Characterization of N-(Adamantan-1-ylmethyl)-5-[(3R-amino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide, a P2X<sub>7</sub> Antagonist in Animal Models of Pain and Inflammation

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Received June 16, 2008; accepted September 2, 2008

ABSTRACT

Recent evidence suggests that the P2X<sub>7</sub> receptor may play a role in the pathophysiology of preclinical models of pain and inflammation. Therefore, pharmacological agents that target this receptor may potentially have clinical utility as anti-inflammatory and analgesic therapy. We investigated and characterized the previously reported P2X<sub>7</sub> antagonist N-(adamantan-1-ylmethyl)-5-[(3R-amino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide, hydrochloride salt (AACBA; GSK314181A). In vitro, AACBA was a relatively potent inhibitor of both human P2X<sub>7</sub>-mediated calcium flux and quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(triemethylammonio)propyl]-diiodide (YO-PRO-1) uptake assays, with IC<sub>50</sub> values of approximately 18 and 85 nM, respectively. Compared with the human receptor, AACBA was less potent at the rat P2X<sub>7</sub> receptor, with IC<sub>50</sub> values of 29 and 980 nM in the calcium flux and YO-PRO-1 assays, respectively. In acute in vivo models of pain and inflammation, AACBA dose-dependently reduced lipopolysaccharide-induced plasma interleukin-6 release and prevented or reversed carrageenan-induced paw edema and mechanical hypersensitivity. In chronic in vivo models of pain and inflammation, AACBA produced a prophylactic, but not therapeutic-like, prevention of the clinical signs and histopathological damage of collagen-induced arthritis. Finally, AACBA could not reverse L<sub>6</sub> spinal nerve ligation-induced tactile allodynia when given therapeutically. Consistent with previous literature, these results suggest that P2X<sub>7</sub> receptors do play a role in animal models of pain and inflammation. Further study of P2X<sub>7</sub> antagonists both in preclinical and clinical studies will help elucidate the role of the P2X<sub>7</sub> receptor in pain and inflammatory mechanisms and may help identify potential clinical benefits of such molecules.

The P2X<sub>7</sub> receptor is a purinergic ion channel that has been implicated in mechanisms related to both pain and inflammation (for reviews, see Donnelly-Roberts and Jarvis, 2007; Lister et al., 2007). It is expressed in many cell types of immunological importance and of hematopoietic lineage as well as being centrally expressed on microglial cells (Surprenant et al., 1996; Collo et al., 1997). Functionally, P2X<sub>7</sub> mediates the release of proinflammatory cytokines known to have roles in inflammatory/immune conditions and pain, including interleukin (IL)-1, tumor necrosis factor (TNF)-α, and IL-6 (Ferrari et al., 1997; Hide et al., 2000; Solle et al., 2001; Gourine et al., 2005). Therefore, its location on immune cells makes it an attractive target for inflammatory and analgesic therapy. We investigated and characterized the previously reported P2X<sub>7</sub> antagonist N-(adamantan-1-ylmethyl)-5-[(3R-amino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide, hydrochloride salt (AACBA; GSK314181A). In vitro, AACBA was a relatively potent inhibitor of both human P2X<sub>7</sub>-mediated calcium flux and quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(triemethylammonio)propyl]-diiodide (YO-PRO-1) uptake assays, with IC<sub>50</sub> values of approximately 18 and 85 nM, respectively. Compared with the human receptor, AACBA was less potent at the rat P2X<sub>7</sub> receptor, with IC<sub>50</sub> values of 29 and 980 nM in the calcium flux and YO-PRO-1 assays, respectively.
and microglial cells and its functional role in modulating the release of inflammatory factors implicates P2X7 in pathological inflammation and pain. Further evidence for a role of P2X7 in inflammation and pain has come from knockout and pharmacological studies. Chessell et al. (2005) described a P2X7 gene-knockout (P2X7−/−) mouse that displayed no development of thermal or mechanical hypersensitivity following partial sciatic nerve ligation and also failed to develop CFA-induced changes in weight bearing compared with wild-type animals. In contrast, these P2X7 knockout mice had no change in baseline nociceptive responses, as measured by the hot-plate assay compared with wild-type animals (Chessell et al., 2005). Furthermore, Labasi et al. (2002) observed a lower incidence and severity of monoclonal antigencolon-induced arthritis in P2X7 knockout mice compared with wild type, suggesting a pathological role for P2X7 in inflammatory/immunological disease. Finally, local administration of oxidized ATP had antihyperalgesic effects in the CFA-induced immunological disease. Further evidence for a role of the P2X7 receptor in rheumatoid arthritis (McInnes et al., 2007). The CREATE study examined the effect of AZD9056, a P2X7 antagonist in rheumatoid arthritis sufferers. This trial demonstrated that significantly more patients receiving AZD9056 achieved American College of Rheumatology 20 scores than those receiving placebo, indicating an improvement of arthritic symptoms. However, no change in the level of C-reactive protein, a well established biomarker of rheumatoid arthritis disease progression in humans, was seen following AZD9056 treatment. Therefore, initial indications from clinical studies indicate that antagonists of the P2X7 receptor may be of clinical benefit to sufferers of rheumatoid arthritis.

The preclinical and clinical evidence suggesting a use of P2X7 antagonists in the treatment of pain and inflammatory diseases necessitates further elucidation of the pharmacology of the P2X7 receptor. The aim of the present study was to further investigate the inflammatory/immunological and pain biology of the P2X7 receptor by further characterizing the P2X7 receptor antagonist AACBA. The effects of this P2X7 antagonist were evaluated in vitro and in vivo assays and preclinical disease models of acute and chronic pain and inflammation.

Materials and Methods

Materials

Unless otherwise noted, all reagents were obtained from Sigma-Aldrich (St. Louis, MO). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). SH-SY5Y cells were purchased from American Type Culture Collection (Manassas, VA). All cells were cultured in standard, tissue-culture-treated plastic ware (Thermo Fisher Scientific, Waltham, MA). Carrageenan was dissolved in saline. LPS, hydroxypropyl-β-cyclodextrin (HPBCD), and methylcellulose was dissolved in distilled water. d-o-Tocopheryl polyethylene glycol 1000 succinate (TPGS; Eastman, Kingsport, TN) was also dissolved in distilled water. Dexamethasone and naproxen were dissolved in 0.5% methylcellulose. Pregabalin (Waterstone Technologies, Carmel, IN) and methotrexate (MTX) were dissolved in 2% DMSO. Isoflurane was obtained from Webster Veterinary (Sterling, MA), and incomplete Freund’s adjuvant was obtained from Difco (Detroit, MI). AACBA (Alcaraz et al., 2000; Fonfria et al., 2005; Lappin et al., 2005) was synthesized in-house as described below and was dissolved in dimethyl sulfoxide (DMSO) as a stock solution for in vitro assays and in a final concentration of 10% HPBCD for in vivo experiments.

Preparation of AACBA

AACBA was prepared according to the procedures described in Alcaraz et al. (2000), with some modifications. Structure and purity of the target compound and intermediates were ascertained by 1H NMR and high-performance liquid chromatography/mass spectrometry.

Preparation of 5-Bromomethyl-2-chloro-benzoic Acid. To a stirred solution of 2-chloro-5-methyl-benzoic acid (25 g) in chloroform (500 ml) at 50°C was added N-bromosuccinimide (27.4 g). The flask...
was purged with nitrogen, and azobisobutyronitrile (0.10 g) was added in one portion. The solution was heated at reflux for 1 h. More azobisobutyronitrile (0.10 g) was added, and the mixture heated a further 3 h. The solution was concentrated in vacuo, redissolved in diethyl ether, and filtered to remove insoluble succinimide. The ether solution was washed with 2 N aqueous hydrochloric acid solution followed by brine and then dried over magnesium sulfate. The solution was concentrated to a volume of 150 ml and then diluted with hexane. After further partial concentration crystallization started, the mixture was allowed to stand in an ice bath for 1 h. The resulting crystals were filtered, washed with hexane, and dried in vacuo to give the title compound (17 g).

**Preparation of N-(Adamantan-1-ylmethyl)-5-bromomethyl-2-chloro-benzamide.** To a stirred solution of 5-bromomethyl-2-chloro-benzoic acid (12.4 g) in dichloromethane (250 ml) and dimethylformamide (0.12 ml) at 0°C was added oxalyl chloride (8.7 ml). The cooling bath was removed, and the solution was warmed to room temperature. Once gas evolution had ceased, the solution was concentrated in vacuo. The residue was redissolved in dichloromethane (300 ml), cooled to 0°C, and treated with di-isopropyl ethylamine (12.4 ml) and adamantylmethylamine (7.54 ml). After 15 min at 0°C the solution was poured into diethyl ether (1 liter) and washed with 1 N aqueous hydrochloric acid followed by brine. The organics were dried over magnesium sulfate and concentrated in vacuo to give the title compound as a white powder (19 g).

**Preparation of N-(Adamantan-1-ylmethyl)-5-[(3R-tert-butoxycarbonylamino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide.** A mixture of N-(adamantan-1-ylmethyl)-5-bromomethyl-2-chloro-benzamide (0.13 g), 3R-tert-butoxycarbonylamino-pyrrolidine (0.074 g), and diisopropylethylamine (6.3 ml) in dimethylformamide (3 ml) was heated at 60°C for 3 h. The mixture was diluted with water (10 ml) and extracted with ethyl acetate (3 × 10 ml). The organic layer was dried over magnesium sulfate and then filtered. The filtrate was concentrated under reduced pressure. The crude material was purified on a silica gel eluting with dichloromethane/ethanol (0–20% ethanol gradient) to afford the title compound as a white foam (0.112 g).

**Preparation of AACBA.** N-(Adamantan-1-ylmethyl)-5-[(3R-tert-butoxycarbonylamino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide (0.080 g) was dissolved in methanol (3 ml), 4 N HCl in dioxane (1 ml) was added, and the mixture was stirred at room temperature for 1.5 h. The solvent was removed under vacuum, and the resulting solid was triturated with ether to afford the title compound as a white powder (0.062 g).

**Animals**

Male Sprague-Dawley rats (all experiments except LPS-induced arthritis) weighing approximately 200 to 250 g at the start of the experiment and male Wistar rats (LPS-induced IL-6 release and collagen-induced arthritis) weighing approximately 200 to 250 g were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Female Lewis rats (collagen-induced arthritis experiments) weighing 125 to 157g at the start of the experiment were obtained from Harlan (Indianapolis, IN). All animals were housed under a 12:12-h light/dark cycle, with lights on at 6:00 AM. Animals were housed in groups of three to four in Plexiglas cages, with corn chip bedding for the duration of the experiment. They were provided with food and water ad libitum. Animals were allowed to habituate to the vivarium for at least 3 days before the start of the experiment. All studies were approved by Neurogen Corporation’s Institutional Animal Care and Use Committee and were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

**Calcium Mobilization Assays**

For human P2X₇ (hP2X₇) calcium mobilization assays, SH-SY5Y cells were seeded from log phase cultures into 96-well plates at a density of 100,000 cells/well, and the plates were incubated at 37°C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 10 mM HEPES. Twenty-four hours later, the culture media were removed from the wells and replaced with 50 μl of sucrose assay buffer (280 mM sucrose, 25 mM HEPES, 5.0 mM KCl, 9.6 mM NaH₂PO₄, 5 mM glucose, 0.5 mM CaCl₂, and 1 mM probenecid, pH 7.4) containing 2.5 mM Fluo 4-acetoxyethyl ester (Invitrogen). The plates were then incubated at 37°C for 1 h. The dye solution was removed, and each well was washed once with 50 μl of sucrose assay buffer. One hundred microliters of sucrose assay buffer containing 1% DMSO with and without AACBA was then added to each well. After 1-h incubation at room temperature, 100 μl of sucrose assay buffer containing BzATP was transferred onto cells, and fluorescence response was monitored for 5 min on a fluorometric imaging plate reader TETRA instrument (Molecular Devices, Sunnyvale, CA). AACBA potency was determined using 80 μM BzATP, the approximate EC₅₀ for BzATP under these conditions.

RBA-2 cells were used in a similar procedure to measure compound activity at rat P2X₇. RBA-2 cells were cultured in F-10 medium containing 10% fetal bovine serum and 10 mM HEPES. Cell seeding densities and dye loading were as described above. The assay buffer was a modified Krebs-Ringer HEPES buffer (115 mM NaCl, 5 mM KCl, 1 mM NaH₂PO₄, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 5 mM glucose, and 25 mM HEPES, pH 7.4) containing 1 mM probenecid. AACBA potency was determined in the RBA-2 assay using 10 μM BzATP, the approximate EC₅₀ value for BzATP under these conditions. To determine the BzATP EC₅₀ value, the maximum fluorescence achieved was plotted as a function of compound concentration. To determine the AACBA IC₅₀ value, the percentage of inhibition of BzATP response was plotted as a function of compound concentration. These data were fit to a sigmoidal equation using Kaleidagraph (Synergy Software, Reading, PA) as described previously (Szallasi et al., 1999). Data are reported as the average ± S.E. of the mean for at least three independent experiments.

**YO-PRO-1 Uptake Assays**

Inhibition of BzATP-induced P2X₇ pore formation by AACBA was tested in blood monocytes. Human whole blood was collected using 0.105 M buffered sodium citrate anticoagulant tubes (BD Vacutainer; BD Biosciences, Franklin Lakes, NJ), and rat blood was collected by cardiac puncture, with K3EDTA as the anticoagulant. Blood samples were used within 4 h of collection. Three milliliters of blood was centrifuged at 1700g for 5 min to pellet cells. The supernatant was removed, and the cells were resuspended to a final volume of 3 ml in RPMI 1640 medium (Invitrogen) supplemented with 5 mM EDTA to deplete divalent cations. Human monocytes were labeled with CD14 antibody conjugated with phycoerythrin-Cy5 (Beckman Coulter, Fullerton, CA). AACBA was added to aliquots of CD14-labeled blood samples (1% final DMSO concentration), and then samples were incubated at room temperature for 30 min. Vehicle control was 1% DMSO (final concentration) alone. YO-PRO-1 (4 μM; Invitrogen) was then added for 5 min before the addition of BzATP. For drug inhibition experiments, human samples received 100 μM BzATP before incubation at 37°C for 20 min, whereas rat samples received 300 μM BzATP and were incubated for 30 min at 37°C. After the 37°C incubation, P2X₇ dye uptake was stopped by adding MgCl₂ to each sample at a final concentration of 10 mM. Samples were washed twice in 2 ml of RPMI 1640 buffer, fixed in 1% paraformaldehyde/phosphate-buffered saline, and resuspended in 2 ml of phosphate-buffered saline. The samples were run on an EPICS XL-MCL flow cytometer (Beckman Coulter), and data were collected and analyzed using EXPo32 software (Beckman Coulter). CD14 staining was used to detect human monocytes, whereas forward and side light scattering were used to identify a rat monocyte-like population. Data from approximately 5000 monocytes were acquired per human and rat sample, and the mean YO-PRO-1 fluorescence intensity for each sample was determined. BzATP EC₅₀ and AACBA IC₅₀ values were determined as described for the calcium mobilization.
assays. Data are reported as the average ± S.E. of the mean for at least three independent experiments.

**LPS-Induced Interleukin-6 Release**

Male Wistar rats were injected intraperitoneally or subcutaneously with drug or vehicle, and 1 h later they were injected with 100 μg/kg i.p. LPS or saline (1-ml volume). Two hours after the LPS injection (3 h after drug injection), all animals were euthanized by CO₂ asphyxiation, and blood samples were obtained by cardiac puncture. IL-6 levels in the plasma were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Data were expressed as the percentage of maximal IL-6 release by using the following equation: [(test) – (mean of vehicle group)]/[mean of LPS alone group] – (mean of vehicle group) × 100 = % maximal IL-6 release.

Statistical analysis was performed by analysis of variance (ANOVA), with post hoc analysis, as necessary, using Fisher’s least significant difference (LSD) test. Statistical significance was accepted as P < 0.05. Statistics for this and all behavioral experiments were performed using SYSTAT (Systat Software, Inc., San Jose, CA).

**Carrageenan-Induced Mechanical Hypersensitivity**

Male Sprague-Dawley rats were individually habituated to handling for the 3 days before testing, with testing performed on day 4. During the first habituation session (day 1), animals were handled but not exposed to the paw pressure apparatus. The last two habituation sessions (days 2 and 3) included habituation to the paw pressure testing apparatus (IITC Inc., Woodland Hills, CA), with the last habituation (day 3) serving as the baseline measurement for the experiment. For habituation, the paw pressure apparatus was applied to each hindpaw in turn with the cone in contact with the plantar surface of the hindpaw until the animal responded. A response was defined as an attempt to withdraw the paw, struggle, or vocalize. The pressure (in grams) at which the rat first responded (the paw pressure threshold) was recorded. A cut-off of 400 g was used to prevent injury to the hindpaw. Three measurements were taken from each hindpaw during each habituation/baseline or test, with a 4 to 5-min interval between trials. On the day of the test (day 4), compound was administered subcutaneously, intraperitoneally, or orally at the stated pretreatment time before testing. One hundred microliters of carrageenan (0.5–1% solution) was administered by intraplantar injection into the left hindpaw 3 h before testing. Testing was performed and hindpaw pressure thresholds were measured for each rat using the same technique as described for baseline measurement. The measurements taken on baseline, and test days were averaged to obtain a mean baseline and a mean test value for each hindpaw. Data were subsequently expressed in two analyses. First, test-baseline analysis was performed by subtracting the averaged baseline value from the averaged test value for each injured (i.e., left) hindpaw. Second, left foot only analysis was performed by simply taking the averaged test value for each injured hindpaw. Statistical analysis was performed by ANOVA, with post hoc analysis, as necessary, using Fisher’s LSD test. Statistical significance was accepted as P < 0.05.

**Carrageenan-Induced Paw Swelling**

On the day of testing, male Sprague-Dawley rats were dosed subcutaneously, intraperitoneally, or orally with test compound at the stated pretreatment time before testing. One hundred microliters of carrageenan (1% solution) was administered by intraplantar injection into the left hindpaw 3 h before testing. For testing, each hindpaw was lowered in turn into a plethysmometer (Ugo Basile, Comerio, Italy), and the volume of the paw was recorded. This procedure was repeated until three readings within 0.1 ml of each other were obtained for each hindpaw. Immediately after testing, all rats were euthanized by CO₂ asphyxiation, and a plasma sample was obtained from a subset of AACBA-treated animals to evaluate plasma drug exposure. Therefore, plasma samples were obtained approximately 2.25 to 2.5 h after AACBA dosing. The volume measurements obtained were averaged to obtain one value for each hindpaw. Data are subsequently expressed as left-right hindpaw volume difference by subtracting the averaged right hindpaw volume from the averaged left (injured) hindpaw volume. Statistical analysis was performed by ANOVA, with post hoc analysis, as necessary, using Fisher’s LSD test. Statistical significance was accepted as P < 0.05.

**Nerve Injury-Induced Tactile Hypersensitivity**

For this study, the L₅ spinal nerve ligation model (SNL) was modified from the method described by Kim and Chung (1992). Before surgery, animals were habituated for 3 days to the von Frey testing apparatus. On the first day, animals were placed in upturned Perspex containers on an elevated wire grid and allowed to acclimate. On the second day, animals were placed in the Perspex containers on the elevated grid again and were habituated to the von Frey testing procedure. In brief, a hand-held von Frey testing device (IITC Inc.) with a 60-g Semmes Weinstein filament attached automatically recorded the highest force achieved upon application to the hindpaw (i.e., the force at which the animal withdraws its paw). The filament was applied to each hindpaw until either a foot-withdrawal response was elicited or the filament was fully bent, indicating that the cut-off force had been achieved. Both the left and right hindpaw received four trials each, with at least a 15-s interval between subsequent trials. On the third day, the presurgery baseline withdrawal threshold was measured, using the same testing procedure as outlined above for habituation day 2.

For SNL surgery, animals were anesthetized using isoflurane and the appropriate area of skin shaved and sterilized using povidone-iodine prep pads (PDI, Inc., Orangeburg, NY). A longitudinal incision was made through the dorsal, lower back skin slightly to the left of midline in the region of the hip. The underlying fascia and paraspinal muscles were separated to expose the left spinous process. The process was then carefully removed using micro-rougeurs to expose the L₅ spinal nerve. The L₅ spinal nerve was tightly ligated using a single 4-0 silk suture. Finally, the muscle was apposed and sutured with monofilament, and the skin was closed using surgical staples. For sham surgery animals, only the skin incision and separation of the fascia were performed before skin closing. Animals were allowed to fully recover from anesthesia before returning to their home room.

After surgery, animals were allowed to develop hindpaw hypersensitivity for at least 5 days before a postsurgery baseline test was performed and drug administration was initiated. Animals were then subjected to postdrug testing at the appropriate time point. Animals were subjected to three experiments, with at least 5 days between the end of one experiment and the start of the next experiment. Each experiment began with a postsurgery (untreated) baseline to ensure animal sensitivity before drug administration. Baseline and testing procedures were performed as outlined above for habituation day 2. Experiments were performed according to the following schedule: On day 10, 5 mg/kg s.c. morphine or saline (saline) was dosed 1 h before testing. On day 17, 30 mg/kg p.o. pregabalin or vehicle (0.5% methylcellulose) was dosed 1 h before testing. On days 22 to 24, 30 mg/kg s.c. AACBA or vehicle (10% hydroxypropyl β-cyclodextrin) was dosed 48, 24, and 1 h before testing (testing for this last experiment occurred on day 24). Testing was performed by a tester blinded to the treatment received by each animal.

Data were analyzed using a repeated measures ANOVA to determine significant differences between groups. Post hoc analysis was performed using the LSD test. Statistical significance was accepted as P < 0.05.

**Collagen-Induced Arthritis, Experiment 1 (Prophylaxis)**

Each experiment lasted for 22 days. Animals were examined for baseline scores (clinical score and paw swelling/plethysmometer),
and collagen-induced arthritis was initiated according to the standard protocol issued by Chondrex, Inc. (Redmond, WA; Protocol for the Successful Induction of Collagen-Induced Arthritis in Rats, version 1.2). In brief, 200 μg s.c. (0.2 ml) of emulsified porcine collagen (Chondrex, Inc.) in incomplete Freund’s adjuvant was injected into the base of the tail of each rat (control rats received a corresponding injection of 0.2 ml of saline). The first injection of collagen (or saline control) was performed on day 1, and all animals were monitored periodically for the next 20 days. A single booster injection of collagen emulsion (100 μg/0.1 ml collagen or 0.1 ml of saline for control rats) was administered on day 8. Dosing of test compounds began on day 1, immediately before collagen injection, and it continued through day 21. Dexamethasone (0.1 mg/kg p.o.), AACBA (30 mg/kg s.c.), and vehicle (10% hydroxypropyl-β-cyclodextrin subcutaneously) were dosed once a day. Five treatment groups, including three control groups were examined in this experiment. A negative control group (Saline/Veh) was dosed with saline (subcutaneously) into the base of the tail (as a control for the collagen injections) and drug vehicle (10% HPBCD). A dexamethasone-alone control group (Saline/Dex) received saline (subcutaneously) into the base of the tail (as a control for the collagen injections) and dexamethasone. A positive control group (CIA/Veh) received collagen and drug vehicle (10% HPBCD) to examine the effects of collagen alone. Two drug-treated groups (CIA/Dex and CIA/AACBA) received collagen and either dexamethasone (CIA/Dex) or AACBA (CIA/AACBA).

Final clinical and behavioral tests were conducted before the last administration of drug or vehicle on day 21. The animals were subsequently euthanized on day 22, and blood plasma and joint tissues were harvested. Tissues collected were the shoulder, knee, and carpal and tarsal joints of one side for each animal. The side chosen for each animal corresponded to the most affected hindpaw according to clinical score (e.g., if the right hindpaw had a larger clinical score than the left hindpaw, then the right front leg and hind leg were collected for analysis). All joints collected were placed in formaldehyde for fixation and sent to Histo-Scientific Research Laboratories (Mt. Jackson, VA) for decalcification, sectioning, and staining with hematoxylin and eosin. Slides were returned to Neurogen Corporation for histological analysis of and scoring of each joint. Scoring was performed by a board-certified veterinary pathologist (R. Ochoa) unaware of animal identity.

All animals were inspected daily to ensure well being and health, and clinical scoring and paw swelling were monitored twice per week for the duration of the experiment. These were performed as follows: clinical scoring of each of the rat’s paws was visually examined by an observer blinded to treatment status and scored using an arbitrary scale according to the standard protocol issued by Chondrex, Inc. (Protocol for the Successful Induction of Collagen-Induced Arthritis in Rats, version 1.2): 0, within normal limits; 1, mild, but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits; 2, moderate redness and swelling of ankle or wrist; 3, severe redness and swelling of the entire paw including digits; and 4, maximally inflamed limb with involvement of multiple joints.

The scores for each individual’s four paws were then totaled to provide a minimum score of 0 and a maximum score of 16 per animal. Clinical scoring data were analyzed using a one-way ANOVA, with follow-up ANOVAs to determine significant separation between groups. Statistical significance was accepted as P < 0.05.

Paw swelling was measured using a plethysmometer (Ugo Basile). Each hindpaw was lowered in turn into the plethysmometer, and the volume of the paw was recorded. This procedure was repeated until three readings within 0.1 ml of each other were obtained for each hindpaw. Plethysmometer data were statistically analyzed by repeated measures ANOVA, with follow-up univariate ANOVAs for significant main effects (time and treatment). Any significant main effects were followed up with LSD post hoc test. Statistical significance was accepted as P < 0.05.

Spontaneous exploratory locomotor activity in a novel open-field environment was measured on day 21, 1 day before euthanasia (day 22). It has been demonstrated previously that in certain paradigms of inflammatory hypersensitivity, measures of spontaneous locomotor activity (exploration of a novel environment) can be suppressed by an inflammatory stimulus (Matson et al., 2007). Spontaneous locomotor activity was measured using a computerized Digiscan-16 Animal Activity Monitor (model 1300JCC/Digi, version 2.4; Omnimetronics, Columbus, OH) equipped with 48 infrared photocell emitters and detectors (2.5 cm between sensors). Each box (41.25 × 41.25 × 30 cm) was constructed of Plexiglas sides and floor. All rats were unfamiliar with the testing boxes; therefore, the boxes represented a novel environment during the test session. The rats were tested in the presence of white noise (62 dB) and red light (60 W), and activity was assessed for one session lasting 15 min. Locomotor activity data were analyzed using a one-way ANOVA for each of the activity measures (movement time, vertical activity, and total distance), with follow-up LSD post hoc test for significant main effects. Statistical significance was accepted as P < 0.05.

Histopathology was assessed by examining longitudinal sections of the shoulder, knee, carpal (front paw), and tarsal (hindpaw) joints for seven progressive signs of collagen-induced morphological evidence of damage. Four longitudinal sections of each joint were observed, representing different levels of the joint. The seven parameters scored were as follows: 1) intra-articular exudates (included the presence of proteinaceous material within the articular joint); this could range from light eosinophilic material to flocculent material that would be more strongly eosinophilic; 2) thickening of the synovial cell layer (represented thickening of the synovial cell layer to more than one cell); in the more severe cases, this thickening included fibrin strands; 3) cell infiltration in the articular space (included the presence of synovial cells, macrophages, and occasionally, in the most severe cases, polymorphonuclear leukocytes within the articular space); 4) inflammatory infiltration of the synovial membrane (included the infiltration of the synovial membrane with macrophages, lymphocytes, neutrophils, and other inflammatory cells); 5) erosion of articular cartilage (change in staining characteristics or thickness of the articular cartilage of the joint or joints in the section); 6) periostial proliferation (defined as the presence of new woven bone radiating from the periosteum of bones adjacent to affected joints); and 7) vascular inflammation (defined as the presence of inflammatory cells within a vessel wall). This was observed only in one animal.

The most severe change was scored in each slide or joint. Changes were rated from 0 to 4 based on the severity of the measure being evaluated and according to the following scale: 0, within normal limits; 1, mild (barely discernible from background changes); 2, moderatesevere (evident due to increase in the number of occurrences or extension of the change to more components); 3, severe (definitively apparent, involving a majority of the joint or section, probably resulting in an interference with function); and 4, maximal (very extensive and complex, often involving periarticular tissues, definitively affecting function).

The correlation between clinical scores and histopathology scores was analyzed using the Spearman rank-order correlation coefficient. Statistical significance was accepted as P < 0.05.

Collagen-Induced Arthritis, Experiment 2 (Therapeutic)

The method of induction, monitoring, and analysis of the second collagen-induced arthritis experiment was identical to the first experiment, with the exception that the dose of collagen used was 400μg for the first injection and 200μg for the booster. Dosing of compounds began on day 13 and continued through day 21. Dexamethasone (0.1 mg/kg p.o.), AACBA (30 mg/kg s.c.), and vehicle (10% hydroxypropyl-β-cyclodextrin) were dosed once a day. Methotrexate (0.75 mg/kg i.p.) was dosed every other day. Five treatment groups, including two control groups, were examined in this experiment. A negative control group (Saline/Veh) was dosed with saline (subcutaneously) into the base of the tail (as a control for the collagen
injections) and drug vehicle (10% HPBCD). A positive control group (CIA/Veh) received collagen as described above and drug vehicle (10% HPBCD) to examine the effects of collagen alone. Three drug-treated groups (CIA/Dex, CIA/MTX, and CIA/AACBA) received collagen and either dexamethasone (CIA/Dex), methotrexate (CIA/MTX), or AACBA (CIA/AACBA) as described above. Clinical scoring and assessment of spontaneous locomotor activity were performed as described for CIA experiment 1. Assessment of spontaneous locomotor activity was performed on day 21 before final dosing of drug, and final clinical scoring was conducted on day 22. Animals were then euthanized, and joint tissues were harvested (only knee and tarsal joint tissue for Saline/Veh, CIA/Veh, and CIA/AACBA groups was collected).

Analysis of Plasma Drug Exposures

Plasma samples collected during testing were stored frozen at approximately −20°C before analysis. Plasma samples were prepared for analysis by the addition of an internal standard followed by protein precipitation with acetonitrile. Denatured protein was removed by centrifugation, and the supernatant was analyzed for AACBA concentration using reverse phase high-performance liquid chromatography with tandem mass spectrometry detection.

Results

Functional Activity of AACBA in Vitro. BzATP is a relatively selective activator of the P2X7 channel. Hence, we were able to design high-throughput assays to measure the potency of inhibitors of P2X7 calcium mobilization in vitro using cells that normally express P2X7, rather than recombinantly expressed stable cell lines (Baraldi et al., 2003; Nelson et al., 2006). As shown in Fig. 2A, BzATP increases calcium-dependent Fluo-4 fluorescence in SH-SY5Y cells (Larsson et al., 2002) and RBA-2 cells (Sun et al., 1999) in a concentration-dependent manner. The EC50 value for BzATP on SH-SY5Y cells was 67.1 ± 4.3 μM (n = 8), whereas on RBA-2 cells, the EC50 value was 13.3 ± 1.1 μM (n = 5). The BzATP-induced calcium response in SH-SY5Y and RBA-2 cells was inhibited by the small molecule P2X7 antagonist AACBA in a concentration-dependent manner (Fig. 2B). The IC50 value for AACBA on SH-SY5Y cells was 18.0 ± 1.2 nM (n = 8), and the IC50 value on RBA-2 cells was 29.2 ± 4.0 nM (n = 8).

P2X7 exhibits the rather unique property of forming a pore upon activation that is permeable to certain nucleic acid-binding dye molecules, such as YO-PRO-1. We exploited this characteristic to devise an assay of P2X7 activity in freshly collected blood cells. Flow cytometry provided a convenient method to detect BzATP-activated YO-PRO-1 uptake into monocytes in blood samples (Fig. 3A). The effect is dose-dependent, exhibiting an EC50 value of 97.5 ± 5.9 μM (n = 6) in human CD14-positive blood cells and 86.5 ± 12.5 μM (n = 6) in rat monocyte-like cells. As is evident in the representative experiment shown in Fig. 3A, the BzATP concentration-response curve in the rat blood cell assay exhibits a low slope (Hill coefficient typically <0.7). Hence, we routinely used 300 μM BzATP when assessing P2X7 antagonist activity in the rat YO-PRO-1 uptake assay to ensure a robust signal to noise. BzATP-mediated YO-PRO-1 uptake in blood cells was inhibited by AACBA, with an IC50 value of 85.0 ± 7.0 nM (n = 4) in human CD14-positive blood cells and 980 ± 110 nM (n = 5) in rat monocyte-like cells, as shown in Fig. 3B.
LPS-Induced Interleukin-6 Release. LPS, when dosed in the absence of drug, produced a large increase in plasma IL-6 levels compared with vehicle-treated animals (Fig. 4). This effect was almost fully prevented by pretreatment with 0.1 mg/kg i.p. dexamethasone. AACBA produced a dose-dependent prevention in the LPS-induced increase in plasma IL-6, with a significant effect at 10 mg/kg s.c. The magnitude of effect seen at 10 mg/kg was not as great as that seen with 0.1 mg/kg dexamethasone.

Carrageenan-Induced Mechanical Hypersensitivity. Carrageenan, when dosed in the absence of drug, produced a drop in the mechanical threshold required to elicit a response. This was calculated by subtracting each animal’s baseline (uninjured) response threshold from its test (injured) response threshold and resulted in a negative test-baseline value (Fig. 5, A and C). AACBA, when dosed subcutaneously 1.5 h following carrageenan (1.5 h before testing) in a therapeutic-like administration, produced a dose-dependent reduction in carrageenan-induced mechanical hypersensitivity (Fig. 5A). The magnitude of reversal seen was slightly lower than that achieved with oral administration of 20 mg/kg naproxen. Analysis of the left foot alone (the response threshold on test day alone) demonstrated a similar dose effect with AACBA to the test-baseline analysis (Fig. 5B).

When dosed 30 min before carrageenan (and 3.5 h before testing) in a prophylactic-like administration, 100 mg/kg AACBA produced a reduction in carrageenan-induced mechanical hypersensitivity (Fig. 5C). This reversal of hypersensitivity was similar in magnitude to that elicited by 10 mg/kg naproxen, dosed 1 h before carrageenan (and 4 h before testing). The effect of prophylactically administered AACBA was also evident in left foot only (test day only) analysis (Fig. 5D).

Carrageenan-Induced Paw Swelling. In the absence of drug, carrageenan produced a significant swelling in the injected paw compared with the uninjected paw (Fig. 6). AACBA, when dosed 1 h following carrageenan (and 2 h before testing) in a therapeutic-like administration produced a dose-dependent reversal in paw swelling (Fig. 6). The full extent of this reversal seemed to be of lesser magnitude than that seen with 20 mg/kg p.o. naproxen dosed 1 h following carrageenan. Plasma exposures of AACBA were determined for a subset of test animals (n = 4 at each dose) and at approximately 2.25 to 2.5 h after drug dosing was found to be 98.7 ± 4.5 ng/ml (245.6 ± 11.3 nM), 490.5 ± 111.0 ng/ml (1220.3 ± 276.1 nM), and 715.5 ± 78.2 ng/ml (1780.0 ± 194.6 nM) for the 3, 10, and 30 mg/kg doses of AACBA, respectively.

Nerve Injury-Induced Tactile Hypersensitivity. When administered by three subcutaneous injections of 30 mg/kg given 48, 24, and 1 h before testing, AACBA did not produce a significant change in mechanical (tactile) hypersensitivity in the SNL-injured hindpaw compared with SNL-injured animals that received vehicle (Fig. 7A). In contrast, in these same animals a single administration of either 30 mg/kg p.o. pregabalin (1 h before testing; Fig. 7B) or 5 mg/kg s.c. morphine (1 h before testing; Fig. 7C) produced a significant increase in force required to elicit a response, indicating a significant reversal of hypersensitivity. After administration of either pregabalin or morphine, the force required to elicit a withdrawal returned to a level similar to that seen in sham-operated animals. It should be noted that the response threshold (gram force) of the sham surgery group dropped in both postsurgery and postdrug reading while testing AACBA (Fig. 7A). This experiment was performed last of the three experiments performed in this study (hence, it was performed on days 23–24). Therefore, this drop is possibly due to an increased sensitivity of the rats to the actual testing procedure, indicating that the rats had become accustomed to the filament application and flinch in response to the application itself rather than a noxious stimulus. However, despite the increased sensitivity of the sham animals, on postdrug test day they were still significantly less sensitive than the animals that had undergone SNL surgeries (both the vehicle-treated and AACBA-treated groups).

Collagen-Induced Arthritis, Experiment 1 (Prophylaxis). Animals that received collagen had a significantly higher combined clinical score than control animals (Fig. 8A). This effect was evident on days 15, 18, and 21 after initial collagen injection. Chronic administration (from day 1) of either dexamethasone or AACBA prevented this elevation in clinical score and produced a score similar to that seen in vehicle control animals (that did not receive collagen). Furthermore, dexamethasone alone (in animals that did not receive collagen; Saline/Dex) had no effect compared with vehicle control animals (that did not receive collagen; Saline/Veh).

Collagen produced a significant increase in paw volume difference (test − baseline paw volume) when examined on days 18 and 21 after the initial injection of collagen (Fig. 8B). Dexamethasone prevented the increase in the paw volume difference compared with animals receiving collagen alone and even significantly reduced the paw volume difference compared with vehicle-treated animals. This reduction may have been due to the lack of normal weight gain seen in dexamethasone-treated animals compared with control animals (Fig. 8C). Indeed, both CIA/Dex- and Saline/Dex-treated groups displayed lower paw volume differences than Saline/Veh-treated animals. AACBA also prevented an increase in the paw volume difference compared with collagen alone-treated animals, indicating a suppression of paw swelling in these animals.

With the exception of CIA/AACBA-treated animals, all
treatment groups gained weight during days 11 to 21 (Fig. 8C). Animals treated with dexamethasone (CIA/Dex and Saline/Dex), although gaining weight at a similar rate to Saline/Veh-treated animals, did have a lower weight on day 11 than they did at baseline. In contrast, CIA/AACBA-treated animals were similar in weight to Saline/Veh and CIA/Veh animals on day 11, but then they did not gain weight between days 11 and 21.

Animals that received collagen alone (CIA/Veh) had significantly reduced movement time and vertical activity measures compared with control animals (Saline/Veh; Fig. 8F).

Chronic administration of dexamethasone (CIA/Dex group) prevented the collagen-induced reduction in activity. In contrast, chronic administration of AACBA (CIA/AACBA group) significantly decreased movement time, vertical activity, and total distance compared with the collagen alone-treated group (CIA/Veh).

Histologically, collagen alone significantly increased histological signs of damage within the joints. This damage was prevented by treatment with either dexamethasone or AACBA (representative photomicrographs are shown in Fig. 9). Comparing histology score (the sum of scores for the seven assessed measures on the hindpaw) to the clinical score obtained on day 21 for the same hindpaw, a significant correlation between the treatment groups was seen ($r_s = 0.376; P = 0.05$; Fig. 8D).

When examining the clinical score and histology scores according to treatment group, the results show a similar pattern in both measurements (Fig. 8G). The CIA/Veh-treated animals showed significant increases in both measures compared with Saline/Veh alone. Dexamethasone or AACBA treatment significantly prevented the collagen-induced increase in clinical score. Furthermore, dexamethasone significantly prevented the collagen-induced increase in histology score, whereas AACBA, although not statistically significant ($P = 0.055$), partially prevented the collagen-induced increase in histology score. When examining the effect of treatment on the number of scores at each histopathology severity level in the hindpaw tarsal joints, a shift from mainly normal and mild in the Saline/Veh group to some scores of moderate (2), severe (3), and maximal (4) in the CIA/Veh group (Fig. 8E). Interestingly, both dexamethasone (CIA/Dex) and AACBA (CIA/AACBA) treatment reduced the number of severe and maximal ratings, indicating a profile closer to that seen in the Saline/Veh animals than that seen with CIA/Veh.

Blood plasma samples were taken by cardiac puncture immediately before necropsy on day 22, approximately 24 h...
Fig. 7. Effect of AACBA on mechanical sensitivity following the L5 SNL model of peripheral neuropathy. Habituation and baseline testing as well as surgery were performed as described under Materials and Methods. Animals were tested with morphine, pregabalin, and AACBA or vehicle sequentially, with a washout period of 5 days between the testing of each drug. Force describes the mean force required per group to elicit a withdrawal response in the injured (left) hindpaw of animals following SNL surgery. A, effect of three subcutaneous injections of vehicle (10% hydroxypropyl-β-cyclodextrin) or 30 mg/kg AACBA administered 48, 24, and 1 h before testing. Animals received SNL surgery and AACBA (SNL/AACBA), SNL surgery and vehicle (SNL/Vehicle), or sham surgery and vehicle (Sham/Vehicle). Testing was performed on day 24 after surgery. B, effect of a single oral dose of vehicle (0.5% methylcellulose) or 30 mg/kg pregabalin administered 1 h before testing. Animals received SNL surgery and pregabalin (SNL/Pregabalin), SNL surgery and vehicle (SNL/Vehicle), or sham surgery and vehicle (Sham/Vehicle). Testing was performed on day 24 after surgery. C, effect of a single subcutaneous injection of vehicle (saline) or 5 mg/kg morphine administered 1 h before testing. Animals received SNL surgery and morphine (SNL/Morphine), SNL surgery and vehicle (SNL/Vehicle), or sham surgery and vehicle (Sham/Vehicle). Testing was performed on day 17 after surgery. *P < 0.05 compared with SNL-injured, vehicle-treated animals (SNL/Vehicle) on that same day.

Discussion

It has widely been suggested that the P2X7 receptor plays a role in the pathophysiology of pain and inflammation. Previous studies have identified such a role in animal models of neuropathic pain (Chessell et al., 2005; Honore et al., 2006; McGaraughty et al., 2007) and rheumatoid arthritis (Labasi et al., 2002). Recent studies have identified novel chemical matter that selectively antagonizes the P2X7 receptor and has effects in disease models (Lappin et al., 2005; Honore et al., 2006; Nelson et al., 2006; Cruwys et al., 2007; McGaraughty et al., 2007) as well as more acute models of pain and inflammation (Lappin et al., 2005; Honore et al., 2006). In the present study, we fully characterized the previously described P2X7 antagonist AACBA (Table 1). In addition to the previously reported selectivity of AACBA for P2X7, compared with P2X1 (P2X1 IC50 > 10 μM; Lappin et al., 2005), we found that AACBA was also selective for P2X7 compared with P2X4 (IC50 > 10 μM; data not shown). However, the complete selectivity profile of this compound is, as yet, unknown. As reported previously (Fonfria et al., 2005; Lappin et al., 2005), AACBA demonstrated greater potency at the human compared with rat P2X7 receptor based on YO-PRO-1 uptake assays.

The effect of AACBA in the LPS-induced IL-6 release assay is consistent with previous reports of the P2X7 receptor playing a pivotal role in cytokine release (Gourine et al., 2005). Although we measured IL-6 levels in the present study, we have also reproduced the observations of Gourine et al. (2005) that P2X7 antagonism by pyridoxal-phosphate-6-azophenyl-2',4'-disulphonate can modulate LPS-induced changes in IL-1 and TNF-α levels (Gourine et al., 2005; data not shown). Other studies have demonstrated a prominent role for P2X7 in the production of other cytokines (Ferrari et al., 1997; Solle et al., 2001). Subsequent investigation has found close links between P2X7 activation and the NALP3 inflammasome, providing a mechanism by which P2X7 plays a role in modulating cytokine release (Mariathasan et al., 2006; Di Virgilio, 2007). These results may suggest that AACBA also inhibits LPS-induced IL-1β and TNF-α levels in vivo, and they support a broad role of P2X7 in the activation and potentiation of inflammatory processes by the modulation of proinflammatory cytokine release into the systemic circulation.

We also found that AACBA produces dose-dependent decreases in both paw swelling and mechanical hypersensitivity after intraplantar carrageenan injection. This effect of P2X7 antagonism occurs both by prophylactic and therapeutic dosing, indicating a role of P2X7 in the development of...
and also in the acute maintenance of inflammation-induced swelling and hypersensitivity. Thus, P2X<sub>7</sub> antagonism can both prevent and reverse the effects of carrageenan injection. These results are consistent with results seen by Honore et al. (2006) in which another P2X<sub>7</sub> antagonist, A-740003, produced significant antihyperalgesic and antiedemic effects after carrageenan injection. Interestingly, unlike A-740003, AACBA seemed to be similarly efficacious at reversing/preventing edema as it was in reversing thermal hyperalgesia. However, this difference may be due to differences in pretreatment time between the present study (1.5 or 2 h before testing for therapeutic dosing and 3.5 h before testing for prophylactic dosing; carrageenan was always dosed 3 h before testing) and the study by Honore et al. (2006) (30 min before testing; carrageenan was dosed 2 h before testing). The present data add to previous literature suggesting a role for the P2X<sub>7</sub> receptor in the development and acute maintenance of inflammatory edema and hypersensitivity.

Why AACBA did not affect L5 SNL-induced tactile hypersensitivity is unknown. Knockout studies suggest that the P2X<sub>7</sub> receptor does play a role in either the development or maintenance of nerve injury-induced behavior (Chessell et al., 2005). Our results suggest that antagonizing the P2X<sub>7</sub>
receptor has no effect, if the hypersensitivity is pre-established. Thus, a therapeutic dosing schedule such as the one performed in this study has little effect. This may be consistent with the observation of Chessell et al. (2005) that the P2X7 receptor was critically active during the development phase of the neuropathy. A potential role or roles for the P2X7 receptor in the development or maintenance of nerve injury-induced behavior can be implied by the presence of the receptor on glial cells. Microglia have been shown to play a crucial role in the development of pathological changes in various models of neuropathic-like injury (for reviews, see Watkins and Maier, 2002; Wieseler-Frank et al., 2004), and P2X7 receptors are involved in the release IL-1β from microglia (Ferrari et al., 1997; Brough et al., 2002). Such mechanisms may be consistent with a role for P2X7 in the development but not the maintenance of nerve injury-induced hypersensitivity because previous reports have suggested that inhibition of microglial activation is important in the development but not maintenance of L5 SNL-induced hypersensitivity (Raghavendra et al., 2003), although astrocytes, which also express P2X7, are also reported to be activated and play a role in nerve injury (Tanga et al., 2004; Wieseler-Frank et al., 2004).

Previous pharmacological studies have demonstrated an effect of P2X7 antagonists in a similar model of nerve injury-induced hypersensitivity (Honore et al., 2006; Nelson et al., 2006; McGaraughty et al., 2007). The reasons for the difference in effect between P2X7 antagonism in these previous studies and the present study are unknown. Our study used a compound dissimilar in structure from previous work. Hence, it may possess properties unsuited to seeing an effect in the L5 SNL-induced hypersensitivity model. For example, AACBA is 30 times less potent in the rat YO-PRO assay (IC50 = 980 nM) compared with rat calcium mobilization (IC50 = 29 nM). This is in contrast to A-740003, which did not have such a large difference in potency in similar assays.
Alternatively, because the glial P2X7 receptor may be the key target for antagonizing nerve injury-induced hypersensitivity, CNS exposure of AACBA may also have played a role in the lack of effect of the compound on L5 SNL-induced hypersensitivity. Plasma exposures measured after acute administration of AACBA in the carrageenan-induced paw swelling experiment indicated sufficient systemic exposure to obtain a therapeutic effect. Yet, AACBA was reported by Lappin et al. (2005) to have a brain-to-blood ratio of approximately 0.46, indicating that CNS levels of drug would be lower than those seen in the plasma. Therefore, the dose of AACBA used in the SNL study may not have achieved sufficient CNS penetration to gain efficacious levels of the drug at the site of action and thus may explain the lack of effect. Pharmacokinetic properties of AACBA may limit its ability to fully probe the role of P2X7 receptor in neuropathic hypersensitivity. Studies using intrathecally administered AACBA may help address this question. The role of the P2X7 receptor in animals models of rheumatoid arthritis have been documented by other groups (Labasi et al., 2002; Cruwys et al., 2007). Our results are consistent with previous work indicating an antiarthritic-like effect with P2X7 antagonism. AACBA not only prevented the

TABLE 1

Summary of in vitro and in vitro characteristics of AACBA
Assays were performed and AACBA dosed as described under Materials and Methods. IC50 values or minimal effective dose (MED) is given where possible. MED was defined as the lowest dose tested that produced a statistically significant effect. Single dose experiments are summarized by the presence or lack thereof of a statistically significant effect at that dose.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
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<tbody>
<tr>
<td>Calcium mobilization</td>
<td>IC50 = 18 nM (hP2X7)</td>
</tr>
<tr>
<td>YO-PRO-1 uptake</td>
<td>IC50 = 29 nM (rP2X7)</td>
</tr>
<tr>
<td>LPS-induced IL-6 release</td>
<td>IC50 = 85 nM (hP2X7)</td>
</tr>
<tr>
<td>Carrageenan-induced mechanical hypersensitivity</td>
<td>IC50 = 890 nM (rP2X7)</td>
</tr>
<tr>
<td>Carrageenan-induced paw swelling</td>
<td>MED = 10 mg/kg</td>
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<tr>
<td>L5 SNL</td>
<td>MED = 30 mg/kg</td>
</tr>
<tr>
<td>Collagen-induced arthritis</td>
<td>MED = 10 mg/kg</td>
</tr>
<tr>
<td></td>
<td>No effect at 30 mg/kg</td>
</tr>
<tr>
<td></td>
<td>Effective prophylactically at 30 mg/kg</td>
</tr>
<tr>
<td></td>
<td>No effect therapeutically at 30 mg/kg</td>
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</table>

a AACBA (30 mg/kg) was dosed three times, at 48, 24, and 1 h before testing.

b AACBA (30 mg/kg) was dosed once a day from day of first collagen injection through day 21.

c AACBA (30 mg/kg) was dosed once a day starting after the initial appearance of clinical signs and symptoms following the second collagen injection (dosing began on day 13) and continued through day 21.

(17 nM in calcium versus 100 nM in YO-PRO; Honore et al., 2006). Alternatively, because the glial P2X receptor may be the key target for antagonizing nerve injury-induced hypersensitivity, CNS exposure of AACBA may also have played a role in the lack of effect of the compound on L5 SNL-induced hypersensitivity. Plasma exposures measured after acute administration of AACBA in the carrageenan-induced paw swelling experiment indicated sufficient systemic exposure to obtain a therapeutic effect. Yet, AACBA was reported by Lappin et al. (2005) to have a brain-to-blood ratio of approximately 0.46, indicating that CNS levels of drug would be lower than those seen in the plasma. Therefore, the dose of AACBA used in the SNL study may not have achieved sufficient CNS penetration to gain efficacious levels of the drug at the site of action and thus may explain the lack of effect. Pharmacokinetic properties of AACBA may limit its ability to fully probe the role of P2X7 receptor in neuropathic hypersensitivity. Studies using intrathecally administered AACBA may help address this question. The role of the P2X7 receptor in animals models of rheumatoid arthritis have been documented by other groups (Labasi et al., 2002; Cruwys et al., 2007). Our results are consistent with previous work indicating an antiarthritic-like effect with P2X7 antagonism. AACBA not only prevented the
onset of signs and symptoms of collagen-induced arthritis but also prevented histopathological damage from occurring. We did, however, see differences in effect with different dosing regimens, with dosing initiation required before the onset of clinical signs and symptoms to see a significant effect. This indicates that P2X7 antagonism is required either before or immediately after the second (booster) injection of collagen, but it cannot reverse pre-established collagen-induced arthritis. Although we have described the effective administration of AACBA as "propylactic" and the ineffective administration paradigm as "therapeutic," the results could be more indicative of the pathology of the collagen-induced arthritis model, rather than indicative of the potential clinical usefulness of the drug itself. This is illustrated by the fact that methotrexate did not produce a significant effect when dosed in a similar therapeutic dosing paradigm even though methotrexate has clear therapeutic benefit in the clinic. It should be noted that methotrexate administered as a propylactic exhibits significant efficacy in a CIA model (Budancamanak et al., 2006). This profile contrasts with the strong therapeutic effect seen here with dexamethasone, a frank immunosuppressant, and suggests that P2X7 antagonism may have an effect more similar to methotrexate. Considering these results, AACBA adds to the literature reporting P2X7 antagonism as being a potentially useful pharmacological intervention for patients with rheumatoid arthritis. Early data from AstraZeneca's AZD9056 is highly suggestive of this potential (McInnes et al., 2007), and further information from forthcoming clinical trials will help to further define the role for P2X7, in rheumatoid arthritis.

In summary, AACBA proved to be a useful tool in the evaluation of P2X7 receptor involvement in pain and inflammation (Table 1). The P2X7 receptor is clearly involved in preclinical models of inflammation and in some models of pain. Further study of the P2X7 receptor with this and other pharmacological antagonists in preclinical studies as well as in disease-specific clinical trials will help evaluate this mechanism for potential therapeutic use in pain and inflammatory/autoimmune diseases.

Acknowledgments
We thank Julia Isabelle, Lauren Danner, and Carolyn Baltazar for expert cell culture assistance and Fenggang Zhu, Jean Simmerman, David Beers, and Drs. Ronda Morgan and Yong Lui for drug plasma exposure analyses.

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