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Recent developments in the field of A_{2A} and A_3 adenosine receptor antagonists

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Abstract

In the last years adenosine receptors have been extensively studied, and mainly at present we understand the importance of A_{2A} and A_3 adenosine receptors. A_{2A} selective adenosine receptors antagonists are promising new drugs for the treatment of Parkinson's disease, while A_3 selective adenosine receptors antagonists have been postulated as novel anti-inflammatory and antiallergic agents; recent studies also indicated a possible employment of these derivatives as antitumour agents. Lately different classes of compounds have been identified as potent A_{2A} and A_3 antagonists. In this article we report the past and present efforts which led to development of more potent and selective A_{2A} and A_3 antagonists. Our group has mainly worked on the pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine nucleus both as A_{2A} and A_3 antagonists, aiming to improve the affinity, selectivity and the hydrophilic profile. In fact, we have synthesised several compounds endowed with high affinity and selectivity versus A_{2A} adenosine receptors, as **2**, **2a**–**c** ($K_iA_{2A} = 0.12$–0.19 nM), or A_3 adenosine receptors, as **4p** ($K_iA_3 = 0.01$ nM) and **4q** ($K_iA_3 = 0.04$ nM).
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Keywords: A_{2A} adenosine receptors; A_3 adenosine receptors; Antagonists; Water-solubility

1. Introduction

Adenosine, an endogenous modulator of a wide range of biological functions, interacts with at least four cell surface receptor subtypes classified as A_1, A_{2A}, A_{2B} and A_3. These receptor subtypes belong to the superfamily of G protein-coupled receptors and have been cloned in several animal species [1,2]. In the central nervous system the A_{2A} adenosine receptors are present with high density in basal ganglia, and are able to determine a cross-talk with dopamine receptors [3]. Several data showing the colocalisation of A_{2A} and D_2 receptors are supporting the concept that blockade of A_{2A} receptors produces direct effects on D_2 receptors [3]. In the peripheral system, A_{2A} receptors are present on numerous tissues including platelets [4], lymphocytes [5], neutrophils [6,7], monocytes, macrophages and mast cells. Moreover, activation of A_{2A} receptors seems to be associated with inhibition of tumour necrosis factor-α, IL-6, IL-8 and elastase release by activated mononuclear phagocytes [8]. It has been also investigated the possible hepatoprotective effects of A_{2A} receptor antagonism in 'in vivo' models of hepatic injury [9]. In the past 10 years, great efforts by medicinal chemists and pharmacologists have been devoted to the design of potent and selective ligands for A_{2A} receptors. Thus, the pyrazolotriazolo pyrimidines SCH 58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-e]1,2,4-triazolo [1,5-c]pyrimidine), SCH 63390 (5-amino-7-(3-phenylpropyl)-2-(2-furyl)pyrazolo[4,3-e]1,2,4-triazolo [1,5-c] pyrimidine) [10] and related compounds which posses hydrophilic groups at *para* and *ortho* position of the aromatic ring have been found to be potent and selective adenosine A_{2A} antagonists [11,12]. Then the availability of the tritiated form of SCH 58261 has helped to characterise A_{2A} receptor subtypes in various tissues

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which are known to be sensitive to adenosine action [13,14]. Blockade of A_{2A} receptor through the use of the selective antagonist SCH 58261 have been found to be effective in animal models of Parkinson's disease and stroke [15,16]. Several efforts have allowed to better understand the specific function of A_{2A} receptor in physiological or altered conditions [17–19]. Recently, selective A_{2A} antagonists showed their ability to fully block agonist stimulated adenylyl cyclase in a model 'in vitro' of Huntington's disease [20]. It has been demonstrated that SCH 58261 is able to block NECA-stimulated adenylyl cyclase in striatal derived cells and subclones engineered to express either normal or mutant huntingtin suggesting possible new pharmacological tools in the treatment of Huntington's disease [21]. It has been also demonstrated that A_{2A} receptors may contribute to cell death in ischemia associated neurodegeneration and that A_{2A} antagonists may indeed prove useful in preventing such damage [22,23]. Several studies indicate that adenosine A_3 receptors may play a basic role in different pathologies such as inflammation [24,25] and neurodegeneration [26,27], ischemic brain damage [28,29], cardiac ischemia [30], hypotension [31], ischemic heart pre-conditioning [32,33], asthma [34] and cancer [35]. The newly discovered A_3 adenosine receptors [36] exert their anti-inflammatory properties by inhibiting specific cell functions in different systems such as human monocytes, macrophages and neutrophils [37]. In the last few years important progress has been made on the development of selective A_3 receptor antagonists which have an interesting profile [38–41]. Recently, it has been identified a series of substituted pyrazolo triazolo pyrimidines as potent and selective antagonists to human A_3 adenosine receptors [42–46]. In particular, one of these compounds, [^{3}H]-5N-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl) pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine ([^{3}H]-MRE 3008F20) has been tritiated and used to characterised the human A_3 adenosine receptors expressed in CHO cells [21]. This new ligand displayed high selectivity (1294-, 165- and 2471-fold) in binding assay to human A_3 versus A_1, A_{2A} and A_{2B} adenosine receptors, respectively. The pharmacological profile of [^{3}H]-MRE 3008F20 binding to hA_3CHO cells was evaluated using known adenosine receptor agonists and antagonists with a rank order of potency consistent with that typically found for interactions with the A_3 adenosine receptors. With the aim of contributing to an evaluation of the molecular mechanism underlying ligand–receptor interactions it has been studied the binding thermodynamic of [^{3}H]-MRE 3008F20 in hA_3CHO cells [47]. Earlier pharmacological works indicate the presence of A_3 receptors in Jurkat cells, a human leukemia line with a pharmacological, biochemical and thermodynamic profile of the human A_3 subtype found in other cell types [48]. Moreover it has been evaluated the presence and the functionality of A_3 receptors in the neutrophil granulocytes and promyeolocytic HL60 cells revealing a potential role of important regulator of the adenosine in acute inflammation [36]. Recently, it has been also reported that human melanoma cell line (A375) expresses high level of A_3 receptors suggesting a potential role for adenosine in modulating tumoural processes [48]. In A375 cells, it has been investigated the effects of adenosine on cell proliferation, colony formation ability and on the balance between cell survival and cell death [49]. A_3 receptors and their ability to regulate cell survival represent a promising therapeutic target in diseases in which excessive cell death is either undesirable such as neurodegeneration or desirable such as cancer and inflammation [50].

The aim of this article is to summarize past and present studies which carried to discovery of more potent and selective A_{2A} and A_3 antagonists. In particular our research group have developed the pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines, a new class of A_{2A} and A_3 antagonists endowed with greater potency and selectivity, and having a better hydrophilic profile.

2. A_{2A} AR antagonists

Historically it is known that A_{2A} ARs antagonists are divided in xanthine and non-xanthine derivatives.

Starting from the naturally occurring methylxanthines (caffeine and theophylline), a number of xanthine derivatives have been synthesised. The substitution of the 8 position with a variety of groups, has led the chemists to discover that the introduction of the styryl group in this position was critical in achieving compounds endowed with selective A_{2A} receptor antagonistic properties [10,51,52]. The result of this effort was the discovery of KF 17837, KW 6002, and CSC, Fig. 1, the pharmacological characteristics of which have been studied extensively [53–55]. One of the first AR antagonists described in the literature as A_{2A} antagonist

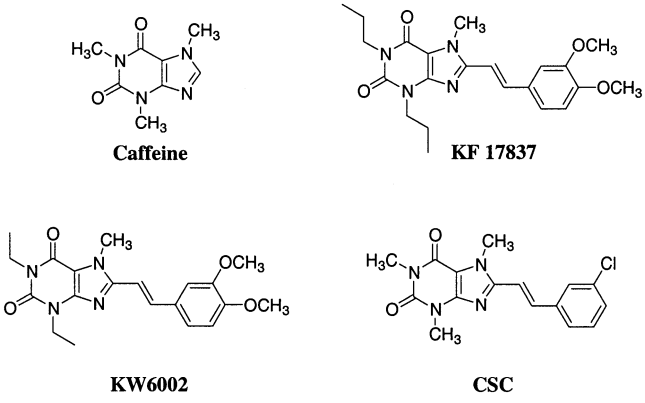


Fig. 1. A2A-selective Ars antagonists containing xantine neucleus.

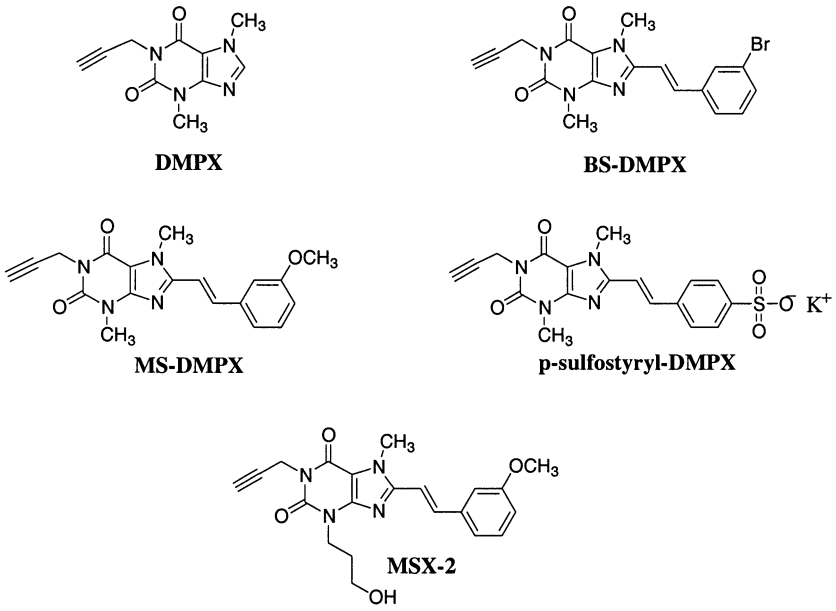


Fig. 2. 3,7-Dimethyl-1-propargyl-8-stylxanthine derivatives designed as A2A-selective Ars antangonists.

was 3,7-dimethyl-1-propargylxanthine (DMPX), Fig. 2, [56,57].

Müller et al. have developed a series of 3,7-dimethyl-1-propargyl-8-styrylxanthine derivatives as A_{2A}-selective ARs antagonists [58], among which 8-(*m*-bromostyryl)-3,7-dimethyl-1-propargylxanthine (BS-DMPX) and 8-(*m*-methoxystyryl)-3,7-dimethyl-1-propargylxanthine (MS-DMPX), Fig. 2. A major problem of all high-affinity A_{2A} antagonists has been their low water-solubility, which limited their usefulness especially for in vivo studies. Therefore Müller et al. have developed two series of water-soluble A_{2A}-selective AR antagonists: *meta*- and *para*-sulfostyryl-DMPX derivatives [59], and phosphate prodrugs of 1-propargyl-8-styrylxanthine derivative, Fig. 2 [60]. It was found that polar functional groups suitable for the attachment of a prodrug moiety were tolerated on the styryl ring, thus 3-(3-hydroxypropyl)-8-(*m*-methoxystyryl)-1-propargylxanthine (MSX-2), which resulted the most potent and A_{2A}-selective compound, was selected for phosphate prodrug formation, Fig. 2.

Among the non-xanthine heterocycles developed as A_{2A} AR antagonists, there are several classes of compounds. Initially 4-amino-8-chloro-1-phenyl-[1,2,4]triazolo[4,3-a]quinoxaline (CP 66 713) and 5-amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-c]quinazoline (CGS 15943) have shown interesting properties, however they also interacted with other adenosine receptors (Fig. 3) [61]. Intensive efforts by Baraldi and co-workers have led to development of the pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines SCH 58261 and SCH 63390, reported in Fig. 3, which have shown to be potent and selective A_{2A} AR antagonists [62,63]. Few years later, to improve the hydrophilic profile, which was the

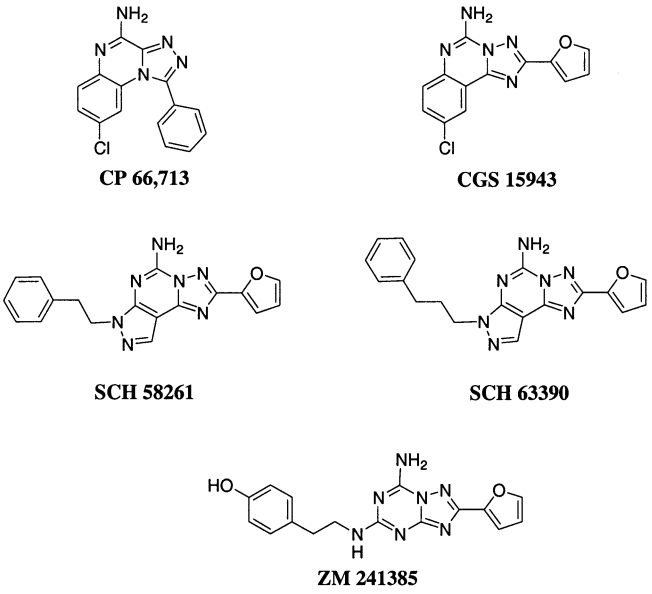


Fig. 3. Non-xanthine heterocycles developed as A2A AR antagonists.

main problem of our previous series, our group has synthesised a second generation of pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines bearing oxygenated substituents on the phenylalkyl chains in the 7-position, the most interesting compounds of this new series were **1**, **1a** and **1b** (Fig. 4) [11].

Going on with our studies in this field, in 2002 our group has presented an extended series of the pyrazolo-triazolo-pyrimidines synthesised with a view to investigate the influence of the substitution on the pyrazole ring [46]. In this study, the choice of the substituents was based on their ability to improve water solubility while retaining high affinity and selectivity at the human A_{2A}

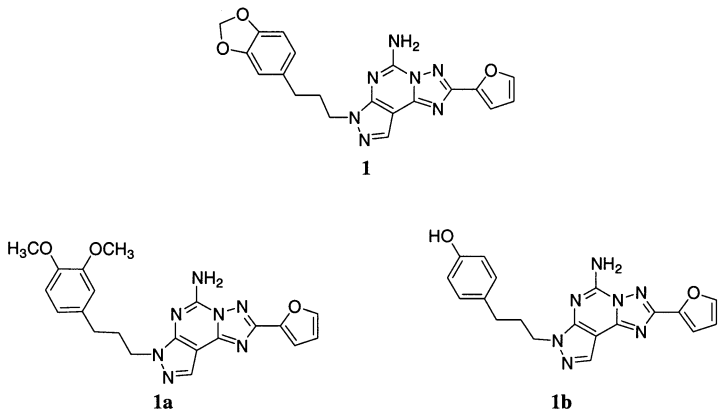


Fig. 4. Second generation of pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines bearing oxygenated substitution/on the phenylalkyl chains at the 7-position.

adenosine receptor subtype: in this series we focused our attention on the nature of the phenyl ring substituent, obtaining the compounds **2**, **2a**–**2c**, Fig. 5, [46].

Our recent research efforts were focused on the design and the synthesis of a C^9-alkylsulfonyl/(cyclo)alkyl-amino/aryl-amino substituted series of pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines of general formula **3**, **3a**–**3j**. Unfortunately this type of structural modification determined a retention of affinity, but a loss of selectivity.

Another interesting compound is ZM241385 [64], which, like SCH 58261, has been derived from prototype CGS 15943, but above all is quite hydrophilic, an interesting characteristic because, usually, the A_{2A} AR antagonist are lipophilic compounds, Fig. 3.

Another class of compounds having properties A_{2A} AR antagonist is the one of the 1,2,4-triazolo[4,3-a]-quinoxalin-1-ones, in particular 4-amino-6-benzyla-mino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]quinox-alin-1-one ($K_iA_{2A} = 6.5$ nM) which is 2700-fold selective antagonist for A_{2A} versus A_1 ARs [65]. Furthermore, recently Müller et al. have developed imidazo[2,1-i]purin-5-ones as A_{2A} and A_3 ARs antagonists [66].

3. A_3 AR antagonists

With regard to A_3 AR, different classes of heterocyclic compounds were identified as potent A_3 antagonists: flavonoid derivatives, 1,4-dihydropyridine and pyridine derivatives, triazoloquinazoline, isoquinoline and quinazoline derivatives and, finally, various heterocycles [43].

In general our group has worked on the classes of pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidnes; our strategy was based on the design of new hybrid molecules between the N^8 substituted pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine derivatives and the phenylcarbamoyl chain present in a series of N^6-(substituted-phenylcarbamoyl)adenosine-5′-uronamides (Fig. 6) previously reported as A_3 agonists [62,67,68].

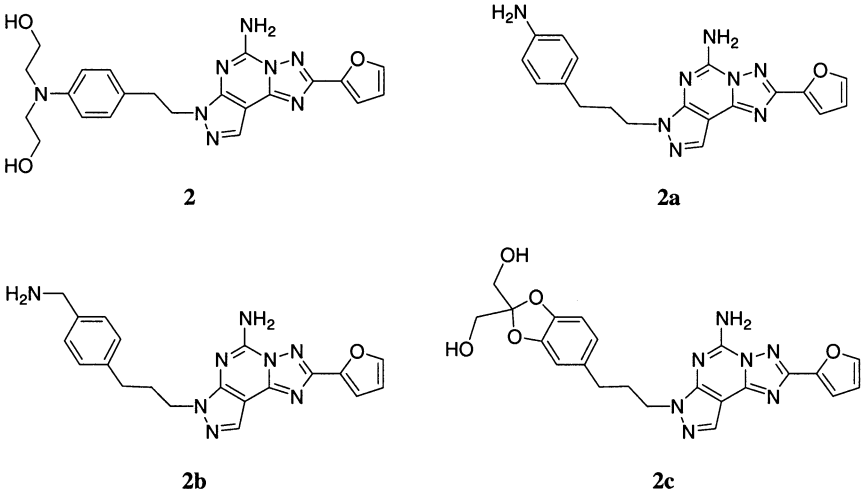


Fig. 5. Pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines modified at 7 position to improve water solubility.

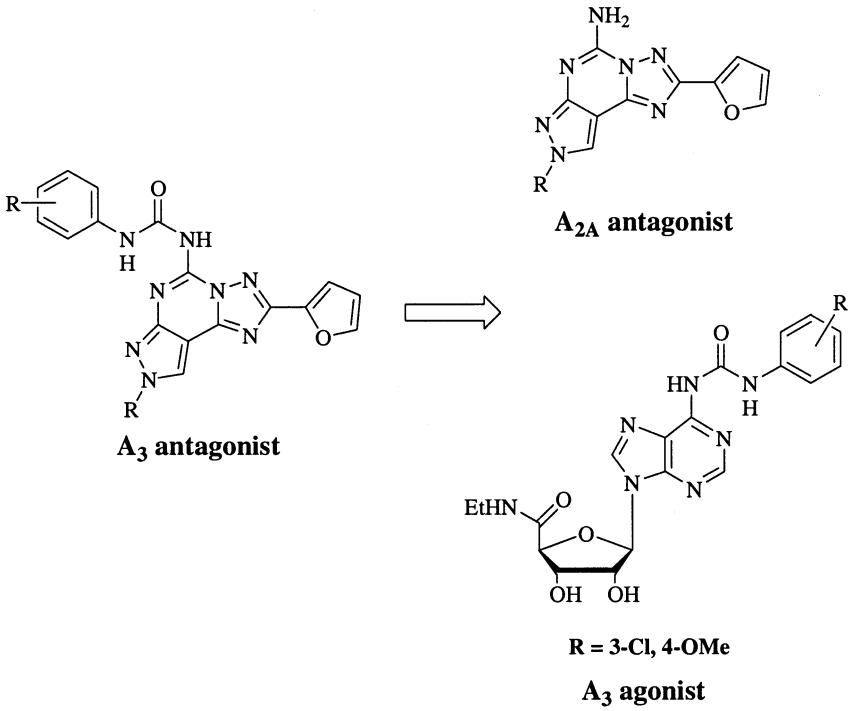


Fig. 6. Design of new hybrid molecules as A3 adenosine antagonists.

Starting from these structures, we optimised the substitution patterns at the phenyl ring of the arylcarbamoyl, and we succeeded in obtaining the first potent, selective and water-soluble human A_3 AR antagonist (compound **4q**) [69].

Furthermore, our group synthesised a new class of irreversible antagonists of A_3 AR [70], which should be an important tool for the study of A_3 receptor properties.

We finally tuned the synthesis of a new series of pyrazolo-triazolo-pyrimidines substituted at 9-position to investigate the effect of the introduction of different substituents in this position of the tricyclic template.

4. Result and discussion

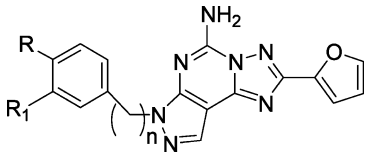
4.1. Pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines as human A_{2A} adenosine receptor antagonists

The classes of A_{2A} AR antagonists so far developed have been numerous, and our group worked mainly on pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines.

As aforesaid, in 1998 our group developed a second generation of pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines: starting from the structure of the SCH 58261, in order to improve the hydrophilic character, and in analogy with ZM 241385 structure, we investigated the effects of variously substituted hydroxylic functions on the phenyl ring of the side chain in the 7-position. The presence of the hydroxylic function in the *para* position of the phenyl ring increases the affinity and in particular A_{2A} AR selectivity; furthermore we confirmed the previous observation [62] that the best length for the side chain is three methylene groups: in fact the compound **1b** showed the highest affinity and selectivity in binding assays ($K_iA_{2A} = 0.94$ nM, $A_1/A_{2A} = 787$). The new compounds synthesised bearing the hydroxyl moiety at the *para* position on the phenyl ring in the side chain, compared versus SCH 58261 and SCH 63390, showed an appreciable improvement in hydrophilic properties, unfortunately this was not enough to make our compounds water-soluble [11] (Table 1).

In 2002, our group has presented an extended series of pyrazolotriazolopyrimidines synthesised with the aim to investigate the influence of the substitutions on the pyrazole ring; in this series we maintained some structural characteristics that are fundamental for the affinity, i.e., tricyclic structure, free amino group at 5-position, furan ring, and substituent at 7-position on the pyrazole ring, but we focused our attention on the nature of the phenyl ring substituent to improve water-solubility, Table 2. In particular, we tried to introduce different substituents, like basic or acidic moieties suitable for the preparation of salts or possible pro-drugs, to modify the physicochemical properties of the compounds. All derivatives synthesised, reported in Table 2, showed a good affinity and selectivity for hA_{2A} AR with a different degree of selectivity versus hA_1. The results of these studies permitted us to confirm our previous observations [43] according to which the pyrazole nitrogens also plays a fundamental role in

Table 1
Biological activity of the second generation of pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines



Compound	*n*	R	R_1	rA_1(K_i, nM) [a]	rA_{2A}(K_i, nM) [b]	hA_3(K_i, nM) [c]	RA_1/rA_{2A}	hA_3/rA_{2A}
SCH 58261	–	–	–	121	2.3	> 10 000	53	> 4347
SCH 63390	–	–	–	504	2.4	> 10 000	210	> 4166
CGS 15943	–	–	–	6.4	0.95	14	6.7	14.5
1	3	OCH_2O	OCH_2O	1841	3.8	> 10 000	484	> 2631
1a	3	OCH_3	OCH_3	2825	5.3	> 10 000	487	> 1886
1b	3	OH	H	741	0.94	> 10 000	787	> 10 638
1c	1	OBn	H	9450	246	> 10 000	38	> 40
1d	2	OBn	H	> 10 000	59	> 10 000	> 169	> 169
1e	3	OBn	H	1460	45	> 10 000	32	> 222
1f	1	OH	H	13 400	53	> 10 000	252	> 188
1g	2	OH	H	444	1.7	> 10 000	261	> 5882
1h	3	OH	OH	2250	49	> 10 000	46	> 204

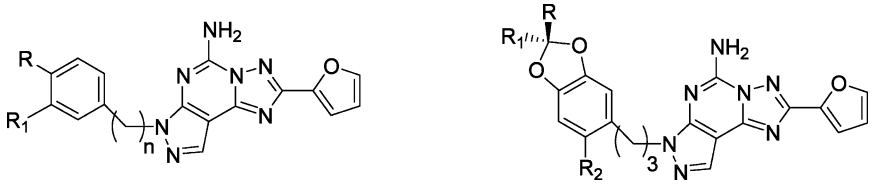
[a] Data are expressed as geometric means, with 95% confidence limits. Displacement of [^{3}H]CHA binding (A_1) at rat cortical membranes.
[b] Displacement of [^{3}H]CGS 21680 binding (A_{2A}) at rat striatal membranes.
[c] Displacement of [^{125}I]AB-MECA binding at human A3 adenosine receptors expressed in HEK-293 cells.

receptor recognition and discrimination and the substitutions of the pyrazolo-triazolo-pyrimidine nucleus modulate affinity and selectivity versus AR subtypes. The sulfonic analogues, compounds **2d**, **2t** (Table 2) appeared to be completely water-soluble, but unfortunately a significant reduction both of the affinity and selectivity was observed. The carboxylic analogue **2x** demonstrated a weakly increase of affinity, while *p*-amino derivative **2a**, showed a very high affinity and selectivity at hA_{2A} AR. The competition curves of these derivatives to human cloned A_1, A_{2A}, A_{2B} and A_3 ARs are depicted in Figs. 7–9. From these results emerged that, probably, there is same interaction between the amino moiety, present in compound **2a**, and an hypothetical carboxylic group sited in the A_{2A} AR.

To retain the water solubility of **2d** and to restore the affinity and selectivity for the A_{2A} ARs, a weaker acidic function on the phenyl ring, such as the carboxylic moiety or its ethyl ester, was introduced producing compounds **2v**–**y**, however also in this case our project of designing a new compound endowed with both water solubility and affinity was not reached. Finally, we decided to introduce an amino group on the phenyl ring, with the aim to obtain soluble salts, even if the basicity of an aromatic amino group is not significantly high. Among these derivatives, the compound **2b** (K_i hA_{2A} = 0.13 nM, hA_1/hA_{2A} = 4430) has shown high affinity and selectivity for the human A_{2A} ARs, but the water solubility was not significantly increased; however **2b** was appeared to be more promising for further pharmacological studies, possibly, like prodrug [46]. The synthesis of compounds reported in Tables 1 and 2 was performed following the general synthetic pathway depicted in Scheme 1 route A: as showed, to obtain only the 7-substituted derivatives we started from the most appropriate hydrazine [11,62,63]. In some cases, is possible to obtain the same compounds following an alternative route by alkylation of a common intermediate which has been synthesised as previously reported [62], Scheme 1 route B.

Finally, as mentioned above, we performed the synthesis of 5-amino-C^9-substitued pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines in order to evaluate the effects of the introduction of different functions at C^9 position. In compounds **3**, **3a**–**3d** we simultaneously considered the effects of C^9 and 5′-position of furan ring substitution. From binding data of the obtained compounds, listed in Table 3, is possible to infer that the C^9-substitution is sometimes compatible with the interaction with the A_{2A} AR, and in particular our best results were obtained introducing at this position an alkylsulfanyl chain. Compound **3c**, bearing a methyl group at 8-position, a methyl-sulfanyl moiety at C^9 and the free amino group at 5-position, resulted to be the best of this series in term of affinity, whereas substitution of the furan ring determined a dramatic loss of affinity. Probably due to unspecific interaction promoted by the C^9-substituent, the selectivity profile of synthesised compounds showed to be rather negative.

Table 2
Biological activity of new series of pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines



Compound	n	R	R_1	R_2	hA_1(K_i, nM) [a]	hA_{2A}(K_i, nM) [b]	hA_{2B}(K_i, nM) [c]	hA_3(K_i, nM) [d]	hA_1/hA_{2A}	$hA_{2B}hA_{2A}$	hA_3/hA_{2A}
2	2	$N(CH_2CH_2OH)_2$	H	–	123	0.12	> 10 000	> 10 000	1025	≫ 10 000	≫ 10 000
2a	3	NH_2	H	–	2160	0.22	> 10 000	> 10 000	9818	≫ 10 000	≫ 10 000
2b	3	CH_2NH_2	H	–	576	0.13	> 10 000	> 10 000	4430	≫ 10 000	≫ 10 000
2c	3	CH_2OH	CH_2OH	H	432	0.19	> 10 000	> 10 000	2273	≫ 10 000	≫ 10 000
2d	2	SO_3H	H	–	190	100	> 10 000	> 10 000	1.9	> 100	> 100
2e	3	NO_2	H	–	1026	1.00	> 10 000	> 10 000	1026	> 10 000	> 10 000
2f	2	$NHCOCH_3$	H	–	419	4.8	> 10 000	> 10 000	87	> 2083	> 2083
2g	3	$N(CH_2CH_2OH)_2$	H	–	558	1.1	> 10 000	> 10 000	507	> 9090	> 9090
2h	3	CN	H	–	6496	86	> 10 000	> 10 000	75.5	> 116	> 116
2I	3	COOEt	H	–	4494	4.0	> 10 000	> 10 000	1123	> 2500	> 2500
2j	2	OCH_2COOEt	H	–	4197	0.43	> 10 000	> 10 000	9760	≫ 10 000	≫ 10 000
2k	3	$C(NOH)NH_2$	H	–	60	6.0	> 10 000	> 10 000	10	> 1666	> 1666
2l	2	NH_2	H	–	75	55	> 10 000	> 10 000	1.36	> 181	> 181
2m	3	$C(NH)NH_2$	H	–	4965	4.4	> 10 000	> 10 000	1128	> 2272	> 2272
2n	3	COOH	H	–	4927	4.63	> 10 000	> 10 000	1064	> 2160	> 2160
2o	2	SO_2NH_2	H	–	2630	1.31	> 10 000	> 10 000	2007	> 7633	> 7633
2p	2	$SO_2N(CH_2CH_2OH)_2$	H	–	2495	0.80	> 10 000	> 10 000	3118	≫ 10 000	≫ 10 000
2q	2		H	–	369	3.8	> 10 000	> 10 000	97	> 2631	> 2631
2r	2	$SO_2N(CH_2CH_2Cl)_2$	H	–	5297	0.59	> 10 000	> 10 000	8977	≫ 10 000	≫ 10 000
2s	2	SO_2NHCH_2COOH	H	–	9330	50.0	> 10 000	> 10 000	186	> 200	> 200
2t	3	SO_3H	H	–	139	140	> 10 000	> 10 000	1	> 714	> 714
2u	3	H	H	SO_3H	6392	75	> 10 000	> 10 000	85	> 133	> 133
2v	3	COOEt	H	H	1793	5.48	> 10 000	> 10 000	327	> 1824	> 1824
2w	3	COOH	COOH	H	9399	120	> 10 000	> 10 000	78	> 83	> 83
2x	3	COOH	H	H	3599	59	> 10 000	> 10 000	61	> 169	> 169
2y	3	COONa	COONa	H	9533	120	> 10 000	> 10 000	79	> 83	> 83

Data are expressed as geometric means, with 95% confidents limits.
[a] Displacement of specific [^{3}H]-DPCX binding at human A_1 receptors expressed in CHO cells ($n = 3–6$).
[b] Displacement of specific [^{3}H]-SCH 58261 binding at human A_{2A} receptors expressed in HEK-293 cells.
[c] Displacement of specific [^{3}H]-DPCPX binding at human A_{2B} receptors expressed in HEK-293 cells ($n = 3–6$).
[d] Displacement of specific [^{3}H]-MRE3008-F20 binding at human A_3 receptors expressed in CHO cells.

4.2. Pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines as human A_3 adenosine receptor antagonists

Recently, our group characterised a new class of A_3 AR antagonists having a pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidinic structure substituted at the 8 position and bearing a phenylureidic function at the 5 position. This structure has been conceived on the basis of the knowledge emerged from SAR studies on the ligands of A_{2A} ARs (in particular of the SCH series) and on the A_3 AR agonists [42,50]. A_{2A} AR antagonists of the SCH series show a free exocyclic amino group at the 5 position, a furan ring and several substituents at the 7-position on the pyrazole ring; while *N*-phenylcarbamoil-5′-uronamidic derivatives of adenosine show a good selectivity versus A_3 AR [42,62,67]. On the basis of these observations, we tried to combine the pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidinic nucleus of the SCH

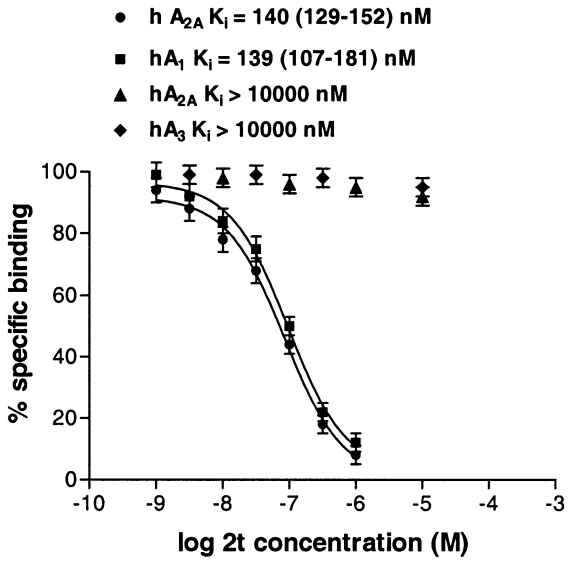


Fig. 7. Competition curves of **2t** compound to human cloned A_1, A_{2A}, A_{2B} and A_3 adenosine receptors. Curves are the mean derived from a series of three independent experiments.

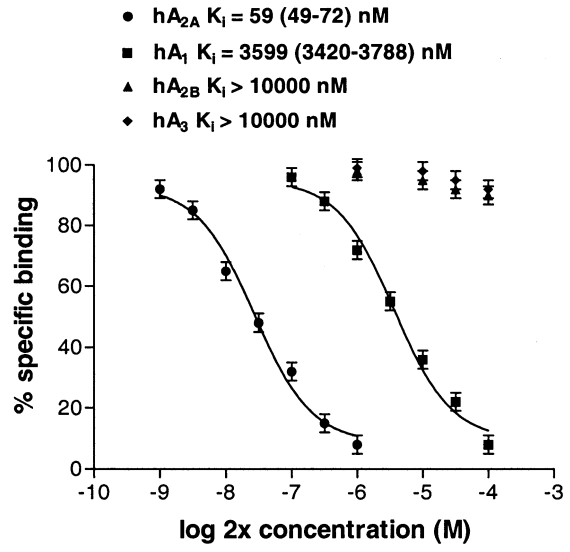


Fig. 8. Competition curves of **2x** compound to human cloned A_1, A_{2A}, A_{2B} and A_3 adenosine receptors. Curves are the mean derived from a series of three independent experiments.

series with the phenylcarbamoyl function of the *N*-phenylcarbamoil-5′-uronamidic derivatives, obtaining a new hybrid structure potent and selective versus A_3 AR, Fig. 6. The choice of the substituents originated from the consideration that the introduction of the 3-chlorophenyl and the 4-methoxyphenyl moieties generated compounds very potent at rat A_3 receptors in the agonists series ($K_i = 4.4$ and 6.6 nM, respectively) [67]. The biological data of the synthesised hybrid compounds indicated the fundamental importance of the phenylcarbamoyl chain for obtaining both potency and selectivity at the human A_3 AR.

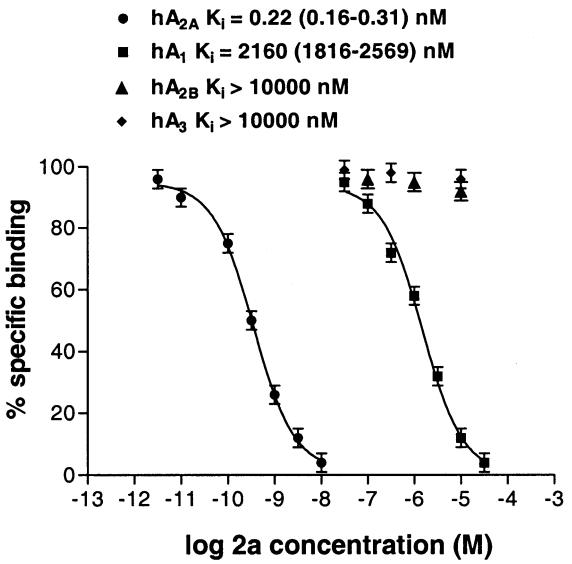
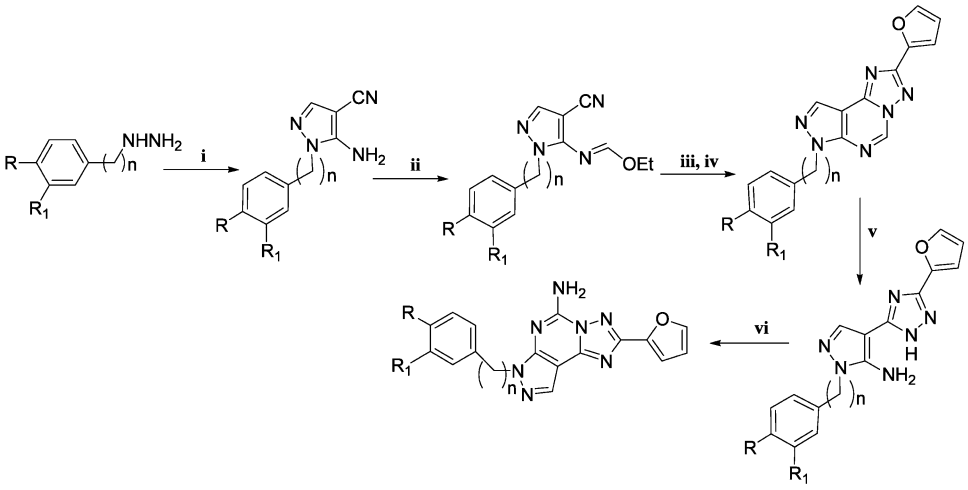


Fig. 9. Competition curves of **2a** compound to human cloned A_1, A_{2A}, A_{2B} and A_3 adenosine receptors. Curves are the mean derived from a series of three independent experiments.

Structure–activity studies carried out by us on the synthesised compounds, indicated a possible correlation between the molecular volume of the subtituents at N^8 pyrazole nitrogen and the experimental K_i values; in particular it was evident that the A_3 affinities decreased with the increasing of molecular volume values at the N^8 position. In fact the compound **4**, Fig. 10, which is the most potent and selective at hA_3 receptor, derived from the combination of the N^8-unsubtituted derivative and the N^5-(4-methoxy)phenylcarbamoyl chain, while a reduction of affinity of about 10- to 100-fold was noticed when the phenylethyl and phenylpropyl chains were introduced at N^8 position (compounds **4a**, **4b**, Fig. 10).

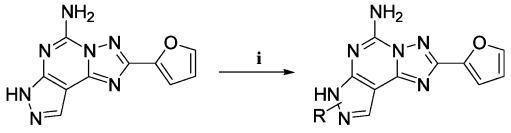
Going on with our studies in this field, we proceeded with the optimisation of substitution patterns at the phenyl ring of the arylcarbamoyl moiety. We focused our attention on the valuation of the effect of both types of substitution and the position of the substituents on the phenyl nucleus. As Table 4 shows, the most interesting compound in terms of both affinity and selectivity was the unsubstituted derivative **4c** ($K_i = 0.16$ nM at hA_3). Docking studies on a new model of hA_3 adenosine receptor deriving from a 2.8 Å resolution crystal structure of bovine rhodopsin [68] gave explanation for the fact that polar substituents in *para* position are not very well tolerated as in the case of sulfonic acid group (see compound **4f** in Table 4). These studies indicated also that a steric control takes place around the *para* position of the phenyl ring: indeed substituents larger than fluorine, chlorine or methyl are not well tolerated, and these substituents cause a decrease of affinity at the hA_3 AR of about 2- to 5-fold with respect to the unsubstituted derivatives. In addition, a steric

Route A



Reagents: (i) (ethoxymethylene)malononitrile; (ii) $HC(OEt)_3$, reflux; (iii) furoic hydrazide, $MeO(CH_2)_2OH$; (iv) Ph_2O, 260 °C; (v) 10% HCl, reflux; (vi) NH_2CN, 1-mehyl-2-pyrrolidone, pTsOH, 140 °C.

Route B



Reagents: (i) RCl, K_2CO_3, DMF, 100 °C, 12h.

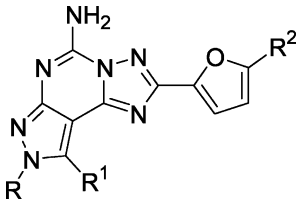
Scheme 1.

repulsion between substituents at the *ortho* and *meta* positions of the phenyl ring and the binding site of the receptor, contributes to reduce the affinity at A_3 receptor (compounds **4m**–**o**).

Another interesting aspect analysed in the same work is the evaluation of derivatives **4r**–**u** listed in Table 4, bearing at N^5 position the phenylacetic chain characteristic of **MRS 1220** [40,71]. In a previous work [43] we reported the synthesis of two hybrid derivatives structurally correlated to **MRS 1220** by the substitution of the phenylacetic chain with the urea moiety, demonstrating that the phenylcarbamoyl moiety and the pyrazole nitrogen play an important role in determining potency and selectivity at human A_3 AR subtype. Interestingly, compounds **4r**–**u** resulted to be about 4- to 8-fold less potent at human A_3 compared to the parent urea derivatives, with consequent loss of selectivity. Our theoretical A_3 receptor model justifies this result suggesting that the N^5 substituent should interact with an hydrophilic region in the hypothetical receptor binding site which consequently shows to be better at accommodating the NH of the phenylcarbamoyl derivatives than the lipophilic CH_2 spacer of the corresponding phenylacetic analogues. Moreover, these results confirmed our previous observations suggesting that the pyrazole nitrogens could modulate the selectivity, while the urea group is able to confer potency versus human A_3 receptor.

Considering that the major problem of pyrazolo-triazolo-pyrimidines was their low water solubility, which has limited their employment as pharmacological and diagnostic tools, an important goal of our group was the synthesis of the first potent, selective, but above all, water-soluble human A_3 AR antagonist [69]. Compound **4c**, reported in Table 4, is characterised by good binding data, but unfortunately, also by a elevated hydrophobicity (Rm = 4.06). Subsequently we tried to improve water solubility of such molecule introducing a sulfonic acid group at the *para* position on the phenyl ring of the carbamoyl moiety: this modification leaded to a marked increase of water-solubility (Rm = 1.66), however a significant loss of selectivity (156-fold) was observed (see compound **4f**, Table 4). Bearing in mind the steric control around the *para* position of the phenyl ring, we assumed that the bioisosteric replacement of the aromatic nucleus with a 4-pyridyl moiety (**4p**) could provide higher water solubility while avoiding the steric hindrance of *para* substituents. The choice of introducing a basic nitrogen was dictated by the opportunity of obtaining the corresponding HCl salt (**4q**) with a further improvement of water solubility. As expected both substances showed very high affinity and good selectiv-

Table 3
Biological activity of pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines substituted on the C^9-position



Compound	R	R_1	R_2	hA_1(K_i,nM) [a]	hA_{2A}(K_i, nM) [b]	hA_{2B}(K_i, nM) [c]	hA_3(K_i, nM) [d]
3	CH_3	$NHCH_2CH_3$	H	50	10	81	225
3a	CH_3	4-methoxy-phenyl-amino	H	260	>1000	>1000	>1000
3b	CH_3	4-methyl-piperazin-1yl	H	30	156	35	>1000
3c	CH_3	SCH_3	H	8.4	1.2	10.3	35
3d	CH_3	$SCH_2CH_2CH_3$	H	9	2.1	69	224
3e	$(CH_2)_3Ph$	SCH_3	H	175	22	31	>1000
3f	CH_3	SCH_3	4-methyl-piperazin-1-yl-methyl	>1000	>1000	>1000	>1000
3g	CH_3	4-hydroxy-phenyl-amino	H	666	1100	2060	308
3h	CH_3	ethyl-amino (HCl)	H	41	10	36	25
3i	CH_3	4-methyl-piperazin-1yl (HCl)	H	48	135	12	>1000
3j	CH_3	SCH_3	4-methyl-piperazin-1-yl-methyl (HCl)	>1000	>1000	>1000	>1000

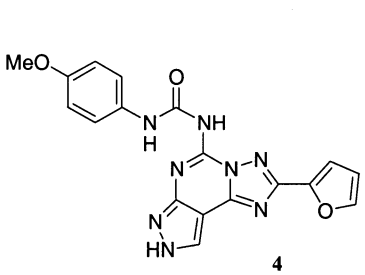
[a] Displacement of specific [^{3}H]-DPCX binding at human A_1 receptors expressed in CHO cells.
[b] Displacement of specific [^{3}H]-SCH 58261 binding at human A_{2A} receptors expressed in CHO cells.
[c] Displacement of specific [^{3}H]-DPCPX binding at human A_{2B} receptors expressed in HEK-293 cells.
[d] Displacement of specific [^{3}H]-MRE3008-F20 binding at human A_3 receptors expressed in CHO cells.

ity at A_3 receptor subtype with K_i values in the picomolar range (10–40 pM) (Table 4). First of all, the hydrochloride salt **4q** showed increased selectivity and affinity with respect to the reference compound **4c** with a notable improvement of water-solubility ($Rm = 2.29$). In view of such results, **4q** could be considered an ideal candidate for pharmacological and clinical investigation of human A_3 adenosine receptor subtype. Competition curves of compounds **4c**, **4f**, **4p**, and **4q** are depicted in Figs. 11 and 12.

Our group newly reported the synthesis of chemically reactive derivatives, which act as irreversible antagonists of A_3 AR (compounds **5a**–**h**, Table 5) [70]. To achieve this purpose we introduced electrophylic groups, specifically sulfonyl fluoride and nitrogen mustard (bis-(β-chloroethyl)-amino) moieties at the 4-position of the phenyl-urea function. The utilization of irreversible antagonists represents a useful approach for the study of receptor features, such as ligand binding site mapping and physiological receptor functioning. The biological evaluation showed that compounds containing a fluorosulfonyl moiety proved to be irreversible antagonists with different degrees of potency at the human A_3 AR (about 70–80% of irreversible inhibition percentage at 100 nM concentration), while the corresponding nitrogen mustard derivatives were unable to covalently bind at this receptor subtype.

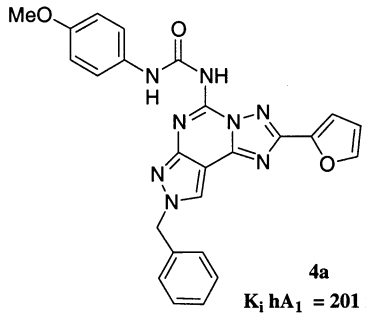
We lately performed the synthesis of a new series of pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines substituted at 9-position to investigate the effect of the introduction of different substituents, such as $-SCH_3$, $-NHCH_2CH_3$ and –4-methyl-pyperazin moieties, in this position of the tricyclic template (Table 6, compounds **6**, **6a**–**6g**). Unfortunately, binding data indicated that such structural innovation induces a loss of both affinity and selectivity toward hA_3 adenosine receptor target. It could be suggested that the introduced substituents cause a repulsion effect, due to the steric hindrance, which hampers the interaction with the binding site of the receptor. Both ureidic (**6**, **6a**–**6c**) and phenylacetic derivatives (**6d**–**g**) of this series of pyrazolo-triazolo-pyrimidines, showed in fact low or totally lacking affinity at the desired hA_3 target, except for compound **6c** (K_i $hA_3 = 9.00$ nM), which displaying nevertheless to be non-selective versus other adenosine receptor subtypes. A surprising element emerging from the pharmacological data, listed in Table 6, is the noteworthy affinity of these derivatives for hA_{2A} subtypes and for hA_{2B} adenosine receptors.

In order to better pharmacologically characterise the hA_3 adenosine receptor, our efforts were focused on the research of labelled derivatives. We carried out the project introducing an allyl chain at N^8 pyrazole nitrogen and then reducing it with tritium gas [72].



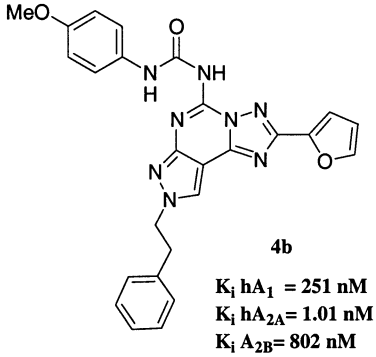
4

K_i hA_1 = 448 nM
K_i hA_{2A}= 520 nM
K_i A_{2B}= 350 nM
K_i A_3 = 0.14 nM
A_1/A_3= 3.2
A_{2A}/A_3= 3.714
A_{2B}/A_3 = 2.5



4a

K_i hA_1 = 201 nM
K_i hA_{2A}= 120 nM
K_i A_{2B}= 81 nM
K_i A_3 = 1.47 nM
A_1/A_3= 137
A_{2A}/A_3= 82
A_{2B}/A_3 = 55



4b

K_i hA_1 = 251 nM
K_i hA_{2A}= 1.01 nM
K_i A_{2B}= 802 nM
K_i A_3 = 19.81 nM
A_1/A_3= 12.6
A_{2A}/A_3= 51
A_{2B}/A_3 = 40

Fig. 10.

The obtained compound, named [^{3}H]-**MRE 3008-F20**, proved to be the first potent and selective labelled antagonist for the human A_3 adenosine receptor showing a K_D value of 0.82 ± 0.08 nM and B_{max} value of 297 ± 28 fmol (mg protein)$^{-1}$.

5. Conclusions

In the last years many research groups have worked to identify and develop new molecular classes of A_{2A} and A_3 adenosine receptors antagonists. The goal of our studies was completely achieved developing the nucleus of pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines variously substituted. This class of compounds allowed us to obtain new A_{2A} and A_3 adenosine receptors antagonists decidedly potent and selective, and some water-soluble derivatives which should be candidate for pharmacological and clinical investigation of human adenosine receptor subtype.

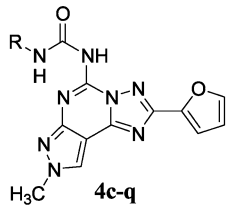
5.1. Pharmacology

5.1.1. Binding and functional assays

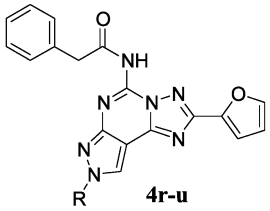
The binding data of the examined compounds were determined to the human A_1, A_{2A}, A_{2B} and A_3 receptors expressed in CHO or HEK 293 cells (Figs. 7–9 and 11).

5.1.1.1. Binding of [^{3}H]-DPCPX to CHO cells transfected with the human recombinant A_1 adenosine receptors. Displacement experiments were performed for 120 min at 25 °C using 1 nM [^{3}H]-DPCPX ([^{3}H]-1,3-dipropyl-8-cyclopentylxanthine), diluted membranes and different concentrations of antagonists studied. Non-specific binding was determined in the presence of 10 μM of DPCPX.

Table 4
Effect of the substitution on the phenyl ring on hA_3 AR affinity



4c-q

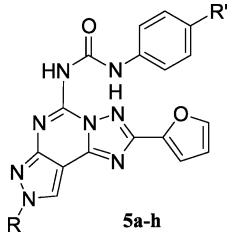


4r-u

Compound	R	*R*m [a]	hA_1(K_i, nM) [b]	hA_{2A}(K_i, nM) [c]	hA_{2B}(K_i, nM) [d]	hA_3(K_i, nM) [e]
4c	Ph	4.06	594	381	222	0.16
4d	3,4-Cl_2–Ph	–	392	143	116	3.4
4e	3,4-OCH_2O–Ph	–	1015	680	142	0.24
4f	4-SO_3H–Ph	1.66	> 10 000	594	> 10 000	25
4g	4-NO_2–Ph	–	1115	695	180	0.43
4h	4-CH_3–Ph	–	731	110	302	0.31
4I	4-Br–Ph	–	600	100	181	0.46
4j	4-F–Ph	–	700	120	226	0.34
4k	4-CF_3–Ph	–	750	140	286	0.74
4l	4-Cl–Ph	–	430	180	128	0.29
4m	2-Cl–Ph	–	400	200	101	0.91
4n	2-OCH_3–Ph	–	450	180	223	0.7
4o	3-OCH_3–Ph	–	500	160	251	0.8
4p	4-pyridyl	3.06	250	60	200	0.04
4q	4-pyridyl (HCl)	2.029	350	100	250	0.01
4r	CH_3	–	702	423	165	0.81
4s	C_2H_5	–	714	335	161	1.03
4t	*n*-C_3H_7	–	351	306	143	1.01
4u	*n*-C_4H_9	–	602	400	101	1.11

[a] The *R*m values of **4c**, **4f**, **4–q** were measured with a mobile phase of different concentration of MeOH/H_2O and are reported as theoretical at 0% organic solvent in the mobile phase (*R*m(0)).
[b] Displacment of specific [^{3}H]-DPCPX binding at human A_1 receptors expressed in CHO cells.
[c] Displacement of specific [^{3}H]-SCH 58261 (**4c–o**, **4r–u**) [^{3}H]-ZM241385 (**4p**–**4q**) and binding at human A_{2A} receptors expressed in CHO cells (**4c–o**) and HEK-293 cells (**4p–u**).
[d] Displacement of specific [^{3}H]-DPCPX binding at human A_{2B} receptors expressed in HEK-293 cells.
[e] Displacement of specific [^{125}I]-AB-MECA (**4c–o**) and [^{3}H]MRE3008-F20 (**4p–u**) binding at human A3 receptors expressed in HEK-293 cells.

Table 5
Irreversible inhibition of radioligand ([^{125}I]-AB-MECA) binding to human adenosine receptors in CHO cell membranes following 1 h incubation with compounds **4a**–**h** at concentrations of 1, 10 and 100 nM



5a-h

Compound	R	R′	Inhibition (% of control)		
			1 nM	10 nM	100 nM
5a	CH_3	$N(CH_2CH_2Cl)_2$	0	11 ± 6	22 ± 5
5b	CH_3	SO_2F	ND	ND	ND
5c	C_2H_5	$N(CH_2CH_2Cl)_2$	0	0	18 ± 17
5d	C_2H_5	SO_2F	4.1 ± 5.1	30 ± 5	65 ± 4
5e	*n*-C_3H_7	$N(CH_2CH_2Cl)_2$	7.8 ± 10	11 ± 9	16 ± 14
5f	*n*-C_3H_7	SO_2F	47 ± 7	70 ± 3	79 ± 8
5g	*n*-C_4H_9	$N(CH_2CH_2Cl)_2$	0	7 ± 4.9	9 ± 7.2
5h	*n*-C_4H_9	SO_2F	31 ± 6	40 ± 11	73 ± 7

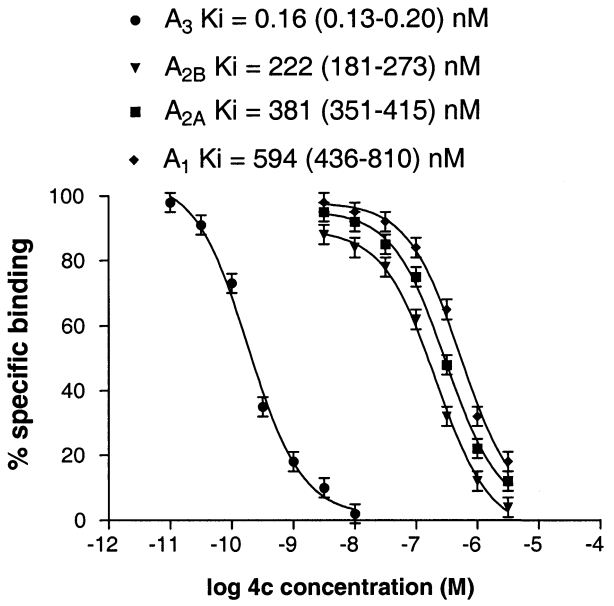


Fig. 11. Competition curves of **4c** compound to human cloned A_1, A_{2A}, A_{2B} and A_3 adenosine receptors. Curves are the mean derived from a series of three independent experiments.

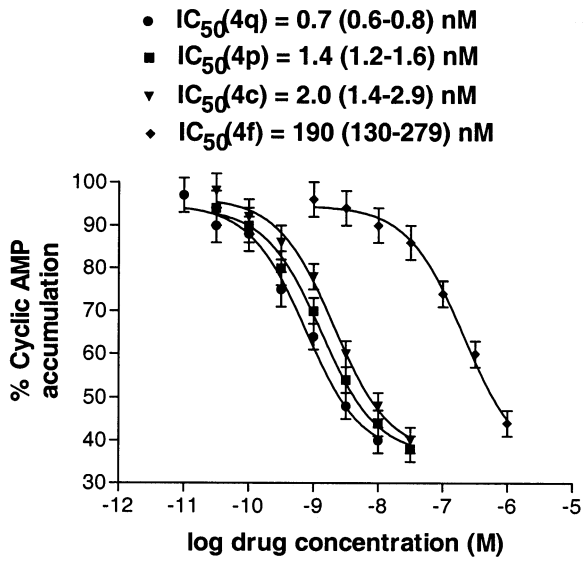


Fig. 12. Inhibitory capability of compounds **4c**, **4f**, **4p**, and **4q** to blockade the effect of 100 nM Cl-IB-MECA induced inhibition of cAMP production in CHO cells expressing human cloned A_3 adenosine receptors. Curves are the mean derived from a series of three independent experiments.

5.1.1.2. Binding of [^{3}H]-ZM 241385 to CHO cells transfected with the human recombinant A_{2A} adenosine receptors. Membrane suspension was incubated using 1 nM [^{3}H]-ZM 241385 ([^{3}H]-4-(2-[7-amino-2 (2-furyl)-[1,2,4]triazolo [2,3-a]-[1,3,5]triazin-5-yl-amino)ethyl)phenol) and different concentrations of antagonists studied for an incubation time of 60 min at 4 °C. Non-specific binding was determined in the presence of 10 μM ZM 241385.

5.1.1.3. Binding of [^{3}H]-DPCPX to HEK-293 cells transfected with the human recombinant A_{2B} adenosine receptors. Membranes were incubated for 60 min at 25 °C using [^{3}H]-DPCPX, diluted membranes and different concentrations of selected compounds. Non-specific binding was determined in the presence of 100 μM of NECA.

5.1.1.4. Binding of [^{3}H]-MRE 3008F20 to CHO cells transfected with the human recombinant A_3 adenosine receptors. Competition experiments were carried using 1 nM [^{3}H]-MRE 3008F20 ([^{3}H]-5-(4-methoxyphenyl-carbamoyl) amino-8-propyl-2-(2-furyl) pyrazolo [4,3-e] 1,2,4-triazolo[1,5-c]), pyrimidine diluted membranes and different concentrations of examined antagonists. Incubation time was 120 min at 4 °C, according to the results of previous time-course experiments. Non-specific binding was defined as binding in the presence of 1 μM MRE 3008F20.

Bound and free radioactivity were separated by rapid filtration through Whattman GF/B glass-fiber filters which were washed three times with ice cold buffer. The filter bound radioactivity was counted in a Beckman LS-1800 spectrometer.

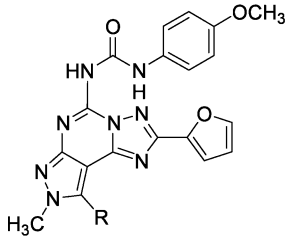
5.1.1.5. cAMP assays. To evaluate the regulation of adenylyl cyclase activity and to test whether the binding parameters correlated with the funcional response, we evaluated in CHO cells transfected with hA_3 adenosine receptors the capability of the antagonists to blockade the effect of Cl-IB-MECA 100 nM induced inhibition of cAMP production (Fig. 12).

Inhibitory binding constant, K_i, values were calculated from those of IC_{50} according to Cheng and Prusoff equation [73] $K_i = IC_{50}/(1+[C^*]/K_D^*)$, where $[C^*]$ is the concentration of the radioligand and K_D^* its dissociation constant. A weighted non-linear least-squares curve fitting program LIGAND [74] was used for computer analysis of inhibition and cAMP experiments. Data are expressed as geometric mean, with 95 or 99% confidence limits in parentheses.

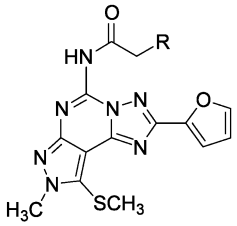
Acknowledgements

Financial support by King Pharmaceutical R&D and Shering Plough is gratefully acknowledged.

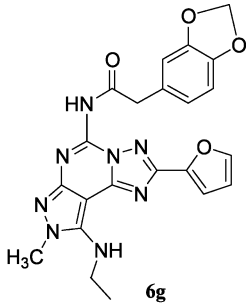
Table 6
Effect of substitution at 9-position on hA_3 adenosine receptor affinity of synthesised compounds



6, 6a-6c



6d-f



6g

Compound	R	hA_1(K_i, nM) [a]	hA_{2A}(K_i, nM) [b]	hA_{2B}(K_i, nM) [c]	hA_3(K_i, nM) [d]
6	4-Me-pyperazin-1-yl	316	> 10 000	26	> 10 000
6a	SCH_3	70	3.1	24	212
6b	$NHCH_2CH_3$	150	21	37	17
6c	$NHCH_2CH_3$ (HCl)	100	16	23	9
6d	4-OCH_3–phenyl	80	15	45	> 10 000
6e	4-isobutyl–phenyl	780	50	190	> 10 000
6f	3,4-OCH_2O–phenyl	70	4.1	30	110
6g	–	136	61	65	183

[a] Displacement of specific [^{3}H]-DPCPX binding at human A_1 receptors expressed in CHO cells.
[b] Displacement of specific [^{3}H]-SCH 58261 binding at human A_{2A} receptors expressed in CHO cells.
[c] Displacement of specific [^{3}H]-DPCPX binding at human A_{2B} receptors expressed in HEK-293 cells.
[d] Displacement of specific [^{3}H]-MRE3008F20 binding at human A3 receptors expressed in CHO cells.

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