Preclinical Pharmacology of AZD2327: A Highly Selective Agonist of the δ-Opioid Receptor

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Received January 18, 2011; accepted March 24, 2011

ABSTRACT

In the present article, we summarize the preclinical pharmacology of 4-[(R)-(3-aminophenyl)[4-(4-fluorobenzyl)piperazin-1-yl]methyl]-N,N-diethylbenzamide (AZD2327), a highly potent and selective agonist of the δ-opioid receptor. AZD2327 binds with sub-nanomolar affinity to the human opioid receptor (Ki = 0.49 and 0.75 nM at the C27 and F27 isoforms, respectively) and is highly selective (>1000-fold) over the human μ- and κ-opioid receptor subtypes as well as >130 other receptors and channels. In functional assays, AZD2327 shows full agonism at human δ-opioid receptors ([35S]GTPγS EC50 = 24 and 9.2 nM at C27 and F27 isoforms, respectively) and also at the rat and mouse δ-opioid receptors. AZD2327 is active in a wide range of models predictive of anxiolytic activity, including a modified Geller-Seifter conflict test and social interaction test, as well as in antidepressant models, including learned helplessness. In animals implanted with microdialysis probes and then given an acute stressor by pairing electric shock delivery with a flashing light, there is an increase in norepinephrine release into the prefrontal cortex associated with this acute anxiety state. Both the benzodiazepine anxiolytic standard diazepam and AZD2327 blocked this norepinephrine release equally well, and there was no evidence of tolerance to these effects of AZD2327. Overall, these data support the role of the δ-opioid receptor in the regulation of mood, and data suggest that AZD2327 may possess unique antidepressant and anxiolytic activities that could make a novel contribution to the pharmacotherapy of psychiatric disorders.

Introduction

Despite a growing number of treatment options available to patients with major depressive disorder, a significant proportion of patients remain untreated or undertreated (Warden et al., 2007). This incomplete treatment response is likely due to a number of factors, including disease heterogeneity, and certainly to a great overlap in the mechanisms of action of the available therapies. It is likely that if the treatment gap is to be filled, mechanistically distinct treatment approaches will be needed.

The involvement of the opioid system in emotional regulation has been suggested for decades (Emrich et al., 1979; Tejedor-Real et al., 1995), although understanding of it is complicated by the multiplicity of receptor subtypes and endogenous ligands and potential hetero-oligomerization of the opioid receptors with each other as well as with other G protein-coupled receptors and ion channels (Walwyn et al., 2009). The three historically defined opioid subtypes are μ-opioid receptors, named after the prototypic agonist morphine; κ-opioid receptors, named after the benzomorphan opioid ketocyclazocine; and δ-opioid receptors, named after observations of effects of agonists in a mouse vas deferens assay. All three opioid receptors are thought to affect nociceptive and emotional responses, albeit in qualitatively different manners. Endorphins and enkephalins [primarily Met-enkephalin (FK 33-824)] are the endogenous ligands for μ-opioid receptors, and μ-opioid receptor agonists produce euphorogenic responses and robust analgesia (both spinal and supraspinal) for acute nociceptive stimuli. Limitations of...
their therapeutic use include abuse liability and marked tolerance to the analgesic effects, requiring increases in dose, but with little or no tolerance to other, undesired effects, such as respiratory depression or decreases in gastrointestinal motility. The clinical use of μ-opioid receptor antagonists has been limited largely to rescue after opiate overdose but has been shown more recently to attenuate the euphorogenic effects of other drugs of abuse, such as alcohol, thereby further suggesting the potential involvement of the opioid system in emotional regulation. The endogenous dynorphins bind to κ-opioid receptors and are thought to negatively regulate mood as well as mediate visceral and spinal analgesic responses. Although agonists are known to produce dysphoric and, in some cases, hallucinogenic effects (Walentiny et al., 2010), there are preclinical data to suggest that κ-opioid receptor antagonists may be useful antidepressants (Carr et al., 2010). Endogenous Leu-enkephalin somewhat preferentially interacts with δ- versus μ-opioid receptors, and a concordance of data would suggest that more selective agonists of the receptor will result in positive modulation of mood, anxiolytic effects (Nieto et al., 2005), and analgesia, which may be related to these affective effects of agonists. Unlike μ-opioid receptor agonists, δ-opioid receptor agonists produce minimal effects on respiratory and gastrointestinal systems (Porreca et al., 1984). Several studies have shown that δ-opioid receptor agonists lack or have minimal reinforcing effects (Negus et al., 1998; Do Carmo et al., 2009), indicating a low liability for abuse. However, δ-opioid receptor agonists do introduce a higher risk of producing convulsions than agonists at the other receptor subtypes. Identification of the δ-opioid receptor as a possible target in the treatment of anxiety and depression began with observations of an anxious and depressive-like phenotype in the δ-opioid receptor knockout mouse (Filliol et al., 2000). Since then, a number of laboratories have demonstrated the efficacy of several peptidic and nonpeptidic opioids of varying selectivities for the δ-opioid receptor in assays for antidepressant activity (reviewed by Jutkiewicz, 2006).

In Vitro Pharmacology. Radioligands. Deltorphin II, FK 33-824, and d-Pro10-dynorphin A(1-11) (DPDYN) were iodinated with [125I]Na and then purified by high-performance liquid chromatography to apparent homogeneity (2200 Ci/mmol) as described previously (Payza, 2003).

Cell lines and membrane preparations. As described previously (Payza, 2003), human embryonic kidney (HEK) 293S cells were used to express all three human opioid receptor subtypes. The human δ-opioid receptors were expressed with both of the prevalent single-nucleotide polymorphisms at position 27 (Adam et al., 2001): one with cysteine (GenBank accession number U07882; C27) and the other with phenylalanine (GenBank accession number U10504; F27). Both clones were used because both are represented in the general population, although the F27 isoform is more prevalent. The C27 clone 11 cell line (Bmax 9.7 pmol/mg protein) was used for receptor binding assays, and lower expressing clone 2 (0.69 pmol/mg protein) was used for [35S]GTPγS assays. In the case of the F27 isoform, clone 4 (Bmax 3.7 pmol/mg protein) was used for both binding and functional assays. Suspension conditions were used for the production of membranes expressing the human δ-opioid receptor isoform C27 as well as the human μ- and κ-opioid receptors. The cells were grown, harvested, and processed into membrane preparations exactly as described previously (Payza, 2003). In the case of the human δ-opioid receptor isoform F27, culture was done under adher-

![Fig. 1](https://jpet.aspetjournals.org/)

**Fig. 1.** Structures of representative δ-opioid receptor agonists. ADL5859 [N,N-diethyl-4-(5-hydroxyspiro[chromene-2,4'-piperidin]-4-yl)benzamide] and closely related ADL5747 [N,N-diethyl-3-hydroxy-4-(spiro[chromene-2,4'-piperidin]-4-yl)benzamide] have been in clinical development as analgesics. AR-M100390 (sometimes referred to as ARM390) is a tool compound from AstraZeneca that has been published previously (Wei et al., 2000; Smagin et al., 2008; Pradhan et al., 2010).
ent conditions in normal Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 2 mM glutamine, and 600 μg/ml Genetica, followed by membrane preparation using the same method as that for suspension cells. Rat brain membranes were prepared as described previously (Fraser et al., 1999).

Affinity binding assays. The Kᵢ values of AZD2327 were determined in membrane-based competitive binding assays exactly as described previously (Payza, 2003). In brief, AZD2327 was tested for its ability to compete against the binding of [³⁵S]Deltorphin II to δ-opioid receptors and against [¹²⁵I]P FK 33-824 and [¹²⁤I]DPDYN for the μ- and κ-opioid receptors, respectively. Radioligands were used at concentrations of 0.03 to 0.05 nM. In all cases, the buffer was 50 mM Tris, 3 mM MgCl₂, and 0.1% bovine serum albumin (pH 7.4), and 10 μM naloxone was used to define nonspecific binding. Internal standards were SNC-80 (for δ), [α-Ala²,N-MePhe⁴,Gly-ol]-enkephalin (for μ), and U69593 (N-methyl-2-phenyl-N-[(4R,7S,8S)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]dec-8-yl]acetamide) (for κ). All of the selectivity ratios were determined using high-affinity binding conditions among each of the receptor subtypes.

[^35S]GTPγ binding assays. AZD2327 was tested for agonism in membrane-based[^35S]GTPγ filtration binding assays using the exact experimental procedure and method to determine EC₅₀ and E₄₅ as described previously for the cloned human δ-opioid receptors (Payza, 2003) and for endogenous δ-opioid receptors in rat brain membranes (Fraser et al., 1999). In brief, the assays were performed in 96-well plates in 50 mM HEPES, 20 mM NaOH, 200 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 1 mM dithiothreitol, and 0.5% bovine serum albumin (pH 7.4), with GDP added a concentration of 3 μM (for C27 and F27 isoforms of the human δ-opioid receptor) or 45 μM (for rat brain membranes). Incubation was for 1 h at room temperature. SNC-80 (10 μM) was used to define 100% E₄₅.

Electrically stimulated tissue assay. Mouse vas deferens, a standard δ-opioid receptor agonism assay, and guinea pig ileum, a standard μ-opioid receptor agonism assay, were performed at CEREP (Poitiers, France) using established methods exactly as described previously (Gengo et al., 2003).

In Vivo Microdialysis. Animals. Sixty-four male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were used in this experiment. Animals were housed in a temperature-controlled vivarium with free access to food and water.

Probe implantation and measurements. Under ketamine (60 mg/kg) and xylazine (8 mg/kg) anesthesia, animals were implanted with a guide cannula with a dummy insert (CMA/12) aimed into the prefrontal cortex using the following coordinates: AP (anterior-posterior) + 3.2 mm, L (lateral) ± 1.6 mm, V (ventral) −1.0 mm. The guide cannulae were anchored to the skull with screws and dental cement. Animals were allowed to recover from surgery for 6 days. Microdialysis probes (CMA/12, 4-mm membrane length) were implanted in the brain 18 h before the experiment and were perfused with artificial cerebrospinal fluid (CMA Microdialysis, North Chelmsford, MA) at a flow rate of 1.1 μl/min. Neurotransmitter levels were assessed by high-performance liquid chromatography (model 5200A Coulombic II detector, MD-150 3 × 150 mm column, model 5041 Amperometric cell; all from ESA, Inc.) with an on-line injector (BAS Bioanalytical Systems, West Lafayette, IN). The mobile phase was 75 mM NaH₂PO₄, 25 mM EDTA, 1.7 mM 1-octanesulphonic acid, 100 μl triethylamine, and 10% acetonitrile (pH 3.0). The potential was set at +0.65 V, and the flow rate was maintained at 0.3 ml/min. Three 20-min samples were collected to define the baseline, animals were given vehicle or compounds, and sample collection was carried out for the next 5 h. Concentrations of neurotransmitters in three samples collected before the administration of compounds or vehicle were averaged and defined as the baseline (100%). Concentrations of neurotransmitters in the subsequent microdialysates then were expressed as percentages of baseline levels. Statistical analyses for microdialysis studies were performed using repeated measures multivariate analysis of variance (ANOVA) followed by Dunnett’s post hoc test.

Stress procedure. Standard avoidance chambers equipped with lights and shockers were used (MED Associates, St. Albans, VT). Boxes were placed in sound-attenuating chambers. The stress conditioning paradigm was conducted over 1 day. Animals were acclimated to the chambers for 18 h before conditioning. Conditioning consisted of turning on and off the houselight in the chamber at a rate of two times per second for 2 s (conditioned stimulus), followed by delivery of electric current to the feet of the rat via the grid floor in the chambers (unconditioned stimulus; 0.5-s duration, 1.5-mA intensity, total 10 shocks). The “no stress” group was exposed to chambers with lights but did not receive shocks. Two hours after the start of the stress paradigm, the light sequence was repeated, but no shocks were administered. The eight experimental groups were assigned as shown in Table 1.

All of the experimental compounds and vehicle (0.9% phosphate-buffered saline) were administered orally 1 h before the stress procedure. The AZD2327 doses that were tested were 0.3, 1, and 3 mg/kg. Diazepam (Sigma-Aldrich, St. Louis, MO) was given at a dose of 3 mg/kg, in a vehicle of Abbott’s cocktail.

To address whether tolerance developed to the neurochemical effects of AZD2327, groups of 16 naive rats were administered orally 1 mg/kg AZD2327 daily for either 1, 14, or 21 days and half of the animals in each group were conditioned as described above 1 h after their last doses, and the other half were placed simply into the conditioning chamber for the same period of time as those groups receiving footshock. Two vehicle control groups also were included: one that received conditioning after a single administration of vehicle and a second that did not receive conditioning after administration of vehicle. Three additional groups of four animals were treated with 3 mg/kg p.o. diazepam for 1, 14, and 21 days and submitted to conditioning. Forty minutes after conditioning, the brains were removed rapidly and snap-frozen. Medial prefrontal cortex was dissected from half of the brains in each treatment group, homogenized, and assayed for norepinephrine (NE) and 3-methoxy-4-hydroxyphenolglycol (MHPG) (NE catabolite) in 0.1 M perchloric acid using electrochemical detection (Coulochem 5011; ESA, Inc., Chelmsford, MA) after separation by high-performance liquid chromatography on a reverse-phase C18 column (UltraspHERE ODS, 4.6 × 250 mm; Beckman Coulter, Inc., Fullerton, CA). Striatal membranes from the remaining brains in each treatment group (except for diazepam-treated rats) were prepared individually and assayed for agonist activity as described above.

Anxiolytic Activity: Modified Geller-Seifert Conflict Test. Animals. Male Long-Evans rats weighing 375 to 425 g were used. Animals were maintained at 80 to 90% of their free-feeding weights by limited feeding after the experimental session. For any given drug test, rats whose responses were most stable were chosen from a larger pool of animals trained as described below. Several doses were tested on a given test day in different subjects. A minimum of eight animals was used for each data point.

Apparatus. Standard two-lever operant chambers were used (MED Associates). The chambers were fitted with two retractable response levers and a stimulus lamp over each of the two levers. A pellet dispenser delivered 45 mg of food pellets (Bio-Serv, Frenchtown, NJ) to a cup located inside the chamber below and between the two response levers. A lamp at the top and back of the chamber served as the houselight. The grid floors of the operant chambers were interfaced to shock generators and scramblers (MED Associates, St. Albans, VT). All of the events in the chambers were controlled and monitored by a microprocessor.

| TABLE 1 |
| Eight experimental groups assigned (n = 8 mice per group) |
| Vehicle: No Stress | Vehicle: Stress |
| **Diazepam (3 mg/kg p.o.): stress** | **AZD2327 (0.1 mg/kg p.o.): stress**  |
| **AZD2327 (3 mg/kg p.o.): no stress** | **AZD2327 (0.3 mg/kg p.o.): stress** |
| **AZD2327 (3 mg/kg p.o.): stress** | **Diazepam (3 mg/kg p.o.): no stress** |
Procedure. There were two components in the procedure: 1) unsuppressed response components (unpunished) 2 min in duration and 2) suppressed response components (punished) 3 min in duration. In unpunished components, the houselights and both stimulus lamps over the response levers were turned on, the lever on the left-hand side of the chamber was extended, and a food pellet was delivered after an average of 17 responses on the lever in the chamber (range, 3–40 responses)—a variable-ratio 17 schedule. The punished components followed unpunished components, and during these, the right-hand lever was extended into the chamber, and the stimulus lamps and houselights were turned on and off at 1-s intervals, in succession, which served as a cue for this component. In the punished component, food was also available under a variable-ratio 17 schedule, but in addition, electrical current (0.5-s duration) was delivered to the grid floor of the chamber under an independent variable-ratio 17 schedule. The level of the current was adjusted for each individual subject until response was reduced in the suppressed component to a level that was approximately 5 to 10% that of the unpunished component and ranged from 0.2 to 0.75 mA. Unpunished and punished components were separated by 10-s time-out periods in which both response levers were retracted and all of the stimulus lamps were turned off. The 2-min unpunished and 3-min punished punished components alternated until five of each were completed. Daily sessions always began with the unpunished response component.

The dependent variables recorded were the rate of response in unpunished and punished components (total responses/total time under the component) and the number of shocks delivered. A selective anxiolytic effect is defined as an increase in response in the punished components with relatively less or no effect on response in unpunished components. The t tests were used to compare the mean of the control group’s rate of response to vehicle for the rats used for a specific dose to the same rats’ means after delivery of each dose of compound (for only the rats used within each dose).

Dosages of the compound were reported as the free base. AZD2327 was dissolved in distilled deionized water/85% lactic acid. Naltrexone was dissolved in saline and was given subcutaneously in combination with AZD2327 given orally. All of the compounds were given in a dose volume of 1 ml/kg b.wt. AZD2327 was tested 1 h after drug administration, and naltrexone was administered 20 min before testing. Drugs were administered on Tuesday and Friday, and vehicle was administered on Thursday. Animals were run in baseline operant sessions on Mondays and Wednesdays.

Social Interaction Test. Animals. Male Long-Evans rats weighing 250 to 300 g were used. Animals were housed individually during the duration of the experiment and allowed free access to food and water, except during experimental testing.

Apparatus. A circular steel arena (33 cm in height 33 cm, 118 cm in diameter) was used. The arena was illuminated to 1000 lux, and activity was monitored from an adjacent room by a video camera.

Procedure. The rats were pre-exposed individually to the test under low (>100 lux) light level for a 10-min period on each of the 2 days preceding the test. The animals then were allocated to test partners on the basis of weight (closely matched) and divided into drug groups. On the test day, the pairs of rats were placed in the center of the area under high light (>1000 lux) and were scored for a 10-min period from a video monitor in an adjacent room in addition to one observer. The following behaviors were scored and classified as active social interaction: sniffing, following, grooming, mounting, and crawling under or over the partner. The total time spent in active interaction then was reported. Mean scores of the drug-treated groups were compared with those of the vehicle-treated groups by Student t tests. AZD2327 (1 and 10 mg/kg) was administered 30 min before the session. Chlordiazepoxide (7.5 mg/kg i.p.) was administered 20 min beforehand. Both rats were administered equivalent doses of the same compound.

Antidepressant Activity: Learned Helplessness. Animals. Sixty-four male Sprague-Dawley rats were used in the present study. Subjects weighed on average 275 to 325 g at the start of the study.

Apparatus. Standard shuttle cages (20 cm in length, 16 cm in width, 21 cm in height) fitted with a grid floor were used. The chambers could be partitioned with a closed partition or with an archway that allowed the animals to pass between the two sides of the cage. A computer controlled and monitored all of the events in the chamber.

Procedure. The procedure consisted of two phases: induction and avoidance training. In the induction phase, mice were enclosed into one side of the shuttle cages, and electrical stimulation (2 mA, 9.5-s duration) was delivered to the floor of the cage every 2.5, or 10 s (randomly selected for each trial) until 90 shocks were delivered. Subjects had no opportunity to escape or to avoid shocks. Induction was conducted for 2 consecutive days. In the avoidance training phase, testing also was conducted in the shuttle cages, except all of the cages then were fitted with a partition with an arch in the center of the cage through which animals could pass between the left and right halves of the cage. The procedure used was a standard shuttle avoidance in which a compound, conditioned stimulus (a 5-s presentation of a tone and turning on of a lamp on the side of the cage that the subject was occupying) served to indicate the impending presentation of electrical current to the floor of the cage. Shock was presented for a 5-s period, 5 s after initiation of the stimulus. Entry into the opposite side of the shuttle cage via the arch partition after shock onset resulted in the end of the trial (avoidance response). If a shock was delivered, then entry into the opposite side of the cage resulted in the termination of the shock and the CS (escape response). A 30-s intertrial interval was used. Forty-minute avoidance training sessions consisting of 50 trials were conducted on 2 consecutive days, beginning 48 h after the final induction session.

Drug administration and preparation. Dosages of all of the compounds were reported as the free base. Imipramine and AZD2327 were dissolved in distilled deionized water and were administered orally in a volume of 1 ml/kg b.wt. Drug was administered twice daily throughout the experiment, immediately after conditioning and training sessions and approximately 7 to 8 h after the first injection, as well as on the day in the middle of the study when no conditioning or training was conducted.

Data analysis. The primary dependent variable was escape failures during avoidance training. In addition, because some δ-opioid receptor agonists have been shown to produce locomotor stimulation, center crossings during avoidance training also were recorded and compared among groups, which allows a gauge of motor activity. An increase in center crossings with respect to the vehicle control suggests that locomotor stimulation may be at least partly responsible for the putative antidepressant effects of the compound. The t tests were used to compare the performance of the vehicle-administered group to those of the drug-treated groups. The no-induction group was used to gauge whether learned helplessness was established by comparison with the vehicle-treated group.

Secondary Pharmacology. All of the in vivo safety pharmacology studies described below were conducted using the standard vehicle (phosphate-buffered saline) with the drug delivered orally. There were no differences in the pharmacokinetics noted among the different formulations used in the present report.

Locomotor Activity. Subjects. Groups of six naive male Sprague-Dawley rats (250–300 g) were used for each data point. Animals were given free access to food and water, except during experimental sessions.

Apparatus. An Open Field Activity System (MED Associates) was used to assess spontaneous locomotor activity in separate groups of animals. The system consisted of eight locomotor activity boxes (ENV 515; MED Associates) of clear acrylic with an inside area of 43.2 × 43.2 × 30.4 cm, housed in melamine sound-attenuating cubicles (ENV 017; MED Associates) with house lights and exhaust fans. Three 16-beam infrared arrays, two placed at 2.5 cm (floor level) and one at 12.5 cm, were used to measure activity. All of the
beam breaks and activity in the boxes were recorded by Activity Monitor software (MED Associates).

Procedure. On the day of the experiment, male Sprague-Dawley rats (Scanbur BK, Sollentuna, Sweden) weighing 200 to 250 g were administered the appropriate doses and placed individually into a locomotor activity box for a 60-min test session. The rats were removed, and the boxes were cleaned after the conclusion of the test session. This procedure was repeated for all of the animals in the experiment. Animals were distributed evenly over doses and time of day. The total mean of ambulatory activity was collected in 5-min intervals during the test session, and ANOVA was used to compare means. Drug was administered orally.

Irwin’s Test. The rat Irwin test was performed to assess central nervous system side effects. Six male Wistar rats were administered orally vehicle or 0.5, 1.5, 5, 15, or 50 mg/kg AZD2327. Each animal was then tested for signs of behavioral, autonomic, neurological, or toxic effects at 15 and 30 min, 1, 2, 4, and 24 h after administration.

Pentylenetetrazole Seizure Thresholds. Six Wistar Han rats weighting approximately 250 to 300 g were used for each data point. Approximately 10 min before the pentylenetetrazole (PTZ) infusion, the rat was placed in a restrainer cage, and a catheter (Neoflon 24G; BD Biosciences, Helsingborg, Sweden) was inserted into the tail vein for the administration of PTZ. The 100 μM/kg per minute infusion of PTZ started 60 min after the oral administration of the test doses of AZD2327. When the first clonic convulsion was observed, the infusion was stopped, and the rat was sacrificed immediately with a bolus infusion of a lethal dose of 100 mg/ml pentobarbital (Arlivningsvåtska; Apoteket AB, Stockholm, Sweden) via the Neoflon catheter. The volume of PTZ infused at the time of the appearance of the first clonic convulsion was read off the injection pump, and the dose of PTZ received was calculated and given in micromoles per kilogram. ANOVA was used to compare means.

Results

Receptor Binding Assays. The activities of AZD2327 at both isoforms of the human δ-opioid receptor and at the human μ- and κ-opioid receptors are shown in Fig. 2. AZD2327 inhibited, in a dose-dependent manner, the binding of [125I]Deltorphin II to the human δ-opioid receptor isoforms C27 and F27 and rat brain δ-opioid receptor with potency Kᵢ values; in contrast, its inhibition of [125I]FK 33-824 and [125I]DPDYN binding to human μ- and κ-opioid receptors was much weaker (Table 2). The selectivity ratios of AZD2327 for the human δ-opioid receptor isoforms C27 and F27 versus human μ- and κ-opioid receptors were in all instances, regardless of which clone was used, >1000-fold. AZD2327 also was tested in a broad screening panel of 130 targets and was without significant activity in the same potency range as its δ-opioid receptor binding (data not shown).

Receptor Agonism Assays. AZD2327 behaved as a moderately potent, full agonist on both human δ-opioid receptor isoforms C27 and F27 as well as on endogenous δ-opioid receptors in rat brain membranes (Fig. 3), with potency values shown in Table 3. In low-affinity conditions (Fraser et al., 1999), the binding potency of AZD2327 was shifted to the right, as expected for G protein-coupled receptor agonists, by 33- and 39-fold for the human δ-opioid receptor isoforms F27 and C27, respectively (data not shown). In the mouse vas deferens, a standard δ-opioid receptor assay, AZD2327 showed full agonism with an EC₅₀ value of 1.7 nM (data not shown). In the guinea pig ileum, a standard μ-opioid receptor assay, it showed very weak activity, eliciting only 33% E₅₀ at a concentration of 10 μM (data not shown).

Neurochemical Assays and Tolerance Development. In microdialysis, delivery of footshock produced a 60 to 70% increase in NE content in the medial prefrontal cortex that was sustained for up to 3 h after the initial stressor (Fig. 4). Consequent increases in MHPG were observed >1 h after the initial increases in NE. Pretreatment with diazepam (3 mg/kg, a dose that was chosen due to consistent efficacy noted historically in our laboratories) fully reversed these effects (Fig. 4). Likewise, 1 and 3 mg/kg AZD2327 reversed the neurochemical changes measured after footshock (Fig. 5). In studies in which AZD2327 was administered for up to 21 days followed by ex vivo neurochemistry and binding and

Table 2

AZD2327 potencies in inhibiting radioligand binding at the human δ-opioid receptor isoforms C27 and F27 and human μ- and κ-opioid receptors stably expressed in HEK293S cells and at rat brain δ-opioid receptors Kᵢ values (geometric means) were determined using the radioligands described under Materials and Methods. pKᵢ = −log Kᵢ (M). n is the number of dose-response curves (each having 10 concentrations of AZD2327 in duplicate).

<table>
<thead>
<tr>
<th></th>
<th>hδC27</th>
<th>hδF27</th>
<th>hμ</th>
<th>hκ</th>
<th>Rat Brain δ</th>
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<tbody>
<tr>
<td>Kᵢ (nM)</td>
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<td>0.75</td>
<td>770</td>
<td>4500</td>
<td>12</td>
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<tr>
<td>Bmax (pmol/mg)</td>
<td>9.7 ± 0.62</td>
<td>3.71 ± 0.72</td>
<td>0.29 ± 0.04</td>
<td>3.4 ± 0.59</td>
<td>0.076</td>
</tr>
<tr>
<td>pKᵢ ± S.E.M.</td>
<td>9.31 ± 0.03</td>
<td>9.13 ± 0.04</td>
<td>6.11 ± 0.03</td>
<td>5.35 ± 0.04</td>
<td>8.92 ± 0.05</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>5</td>
<td>14</td>
<td>13</td>
<td>6</td>
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</table>

Ethics. This study was conducted under protocols that have been approved by an ethical committee. The animals were kept and experiments were performed at AstraZeneca Research and Development (Wilmington, DE), which has accreditation from Association for the Assessment and Accreditation of Laboratory Animal Care and is approved by the AstraZeneca Global Veterinary Council for study conduct.

Fig. 2. Representative displacement curves for AZD2327 with various membrane preparations of HEK293S stable cell lines expressing the human δ-opioid receptor F27 isoform in high-affinity (△) and low-affinity conditions (●), the human μ-opioid receptor (▲), and the human κ-opioid receptor (○). Points represent the means of duplicates. [125I]Deltorphin II was used to label δ-opioid receptors under high-affinity conditions and [125I]AR-M106613 was used under low-affinity conditions, [125I]FK 33-824 was used to label μ-opioid receptors, and [125I]DYN was used to label κ-opioid receptors. Further details are summarized in Table 3 and in the text.
Agonist activity of AZD2327 in [{35S}GTPγS] binding assays performed on membrane preparations of HEK293S stable cell lines expressing the F27 isoform of the human δ-opioid receptor (●) and on rat brain membrane expressing endogenous δ-opioid receptor (○). Points represent the means of duplicates.

TABLE 3
Agonist activity of AZD2327 in [35S]GTPγS binding assays performed on membrane preparations of HEK293S cell lines expressing human δ-opioid receptor isoforms C27 and F27 and human μ- and κ-opioid receptors and rat brain membranes expressing δ-opioid receptors endogenously.

<table>
<thead>
<tr>
<th></th>
<th>hC27</th>
<th>hF27</th>
<th>Rat Brain δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (nM)</td>
<td>24</td>
<td>9.2</td>
<td>18</td>
</tr>
<tr>
<td>%E_{max} ± S.E.M.</td>
<td>91 ± 2</td>
<td>101 ± 1</td>
<td>91 ± 1</td>
</tr>
<tr>
<td>pEC50 ± S.E.M.</td>
<td>7.63 ± 0.03</td>
<td>8.04 ± 0.09</td>
<td>7.65 ± 0.06</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Efficacy measurements, there was no evidence of tolerance development over the course of drug administration. NE turnover, expressed as the ratio of metabolite (MHPG) to NE, was elevated in animals that were stressed by being subjected to the conditioning paradigm used for the in vivo microdialysis studies relative to that in unstressed animals (Fig. 6A, left). This elevation in turnover was reversed by a single administration of AZD2327 and after 7, 14, and 21 days of administration (open squares). Diazepam, used as a positive control in this study, also dampened the stress-induced changes in turnover over the entire period of administration. By 14 days of administration, AZD2327 resulted in a lowering of the ratio to below control (unstressed) levels, although the functional significance of this is not clear. In tissues taken from a subset of animals in this study, both potency (Fig. 6B) and efficacy (Fig. 6C) were unchanged by repeated administration of AZD2327, regardless of whether they were unstressed by shock administration (Fig. 6, B and C, left) or stressed (Fig. 6, B and C, right), indicating no change in signaling through the receptor at least up to 21 days of drug administration.

Anxiolytic Assays. In the conflict test, rates of response averaged 0.04 ± 0.01 and 2.03 ± 0.12 responses/s in the unsuppressed component under baseline conditions. Administration of ≥1 mg/kg AZD2327 was as effective as diazepam in producing increases in response with respect to the control in the suppressed component of the schedule (Fig. 7, left) and did so with a potency that was an order of magnitude greater than that of diazepam. The effects of both agents upon suppressed response were specific to this component, because neither altered response in the unsuppressed component (Fig. 7, right). Evidence that the anxiolytic effects measured in this test were in fact mediated by the δ-opioid receptor is shown in Fig. 8. Pretreatment subcutaneously with 1 mg/kg of the selective δ-opioid receptor antagonist naltrexone was able to fully reverse the effects of 1 mg/kg AZD2327.

The effects of AZD2327 in the social interaction test are summarized in Table 4. ADZ2327 produced a dose-related increase in the amount of time that pairs of rats spent in
social interaction, achieving a maximal effect over the doses tested of doubling the amount of social interaction at baseline condition (ANOVA, Dunnett's post hoc test), and daggers indicate significant differences from vehicle-treated animals that were not stressed. B, effect of repeated administration of AZD2327 in control and stressed rats on rat δ-opioid receptor potency in rat striatum. Points are means of ratios from three independent experiments. Data from each individual rat striatum is shown and categorized in respective group (VEH, vehicle; D, day; S, stressed rats). C, maximal efficacy was determined by one-point [35S]GTP\textgamma\ binding using 2 μM AZD2327. Fold stimulation is defined as a ratio between maximal stimulation (2 μM) over basal condition (cpm with AZD2327/cpm basal condition). As in B, points are means of ratios from three independent experiments ± S.D. ANOVAs were performed separately on tissue from stressed (left sides of figures) and unstressed animals (right sides of figures). No main effect for treatment duration versus vehicle was noted.

Discussion

The suggested potential therapeutic applications of δ-opioid receptor agonists have spanned a number of different areas, including neuroprotection (Borlongan et al., 2000; Zhang et al., 2002), cardioprotection (Patel et al., 2002), Parkinson's disease (Hudzik et al., 2000; Hille et al., 2001), and pain management (Porreca et al., 1984; Pradhan et al., 2009). Although there is clear merit as well as a high level of medical need for each of these therapeutic approaches, the potential use of greatest interest to us was an application in psychiatry, based in part upon observations that the δ-opioid receptor knockout mouse had a phenotype that was consistent with both anxiety and depressive-like symptoms (Filliol et al., 2000). This led us to begin testing the potential utility of agonists in psychiatric disease. A number of chemical approaches were undertaken to develop compounds that had a high degree of selectivity for the receptor with otherwise drug-like properties and across several different chemical series (Plobeck et al., 2000; Wei et al., 2000; Griffin et al., 2009). One product of this medicinal chemistry effort has been AZD2327, the activity of which is reviewed in the present article. Unlike the standard SNC-80, AZD2327 is active by the oral route of administration.

AZD2327 possesses equally robust anxiolytic and antidepressant activities. AZD2327 is active in the conflict test—a very stringent test for anxiolytic activity. The activity therein was observed when AZD2327 was administered alone. The doses of PTZ [mean (±S.E.M.)] required to induce clonic seizures were 204.0 (8.0) after vehicle alone, 221.2 (7.1) after 0.6 mg/kg, 187.2 (11.2) after 1.5 mg/kg, 163.8 (12.4) after 5.6 mg/kg, and 137.8 (5.8) after 15.6 mg/kg. Thus, AZD2327 caused a dose-dependent decrease of the threshold to PTZ-induced seizures. At doses ≥5.6 mg/kg, this change in seizure threshold was significantly different from that of the vehicle-treated rats (P = 0.03 and 0.0002, respectively).
antidepressant potential. The relative potency of AZD2327 in anxiolytic tests appears to be greater than that in antidepressant tests, indicating specificity of the drug’s effects across the different assays and therefore arguing against some secondary effect of the drug as the sole explanation for its effects.

The evidence that agonists for the δ-opioid receptor would be antidepressants has been accruing rapidly over the last decade and comprehensively reviewed by Jutkiewicz (2006). For example, peptidic agonists, such as [D-Pen²,D-Pen⁵]-enkephalin (Broom et al., 2002a), and nonpeptidic agonists, such as SNC-80 (Saitoh et al., 2004), have shown activity in antidepressant screens, most typically the forced swim test. Although this test is useful in detecting potential antidepressant activity, demonstrating activity in additional, qualitatively different tests, such as learned helplessness, strengthens the evidence for antidepressant activity. The evidence that δ-opioid receptor agonists are anxiolytic is sparser. Perrine et al. (2003) showed that SNC-80 has diazepam-like activity in the elevated plus maze test (increased time in the open arms of the maze) as well as in the defensive burying test and additionally showed that these effects were not purely due to increases in locomotion. The effects of AZD2327 in the conflict test, social interaction test, and stress/microdialysis paradigm in the present article further support the notion that δ-opioid receptor agonists are anxiolytic.

After 21 days of administration, tolerance was not noted to the neurochemical effects of AZD2327, at least as determined by measurement of NE turnover ex vivo in response to a stressor, nor when tissue from the same set of animals was assayed for agonist potency and activity by [35S]GTPγS binding. This observation is consistent with the lack of effect on behavioral testing in the present article, although the lack of effect could also be due to insufficient exposure to AZD2327.

TABLE 4
Average ± S.E. amount of time (minute) engaging in social interaction counts during a 10-min observation period. Chlordiazepoxide was used as a positive control.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.35</td>
<td>0.29</td>
</tr>
<tr>
<td>AZD2327 (1 mg/kg)</td>
<td>2.49</td>
<td>0.51</td>
</tr>
<tr>
<td>AZD2327 (10 mg/kg)</td>
<td>3.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Chlordiazepoxide (20 mg/kg)</td>
<td>2.83</td>
<td>0.75</td>
</tr>
</tbody>
</table>

TABLE 5
Mean ± S.E. escape failures in the learned helplessness test. Effects of doses of AZD2327 upon dependent variables in the learned helplessness paradigm. P value is associated with t test versus the inescapable shock + saline group.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean Escape Failures (±S.E.M.)</th>
<th>P Value</th>
<th>Mean Center Crossings (±S.E.M.)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inescapable shock + saline</td>
<td>16.2 (3.9)</td>
<td>&lt;0.003*</td>
<td>32.9 (2.1)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>No inescapable shock + saline</td>
<td>5.4 (1.4)</td>
<td>&lt;0.002*</td>
<td>30.2 (1.1)</td>
<td>&lt;0.02*</td>
</tr>
<tr>
<td>Imipramine (20 mg/kg)</td>
<td>2.3 (1)</td>
<td>0.06</td>
<td>28.6 (0.85)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AZD2327 (0.1 mg/kg)</td>
<td>6.1 (3.4)</td>
<td>&lt;0.004*</td>
<td>30.9 (2.9)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AZD2327 (1 mg/kg)</td>
<td>3.1 (1.7)</td>
<td>&lt;0.01*</td>
<td>26.9 (0.7)</td>
<td>&lt;0.02*</td>
</tr>
<tr>
<td>AZD2327 (10 mg/kg)</td>
<td>4.9 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* statistically significant values.
vation is consistent with prior studies demonstrating a lack of tolerance development to the antidepressant effects of δ-opioid receptor agonists (Jutkiewicz et al., 2005).

Although the behavioral pharmacology strongly indicates an antidepressant and anxiolytic profile for δ-opioid receptor agonists, the mechanistic underpinning for such a response is less clear. Standard antidepressants such as selective serotonin reuptake inhibitors or serotonin norepinephrine reuptake inhibitors act, at least initially, via enhancement of monoaminergic neurotransmission. Although some synergy with dopamine agonists on locomotor endpoints can be noted when combined with δ-opioid receptor agonists (Hudzik et al., 2000), the locomotor stimulant effects are not blocked by dopamine antagonists, suggesting a modulatory effect perhaps downstream from the dopamine receptor (Longoni et al., 1998). In the present article as well as an earlier publication using a different δ-opioid receptor agonist compound with a pharmacology and structure similar to those of AR-M100390 (Smagin et al., 2008), no effects on basal NE, dopamine, or serotonin were noted after administration of agonists (data not shown). It could be argued that the antidepressant effects of δ-opioid receptor agonists are due to their proconvulsant effects, given the high level of efficacy of treatments such as electroconvulsive therapy. However, not all of the agonists produce convulsions, and further, specifically, AZD2327 is markedly more potent in the efficacy tests than in producing convulsions, because it did not produce convulsions in rats in the present study up to doses that were >50- to 100-fold higher. After repeated administration, antidepressants evoke changes in neurotrophic factor expression and provoke associated neurogenesis (Nibuva et al., 1995; Dranovsky and Hen, 2006). Although there is somewhat mixed evidence whether δ-opioid receptor agonists may be enhancing monoaminergic transmission, there is evidence that δ-opioid receptor agonists will evoke increases in brain-derived neurotrophic factor expression and provoke separations of locomotor stimulant effects of SNC-80 from effects in the forced swim test, for example, has been noted (Broom et al., 2002a; Jutkiewicz et al., 2005; Jutkiewicz, 2006). In addition, many of the agonists that have been tested during the development of AZD2327 appeared to lack locomotor stimulant effects but retain anxiolytic activity (T. Hudzik, unpublished observations; Gengo et al., 2003). It is interesting to speculate as to why differences among agonists can be observed. It is known, for example, that different agonists can differentially traffic the receptor (Pradhan and Clarke, 2005; Pradhan et al., 2009, 2010) and that different agonists can signal differentially, as suggested by the appearance of pharmacologic subtypes of the receptor. It is possible that these and other mechanisms can contribute to agonists’ differences.

The appearance of convulsions after administration of many δ-opioid receptor agonists remains an obstacle to their development as a new pharmacologic class. Convulsions, when noted with δ-opioid receptor agonists are usually brief (<1 min in duration) and self-limiting (Comer et al., 1993; Broom et al., 2002b; Jutkiewicz et al., 2006). In addition, tolerance develops rapidly to the convulsant effects of SNC-80 and other agonists (Broom et al., 2002b; T. Hudzik and R. Caccese, unpublished observations). Furthermore, not all of the agonists have an equal liability to produce convulsions. Although SNC-80 appears to produce convulsions quite readily in rats (Broom et al., 2002b; Jutkiewicz et al., 2005), AZD2327 has been tested up to doses that are >150-fold over its “therapeutic” doses, and it does not produce convulsions in rats, as noted in the present article. However, AZD2327 does have proconvulsant effects (as measured by decreases in PTZ seizure thresholds) after doses that are several-fold over those indicative of anxiolytic or antidepressant activity. Because convulsion with δ-opioid receptor agonists generally occurs within minutes of injection (Jutkiewicz et al., 2006), it is unlikely to have been missed among all of the present studies. Although direct convulsion has not been noted in rats with AZD2327, it is interesting to note that it can be noted in other species, such as mice, dogs, and squirrel monkeys (T. Hudzik, unpublished observations; C. A. Paronis, personal communication). The extensive safety pharmacology of AZD2327 with respect to seizures will be reviewed in a separate article (T. Hudzik, B. Bell, C. A. Paronis, M. Swedberg, A. Manninen, M. Quirk, K. Bui, A. Easter, M. Pietras, R. Caccese, et al., manuscript in preparation).

The notion of differences among agonists’ pharmacologies is not limited to convulsions. For example, some of the agonists, but not all, appear to have locomotor stimulant effects. It is noteworthy that AZD2327 can produce both anxiolytic and antidepressant effects at doses 3- to 10-fold lower than those producing any motor effects, thus removing this potential confound in the interpretation of efficacy data. Significantly less separation of locomotor stimulant effects of SNC-80 from effects in the forced swim test, for example, has been noted (Broom et al., 2002a; Jutkiewicz et al., 2005; Jutkiewicz, 2006). In addition, many of the agonists that have been tested during the development of AZD2327 appeared to lack locomotor stimulant effects but retain anxiolytic activity (T. Hudzik, unpublished observations; Gengo et al., 2003). It is interesting to speculate as to why differences among agonists can be observed. It is known, for example, that different agonists can differentially traffic the receptor (Pradhan and Clarke, 2005; Pradhan et al., 2009, 2010) and that different agonists can signal differentially, as suggested by the appearance of pharmacologic subtypes of the receptor. It is possible that these and other mechanisms can contribute to agonists’ differences.

Taken together, the data demonstrate that AZD2327 possesses a relatively unique pharmacological profile that overlaps with those of benzodiazepines (anxiolytic-like) and antidepressants (e.g., monoamine oxidase inhibitors or triple reuptake inhibitors). Its pharmacology differs from those of μ- and κ-opi-
oid receptor agonists, which are not active in models predictive of anxiolytic or antidepressant activity. These data support the hypothesis that δ-oid receptor agonists in general and AZD2327 in particular may have utility in the treatment of depression and, given the mixed anxiolytic/antidepressant profile, possibly depression with concomitant anxiety (“anxious depression”). This is significant given the very high level of medical need—AZD2327 could represent possibly an important advancement in the treatment of this group of patients. Ongoing clinical studies with this compound will further elucidate whether this hypothesis is correct.

Acknowledgments

We thank Lynn Hudzik for editorial and other helpful comments on the manuscript and Mylene Gosselin, Leilja Hodzie, and Gabrielle Mankiewicz for performing some of the in vitro pharmacology experiments.

Authorship Contributions

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Conducted experiments: Hudzik, Maciag, Caccece, Pietras, Coupal, Adam, Song, and Swedberg.

Contributed new reagents or analytic tools: Payza, Griffin, and Brown.

Performed data analysis: Hudzik, Coupal, Adam, Payza, Song, and Swedberg.

Wrote or contributed to the writing of the manuscript: Hudzik, Maciag, Smith, Coupal, Adam, Payza, Smaign, and Swedberg.

References


Contributed new reagents or analytic tools: Payza, Griffin, and Brown.

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