

AZD3355, a novel GABA_B receptor agonist, inhibits transient lower esophageal sphincter relaxation through a peripheral mode of action

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Nonstandard abbreviations: CNS – central nervous system; GABA – γ -aminobutyric acid; GAT – GABA transporter; GERD – gastroesophageal reflux disease; i.g. –intra-gastric; LES – lower esophageal sphincter; PPI – proton pump inhibitor; TLESR – transient lower esophageal sphincter relaxation

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Abstract

Gastroesophageal reflux disease (GERD) affects >10% of the Western population.

Conventionally, GERD is treated by reducing gastric acid secretion, which is effective in most patients but inadequate in a significant minority. We describe a new therapeutic approach for GERD, based on inhibition of transient lower esophageal sphincter relaxation (TLESR) with a proposedly peripherally acting γ -aminobutyric acid type B (GABA_B) receptor agonist, AZD3355 [(R)-(3-amino-2-fluoropropyl) phosphinic acid]. AZD3355 potently stimulated recombinant human GABA_B receptors and inhibited TLESR in dogs with a biphasic dose-response curve. In mice, AZD3355 produced considerably less central side-effects than the prototypical GABA_B receptor agonist baclofen, but evoked hypothermia at very high doses (blocked by a GABA_B receptor antagonist and absent in GABA_B^{-/-} mice). AZD3355 and baclofen differed markedly in their distribution in rat brain; AZD3355, but not baclofen, was concentrated in circumventricular organs as a result of active uptake (shown by avid intracellular sequestration), and related to binding of AZD3355 to native GABA transporters in rat cerebrocortical membranes. AZD3355 was also shown to be transported by all four recombinant human GABA transporters. AR-H061719 (the racemate of AZD3355) inhibited the response of ferret mechanoreceptors to gastric distension, further supporting its peripheral site of action on TLESR. In summary, AZD3355 likely inhibits TLESR through stimulation of peripheral GABA_B receptors and may offer a potential new approach to treatment of GERD.

Introduction

The γ -aminobutyric acid type B (GABA_B) receptor was originally defined pharmacologically by virtue of its insensitivity to the GABA_A receptor antagonist bicuculline and sensitivity to the GABA analog baclofen (Bowery et al., 1980). The GABA_B receptor, a member of family C of the G protein coupled receptors, is characterized by its large, ligand-binding extracellular N-terminal domain. It couples negatively to adenylyl cyclase and to voltage-gated calcium channels, and positively to inwardly rectifying potassium channels (Bettler et al., 2004).

Based on a wealth of preclinical data, the GABA_B receptor has been proposed as a therapeutic target for a number of diseases including gastroesophageal reflux disease (GERD) (Lehmann, 2009), one of the most common diseases of the upper gastrointestinal tract. Inhibition of gastric acid secretion with proton pump inhibitors (PPIs) is the mainstay of GERD therapy. However, symptoms do not resolve in all patients despite efficient suppression of acid secretion with a PPI (Fass et al., 2005), and this may be partly explained by reflux of weakly acidic/alkaline or neutral gastric contents. Consequently, pharmacotherapies beyond acid suppression are needed. A logical approach to this problem is to target the major barrier against reflux, the lower esophageal sphincter (LES), together with the partly superimposed crural diaphragm. Combined pH-metric and manometric studies have demonstrated that transient lower esophageal sphincter relaxation (TLESR) accounts for most reflux episodes in healthy subjects and in those with GERD (Mittal et al., 1995). Inhibitors of TLESR may therefore afford symptomatic relief in this population. Baclofen, for example, the prototypical GABA_B receptor agonist, has been demonstrated to inhibit TLESR and acid reflux in those with GERD (van Herwaarden et al., 2002; Zhang et al., 2002); in turn, GERD symptoms are mitigated (Ciccaglione and Marzio, 2003; Koek et al., 2003; Vela et al., 2003). Another beneficial effect of baclofen is its enhancing effect on basal LES

pressure (Zhang et al., 2002; Lee et al., 2003), which may be therapeutically relevant in some patients.

Despite clinical ‘proof of concept’, central side-effects such as dizziness limit the usefulness of baclofen in patients with GERD. Consequently, research efforts have focused on GABA_B receptor agonists with minimal side-effects. Here, we characterize the *in vitro* and *in vivo* pharmacology of AZD3355 [(R)-(3-amino-2-fluoropropyl) phosphinic acid] (Alstermark et al., 2008), a potent and selective GABA_B receptor agonist with a preclinical therapeutic window superior to baclofen.

Methods

All animal experiments were approved by the relevant local ethical committees for animal experiments. Further details on certain methods outlined below, along with chemical structures of compounds used in the experiments, can be found in Supplemental Information (Supplemental Figure 1).

Determination of binding affinities of GABA agonists at GABA_A and GABA_B receptor sites in brain membranes

Preparation of Sprague-Dawley rat brain synaptic vesicles was performed as described elsewhere (Jensen et al., 2002). The whole brain from beagles (Rååhöjden's kennel, Rååhöjden, Sweden) was isolated and membranes were prepared using the same method as used for the rat brain membranes.

Inhibition of [³H]GABA binding at GABA_B receptor sites in rat or dog brain membranes by test compounds was measured using a filtration binding assay. Displacement curves to determine IC₅₀ values were constructed by fitting the 4-parameter logistic equation to the data. Binding affinity (K_D) for GABA was determined on each preparation by homologous competition and used to calculate K_i values from IC₅₀ determinations on that particular preparation using the Cheng-Prusoff equation. The average K_D for GABA was 110 ± 21 nM (mean ± S.E.M., n=16 preparations).

The affinity of test compounds at GABA_A receptor sites in rat brain membranes was measured using a [³H]muscimol radioligand binding assay (Lehmann et al., 2005).

Determination of *in vitro* potency and efficacy of GABA_B receptor agonists on GABA_B receptor-mediated intracellular calcium release

The effect of test compounds on GABA_B receptor-mediated intracellular calcium release was determined in CHO-K1 cells stably co-expressing a GABA_{B(1a)}-G_qi5 fusion protein and GABA_{B(2)} using fluorescence imaging, as described in detail elsewhere (Lehmann et al., 2005).

Comparison of the effect of GABA_B receptor agonists on various GABA_{B(1)} splice variants

The human GABA_{B(1)} splice variants GABA_{B(1a)}, GABA_{B(1b)}, GABA_{B(1e)}, GABA_{B(1g)}, GABA_{B(1m)} and GABA_(1o), as well as GABA_{B(2)}, were cloned from a human brain cDNA library as previously described (Lehmann et al., 2005) and subcloned into pCI-Neo expression vectors. Transient co-transfections of GABA_{B(1)} splice variants and GABA_{B(2)} in CHO-G_qi5 cells were performed using lipofectamin (Life Technologies, Paisley, Scotland) according to the manufacturer's protocol. Thereafter, the effect of agonists on GABA_B receptor-mediated calcium release was determined using fluorescence imaging (Lehmann et al., 2005). Details concerning saturation binding analysis with [³H]CGP54626 (0.1-30 nM) (Tocris, Bristol, UK) can be found in Supplemental Information. Non-specific binding was determined by the inclusion of 3 mM GABA in the assay buffer. K_D and B_{max} values were calculated by fitting binding data to the equation $B = B_{\max} / (1 + K_D / [L])$.

Uptake of radiolabeled GABA_B receptor agonists in rat cerebrocortical slices

Rat cerebrocortical slices were prepared and incubated as previously described (Oja and Kontro, 1980), with 0.1, 1.0, or 10.0 μM of labeled compounds for 5, 10, and 30 min. The intracellular uptake of [³H]GABA (Amersham International, Bristol, UK; specific activity 3.40 TBq/mol,

positive control), [^{14}C]baclofen (3.2 TBq/mol, radiochemical purity 99%), and [^{14}C]AZD3355 (5.8 TBq/mol, radiochemical purity 96%) was calculated after subtraction of the amount of label in the extracellular spaces, estimated with the hydroxyl[^{14}C -methyl]inulin (Amersham) method.

GABA transporter (GAT) binding competition assay in rat cerebrocortical membranes

The method of Shank et al. was used to determine competition between GABA_B receptor agonists and [^3H]GABA for binding to the native GATs in rat cerebrocortical membranes (Shank et al., 1990). The assay was performed in the presence of 10 μM isoguvacine and 10 μM baclofen to block binding to GABA_A and GABA_B receptors, respectively. The specific binding of ligands was defined as the difference between the total binding and the non-specific binding in the presence of 1 mM GABA. The IC₅₀ values and Hill coefficients (n_H) were determined by non-linear regression analysis of the competition curves using Hill equation curve fitting. Baclofen and AZD3355 were evaluated in this assay using nipecotic acid as a positive control.

[^3H]GABA uptake assay and FLIPR[®] membrane potential assay in mammalian cells transiently expressing the human GATs

The experiments were performed as described previously (see Supplemental Information). Briefly, tsA201 cells were transfected with constructs encoding each of the four human GATs (hGAT-1, hBGT-1, hGAT-2, and hGAT-3). [^3H]GABA uptake in the absence or presence of drugs was measured after 3-min incubation at 37°C, and cold GABA was included as a positive control. GAT-mediated transport of the ligands was measured by using the FLIPR[®] membrane potential assay (Molecular Devices, Crawley, UK). The assay is based on the depolarizing effects of co-transport of a substrate and sodium and chloride ions. This assay, in contrast to the

[³H]GABA uptake assay, determines whether or not the test compounds are actively transported by the GATs.

Vagal afferent recordings

Single fibers were dissected from the main vagal trunks with receptive fields in the esophagus and stomach, as previously described in isolated superfused preparations of ferret tissue (Page and Blackshaw, 1999). The effects of AR-H061719 (racemate of AZD3355) on mucosal and tension receptors were studied after cumulative dosing. The racemate was used in this experiment since the R-enantiomer (AZD3355, the most active enantiomer) was not available at the time of the experiment.

TLESR in dogs

The method for quantitation of gastric distension-induced TLESR in dogs has been described in detail (Lehmann et al., 1999). In short, adult Labrador retrievers were equipped with a cervical esophagostomy. Manometry was performed using a Dentsleeve multilumen assembly (Dentsleeve, Wayville, Australia), and TLESRs were stimulated by infusion of an acidified liquid nutrient followed by insufflation of air. Acid reflux was measured with an antimony pH catheter positioned 3 cm above the LES. The total stimulation period was 45 min, and each dog served as its own control. The following compounds were evaluated: AZD3355, AR-H061719, baclofen, and GABA. Compounds were given i.v. (via a foreleg vein) or intragastrically (i.g., via the Dentsleeve assembly) 10 and 30 min before nutrient infusion, respectively. GABA was infused continuously i.v. starting 10 min before nutrient infusion. The doses of GABA were chosen according to Thirlby et al. (Thirlby et al., 1988).

The potential for agonists to induce tolerance was addressed in a 2-week study with once-daily administration of AZD3355 to dogs. The study design, results, and interpretation can be found in Supplemental Information (Supplemental Figure 2).

TLESR in ferrets

Experiments were performed according to Staunton et al. (Staunton et al., 2000) on adult female ferrets (weight range 0.6–0.8 kg) obtained from the Institute for Medical and Veterinary Science, Adelaide, Australia (see Supplemental Information). In brief, chronic lateral cervical mucosa-to-skin esophagostomies were constructed under anesthesia, and manometric studies were begun at least a month after surgery. Animals were fasted overnight before studies. On the day of study, 60 min before gastric infusions, an intraperitoneal injection of saline (1 mL/kg) or drug was given. The pH/manometric assembly was then introduced, and the ferret was placed into the recording chamber and allowed to adapt to study conditions for 10 min before data acquisition commenced. A gastric load (25 mL of 10% D-glucose, pH 3.5) was then administered after a 30-min baseline period. This was immediately followed by 2 mL/min air for 10 min, then a rest period of 10 min, and finally another similar infusion of air. Only AR-H061719 was evaluated in the ferret model.

Hypothermia after s.c. administration of GABA_B receptor agonists in mice

The measurement of changes in body temperature using thermosensitive chips has been previously described elsewhere (Quéva et al., 2003). The nadir temperature after drug treatment adjusted for any changes in vehicle-treated controls was used. For comparisons between compounds, ED_2 (the dose producing a 2°C drop in temperature) was calculated (see Supplemental Information). AZD3355 (doses in $\mu\text{mol/kg}$ s.c.: 500, n=7; 750, n=3; 1000, n=7 and

2000, n=7), AR-H061719 (doses in $\mu\text{mol/kg}$ s.c.: 0.75, n=4; 7.5, n=4; 15, n=4; 30, n=4; 100, n=4; 500, n=4; 1000, n=4; 2000, n=4 and 5000, n=4) and baclofen (doses in $\mu\text{mol/kg}$ s.c.: 14.6, n=5; 28, n=2; 29.3, n=11; 32, n=14; 44, n=7; 58.8, n=12 and 117, n=5) were tested in this model. The GABA_B receptor antagonist AR-H040551 or vehicle was injected 20 min before administration of AZD3355 or vehicle. AR-H040551 is a 50/50 mixture of the centrally acting antagonist CGP62349 and its epimer 3-[(1S)-1-[[[(2S)-2-hydroxy-3-[hydroxy[(4-methoxyphenyl)methyl]phosphinyl]propyl]amino]ethyl]-benzoic acid.

Central nervous system autoradiography in rats

Autoradiographic studies in rats were performed using standard methods of sectioning and visualization (see Supplemental Information), with particular focus on distribution in the central nervous system (CNS).

Pharmacokinetic studies

For analysis of plasma concentrations of AZD3355, blood was drawn from a carotid cannula after i.v. or oral administration (7 $\mu\text{mol/kg}$) in female Sprague-Dawley rats. Separate animals were used in the i.v. and oral administration experiments. In dogs, blood samples for pharmacokinetic analysis were drawn in conjunction with the pharmacodynamic experiments outlined above. In both studies, the plasma concentration of AZD3355 was determined according to Fakt et al. (Fakt et al., 2003). Plasma protein binding was determined in rat and human plasma using ultrafiltration.

Statistical analysis

The hypothermia results were analyzed using unpaired Student's t-test. To avoid multiple comparisons, only nadir temperatures were analyzed. Data on vagal afferent firing were analyzed statistically by 2-way ANOVA of stimulus-response functions.

Results

Binding affinities and agonistic properties of GABA_B receptor agonists at native rat and dog and recombinant human GABA_B receptors

The binding affinities (K_i) and agonistic effects (EC_{50}) of the test compounds are summarized in Table 1. Overall, there was a good correlation between the affinity for the native rat GABA_B receptor and the agonistic potency of test compounds at the human recombinant GABA_{B(1a,2)} heterodimer. Similar to baclofen, both AZD3355 and AR-H061719 were full agonists at the GABA_B receptor.

The affinity (K_i) of AZD3355 for rat and dog GABA_B receptors was comparable, as measured by displacement of [³H]GABA binding in brain membranes: 5.1 ± 1.2 nM (Table 1) and 5.7 ± 0.2 nM (mean \pm S.E.M., $n=5$), respectively. In rat brain membranes, AZD3355 was almost 300 times more selective for GABA_B versus the GABA_A receptor (Table 1).

Effects of AZD3355 and GABA on human GABA_B receptor splice variants

The potency and maximal response between different splice variants were similar for AZD3355 and baclofen (Supplemental Table 1). The binding affinities (K_D) for the antagonist CGP54626 were similar for all splice variants (Supplemental Table 2), and comparable to the K_D obtained at native receptors (Kaupmann et al., 1997; Quéva et al., 2003). The receptor densities (B_{max}) at the cell surface, measured in the same cells as used for functional measurements, did not differ between the 1a, 1b, 1e, 1g, and 1m variants but were somewhat lower for the 1o isoform.

Uptake of GABA_B agonists in rat cerebrocortical slices

GABA was efficiently taken up by brain slices, while accumulation of baclofen was considerably lower and uptake of AZD3355 was intermediary (Table 2). The differences were evident not only with respect to maximal uptake but also for the rate of uptake.

Binding of AZD3355 and baclofen to native GATs in rat cerebrocortical membranes

AZD3355 inhibited GABA binding with a mean IC₅₀ of 0.67 mM ($n_H=0.8$), but baclofen was unable to displace GABA (Figure 1). Mean IC₅₀ for the positive control nipecotic acid was 6.4 μM ($n_H=0.6$).

Effects of GABA, AZD3355, and baclofen on [³H]GABA uptake and membrane potential in tsA201 cells expressing the human GATs

AZD3355 displayed pronounced competition of [³H]GABA transport at all four human GATs (Table 3). At hBGT-1 the IC₅₀ value of AZD3355 was found to be 3–4 times higher than for GABA. The effect of AZD3355 at the three remaining subtypes was too weak to establish an IC₅₀ value, but approximately 50% inhibition of [³H]GABA transport was observed at the maximal tested concentration (3 mM). In contrast, baclofen displayed only minor competition of [³H]GABA transport at all four GATs.

The FLIPR[®] membrane potential assay was used to establish that AZD3355 is actively transported by the human GATs. Concentration-dependent cell depolarization was observed for all four subtypes and again, the effect of AZD3355 was most distinct at the hBGT-1 subtype (Table 3). Baclofen was ineffective in depolarizing cells expressing either of the human GATs.

Neither AZD3355 nor baclofen at concentrations up to 3 mM had any effect on the human glutamate transporter EAAT3 in the two cellular assays (data not shown).

Effects of AR-H061719 on firing of ferret gastric vagal mechanoreceptors *in vitro*

Two populations of vagal afferent endings exist in the ferret stomach. Mucosal receptors responded to tactile stimulation of the epithelium, whose responses were unaffected by AR-H061719 at any concentration tested (Figure 2a). However, receptors in the smooth muscle responded to distension proportional to the load applied. AR-H061719 reduced stimulus-response function of tension receptors in a concentration-dependent manner (Figure 2b). The effect of the compound was observed at all tensions applied. Basal firing rate of tension receptors was also suppressed by AR-H061719. Time control experiments were performed with the vehicle over the same time course to the drug experiments, and no changes in afferent sensitivity were seen with $n \geq 5$ experiments at any time point.

Effect of AZD3355, baclofen and GABA on TLESR in dogs

Despite the very potent effect of AZD3355 on GABA_B receptors, the dose-response curve for this compound differed markedly from that of baclofen (Figure 3a). Even at high doses of AZD3355 (up to 100 $\mu\text{mol/kg}$ i.g.), only partial inhibition was observed, and there was only a small increase in effect in the dose range 3–100 $\mu\text{mol/kg}$. However, at 300 $\mu\text{mol/kg}$ of AZD3355, close to full inhibition of TLESR was achieved. This dose did not produce any visible side-effects, but pilot experiments showed that at 500 $\mu\text{mol/kg}$, AZD3355 induced typical GABA_B-related side-effects such as sedation and muscle relaxation. An i.v. dose of 7 $\mu\text{mol/kg}$ of AZD3355 induced a similar level of inhibition as the same dose

given i.g. (Figure 3a), consistent with the oral bioavailability of 88%. The maximal level of inhibition achieved during i.v. infusion of GABA was approximately 50% (Figure 3a). In contrast, baclofen afforded almost 90% inhibition at 7 $\mu\text{mol/kg}$ (Figure 3a). This is very close to the dose provoking typical GABA_B side-effects in dogs (10 $\mu\text{mol/kg}$, unpublished observations). The slope of the dose-response curve for baclofen was markedly steeper than that of AZD3355 and GABA. There were no effects of any compound on basal LES pressure (data not shown).

Effect of AR-H061719 on TLESR in ferrets and dogs

The profile of the dose-response curve for AR-H061719 in the ferret was somewhat different from that in the dog, in that the compound slightly reduced the incidence of TLESRs up to approximately 30% at the second highest dose, 250 $\mu\text{mol/kg}$ (Figure 3b). However, the interindividual variation was marked. At 500 $\mu\text{mol/kg}$, TLESRs were abolished in all ferrets. At this dose, there was some retching as seen in other studies of GABA_B agonists (Blackshaw et al., 1999; Staunton et al., 2000), but no other typical GABA_B-related side-effects were noted. The highest dose of AR-H061719 evaluated in the dog was 35 $\mu\text{mol/kg}$, and maximal inhibition in the present dose-range was seen already at 3 $\mu\text{mol/kg}$ (Figure 3b). Basal LES pressure was unaffected by AR-H061719 in both species (data not shown).

Hypothermia after s.c. administration of GABA_B receptor agonists in mice

*ED*₂ values were calculated from the dose-response experiments, and there was a remarkable difference between AZD3355 and AR-H061719 on the one hand, and

baclofen on the other. Despite the fact that the two former compounds were much more potent on the GABA_B receptor, baclofen was more potent in inducing hypothermia (Figure 4a).

To ascertain that the hypothermic effects of AZD3355 (1 mmol/kg) are mediated by the GABA_B receptor, the selective GABA_B receptor antagonist AR-H040551 (2 mg/kg) was administered 20 min before injection of AZD3355. AR-H040551 did not have any effects on body temperature in its own right, but it completely prevented hypothermia induced by AZD3355 (Figure 4b).

AZD3355 at 1 mmol/kg produced a marked hypothermic effect in GABA_B^{+/+} and GABA_B^{+/-} mice. However, there was no effect on body temperature in GABA_B^{-/-} mice (Figure 4c).

CNS autoradiography in rats

Both baclofen and AZD3355 were found at low levels in the CNS (Figure 5), and baclofen was distributed evenly. In contrast, while AZD3355 was evenly distributed in areas within the blood-brain barrier, high levels were observed in the circumventricular organs such as the area postrema and median eminence. The pineal gland was also strongly labeled after administration of AZD3355 but not after dosing with baclofen. The high levels in circumventricular organs were not restricted to those areas; a concentration gradient into the adjacent regions was also apparent.

Pharmacokinetic studies

The pharmacokinetics of AZD3355 were characterized by high oral availability (88% in the dog and 100% in the rat) and relatively low systemic clearance (Supplemental Table 3 and Supplemental Figure 3). The plasma levels in dogs and rats were dose-linear (not shown).

Plasma protein binding was <1% in rat and human plasma.

Discussion

The troublesome symptoms of GERD often persist despite acid-suppressive therapy (Fass et al., 2005). Evidence from animal and human studies (considerations regarding extrapolation of results across species are in Supplemental Information) indicates that TLESR inhibition by GABA_B receptor agonists could address this unmet need, although the search for new GABA_B receptor agonists with a benign side-effect profile has proved challenging. AZD3355 is one of the most potent GABA_B agonists described, and its CNS side-effect liability is minimized by the compound's unusual disposition. The marked differences between baclofen and AZD3355 *in vivo* can be explained by GAT-mediated transport of AZD3355, but not baclofen. Sequestration of AZD3355 by CNS neurons/glia cells prevents stimulation of central GABA_B receptors, while concentrations sufficient to activate GABA_B receptors can be maintained peripherally. The basis for this conclusion is discussed below.

Selectivity for the GABA_B receptor and GABA_{B(1)} splice variants

Binding studies showed that AZD3355 had a higher selectivity for GABA_B than GABA_A receptors. Accordingly, AZD3355 was completely selective for GABA_B receptors at doses administered *in vivo*, as shown by a lack of effect of AZD3355 on body temperature in GABA_{B(1)}^{-/-} mice, in whom GABA_A receptor stimulation induces an exaggerated hypothermic response (Quéva et al., 2003). Additionally, the GABA_B receptor antagonist AR-H040551 abolished the effects of AZD3355 in wild-type mice. Our data therefore show that the hypothermic action of AZD3355 is not an off-target effect.

Similar to other GABA_B receptor agonists, AZD3355 stimulated different human recombinant GABA_{B(1)} splice variants with no conspicuous differences in potency and efficacy. Thus, the

unique profile of AZD3355 *in vivo* is unrelated to selectivity for any splice variants. This also demonstrates that the potency of AZD3355 on splice variants is virtually identical to the two predominant variants, GABA_{B(1a)} and GABA_{B(1b)}. Moreover, the GABA_B receptor antagonist [³H]CGP54626 bound with similar affinity to all receptor isoforms, reflecting the pharmacological similarities between the splice variants. Notably, AZD3355 is a full agonist *in vitro*, so the *in vivo* properties do not reflect partial agonism.

Sequestration of AZD3355 by CNS neural cells

Both baclofen and AZD3355 are metabolically stable in rats (unpublished observations), so the distribution of radioactivity in autoradiographic experiments largely reflects that of the parent compounds. There were some differences in the distribution of AZD3355 and baclofen in peripheral tissues. However, the most striking difference was observed for concentrations in and around circumventricular organs. AZD3355 was present at high levels in the basomedial hypothalamus and the median eminence, as well as the dorsomedial brain stem within and in the vicinity of the area postrema. AZD3355, but not baclofen, also strongly labeled the pineal body, which has an avid GABA uptake system (Rosenstein et al., 1990). Interestingly, an earlier report described very similar differences between baclofen and GABA distribution in the mouse brain (Kuroda et al., 2000). This supports the view that AZD3355 is more similar to GABA than baclofen is, and that it is taken up in circumventricular organs as a result of active uptake. The hypothesis that AZD3355 is sequestered by neural cells was investigated directly using rat brain cortical slices. While baclofen uptake was very modest, accumulation of AZD3355 was considerable, although not as high as that of GABA. The notion that uptake into brain slices was mediated by GATs was confirmed by the finding that AZD3355, but not baclofen, inhibited binding of [³H]GABA to GATs in rat brain membranes. Earlier data also suggested that baclofen

has only a low affinity for native GATs in rat cerebral cortex (Pallo et al., 2007), and for the recombinantly expressed rat brain GABA transporter GAT-1 (Corey et al., 1994). Importantly, since we hypothesized that AZD3355 affinity for GAT explains its favorable side-effect profile in laboratory animals, it is critical to demonstrate that this mechanism is valid for human GATs. This was confirmed in recombinantly expressed human GATs, and AZD3355 was transported by all four human GATs with some preference for hBGT-1. There were major differences in the concentrations used in the three different *in vitro* models used to study uptake or GAT interaction, with the rat brain membrane assay showing the lowest sensitivity. The observations that AZD3355 uptake could be demonstrated in brain slices at 0.1 μM and that the compound was only 3–4 times less active than GABA in recombinant human BGT-1 clearly support the notion that uptake mechanisms control extracellular levels *in vivo*. Moreover, the results strongly suggest that BGT-1, but not the other GATs, plays an important role in this regard. CGP27492 (the desfluoro analog of AZD3355) is an unexpectedly weak agonist in brain slices compared with baclofen when actual potency at the receptor level is considered (Ong and Kerr, 1998). It has been proposed that this is due to cellular uptake in brain slices, since NO-711 (a selective inhibitor of GAT-1) potentiates the electrophysiological effects of CGP27492 but not baclofen (Ong and Kerr, 1998). Our data support this notion and provide the first direct evidence for active uptake of 3-aminopropylphosphinic acid derivatives.

The mechanism of action AZD3355 on TLESR

Baclofen produced full inhibition of TLESR with a monophasic dose-response curve [see also (Lehmann et al., 1999) and (Blackshaw et al., 1999)]. In contrast, the AZD3355 dose-response curve in dogs was biphasic, with 50% inhibition achieved at 3–7 $\mu\text{mol/kg}$ without much further

increase up to 100 $\mu\text{mol/kg}$. At 300 $\mu\text{mol/kg}$, there was almost complete inhibition. A similar dose-response relationship was observed for the racemate (AR-H061719) in ferrets. At the doses tested, both GABA and AR-H061719 afforded approximately 50% inhibition of TLESR in dogs. Abolition of TLESR with baclofen was seen at 7 $\mu\text{mol/kg}$ in dogs, but at 10 $\mu\text{mol/kg}$ visible side effects occurred (sedation, vomiting, and hypothermia; unpublished observations). AZD3355 500 $\mu\text{mol/kg}$ led to an identical side-effect profile (results not shown), which suggests that GABA_B receptor agonists can abolish TLESR only at doses affecting the CNS.

As previously reported for baclofen and CGP27492 (Page and Blackshaw, 1999; Smid et al., 2001), AR-H061719 inhibited tension-induced firing of ferret gastric vagal mechanoreceptors *in vitro*. This is consistent with the dense expression of GABA_{B(1)} in retrogradely labeled gastric neurons of the ferret nodose ganglion (Smid et al., 2001). We also found that GABA_{B(2)}, which is required for functional GABA_B receptors, is expressed in the nodose ganglion (unpublished observations). Notably, the potency of GABA_B receptor agonists on ferret vagal afferents was considerably lower than in most other functional assays *in vitro*. Whether this is due to differences in signal transduction efficiency, disproportionate expression of GABA_{B(1)} versus GABA_{B(2)}, low receptor density, or poor tissue penetration remains unknown.

Given these considerations, the dissimilarities in TLESR dose-response curves for AZD3355, AR-H061719, and GABA on one hand, and baclofen on the other, may be explained as follows. The former group of compounds inhibit TLESR at moderate doses by acting exclusively on GABA_B receptors on peripheral gastric vagal mechanoreceptors. The comparatively low potency of GABA_B receptor agonists in vagal afferents, as reported in the present study and elsewhere (Smid et al., 2001), is compatible with the moderate effect on TLESR even if plasma concentrations (micromolar levels) would be sufficient to fully activate GABA_B receptors in

recombinant systems (nanomolar levels). At doses of AZD3355 close to those producing central side effects, complete TLESR inhibition occurs due to GAT saturation followed by elevation of brain interstitial AZD3355 concentrations. In addition, due to competition for the GATs, extracellular GABA concentrations may increase to levels at which GABA_B receptors are activated. As a consequence of central GABA_B receptor stimulation, possibly those expressed on central terminations of vagal afferents, excitatory transmitter release would be inhibited and the vagovagal pathway underlying TLESR dampened. The proposal is also consistent with the finding that, in ferrets, baclofen inhibits gastric vagal tension-sensitive afferents and their central connections with vagal efferents, and that the latter are more sensitive (Partosoedarso et al., 2001). This clearly suggests that a central effect is required to achieve maximal inhibition of the TLESR efferent response. Notably, pharmacokinetic differences between baclofen and AZD3355 cannot explain the pharmacodynamic disparities. For instance, there was a linear relationship between dose and exposure, while AZD3355 plasma protein binding was negligible.

Alternatively, AZD3355 could inhibit TLESR through an effect on presynaptic GABA_B receptors on vagal motoneurons. Baclofen and CGP27492 reduce relaxation of ferret LES *in vitro* after electrical vagal stimulation (Smid and Blackshaw, 2000), supporting this hypothesis. However, since swallow-induced LES relaxation is thought to share the same motor pathway as TLESR, swallow-induced LES relaxation would also be expected to decrease. Since such an effect has never been seen after administration of any GABA_B receptor agonist, presynaptic inhibition is unlikely to play any significant role in the mechanism of action of AZD3355.

In summary, we have shown that the GABA_B receptor agonist AZD3355 inhibits TLESR in dogs by a peripheral mechanism different from that of baclofen (which mainly acts centrally).

AZD3355 therefore represents a promising candidate for the treatment of GERD, and recent

clinical data confirm this proposal (Boeckxstaens et al., 2009a; Boeckxstaens et al., 2009b; Boeckxstaens et al., 2009c).

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Footnotes

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Legends for Figures

Figure 1. Displacement of GABA from the GAT in rat cortical membranes by AZD3355 and baclofen. IC_{50} for AZD3355 was 0.67 mM but no IC_{50} could be calculated for baclofen. Each concentration was tested in duplicate and the means are presented.

Figure 2. Effects of AR-H061719 on firing of ferret mucosal (a) and muscular (b) mechanosensitive gastroesophageal vagal afferents. Results are mean+S.E.M.; n=3 (control) and n=4 (AR-H061719).

Figure 3. Effects of AZD3355, baclofen, and GABA on TLESR in the dog (a), and effects of AR-H061719 on TLESR in the dog and ferret (b). In (a), the compounds were administered intragastrically (AZD3355 and baclofen) or as a continuous i.v. infusion (GABA; the dose on the x axis represents the dose given over the duration of the experiment). In (b), AR-H061719 was given intragastrically in dogs and intraperitoneally in ferrets. Results are means; bars represent S.E.M., n=3–6 for each dose (dog) and n=6 (ferret).

Figure 4. The relationship between *in vitro* potency on recombinant human $GABA_B$ receptors and hypothermia in the mouse hypothermia model (a); ED_2 was calculated as the dose producing lowering of body temperature by 2°C. Panels (b) and (c) show the effects of AZD3355 on mouse core body temperature in $GABA_B$ null mutants, and inhibitory effects of AR-H040551 on the hypothermic action of AZD3355, respectively. Results are mean+S.E.M.; n=3–4 (a) and n=5–12 (b). **p < 0.01; ***p < 0.001 (unpaired Student's t-test, only nadir temperatures were analyzed and compared to vehicle).

Figure 5. Autoradiograms for radiolabeled AZD3355 (a) and baclofen (b) in the rat. Arrows in (a) denote, from left to right, ventricular space, the hypothalamus, the pineal body and the area postrema.

Table 1: Binding affinity (K_i) of AZD3355, AR-H061719, baclofen, and GABA for rat brain GABA receptors and agonist activity (EC_{50}) at human recombinant GABA_B receptors

	GABA_B affinity K_i (nM)	GABA_A affinity K_i (μM)	GABA_B/GABA_A selectivity	GABA_B agonism EC₅₀ (nM)	GABA_B agonism Intrinsic Activity
AZD3355	5.1 ± 1.2 (10)	1.4 ± 0.3 (3)	274	8.6 ± 0.77 (4)	1.0 ± 0.1 (4)
AR-H061719	10 ± 1.9 (12)	4.3 ± 0.7 (3)	430	15 ± 1.5 (5)	1.1 ± 0.1 (5)
Baclofen	220 ± 50 (6)	n.d.	n.d.	750 ± 150 (6)	1.0 ± 0.02 (6)
GABA	110 ± 21 (16)	0.097 ± 0.001 (3)	0.9	160 ± 10 (94)	1

Data are the mean ± S.E.M. of (n) experiments. n.d., not determined.

Table 2: Intracellular uptake ($\mu\text{mol/kg}$ tissue) of radiolabeled AZD3355, baclofen, and GABA in rat cerebrocortical slices

Concentration (μM)	5 min	30 min	60 min
AZD3355			
0.1	0.18 ± 0.01	1.40 ± 0.03	2.23 ± 0.03
1.0	2.01 ± 0.10	11.07 ± 0.28	19.38 ± 0.45
10.0	21.26 ± 1.13	123.03 ± 9.13	195.66 ± 4.89
Baclofen			
0.1	0.11 ± 0.00	0.18 ± 0.01	0.20 ± 0.01
1.0	0.99 ± 0.03	1.77 ± 0.01	1.64 ± 0.10
10.0	10.06 ± 0.17 (7)	16.73 ± 0.70 (12)	16.62 ± 0.58
GABA			
0.1	1.54 ± 0.14 (7)	5.69 ± 0.29	6.66 ± 0.62
1.0	15.37 ± 1.07	52.11 ± 4.60	68.84 ± 4.35
10.0	128.42 ± 8.99 (11)	471.59 ± 16.65 (12)	631.36 ± 31.45 (12)

Data are the mean \pm S.E.M. of 8 experiments, unless otherwise stated in parentheses.

Table 3: Effects of GABA, AZD3355, and baclofen on [³H]GABA uptake and membrane potential in tsA201 cells transiently expressing the four human GATs

Compound	³ H]GABA uptake assay				FLIPR [®] membrane potential assay			
	IC ₅₀ (pIC ₅₀ ± S.E.M.) (μM)				EC ₅₀ (pEC ₅₀ ± S.E.M.) (μM)			
	hGAT-1	hBGT-1	hGAT-2	hGAT-3	hGAT-1	hBGT-1	hGAT-2	hGAT-3
GABA	24 (4.6 ± 0.02)	30 (4.5 ± 0.07)	15 (4.8 ± 0.02)	12 (5.0 ± 0.1)	11 (4.9 ± 0.07)	31 (4.5 ± 0.05)	6.6 (5.2 ± 0.08)	10 (5.0 ± 0.05)
AZD3355	>1000 ^a	110 (4.0 ± 0.06)	>1000 ^a	>1000 ^a	440 (3.4 ± 0.06) ^b	110 (4.0 ± 0.01)	600 (3.2 ± 0.07) ^b	770 (3.1 ± 0.07) ^b
Baclofen	>3000 ^a	>3000 ^a	>3000 ^a	>3000 ^a	>3000	>3000	>3000	>3000

^aThe compounds displayed less than 75% inhibition of the indicated GABA transporters at the maximal tested concentration and it was therefore not possible to generate concentration-inhibition curves. These compounds exhibited the following maximal inhibition at 3 mM (mean ± S.E.M.): AZD3355 at hGAT-1, 54 ± 1.1%; AZD3355 at hGAT-2, 46 ± 1.2%; AZD3355 at hGAT-3, 46 ± 5.3%; Baclofen at hGAT-1, 4.9 ± 1.2%; Baclofen at hBGT-1, 11 ± 1.1%; Baclofen at hGAT-2, 10 ± 2.8%; Baclofen at hGAT-3, 5.1 ± 3.0%.

^bIt was not possible to generate fully completed concentration-response curves and the curves were therefore fitted to the maximal response of GABA (3 mM).

Figure 1

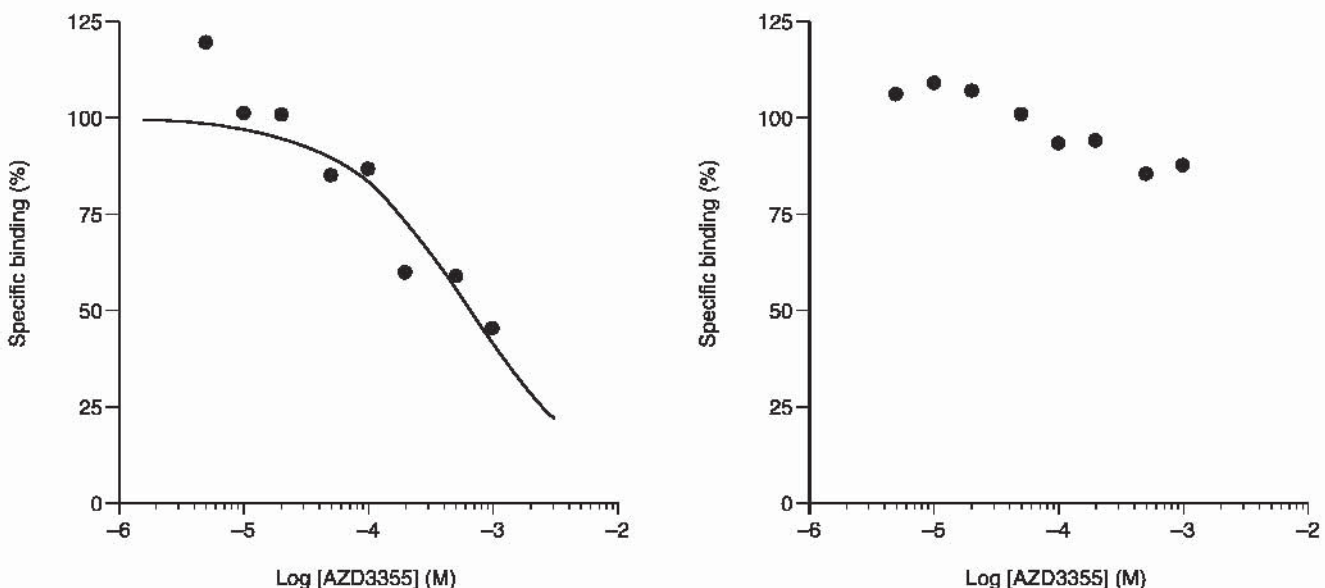


Figure 2

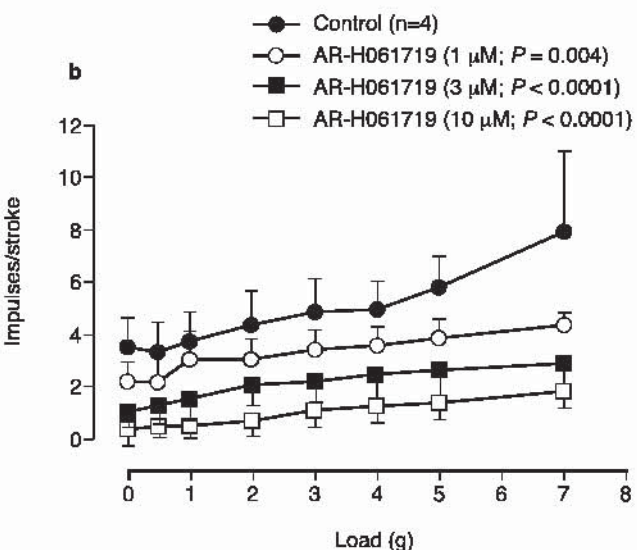
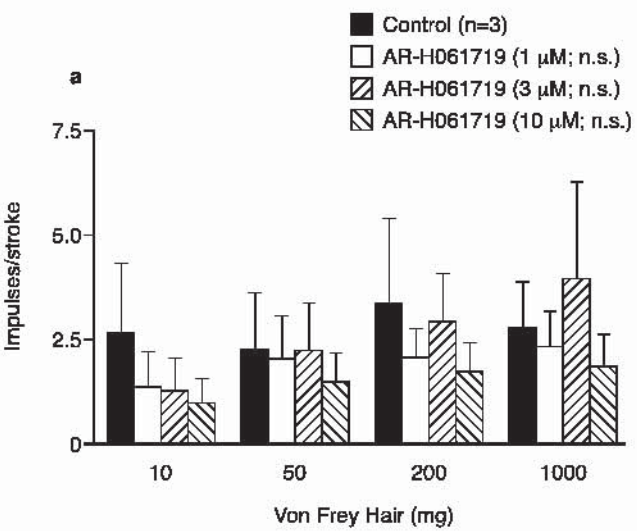


Figure 3

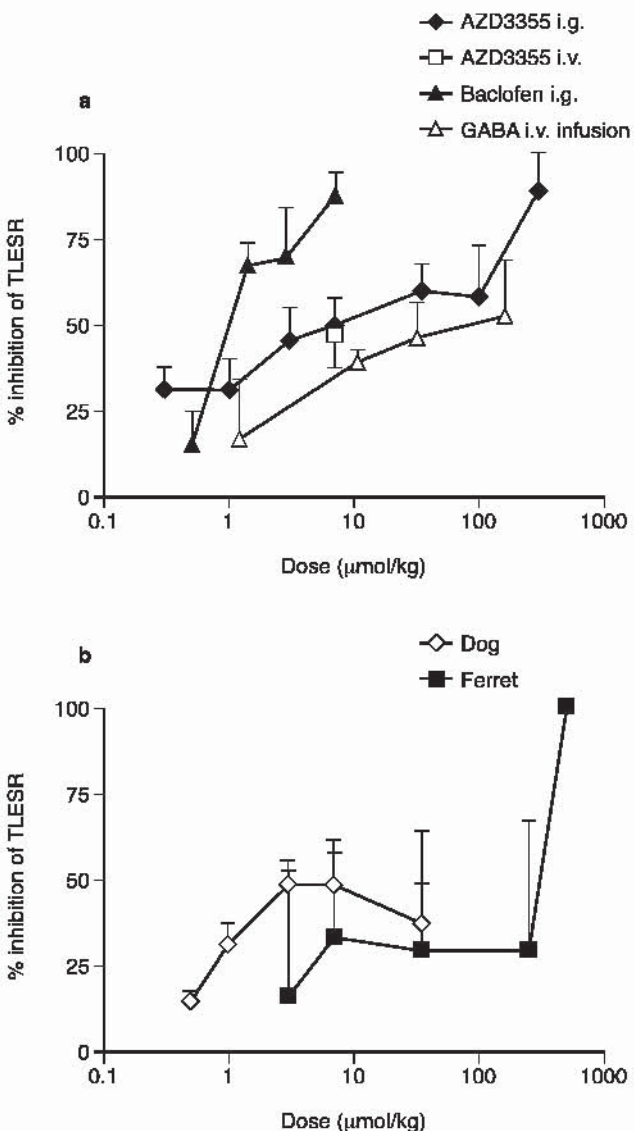


Figure 4

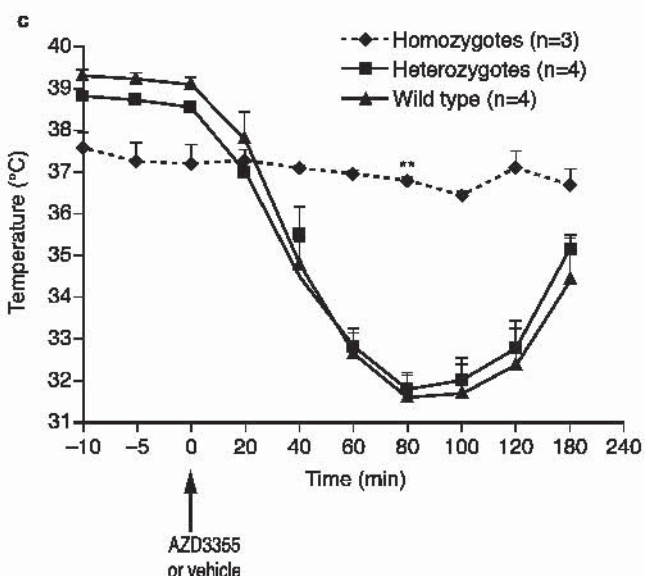
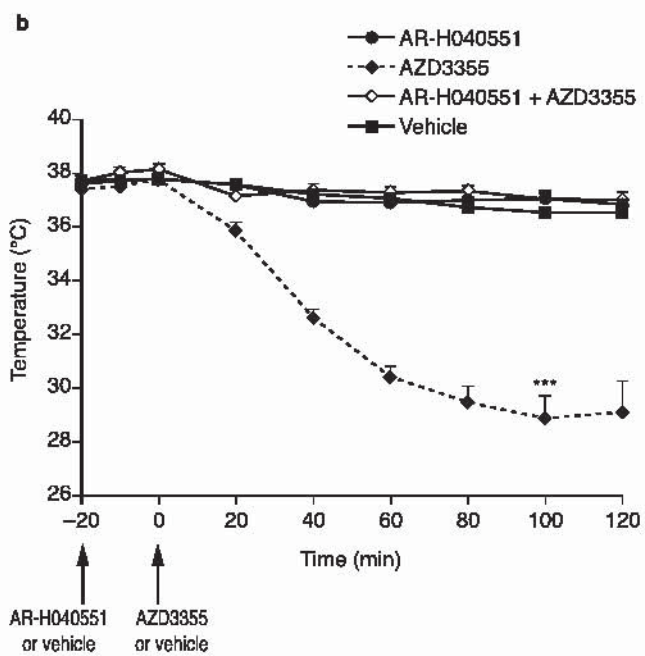
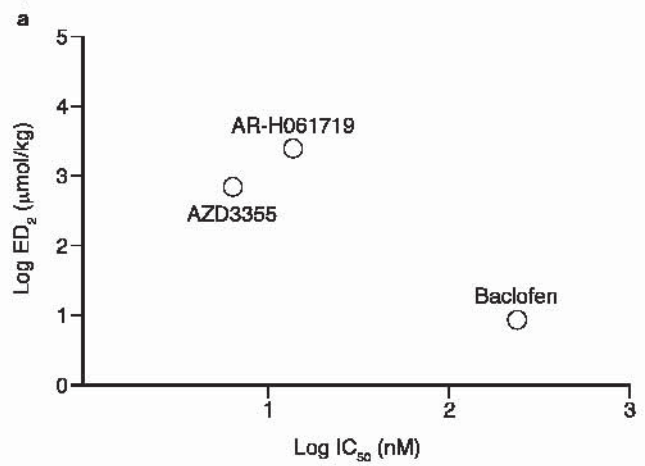
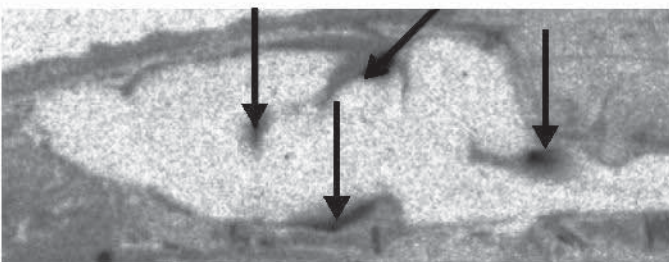
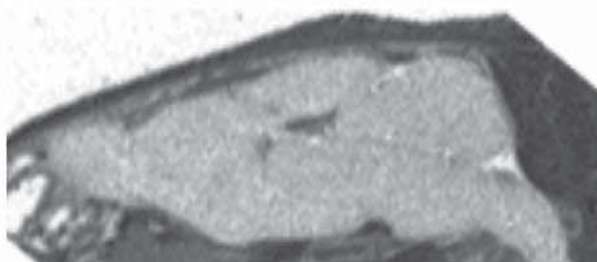


Figure 5



AZD3355



Baclofen