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New P2X3 receptor antagonists. Part 1: Discovery and optimization of tricyclic compounds

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ABSTRACT

Purinergic P2X3 receptors are trimeric ligand-gated ion channels whose antagonism is an appealing yet challenging and not fully validated drug development idea. With the aim of identification of an orally active, potent human P2X3 receptor antagonist compound that can penetrate the central nervous system, the compound collection of Gedeon Richter was screened. A hit series of tricyclic compounds was subjected to a rapid, two-step optimization process focusing on increasing potency, improving metabolic stability and CNS penetrability. Attempts resulted in compound **65**, a potential tool compound for testing P2X3 inhibitory effects in vivo.

The theory of extracellular signaling via receptors that bind adenosine 5′-tri-phosphate (ATP) as a transmitter was proposed almost half a century ago by Burnstock.[1] Since that time not only has the existence of a membrane receptor family activated by ATP been certified, but the possible roles of the members of this family in various disease conditions have also been recognized and widely studied.[2] Purinergic receptors comprise two subfamilies, namely the P2Y subfamily belonging to the metabotropic G protein-coupled receptors, and the P2X subfamily, which contains receptors that are cation channels gated by extracellular ATP. These latter ionotropic receptors are expressed on both neuronal and non-neuronal cells, and mediate fast current responses upon ligand binding by permitting the passage of cations along their electrochemical gradients. In excitable cells this leads to a great influx of Na^+ and Ca^{2+} ions and resulting in a depolarization of the cell and triggering downstream Ca^{2+} signaling. Functional P2X receptors are homo- or heterotrimers formed by subunits with two transmembrane domains and intracellular N- and C-termini. Until now seven P2X receptor genes have been identified and cloned, encoding receptor subunits termed P2X1–7.

The idea of specific targeting of the P2X3 receptor has raised much pharmaceutical interest due to the restricted tissue distribution of the protein and the promise of therapeutic potential of its antagonism suggested by experimental studies ranging from preclinical observations to proof of concept studies in humans. Significant attention has been paid to the rational of P2X3 receptor inhibition in pain management due to the unmet medical need, which is still definite on this therapeutic field, and to the supportive findings of numerous preclinical studies.[3] However, the relatively poor translatability of rodent studies to humans poses unacceptably high risk to pursue compounds to clinic in indications associated with chronic pain. Fortunately, there are many other possible indications associated with airways hyperreactivity, overactive bladder or even cardiovascular problems in which pathological cellular mechanisms are ignited by P2X3 receptor activation, and the blockade of P2X3 receptors may be converted into efficacy and effectiveness in clinical settings.[4,5]

Channels containing the P2X3 subunits are either homo- (P2X3) or heterotrimers (P2X2/3), which may complicate in vitro assaying and, not negligibly, the evaluation of the role of P2X3 at the level of individual organisms. It is suggested however, that there is a clear difference in distribution of P2X2/3 receptors between rodents and primates due to the absence of functional P2X2/3 receptors in primate sensory neurons,[6] which justifies the search for pure P2X3 antagonists.

To date, a number of patents comprising P2X3-related compounds have been filed,[7] some of them molecules from Abbot (**1**), Roche (**2**, **3**) and Astra Zeneca (**4**). Nowadays among them only

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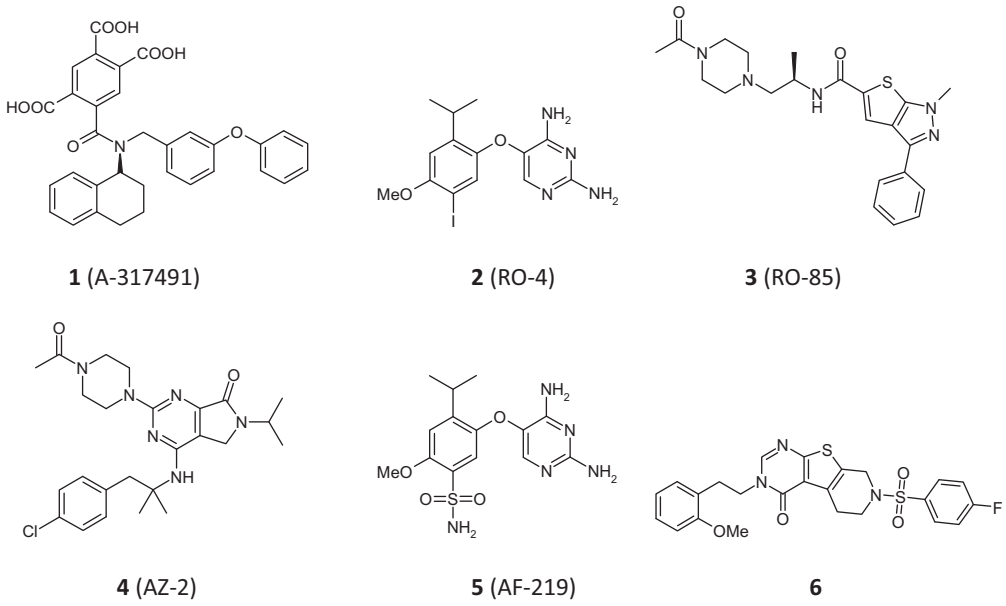
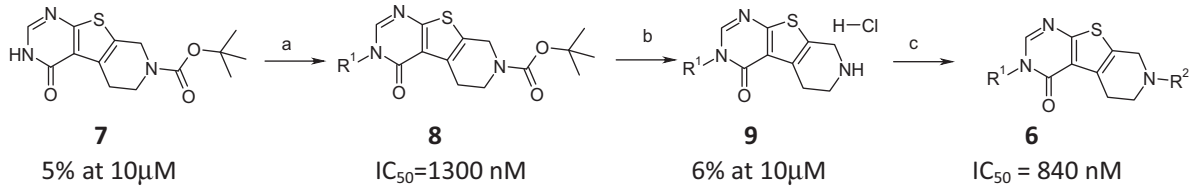


Figure 1. Structures of P2X3 receptor antagonists (**1**–**5**) and HTS hit (**6**).



Scheme 1. Synthesis of **6** with a general synthetic method (R^1 = 2-MeO-phenethyl, R^2 = 4-F-phenylsulfonyl). Reagents and conditions: (a) R^1–X (X = Br, Cl, OMs), Cs_2CO_3, DMF, 60 °C, 2–3 h; 30–60% yield (b) HCl/dioxane or TFA/DCM, rt, 2–24 h, 60–98% yield; (c) R^2–Y (Y = OH, Cl, Br), DIPEA, DCM, rt, 2–24 h, 33–90% yield.

AF-219 (**5**) is in Phase 2 clinical trial[8,9] to demonstrate improvements in refractory chronic cough[4] (Fig. 1).

Our goal was the identification of an orally available, CNS-penetrant, potent human P2X3 receptor antagonist compound. Gedeon Richter compound collection served as a starting point of an HTS campaign in which a fluorometric $[Ca^{2+}]_i$-assay using a cell line expressing the hP2X3 receptor in an inducible manner was deployed.[10]

On our ongoing studies, a HTS campaign[11] was performed and we identified **6** as the best hit (Fig. 1) with an inhibitory action on hP2X3 with an IC_{50} value of hardly less than 1 micromole (IC_{50}: 840 nM). The next step was to find and identify a more potent P2X3 antagonist molecule having lower molecular weight (M.W. < 500) and better solubility (≫1.7 μM). For the exploration of the chemical space around **6**, resynthesis was carried out in the following route: we started from known-tricycle **7**,[12] and after alkylation we obtained intermediate **8**. Removal of the *t*-butoxycarbonyl (BOC) protecting group yielded **9**, from which, by sulfonation, we successfully prepared target compound **6**. Surprisingly, intermediate **8** also showed significant P2X3 antagonist activity (IC_{50}: 1300 nM), which allowed us to perform a two-step SAR investigation. First, we examined the role of the left side of the molecule having BOC-group, then we moved on to explore the right side with various acylating agents (Scheme 1).

Starting with the left side of the molecule, we investigated the optimal distance between the tricycle and the *o*-metoxyphenyl group. In case of both longer (**8a**) and shorter (**8b**) chain, we observed a dramatic drop of activity. So, keeping the most effective two carbon atom length chain, we synthesized analogues having different groups on the far-end of the linker. In case of unsubstituted phenyl (**8c**) the activity dropped, while having pyridyl, pyrrole, piperidine or *i*-propyl (**8d**–**8g**) we observed the complete loss of activity.

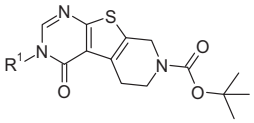
Introducing *o*-F or *o*-NO_2 substituents to the phenyl ring (**8h**, **8i**) the activity remained in the same few micromolar range as in case of *m*- and *p*-methoxy groups (**8j**, **8k**). However, introducing a second methoxy group had a great impact on the P2X3 antagonist activity; in case of 3,4- and 2,3-dimethoxy substitution (**8l**, **8m**) there were no big changes in the activity, but in contrast, 2,4- and 3,5-dimethoxy analogues (**8n**, **8o**) had sub-micromolar IC_{50} values. Conversely, 2,4,6-trimethoxy substitution (**8p**) resulted in drop of activity (Table 1).

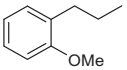
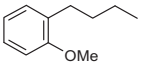
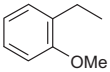
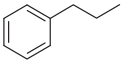
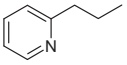
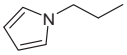
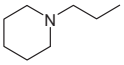
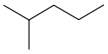
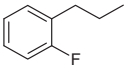
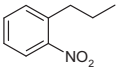
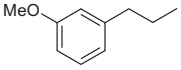
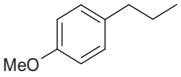
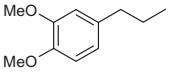
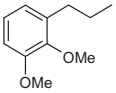
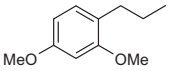
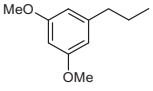
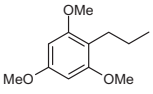
Considering these results, it seems that this region of the receptor has two independent hydrophobic pockets, and therefore, the left side of this large tricycle can bind there only with a proper size and substituent pattern.[13]

Subsequently, we investigated the role of the substituents on the right side. After preparing **9** from **8** by removing the protecting group, it was reacted with various acylating agents. Surprisingly, the alkylated derivatives were chemically unstable, which hampered their further investigation (data not shown).

Replacing the *t*-butoxy group of **8** with *t*-butylamino group (**10**), the molecule became fivefold less potent. In case of the *i*-propylamino group (**11**), the P2X3 activity dropped even more, however, the dimethylamino group (**12**) turned out to be equipotent (IC_{50} = 1300 nM) with **8**. When we increased the size of the cyclic amine attached to the carbonyl-group, the P2X3 activity decreased accordingly (**13**–**15**). The phenylsulfonyl (**16**) and piperidinosul-

Table 1
Exploration of the R^1 position of the tricyclic

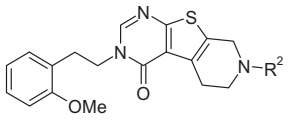


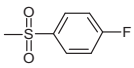
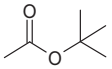
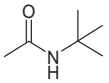
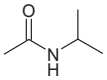
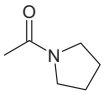
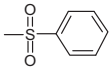
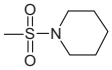
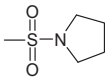
Ex	R^1	hP2X3[a] (IC_{50}, nM)
8		1300
8a		7500
8b		6300
8c		9900
8d		4% at 10 μM
8e		6% at 10 μM
8f		11% at 10 μM
8g		36% at 10 μM
8h		5000
8i		5700
8j		3700
8k		7500
8l		3700
8m		2400
8n		580
8o		390
8p		38% at 10 μM

[a] Ca^{2+} assay: mean IC_{50}, hP2X3, HEK293 cells loaded with Fluo-4 and stimulated with α,β-me-ATP; IC_{50} values are the mean of at least three experiments, with SD of ±20%.

fonyl (**17**) analogues were equipotent with the fluorophenylsulfonyl **6**. Attempts to reduce the size of the sulfonamides, resulted in the same tendency with respect to inhibitory effect on P2X3 receptors, observed with amides; i.e. the substitution of the sulfonyl group with pyrrolidine **18** was better than piperidine **17**, while dimethylamino derivative **19** was the most potent analogue which yielded an IC_{50} value of 440 nM for inhibiting the P2X3 receptors (Table 2).

Table 2
Exploration of the R^2 position of the tricyclic



Ex	R^2	hP2X3[a] (IC_{50}, nM)	M.W.	Kin. sol.[b] (μM)
6		840	500	1.7
8		1300	442	13.9
10		7500	441	56.9
11		10,700	427	91.5
12		1300	413	100
13		1600	439	83.1
14		2100	453	47.2
15		3800	455	100
16		810	482	1.2
17		850	489	7.7
18		720	475	19.6
19		440	449	40.4

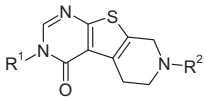
[a] Ca^{2+} assay: mean IC_{50}, hP2X3, HEK293 cells loaded with Fluo-4 and stimulated with α,β-me-ATP; IC_{50} values are the mean of at least three experiments, with SD of ±20%.

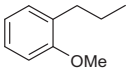
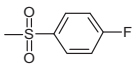
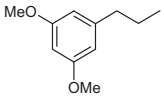
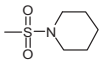
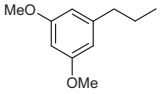
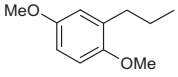
[b] For determination of the kinetic solubility 10 μM DMSO stock solution was used in a pH 7.4 phosphate buffer.

These data suggest that there must be a new, unknown pocket in that direction of the receptor, where a powerful hydrogen-bridge bond is combined with a hydrophobic pocket. Luckily, kinetic solubility could be improved in parallel with lowering of the IC_{50} value of inhibition of P2X3 receptors (**6** < **18** < **19**).

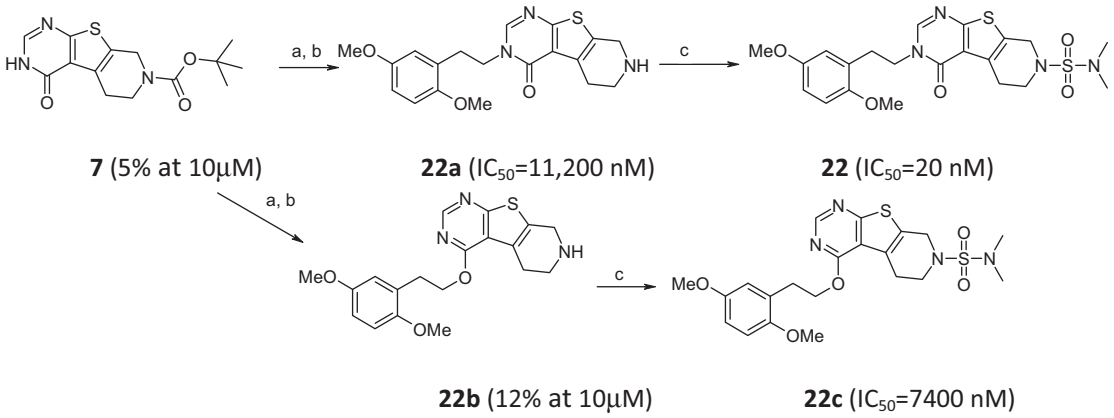
The supraadditivity worked excellently when we combined the best R^1 group (3,5-dimethoxyphenethyl: **8o**) with the most promising R^2 groups (piperidinosulfonyl **17** and dimethylaminosulfonyl **19**): the IC_{50} was 150 nM for compound **20**, and 50 nM for **21**. When the left side of this latter compound was modified by introducing a 2,5-dimethoxyphenethyl side chain, which had not been examined previously, the obtained molecule **22** yielded an IC_{50} of 20 nM for inhibiting P2X3 receptors and, not negligibly, possessed a kinetic solubility value, higher than fifty micromoles (Table 3).

Table 3
Combination of the selected R^1 and R^2 group



Ex	R^1	R^2	hP2X3[a] (IC_{50}, nM)	M.W.	Kin. sol.[b] (μM)	hCLint[c] (μL/min/mg)
1	**A-317491**		220	566	500	0.2
2	**RO-4**		20	400	⩾100	8
3	**RO-85**		2400	425	407	4
4	**AZ-2**		25	484	79	13
5	**AF-219**		45	353	⩾100	0.2
6			840	500	1.7	315
20			150	519	3.8	287
21			50	479	10.6	166
22			20	479	59.3	167

[a] Ca^{2+} assay: mean IC_{50}, hP2X3, HEK293 cells loaded with Fluo-4 and stimulated with α,β-me-ATP; IC_{50} values are the mean of at least three experiments, with SD of ±20%.
[b] For determination of the kinetic solubility 10 μM DMSO stock solution was used in a pH 7.4 phosphate buffer.
[c] Metabolic stability performed in human liver microsomes in the presence of NADPH at 37 °C.



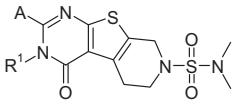
Scheme 2. Synthesis of **22** and its derivatives. Reagents and conditions: (a) 2,5-dimethoxyphenylethyl mesylate, Cs_2CO_3, DMF, 60 °C, 2 h; (b) HCl/dioxane, rt, 2 h; (c) Me_2NSO_2Cl, DIPEA, DCM, rt, 24 h.

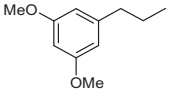
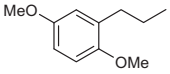
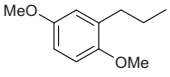
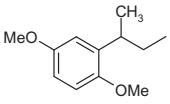
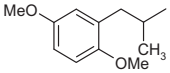
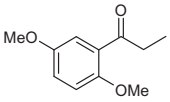
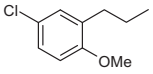
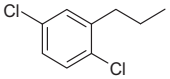
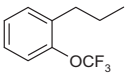
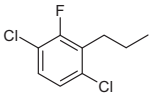
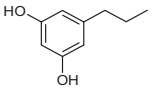
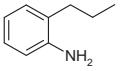
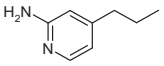
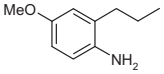
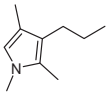
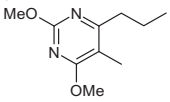
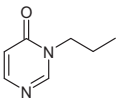
Compound **22** was obtained by utilizing the route depicted on Scheme 1, but in this case we isolated both the *N*-alkyl and *O*-alkyl intermediates (**22a** and **22b**). The synthesis was continued by using both analogues. After testing these molecules, it turned out that the *O*-alkyl derivative is 370-fold less potent at the P2X3 receptor than the *N*-alkyl derivative, so, later on, we stopped isolating these analogues (Scheme 2).

As both water solubility and penetrability properties of compound **22** were deemed acceptable, with values of 59 μM and 31.1×10^{-6} cm/s, respectively, together with lack of an excessive outward penetration property (having a PDR value of 0.9),[14] compound **22** was selected to serve as a lead for further optimization. Compounds **6**, **20**–**22** were suffering from poor stability in human microsomes[15] (hCLint; Table 3). Consequently, the course of later optimization work was focused on improving this property.

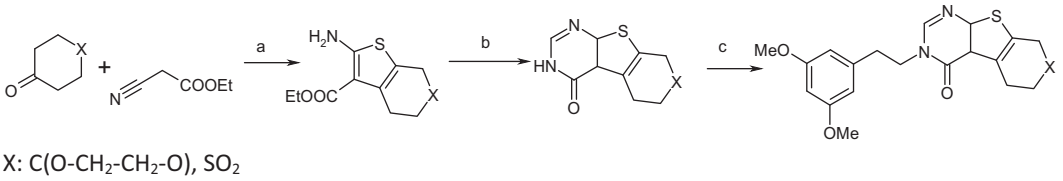
Analysis of metabolic pathways of compounds **20**–**22** clarified that oxidation and demethylation were the primary reason for the poor metabolic stability. To avoid oxidation, efforts were made to introduce a sterical hindrance, while to avoid the demethylation of the methoxy group, replacement of the methoxys by halogens was attempted. By introducing a methyl group between the two nitrogens of the tricycle (in *A* position of the structure in Table 4) the inhibition of P2X3 activity decreased dramatically (**23**), yielding an IC_{50} value for inhibiting the receptor almost in the micromolar range compared to the anticipated low nanomolar range. The P2X3 activity turned out to be even lower, when methyl or oxo group was introduced to the side chain (**24**, **25** and **26**). Although 5-chloro-2-methoxy analogue **27** was very potent (IC_{50} = 35 nM), halogenated molecules not having methoxy groups were five to tenfold less active (**28**, **29** and **30**) than **27**. In spite of these efforts, not only did the microsomal stability of compounds remain very

Table 4
Modification of the side-chain of **22**



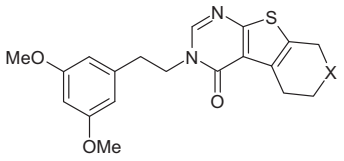
Ex	R^1	A	hP2X3[a] (IC_{50}, nM)	*c*log*P*[b]	hCLint[c] (μL/min/mg)
21		H	50	1.8	166
22		H	20	1.8	167
23		CH_3	590	1.8	144
24		H	1400	2.2	174
25		H	3300	2.2	282
26		H	13% at 10 μM	1.2	84
27		H	35	2.6	266
28		H	280	3.3	211
29		H	180	3.4	248
30		H	380	3.5	267
31		H	inactive	1.4	51
32		H	38% at 10 μM	1.2	8
33		H	19% at 10 μM	0.6	4
34		H	7300	1.1	55
35		H	10% at 10 μM	1.2	39
36		H	7500	1.8	238
37		H	inactive	−0.5	1

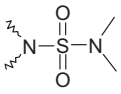
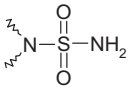
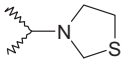
[a] Ca^{2+} assay: mean IC_{50}, hP2X3, HEK293 cells loaded with Fluo-4 and stimulated with α,β-me-ATP; IC_{50} values are the mean of at least three experiments, with SD of ±20%.
[b] Calculated log*P* was determined by MarwinSketch 16.2.1.0 (ChemAxon®) program.
[c] Metabolic stability performed in human liver microsomes in the presence of NADPH at 37 °C.



Scheme 3. Synthesis of tricyclic analogues. Reagents and conditions: (a) S_8, morpholine, EtOH, rt, 24 h; (b) Form-amide, ammonium formiate, 140 °C, 16 h; (c) 3,5-dimethoxyphenylethyl mesylate, Cs_2CO_3, DMF, 60 °C, 2 h.

Table 5
Tricyclic analogues (**38**–**43**) with different polar moieties



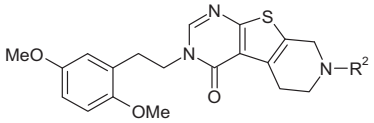
Ex	X	hP2X3[a] (IC_{50}, nM)	hCLint[b] (μL/min/mg)
21		50	166
38		20% at 10 μM	N.D.
39		650	19
40		65	66
41		6800	N.D.
42		140	170
43		1300	45

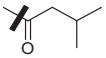
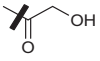
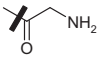
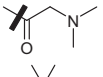
N.D. means not determined.
[a] Ca^{2+} assay: mean IC_{50}, hP2X3, HEK293 cells loaded with Fluo-4 and stimulated with α,β-me-ATP; IC_{50} values are the mean of at least three experiments, with SD of ±20%.
[b] Metabolic stability performed in human liver microsomes in the presence of NADPH at 37 °C.

low, but also the inhibitory potential of compounds on the activity of P2X3 receptors was dampened (Table 4).

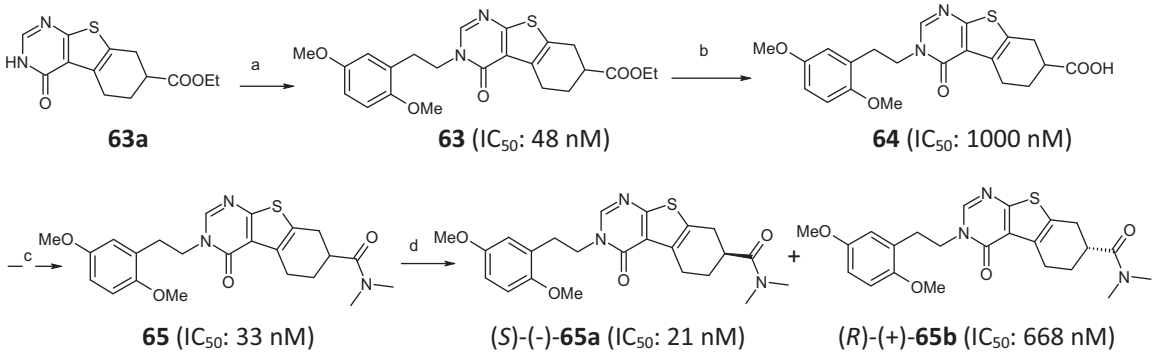
Applying a different approach, we tried to improve microsomal stability by increasing the polarity (and lowering the log*P*) of the left side of **22**. Indeed, by replacing the 3,5-dimethoxyphenyl (**21**) moiety with 3,5-dihydroxyphenyl group (**31**), metabolic stability could be improved, but, simultaneously, the P2X3 inhibitory action of the compound was completely lost. In case of aminophenyl and aminopiridyl analogues (**32** and **33**), the metabolic stability on human microsomes was excellent, however, activities on the P2X3 receptors were unacceptable. Although introduction of a methoxy group to compound **32** improved the value of IC_{50} for inhibiting the P2X3 receptors (**34**), but in return, it also lessened the metabolic stability of the compound. The same result was observed in case of trisubstituted pyrrolo (**35**) and pyrimidine (**36**) analogues. Investigation of the pyrimidinone analogue with highly polar properties (**37**, *c*log*P*: −0.5) revealed that though the metabolic stability of the compound was excellent, its P2X3

Table 6
N-Acyl and *N*-alkylsulfonyl derivatives of **22a**



Ex	R^2	hP2X3[a] (IC_{50}, nM)	hCLint[b] (μL/min/mg)
22a	H	11,200	N.D.
22	SO_2–$N(CH_3)_2$	20	167
44	CO–CH_3	140	14
45		35	44
46		25	59
47		15	79
48		10	133
49		40	196
50		15	191
51		40	138
52		15	177
53		15	139
54		180	9
55		85	29
56		55	16
57		1400	2
58		1000	5
59		640	3
60	SO_2-Me	40	36
61	SO_2-*i*-Pr	20	135
62	SO_2-*c*-Pentyl	40	190

N.D. means not determined.
[a] Ca^{2+} assay: mean IC_{50}, hP2X3, HEK293 cells loaded with Fluo-4 and stimulated with α,β-me-ATP; IC_{50} values are the mean of at least three experiments, with SD of ±20%.
[b] Metabolic stability performed in human liver microsomes in the presence of NADPH at 37 °C.



Scheme 4. Synthesis of **65**. Reagents and conditions: (a) 2,5-dimethoxyphenylethyl mesylate, Cs_2CO_3, DMF, 60 °C, 3 h; 62% yield; (b) 30% aqueous NaOH, EtOH, 60 °C, 2 h, 91% yield; (c) $Me_2NHxHCl$, HOBt, EDCxHCl, trimethyl amine, DMF, rt, 24 h, 70% yield; (e) Chiral chromatography.[18]

Table 7
Properties of the best references and new molecules

	2 (RO-4)	5 (AF-219)	22	56	65
hP2X3[a] (IC_{50}, nM)	20	45	20	55	33
M.W.	400	353	479	458	442
*c*log*P*[b]	3.5	1.1	1.8	2.8	3.7
Kin. sol.[c] (μM)	⩾100	⩾100	59.3	⩾100	49.6
hCLint[d] (μL/min/mg)	8	0.2	167	16	11
VB-Caco-2[e] (x10^{-6} cm/s)	30.6	0.7	31.1	39.7	46.8
PDR[e] (efflux/influx)	0.7	12.1	0.9	1	0.78
hERG selectivity (fold)	>500	>600	>1500	>500	>300

[a] Ca^{2+} assay: mean IC_{50}, hP2X3, HEK293 cells loaded with Fluo-4 and stimulated with α,β-me-ATP; IC_{50} values are the mean of at least three experiments, with SD of ±20%.
[b] Calculated log*P* was determined by MarwinSketch 16.2.1.0 (ChemAxon®) program.
[c] Kinetic solubility measured used in a pH 7.4 buffer using HPLC with UV detection for quantification.
[d] Metabolic stability performed in human liver microsomes in the presence of NADPH at 37 °C.
[e] VB-Caco-2 measurements and PDR determination based on the literature.[15]

antagonist activity was completely lost. These data support the earlier published presumption, that there are hydrophobic pockets in this part of the receptor,[14] and that is the reason, why polar analogues bind very weakly, if they bind at all (Table 4).

Considering these results, it seemed that it was not possible to cope with the metabolic liability issue by changing the left side of the molecule, so we carried on to explore the right side in more details. Since we could identify demethylated sulfonamide group among the major metabolites of **21**–**22**, we investigated what polarity increase the right side of the molecule could tolerate where the activity remained, but the metabolism decreased. Utilizing literature examples,[16] the NH group of the tricycle was replaced with other polar groups (carbonyl, sulfoxide, Scheme 3).

First, we investigated the close analogues of **21** (IC_{50} = 50 nM). Its intermediate **38**, as it was expected, demonstrated only weak activity on P2X3 receptors. No higher potency on P2X3 inhibition with the di-desmethyl analogue **39** compared to compound **21** was seen either (IC_{50} value of 650 nM for **39**). However, the metabolic stability turned out to be significantly better (hCLint: 19 μL/min/mg) compared to that of **21**, which was only 166 μL/min/mg indicating that the methylated sulfonamide was indeed vulnerable by the aspect of metabolic stability. Intermediate acetal **40** possessed a favorable inhibitory activity on P2X3 receptors with an IC_{50} value of 65 nM, while the ketone **41** obtained from it by hydrolysis turned out to be 2 orders of magnitude less potent than **40**. Reacting it with thiazole, the intermediate **42** was obtained to be active on P2X3 receptors, but to be unstable metabolically (hCLint: 170 μL/min/mg). Tricyclic sulfon analogue **43** was 10-fold less potent than **42**, but despite the presence of a polar group in the molecule, the metabolic stability did not improve significantly. These data may further indicate the presence of a binding pocket other than identified so far on the P2X3 receptor. The SAR information we have reached suggests that in order to achieve a strong P2X3 inhibitory activity, a group (CO_2, S, SO_2) being able to form hydrogen-bridge bond is essential, which needs to be 'covered' with a smaller lipophilic group that fills the hydrophobic pocket of the receptor (Table 5).

Findings with the *N*-acyl analogues of intermediate **22a** are also in accordance with the proposed theory. The subsequent work was directed toward acylation of **22a** with various small acid chlorides and sulfonyl chlorides. As a result, compound **44** possessed a sufficient stability on human microsomes (14 μL/min/mg), while the P2X3 inhibitory activity (IC_{50}: 140 nM) was worse than that of compound **22**. Although the P2X3 inhibitory action of compounds could be improved (Me < Et < *i*-Pro < *c*-Pro < *t*-Bu) by increasing the size of R^2, deterioration of the metabolic stability was found in that group of compounds (**45**–**48**). The most potent P2X3 antagonist molecule that was synthesized in this series was the *tert*-butoxy **48** with having an IC_{50} value of 10 nM, which seemed to have the optimal size of side chains to fit in to the binding pocket of the P2X3 receptor. In case of larger groups (**49**–**53**) the activity began to decrease compared to **48**. To improve the metabolic stability, we introduced polar OH and NH groups to the small alkyl chain. Compounds having amino groups (**57**–**59**) had excellent stability, however, their inhibitory activity on human P2X3 receptors was only weak with IC_{50} values of ranging between 640 and 1400 nM. Analogues having OH groups were more active in terms of P2X3 inhibition, so **56** was the first compound, which yielded an acceptable metabolic stability (16 μL/min/mg) and, in parallel, an IC_{50} on P2X3 receptors of 55 nM, which was below the desired 100 nM level. Although the potencies of these alkylsulfonyl derivatives (**60**–**62**) were tended to be better (i.e. IC_{50} were lower than

40 nM) than that of compound **56**, alkylsulfonyl derivatives were found to suffer from poor metabolic stability, similarly to the acyl analogues (Table 6).

As a last attempt, we changed the NH to a CH_2 group and introduced a carboxyl-group in the right side of the tricycle. It was assumed that converting it to an amide with dimethylamine, a molecule that is as shaped as compound **22** could be achieved. The synthesis was carried out by applying the route depicted in Scheme 4 by utilizing the known intermediates **63a**.[17]

All results obtained fulfilled the minimum requirements of the project: the P2X3 inhibitory action of the ester **63** was in accordance with our expectations with having an IC_{50} value of 48 nM, while of the same value for compound **65** turned out to be even better, i.e. 33 nM. The metabolic stability of this compound succeeded the desired 11 μL/min/mg value on human microsome.[15] Compared to **22**, the human microsomal stability is far better, albeit the higher *c*log*P* value of **65**. The kinetic solubility and the penetration properties of compound **65** are also favorable and, in contrast to AF-219, which is suffering from properties making the molecule probable to be a substrate of efflux transporters (PDR value of AF-219 is higher than 10), it is expected that compound **65** may exert its pharmacodynamic effects also in the central nervous system in addition to its actions in the periphery (Table 7).

As compound **65** carries a chiral center, separation of the enantiomers has further improved the inhibitory potency of **65** on P2X3 receptors (Scheme 4). The absolute configuration of compound (−) **65a** was assigned as (*S*) by comparison of its specific rotation with the literature data of a structure analogue.[19] This enantiomer was much more active than the (+) (*R*) **65b** enantiomer (IC_{50}: 21 nM vs 668 nM).[20]

In summary, we have discovered compounds with P2X3 antagonistic properties based on a tricyclic core. Starting from a mildly active HTS hit with poor physicochemical properties compounds with favorable P2X3 potency, solubility and metabolic stability could be achieved by the end of the optimization process. On the basis of the SAR described in the present Letter, proposal for a new (allosteric) binding site on the P2X3 receptor has been made. This series of compounds provided us with useful pharmacological tools, such as **65**.

Acknowledgements

The authors greatly appreciate Gedeon Richter R&D management for the strong support, P2X3 project team members for their contributions, Compound Profiling Laboratory for analytics, kinetic solubility and chiral separation of compounds and Spectroscopic Research Division for their support on analytical studies in confirmation of the structures.

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2. Burnstock, G.; Krügel, U.; Abbracchio, M. P.; Illes, P. *Prog. Neurobiol.* **2011**, *95*, 229.
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10. hP2X3 Ca^{2+} assay protocol: recombinant HEK293 Tet-On cells expressing human P2X3 receptors were loaded with Fluo-4 and stimulated with α,β-methylene-ATP; IC50 values are the mean of at least three experiments, with SD of ±20%. All reference compounds **1**–**5** were synthesized and measured in our test.
11. A screening concentration of 10 μM with 0.5% DMSO was applied. The campaign was run in singletons on 384-well plates and yielded an average Z′ of 0.63 ± 0.08. The selection criteria were 45% median-polished activity (0.55% active rate).
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13. Human P2X3 is known to have a high sequence identify at the ATP binding site with zebrafish P2X4 (see: Kawate, T.; Michel, J.C.; Birdsong, W.T.; Gouaux, E. Nature, 2009, 460, 592) but until know there are only speculations about the structure of P2X3 receptor binding site: i.e. "suspect that 2 methoxy groups occupy separate hydrophobic pocket" in the article about the discovery of RO-4 (Carter, D. S.; Alam, M.; Cai, H.; Dillon, M. P.; Ford, A. P. D. W.; Gever, J. R.; Jahangir, A.; Lin, C.; Moore, A. G.; Wagner, P. J.; Zhai, Y. Bioorg. Med. Chem. Lett. 2009, 19, 1628).
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15. 5 mg/mL human liver microsomes ($n = 3$) were incubated with the test compound (at 1–2.5 μM) in the presence of NADPH at 37 °C. Clearance classification (μL/min/mg): low: <11, Medium: between 11 and 38; High: >38.
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18. Analytical chiral separation was performed on Chromasil 5-Cellucoat RP 4.5 × 150 mm, Eluents: water/80%ACN-20%IPA; Ret.time of enantiomers: (*R*): 8.96 and (*S*): 9.70 min.
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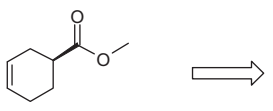
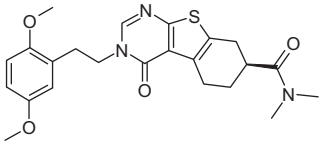
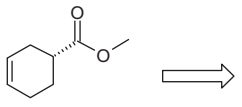


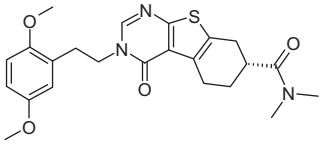
(*S*)-(-) $[a]_D^{25}$= -80.3 (c 1.0, $CHCl_3$)



(*S*)-**65a**
$[a]_D^{25}$= -45.8 (c 0.1, pyridine)



(*R*)-(+) $[a]_D^{25}$= +86.5 (c 1.0, $CHCl_3$)



(*R*)-**65b**
$[a]_D^{25}$= +40.5 (c 0.1, pyridine)

20. Literature findings suggest that the human P2X3 receptor is very sensitive for chirality. Reports claim that huge differences of the P2X3 antagonistic effect between the enantiomers exist.
    a. the first non-nucleotide antagonist, A-317491 (1, S-enantiomer) is more potent than R-enantiomer, A-317344 (Ki: 9 nM vs 1,100 nM) in PNAS, 2002, 17179.
    b. in a pyrido[3,4-d]pyrimidine family in WO 2008/130481 patent by Renovis: IC50 of Ex.58 (R-enantiomer) = 10 nM vs Ex.87 (S-enantiomer) = 481 nM.
    c. in a hydroxy-pyrrolone family in EP 2 336 109, 2010 patent by Shionogi: IC50 of Ex.I-206 (S-enantiomer) = 630 nM vs -Ex.I-207 (R-enantiomer): 8 nM.
    d. in pyrrolo-pyrimidinones published by AstraZeneca in Bioorg. Med. Chem. Lett. 2012, 22, 2565, see reference 15 (“In all cases the S-enantiomer was the only one active. The R-enantiomer was consistently inactive.”).