Supplementary information

Targeting the P2X7 receptor in rheumatoid arthritis: biological rationale for P2X7 antagonism

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Supplementary methods

In vitro culture methodology

BzATP-induced release of IL-1β and IL-18 from human isolated peripheral blood monocytes: Human peripheral blood monocytes were prepared from the blood of healthy human volunteers collected in EDTA blood tubes (product code 021066001; Sarstedt Monovette®, Germany). Dextran T500 (product code 17-0320-01; Amershams Pharmacia Biotech, Sweden) was added to each 9 mL tube. The tube was mixed by inversion and left to stand for 45 minutes at room temperature, allowing the red blood cells to sediment. The upper, straw-coloured, white blood cell (WBC)-containing layer was layered (typically 5 mL) onto 2.5 mL buffer (1% [v/v] DMSO in RPMI assay buffer containing fetal bovine serum [FBS] (10% v/v), Sigma C8051), and incubated at 37°C (5% CO2, 95% humidity). After this time, supernatants were transferred to a 96-well Sero-Wel plate and frozen for subsequent cytokine measurements using anti-human interleukin-1β (hIL-1β) capture antibody, lyophilised reconstituted hIL-1β standard and biotinylated anti-hIL-1β (all in product code OptELISA set 2687KI) or IL-18 enzyme-linked immunosorbent assay (ELISA) (product code MBL7620; R&D systems, UK).

ATP-induced release of IL-1β from human blood: Blood was collected into lithium heparin-coated (final concentration 10–30 IU/mL) tubes (product code 02.1065.001; Sarstedt, Leicester, UK) by venepuncture from each of 11 blood donors from the AstraZeneca R&D Charnwood healthy volunteer panel. Heparinised blood was diluted 1:1 with HEPES-buffered RPMI. Following addition of LPS (1 ng/mL, Sigma L4391), aliquots (160 μL/well) of diluted blood were added to 96-well plates (product code 611F96; Bibby Sterlin, Stone, UK) and incubated for 160 minutes at 37°C. Vehicle (1% [v/v] DMSO in HEPES-buffered RPMI) or the appropriate solution of AZD9056 in 1% DMSO in HEPES-buffered RPMI was added in a volume of 20 μL to the appropriate wells and the incubation continued for a further 20 minutes. ATP (3 mM) was then added and cells were incubated for 30 minutes at 37°C. The final concentration of DMSO in all wells was 0.1%. All incubations were performed in duplicate. At the end of the incubation period, the plates were centrifuged at 700 g for 10 minutes. The supernatant was removed from the wells and stored at –20°C until measurement of IL-1β levels by ELISA using anti-hIL-1β (MAB601), biotinylated anti-hIL-1β (BAF201) and hIL-1β standard (2011LB) from R&D Systems, Minneapolis, USA.

BzATP-induced release of IL-1β from human rheumatoid arthritis synovial cells: Synovial tissue (rheumatoid joint synovium from either hip, knee or elbow replacement surgery) was transported in assay buffer (DMEM containing L-glutamine [2 mM], penicillin [200 units/mL] and streptomycin [0.2 mg/mL]) either at room temperature (King’s Mill Hospital) or on ice (Western General Hospital, Edinburgh, UK) and, upon receipt, was stored in a refrigerator (4°C) awaiting isolation of cells. Cells were isolated between 12 and 48 hours after surgery.

Cell isolation

The tissue was finely chopped using a sterile scalpel and placed in 25 mL assay buffer containing fetal bovine serum (FBS) (10% v/v, Sigma CR0848) and collagenase Type H (2 mg/mL, Sigma CR051), and incubated at 37°C for 2 hours in a CO2 incubator. The cell suspension was passed through a sterile mesh filter (100 μM) (product code 35-2360; Becton Dickinson Labware, New Jersey, USA) into 50 mL sterile centrifuge tubes (product code 62-547-254; Sarstedt), which were then centrifuged at 400 g at room temperature for 5 minutes. The supernatant was discarded and the cells re-suspended with gentle agitation in assay buffer (10 mL) containing FBS (10% v/v). The volume of the cell suspension was adjusted to give a cell count of 3 x 106 WBC/mL. The cell suspension was added to either 48 (0.25 mL/well)- or 96- (0.2 mL/well) well tissue culture plates (product codes 3524 and 3598; Costar) and incubated overnight in a CO2 incubator. Non-adherent cells were removed from the wells and adherent cells were washed three times with assay buffer at
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37°C. Assay buffer (160 μL) was added to all wells, followed by addition of vehicle (1% [v/v] DMSO in assay buffer, 20 μL) or the appropriate concentration of AZD9056 in 1% (v/v) DMSO assay buffer. The final concentration of DMSO in all wells was 0.1% (v/v). The cells were incubated at 37°C in a CO<sub>2</sub> incubator for 10 minutes. BzATP (20 μL) was then added to each well to give a final concentration of 1 mM. Cells were then incubated at 37°C in a CO<sub>2</sub> incubator for a further 20 minutes. Media was removed from the wells and stored at -20°C awaiting measurement of IL-1β levels by ELISA using anti-hIL-1β (MAB601), biotinylated anti-hIL-1β (BAF201) and hIL-1β standard (201LB) from R&D Systems (Minneapolis, USA).

SCW rat arthritis model procedures
Mono-articular streptococcal cell wall (SCW)-induced arthritis was induced as described by Carlson and Jacobsen (1). In this study, the effects of AZ11657312 on early (day 3) and late (day 6) phases of SCW-induced arthritis were assessed. Animals were anaesthetised with isoflurane and then sensitised by intra-articular injection of SCW (5 μg in 20 μL) into the left ankle. Ankle swelling was assessed 3 days after injection and non-responders (animals with no apparent ankle swelling) were rejected. Responding animals were randomly allocated to the test groups. Arthritis was induced 21 days after sensitisation by intravenous injection of SCW (100 μg in 500 μL saline). Animals were monitored and assessed on a daily basis through to termination 3 or 6 days after induction. AZ11657312 (10, 30 and 60 mg/kg) was administered by oral prophylactic dosing, starting at 09:00 1 day before (day -1) induction of arthritis through to termination on day 3 or day 6 post-induction. Ankle diameters were measured with vernier callipers on a daily basis from day -1. Mechanical thresholds were assessed using von Frey filaments on days -1, 1, 3 and 5. The filaments were applied in increasing weights to the ankle region on the footpad of both feet. The first filament to induce a withdrawal response was considered to be the threshold. Terminal blood samples were taken into EDTA for pharmacokinetic analysis on day 3 or 6. Fifty percent of animals from each group were dosed at 09:00 on day 6 in order to obtain a peak (2-hour) sample. The remaining animals were dosed at 19:00 on day 5 to obtain a trough (18-hour) sample. The left hind limbs were then X-rayed and then fixed in 10% formalin for histological assessment.

For radiology, the tibio-tarsal compartment was examined. The articular compartment was divided into quadrants from the centre of the talus. The top-right quadrant covered the head of the tibia to the epiphysis, the bottom right the centre superficial surface of the calcaneus, the top left the proximal facet of the talus, and the bottom left the distal facet of the talus and the distal calcaneus. Images showing torsion of the joint or masking, suggestive of changes in baseline image intensity, were excluded from the analysis. There was no scoring of severity. Scoring was based on the presence or absence of a lesion in a quadrant and then a sum of scores made. The quadrant was used to facilitate correct anatomical alignment of the areas from sample to sample. The following radiographical features were recorded: loss of cartilage/subchondral bone margin; erosions; osteopenic change in trabecular bone; calcification of tendons; and osteogenesis. The sum of radiographical scores were then combined for each joint and analysed by a Mann-Whitney U test. All other results are expressed as mean ± standard error of the mean (SEM). von Frey thresholds are described as the mean filament weight (g) and graphically presented as log10 of the force exerted. In addition to graphical representation of absolute changes with time, effects on ankle swelling and mechanical threshold were also calculated on an area under the concentration-time curve basis, as the sum of the differences from individual day -1 values. Data analysis was by one-way analysis of variance followed by Dunnett’s test (ankle diameter) or Dunn’s test (von Frey threshold) on the raw data (GraphPad Instat).

Histology analyses
For haematoxylin and eosin analysis, sections were de-waxed and re-hydrated into distilled water. Sections were then incubated in Gills Haematoxylin (Pioneer Research Chemicals Ltd) for 10 minutes and then washed in running tap water for 5 minutes. Sections were next incubated in 1% Eosin Y (Acros Organics, Belgium) for 2 minutes, followed by a quick wash in water before being dehydrated in absolute alcohols. Sections were then cleared in xylene and mounted in a non-aqueous media (Histomount, Leica), and examined by light microscopy. For immunohistochemistry, sections were de-waxed and re-hydrated with PBS in 0.05% Tween 20 (pH 7.4). Non-specific Ig-binding sites were blocked with 20% normal goat serum in PBS with 1% bovine serum albumin (BSA) (pH 7.4, Sigma) for 30 minutes at room temperature. The sections were then analysed using the goat anti-rabbit Envision-AP labelled polymer system (DakoCytomation, Denmark) according to the manufacturer’s instructions. Briefly, sections were incubated overnight at 4°C with a rabbit polyclonal antibody raised against a synthetic peptide corresponding to residues 576–595 of rat P2X<sub>7</sub> receptor (3 mg/mL; Alomone Labs, Israel). For negative control, 1 mg of primary antibody was pre-incubated with 1 mg of its corresponding antigen peptide for 1 hour at room temperature. This antibody had been used in previous studies. In-house immunocytochemical analysis revealed that this anti-P2X<sub>7</sub> antisemirum recognised a membrane-bound protein from both human and rat HEK cells transfected with P2X<sub>7</sub> subunits. This confirmed the validity and specificity of the polyclonal antibody. The antibody was diluted in PBS with 1% BSA (pH 7.4, Sigma) and all washes were carried out in PBS containing 0.05% Tween 20. Following primary antibody incubation, sections were washed in buffer and a labelled polymer to AP was applied for 30 minutes at room temperature. To allow visualisation, sections were incubated with fast red chromagen (Sigmafast, Sigma) for 20 minutes at room temperature. Sections were counterstained
Supplementary Fig. 1. Synovial P2X7 distribution in rheumatoid arthritis. a) Rat negative control. b) P2X7 expression in SCW – a pilot study demonstrated that P2X7 receptor expression in SCW arthritic joints was predominantly on macrophage and osteoclastic cells. c) Human synovial tissue expression.

Supplementary Fig. 2. Proposed mechanism of action of P2X7 antagonists.
with Gills Haematoxylin (Pioneer Research Chemicals Ltd, UK) to delineate nuclear morphology. All sections were mounted in aqueous media (Aqua-Pern, Shandon), dried and examined by light microscopy. Histology evaluation: the movement from a low grade lesion to a higher grade lesion was based upon the evolution of the pathology from focal, *i.e.* present in a few areas, to diffuse, *i.e.* widespread distribution. Moderate severity was largely multi-focal in nature.

**Synovitis** was assessed on the basis of overall thickness of the synovial layer, together with degree of cellularity of that layer. **Inflammation of the synovial sub-lining** was assessed on the basis of inflammatory cell infiltration and occupation of this region, together with evidence of inflammatory change (vasculitis) of the small blood vessels. **Chondronecrosis** was assessed on the basis of lytic resorption and associated areas of cell death in the cartilage region, both by expansion of the stromal cavities within the cartilage plate and by adhesion and intrusion of the inflamed synovium into the cartilage. **Bone resorption** was assessed on the basis of the expansion of lytic areas of (essentially) osteoclastic resorption in the stromal cavities and within the bone cartilage interface, and upon the cancellous bone abutting onto the epi-physis and cartilage plates.

**References**