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RESEARCH PAPER

Different *in vitro* and *in vivo* profiles of substituted 3-aminopropylphosphinate and 3-aminopropyl(methyl) phosphinate $GABA_B$ receptor agonists as inhibitors of transient lower oesophageal sphincter relaxation

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BACKGROUND AND PURPOSE

Gastro-oesophageal reflux is predominantly caused by transient lower oesophageal sphincter relaxation (TLOSR) and $GABA_B$ receptor stimulation inhibits TLOSR. Lesogaberan produces fewer CNS side effects than baclofen, which has been attributed to its affinity for the GABA transporter (GAT), the action of which limits stimulation of central $GABA_B$ receptors. To understand the structure–activity relationship for analogues of lesogaberan (3-aminopropylphosphinic acids), and corresponding 3-aminopropyl(methyl)phosphinic acids, we have compared representatives of these classes in different *in vitro* and *in vivo* models.

EXPERIMENTAL APPROACH

The compounds were characterized in terms of $GABA_B$ agonism *in vitro*. Binding to GATs and cellular uptake was done using rat brain membranes and slices respectively. TLOSR was measured in dogs, and CNS side effects were evaluated as hypothermia in mice and rats.

KEY RESULTS

3-Aminopropylphosphinic acids inhibited TLOSR with a superior therapeutic index compared to 3-aminopropyl(methyl)phosphinic acids. This difference was most likely due to differential GAT-mediated uptake into brain cells of the former but not latter. In agreement, 3-aminopropyl(methyl)phosphinic acids were much more potent in producing hypothermia in rats even when administered i.c.v.

CONCLUSIONS AND IMPLICATIONS

An enhanced therapeutic window for 3-aminopropylphosphinic acids compared with 3-aminopropyl(methyl)phosphinic acids with respect to inhibition of TLOSR was observed and is probably mechanistically linked to neural cell uptake of the former but not latter group of compounds. These findings offer a platform for discovery of new $GABA_B$ receptor agonists for the treatment of reflux disease and other conditions where selective peripheral $GABA_B$ receptor agonism may afford therapeutic effects.

Abbreviations

FLIPR, fluorescence image plate reader; GAT, GABA transporter; GORD, gastro-oesophageal reflux disease; i.g., intragastric; LOS, lower oesophageal sphincter; SAR, structure–activity relationship; TLOSR, transient lower oesophageal sphincter relaxation

Introduction

Transient lower oesophageal sphincter relaxation (TLOSR) has been identified as the major mechanical factor behind gastro-oesophageal reflux (Mittal *et al.*, 1995) and is therefore of great importance in the pathophysiology of gastro-oesophageal reflux disease (GORD). Although it can be triggered spontaneously at a low rate, TLOSR typically occurs post-prandially as a result of gastric distention (Mittal *et al.*, 1995). This stimulus causes gastric vagal mechanoreceptors to fire, which is followed by excitation of brainstem vagal motoneurons. The subsequent efferent signal powerfully activates enteric inhibitory neurons that relax the smooth muscle cells of the lower oesophageal sphincter (LOS). While gastric distension is the principal driver of TLOSRs, several factors appear to modulate them. These include posture (van Wijk *et al.*, 2010), adiposity (Wu *et al.*, 2007), intragastric (i.g.) pH (Stakeberg and Lehmann, 1999) and colonic feedback (Piche *et al.*, 2000). A promising therapeutic strategy in the treatment of GORD is therefore to reduce the excitability of afferent vagal fibres, and this has been successfully accomplished with $GABA_B$ receptor agonists such as baclofen (Lehmann *et al.*, 2010). However, baclofen produces a number of central side effects and is not an agent recommended in the management of GORD. In some patients, a low basal LOS pressure accounts for significant reflux (van Herwaarden *et al.*, 2000). Notably, $GABA_B$ receptor agonists elevate basal LOS pressure in humans (in contrast to dogs or ferrets; reviewed by Lehmann *et al.*, 2010) so $GABA_B$ receptor agonists may reduce reflux through two mechanisms.

We have discovered that the substituted 3-aminopropylphosphinic acid (2*R*)-(3-amino-fluoropropyl) phosphinic acid (AZD3355, lesogaberan, compound 10 in Figure 1) reduces the incidence of TLOSR and reflux in dogs with a broader therapeutic window than baclofen (Alstermark *et al.*, 2008; Lehmann *et al.*, 2009; Brändén *et al.*, 2010). This differentiation was attributed to the affinity of lesogaberan but not baclofen for the GABA transporter (GAT) in the CNS (Lehmann *et al.*, 2009). Lesogaberan has been shown to reduce TLOSR in healthy volunteers (Boeckxstaens *et al.*, 2010b) and to inhibit both TLOSRs and reflux episodes as measured with pHmetry and impedance in GORD patients not responding fully to proton pump inhibitors (the target patient population for reflux inhibitors; Boeckxstaens *et al.*, 2010a). Importantly, a 1 month phase IIa study demonstrated symptomatic relief in GORD patients with a partial response to proton pump inhibitors with an acceptable side effect profile (Boeckxstaens *et al.*, 2011).

The potential of discovering novel $GABA_B$ receptor agonists in the 3-aminopropylcarboxylic acid class may have been exhausted from a drug discovery viewpoint. However, 3-aminopropyl(methyl)phosphinic acids are more interesting in this context, and their effect on TLOSR has not been assessed in any detail with the exception of 3-aminopropyl (methyl)phosphinic acid (SK&F97541; Blackshaw *et al.*, 1999). The primary goal of the current investigation was to compare 3-aminopropylphosphinic and 3-aminopropyl (methyl)phosphinic acids as reflux inhibitors. The structure–activity relationship (SAR) for most of the compounds used in the present study with respect to affinity for and activation of the $GABA_B$ receptor has been published before (Alstermark *et al.*, 2008). There is a reasonable correlation both for 3-aminopropylphosphinic and for 3-aminopropyl(methyl) phosphinic acids between the affinity for the native rat $GABA_B$ receptor and the agonistic potency on the human recombinant $GABA_{B(1a)}$–$GABA_{B(2)}$ receptor. Both the 3-aminopropylphosphinic and 3-aminopropyl(methyl) phosphinic acids are full agonists at the $GABA_B$ receptor (Alstermark *et al.*, 2008). In general, there are no major differences in either affinity or potency between the 3-aminopropylphosphinic acids and the corresponding 3-aminopropyl(methyl)phosphinic acids. One exception is the 2-keto substituted analogues where the 3-aminopropyl (methyl)phosphinic acid displays lower affinity and potency than the corresponding 3-aminopropylphosphinic acid (Alstermark *et al.*, 2008).

In the current study, particular emphasis was placed on the therapeutic index of the compounds. Since it was found that 3-aminopropylphosphinic acids carrying a small (O, OH, F) but not large (4-chlorophenyl, 5-chlorothienyl) substituent in the 2-position had a superior therapeutic margin, we next determined whether this was related to intracellular uptake in the CNS secondary to binding to the GAT. The selection of compounds to evaluate in this context is fairly narrow since most modifications of the 3-aminopropyl backbone render the molecules inactive at the $GABA_B$ receptor (Froestl *et al.*, 1995; Alstermark *et al.*, 2008). The results of this study helped us to delineate a SAR for these two groups of compounds as regards the therapeutic index, and based on their functional differences, we now refer to them as group I (3-aminopropyl (methyl)phosphinic acids and 3-aminopropylphosphinic acids with a large substituent in the 2-position) and group II

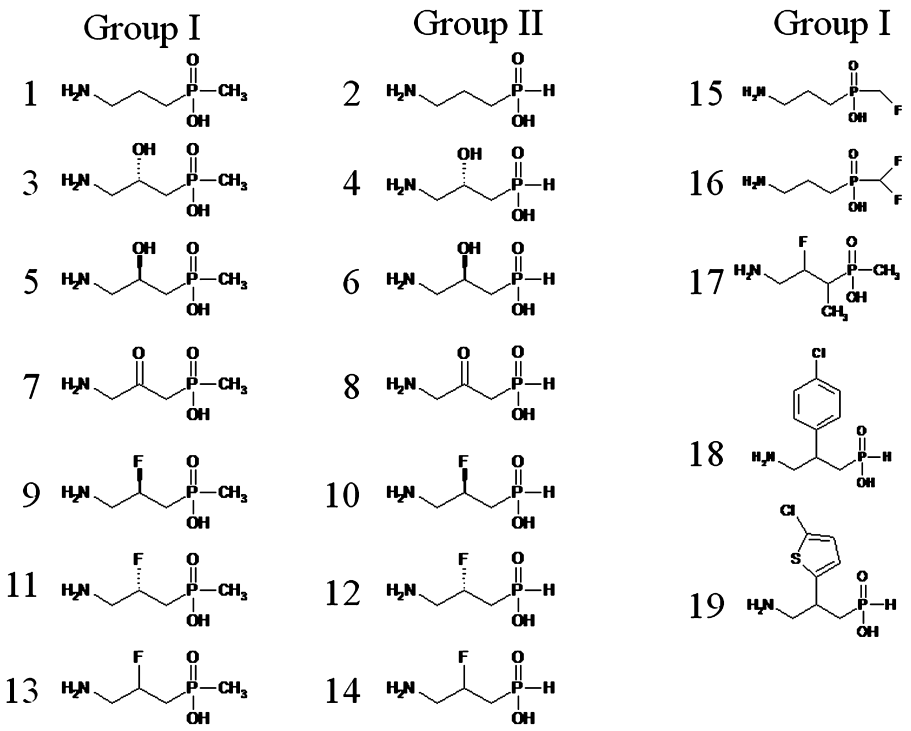

Figure 1
Structures of the compounds studied. The compounds are, when applicable, organized pairwise (compounds 1–14).

$GABA_B$ agonists (3-aminopropylphosphinic acids with a small substituent in the 2-position; see Figure 1).

Methods

Animal care

Unless otherwise noted, the following applied for rodent and dog experiments. All animal care and experimental procedures were approved by the Ethical Committee for Animal Experiments of the Göteborg Region. A license (No. 31-8825/08) for housing and using laboratory animals was obtained from the Swedish Board of Agriculture, and EU directive 609/86 was followed. Dogs were housed in compatible (double) pairs in dog holding rooms. Dogs were fed complete Good Laboratory Practice fixed pelleted formula from Dechra (Uldum, Denmark). Fresh water was supplied *ad libitum* through multiple drinking nozzles in the holding room. The size of the areas the dogs accessed was above the minimum requirements of the Swedish national regulations. Temperature and humidity of the dog holding rooms were generally kept at 17–20°C and 30–60% relative humidity. The number of air changes per hour was approximately 15. The animals had daylight through windows and electric light from fluorescent lamp fittings. Light was regulated to give 12 h each of daylight and darkness (night).

Rodents were housed in solid-bottomed macrolon cages. The number of animals per cage was equal to or less than maximum number according Swedish and EU regulations on housing space requirements. The cage bottoms were covered with aspen bedding material. Rodents received *ad libitum* pelleted rodent diet R3 from Lantmännen (Kimstad, Sweden). Municipal drinking water was available from plastic bottles with stainless steel sipper tubes. Temperature and humidity of the rodent holding rooms were generally kept at 20–23°C and 40–60% relative humidity, and the air was changed 15 times h^{-1}. Light was provided from fluorescent lamp fittings or light bulbs and regulated to give 12 h each of daylight and darkness (night).

Binding of ligands to $GABA_B$ receptors in rat and dog brain membranes and to $GABA_A$ receptors in rat brain membranes

The methods to assess binding to GABA receptors have been described previously (Lehmann *et al.*, 2005, 2009). In brief, binding of ligands to $GABA_B$ receptors was determined by displacement of [^{3}H]-GABA in the presence of isoguvacine from Sprague–Dawley rat or Beagle dog cerebrocortical membranes, employing a filtration binding assay in 96-well plates.

Membranes and [^{3}H]-GABA were incubated on a Denley microplate shaker (Colchester, UK) for 20 min at room temperature in the presence or absence of test compound, followed by rapid filtration through a glass fibre filter (Printed filtermat B filters, Wallac, Turku, Finland) using a TOMTEC cell harvester (Orange, CT). Radioactivity on the filters was determined in a Microbeta scintillation counter (Wallac). [^{3}H]-GABA displacement curves to determine IC_{50} values were constructed by fitting the four-parameter logistic equation to the data. 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 and Prusoff, 1973).

Binding to rat brain $GABA_A$ receptors was assessed in a similar manner as for the $GABA_B$ binding but using [^{3}H]-muscimol as a radioligand and without the presence of isoguvacine in the incubation buffer (Lehmann *et al.*, 2005, 2009). The $GABA_A$/$GABA_B$ selectivity was determined for compounds 1–2, 7–10 and 13–14.

Effect of compounds 15–19 on human $GABA_{B1(a)}$/$GABA_{B2}$ receptors

The potency of $GABA_B$ receptor agonists to stimulate intracellular calcium release was assessed in CHO-K1 cells stably co-expressing a $GABA_B$–G_{qi5} fusion protein and $GABA_{B2}$ using a fluorescence imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA) as described previously (Lehmann *et al.*, 2005, 2009). Only compounds 15–19 were studied since the effects of the other agonists in this assay have been reported earlier (Alstermark *et al.*, 2008). Briefly, $GABA_{B1}$-G_{qi5}/$GABA_{B2}$ cells, seeded in black-walled poly-D-lysine coated 96-well plates (Becton Dickinson, Bedford, UK), were loaded with the calcium sensor dye Fluo-3. The cells were then assayed in the FLIPR in the presence or absence of test compound. 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 two to four determinations at each concentration of test compound were used to construct concentration–response curves for EC_{50} determinations by fitting the four-parameter logistic equation to the data.

Effect of $GABA_B$ receptor agonists on splice variants of the $GABA_{B1}$ receptor

The human splice variants $GABA_{B1(a)}$, $GABA_{B1(b)}$, $GABA_{B1(e)}$, $GABA_{B1(g)}$, $GABA_{B1(m)}$, $GABA_{B1(o)}$ and $GABA_{B2}$ were cloned from a human brain cDNA, as specified in Lehmann *et al.* (2005) and Ekstrand (2002), and subcloned into pCI-Neo expression vectors. Transient co-transfections of $GABA_{B1}$ splice variants and $GABA_{B2}$ in CHO-G_{qi5} cells were performed using lipofectamin (Life Technologies, Paisley, Scotland) as specified in the manufacturer's protocol. The potency of the compounds evaluated was then determined using FLIPR technology as described above. The details of these experiments have been described in Lehmann *et al.* (2009). The following pairs of group I and II agonists were tested: compounds 1 and 2, 7 and 8 as well as 13 and 14.

Sequestration of radiolabelled $GABA_B$ receptor ligands in slices from the rat cortex

Superficial slices were manually prepared from the cerebral cortices of young adult 3 month old Wistar rats (Orion, Espoo, Finland). The experiments were made according to the EU directive 609/86 on the protection of laboratory animals. They were approved by the Tampere University Committee for Animal Experiments.

The animals were housed individually in separate cages under 12 h/12 h cycles of light and dark and received food and water *ad libitum* before the experiments. The slices were incubated for varying periods with the radioactively labelled compounds in standard Krebs–Ringer–HEPES medium containing (mmol L^{-1}) NaCl 127, KCl 5, $CaCl_2$ 0.75, $MgSO_4$1.3, Na_2HPO_4 1.3, HEPES 15, D-glucose 10, pH adjusted to 7.4 with 1 M NaOH. The extracellular space in the slices were estimated with [^{3}H]-inulin, and the label retained in them was subtracted to obtain the rates of intracellular penetration of the labelled compounds.

The following isotopes were used (for structures, see Figure 1):

[^{3}H]-compound 1, radiochemical purity 99%, specific activity 1623 kBq $nmol^{-1}$
[^{14}C]-compound 4, radiochemical purity 98%, specific activity 2.06 kBq $nmol^{-1}$
[^{14}C]-compound 8, radiochemical purity 92%, specific activity 2.1 kBq $nmol^{-1}$
[^{14}C]-compound 12, radiochemical purity 92%, specific activity 5.8 kBq $nmol^{-1}$

The *in vivo* distribution of [^{14}C]-compound 8 was also studied in the rat (supplementary information online) where emphasis was placed on the uptake into the CNS.

Binding of $GABA_B$ receptor ligands to the rat GAT

Competition for binding to the GAT between [^{3}H]-GABA and different $GABA_B$ receptor agonists in Wistar rat brain membranes was investigated using the method of Shank *et al.* (1990). Binding to GABA receptors was prevented by adding muscimol (10 μM) and baclofen (10 μM), and each concentration was tested in duplicate. The following compounds were evaluated in this assay: 1–3, 8–9, 11–12 and 16.

Quantification of TLOSR in dogs

TLOSRs were measured in adult Labrador retrievers of either gender using Dentsleeve manometry as described previously (Lehmann *et al.*, 1999).

TLOSRs were stimulated for 45 min by infusion of an acidified nutrient solution into the stomach followed by insufflation of air. As the incidence of TLOSR varies between dogs, each animal was used as its own control. The number of acid reflux episodes was also quantified using intra-oesophageal pHmetry, but since this parameter is much more variable than TLOSR (e.g. a reflux episode may pass undetected if a previous reflux event has acidified the oesophagus), data on reflux are not presented. All compounds were given i.g. 30 min before stimulation of TLOSR, respectively, as indicated in Figure 5. The central channel of the Dentsleeve assembly was used for i.g. administration of drugs and liquid nutrient and for insufflation of air. The following agonists were tested in this model: 1–8, 11–12 and 15–19. The effect of compound 8 on TLOSR was also evaluated in ferrets (supplementary information on-line).

Hypothermia after i.c.v. administration of $GABA_B$ receptor agonists in rats

Adult female Sprague–Dawley rats (M&B A/S, Ry, Denmark) weighing 250–300 g were used in all experiments. Throughout the experiments, the animals were housed individually in the care of AstraZeneca Laboratory Animal Resources, where

they were maintained on standard light–dark cycle (12:12) and received food and water *ad libitum*.

One day before the surgical procedure, and 7 days after surgery, the animals were treated with Bactrim® (0.2 mL per animal; Roche, Stockholm, Sweden) to reduce the risk of infection. Animals were stereotaxically implanted with an intracerebral microinjection guide cannula (26 gauge cannula from Plastics One, Roanoke, VA). On the day of surgery, the rats were anaesthetized with an i.p. injection of an 8:1 mixture of Ketalar® (ketamine, 50 mg·mL^{-1}, Pfizer, Sollentuna, Sweden) and Rompun® (xylazine, 20 mg·mL^{-1}, Bayer, Animal Health Care, Lyngby, Denmark) 8:1, 5 mL·kg^{-1} and subsequently mounted in a stereotaxic apparatus (Kopf, Tujunga, CA). The stereotaxic co-ordinates for the guide cannula were 1.0 mm anterior to the bregma, 1.5 mm lateral to the sagittal suture and 4.0 mm ventral to the cortical surface (Paxinos and Watson, 1982). The guide cannula and a stainless steel screw were anchored to the skull with acrylic dental cement. After 7 days of recovery, the accuracy of the implantation was tested using the angiotensin II (Sigma, St. Louis, MO) drinking test. An i.c.v. injection of angiotensin II (100 ng per animal) was given, and animals not demonstrating a prompt and sustained drinking response were excluded. The placement of the cannula was also verified in some animals at the end of the study by the injection of Evans Blue, removal of the brain and macroscopic examination of coronal brain slices.

A temperature-sensitive microchip (Implantable Programmable Temperature Transponder, IPTT 200, PLEXX, Elst, The Netherlands) was implanted s.c. in the interscapular region in animals that passed the angiotensin II test. Compounds were administered via an internal cannula (Plastics One, Inc.) projecting 1 mm below the tip of the guide cannula. All injections were performed manually with a Hamilton microsyringe in a volume of 5 µL during 60 s. Before each i.c.v. injection, the animals temperature was monitored to set a baseline level (three measurements during 20 min). Immediately after the injections, rats were placed in their home cage, and their temperature was measured for 180 min.

All compounds were dissolved in 0.9% saline, and i.c.v. treatments were given at least 7 days apart and between 0800 h and 1400 h. The hypothermic effects of compounds 7–10 were evaluated.

Hypothermia after administration of $GABA_B$ receptor agonists to mice

The effects of $GABA_B$ receptor agonists on body temperature in C57Bl mice were determined using interscapular thermosensitive chips as described previously (Lehmann *et al.*, 2009). All compounds were administered s.c. For each substance and time, an exponential model, given by

$$\Delta T_{j,k} = a \cdot \exp(b \cdot \text{dose}_k) + \varepsilon_{j,k}, \quad \text{dose}_k > 0, \quad \varepsilon_{j,k} \sim N(0, \sigma^2),$$

was fitted to the data. The response $\Delta T_{j,k}$ was defined as the j^{th} mouse's temperature change from baseline adjusted with the average change from baseline of all animals that were given placebo. The baseline value was calculated as the average of all pre-drug administration data points. In this model, α represents the change from baseline for vehicle, and e^{β} denotes the change in ΔT when the dose increases with one unit, that is, $\Delta T_k/\Delta T_{k+1}$ when $\text{dose}_{k+1} - \text{dose}_k = 1$. This model was fitted assuming homogeneous normal errors, $\varepsilon_{j,k} \sim N(0,\sigma^2)$. The model was then used to calculate ED_2, defined as the dose producing a 2°C drop in temperature, which reflects central actions of $GABA_B$ receptor agonists. The following compounds were evaluated in the mouse hypothermia model: 1, 7–10, 13–14 and racemic baclofen. The findings on compounds 10, 14 and baclofen have been published before (Lehmann *et al.*, 2009), but they are included in the current data set to further underscore the differences between group I and II agonists.

Pharmacokinetics in dogs and rats

The purpose of the pharmacokinetic experiments was to ascertain that the kinetics (e.g. oral availability and plasma half-life) were appropriate for the pharmacodynamic experiments. A secondary aim was to determine whether there were any systematic differences between group I and II agonists that could explain any pharmacodynamic disparities. The pharmacokinetics of different $GABA_B$ receptor agonists were determined using routine methods as described in Lehmann *et al.* (2009). Both female Sprague–Dawley rats and Labrador retrievers were used. In the rat experiments, blood was drawn from a catheter placed in the carotid artery after i.v. or i.g. dosing. Blood samples from dogs were taken from a leg vein.

Chemicals

All compounds except for compound 1 (Tocris, Bristol, UK) were synthesized at AstraZeneca R&D Mölndal or at Albany Molecular Research, Albany, NY. The synthetic procedures were according to those described in Dingwall *et al.* (1989) (compounds 2 and 18), Froestl *et al.* (1995) (compounds 3, 5 and 16), Alstermark *et al.* (2008) (compounds 4, 6, 7, 8, 9, 10, 11, 12, 13 and 14), European patent 399949 (compound 15) and US patent 6596711 (compound 17). Compound 19 was synthesized according to the procedure described for compound 18 in Dingwall *et al.* (1989) but using 2-chloro-5-(2-nitroethenyl)thiophene as starting material instead of 1-(4-chlorophenyl)-2-nitroethene. All agonists were prepared in their zwitterionic form. The radiolabelled compounds were synthesized by Roger Simonsson, AstraZeneca R&D Mölndal. The compounds were stored at room temperature protected from light, and all formulations were prepared freshly before the experiments using 0.9% NaCl as vehicle.

Selection of compounds for evaluation in different assays and models

The current series of experiments involved 19 compounds and 13 different *in vitro* assays or *in vivo* models. Obviously then, the experimental period stretched over a number of years, and therefore, not all compounds and models were available at the same time. Because of this shortcoming, selection of agonists to test had to be done based on the availability of test compound at any given time rather than on an optimal experimental strategy. In addition, due to high complexity and low yield in the synthesis of some compounds and requirement of large amounts of compounds in the dog experiments, the choice of dose levels was restricted in some cases.

Table 1

$GABA_B/GABA_A$ selectivity for four pairs of group I/II compounds

Compound	$GABA_B$ affinity K_i (nM)	$GABA_A$ affinity K_i (μM)	K_i $GABA_A/K_i$ $GABA_B$ Selectivity
1	33 ± 2.3 (87)	520 ± 43 (3)	16 000
2	15 ± 3.2 (11)	1.1 ± 0.9 (3)	70
7	200 ± 50 (11)	1 800 ± 100 (3)	9 000
8	48 ± 8.8 (12)	33 ± 7.2 (3)	690
9	4.3 ± 1.3 (3)	330 ± 22 (3)	77 000
10	5.1 ± 1.2 (10)	1.4 ± 0.3 (3)	270
13	14 ± 3.2 (9)	820 ± 11 (3)	59 000
14	10 ± 1.9 (12)	4.3 ± 0.7 (3)	430

Binding affinities (K_i) of ligands at the rat brain membrane $GABA_B$ and $GABA_A$ receptors were measured as detailed in Methods. Data are the mean ± SEM of (n) experiments. Results for $GABA_B$ affinity are from Alstermark *et al.* (2008).

Nomenclature

The nomenclature regarding receptors conforms to that of The British Journal of Pharmacology's *Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Statistical analysis

When appropriate, the data were analysed using Student's unpaired two-tailed *t*-test or by ANOVA followed by Hartley's sequential method of testing of individual means as indicated in the tables/figures. The null hypothesis was rejected at $P < 0.05$.

Results

Binding of $GABA_B$ ligands to rat $GABA_A$ and $GABA_B$ receptors and to dog $GABA_B$ receptors

Binding affinity to the $GABA_A$ receptor in rat brain membranes of compounds 1–2, 7–10 and 13–14 is reported in Table 1 where data from Alstermark *et al.* (2008) on affinity for the $GABA_B$ receptor also are given to facilitate comparison. While all compounds showed selectivity for the $GABA_B$ receptor, the group I compounds were 1–2 orders of magnitude more selective for the $GABA_B$ receptor than the corresponding group II analogues. Thus, methylation at the phosphorous greatly increases the selectivity for the $GABA_B$ receptor. The IC_{50} values for displacement of [^{3}H]-GABA from rat brain membrane $GABA_B$ receptors of compounds 15–19 are summarized in Table 2. In an attempt to explore SAR of 3-aminopropyl(methyl)phosphinates and 3-aminopropylphosphinates further, the hydrogen atoms at the methyl-phosphinic group were substituted by fluoro atoms. While mono-fluorination at the methyl-phosphinic group (compound 15) reduced the affinity for the rat brain $GABA_B$ receptor by approximately three times, di-fluorination (compound 16) produced a ninefold drop in affinity. This was also found to be true after methylation of compound 13 in the 1-position (compound 17; seventeenfold less potent). Larger substituents in the 2-position of compound 2, like the 4-chlorophenyl (compound 18) or 5-chloro-2-thienyl (compound 19), decreased affinity by a factor of approximately 40.

Table 2

Affinity for the native rat brain $GABA_B$ receptor and potency on the recombinant human $GABA_B$ receptor for compounds 15–19

Compound	Binding assay $GABA_B$ IC_{50} (nM)	FLIPR assay $GABA_B$ EC_{50} (nM)
15	93 ± 39	80 ± 14
16	300 ± 67	370 ± 120
17	240 ± 15	440 ± 100
18	650 ± 150	150 ± 32
19	590 ± 180	350 ± 35

Binding affinities (IC_{50}) and agonistic properties (EC_{50}) of compounds at the $GABA_B$ receptor were measured as detailed in the Methods. Data are the mean ± SEM of three independent experiments. All compounds shown in the table had an intrinsic activity = 1 with the exception of 16, which had an intrinsic activity of 0.25.

The affinities for two pairs of group I/II $GABA_B$ receptor agonists (compounds 3 and 4 and compounds 9 and 10, respectively) at the rat and dog receptors did not differ consistently as measured by displacement of [^{3}H]-GABA binding form $GABA_B$ receptor sites in brain membranes, although compound 3 had a higher, and compound 10 a lower, affinity for the dog receptor (Table 3).

Agonistic potency of compounds 15–19 on human $GABA_B$ receptors

These five compounds all proved to be active as $GABA_B$ receptor agonists with potencies (EC_{50}) at the human receptor, ranging from 80–440 nM and binding affinity at the rat $GABA_B$ receptor between 93 and 650 nM (Table 2). Consequently, the activity of these agonists was high enough to justify TLOSR studies in dogs.

Table 3

Affinity for two pairs of group I/II $GABA_B$ agonists for the native dog and rat $GABA_B$ receptor

Compound	IC_{50} dog brain (nM)	IC_{50} rat brain (nM)
3	89 ± 6.9 (3)**	200 ± 21 (3)
4	58 ± 6.2 (4)	67 ± 6.7 (3)
9	4.6 ± 0.2 (3)	3.5 ± 1.3 (3)
10	5.7 ± 0.3 (4)**	3.6 ± 0.4 (5)

The data represent mean ± SEM; number of independent replicates is given in parentheses. **Denotes statistically significant differences ($P < 0.01$) between IC_{50} in dog and rat brain membranes (Student's unpaired *t*-test).

Effects of group I and II agonists on splice variants of the human $GABA_B$ receptor

Three different human $GABA_{B1}$ receptor splice variants, $GABA_{B1(a)}$, $GABA_{B1(b)}$ and $GABA_{B1(e)}$ [also named $GABA_{B1(c)}$] (GenBank no AJ012187), have previously been shown to form functional heterodimers with $GABA_{B2}$. In addition to these, we also tested the functionality of three other putative splice variants, named $GABA_{B1(g)}$, $GABA_{B1(m)}$ and $GABA_{B1(o)}$. All the tested splice variants were functional when co-expressed with $GABA_{B2}$. The agonistic activity for the 3-aminopropylphosphinates (compounds 2, 8 and 14) and corresponding 3-aminopropyl(methyl)phosphinates (compounds 1, 7 and 13) did not differ between the various splice variants (Figure 2), with the exception that there was a trend for compound 8 to be more active than compound 7 on all splice variants ($P < 0.05$ for $GABA_{B1(b)}$, $GABA_{B1(e)}$ and $GABA_{B1(o)}$; not significant for $GABA_{B1(a)}$, $GABA_{B1(g)}$ and $GABA_{B1(m)}$ using Student's unpaired *t*-test), in agreement with the findings of Alstermark *et al.* (2008).

Accumulation of $GABA_B$ agonists in rat cerebrocortical slices

One group I (compound 1) and three group II (compounds 4, 8 and 12) agonists were evaluated in this assay. There was a time- and concentration-dependent uptake of the compounds, but the uptake of compound 1 was markedly lower than that of the others at all concentrations and time points (Figure 3). Among the group II agonists, the uptake of compound 4 appeared lower than that of the two other agonists.

Binding of $GABA_B$ receptor agonists to rat brain membrane GAT

Eight $GABA_B$ agonists (compounds 1–3, 8–9, 11–12 and 16) were characterized in the GAT rat brain membrane binding assay. While none of the group I agonists was able to displace GABA from binding to the GAT at concentrations up to 1 mM, all group II compounds produced a concentration-dependent inhibition (Figure 4), with compound 12 having the highest affinity.

TLOSR in dogs

The effects of group I and II compounds are shown pairwise (when applicable) in Figure 5. In agreement with previous

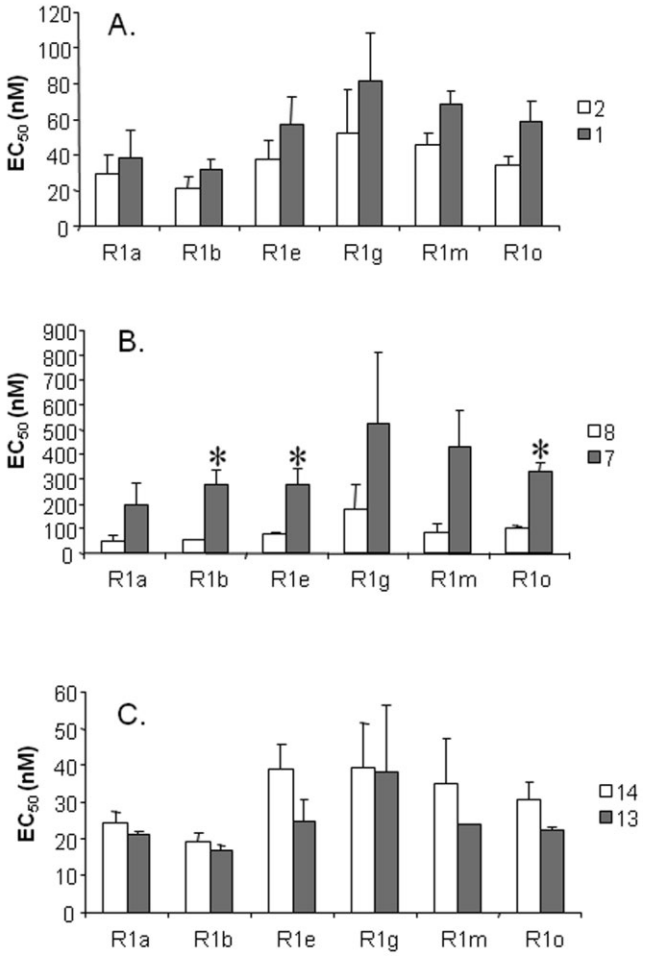

Figure 2

Potencies of three pairs (A: compounds 1 and 2, B: compounds 7 and 8 and C: compounds 13 and 14) of group I/II $GABA_B$ receptor agonists on human recombinant for $GABA_B$ splice variants. The effects of the ligands at the $GABA_{B1(a)}$, $GABA_{B1(b)}$, $GABA_{B1(e)}$, $GABA_{B1(g)}$, $GABA_{B1(m)}$ and $GABA_{B1(o)}$ receptor splice variants were tested as detailed in Methods. Results are the mean ± SEM of two to three experiments; *$P < 0.05$ (Student's unpaired two-tailed *t*-test).

studies, none of the compounds studied had any effect on basal LOS pressure (data not shown; Blackshaw *et al.*, 1999; Lehmann *et al.*, 1999). Despite the finding that most group II agonists were equally or even more active at the $GABA_B$ receptor than their corresponding group I counterpart *in vitro*, the latter were consistently much more potent and efficacious in inhibiting TLOSR. For instance, while the dose of lesogaberan (compound 10) producing some 50% inhibition of TLOSR approximates 7 $\mu mol \cdot kg^{-1}$ in the dog (Lehmann *et al.*, 2009), the group I comparator compound 9 produced 51 ± 4% (n = 4) inhibition at 0.015 $\mu mol \cdot kg^{-1}$. In addition, while close to full inhibition of lesogaberan is achieved at 300 $\mu mol \cdot kg^{-1}$ (Lehmann *et al.*, 2009), compound 9 abolished TLOSR at 0.15 $\mu mol \cdot kg^{-1}$ (100 ± 0%, n = 2). With the limitation that some compounds only were tested at one or two doses, similar striking differences in potency and efficacy were seen for all other pairs. Interestingly, as has been reported for

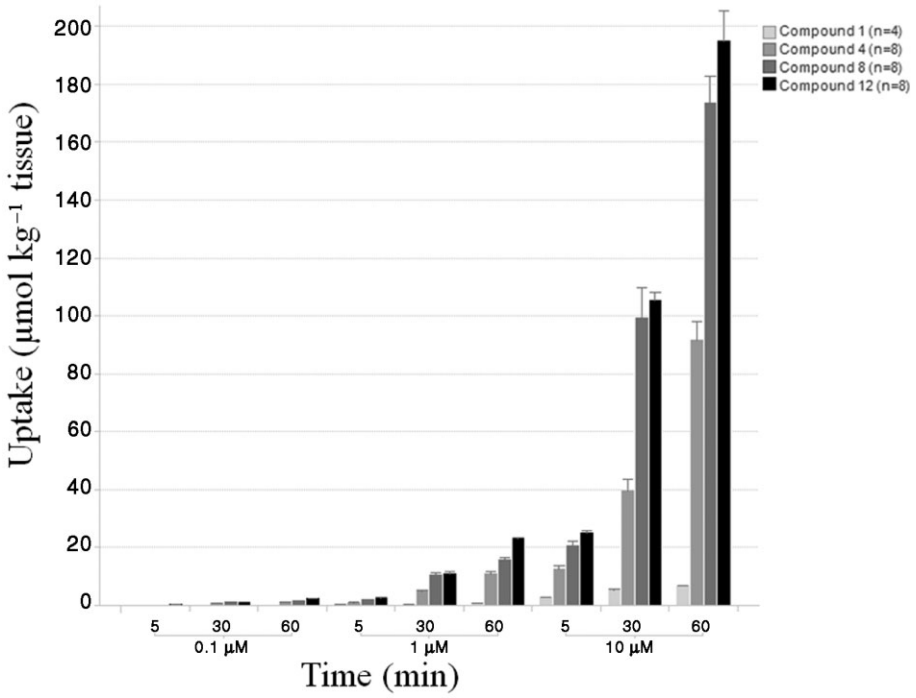

Figure 3

Uptake of $GABA_B$ receptor agonists in rat cerebrocortical slices as a function of time and concentration. Slices were incubated with radiolabelled compounds at the times and concentrations indicated, and cellular uptake was calculated from total uptake-uptake in the extracellular space. The uptake of the group I agonist compound 1 was markedly lower than that of the group II agonists compounds 4, 8 and 12 at all concentrations and times ($P < 0.01$; ANOVA followed by Hartley's sequential method of testing individual means).

lesogaberan (Lehmann *et al.*, 2009), the effects of the group II but not group I agonists appeared to reach a plateau at some 50% inhibition. Whether or not they would display biphasic dose–response curves with full inhibition at very high doses like lesogaberan (Lehmann *et al.*, 2009) was not possible to evaluate due to limited supply of the compounds.

The rank order of *in vivo* activity of the group I compounds clearly correlated with their *in vitro* activities. For example, the S-enantiomer compound 11 (EC_{50} at the human $GABA_B$ receptor = 1700 nM; Alstermark *et al.*, 2008) produced 60% inhibition of TLOSR at 2.8 µmol·kg^{-1}, while the R-enantiomer compound 9 (EC_{50} at the human $GABA_B$ receptor = 14 nM; Alstermark *et al.*, 2008) provided 51% inhibition at 0.015 µmol·kg^{-1}. Partly due to the relative incompleteness of the dose–response curves for the group II ligands, a similar conclusion was difficult to reach for this group. However, the S-enantiomer compound 12 was less active *in vivo* than the R-enantiomer lesogaberan (Lehmann *et al.*, 2009), which is some 30 times more active *in vitro* (Alstermark *et al.*, 2008). Also, the R-enantiomer compound 6, which binds 42 times less avidly to the rat $GABA_B$ receptor than the S-enantiomer compound 4, did not have any effect at all at 10 µmol·kg^{-1}, a dose approximating ID_{50} for compound 4.

Of note is the observation that compounds 18 and 19 afforded 85% and 90% inhibition of TLOSR, respectively. The efficacy of these compounds clearly suggests that they can be classified as group I agonists despite the fact that they are 3-aminopropylphosphinic acids.

None of the group I and II agonists produced any visible side effects at the doses tested. Compound 8 was also tested in ferrets with findings similar to those in dogs (supplementary material online).

Effects of i.c.v. administration of $GABA_B$ receptor agonists on body temperature in rats

Two pairs of group I and II agonists were evaluated: compounds 7 and 8, and 9 and 10. All compounds induced a statistically significant, dose-dependent hypothermia that usually reached its nadir 30 min after administration (Figure 6). However, there was a large discrepancy between the ability of the compounds to produce hypothermia. Despite being comparably active *in vitro*, the group I agonist 7 was approximately 3 orders of magnitude more active than the corresponding group II agonist 8. There seemed to be a steep dose–response for the latter compound since there was no effect at 100 nmol, but the maximal effect was observed at 300 nmol with no further decrease at 3000 nmol. Likewise, the group I agonist 9 was 3 orders of magnitude more active than the corresponding group II agonist 10.

Hypothermic effects of $GABA_B$ receptor agonists in mice

There was a clear relationship between the activity of $GABA_B$ receptor agonists at the human recombinant $GABA_B$ receptor

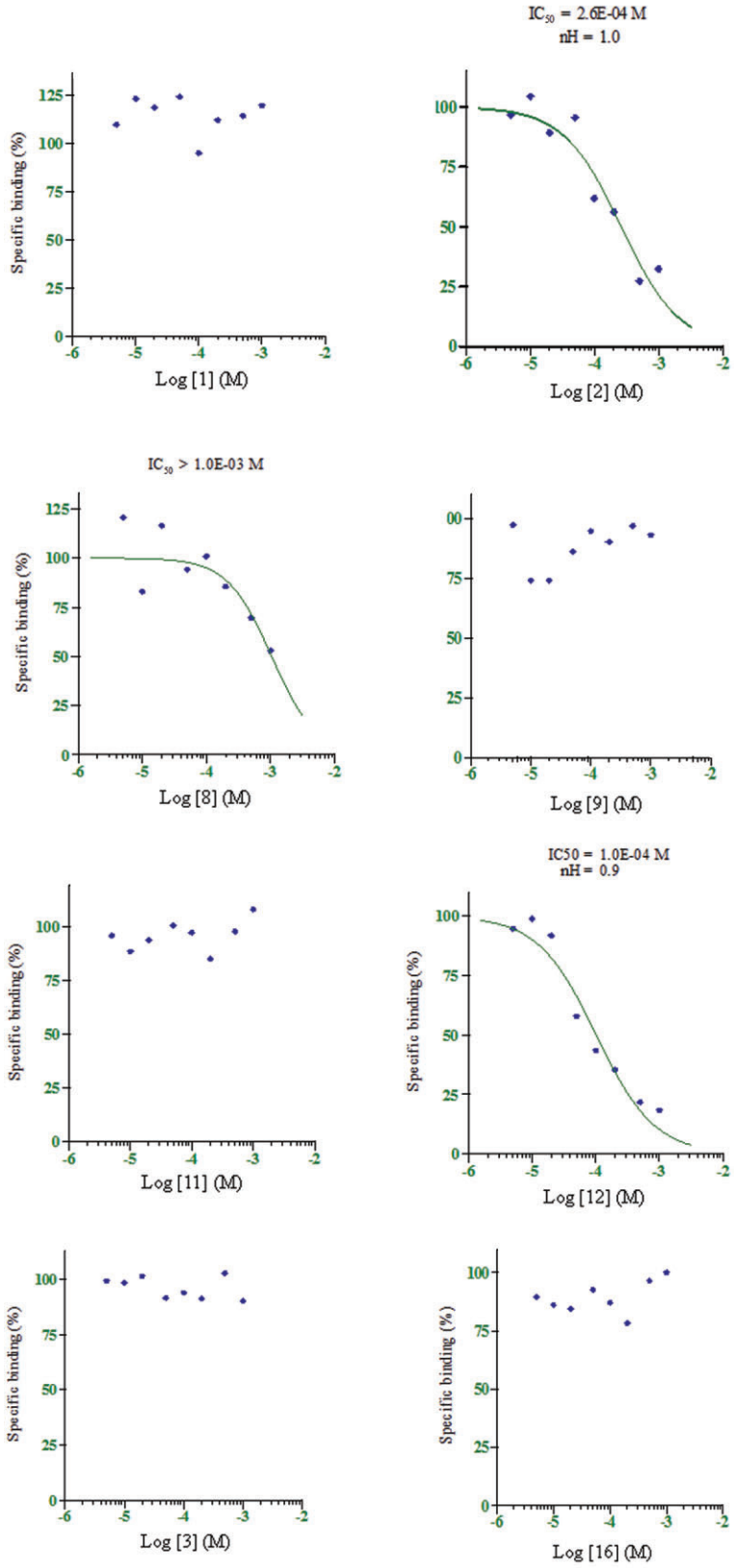

Figure 4

Displacement by $GABA_B$ agonists of binding of [^{3}H]-GABA to GAT in rat brain membranes. Note that all group II agonists (compounds with even numbers except for compound 16) were active in contrast to the group I agonists. Each concentration was tested in duplicate, and the data points represent the average.

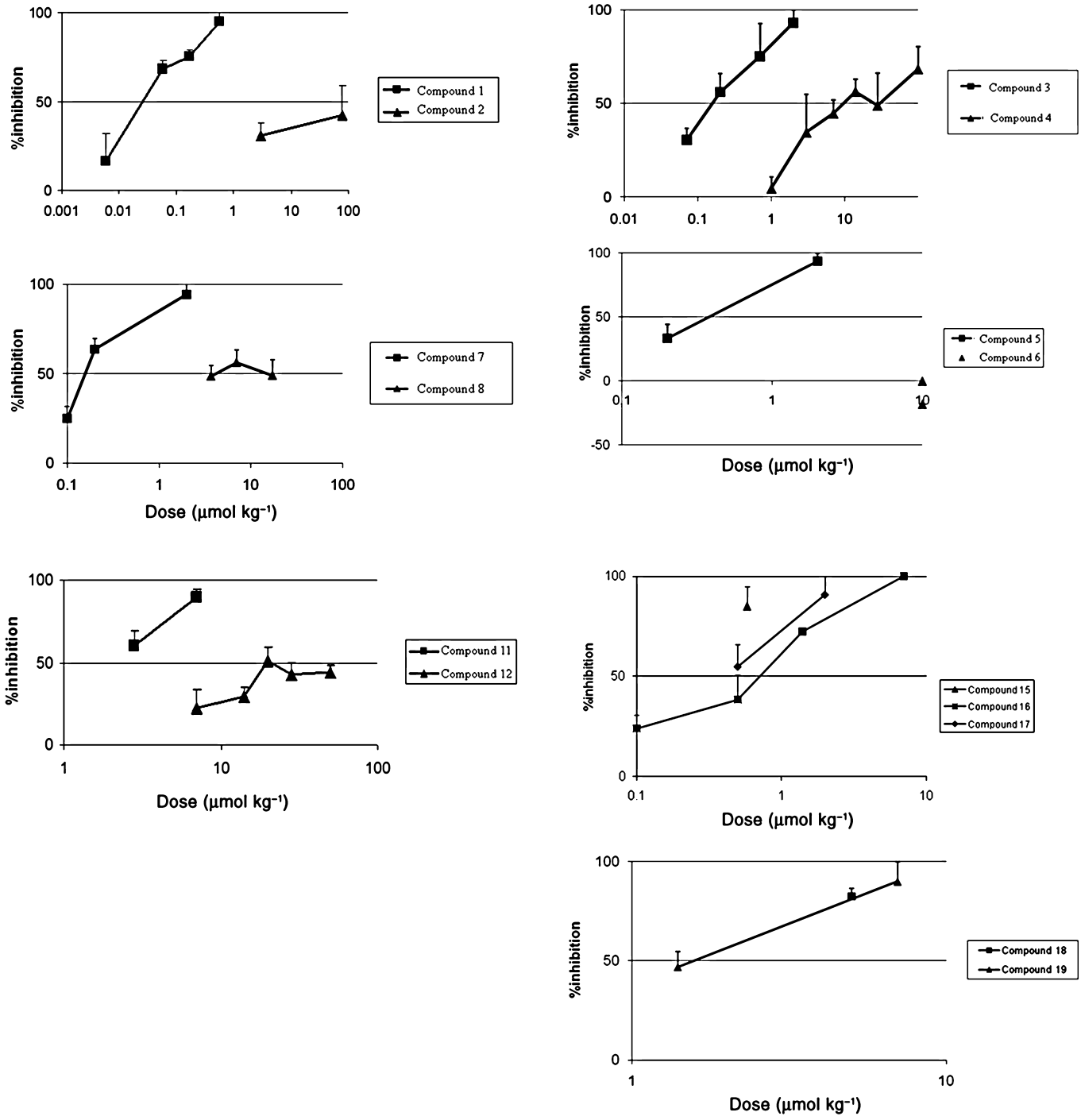

Figure 5

Effects of $GABA_B$ agonists on TLOSR in dogs. TLOSRs were scored during 45 min of gastric distension, and all drugs were administered i.g. 30 min before onset of gastric distension. Inhibition was calculated as the ratio between the number of TLOSRs after drug/the number of TLOSRs after vehicle as determined in separate experiments. The results represent means ± SEM; n = 2–12.

and their ability to induce hypothermia in mice (Figure 7). However, this relationship was only seen within the two groups of compounds. The group I agonists were considerably more active in producing a reduction in body temperature. For instance, the group I agonist 9 was equipotent to its group II analogue 10 *in vitro*, but it was more than 3 orders of magnitude more potent at reducing body temperature in mice.

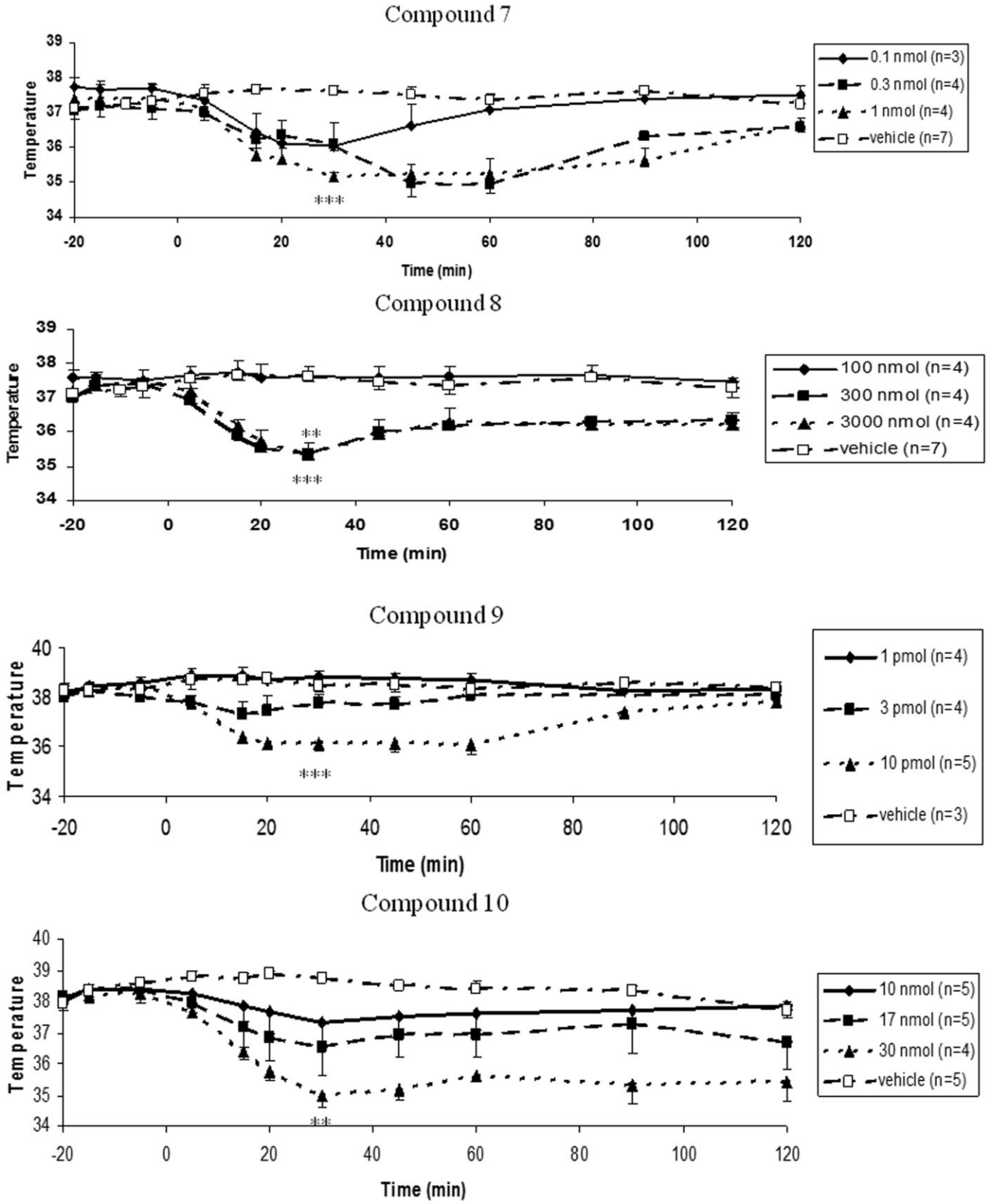

Figure 6

Effects of $GABA_B$ agonists on body temperature in rats after i.c.v. injection. Temperature was measured telemetrically using interscapular thermosensitive chips. The results reflect means ± SEM of three to seven experiments as indicated. For some data points, SEM is not displayed since it was within the resolution of the symbol. ***$P < 0.001$ versus vehicle (only nadir temperature was analysed), **$P < 0.01$.

Table 4

Pharmacokinetic parameters for $GABA_B$ receptor agonists in female dogs following i.v. and p.o. dosing, individual data

Compound	Dose (μmol·kg^{-1}) i.v. and p.o.	*N* i.v. + p.o.	CL (mL·min^{-1}·kg^{-1})	$t_{1/2}$ (h)	V_{ss} (L·kg^{-1})	*F* (%)
Group II						
2	2	2 + 2	16, 25	0.51, 0.54	0.34, 0.88	8.5, 12
4	3	2 + 2	5.9, 7.5	0.37, 0.68	0.22, 0.28	82, 90
8	3	2 + 2	6.5, 10	2.5, 2.6	0.55, 0.98	55, 65
18	3	2 + 2	3.3, 4.9	2.2, 2.4	0.42, 0.58	47, 103
Group I						
3	2	2 + 2	3.5, 3.7	0.91, 1.02	0.23, 0.27	68, 77
7	1	2 + 2	2.0, 2.9	0.85, 0.94	0.15, 0.16	67, 76
13	0.5	2 + 2	2.2, 2.7	0.94, 0.95	0.16, 0.20	60, 88

CL, clearance; $t_{1/2}$, half-life; V_{ss}, volume of distribution at steady state; *F*, oral availability.

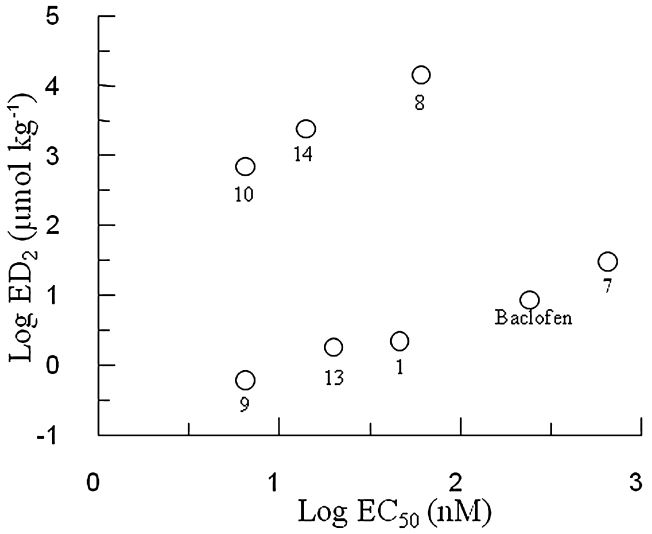

Figure 7

Hypothermic effects (log ED_2) in mice as a function of potency on recombinant human $GABA_B$ receptors (log EC_{50}). Data on compounds 10, 14 and baclofen are from Lehmann *et al.* (2009). Note that the potency of the compounds to produce hypothermia is related to the potency at the $GABA_B$ receptor, but that group II agonists are much less potent at inducing hypothermia than group I compounds at comparable *in vitro* potencies.

Pharmacokinetics in dogs and rats

The plasma half-life and oral availability (Table 4) of the compounds studied in dogs were compatible with a 45 min recording period of TLOSR and with i.g. administration. There was no general difference in the pharmacokinetics in dogs (Table 4) or rats (Table 5) between 3-aminopropylphosphinic and 3-aminopropyl(methyl)phosphinic acids that could explain the disparate *in vivo* profiles. In general, oral availability was high for both classes of agonists.

Discussion and conclusions

In this study, evidence is presented to suggest that $GABA_B$ receptor agonists with a phosphinic acid functionality can be subdivided into two groups based on their *in vivo* properties (group I: full inhibition of TLOSR at a narrow therapeutic range; group II: partial inhibition of TLOSR at a broad therapeutic range). We have not formally established the therapeutic range as defined by the potency to produce side effects compared with the potency to inhibit TLOSR. However, the span between doses producing some 50% inhibition of TLOSR and the lowest dose producing visible side effects in dogs is lower for baclofen than for lesogaberan (Lehmann *et al.*, 2009). The prediction that a group II agonist (lesogaberan) does not produce typical central $GABA_B$-related side effects at therapeutic exposures has now been confirmed in humans (Boeckxstaens *et al.*, 2011). Consequently, the suggestion that there is a difference with respect to therapeutic window between the group I and II agonists reported here is speculative and based on extrapolation from comparisons between baclofen and lesogaberan.

The substituent on the phosphorus atom dictates whether any compound of the current series is a group I or II compound so that 3-aminopropyl(methyl)phosphinic acids belong to group I, while 3-aminopropylphosphinic acids belong to group II agonists (provided that other structural features are compatible with $GABA_B$ receptor agonism). However, there is one important exception to this classification in that 3-aminopropylphosphinic acids with a large 2-substituent such as 4-chlorophenyl (compound 18) have a profile similar to the 3-aminopropyl(methyl)phosphinic acids. This conclusion is limited by the fact that it is only based on the high efficacy and potency (in relation to the *in vitro* potency) on TLOSR in the dog of compounds 18 and 19. We propose that the critical difference between group I and II agonists can be explained by the affinity of group II but not group I for the GAT, a hypothesis consistent with all results obtained in our study. Thus, methylation of the phosphorous atom, or a large substituent in the 2-position of

Table 5

Pharmacokinetic parameters for $GABA_B$ receptor agonists in female rats following i.v. and p.o. dosing, individual data or mean ± SD

Compound	Dose ($\mu mol \cdot kg^{-1}$) i.v./p.o.	N i.v. + p.o.	CL ($mL \cdot min^{-1} \cdot kg^{-1}$)	$t_{1/2}$ (h)	V_{ss} ($L \cdot kg^{-1}$)	F (%)
Group II						
2	2/4	2 + 3	71, 119	0.09, 0.18	0.40, 0.96	33, 41, 42
4	7	4 + 5	15 ± 2	1.8 ± 0.3	1.1 ± 0.1	77 ± 13
8	3	2 + 2	23, 28	3.0, 3.5	2.9, 3.6	89, 97
10	7	2 + 3	13, 12	2.8, 2.8	2.2, 2.3	79, 110, 119
14	7	3 + 3	26, 28, 30	1.9, 1.8, 1.6	2.8, 2.9, 2.5	72, 79, 94
Group I						
13	0.5	2 + 3	13, 13	0.34, 0.34	0.38, 0.34	74, 89

CL, clearance; $t_{1/2}$, half-life; V_{ss}, volume of distribution at steady state; *F*, oral availability.

3-aminopropylphosphinic acids, prevents binding to the GAT while binding to the $GABA_B$ receptor is retained. Sequestration of group II agonists in neural cells reduces the extracellular levels in the brain and protects against side effects caused by excessive central $GABA_B$ activation. Systemic pharmacokinetics can be excluded as a differentiating factor since they are broadly comparable for group I and II agonists in two different species. Incidentally, while compounds 1–3 have been widely used in different *in vivo* experiments before, this is to our knowledge the first report on their pharmacokinetics. The discussion below attempts to join all results into a unifying hypothesis to explain the *in vivo* differences between group I and II $GABA_B$ receptor agonists.

Species differences

The results in the present study are based on studies performed on human recombinant $GABA_B$ receptors, rat and dog brain membranes, as well as *in vivo* experiments in the rat, mouse, ferret and dog. There were several reasons for using different species. Rats were used in most experiments since those assays were developed and optimized for rats. Mice were used in hypothermia experiments after systemic administration of drugs since we have used this species for this purpose in several other studies with the aim of having a homogenous data base. Human recombinant $GABA_B$ receptors were utilized for translational purposes. Finally, as TLOSRs would be difficult to measure in rodents, and since indirect evidence suggests that they lack this reflex, dogs were used. In this context, some experiments were done using dog brain membranes to verify that the agonists indeed do have similar effects on canine $GABA_B$ receptors as on human and rat $GABA_B$ receptors. While the use of many different species in different models may be viewed as a disadvantage, it can just as well be considered a strength since it demonstrates to some extent that there is universal and not species-specific validity of the results. In order to be able to make any cross-species comparisons, the similarities of the $GABA_B$ receptors between species have to be assessed. The $GABA_B$ receptor is one of the most highly conserved GPCRs, and the amino acid residues of $GABA_{B1}$ participating in binding of ligands (Lehmann *et al.*, 2010) are identical in humans, dogs, rats and mice [sequence alignment using ClustalX; sequences retrieved from Swissprot (human, rat, mouse) and REFSEQ (dog)]. Furthermore, as shown in the present work, displacement of [^{3}H]-GABA from dog and rat brain membranes was almost identical for most agonists tested. Previous work has shown that baclofen has, at comparable doses, very similar potency 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) as an inhibitor of TLOSRs. In addition, lesogaberan has similar effects on TLOSR in dogs and humans at comparable plasma exposures (Lehmann *et al.*, 2010). It therefore seems reasonable to extrapolate results obtained in one species to the other species. Indeed, apart from expected differences related to body mass-dependent disparities in pharmacokinetics, the only species differences that have been demonstrated in this regard pertain to the expression of some splice variants in the rat but not in humans (Pfaff *et al.*, 1999). However, these variants have a pharmacology similar to that of the other splice variants (Pfaff *et al.*, 1999).

Different selectivity for the $GABA_B$ versus $GABA_A$ receptor or for $GABA_{B1}$ splice variants does not explain the disparities between group I and II agonists

Both group I and II agonists were found to be highly selective for $GABA_B$ receptors, but the former had a considerably higher selectivity for the $GABA_B$ compared with the $GABA_A$ receptor. However, the group II agonist lesogaberan was completely selective for the $GABA_B$ receptor at the doses administered *in vivo* as shown by the absence of an effect of the compound on body temperature in $GABA_{B1}^{-/-}$ mice (Lehmann *et al.*, 2009). In addition, the $GABA_B$ receptor antagonist CGP62349 abolished the effects of lesogaberan in wild-type mice (Lehmann *et al.*, 2009). Others have shown that compound 2 is less selective for the $GABA_B$ receptor than its 3-aminopropyl(methyl)phospinic acid congener compound 1 (Froestl *et al.*, 1995). The most likely explanation for the lower $GABA_B$ selectivity of group II compounds is that the

properties of the phosphinic acid group are more similar to the carboxylic acid group of GABA than the methylphosphinic acid group. GABA is only slightly more selective for the $GABA_B$ versus the $GABA_A$ receptor (Froestl *et al.*, 1995), so it is clear that the phosphinic, and particularly so the methylphosphinic acid group, confers $GABA_B$ receptor selectivity. There are no data to suggest that the differential selectivity between group I and II agonists for $GABA_B$/$GABA_A$ has any relevance to their different *in vivo* profiles.

Although some minor disparities were observed, there was no consistent difference between group I and II agonists as regards selectivity for different human recombinant $GABA_{B1}$ splice variants, which suggests that the differences in terms of the *in vivo* effects are unrelated to differential activation of splice variants. This set of data also demonstrates that the potency of the agonists on the splice variants is virtually identical to that on the two predominant splice variants, the $GABA_{B1(a)}$ and $GABA_{B1(b)}$. Furthermore, it should be underscored that both group I and II compounds used in the present study are all full agonists *in vitro* so differences in terms of efficacy at the receptor level can be excluded in the explanation of disparate *in vivo* profiles. In fact, we found that the group I agonist 16, which is a partial agonist *in vitro* (Froestl *et al.*, 1995; Knight and Bowery, 1996), acted as a full agonist in the dog TLOSR model. This suggests that there is a large $GABA_B$ receptor reserve, and that only a fraction of the receptors has to be stimulated to produce full inhibition of TLOSRs.

Penetration across the blood–brain barrier does not determine the in vivo *characteristics of $GABA_B$ receptor agonists*

Any differences between group I and II agonists, which are related to a divergent ability to cross the blood–brain barrier, would be negated after central administration of the compounds. Using two pairs of group I and II agonists (compound 7 and 8, and 9 and 10), we found that there was an extremely large difference in the ability to evoke hypothermic effects in rats. The *in vitro* activity was similar within the pairs, but the group I agonists were 2–3 orders of magnitude more active when given i.c.v. This would indicate that the difference in activity with respect to CNS side effects seen between group I and II agonists is related to the disposition of the compound within the CNS and not to the permeation across the blood–brain barrier.

Efficient sequestration of group II but not group I agonists in neural cells provides an explanation for differences in in vivo *properties of the compounds*

The hypothesis that group II agonists are sequestered by neural cells was investigated directly using rat brain membrane GAT and cerebrocortical slices. All group II agonists evaluated displaced GABA from GAT, while none of the group I ligands had any effect. Consistent with this, the uptake of group II compounds in brain slices was considerably more extensive than that of compound 1. Incidentally, piperidinyl-3-phosphinic acids, which have no affinity for any GABA receptor, are only active as GABA transporter inhibitors if they carry a phosphinic acid but not a methylphosphinic acid group in the 3-position (Kehler *et al.*, 1999). The *in vitro* results were corroborated by findings on the CNS distribution of compound 8 (supplementary information online).

The mechanism of action of group I and II agonists on TLOSRs

Group I agonists produced full inhibition of TLOSRs largely according to their *in vitro* potency. In addition, the dose-response curves appeared regular and monophasic (see also Lehmann *et al.*, 1999 and Blackshaw *et al.*, 1999). In contrast, none of the group II agonists afforded more than 50% inhibition of TLOSR in dogs even at high doses. Whether they would produce biphasic dose–response curves like lesogaberan (Lehmann *et al.*, 2009) could not be investigated due to limited availability of the compounds.

Baclofen and compounds 2 (Smid *et al.*, 2001) and 14 (Lehmann *et al.*, 2009) inhibit tension-induced firing of ferret gastric vagal mechanoreceptors *in vitro*. Such an effect is in agreement with the dense expression of $GABA_{B1}$ in retrogradely labelled gastric neurons of the ferret nodose ganglion (Smid *et al.*, 2001). This forms a possible morphological correlate to the proposed peripheral actions of group II agonists in inhibiting TLOSR.

Given the considerations above, the dissimilarities in dose–response curves on TLOSRs for group I and II agonists may be explained in the following way. Group II agonists act at moderate doses exclusively on gastric endings of vagal mechanoreceptors. The relatively low potency and/or efficacy of $GABA_B$ agonists on $GABA_B$ receptors in vagal afferents (Smid *et al.*, 2001) is compatible with the moderate effect on TLOSRs, even if the plasma concentrations were sufficient to fully activate $GABA_B$ receptors in recombinant systems, or to saturate native brain $GABA_B$ receptors. In contrast, group I agonists act mainly on central $GABA_B$ receptors, possibly those that may be expressed on central terminations of vagal afferents. Their effect is proposedly explained by inhibition of release of excitatory transmitters leading to a dampening of the vagovagal pathway underlying TLOSR. This notion is also consistent with the finding that baclofen given to ferrets *in vivo* inhibits gastric vagal tension-sensitive afferents and 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 efferent response that controls TLOSRs.

Baclofen and compound 2 are antitussive in different animal models (Bolser *et al.*, 1993, 1994; Chapman *et al.*, 1993; Hey *et al.*, 1995). Their mechanism of action has been extensively investigated, and it has been suggested that baclofen has a central site of antitussive action, while compound 2 inhibits cough peripherally. Such a suggestion, although limited by the fact that only one group II agonist was evaluated, agrees well with the present findings.

There is now supportive translational data from clinical trials to show not only that lesogaberan (compound 10) reduces TLOSR and gastro-oesophageal reflux (Boeckxstaens *et al.*, 2010a; b), but, more importantly, a 1 month phase IIa study (Boeckxstaens *et al.*, 2010a) demonstrated that lesogaberan provides symptomatic relief in GORD patients with an incomplete response to proton pump inhibitors in the absence of the CNS side effects of baclofen (a group I compound). Together, the clinical studies provide evidence for a mechanistic link between inhibition of TLOSR and reflux

episodes on the one hand, and amelioration of GORD-related symptoms on the other. A shortcoming with the group II $GABA_B$ receptor agonists as reflux inhibitors is that their postulated superior therapeutic index compared with baclofen occurs at the expense of a lower level of inhibition of TLOSR and reflux. While this is a reasonable compromise for a GORD therapy where a benign side effect profile is of paramount importance, additional clinical studies are needed to determine both the minimal level of TLOSR inhibition required to achieve a clinically significant symptomatic response as well as identification of target patient population(s). For instance, patients with functional heartburn (typical symptoms not associated with reflux) are not expected to benefit from a reflux inhibitor, and they may constitute a significant proportion of the incomplete responders to proton pump inhibitor therapy (Zerbib *et al.*, 2006). In contrast, patients whose main symptom is regurgitation may be an ideal population for a reflux inhibitor therapy. Similarly, volume regurgitation in infants, which presents a major clinical challenge for which no effective treatment exists, may be another population in which reflux inhibitors may afford beneficial effects (Omari *et al.*, 2006).

In summary, we have shown that $GABA_B$ receptor agonists can be divided into two functional groups. The major drawback of baclofen is its central side effects, and this, together with the lack of receptor subtype selective compounds, has encouraged the discovery of new $GABA_B$ receptor agonists. The present report suggests that group II $GABA_B$ agonists may have a therapeutic utility in GORD (Kuo and Holloway, 2010). The finding that 3-aminopropylphosphinic acids generally have high oral availability and favourable plasma half-life makes these compounds highly interesting from a drug discovery and development point of view.

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Conflicts of interest

LAB receives financial research support from AstraZeneca.

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