Supplementary information

Targeting the P2X$_7$ receptor in rheumatoid arthritis: biological rationale for P2X$_7$ 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; Amersham Pharmacia Biotech, Sweden) solution (1 mL of a 6% [w/v] solution in 0.9% [w/v] NaCl) 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 Nycoprep 1.068 (product code 1002351; Nycomed Pharma, Switzerland) in 15 mL centrifuge tubes (Costar 430791). Tubes were centrifuged at 600 g for 15 minutes (brake off). The upper plasma layer (typically 2–3 mL) and the remaining buffy coat layer (typically 4–5 mL) were removed and collected separately into 50 mL centrifuge tubes (Costar 62-547-254). Tubes were centrifuged at 700 g for 10 minutes. To reduce platelet contamination, the buffy coat pellet was re-suspended in 15 mL autologous plasma (5% [v/v] in phosphate-buffered saline [PBS]), made up from the supernatant of the centrifuged plasma. Tubes were centrifuged at 78 g for 10 minutes (brake off). This was repeated three more times. After the final spin, the cells were re-suspended in RPMI priming buffer at a concentration of 0.5×10^6 cells/mL. Lipopolysaccharide (LPS) (10 ng/mL, Sigma L4391, Lot 89H4001) was added to the cell suspension and 200 μL was then transferred to the wells of a U-bottomed tissue culture-treated 96-well plate (product code 3799; Costar) and the plate was incubated for 4 hours at 37°C (5% CO_2, 95% humidity). The supernatants were removed by aspiration, the plate blot-dried and RPMI assay buffer (160 μL) added to each well. Agonist benzoylbenzoyl adenosine triphosphate (BzATP) (20 μL, pre-diluted in RPMI assay buffer from a 100 mM stock) and either vehicle (1% [v/v] DMSO in RPMI assay buffer, 20 μL) or the appropriate concentration of AZD9056 (20 μL in 1% [v/v] DMSO in RPMI assay buffer) were added simultaneously in a total volume of 40 μL (from premixed combinations of the appropriate solutions). Plates were incubated in a water tray for 30 minutes at 37°C (5% CO_2, 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 recombinant hIL-1β standard and biotinylated anti-hIL-1β (all in product code OptEIA 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 Sterilin, 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 (201LB) 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 C8051), and incubated at 37°C for 2 hours in a CO_2 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 3×10^6 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 CO_2 incubator. Non-adherent cells were removed from the wells and adherent cells were washed three times with assay buffer at

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_2 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_2 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

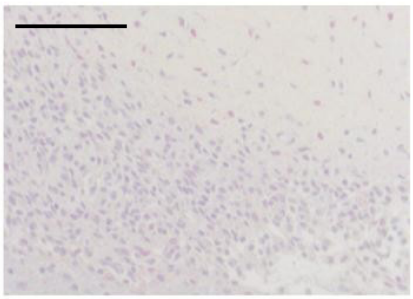
Mono-articular streptococcal cell wall (SCW)-induced arthritis was induced as described by Carlson and Jacobson (1). In this study, the effects of AZ11657312 on early (day 3) and late (day 6) phases of SCW-induced arthritis were assessed. Animals were anaesthetised with isoflurane and then sensitised 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 taken, 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 quandrants 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

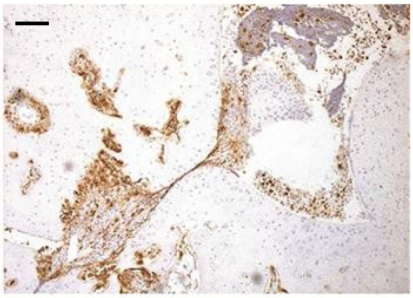
For haematoxylin and eosin analysis, sections were de-waxed and re-hydrated into distilled water. Sections were then incubated in Gills Haematoxylin (Pioneer Research Chemicals Ltd) for 10 minutes and then washed in running tap water for 5 minutes. Sections were next incubated in 1% Eosin Y (Acros Organics, 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 antigen (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 $P2X_7$ 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-$P2X_7$ antiserum recognised a membrane-bound protein from both human and rat HEK cells transfected with $P2X_7$ 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

(a)



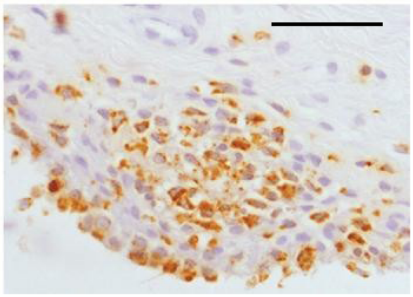
Rat negative control (bar = 500µm). Microphotograph showing lack of staining using control antibody.

(b)



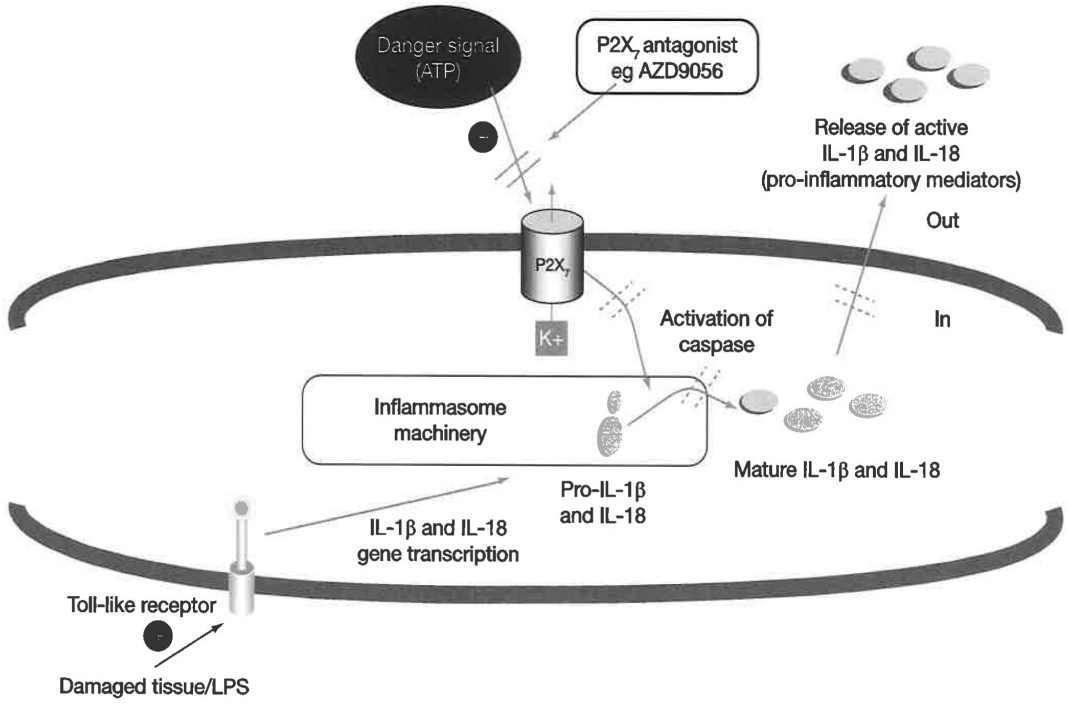
P2X7r in rat scw arthropathy (bar = 500µm). P2X7r expression in regions of synovitis and active bone resorption. The dominant expression is associated with macrophages and osteoclasts. Although co-localisation studies were not carried out, the cytological forms of these cells were very clear. Macrophages showed enlarged, dense cytoplasm with prominent cytosolic vacuolation and a an irregular cell membrane, often showing multiple processes. Osteoclasts showed a similar morphology and were associated with 'lytic' areas on bone and cartilage. Loss of stain intensity was a prominent feature of these lytic regions.

(c)



P2X7r expression in human rheumatoid arthritis (bar = 500µm). Higher power view showing marked macrophage and synoviocyte expression on synovial margin. The macrophages showed a typical 'active' form, with enlarged apparent cell volume, dense cytosolic vacuolation and irregular cell membranes. There are several examples of macrophages showing focal concentration of vacuoles, suggestive of active phagocytosis. Border synoviocyte staining was irregular, and confined to dense, vacuolated cells. Co-localisation studies were not carried out and so the relative distribution of receptor expression on cell types cannot be determined. Negative antibody control was run on parallel tissue sections and showed no specific staining (data not shown). Human tissue samples employed were: 4 RA, 2 OA and 2 normal

Supplementary Fig. 1. Synovial P2X$_7$ distribution in rheumatoid arthritis. **a)** Rat negative control. **b)** P2X$_7$ expression in SCW – a pilot study demonstrated that P2X$_7$ 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 P2X$_7$ antagonists.

with Gills Haematoxylin (Pioneer Research Chemicals Ltd, UK) to delineate nuclear morphology. All sections were mounted in aqueous media (Aqua-Perm, 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 epiphysis and cartilage plates.

References

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