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Bioorganic & Medicinal Chemistry Letters 14 (2004) 5709–5712
Bioorganic & Medicinal Chemistry Letters

Synthesis, radiolabeling, and preliminary biological evaluation of [^{3}H]-1-[(*S*)-*N*,*O*-bis-(isoquinolinesulfonyl)-*N*-methyl-tyrosyl]-4-(*o*-tolyl)-piperazine, a potent antagonist radioligand for the $P2X_7$ receptor

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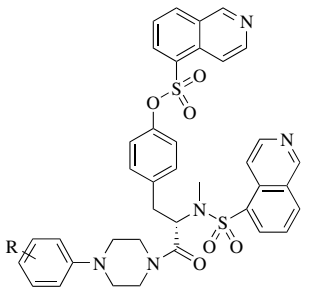
Received 18 June 2004; accepted 22 July 2004
Available online 16 September 2004

Abstract—The design, synthesis, and preliminary biological evaluation of the first potent radioligand antagonist for the $P2X_7$ receptor, named [^{3}H]-1-[(*S*)-*N*,*O*-bis-(isoquinolinesulfonyl)-*N*-methyl-tyrosyl]-4-(*o*-tolyl)-piperazine (compound **13**), are reported. This compound bound to human $P2X_7$ receptors expressed in HEK transfected cells with K_D and B_{max} value of 3.46 ± 0.1 nM and 727 ± 73 fmol/mg of protein, respectively. The high affinity and facile labeling makes it a promising radioligand for a further characterization of $P2X_7$ receptor subtype.

The $P2X_7$ receptor ($P2X_7R$) is an unusual nondesensitizing, cation selective, ion channel directly gated by extracellular ATP, and a few pharmacological ATP analogs.[1] This receptor, previously known as P2Z, was initially described in lymphocytes and macrophages and thought to be restricted to cells involved in inflammation and immunity.[2] However, we now know that, despite its elective expression in immunocytes, $P2X_7$ is also present in cells not primarily participating in immunomodulation.[3] The structural feature that most characteristically differentiates $P2X_7$ from the other six members of the P2X subfamily is the long (240 aa) cytoplasmic carboxy-terminal tail.[4] It is understood that many of the typical (and possibly exclusive) functional responses elicited by $P2X_7$ stimulation depend on this extended amino acid stretch.[5]

Expression of $P2X_7$ message or protein has been reported in cell types as different as macrophages[6] central neurons,[3] lymphocytes[7] epithelial cells,[8] endothelial cells,[9] and fibroblasts.[10] However, it is also well established that mononuclear phagocytes (macrophages, dendritic cells) express $P2X_7$ to the highest level, and these are the cells in which $P2X_7R$ functions have been best characterized.[2] There are few doubts that this receptor is one of the most potent stimuli for the release of



1a, KN 62, R=H
1b, R=o-CH_3

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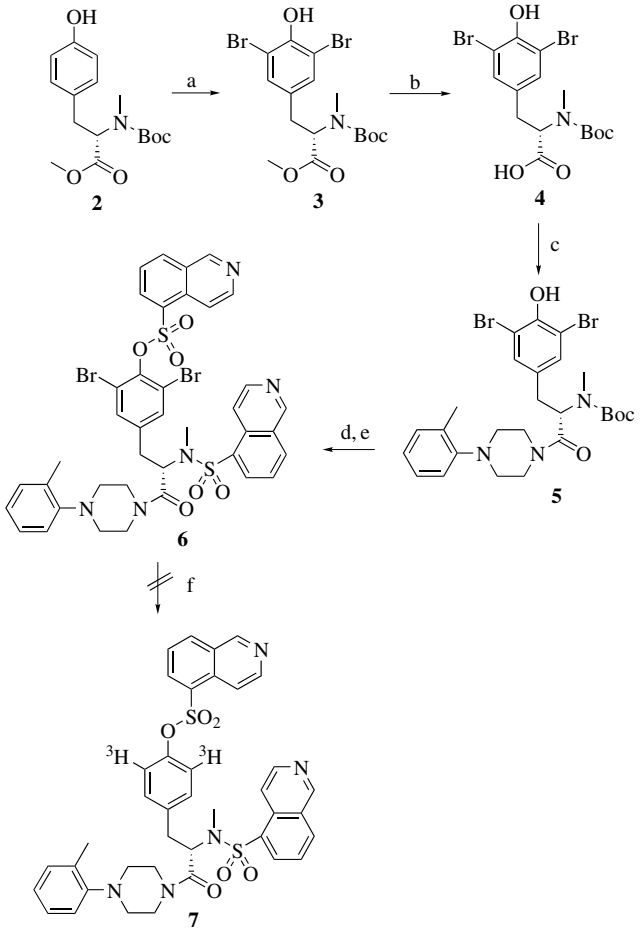
doi:10.1016/j.bmcl.2004.07.095



Scheme 1. Reagents and conditions: (a) Br_2, CH_2Cl_2, rt, 30 min; (b) LiOH, THF, H_2O, MeOH, rt, 1 h; (c) *o*-tolyl piperazine, HOBt, EDC, DMF, rt, 24 h; (d) TFA, CH_2Cl_2, rt; (e) isoquinoline sulfonyl chloride, TEA, CH_2Cl_2, rt, 18 h; (f) tritium gas, 10% Pd/C, EtOH.

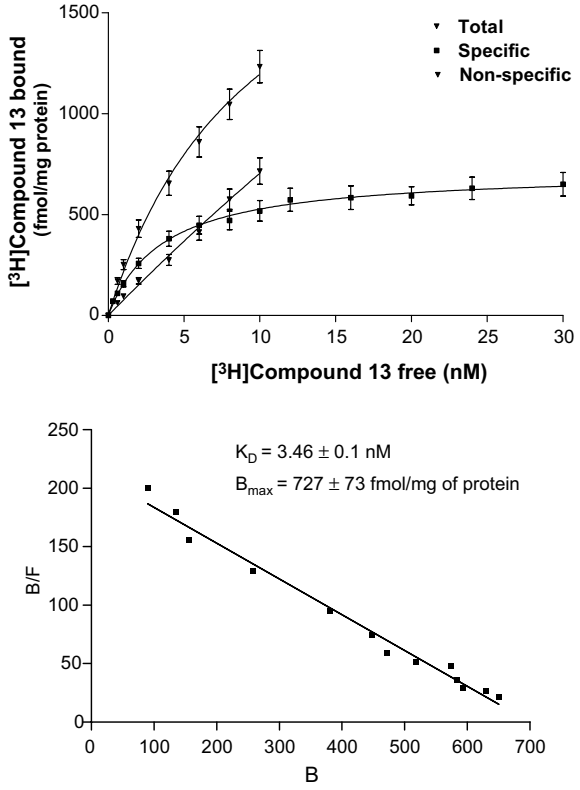
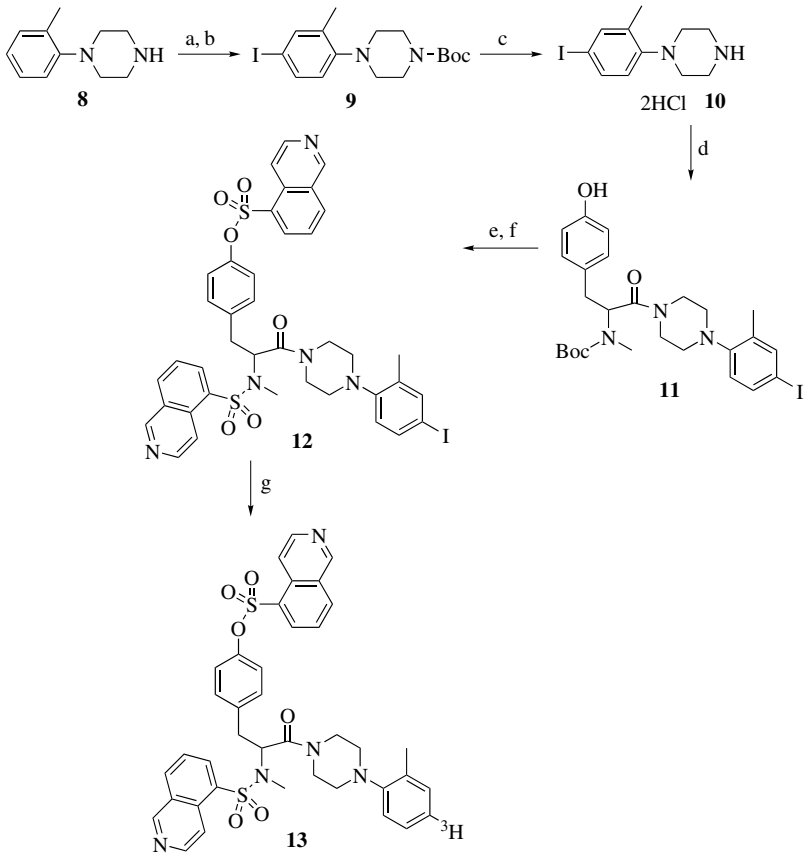


Figure 1. Saturation of **13** binding to human HEK $P2X_7$ transfected cells. In saturation experiments, 100 μL of membrane homogenate (80 μg protein/assay) was incubated in duplicate with 10–12 different concentrations of 13 in the range 0.3–30 nM. Nonspecific binding, defined as binding in the presence of 1 μM of **1b**, at the K_D value for the radioligand was 40% of total binding. Incubation time was 60 min at 25 °C to allow equilibrium to be reached. Values are the means and vertical lines ± SE mean of four separate experiments performed in triplicate. In the lower panel the Schatchard plot of the same data is shown. K_D value was 3.46 ± 0.1 nM and B_{max} value was 727 ± 73 fmol/mg of protein.

mature, biologically active, Interleukin-1β (IL-1β).[11] Besides IL-1β, $P2X_7$ also drives the release of other key pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and probably IL-6.[12] These features make $P2X_7$ a new potential immunomodulatory receptor.

Until recently, the lack of potent and selective ligands has hampered the development of radioligands to devise useful receptor binding assays. Our group has recently reported the identification and the functional antagonistic properties of a novel series of KN-62-related compounds with general structure **1**, focusing our attention on the systematic modification of the phenyl piperazine residue, by examining the effects due to the insertion of different chemical functionalities on the phenyl ring linked to the piperazine nitrogen.[13] The biological response investigated was the ATP-dependent Ca^{2+} influx across the plasma membrane of human monocytes. KN-62 (**1a**, $IC_{50} = 51$ nM) is characterized by the presence of a phenyl-piperazine moiety. In the series of synthesized compounds, which possess different substituents on the phenyl ring, the presence of a methyl group in the *ortho* position gives one of the most potent compounds of the whole series (**1b**, $IC_{50} = 15$ nM), which results 3-fold more potent than KN-62.[14] Starting from this result, we focused our attention on the radiolabeling of **1b** with tritium, which could furnish a useful tool to investigate the receptor distribution and pharmacology. The incorporation of tritium into the carbon framework of **1b** is, however, not easily performed.

The first approach to the synthesis of the radiolabeled form of **1b** is shown in Scheme 1, and uses (L)-*N*-Boc-*N*-methyl tyrosine methyl ester (**2**) as starting material.[15] Compound **2** was dibrominated at the *ortho* positions to the phenolic hydroxyl group to yield the derivative **3**. This intermediate was converted into the corresponding carboxylic acid derivative **4** after saponification. The subsequent condensation with *o*-tolyl piperazine furnished the amide **5** in good yield. The protecting Boc-group in **5** was conveniently removed by the use of TFA and furnished the corresponding free amine, which was then coupled with an excess of 5-isoquinolinesulfonyl chloride[16] to deliver the precursor **6**. Unfortunately, the dehalogenation with tritium gas in presence of 10% Pd/C, did not furnish the final compound **7**, affording



Scheme 2. Reagents and conditions: (a) $(Boc)_2O$, TEA, DCM, 24 h; (b) IPy_2BF_4, CH_2Cl_2, rt, 1 h; (c) HCl/MeOH, rt, 2 h; (d) Boc-*N*-methyl tyrosine, HOBt, EDC, DMF, rt, 24 h; (e) TFA, CH_2Cl_2, rt; (f) TEA, isoquinoline sulfonyl chloride, CH_2Cl_2, rt, 18 h; (g) tritium gas, 10% Pd/C, EtOH.

instead a degradation product in which one of the isoquinolines was lost.

An alternative approach was employed with the aim of incorporating the label in a different position of compound **1b** in the last step of the synthesis (Scheme 2). Commercially available *o*-tolyl piperazine **8** was converted quantitatively into the corresponding *N*-Boc derivative and then efficiently monoiodinated on the aromatic ring using IPy_2BF_4[17] to furnish the corresponding *p*-iodo-*o*-tolyl *N*-Boc piperazine **9**. The subsequent *N*-Boc deprotection of **9**, to yield **10**, followed by condensation with *N*-Boc-*N*-methyl tyrosine, gave the amide **11**. Removal of the Boc-group was performed with TFA, followed by condensation with an excess of 5-isoquinolinesulfonyl chloride to provide the precursor **12**.[18] The final compound **13** ([^{3}H]-**1b**) was obtained by dehalogenation of **12** with tritium gas in the presence of 10% Pd/C followed by HPLC purification in good yield (60%) and with a radiochemical purity >95%.

Figure 1 shows a saturation curve of the binding of [^{3}H]-**1b** (compound **13**) to the human $P2X_7$ receptors expressed in HEK 293 cells. The linearity of the Scatchard plot in the inset is indicative, in our experimental conditions, of the presence of a single class of binding sites with a B_{max} value of 727 ± 73 fmol/mg of protein and K_D value of 3.46 ± 0.1 nM ($n = 4$), comparable to that determined by kinetic experiments. Kinetic studies showed that at 25 °C, compound **13** binding reached equilibrium within 50 min. Association and dissociation were fitted to a one-component model significantly better than a two-component model ($P < 0.05$). Calculations based on the kinetic data gave an observed association constant (K_{obs}) of $0.046 \pm 0.002\,min^{-1}$ with a corresponding association rate constant (k_{+1}) of $0.007\,min^{-1}\,nM^{-1}$ in HEK 293 cells. Compound **13** binding was rapidly reversed by the addition of 1 μM **1b**, giving a dissociation rate constant (k_{-1}) of $0.025 \pm 0.001\,min^{-1}$. From the rates of association and dissociation, the equilibrium constant $K_D = k_{-1}/k_{+1}$ was estimated to be 3.57 nM.

Displacement studies of [^{3}H]-**1b** (compound **13**) with the nonlabeled **1b** led to an IC_{50} value of the same order of magnitude as in the functional assay. In summary, based upon these findings, the in vitro characterization of compound **13** indicates that this new radioligand should be considered a very useful tool to study the human $P2X_7$ receptor subtype.

Acknowledgements

The authors would like to thank King Pharmaceuticals Research and Development, Inc. for financial support and Amersham Biosciences for tritium gas

dehalogenation of precursor **12** and Prof. Alan North, Institute of Molecular Physiology, University of Sheffield, Sheffield, England, for supplying human HEK $P2X_7$ transfected cells.

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18. Characterization of compound **12**. White solid, mp = 110–112 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.21 (s, 3H), 2.33 (m, 1H), 2.52 (dd, J = 12 and 4.2 Hz, 1H), 2.65 (m, 2H), 3.04 (s, 3H), 3.28 (m, 2H), 3.56 (m, 4H), 5.14 (dd, J = 10.2 and 4.4 Hz, 1H), 6.58 (d, J = 8.4 Hz, 1H), 6.82 (d, J = 8.6 Hz, 2H), 7.00 (d, J = 8.6 Hz, 2H), 7.48 (t, J = 7.8 Hz, 1H), 7.51 (s, 1H), 7.59 (t, J = 7.8 Hz, 1H), 7.71 (t, J = 7.8 Hz, 1H), 8.33 (dt, J = 8.4 and 6.0 Hz, 4H), 8.43 (d, J = 6.0 Hz, 1H), 8.52 (d, J = 6.0 Hz, 1H), 8.69 (d, J = 6.0 Hz, 1H), 8.84 (d, J = 6.0 Hz, 1H), 9.36 (s, 1H), 9.43 (s, 1H).