Bioorganic & Medicinal Chemistry Letters 18 (2008) 3848–3851

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and activity of *N*-cyanoguanidine-piperazine $P2X_7$ antagonists

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ARTICLE INFO

Article history:
Received 13 May 2008
Revised 16 June 2008
Accepted 17 June 2008
Available online 20 June 2008

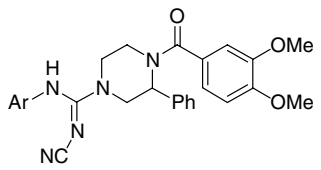
Keywords:
P2X7
Antagonists
Cyanoguanidine
Piperazine

ABSTRACT

A novel series of cyanoguanidine-piperazine $P2X_7$ antagonists were identified and structure–activity relationship (SAR) studies described. Compounds were assayed for activity at human and rat $P2X_7$ receptors in addition to their ability to inhibit IL-1β release from stimulated human whole blood cultures. Compound **27** possesses potent activity (0.12 μM) in this latter assay and demonstrates moderate clearance in-vivo.

The $P2X_7$ receptor is an ATP-activated ligand-gated ion channel that represents a promising new therapeutic target with potential applications in the treatment of pain and inflammation.[1–5] Expression of the $P2X_7$ receptor is primarily on cells that regulate the immune system such as mast cells, macrophages, and lymphocytes. A clear role has been defined for ATP agonism of the $P2X_7$ receptor leading to caspase-1 activation, a protease required for the processing and release of the pro-inflammatory cytokines IL-1β and IL-18.[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 inhibit IL-1β and IL-18 release in-vitro and/or in-vivo.[8–12] A study using $P2X_7R$ knockout mice established that joint inflammation was substantially decreased in the anti-collagen Ab-induced mouse model of arthritis.[13] The receptor is also expressed in the central nervous system on microglia[14] and astrocytes[15] and this suggests a role for the development and maintenance of neuropathic pain as evidenced in $P2X_7$ knock-out mice that showed protection from inflammatory pain and partial nerve ligation-induced neuropathic pain.[16] These data are in agreement with the efficacy profile of $P2X_7$ antagonists in models of chronic inflammatory and neuropathic pain.[8–10,12,17]

The role of the $P2X_7$ receptor in the processing and release of both IL-1β and IL-18 represents an attractive approach to inhibiting levels of these cytokines to provide a potential treatment for inflammatory conditions such as rheumatoid arthritis. We recently published initial structure–activity relationships of a novel series of cyanoguanidine-piperazine $P2X_7$ antagonists exemplified by the amides **1** and **2** with sub-100-nM potency against the human $P2X_7$ receptor.[18] A general limitation of these amides was the low potency observed in LPS/ATP stimulated human whole blood cultures (data not shown) that precluded further advancement. This letter describes significant further optimization of this novel series of cyanoguanidine-piperazine P2X7 antagonists resulting in urea-linked compounds with less than 250 nM potency in human whole blood.



1, Ar = *o*-Tolyl ($hP2X_7$ IC_{50} = 94 nM)
2, Ar = 5-Quinolinyl ($hP2X_7$ IC_{50} = 59 nM)

The amides in this letter were prepared using the general synthetic methods shown in Scheme 1 and illustrated for the synthesis of target molecule **1**. Reaction of the *o*-tolyl isothiocyanate I with

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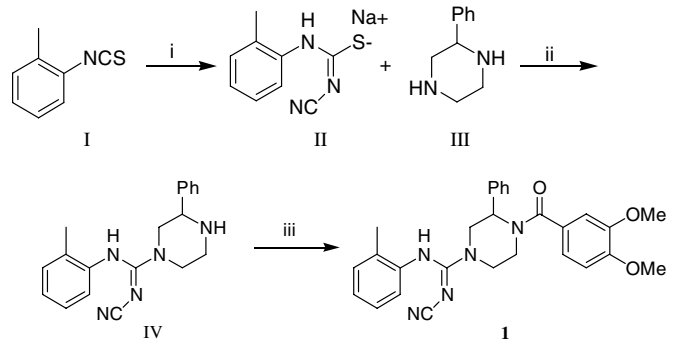
doi:10.1016/j.bmcl.2008.06.055



Scheme 1. Reagents and conditions: (i) NaHNCN, DMF; (ii) EDC, DMF; (iii) EDC, DMF, 3,4-dimethoxybenzoic acid.

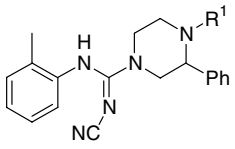
sodium cyanamide gave the thiocyanoimidate intermediate II. In-situ coupling with the phenyl piperazine III constructed the cyanoguanidine IV at the less sterically congested nitrogen. Standard amide-coupling protocols provided the final product **1** by reaction at the more hindered nitrogen. Alternatively, ureas such as **4** and **5** were accessed by reacting intermediates such as IV with the appropriate isocyanate. To prepare analogs shown in Table 4 with the R^2 group proximal to the cyanoguanidine and distal to the amide or urea (such as **13**), the sequence of coupling reactions was reversed making the cyanoguanidine as the last step.

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 3′-*O*-(4-benzoylbenzoyl) ATP (BzATP) as the agonist.[9] Selected compounds were subsequently profiled for their ability to inhibit IL-1β release from stimulated human whole blood cultures.

We initially probed the requirement for R^1 as shown in Table 1. The tolylcyanoguanidine substitution was selected for these investigations based on the potency of compound **1**. Although the sulfonamide **7** provided a moderate level of potency at $hP2X_7$, greater than 8-fold less activity was observed at the rat receptor. Replacing the amide with a urea (**4** and **5**) provided a useful pharmacophore as these had comparable potency to **1**, both at $hP2X_7$ and at $rP2X_7$. Methylation of the urea provided compound **6** that exhibited eroded potency compared to its progenitor **5**. Analysis of these data is consistent with a preference for a hydrogen-bond donor in this region of the molecule.

With the promising activity of urea **5**, additional exploration was conducted on the cyanoguanidine replacements to ascertain

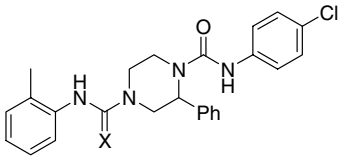
Table 1



	R^1	$rP2X_7IC_{50}$[a](μM)	$hP2X_7$[a]IC_{50} (μM)
1	–CO-(3,4-dimethoxyphenyl)	0.29	0.094
3	$-COCH_2-$(3,4-dimethoxyphenyl)	0.14	0.63
4	–CONH-(3,4-dimethoxyphenyl)	0.178	0.025
5	–CONH-(4-chlorophenyl)	0.095	0.132
6	–CONMe-(4-chlorophenyl)	0.29	1.03
7	$-SO_2-$(4-chlorophenyl)	4.06	0.54
8	$-CH_2-$(4-chlorophenyl)	>10	>10

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

Table 2



	X	$rP2X_7IC_{50}$[a] (μM)	$hP2X_7IC_{50}$[a] (μM)
9	$CHNO_2$	3.11	0.31
10	$NCONH_2$	10.3	6.4
11	O	0.62	0.78

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

if further improvements in activity could be attained. The results of this exercise can be illustrated with just three examples (Table 2) as all analogs exhibited a reduction in potency compared to the cyanoguanidine.

Having established that compound **5** represented our lead compound, we sought to profile it more widely. To study the effect of $P2X_7$, antagonists on ICE-dependent cytokines, compound **5** was tested for its ability to inhibit both IL-1β and IL-18 in LPS/ATP stimulated human whole blood cultures. In accord with its mechanism of action, compound **5** inhibited the secretion of both cytokines with similar concentration–response relationships at an IC_{50} of 1.19 and 1.26 μM, respectively (data not shown).

Target validation studies required a compound with cellular activity married with the ability to achieve good oral exposure. Profiling of compound **5** in liver microsomal preparations (rat and human) indicated a high rate of oxidative metabolism, an observation reinforced by the high clearance and short half-life of compound **5** after iv administration in rats shown in Table 3. Combination of these data and the whole blood potency suggested that analogs with an improved profile were needed.

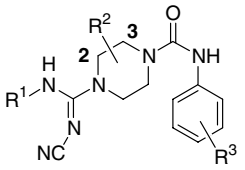
Our initial focus was to identify groups that would impart greater human whole blood potency by exploring further cyanoguanidine substitutions (R^1 in Table 4). In contrast to the data in the recombinant human receptor, human whole blood data for the inhibition of IL-1β indicate that the phenyl group and the isopropyl group are not interchangeable in the 2- or 3-position. For example, in human whole blood, the 2-isopropyl is preferred over the 3-isopropyl as seen for the quinolines **14** and **15**with the former being fourfold more potent. In addition, there is a profound effect in this assay for the 3-phenyl group in indole **17** compared to its 2-isopropyl analog **16** with the latter being greater than 30-fold more potent. The reasons for this discrimination are currently unclear. One common feature of the three most potent compounds in the whole blood assay (**14**, **23**, and **24**) is that each contains a basic residue at R^1.

Our attention turned to the 5-quinolyl analog, compound **14**, as this represented a 10-fold increase in potency compared to compound **5** in the key human whole blood assay. Unfortunately **14** suffered from microsomal instability (43% parent remaining after 25 min in rat liver microsomes) and high in-vivo clearance in rats (3.2 l/h/kg). In mouse liver microsomes, the primary metabolic route is oxidation of the quinoline to the N-oxide with no impact on the cyanoguanidine. We were also able to ascertain that the quinoline N-oxide was the major metabolite formed in-vivo.

Table 3
Rat pharmacokinetic parameters at 5 mg/kg iv for compound **5**

Vss (l/kg)	Clp (l/h/kg)	$t_{1/2}$ (h)
5.3	4.1	0.9

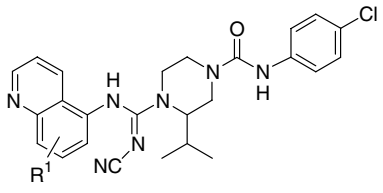
Table 4



	R^1	R^2	R^3	$rP2X_7IC_{50}$[a] (μM)	$hP2X_7IC_{50}$[a] (μM)	Whole Blood[a] (μM)
5	*o*-Tolyl	3-Ph	*p*-Cl	0.095	0.132	1.19
12	*o*-Tolyl	2-*i*Pr	*p*-Cl	0.125	0.06	NT
13	*o*-Tolyl	3-*i*Pr	*p*-Cl	0.11	0.112	0.98
14	5-Quinolinyl	2-*i*Pr	*p*-Cl	0.057	0.04	0.121
15	5-Quinolinyl	3-*i*Pr	*p*-Cl	0.093	0.067	0.88
16	4-Indolyl	2-*i*Pr	*p*-Cl	0.11	0.045	0.47
17	4-Indolyl	3-Ph	*p*-Cl	0.056	0.13	12.2
18	4-Indazolyl	2-*i*Pr	*m*-F	NT	0.25	1.59
19	4-Benzofuranyl	2-*i*Pr	*m*-F	NT	24.9	NT
20	2-(CH_2NMe_2)Ph	3-Ph	*p*-Cl	1.07	0.48	NT
21	1-*N*-Methyl-4-benzimidazoyl	3-Ph	*p*-Cl	5.19	1.66	NT
22	3-Pyridyl	3-Ph	*p*-Cl	2.04	1.33	NT
23	5-Tetrahydro quinolinyl	2-*i*Pr	*m*-F	NT	0.06	0.46
24	5-Tetrahydro *iso*-quinolinyl	2-*i*Pr	*p*-Cl	0.18	0.11	0.21

[a]Values are means of 2–3 experiments. NT, not tested. Compounds tested at the recombinant human receptors as described.[9]

Table 5



	R^1	Whole blood[a] (μM)	Microsomal stability[b] (% parent remaining)
14	H	0.121	43
25	2-Cl	0.87	NT
26	2-F	2.64	60
27	2-Me	0.12	74
28	8-Me	3.6	64

NT, not tested.
[a] Values are means of 2–3 experiments.
[b] Rat microsomes after 1 h incubation.

This route of metabolism prompted an effort to block this pathway as shown in Table 5. Incorporation of flanking groups at either the 2- or 8-position of the quinoline provided compounds with an increased stability in microsomal preparations. All but one of these compounds suffered from a decrease in potency in human whole blood (4-30-fold) compared to quinoline **14**. In contrast to its 8-methyl counterpart **28**, the regioisomeric 2-methyl analog **27** maintained potency in this assay and was also equally potent for both the rat $P2X_7$ (0.151 μM) and the human $P2X_7$ (0.093 μM). It is also worthwhile noting that both enantiomers of the 2-methyl quinoline **27** were profiled in both rat and human $P2X_7$ receptor assays and were indistinguishable from the racemate (data not shown). In accord with its increased microsomal stability, quinoline **27** exhibited improved intravenous pharmacokinetic parameters to compound **14** (Table 6), with reduced clearance and a half-life in excess of 4 h in rats. Unfortunately compound **27** had a low oral bioavailability of 5% in rats, a finding that is likely due to solubility limited absorption as it exhibited a low aqueous solubility of 19 μg/mL. This is further supported by the moderate clearance value that reduces the likelihood of a major first-pass effect leading to low systemic concentrations. Compound **27** was 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 6
Rat pharmacokinetic parameters at 5 mg/kg iv for compound **27**

Vss (l/kg)	Clp (l/h/kg)	$t_{1/2}$ (h)
11.8	1.4	4.4

In summary, a novel series of cyanoguanidine-piperazines were discovered with potent activity at $P2X_7$ and in a human whole blood assay measuring the inhibition of the pro-inflammatory cytokine IL-1β. It was found from these studies that, with suitable structural modifications, compounds with increased whole blood potency and moderate in-vivo clearance could be identified. Most analogs displayed approximately 3-5-fold greater potency for $hP2X_7$ over $rP2X_7$; however, compound **27** was highly potent at both species. These findings indicate that substantial flexibility around the pharmacophore of **1** and **27** exists to incorporate structural changes with retention of potent $P2X_7$ antagonism. Additional studies describing structural modifications around **27** to improve the solubility and oral bioavailability in this series will be the subject of future reports.

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