Journal of Medicinal Chemistry

Brief Article

One-Pot Reaction to obtain N,N'-disubstituted Guanidines of Pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine Scaffold as Human A3 Adenosine Receptor Antagonists

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J. Med. Chem., **Just Accepted Manuscript** • Publication Date (Web): 05 Jun 2015

One-Pot Reaction to obtain *N,N'*-disubstituted Guanidines of Pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine Scaffold as Human A_3 Adenosine Receptor Antagonists

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ABSTRACT: In this paper we describe an extension SAR study of pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine nucleus as A_3AR antagonist. Our initial aim was to replace the phenylcarbamoyl moiety at the 5 position of PTP nucleus with a thiourea functionality, in order to evaluate the contribute of new structural modification against the A_3AR. The synthesized compounds **12-25** were not characterized by the predicted side chain but with a 1,3 disubstituted guanidine, which showed to be interesting A_3AR antagonists.

INTRODUCTION

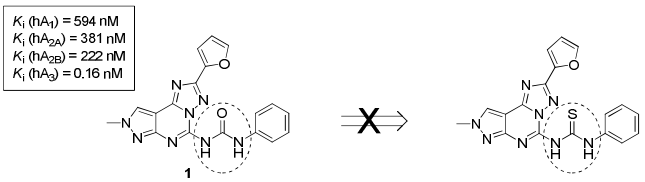
Adenosine, a ubiquitous nucleoside, regulates many physiological functions through the activation of four specific receptor subtypes, classified as A_1, A_{2A}, A_{2B} and A_3 (ARs), belonging to the superfamily of G-protein-coupled receptors (GPCRs). The A_3AR subtype is the most recently characterized member of the family.[1] Activation of A_3AR subtype has been displayed to inhibit adenylate cyclase, to increase the activity of phospholipase C and D, to promote intracellular Ca^{++} and IP_3 (inositol 1,4,5-trisphosphate) levels and to improve the release of inflammatory and allergic mediators from mast cells.[2,3] The potential therapeutic application, resulting from the activation of this receptor subtype, have been recently reviewed.[4]

The A_3AR antagonists are presently undergoing biological testing for their potential use in the treatment of stroke, neurodegenerative diseases, allergy, asthma and COPD (Chronic Obstructive Pulmonary Disease,Thomson Reuters Integrity source). It is becoming increasingly evident that the A_3AR antagonists could be therapeutically useful for the acute treatment of glaucoma.[5] It has been also observed that in central nervous system, the prolonged A_3AR stimulation decrease the currents generated by gamma aminobutyrric acid in epileptic tissues suggesting that A_3AR antagonists may offer therapeutic opportunities in various forms of human epilepsy.[6]

The pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine (PTP) nucleus has been revealed constantly to provide as an attractive scaffold for the preparation of adenosine receptor antagonists.

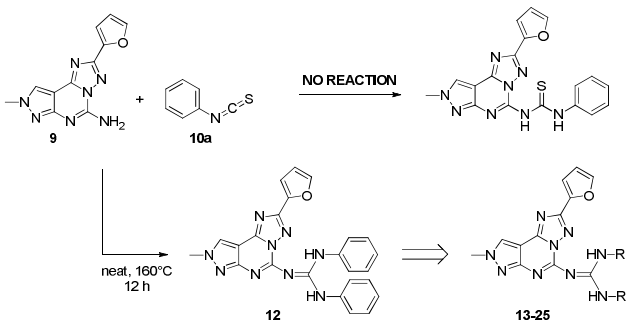
Our research group synthesized a large number of compounds,[7-9] characterized by the PTP nucleus, with very good binding profile as ligands for ARs. To perform a structure-activity relationship (SAR) investigation, the effect of substitutions at various positions of this scaffold was evaluated. From these studies substituents responsible of affinity for the human A_3AR were identified. In particular the best potency and affinity towards the hA_3AR was obtained by substitution at the C-2 position with a 2-furyl or substituted phenyl moieties, combined with small alkyl chain at the N-8 and substituted phenylcarbamoyl moiety at the C-5 position.[7,10] The 2-furyl moiety at position C-2, the methyl group at position N-8 combined with the phenylcarbamoyl moiety at the C-5 position allowed us to identified one of the best compound with high affinity and selectivity for A_3AR (**1**, Chart 1, hA_3 = 0.16 nM, hA_1 = 594 nM, hA_{2A} = 381 nM, hA_{2B}= 222 nM).[11]

Chart 1. Preliminary rational design for the synthesis of new A_3 antagonists.



In light of this, our aim was to synthesize new hA_3AR antagonists by replacing the urea group with a thiourea moiety at C-5 position of PTP scaffold to evaluate the effect of this substitution on biological activity.

Chart 2.



Nevertheless, applying the classical synthetic approach used for the synthesis of the urea derivatives (appropriated isocyanate, THF/ Rfx or dioxane/ Rfx)[11] for the preparation of thiourea compounds, in presence of the appropriate isothiocyanate, no reaction was observed (Chart 2).

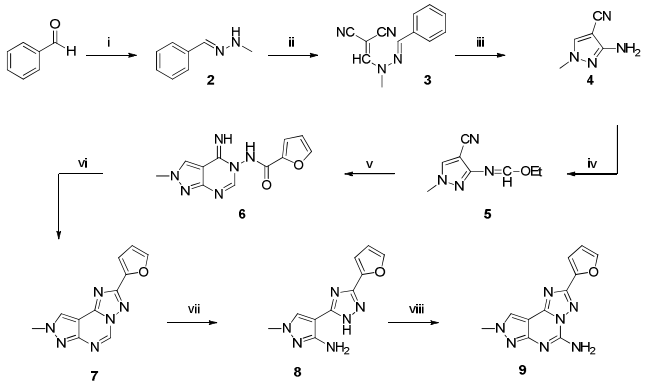
In the attempt to force the reaction by employing stronger conditions, high temperature (160 °C) and an excess of phenyl isothiocyanate, compound **12** was achieved. The structural investigation of the new compound showed the presence of a guanidine moiety at the 5 position of PTP nucleus rather than the predicted thiourea functionality. Applying the new stronger conditions to commercial appropriate isothiocyanate or available carbodiimide, choosen in agreement with the best results obtained in phenylcarbamoyl series, compounds **13-23** and **23-25** were obtained according to method A or method B respectively.

The guanidine functionality represents an important moiety which turns up as a structural part in various drug and agrochemicals and are actually top sold pharmaceuticals appearing moreover in the top 200 prescription list of 2010.[12] Considering the importance of the new structural motif (*N,N'*-disubstituted guanidines) we tested these derivatives in binding and cAMP functional assays as hA_3AR antagonists, using Chinese Hamster Ovary (CHO) cells.

CHEMISTRY

All final compounds were synthesized starting from 2-(2-furyl)-8-methyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (**9**) prepared by known regioselective procedure reported in Scheme 1 (for experimental section see supporting information).[13,14]

Scheme 1



[a]**Reagents and conditions:** i) CH_3NHNH_2, EtOH, 80 °C, 3h; ii) ethoxymethylenemalononitrile, benzene, 80 °C, 1h; iii) EtOH, HCl 37%, 80 °C, 1h; iv) $(EtO)_3CH$, reflux, 20h; v) 2-furoic hydrazide, 2-methoxyethanol, reflux, 24h; vi) Ph_2O, 260 °C, 1h; vii) HCl aq. 10%, reflux, 1h; viii) 1-methyl-2-pyrrolidone, pTsOH, cyanamide, 160 °C, 6h.

The reaction between benzaldehyde and N-methylhydrazine, commercially available, gave the corresponding hydrazone **2**. Subsequent reaction with ethoxymethylenemalononitrile and hydrolysis with concentrated HCl provided **4** in good yield. This synthetic procedure starting from benzaldehyde allowed us to obtain only one isomer of 3-amino-1-methyl-1*H*-pyrazole-4-carbonitrile (**4**), to the contrary of which could be obtain by classical pyrazole alkylation route.[15] Consequently this synthetic approach furnished us only the 2-(2-furyl)-8-methyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (**9**) and not two isomers of tricycle amine N-7 and N-8 methylalkylated respectively.[16]

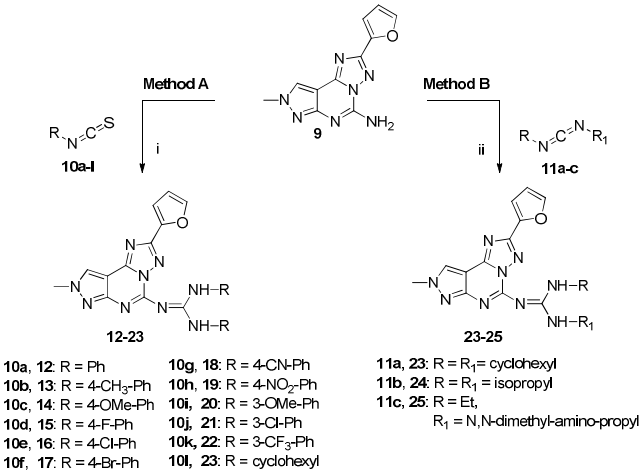
The free amino group of **4** was transformed into the imidate **5** by refluxing in $HC(OEt)_3$ that was reacted with 2-furoic acid hydrazide in refluxing 2-methoxyethanol to provide **6**, following the method of Gatta *et al*.[17] The pyrazolo[4,3-*e*]pyrimidine derivative **6** was converted through a thermally-induced ciclyzation in diphenylether to the tricyclic derivative **7** in good yield. Treatment of **7** with diluted HCl induced pyrimidine ring opening to give **8** which was converted in **9** by treatment with an excess of cyanamide and 1-methyl-2-pyrrolidone at 160 °C.

The synthetic route to achieve *N''*-[2-(2-furyl)-8-methyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-yl]-*N,N'*-disubstituted guanidine **12-25** is depicted in Scheme 2 [Method A, via isothiocyanate (**12-23**) or Method B, via carbodiimide(**23-25**)].

In view of the first attempt in which **9** was reacted with phenyl isothiocyanate (**10a**) in mild condition[11,13] without achieving any product, the reaction was carried out in stronger condition. Indeed, performing the reaction neat (without solvent) at high temperature (160 °C) and using an excess of commercial phenyl isothiocyanate yielded the guanidine derivative **12**. Applying the same approach and different isothiocyanates, compounds **13-23** were syn-

thesized, all characterized by 1,3-disubstituted guanidine moiety at the 5 position of PTP nucleus.

Scheme 2



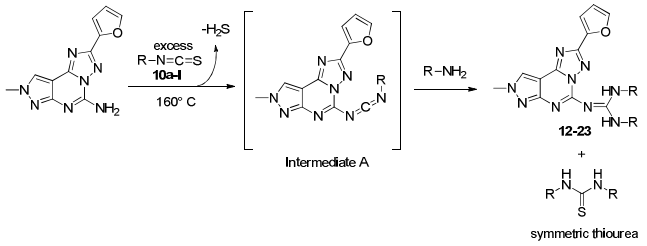
[a]**Reagents and conditions:** i) neat, 160 °C, 18 h (except for **23**, 36 h); ii) neat, 160 °C, 6h (except for **25**, 12h).

To confirm the chemical structure of the new molecules, we decided to perform the reaction of **9** in presence of *N,N'*-dicyclohexylcarbodiimide (DCC, **11a**) which permitted us to obtain compound **23**, previously achieved using cyclohexyl isothiocyante **10l**. The analytical data of **23**, reached both via isothiocyanate (method A, 45% yield) and via carbodiimide (method B, 60% yield), matched. We extended this methodology using other commercially available carbodiimide as starting material which led to compounds **24** and **25** (Scheme 2, method B).

All final compounds **12-25** were obtained in moderate yield and was not observed the complete conversion of the starting material **9** which was recovered as well.

In Scheme 3 is reported the proposed reaction mechanism to achieve compounds **12-23**. During the reaction between **9** and **10a-l**, sulfidric acid was released (whose characteristic smell was developed during Schlenk opening) and the intermediate A, with PTP structure and appropriate carbodiimidic functionality at position 5, was observed by TLC and MS monitoring. In addition, the strong conditions are probably responsible for the release of the amine from the corresponding isothiocyanate. Subsequent addition of the amine species to the carbodiimidic intermediate provided the final compounds *N,N'*-disubstituted guanidine (**12-23**). This mechanism was further supported by formation of corresponding 1,3-disubstituted symmetric thiourea as side product which contributes to the low yield of the final compounds.

Scheme 3. Proposed reaction mechanism to achieve compound 12-23



RESULTS AND DISCUSSION

All the final compounds *N''*-[2-(2-furyl)-8-methyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-yl)-*N,N'*-disubstituted guanidine (**12-24**, except **25**) are characterized by a symmetric 1,3-guanidine moiety at the 5 position of PTP.

The synthesized compounds (**12-25**) were evaluated in radioligand binding assays to determine their affinities for hA_1, A_{2A}, and A_3ARs using [^{3}H]DPCPX (1,3-dipropyl-8-cyclopentylxanthine), [^{3}H]ZM241385 ([4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol), [^{3}H]MRE-3008-F20 (5-N-(4-methoxyphenylcarbamoyl) amino-8-propyl-2-(2-furyl)pyrazolo-[4,3-*e*]-[1,2,4]triazolo[1,5-*c*]pyrimidine), as radioligands, respectively. Efficacy of the compounds versus hA_{2B}AR was investigated by evaluating their capability to inhibit NECA (5′-(*N*-ethylcarboxamido)adenosine)-stimulated (100 nM) cAMP production. Antagonism of selected ligands versus hA_3AR was also assessed through cAMP experiments evaluating their capability to block the inhibitory effect mediated by 100 nM Cl-IBMECA (2-chloro-N^6-(3-iodobenzyl)adenosine-5′-N-methylcarboxamide).

Affinity data expressed as K_i values for A_1, A_{2A}, and A_3 receptors, and K_B values for hA_{2B} and hA_3 in the functional assays are listed in Table 1.

All the *N''*-[2-(2-furyl)-8-methyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-yl)-*N,N'*-disubstituted guanidine **12-25** exhibited a good selectivity towards the other AR subtypes (hA_1, hA_{2A}: K_i >1000, hA_{2B}: K_B >1000) and 12 out of 18 showed a K_i values for hA_3AR lower than 89 nM. Derivative **12** is characterized by an unsubstituted phenyl ring at 1,3 positions of guanidine moiety and presented a good binding profile against hA_3AR (hA_3: K_i = 79 nM, hA_1, hA_{2A}: K_i > 1000, hA_{2B}: K_B >1000). The introduction of different substituents both electron withdrawing and electron donating functions have been examined. The substitution of position 4 of phenyl ring with electron donating moieties maintained or improved the hA_3AR affinity (**13**, Me, hA_3: K_i = 63 nM and **14**, OMe, hA_3: K_i= 42 nM) respect to the unsubstituted **12** (hA_3: K_i= 79 nM).

The presence of a chlorine residue at the para position (**16**, 4-Cl-Ph, hA_3: *K*i= 71 nM) did not show any improvements in binding affinities respect to the unsubstituted analogue **12**. The introduction of different halogens, such

Table 1. Biological Data of the Synthesized Compounds

compd	R	R_1	hA_1ARs K_i (nM)[a]	$hA_{2A}ARs$ K_i (nM)[b]	$h_{A3}ARs$ K_i (nM)[c]	hA_3ARs K_B (nM)[d]	$hA_{2B}ARs$ K_B (nM)[d]
12	Ph	Ph	>1000 (10%)	>1000 (2%)	79 ± 8	67 ± 6	>1000 (1%)
13	4-Me-Ph	4-Me-Ph	>1000 (6%)	>1000 (1%)	63 ± 5	57 ± 5	>1000 (3%)
14	4-OMe-Ph	4-OMe-Ph	>1000 (7%)	>1000 (1%)	42 ± 5	42 ± 4	>1000 (4%)
15	4-F-Ph	4-F-Ph	>1000 (1%)	>1000 (16%)	122 ± 10	129 ± 10	>1000 (1%)
16	4-Cl-Ph	4-Cl-Ph	>1000 (8%)	>1000 (11%)	71 ± 6	63 ± 5	>1000 (2%)
17	4-Br-Ph	4-Br-Ph	>1000 (1%)	>1000 (10%)	345 ± 36	362 ± 34	>1000 (1%)
18	4-CN-Ph	4-CN-Ph	>1000 (1%)	>1000 (1%)	30± 3	33 ± 3	>1000 (1%)
19	4-NO2-Ph	4-NO2-Ph	>1000 (1%)	>1000 (1%)	18 ± 2	13 ± 2	>1000 (1%)
20	3-OMe-Ph	3-OMe-Ph	>1000 (1%)	>1000 (18%)	155 ± 11	141 ± 12	>1000 (1%)
21	3-Cl-Ph	3-Cl-Ph	>1000 (17%)	>1000 (1%)	89 ± 8	74 ± 6	>1000 (1%)
22	3-CF3-Ph	3-CF3-Ph	>1000 (3%)	>1000 (1%)	520 ± 57	537 ± 49	>1000 (2%)
23	cyclohexyl	cyclohexyl	>1000 (25%)	>1000 (3%)	29 ± 3	32 ± 3	>1000 (4%)
24	isopropyl	isopropyl	>1000 (4%)	>1000 (1%)	33 ± 4	36 ± 3	>1000 (3%)
25	ethyl	N,N-dimethyl-aminopropyl	>1000 (22%)	>1000 (3%)	>1000 (19%)	>1000 (3%)	>1000 (1%)

Affinity values obtained from displacement of specific [^{3}H]DPCPX (a), [^{3}H]ZM241385 (b) or [^{3}H]MRE3008F20 (c) binding to hA_1 ARs, hA_{2A} ARs or h A_3 ARs, respectively (n=3-6). [d]Potency (K_B) calculated by using the modified Cheng-Prusoff analysis proposed by Leff and Dougall.[18] In parentheses are reported the % of inhibition to hA_1, A_{2A}, A_{2B} or A_3 CHO cells. Data are expressed as means ± SEM.

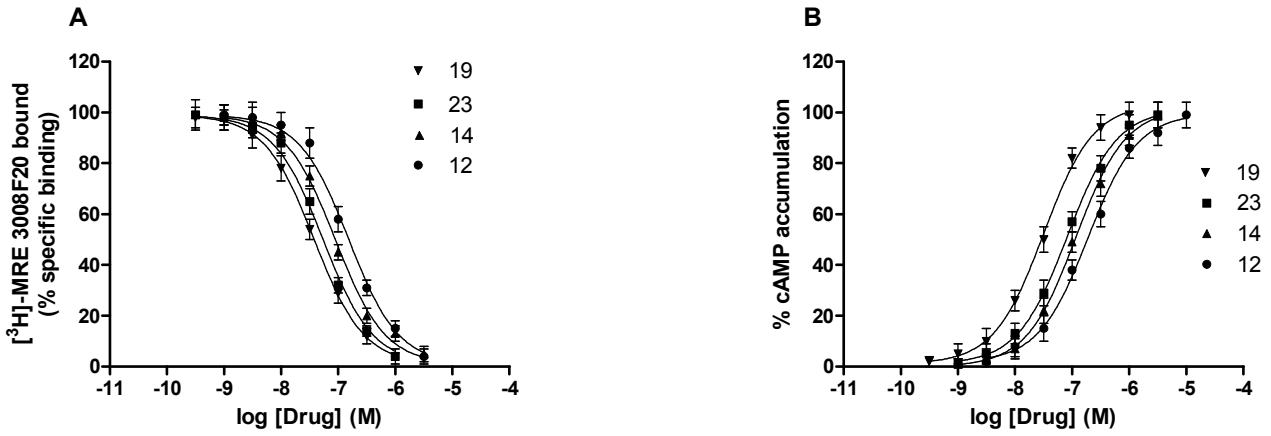


Figure 1. Competition curves of specific [^{3}H]MRE 3008F20 binding to hA_3AR by selected compounds (A). Inhibition curves representing the capability of the antagonists to block the inhibitory effect of 100 nM Cl-IB-MECA on Forskolin-induced cAMP accumulation (B).

The functionalization of the 3 position of the phenyl ring was also investigated and from the binding data emerged that the substitution in 4 is preferred for the hA_3AR affinity (**20**, 3-OMe-Ph, hA_3: K_i = 155 nM *vs* **14**, 4-OMe-Ph, hA_3: K_i = 42 nM). Compound **22** (3-CF3-Ph, hA_3: K_i = 520 nM) functionalized with a sterically hindrance group, showed a dropped affinity against hA_3AR.

We evaluated the introduction of an alkyl cyclic substituent, such as cyclohexyl, that conferred a greater affinity for A_3 than the corresponding unsaturated cycle. In fact, the cyclohexyl derivative **23** showed 2,5 folds high affinity than that of compound **12** (**23**, hA_3: K_i = 29 nM *vs* **12**, hA_3: K_i = 79 nM). Also compound **24** bearing an isopropyl alkyl chain, exhibited comparable affinity for A_3AR with a K_i value of 33 nM.

The asymmetric alkyl guanidine **25**, resulted completely inactive against all four ARs subtypes (hA_3, hA_1, hA_{2A}: K_i > 1000, hA_{2B}: K_B >1000).

Functional experiments showed that all examined derivatives proved no activity at the $hA_{2B}AR$ with K_B values major than 1 μM (Table 1). Moreover, a good correlation between affinity and potency values to hA_3AR was detected (Figure 1). The majority of the novel compounds displayed good affinity for the A_3AR subtype and high potency as expressed by the K_B values. Compound **19** is the most affine, potent and selective of this series as indicated by the K_i value of 18 nM vs hA_3ARs closely similar to the K_B value of 13 nM. Interestingly this compound is not able to bind the other AR subtypes (Table 1) showing a complete selectivity versus hA_3ARs.

CONCLUSION

We synthesized by one-pot reaction a series of new compound characterized by a guanidine function at position 5 of the PTP scaffold. From our biological data emerged that the synthesized molecules showed a good affinity and potency for the hA_3AR with a complete selectivity versus the other subtypes (hA_1, hA_{2A}: K_i > 1000, hA_{2B}: K_B > 1000).

EXPERIMENTAL SECTION

Chemistry. Materials and Methods. All commercially available reagents and anhydrous solvents were furnished by Sigma-Aldrich or Alfa Aesar and used without further purification. Reaction progress and product mixtures were monitored by TLC on silica gel (precoated F_{254} Macherey-Negel plates) and visualized by UV lamp (254 nm light source) or with ninidrine ethanolic solution. Chromatoghraphy was performed on Merck 230-400 mesh silica gel. The organic layer obtained after extraction from acquous phases were dried over anhydrous sodium sulfate. ^{1}H-NMR data were resolute in DMSO-d_6 solution with a Varian VXR 200 spectrometer and peack position are given in ppm (δ) downfield from tetramethylsilane as internal standard, and *J* values are given in Hertz. All of the identified signals were in accordance with the proposed structure and the splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad. Electrospray ionization mass spectrometry (ESI/MS) were performed with an Agilent 1100 series LC/ MSD in positive scan mode using a direct injection of the purified compound solution (MH^+). Elemental analyses were performed by the microanalytical laboratory of Dipartimento di Scienze Chimiche e Farmaceutiche, University of Ferrara, and were within ± 0.4% of the theoretical values for C, H, and N. All final compounds revealed a purity of not less than 95%. Melting points for purified derivatives were determined in a glass capillary on a Stuart Scientific electrothermal apparatus SMP3 and are uncorrected.

General Procedures for the Synthesis of N''-[2-(2-furyl)-8-methyl-8H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-yl]-N,N'-disubstituted guanidine (12-25)

*Method A (via isothiocyanate, **12-23**)*

A mixture of 2-(2-furyl)-8-methyl-8*H*-pyrazolo[4,3-*e*][1,2,4]-triazolo[1,5-*c*]pyrimidin-5-amine **9** (0.2 mmol) and the appropriate substituted aryl/ alkyl isothiocyanates (**10a-k** and **10l** respectively, 1.0 mmol except for compound **23** which required 2.0 mmol) was stirred neat (without solvent) at 160 °C in a Schlenk tube for 18 hours (except for compound **23** for which the reaction time increased to 36 hours). The final compounds (**12-23**) were purified by flash chromatography, eluting with EtOAc (ethyl acetate) /Petroleum Ether (7:3).

N''-[2-(2-Furyl)-8-methyl-8H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-yl]-N,N'-diphenylguanidine (12)

Pale white solid; $C_{27}H_{19}N_9O$; 40% yield; mp 241 °C. MS (ESI): $[MH]^+$ = 450.5. ^{1}H NMR (200 MHz, DMSO-d_6): δ 4.05 (s, 3H), 6.70-6.73 (m, 1H), 7.03-7.10 (m, 2H), 7.16-7.19 (m, 1H), 7.27-7.35 (m, 4H), 7.54-7.58 (m, 4H), 7.93-7.96 (m, 1H), 8.59 (s, 1H), 10.15 (bs, 2H).

Experimental data for compounds **13-23** are reported in the SI (S5-S6).

*Method B (via carbodiimide, **23-25**)*

A mixture of 2-(2-furyl)-8-methyl-8*H*-pyrazolo[4,3-*e*][1,2,4]-triazolo[1,5-*c*]pyrimidin-5-amine **9** (0.2 mmol) and the appropriate commercial carbodiimide (**11a-b**, 0.6 mmol; **11c**, 1.0 mmol) was stirred neat at 160 °C in a Schlenk tube for 6 hours (except for compound **25** for which the reaction time increased to 12 hours). The final desired compounds (**23-25**) were purified by flash chromatography, eluting with EtOAc /Petroleum Ether (7:3).

N''-[2-(2-Furyl)-8-methyl-8H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-yl]-N,N'-diisopropylguanidine (24)

White solid; $C_{18}H_{23}N_9O$; 44% yield; mp 135-136 °C. MS (ESI): $[MH]^+$ = 382.4. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.20 (d, 12H, *J* = 6.4 Hz), 3.84- 3.87 (m, 2H), 3.99 (s, 3H), 4.12 (bs, 2H) 6.66-6.69 (m, 1H), 7.13-7.15 (m, 1H), 7.83-7.85 (m, 1H), 8.42 (s, 1H).

Experimental data for compound **25** is reported in the SI (S6).

Biology Experiments. Materials. [^{3}H]DPCPX ([^{3}H]1,3-dipropyl-8-cyclopentyl-xanthine, specific activity, 120 Ci/mmol) was obtained from Perkin Elmer Research Products (Boston, MA). [^{3}H]ZM 241385 ([^{3}H](4-(2-[7-amino-2-(2-furil)[1,2.4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol), specific activity, 17 Ci/mmol) was obtained from Biotrend (Cologne, Germany). [^{3}H]MRE-3008-F20 ([^{3}H]5-N-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-e]-1,2,4-triazolo [1,5-c]pyrimidine, specific activity, 67 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK).

DPCPX (1,3-dipropyl-8-cyclopentyl-xanthine), R-PIA ((R)-N^6-(L-2-Phenylisopropyl)adenosine) and CCPA (2-chloro-N^6-cyclopentyladenosine) were obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade and obtained from commercial sources.

CHO Membranes Preparation. The human A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (ARs) were transfected in CHO cells which were grown adherently and maintained in Dulbecco's modified Eagles medium with nutrient mixture F12 (DMEM/F12) without nucleosides, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and Geneticin (G418, 0.2 mg/ml) at 37 °C in 5% CO_2, 95% air. For membrane preparation the culture medium was removed and the cells were washed with PBS and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with Polytron and the homogenate was spun for 10 min at 1000 x g. The supernatant was then centrifuged for 30 min at 100000 x g. The membrane pellet was suspended in: a) 50 mM Tris HCl buffer pH 7.4 for A_1ARs; b) 50 mM Tris HCl, 10 mM $MgCl_2$ buffer pH 7.4 for A_{2A}ARs; c) 50 mM Tris HCl, 10 mM $MgCl_2$, 1 mM EDTA buffer pH 7.4 for A_3ARs.[19] The cell suspension were incubated with 2 UI/ml of adenosine deaminase for 30 min at 37 °C. The membrane preparation was used to perform competition binding experiments.

Competition binding experiments to ARs. All synthesized compounds have been tested for their affinity to hA_1, A_{2A} and A_3ARs. Displacement binding experiments of [^{3}H]-DPCPX (1 nM) to hA_1CHO membranes (50 μg of protein/assay) and at least 6-8 different concentrations of antagonists studied were performed for 120 min at 25 °C. Non-specific binding was determined in the presence of 1 μM of DPCPX and this was always ≤ 10% of the total binding.[20]

Inhibition binding experiments of [^{3}H]-ZM 241385 (2 nM) to hA_{2A}CHO membranes (50 μg of protein/assay) and at least 6-8 different concentrations of antagonists studied were performed for 60 min at 4 °C. Non-specific binding was determined in the presence of 1 μM ZM 241385 and was about 20% of total binding.[21]

Competition binding experiments of [^{3}H]-MRE 3008F20 (1 nM) to hA_3CHO membranes (50 μg of protein/assay) and at least 6-8 different concentrations of examined ligands were performed for 120 min at 4 °C. Non-specific binding was defined as binding in the presence of 1 μM MRE 3008F20 and was about 25% of total binding.[19]

Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fiber filters using a Brandel cell harvester (Brandel Instruments, Unterföhring, Germany). The filter bound radioactivity was counted by Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

Effect of the novel compounds in cyclic AMP assays. CHO cells transfected with hA_{2B} or A_3ARs were washed with phosphate-buffered saline, diluted trypsine and centrifuged for 10 min at 200 g. The pellet containing CHO cells ($1x10^6$ cells /assay) was suspended in 0.5 ml of incubation mixture (mM): NaCl 15, KCl 0.27, NaH_2PO_4 0.037, $MgSO_4$ 0.1, $CaCl_2$ 0.1, Hepes 0.01, $MgCl_2$ 1, glucose 0.5, pH 7.4 at 37°C, 2 IU/ml adenosine deaminase and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as phosphodiesterase inhibitor and preincubated for 10 min in a shaking bath at 37 °C. The potencies of antagonists to hA_{2B}ARs were determined by antagonism of NECA (100 nM)-induced stimulation of cyclic AMP levels.[21] The potency of the antagonists to hA_3ARs was determined in the presence of Forskolin 1μM by antagonism of Cl-IB-MECA (100 nM)-induced inhibition of cyclic AMP levels.[19] The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged at 2000 g for 10 min at 4 °C and the supernatant was extracted four times with water saturated diethyl ether. The final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay. Samples of cyclic AMP standard (0-10 pmoles) were added to each test tube containing the incubation buffer (trizma base 0.1 M, aminophylline 8.0 mM, 2 mercaptoethanol 6.0 mM, pH 7.4) and [^{3}H] cyclic AMP. The binding protein previously prepared from beef adrenals, was added to the samples previously incubated at 4 °C for 150 min, and after the addition of charcoal were centrifuged at 2000 g for 10 min. The clear supernatant was counted in a 2810-TR Packard scintillation counter.

Data Analysis. The protein concentration was determined according to a Bio-Rad method[22] with bovine albumin as a standard reference. Inhibitory binding constants, Ki values, were calculated from those of IC_{50} according to Cheng & Prusoff equation[23] $K_i = IC_{50}/(1+[C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D* its dissociation constant. A weighted non-linear least-squares curve fitting program LIGAND[24] was also used for computer analysis of inhibition experiments. IC_{50} values obtained in cyclic AMP assays were calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve (Graph PAD Prism, San Diego, CA, USA). Potency values (K_B) were calculated by using the modified Cheng-Prusoff analysis proposed by Leff and Dougall.[18] Affinity (K_i) and potency (K_B) values are expressed as the arithmetic mean ± SEM.

ASSOCIATED CONTENT

Supporting Information.

Experimental details for chemical synthesis of 2-(2-furyl)-8-methyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine(**9**); experimental data for compounds 13-23 and 25. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing finantial interest.

ABBREVIATIONS

hA_1AR, human A_1 adenosine receptor; $hA_{2A}AR$, human A_{2A} adenosine receptor; $hA_{2B}AR$, human A_{2B} adenosine receptor; hA_3AR, human A_3 adenosine receptor.

REFERENCES

(1) Fredholm, B. B.; AP, I. J.; Jacobson, K. A.; Linden, J.; Muller, C. E. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors: an update. *Pharmacol. Rev*. **2011**, *63*, 1–34.

(2) Van Schaick, E. A.; Jacobson, K. A.; Kim, H. O.; IJzerman, A. P.; Danhof, M. Hemodynamic effects and histamine release elicited by the selective adenosine A3 receptor agonist Cl-IB-MECA in conscious rats. *Eur. J. Pharmacol.* **1996**, *308*, 311–314.

(3) Hannon, J. P.; Pfannkuche, H. J.; Fozard, J. R. A role for mast cells in adenosine A_3 receptor-mediated hypotension in the rat. *Br. J. Pharmacol*. **1995**, *115*, 945–952.

(4) Borea, P.A., Varani, K., Vincenzi, F., Baraldi, P.G., Aghazadeh Tabrizi, M., Merighi, S., Gessi, S. The A_3 Adenosine Receptor: History and Perspectives. *Pharmacol. Rev.* **2015**, *67*, 74–102.

(5) Baraldi, P.G.; Preti, D.; Borea, P.A.; Varani K. Medicinal chemistry of A_3 adenosine receptor modulators: pharmacological activities and therapeutic implications. *J. Med. Chem*. **2012**, 55, 5676-5703.

(6) Boison D. The adenosine kinase hypothesis of epileptogenesis. *Prog. Neurobiol*., **2008**, *84*, 249-262.

(7) Baraldi, P. G.; Tabrizi, M. A.; Gessi, S.; Borea, P. A. Adenosine receptor antagonists: translating medicinal chemistry and pharmacology into clinical utility. *Chem. Rev.* **2008**, *108*, 238–263.

(8) Baraldi, P. G.; Saponaro, G.; Tabrizi, M. A.; Baraldi, S.; Romagnoli, R.; Moorman, A. R.; Varani, K.; Borea, P. A.; Preti, D. Pyrrolo- and pyrazolo-[3,4-e][1,2,4]triazolo[1,5-c]pyrimidines as adenosine receptor antagonists. *Bioorg. Med. Chem.* **2012**, *20*, 1046-1059.

(9) Baraldi, P. G.; Saponaro, G.; Romagnoli, R.; Tabrizi, M. A.; Baraldi, S.; Moorman, A. R.; Cosconati, S.; Di Maro, S.; Marinelli, L.; Gessi, S.; Merighi, S.; Varani, K.; Borea, P. A.; Preti, D. Water-Soluble Pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidines as Human A3 Adenosine Receptor Antagonists. J. Med. Chem. **2012**, 55, 5380-5390.

(10) Cheong, S. L.; Dolzhenko, A.; Kachler, S.; Paoletta, S.; Federico, S.; Cacciari, B.; Dolzhenko, A.; Klotz, K.-N.; Moro, S.; Spalluto,G.; Pastorin , G. The Significance of 2-Furyl Ring Substitution with a 2-(para-substituted) Aryl Group in a New Series of Pyrazolo-triazolo-pyrimidines as Potent and Highly Selective hA_3 Adenosine Receptors Antagonists: New Insights into Structure-Affinity Relationship and Receptor-Antagonist Recognition. *J. Med. Chem.* **2010**, *53*, 3361–3375.

(11) Baraldi, P. G.; Cacciari, B.; Moro, S.; Spalluto, G.; Pastorin, G.; Da Ros, T.; Klotz, K. N.; Varani, K.; Gessi, S.; Borea, P. A. Synthesis, biological activity and molecular modelling investigation of new pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine derivatives as human A_3 adenosine receptor antagonists. *J. Med. Chem*. **2002**, *45*, 770-780.

(12) Rauws, T. R. M.; Maes, B. U. W. Transition metal-catalyzed N-arylations of amidines and guanidines. *Chem. Soc. Rev*. **2012**, *41*, 2463–2497.

(13) Baraldi, P. G.; Cacciari, B.; Romagnoli, R.; Spalluto, G.; Klotz, K. N.; Leung, E.; Varani, K.; Gessi, S.; Merighi, S.; Borea, P. A. Pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]- pyrimidine derivatives as highly potent and selective human A_3 adenosine receptor antagonists *J. Med. Chem*. **1999**, *42*, 4473-4478.

(14) Baraldi, P.G.; Bovero, A.; Fruttarolo, F.; Romagnoli, R.; Aghazadeh Tabrizi, M.; Preti, D.; Varani, K.; Borea, P.A.; Moorman A. R. New strategies of the synthesis of A_3 adenosine receptors antagonists. *Bioorg. Med. Chem*. **2003,** *11*, 4161-4169.

(15) Baraldi, P.G.; Chiarini, A.; Budriesi, R.; Roberti, M.; Casolari, A.; Manfredini, S.; Simoni, D.; Zanirato, V; Varani, K.; Borea P.A. Synthesis and Calcium antagonist activity of dialkyl 1,4-dihydro-2,6-dimethyl-4-(pyrazolyl)-3,5-pyridine-dicarboxylate.1 *Drug design and delivery* **1989**, 5, 13-29.

(16) Baraldi, P.G.; Manfredini, S.; Simoni, D.; Zappaterra, L.; Zocchi, C.; Dionisotti, S.; Ongini, E. Synthesis of new pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines and 1,2,3 triazolo[4,5-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines displaying potent and selective activity as A_{2A} Adenosine Receptor Antagonists. *Bioorg. Med. Chem. Lett*. **1994**, 4(21), 2539-2544.

(17) Gatta, F.; Del Giudice, M. R.; Borioni, A.; Borea, P. A.; Dionisotti, S.; Ongini, E. 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. *Eur. J. Med. Chem.* **1993**, *28*, 569-576.

(18) Leff P. & Dougall IG. Further concerns over Cheng Prusoff analysis. *Trends Pharmacol Sci*, **1993**, *14*, 110-112.

(19) Varani, K.; Merighi, S.; Gessi, S.; Klotz, K.N.; Leung, E.; Baraldi, P.G.; Cacciari, B.; Romagnoli, R.; Spallato, G.; Borea, P.A. [^{3}H]-MRE 3008-F20: a novel antagonist radioligand for the pharmacological and biochemical characterization of human A_3 adenosine receptors. *Mol. Pharmacol.* **2000**, 57, 968-975.

(20) Borea P.A., Dalpiaz A., Varani K., Gessi S., Gilli G. Binding thermodynamics at A_1 and A_{2A} adenosine receptors. *Life Sciences* **1996**, 59, 1373-1388.

(21) Varani, K.; Gessi, S.; Merighi, S.; Vincenzi, F.; Cattabriga, E.; Benini, A.; Klotz, K.N. ; Baraldi, P.G.; Tabrizi, M.A.; Mac Lennan, S.; Leung, E.; Borea, P.A. Pharmacological Characterization of novel adenosine ligands in recombinant and native human A_{2B} receptors. *Biochem. Pharmacol*. **2005**, 70, 1601-1612.

(22) Bradford, M.M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* **1976**, *72*, 248-254.

(23) Cheng, Y.C.; Prusoff, W.H. Relationships between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099-3108.

(24) Munson, P.J., Rodbard, D. Ligand: a versatile computerized approach for the characterization of ligand binding systems. *Anal. Biochem*. **1980**, *107*, 220-239.

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