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RESEARCH PAPER

Evaluation of peripheral versus central effects of $GABA_B$ receptor activation using a novel, positive allosteric modulator of the $GABA_B$ receptor ADX71943, a pharmacological tool compound with a fully peripheral activity profile

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BACKGROUND AND PURPOSE

The $GABA_B$ receptor agonist, baclofen, has shown promising effects in patients suffering from pain, post-traumatic stress disorder, alcoholism, overactive bladder and gastroesophageal reflux disease. However, baclofen's short duration of action and side effects limit its wider use. Here we characterized a novel, $GABA_B$ receptor positive allosteric modulator (PAM) ADX71943.

EXPERIMENTAL APPROACH

In vitro, ADX71943 was assessed for pharmacological activity and selectivity using recombinant and native $GABA_B$ receptors. *In vivo* ADX71943 was assessed in the acetic acid-induced writhing (AAW) test in mice and formalin tests (FTs) in mice and rats. Marble burying (MB) and elevated plus maze (EPM) tests, rotarod, spontaneous locomotor activity (sLMA) and body temperature (BT) tests in mice and rats were used to investigate centrally-mediated effects.

KEY RESULTS

In vitro, in the presence of GABA, ADX71943 increased the potency and efficacy of agonists and showed selectivity at the $GABA_B$ receptor. ADX71943 reduced pain-associated behaviours in AAW; an effect blocked by $GABA_B$ receptor antagonist CGP63360. ADX71943 reduced pain in the FT in mice and rats, but was inactive in the MB and EPM despite reaching high concentrations in plasma. ADX71943 had no effect on BT, rotarod and sLMA.

CONCLUSIONS AND IMPLICATIONS

ADX71943 showed consistent and target-related efficacy in tests of disorders that have a significant peripheral component (acute and chronic pain), while having no effect in those associated with centrally-mediated anxiety-like reactivity and side effects. Thus, ADX71943 is a useful pharmacological tool for delineation of peripherally- versus centrally-mediated effects of $GABA_B$ receptor activation.

Abbreviations

AAW, acetic acid-induced writhing; ADX71943, N-(5-(4-(4-cyano-3-methoxybenzyl)-6-methoxy-3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl)-2-fluorophenyl)acetamide; BT, body temperature; CMC, carboxymethyl cellulose; EPM, elevated plus maze; GERD, gastroesophageal reflux disease; MB, marble burying; MED, minimum effective dose; mGlu, metabotropic glutamate receptor; MIA, monosodium iodoacetate; OA, osteoarthritis; PAM, positive allosteric modulator; PgP, P-glycoprotein; sLMA, spontaneous locomotor activity

Table of Links

TARGETS	LIGANDS
$GABA_B$ receptor	Baclofen
mGlu receptors	CGP7930
	Chlordiazepoxide
	Diazepam
	GTPγS

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

The $GABA_B$ receptor mediates slow, modulatory neurotransmission of GABA, the major inhibitory neurotransmitter in the mammalian CNS. The $GABA_B$ receptor is widely expressed in the brain, spinal cord, dorsal root ganglia and in peripheral organs, including heart, stomach, intestine, spleen and bladder (Bettler *et al.*, 2004). Significant progress in understanding the physiological role of the $GABA_B$ receptor and its relevance to the pathophysiology of several disorders is linked to accumulating preclinical and clinical evidence involving its prototypic agonist, baclofen (β-p-chlorophenyl-GABA). Introduced into the clinic for its muscle-relaxant properties to treat spasticity four decades ago, baclofen has since shown efficacy in a number of clinical trials. For example, baclofen reduced signs of overactive bladder, gastroesophageal reflux disease (GERD), chronic cough and asthma (Cange *et al.*, 2002; Van Herwaarden *et al.*, 2002; Zhang *et al.*, 2002; Xu *et al.*, 2007; 2012). Baclofen also reduced neuropathic and musculoskeletal pain, as well as pain associated with spinal cord injury or stroke (Fromm, 1994; Loubser and Akman, 1996; Becker *et al.*, 2000; Slonimski *et al.*, 2004). Furthermore, baclofen reduced signs of anxiety in patients with panic disorders (Breslow *et al.*, 1989), post-traumatic stress disorder (Drake *et al.*, 2003) and alcohol withdrawal syndrome (Addolorato *et al.*, 2002). Despite these effects, clinical use of baclofen has been limited by its short duration of action, narrow therapeutic margin and side effects, including sedation, dizziness, nausea, muscle weakness and mental confusion (Bowery, 2006).

As baclofen freely passes through the brain–blood barrier, its effects are mediated by the central as well as peripheral $GABA_B$ receptors. One way to understand the role of central versus peripheral $GABA_B$ receptors is to investigate effects of its activators that have a peripheral mode of action. One such compound, a novel $GABA_B$ receptor agonist, AZD3355 (lesogaberan), binds to GABA transporters, which markedly limits its CNS penetration (Lehmann *et al.*, 2009). Furthermore, $GABA_B$ receptor activators with a peripheral mode of action can be a potential treatment for medical conditions with a significant peripheral component, while being free from any centrally-mediated side effects. Indeed, AZD3355 reduced frequencies of transient lower esophageal sphincter relaxation, the major cause of GERD, in an animal model, healthy volunteers and GERD patients (Lehmann *et al.*, 2009; Boeckxstaens *et al.*, 2010a,b).

Here we present characterization of N-(5-(4-(4-cyano-3-methoxybenzyl)-6-methoxy-3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl)-2-fluorophenyl)acetamide (ADX71943), a novel, potent and selective $GABA_B$ receptor PAM (positive allosteric modulator) with a peripheral mode of action (Figure 1). ADX71943 was initially in development, but after

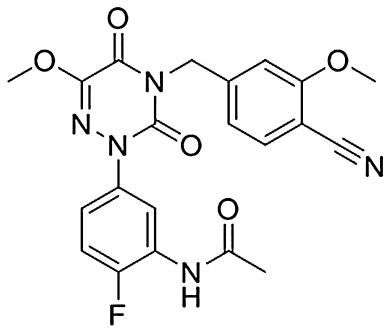

Figure 1
The chemical structure of ADX71943.

obtaining an inadequate safety profile, it was further characterized as a pharmacological tool compound for investigation of peripheral versus central efficacy of $GABA_B$ receptor PAMs. ADX71943 was discovered through lead optimization of a chemical series, identified from a high-throughput screening campaign of the corporate chemical library of Addex Therapeutics (L. Tang, unpubl. data). ADX71943 was confirmed to be a substrate for P-glycoprotein (PgP) which resulted in an active efflux of the compound out of the CNS (H. Haddouk *et al.*, in preparation). Here, after confirming the PAM properties and selectivity of the compound, we tested it in models of pain, the acetic acid-induced writhing (AAW) test in mice and formalin test (FT) in mice and rats. The compound was also tested for anxiety-like behaviours in mice using the marble burying (MB) and the elevated plus maze (EPM) procedures. We chose the anxiety domain to confirm the lack of centrally-mediated effects with ADX71943, since in our laboratory MB and EPM tests were shown to be sensitive to ADX71441, a $GABA_B$ PAM with a balanced central-peripheral profile (M. Kalinichev *et al.*, unpublished observations). ADX71943 was also tested in the body temperature (BT) test as well as in spontaneous locomotor activity (sLMA) tests in mice and rats (Figure S1) and in the rotarod test in mice (Table S1) (rotarod and sLMA data are included in the Supplementary Information). ADX71943 showed dose-dependent reductions in pain-associated behaviours in AAW and FT tests, albeit falling short of full efficacy shown by baclofen. ADX71943 had no effect in the MB and EPM tests, despite reaching high concentrations in plasma, also it had no effect on the BT, rotarod and sLMA in mice or rats. Thus, ADX71943 represents a useful pharmacological tool for further delineation of peripherally- versus centrally-mediated effects of $GABA_B$ receptor activation.

Methods

Stable cell lines

The cDNAs encoding the two subunits of the human $GABA_B$ receptor, $GABA_{B1(a)}$ and $GABA_{B2}$, were subcloned into two expression vectors also containing the hygromycin resistance gene. For intracellular calcium flux measurement, the cDNA encoding a chimeric Gα protein allowing redirection of the activation signal to intracellular calcium flux was subcloned into a different expression vector also containing the puromycin resistance gene, and both of these vectors were cotransfected into HEK293 cells with PolyFect reagent (Qiagen, Basel, Switzerland). Subsequently, hygromycin and puromycin treatment allowed selection of antibiotic resistant clones that had stably integrated one or more copies of both plasmids. Positive functional cellular clones expressing $hGABA_B$ receptor and the chimeric Gα protein were identified by measuring intracellular Ca^{2+} changes in response to the orthosteric agonist GABA (Tocris Bioscience, Abingdon, UK) or the selective known $GABA_B$ receptor antagonist, CGP63360, and the $GABA_B$ receptor PAM, CGP7930. HEK293 cells expressing $hGABA_B$ receptor were maintained in media containing DMEM + GlutaMAX, decomplemented FBS (10%), penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$), geneticin (100 $\mu g \cdot mL^{-1}$), hygromycin B (40 $\mu g \cdot mL^{-1}$) and puromycin (1 $\mu g \cdot mL^{-1}$) at 37°C with 5% CO_2 in a humidified atmosphere.

Fluorescent cell-based Ca^{2+} mobilization assay

This assay was performed in a pH 7.4 buffered solution containing 20 mM HEPES, 143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.125 mM sulfinpyrazone and 0.1% glucose. Twenty-four hours before the pharmacological experiment, $hGABA_B$ receptor-transfected HEK293 cells were plated out at a density of 30 000 cells per well in black-well/clear-bottomed and poly-L-ornithine-coated 384-well plates in glutamine/glutamate-free DMEM containing 10% decomplemented FBS, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin, supplemented with 1 $\mu g \cdot mL^{-1}$ doxycycline. Cells were incubated overnight at 37°C with 5% CO_2 in a humidified atmosphere. On the day of the assay, the cells were loaded with a 3 μM dye solution of Fluo4-AM (Invitrogen, Lucerne, Switzerland) in assay buffer containing 0.03% pluronic acid. After 1 h at 37°C with 5% CO_2 in a humidified atmosphere, the extracellular dye was removed by washing the cell plate three times with 1x PBS (Invitrogen). Assay buffer was added to cells, and cells were left for 3 h in the dark at room temperature. Calcium flux was then measured using a fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA, USA). After 10 s of basal fluorescence recording, compounds to be tested were added to cells in a concentration-dependent manner, and incubated with the cells for 170 s. During that time, changes in fluorescence levels were monitored to detect any agonist activity of the compounds. The cells were then stimulated by an EC_{20} of GABA (concentration giving 20% of the maximal GABA response) for an additional 170 s to measure enhancement activities of the compounds.

Cortical membrane preparation

For rat cortical membranes, cortices were dissected out from brains of 200–300 g Sprague-Dawley rats (Charles River Laboratories, L'Arbresle, France). The tissues was homogenized in 10 vol ($vol \cdot wt^{-1}$) of ice-cold 50 mM HEPES-NaOH (pH 7.4) using a Polytron disrupter (Kinematica AG, Luzern, Switzerland) and centrifuged for 30 min at 40 000× *g* at 4°C. The supernatant was discarded and the pellet washed twice by resuspension in 10 vol of 50 mM HEPES-NaOH. Membranes were then collected by centrifugation and washed before final resuspension in 10 vol of 20 mM HEPES-NaOH, pH 7.4. Protein concentration was determined by the Bradford method (Bio-Rad protein assay kit; Reinach, Switzerland) with BSA as standard. For human cortical membranes, P2 membrane fractions were prepared from the human cerebral cortex by Analytical Biological Services (Wilmington, DE, USA) based on the following protocol. The extracted tissue was thawed in 10× vol of ice-cold buffer A (320 mM sucrose, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.02% Na Azide). The tissue was homogenized and centrifuged at 1881× *g* for 10 min at 4°C. The supernatant was kept after decantation. Another 10× vol of buffer A was added to the pellet. The tissue was again homogenized and centrifuged at 1881× *g* for 10 min at 4°C. Both supernatants were pooled and centrifuged at 32 500× *g* for 30 min at 4°C. The pellet was resuspended in buffer B (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.02% Na Azide) and frozen at –20°C for 30 min. The sample was thawed in a 37°C water bath and centrifuged at 32 500×

g for 30 min at 4°C. These steps were repeated three times (resuspension of pellet in buffer B, freezing, thawing and centrifugation). The final pellet was resuspended in buffer B. The quantity of protein was determined using the Bradford assay with BSA as standard.

[^{35}S]-GTPγS binding

[^{35}S]-GTPγS binding studies were performed on cortical membranes of rat or human origin. Membranes (1.5 µg per well) were incubated for 30 min at 30°C with test compounds in 25 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2 mM EGTA, 10 µg·mL^{-1} saponine and 10 µM GDP (Sigma Aldrich, Buchs, Switzerland), pH 7.4, in the presence of 10 µM of the selective $GABA_B$ receptor agonist baclofen (Tocris Bioscience) corresponding to an EC_{50} (concentration giving 50% of the maximal baclofen response). Then 0.16 nM [^{35}S]-GTPγS (PerkinElmer, Oftringen, Switzerland) was added for a further incubation of 30 min at 30°C. The assay was terminated by rapid vacuum filtration over Unifilter GF/B plate (PerkinElmer) using the Filtermate harvester system (Perkin-Elmer; Downers Grove, IL, USA) with a washing buffer containing 25 mM HEPES and 100 mM NaCl. Plates were dried and MicroScint-20 (PerkinElmer) was added to each well, followed by counting in a TopCount scintillation counter (Perkin-Elmer).

In vitro *selectivity*

Up to 30 µM ADX71943 was functionally tested as an agonist positive or negative allosteric modulator of rat or human members of the metabotropic glutamate receptor family ($mGlu_1$ to $mGlu_8$) using the above-described fluorescent cell-based Ca^{2+} mobilization assay. In addition, ADX71943 was tested at 10 µM in competition radioligand binding assays on a panel of 71 targets, including receptors, transporters, enzymes and ion channels (ADDEX 71 Profile; Cerep, Poitiers, France).

Animals

Adult male C57Bl6/J mice (24–30 g) and Sprague-Dawley rats (250–350 g; Charles River Laboratories) were used. Mice were housed 5 per cage in type II cages (16 × 22 × 24 cm), while rats were housed 2 per cage in type III cages (22 × 37 × 18 cm). Animals were maintained on a 12 h light/dark cycle (lights on from 07:00 to 19:00 h) under constant temperature (22 ± 2°C) and humidity (>45%) conditions with food and water available *ad libitum*. Animals were acclimatized for at least 10 days before experimentation. All procedures were approved by the Ethical Committee of Addex Therapeutics and performed in full compliance with international European ethical standards (86/609-EEC) and the French National Committee for the care and use of laboratory animals (décret 87/848). A total of 513 mice and 186 rats were used in this study. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010)

In vivo *pharmacokinetic studies in rats*

Plasma and CSF concentrations of ADX71943 were assessed following p.o. administration of 10 mg·kg^{-1}, p.o., ADX71943 (n = 6). Rats were anaesthetized with isoflurane administered via a mouth mask and were maintained under light anaesthesia during the whole duration of surgery. The depth of anaesthesia was monitored by squeezing the animal's foot with forceps and by observing its breathing. Also, the eyes of the animal were kept moist by application of physiological saline. For blood sampling, rats were surgically implanted with jugular vein catheters. The same animals were also implanted with a cannula in the cisterna magna for CSF sampling. Plasma and CSF samples were collected 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h following dosing in the same animal. Plasma and CSF samples were analysed as described in the Supplementary Information.

Selection of doses of ADX71943 and reference drugs for the in vivo *experiments*

The doses of ADX71943 for each assay were selected based on a series of preliminary experiments performed in our laboratory. Our aim was to obtain a complete range of responses and to assess the dose -dependency and the *in vivo*/*in vitro* relationship in each assay. Based on our preliminary data, in some experiments, smaller or subtly different dose ranges were used; it should be noted that lower doses were used in the experiments assessing pain (AAW and FT), while higher doses were used in those tests assessing anxiety-like reactivity (MB and EPM).

As the premise of the study was to characterize the peripherally-mediated effects of ADX71943, we used baclofen, the orthosteric agonist of the $GABA_B$ receptor with a balanced central-peripheral profile, as a reference drug in the majority of experiments described below. The doses of baclofen were selected based on a large body of internal data. In mice and rats, 6 and 3 mg·kg^{-1} of baclofen, respectively, were chosen to obtain a near-maximal suppression of pain-associated behaviours, without causing a reduction in rotarod activity presumably due to muscle-relaxant properties (see Supporting Information). In our preliminary studies, as seen by others (Dalvi and Rodgers, 1996), baclofen lacked clear efficacy in the MB and EPM and showed a confounding reduction of general locomotor activity at higher doses. Therefore, we chose chlordiazepoxide and diazepam as the reference compounds for the MB and EPM respectively.

AAW test in mice

In the dose–response experiment, mice (n = 11–12 per group), fasted overnight, were treated via gavage (p.o.) with ADX71943 (0.3, 1, 3, 10 and 30 mg·kg^{-1}), baclofen (3 mg·kg^{-1}) or their corresponding vehicles, plasdone (PD) and 1% carboxymethyl cellulose (CMC) respectively. Seventy minutes later, animals received an i.p. injection of 0.7% acetic acid (AA) and were placed into observation (modified type II) cages. Observation of animals began 10 min following administration of AA and lasted 10 min. The writhe was defined as a contraction of the abdomen or stretch of the trunk with or without extension of the limbs.

The rationale of the follow-up combination treatment experiment was to confirm the receptor specificity of the effect seen with ADX71943 in the mouse AAW test. To achieve this, we used a $GABA_B$ receptor selective antagonist CGP63360 to block the effect of ADX71943. In this target

engagement experiment, mice were treated p.o. with PD or ADX71943 (10 mg·kg^{-1}) as treatment 1, followed 40 min later with p.o. treatment of saline or the $GABA_B$ antagonist CGP63360 (1 mg·kg^{-1}) as treatment 2. Thus, the following experimental groups were formed: PD/saline, PD/CGP63360, ADX71943/saline and ADX71943/CGP63360 (n = 11–14 per group). Thirty minutes after treatment 2 (and 70 min after treatment 1), all animals received 0.7% AA (i.p.) before being placed into observation cages and monitored for writhes as described above. Terminal blood samples were collected from all ADX71943-treated animals at the end of experiments. All plasma samples from these and other experiments (see below) were analysed as described for the pharmacokinetic studies in the Supplementary Information. The effect of ADX71943 on the number of writhes was analysed by one-way ANOVA followed by Dunnett's test or by planned comparisons. The effect of baclofen on the number of writhes was analysed by t-test.

FT in mice and rats

Mice (n = 10–14 per group) were treated p.o. with ADX71943 (0.3, 1, 3, 10, 30 and 100 mg·kg^{-1}), baclofen (6 mg·kg^{-1}) or their corresponding vehicles (PD and 1% CMC). Fifty minutes later, they were individually placed into observation cages for a 10 min acclimatization period, following which they received an injection of 5% formalin (20 µL) into the dorsal part of the right hind paw (using a 100 µL Hamilton syringe; Hamilton Bonaduz AG, Bonaduz, Switzerland, and a 27 gauge needle) and observed for 40 min. The nociceptive behaviours (see below) were scored between 0 and 5 min (as phase I) and between 30 and 40 min (as phase II) after formalin administration.

Rats (n = 10 per group) were treated p.o. with PD, ADX71943 (1, 5, 15 and 50 mg·kg^{-1}) or baclofen (3 mg·kg^{-1}). Fifty minutes later, they were individually placed into observation cages (modified type III cages) for a 10 min acclimatization period, following which they received injection of 5% formalin (50 µL) into the dorsal part of the right hind paw (using a 100 µL Hamilton syringe and a 27 gauge needle) and observed for 40 min. The nociceptive behaviours were scored during 0–2 min (as phase I), and as a total on 18–20, 28–30 and 38–40 min (as phase II). The nociceptive behaviours were defined as lifting, flexing, twitching or licking the injected paw. Terminal blood samples were collected from all ADX71943-treated animals at the end of experiments and were analysed as described for the pharmacokinetic studies (see Supplementary Information). The effect of ADX71943 on the total number of nociceptive behaviours for phase I and phase II was analysed by one-way ANOVA followed by Dunnett's test. The effect of baclofen on the same measures was analysed by t-test.

MB test in mice

The test was performed as described previously (Kalinichev *et al.*, 2013). Animals (n = 10 per group) were treated p.o. with PD, ADX71943 (0.3, 1, 3, 10, 30 and 100 mg·kg^{-1}), chlordiazepoxide (30 mg·kg^{-1}) or the vehicle for chlordiazepoxide (saline). Sixty minutes following treatment, animals were individually placed into experimental cages and were left undisturbed for 30 min, following which numbers of buried marbles were counted. Terminal blood samples were collected from all ADX71943-treated animals at the end of the experiments and plasma was analysed as described for the pharmacokinetic studies (see Supplementary Information). The effect of ADX71943 on the number of buried marbles was analysed by Kruskal–Wallis test followed by Dunn's multiple comparisons. The effect of chlordiazepoxide was analysed by the Mann–Whitney U-test.

EPM test in mice

This test was performed as described previously (Kalinichev *et al.*, 2013). Mice (n = 9–10 per group) were treated p.o. with 1% CMC, ADX71943 (3, 10, 30 and 100 mg·kg^{-1}) or diazepam (1.5 mg·kg^{-1}; dissolved in PEG400/water solution, 30/70%). Sixty minutes later, animals were individually placed in the centre of the maze and were left to explore for 5 min. The effect of ADX71943 on the number of entries into open and closed arms as well as the time spent in open arms of the maze was analysed by one-way ANOVA followed by planned comparisons. The effect of diazepam was analysed by t-test.

BT test in mice and rats

Experiments in mice and rats were performed as described previously (M. Kalinichev *et al.*, submitted). After assessment of the basal BT before treatment, at T0, animals were allocated to treatment groups with equal means. The BT was assessed again 60 min (T60), 120 min (T120) and 240 min (T240) following treatment. Mice (n = 10 per group) were treated p.o. with 1% CMC, ADX71943 (3, 10, 30 and 100 mg·kg^{-1}) or baclofen (10 mg·kg^{-1}). Rats (n = 10 per group) were treated p.o. with PD, ADX71943 (10, 30 and 100 mg·kg^{-1}) or baclofen (3 and 6 mg·kg^{-1}). The effect of ADX71943 on the BT was analysed by two-way ANOVA followed by planned comparisons. The effect of baclofen was analysed by two-way ANOVA followed by planned comparisons.

Nomenclature

The nomenclature regarding receptors fully conforms to that of *The British Journal of Pharmacology's Concise Guide to PHARMACOLOGY* (Alexander *et al.*, 2013).

Drugs

ADX71943 was synthesized at Addex Therapeutics. (*R*)-baclofen ((R)-4-amino-3-(4-chlorophenyl) butanoic acid), CGP63360, chlordiazepoxide and diazepam were purchased from Sigma Aldrich. The suspensions were homogenized with stainless steel balls for 30 min at 30 Hz in a 2 mL Eppendorf tube (Vaudaux-Eppendorf AG, Basel, Switzerland), then vortexed and sonicated for 10 min.

ADX71943 was dissolved in PD or suspended in 1% CMC and administered p.o. at 10 or 5 mL·kg^{-1} vol in mice and rats respectively. (*R*)-baclofen was suspended in 1% CMC and administered p.o at 10 or 5 mL·kg^{-1} vol in mice and rats respectively. AA was dissolved in saline and administered i.p. at 3 mL·kg^{-1} vol. CGP63360 was dissolved in saline and administered p.o. at 10 mL·kg^{-1} vol. Chlordiazepoxide was dissolved in saline and administered p.o. at 10 mL·kg^{-1} vol. Diazepam was dissolved in PEG400/water solution (30/70%) and administered p.o. at 10 mL·kg^{-1} vol. All solutions and suspensions were prepared fresh daily. All doses of pharmacological agents are expressed as free base.

Results

Identification and in vitro *pharmacological characterization of ADX71943 on recombinant and native GABA$_B$ receptors*

In the Ca^{+2} mobilization assay, the compound was tested in a HEK293 stable cell line co-expressing the two subunits of the human $GABA_B$ receptor, $GABA_{B1(a)}$ and $GABA_{B2}$, with a chimeric Gα protein allowing redirection of receptor activation onto calcium signalling. A concentration–response curve of this compound was found to enhance an EC_{20} of the endogenous orthosteric agonist GABA with an efficacy of 181% and an EC_{50} of 96 ± 15 nM (Figure 2A; Table 1). When tested in the absence of GABA in this assay, the compound did not show any activity in this assay. Naturally, $GABA_B$ receptor is coupled to Gαi leading to cAMP signalling. Therefore, to demonstrate the activity of ADX71943 under native conditions, with the endogenously expressed receptor using its natural G-protein coupling, the compound was tested on cortical membranes of rat and human origins using the [^{35}S]-GTPγS binding assay. In the presence of an EC_{50} of the selective $GABA_B$ receptor agonist baclofen, ADX71943 enhanced the binding of [^{35}S]-GTPγS on $GABA_B$ receptor in a concentration-dependent manner, with the efficacy of 280% and EC_{50} values of 28 ± 2 nM when rat cortical membranes were used (Figure 2B; Table 1). In a follow-up experiment, where ADX71943 was tested in the absence of an agonist, it had no effect on [^{35}S]-GTPγS binding at low concentrations, while increasing the binding at the three highest concentrations (3, 10 and 30 μM; Figure 2C). Testing only the buffer without ADX71943 led to no [^{35}S]-GTPγS binding, while 30 μM baclofen increased binding (Figure 2C). When [^{35}S]-GTPγS binding assay was performed using human cortical membranes, ADX71943 enhanced the binding of [^{35}S]-GTPγS on $GABA_B$ receptor in a concentration-dependent manner, with the efficacy of 261% and EC_{50} values of 116 ± 32 nM (Figure 2D). As seen previously, the highest concentration of ADX71943 (30 μM) tested without an agonist produced ~110% increase in [^{35}S]-GTPγS binding (Figure 2D).

Using a cell-based calcium mobilization assay, ADX71943 showed no detectable activity (agonist, antagonist or allosteric effects) in cell lines overexpressing $mGlu_1$-$mGlu_8$ (data not included). In addition, in binding experiments against a panel of 71 receptors, transporters, enzymes and ion channels (ADDEX 71 profile), ADX71943 exhibited no detectable reference radioligand displacement at 10 μM.

In vivo *pharmacokinetic properties of ADX71943*

Table 2 presents the mean pharmacokinetic parameters after p.o. administration of ADX71943. Following p.o. administra-

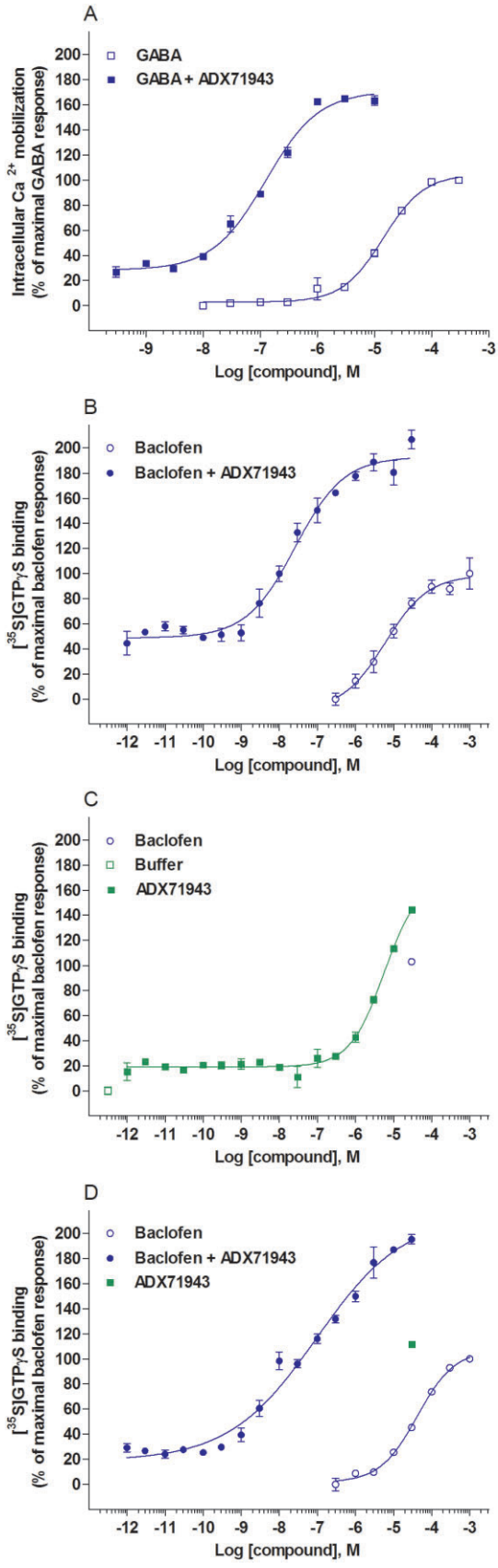

Figure 2

Concentration–response curve of ADX71943 on recombinant $hGABA_B$ receptor in intracellular Ca^{2+} mobilization assay in the presence of an EC_{20} of GABA (A). Concentration–response curve of ADX71943 on native $GABA_B$ receptor expressed in rat (B, C) or in human (D) cortical membranes in [^{35}S]-GTPγS binding assay in the presence of an EC_{50} of baclofen. Data are representative of at least three independent experiments performed in duplicate.

Table 1

Summary of functional pharmacological activity of ADX71943 on recombinant and native $GABA_B$ receptors

	FLIPR Ca^{2+} assay versus GABA EC_{20}	[^{35}S]-GTPγS binding assay versus baclofen EC_{50}	
Activity	**Recombinant human $GABA_B$ receptor**	**Rat cortical membranes**	**Human cortical membranes**
EC_{50} ± SEM (nM)	96 ± 15	28 ± 2	116 ± 32
Efficacy (%)	181	280	261

Values are expressed as mean from at least three independent experiments performed in duplicate.

tion of 10 mg·kg^{-1} to rats, plasma and CSF concentrations of ADX71943 reached maximum after approximately 3.6 and 4.4 h respectively (Table 2). The maximal concentration (C_{max}) of ADX71943 in plasma and CSF was 263 and 5.21 ng·mL^{-1}. This resulted in C_{max} CSF/plasma of 2.03% (Table 2).

AAW test in mice

ADX71943 (0.3–30 mg·kg^{-1}, p.o.) dose dependently reduced the number of writhes [$F(5, 64) = 7.19$, $P < 0.001$]. Specifically, 30% reductions ($P < 0.05$) at 1 and 3 mg·kg^{-1} ADX71943 were followed by 43 and 62% (both $P < 0.001$) reductions at 10 and 30 mg·kg^{-1} respectively (Figure 3A). The plasma concentrations of ADX71943 in treated animals are presented in Table 3. Baclofen (3 mg·kg^{-1}) caused 80% ($P < 0.001$) reduction in numbers of writhes compared to its vehicle (1% CMC; Figure 3A).

In the target engagement study there was overall effect of treatment [$F(3, 47) = 3.7$, $P < 0.05$]. When co-administered with saline, ADX71943 (10 mg·kg^{-1}) resulted in 40% ($P < 0.01$) reduction in the number of writhes compared to PD-saline treatment, while PD/CGP63360 (1 mg·kg^{-1}) treatment had no effect (Figure 3B). Following CGP63360/ADX71943 co-administration, the number of writhes did not differ from those shown by animals treated with PD/saline while being 1.7-fold ($P = 0.07$) higher than those seen in saline/ADX71943-treated animals (Figure 3B). The plasma concentrations of ADX71943 and CGP63360 at the end of the experiment are presented in Table 4.

FT in mice and rats

In mice, ADX71943 (0.3–100 mg·kg^{-1}, p.o.) dose dependently reduced [$F(6, 76) = 21.5$, $P < 0.0001$] nociceptive behaviours in phase I (Figure 4A). Specifically, 20% reduction ($P < 0.05$) at 1 mg·kg^{-1} was followed by approximately 27, 35, 40 and 60% (all $P < 0.001$) reductions in response to 3, 10, 30 and 100 mg·kg^{-1} respectively (Figure 4A). ADX71943 also dose dependently reduced [$F(6, 76) = 21.5$, $P < 0.0001$] nociceptive behaviours in phase II, where 27% reductions ($P < 0.05$) at 3 mg·kg^{-1} were followed by approximately 35, 50 and 80% reductions (all $P < 0.001$) at 10, 30 and 100 mg·kg^{-1} respectively (Figure 4B). In mice, baclofen (6 mg·kg^{-1}, p.o.) resulted in 80–90% ($P < 0.001$) reductions in nociceptive behaviours in both phases (Figure 4A and B).

In rats, acute ADX71943 (1–50 mg·kg^{-1}, p.o.) was inactive in phase I, while causing dose-dependent reductions [$F(5, 54) = 8.91$, $P < 0.0001$] in nociceptive behaviours in phase II.

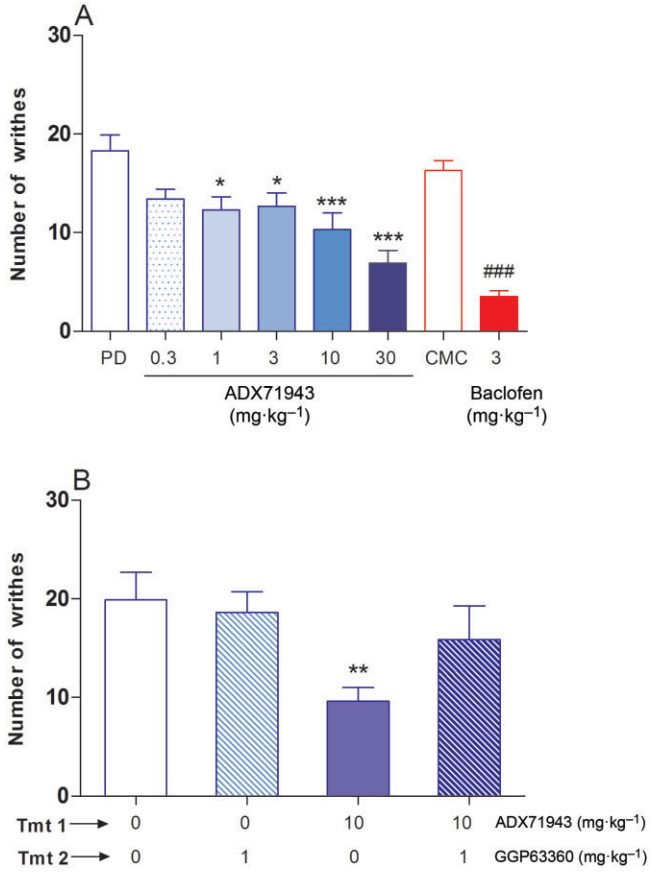

Figure 3

Effects of ADX71743 on the number of acetic acid-induced writhes (see text). In the dose–response experiment, (A) C57Bl6/J mice (n = 11–12 per group) were treated p.o. with PD, ADX71943 (0.3, 1, 3, 10 and 30 mg·kg^{-1}), 1% CMC or baclofen (3 mg·kg^{-1}). In the target engagement experiment, (B) C57Bl6/J mice (n = 11–14 per group) were treated p.o. with PD or ADX71943 (10 mg·kg^{-1}) as treatment 1 (Tmt 1) followed by p.o. treatment with CGP63360 (1 mg·kg^{-1}) or saline (Sal) as treatment 2 (Tmt 2; see text). Each point represents the observed mean (± SEM). **$P < 0.01$ compared with the PD/Sal-treated group.

Specifically, 30% ($P < 0.05$) and 43% ($P < 0.001$) reductions were seen at 15 and 50 mg·kg^{-1} respectively (Figure 4D). In rats, baclofen had no effect on pain in phase I, but in phase II it reduced nociceptive behaviours by 63% ($P < 0.001$;

Table 2
Mean pharmacokinetic parameters following p.o. administration of ADX71943 in male Sprague-Dawley rats

Species	Dose ($mg \cdot kg^{-1}$)	Route	Plasma				CSF			
			$t_{1/2}$ (h)	$AUC_{last} \pm SD$ ($ng \cdot h \cdot mL^{-1}$)	T_{max} (h)	C_{max} ($ng \cdot mL^{-1}$)	$t_{1/2}$ (h)	$AUC_{last} \pm SD$ ($ng \cdot h \cdot mL^{-1}$)	T_{max} (h)	C_{max} ($ng \cdot mL^{-1}$)	C_{max} CSF/plasma (%)
Rats	10	p.o.	9.6 ± 8.4	290 ± 31	3.6 ± 0.9	263 ± 70.8	3.97	3.17 ± 0.83	4.40 ± 0.89	5.21 ± 1.01	2.03 ± 0.30

Figure 4C and D). Plasma concentrations of ADX71943 at the end of experiments are presented in Table 3.

MB test in mice

ADX71943 (0.3–100 $mg \cdot kg^{-1}$, p.o.) had no effect on MB, despite dose-proportional increases in plasma concentrations of the compound (Figure 5). Chlordiazepoxide (30 $mg \cdot kg^{-1}$) resulted in a near-complete reduction (95%; $P < 0.001$) in MB compared to saline (Figure 5).

EPM test in mice

ADX71943 (3–100 $mg \cdot kg^{-1}$, p.o.) had no effect on open-arm activity (Table 5). Diazepam caused 2.5- to 3-fold increases ($P < 0.001$) in open-arm entries and time spent on those arms compared to vehicle (Table 5)

BT in mice and rats

In mice, acute ADX71943 (3–100 $mg \cdot kg^{-1}$, p.o.) had no effect on BT across the experiment (Table 6). At 10 $mg \cdot kg^{-1}$, baclofen reduced BT 60, 120 and 120 min following treatment (all $P < 0.001$).

In rats, ADX71943 (10 and 30 $mg \cdot kg^{-1}$, p.o.) had no effect on BT across the experiment (Table 6). At the highest dose (100 $mg \cdot kg^{-1}$), ADX71943 resulted in mild 0.4°C reduction ($P < 0.01$) of BT 120 min following administration, but not at other time points (Table 6). At 3 $mg \cdot kg^{-1}$, baclofen reduced BT 60 min ($P < 0.05$) and 120 min ($P < 0.001$) following treatment, while at 6 $mg \cdot kg^{-1}$ reductions in BT (all $P < 0.001$) were seen across the entire test (Table 6).

Efficacy and PK/PD

Evaluation of minimum effective doses (MEDs) of ADX71943, associated plasma and estimated unbound concentrations across several experiments showed a good *in vitro*/*in vivo* correlation in models of pain (Table 7). Specifically, following acute p.o. administration, the MEDs of ADX71943 were associated with mean plasma concentrations in the range of 109–255 $ng \cdot mL^{-1}$ that corresponded to calculated unbound concentration over *in vitro* EC_{50} ratios between 1.4 and 3.2 in mice. In contrast, in an anxiety model, the MB test, even 100 $mg \cdot kg^{-1}$ and associated plasma concentration of 3370 $ng \cdot mL^{-1}$ did not lead to efficacy. The same dose (100 $mg \cdot kg^{-1}$) failed to result in efficacy in tests relevant to centrally mediated side effects (Table 7).

Discussion

To our knowledge, ADX71943 is the first $GABA_B$ receptor PAM with a fully peripheral activity profile. After the discovery of an inadequate safety profile for ADX71943, its further development was stopped and the compound was characterized as a pharmacological tool for delineation of peripherally- versus centrally-mediated functions of $GABA_B$ receptor. Previously, we confirmed that ADX71943 is a PgP substrate that results in an active efflux of the molecule from the CNS, leading to peripheral mode of action of the molecule. Here, measured concentration of ADX71943 in plasma and CSF revealed that at C_{max}, the ratio was 2%, confirming virtually full absence of

Table 3

Plasma concentrations of ADX71943 in mice and rats at the end of *in vivo* tests

Test	Treatment	Species	Route	Dose ($mg \cdot kg^{-1}$)	*n*	Plasma concentration ($ng \cdot mL^{-1}$) (mean ± SD)
AAW	PD	Mice	p.o.	0	11	
	ADX71943	Mice	p.o.	0.3	11	37 ± 15
	ADX71943	Mice	p.o.	1	12	109 ± 21
	ADX71943	Mice	p.o.	3	12	357 ± 89
	ADX71943	Mice	p.o.	10	12	809 ± 197
	ADX71943	Mice	p.o.	30	12	1618 ± 305
FT	PD	Mice	p.o.	0	10	
	ADX71943	Mice	p.o.	0.3	11	27 ± 10
	ADX71943	Mice	p.o.	1	12	99 ± 13
	ADX71943	Mice	p.o.	3	14	255 ± 46
	ADX71943	Mice	p.o.	10	12	1020 ± 195
	ADX71943	Mice	p.o.	30	12	1879 ± 310
	ADX71943	Mice	p.o.	100	12	3622 ± 994
FT	PD	Rats	p.o.	0	10	
	ADX71943	Rats	p.o.	1	10	21 ± 5
	ADX71943	Rats	p.o.	5	10	65 ± 15
	ADX71943	Rats	p.o.	15	10	124 ± 50
	ADX71943	Rats	p.o.	50	10	217 ± 56
MB	PD	Mice	p.o.	0	10	
	ADX71943	Mice	p.o.	0.3	10	25 ± 5
	ADX71943	Mice	p.o.	1	10	116 ± 33
	ADX71943	Mice	p.o.	3	10	299 ± 63
	ADX71943	Mice	p.o.	10	10	1128 ± 218
	ADX71943	Mice	p.o.	30	10	2044 ± 392
	ADX71943	Mice	p.o.	100	10	3370 ± 268

Table 4

Plasma concentrations of ADX71943 (ADX) and a $GABA_B$ receptor antagonist CGP63360 (CGP) at the end of the AAW test when the compounds were administered alone (i.e. with vehicles) or co-administered

Treatment 1	Treatment 2	Route	*n*	ADX71441 plasma conc ($ng \cdot mL^{-1}$) (mean ± SD)	CGP63360 plasma conc ($ng \cdot mL^{-1}$) (mean ± SD)
PD	Saline	p.o.	11	–	–
PD	1 $mg \cdot kg^{-1}$ CGP	p.o.	14	–	245 ± 77
10 $mg \cdot kg^{-1}$ ADX	Saline	p.o.	14	744 ± 176	–
10 $mg \cdot kg^{-1}$ ADX	1 $mg \cdot kg^{-1}$ CGP	p.o.	12	652 ± 192	219 ± 93

the compound from the CNS. *In vitro*, in HEK293 cell line, ADX71943 showed increases in efficacy and potency of GABA, as seen previously for $GABA_B$ receptor PAMs, CGP7930, GS39783, rac-BHFF (Urwyler *et al.*, 2001; 2003; Malherbe *et al.*, 2008) and recently described PAM, ADX71441 (M. Kalinichev *et al.*, unpublished observations). The observed increase in binding produced by high concentration of ADX71943 in the absence of GABA or baclofen is likely to be an artefact linked to very low levels of endogenous GABA still present in the cortical membranes. In accordance with this hypothesis, ADX71943 showed no intrinsic activity in the intracellular Ca^{2+} mobilization assay, in which a recombinant cell line (expressing human $GABA_B$), fully devoid of endogenous GABA, was used. Interestingly, we

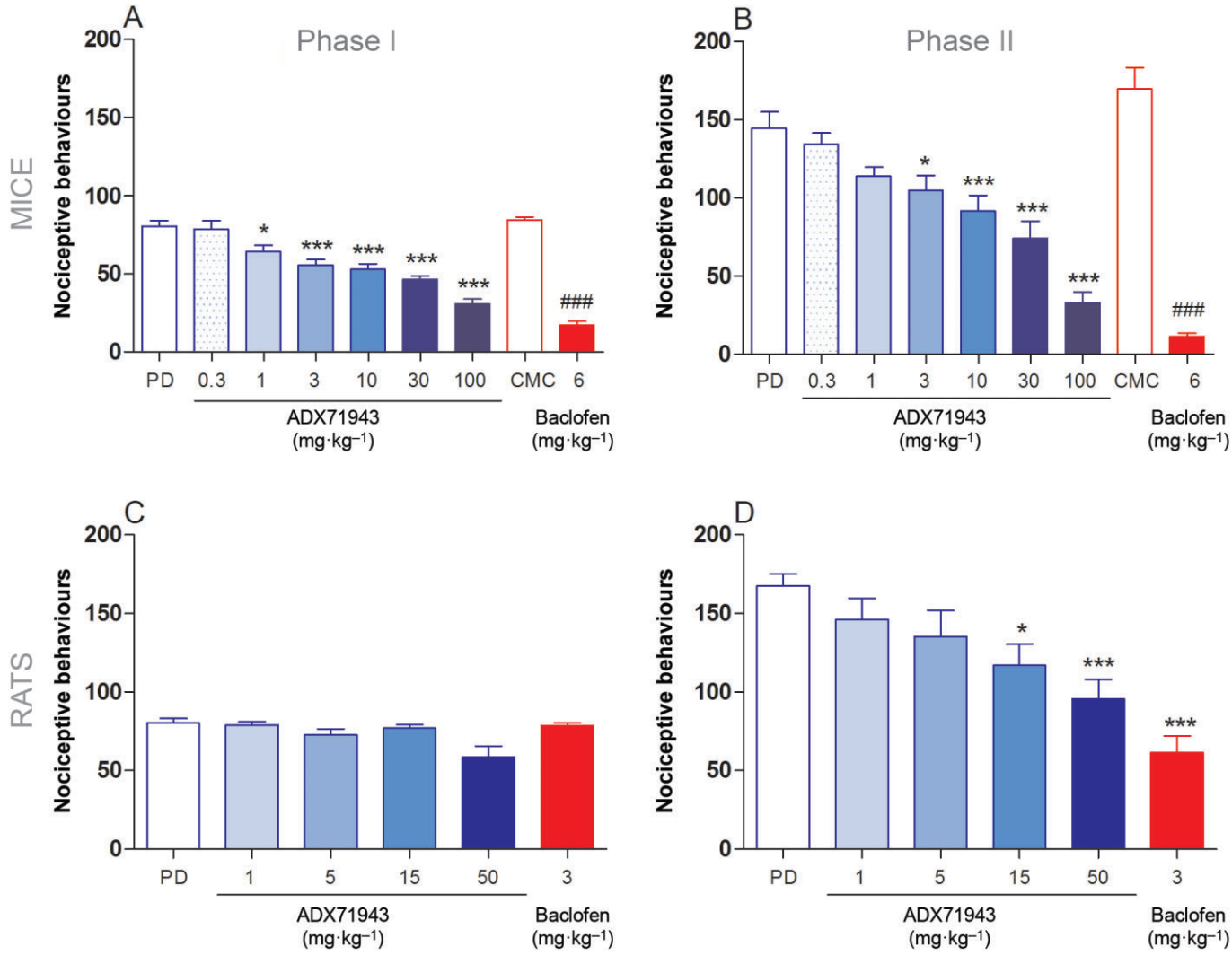

Figure 4

Nociceptive behaviours in C57Bl6/J mice (A, B) and Sprague-Dawley rats (C, D) monitored during phase I (A, C) and phase II (B, D) of the formalin test (see text). Mice (n = 10–14 per group) were pretreated p.o. with PD, ADX71943 (0.3, 1, 3, 10, 30 and 100 mg·kg^{-1}), saline (Sal) or baclofen (6 mg·kg^{-1}). Rats (n = 10 per group) were pretreated p.o. with PD, ADX71943 (1, 5, 15 and 50 mg·kg^{-1}) or baclofen (3 mg·kg^{-1}). Each point represents the observed mean (± SEM). *P < 0.05, ***P < 0.001 compared with PD. $^{###}P$ < 0.001 compared with Sal.

Table 5

Activity in the mouse EPM test

Treatment	Route	Dose (mg·kg^{-1})	n	Open arm entries	Time (s) in open arms	Closed arm entries
1% CMC	p.o.	0	10	3.2 ± 0.4	17 ± 2.4	12 ± 0.8
ADX71943	p.o.	3	10	4.0 ± 0.6	23 ± 5.3	12 ± 1.1
ADX71943	p.o.	10	9	3.8 ± 0.6	23 ± 3.9	12 ± 0.7
ADX71943	p.o.	30	10	4.2 ± 0.4	25 ± 3.5	13 ± 0.5
ADX71943	p.o.	100	10	4.1 ± 0.8	31 ± 6.4	12 ± 0.8
Diazepam	p.o.	1.5	10	8.5 ± 0.5***	54 ± 7.0***	15 ± 1.3

Results are presented as the mean ± SEM; ***P < 0.001 compared to 1% CMC.

Table 6

Body temperature in mice and rats

Treatment	Species	Route	Dose ($mg \cdot kg^{-1}$)	*n*	Body temperature T0	Body temperature T60	Body temperature T120	Body temperature T240
1% CMC	Mice	p.o.	0	10	37.21 ± 0.26	37.20 ± 0.11	37.03 ± 0.11	36.60 ± 0.17
ADX71943	Mice	p.o.	3	10	37.14 ± 0.15	37.20 ± 0.08	37.00 ± 0.09	36.77 ± 0.15
ADX71943	Mice	p.o.	10	10	37.19 ± 0.16	37.31 ± 0.10	36.88 ± 0.28	36.88 ± 0.18
ADX71943	Mice	p.o.	30	10	37.28 ± 0.24	37.51 ± 0.15	37.52 ± 0.14	37.10 ± 0.25
ADX71943	Mice	p.o.	100	10	37.22 ± 0.10	37.12 ± 0.15	37.00 ± 0.10	36.72 ± 0.12
Baclofen	Mice	p.o.	10	10	37.82 ± 0.11	34.35 ± 0.48***	32.85 ± 0.39***	35.15 ± 0.42***
PD	Rats	p.o.	0	10	36.92 ± 0.06	37.18 ± 0.06	37.09 ± 0.04	37.05 ± 0.17
ADX71943	Rats	p.o.	10	10	36.94 ± 0.07	36.98 ± 0.09	36.93 ± 0.07	36.96 ± 0.07
ADX71943	Rats	p.o.	30	10	36.91 ± 0.01	36.98 ± 0.07	36.96 ± 0.07	37.16 ± 0.05
ADX71943	Rats	p.o.	100	10	37.02 ± 0.07	36.90 ± 0.09	36.69 ± 0.08##	37.18 ± 0.02
Baclofen	Rats	p.o.	3	10	37.03 ± 0.06	36.82 ± 0.15#	36.49 ± 0.11###	37.04 ± 0.04
Baclofen	Rats	p.o.	6	10	37.02 ± 0.06	36.24 ± 0.16###	36.12 ± 0.11###	36.34 ± 0.13###

Results are presented as the mean ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to 1% CMC; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared to PD.

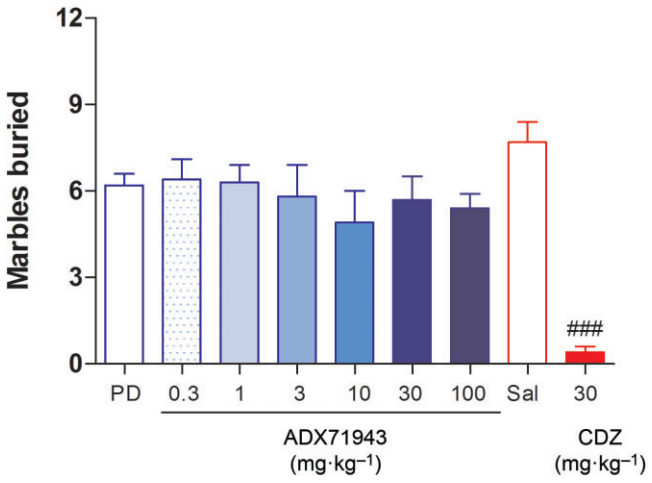

Figure 5

Number of buried marbles in C57Bl6/J mice (n = 10 per group) following p.o. treatment with PD, ADX71943 (0.3, 1, 3, 10, 30 and 100 $mg \cdot kg^{-1}$), saline (Sal) or chlordiazepoxide (CDZ; 30 $mg \cdot kg^{-1}$). Each point represents the observed mean (± SEM). ###$P < 0.001$ compared with Sal.

saw a similar difference in *in vitro* activity of another $GABA_B$ receptor PAM ADX71441 on recombinant versus native $GABA_B$ receptor (M. Kalinichev *et al.*, in review).

The *in vitro* characterization studies, however, did not include a $GABA_B$ antagonist that could have added an additional value to these experiments. ADX71943 showed selectivity for the $GABA_B$ receptor against all mGlus and a panel of 71 targets. Before testing the compound *in vivo*, in a series of experiments we tested the solubility of ADX71943 in 29 solvents (Ayad *et al.*, 2013). As PD provided a high solubility for the compound and was acceptable for *in vivo* studies, it was chosen as a vehicle for ADX71943 for the majority of experiments in mice and rats.

In vivo, in the mouse AAW, a commonly used test to evaluate acute generalized visceral pain (Collier *et al.*, 1968), ADX71943, dose dependently reduced writhes and showed a good *in vitro*/*in vivo* correlation, indicative of target-related antinociceptive efficacy. The specificity of the target was confirmed by the target engagement experiment in which effects of ADX71943 were reversed by a GABA receptor selective antagonist CGP63360. The reversal of the effect of ADX71943 by CGP63360 was due to pharmacodynamic rather than pharmacokinetic interaction between the compounds, as their plasma concentrations, when administered separately and when co-administered, were virtually identical. The antinociceptive efficacy of ADX71943 was further confirmed in the FT, which is characterized by the biphasic pattern of pain-associated behaviours. It has been suggested that phase I, characterized by acute pain, is mediated by C and Aδ fibres, while phase II, characterized by persistent pain, is mediated primarily by inflammation of the peripheral tissue (Tjölsen *et al.*, 1992). ADX71943 dose dependently reduced the number of nociceptive behaviours in both phases in mice, showing a good *in vitro*/*in vivo* correlation. In rats, ADX71943 dose dependently inhibited nociceptive behaviours only in the phase II, while being inactive in phase I. Interestingly, baclofen, used as a positive control, also showed an activity in both phases in mice, while showing activity only in phase II in rats. We can speculate that there are species differences either in the number, distribution or regulation of the $GABA_B$ receptor in regions that regulate chemically induced acute versus inflammatory pain. We also need to point to the fact that the efficacy of ADX71943 in the phase II of FT was lower in rats (MED 15 $mg \cdot kg^{-1}$) than in mice (MED 3 $mg \cdot kg^{-1}$). This is probably linked to the differences in pharmacokinetic

Table 7

MEDs in several mouse and rat efficacy tests, plasma concentration, unbound plasma concentration and unbound plasma concentration/EC_{50} (*in vitro*) values at the end of each experiment

Test	Domain	Species	MED dose ($mg \cdot kg^{-1}$)	Plasma exposure ($ng \cdot mL^{-1}$)	Plasma exposure (nM)	Unbound plasma exposure[a,b] ($ng \cdot mL^{-1}$)	Unbound plasma exposure[a] (nM)	Unbound plasma explosure/ EC_{50} (*in vitro*)
AAW	Pain	Mice	1	109	248	20	46	1.4
FT[a]	Pain	Mice	3	255	581	47	107	3.2
FT[a]	Pain	Rats	15	124	282	23	52	1.5
MB	Anxiety	Mice	>100	3370	7677	623	1420	42
EPM	Anxiety	Mice	>100					
BT	Side effect	Mice	>100					
BT	Side effect	Rats	>100					
Rotarod	Side effect	Mice	>100					
sLMA	Side effect	Mice	>100					
sLMA	Side effect	Rats	>30					

[a]Phase II.
[b]Unbound plasma exposure is calculated from the plasma exposure using mice plasma protein binding (PPB; fraction unbound = 18.5%).

profile of ADX71943 in rats versus mice after p.o. administration, as the mean plasma exposure associated with MEDs was similar in these species (124 $ng \cdot mL^{-1}$ in mice and 255 $ng \cdot mL^{-1}$ in rats). Reflecting its fully peripheral profile, ADX71943-mediated antinociception was devoid of any confounding sedative or muscle-relaxant effects, as confirmed by the sLMA and rotarod studies. To summarize, $GABA_B$ receptor PAMs with peripheral activity profile may represent a promising therapeutic approach for treatment of acute and inflammatory pain.

Across several experiments in mice and rats, the assessment of efficacy of ADX71943 in models of pain was characterized with a consistent *in vivo*/*in vitro* correlation. Specifically, the MEDs of the compound were associated plasma concentration of 109–255 $ng \cdot mL^{-1}$ that corresponded to calculated unbound concentration over *in vitro* EC_{50} ratios of 1.4–3.2. It also needs to be emphasized that the magnitude of the effect seen with ADX71943 in the AAW test, even at its highest dose (30 $mg \cdot kg^{-1}$), was moderate (~60% reduction), falling behind a more robust suppression (~80%), seen with baclofen (6 $mg \cdot kg^{-1}$). As seen in the AAW test, the maximal suppression of nociceptive behaviours induced by ADX71943 even at its highest doses (100 and 50 $mg \cdot kg^{-1}$ in mice and rats, respectively) was more modest than those induced by low doses of baclofen (6 and 3 $mg \cdot kg^{-1}$ in mice and rats respectively). Furthermore, in a monosodium iodoacetate (MIA) model of chronic osteoarthritic (OA)-like pain in the rat, the anti-hyperalgesic effects of ADX71943 were more modest than those induced by a non-steroidal anti-inflammatory drug, a COX-2 inhibitor, celecoxib (M. Kalinichev *et al.*, submitted). Reflecting its peripheral mode of action, the antinociceptive effects of ADX71943 are likely to be mediated solely by the peripheral mechanisms, which may not be sufficient for the full suppression of either acute or chronic pain. We can hypothesize that a $GABA_B$ receptor PAM with a balanced central-peripheral mode of action can offer a more complete suppression of pain. Indeed, a novel, potent and selective $GABA_B$ receptor PAM ADX71441 with a balanced central-peripheral profile resulted in near-complete (~90%) suppression of writhing behaviour in a manner similar to that seen with baclofen (M. Kalinichev *et al.*, submitted). Furthermore, in the MIA-induced model of chronic OA pain in rats, the anti-hyperalgesic effects of acute ADX71441 were up to 1.5-fold higher than those of celecoxib (M. Kalinichev *et al.*, unpublished observations).

Reflecting its peripheral activity profile, ADX71943 was inactive in the MB test in mice even when very high plasma concentrations of the compound were achieved (>3000 $ng \cdot mL^{-1}$ at 100 $mg \cdot kg^{-1}$). ADX71943 was also inactive in the mouse EPM test, further confirming its lack of anxiolytic-like efficacy. These data support the notion that $GABA_B$ receptors localized in the brain (rather than periphery) regulate emotional-like reactivity and mediate anxiolytic-like efficacy of $GABA_B$ activators. Previously, ADX71441 showed anxiolytic-like effects in the mouse MB test and in the EPM test in mice and rats (M. Kalinichev *et al.*, submitted). Furthermore, a broad anxiolytic-like profile has been seen with CGP7930 and GS39783 (Cryan *et al.*, 2004; Mombereau *et al.*, 2004; Frankowska *et al.*, 2007; Jacobson and Cryan, 2008).

In rodents, the muscle-relaxant properties of baclofen and other $GABA_B$ receptor agonists are typically evaluated by using the rotarod test. It is believed that the muscle-relaxant effects are driven by the $GABA_B$ receptors localized in the ventral horn of the spinal cord (Curtis and Lacey, 1994; Malcangio and Bowery, 1996). Reflecting its lack of CNS penetration, ADX71943 did not reduce rotarod activity in mice even when administered at high doses (100 $mg \cdot kg^{-1}$; Supplementary Information). Interestingly, reflecting its narrow therapeutic window, baclofen had no effect in the rat rotarod test at 1 and 3 $mg \cdot kg^{-1}$ while causing the full suppression of activity at 6 $mg \cdot kg^{-1}$. ADX71943 also had no effect on sLMA

in a novel environment when tested up to 100 $mg \cdot kg^{-1}$ in mice and rats (Supplementary Information). These results are well aligned with its peripheral mode of action as several factors that typically result in reductions in sLMA, sedative/hypnotic, exploratory and emotional-like (Jähkel *et al.*, 2000; Tanaka *et al.*, 2012) are typically linked to centrally mediated effects of the compound. Again, reflecting its narrow therapeutic window, baclofen had no effect in the sLMA at 0.3 and 1 $mg \cdot kg^{-1}$, while markedly reducing activity at 3 $mg \cdot kg^{-1}$.

In rodents, baclofen causes consistent decreases in BT (Gray *et al.*, 1987; Jacobson and Cryan, 2005; Koek *et al.*, 2010), presumably acting on the population of $GABA_B$ receptor in the medial preoptic area in the forebrain. As expected, ADX71943 also had no effect on BT when tested up to 100 $mg \cdot kg^{-1}$ in mice and rats. A modest reduction in BT in rats at 100 $mg \cdot kg^{-1}$ is likely to be a statistical artefact, as it was observed 120 min following treatment, but not at earlier or later time points. While the hypothermic effects of baclofen in rodents are well documented, similar effects in humans are seen only in the case of severe baclofen overdose (Perry *et al.*, 1998), but not under normal treatment conditions.

To summarize, here we present a comprehensive characterization of a novel, potent and selective $GABA_B$ receptor PAM ADX71943 with a fully peripheral activity profile. The compound shows consistent effects in rodent models of acute and persistent pain, albeit with maximal responses that are more modest compared to those seen with baclofen. While potential therapeutic application of such compounds can be considered for largely peripheral indications (GERD, asthma, cough), other indications with a significant central component (pain, anxiety, Fragile X) are likely to benefit from a PAM with a balanced central-peripheral profile.

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Author contributions

T. D-R., F. G., E. R., M. R., B. B., H. H. and S. P. participated in research design. T. D-R., F. G., E. R., M. R., B. B. and H. H. conducted the experiments. M. R., B. B. and H. H. contributed new reagents or analytic tools. M. K., T. D-R., F. G., E. R., M. R., B. B. and S. P. performed the data analysis. M. K., H. H., V. M. and S. P. wrote or contributed to the writing of the manuscript.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Locomotor activity (distance travelled; cm) in C57Bl6/J mice (A) or Sprague-Dawley rats (B) travelled and expressed as time courses in 5 min intervals or total distances travelled (insets) during 60 min in mice and 30 min in rats. Mice (n = 10 per group) were pretreated p.o. with 1% CMC, ADX71943 (3, 10, 30 and 100 $mg{\cdot}kg^{-1}$) or baclofen (10 $mg{\cdot}kg^{-1}$). Rats (n = 10 per group) were pretreated p.o. with plasdone (PD), ADX71943 (3, 10 and 30 $mg{\cdot}kg^{-1}$), 1% CMC or baclofen (0.3, 1 and 3 $mg{\cdot}kg^{-1}$). All treatments were administered 60 min before commencement of the test. Each point represents the observed mean (± SEM). ***$P < 0.001$ compared with corresponding vehicles.
Table S1 Rotarod in mice.