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Synthesis and in vitro activity of *N*′-cyano-4-(2-phenylacetyl)-*N*-*o*-tolylpiperazine-1-carboximidamide $P2X_7$ antagonists

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Abstract—A novel series of cyanoguanidine-piperazine $P2X_7$ antagonists was designed based upon the structure of A-740003. Structure–activity relationship (SAR) studies focused on the piperazine moiety and the right hand side substitution. Compounds were assayed for activity at human and rat $P2X_7$ receptors and compound **29** was found to possess potent activity (IC_{50} = 30–60 nM) at both species.

The $P2X_7$ receptor has drawn increasing attention in recent years as a promising new therapeutic target for small molecule drug research with potential applications in the treatment of pain and inflammation.[1–5] This ligand-gated ion channel is primarily expressed on cells of the immune system such as mast cells, macrophages, lymphocytes, and glia. Acute activation of the $P2X_7$ receptor by its endogenous ligand ATP gives rise to a cascade of biochemical signaling events including reversible channel opening, activation of caspases and proinflammatory cytokine (IL-1β, IL-18, TNF-α) release.[1,5,6] Macrophages from $P2X_7$ knock-out (KO) mice show an impaired ability to release IL-1β in response to stimulation with lipopolysaccaride (LPS) and ATP both in vitro and in vivo.[7] Small molecule $P2X_7$ antagonists have similarly been found to block IL-1β and IL-18 release in vitro and/or in vivo.[8–12] In a model of collagen-induced arthritis, $P2X_7$ KO mice additionally showed a reduction in the incidence and severity of arthritic symptoms compared to wild-type controls.[13] Collectively, these data suggest that $P2X_7$ antagonists may offer a new approach to the treatment of chronic inflammatory conditions such as rheumatoid arthritis. AZ9056, a $P2X_7$ antagonist of undisclosed structure, is reportedly under clinical investigation for rheumatoid arthritis and inflammatory bowel disease.[14] In the pain area, $P2X_7$ KO mice displayed reduced sensitivity to inflammatory or nerve insult[15] in agreement with the efficacy profile of $P2X_7$ antagonists in models of chronic inflammatory and neuropathic pain.[8–10,12,16] These observations may be related to the involvement of $P2X_7$ in modulating either inflammatory cytokine or glutamate release.[5,17]

The potential therapeutic applications for a potent and selective $P2X_7$ antagonist has led to a flurry of recent reports both in the patent and scientific literature describing a diverse array of small molecule ligands.[2–5] The cyanoguanidine $P2X_7$ antagonists **1** (A-740003) and **2** provided an attractive starting point for the design of additional novel agents due to their potent activity at both rat and human receptors as well as their reported selectivity over other P2X and P2Y receptors.[9,16] An early area of focus was to explore the potential to replace the unusual aminal unit present in **1** and **2** with a piperazine moiety as shown. Described below are structure–activity relationship studies that resulted in the discovery of a novel series of cyanoguanidine-piperazine $P2X_7$ antagonists around the general structure **A**.

Compounds were prepared using the general synthetic methods shown in Scheme 1; here illustrated for the synthesis of target molecules V with the R^1 group proximal

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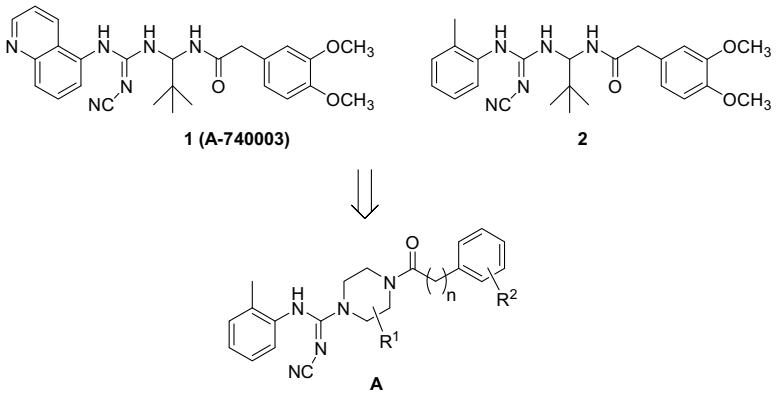
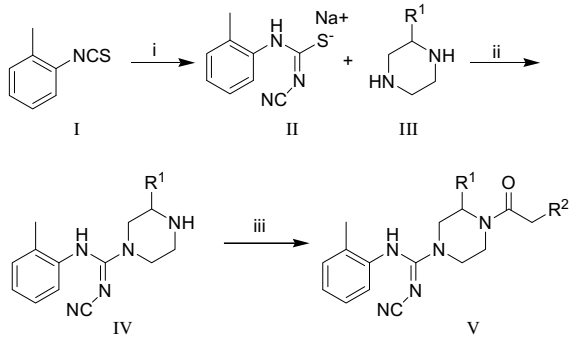


to the amide and distal to the cyanoguanidine. The cyanoguanidine functionality is first constructed by coupling intermediate II with intermediate III where bond formation occurs predominantly at the less sterically congested nitrogen. Subsequently, the intermediate IV is reacted at the more hindered nitrogen to form the amide bond using similar coupling conditions and giving rise to final products V. To access analogs with the R^1 group proximal to the cyanoguanidine and distal to the amide, the sequence of coupling reactions was reversed making the amide first and the cyanoguanidine second.

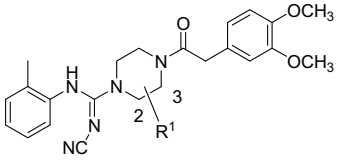
In vitro $P2X_7$ activity was assessed using the recombinant rat and human receptors. Antagonist potencies were determined by measuring the inhibition of Ca^{2+} flux with a fluorometric imaging plate reader (FLIPR) using Fluo-4 as the dye and benzoylbenzoylATP (BzATP) as the agonist.[9]

Initial exploration around the general structure **A** began with the evaluation of a variety of substituted piperazines as shown in Table 1. The left and right hand aromatic substitutions selected for these investigations were based upon those which provided potent activity for compound **2**. Although the early alkyl substituted analogs **3**–**6** provided a moderate level of potency at $hP2X_7$, activity at the rat receptor was weaker and in general potency fell short of that seen with **1** and **2**. Replacing the alkyl groups with phenyl in either the 2- or 3-position (**7**, **8**), however, resulted in a significant improvement in potency at both rat and human receptors. The potency at $hP2X_7$ for **7** and **8** was comparable to both **1** and **2**.



Scheme 1. Reagents: (i) NaHNCN, DMF; (ii) EDC, DMF; (iii) EDC, DMF, $R^2CH_2CO_2H$.

Table 1. SAR on the central piperazine ring

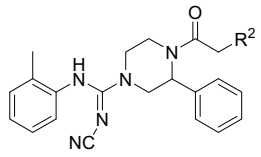


Compound	R^1	$rP2X_7$ IC_{50} (μM)[a]	$hP2X_7$ IC_{50} (μM)
1	—	0.018	0.044
2	—	0.014	0.12
3	*cis*-3,5-Dimethyl	19	0.46
4	(*R*)-2-Isopropyl	16	1.3
5	(*R*)-2-Isobutyl	4.5	0.43
6	3-*tert*-Butyl	0.42	0.19
7	3-Phenyl	0.14	0.058
8	2-Phenyl	0.22	0.051

[a]Values are means of 2–3 experiments. Compounds tested at the recombinant human and rat $P2X_7$ receptors as described.[9]

With the promising activity of compound **7**, additional exploration was conducted on the right hand side (RHS) in order to ascertain if further improvements in activity could be attained by replacement of the 3,4-dimethoxyphenyl with other aromatic groups. As shown in Table 2, several heteroaryl replacements were found to possess similar potency as **7** and **8**, in particular the pyridyl (**12**, **13**), thiophene (**14**, **15**), and isoxazole (**18**) analogs. The pyrazole compounds **20** and **21** provided potent activity at $hP2X_7$ but were almost 10-fold weaker at $rP2X_7$. Some sensitivity to the attachment point of the heteroaryl group was noted for the isomeric isoxazoles **18** and **19** with attachment at the 5-position of the isoxazole giving ~10-fold better potency than at the 3-position. Other heteroaryls such as furan (**16**, **17**) and indole (**23**) were slightly weaker whereas the more basic imidazole (**22**) was considerably weaker. The phenyl (**9**), 4-chlorophenyl (**10**), and naphthyl (**11**) analogs also displayed slightly attenuated activity compared to **7**. Interestingly, adamantyl substitution, which is a common feature of many $P2X_7$ antagonists,[3,4] was not found to provide potent activity in this series (e.g., **24**).

Table 2. SAR on the right hand side aromatic group



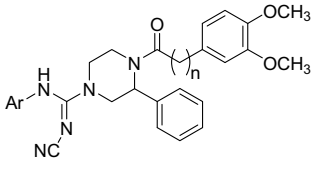
Compound	R^2	$rP2X_7$ IC_{50} (μM)[a]	$hP2X_7$ IC_{50} (μM)
9	Phenyl	0.38	0.088
10	4-Chlorophenyl	0.11	0.14
11	2-Naphthyl	0.14	0.31
12	4-Pyridyl	0.15	0.036
13	3-Pyridyl	0.39	0.040
14	2-Thienyl	0.15	0.046
15	3-Thienyl	0.27	0.071
16	2-Furanyl	0.52	0.11
17	3-Furanyl	0.25	0.22
18	3-Methylisoxazol-5-yl	0.17	0.046
19	5-Methylisoxazol-3-yl	0.61	0.41
20	5-Methyl-1H-pyrazol-1-yl	0.56	0.060
21	3-Methyl-1H-pyrazol-1-yl	0.32	0.040
22	1H-Imidazol-4-yl	14	1.0
23	1H-Indol-3-yl	0.21	0.10
24	1-Adamantyl	0.63	1.5

[a] Values are means of 2–3 experiments. Compounds tested at the recombinant human and rat $P2X_7$ receptors as described.[9].

The influence of the length of the tether connecting the piperazine with the right hand aromatic group was also interrogated (Table 3, **25**–**28**). Surprisingly, the length of the tether had little effect on activity at $hP2X_7$, with all compounds displaying IC_{50}s between 0.05 and 0.1 μM. An expanded panel of directly attached aromatic amides ($n = 0$) that overlapped most of the substitutions in Table 2 was also investigated. As seen for **25**, these additional directly attached amides displayed equivalent to slightly reduced (2- to 3-fold) potency when compared with their counterparts in Table 2 (data not shown). At $rP2X_7$ direct attachment of the aromatic group (**25**) gave the weakest activity.

Although extensive SAR studies on the left hand aromatic group of the pharmacophore were not conducted as part of these early investigations, a limited foray in this area did prove fruitful. Replacement of the *o*-tolyl group with the 5-quinolinyl moiety as in A-740003 was investigated. Gratifyingly, compound **29** proved to be the most potent compound at $rP2X_7$ from this series, with an IC_{50} of 30 nM. This was an especially encouraging result given the previous observations that direct attachment of the right hand aromatic group was not optimal for potency at the rat receptor. Compound **29** was also found to be selective for $P2X_7$ over other P2 receptors, as evidenced by the lack of activity at $P2X_{2/3}$, $P2X_3$, $P2X_4$, and $P2Y_2$ at 10 μM.

Table 3. Modification of the chain length on the right hand side and replacement of *o*-tolyl with 5-quinolinyl



Compound	Ar	*n*	$rP2X_7$ IC_{50} (μM)[a]	$hP2X_7$ IC_{50} (μM)
25	*o*-Tolyl	0	0.29	0.094
26	*o*-Tolyl	1	0.14	0.058
27	*o*-Tolyl	2	0.076	0.057
28	*o*-Tolyl	3	0.095	0.070
29	5-Quinolinyl	0	0.030	0.059

[a] Values are means of 2–3 experiments. Compounds tested at the recombinant human and rat $P2X_7$ receptors as described.[9].

In summary, a novel series of cyanoguanidine-piperazines was discovered with potent and selective activity at $P2X_7$. It was found from these early studies that, with suitable structural modifications, potency could be achieved comparable to that of A-740003. Most analogs displayed approximately 3- to 5-fold greater potency for $hP2X_7$ over $rP2X_7$, however, compound **29** was highly potent at both species. These findings indicate that substantial flexibility around the pharmacophore of **1** and **29** exists to incorporate structural changes with retention of potent $P2X_7$ antagonism. Additional studies further describing structural modifications around **7** and **29** will be the subject of future reports.

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