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Effects of (2*R*)-(3-amino-2-fluoropropyl)sulphinic acid (AFPSiA) on transient lower oesophageal sphincter relaxation in dogs and mechanism of hypothermic effects in mice

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1 The effects of the novel GABA analogue (2*R*)-(3-amino-2-fluoropropyl)sulphinic acid (AFPSiA) on transient lower oesophageal sphincter relaxations (TLOSRs) were studied in the dog. In addition, the $GABA_A/GABA_B$ selectivity was determined *in vitro* and *in vivo*, and the pharmacokinetics and the metabolism of the compound were studied in the dog and rat.

2 TLOSRs were reduced by $55\pm8\%$ after intragastric administration of AFPSiA at $14\,\mu mol\,kg^{-1}$ and did not decrease further at higher doses. When evaluated 2 and 4 h after administration, the effect declined to 37 ± 6 and $16\pm9\%$, respectively. Spontaneous swallowing was only significantly inhibited at $100\,\mu mol\,kg^{-1}$.

3 The oral availability of AFPSiA was 52 ± 17 and $71\pm4\%$ in the dog and rat, respectively. A fraction of AFPSiA was oxidised to the corresponding sulphonate, (2*R*)-(3-amino-2-fluoropropyl)-sulphonic acid (AFPSoA) after oral administration to the rat and dog.

4 In rat brain membranes, AFPSiA was found to have ten times higher affinity for rat brain $GABA_B$ ($K_i=47\pm4.4$ nM) compared to $GABA_A$ ($K_i=430\pm46$ nM) binding sites. The compound was a full agonist at human recombinant $GABA_{B(1a,2)}$ receptors ($EC_{50}=130\pm10$ nM). In contrast, the metabolite AFPSoA was considerably more selective for binding to rat brain $GABA_A$ ($K_i=37\pm3.1$ nM) vs $GABA_B$ ($K_i=6800\pm280$ nM) receptors.

5 In the mouse, high doses (1–8 $mmol\,kg^{-1}$) of AFPSiA induced a rapid and mild hypothermia followed by a profound and sustained hypothermia at the higher doses tested (6 and 8 $mmol\,kg^{-1}$). This effect was unaffected by the selective $GABA_B$ receptor antagonist CGP62349. AFPSoA (1 and 2 $mmol\,kg^{-1}$) produced transient and moderate hypothermia while the hypothermic response was considerably larger at 4 $mmol\,kg^{-1}$.

6 It is concluded that AFPSiA inhibits but does not abolish TLOSRs in the dog. High doses of the compound induce hypothermia in the mouse, which probably is attributable to activation of the $GABA_A$ receptor. The latter effect may be caused both by AFPSiA and its oxidised sulphonic acid metabolite AFPSoA.

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Abbreviations: AFPSiA, (2*R*)-(3-amino-2-fluoropropyl)sulphinic acid; AFPSoA, (2*R*)-(3-amino-2-fluoropropyl)sulphonic acid; EDTA, ethylenediaminetetraacetic acid; FLIPR, fluorescence imaging plate reader; GORD, gastro-oesophageal reflux disease; HBSS, Hank's buffered salt solution; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonicacid); PBS, phosphate-buffered saline; TLOSR, transient lower oesophageal sphincter relaxation

Introduction

Gastro-oesophageal reflux disease (GORD) is a highly prevalent condition and a major burden to the society as well as the afflicted individual. GORD is nowadays in most cases successfully treated with proton pump inhibitors, but a number of patients do not receive full symptomatic allieviation (Klinkenberg-Knol & Meuwissen 1990; Vakil, 2004). In many of these patients, reflux of bile and other nonacid contents from the stomach is believed to generate symptoms (Koek *et al*., 2003). Since transient lower oesophageal sphincter relaxation (TLOSR) is the dominant cause of reflux, interventions aimed at inhibiting TLOSRs may have a therapeutic role to play in GORD (Mittal *et al*., 1995; Hirsch *et al*., 2002), particularly when acid secretion suppression alone is not sufficiently successful.

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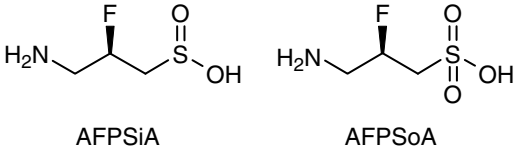

Figure 1 Structure of AFPSiA and AFPSoA.

The pharmacology of TLOSR has evolved rapidly during the past few years. Among compounds inhibiting TLOSRs, $GABA_B$ receptor agonists are the most promising. The skeletal muscle relaxant baclofen, the only $GABA_B$ agonist approved for clinical use, reduces TLOSRs in dogs (Lehmann *et al*., 1999), ferrets (Blackshaw *et al*., 1999), cats (Liu *et al*., 2002) and humans (Lidums *et al*., 2000; Zhang *et al*., 2002). Importantly, data have emerged to show that baclofen also provides reduction in reflux of both acid and nonacid gastric contents and symptomatic relief in GORD patients (Cange *et al*., 2002; Ciccaglione & Marzio, 2003; Koek *et al*., 2003; Vela *et al*., 2003).

While being the preferred pharmacological option in the treatment of spasticity, baclofen may be less useful in GORD due to its central side effects. The need for novel $GABA_B$ agonists with an improved safety and tolerability profile is therefore obvious. GABA analogues in which a sulphinic acid group replaces the carboxylic acid group of GABA have not previously been evaluated to any great extent. The sulphinic acid analogue of baclofen ('siclofen', Carruthers *et al*., 1995) and some other 3-aminopropyl sulphinic acids with a large lipophilic group in the 2-position have properties very similar to baclofen with central side effects appearing at doses close to those inhibiting TLOSRs (unpublished own findings). We have synthesised 3-aminopropyl sulphinates with small substituents in the 2-position (for instance hydroxy and keto groups). Based on the potency of the compounds to activate recombinant human $GABA_B$ receptors, we selected the most potent for further *in vivo* studies. This compound, (2*R*)-(3-amino-2-fluoropropyl)sulphinic acid (AFPSiA; Figure 1), was evaluated with respect to its effect on TLOSRs in the dog and $GABA_A$/$GABA_B$ selectivity *in vitro*. Further, the pharmacokinetics of AFPSiA were determined in dogs and rats. Moreover, the side effect profile of AFPSiA was assessed *in vivo* in mice, and the relation between side effects and formation of a major metabolite [(2*R*)-(3-amino-2-fluoropropyl)sulphonic acid, AFPSoA] of the compound was investigated.

Methods

Synthesis of AFPSiA and AFPSoA

AFPSiA was synthesised from the *tert*-butyl [(2*R*)-2-fluoro-3-hydroxypropyl]carbamate. This hydroxy intermediate was transformed into mesylate by the reaction with methanesulphonyl chloride and this mesylate was then reacted with *tert*-butyl thiol to form the corresponding sulphide. Oxidation of the sulphide by 3-chloroperoxybenzoic acid gave the sulphone, which was reacted with trifluoromethanesulphonic acid to yield AFPSiA by the simultaneous deprotection of the amino group and the unmasking of the sulphinic acid moiety.

AFPSoA was synthesised from the iodide intermediate, *tert*-butyl [(2*R*)-2-fluoro-3-iodopropyl]carbamate. The iodide was transformed into thioacetate through the reaction with potassium thioacetate, and the subsequent reaction with trifluoroacetic acid and hydrogen peroxide then gave AFPSoA.

Determination of binding affinities of AFPSiA and AFPSoA at $GABA_A$ and $GABA_B$ receptor sites in rat brain membranes

Preparation of rat brain synaptic vesicles was performed as described (Jensen *et al*., 2002). Inhibition of [^{3}H]GABA binding at $GABA_B$ receptor sites in rat brain synaptic membranes by test compounds was measured using a filtration binding assay as described (Jensen *et al*., 2002). Displacement curves to determine IC_{50} values were constructed by fitting the 4-parameter logistic equation to the data using XLfit for Microsoft Excel. K_D for GABA was determined on each preparation by homologous competition and used to calculate K_i values from IC_{50} determinations on that particular preparation using the Cheng–Prusoff equation (Cheng & Prusoff, 1973). The average K_D for GABA was 110 ± 21 nM (mean $\pm$ s.e.m., $n = 16$ preparations).

The affinity of test compounds at $GABA_A$ receptor sites in rat brain membranes was measured using a [methylamine-^{3}H]-muscimol radioligand binding assay (Martini *et al*., 1983). This assay was performed as described above for the [^{3}H]GABA assay, with the exception that 10 nM [^{3}H]muscimol (274 GBq mmol^{-1}, Amersham Biosciences, Uppsala, Sweden) was used instead of GABA and that isoguvacin was excluded from the incubation buffer. The binding affinity (K_D) for muscimol at $GABA_A$ receptor sites in the rat brain synaptic membrane preparation was determined by saturation binding analysis to 14 ± 1.4 nM (mean $\pm$ s.e.m., $n = 3$) and used to calculate K_i values from IC_{50} determinations of test compounds using the Cheng–Prusoff equation.

Determination of effects of $GABA_B$ receptor agonists on $GABA_B$-receptor-mediated intracellular calcium release

The effect of compounds on $GABA_B$-receptor-mediated intracellular calcium release was determined in CHO-K1 cells stably coexpressing a $GABA_{B(1a)}$–$G_{\alpha qi5}$ fusion protein and $GABA_{B(2)}$ using a fluorescence imaging plate reader (FLIPR, Molecular Devices). $GABA_{B(1a)}$ and $GABA_{B(2)}$ were cloned from human brain cDNA and subcloned into pCI-Neo (Promega, Madison, WI, U.S.A.) and pcDNA3.1(−)/Zeo (Invitrogen, Basel, Switzerland), respectively. A $GABA_{B(1a)}$-$G_{\alpha qi5}$ fusion protein expression vector was constructed using the pCI-Neo-$GABA_{B(1a)}$ cDNA plasmid and pLEC1-$G_{\alpha qi5}$ (Molecular Devices, Menlo Park, CA, U.S.A.). In order to make the $G_{\alpha qi5}$ pertussis toxin insensitive, Cys356 was mutated to Gly using standard PCR methodology with primers 5′-GGATCCATGGCATGCTGCCTGAGCGA-3′ (forward) and 5′-GCGGCCGCTCAGAAGAGGCCGCCGTCCTT-3′ (reverse). The $G_{\alpha qi5mut}$ cDNA was ligated into the *Bam*HI and *Not*I sites of pcDNA3.0 (Invitrogen). The $GABA_{B(1a)}$ coding sequence was amplified by PCR from pCI-Neo- $GABA_{B(1a)}$ using primers, 5′-GGATCCCCGGGGAGCCGGGCCC-3′ (forward) and 5′-GGATCCCTTATAAAGCAAATGCACTCGA-3′ (reverse) and subcloned into the *Bam*HI site of pcDNA3.0-$G_{\alpha qi5mut}$.

For generation of a stable cell line expressing the $GABA_B$ receptor heterodimer, $GABA_{B(1a)}$–$G_{\alpha qi5mut}$ plasmid DNA (8 μg), $GABA_{B(2)}$ plasmid DNA (8 μg) and 24 μl lipofectamine (Life Technologies, Paisley, U.K.) were mixed in 5 ml OptiMEM (Life Technologies), and incubated for 45 min at room temperature. CHO-K1 cells were grown in culture medium containing Nut mix F-12 (Ham) with Glutamax-1 (Life Technologies) supplemented with 10% fetal bovine calf serum (Life Technologies) 100 U ml^{-1} penicillin (Life Technologies) and 100 $\mu g\,ml^{-1}$ streptomycin (Life Technologies) at 37°C in a humidified CO_2-incubator. The cells were detached with 1 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, St Louis, MO, U.S.A.) in phosphate-buffered saline (PBS) without calcium and magnesium (Life Technologies) and 1 million cells were seeded in 100 mm Petri dishes. After 24 h the culture medium was replaced with OptiMEM and incubated for 1 h in a CO_2 incubator. The cells were exposed to the transfection medium for 5 h, which was then replaced with culture medium. After 48 h, the cells were detached and seeded in six-well plates 2000 cells $well^{-1}$, and grown in culture medium supplemented with geneticin (400 $\mu g\,ml^{-1}$) and zeocin (250 $\mu g\,ml^{-1}$). After 4 days, cells from single colonies were collected and transferred to a 24-well plate. After 10 days, the cell clones were seeded in T-25 flasks and grown for another 16 days before they were tested for $GABA_B$-receptor-mediated functional response in the FLIPR assay described below. The clones that showed the highest peak response were collected and subcloned by seeding the cells in six-well plates 1000 cells $well^{-1}$ and repeating the steps described above. The clonal cell line that gave the highest peak response in the FLIPR was used in the present study.

Measurement of $GABA_B$ receptor dependent release of intracellular calcium in the FLIPR was performed as described by Coward *et al*. (1999), with some modifications. CHO cells transfected with $GABA_{B(1a)}$–$G_{\alpha qi5mut}$ and $GABA_{B(2)}$ were cultivated in culture medium with the addition of 250 $\mu g\,ml^{-1}$ zeocin and 400 $\mu g\,ml^{-1}$ geneticin. At 24 h prior to the experiment, the cells (35,000 cells $well^{-1}$) were seeded in black-walled poly-D-lysine-coated 96-well plates (Becton Dickinson, Bedford, U.K.) in culture medium without antibiotics. The cell culture medium was aspirated and 100 μl of Fluo-3 (Molecular Probes, Eugene, OR, U.S.A.) loading solution (4 μM Fluo-3, 2.5 mM probenecid [Sigma-Aldrich] and 20 mM HEPES (Life Technologies) in Nut Mix F-12 Ham) was added. After incubation for 1 h at 37°C in a 5% CO_2 incubator, the dye solution was aspirated and the cells were washed two times with 150 μl of wash solution (2.5 mM probenecid and 20 mM HEPES in Hank's buffered salt solution (HBSS, Life Technologies) followed by addition of 150 μl of wash solution. The cells were then assayed in the FLIPR. The test compounds were diluted in HBSS containing 20 mM HEPES and added to a volume of 50 μl wash solution (final volume in plate well = 200 μl). The fluorescence was sampled every second for 60 s (10 s before and 50 s after the addition of test compound) and then every sixth second for 120 s. The peak response values from 2 to 4 determinations at each concentration of test compound and experiment were calculated, after subtraction of basal fluorescence, as the percentage of the maximum response obtained with GABA run in the same experiment and used to construct concentration–response curves for EC_{50} determinations by fitting the 4-parameter logistic equation to the data using XLfit for Microsoft Excel. Intrinsic activities were calculated by dividing the peak response obtained by test compounds with the peak response obtained by GABA.

Effects on TLOSRs and spontaneous swallowing in the dog

The method used has been described previously (Stakeberg & Lehmann, 1999). In brief, adult Labrador retrievers of both sexes were equipped with an oesophagostomy. After recovery, they were intubated with a water-perfused multilumen Dentsleeve assembly to record pressure of the oesophagus, lower oesophageal sphincter and stomach. An antimony pH catheter was placed alongside the manometric assembly to measure reflux episodes. A thin air-perfused catheter was placed retrogradely in the hypopharynx to measure swallows. Only pharyngeal contractions followed by a peristaltic wave were included in the analysis. TLOSRs were stimulated by infusion of an acidified nutritious soup followed by insufflation of air. All dogs had been used for other studies previously, but there was always a wash-out period of at least 3 days between experiments in which drugs had been administered. The data were related to the average of the five preceding control experiments in each dog, and every fourth experiment was designated as a control. The database generated over a large number of years shows that there is no consistent change with time with regard to TLOSR incidence. AFPSiA (dissolved in 0.9% NaCl) was given intragastrically through the assembly at 2 ml kg^{-1} 30 min before infusion of soup. In effect duration studies, AFPSiA was administered at 14 $\mu mol\,kg^{-1}$ 2 and 4 h before infusion of soup.

AFPSoA was administered i.v. in two dogs at 13 $\mu mol\,kg^{-1}$. However, the compound proved to be emetogenic and no further experiments were performed.

Effects on behaviour and body temperature in the mouse

Measurement of body temperature was done as described by Quéva *et al*. (2003). In summary, temperature-sensitive chips were implanted subcutaneously in C57/Bl mice. Drugs were injected subcutaneously and temperature was measured at regular intervals with a transponder so as to produce minimal disturbance to the animals. The centrally active $GABA_B$ receptor antagonist CGP62349 (Froestl *et al*., 1996) was not a pure diastereomer, but a 50/50 mixture of CGP62349 and its epimer 3-[(1*S*)-1-[[(2*S*)-2-hydroxy-3-[hydroxy[(4-methoxyphenyl)methyl]phosphinyl]propyl]amino]ethyl]-benzoic acid, but for the sake of simplicity, this mixture was referred to as CGP62349. This compound was synthesised in-house.

Pharmacokinetics

For analysis of plasma concentrations of AFPSiA in dogs, blood was drawn from a foreleg vein after intravenous administration (7 $\mu mol\,kg^{-1}$) in separate experiments and after intragastric administration (7 and 14 $\mu mol\,kg^{-1}$) in the manometric experiments. Female Sprague–Dawley rats were dosed orally or intravenously at 7 $\mu mol\,kg^{-1}$ and blood samples were drawn from an arterial catheter.

The blood samples for analysis of both AFPSoA and AFPSiA were taken from dogs as described above (dose

56 μmol kg^{-1}, intragastrically) and from the orbital plexus of female Wistar rats after adminstration of AFPSiA (350 μmol kg^{-1}) by gavage.

For collection of urine, female Sprague–Dawley rats ($n=2$) were placed in metabolism cages immediately after intravenous administration at 14 μmol kg^{-1} and urine was collected quantitatively for 48 h.

AFPSiA, AFPSoA and an internal standard were isolated from plasma by precipitation of the plasma proteins, separated by liquid chromatography on a BioBasic AX column and measured by tandem mass spectrometry. Determination of AFPSiA in urine was performed after dilution of the urine with the mobile phase. The methods were similar to those reported by Fakt *et al*. (2003).

The pharmacokinetic parameters of AFPSiA were calculated by non-compartment analysis using WinNonlin Professional (Pharsight Corporation, CA, U.S.A.). The area under the plasma concentration–time curve ($AUC_{(0-t)}$) was calculated by a combination of the linear and logarithmic trapezoidal rule from the time of administration to the last sampling time or the sampling time with the last detectable plasma concentration. $AUC_{(0-t)}$ was extrapolated to infinity (AUC) by adding the residual area Ct/k, where Ct is the predicted plasma concentration at time t and k is the elimination rate constant. Ct and k were obtained by linear least-square regression analysis of the logarithm of the last 3–5 plasma concentrations *versus* time. The plasma clearance (CL) and volume of distribution at steady state (V_{ss}) were calculated after intravenous administration. The half-life in plasma was calculated as

$$t_{1/2} = \ln 2k^{-1}$$

After oral administration, the oral bioavailability (F) of AFPSiA was calculated as

$$F(\%) = (AUC_{oral}/AUC_{iv}) \times (Dose_{iv}/Dose_{oral}) \times 100$$

For dogs, the indiviual bioavailability was calculated using individual values for AUC_{iv}, whereas a mean value was used for rats.

All animal experiments were approved by the local Ethical Committee for Animal Experiments.

Statistics

Student's t-test was used to identify statistically significant differences between placebo- and drug-treated groups. In the hypothermia experiments, only nadir temperature was compared between placebo and drug groups.

Results

Binding affinities and agonistic properties of AFPSiA and AFPSoA at native and recombinant $GABA_B$ receptors

The binding affinities (K_i) and agonistic effects (EC_{50} and intrinsic activity) of the compounds are summarised in Table 1. In summary, AFPSiA was found to have a nine-fold selectivity for $GABA_B$ compared with $GABA_A$ receptors while AFPSoA was almost 200 times more selective for the latter. AFPSiA, but not AFPSoA, was a full agonist at $GABA_B$ receptors.

TLOSRs and swallows

The number of TLOSRs in placebo experiments was similar to what has been reported previously (Lehmann *et al*., 1999; Figure 2). AFPSiA reduced the frequency of TLOSRs by 58$\pm$22% at 100 μmol kg^{-1} (NS, Figure 2a). However, the maximal effect seemed to be achieved already at 14 μmol kg^{-1} which produced 55$\pm$8% inhibition. The inhibition was statistically significant even at 7 μmol kg^{-1}. In contrast, spontaneous swallowing was only significantly inhibited at 100 μmol kg^{-1} of AFPSiA and there was no trend for a dose dependency at lower doses (Figure 2b). Basal lower oesophageal sphincter pressure and duration of TLOSR were unaffected (data not shown), and the effect on acid reflux (defined as an oesophageal pH drop <4) was variable (not shown). The effect of AFPSiA declined with time, and amounted to 37$\pm$6 and 15$\pm$9%, 2 and 4 h after dosing, respectively (Figure 3). There were no visible side effects in any experiment after administration of AFPSiA to dogs.

Hypothermia in the mouse

Despite the potency of AFPSiA at the $GABA_B$ receptor, high doses of the compound were required to decrease body temperature in the mouse. At 1 and 2 mmol kg^{-1}, there were brief and minor effects, while 4 mmol kg^{-1} produced a mild but more sustained hypothermia (Figure 4a). In contrast, 6 and 8 mmol kg^{-1} of AFPSiA induced an initial moderate drop in body temperature which remained at a relatively stable level for about 100 min and then continued to decrease substantially for the remainder of the experiment.

The oxidised metabolite of AFPSiA, AFPSoA, induced a dose-dependent hypothermia (Figure 4b) and was more potent than the parent compound. At the highest dose tested (4 mmol kg^{-1}) the reduction in body temperature was so prominent that the mice had to be sacrificed 1 h after dosing on ethical grounds.

Table 1 Binding affinities and agonistic properties of AFPSiA and AFPSoA at $GABA_B$ and $GABA_A$ receptors

Compound	*$GABA_B$ affinity* K_i (nM)	*$GABA_A$ affinity* K_i (nM)	*$GABA_B$ agonism EC_{50}* (nM)	*$GABA_B$ intrinsic activity*
AFPSiA	47$\pm$4.4 (10)	430$\pm$46 (3)	130$\pm$10 (6)	1.1$\pm$0.04 (6)
AFPSoA	6800$\pm$280 (7)	37$\pm$3.1 (3)	28,000$\pm$1300 (7)	0.6$\pm$0.08 (3)

Binding affinities (K_i) and agonistic properties (EC_{50} and intrinsic activity) of AFPSiA and AFPSoA at the $GABA_B$ and $GABA_A$ receptors were measured as detailed in the 'Methods'. Data are means$\pm$s.e.m. of (n) experiments.

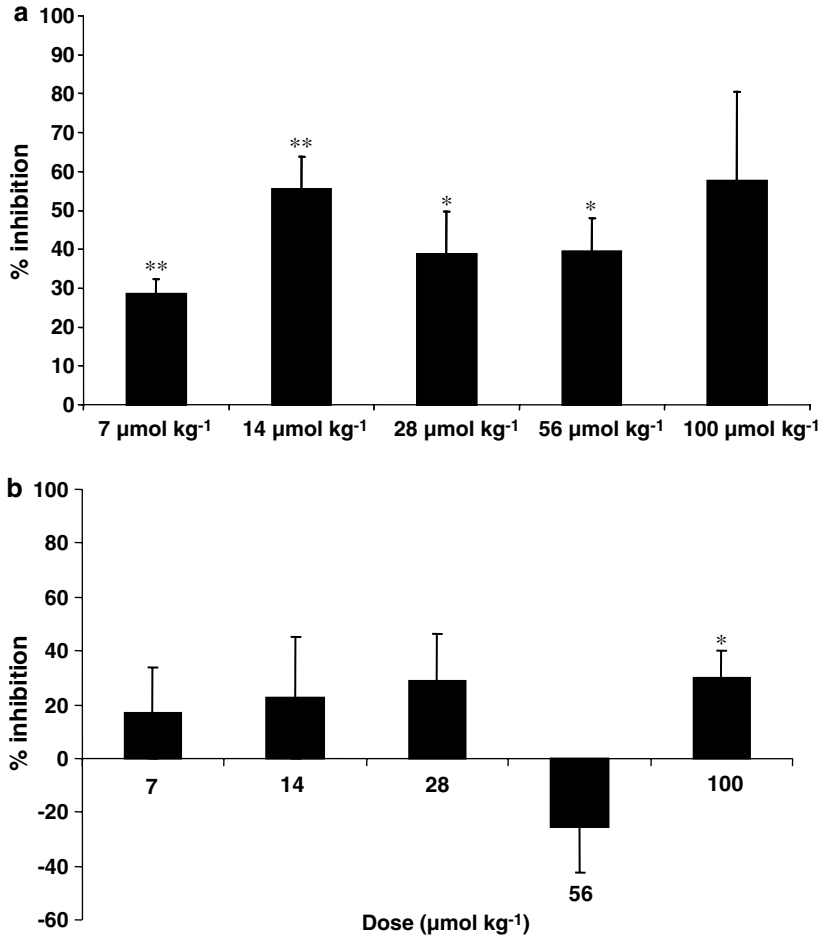

Figure 2 Dose–response curve for the effects of AFPSiA on TLOSRs (a) and spontaneous swallowing (b) in dogs. Note that the maximal inhibition of TLOSRs is about 60% and does not increase in the range of 14–100 μmol kg^{-1}. *$P<0.05$; **$P<0.01$, n (number of dogs) 3 (100 μmol kg^{-1}), 6 (14 μmol kg^{-1}) and 4 (the other groups). The control number (mean $\pm$ s.e.m.) of TLOSRs/45 min for 7, 14, 28, 56 and 100 μmol kg^{-1} was 5.9 $\pm$ 0.5, 5.9 $\pm$ 0.7, 5.5 $\pm$ 0.8, 5.3 $\pm$ 0.8 and 5.7 $\pm$ 0.5, respectively.

The $GABA_B$ receptor antagonist CGP62349 (4.9 μmol kg^{-1}) neither affected body temperature in its own right nor the pronounced reduction in body temperature seen after 6 mmol kg^{-1} of AFPSiA (Figure 5).

Pharmacokinetics

AFPSiA was rapidly absorbed from the gastrointestinal tract and the plasma peak appeared within an hour after administration in dogs and rats and declined with a half-life of 0.5 and 1.5 h, respectively (Table 2). The oral bioavailability was 52% in dogs and 71% in rats (Table 2). In rats, 40% of the administered dose of AFPSiA was recovered in urine after intravenous administration. The plasma concentration time profile for APFSiA and the sulphonate metabolite are presented in Figure 6a (dogs) and Figure 6b (rats administered 350 μmol kg^{-1}). There was a delayed increase in the concentration of AFPSoA, and the levels were below the limit of quantification in some of the early samples (Figure 6). However, the lower limit of quantification was at least ten times higher for AFPSoA than for the parent compound.

Discussion

The primary aim of the present work was to assess the effects of the GABA analogue AFPSiA on TLOSRs in dogs. The interest in this effect stems from the well-documented inhibitory actions on TLOSRs that baclofen and other $GABA_B$ agonists have been shown to produce. However, since all such compounds tested previously carry the burden of producing central side effects at low doses, a secondary aim was to study the side-effect profile of AFPSiA. For ethical reasons, this could not be done in dogs. Mice were chosen since hypothermia in our experience is more pronounced in mice than in rats, for instance, and smaller quantities of compound are required. In addition, the suspicion that the sulphinate group may be converted to sulphonate *in vivo* motivated an investigation into the pharmacokinetics of AFPSiA and possible formation of the corresponding sulphonate, AFPSoA. Since repeated blood sampling is difficult to perform in mice, the pharmacokinetics and metabolite studies were performed in dogs and rats.

In contrast to baclofen and other centrally acting $GABA_B$ agonists that can abolish TLESRs (Blackshaw *et al*., 1999; Lehmann *et al*., 1999), AFPSiA reduced them maximally by

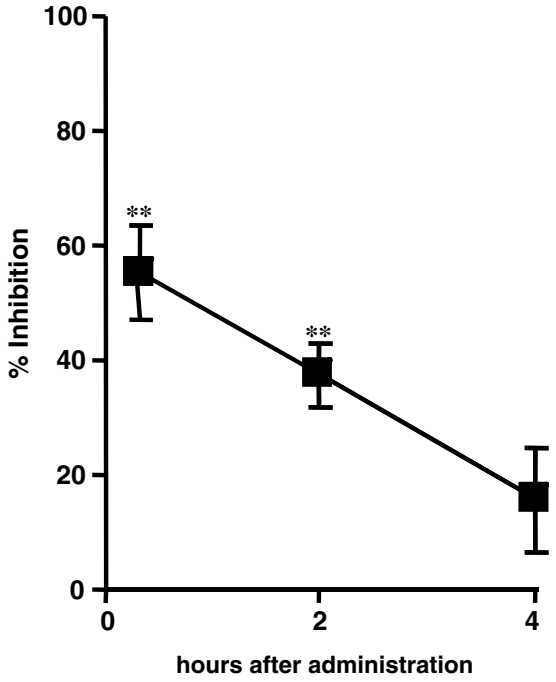

Figure 3 Effect duration of AFPSiA (14 μmol kg^{-1}) on TLOSRs in dogs. The inhibitory effect was abolished 4 h after administration. $^{**}P<0.01$, n (number of dogs) = 6. The control number (mean ± s.e.m.) of TLOSRs/45 min for 1, 2 and 4 h was 5.9 ± 0.5, 5.9 ± 0.6 and 5.8 ± 0.6, respectively.

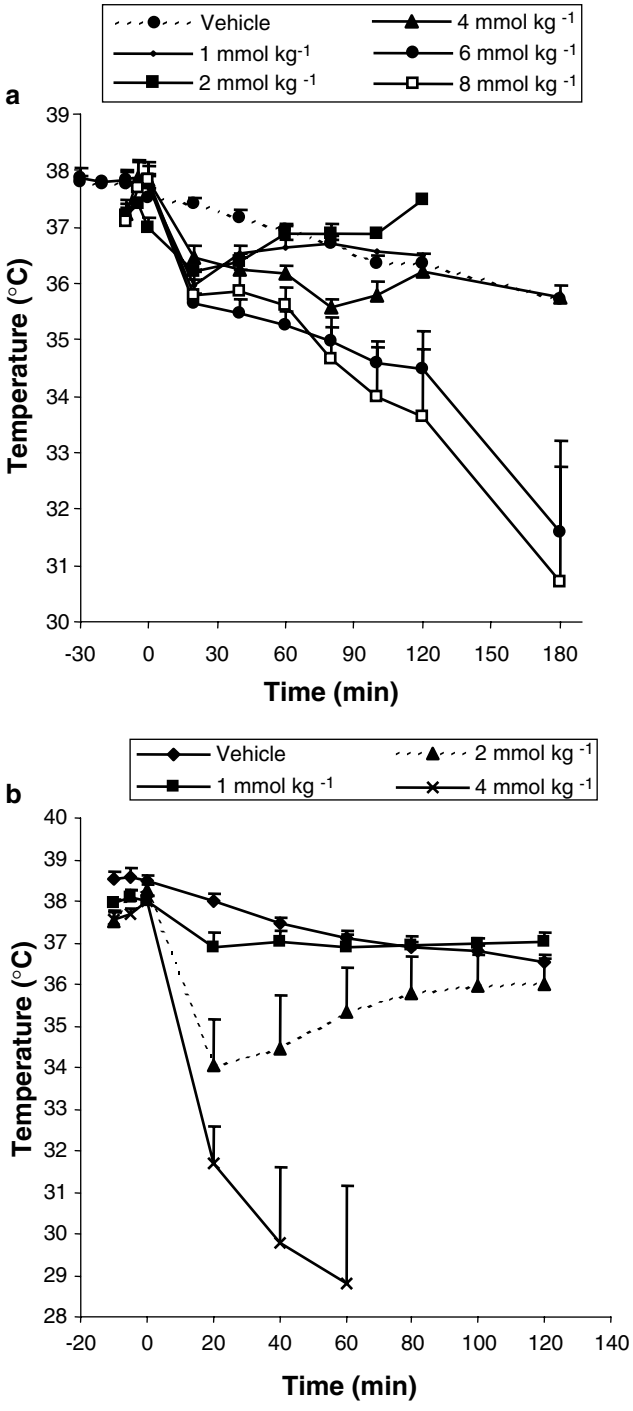

Figure 4 Dose–response curve for the hypothermic effects of AFPSiA (a) and AFPSoA (b) in the mouse. AFPSiA 1 mmol kg^{-1} vs vehicle $P<0.01$; 2 mmol kg^{-1} vs vehicle $P<0.05$; 4 mmol kg^{-1} vs vehicle $P<0.05$; 6 mmol kg^{-1} vs vehicle $P<0.001$; 8 mmol kg^{-1} vs vehicle $P<0.01$ (all comparisons at 20 min after dosing); $n=4$–6. AFPSoA 1 mmol kg^{-1} vs vehicle $P<0.01$; AFPSoA 2 and 4 mmol kg^{-1} vs vehicle $P<0.001$ (all comparisons at 20 min after dosing); $n=5$–6. The mice were killed for ethical reasons 60 min after the highest dose.

approximately 60%. However, the compound was found to have a markedly better safety profile in that only extremely high doses in mice could induce CNS effects. We have found in other studies that the threshold dose of baclofen to produce hypothermia in C57/Bl mice is about 20–30 μmol kg^{-1} s.c., so the threshold dose for AFPSiA was approximately 30–50 times higher. Such a profile is consistent with a peripheral site of action of AFPSiA to inhibit TLOSRs. Presumably, this effect is exerted at the peripheral endings of the gastric vagal mechanosensitive afferents (Partosoedarso *et al*., 2001; Smid *et al*., 2001). Racemic baclofen is in our hands approximately five times less potent on the human $GABA_{B(1a,2)}$ receptor compared with AFPSiA, yet, baclofen is about 10 times more potent on TLOSRs in the dog (Lehmann *et al*., 1999). Since the potency of $GABA_B$ agonists on vagal afferents (Smid *et al*., 2001) is considerably lower than that reported for central preparations (Knight & Bowery, 1996), it may be suggested that AFPSiA inhibits TLOSRs by a selective action on the peripheral branches of vagal afferents, while baclofen preferentially acts centrally, possibly on the central terminals of vagal afferents. If this assumption is correct, inhibition of vagal afferents peripherally would then maximally reduce TLOSRs by 50–60%, while centrally mediated inhibition can abolish TLOSRs. Consequently, the advantage of AFPSiA in terms of safety window compared with baclofen is to some extent counterbalanced by the limited maximal effect on TLOSRs. However, for a benign disease such as GORD, safety outweighs efficacy, and inhibition of TLOSRs in humans by some 60% by baclofen (Lidums *et al*., 2000) has been shown not only to reduce acid (Ciccaglione & Marzio, 2003), nonacid (Vela *et al*., 2003) and bile reflux (Koek *et al*., 2003), but also to relieve GORD symptoms (Ciccaglione & Marzio, 2003; Koek *et al*., 2003; Vela *et al*., 2003). Another finding arguing for a peripheral site of action of AFPSiA was the lack of an effect on swallowing except for after the highest dose. Previous studies have shown that both baclofen and

CGP44532, two centrally acting $GABA_B$ receptor agonists, inhibit swallowing (Lehmann *et al*., 2002), presumably through an action on the central pattern generator for swallowing. The absence of an effect of AFPSiA on swallowing can be considered advantageous since primary peristalsis is the major mechanism for clearance of gastric refluxate (Allen *et al*., 1996). Compatible with the relatively short half-life of AFPSiA, the duration of the effect was comparatively brief and essentially absent 4 h after administration of the compound.

Stimulation of the $GABA_B$ receptor on vagal efferents to the lower oesophageal sphincter has been shown to inhibit evoked lower oesophageal sphincter relaxation (Smid & Blackshaw, 2000), and vagal motoneurons innervating the lower oesophageal sphincter express $GABA_{B(1)}$ receptors abundantly (McDermott *et al*., 2001). However, it appears unlikely that this effect underlies the inhibitory action of AFPSiA on TLOSRs since TLOSRs are discrete events, and a reduction in transmitter release from vagal efferents would presumably produce a graded inhibition of the depth of TLOSRs and not a diminution in incidence. In the present experiments, there were no indications of hypothetical 'partial TLOSRs' and, indeed, such phenomena have never been reported after pharmacological interventions.

There was a conversion of AFPSiA to AFPSoA both in dogs and rats and the plasma AUC for the latter was approximately half of that of the former. In the dog, AFPSoA appeared later in blood than AFPSiA and quantifiable levels could only be detected 90 min after administration of the parent compound, that is, after the measurement of TLOSRs. It can thus be

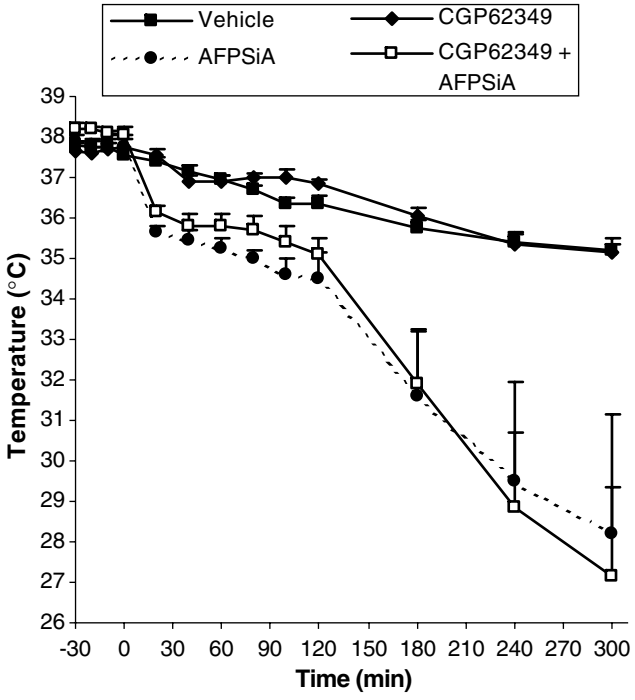

Figure 5 Lack of an effect ($P>0.05$) of CGP62349 (4.9 μmol kg^{-1}) on AFPSiA (6 mmol kg^{-1})-induced hypothermia in the mouse; $n=6$ (vehicle group) and $n=5$ (all other groups).

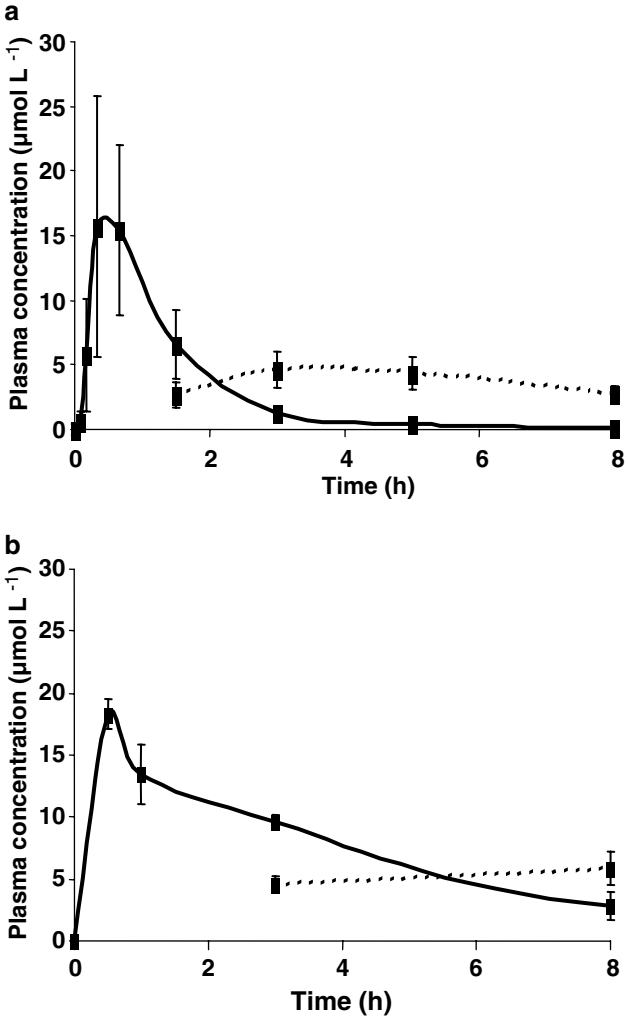

Figure 6 Plasma levels of AFPSiA (solid line) and AFPSoA (interrupted line) after intragastric administration of AFPSiA to dogs (a; 56 μmol kg^{-1}, $n=4$) and rats (b; 350 μmol kg^{-1}, $n=3$). In the dog experiments, the levels of AFPSoA were below the limit of quantification until 90 min after dosing, and in the rat study, AFPSoA was below the limit of quantification at 30 and 60 min after dosing.

Table 2 Pharmacokinetic constants for AFPSiA

Species	*Dose* (µmol kg^{-1})	*CL* (ml min^{-1} kg^{-1})	V_{ss} (L kg^{-1})	$t_{1/2\lambda z}$ (h)	F (%)	f_e
Rat	7 (iv/po)	40±6 (3)	3.0±0.8 (3)	1.5±0.1 (3)	71±4 (4)	0.4[a] (2)
Dog	7 (iv/po)	19±6 (4)	0.6±0.1 (4)	0.5±0.2 (4)	52±17 (4[b])	

The data represent arithmetical means±s.d. (*n*).
[a]After an intravenous dose of 14 μmol kg^{-1}.
[b]*F* is calculated after an oral dose of 14 μmol kg^{-1} for two dogs.

concluded that it is unlikely that AFPSoA contributes to the inhibition of TLOSRs after administration of AFPSiA. This notion is also based on the finding that the selective $GABA_A$ receptor agonist muscimol has no effect on TLOSRs in the ferret (Blackshaw *et al*., 1999). The hypothesis that the effect of AFPSiA on TLOSRs was mediated by $GABA_B$ receptors with no contribution from $GABA_A$ receptors could not be further tested using antagonists since $GABA_B$ antagonists are emetogenic in dogs in the presently used experimental setting and since $GABA_A$ antagonists are epileptogenic. While the possibility that AFPSiA also may act on other targets cannot be excluded, this seems unlikely since other $GABA_B$ agonists based on the GABA structure have never been shown to have any affinity for non-$GABA_B$(/$GABA_A$) proteins.

Hypothermia in the mouse accompanied administration of high doses of AFPSiA and AFPSoA. The time course after administration of high doses of AFPSiA was different from that seen for, for example baclofen, γ-hydroxybutyrate and muscimol (Quéva *et al*., 2003), which produce hypothermia that peaks within an hour and then rapidly returns towards control levels. Also, lower doses of AFPSiA and AFPSoA provoked a rapid and transient hypothermia. When AFPSiA was given at 6 or 8 mmol kg^{-1}, the hypothermic response appeared to consist of two phases. The first phase was characterised by a rapid and moderate hypothermia, and the second by a sustained and profound hypothermia. Notably, the second phase coincided with a prominent increase in plasma levels of AFPSoA noted in the rat experiments. It is suggested that the first phase of hypothermia is related to AFPSiA, and the second both to AFPSiA and AFPSoA. At lower doses, the formation of AFPSoA might not have been large enough to affect body temperature.

Despite the fact that AFPSiA was found to be a potent $GABA_B$ agonist *in vitro*, the mouse experiments clearly showed that hypothermia was exclusively induced by stimulation of $GABA_A$ receptors or some other non-$GABA_B$-receptor-mediated mechanism. The latter alternative seems less probable since there is no report on any affinity of $GABA_B$ receptor agonists for other receptors. We have found (unpublished) that CGP62349 at the dose used here completely prevents hypothermia produced by other $GABA_B$ agonists in the mouse. Additionally, CGP62349 inhibits baclofen-induced hypothermia in mice at a four-fold lower dose (Froestl *et al*., 1996). The dose of CGP62349 used in the present study should therefore have blocked central $GABA_B$ receptors sufficiently to prevent any $GABA_B$-mediated hypothermia.

In analogy with the low selectivity of GABA for $GABA_B$ over $GABA_A$ receptors, the unsubstituted sulphinate analogue of GABA, 3-aminopropylsulphinic acid or homohypotaurine, has a low selectivity for the $GABA_B$ receptor (Shue *et al*., 1996). Substitution with fluorine at the 2-position apparently does not alter this profile significantly, in fact, the selectivity profile of AFPSiA is identical to that of its unsubstituted congener. It may also be noted that replacement of the sulphinate group with a sulphonate group markedly directs the selectivity towards the $GABA_A$ receptor. This finding is supported by the fact that 3-aminopropylsulphonic acid or homotaurine is a selective $GABA_A$ receptor agonist (Shue *et al*., 1996) with a selectivity almost identical to AFPSoA.

While it was suspected that AFPSiA would be oxidised very rapidly and extensively to AFPSoA, AFPSiA was found to have a relatively high oral availability both in rats and dogs. Moreover, 40% of the given dose was recovered in urine in rats. This suggests that substituted 3-aminopropylsulphinic acids represent a group that may have a potential in drug development, particularly since at least AFPSiA may have a peripheral site of action and a low propensity to provoke central side effects. In addition to GORD, other indications of interest include, but are not restricted to, asthma and coughing (Chapman *et al*., 1993) as well as prevention of metastases (Ortega, 2003).

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References

ALLEN, M.L., CASTELL, J.A. & DIMARINO JR, A.J. (1996). Mechanisms of gastroesophageal acid reflux and esophageal acid clearance in heartburn patients. *Am. J. Gastroenterol.,* **91,** 1739–1744.

BLACKSHAW, L.A., STAUNTON, E., LEHMANN, A. & DENT, J. (1999). Inhibition of transient LES relaxations and reflux in ferrets by GABA receptor agonists. *Am. J. Physiol.,* **277,** G867–G874.

CANGE, L., JOHNSSON, E., RYDHOLM, H., LEHMANN, A., FINIZIA, C., LUNDELL, L. & RUTH, M. (2002). Baclofen-mediated gastro-oesophageal acid reflux control in patients with established reflux disease. *Aliment. Pharmacol. Ther.,* **16,** 1–5.

CARRUTHERS, N.I., SPITLER, J.M., WONG, S.-C., BLYTHIN, D.J., CHEN, X., SHUE, H.-J. & MITTELMAN, S. (1995). Synthesis and resolution of beta-(aminomethyl)-4-chlorobenzeneethanesulfinic acid a potent GABA(B) receptor ligand. *Bioorg. Med. Chem. Lett.,* **5,** 237–240.

CHAPMAN, R.W., HEY, J.A., RIZZO, C.A. & BOLSER, D.C. (1993). $GABA_B$ receptors in the lung. *Trends Pharmacol. Sci.,* **14,** 26–29.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}). *Biochem. Pharmacol.,* **22,** 3099–3108.

CICCAGLIONE, A.F. & MARZIO, L. (2003). Effect of acute and chronic administration of the GABA B agonist baclofen on 24 h pH metry and symptoms in control subjects and in patients with gastro-oesophageal reflux disease. *Gut.,* **52,** 464–470.

COWARD, P., CHAN, S.D.H., WADA, H.G., HUMPHRIES, G.M. & CONKLIN, B.R. (1999). Chimeric G proteins allow a high-throughput signaling assay of G(i)-coupled receptors. *Anal. Biochem.,* **270,** 242–248.

FAKT, C., JACOBSON, B.-M., LEANDERSSON, S., OLSSON, B.-M. & PERSSON, B.-A. (2003). Determination of a small, highly polar aminopropylphosphinic acid as racemate in plasma and urine and as separated enantiomers in plasma by liquid chromatography and tandem mass spectrometry. *Anal. Chim. Acta,* **492,** 261–269.

FROESTL, W., MICKEL, S.J., SCHMUTZ, M. & BITTIGER, H. (1996). Potent, orally active $GABA_B$ receptor antagonists. *Pharmacol. Res. Comm.,* **8,** 127–133.

HIRSCH, D.P., TYTGAT, G.N. & BOECKXSTAENS, G.E. (2002). Transient lower oesophageal sphincter relaxations – a pharmacological target for gastro-oesophageal reflux disease? *Aliment. Pharmacol. Ther.,* **16,** 17–26.

JENSEN, A.A., MOSBACHER, J., ELG, S., LINGENHOEHL, K., LOHMANN, T., JOHANSEN, T.N., ABRAHAMSEN, B., MATTSSON, J.P., LEHMANN, A., BETTLER, B. & BRÄUNER-OSBORNE, H. (2002). The anticonvulsant gabapentin (Neurontin) does not act through γ-aminobutyric acid-B receptors. *Mol. Pharmacol.,* **61,** 1377–1384.

KLINKENBERG-KNOL, E.C. & MEUWISSEN, S.G.M. (1990). Combined gastric and oesophageal 24-hour pH monitoring and oesophageal manometry in patients with reflux disease, resistant to treatment with omeprazole. *Aliment. Pharmacol. Ther.,* **4,** 485–495.

KNIGHT, A.R. & BOWERY, N.G. (1996). The pharmacology of adenylyl cyclase modulation by $GABA_B$ receptors in rat brain slices. *Neuropharmacology,* **35,** 703–712.

KOEK, G.H., SIFRIM, D., LERUT, T., JANSSENS, J. & TACK, J. (2003). Effect of the GABA(B) agonist baclofen in patients with symptoms and duodeno-gastro-oesophageal reflux refractory to proton pump inhibitors. *Gut,* **52,** 1397–1402.

LEHMANN, A., ANTONSSON, M., BREMNER-DANIELSEN, M., FLÄRDH, M., HANSSON-BRÄNDÉN, L. & KÄRRBERG, L. (1999). Activation of the $GABA_B$ receptor inhibits transient lower esophageal sphincter relaxations in dogs. *Gastroenterology,* **117,** 1147–1154.

LEHMANN, A., BREMNER-DANIELSEN, M., BRÄNDÉN, L. & KÄRRBERG, L. (2002). Effects of $GABA_B$ receptor agonists on swallowing in the dog. *Eur. J. Pharmacol.,* **448,** 67–70.

LIDUMS, I., LEHMANN, A., CHECKLIN, H., DENT, J. & HOLLOWAY, R.H. (2000). Control of transient lower esophageal sphincter relaxations and reflux by the $GABA_B$ agonist baclofen in normal subjects. *Gastroenterology,* **118,** 7–13.

LIU, J., PEHLIVANOV, N. & MITTAL, R.K. (2002). Baclofen blocks LES relaxation and crural diaphragm inhibition by esophageal and gastric distension in cats. *Am. J. Physiol. Gastrointest. Liver Physiol.,* **283,** G1276–G1281.

MARTINI, C., RIGACCI, T. & LUCACCHINI, A. (1983). [^{3}H]muscimol binding site on purified benzodiazepine receptor. *J. Neurochem.,* **41,** 1183–1185.

MCDERMOTT, C.M., ABRAHAMS, T.P., PARTOSOEDARSO, E., HYLAND, N., EKSTRAND, J., MONROE, M. & HORNBY, P.J. (2001). Site of action of $GABA_B$ receptor for vagal motor control of the lower esophageal sphincter in ferrets and rats. *Gastroenterology,* **120,** 1749–1762.

MITTAL, R.K., HOLLOWAY, R.H., PENAGINI, R., BLACKSHAW, L.A. & DENT, J. (1995). Transient lower esophageal sphincter relaxation. *Gastroenterology,* **109,** 601–610.

ORTEGA, A. (2003). A new role for GABA: inhibition of tumor cell migration. *Trends Pharmacol. Sci.,* **24,** 151–154.

PARTOSOEDARSO, E.R., YOUNG, R.L. & BLACKSHAW, L.A. (2001). GABA(B) receptors on vagal afferent pathways: peripheral and central inhibition. *Am. J. Physiol.,* **280,** G658–G668.

QUÉVA, C., BREMNER-DANIELSEN, M., EDLUND, A., EKSTRAND, A.J., ELG, S., ERICKSON, S., JOHANSSON, T., LEHMANN, A. & MATTSSON, J.P. (2003). Effects of GABA agonists on body temperature regulation in $GABA_{B(1)}^{-/-}$ mice. *Br. J. Pharmacol.,* **140,** 315–322.

SHUE, H.-J., CHEN, X., BLYTHIN, D.J., CARRUTHERS, N.J., SPITLER, J.M., WONG, S.-C., CHAPMAN, R.W., RIZZO, C., WEST, R. & SHE, H.S. (1996). A study of 3-amino-N-hydroxypropanesulfonamide derivatives as potential $GABA_B$ agonists and their fragmentation to 3-aminopropanesulfinic acid. *Bioorg. Med. Chem. Lett.,* **6,** 1709–1714.

SMID, S.D. & BLACKSHAW, L.A. (2000). Vagal neurotransmission to the ferret lower oesophageal sphincter: inhibition via $GABA_B$ receptors. *Br. J. Pharmacol.,* **131,** 624–630.

SMID, S.D., YOUNG, R.L., COOPER, N.J. & BLACKSHAW, L.A. (2001). $GABA_B$R expressed on vagal afferent neurones inhibit gastric mechanosensitivity in ferret proximal stomach. *Am. J. Physiol.,* **281,** G1494–G1501.

STAKEBERG, J. & LEHMANN, A. (1999). Influence of different intragastric stimuli on triggering of transient lower oesophageal sphincter relaxation in the dog. *Neurogastroenterol. Mot.,* **11,** 125–132.

VAKIL, N. (2004). New pharmacological agents for the treatment of gastro-oesophageal reflux disease. *Aliment. Pharmacol. Ther.,* **19,** 1041–1049.

VELA, M.F., TUTUIAN, R., KATZ, P.O. & CASTELL, D.O. (2003). Baclofen decreases acid and non-acid post-prandial gastro-oesophageal reflux measured by combined multichannel intraluminal impedance and pH. *Aliment. Pharmacol. Ther.,* **17,** 243–251.

ZHANG, Q., LEHMANN, A., RIGDA, R., DENT, J. & HOLLOWAY, R.H. (2002). Control of transient lower oesophageal sphincter relaxations and reflux by the $GABA_B$ agonist baclofen in patients with gastro-oesophageal reflux disease. *Gut,* **50,** 19–24.

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