Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 681–684

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis of Conformationally Constrained Analogues of KN62, a Potent Antagonist of the $P2X_7$-Receptor

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Received 16 November 1999; accepted 3 February 2000

Abstract—Conformationally constrained analogues of KN62 containing 1,2,3,4-tetrahydro-7-hydroxyisoquinoline-3-carboxylic acid with *S* configuration in position 3 were synthesized and their antagonist activities were tested on human macrophage cells. While KN62 is a potent antagonist of the $P2X_7$ receptor, these analogues were inactive as antagonists and only one compound showed appreciable activity as $P2X_7$ antagonist, which was 30 times weaker than that reported for KN62.

Introduction

It is increasingly recognized that extracellular ATP acts as an extracellular messenger and mediates a wide range of effects by stimulating purinergic P2 receptors,[1] which are considered a promising new target for anti-inflammatory drug development. P2 receptor subtypes comprise an ionotropic ($P2X_{1-7}$) and a metabotropic ($P2Y_{1-11}$) family, and increasing evidence suggests that they are involved in the modulation of the immune and inflammatory responses. Unfortunately, characterization of native and recombinant P2 receptor continues to be hindered by the lack of specific and subtype-selective antagonists.

One of the most interesting members of the ionotropic P2X family is $P2X_7$. This receptor is mainly, if not exclusively, expressed by mononuclear phagocytes, where it mediates cytotoxic responses, cytochine release and cell fusion.[2,3] The $P2X_7$ receptor is formed by the assembly of an unknown number of subunits each 595 AA long, and upon stimulation by extracellular ATP generates a nonselective membrane pore permeable to hydrophilic molecules with molecular weight up to 900 D.[4] Due to its likely involvement in immunomodulation, it would be of the most importance to develop selective $P2X_7$ antagonists. Human macrophages have been proven very useful for the evaluation of $P2X_7$ agonists and antagonists, and among these latter the KN62 (1-(*N*,0-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosil)-4-phenylpiperazine) compound is, to the best of our knowledge, the most potent with IC_{50}, for inhibition of ATP-stimulated Ca^{2+} influx into fura-2 loaded human macrophage cells, of 13.4 nM and complete inhibition at a concentration of 500 nM.[5] The same compound selectively inhibited Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II)[6] activity and, at non-cytotoxic concentrations (2 μM), it enhanced etoposide cytotoxicity in Adriamycin-resistant cells (HL-60).[7]

In an attempt to improve the activity and to study the structure–activity relationship of KN62 inhibition on $P2X_7$ receptor, tethering the *N*-methyl of the tyrosine backbone to the *ortho*-position of the phenyl ring has resulted in a series of conformationally constrained KN62 analogues with the formula **1**–**5**. These derivatives have been synthesized in order to restrict conformational freedom and to stabilize the desired bioactive conformation of KN62. The derivatives **1**–**5** possess 1,2,3,4-tetrahydro-isoquinoline (Tic) moiety, that can be considered as a cyclic tyrosine in which the side chain orientation has been fixed by the methylene unit which bridges the 2′ position in the aromatic ring and the α-nitrogen. While the compound **1** contains the isoquinoline-5-sulfonyl moiety which acts as "ATP mimic",[8] in the derivatives **2** and **3** this function has been substituted with two isomers corresponding to

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0960-894X/00/$ - see front matter
PII: S0960-894X(00)00083-4

quinoline-5 and 8-sulfonyl, respectively. With the aim to study the binding capacity of the isoquinoline nitrogen to the $P2X_7$ receptor, we have considered the replacement of one or both of these moieties with the same number of naphtalenic rings to obtain the derivatives **4** and **5**, respectively.

Chemistry

The Pictet–Spengler reaction has been widely used for the synthesis of a variety of alkaloids because of generally good yields and mild reaction conditions.[9] This reaction has been applied as a key step to access to the tetrahydroisoquinoline moiety present in the conformationally constrained analogues **1**–**5** of KN62.

The preparation of **1**–**5** has been accomplished by a synthetic sequence according to Scheme 1. The compound **6** has been easily obtained by already published procedure[10] and transformed into the corresponding benzyloxycarbonyl (Z or CBz) derivative **7** by action of Z-OSu/TEA system in dry DMF. The protected precursor **7** was converted to the activated 1-hydroxy-1, 2,3-benzotriazole (HOBT) ester and condensed with the *N*-phenylpiperazine to give rise the amide **8**. This latter compound has been dehalogenated by catalytic hydrogenation with 10% Pd/C and in presence of TEA, without removal of Z protecting group, furnishing the *N*-CBz-protected **9**. The compounds **10**–**14** were prepared from the anion of **9** (generated with NaH) by treatment with a CH_2Cl_2 solution of the appropriate sulfonyl chloride.[11] The protecting CBz group in **10**–**14** was conveniently cleaved off by the use of 3 equiv of $(CH_3)_3SiI$ in acetonitrile[12] and furnished **15**–**19**, respectively, in good yields. Finally, the sulphonamides **1**–**5** were synthesized from the amines **15**–**19** by treatment with the appropriate sulfonyl chloride.[11]

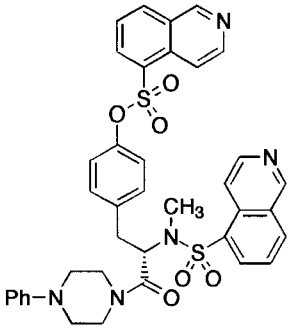
Results and Discussion

The antagonist activity of KN62 and derivatives **1**–**5** was determined by the measurements of the ATP-stimulated Ca^{2+} influx into macrophages loaded with the fluorescent indicator fura-2-acetoxymethyl ester (fura-2-AM) as described previously.[13] Results are expressed as percentage inhibition of maximal response to 1 mM ATP in the absence of inhibitor, which was defined as 100% response.

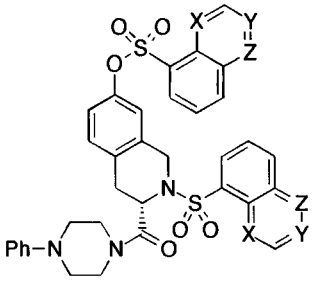
The compounds **1**–**5** synthesized had no effect on the $P2X_7$ receptor and for the derivatives **1**–**3** of this series, their inhibitory effects at this receptor was reversed when their concentrations were increased. This phenomenon could be explained from the appearance of cytotoxic effects or precipitation at high concentrations. The reference compound KN62 was considerably more potent than **5** (IC_{50}, 13.4 nM for KN62 versus 316 nM for **5**), whereas **1**–**4** are inactive. As can be seen in Table 1, the compound **5** was the most active antagonist in this series and blocked more markedly the $P2X_7$ receptor than did the other compounds **1**–**4**. It is noteworthy that the constrained form of KN62, corresponding to derivative **1**,[14] lost completely antagonist properties of the parent compound. In fact, the compound **1**, at the concentration of 50 nM, only inhibited the Ca^{2+} influx by 1.66%, while at the same concentration, KN62 shows a 94% of inhibition.

Concerning the derivative **2**, where the isoquinoline-5-sulfonyl moiety has been substituted with quinoline-5-sulfonyl, at the concentration of 50 nM it shows a 59% inhibition, i.e. a value slight lower than that reported for KN62 at the same concentration. The compound **3**, which possess the quinoline-8-sulfonyl instead of isoquinoline-5-sulfonyl moiety, shows a behaviour comparable than that of **1**, since its antagonist activities decreased with the increasing of the concentration. In the compound **1**, the substitution of the 5-isoquinolinesulfonyl residue linked to the nitrogen with a naphthalene lead to the derivative **5**, which is, if compared to the other derivatives **1**–**4**, the most potent inhibitor in this series. Nevertheless, the IC_{50} value of **5** was 316 nM, almost 30-fold higher than that reported for KN62. Replacing the isoquinoline with naphthalene provides **4**, which shows no capability to inhibit the Ca^{2+} influx at any concentration.

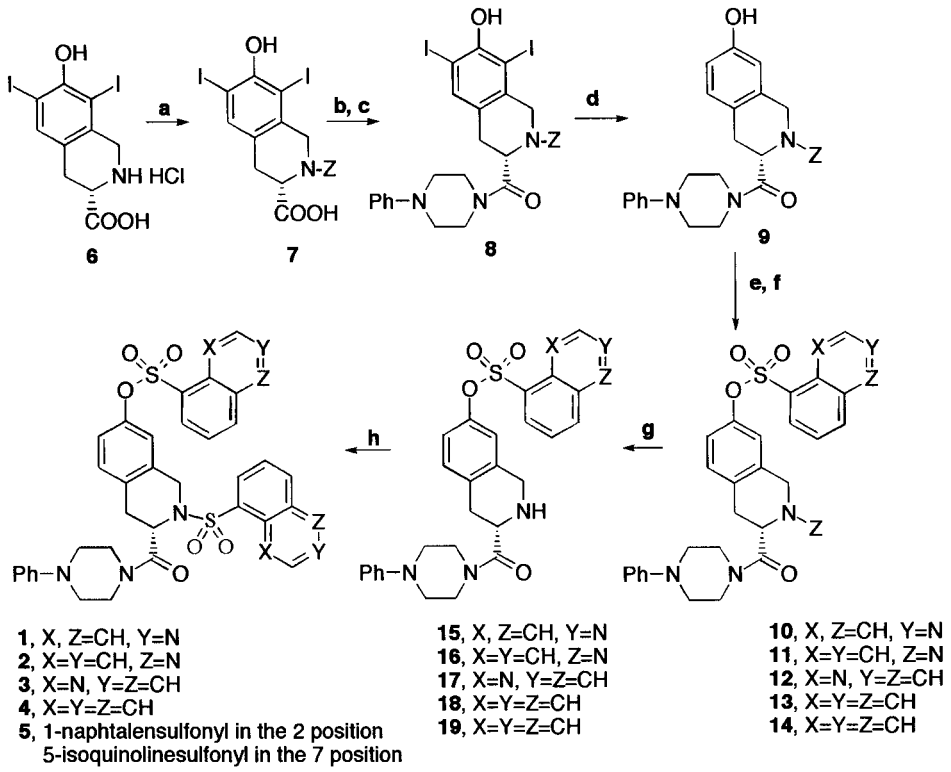
Comparing the antagonist activities of **1** and KN62, the results confirm that an extended rather than folded conformation of KN62 is preferred at $P2X_7$ receptor.



KN62



1, X, Z=CH, Y=N
2, X=Y=CH, Z=N
3, X=N, Y=Z=CH
4, X=Y=Z=CH
5, 1-naphtalensulfonyl in the 2 position
5-isoquinolinesulfonyl in the 7 position



Scheme 1. Reagents: (a) Z-Osu, TEA, DMF, rt; (b) HOBt, DCC, DMF, 70°C, 4 h; (c) 4-Pheylpiperazine, rt, 18 h; (d) H_2, Pd/C, TEA, dioxane/EtOH, rt, 6 h; (e) NaH, dioxane, rt, 15 min; (f) appropriate solfonyl chloride (see ref 11), TEA, dioxane, rt, 18 h; (g) $(CH_3)_3Sil$, CH_3CN, 1 h at 0°C and then 4 h at rt; (h) appropriate solfonyl chloride (see ref 11), TEA, dioxane, rt, 18 h.

Table 1. Activities of synthesized compounds **1**–**5** and KN62 on the calcium influx

Compound	% Inhibition of Ca^{2+} influx with different concentration of inhibitor			IC_{50}[a] (nM)
	50 nM	500 nM	1000 nM	
1	1.66 ± 0.42	34.48 ± 5.26	25.80 ± 3.26	n.d.[b]
2	59.05 ± 8.04	72.36 ± 8.11	66 ± 5.58	n.d.
3	6.90 ± 1.11	3.40 ± 0.48	0	n.d.
4	0	0	0	n.d.
5	17.63 ± 2.36	58.80 ± 7.18	58.80 ± 8.22	316 ± 38.6
KN62	94.4 ± 4.7	100	100	13.4 ± 1.4

[a]IC_{50} = 50% inhibitory concentration represents the mean from dose–response curves of at least three experiments. All experiments were repeated three times.
[b]n.d. = not determined.

Therefore in the synthesized compounds **1**–**5**, the incorporated conformational constraint probably completely inhibit the interaction with the $P2X_7$ receptor. The resulting compounds **1**–**5** were shown not to be $P2X_7$ antagonists and constraining the relatively mobile phenyl ethyl group of the tyrosine onto the framework of TIC did not yield the desired results.

Acknowledgement

We thank Ministero Università e Ricerca Scientifica (MURST) (60%) for generous financial support of this work.

References and Notes

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13. Di Virgilio, F.; Fasolato, C.; Steinberg, T. H. *Biochem. J.* **1988**, *256*, 959. Briefly, washed cells ($10^6 \times mL^{-1}$) suspended in HEPES buffered saline were loaded with 4 μM fura-2-acetoxymethyl ester and incubated in a thermostat-controlled (37 °C) for 20 min in the dark and magnetically stirred fluorometer cuvette. Cell suspension was incubated with KN62 or compounds **1**–**5** (25–500 nM) for 5 min at 37 °C before fluorimetric analysis in a stirred cuvette at 37 °C. Intracellular Ca^{2+} concentration was determined with the 340:380 nm excitation ratio at an emission wavelenght of 500 nm. All experiments were repeated three times.

14. Selected data for the compound **1.** Off white solid; mp (uncorrected): 142–146 °C (dec.); $[\alpha]_D^{25}$ (dioxane): +27.7 ($c = 0.6$). ^{1}H NMR (DMSO-d_6) δ: 2.96 (m, 2H), 3.22 (m, 4H), 3.76 (m, 4H), 4.49 (d, $J = 11$ Hz, 1H), 4.60 (d, $J = 12.6$ Hz, 1H), 5.40 (m, 1H), 6.46 (m, 2H), 6.83 (m, 4H), 7.23 (t, $J = 8$ Hz, 2H), 7.80 (t, $J = 7.2$ Hz, 1H), 7.87 (t, $J = 7.2$ Hz, 1H), 8.42 (m, 4H), 8.61 (t, $J = 8$ Hz, 2H), 8.82 (t, $J = 6.4$ Hz, 2H), 9.42 (s, 1H), 9.58 (s, 1H). IR (KBr) cm^{-1}: 3434, 1647, 1458, 1378, 1225, 1174, 828. Elemental analysis (calcd): C. 63.40; H. 4.62; N. 9.73; S. 8.91 (found): C. 62.98; H. 4.43; N. 9.42; S. 8.71.