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Synthesis and structure–activity relationship studies of tyrosine-based antagonists at the human $P2X_7$ receptor

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Abstract—Analogues of the $P2X_7$ receptor antagonist KN-62, modified at the piperazine and arylsulfonyl groups, were synthesized and assayed at the human $P2X_7$ receptor for inhibition of BzATP-induced effects, that is, uptake of a fluorescent dye (ethidium bromide) in stably transfected HEK293 cells and IL-1β release in differentiated THP-1 cells. Substitution of the arylsulfonyl moiety with a nitro group increased antagonistic potency relative to methyl substitution, such that compound **21** was slightly more potent than KN-62. Substitution with D-tyrosine in **36** and sterically bulky tyrosyl 3,5-dimethyl groups in **9** enhanced antagonistic potency.
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The P2 purinergic receptor family is divided into P2X and P2Y subfamilies. The P2X receptors are ligand-gated cation channels activated by extracellular ATP (adenosine-5-triphosphate). P2X receptors are further classified into seven receptor subtypes, $P2X_1$–$P2X_7$.[1,2] In particular, the $P2X_7$ receptor, formerly designated as the P2Z receptor,[3,4] is highly expressed in cells of the immune system such as macrophages,[5] lymphocytes,[6] mast cells,[7] and microglia.[8] The $P2X_7$ receptor, which displays 35–40% structural homology with other subtypes of P2X receptors, consists of two hydrophobic membrane-spanning domains (M1, M2), a large extracellular loop, a short intracellular N-terminus, and a long intracellular C-terminus.[9] Interestingly, the C terminus of the $P2X_7$ receptor is 239 amino acids longer (AA 352–595) than that of other P2X receptor subtypes. The long intracellular C-terminal domain of the $P2X_7$ receptor modulates several functions, including signal transduction events and cytolytic pore formation.[10–12] The activation of $P2X_7$ receptors results in two distinct responses, depending on the exposure time to agonist. A brief stimulation of the receptor by extracellular ATP opens a membrane channel permeable to small cations (Na^+, Ca^{2+}, K^+). However, repeated or sustained activation by ATP induces the formation of a pore permeable to large molecular weight molecules/ions up to 900 Da in mass, such as ethidium bromide (314 Da) and YO-Pro-1 (376 Da), leading to cellular death or cell lysis.[13–15]

The $P2X_7$ receptor is implicated in the regulation of the expression and secretion of cytokines and inflammatory mediators including interleukin (IL)-1,[16] IL-2,[17] IL-18,[18] and tumor necrosis factor (TNF)-α.[19] Notably, the $P2X_7$ receptor plays an important role in the processing and release of the proinflammatory cytokine IL-1β in the immune system by a complex sequence of events: Initially, the activated $P2X_7$ receptor causes the depletion of K^+ leading to the stimulation of IL-1β converting enzyme (caspase-1), which converts LPS-activated pro-IL-1β to mature IL-1β.[16] Therefore, development of antagonists for inhibition of the $P2X_7$ receptor could be a therapeutic strategy to treat inflammatory diseases (Fig. 1).

Among potent and specific non-competitive antagonists at the human (h) $P2X_7$ receptor developed so far[20] is a

Keywords: $P2X_7$ receptor; Tyrosine-based antagonists; Ethidium bromide uptake; IL-1β release.
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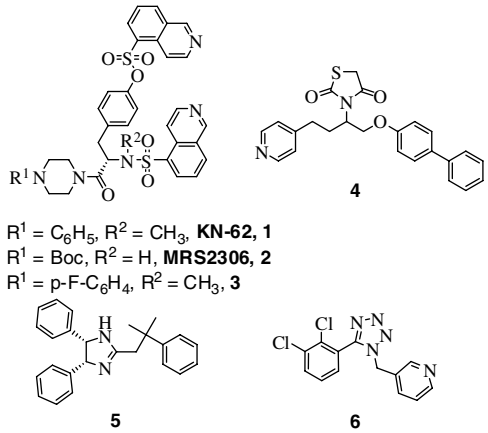
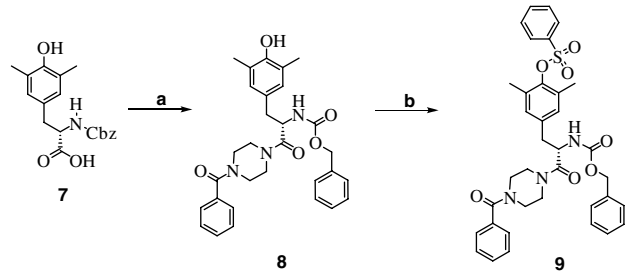


Figure 1. $P2X_7$ receptor antagonists.[21–27]

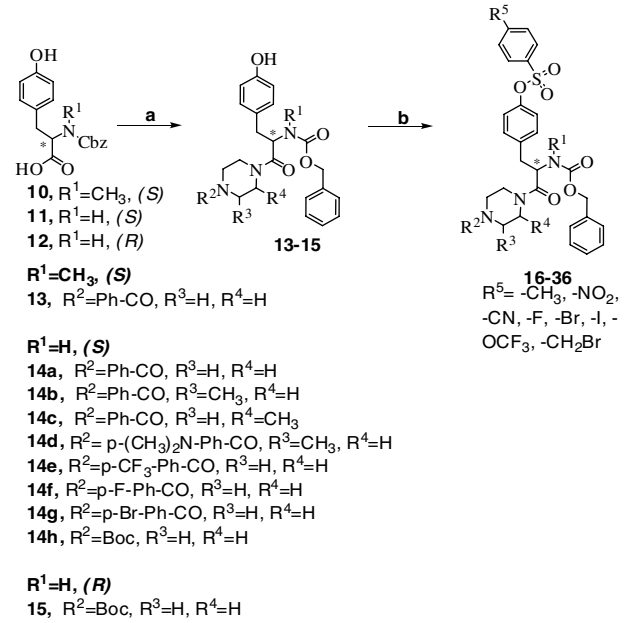
tyrosyl derivative, the isoquinoline KN-62 (1-(*N*,*O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl)-4-phenylpiperazine) **1**, first known as an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II).[21] Compound **2** (MRS2306), modified from KN-62, with the general structure R^1-Tyr(OR^2)-piperazinyl-R^3, exhibited an IC_{50} value of 40 nM and was more potent than KN-62 in an ATP-induced K^+ efflux assay in $hP2X_7$-expressing HEK293 cells.[22] Another series of conformationally constrained and *N*-arylpiperazine-modified analogues was reported.[23,24] An *N*-*p*-fluorophenylpiperazine analogue, **3**, displayed higher antagonistic potency than KN-62 in an ethidium bromide uptake assay and significantly inhibited ATP-stimulated secretion of IL-1β from human macrophages. Other $P2X_7$ receptor antagonists, such as the thiazolidine-2,4-dione derivative **4**, the 4,5-diarylimidazoline derivative **5**, and the 1-benzyl-5-phenyltetrazole derivative **6**, have been reported by several industrial pharmaceutical laboratories.[25–27]

In the present study, we report the structure–activity relationship (SAR) of a novel series of Cbz-substituted tyrosyl derivatives having substituted piperazine and arylsulfonyl groups. The compounds were evaluated in an assay of the uptake of a fluorescent dye ethidium bromide induced by 2′- and 3′-*O*-(4-benzoyl-benzoyl)-ATP (BzATP), which activates the $hP2X_7$ receptor stably expressed in HEK293 cells, and in an ELISA for IL-1β release from differentiated THP-1 cells.

Two variable positions of the skeleton, that is, the arylpiperazine and arylsulfonate moieties, were systematically modified through facile acylation and sulfonylation reactions as shown in Schemes 1–3. The starting materials, the compounds **7**, **10**, **11**, and **12**, were first reacted with a variably substituted arylpiperazine or Boc-piperazine to produce an amide in the presence of coupling reagents such as EDC·HCl or Bop-Cl. Then, the phenol was derivatized with various sulfonamide groups by the reaction of a *para*-substituted arylsulfonyl chloride in the presence of DMAP as catalyst. Compound **21** was used as the starting material in a synthetic route that led to the dimerized compound **40** (Scheme 3). This symmetric dimer was designed for the purpose



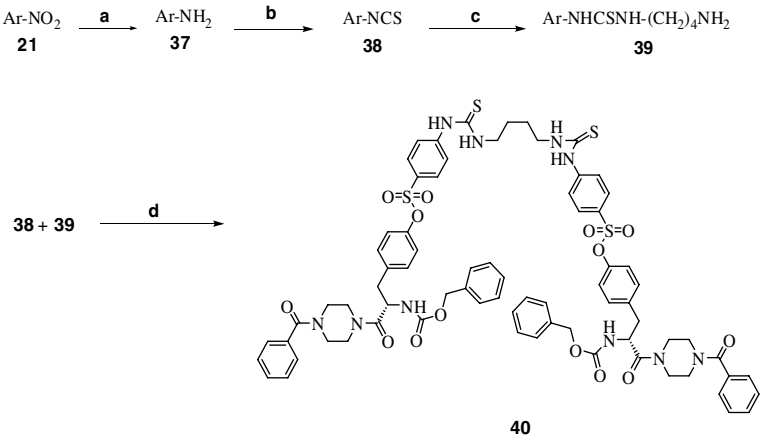
Scheme 1. Synthesis of a representative $P2X_7$ receptor antagonist **9** consisting of a 3,5-dimethyltyrosyl derivative. Reagents and conditions: (a) benzoyl piperazine, Et_3N, Bop-Cl, rt, 95%; (b) *p*-methylbenzenesulfonyl chloride, DMAP, CH_2Cl_2, rt, 77%.



Scheme 2. Synthesis of various $P2X_7$ receptor antagonists consisting of tyrosyl derivatives. Reagents and conditions: (a) substituted arylpiperazine or Boc-piperazine, HOBt, EDC·HCl, DMF, rt, 93–98%; (b) substituted benzenesulfonyl chloride, DMAP, CH_2Cl_2, rt, 70–90%.

of probing the proximity of adjacent binding sites on the receptor.[22] The nitro group of compound **21** was reduced with $NaBH_4$-copper (II) bisacetylacetonate to an amine, which was subsequently converted to the isothiocyanate in compound **38** by a reaction with thiophosgene. The isothiocyanate group in **38** was reacted with 1,4-diaminobutane to produce the primary amine congener **39**. Finally, to make the dimer **40** a R^5–R^5 linker was created by a coupling reaction between **38** and **39**.

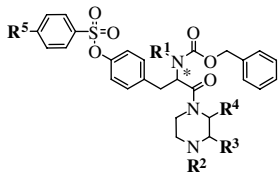
The SAR studies of tyrosine-based derivatives **9**, **16**–**36**, and **40**, which have various substitutions at the toluenesulfonyl, substituted piperazine, and benzyloxycarbonyl (Cbz) groups, were assessed in the ethidium$^+$ accumulation assay. Experiments were performed using HEK293 cells stably transfected with cDNA encoding the $hP2X_7$ receptor. As shown in Table 1, compound **9** with two methyl groups at the phenyl ring of tyrosine displayed a 3-fold increase of the antagonistic activity compared



Scheme 3. Dimerization of $P2X_7$ receptor antagonists through formation of a thiourea linkage. Reagents and conditions: (a) $NaBH_4$, $Cu(acac)_2$, methanol, rt, 80–85%; (b) $CSCl_2$, chloroform, water, sodium bicarbonate, 70–85%; (c) 1,4-diaminobutane, CH_2Cl_2, rt, 40–45%; (d) dry DMF, at 80 °C, 52%.

with compound **16**. In addition, when the methyl group was adjacent to the benzoyl piperazine group (e.g., **17** and **18**), the antagonist effects were also increased by 2-fold compared with compound **16** regardless of the stereochemistry of the chiral carbon of the piperazine group. Thus, the steric effects near the sulfonyl ester of the phenyl group of tyrosine and the benzoyl group of piperazine ring were tolerated in the antagonism of the receptor. However, unlike compound **17** or **18**, their regioisomer, **19**, contained a methyl group adjacent to the α-

Table 1. Activities of synthesized tyrosine-based derivatives on ethidium accumulation in $hP2X_7$-expressing HEK293 cells[28]



Compound	R/S	R^1	R^2	R^3	R^4	R^5	IC_{50}[a] ± SE (nM)
KN-62 (positive control)							175 ± 35
9[b]	(*S*)	H	Ph-CO	H	H	CH_3	219 ± 69
16	(*S*)	H	Ph-CO	H	H	CH_3	599 ± 22
17	(*S*)	H	Ph-CO	(*S*)CH_3	H	CH_3	260 ± 77
18	(*S*)	H	Ph-CO	(*R*)CH_3	H	CH_3	256 ± 96
19	(*S*)	H	Ph-CO	H	(*R*)CH_3	CH_3	504 ± 136
20	(*S*)	CH_3	Ph-CO	H	H	CH_3	1360 ± 580
21	(*S*)	H	Ph-CO	H	H	NO_2	117 ± 44
22	(*S*)	H	*p*-$(CH_3)_2$N-Ph-CO	(*S*)CH_3	H	CN	539 ± 90
23	(*S*)	H	*p*-$(CH_3)_2$N-Ph-CO	(*S*)CH_3	H	I	55 ± 5%[c]
24	(*S*)	H	*p*-$(CH_3)_2$N-Ph-CO	(*S*)CH_3	H	CH_3	54 ± 3%[c]
25	(*S*)	H	*p*-$(CH_3)_2$N-Ph-CO	H	H	CH_3	2010 ± 340
26	(*S*)	H	*p*-CF_3-Ph-CO	H	H	CH_3	724 ± 171
27	(*S*)	H	*p*-F-Ph-CO	H	H	CH_3	319 ± 65
28	(*S*)	H	*p*-Br-Ph-CO	H	H	CH_3	481 ± 41
29	(*S*)	H	Boc	H	H	F	5880 ± 3280
30	(*S*)	H	Boc	H	H	Br	517 ± 18
31	(*S*)	H	Boc	H	H	I	3540 ± 907
32	(*S*)	H	Boc	H	H	CN	780 ± 107
33	(*S*)	H	Boc	H	H	OCF_3	725 ± 118
34	(*S*)	H	Boc	H	H	CH_2Br	2460 ± 470
35	(*S*)	H	Boc	H	H	CH_3	1540 ± 102
36	(*R*)	H	Boc	H	H	CH_3	861 ± 123
40[d]							28 ± 1%[c]

[a] IC_{50} = 50% inhibitory concentrations were obtained from concentration–response curves. Data values are expressed as means ± SD. All experiments were repeated at least 3–11 times.
[b] This compound has two methyl groups at the phenyl ring of the tyrosyl moiety (see Scheme 1).
[c] The compounds **23**, **24**, and **40** represented the % inhibition at 10 μM concentration level.
[d] This compound is a dimer linked through formation of a thiourea linkage (see Scheme 3). **16**, MRS2427; **21**, MRS2447; **27**, MRS2544.

Table 2. Antagonistic effects of tyrosyl derivatives against BzATP-stimulated IL-1β release in LPS/IFNγ-differentiated human THP-1 cells[29]

Compound	% inhibition[a]	Compound	% inhibition
KN-62	83 ± 9	**26**	65 ± 6
9	87 ± 8	**27**	68 ± 10
16	70 ± 14	**28**	78 ± 6
17	69 ± 12	**29**	56 ± 13
18	65 ± 3	**30**	68 ± 12
19	45 ± 4	**31**	72 ± 4
20	47 ± 12	**32**	60 ± 8
21	48 ± 7	**33**	46 ± 9
22	59 ± 11	**34**	52 ± 5
23	61 ± 6	**35**	72 ± 15
24	68 ± 7	**36**	56 ± 5
25	65 ± 12	**40**	37 ± 20

[a] The values represent the % inhibition at a 1 μM concentration expressed as means ± SD against 1 mM Bz-ATP (n = 3).

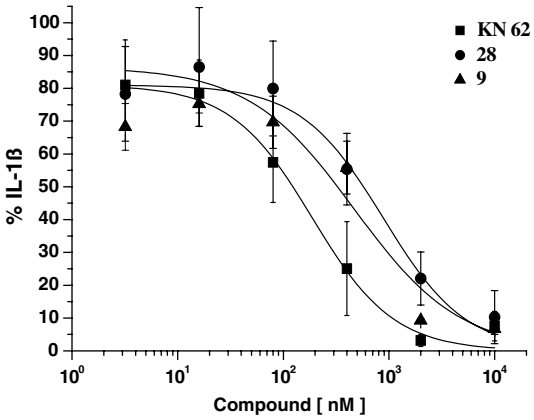


Figure 2. Concentration-dependent inhibition of BzATP-stimulated IL-1β release in LPS/IFNγ-differentiated human THP-1 cells by compounds **9**, **28**, and KN-62. Data points represent means ± SD of values obtained (n = 3).

carbonyl of the tyrosyl moiety, which reduced the antagonistic effect. In a comparison between compounds **20** and **16**, the presence of a methyl group at the tyrosyl α-nitrogen decreased the antagonistic activity by 2-fold at the $hP2X_7$ receptor, which is consistent with previously reported results.[23] The 4-nitro group at the R^5 position (compound **21**) increased the antagonistic effect by 5-fold compared with the corresponding 4-methyl group of **16**; compound **21** was slightly more potent than KN-62.

The rank order of potency according to IC_{50} values of *para*-substituents at the benzoyl group was F (**27**) > Br (**28**) > CF_3 (**26**) > $(CH_3)_2N$ (**25**). Comparing compounds **17** versus **24** and **16** versus **25** show that a 4-dimethylamino group significantly reduced the antagonistic activity. Thus, basic or bulky groups seem to be inappropriate recognition motifs for the antagonism. Compounds that were Boc-substituted at the R^2 position showed different antagonistic effects depending on phenyl sulfonyl ring *para*-substitution (i.e., R^5-substituent) with the following order of potency: Br (**30**) > OCF_3 (**33**) > CN (**32**) >> CH_3 (**35**) > CH_2Br (**34**) > I (**31**) > F (**29**).

In the study of the effect of chirality of the tyrosine by comparing *R*- and *S*-isomers **35** and **36**, the *R*-(D)-isomer was the more potent $P2X_7$ receptor antagonist. The substitution with a D-tyrosyl residue might add stability to the compound in biological systems. The dimeric compound **40** showed only marginal antagonism at a 10 μM concentration.

The functional antagonism of the tyrosine-based derivatives (**9**, **16**–**36**, and **40**) was evaluated with an ELISA for the detection of BzATP-activated IL-1β release in LPS/IFNγ-differentiated human THP-1 cells (Table 2). Most of the compounds with low submicromolar IC_{50} values in the ethidium accumulation assay (e.g., **9**, **17**, **18**, **27**, **28**, etc.) except for **21**, resulted in the parallel correlation with the inhibitory activity against the release of IL-1β by BzATP. However, compounds **24** and **31**, which had very low antagonistic activity in the ethidium accumulation assay, showed a relatively high inhibitory effect on the release of IL-1β. The methyl group at the R^4 position of **19** and the methyl group on the α-nitrogen of **20** seem to affect the inhibitory effect on the release of IL-1β as a comparison between them and compound **16** shows.

Compounds **9** and **28**, which showed inhibitory effects similar to KN-62 against the release of IL-1β by BzATP, were further evaluated with full concentration–response curves to compare IC_{50} values (Fig. 2). The IC_{50} values of compounds **9** (481 nM) and **27** (881 nM) were 2- and 4-fold higher than that of KN-62 (188 nM).

In conclusion, a novel series of tyrosine-based $P2X_7$ receptor antagonists with modifications at the piperazine and arylsulfonyl groups were synthesized and evaluated in ethidium accumulation assays and ELISAs of the release of IL-1β. Substitution of the arylsulfonyl moiety with a nitro group, in comparison to a methyl group, increased antagonistic potency in compound **21**, which was slightly more potent than KN-62. Several derivatives, including the dimethylphenylsulfonate analog **9** and the methylpiperazine analogs **17** and **18**, were nearly equipotent to the parent compound KN-62 and their functional inhibitory activity against the release of the inflammatory cytokine, IL-1β, was confirmed. Substitution with D-tyrosine in **36** and sterically bulky tyrosyl 3,5-dimethyl groups in **9** favored $P2X_7$ receptor antagonistic potency.

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28. *Ethidium accumulation in hP2X$_7$-expressing HEK 293 cells.* All experiments were performed using adherent HEK293 cells stably transfected with cDNA encoding the human P2X$_7$ receptor. Synthesized tyrosine-based derivatives was added to each well of 96-well plate (black, clear bottom). hP2X$_7$-expressing HEK293 cells were then re-suspended at 2.5×10^6 cells/ml in Hepes-buffered salt solution that comprised (in mM): ethidium bromide 0.1, ethylene diamine tetraacetic acid (EDTA) 1, glucose 5, Hepes 20, and potassium chloride 140 (pH 7.4). The cell suspension was treated to the wells of 96-well plate followed by addition of BzATP. The plates were incubated at 37 °C for 120 min, and cellular accumulation of ethidium$^+$ was determined by measuring fluorescence with a fluorescent plate reader (excitation filter of 530/20 and emission filter of 590/20).
29. *Inhibition effects of BzATP-mediated IL-1β release by ELISA measurements.* IL-1β release was measured in differentiated THP-1 cells primed for 3 h with 25 ng/ml LPS and 10 ng/ml IFNγ, and then was stimulated with 1 mM BzATP for 30 min. Synthesized tyrosine-based derivatives at 1 μM were treated for 30 min prior to BzATP. Supernatants were collected by centrifugation at 1000 rpm for 5 min and assayed for the presence of mature human IL-1β using an ELISA kit.