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Structure-based identification and characterisation of structurally novel human P2X7 receptor antagonists

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ABSTRACT

The P2X7 receptor (P2X7R) plays an important role in diverse conditions associated with tissue damage and inflammation, meaning that the human P2X7R (hP2X7R) is an attractive therapeutic target. The crystal structures of the zebrafish P2X4R in the closed and ATP-bound open states provide an unprecedented opportunity for structure-guided identification of new ligands. The present study performed virtual screening of ~100,000 structurally diverse compounds against the ATP-binding pocket in the hP2X7R. This identified three compounds (C23, C40 and C60) out of 73 top-ranked compounds by testing against hP2X7R-mediated Ca^{2+} responses. These compounds were further characterised using Ca^{2+} imaging, patch-clamp current recording, YO-PRO-1 uptake and propidium iodide cell death assays. All three compounds inhibited BzATP-induced Ca^{2+} responses concentration-dependently with IC_{50}s of 5.1 ± 0.3 μM, 4.8 ± 0.8 μM and 3.2 ± 0.2 μM, respectively. C23 and C40 inhibited BzATP-induced currents in a reversible and concentration-dependent manner, with IC_{50}s of 0.35 ± 0.3 μM and 1.2 ± 0.1 μM, respectively, but surprisingly C60 did not affect BzATP-induced currents up to 100 μM. They suppressed BzATP-induced YO-PRO-1 uptake with IC_{50}s of 1.8 ± 0.9 μM, 1.0 ± 0.1 μM and 0.8 ± 0.2 μM, respectively. Furthermore, these three compounds strongly protected against ATP-induced cell death. Among them, C40 and C60 exhibited strong specificity towards the hP2X7R over the hP2X4R and rP2X3R. In conclusion, our study reports the identification of three novel hP2X7R antagonists with micromolar potency for the first time using a structure-based approach, including the first P2X7R antagonist with preferential inhibition of large pore formation.

1. Introduction

P2X receptors are a family of trimeric protein complexes that function as ATP-gated calcium-permeable, nonselective cationic channels [32]. The P2X7 receptor (P2X7R), the last identified member of the P2X receptor family, acts as an ion channel during brief stimulation. However, prolonged receptor activation can induce the formation of large pores which allow the passage of molecules of up to 900 Da in size which ultimately results in cell death. Because of this, the P2X7R is known as a cytolytic receptor [42]. The P2X7R is found in almost all tissues of the body, being expressed in particular in cells of haematopoietic origin [22]. It plays an important role in a diversity of conditions associated with tissue damage or inflammation including chronic pain, rheumatoid arthritis and age-related macular degeneration, making the P2X7R an attractive therapeutic target [33,38,1]. Huge drug discovery efforts over the past few years have led to the discovery of numerous P2X7R antagonists [17,41,27]. Whilst clinical trials of the first two P2X7R antagonists against rheumatoid arthritis showed disappointing outcomes [26,39], a more recent clinical trial shows promise in the therapeutic use of a P2X7R antagonist (AZD9056) in the treatment of moderate-to-severe Crohn's disease [10].

In order to exploit the full potential of P2X7R antagonists as therapeutics, further efforts are inevitably required to develop more cost-effective and efficient drug discovery approaches. These will help to expand the structural diversity of P2X7R antagonists as well as to develop tools which will help us better understand the role of the P2X7R in disease mechanisms, particularly its large pore formation [37,43,14]. None of the known antagonists can

Abbreviations: zfP2X4R, zebrafish P2X4 receptor; P2X7R, P2X7 receptor; hP2X7R, human P2X7R; rP2X7R, rat P2X7R; BzATP, 2,3-O-(4-benzoylbenzoyl)-ATP; PI, propidium iodide; BBG, brilliant blue G; TNP-ATP, trinitrophenyl-ATP; HEK293 cells, human embryonic kidney 293 cells.

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definitively discriminate between the ion channel and large pore-forming functionalities of the P2X7R. Furthermore, all the currently available P2X7R antagonists have been discovered by high-throughput screening which involves the random testing of large compound libraries against the receptor; a process that is often time-consuming, costly and inefficient. A relatively recent breakthrough in the study of P2X receptors is the determination of the atomic structures of the zebrafish P2X4R (zfP2X4R) in both the apo, closed, and ATP-bound, open, states [18]. Homology models of the P2X7R have been useful in providing structural insights into the effects of disease-associated mutations on P2X7R function [7,35,2,24] and the striking species difference of P2X7R antagonists [6]. The key residues coordinating ATP binding identified in the ATP-bound zfP2X4R structure are almost completely conserved through the mammalian P2X receptors [4] but not all the residues participating in the formation of the ATP-binding pocket are identical [24]. The ATP binding pocket in itself is unconventional, not consisting of a well-known ATP binding motif such as the Walker motif. As such, this region is a favourable target for therapeutic compounds due to its reduced likelihood of interactions with other vital ATP-binding proteins. Such structural features offer a feasible opportunity permitting the structure-based identification of small molecules which bind to this ATP-binding pocket with receptor-subtype specificity. In the present study, we used a sphere 10 Å in diameter encompassing the ATP-binding pocket in the hP2X7R homology model in the virtual screening of a structurally diverse compound database. In combination with functional assays, we have identified three structurally novel hP2X7R antagonists, including the first hP2X7R antagonist which displays selective inhibition of the large pore formation without having an effect on the ion channel function.

2. Materials and methods

2.1. Chemicals

General chemicals used in the study, ATP, 2,3-O-(4-benzoylbenzoyl)-ATP (BzATP), trinitrophenyl-ATP (TNP-ATP), brilliant blue G (BBG), YO-PRO-1 iodide, propidium iodide (PI) and G418 were purchased from Sigma (Dorset, UK). AZ11645373 and 5-BDBD were from Tocris Bioscience (Bristol, UK). All 73 compounds tested in the study (C1-C73) were sourced from Enamine (Ukraine) with a purity of $\geqslant$93% determined by high performance liquid chromatography analysis.

2.2. Homology modelling

Structural models of the human and rat P2X7R were produced based on the atomic structures of the zfP2X4R in the apo, closed state and ATP-bound open state (Protein Data Bank code 4DW0 and 4DW1, respectively) using Modeller version 9.12 [11], as described in our previous studies [21,6]. In brief, one hundred versions of the P2X7R were generated for each model and the five with the lowest energy were analysed using MolProbity [8]. Those with the greatest percentage of residues in allowed regions of the Ramachandran plot were selected for use in further investigations. The non-conserved loop region between the β2 and β3 strands was modelled de novo using the ModLoop server [13].

2.3. Virtual screening

Virtual screening was carried out using eHiTS version 12 [45]. The ATP-binding pocket file used in the screening was produced in SPROUT [16] and consisted of a sphere 10 Å in diameter centred on the bound ATP molecule. The ZINC12 database, containing approximately 100,000 structurally diverse compounds [20], were docked to this ATP-binding pocket. 500 of the best eHiTS scoring compounds were further scored using SPROUT. 42 of the 50 compounds (C1–C42) ranked with the highest predicted energy binding scores were commercially available and were tested in the initial functional assay. Further screening of the ZINC12 database was performed based on structural similarity using the ZINC12 website search function in order to carry out 'structure-activity relationships by catalogue' against the common shapes and functional groups of the two hits (C23 and C40). The top 31 compounds (C43–C73) with >80% structural similarity were tested in functional assays. PyMOL [9] was used for the visual inspection of ligand-receptor interactions and later graphical representations.

2.4. Cell culture

Human embryonic kidney (HEK) 293 cells stably expressing C-terminally EE-tagged human or rat P2X7R [42,34], human P2X4R [15] and rat P2X3R [28] were generated in previous studies. A stable HEK293 cell line expressing C-terminally His-tagged human P2X7R was generated in this study and there was no difference in the experimental results using EE-tagged and His-tagged human P2X7R. These cells were maintained in Dulbecco's Modified Eagle Medium (Life Technologies) supplemented with 10% foetal bovine serum and 200 ng/ml G418 in a tissue culture incubator at 37°C and 5% CO_2 under humidified conditions. Cells were cultured in T25 flasks and split (every 3–4 days) once confluent.

2.5. Calcium measurements

FlexStation II and III (Molecular Devices) were used to measure intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). Human or rat P2X7R-expressing HEK293 cells were plated in poly-D-lysine-coated 96-well plates (Molecular Devices) with 50,000 cells per well 24 h prior to use. Cells were rinsed with standard buffer solution (SBS) containing: 134 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$ and 1.5 mM $CaCl_2$, 8 mM glucose, 2.4 mM HEPES, pH 7.4. After 100 μl of loading buffer consisting of SBS containing 1 μM Fura-2/AM (Life Technologies) and 0.01% (v:v) pluronic acid (ThermoFisher) was added to each well, the plate was incubated at 37 °C for 45 min. Cells were rinsed once with SBS, after which 160 μl of SBS containing either 0.01–0.02% dimethyl sulphoxide (DMSO) used to prepare compound stock solutions or test compound were added to each well. The plate was incubated at 37 °C for 30 min. Cells were excited at 340 nm and 380 nm alternatively and the emission at 510 nm recorded using a FlexStation. The ratio of fluorescence intensity (F340/F380) was used to indicate the $[Ca^{2+}]_i$. The basal $[Ca^{2+}]_i$ was recorded for 60 s prior to the exposure to agonist and recording was continued for a further 120 s. BzATP at 300 μM was used to evoke maximal activation of human and rat P2X7R (e.g. [3]), and ATP at 100 μM at the hP2X4R and rP2X3R [28,15]. The maximal changes in F340/F380 (ΔF340/380) were used for quantitative analysis.

2.6. Patch-clamp recording

Membrane ionic currents were measured using patch-clamp recording in the whole-cell configuration at room temperature using an Axopatch 200B amplifier and analysed with pClamp 10.3 software (Axon Instruments) as described in our previous study [2]. In brief, HEK293 cells expressing the hP2X7R were seeded onto 10-mm glass cover slips prior to use. The cover slip was placed in a recording chamber connected to a solution exchange system driven by a gravity feed at a rate of 5 ml min^{-1}. Patch microelectrodes with a resistance of approximately 1–5 MΩ were produced using borosilicate glass capillaries (World

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