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Letter

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Synthesis and Pharmacological Characterization of two Novel, Brain Penetrating $P2X_7$ Antagonists

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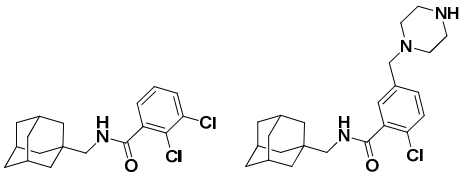
KEYWORDS $P2X_7$, neuro-inflammation, depression.

ABSTRACT: The synthesis and preclinical characterization of two novel, brain penetrating $P2X_7$ compounds will be described. Both compounds are shown to be high potency $P2X_7$ antagonists in human, rat and mouse cell lines and both were shown to have high brain concentrations and robust receptor occupancy in rat. Compound **7** is of particular interest as a probe compound for the pre-clinical assessment of $P2X_7$ blockade in animal models of neuroinflammation.

The $P2X_7$ ion channel is a member of a large purinoreceptor family that includes both P2X ionotropic and P2Y metabotropic receptors.[1] There are seven known P2X receptor subtypes, and of that group, $P2X_7$ has been shown to be involved in the release pro-inflammatory cytokines, including IL-1β.[2] As such, numerous reports have appeared in the literature describing the role of $P2X_7$ in a variety of pro-inflammatory disease states including pain, osteoarthritis, rheumatoid arthritis and pathology associated with neuro-inflammation such as in epilepsy, multiple sclerosis and a variety of neurodegenerative states including Alzheimer's disease.[3] Given that the $P2X_7$ receptor is expressed in the CNS on astrocytes and microglial cells, and that the expression and activation of $P2X_7$ in glial cells may regulate glutamate and IL-1β release, our interest in this target has focused on the role of $P2X_7$ in neuro-immune modulation. To that end, it has been shown by various groups that the pro-inflammatory cytokine IL-1β is involved in chronic stress models of affective disorders.[4-7] Furthermore, $P2X_7$ antagonism has been reported to be efficacious in animal models of mood disorders and mouse/human genetics study link $P2X_7$ locus to mood disorders.[8,9] We were particularly interested in recent reports showing evidence that IL-1 receptor blockade may be an effective approach for the treatment of depression, suggesting that a brain-penetrating $P2X_7$ antagonist that blocked IL-1β release in glial cells might be efficacious in mood disorders.[10]

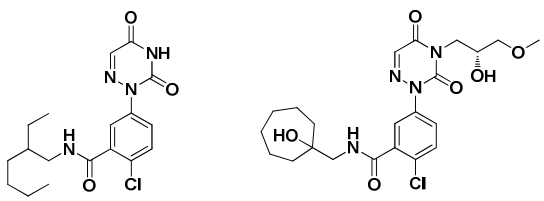
A wide variety of $P2X_7$ antagonists have been disclosed over the past several years[11] including numerous interesting benzamides. Some early examples of drug-like $P2X_7$ antagonists disclosed by the Astra-Zeneca group are the adamantyl-based compounds **1**[12] and **2**[13] shown below. Both are potent $P2X_7$ antagonists as demonstrated by inhibition of BzATP induced IL1-β release in human peripheral blood monocytes (pA_2's=8.8 and 8.0).



1, h $P2X_7$ pA_2=8.8

2, h $P2X_7$ pA_2=8.0
r $P2X_7$ pA_2=6.4

Pfizer also reported on a series of 2-chlorobenzamides discovered via high throughput screening. Compound **3** was reported to be a relatively weak screening hit ($P2X_7$ Yo-Pro IC_{50}=1.1μM), however medicinal chemistry efforts lead to the identification of an analog (CE-224,535), a very potent $P2X_7$ antagonist that became a clinical candidate.[14] Pfizer recently reported clinical results with CE-224,535 in rheumatoid arthritis patients inadequately controlled by methotrexate.[15]



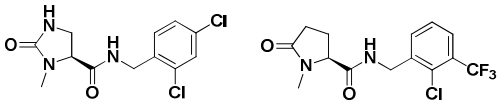
3, h $P2X_7$ Yo-Pro IC_{50}=1.1μM
h IL-1β IC_{50}=0.8μM

4, CE-224,535
h IL-1β IC_{50}=1.4 nM

While the compound did not show efficacy in a three month rheumatoid arthritis trial it is of note that Pfizer reported that the compound exposure exceeded the amount required for sustained inhibition of $P2X_7$ (as measured by IL-1β inhibition) for the entire three month period. This data indicates that $P2X_7$ inhibition is un-

likely to be a viable approach to the treatment of rheumatoid arthritis; however it also indicates that sustained inhibition of $P2X_7$ in humans is not likely to be plagued with mechanism-based adverse events.

Examples of other interesting small, drug-like $P2X_7$ antagonists include the imidazolidinecarboxamides and pyroglutamates recently reported by Glaxo SmithKline.[16,17] While these compounds are potent human $P2X_7$ antagonists, they also have some activity at the rat $P2X_7$ receptor and therefore could be used for pre-clinical efficacy studies. The authors report that brain penetrating $P2X_7$ antagonists such as those shown below are efficacious in pre-clinical pain models.

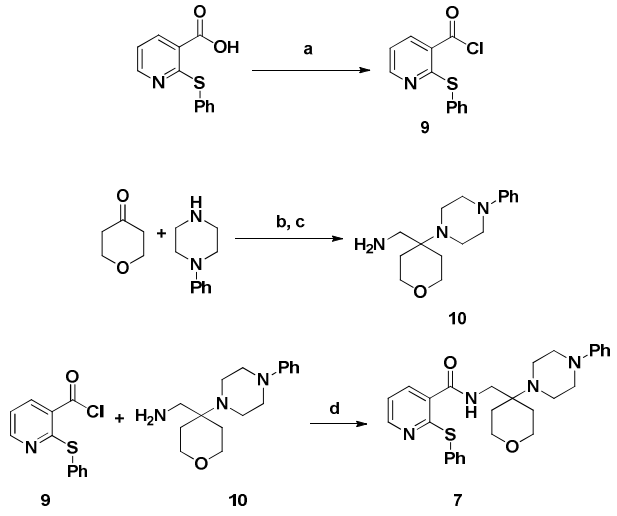


5, h $P2X_7$ pIC_{50}=8.0
r $P2X_7$ pIC_{50}=6.4

6, h $P2X_7$ pIC_{50}=8.5
r $P2X_7$ pIC_{50}=6.5

Our interest in $P2X_7$ antagonists focused on the identification of potent, brain penetrating compounds with high affinity for both the rat and human $P2X_7$ in order to assess their utility for the treatment of CNS disorders, including depression. Towards this end, we now report the discovery of N-((4-(4-phenyl-piperazin-1-yl)tetrahydro-2H-pyran-4-yl)methyl)-2-(phenyl-thio) nicotinamide (**7**) and 2-methyl-N-((1-(4-phenylpiperazin-1-yl)cyclohexyl)methyl)-1,2,3,4-tetrahydroisoquinoline-5-carbox-amide (**8**). These compounds were identified after extensive medicinal chemistry efforts following an HTS campaign that showed that simple *N*-(cyclohexylmethyl)benzamides were weak $P2X_7$ antagonists. The compounds were synthesized as shown in Schemes 1 and 2. For compound **7**, 2-(phenylthio)nicotinic acid was first converted to the acid chloride **9**. Next, dihydro-2H-pyran-4(3H)-one was condensed with 1-phenylpiperazine and potassium cyanide then reduced to form the amine **10**. Condensing **9** and**10** then provided compound **7** in reasonable overall yield.

Scheme 1. Synthesis of Compound 7[a]

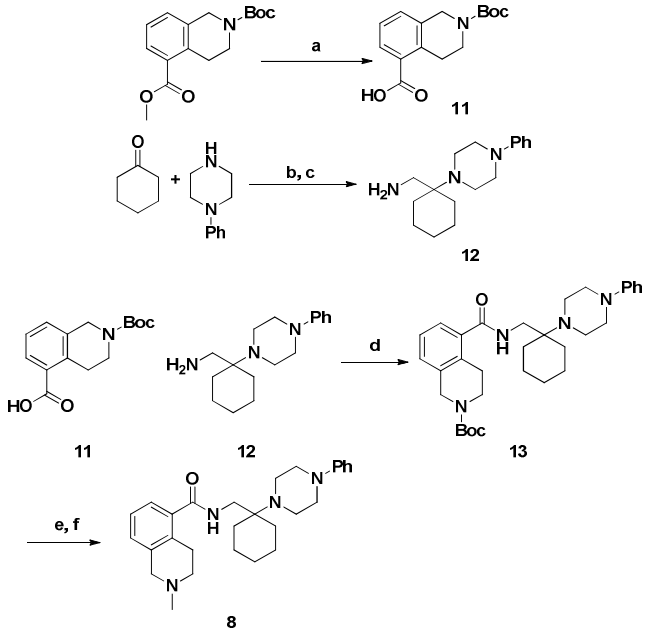


[a]Reagents and conditions: (a) oxalyl chloride, dichloromethane, dimethylformamide, 100%, (b) KCN, H_2O, pH=3, (c) lithium aluminum hydride, tetrahydrofuran, 75%, (d) dichloromethane, 53%.

Similarly, compound **8** was prepared from 2-*tert*-butyl 5-methyl 3,4-dihydroisoquinoline-2,5(1H)-dicarboxylate and the amine **12**, which was formed by reaction of cyclohexanone with 1-phenylpiperazine as shown in Scheme 2.

In vitro data for compounds **7** and **8** are detailed on Table 1. Both compounds are potent $P2X_7$ antagonists in human and rodent cell lines and both compounds show inhibition of Bz-ATP induced IL-1β secretion in human peripheral blood monocytes and in human whole blood. Because both compounds have good functional activity and binding affinity in rat, we chose to further characterize these compounds in vivo with the intention of using one or both of these as tool compounds for pharmacodynamic studies.

Scheme 2. Synthesis of Compound 8[a]



[a]Reagents and conditions: (a) methanol, H_2O, NaOH, 100%, (b) KCN, H_2O, pH=3, (c) lithium aluminum hydride, tetrahydrofuran, 75%, (d) BOP, triethyl amine, dichloromethane, 77%, (e) Trifluoroacetic acid, (f) formaldehyde, sodium triacetoxyborohydride, 74%.

During the course of this characterization we discovered that compound **8** is a high affinity SERT inhibitor, both in human and rat (Table 1). Both compounds were also screened in a commercial panel of 50 receptor, ion channel and transporter assays (CEREP, www.cerep.com) at 1 μM. Compound **7** had a <50% effect at all targets tested, whereas compound **8** had >50% inhibition on human NK2 (68% inhib.), human DAT (93% inhib.) and r NaCH (54% inhib.) IC_{50}'s were generated for DAT (290 nM) and r NaCH (910 nM). Recognizing that the SERT affinity observed with **8** may be an issue when attempting to assess the antidepressant effects of our $P2X_7$ inhibitors, most of our subsequent efforts were focused on compound **7**, however we continued to profile both lead molecules in order to assess their ability to distribute into the brain.

Prior to any *in vivo* work, we obtained preliminary DMPK and developability measurements for each compound. The data are shown in Table 2. Both compounds

are relatively drug-like with reasonable physical properties, solubility, protein binding, and permeability, however both suffer from very high extraction ratios in human and rat liver microsomes indicating that they are very unlikely to be suitable for oral delivery. Because sub-cutaneous dosing is preferred for many *in vivo* models, we decided to assess the plasma and brain exposures following sub-cutaneous dosing.

Table 1. In Vitro Pharmacology for Compounds 7 and 8.

	human FLIPR pIC_{50}[b]	rat FLIPR pIC_{50}[c]	mouse FLIPR pIC_{50}[d]	human pK_i	rat pK_i	rat/human SERT[e] pK_i (nM)	human PBMC pIC_{50}[a]	human WB pIC_{50}[f]
7	8.3±0.08	7.2±0.08	7.5±0.1	7.9±0.08	8.7±0.08	5.5/n.d.	7.6±0.07	6.7±0.07
8	7.7±0.07	7.8±0.1	7.1±0.2	7.9±0.08	9.1±0.07	7.7 (n=2)/7.8	7.4±0.07	6.7±0.09

[a]human peripheral blood monocyte pIC_{50} for IL-1b inhibition , [b]human FLIPR pIC_{50} measured in a Ca^{2+} flux assay, [c]rat FLIPR pIC_{50} measured in a Ca^{2+} flux assay, [d]mouse FLIPR pIC_{50} measured in a Ca^{2+} flux assay, [e]rat and human SERT K_i, [f]human whole blood pIC_{50}, all data are the result of at least three assays run in triplicate ± SEM.

Table 2. Physical Properties and In Vitro DMPK Parameters for Compounds 7 and 8.

	MW	c log P	human ER[a]	rat ER[a]	mouse ER[a]	human/rat ppb[b]	Caco-2 A to B/B to A[c]	brain pb[d]	Solubility pH2/pH7[e]
7	488.7	4.3	>0.95	>0.92	>0.93	97.8/97.1	--	98.3	400/31
8	446.6	5.1	0.90	0.86	0.90	83.5/95.6	11/2.1	97.6	95/90

[a]human, rat or mouse extraction ratio as measured in a microsomal preparation, [b]human or rat protein binding reported as % bound, [c]P_{app} reported in units of cm/sec x 10^{-6} (data generated at CEREP, www.cerep.com), [d]rat brain protein binding reported as % bound, [e]Reported in μM.

Figure 1. *Ex Vivo* $P2X_7$ Receptor Occupancy, Plasma and Brain Levels with Compound 7 (30 mg/kg) in Rat Brain: Time Dependency after Subcutaneous Administration (n = 3 per time point ± SEM).

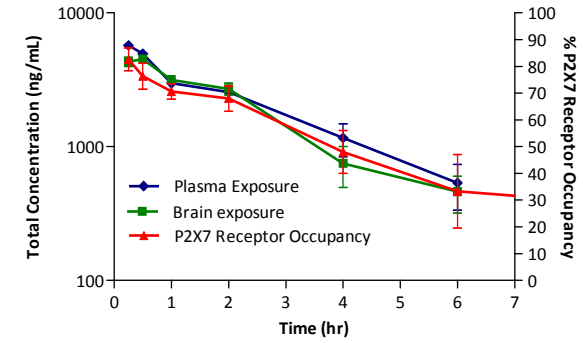
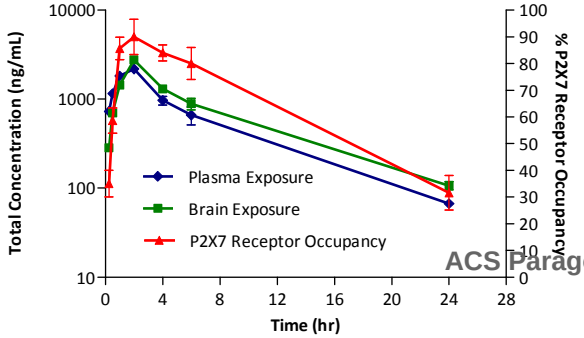


Figure 2. *Ex Vivo* $P2X_7$ Receptor Occupancy, Plasma and Brain Levels with Compound 8 (10 mg/kg) in Rat Brain: Time Dependency after Subcutaneous Administration (n = 3 per time point ± SEM).



Initial experiments are shown in Figures 1 and 2. In these experiments, the compound was dosed subcutaneously (s.c.) and plasma and brain concentrations were measured over time, along with central $P2X_7$ receptor occupancy as assessed by *ex vivo* autoradiography. The results for compound **7** are shown in Figure 1. High $P2X_7$ receptor occupancy was achieved following a single dose of 30 mg/kg s.c. and greater than >50% occupancy remained for 2 hours at this dose, indicating robust target engagement. Similar results were seen with 10 mg/kg s.c. of compound **8** (Figure 2).

The dose dependency of central receptor occupancy was assessed in a second series of experiments (Figures 3 and 4). Central occupancy was measured at the t_{max} for each compound (15 min for Compound 7 and 120 min for Compound 8; data not shown). Compound **7** had an ED_{50} for occupancy of 2.5 mg/kg whereas compound **8** had a nearly 10-fold lower ED_{50} of 0.3 mg/kg. In separate *ex -vivo* autoradiography studies compound **8** was

determined to have ED_{50} for SERT occupancy of 24 mg/kg, indicating that SERT target engagement in rat was not significant at doses where robust $P2X_7$ receptor occupancy was observed.

Figure 3. *Ex Vivo* $P2X_7$ Receptor Occupancy with Compound 7 in Rat Brain: Dose Dependency Following Subcutaneous Administration (n = 3 per dose ± SEM). $P2X_7$ occupancy was measured 15 min after drug administration.

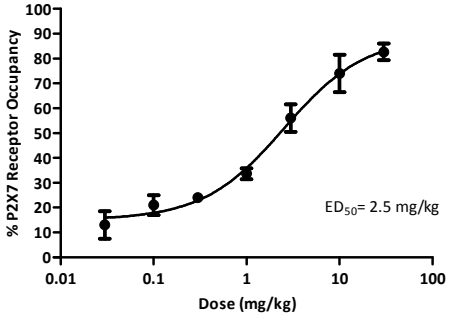
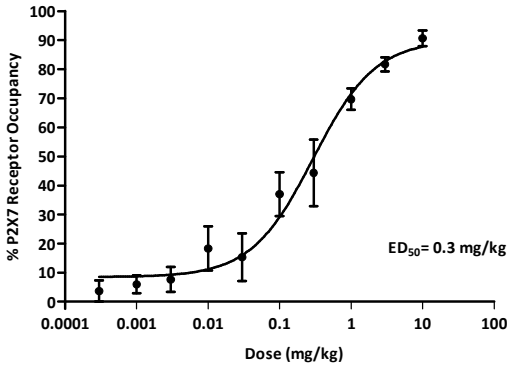


Figure 4. *Ex Vivo* $P2X_7$ Receptor Occupancy with Compound 8 in Rat Brain: Dose Dependency Following Subcutaneous Administration (n = 3 per dose ± SEM). $P2X_7$ occupancy was measured 120 min after drug administration.



In conclusion, we have demonstrated that compounds **7** and **8** are high affinity rat, mouse and human $P2X_7$ receptor antagonists and that both compounds have DMPK properties suitable for pre-clinical pharmacodynamics studies. At appropriate doses, both compounds were shown to occupy central $P2X_7$ receptors in vivo, as assessed by ex-vivo autoradiography studies. Future reports on the pharmacology of these interesting $P2X_7$ compounds will focus on the characterization of compound **7** in a variety of pre-clinical models of depression and related mood disorders.

ASSOCIATED CONTENT

Supporting Information. Supporting information includes detailed synthetic procedures for all intermediates and products and descriptions of assays used to characterize compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ABBREVIATIONS

IL-1β interleukin-1β, Bz-ATP benzoyl adenosine triphosphate, SERT serotonin transporter, DAT dopamine transporter.

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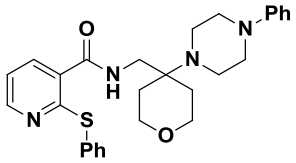
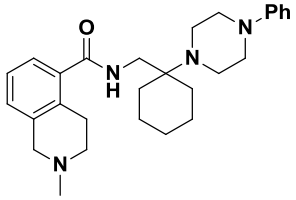


7

$P2X_7$ human pK_i=7.9
$P2X_7$ rat pK_i=7.9
$P2X_7$ human whole blood pK_i=6.9



8

$P2X_7$ human pK_i=7.9
$P2X_7$ rat pK_i=9.1
$P2X_7$ human whole blood pK_i=6.7