 4 h cyanamide (2.5 g, 60 mmol) was once again added and heating continued overnight. The mixture was quenched in hot water (200 ml) and the precipitated solid was collected by filtration, thoroughly washed with water and ethanol, then crystallized.

5-Amino-7-(2-fluorobenzyl)-2-phenyl-pyrazolo[4,3-e]1,2,4-triazolo-[1,5-c]pyrimidine ***13c.*** Yield: 1.6 g (45%), mp: 245–248°C (dimethylformamide/ethanol). Anal $C_{19}H_{14}FN_7$ (C, H, N). ^{1}H-NMR (DMSO–d_6), δ: 8.20 (s, partially overlapped by aromatic and NH_2 protons, 9-CH), 8.30–7.95 (m, 5H, 3H after D_2O exchange, 2 aromatic protons, NH_2, 9-CH), 7.60–7.00 (m, 7H, aromatic protons), 5.54 (s, 2H, benzyl-CH_2). MS: *m/z* 359 (M^+).

5-Amino-7-(4-fluorobenzyl)-2-phenyl-pyrazolo[4,3-e]1,2,4-triazolo-[1,5-c]pyrimidine ***13d.*** Yield: 1.9 g (53%), mp: 263–265°C (methanol). Anal $C_{19}H_{14}FN_7$ (C, H, N).

5-Amino-7-(2-fluorobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo-[1,5-c]pyrimidine ***13e.*** Yield: 2.2 g (63%), mp: 258–261°C (dimethylformamide/ethanol). Anal $C_{17}H_{12}FN_7O$ (C, H, N).

5-Amino-7-(4-fluorobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo-[1,5-c]pyrimidine ***13f.*** Yield: 2.0 g (58%), mp: 272–274°C (methanol). Anal $C_{17}H_{12}FN_7O$ (C, H, N).

3-Amino-4-cyanopyrazoles ***14a,b***

3-Amino-4-cyanopyrazole (2.2 g, 20 mmol) [12] was added to a stirred suspension of anhydrous potassium carbonate (4.1 g, 30 mmol) in anhydrous *N,N*-dimethylformamide (50 ml). After stirring 30 min at 70°C, the appropriate fluorobenzylchloride (3.6 g, 25 mmol) was added dropwise. The mixture was stirred for 2 h at 70°C, then after cooling, poured into water. The precipitate was collected and twice crystallized from ethyl acetate/hexane.

3-Amino-4-cyano-1- (2-fluorobenzyl)pyrazole ***14a.*** Yield: 2.10 g (49%), mp: 149–151°C. Anal $C_{11}H_9FN_4$ (C, H, N). ^{1}H-NMR (DMSO–d_6) δ: 8.17 (s, 1H, 5-CH), 7.35–7.05 (m, 4H, aromatic protons), 5.53 (bs, 2H, exchangeable with D_2O, NH_2), 5.07 (s, 2H, benzyl-CH_2).

3-Amino-4-cyano-1-(4-fluorobenzyl)pyrazole ***14b.*** Yield: 1.60 g (37%), mp: 140–142°C. Anal $C_{11}H_9FN_4$ (C, H, N).

5-Amino-4-cyanoimidazoles ***15a,b***

These compounds were prepared from aminomalononitrile-*p*-toluenesulfonate (5.4 g, 20 mmol), trimethyl orthoformate and 2- or 4-fluorobenzylamine according to the synthetic pathway described for the corresponding 1-benzyl derivative [13].

5-Amino-4-cyano-1-(2-fluorobenzyl)imidazole ***15a.*** Yield: 2.70 g (63%), mp: 167–169°C (ethyl acetate/hexane). Anal $C_{11}H_9FN_4$ (C, H, N).

5-Amino-4-cyano-1-(4-fluorobenzyl)imidazole ***15b.*** Yield: 2.60 g (61%), mp: 179–181°C (ethyl acetate). Anal $C_{11}H_9FN_4$ (C, H, N).

3-Amino-4-(1H-1,2,4-triazol-5-yl)pyrazoles ***16c–f****; 5-amino-4-(1H-1,2,4-triazol-5-yl)imidazoles* ***17c–f***

A mixture of each compound **14** or **15** (4.3 g, 20 mmol) and benzhydrazide (2.9 g, 22 mmol) or 2-furoic acid hydrazide (2.5 g, 22 mmol) in diphenyl ether (50 ml) was refluxed while stirring with a Dean–Stark trap to remove water. The reaction was monitored by TLC (ethyl acetate as eluent) and was complete after ≈ 2–4 h. The precipitate obtained according to the procedure described for compounds **7** was purified by chromatography on a silica gel column by eluting with ethyl acetate/hexane (1:1 v/v).

3-Amino-1-(2-fluorobenzyl)-4-(3-phenyl-1H-1,2,4-triazol-5-yl)-pyrazole ***16c.*** Yield: 3.8 g (57%), mp: 210–213°C (methanol). Anal $C_{18}H_{15}FN_6$ (C, H, N). ^{1}H-NMR (DMSO–d_6), δ: 13.65 (broad, 1H exchangeable with D_2O, NH), 8.15–7.85 (m, 3H, 2H aromatic protons and 5-CH), 7.65–7.00 (m, 7H, aromatic protons), 5.60 (bs, 2H, exchangeable with D_2O, NH_2), 5.27 (s, 2H, benzyl-CH_2).

3-Amino-1-(4-fluorobenzyl)-4-(3-phenyl-1H-1,2,4-triazol-5-yl)-pyrazole ***16d.*** Yield: 4.4 g (66%), mp: 122–124°C (methanol). Anal $C_{18}H_{15}FN_6$ (C, H, N).

3-Amino-1-(2-fluorobenzyl)-4-[3-(2-furyl)-1H-1,2,4-triazol-5-yl]pyrazole ***16e.*** Yield: 2.0 g (31%), mp: 188–190°C (methanol). Anal $C_{16}H_{13}FN_6O$ (C, H, N).

3-Amino-1-(4-fluorobenzyl)-4-[3-(2-furyl)-1H-1,2,4-triazol-5-yl]pyrazole ***16f.*** Yield: 2.8 g (43%), mp: 115–117°C (methanol). Anal $C_{16}H_{13}FN_6O$ (C, H, N).

5-Amino-1-(2-fluorobenzyl)-4-(3-phenyl-1H-1,2,4-triazol-5-yl)imidazole ***17c.*** Yield: 5.0 g (74%), mp: 244–246°C (chloroform). Anal $C_{18}H_{15}FN_6$ (C, H, N). ^{1}H-NMR (DMSO–d_6), δ: 13.70 (bs, 1H exchangeable with D_2O, NH), 8.07–7.80 (m, 3H, 2 aromatic protons and 2-CH), 7.60–6.90 (m, 7H, aromatic protons), 5.77 (bs, 2H, exchangeable with D_2O, NH_2), 5.23 (s, 2H, benzyl-CH_2).

5-Amino-1-(4-fluorobenzyl)-4-(3-phenyl-1H-1,2,4-triazol-5-yl)-imidazole ***17d.*** Yield: 4.6 g (69%), mp: 230–232°C (chloroform). Anal $C_{18}H_{15}FN_6$ (C, H, N).

5-Amino-1-(2-fluorobenzyl)-4-[3-(2-furyl)-1H-1,2,4-triazol-5-yl]imidazole ***17e.*** Yield: 4.9 g (73%), mp: 240–242°C (chloroform). Anal $C_{16}H_{13}FN_6O$ (C, H, N).

5-Amino-1-(4-fluorobenzyl)-4-[3-(2-furyl)-1H-1,2,4-triazol-5-yl]imidazole ***17f.*** Yield: 4.3 g (65%), mp: 253–255°C (chloroform). Anal $C_{16}H_{13}FN_6O$ (C, H, N).

5-Amino-8-(2- or 4-fluorobenzyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines ***18c–f****; 5-amino-9-phenyl-[or 9-(2-furyl)]-1,2,4-triazolo[5,1-i]purines* ***19c–f***

These compounds were synthesized starting from **16** or **17** (20 mmol) following exactly the aformentioned procedure described for the preparation of compounds **13**.

5-Amino-8-(2-fluorobenzyl)-2-phenyl-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine ***18c.*** Yield: 2.8 g (39%), mp: 265–267°C (methanol). Anal $C_{19}H_{14}FN_7$ (C, H, N). ^{1}H-NMR (DMSO–d_6), δ: 9.66 (s, 1H, 9-CH), 8.30–8.10 (m, 2H, aromatic protons), 7.70–7.05 (m, 9H, 7H after D_2O exchange, aromatic protons and NH_2), 5.55 (s, 2H, benzyl-CH_2).

5-Amino-8-(4-fluorobenzyl)-2-phenyl-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine ***18d.*** Yield: 3.9 g (50%), mp: 270–273°C (methanol). Anal $C_{19}H_{14}FN_7$ (C, H, N).

5-Amino-8-(2-fluorobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine ***18e.*** Yield: 2.4 g (34%), mp: 284–287°C (methanol). Anal $C_{17}H_{12}FN_7O$ (C , H , N).

5-Amino-8-(4-fluorobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine ***18f.*** Yield: 3.2 g (46%), mp: 285–288°C (ethyl acetate). Anal $C_{17}H_{12}FN_7O$ (C, H, N).

5-Amino-3-(2-fluorobenzyl)-8-phenyl-1,2,4-triazolo[5,1-i]-purine ***19c.*** Yield: 2.2 g (31%), mp: 265–267°C (methanol). Anal $C_{19}H_{14}FN_7$ (C, H, N). ^{1}H-NMR (DMSO–d_6), δ: 8.40–8.05 (m, 2H aromatic protons), 8.10 (s, 1H, partially overlapped by aromatic protons 2-CH), 7.80 (bs, 2H, exchangeable with D_2O, NH_2), 7.65–7.00 (m, 7H, aromatic protons), 5.50 (s, 2H, benzyl-CH_2).

5-Amino-3-(4-fluorobenzyl)-8-phenyl-1,2,4-triazolo[5,1-i]purine ***19d.*** Yield: 2.0 g (28%), mp: 274–276°C (methanol). Anal $C_{19}H_{14}FN_7$ (C, H, N).

5-Amino-3-(2-fluorobenzyl)-8-(2-furyl)-1,2,4-triazolo[5,1-i]-purine ***19e.*** Yield: 2.0 g (28%), mp: 290–292°C (dimethylformamide). Anal $C_{17}H_{12}FN_7O$ (C, H, N).

5-Amino-3-(4-fluorobenzyl)-8-(2-furyl)-1,2,4-triazolo[5,1-i]-purine ***19f.*** Yield: 2.5 g (35%), mp: 292–294°C (methanol). Anal $C_{17}H_{12}FN_7O$ (C, H, N).

Biological assays

Reference materials

Adenosine diphosphate (ADP) was supplied by Boehringer Mannheim GmbH (Mannheim, FRG). 5'-*N*-Ethylcarboxamidoadenosine (NECA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), *R*(–)-N^6-(2-phenylisopropyl)adenosine (R-PIA), forskolin and Ro 20-1724 were from Research Biochemicals Inc (Natick, MA, USA). Adenosine deaminase Type VI and theophylline were from Sigma Chemical (St Louis, MO, USA). 5-Amino-9-chloro-2-(2-furyl)1,2,4-triazolo[1,5-*c*]quinazoline (CGS 15943) was kindly supplied by Ciba-Geigy (Summit, NJ, USA).

[^{3}H]-N^6-Cyclohexyl-adenosine ([^{3}H]-CHA) (specific activity 13.5 Ci/mmol) and 2-[*p*-(2-carboxyethyl)-phenethylamino]-5'-*N*-ethylcarboxamido-adenosine ([^{3}H]-CGS 21680) (spec act 48.1 Ci/mmol) were NEN Research Products (Boston, MA, USA). [^{3}H]-Cyclic adenosine monophosphate ([^{3}H]-cAMP) (spec act 24.0 Ci/mmol) was from Amersham International (Little Chalfont, Buckinghamshire, UK). Instagel and Ready Gel were from Beckman Instruments Inc (Fullerton, CA, USA). All other reagents were from commercial sources.

A_1 and A_2 receptor binding

Male Wistar rats (Charles-River, Calco, Italy) weighing 150–200 g were killed by decapitation and the whole brain (minus brainstem and cerebellum) was dissected on ice. The tissue was disrupted in a Polytron PTA 10 Probe (setting 5.20 s) in 20 vol of 50 mM Tris–HCl buffer, pH 7.4. The homogenate was centrifuged (48 000 *g* at 4°C for 10 min) and resuspended in Tris–HCl containing 2 IU/ml of adenosine deaminase (Type VI, Sigma). After 30 min incubation at 37°C the membranes were centrifuged and the pellet was stored at –70°C.

Adenosine A_1 receptor binding assay was performed according to Bruns *et al* [2] by using [^{3}H]-CHA as radioligand. Experiments were carried out in 1 ml buffer which contained 1 nM [^{3}H]-CHA, membranes from 15 mg (wet weight) of brain tissue and compounds to be tested. After 120 min incubation at 25°C, separation of bound from free ligand was performed by rapid filtration through Whatman GF/B filters under reduced pressure using a cell harvester (Brandel, Gaithersburg, MD, USA). Filters were washed 3 times with ice-cold buffer, dried and treated with 5 ml acidified Instagel. Radioactivity was determined using a LS-1800 Beckman liquid scintillation counter (Beckman Instruments Inc, Fullerton, CA, USA) at an efficiency ranging from 40 to 50%. Non-specific binding was defined as binding in the presence of R-PIA 10 μM and was ≤ 10% of total binding.

Adenosine A_2 receptor binding assay was performed essentially according to Jarvis *et al* [9] by using [^{3}H]-CGS 21680 as radioligand. Experiments were carried out in 1 ml buffer containing 10 nM [^{3}H]-CGS 21680, membranes from 5 mg (wet weight) of striatum brain tissue, 10 mM $MgCl_2$ and compounds to be tested. The method was the same as used for A_1 receptor binding. Non-specific binding was defined as binding in the presence of NECA 100 μM and was ≤ 10% of total binding.

The IC_{50} values were calculated by probit analysis based on at least 6 concentrations of each compound and were the mean of 3–4 separate experiments.

Adenylate cyclase assay

Washed human platelets from peripheral blood of healthy volunteers was prepared as previously described by Korth *et al* [17]. The final suspending medium was a Tyrode's buffer, pH 7.4, of the following composition (mM): NaCl 137; KCl 2.68; $NaHCO_3$ 11.9; $MgCl_2$ 1.0; $NaHPO_4$ 0.4; glucose 5.5. Platelets (6–8 x 10^7 cells) were suspended in 0.5 ml incubation mixture (Tyrode's buffer containing BSA 0.25%, adenosine deaminase 1 UI/ml and 0.5 mM Ro 20-1724 as a phosphodiesterase inhibitor) and preincubated for 10 min in a shaking bath at 37°C. Then, the synthesized compounds, NECA (1 μM) and forskolin (1 μM) were added, and the incubation continued for a further 5 min. The reaction was terminated by the addition

of cold 6% trichloroacetic acid (TCA). TCA suspension was centrifuged at 2000 *g* for 10 min at 4°C and the supernatant was extracted 4 times with water-saturated diethyl ether. The final aqueous solution was tested for cAMP levels by a competitive protein binding assay carried out essentially according to Brown *et al* [18] and Nordstedt and Fredholm [19]. Samples of cAMP standards (0–10 pM) were added to each test-tube containing buffer Brown pH 7.4 (trizma base 0.1 M; aminophylline 8.0 mM; 2-mercaptoethanol, 6.0 mM) and [^{3}H]-cAMP, in a total volume of 0.5 ml. Binding protein, previously prepared from bovine adrenals, was added to the samples which were incubated at 4°C for 150 min and, after the addition of charcoal, were centrifuged at 2000 *g* for 10 min. Clear supernatant (0. 2 ml) was mixed with 4 ml of Ready Gel and counted in a LS-1800 Beckman scintillation counter. IC_{50} values were obtained from concentration–response curves by linear regression analysis after log transformation.

Platelet aggregation

New Zealand White male rabbits from Bettinardi Farm (Alzate, Novara, Italy) were housed in temperature and light-controlled room and maintained on food pellets and water *ad libitum*. Animals were acclimatized for at least 1 wk before experiments. After anaesthesia with pentobarbital (20 mg/kg iv), blood was collected from the left carotid artery and put into plastic tubes containing 1/10 volume 3.8% trisodium citrate. To prepare platelet-rich plasma (PRP), the blood was centrifuged at room temperature for 18 min at 150 *g* and the upper two-thirds of the PRP layer was withdrawn. Platelet poor plasma (PPP) was prepared by centrifugation of the packed erythrocyte layer at 3 000 *g* for 15 min. Rabbit PRP was adjusted to a platelet count of 4.0–5.0 x 10^8/ml using autologous PPP.

Platelet aggregation was performed according to the Born turbidimetric technique [20] using a DIC PA-3220 Aggrecorder (Kyoto Daiichi Kagaku Co, Japan). Compounds were dissolved in saline containing 10% dimethyl sulphoxide (DMSO), which was present in the PRP at a final concentration of 0.5%. PRP (285 µl) was preincubated with the test compounds (5 µl) for 2 min in siliconized cuvettes at 37°C with continuous stirring (1000 rpm). Separate experiments using different time periods of preincubation (0, 2 and 5 min) indicated that the activity of the compounds examined was not influenced by preincubation time. Then, NECA was added at a concentration (0.3 or 1.0 µM) inducing a platelet aggregation inhibition of ≈ 50%. After 3-min incubation, platelet aggregation was induced by adding ADP (25 µl) to give a final concentration of 5 µM. All experiments were carried out within 2 h of blood collection. The maximal amplitude of aggregation, recorded 5 min after the addition of ADP, was used for the quantitative evaluation of the aggregation process. As for statistical analysis, 3 different concentrations of the test compounds were assayed in duplicate on at least 3 different animals. The dose of antagonist reducing by 50% the effect induced by NECA was calculated by linear regression analysis.

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