A_{2B} Adenosine Receptor Antagonists: Recent Developments

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Abstract: There are pharmacological evidences that A_{2B} receptors are involved in inflammatory processes, such as asthma. For this reason, many efforts has been made for identifying selective A_{2B} antagonists as anti-asthmatic agents. The updated material related to this field has been rationalised and arranged in order to offer an overview of the topic.

INTRODUCTION

Adenosine is an endogenous nucleoside distributed in several tissues in the mammalian organisms, which modulates different important physiological functions [1]. Adenosine could be considered a neurotransmitter due to the fact that: i) it exerts its actions through the interaction with receptors and its actions can be blocked by specific antagonists; ii) adenosine-producing enzymes are present in synapses; iii) its actions are terminated by an efficient reuptake system and a metabolising system. Nevertheless, adenosine is usually considered a neuromodulator because there are no clear evidences that it is stored in or released by specific purinergic nerves [2]. The action of adenosine occurs through the stimulation of the purinergic receptors that were classified as P1 receptors, while the receptors activated by nucleotides like ATP were classified as P2 receptors [3]. P1 Receptors were initially subdivided into A_1 and A_2 subtypes based on their ability to inhibit or stimulate adenylyl cyclase, respectively [4,5]. The further division of A_2 receptors into two subtypes was proposed originally by Daly *et al*. [6], based on the finding of high affinity A_2 receptors in rat striatum and low affinity A_2 receptors throughout the brain, both of which activated adenylyl cyclase. The existence of subtypes of A_2 receptors was also suggested by the finding of high affinity A_2 receptors in cultured neuroblastoma cells and low affinity A_2 receptors in glioma cells. These high and low affinity receptor subtypes were later designated as A_{2A} and A_{2B}, respectively [7]. The classification of P1 receptors has been validated by the recent success in molecular cloning and expression of all four A_1, A_{2A}, A_{2B} and A_3 adenosine receptors subtypes.

This classification has been endorsed by IUPHAR Committee on Receptor Nomenclature and Drug Classification [1,8,9]. With the combination of pharmacological data, using selective ligands, important progresses have been made towards an explanation of the role of adenosine receptors in a variety of patho-physiological conditions.

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A_{2B} ADENOSINE RECEPTORS

Initial studies indicated that the A_{2B} receptor expression was restricted only in peripheral organs such as bowel, bladder, lung, epididymis and vas deferens as well as to spinal cord and brain [10]. Instead, further studies clearly indicated a wide distribution of this receptor subtype. In the brain, functional A_{2B} receptors mRNA and protein were found in hippocampal CA_1 and CA_3 pyramidal neurones of guinea-pig and rats [11], and glial cells [12]. Functional A_{2B} receptors have also been found in fibroblasts [13], in various vascular beds [14], in haematopoietic cells [15], mast cells, [16] myocardial cells, [17] muscle cells [18] and endothelium cells [19]. The biochemical processes activated by the A_{2B} receptor stimulation involve the activation of adenylyl cyclase by directly coupling to Gs intracellular proteins. However, additional intracellular signalling pathways have been found to be functionally coupled to A_{2B} receptors [20]. The stimulation of A_{2B} receptors results in accumulation of intracellular Ca^{2+}; the process is thought to be mediated *via* different mechanisms such as potentiating P-type channels in pyramidal neurones of guinea pig hippocampus *via* coupling with Gs protein, that is blocked by cAMP dependent protein kinases [21]. Another mechanism involved could be independent of cAMP requiring Gs protein coupling or through phospholipase C (PLC) activation [22] (Fig. **1**).

Consequently, it has been found that A_{2B} receptors are also coupled to phosphatidylinositol-specific PLC *via* G protein of the Gq family and the activation of this pathway results in an increase of diacylglycerol which activates protein Kinase C (PKC) and IP_3 [20]. Moreover, the intracellular signalling of A_{2B} receptors can be modulated by interaction with other receptor systems [8]. In fact, it has been demonstrated that agents which increase intracellular calcium or activate PKC significantly potentiate A_{2B}-mediated cAMP production in various cells [23]. On the

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contrary, bradykinin-stimulated calcium entry causes inhibition of A_{2B} receptor-stimulated adenylyl cyclase in astrocytoma cells, while direct stimulation of protein kinase C enhances the A_{2B} response [24]. It is of interest that, as far as we consider intracellular pathways, A_{2B} receptors have as much in common with A_1 or A_3 receptors (activation of phospholipase C), as with A_{2A} receptors (activation of adenylyl cyclase). Unfortunately, the lack of selective agonists and antagonists of the A_{2B} receptors has precluded, till now, the pharmacological characterisation of this receptor system and it remains to be elucidated whether all the mechanisms described herein pertain to every cell type expressing A_{2B} receptors. Despite the widespread distribution of A_{2B} receptors in central nervous system, little information is available with regards to their functions. Adenosine agonists have been shown to increase the release of excitatory amino acids and acetylcholine and to decrease GABA efflux in rat cerebral cortex [25]. Adenosine agonists potentiate also a P-type Ca^{2+} current in pyramidal neurons from the CA_3 region of guinea pig hippocampus with a pharmacological profile consistent with A_{2B} receptors [21], while they induce a long-term potentiation in the CA_1 region of rat hippocampus [11].

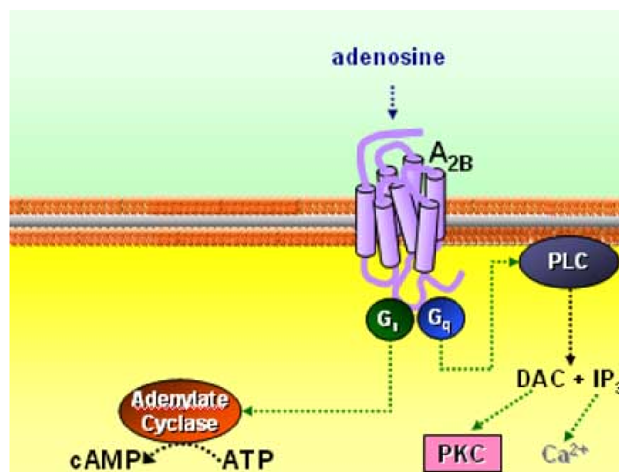

Fig. (1). Molecular pharmacology of the human A_{2B} receptor.

The presence of A_{2B} receptors in some vascular beds raises the possibility that they participate in the regulation of vascular tone [26]. However it has been suggested that the endothelium contributes to the vasodilatory effects of intravascular adenosine. Both A_{2B} and A_{2A} receptors regulate cAMP production in human aortic [19] and human umbilical vein endothelial cells, [27] and A_{2B} receptor mRNA has been detected in human aortic endothelial cells. Recent evidences suggest that adenosine may also play a long-term modulatory role on smooth muscle growth [27]. In fact, A_{2B} receptors inhibit rat aortic smooth muscle cell growth induced by fetal calf serum [14].

On the other hand, some reports implicate A_{2B} receptors in mediating human retinal endothelium cells growth by inducing the vascular growth factor (VEGF). The high levels of A_{2B} receptor expression found in different parts of the intestinal tract motivated great interest in defining their function. Adenosine elicits relaxation of dispersed guinea pig longitudinal muscle cells from small intestine by means of A_{2B} receptors coupled to adenylyl cyclase [28]. In rat duodenum, A_{2B} receptors cause relaxion of longitudinal muscle but contraction of muscularis mucosae [18]. Recently, there has been investigated the action of A_{2B} receptors on gastric mucosal cells [29]. There are some evidences that A_{2B} receptors residing on mast cells are involved in inflammatory processes, such as asthma [30]. This has led to a research effort aimed at identifying selective A_{2B} antagonists endowed with anti-asthmatic properties [31].

A_{2B} ADENOSINE RECEPTOR ANTAGONISTS

Considering the potential therapeutic applications of A_{2B} adenosine receptor ligands, many efforts have been made in the last few years for searching potent and selective antagonists versus this receptor subtype. In particular, different classes of heterocyclic compounds have been identified as A_{2B} adenosine antagonists, which can be classified in two great families of derivatives: i) Xanthine derivatives; ii) Non-xanthinic derivatives [32].

Xanthine Derivatives

Natural xanthines (e.g. theophylline, caffeine) could be considered the natural antagonists for adenosine receptors. For this reason, the xanthine core has been strongly modified also for obtaining A_{2B} adenosine receptor antagonists [33,34]. A large number of substitutions have been performed at the 1, 3 and 8 positions to design a SAR profile for this receptor subtype. In particular, the introduction of a substituent at the 8-position led to an increased affinity at the A_{2B} adenosine receptors. In fact, the A_1 adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, **1**) resulted to be very potent, but not selective, versus the A_{2B} adenosine receptor subtype [35].

Further studies permitted to observe that the 1,3 unsubstituted derivatives bearing phenyl ring at the 8-

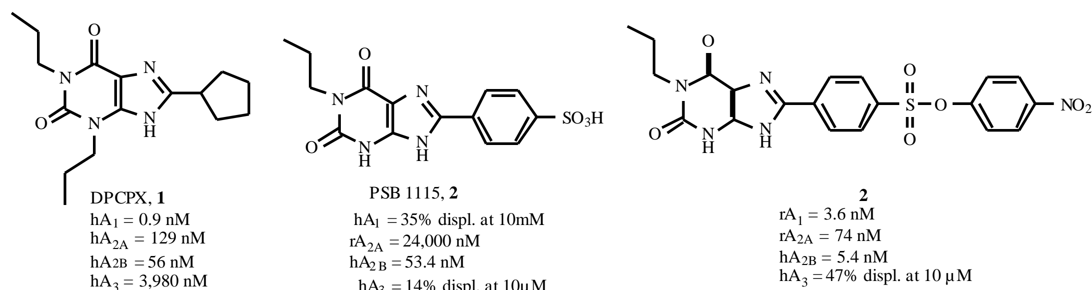

Fig. (2). Structures and binding affinities of 8-substituted xantines as A_{2B} adenosine receptor antagonists.

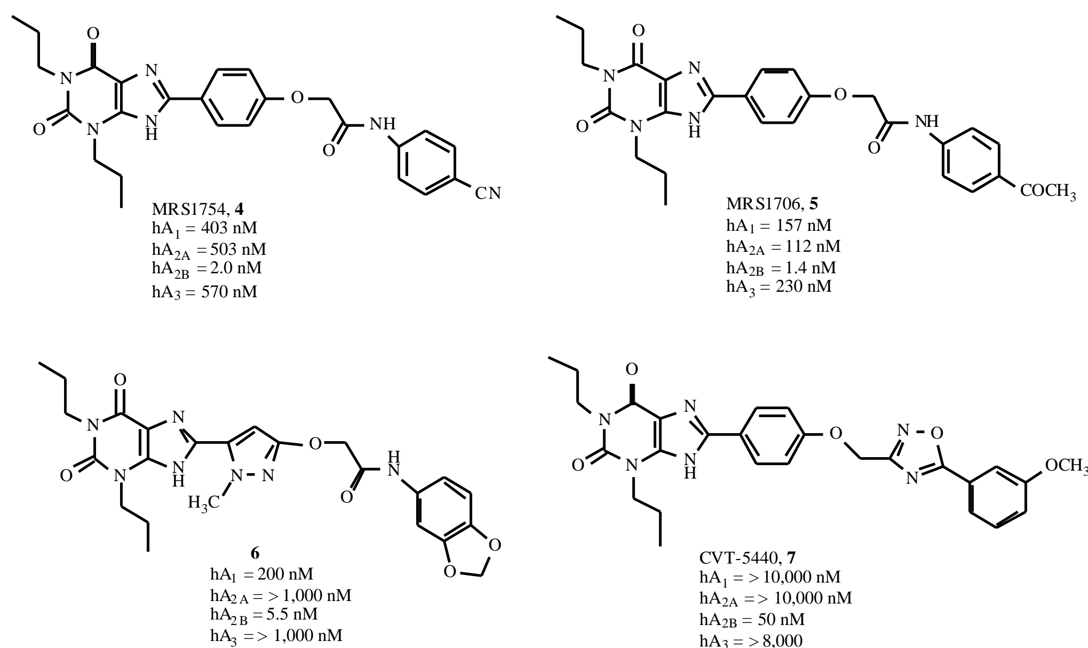

Fig. (3). Structures and binding affinities of 8-phenylxanthine functionalized congeners.

position possess good selectivity but poor potency at the A_{2B} adenosine receptor subtype [33]. An optimisation of this structure led to the discovery of 1-propyl-8-(4-sulphophenyl) xanthine PSB 1115 (**2**) which was found to be a quite potent and selective A_{2B} adenosine receptor antagonist [34]. This result led also to the synthesis of a 4-nitrophenylester of PSB 1115 (**3**) as potential pro-drug: Unfortunately, this derivative could not be considered a classic pro-drug, considering that this compound showed significant binding affinities for the adenosine receptor subtypes. In fact, a retention of selectivity vs A_{2A} and A_3 receptors, but a complete loss of selectivity was observed versus the A_1 subtype [36] (Fig. **2**).

In the series of 8-phenyl xanthine derivatives, a large number of amides derived from the 8-{4-[(carboxymethyl)oxy]phenyl}-1,3-dipropylxanthine have been prepared and tested as A_{2B} adenosine receptor antagonists [35,37]. This study led to the discovery of the ([N-(4-cyanophenyl)- 2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy] acetamide] (**4**, MRS1754) and ([N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy] acetamide] (**5**, MRS 1706), which proved to be the most potent and selective human A_{2B} adenosine receptor antagonists [38]. In fact, for derivative **4** the tritium labelled form has been prepared and utilised in radioligand binding studies [39] (Fig. **3**).

A very similar approach has been utilised recently by Baraldi and coworkers which replaced the phenyl ring with a pyrazole moiety obtaining the N-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]acetamide**6**, which demonstrate a quite similar A_{2B} affinity with respect to the phenyl series [40]. Also in this case the tritium labelled form has been prepared and characterised, demonstrating a promising profile for the characterisation of A_{2B} adenosine receptor subtype (Fig. **3**) [41].

Also a further modification of the chain by inserting the amide bond into an oxadiazole cycle has been investigated very recently. This led to quite potent compound (**7**) (high nanomolar range) but the level of selectivity is very significant (Fig. **3**) [42].

In the xanthine family, very recently there has been reported a new class of deaza-analogues of general formula **8**, which displays affinity for A_{2B} adenosine receptors in the micromolar range, but poor selectivity versus the A_{2A} subtype (Fig. **4**) [43].

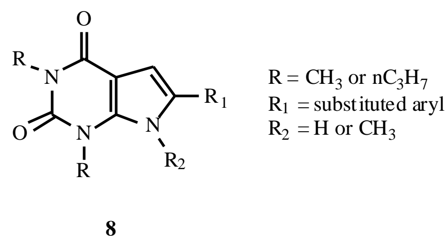

Fig. (4). General structure of xanthine deaza-analogs as potential A_{2B} adenosine receptor antagonists.

Non-Xanthinic Derivatives

In this family of compounds, quite different compounds have been structurally modified for searching new A_{2B} adenosine receptor antagonists. These compounds could be classified in five subclasses of derivatives: i) Triazolo-quinazoline derivatives; ii) Pyrazolo-triazolo-pyrimidine derivatives; iii) Triazolo-triazine derivatives; iv) Adenine derivatives; v) Quinazoline derivatives.

Triazolo-Quinazoline Derivatives

Starting from the experimental observation that the non-selective A_{2A} adenosine receptor antagonist 5-amino-9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazoline (**9**,

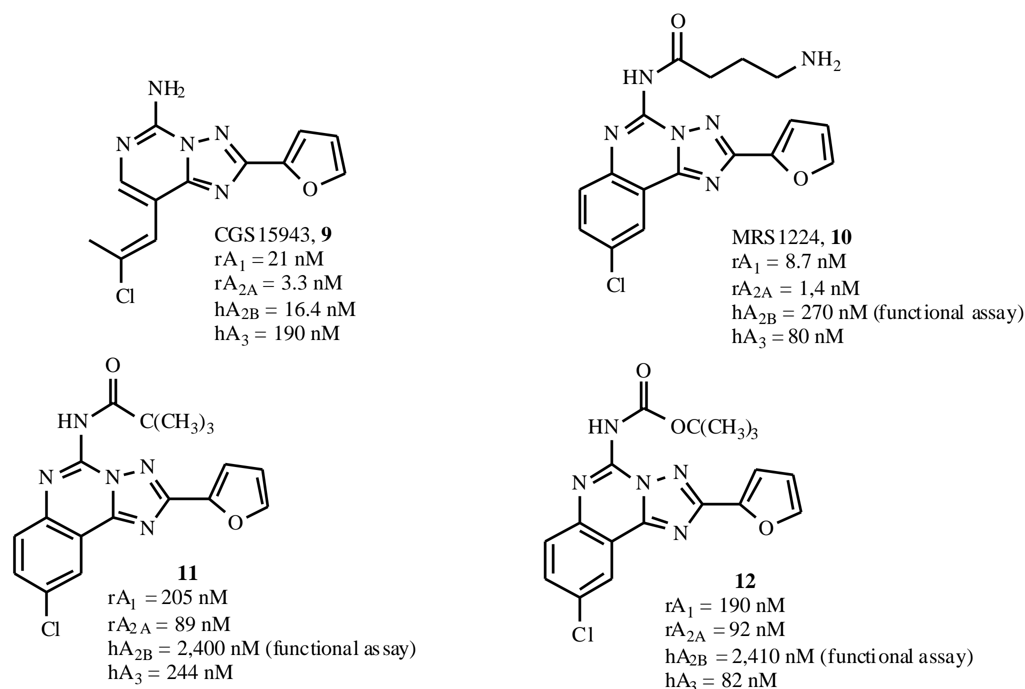

Fig. (5). Structures and binding affinities of triazolo-quinazolines as A_{2B} adenosine receptor antagonists.

CGS15943) displays also good A_{2B} antagonistic properties both in functional and binding studies, Jacobson and coworkers introduced a large number of acyl moieties at the N5 position (Fig. **5**).

They observed that while the introduction of polar moieties such as -aminobutyryl amide (**10**) induces an increase in potency at the A_{2B} adenosine receptors but a complete lack of selectivity versus the A_1 and A_{2A} subtypes, the presence of apolar chains such as the N5-pivaloyl (**11**) or the N5-tert-butyloxycarbonyl derivative (**12**) displays less potency than derivative **10** at the A_{2B} adenosine receptors but an increased selectivity vs the other receptor subtypes, indicating a preliminary SAR profile on this class of

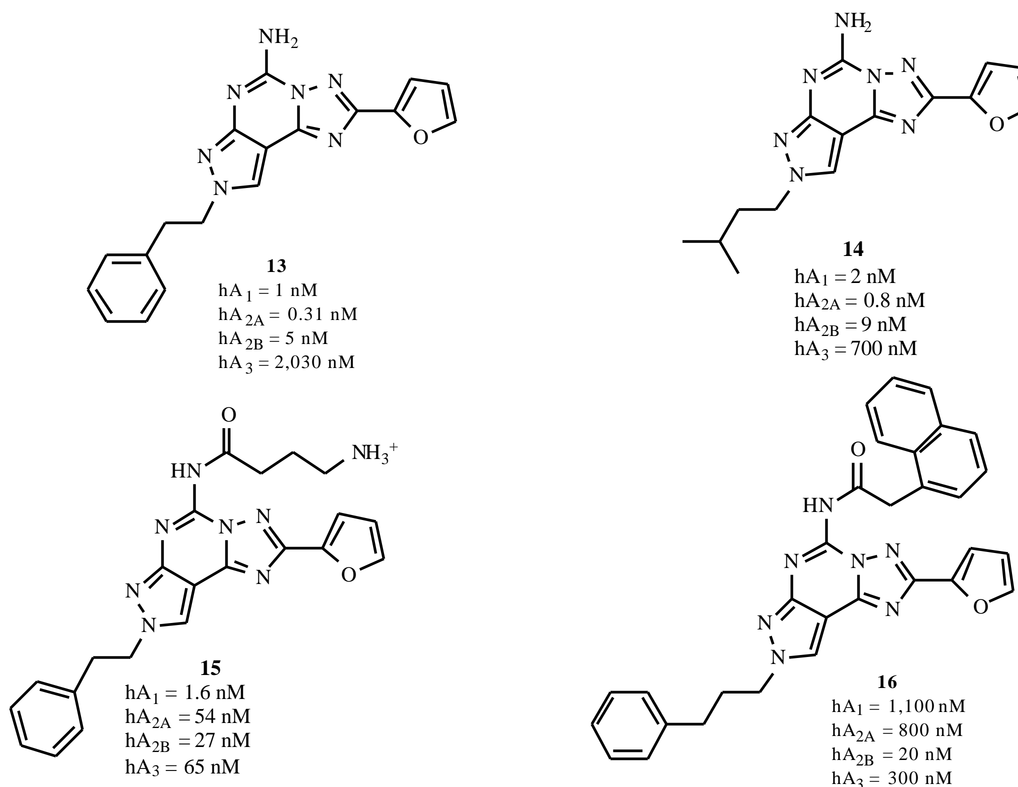

Fig. (6). Structures and binding affinities of pyrazolo-triazolo-pyrimidines as A_{2B} adenosine receptor antagonists.

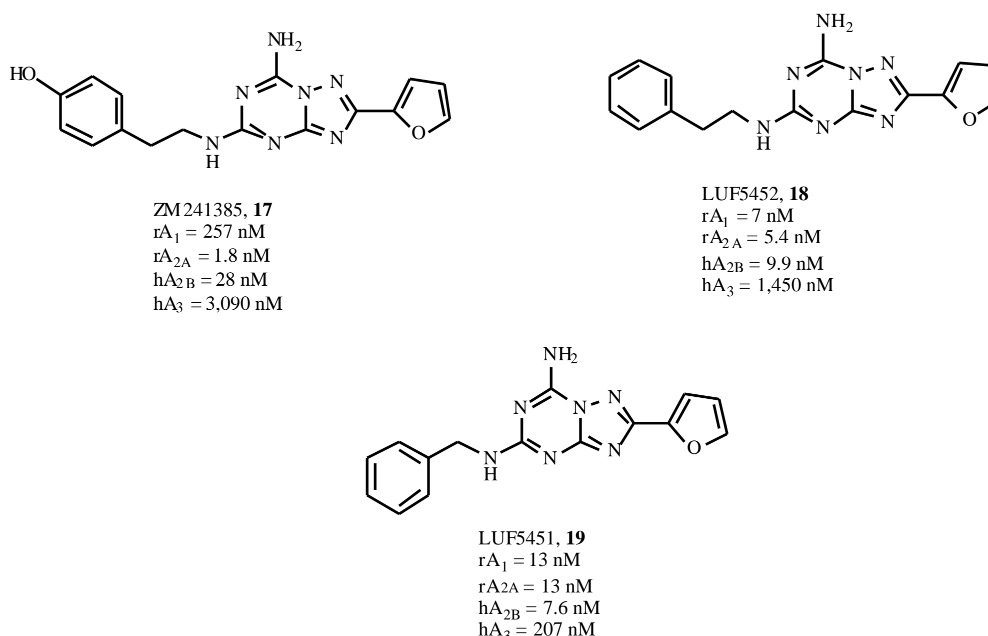

Fig. (7). Structures and binding affinities of triazolo-triazines as A_{2B} adenosine receptor antagonists.

compounds as A_{2B} adenosine receptor antagonists (Fig. **5**) [44].

Pyrazolo-Triazolo-Pyrimidine Derivatives

Considering the structural similarity between the pyrazolo-triazolo-pyrimidine nucleus and the CGS15943 (9), our group acylated the N5 position with different moieties as mentioned above for the triazolo-quinazolines. We observed that the N-5 unsubstituted derivatives (13,14) possess high affinity at the hA_{2B} adenosine receptors but a complete lack of selectivity (Fig. **6**).

The N5 substitution with a -aminobutyryl amide (15) produced on the contrary a decrease of affinity at the A_{2B} adenosine receptors but they resulted quite selective vs the A_{2A} subtype [45]. An improvement of this work on this class of compounds led us to optimise a pattern of substitutions at the N5 and N8 positions permitting to obtain compound (16) which resulted quite potent and selective for the A_{2B} adenosine receptors. These results seem to suggest that bulky substituents at both N5 and N8 position could lead to potent and selective A_{2B} adenosine receptor antagonists (Fig. **6**) [46].

Triazolo-Triazine Derivatives

It is well known that the potent and selective A_{2A} adenosine receptor antagonist 7-amino-2-(2-furyl)-5-[2-(4-hydroxy-phenyl) ethyl] amino [1,2,4]-triazolo[1,5-a][1,3,5]triazine (ZM241385, 17) proved to be also quite potent at the A_{2B} adenosine receptors, in fact its tritiated form is usually utilised in radioligand binding studies [47]. On these bases, several modifications at the 5 position of the triazolo triazine nucleus have been performed. In particular, it has been observed that not only the hydroxyl group replacement (18) enhances the A_{2B} affinity but also the substitutions of phenylethyl chain with benzyl group (19) produce the same effect. Nevertheless the great problem of selectivity is still unsolved in this class of compounds (Fig. **7**) [48].

Adenine Derivatives

Very interesting results were recently obtained by Cristalli and coworkers on this class of derivatives [49]. A detailed investigation on this class of compounds permitted to partially optimise the pattern of substitution on adenine nucleus for enhancing both potency and selectivity for the

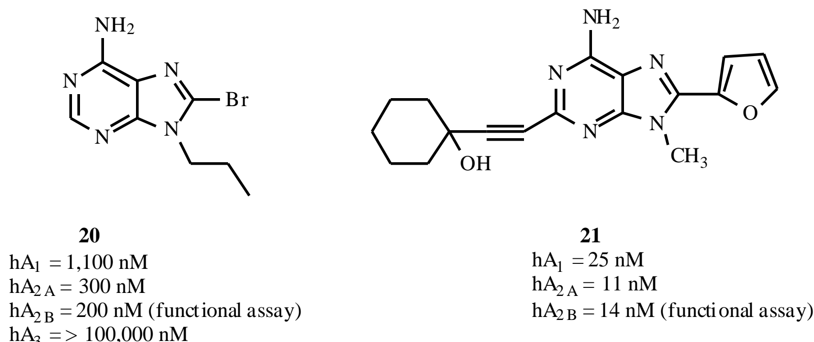

Fig. (8). Structures and binding affinities of adenines as A_{2B} adenosine receptor antagonists.

A_{2B} adenosine receptor subtype. In particular the presence of a propyl group at the 9-position and a bromine at the 8-position (20) increases potency and selectivity, but even the presence of alkynyl moiety at the 2-position and the presence of a furyl ring at the 8-position led to a very promising potent and selective A_{2B} antagonist (21) [50]. This data suggest that further optimisation of the pattern of substitutions at these positions could lead to the discovery of a highly potent and selective A_{2B} adenosine receptor antagonist (Fig. **8**).

Quinazoline Derivatives

In a screening program focused on the searching of new tools as adenosine receptor antagonists a quinazoline derivative, named CMB 6446 (2-(7-methoxy-4-methylquinazolin-2-ylamino)-4,5-dihydro-3H-imidazol-4-one) (**22**) proved to be quite potent and selective at the A_{2B} adenosine receptor subtype with a binding K_i value of 112 nM (Fig. **9**) [51].

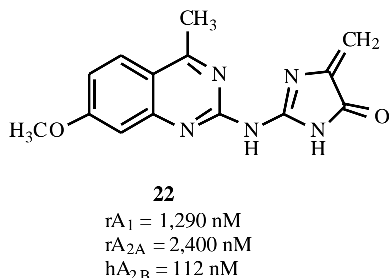

Fig. (9). Quinazoline derivative as A_{2B} adenosine receptor antagonist.

Nevertheless, also if a strong synthetic work was made on this class of compounds, no enhancement of A_{2B} receptor affinity of **22** was achieved.

TOPOLOGY OF HUMAN A_{2B} RECEPTOR

The human A_{2B} receptor shares 86 to 87% amino acid sequence identity with the rat and mouse A_{2B} receptors [52,53], 46% amino acid sequence identity with both human A_1 and A_{2A} receptors, and only 38% with the human A_3 receptor. As for all the other members of Family A-GPCRs, the general topology of the human A_{2B} receptor is preserved consisting in a typical 3-4 type helix-helix contact associated with optimal interactions between nearly parallel aligned helices. However, comparing the primary sequences of the four subtypes of adenosine receptors, several amino acids mutations are detectable, in particular in the putative ligand binding cavity. Evidently, some of these mutations might play a role on the recognition process for both agonists and antagonists. To elucidate the pharmacological differences among all receptors subtypes, in the recent past we theoretically depicted the general topology of all four human adenosine receptors. Following our recently reported modelling approach [54-56], we have built a theoretical model of the human A_{2B} receptor, using the bovine rhodopsin crystal structure as template. A special carefulness had to be given to the second extracellular (EL2) loop, which has been described in bovine rhodopsin to fold back over transmembrane helices, and, therefore, it limits the size of the active site. In fact, two cysteine residues (one in TM3 and one in EL2), which are conserved in most GPCRs, form a disulfide link which could be crucial for the packing and for the stabilisation of a restricted number of conformations of these seven TMs. Interestingly, as reported in Fig. (**10**), EL2 represents the less conserved ligand-recognition region comparing all the human adenosine receptors; consequently we have speculated its implication in determining ligand selectivity among the different receptor subtypes.

Consistently, as Jacobson and co-authors have demonstrated [57,58], amino acids of this loop could be involved in direct interactions with the ligands in both human A_{2A} and A_3 receptors. As already reported for the human A_3 receptor [54-56], the recognition of known A_{2B} antagonists seems to occur in the upper region of the TM helical bundle. TMs 3, 5, 6 and 7 seem to be crucial for the recognition of both agonists and antagonists. A number of amino acid residues in the transmembrane domains 3, 5, and the second extracellular loop (EL2) were individually replaced with Ala and other amino acids [58]. These residues are homologous in human A_{2B} receptor to those that have been predicted to be involved in the ligand recognition in previous pharmacological and theoretical studies of both A_{2A} and A_3 receptors, including: Gln90 (His95 in hA_3), Trp247 (Trp243 in hA_3), His251 (Ser247 in hA_3), Asn254 (Asn250 in hA_3), and Lys147 (Lys152 in hA_3) (Fig. **11**).

We have recently confirmed that all these amino acids could interact with pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine derivatives in which both N-5-(pyrimidinyl) and N-8-(pyrazolyl) positions are appropriately substituted (see derivative **16** in Fig. **6**) [46]. This chemical class represents a potentially new generation of A_{2B} antagonists. In particular, we observed that the introduction at the N5 position of a phenyl acetyl moiety produces a significant decrease of affinity at the hA_3 adenosine receptors with respect to the aryl carbamoyl moiety typical of hA_3 adenosine receptor antagonists, with a simultaneous retention or a slight increase of affinity at the hA_{2B} adenosine receptor subtype. Referring specifically to human A_3 receptor, we have underlined a few interesting differences: a) considering the topological differences in the TMs cavity between hA_{2B} and hA_3 adenosine receptors, bulkiness substituents in both N5 and N8 positions are well tolerated

A_1	FGWNNLSAVERAWAAN	GSMGEP	VIK**C**EFEKVIS
A_{2A}	FGWNNCGQPKEGKNHS	QGCGEG	QVA**C**LFEDVVP
A_{2B}	LGWNSKDSATNNCTEPWDGTTNESCCLVK**C**LFENVVP		
A_3	FGWNMKLTSEYHRNVT		FLS**C**QFVSVMR

Fig. (10). Second extracellular loop (EL2) sequence alignment of all four adenosine receptor subtypes. The highly conserved cisteine involved in the disulfide bridge between TM3 and EL2 is bolded.

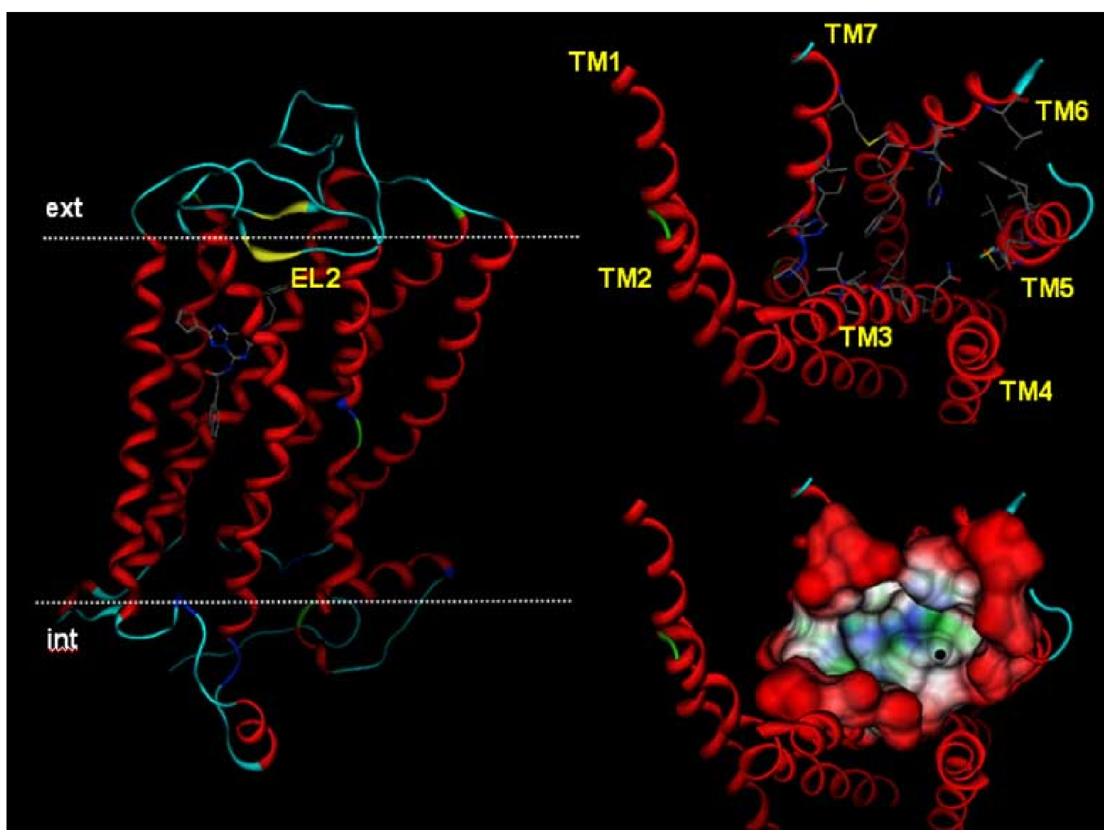

Fig. (11). *On the left:* general topology of the modeled human A_{2B} receptor. Extracellular loop 2 (EL2) is highlighted. *On the right:* rearrangement of trans membrane region. Side chains of amino acids delimiting the ligand recognition cavity are highlighted (*top*). The ligand recognition cavity surface is also represented (*bottom*).

at hA_{2B} adenosine receptor only if an acetyl moiety is present at the N5 position; b) *vice versa* bulky substituents in both N5 and N8 positions are not well tolerated at hA_3 adenosine receptor; c) the bioisosteric replacement of CH_2 with an NH group moiety at the N5 position induces a complete inversion of affinity from A_{2B} to A_3 adenosine receptors; d) in hA_3 receptor, the hydrophilic environment created by Ser243 (TM6) and Ser271 (TM7) might be responsible for the better accommodation of the NH of the phenyl-carbamoyl derivatives in the hypothetical binding site with respect to the CH_2 present in the phenyl-acetyl derivatives; e) conformational adaptability of EL2 on the top of the TM bundle could modulate the recognition (affinity and selectivity) of both agonist and antagonists. We are running an extensive molecular modelling study to demonstrate that this could represent a common pharmacophore model for other structurally diverse A_{2B} antagonist. Nowadays, the data herein collected provide helpful information for designing new more potent and selective hA_{2B} adenosine receptor antagonist.

CONCLUSIONS

In conclusion, the herein presented update clearly indicates the significant improvements made in the field of A_{2B} adenosine receptor antagonists. Nevertheless the discovery of highly potent and selective A_{2B} adenosine receptor antagonist (sub-nanomolar range) has not been achieved yet. Anyway, the high cooperation between synthetic and computational chemists could solve this lack in a very short time, as clearly described.

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