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Discovery and optimization of RO-85, a novel drug-like, potent, and selective $P2X_3$ receptor antagonist

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ABSTRACT

Despite the extensive literature describing the role of the ATP-gated $P2X_3$ receptors in a variety of physiological processes the potential of antagonists as therapeutic agents has been limited by the lack of drug-like selective molecules. In this paper we report the discovery and optimization of RO-85, a novel drug-like, potent and selective $P2X_3$ antagonist. High-throughput screening of the Roche compound collection identified a small hit series of heterocyclic amides from a large parallel synthesis library. Rapid optimization, facilitated by high-throughput synthesis, focusing on increasing potency and improving drug-likeness resulted in the discovery of RO-85.

P2X receptors are ligand gated ion channels activated by adenosine 5′-triphosphate (ATP) and related di- and tri-phosphate nucleotides. Seven P2X receptor subunits have been identified $P2X_{1-7}$ and each channel shown to be assembled from three subunits.[1] Understanding the pharmacology of the channels is complex in that, with the exception of $P2X_6$, in addition to homomeric channels, each subunit also has the ability to form heteromeric channels and a total of seven heteromeric P2X family members have been identified.[2] Homomeric $P2X_3$, and the closely related heteromultimeric $P2X_{2/3}$, receptors are predominantly co-localized on small to medium diameter sensory afferent neurons and have become increasingly recognized as playing a major role in mediating the primary sensory effects of ATP. $P2X_3$-KO mice demonstrate a reduced sensitivity to thermal stimuli and decreased pain behaviors.[3] Furthermore reduction of $P2X_3$ expression through intrathecal administration of $P2X_3$-selective antisense,[4] and more recently siRNA,[5] also causes a significant decrease in pain behaviors in mice. By eliminating the $P2X_3$ subunit these gene deletion and knockdown techniques also eliminate the $P2X_{2/3}$ receptor so a clear understanding of the receptor responsible for these effects is hard to ascertain.

Despite significant advances in elucidating the pharmacology of $P2X_3$ receptors, the field has suffered from a lack of drug-like, potent and selective ligands. Many of the reported selective $P2X_3$ antagonists (Fig. 1), including the most recently reported highly potent peptide antagonist Spinorphin (**1**),[6] either do not report selectivity over $P2X_{2/3}$ or are dual $P2X_3$/$P2X_{2/3}$ antagonists like TNP-ATP (**2**)[7] and A-317491 (**3**),[8] as well as our recently published diaminopyrimidines, (**4a**) and (**4b**).[9,10]

In addition to the unknown, or lack of selectivity over $P2X_{2/3}$ making their pharmacology hard to understand, these ligands also show poor drug-like properties. TNP-ATP and A-317491, similar to many of the non-selective $P2X_3$ receptor antagonists, contain polyacidic functional groups that limit their bioavailability and overall potential as drug candidates. Despite this A-317491 has been shown to be active in a number of in vivo models of chronic inflammatory pain, neuropathic pain[8] and overactive bladder.[11] No data has been published on the drug-like properties of spinorphin which has also been shown to be effective in the tail pinch assay following intracerebroventricular (ICV) administration.[12] In this Letter we report the discovery and optimization of a series of drug-like potent and selective $P2X_3$ antagonists.

A high-throughput screening campaign of the Roche compound collection using the rat recombinant $P2X_3$ receptor was performed. Disappointingly, in addition to a very overall low hit rate of 0.01%, many of the more potent sub-micromolar hits contained similar

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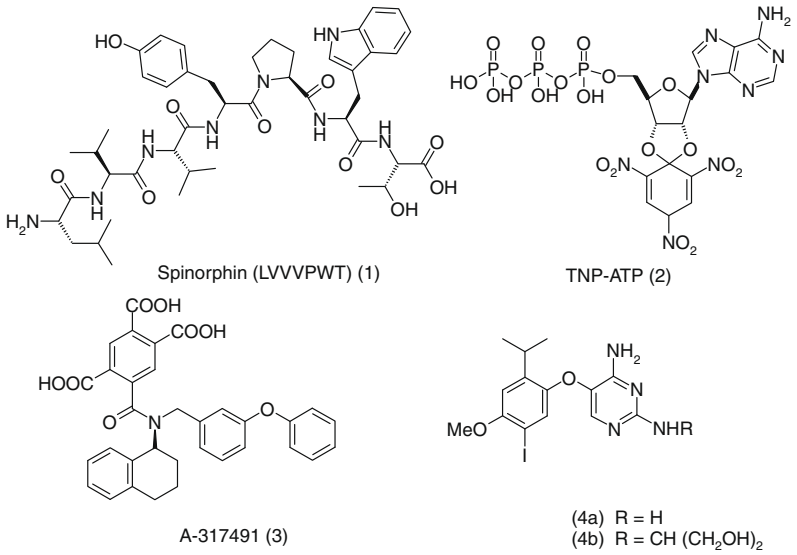


Figure 1. Structures of known $P2X_3$ antagonists.

poly-acidic functionality to the known antagonists with their implied poor drug-like properties. These were not deemed suitable starting points for a medicinal chemistry program aimed at producing potential therapeutic agents so a careful analysis of the less potent hits was undertaken. Four compounds, initially synthesized as part of a ~4500 member parallel synthesis library, were identified as hits. The library was based on a 2,4-disubstituted-oxazole-5-carboxylic acid amide core structure. The hits were characterized by a 2-phenyl substituent on the oxazole core and, more specifically, the presence of a 1-methyl-2-(4-pyrimidin-2-yl-piperazin-1-yl)-ethylamine amide (Fig. 2).

A detailed pharmacological and drug-likeness characterization of (**5a**) was undertaken. (**5a**) was shown to be a selective antagonist of $rP2X_3$ (pIC_{50} 6.6) with no antagonist activity at $P2X_{2/3}$ or other family members tested (pIC_{50} <5). In a standard CEREP profile of 80 other enzymes, receptors and ion channels only five other activities were identified in the 5–10 μM range.[13] In an assessment of drug-likeness; with positive attributes of moderate molecular weight (434) and calculated log *P* (2.7), and high permeability without efflux it was disappointing to find high in vitro clearance in rat and human microsomes and hepatocytes, and >99% protein binding in rat plasma. In addition (**5a**) was a moderate inhibitor of the CYP450 isozymes 3A4 and 2D6 with IC_{50}'s <10 μM. A parallel optimization approach exploring replacement of the oxazole core and its substituents, and the amide portion of the molecule was undertaken.

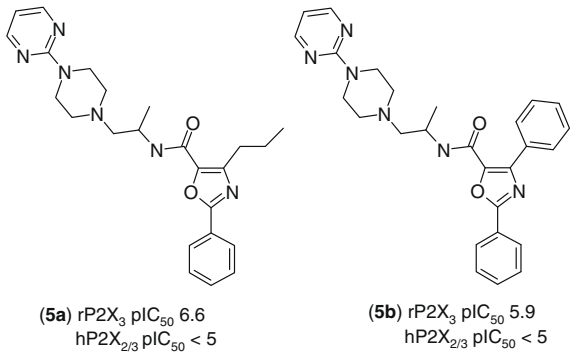


Figure 2. Hits structures identified by high-throughput screening and their pIC_{50}'s at the rat $P2X_3$ receptor.

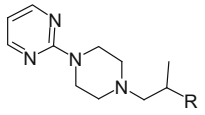
The role of the oxazole scaffold was investigated using both a general collection of acids fitting a similar motif and by a traditional iterative SAR approach. A representative set of similar amides is shown in Table 1. For five-membered scaffolds, pyrazole amides (**6a**) and (**6b**) and furans (**6c**) and (**6d**) had similar activity to one of the other initial hits (**5b**). Substitutions that altered the geometry of the amide bond, (**6g**) and (**6h**) were not tolerated. Substitution of the butyl or phenyl group for a methyl gave a significant drop in activity (**6i**) versus (**5a**) and (**5b**). Interestingly, substitution of the phenyl groups in a 3,4 relationship as in (**6j**) and (**6f**) led to no loss in activity.

A six-membered ring scaffold was also accepted, as shown in examples (**8a**–**8f**), even with only one aryl substituent. An interesting 5-5-bicylic core, 1-methyl-3-phenyl-1*H*-thieno[2,3-*c*] pyrazole-5-carboxylic amide (**7**), discovered using parallel synthesis was found to show promising opportunities by allowing truncation of the propyl group to a methyl group.

Exploration of the piperazinyl-ethylamine linker of the molecule showed little opportunity for modification (see Fig. 3) with the exception of a preference for the R-stereochemistry (Table 2). The pyrimidine group could, however be modified with other heterocycles, without losing activity at the $P2X_3$ receptor (**9a**–**9f**) as long as a nitrogen was present in the 2-position to act as an elector acceptor (see Table 2). The *m*-pyridine and *p*-pyridine were inactive (**9g** and **9h**). Interestingly, a simple carbonyl group was shown to be sufficient for good affinity as long as the group remained planar. For example, acetyl amide (**10a**), thioacetyl amide (**10c**) and urea (**10d**) were all active, whereas the cyclopropylcarbonylamide (**10f**) and other amides not shown here, the substituted urea (**10e**), sulfonamide (**10g**) and ethyl carbamate (**10h**) were inactive. Of particular significance was the promising activity of (**10a**) with a concurrent improvement in molecular weight and lipophilicity. These analogs were synthesized easily starting from Boc-D-alaninal as shown in Scheme 1.

Reaction of Boc-D-alaninal with *N*-acetylpiperazine in the presence of $NaBH(OAc)_3$ afforded intermediate (**15**) in 85% yield. Removal of the Boc group with TFA, followed by coupling with commercially-available 1-methyl-3-phenyl-1*H*-thieno[2,3-*c*]pyrazole-5-carboxylic acid using a HBTU coupling afforded the amide, which was converted the oxalate salt (**16**).[14] Similar reductive amination conditions, followed by deprotection were used to prepare *N*-arylpiperidines (**14a**–**14h**), which were coupled to 2-(4-fluorophenyl)-4-propyl-oxazole-5-carboxylic acid[15] using DCC and HOBT

Table 1
Heterocyclic core SAR



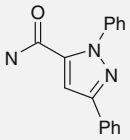
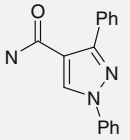
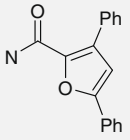
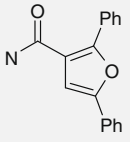
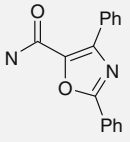
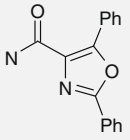
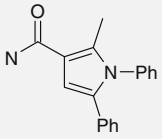
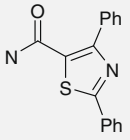
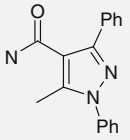
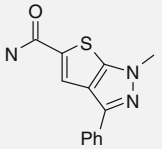
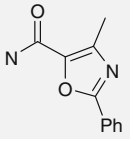
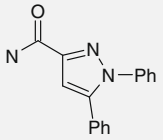
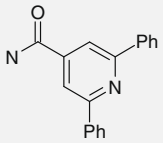
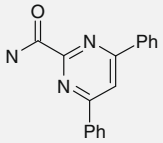
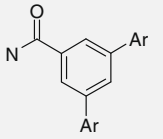
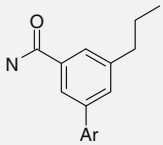
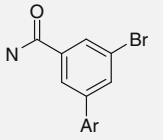
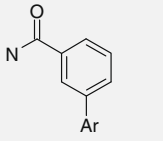
No.	R	pIC_{50}[a,b,c]
6a		6.0
6b		6.0
6c		6.2
6d		5.7
5b		5.9
6e		5.4
6f		5.7
6g		<5
6h		<5
7		6.7
6i		5.3

Table 1 (*continued*)

No.	R	pIC_{50}[a,b,c]
6j		5.9
8a		5.9
8b		5.3
8c		6.4
8d		6.3
8e		6.3
8f		6.9

Ar = *p*-F-phenyl
[a] FLIPR: mean pIC_{50}, $rP2X_3$ CHO cell.
[b] FLIPR: mean pIC_{50} <5.0, $hP2X_{2/3}$ 1321n1c cells.
[c] pIC_{50} values are the mean of at least three experiments performed in triplicates, standard deviation +20%.

to afford the corresponding amides (**9a**–**9h**). Intermediate (**17**), prepared by reductive amination of Boc-D-alaninal with Cbz-piperazine, was coupled the same acid to provide intermediate (**18**). Removal of the Cbz group by catalytic hydrogenation afforded the free piperazine, which was functionalized to afford (**10a**–**10f**) using standard conditions.

The thienopyrazole template, discovered with analog (**7**) and which had the advantage of having good drug-likeness and for not requiring a longer alkyl chain with the potential metabolic liabilities, was explored further. The results are shown in Table 3. Substitution of the phenyl ring in the 3-position with an *ortho* fluorine also led to active analog (**11a**), however an *ortho*-methyl substituent was less tolerated (**11b**). Increasing the size of the N-1 substituent to an ethyl slightly decreased activity (**11c**) and introduction of a phenyl group led to an inactive analog (**11d**). Changing the sulfur to an oxygen to give (**12**) led to an inactive molecule.

Combining the substitution of an acetyl for a pyrimidyl group and the thienopyrazole core template led to potent molecules (**11f**–**11h**, Table 3). Resolution of the amide side chain resulted in the identification of (*R*) as the preferred stereochemistry; thus 1-methyl-3-phenyl-1*H*-thieno[2,3-*c*]pyrazole-5-carboxylic acid [(*R*)-

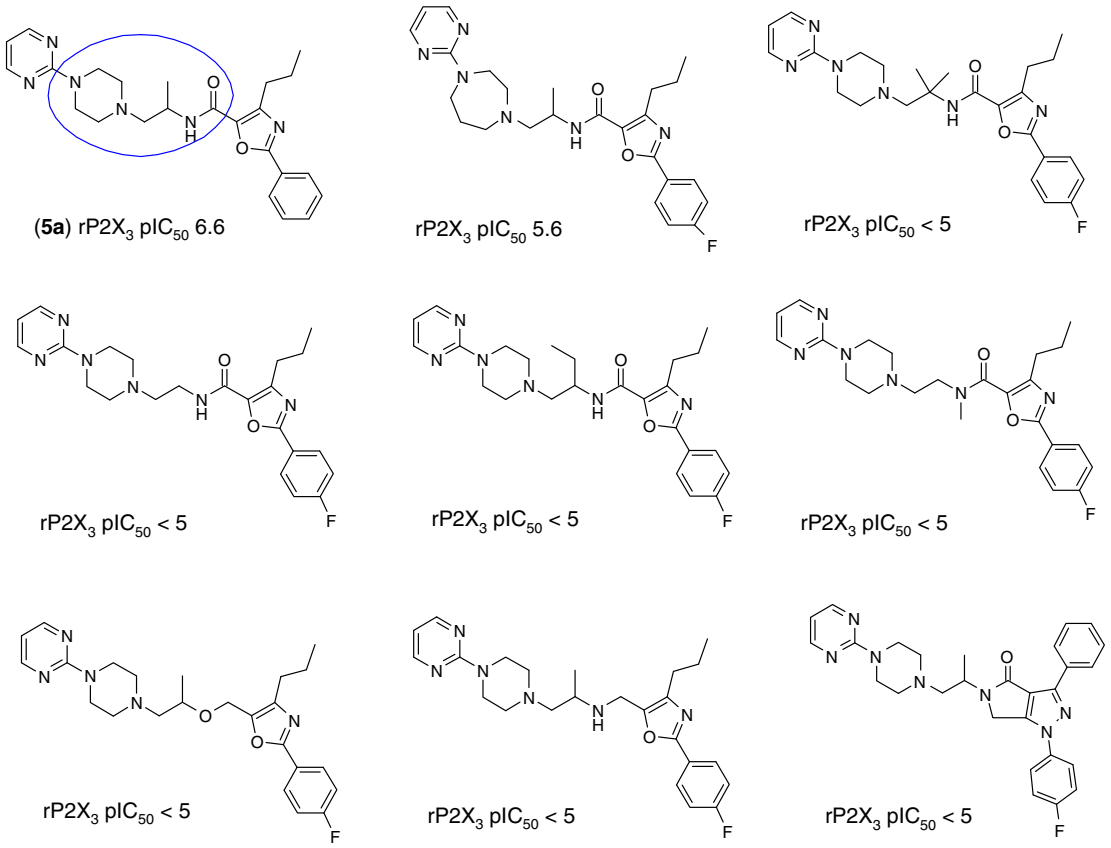
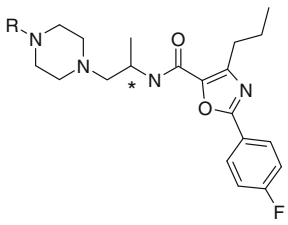


Figure 3. Linker modifications and their pIC_{50}'s at the rat $P2X_3$ receptor.

Table 2
Acceptor group SAR

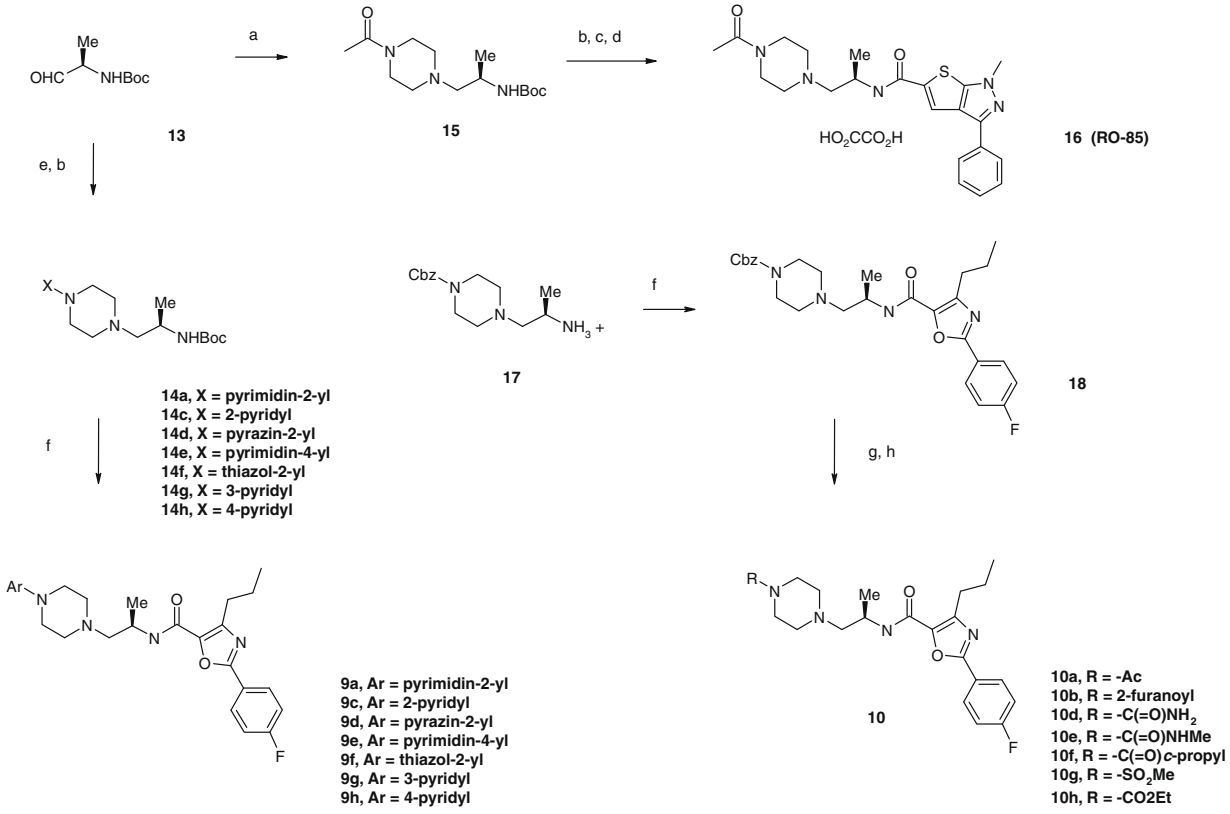


No.	R	Stereo-chem.	pIC_{50}[a,b,c]
9a		R	7.3
9b		S	5.7
9c		R	5.8
9d		R	6.3
9e		R	5.9
9f		R,S	6.2

Table 2 (*continued*)

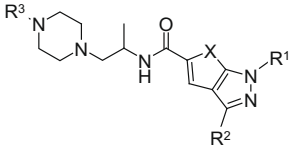
No.	R	Stereo-chem.	pIC_{50}[a,b,c]
9g		R,S	<5
9h		R	<5
10a	Ac	R	6.4
10b		R	5.6
10c		R,S	7.0
10d		R	6.0
10e		R	<5
10f		R	<5
10g	$-SO_2Me$	R	<5
10h	$-CO_2Et$	R	<5

[a] FLIPR: mean pIC_{50}, $rP2X_3$ CHO cell.
[b] FLIPR: mean pIC_{50} <5.0, $hP2X_{2/3}$ 1321n1c cells.
[c] pIC_{50} values are the mean of at least three experiments performed in triplicates, standard deviation +20%.



Scheme 1. Reagents and conditions: (a) *N*-acetylpiperidine, 1.2 equiv, DCE, $NaHB(OAc)_3$, 2 equiv, 20 h ; (b) TFA, CH_2Cl_2 2 h; (c) HBTU, 1.5 equiv, excess DIEA, THF; (d) oxalic acid, 1.1 equiv, ether; (e) CH_2Cl_2; *N*-arylpiperazines (2-piperazin-1-ylpyrimidine, 1-pyridin-2-ylpiperazine, pyrazin-2-ylpiperazine, 4-piperazin-1-ylpyrimidine, 1-thiazol-2-yl-piperazine, 1-pyridin-3-yl-piperazine, or 1-pyridin-4-yl-piperazine), 1.2 equiv, DCE, $NaBH(OAc)_3$, 2 equiv; (f) (i) 2-(4-fluorophenyl)-4-propyl-oxazole- 5-carboxylic acid, DCC on resin, HOBT, CH_2Cl_2, 2 h, (ii) amine (**14a**–**14h**, **17**), CH_2Cl_2, DIEA 20 h; (g) Pd/C (10% Degussa), H_2, EtOH; (h) for **10b** 2-furoylchloride, CH_2Cl_2, DIEA; for **10d** KOCN, AcOH, H_2O; for **10e** CH_3NCO, CH_2Cl_2; for **10f** c-C_3H_5COCl, CH_2Cl_2, DIEA, for **10g** CH_3SO_2Cl, CH_2Cl_2, DIEA, for **10h** $ClCO_2Et$, CH_2Cl_2, DIEA.

Table 3
SAR of 1H-thieno[2,3-*c*]pyrazole-5-carboxamides



No.	X	R^1	R^2	R^3	pIC_{50}
7	S	Me	Ph	Pyrimidyl	6.7
11a	S	Me	*o*-F-Ph	Pyrimidyl	7.2
11b	S	Me	*o*-Me-Ph	Pyrimidyl	5.7
11c	S	Et	Ph	Pyrimidyl	6.3
11d	S	Ph	Ph	Pyrimidyl	<5
11e	S	Ph	Me	Pyrimidyl	5
11f	S	Me	Ph	Acetyl	7.0
11g	S	Me	*p*-Me-Ph	Acetyl	7.4
11h	S	Me	*o*-F-Ph	Acetyl	7.4
12	O	Me	Ph	Pyrimidyl	<5

2-(4-acetyl-piperazin-1-yl)-1-methyl-ethyl]-amide (**16**), identified as RO-85 was discovered.

RO-85 is a potent $P2X_3$ receptor antagonist at the rat and human receptors ($rP2X_3$ $pIC_{50} = 7.5$; $hP2X_3 = 6.4$) selective over $hP2X_{2/3}$ and the other P2X family members tested (pIC_{50} <5). No other pharmacological activity was seen in a standard CEREP panel of 80 other enzymes, receptors and ion channels (no activity >50% at 10 μM).[13] Favorable molecular properties comparable to the original hit structure were also retained (MW 425, c log *P* 2.8). RO-85 showed significantly higher in vitro metabolic stability[16] in rat and human liver microsomes with 89% protein binding in rat plasma. No inhibition of the CYP450 isozymes was seen at concentrations <15 μM. When dosed orally to rats RO-85 was 89% orally bioavailable with a half-life of 1.6 h.

In summary we have identified a novel, potent and selective $P2X_3$ receptor antagonist RO-85. This was discovered through the rapid optimization of a hit series identified by high-throughput screening facilitated by parallel synthesis. RO-85 is exquisitely selective for the $P2X_3$ receptor subtype over other P2X family members and other pharmacological targets and has suitable pharmacokinetic properties to explore the therapeutic potential of $P2X_3$ antagonists in vivo. Additional SAR studies will be published in the future.

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