

# Pyrazolo-triazolo-pyrimidines as adenosine receptor antagonists: A complete structure–activity profile

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**Abstract** In the last 5 years, many efforts have been conducted searching potent and selective human A<sub>3</sub> adenosine antagonists. In this field several different classes of compounds, possessing very good affinity (nM range) and with a broad range of selectivity, have been proposed. Recently, our group synthesized a new series of pyrazolo-triazolo-pyrimidines bearing different substitutions at the N<sup>5</sup> and N<sup>8</sup> positions, which have been described as highly potent and selective human A<sub>3</sub> adenosine receptor antagonists. The present review summarizes available data and provides an overview of the structure–activity relationships found for this class of human A<sub>3</sub> adenosine receptor antagonists.

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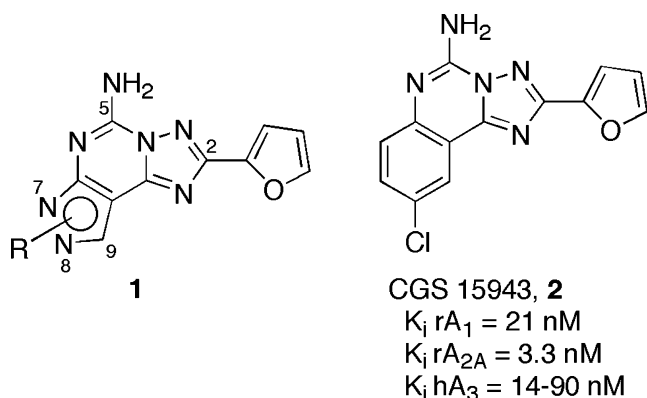
**Key words** adenosine receptors · antagonists · binding · molecular modeling · pyrazolo-triazolo-pyrimidines

## Abbreviations

CGS15943	(9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine)
8FB-PTP	5-amino-8-(4-fluorobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
SCH 58261	5-amino-7-(β-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
SCH 442416	5-amino-7-[3-(4-methoxyphenyl)propyl]-2-(2-furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
PET	positron emission tomography
MRS 1220	N-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-yl]benzene-acetamide
MRE3008-F20	5-[[4-(4-Methoxyphenyl)amino]carbonyl]amino-8-propyl-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine
TM	transmembrane domain

## Introduction

In the last 10 years the pyrazolo-triazolo-pyrimidine nucleus (1) represented an attractive key intermediate for obtaining adenosine receptor antagonists due to its strong structural correlation with the nonselective adenosine receptor antagonist CGS15943 (2) [1, 2] (Figure 1).



**Figure 1** Structural similarities between pyrazolo-triazolo-pyrimidines and reference compound CGS15943 [1]

The great advantage of this nucleus with respect to the reference compound (2) is related to a large number of substitutions that could be done on this nucleus, such as in positions N<sup>7</sup>, N<sup>8</sup>, N<sup>5</sup>, C<sup>9</sup>, or C<sup>2</sup> opening kaleidoscopic possibilities for different substituted heterocycles as adenosine receptor antagonists. The aim of this review was to briefly summarize the great efforts made on this class of compounds, resulting in a complete structure–activity relationship (SAR) profile of the pyrazolo-triazolo-pyrimidine family.

### Substitutions at the N<sup>7</sup> and N<sup>8</sup> positions

The first example of adenosine receptor antagonists with the pyrazolo-triazolo-pyrimidine nucleus was reported by Gatta and coworkers [3], who proposed a compound named 8FB-PTP (3), which displayed potent binding to but no selectivity versus A<sub>2A</sub> adenosine receptors (Figure 2).

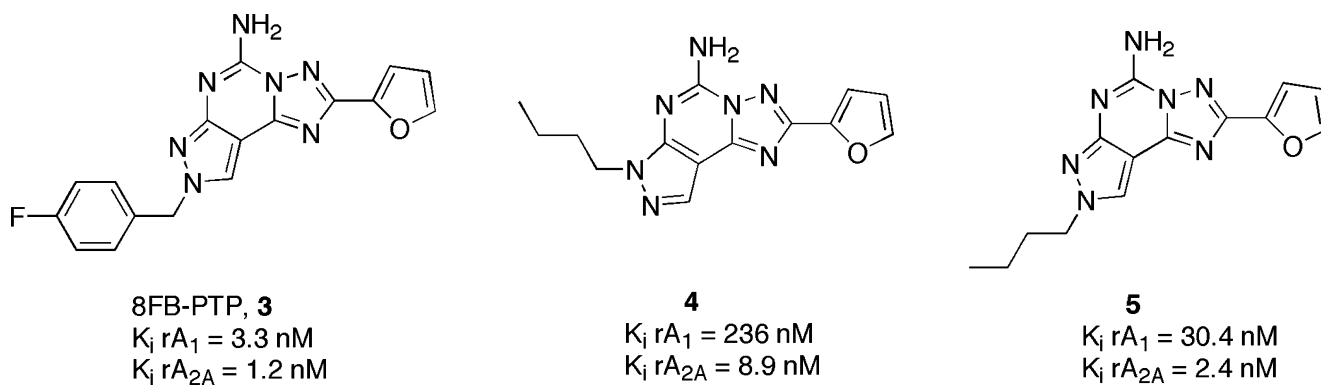
Taking into account this experimental observation and considering the limited number of compounds reported by Gatta et al., our group decided to extensively investigate the effect of substitutions on the pyrazole moiety. From earlier

studies, the importance of the free amino group at 5-position and the effect of the substituent on the pyrazole ring seems to be fundamental for both high affinity and selectivity for the A<sub>2A</sub> adenosine receptor subtype. In particular, substitutions at 7-position improve the selectivity for the A<sub>2A</sub> adenosine receptor whereas the same substitutions at 8-position increase the potency both at the A<sub>1</sub> and A<sub>2A</sub> receptors with low levels of selectivity as a consequence, as indicated for the N<sup>7</sup>-n-butyl (4) and the N<sup>8</sup>-n-butyl (5) derivatives [4, 5] (Figure 2).

An accurate investigation of the effect of the chain at the N<sup>7</sup> position clearly demonstrated that the presence of an aralkyl chain seems to be essential for both potency and selectivity at the A<sub>2A</sub> adenosine receptors. In fact, two selected compounds, named SCH 58261 (6) and SCH 63390 (7) proved to be the most potent and selective A<sub>2A</sub> adenosine receptor antagonists ever reported, both in rat and human models [5] (Figure 3).

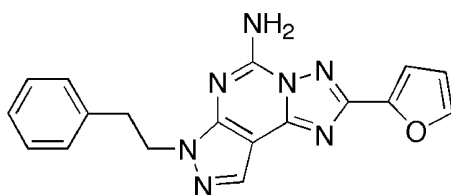
Nevertheless, this class of compounds presents a significant problem in relation to the poor water solubility, which compromises the use of this compound as pharmacological tool. To overcome this problem, several polar moieties on the side chain of the pyrazole nucleus have been introduced. In particular, the introduction of a hydroxyl function at the para position of the phenyl ring of compounds (6) and (7), as in one of the most potent and selective A<sub>2A</sub> antagonist ZM241385 (4-[2-[[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a] [1,3,5]triazin-5-yl]amino]ethyl]phenol) [6a], led to derivatives (8) (5-amino-7-[β-(4-hydroxyphenyl) ethyl]-2-(2-furyl)pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine) and (9) (5-amino-7-[3-(4-hydroxyphenyl)propyl]-2-(2-furyl)pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine), which not only showed a better hydrophilic character but also a significant increase of both affinity and selectivity for the A<sub>2A</sub> adenosine receptor subtype, suggesting that most probably, a hydrogen bond is involved in the receptor recognition via this part of the ligand [6] (Figure 4).

To understand the nature of such a hypothetical hydrogen bond, compound SCH 442416 (10) was synthe-

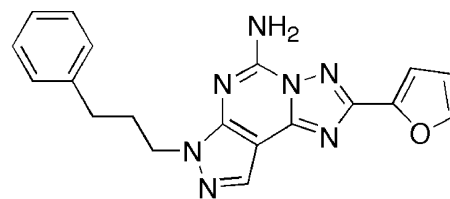


**Figure 2** Structures and binding affinities of the first synthesized pyrazolo-triazolo-pyrimidines: effect of the substituent position [3, 5]

**Figure 3** First generation of pyrazolo-triazolo-pyrimidines as potent and selective  $A_{2A}$  adenosine receptor antagonists [5, 6b, 8]

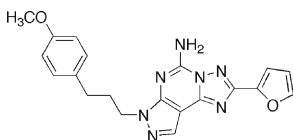


SCH 58261, **6**  
 $K_i$   $rA_1$  = 121 nM  
 $K_i$   $rA_{2A}$  = 2.3 nM  
 $K_i$   $hA_{2B}$  = > 10,000 nM  
 $K_i$   $hA_3$  = > 10,000 nM



SCH 63390, **7**  
 $K_i$   $rA_1$  = 504 nM  
 $K_i$   $rA_{2A}$  = 2.4 nM  
 $K_i$   $hA_3$  = > 10,000 nM

sized. This derivative showed even higher affinity and selectivity for the  $A_{2A}$  adenosine receptor, which makes it a candidate as a tool for positron emission tomography (PET) studies in its  $^{11}C$ -labeled form [7]. In addition, this derivative confirms the role of a hydrogen bond via the pyrazolo side chain.

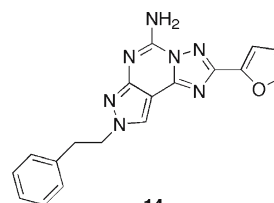


SCH 442416, **10**  
 $K_i$   $hA_1$  = 1,111 nM [7]  
 $K_i$   $hA_{2A}$  = 0.048 nM [7]  
 $K_i$   $hA_{2B}$  = > 10,000 nM [7]  
 $K_i$   $hA_3$  = > 10,000 nM [7]

However, the introduction of oxygenated groups could not be considered sufficient to confer water solubility. For this reason, carboxylic (*11*) and sulfonic (*12*) moieties were introduced, which contributed highly to the water solubility, in particular, the sulfonic moiety. However, in some cases, a loss of affinity with respect to reference compounds (*8*, *9*) for the  $A_{2A}$  adenosine receptor was the consequence. In contrast, the introduction of an amino group at para position of the phenyl ring in the side chain (*13*) gave better results in terms of affinity and selectivity for the  $A_{2A}$  adenosine

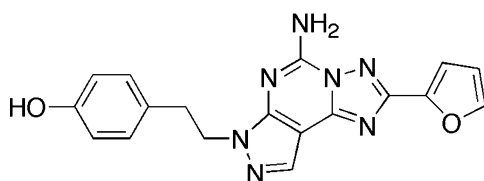
receptor subtype, but unfortunately, the water solubility was not optimal for a possible therapeutic use. From these advanced studies, it seemed very hard to find a useful combination of affinity, selectivity, and acceptable characteristics of bioavailability [8] (Figure 5).

The binding data for these compounds revealed another relevant aspect relating to the  $N^7$  and  $N^8$  pattern of substitutions (e.g., compounds *6* and *14*) (see also Figure 3) [5]. It is quite evident that the  $N^7$  derivative (*6*) is totally inactive at the human  $A_{2B}$  and  $A_3$  receptors whereas the  $N^8$  isomer (*14*) showed a slight affinity profile for these two receptor subtypes.

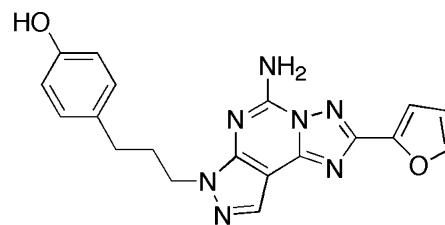


**14**  
 $K_i$   $hA_1$  = 1 nM [12,13]  
 $K_i$   $hA_{2A}$  = 0.31 nM [12,13]  
 $K_i$   $hA_{2B}$  = 5 nM [12,13]  
 $K_i$   $hA_3$  = 2,030 nM [12,13]

**Figure 4** Hydrophilic pyrazolo-triazolo-pyrimidines as  $A_{2A}$  adenosine receptor antagonists [6b]

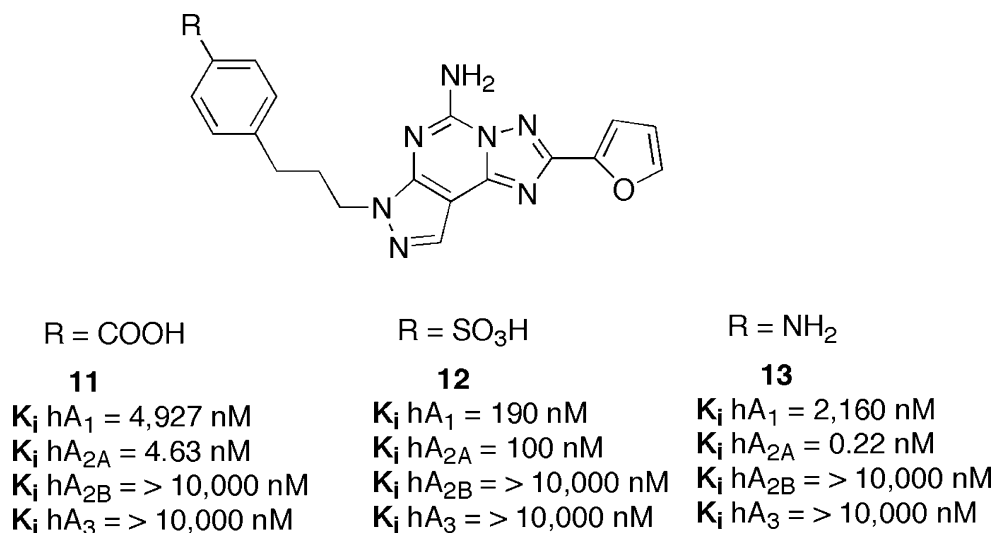


**8**  
 $K_i$   $rA_1$  = 444 nM  
 $K_i$   $rA_{2A}$  = 1.7 nM  
 $K_i$   $hA_3$  = > 10,000 nM



**9**  
 $K_i$   $rA_1$  = 741 nM  
 $K_i$   $rA_{2A}$  = 0.94 nM  
 $K_i$   $hA_3$  = > 10,000 nM

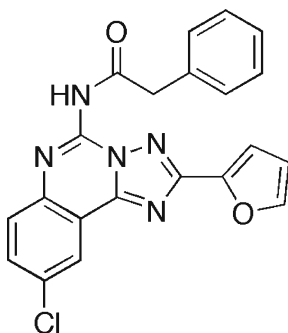
**Figure 5** Structures and binding affinities of different water-soluble pyrazolo-triazolo-pyrimidines [8]



This experimental observation strongly suggests a fundamental role of space occupancy of the substitutions on the pyrazole nucleus for recognition of the different receptor subtypes, and it represented a starting point for further studies.

### Substitution at the N<sup>5</sup> position

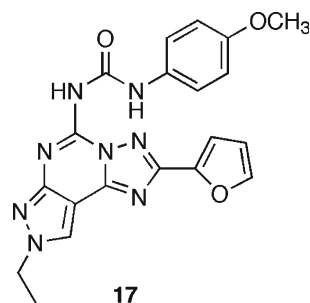
These results described above led us to thoroughly investigate this structure as a possible template for other subtype selective ligands, in particular, versus the human A<sub>3</sub> and A<sub>2B</sub> adenosine receptors. Taking into account these data and the fact that MRS 1220, (15) [9] was the most potent but not highly selective A<sub>3</sub> adenosine receptor antagonist reported in the literature, and considering the structural relation to CGS15943 (1) and the pyrazolo-triazolo-pyrimidine core, we modified the N<sup>5</sup> position of this nucleus in an attempt to obtain antagonists for the human A<sub>3</sub> adenosine receptor.



**MRS 1220, 3**  
 $K_i$  rA<sub>1</sub> = 52.7 nM [9]  
 $K_i$  rA<sub>2A</sub> = 10.3 nM [9]  
 $K_i$  hA<sub>3</sub> = 0.65 nM [9]

The planned modification was based on the introduction of a phenylcarbamoyl chain (4-OMe or 3-Cl substituted) present in a previously reported series of N<sup>6</sup> (substituted phenylcarbamoyl) adenosine-5'-uronamides, as A<sub>3</sub> agonists, of general formula (16), [4, 5], at the N<sup>5</sup> position of the pyrazolo-triazolo-pyrimidine scaffold [10, 11] (Figure 6).

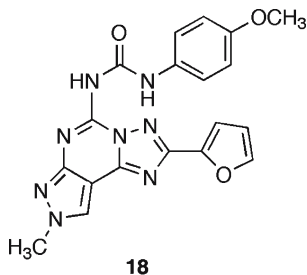
A preliminary study led to the discovery of compound (5-[[[4-methoxyphenyl]amino]carbonyl]amino-8-ethyl-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine (17), which turned out to be the most potent and selective human A<sub>3</sub> adenosine receptor antagonist ever reported and yet was totally inactive in a rat model [12].



**17**  
 $K_i$  hA<sub>1</sub> = 1,026 nM [13]  
 $K_i$  hA<sub>2A</sub> = 1,045 nM [13]  
 $K_i$  hA<sub>2B</sub> = 245 nM [13]  
 $K_i$  hA<sub>3</sub> = 0.60 nM [13]

This result represented the starting point for an intensive investigation of this new class of human A<sub>3</sub> adenosine receptor antagonist. A classic SAR approach for studying this new class of human A<sub>3</sub> adenosine receptor antagonists was performed. We found an interesting correlation between the calculated molecular volume of pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine derivatives and their experimental  $K_i$  values. In fact, it was observed that the A<sub>3</sub>

affinities decrease with increasing molecular volume values at the N<sup>8</sup> position. All these observations suggested that small alkyl groups at the N<sup>8</sup> pyrazole nitrogen combined with the N<sup>5</sup>-(4-methoxy)phenylcarbamoyl substitution afford the best compounds in terms of affinity and selectivity at the human A<sub>3</sub> adenosine receptors [13].



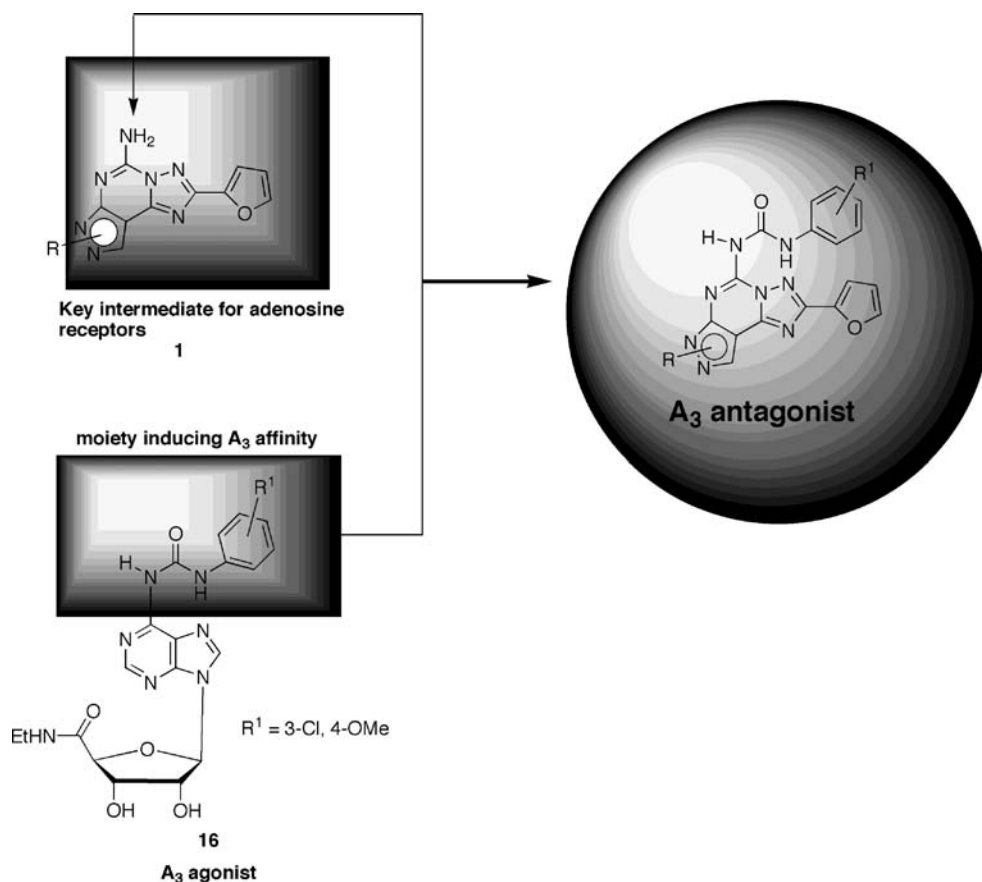
$K_i$  hA<sub>1</sub> = 1,097 nM [13]  
 $K_i$  hA<sub>2A</sub> = 1,390 nM [13]  
 $K_i$  hA<sub>2B</sub> = 261 nM [13]  
 $K_i$  hA<sub>3</sub> = 0.20 nM [13]

In particular, when the N<sup>8</sup>-methyl and N<sup>5</sup>-(4-methoxy)phenylcarbamoyl substitutions were combined, the most potent and selective human A<sub>3</sub> adenosine antagonist (**18**) was obtained [13]. A molecular modeling investigation, reported in the next session/paragraph, was derived in order

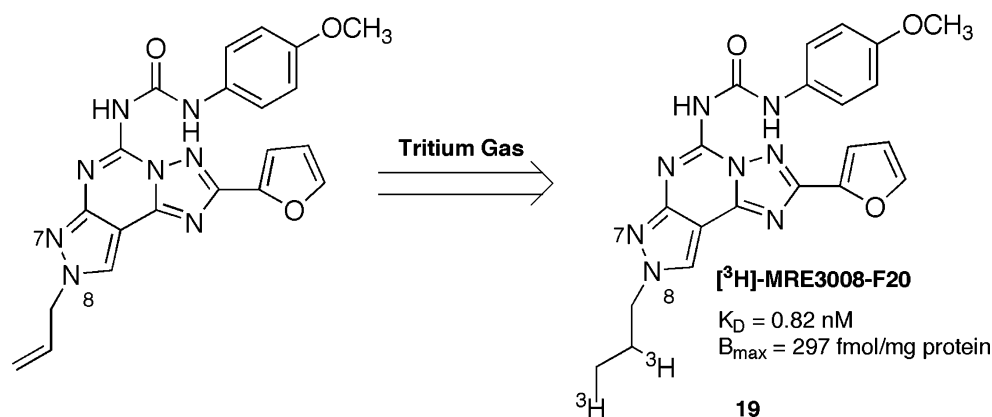
to rationalize these experimental results. This study also permitted the introduction of an allyl chain at the N<sup>8</sup> position that, after reduction with tritium, afforded a labeled form named MRE3008-F20 (**19**), which was the first antagonist radioligand utilized for studying the human A<sub>3</sub> adenosine receptor, showing a  $K_D$  value of 0.82 nM and a  $B_{max}$  of 297 fmol/mg protein (Figure 7) [14, 15].

As for the A<sub>2A</sub> antagonists, these derivatives also showed very low water solubility, which limits their use for therapeutic purpose. Considering that only the phenyl ring is a suitable core for the introduction of polar substituents, we investigated which position (*ortho*, *meta*, *para*) and substituent (e.g., Br, SO<sub>3</sub>H, F, H, CH<sub>3</sub>, CF<sub>3</sub>) could be suitable to solve this problem while maintaining a small substituents (methyl) at the N<sup>8</sup> position. The molecular modeling study performed on the synthesized compounds suggested that the steric factors at the *ortho* or *para* position on the phenyl ring play a fundamental role for affinity at the human A<sub>3</sub> receptors, and a very rigid steric control is present at the *meta* position. In fact, compound (**20**) was found to be the best compound of the series whereas derivative (**21**), which is completely water soluble, showed a dramatic loss of potency and selectivity [16] (Figure 8).

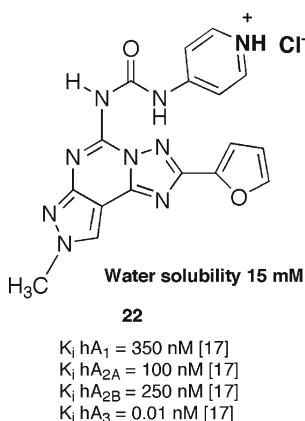
**Figure 6** Rational design of the human A<sub>3</sub> adenosine receptor antagonists



**Figure 7** [ $^3\text{H}$ ]-MRE3008-F20, the first radioligand for the human  $A_3$  adenosine receptor [14, 15]



Starting from experimental observation that bulky substituents are not well tolerated on the phenyl ring (see compounds **20** and **21**), the bioisosteric replacement of the phenyl ring with the 4-pyridyl moiety was performed, thus providing higher water solubility while avoiding the steric hindrance of a substituent at the *para* position, which seemed to be responsible for the reduction of human  $A_3$  adenosine receptor affinity. This approach led us to the discovery of the most potent (pM range), selective ( $>10,000$ )—and most importantly—water-soluble (15 mM) (**22**) human  $A_3$  adenosine receptor antagonist.

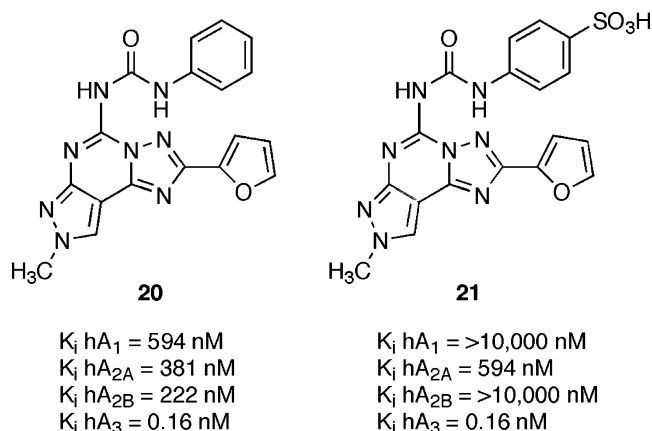


The high potency of this compound was confirmed by functional assay. An analysis of the antagonism of agonist-induced inhibition of cyclic adenosine monophosphate (cAMP) production in Chinese hamster ovary (CHO) cells expressing the human  $A_3$  receptor indicated a  $K_B$  value of 0.20 nM for compound (**22**) [17]. In this case, the support of molecular modeling was fundamental not only for the design of this derivative but also for understanding why the presence of an endocyclic nitrogen was capable of inducing a significant increase of potency in binding interaction. In fact, strong electrostatic interactions appeared to occur between the positively charged pyridinium moiety of (**22**) and the carbonyl oxygen atoms of Asn274 ( $\text{N}^+\text{H}\cdots\text{OC}$  distance=2.5 Å) and Asn278 ( $\text{N}^+\text{H}\cdots\text{OC}$  distance=3.1 Å),

both located on transmembrane (TM)7, which could be considered responsible for the increase in affinity

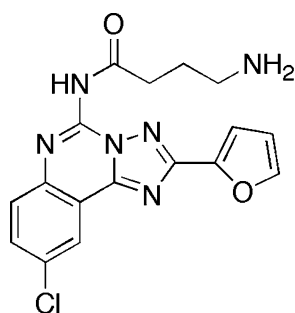
A quite similar approach was utilized for the improvement of the affinity at human  $A_{2B}$  adenosine receptors. In fact, CGS15943 not only binds to human  $A_3$  receptors but displays good affinity for the  $A_{2B}$  subtype. Jacobson and coworkers observed that the introduction of polar moieties, such as  $\gamma$ -aminobutyryl amide (**23**), results in increased potency at the  $A_{2B}$  adenosine receptors but a complete lack of selectivity versus the  $A_1$  and  $A_{2A}$  subtypes. However, the presence of apolar chains, such as the  $\text{N}^5$ -pyvaloyl (**24**) or the  $\text{N}^5$ -tert-butyloxycarbonyl derivative (**25**), displayed less potency than derivative (**23**) at the  $A_{2B}$  adenosine receptors but an increased selectivity versus the other receptor subtypes, indicating a preliminary SAR profile of this class of compounds as  $A_{2B}$  adenosine receptor antagonists (Figure 9) [18].

Considering these data, we performed a similar approach on the pyrazolo-triazolo-pyrimidines. We observed that the  $\text{N}^5$  unsubstituted derivatives (**14**, **26**) possess high affinity at the human  $A_{2B}$  adenosine receptors but a complete lack of selectivity. Instead, its substitution with a  $\gamma$ -amino-



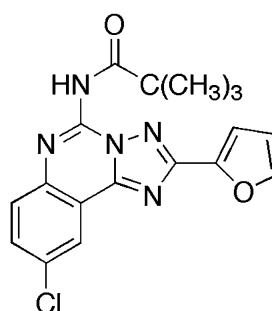
**Figure 8** Structures and binding affinities of the most interesting compounds modified at the phenyl ring [16]

**Figure 9** Structures and binding affinities of triazolo-quinazolines as  $A_{2B}$  adenosine receptor antagonists [18]



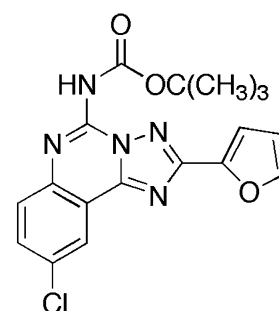
MRS1224, **23**

$K_i$   $rA_1$  = 8.7 nM  
 $K_i$   $rA_{2A}$  = 1.4 nM  
 $IC_{50}$   $hA_{2B}$  = 270 nM  
 $K_i$   $hA_3$  = 80 nM



**24**

$K_i$   $rA_1$  = 205 nM  
 $K_i$   $rA_{2A}$  = 89 nM  
 $IC_{50}$   $hA_{2B}$  = 2,400 nM  
 $K_i$   $hA_3$  = 244 nM



**25**

$K_i$   $rA_1$  = 190 nM  
 $K_i$   $rA_{2A}$  = 92 nM  
 $IC_{50}$   $hA_{2B}$  = 2,410 nM  
 $K_i$   $hA_3$  = 82 nM

butyryl amide (27) produces a decrease of affinity at the  $A_{2B}$  adenosine receptors but was found to be slightly selective versus the  $A_{2A}$  subtype [19] (Figure 10).

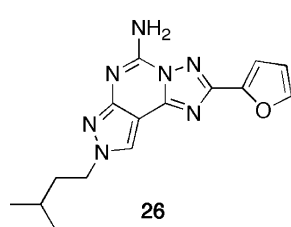
An improvement of this class of compounds was achieved by an optimized pattern of substitutions at the  $N^5$  and  $N^8$  positions. In fact, in parallel studies on human  $A_3$  adenosine antagonists, we observed that replacement of the phenylcarbamoyl moiety at the  $N^5$  position with a phenylacetyl group (e.g., compound 28) produces a decrease in affinity at the human  $A_3$  adenosine receptor affinity and a retention or improvement versus the  $A_{2B}$  subtype. In fact, a combination of a naphthyl acetyl moiety at the  $N^5$  position and a phenylpropyl group (characteristic of  $A_{2A}$  antagonists) at the  $N^8$  position led to compound (29), which was found to be quite potent and selective for the  $A_{2B}$  adenosine receptors [20] (Figure 11).

These results seem to suggest that bulky substituents at both the  $N^5$  and  $N^8$  positions could lead to potent and selective  $A_{2B}$  adenosine receptor antagonists [20]. A theoretical comparison of the putative TM binding motif of compound (29) on both human  $A_{2B}$  and human  $A_3$  receptors was able to elucidate these results, explaining

why such small modifications could significantly change the biological profile of this class of compounds.

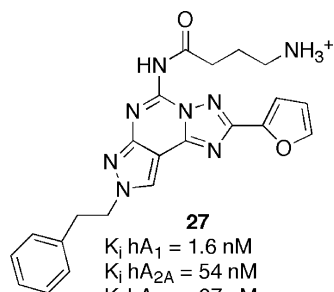
### Molecular modeling

We recently developed a combined target-based and ligand-based drug design approach to define a pharmacophore model of human  $A_3$  receptor antagonists, in order to lead the discovery and the structural refinement of new potent and selective human  $A_3$  receptor antagonists. In particular, we developed a rhodopsin-based model of the human  $A_3$  receptor to provide more accurate information about the putative binding site of the pyrazolo-triazolo-pyrimidines [16, 17, 20–24]. As already reported, this binding site for  $A_3$  agonists and antagonists was located in the upper region of the TM helical bundle of the receptor. The human  $A_3$  receptor model revealed a central pocket surrounded by helices 1–7 and 5–6, as TM4 is not part of the cavity wall. TMs 3, 5, 6, and 7 seem to be crucial for the recognition of  $A_3$  receptor ligands, and particular care has been given to the second extracellular loop (EL2). EL2, as for rhodopsin,



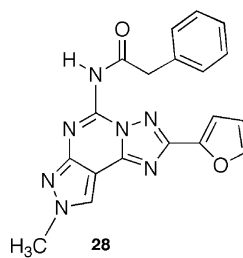
**26**

$K_i$   $hA_1$  = 2 nM  
 $K_i$   $hA_{2A}$  = 0.8 nM  
 $K_i$   $hA_{2B}$  = 9 nM  
 $K_i$   $hA_3$  = 700 nM



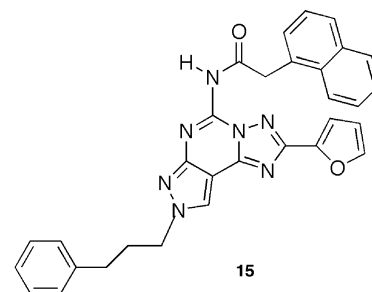
**27**

$K_i$   $hA_1$  = 1.6 nM  
 $K_i$   $hA_{2A}$  = 54 nM  
 $K_i$   $hA_{2B}$  = 27 nM  
 $K_i$   $hA_3$  = 65 nM



**28**

$K_i$   $hA_1$  = 702 nM  
 $K_i$   $hA_{2A}$  = 423 nM  
 $K_i$   $hA_{2B}$  = 165 nM  
 $K_i$   $hA_3$  = 0.81 nM



**15**

$K_i$   $hA_1$  = 1,100 nM  
 $K_i$   $hA_{2A}$  = 800 nM  
 $K_i$   $hA_{2B}$  = 20 nM  
 $K_i$   $hA_3$  = 300 nM

**Figure 10** Structures and binding affinities of pyrazolo-triazolo-pyrimidines as  $A_{2B}$  adenosine receptor antagonists [19]

**Figure 11** Structures and binding affinities of  $N^5$  arylacetyl pyrazolo-triazolo-pyrimidines as  $A_{2B}$  adenosine receptor antagonists [20]

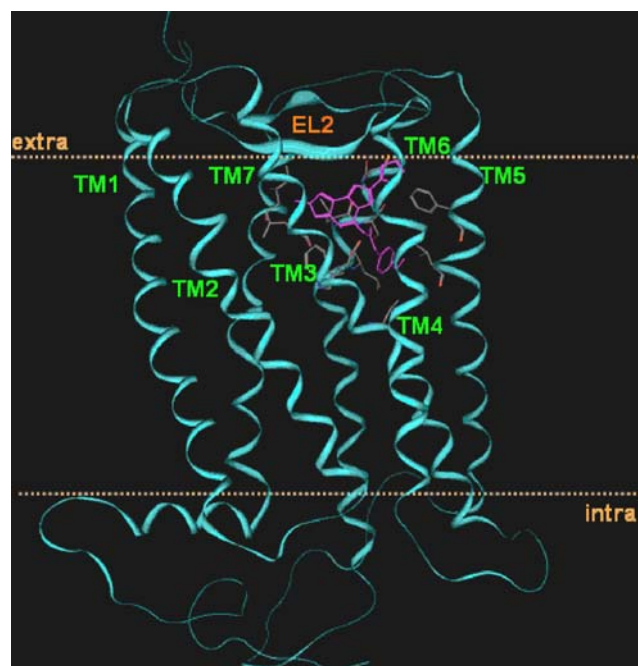
folds back over TM helices, limiting the size of the ligand recognition site and preventing access to the cavity from the periplasm [25]. Docking studies, validated by site-directed mutagenesis analysis, pointed out several residues in transmembrane domains 3 and 5 and in the EL2 that seem to be critical for ligand recognition, including His95, Trp243, Ser247, Asn250, and Lys152 [23, 24, 26]. The molecular docking study was carried out on more than 200 pyrazolo-triazolo-pyrimidine derivatives to recognize the hypothetical binding motif of human A<sub>3</sub> receptor antagonists and to define a target-based pharmacophore model. Consequently, to generate a three-dimensional (3D)-driven pharmacophore hypothesis, the docked low energy conformations of pyrazolo-triazolo-pyrimidine were used as a structural template. Therefore, we identified the hypothetical binding site of these derivatives and proposed a “Y-shaped” pharmacophore model [22]. Interestingly, all derivatives share a common binding motif inside the TM region of human A<sub>3</sub> receptors: the pyrazolo-triazolo-pyrimidine moiety is surrounded by TMs 3, 5, 6, and 7, with the furan ring and the N<sup>8</sup> substituents pointing toward the EL2 and the carbamoyl moiety in the 5-position oriented toward the intracellular environment. The Y-shape binding motif is defined by a peculiar and highly conserved binding mode: the furan ring is positioned between TM5 and TM3 whereas the N<sup>8</sup> substituents are surrounded by TM2 and TM7.

Moreover, analyzing in detail the 3D pharmacophore model, we identified five regions that seem to be crucial for antagonists recognition. In particular, all pyrazolo-triazolo-pyrimidine derivatives present the carbamoyl moiety in the 5-position such that it is surrounded by two polar amino acids: His95 (TM3) and Ser247 (TM6). The major structural difference between the hypothetical binding sites in adenosine receptor subtypes is that the A<sub>3</sub> receptor does not contain the histidine residue in TM6 common to all A<sub>1</sub> (His251 in hA<sub>1</sub>) and A<sub>2</sub> (His250 in hA<sub>2A</sub>) receptors. This histidine has been shown to participate in both agonist and antagonist binding to A<sub>2A</sub> receptors whereas in the A<sub>3</sub> receptor, the histidine is replaced with a serine residue (Ser247 in hA<sub>3</sub>) [27, 28]. The polar amino acids stabilize interactions with the carbamoyl moiety, and consequently, the carbamoyl phenyl ring is oriented in the middle of the TM bundle (Figure 12).

In particular, 2.6 Å separate the N-H of His95 (TM3) and the oxygen atom of the carbamoyl group, appropriately oriented to form a H-bonding interaction. The side chain of Ser247 (TM6) is within hydrogen-bonding distance of NH of the carbamoyl group at 2.9 Å. The phenyl ring of the carbamoyl moiety takes place in a receptor region that is hydrophobic and characterized by three nonpolar amino acids: Ile98 (TM3), Ile186 (TM5), and Leu244 (TM6). As a consequence of this, polar substituents, such as a sulfonic

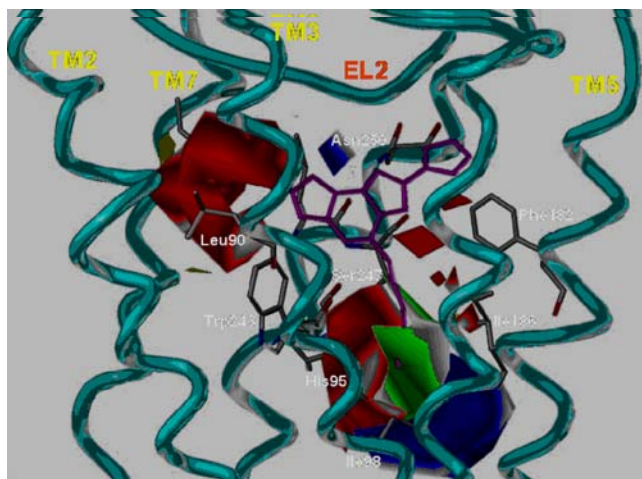
acid group at the para position of the phenyl ring, are not very well tolerated [16]. Moreover, around this *para* position, it seems that a steric control is present because of the very limited empty space between TM5 and TM6. In fact, there is a reduction in the affinity at the human A<sub>3</sub> adenosine receptors of the N<sup>5</sup> phenyl ring derivatives substituted at the para position [16, 17]. In the case of *meta* substitutions, we observed a similar steric control when the meta residues are larger than hydrogen: a significant affinity reduction at the human A<sub>3</sub> receptor is due to the strong steric repulsion among the meta substituents and amino acid side chains of TM6 and TM7. In contrast, the residues at the *ortho* position seem to occupy an empty region of the binding cavity.

Another important pharmacophore feature is located in a highly conserved region delimited by Phe168 (EL2) and Phe182 (TM5). There is a probable  $\pi$ - $\pi$  interaction between the furan ring of the pyrazolo-triazolo-pyrimidine and these two phenylalanine residues. In particular, the amino acids corresponding to Leu90 (TM3) and Phe182 in the human A<sub>2A</sub> receptor was found to be essential for the binding of both agonists and antagonists [28]. Moreover, the nonpolar amino acid Leu90, together with Leu246 (TM6) and Ile268 (TM7), delimited a peculiar hydrophobic pocket where all the substituents at the N<sup>8</sup> position are located, with a good tolerance for the hydrophobic ones. However, in the presence of appropriate substituents, hydrogen bonding can be established with two hydrophilic



**Figure 12** General topology of the human A<sub>3</sub> receptor model. Reference compound **20** (colored in magenta) is docking inside the transmembrane recognition site. Side chains of some amino acids important for ligand recognition are highlighted





**Figure 13** Side view of the 20- $A_3$  complex model. The side chains of some crucial important residues in proximity ( $\leq 5$  Å) to the docked pyrazolo-triazolo-pyrimidine molecule are *highlighted and labeled*: Leu90 (TM3), His 95 (TM3); Phe182 (TM5), Ile186 (TM5); Trp243 (TM6); Ser247 (TM6), Asn250 (TM6), Ser271 (TM7), His272 (TM7), Ser275 (TM7). The steric and the electrostatic contour plots, obtained from the comparative molecular field analysis (CoMFA), are included into ligand binding cavity. The *yellow* and the *blue* polyhedra correspond to regions of the field that are predicted to decrease the  $A_3$  receptor affinity whereas the *green* and the *red* regions are predicted to increase binding affinity

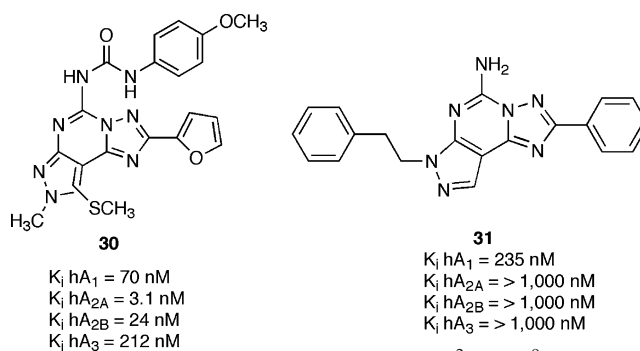
amino acids, namely, Ser175 (EL2) and the His272 (TM7), located on the border of this hydrophobic pocket. [13] However, other aromatic rings, such as phenyl or thiophenyl, seem to be less well tolerated by the receptor-binding pocket, probably due to their less favorable steric complementarities [29].

Another strong hydrogen bonding interaction occurs between Asn250 (TM6) and the triazolo ring of the pyrazolo-triazolo-pyrimidine moiety. The N-H of Asp250 and the  $N^4$  of the triazolo ring group are, in fact, separated by 2.5 Å and appropriately oriented to form an H-bonding interaction. The two polar amino acids Thr94 (TM3) and Ser97 (TM3) were shown to be important for the coordination of agonist but not for antagonist binding at  $A_1$  and  $A_{2A}$  receptors, respectively. Therefore, no direct interactions have been predicted between them and the antagonist structure.

In parallel with the docking studies and the target-based pharmacophore model generation, we derived a comparative molecular field analysis (CoMFA) on the pyrazolo-triazolo-pyrimidine analogue binding data, as alternative scoring function for the prediction of ligand-receptor-binding affinity [22, 24]. The analysis was performed considering the previous results of the docking approach. The CoMFA approach consists of the computation of steric and electrostatic field variables calculated at grid points surrounding the whole molecule, followed by a partial least squares (PLS) on these 3D structure descriptors and on the biological activity. The PLS analysis, followed by cross-

validation, showed very good values of correlation, meaning high predictivity power of the quantitative structure activity relationship (QSAR) model. The CoMFA results can be observed and analyzed as a contour plot and regression plots. The contour plot consists of colored polyhedra that describe the regions of space where the steric and electrostatic fields are predicted to have an effect on binding affinity. The yellow and the blue contours correspond to regions of the field where increase steric bulk and electronegative character is associated with a decrease of receptor affinity. In particular, we observed that the contour map displays a green polyhedron nearby the  $N^5$ -carbamoyl moiety, suggesting enhanced  $A_3$  receptor affinity if we introduce sterically bulky substituents in this position. As already described, this steric-bulk-favorable region nicely fits with the receptor region around the phenyl ring of the carbamoyl moiety, characterized by the three nonpolar amino acids Ile98(TM3), Ile187(TM5), and Leu244(TM6). On the other side, we observed two red polyhedra located around to  $N^8$  substituents and to the  $N^5$ -carbamoyl moiety, complementary to the polar amino acids Ser175 (EL2), His95 (TM3), Ser247 (TM6), and His272 (TM7) present in this region in the  $A_3$ -receptor-binding-site model. Similarly, the blue polyhedron close to the  $N^5$  phenyl ring is complementary to the nonpolar residues Ile98 (TM3), Ile186 (TM5), Phe239 (TM6), and Leu244 (TM6) (Figure 13).

In Figure 13, we present the CoMFA contour plot of a pyrazolo-triazolo-pyrimidine derivative docked inside the human  $A_3$ -receptor-binding-site model. We can see that all steric and electrostatic features are coherent with the SAR previously described in the molecular docking analysis. This combined approach between a very consolidated target-based approach, such as molecular docking, and a solid quantitative ligand-based methodology, such as CoMFA, is very advantageous for the identification of common structural features of pyrazolo-triazolo-pyrimidines important for the affinity at the  $A_3$  receptor. We have, in fact, had really good results in validation of this



**Figure 14** Structures and binding profile of  $C^2$  and  $C^9$  substituted pyrazolo-triazolo-pyrimidines [30]

combined approach, predicting with high precision the binding affinity values of new derivatives with a different spectrum of affinity at the human  $A_3$  receptor. [22, 24]

### Substitutions at the $C^2$ and $C^9$ positions

Very recently, a number of pyrazolo-triazolo-pyrimidines with classic substituents at  $N^7$  and  $N^5$  positions have been modified at the  $C^2$  or  $C^9$  positions. In general, it has been observed that the introduction of a substituent on the  $C^9$  position (e.g.,  $SCH_3$ ,  $NHCH_2CH_3$ ) led to retention of a receptor affinity but a complete loss of selectivity between different receptor subtypes. Indeed, substitution of the furan ring with phenyl or alkoxyphenyl rings led to a loss of affinity at  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$  adenosine receptors only. At the  $A_1$  receptor, a high nanomolar affinity is still present. Also, the introduction of polar substituents (by Mannich reaction) on the furan ring led to complete inactivity. Some examples (30, 31) are depicted in Figure 14.

These data clearly indicate that position 9 plays a role for selectivity whereas the presence of an unsubstituted furan ring at the  $C^2$  position plays a fundamental role in ligand-receptor recognition [30].

### Concluding remarks

All the work performed on the pyrazolo-triazolo-pyrimidine nucleus resulted in potent and selective ligands for  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$  adenosine receptor subtypes whereas for the  $A_1$  subtype, potency was achieved but selectivity was very poor. All these experimental observations resulted in a quite significant SAR profile of this nucleus for the adenosine receptor subtypes. In addition, in the case of  $A_3$  adenosine receptor, all these studies allowed development of a receptor-based pharmacophore model with high levels of predictivity for newly designed compounds, as recently reported.

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