Preclinical pharmacology of AZD2327- a highly selective agonist of the δ opioid receptor.


Astrazeneca R & D, 1800 Concord Pike, Wilmington, DE (TJH¹, CM, MAS, RC, MRP,KHB, GS², DS²), Astrazeneca R & D, Montreal (KP, AG, WB, MC, LA), Astrazeneca R & D, Sodertalje (MS).
Running Title: Pharmacology of AZD2327

Corresponding Author: Thomas Hudzik, Global Pharmaceutical Research and Development, R468, Abbott Laboratories, 100 Abbott Park Rd, Abbott Park, IL 60064. tel: (847) 937-3626; (847) 772-8252. Thomas.Hudzik@Abbott.com.

List of non-standard abbreviations:

AR-M100390 : N,N-diethyl-4-[phenyl(piperidin-4-ylidene)methyl]benzamide
SNC-80 : 4-[(R)-[(2S,5R)-4-allyl-2,5-dimethyl-piperazin-1-yl]-(3-methoxyphenyl)methyl]-N,N-diethyl-benzamide
ADL 5859 : N,N-diethyl-4-(5-hydroxyspiro[chromene-2,4'-piperidin]-4-yl)benzamide
HK 08144: (+)-4-[(aR)-a-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-iodobenzyl]-N,N-diethylbenzamide
MHPG - 3 -methoxy-4-hydroxyphenolglycol

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Abstract
In the present paper, we summarize the preclinical pharmacology of AZD2327 (4-{((R)-(3-aminophenyl)[4-(4-fluorobenzyl)-piperazin-1-yl]methyl}-N,N-diethylbenzamide), a highly potent and selective agonist of the δ opioid receptor. AZD2327 binds with sub-nanomolar affinity to the human form of the δ opioid receptor (Ki = 0.49nM at hδ isoform C27; 0.75nM at isoform F27) and is highly selective (>1000-fold) over the human μ and κ opioid receptor subtypes, as well as more than 130 other receptors and channels. In functional assays, AZD2327 shows full agonism at human δ opioid receptors (GTPγS EC50 = 24nM at hδ C27; 9.2nM at F27) and also at the rat and mouse δ receptors. AZD2327 is active in a wide range of models predictive of anxiolytic activity, including a modified Geller-Seifter conflict test, 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 norepinepherine (NE) release into the prefrontal cortex associated with this acute anxiety state. Both the benzodiazepine anxiolytic standard diazepam and AZD2327 blocked this NE 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 activity which 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 under-treated (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 mechanism of action of the available therapies. It is likely that if the treatment gap is to be filled, that mechanistically distinct treatment approaches will be needed.

The involvement of the opioid system in emotional regulation has been suggested for decades (Emrich et al., 1970; Tejedor-Real et al., 1995), although understanding of it is complicated by the multiplicity of receptor subtypes and endogenous ligands, and potential heterooligomerization of the opioid receptors with each other as well as with other GPCRs and ion channels (Walwyn et al., 2009). The 3 historically defined opioid subtypes are μ-receptors, named after the prototypic agonist morphine, κ-receptors, after the benzomorphan opioid ketocyclazocine, and δ-receptors, named following observations of effects of agonists in a mouse vas deferens assay. All 3 opioid receptors are thought to affect nociceptive and emotional responses, albeit in qualitatively different manners. Endorphins and enkephalins (primarily met-enkephalin) are the endogenous ligands for μ opioid receptors, and μ agonists produce euphorogenic responses and robust analgesia (both spinal and supraspinal) for acute nociceptive stimuli. Limitations of their therapeutic use include abuse liability, 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 GI motility. The clinical use of μ antagonists has been largely limited to rescue following opiate overdose, but has more recently been shown 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. While agonists are known to produce dysphorogenic and in some cases, hallucinogenic effects (Walentiny et al., 2010), there are preclinical data to suggest that κ antagonists may be useful.
antidepressants (Carr et al., 2010). Endogenous leu-enkephalin somewhat preferentially interacts with δ vs μ receptors, and a concordance of data would suggest 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 receptor agonists, δ receptor agonists produce minimal effects on respiratory and GI systems (Porecca, et al., 1984). Several studies have shown that δ agonists lack or have minimal reinforcing effects (Negus at al., 1998; DoCarmo et al., 2009), indicating 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, a number of laboratories have demonstrated efficacy of several peptidic and non-peptidic opioids of varying selectivities for the δ opioid receptor agonists in assays for antidepressant activity (reviewed by Jutkiewitz, 2006).

AZD2327 (4-{(R)-(3-aminophenyl][4-(4-fluorobenzyl)-0piperazin-1-yl]methyl}-${N},-{N}$-diethylbenzamide) is a non-peptidic, full and highly selective agonist at the δ opioid receptor, among only a handful to be described in the literature over the last few decades since the receptor was first described (Plobeck et al., 2000; Wei et al., 2000). Its structure, along with those of a number of other non-peptidic δ opioid agonists, is shown in Figure 1. All compounds shown have been described as full δ opioid agonists, but can have some important differences in their pharmacology, nevertheless. For example, SNC-80 has been shown to reliably stimulate locomotor activity and to produce convulsions in a number of preclinical species (Broom et al., 2002; Jutkiewicz, et al., 2005), whereas there have been no reports of either seizure activity or alteration in locomotor activity with AR-M100390 (Pradhan, et al. 2005; Smagin et al., 2008; T. Hudzik, in-house observations), which may represent the opposite end of the spectrum of activity. In the present paper, we focus upon the biochemical and behavioral pharmacology of a novel compound, AZD2327, and the work conducted to determine the clinical utility
of the compound in the treatment of psychiatric disorders. The safety pharmacology of AZD2327 will be addressed in a separate report.

**Methods**

**In Vitro Pharmacology**

**Radioligands.** Deltorphin II, FK 33-824, and d-Pro\(^{10}\)-Dynorphin A[1-11] (DPDYN) were iodinated with Na\(^{125}\)I and then HPLC-purified to apparent homogeneity (2200 Ci/mmole) as described previously (Payza, 2003).

**Cell lines & Membrane Preparations.** As described previously (Payza 2003), HEK293S cells were used to express all 3 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 (Gene Bank #U07882; C27) and the other with phenylalanine (Gene Bank #U10504; F27). Both clones were used as both are represented in the general population, although the F27 isoform is more prevalent. The C27 clone 11 cell line (Bmax 9.7pmol/mg protein) was used for receptor binding assays, and a lower expressing clone 2 (0.69pmol/mg) was used for GTP\(\gamma\)[\(^{35}\)S] assays. In the case of the F27 isoform, clone 4 (Bmax 3.7pmol/mg) was used for both binding and functional assays. Suspension conditions were used for production of membranes expressing the hδ C27, as well as the hµ and the hκ receptors. The cells were grown, harvested and processed into membrane preparations exactly as described previously (Payza 2003). In the case of the hδ F27, culture was done under adherent conditions in normal DMEM, 10% fetal bovine serum, 2mM glutamine, and 600µg/ml of geneticin, followed by membrane preparation using the same method as 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). Briefly, AZD2327 was tested for its ability
to compete against the binding of $[^{125}\text{I}]-\text{Deltorphin II}$ to $\delta$ receptors, and against $[^{125}\text{I}]-\text{FK 33-824}$, and $[^{125}\text{I}]-\text{D-Pro10-Dynorphin A[1-11]}$ ($[^{125}\text{I}]-\text{DPDYN}$) for the $\mu$ and $\kappa$ receptors, respectively. Radioligands were used at 0.03 to 0.05 nM. In all cases, the buffer was 50 mM Tris, 3 mM MgCl$_2$, and 0.1% BSA, pH 7.4, and 10 $\mu$M of naloxone was used to define non-specific binding. Internal standards were SNC80 (for $\delta$), DAMGO (for $\mu$) and U69593 (for $\kappa$). All selectivity ratios were made utilizing high-affinity binding conditions among each of the receptor subtypes.

**GTP$\gamma^{[35}\text{S}]$ Binding Assays.** AZD2327 was tested for agonism in membrane-based GTP$\gamma^{[35}\text{S}]$ filtration binding assays using the exact experimental procedure and method to determine EC$_{50}$ and Emax as described previously for the cloned human $\delta$ receptors (Payza 2003) and for endogenous $\delta$ receptors in rat brain membranes (Fraser 1999). Briefly, the assays were performed in 96-well plates in 50 mM Hepes, 20 mM NaOH, 200 mM NaCl, 1 mM EDTA, 5 mM MgCl$_2$, 1 mM DTT, 0.5% BSA, pH 7.4, with GDP added at 3 $\mu$M (for h$\delta$ C27 and h$\delta$ F27 receptors) or 45 $\mu$M (for rat brain membranes). Incubation was for 1 hr at room temperature. SNC80 (10 $\mu$M) was used to define 100% Emax.

**Electrically-Stimulated Tissue Assay.** Mouse vas deferens, a standard $\delta$ agonism assay, and guinea pig ileum, a standard $\mu$ agonism assay, were performed at CEREP (France) using established methods exactly as described previously in this journal (Gengo et al., 2003).

**In Vivo Microdialysis.**

**Animals.** 64 Male Sprague Dawley rats (Charles River Laboratories) 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 PFC using the following coordinates. AP +3.2 mm, L +/- 1.6 mm, V –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 hrs before
the experiment and were perfused with artificial CSF (aCSF, CMA Microdialysis AB) at a flow rate of 1.1 μL/min. Neurotransmitter levels were assessed by HPLC (Model 5200A Coulochem II detector, MD-150 3 x 150 mm column, model 5041 Amperometric cell (all from ESA Inc) and on-line injector (From BAS Inc). The mobile phase was: 75 mM Na₂HPO₄, 25 mM EDTA, 1.7 mM 1-octanesulphonic acid, 100 μl/L triethylamine, 10% acetonitrile, pH 3.0. The potential was set at +0.65V, and 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 on for the next 5 hrs. Concentrations of neurotransmitters in 3 samples collected before administration of compounds/vehicle were averaged and defined as baseline (100%). Concentrations of neurotransmitters in the subsequent microdialysates were then expressed as percentage of baseline levels. Statistical analyses for microdialysis studies were performed using repeated measures MANOVA followed by Dunnett’s post hoc test.

**Stress procedure.** Standard avoidance chambers were used, equipped with lights, and shockers, (Med Associates, Inc). Boxes were placed in sound-attenuating chambers. The stress conditioning paradigm was conducted over one day. Animals were acclimated to the chambers for 18 hr, prior to conditioning. Conditioning consisted of turning on and off the houselight in the chamber at a rate of 2 times per second for 2 sec (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 sec duration, 1.8 mA intensity, total 10 shocks). The “no stress” group was exposed to chambers with lights, but did not receive shocks. Two hr after the start of the stress paradigm the light sequence was repeated (CS+), but no shocks were administered.

Eight experimental groups (n=8/group) were thus assigned:

<table>
<thead>
<tr>
<th>VEH-No Stress</th>
<th>VEH-Stress</th>
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<tbody>
<tr>
<td>Diazepam-Stress (3 mg/kg, PO)</td>
<td>AZD2327 (0.1 mg/kg PO) –Stress</td>
</tr>
<tr>
<td>AZD2327 (3 mg/kg PO) – No Stress</td>
<td>AZD2327 (0.3 mg/kg PO) –Stress</td>
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</table>
AZD2327 (3 mg/kg PO) – Stress | Diazepam (3 mg/kg PO) - No stress

All experimental compounds and vehicle (0.9% phosphate-buffered saline) were administered p.o. 1 hr prior to the stress procedure. The AZD2327 doses that were tested were 0.3, 1 and 3 mg/kg, corresponding to 0.3, 1 and 3 mg/kg. Diazepam (sigma chemical) was given at a dose of 3 mg/kg, in a vehicle of Abbott’s cocktail.

In order to address whether tolerance developed to the neurochemical effects of AZD2327, groups of 16 naive rats were administered AZD2327 1 mg/kg, po. daily for either 1, 14 or 21 days, and half of animals in each group were conditioned as described above, 1 hr following their last dose, and the other half simply placed into the conditioning chamber for the same period of time as those groups receiving footshock. Two vehicle control groups were also included: one receiving conditioning following a single administration of vehicle, and a second which did not receive conditioning following vehicle. Three additional groups of 4 animals were treated with 3 mg/kg po. diazepam for 1, 14, and 21 days, and submitted to conditioning. Forty min after conditioning, the brains were rapidly removed and snap frozen. mPFC was dissected from half of the brains in each treatment group, homogenized, and assayed for NE and MHPG (an NE catabolite) in 0.1 M perchloric acid using electrochemical detection (ESA Coulorchem 5011, Bedford, MA) after separation by HPLC on a reversed-phase C18 column (Ultrasphere ODS, 4.6 x250 mm, Beckman Instruments, Fullerton, CA. Striatal membranes from the remaining brains in each treatment group (except for diazepam-treated rats) were individually prepared and assayed for agonist activity as described above.

**Anxiolytic Activity: Modified Geller-Seifter Conflict Test**

**Subjects.** Male Long-Evans rats, weighing 375-425g were used. Animals were maintained at 80-90% of their free-feeding weights by limited post-experimental session feeding. For any given drug test, rats whose responding was 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 8 animals were used for each data point.

**Apparatus.** Standard 2-lever operant chambers were used (Med Associates). The chambers were fitted with 2 retractable response levers and a stimulus lamp over each of the 2 levers. A pellet dispenser delivered 45 mg food pellets, (Bio Serv) to a cup located inside of the chamber below and between the 2 response levers. A lamp at the top and back of the chamber served as houselights. The grid floors of the operant chambers were interfaced to shock generators and scramblers (Med Associates). All events in the chambers were controlled and monitored by a microprocessor.

**Procedure.** There were 2 components in the procedure: 1) unsuppressed responding components (unpunished) with 2 min in duration 2) suppressed responding components (punished) with 3 minutes 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 extended, and a food pellet was delivered following an average of 17 responses on the lever in the chamber (range 3 to 40 responses) – a variable-ratio 17 schedule (VR17). 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 VR17 schedule, but in addition, electrical current (0.5 s duration) was delivered to the grid floor of the chamber under an independent VR17 schedule. The level of the current was adjusted for each individual subject until responding was reduced in the suppressed component to a level that was about 5% to 10% that of the unpunished component, and ranged from 0.2 mA 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 stimulus lamps turned off. Two-min unpunished and 3-min punished components alternated until 5 of each were completed. Daily sessions always began with the unpunished responding component.
The dependent variables recorded were the rate of responding 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 responding in the punished components with relatively less or no effect on responding in unpunished components. T-tests were used to compare the mean of the control group’s rate of responding on vehicle day of the rats used for a specific dose to the same rats means following 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 (po) was dissolved in distilled deionized water/85% lactic acid. Naltrindole was dissolved in saline and was given sc in combination with AZD2327 (po). All compounds were given in a dose volume of 1 mL/kg body weight. AZD2327 was tested at 1 hr following drug administration and naltrindole was dosed 20 min before testing. Drugs were administered on Tuesday and Friday and vehicle on Thursday. Animals were run in baseline operant sessions on Mondays and Wednesdays.

Social interaction test.

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

Apparatus. A circular, steel arena (Height 33 cm, diameter 118 cm) 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 individually pre-exposed 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 were then 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 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 was then reported. Mean scores following
drug – treated groups were compared to vehicle-treated groups by Student’s t tests. AZD2327 (1 and 10
mg/kg); was administered 30 min prior to the session. Chlordiazepoxide (CDP, 7.5 mg/kg ip) was
administered 20 min prior. Both rats were administered equivalent doses of the same compound.

**Antidepressant Activity: Learned Helplessness**

**Subjects.** 64 male Sprague Dawley rats were used in the present studies. Subjects weighed on average
275-325 g at the start of the study.

**Apparatus.** Standard shuttle cages (20 L X 16 W X 21 cm H) were 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 2 sides of the cage. A computer controlled and monitored all events in the chamber.

**Procedure.** The procedure consisted of two phases induction and avoidance training. In the induction
phase, subjects were enclosed into one side of the shuttle cages, and electrical stimulation (2mA, 9.9 sec
duration) was delivered to the floor of the cage every 2, 5 or 10 sec (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 was also conducted in the
shuttle cages, except all cages were then 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 employed
was a standard shuttle avoidance in which a compound, conditioned stimulus (a 5-sec presentation of a
tone and turning on of a lamp on the side of the cage that the subject was occupying) served to indicate
impending presentation of electrical current to the floor of the cage. Shock was presented for a 5 sec
period, 5 sec after initiation of the stimulus. Entry into the opposite side of the shuttle cage via the arched
partition prior to shock onset resulted in the end of the trial (avoidance response). If shock was delivered,
entry into the opposite side of the cage resulted in termination of the shock and the CS (escape response).
A 30-sec intertrial interval was employed. Forty min avoidance training sessions, consisting of 50 trials were conducted on 2 consecutive days, beginning 48 hr after the final induction session.

**Drug administration and preparation.** Dosages of all compounds are reported as the free base. Imipramine and AZD2327 were dissolved in distilled deionized water and were administered in a volume of 1 mL/kg body weight, p.o. Drug was administered twice daily throughout the experiment: immediately following conditioning and training sessions and approximately 7-8 hrs 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. Additionally, because some δ opioid agonists have been shown to produce locomotor stimulation, center crossings during avoidance training were also recorded and compared among groups, which allows a gauge of motor activity. An increase in center crossings with respect to vehicle control suggests that locomotor stimulation may be at least partly responsible for the putative antidepressant effects of the compound. T-Tests were used to compare the performance of the vehicle-administered group to drug treated groups. The no-induction group was used to gauge whether learned helplessness was established, by comparison to the vehicle treated group.

**Secondary Pharmacology**

All in vivo safety pharmacology studies described below were conducted using the standard vehicle, PBS, with 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 6 naïve, 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.
An Open Field Activity System (MED Associates) was used to assess spontaneous locomotor activity in separate groups of animals. The system consisted of 8 locomotor activity boxes (ENV 515) of clear acrylic with an inside area of 43.2 x 43.2 x 30.4 cm, housed in Melamine Sound Attenuating Cubicles (ENV 017) with house lights and exhaust fans. Three 16-beam I/R arrays, two placed at 2.5 cm (floor level) and one at 12.5 cm were used to measure activity. All beam breaks and activity in the boxes were recorded by Activity Monitor software. On the day of the experiment, male Sprague-Dawley rats (Scanbur BK, Sollentuna, Sweden) weighing 200-250 g were administered the appropriate doses and individually put 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 animals in the experiment. Animals were evenly distributed over doses and time of day. The total mean of ambulatory activity was collected in 5-minute intervals during the test session, and ANOVA used to compare means. Drug was given p.o.

**Irwin’s Test.** The rat Irwin test was performed to assess CNS side effects. Six Male Wistar rats were dosed orally with vehicle or 0.5, 1.5, 5, 15 or 50 mg/kg of 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 (PTZ) -Seizure Thresholds.** Six Han-Wistar rats weighting approximately 250-300 g were used for each data point. Approximately ten min prior to the PTZ infusion the rat was placed in a restrainer cage and a catheter (Neoflon gul 24 G, Becton Dickinson, Helsingborg, Sweden) was inserted into the tail vein for the administration of PTZ. The 100 µmol/kg per min infusion of PTZ started 60 min following the oral administration of the test doses of AZD2327. When the first clonic convulsion was observed the infusion was stopped and the rat was immediately sacrificed with a bolus infusion of a lethal dose of pentobarbital 100 mg/mL (Avlivningsvätska, 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 as µmol/kg. ANOVA were used to compare means.

Results

**Receptor Binding Assays.** The activity of AZD2327 at both isoforms of the human δ receptor and at the human µ and κ receptor is shown in Figure 2. AZD2327 inhibited, in a dose-dependent manner, the binding of $[^{125}\text{I}]-\text{Deltorphin II}$ to the hδ C27, hδ F27 and rat brain δ receptor with potent $K_i$ values; in contrast, its inhibition of $[^{125}\text{I}]-\text{FK33824}$ and $[^{125}\text{I}]-\text{DPDYN}$ binding to hµ and hκ receptors was much weaker (Table 1). The selectivity ratios of AZD2327 for hδ C27 or hδ F27 receptor vs. hµ and hκ receptors were in all instances, regardless of which clone was used, greater than 1000-fold. AZD2327 was also tested in 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 C27 and δ F27 isoforms, as well as on endogenous δ receptors in rat brain membranes (Figure 3), with potency values shown in Table 2. In low affinity conditions (Fraser et al, 1999), the binding potency of AZD2327 was shifted to the right, as expected for GPCR agonists, by 33 and 39 fold at the hDelta F27 and C27 isoforms, respectively (data not shown). In the mouse vas deferens, a standard δ receptor assay, AZD2327 showed full agonism with an EC50 value of 1.7nM (data not shown). In the guinea pig ileum, a standard µ receptor assay, it showed very weak activity, eliciting only 33% Emax at 10µM (data not shown).

**Neurochemical Assays and tolerance development**

In microdialysis, delivery of footshock produced a 60-70% increase in NE content in medial prefrontal cortex which was sustained for up to 3 hrs after the initial stressor (Figure 4). Consequent increases in MHPG were observed more than an hour after the initial increases in NE. Pretreatment with diazepam (3
mg/kg), which was a dose chosen because consistent efficacy noted historically in our labs, fully reversed these effects. Similarly, 1 and 3 mg/kg AZD2327 reversed the neurochemical changes measured following footshock (Figure 5). In studies in which AZD2327 was administered for up to 21 days followed by ex-vivo neurochemistry and binding and 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 unstressed animals (Figure 6, A, left side). 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 the potency (Figure 6, panel B) and efficacy (Figure 6, Panel C) was unchanged by repeated administration of AZD2327, regardless of whether they were unstressed by shock administration (left half of panels B and C or stressed; right half of panels B and C), 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 responding averaged 0.04 ± 0.01 responses/sec, and 2.03 ± 0.12 responses/sec in the unsuppressed component under baseline conditions. Administration of 1 mg/kg or higher of AZD2327 was as effective as diazepam in producing increases in responding with respect to control in the suppressed component of the schedule (Figure 7, left side), and did so with a potency that was an order of magnitude greater than that of diazepam. The effects of both agents upon suppressed responding was specific to this component, as neither (Figure 7, right side) altered responding in the unsuppressed component. Evidence that the anxiolytic effects measured in this test were in fact mediated by the δ
opioid receptor is shown in Figure 8. Pretreatment with 1 mg/kg of the selective δ opioid receptor antagonist naltrindole, s.c. 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 3. ADZ2327 produced a dose-related increase in the amount of time pairs of rats spent in social interaction, achieving a maximal effect over the doses tested of doubling the amount of social interaction at baseline conditions. These effects were comparable to those of the benzodiazepine, chlordiazepoxide.

**Antidepressant Assay**

The effects of AZD2327 in the learned helplessness test for potential antidepressant activity are summarized in Table 4. Inescapable shock (IS) produced, as designed, an increase in escape failures in rats treated with vehicle and subsequently given the opportunity to escape in avoidance training. Vehicle-treated animals averaged 16 escape failures in avoidance training. Treatment with the positive control imipramine after IS resulted in a decrease in escape failures to an average of 2.3. Administration of 1 mg/kg or more of AZD2327 resulted in decreased escape failures. Stimulation of locomotor activity, as measured by center crossings in the avoidance chambers during intertrial intervals, was unchanged by any treatment.

**Locomotor Activity, Irwin’s test, and PTZ Seizure Thresholds.**

In the locomotor activity studies, there was a main effect of dose; F(3,20)=20.3, P<0.0001; F(3,20)=35.8, P<0.0001 in the first and second studies, respectively (Figure 9). All doses were active, with the exception of 1.5 mg/kg, which was not significantly different from vehicle. Generally, locomotor effects reached a plateau above 15.6 mg/kg. The Irwin’s test (data not shown) did not reveal any significant behavioral or physiological changes at any of the doses tested. Most significantly, no signs of convulsions were 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 15.6mg/kg. Thus, AZD2327 caused a dose dependent decrease of the threshold to PTZ-induced seizures. At 5.6 mg/kg and higher, this change in seizure threshold was significantly different from the vehicle-alone treated rats (p: 0.03 and 0.0002, respectively).
Discussion

The suggested potential therapeutic applications of δ opioid agonists has spanned a number of different areas, including neuroprotection (Borlongan et al., 2000, 2004, Zhang et al., 2002), cardioprotection (Patel et al, 2002;), Parkinson’s disease (Hille, et al, 2001, Hudzik et al, 2000), and pain management (Porreca, et al, 1984; Pradhan et al, 2009). While there is clear merit in each of these therapeutic approaches, as well as a high level of medical need, the potential use of greatest interest to us was application in psychiatry, based in part upon observations that the δ opioid receptor knockout mouse had a phenotype which was consistent with both anxiety and depressive-like symptoms (Filliol et. al, 2000). This led us to begin testing hypotheses around the potential utility of agonists in psychiatric disease. A number of chemical approaches were undertaken to develop compounds which had a high degree of selectivity for the receptor with otherwise drug-like properties and across several different chemical series (Plobeck et al., 2000; Griffin et al, 2009; Wei et al., 2000). One product of this medicinal chemistry effort has been AZD2327, the activity of which is reviewed in the present paper. Unlike the standard, SNC-80, AZD2327 is active by the oral route of administration.

AZD2327 possesses equally robust anxiolytic and antidepressant activity. AZD2327 is active in the conflict test – a very stringent test for anxiolytic activity. The activity therein was shown to be reversed by pretreatment with the δ opioid antagonist, naltrindole, strongly arguing that the effects of the AZD2327 are mediated by the δ opioid receptor. AZD2327 is also active in the learned helplessness test for antidepressant activity, an equally stringent test. Furthermore, AZD2327 is active in the social interaction test, which reflects anxiolytic activity, but also, if one considers that social withdrawal is also a symptom of major depressive disorder as well, may also reflect antidepressant potential. The relative potency of AZD2327 in the anxiolytic tests appears to be greater than 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 antidepressant has been rapidly accruing over the last decade, and comprehensively reviewed by Jutkiewicz (2006a). For example, peptide agonists such as DPDPE (Broom et al., 2002), and non-peptide agonists such as SNC-80 (Saitoh et al., 2004), have shown activity in antidepressant screens, most typically the forced swim test. While 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 more sparse. Perrine et al (2006) showed that SNC-80 has diazepam-like activity in the elevated plus maze test (increase time in the open arms of the maze), as well as in the defensive burying test, and additionally showed that these effects were not due purely to increases in locomotion. The effects of AZD2327 in the conflict test, social interaction test as well as in the stress/microdialysis paradigm in the present paper, further support the notion that δ opioid agonists are anxiolytic. Following 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 GTPγ35S binding. This observation is consistent with prior studies demonstrating a lack of tolerance development to the antidepressant effects of δ agonists (Jutkiewicz et al, 2005).

While the behavioral pharmacology strongly indicates an antidepressant and anxiolytic profile for δ opioid receptor agonists, the mechanistic underpinnings for such a response is less clear. Standard antidepressants such as selective serotonin reuptake inhibitors (SSRIs) or serotonin norepinepherine reuptake inhibitors (SNRIs) act, at least initially, via enhancement of monoaminergic neurotransmission. While some synergy with DA agonists on locomotor endpoints can be noted when combined with δ opioid agonists (Hudzik et al, 2000), the locomotor stimulant effects are not blocked by dopamine antagonists, suggesting a modulatory effect perhaps downstream from the DA receptor (Lonogni et al., 1998). In the present paper, as well as an earlier publication utilizing a different δ opioid agonist compound with a pharmacology and structure similar to AR-M100390 (Smagin et al, 2008), no effects on
basal NE, DA or 5-HT were noted following administration of agonists (data not shown). It could be argued that the antidepressant effects of δ opioid agonists are due to their pro-convulsant effects, given the high level of efficacy of treatments such as ECT. However, not all agonists produce convulsions, and further, specifically, AZD2327 is markedly more potent in the efficacy tests than in producing convulsions, as it did not produce convulsions in rats in the present study up to doses which were more than 50- to 100-fold higher. Following repeated administration, antidepressants evoke changes in neurotrophic factor expression, and provoke associated neurogenesis (Nibuya et al., 1995, Dranovsky and Hen, 2006). While, there is somewhat mixed evidence whether δ opioid agonists may be enhancing monoaminergic transmission, there is evidence that δ opioids will evoke increases in BDNF, indicating that there may be some overlap with standard antidepressants’ actions (Torregrossa et al., 2006).

The appearance of convulsions following administration of many δ opioid agonists remains an obstacle to their development as a new pharmacologic class. Convulsions, when noted with δ opioid agonists are usually brief (<1 min in duration) and self-limiting (Broom et al., 2002b; Comer et. al, 1993; Jutkiewitz et al., 2006b). Additionally, tolerance develops rapidly to the convulsant effects of SNC-80 and other agonists (Broom et al, 2002b, unpublished observations). Further, not all agonists have an equal liability to produce convulsions. While SNC-80 appears to produce convulsions quite readily in rats (Broom et al., 2002b; Jutkiewitz et al, 2005), AZD2327 has been tested up to doses which are more than a 150-fold over its ‘therapeutic’ doses and it does not produce convulsions in rats, as noted in the present paper. AZD2327, however, 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. As convulsion with δ opioids generally occurs within minutes of injection (Jutkiewitz, et al., 2006b), it is unlikely to have been missed among all of the present studies. And, while direct convulsion has not been noted in rats with AZD2327, interestingly, in can be noted in other species, such as mice, dogs, and squirrel monkeys (in-house observations, Carol Paronis, personal communication). The
extensive safety pharmacology of AZD2327 with respect to seizures will be reviewed in a separate manuscript (in preparation).

The notion of differences among agonists’ pharmacologies is not limited to convulsions. For example, some, but not all agonists appear to have locomotor stimulant effects. Interestingly, 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 interpretation of efficacy data. Significantly less separation of motor-stimulant effects of SNC-80 from effects in the forced swim test, for example, has been noted (Jutkiewitz, et al., 2005, 2006a; Broom et al., 2002a). Additionally, many of the agonists that have been tested during the development of AZD2327 appeared to lack locomotor stimulant effects, but retain anxiolytic activity (in house 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, et al., 2005, 2009, 2010), and that different agonists can signal differentially, suggested by the appearance of pharmacologic subtypes of the receptor. Likely these and other mechanisms can contribute to agonists’ differences.

Taken together, the data demonstrate that AZD2327 possesses a relatively unique pharmacological profile which overlaps with benzodiazepines (anxiolytic-like), and antidepressants (e.g, MAOIs or triple reuptake inhibitors). Its pharmacology differs from that of μ and κ agonist opioids, which are not active in models predictive of anxiolytic or antidepressant activity. These data support the hypothesis that δ opioid 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 possibly represent an important advancement in the treatment of this group of patients. Ongoing clinical studies with this compound will further elucidate whether the hypothesis is correct.
Acknowledgements - The authors wish to acknowledge Lynn Hudzik for editorial and other helpful comments on this manuscript, and Mylene Gosselin, Lejla Hodzic and Gabrielle Mankiewicz for performing some of the \textit{in vitro} pharmacology experiments. This study was conducted under protocols that have been approved by an ethical committee. The animals were kept and experiments were performed at AstraZeneca R&D Wilmington, which has accreditation from AAALAC (Association for the Assessment and Accreditation of Laboratory Animal Care) and/or is approved by AZ GVC (AstraZeneca Global Veterinary Council) for study conduct.
Authorship responsibility

*Participated in research design:* Smagin, Hudzik, Bui, Coupal, Adam, Payza, Maciag, Swedberg, Brown.

*Conducted experiments:* Song, Swedberg, Hudzik, Maciag, Caccese, Pietras, Coupal, Adam.

*Contributed new reagents or analytic tools:* Brown, Griffin, Payza

*Performed data analysis:* Song, Swedberg, Hudzik, Payza, Coupal, Adam

*Wrote or contributed to the writing of the manuscript:* Hudzik, Smith, Maciag, Coupal, Adam, Payza, Swedberg, Smagin.
REFERENCES


FOOTNOTES:

1Current Address for TJH: Global Pharmaceutical Research and Development, Dept of Toxicology, Abbott Labs, 100 Abbott Park Rd, Abbott Park, IL 60064;

2Current Address For GS and DS: Lundbeck Research USA, 215 College Rd, Paramus, NJ 07652.
Legends for Figures

Figure 1. Structures of representative δ opioid agonists. ADL5859 and closely related ADL5747 have been in clinical development as analgesics. AR-M100390 (sometimes referred to as ARM390) is a tool compound from AstraZeneca that has been previously published on (Smagin et al, 2008; Wei, et al., 2000; Pradhan, et al., 2010).

Figure 2. Representative displacement curves for AZD2327 with various membrane preparations of HEK293S stable cell lines expressing the hδ F27 receptor in high (○) and low affinity conditions (□), the hδC27 receptor in high (▲) and low affinity conditions (■), the human μ receptor (♦), the human κ receptor (▼), and on membranes prepared from rat brain (●). Points represent mean of duplicates. The 125I-labeled Deltorphin II was used to label δ opioid receptors, [125I]-FK 33-824 (met-enkephalin) used to label μ opioid receptors, and [125I]-DPDYN to label κ opioid receptors. Further details are summarized in Table 2 and in the text.

Figure 3. Representative GTPγS binding curves of AZD2327 on membrane preparations of HEK293S stable cell lines expressing the F27 isoform of the human δ receptor (▲), the C27 isoform of the human δ receptor (▼), and on rat brain membrane expressing endogenously δ receptor (●). Points represent mean of duplicates.

Figure 4. Mean (± SE) % of control cortical norepinepherine (left figure) and MHPG (right figure) in 10 in cerebral microdialysate as a function of time. Vehicle or diazepam (3 mg/kg) were administered po (demarcated by the arrow) 60-min prior to conditioning (footshock + light
pairings, demarcated by FS+L). 60 min thereafter, the CS (L) alone was presented. * indicates significant difference among groups by repeated measures 2-way ANOVA (treatment X time).

Figure 5. Mean (± SE) % of control cortical norepinepherine (left figure) and MHPG (right figure) in cerebral microdialysate as a function of time. Vehicle or compounds were administered (demarcated by the arrow) po 60-min prior to conditioning (Footshock+Light pairings, demarcated by FS+L). 60 min thereafter, the CS (L) alone was presented. * indicates significant difference from the vehicle-treated group by repeated measures 2-way ANOVA (treatment X time). 1 and 3 mg/kg, but not 0.3 mg/kg AZD2327 reversed the effects of footshock upon both NE and MHPG in cortex.

Figure 6. Effects of repeated administration (1, 7, 14 and 21 days) of AZD2327 on both ex-vivo NE turnover (Panel A) and on agonist potency and activity (Panels B and C, respectively).

Panel A: Veh points represent values from stressed (▲) or non-stressed (♦) rats derived from cortex taken from vehicle treated animals pooled across each of the different time points of vehicle administration, as they did not differ. Points along the curves represent average of cortex from 4 animals following either AZD2327 administration (1 mg/kg, □) or diazepam administration (3 mg/kg, po, ■). * indicates significant difference from vehicle-stress condition (ANOVA, Dunnett’s post-hoc test), and † indicates a significant difference from vehicle treated animals which were not stressed. Panel B. Effect of repeated administration of AZD2327 in control and stressed rats on rat δ receptor potency in rat striatum. Points are means pEC50 values ± S.D. determined by a GTPγ35S binding dose response curve in 3 independent experiments. Data from each individual rat striatum is shown and categorized in respective group (VEH: vehicle, D:day, S:
stressed rats). Maximal efficacy (Panel C) was determined by one point GTPγS binding using 2 μM of AZD2327. Fold stimulation is defined as a ratio between maximal stimulation (2 μM) over basal condition (cpm with AZD2327 / cpm basal condition. As in panel B, points are means of ratio from 3 independent experiments ± S.D. ANOVA were performed separately on tissue from stressed (left half of figures) and non-stressed animals (right half of figures). No main effect for treatment duration vs vehicle was noted.

Figure 7. Effects of doses of AZD2327 and diazepam in the suppressed (left figure) and unsuppressed (right figure) components of the modified Geller-Seifter conflict test for anxiolytic activity in rats. Drug was administered po 1 hr prior to initiation of the operant session. * indicates significant difference from vehicle control.

Figure 8. Antagonism of the anxiolytic effects of AZD2327 in the suppressed component of the conflict test (left figure) without altering the effect in the unsuppressed component. * indicates significant difference from control.

Figure 9. Ambulatory distance (cm) traveled within locomotor activity chambers in a 90-min period following administration of lower doses (A) and higher doses (B) of AZD2327. All doses in both studies were significantly different from vehicle (Bonferoni’s post-hoc test), with the exception of 1.5 mg/kg dose.
Tables.

Table 1. AZD2327 potencies in inhibiting radioligand binding at the hδC27, hδF27, hμ, and hκ receptors stably expressed in HEK293S cells, and at rat brain δ receptors

<table>
<thead>
<tr>
<th></th>
<th>hδC27</th>
<th>hδF27</th>
<th>hμ</th>
<th>hκ</th>
<th>Rat brain δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki (nM)</td>
<td>0.49</td>
<td>0.75</td>
<td>770</td>
<td>4500</td>
<td>1.2</td>
</tr>
<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±SEM</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>
</tr>
</tbody>
</table>

Ki values (geometric means) were determined using the radioligands described in the Methods

pKi: -log Ki (M)

SEM: Standard error of the mean pKi

N: number of dose-response curves (each having 10 concentrations of AZD2327 in duplicate)
Table 2. Agonist activity of AZD2327 in GTPγS binding assays performed on membrane preparations of HEK293Scell lines expressing hδC27, hδF27, hμ, and hκ receptors, and rat brain membranes expressing δ receptors endogenously.

<table>
<thead>
<tr>
<th></th>
<th>hδC27</th>
<th>hδF27</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>%Emax±SEM</td>
<td>91±2</td>
<td>101±1</td>
<td>91±1</td>
</tr>
<tr>
<td>pEC50±SEM</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>

EC50 values shown are the geometric means of individual results

%Emax values (arithmetic mean) are relative to 10 µM SNC80

pEC50: -log EC50 (M)

SEM: Standard error of the mean pEC50

N: number of dose-response curves (each having 10 concentrations of AZD2327 in duplicate)
Table 3.

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

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh-</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>CDP 20 mg/kg</td>
<td>2.83</td>
<td>0.75</td>
</tr>
</tbody>
</table>
TABLE 4. Mean ± SE escape failures in the learned helplessness test.

<table>
<thead>
<tr>
<th></th>
<th>Mean Escape Failures ± (SEM)</th>
<th>P Value</th>
<th>Mean Center Crossings ± SEM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS + Saline</td>
<td>16.2 (3.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No IS + Saline</td>
<td>5.4 (1.4)</td>
<td>&lt;0.003*</td>
<td>32.9 (2.1)</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Imipramine 20 mg/kg</td>
<td>2.3 (1)</td>
<td>&lt;0.002*</td>
<td>30.2 (1.1)</td>
<td>&lt;0.02*</td>
</tr>
<tr>
<td>AZD2327 0.1 mg/kg</td>
<td>6.1 (3.4)</td>
<td>= 0.06</td>
<td>26.6 (0.85)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AZD2327 1 mg/kg</td>
<td>3.1 (1.7)</td>
<td>&lt;0.004*</td>
<td>30.9 (2.9)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AZD2327 10 mg/kg</td>
<td>4.9 (2)</td>
<td>&lt;0.01*</td>
<td>26.9 (0.7)</td>
<td>&lt;0.02*</td>
</tr>
</tbody>
</table>

Effects of doses of AZD2327 upon dependent variables in the learned helplessness paradigm. IS = inescapable shock. P value is associated with T test vs. IS + vehicle group. All treatments were given p.o., BID, each 12 hr following initial conditioning, and 60 min prior to avoidance training (2 days after inescapable shock).
Figure 1

AZD2327

SNC-80

ADL5859

AR-M100390
Figure 2

% Specific Binding

$\log_{10} [\text{AZD2327}]\ M$

-10 -11 -12

-10 -9 -8 -7 -6 -5 -4
Figure 4

NOREPINEPHRINE

MHPG

% of Baseline

Time (minutes)

Vehicle
Diazepam

Vehicle
Diazepam

FS+L
L

FS+L
L

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Figure 5

NOREPINEPHRINE

- Vehicle
- AZD2327, 3 mg/kg
- AZD2327, 1 mg/kg
- AZD2327, 0.3 mg/kg

% of Baseline

Time (minutes)

MHPG

- Vehicle
- AZD2327, 3 mg/kg
- AZD2327, 1 mg/kg
- AZD2327, 0.3 mg/kg

% of Baseline

Time (minutes)
Figure 7

Geller-Seifter Conflict Test

% of Control Rate of Responding

Suppressed Component

AZD2327

Diazepam

Unsuppressed Component

% of Control Rate of Responding

AZD2327

Diazepam

Dose - (mg/kg, p.o. - 60 min)
Figure 8

% of Control Rate of Responding

Suppressed Component

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100</td>
</tr>
<tr>
<td>AZD2327 1 mg/kg, p.o.</td>
<td>120*</td>
</tr>
<tr>
<td>Naltrindole 1 mg/kg + AZD2327 1 mg/kg, p.o.</td>
<td>150</td>
</tr>
</tbody>
</table>

Unsuppressed Component

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100</td>
</tr>
<tr>
<td>AZD2327 1 mg/kg, p.o.</td>
<td>100</td>
</tr>
<tr>
<td>Naltrindole 1 mg/kg + AZD2327 1 mg/kg, p.o.</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 9
Locomotor Activity

Mean ± S.E.M. Distance Traveled (cm)

Dose (mg/kg, po)

veh  1.52  5.2  15.6

Dose (mg/kg, p.o.)

veh  15.6  52  156