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Synthesis and structure–activity relationships of pyrazolodiazepine derivatives as human P2X$_7$ receptor antagonists

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ABSTRACT

Screening of library compounds has yielded pyrazolodiazepine derivatives with P2X$_7$ receptor antagonist activity. To explore the structure–activity relationships (SAR) of these pyrazolodiazepines as human P2X$_7$ receptor antagonists, derivatives were synthesized by substitutions at positions R^2 and R^3 of the pyrazolodiazepine skeleton. Using a 2′(3′)-*O*-(4-benzoylbenzoyl)ATP (BzATP)-induced fluorescent ethidium uptake assay, the activities of these derivatives were tested in HEK-293 cells stably expressing human P2X$_7$ receptors. Moreover, the effect of these derivatives was assessed by measuring their effect on IL-1β release induced by BzATP-induced activation of differentiated THP-1 cells. A 2-phenethyl pyrazolodiazepine derivative with a 1-methyl-1*H*-3-indolyl group at position R^2 had fivefold greater activity than the derivative with a 5-isoquinolinyl at R^2. Moreover, a benzyl moiety at R^3 had fivefold greater activity than a bicyclic moiety. The stereochemical effect at C-6 showed a preference for the (*R*)-isomer. Among the series of active derivatives, compound **23b**, with a phenethyl group at R^1, a 3-methyl indole at R^2, and a benzyl at R^3, exhibited activity similar to that of the positive control, KN-62, as shown by the inhibitory effects of IL-1β release.

The P2X receptors are ligand-gated ion channels belonging to the purinergic P2 receptor family. Seven subtypes of P2X$_1$-P2X$_7$ receptor have been identified to date, all of which are activated by extracellular ATP (adenosine-5-triphosphate).[1,2] Among these seven subtypes, P2X$_7$ receptor (P2X$_7$R) is the most distinctive in molecular structure, function and pharmacology. Receptors of this subtype are homomeric[3] and are activated by much higher ATP concentrations (EC$_{50}$ = ~1 mM)[4] than are the other P2X receptor subtypes (EC$_{50}$ <1 μM). Molecularly, P2X$_7$ receptors are distinguished by their long C terminal tails (242 residues),[5] which play a role in the formation of non-selective plasma membrane pores during extended receptor stimulation.[6] The pore function was established by studies of the uptake of large inorganic and organic cations, to molecular weight 900 Da, such as ethidium bromide, propidium bromide, and 4-[(3-methyl-2-(3*H*)-benzoxazolylidene)methyl]-1-[3-(triethylammonio)propyl]di-iodide (Yo-Pro1).[7,8] P2X$_7$R is expressed mainly in hematopoietic cells, including mast cells, lymphocytes, erythrocytes, and macrophages, the human monocyte cell line THP-1, epidermal Langerhans cells, fibroblasts, and cells in the central nervous system such as microglia and Schwann cells, suggesting that these receptors are involved in the pathophysiology of various diseases, such as chronic inflammation, neurodegeneration, and chronic pain.[9–11] Particularly, P2X$_7$R expressed by most immune system cells has been found to play an important role in the processing of caspase-1 and the secretion of cytokines including IL-1β by recruiting accessory proteins such as pannexin-1.[12] In addition, the activation of P2X$_7$R triggers several signaling cascades, which ultimately lead to macrophage fusion,[13] superoxide production in microglia,[14] lymphoid cell proliferation,[15] and apoptosis/necrosis.[16] Thus, therapeutic interventions targeting P2X$_7$R have been explored as a novel approach for the prevention or treatment of inflammatory disorders such as arthritis,[17] chronic inflammatory pain,[18] neuropathic pain,[19] and neurodegenerative diseases.[9]

To date, several P2X$_7$R antagonists have been described, and their structure–activity relationships (SAR) have been studied. A tyrosine derivative, KN-62 (1-(*N*,*O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-l-tyrosyl)-4-phenylpiperazine, compound **1**, Figure 1),[20] has been shown to be one of the most potent non-competitive antagonists for human P2X$_7$ receptors (hP2X$_7$R), and attempts to enhance its antagonistic activity have yielded the more potent compounds **2**[21] and **3**.[22] These KN-62 derivatives, however, are not appropriate for therapeutic use, due to their high molecular weight, high lipophilicity and the presence of metabolically labile sulfonate groups. High throughput screening of drug-like small molecules has yielded an adamantine-based derivative **4** and its analog, AZD9056,[23] the 1-benzyl-5-phenyltriazole derivative **5**

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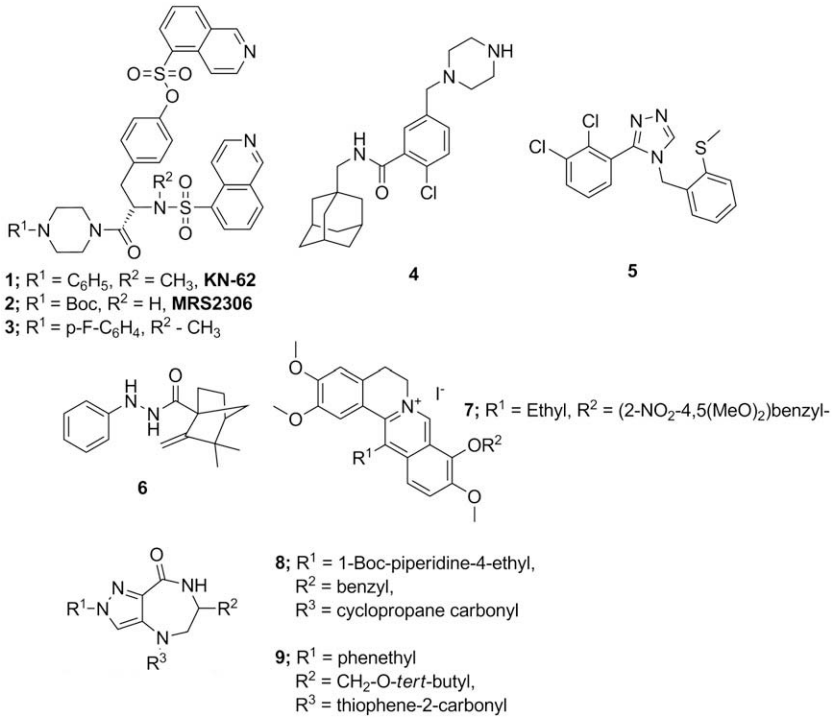
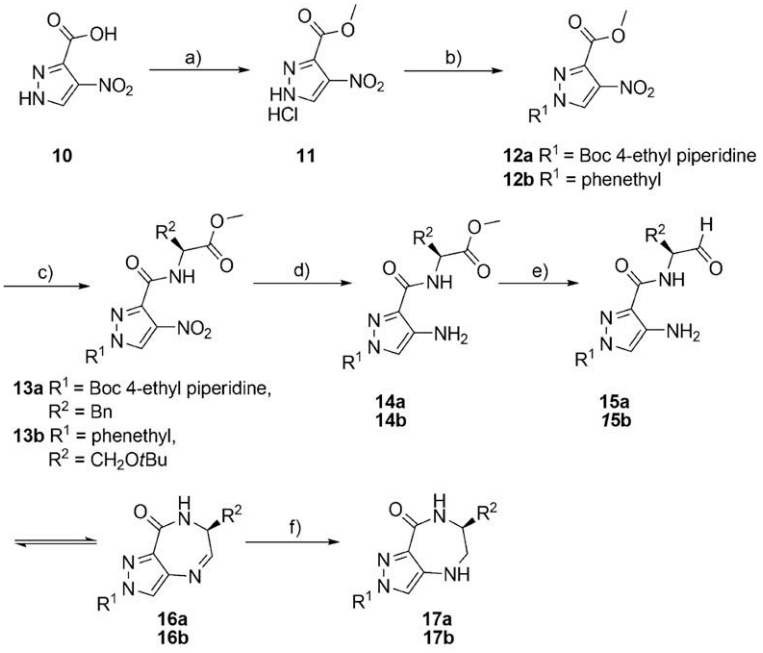


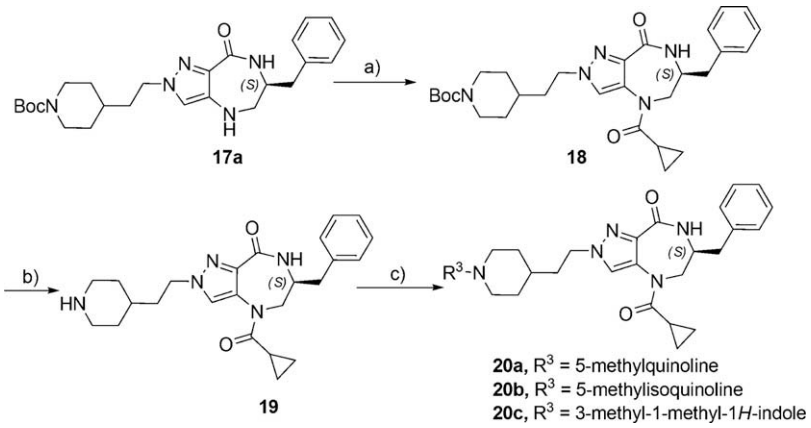
Figure 1. $P2X_7$ receptor antagonists.

and the terpene-derived acyl hydrazide **6**. Compound **4** showed improved solubility and appreciable stability to metabolism,[24] whereas compounds **5** and **6** effectively attenuated allodynia in a rat model of neuropathic pain.[25,26] Compound **7**, an iminium quaternary protoberberine alkaloid (QPA) was reported as a new $hP2X_7R$ antagonist by our research group.[27]

Recently, we demonstrated that pyrazolodiazepine skeleton could be a potential privileged structure by identifying the biological activity of various analogs at different class of target proteins, including melanocortin-4 receptor, β-secretase and $hP2X_7R$.[28] As an extension of this work, we report here the full SAR analysis and optimization of the antagonistic profile of various pyrazolodiazepine derivatives at the $hP2X_7R$. We tested the ability of these derivatives to inhibit fluorescent ethidium ion uptake into HEK293 cells stably expressing $hP2X_7R$ and to block IL-1β release by differentiated THP-1 cells after receptor activation by 2′(3′)-*O*-(4-benzoylbenzoyl)-ATP (BzATP), a selective $P2X_7R$ agonist.



Scheme 1. General synthetic scheme for the preparation of 6,2-substituted tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-ones. Reagents and conditions: (a) CH_3OH, acetyl chloride, 24 h, 95%; (b) phenethyl bromide or 1-Boc 4-(2-(methylsulfonyloxy)ethyl)piperidine, NaH, DMF, 12 h, 65–84%; (c) (i) 1 m NaOH MeOH, 1 h, 98–99%; (ii) (1) L-Ser(*t*Bu)-methyl ester, (2) L-Phe-methyl ester, EDC, HOBt, TEA (triethylamine), DCM, 8 h, 78%; (d) Pd/C, H_2, MeOH, 4 h, 98%; (e) DIBAL-H, toluene, 3 h, 60–70%; (f) $NaBH(OAc)_3$, 1% AcOH, DCM, 55–60%.



Scheme 2. Synthesis of 2-piperidinylethyl pyrazolodiazepine derivatives of $P2X_7$ antagonist. Reagents and condition: (a) cyclopropane carbonyl chloride, TEA (triethylamine), DCM, 80%; (b) 20% TFA, anisole, DCM, 97%; (c) R^3–CHO, $NaBH(OAc)_3$, DCM, 67%.

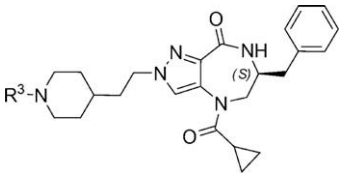
Although the initial compounds were weakly active (e.g., **8**, IC_{50} = 4.31 μM, **9** = 18.6 μM), screening of library compounds showed that the combinations of Boc-piperidine 4-ethyl at R^1 and benzyl at R^2 and phenethyl at R^1 and *tert*-butoxymethyl at R^2 were preferred pharmacophores for antagonistic activity. Using the structural information obtained from compounds **8** and **9**, we established a strategy for further design of derivatives of the pyrazolodiazepine skeleton. We assessed the effect of stereochemistry at the C-6 position and the structure–activity relationships of substituted groups at the N-1 position of the piperidine of compound **8**. Boc was changed to a bicyclic aryl group, which is present as a pharmacophore in the structures of other $hP2X_7R$ antagonists. We also evaluated the importance of the thiophene carbonyl and *tert*-butoxymethyl groups by replacing more flexible amine moiety instead of tertiary amide at the N-4 position and the methyl bicyclic ester at C-6 of the diazepine structure in compound **9**.

The pyrazolodiazepine-8-one skeleton was synthesized by a standard procedure (Scheme 1).[28] The N-2 position of the pyrazole ring of methyl 4-nitro-1*H*-pyrazole-3-carboxylate **11** was alkylated with 1-Boc 4-(2-(methylsulfonyloxy)ethyl)piperidine or phenethyl bromide since library screening showed that these moieties at the R^1 position contributed to their antagonistic activity (compounds **8** and **9**). The resulting carboxylic acid group from hydrolysis of ester **12** was coupled with L-Ser or L-Phe esters. After reduction of nitro group of **13** to amine, ester **14** was subjected to reductive intramolecular cyclization and subsequent reduction of imine **16** afforded the tetrahydro-1,4-pyrazolodiazepin-8-one skeleton **17**.

The piperidine moiety of the pyrazolodiazepine-8-one analogue, **17a**, was further derivatized, as shown in Scheme 2. The N-4 position of the diazepine ring was first substituted with cyclopropane carbonyl chloride to yield the tertiary amide, **18**, and the Boc group of **18** was deprotected with 20% TFA using anisole as a scavenger to yield **19** for the introduction of bicyclic aryl group at the piperidine moiety. The desired substituted piperidine-based pyrazolodiazepine-8-one analogs, **20a–c**, were obtained by reacting **19** with 5-quinoline and 5-isoquinoline carboxaldehydes, both of which are building blocks for $P2X_7R$ antagonists, and 3-methyl-1*H*-indole carboxaldehyde.

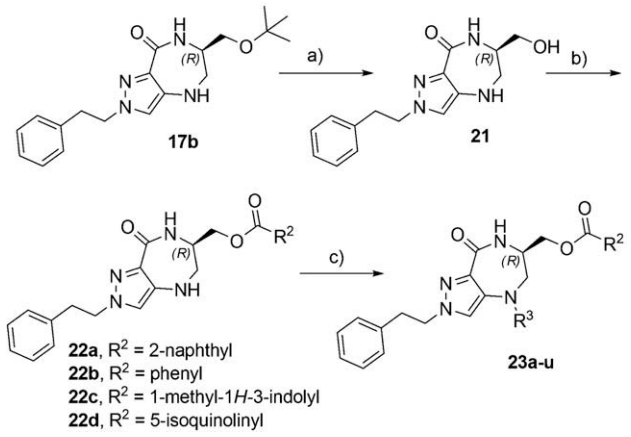
Initial SAR-studies around compound **8** were aimed at investigating the effect on $P2X_7R$ antagonism of the substituents, known heterocyclic pharmacophore of $P2X_7R$ antagonists, in the piperidine N-1 and the stereochemistry of C-5 (*R* to *S*). As shown in Table 1, compound **18**, an enantiomer of **8**, where N-4 substitution of the diazepine ring was fixed with cyclopropane carbonyl and the stereochemistry of C-6 was changed to the *S*-configuration, showed a threefold decrease in antagonistic activity, with an IC_{50} of 13 μM. Thus, the *R*-configuration of the diazepine skeleton was more beneficial than the *S*-configuration. Removal of the Boc group of the piperidine moiety, **19**, dramatically decreased the activity, showing only 13% inhibition at 10 μM. Loss of activity was also observed for all the analogs of **20**, suggesting that the N-1 position of piperidine is not an appropriate site for functionalization with heterocyclic pharmacophores such as isoquinoline and indole groups. Thus, these alterations did not result in any increase in antagonistic activity at $P2X_7R$.

Table 1
Activities of synthesized compounds **18**–**20c** and KN-62 on the ethidium accumulation in $hP2X_7$-expressing HEK293 cells[a]



Compds	R^3	% Inhibition (10 μM)	IC_{50}[b] (μM)
KN62 (positive control)		85 ± 2	0.11 ± 0.02
(*R*)-8	Boc	70 ± 3	4.31 ± 0.50
18	Boc	61 ± 2	13.0 ± 2.4
19	H	13 ± 6	
20a	5-Methylquinoline	24 ± 3	
20b	5-Methylisoquinoline	27 ± 4	
20c	3-Methyl-1-methyl-1*H*-indole	39 ± 7	

[a] Data are expressed as means ± SD. All experiments were repeated three times.
[b] IC_{50} = 50% inhibitory concentration, representing the mean from dose–response curves.

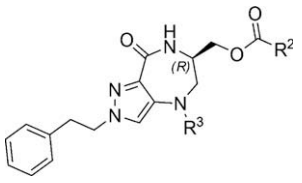


Scheme 3. Synthesis of 2-phenethyl pyrazolodiazepine derivatives of $P2X_7$ antagonist. Reagents and conditions: (a) 50% TFA, anisole, DCM, 95%; (b) substituted carboxylic acid, EDC, DMAP, DCM, 75%; (c) substituted aryl halide, DBU, DMSO, heating, 57% or aryl aldehyde, $NaBH(OAc)_3$, DCM, 45%.

In a second attempt to optimize activity, the N-2 position of the pyrazole ring was fixed with a phenethyl group, as in compound **9**, and the SAR of the remaining two diversity points, the N-4 position and the OH group originating from the serine side chain of the diazepine ring, was assessed. The phenethyl-based pyrazolodiazepine-8-one derivatives were synthesized by three-step reactions, as shown in Scheme 3. The *tert*-butyl group of compound **17b** was removed by treatment with 50% trifluoroacetic acid in dichloromethane to yield the free hydroxyl analog, **21**, which was subsequently functionalized with 2-naphthalene carbonyl **22a**, benzoyl **22b**, 1-methyl-1*H*-3-indole carbonyl **22c**, and 5-isoquinoline carbonyl **22d** under standard coupling conditions using EDAC. Finally, a series of compounds **23a–u** was synthesized by alkylation with various substituted aryl halides using DBU as base, or by reductive alkylation with various substituted aryl aldehydes using standard reducing agents.

SAR for the R^2 position was initially assessed for a small group of compounds (**22a**-**23c**), as shown in Table 2. In general, the heterocyclic group-containing compounds **22c**, **22d**, **23b** and **23c** (1-methyl-1*H*-3-indolyl, and 5-isoquinolinyl, >68% inhibition at 10 μM) had greater antagonistic activity than the simple aromatic ring-containing compounds **22a**, **22b**, and **23a** (naphthyl and phenyl, <46% inhibition at 10 μM). Therefore, subsequent SAR study assays utilized a combination of substitutions at the R^3 position, together with 1-methyl-1*H*-3-indolyl, and 5-isoquinolinyl substitutions at the R^2 position. In the case of 5-isoquinolinyl analogs (**22d**, **23c–i**), despite the appreciable activity (IC_{50} = 0.79 μM) of **22d**, where there is no substitution at R^3 position, neither substitution of a benzyl (**23c**–**g**) or 1-methyl indole (**23h**) moiety enhanced activity. However, a *p*-OH-*m*-CH_3-benzyl moiety at the R^3 position (**23i**) had comparable activity (IC_{50} = 0.74 μM) as **22d**. In contrast, for the 1-methyl-1*H*-3-indolyl analog, **23b**, introduction of a benzyl group at the R^3 position dramatically increased the inhibitory

Table 2
Activities of synthesized compounds **22a–23u** and KN-62 on ethidium accumulation in $hP2X_7$-expressing HEK293 cells and on IL-1β release by LPS/IFNγ-differentiated human THP-1 cells[a]



Compd	R^2	R^3[b]	% Inhibition (10 μM)		IC_{50}[c] on HEK293 cells (μM)
			HEK293 cells[29]	THP-1 cells[30]	
KN62 (positive control)			92 ± 3	103 ± 5	0.11 ± 0.02
22a	1Nph	H	46 ± 13		
22b	phenyl	H	33 ± 6		
22c	3MIn	H	43 ± 3		
22d	5-IQ	H	71 ± 5	44 ± 26	0.79 ± 0.19
23a	1Nph	Bn	44 ± 0	—	
23b	3MIn	Bn	94 ± 4	105 ± 9	0.31 ± 0.08
23c	5-IQ	Bn	68 ± 9	42 ± 37	1.45 ± 0.18
23d	5-IQ	*p*-F-Bn	75 ± 2	65 ± 24	1.28 ± 0.23
23e	5-IQ	*p*-Br-Bn	38 ± 14		
23f	5-IQ	*p*-CH_3-Bn	69 ± 19	48 ± 12	1.18 ± 0.28
23g	5-IQ	*m*-CH_3-Bn	42 ± 32		
23h	5-IQ	1-methyl indole	33 ± 2		
23i	5-IQ	*p*-OH-*m*-CH_3-Bn	84 ± 4	51 ± 17	0.74 ± 0.05
23j	3MIn	*p*-F-Bn	39 ± 9		
23k	3MIn	*p*-Cl-Bn	94 ± 10	55 ± 10	0.25 ± 0.20
23l	3MIn	*p*-Br-Bn	88 ± 9	50 ± 22	0.85 ± 0.36
23m	3MIn	*p*-CH_3-Bn	87 ± 5	38 ± 27	0.52 ± 0.04
23n	3MIn	*p*-OCH_3-Bn	29 ± 3		
23o	3MIn	*p*-NO_2-Bn	65 ± 9	41 ± 16	1.42 ± 0.29
23p	3MIn	*m*-CH_3-Bn	46 ± 11		
23q	3MIn	*m*-F-Bn	45 ± 14		
23r	3MIn	*p*-OH-*m*-CH_3-Bn	88 ± 4	37 ± 18	0.18 ± 0.04
23s	3MIn	1-Methyl indole	68 ± 1	65 ± 15	1.07 ± 0.24
23t	3MIn	1-Methyl naphthyl	48 ± 10		
23u	(*S*)-3MIn	*p*-OH-*m*-CH_3-Bn	30 ± 5		

[a] Data are expressed as means ± SDs. All experiments were repeated at least 2–3 times.
[b] Abbreviations: 1Nph, 1-naphthyl; 3Min, 1-methyl-1*H*-3-indolyl; 5-IQ, 5-isoquinolinyl.
[c] IC_{50} = 50% inhibitory concentration, representing the mean from dose–response curves of at least three experiments.

activity from 43% to 94% at 10 μM, showing an IC_{50} value of 0.31 μM. Therefore, the effect of positional substitutions of benzyl group at R^3 position was assessed by comparing compounds **23j–r** with **23b**.

The antagonistic activity of *p*-F and *m*-F substituted compounds **23j** and **23q** was significantly reduced. In the case of methyl substitutions, the compound containing a *p*-CH_3 benzyl group (**23m**) had an IC_{50} of 0.52 μM, whereas the *m*-CH_3 substituted compound (**23p**) showed reduced activity. Interestingly, the *p*-methoxy analog, **23n**, showed a significant decrease in activity, with only 29% inhibition at 10 μM. The activity of the *p*-nitro substituted compound (**23o**) was somewhat decreased. Unlike the compound with fluoro substitution, compounds with *p*-Cl (**23k**) and *p*-Br-benzyl (**23l**) groups recovered activity. In particular, the *p*-Cl-substituted analog, **23k**, showed slightly higher activity than **23b** with an IC_{50} of 0.25 μM. Notably, the *para* and *meta* disubstituted benzyl moiety (**23r**) was the most potent antagonist among the series of pyrazolodiazepine-8-ones we tested. In the EtBr-uptake inhibition assay, this compound had an IC_{50} of 0.18 μM, and there was an apparent enantiomeric preference for the (*R*)-isomer of the 1-methyl-1*H*-3-indolyl group when compared with the corresponding (*S*)-isomer, **23u**.

As a second assay for functional antagonism, compounds displaying >60% inhibition at 10 μM in the EtBr uptake inhibition assay were also investigated for their ability to inhibit IL-1β release from 1 mM BzATP-activated LPS/IFNγ-differentiated human THP-1 cells. Although the activity profiles of these analogs differed in the two assays, one of the potent compounds **23b** showed parallel functional activity, displaying ~100% inhibition of IL-1β release at 10 μM. However, the most potent analog in the EtBr uptake assay, compound **23r**, showed only 37% inhibition of IL-1β release at 10 μM. Compounds **23d** and **23s**, both of which had moderate activity in the EtBr uptake assay, showed parallel inhibitory activity in the IL-1β release assay, with over 65% inhibition at 10 μM. Figure 2 shows that compound **23b** exhibited dose-dependent inhibition of IL-1β release, and similar potency in the EtBr uptake assay (IC_{50} = 207 nM) as KN-62 (IC_{50} = 166 nM).

In conclusion, we have synthesized a new series of pyrazolodiazepine derivatives that are potent antagonists of $hP2X_7R$. SAR studies of the pyrazolodiazepine-8-one skeleton for $P2X_7R$ antagonism revealed that (1) the *R*-configuration of the C-6 position of the diazepine ring was preferred to the *S*-configuration, (2) a 1-methyl-1*H*-3-indolyl moiety brought an improvement and preferable to a 5-isoquinolinyl moiety for antagonist activity, and (3) the size and position of substituents on the benzyl moiety at the R^3 position had an important effect on the biological activity of 1-methyl-1*H*-3-indolyl pyrazolodiazepine derivatives. In particular, compound **23b**, which contains a phenethyl group at R^1, a 1-methyl-1*H*-3-indolyl group at R^2 and a benzyl group at R^3, presented a combination of potent antagonism, for both IL-1β release and EtBr uptake. These results indicate that modification of the pyrazolodiazepin-8-one skeleton may be useful in designing compounds for therapeutic intervention in $P2X_7$ receptor related diseases.

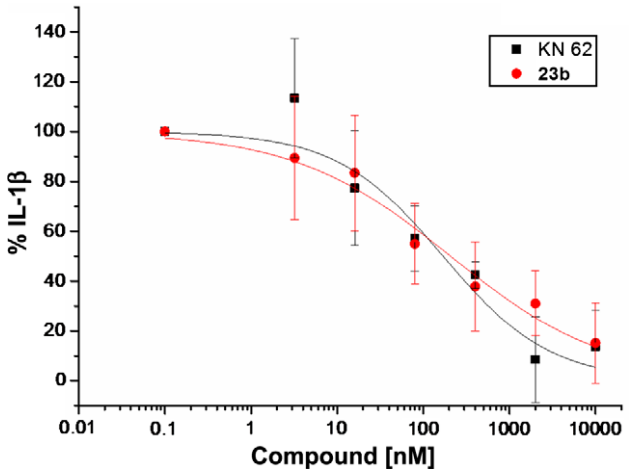


Figure 2. Concentration-dependent inhibition of BzATP-stimulated IL-1β release in LPS/IFNγ-differentiated human THP-1 cells by compound **23b** and KN-62. Data points represent means ± SD of values obtained (n = 3).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.09.053.

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29. All experiments were performed using adherent HEK293 cells stably transfected with cDNA encoding the human $P2X_7$ receptor. Synthesized pyrazolodiazepin-8-one derivatives were 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).
30. 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 pyrazolodiazepin-8-one derivatives at 10 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.