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RESEARCH PAPER

Intestinal gaboxadol absorption via PAT1 (SLC36A1): modified absorption *in vivo* following co-administration of L-tryptophan

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Background and purpose: Gaboxadol has been in development for treatment of chronic pain and insomnia. The clinical use of gaboxadol has revealed that adverse effects seem related to peak serum concentrations. The aim of this study was to investigate the mechanism of intestinal absorption of gaboxadol *in vitro* and *in vivo*.
Experimental approach: *In vitro* transport investigations were performed in Caco-2 cell monolayers. *In vivo* pharmacokinetic investigations were conducted in beagle dogs. Gaboxadol doses of 2.5 mg·kg^{-1} were given either as an intravenous injection (1.0 mL·kg^{-1}) or as an oral solution (5.0 mL·kg^{-1}).
Key results: Gaboxadol may be a substrate of the human proton-coupled amino acid transporter, hPAT1 and it inhibited the hPAT1-mediated L-[^{3}H]proline uptake in Caco-2 cell monolayers with an inhibition constant K_i of 6.6 mmol·L^{-1}. The transepithelial transport of gaboxadol was polarized in the apical to basolateral direction, and was dependent on gaboxadol concentration and pH of the apical buffer solution. In beagle dogs, the absorption of gaboxadol was almost complete (absolute bioavailability, F_a, of 85.3%) and T_{max} was 0.46 h. Oral co-administration with 2.5–150 mg·kg^{-1} of the PAT1 inhibitor, L-tryptophan, significantly decreased the absorption rate constant, k_a, and C_{max}, and increased T_{max} of gaboxadol, whereas the area under the curve and clearance of gaboxadol were constant.
Conclusions and implications: The absorption of gaboxadol across the luminal membrane of the small intestinal enterocytes is probably mediated by PAT1. This knowledge is useful for reducing gaboxadol absorption rates in order to decrease peak plasma concentrations.
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Keywords: Pharmacokinetics; hPAT1 (SLC36A1); gaboxadol; tryptophan; modified intestinal absorption; drug delivery; Caco-2 cells; dog

Abbreviations: ASC, amino acid transport system ASC; DMEM, Dulbecco's modified Eagle's medium; gaboxadol/THIP, [4,5,6,7-tetrahydroisoxazolo (5,4-c) pyridine-3-ol]; HBSS, Hanks' balanced salt solution; hPAT1, human proton-coupled amino acid transporter 1; MES, 2-(N-morpholino)ethanesulphonic acid; THPO, 4,5,6,7,-tetrahydroisoxazolo(4,5-c)-pyridin-3-ol

Introduction

The $GABA_A$ receptor agonist gaboxadol [4, 5, 6, 7-tetrahydroisoxazolo (5,4-c) pyridine-3-ol; THIP] has been in clinical development for the treatment of insomnia (Lancel and Faulhaber, 1996; Ebert *et al.*, 2006). At therapeutically relevant concentrations gaboxadol exclusively activates the extra-synaptic $GABA_A$ receptors (Ebert and Wafford, 2006) and has therefore been characterized as a selective extra-synaptic $GABA_A$ agonist (Nutt, 2005). Earlier preclinical studies with radiolabelled gaboxadol have shown that gaboxadol is almost completely absorbed (84–93%) after oral administration in rat, mouse and human (Schultz *et al.*, 1981). Single gaboxadol doses of 10 or 20 mg, taken as a tablet 30 min. before bedtime, have been shown to improve sleep maintenance and enhance slow wave sleep in humans (Lundahl *et al.*, 2007). Nevertheless, development of dose-related adverse events has been reported, in a small open-labelled study in patients with moderate to severe chronic pain, with morning dosing and intramuscular administration of 5–30 mg gaboxadol. These adverse events were closely correlated to peak serum

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concentrations, leading the authors to conclude that if the compound was to be used in the treatment of pain in cancer, peak serum concentrations above 0.55 μmol·L^{-1} should be avoided (Kjaer and Nielsen, 1983). Obviously some adverse events are related to the specific disease; however, the need for reduced serum peak concentrations may very well be relevant for other indications of gaboxadol as well. The intestinal absorption mechanism for gaboxadol across the intestinal epithelium is still unknown.

The human proton-dependent amino acid transporter 1 (hPAT1) of the solute carrier family SLC36 (SLC36A1), is an absorptive intestinal transporter for small zwitterionic amino acids and amino acid mimetics such as GABA (Thwaites *et al.*, 1995b). The *PAT1 cDNA* was first cloned as the lysosomal amino acid transporter 1 (LYAAT1) from rat brain in 2001 by Sagné *et al.* (2001), and later from mouse intestine (Boll *et al.*, 2002) and Caco-2 cells (Chen *et al.*, 2003). *hPAT1 mRNA* expression has been detected in most parts of the human gastrointestinal tract with maximal expression in small intestinal tissues (Chen *et al.*, 2003; Anderson *et al.*, 2004). hPAT1 has a relatively broad substrate specificity, and drug substances such as vigabatrin (Abbot *et al.*, 2006) and D-cycloserine (Thwaites *et al.*, 1995a) have also been shown to be transported via the transporter. Furthermore, is has been demonstrated that 5-hydroxytryptamine, L-tryptophan and tryptamine are effective inhibitors of amino acid transport via PAT1 (Metzner *et al.*, 2005). Recently, two GABA analogues, muscimol, a $GABA_A$ receptor agonist, and 4,5,6,7,-tetrahydroisoxazolo(4,5-c)-pyridin-3-ol (THPO), a GABA uptake inhibitor, were also identified as substrates for hPAT1 (Larsen *et al.*, 2008). Both analogues are heterocyclic compounds containing a 3-isoxazolol moiety, which has been shown to function as a carboxylic acid bioisostere for binding to the GABA receptors and transporters present in the brain (Corey *et al.*, 1994; Krogsgaard-Larsen *et al.*, 1994) and now also for hPAT1 substrate recognition. Based upon these findings and the structural similarities of muscimol, THPO and gaboxadol, it is hypothesized that gaboxadol is a substrate for PAT1 and that PAT1 is involved in the *in vivo* absorption of gaboxadol.

The aim of the present study was to identify the intestinal transport mechanism of gaboxadol *in vitro* and *in vivo*. During the *in vitro* studies it was found that gaboxadol was a ligand for hPAT1. Further transport studies suggested that hPAT1-mediated transport of gaboxadol across the apical membrane was important for the overall transepithelial transport. Therefore, a further aim was to investigate if PAT1 could be utilized for modifying the intestinal absorption of gaboxadol and subsequently alter the pharmacokinetic profile of gaboxadol. A pharmaceutical formulation approach based on co-administration of gaboxadol and tryptophan was successfully applied to decrease the absorption rate constant of gaboxadol.

Methods

Cell culture experiments

Protocols for cell culturing and *in vitro* experiments were as previously described (Larsen *et al.*, 2008). Caco-2 cells of passages 20 through 29 were seeded onto Transwell™ inserts (1.12 cm^2, 0.4 μm pore size) and experiments were conducted on day 25–28 after seeding. The apical uptake and the transepithelial transport of gaboxadol across Caco-2 cell monolayers in apical to basolateral direction (A–B) and basolateral to apical direction (B–A) were measured in Hanks' balanced salt solution (HBSS) buffers. In all experiments, buffer applied to the basolateral side was pH 7.4. Unless otherwise stated, buffer applied in the apical chamber was adjusted to pH 6.0 after the addition of gaboxadol hydrochloride or 35 mmol·L^{-1} tryptophan. The transport of 0.34, 3.5 or 7.0 mmol·L^{-1} gaboxadol was investigated. These concentrations were selected based on a single bedtime oral dose of 15 mg gaboxadol to humans, which provides an approximate luminal concentration of 0.34 mmol·L^{-1}, and the obtained gaboxadol affinity for hPAT1. Apical uptake experiments were initiated by adding fresh apical HBSS medium with 12.5 nmol·L^{-1} (0.5 μCi) L-(^{3}H)proline and 0–30 mmol·L^{-1} gaboxadol or 0–35 mmol·L^{-1} tryptophan to the apical chamber. The apical uptake experiments were terminated after 5 min. Samples were analysed by scintillation counting.

In vivo *experiments*

Absorption of gaboxadol in dogs. All animal care and experimental studies were approved by the Animal Welfare Committee, appointed by the Danish Ministry of Justice, and were carried out in compliance with EC Directive 86/609/EEC, the Danish law regulating experiments on animals and NIH Guidelines for the Care and Use of Laboratory Animals. Six full-grown male beagle dogs (body weight 15.9–21.7 kg) were selected and allocated into a Roman quadrant design and assigned to receive all the six formulations of gaboxadol hydrochloride randomly during 6 weeks. The dogs were fasted for 20–24 h before the initiation of the experiment and fed again 10 h after the administration. The gaboxadol dose was given either as an intravenous injection (1.0 mL·kg^{-1}) or as an oral solution given by gavage (5.0 mL·kg^{-1}) directly into the stomach using a soft tube. All dogs received 2.5 mg·kg^{-1} gaboxadol. In addition to gaboxadol, the oral formulations contained 0, 2.5, 10, 50 or 150 mg·kg^{-1} of tryptophan to ensure simultaneous co-administration of the two compounds. All solutions were adjusted to a pH of 5.2, and osmolarity was checked with a Vapro vapor pressure osmometer (model 552O, Wescor Inc., Logan, UT, USA), the intravenous solutions were adjusted to iso-osmolarity with glucose. Blood samples (2 mL) were taken from the cephalic vein by individual venepuncture and collected into Eppendorf tubes containing 200 IE heparin as an anticoagulant. Samples were collected before administration of gaboxadol and after 5, 15, 30, 60, 90 min, and 2, 3, 4, 6, 8 and 10 h after gaboxadol administration. The plasma was harvested immediately by centrifugation for 15 min at 2200 g and 4–8°C and stored at –80°C until further analysis. The animals had a 6-day washout period between treatments.

Investigation of gastric emptying in dog. A protocol similar to the one described earlier using paracetamol as a marker was used to evaluate the influence of tryptophan on the gastric emptying rate in dogs. Six dogs (body weight 16.1–21.5 kg)

were selected and randomly allocated to receive three formulations of paracetamol in a crossover study. The dogs received 50 mg·kg^{-1} paracetamol as an intravenous injection (1 mL·kg^{-1}) or as an oral solution (5 mL·kg^{-1}) containing 2.5 mg·kg^{-1} gaboxadol and 0 or 150 mg·kg^{-1} tryptophan. Fasting of the dogs, drug administration, blood sampling and washout were done as described earlier.

Analytical methods

Quantification of gaboxadol in plasma and buffer: Gaboxadol was extracted from plasma and buffer samples by liquid extraction. 100 µL HBSS or plasma samples were mixed with 25 µL internal standard (d_4-gaboxadol) and 25 µL purified water. Protein precipitation was carried out by addition of 400 µL cold acetonitrile. After centrifugation at 10 000 g for 15 min, 425 µL of supernatant was transferred to glass tubes and evaporated to dryness under nitrogen at 45°C. The samples were redissolved in 80 µL of methanol/acetonitrile (30:70), whirl-mixed for 10 min and centrifuged for 3 min at 3300× *g*. Gaboxadol was subsequently quantified by hydrophilic interaction chromatography followed by tandem mass spectrometry (MS/MS) detection using a protocol modified from Kall *et al*. (2007). The liquid chromatography (LC) system comprised by an Agilent 1100 series pump and degasser. An Asahipak amino column, (NH_2P-50, 150 × 2 mm) from Phenomenex (Torrance, CA, USA) was used with a mobile phase of 20.0 mmol·L^{-1} ammonium acetate (pH 4): acetonitrile (30:70) and a flow rate of 0.2 mL·min^{-1}. Twenty-microlitre samples were injected onto the column, which was kept at room temperature. The total run time was 10 min with the first 5 min of elution let to waste. The elution time of gaboxadol on the column was approximately 8 min. The MS/MS system used consisted of a Sciex API 4000 MS/MS detector with a Turbo Ion Spray and Turbo V source (Applied Biosystems, Foster City, CA, USA). The signals were linear between 0.5 and 2500 ng·mL^{-1}, and the limit of quantification by this procedure was 0.5 ng·mL^{-1}. The software was from Analyst™ (Applied Biosystems, version 4.0).

Quantification of paracetamol in plasma samples: 100 µL plasma were added to 60 mg sodium chloride, 50 µL purified water and 25 µL internal standard solution (25 µg·mL^{-1} theophylline in water) before protein precipitation and extraction with 1000 µL ethyl acetate was performed. The Eppendorf tubes were mixed for 20 s on a vortex mixer and centrifuged in a Labofuge 400 from Function Line (Heraeus Instruments, Hanau, Germany) at room temperature for 5 min at 6000 g. The supernatant was evaporated to dryness under a stream of nitrogen at 60°C, and subsequently the residue was resolved in 100 µL of mobile phase. Twenty-five microlitres was injected into the column. Analysis was performed in Merck–Hitachi equipment with a Merck–Hitachi L-4250 UV detector at a wavelength of 245 nm. The column was an XBridge C18, 3.5 µm, 4.6 × 150 mm and the Merck L-5025 column thermostat was heated to 40°C. The mobile phase consisted of 0.025 M phosphate buffer (pH 6.0), acetonitrile (95:5), and the flow rate was 1.0 mL·min^{-1}. The limit of quantification by this procedure was 0.7 µg·mL^{-1} and the assay was linear between 0.7 µg·mL^{-1} and 147 µg·mL^{-1}.

Data analysis

The flux, *J*, of gaboxadol (mass·time^{-1}·area^{-1}) across the Caco-2 cell monolayer was calculated from Fick's first law. The mass of substrate, Q, accumulating in the receptor compartment per time, *t*, and as a function of area, *A*, at steady-state conditions was as follows

$$J = \frac{dQ}{dt} \cdot \frac{1}{A} = P_{app} \cdot C \quad (1)$$

where C is the initial concentration of the gaboxadol in the donor chamber and P_{app} is the apparent permeability coefficient of gaboxadol across the cell monolayer. Sink conditions were confirmed by assuring that solubility was not exceeded and no more than 10% of the initial concentration of gaboxadol was accumulated in the receptor chamber. The steady state flux values of gaboxadol were calculated as mean flux values from 20 to 100 min. The 50% inhibitory concentration (IC_{50}) value of tryptophan was the concentration at which the apical uptake of proline (a prototypical hPAT1 substrate), *U*, was reduced to 50% of the control value. This value was determined using the equation

$$U = U\min + \frac{U\max - U\min}{1 + 10^{(\log IC50 - \log[I])}} \quad (2)$$

where *U* is the uptake of proline (0–100%) at concentration [*I*] of the inhibitor, in this case tryptophan. U_{min} is the minimal uptake of proline (at the highest value of [*I*]), and U_{max} is the control uptake ([*I*] = 0), both given as % values. IC_{50} values were converted to K_i values assuming competitive kinetics as described by Cheng and Prusoff (1973):

$$IC_{50} = K_i \cdot \left(1 + \frac{[S]}{K_m}\right) \quad (3)$$

[*S*] is the gaboxadol or tryptophan concentration, K_m is the Michaelis constant for proline uptake (mmol·L^{-1}). All results are expressed as mean ± standard error of the mean (SEM) of 3–4 independent cell passages. The data were fitted using non-linear regression in order to allow IC_{50} values to be obtained by GraphPad Prism software (version 4.02; GraphPad Prism Software Inc., San Diego, CA, USA).

Pharmacokinetic parameters from the *in vivo* studies on beagle dogs were calculated using WinNonlin Professional (version 5.2, Pharsight Corporation, Mountain View, CA, USA). The plasma concentration profiles of both gaboxadol and paracetamol after intravenous dosing were fitted to a two-compartment model whereas a non-compartmental model was used to analyse plasma concentration profiles from oral dosing. The area under the curve (AUC) was determined using the linear trapezoidal method and extrapolating the last measured plasma concentration to infinity. The size of the residual varied between 0.54 and 1.5% of the AUC. The analysis of the elimination rate constant was obtained by linear regression of at least three time points towards the end of the experiment. The bioavailability, F_a, of gaboxadol from the oral solutions was calculated for the individual animal using the following equation (Rowland and Tozer, 1995)

$$Bioavailability,\ F_a = \left(\frac{AUC(x)}{AUC(IV_A)}\right) \cdot \left(\frac{Dose(IV_A)}{Dose(x)}\right) \quad (4)$$

where $AUC(IV_A)$ is the area under the curve of treatment A, the intravenous gaboxadol administration in the absence of tryptophan, *x* is one of the oral solutions containing gaboxadol and 0–150 mg·kg^{-1} tryptophan, B-F. Results are expressed as mean ± SEM of the six animals.

In the present study, numerical deconvolution as shown by Langenbucher (Langenbucher, 1982) was used and approximated as:

$$I(x_i) = \frac{\left[R(t)/T - \sum_{k=1}^{n} I(x_k)\cdot W(x_{n-k+1})\right]}{W(x_i)} \quad (5)$$

where *T* is the time interval, $I(X_k)$ and $W(X_k)$ the average input rate and weight between the times X_{k-1} and X_k The plasma concentration profiles obtained in the present study were used for numerical deconvolution supplemented with some terminal plasma concentrations estimated by linear regression, as the method requires a constant time interval. The input function $I(t)$ represents the absorption process, whereas $R(t)$ is the obtained plasma concentration following oral administration, and $W(t)$ is the plasma concentration following the IV dose (Langenbucher, 1982).

The mean cumulative fraction of absorbed gaboxadol and paracetamol per time unit were calculated by deconvolution of the obtained plasma concentrations, which provided a dynamic profile of the theoretical first-order absorption process as 0–100% absorbed for all treatments in every single animal. The cumulative absorption of paracetamol is used to estimate the gastric emptying rate (Calbet and MacLean, 1997; Sunesen *et al.*, 2005). Profiles of both paracetamol and gaboxadol absorption were used to estimate the rate coefficient of absorption (k_a). k_a was found as the slope of the deconvolution curves when converted to a first-order function by the equation:

$$A_t = A_{max}\cdot(1 - e\hat{}(-ka\cdot t)) \text{ or } k_a\cdot t = -\ln\left(1 - \frac{A_t}{A_{max}}\right) \quad (6)$$

A_t was obtained from the deconvolution profiles and was the cumulative fraction gaboxadol/acetaminophen absorbed at a given time *t* and A_{max} is 100%. The mean slope and SEM were obtained by linear regression analysis of the linear absorption curves from six animals (A_t vs. time) performed in GraphPad Prism.

Statistical analysis

Statistical analysis was performed in Sigma Stat (version 3.0.1, SPSS Inc. Chicago, IL, USA) using a Students *t*-test or a one-way analysis of variance (ANOVA) followed by multiple comparisons versus control group (Holm-Sidak method), $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Materials

The chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Caco-2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and Dulbecco's modified Eagle's medium (DMEM) was obtained from Life Technologies (Høje Taastrup, DK). HBSS with calcium and magnesium was purchased from GIBCO, Invitrogen (Paisley, UK) and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) from Applichem (Darmstadt, Germany). Cell culture plastic ware was obtained from Corning Inc., Life Sciences (PA, USA). (2,3,4,5-^{3}H)-L-Proline (80 Ci·mmol^{-1}) was purchased from Larodan, (Malmö, Sweden). Gaboxadol hydrochloride [4,5,6,7-tetrahydroisoxazolo(5,4-*c*)pyridin-3-ol hydrochloride] and the deutero-substituted form d_4-gaboxadol [4,4,5,5-tetradeutero-6,7-dihydro-isoxazolo(5,4-c)pyridin-3-ol hydrochloride] were synthesized by H. Lundbeck A/S (Valby, Denmark). Heparin, 5000 IE·mL^{-1}, was purchased from LEO (Ballerup, Denmark). Orthophosphoric acid 85%, disodium hydrogen phosphate, potassium hydrogen phosphate and acetic acid were obtained from Merck (Darmstad, Germany). All chemicals were of analytical reagent grade.

Results

Characterization of gaboxadol transport via hPAT1 in vitro

The interaction between gaboxadol and hPAT1 was investigated by measuring the apical uptake of the hPAT1 substrate proline into Caco-2 cell monolayers in the presence of increasing gaboxadol concentrations (Figure 1). Gaboxadol decreased apical proline uptake in Caco-2 cell monolayers with an estimated inhibitor affinity (K_i value) of 6.6 mmol·L^{-1}. Similarly, the known PAT1 inhibitor, tryptophan, also decreased the apical uptake of proline with a K_i value of 7.7 mmol·L^{-1}. The transepithelial (A-B) flux of gaboxadol transport across Caco-2 cell monolayers was investigated at three apical concentrations (0.34, 3.5 and 7.0 mmol·L^{-1}). The mean P_{app} values of gaboxadol transport were 8.1×10^{-6} cm·s^{-1}, 6.1×10^{-6} cm·s^{-1} and 5.6×10^{-6} cm·s^{-1} for 0.34, 3.5 and

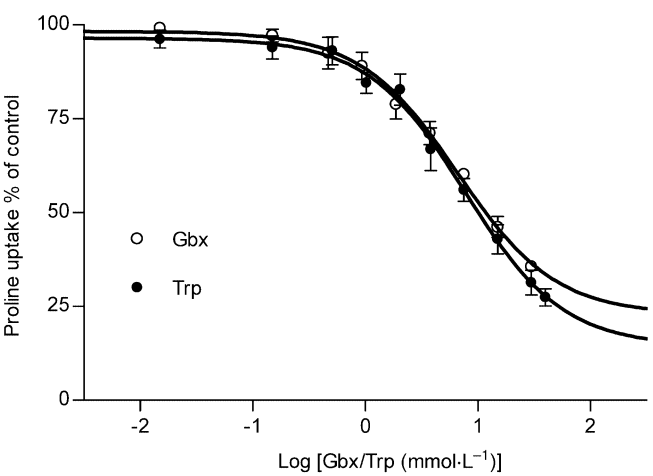

Figure 1 Inhibition of apical L-[^{3}H]proline uptake via hPAT1 by 0.015–30 mmol·L^{-1} gaboxadol (Gbx) and 0.03–40 mmol·L^{-1} tryptophan (Trp) in Caco-2 cell monolayers at days 25–28 after seeding. The apical concentration of L-[^{3}H]proline was 12.5 nmol·L^{-1} (1 μCi·mL^{-1}). Uptake of L-proline was measured for 5 min with an apical pH of 6.0 and a basolateral pH of 7.4. Values are means ± standard error of the mean (SEM) of 3–4 independent cell passages. Fifty percent inhibitory concentration (IC_{50}) values (pIC_{50} ± SEM) gaboxadol 6.6 mmol·L^{-1} (2.18 ± 0.08), tryptophan 7.7 mmol·L^{-1} (2.11 ± 0.08).

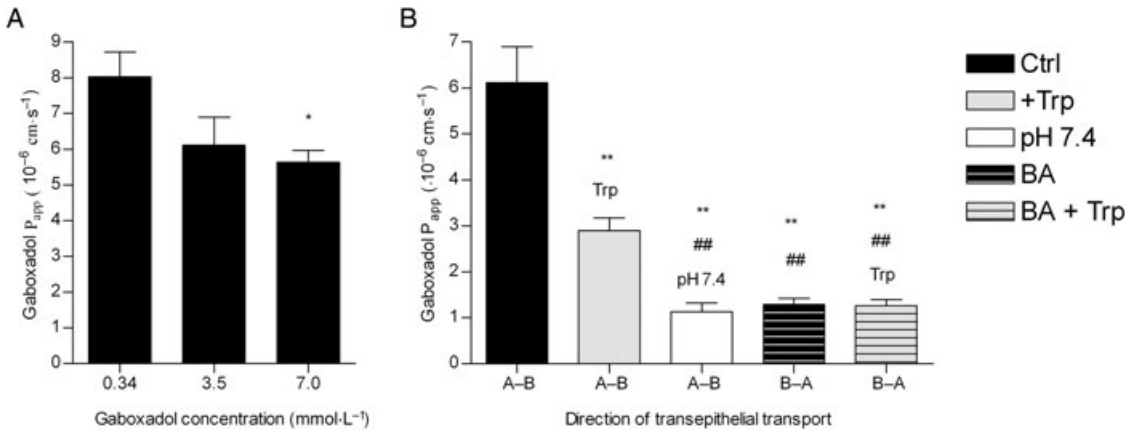

Figure 2 Transepithelial transport of 0.34, 3.5 and 7 mmol·L^{-1} gaboxadol across Caco-2 cell monolayers. (A) Apparent permeability coefficients, P_{app}, calculated from flux data (A–B) shown as a function of apical gaboxadol concentration. The apical pH was 6.0 and basolateral pH was 7.4. Statistically significant difference from the P_{app} of 0.34 mmol·L^{-1} gaboxadol observed by Student's *t*-test, *$P < 0.05$. (B) P_{app} of 3.5 mmol·L^{-1} gaboxadol at various conditions. P_{app} of 3.5 mmol·L^{-1} gaboxadol was measured in absence or presence of 35 mmol·L^{-1} tryptophan (Trp) at an apical pH of 6.0 and basolateral pH of 7.4 or at pH 7.4 at both sides. Each data point represents the mean ± standard error of the mean obtained in 3–4 independent cell passages. Statistically significant difference observed by Student's *t*-test: difference from 3.5 mmol·L^{-1} gaboxadol (A-B), **$P < 0.005$ or difference from 3.5 mmol·L^{-1} gaboxadol in presence of tryptophan (Trp; A–B), ##$P < 0.005$.

7 mmol·L^{-1} gaboxadol, respectively (Figure 2). Thus, the gaboxadol permeability across Caco-2 cell monolayers decreased with increasing gaboxadol concentrations ($P < 0.05$). The gaboxadol transport across Caco-2 cell monolayers using an apical concentration of 3.5 mmol·L^{-1} gaboxadol was investigated in the presence of 35 mmol·L^{-1} tryptophan, with or without a pH gradient, and bidirectional (Figure 2). Transport of gaboxadol in the A–B direction was approximately five times higher than in the B–A direction ($P < 0.005$). The presence of tryptophan reduced the permeability of gaboxadol by 53% (to a P_{app} of 2.9×10^{-6} cm·s^{-1}, $P < 0.005$). In the absence of a proton gradient across the monolayer, the permeability of gaboxadol was reduced by 82% to 1.1×10^{-6} cm·s^{-1} ($P < 0.005$). Gaboxadol permeability in the presence of tryptophan, in absence of a proton gradient, and in the B–A direction was similar to the permeability of [^{3}H]-mannitol (P_{app} of $1.6 \pm 0.36 \times 10^{-6}$ cm·s^{-1}). Furthermore, the presence of gaboxadol and tryptophan in the transport experiments did not change the permeability of metoprolol or mannitol transport across Caco-2 cell monolayers. The permeabilities of mannitol and metoprolol were $1.6 \pm 0.36 \times 10^{-6}$ cm·s^{-1} and $6.9 \pm 0.99 \times 10^{-6}$ cm·s^{-1}, respectively. In summary, the transepithelial gaboxadol transport across Caco-2 cell monolayers was pH-dependent, could be inhibited by tryptophan and was polarized in the A-B direction. Together these observations suggest that hPAT1 mediates gaboxadol transport across the luminal membrane of human intestinal epithelial cells, and that this transport step to a large degree determines the resulting transepithelial transport of gaboxadol.

Pharmacokinetic analysis of oral gaboxadol absorption in dog

Gaboxadol plasma concentration profiles following oral or intravenous administration of 2.5 mg·kg^{-1} gaboxadol in beagle dogs were monitored over 10 h (Figure 3). The bioavailability, F_a, of gaboxadol following oral administration in dog was high (over 80%) (Table 1). Oral co-administration of 2.5–150 mg·kg^{-1} tryptophan did not change the AUC of gaboxadol significantly, and the mean relative bioavailability of the formulations varied between 75 (10 mg·kg^{-1} tryptophan) and 86.1% (2.5 mg·kg^{-1} tryptophan). Also, the elimination rate constant (k_e) and the clearance (CL) of gaboxadol did not change with co-administration of tryptophan. However, co-administration of 150 mg·kg^{-1} tryptophan decreased the maximal gaboxadol plasma concentration, C_{max}, from 2502 to 1419 ng·mL^{-1}, that is, 57%. Furthermore, the time required to reach the maximal plasma concentration, T_{max}, was increased from 0.46 h to 1.5 h ($P < 0.01$). The C_{max} values of the five dose groups were subsequently fitted to a dose-response curve (Figure 4), which indicated a direct interaction between gaboxadol absorption and tryptophan concentration. The *in vivo* IC_{50} value of tryptophan on gaboxadol C_{max} was estimated to be 12.6 mg·kg^{-1}, which is equivalent to a concentration of 12.3 mmol·L^{-1} tryptophan (not corrected for dilution in gastric and intestinal fluids).

Absorption rate constants of gaboxadol and paracetamol

Co-administration of increasing tryptophan doses gradually changed the mean cumulative fraction of absorbed gaboxadol as seen in the deconvolution profiles in Figure 5A. The absorption of gaboxadol in the presence of 150 mg·kg^{-1} tryptophan was significantly decreased at time points 0.5–1.25 h compared with the absorption of gaboxadol alone. An oral dose of 91.5 ± 3.3% of paracetamol was absorbed after 60 min (Figure 5B) indicating that gastric emptying happens mainly within the first hour after administration. Co-administration of 150.0 mg·kg^{-1} of tryptophan did not significantly change the gastric emptying rate, as the fraction of absorbed paracetamol in the absence or presence of tryptophan was not significantly different at the time points tested. The pharmacokinetic parameters T_{max}, AUC and CL of plasma paracetamol concentrations were not significantly different from parameters obtained after co-administration of paracetamol and

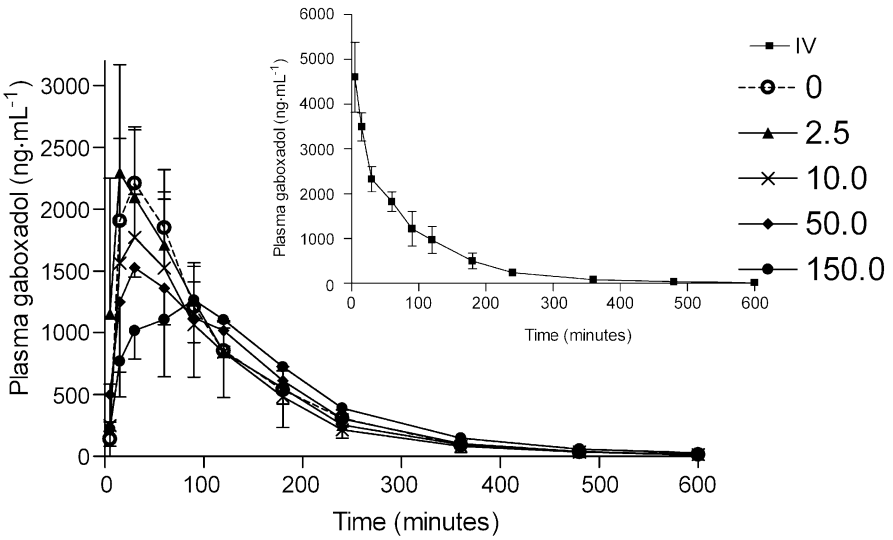

Figure 3 Plasma concentration versus time profiles after intravenous (insert) or oral dosing of gaboxadol to male beagle dogs during 10 h. The dogs received 2.5 mg·kg^{-1} gaboxadol intravenously or orally (2.5 mg·kg^{-1}; 3.5 mmol·L^{-1}) with tryptophan. Tryptophan was given as a co-administration together with gaboxadol. Data shown are means ± standard error of the mean (n = 6).

Table 1 Pharmacokinetic parameters of gaboxadol in male beagle dogs

Group	*A (IV)*	*B*	*C*	*D*	*E*	*F*
Tryptophan (mg·kg^{-1})	0	0	2.5	10	50	150
k_e (h^{-1})	1.02 ± 0.14	0.46 ± 0.02	0.50 ± 0.03	0.44 ± 0.03	0.50 ± 0.03	0.48 ± 0.07
AUC (h·ng·mL^{-1})	5618 ± 377	4715 ± 248	4760 ± 350	4032 ± 339	4294 ± 211	4405 ± 801
T_{max} (h)	–	0.46 ± 0.12	0.35 ± 0.13	0.54 ± 0.15	0.46 ± 0.12	1.50 ± 0.39**
C_{max} (ng·mL^{-1})	5489 ± 404	2502 ± 43	2473 ± 178	1868 ± 114**	1662 ± 37***	1419 ± 161***
Clearance (mL·h^{-1}·kg^{-1})	456 ± 32	538 ± 29	537 ± 34	645 ± 59	589 ± 26	700 ± 152
F_a	–	85.3 ± 5.7	86.1 ± 6.7	75.0 ± 10.4	78.2 ± 6.3	79.7 ± 14.5

Pharmacokinetic parameters of gaboxadol in male beagle dogs after intravenous (A) and oral (B–F) administration of 2.5 mg·kg^{-1} gaboxadol co-administered with increasing amounts of tryptophan. Results are expressed as mean ± standard error of the mean (n = 6).
**P < 0.01, ***P < 0.001, significantly different; one-way ANOVA followed by multiple comparisons versus control group.

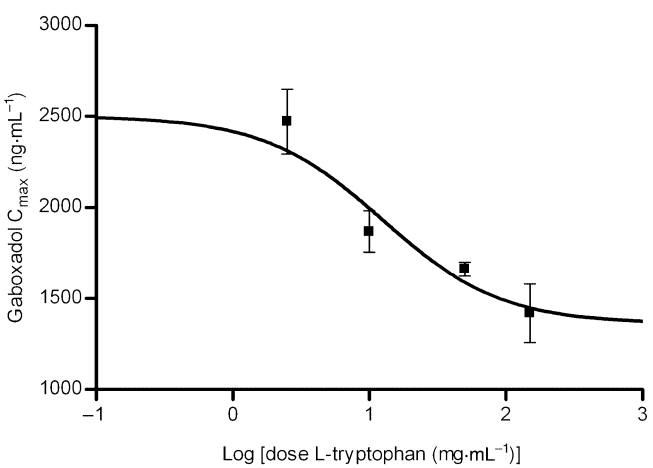

Figure 4 Dose-response curves for the maximal plasma concentration (C_{max}) of gaboxadol after co-administration of an oral solution of 2.5 mg·kg^{-1} gaboxadol and increasing concentrations of tryptophan. Maximum of the curve is the mean C_{max} (2502 ng·mL^{-1}) of the control group that was dosed with gaboxadol alone. Each point is determined separately as mean ± standard error of the mean in six dogs.

tryptophan (results not shown). Based on the profiles shown in Figure 5A, the absorption rate constant, k_a, of gaboxadol were calculated and these are depicted as a function of the logarithmic tryptophan dose in Figure 6A. The k_a of gaboxadol was decreased by co-administration of tryptophan with an *in vivo* IC_{50} value on gaboxadol absorption of 10.3 mg·kg^{-1}, which corresponds to an oral solution with a concentration of 10.1 mmol·L^{-1} tryptophan. Figure 6B shows that 150 mg·kg^{-1} of the PAT1 inhibitor tryptophan significantly decreased the absorption rate constant of gaboxadol (P < 0.01), whereas it had no significant effect on the absorption rate constant of paracetamol.

Discussion and conclusions

The high oral bioavailability of gaboxadol was shown decades ago (Schultz *et al.*, 1981). However, the reason for the high intestinal permeability of gaboxadol, despite its unfavourable physiochemical properties, has never been investigated. The interaction of PAT1 with other structurally similar GABA analogues (Larsen *et al.*, 2008) led to the hypothesis investigated

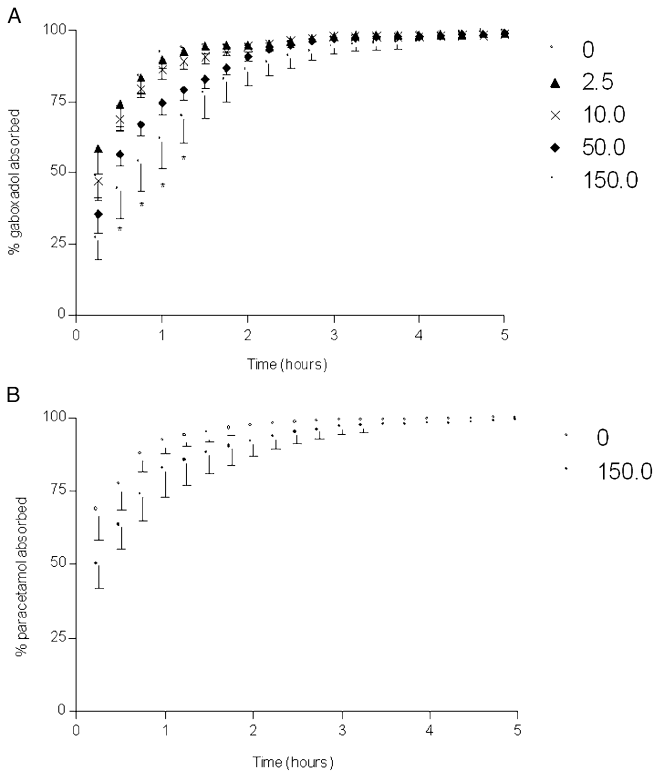

Figure 5 Deconvolution profiles of gaboxadol absorption (A) and paracetamol absorption (B; gastric emptying) following oral administration. (A) The dogs received orally gaboxadol (5.0 mL·kg^{-1}, 2.5 mg·kg^{-1}) together with 0–150 mg·kg^{-1} tryptophan given as a co-administration. (B) The dogs received 2.5 mg·kg^{-1} of gaboxadol with 50 mg·kg^{-1} of paracetamol with or without tryptophan. Data points are mean ± standard error of the mean (n = 6). Significantly different *, $P < 0.05$ using Student's t-test.

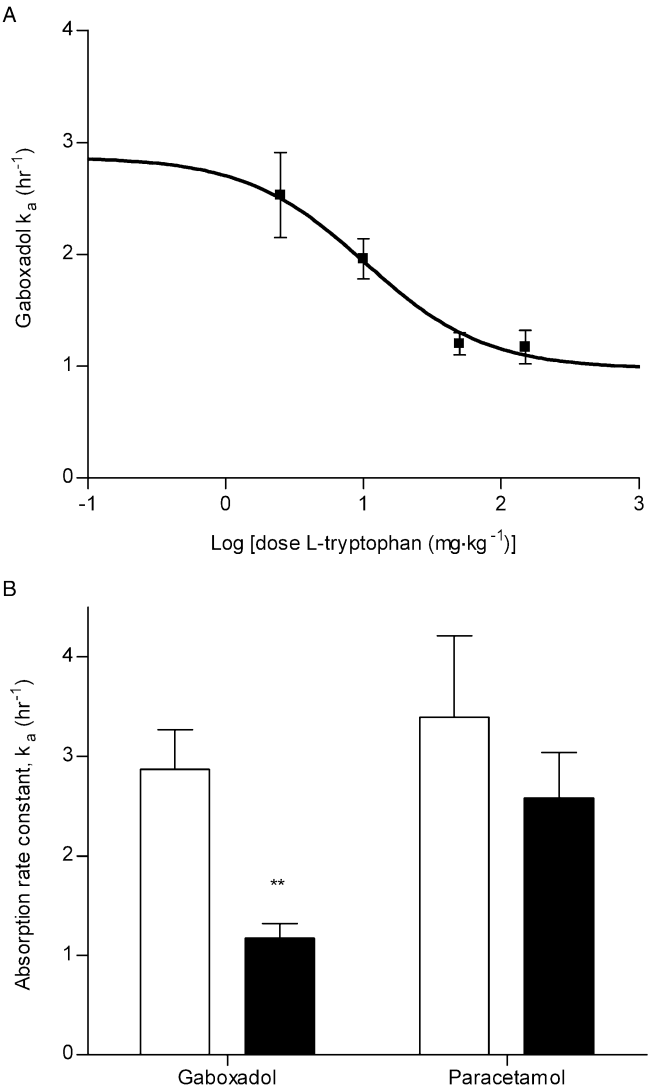

Figure 6 (A) Dose-response curves for the estimated absorption rate constant (k_a) of gaboxadol after co-administration of an oral solution of 2.5 mg·kg^{-1} gaboxadol with increasing concentrations of tryptophan. The maximum of the fitted curve is the mean k_a at zero tryptophan concentration, 2.87%·h^{-1}. (B) Absorption rate constants, k_a, of gaboxadol and paracetamol in the absence or presence of 150 mg·kg^{-1} tryptophan. Each point is determined separately as mean ± standard error of the mean in six dogs.

in the present study, namely that PAT1 transport proteins could be involved in the intestinal absorption of gaboxadol. The present study suggests for the first time the involvement of the proton-coupled amino acid transporter PAT1 in the intestinal absorption of gaboxadol *in vitro* and *in vivo*. Based on this, a formulation approach was used to reduce gaboxadol absorption rates in order to decrease peak plasma concentrations. The present study is to our knowledge the first report, where PAT1 has been shown to be relevant in the *in vivo* absorption of a drug.

Gaboxadol is a substrate for the proton-coupled amino acid transporter, hPAT1, in Caco-2 cell monolayers

Gaboxadol inhibited the apical uptake of the hPAT1 substrate proline in Caco-2 cell monolayers with a K_i value of 6.6 mmol·L^{-1}. This affinity is comparable with those recently observed for other hPAT1 substrates such as GABA (3.1 mmol·L^{-1}) and the GABA analogues muscimol (1.7 mmol·L^{-1}) and THPO (11.3 mmol·L^{-1}) (Larsen *et al.*, 2008). The affinity of tryptophan was found to be quite similar to gaboxadol, that is, 7.7 mmol·L^{-1}. Metzner *et al.* (2005) previously characterized tryptophan as an inhibitor of PAT1, and reported a K_i value of 4.7 mmol·L^{-1} for proline uptake via hPAT1 in Caco-2 cells. Considering minor differences in the proline affinities for hPAT1 between the two laboratories; Metzner *et al.* reports a K_t of 1.4 mmol·L^{-1} whereas Larsen *et al.* reports a K_m of 3.6 mmol·L^{-1}, respectively, the affinities of tryptophan for hPAT1 are quite comparable between the two studies (Metzner *et al.*, 2005; Larsen *et al.*, 2008). In accordance with results published by other groups (Thwaites *et al.*, 1993; Metzner *et al.*, 2005), we found that the majority of apical proline transport in Caco-2 cells was mediated by hPAT1, and no evidence of other sodium-dependent or sodium-independent transporters of proline was observed (Larsen *et al.*, 2008). Other amino acid transporters such as the apical sodium-dependent amino acid transporters B^0 (B^0AT1), $B^{0,+}$ ($ATB^{0,+}$) and system (ASC) (ASCT2)

are not likely to be involved in the transport of gaboxadol. They are all present in Caco-2 cells but their translocation of substrate is not proton-coupled. Furthermore, these transporters are characterized by higher affinity values for their substrates than observed for PAT1, for example, ASC (ASCT2), approximately 100 μmol·L^{-1} (Uchiyama *et al.*, 2005); B^0 (B^0AT1), 500–700 μmol·L^{-1} (Broer *et al.*, 2004) and $B^{0,+}$ ($ATB^{0,+}$), approximately 150 μmol·L^{-1} (Hatanaka *et al.*, 2002).

The transepithelial transport of gaboxadol across Caco-2 cell monolayers was polarized in the apical to basolateral direction. Gaboxadol transport could be inhibited by tryptophan and was dependent on the pH of the apical donor solution. Furthermore, the P_{app} of gaboxadol in the apical–basolateral direction decreased with increasing gaboxadol concentration. This is consistent with transport of gaboxadol via hPAT1, and this pathway accounted for approximately 80% of the total transepithelial transport. The amino acid transport system $b^{0,+}$ has been identified in the small intestine of mouse and in Caco-2 cells, where it accounts for 15–85% of the total transport of alanine and arginine respectively (Wenzel *et al.*, 2001; Dave *et al.*, 2004). However, tryptophan binding to system $b^{0,+}$ has not been unequivocally shown (Su *et al.*, 1992; Tate *et al.*, 1992), and furthermore cationic amino acids, zwitterionic amino acids and cystine have μmol·L^{-1} affinities for system $b^{0,+}$ (Palacin, 1994). Therefore, if gaboxadol is transported via system $b^{0,+}$ to any significant degree, it should be evident in Caco-2 cells. *In vitro* the transepithelial gaboxadol transport was furthermore pH-dependent, and PAT1 is the only currently known proton-coupled amino acid transporter in the intestine. The permeability of gabapentin (also a zwitterionic γ-amino analogue) in rat small intestine was shown to be proton independent (Nguyen *et al.*, 2007), hence different apical transport mechanisms exist for gaboxadol and gabapentin. The results indicate that gaboxadol is a substrate for hPAT1 in Caco-2 cell monolayers, and that hPAT1 mediates gaboxadol transport across the luminal membrane of the intestinal enterocytes, which appear to be important for the resulting transepithelial transport. The mechanism for gaboxadol efflux across the basolateral membrane is still unknown.

In vivo *absorption of gaboxadol in dogs*

The *in vivo* absorption of gaboxadol in dogs occurred rapidly following oral administration with a T_{max} of approximately 0.46 h and a high bioavailability of 85%. These observations are consistent with previous studies on the oral absorption in humans showing a gaboxadol T_{max} of approximately 0.5 h and a bioavailability >90% (Schultz *et al.*, 1981; Lund *et al.*, 2006). Once absorbed, gaboxadol is mainly excreted in the urine in the form of gaboxadol, whereas a minor fraction is excreted in the form of a glucuronic acid conjugate comprising of 2–7% in rat and mouse and 30–35% in two human subjects (Schultz *et al.*, 1981; Lund *et al.*, 2006; Shadle *et al.*, 2006). Collectively, this indicates that in dogs gaboxadol is quickly and completely absorbed, probably in the proximal small intestine, with a minimal post-absorptive metabolism.

In vivo *absorption of gaboxadol following co-administration of tryptophan*

Co-administration of the hPAT1 inhibitor tryptophan had a dose-dependent effect on the absorption profile of gaboxadol resulting in a decreased C_{max} and an increased T_{max}. A reduction in the gaboxadol absorption rate could be caused by an alteration of the gastric emptying rate. In humans, the rate of gastric emptying decreases as a function of the number of calories in an ingested meal (Calbet and MacLean, 1997; Sunesen *et al.*, 2005), and also in dogs the composition of meals has been shown to prolong gastric emptying (Mizuta *et al.*, 1990). To rule out that the observed effect on gaboxadol absorption was a result of altered gastric emptying, the cumulative absorption curve of paracetamol, which is often used as a marker of gastric emptying (Calbet and MacLean, 1997; Sunesen *et al.*, 2005), was investigated in the presence of tryptophan. The gastric emptying of paracetamol was not significantly affected by high doses of tryptophan. However, a significant effect of tryptophan on the k_a of gaboxadol was observed. As co-administration of tryptophan changed gaboxadol T_{max}, C_{max} and k_a whereas the F_a, k_e and the AUC were constant, the effect of tryptophan is likely to be a result of interaction between tryptophan and gaboxadol at the site of absorption, and not due to changes in gastric emptying. From other studies it is known that gaboxadol has a low degree of plasma protein binding and is not metabolized by cytochrome P-450s (Lund *et al.*, 2006). Thus, on the basis of the findings *in vitro* suggesting that hPAT1 mediates the majority of the luminal gaboxadol transport in Caco-2 monolayers, it seems likely that also the *in vivo* observation can be explained by a PAT1-mediated gaboxadol absorption in dogs, which is reduced by the co-administration of tryptophan. The observed *in vivo* affinity values of tryptophan inhibition of gaboxadol intestinal transport were estimated from either the effect of tryptophan on gaboxadol C_{max} or on the intestinal absorption rate constant, k_a. This gave IC_{50} values of 10.1 and 12.6 mmol·L^{-1}, respectively. As discussed earlier, the *in vitro* affinity of hPAT1 for tryptophan measured as inhibition of proline transport via hPAT1 was 7.7 mmol·L^{-1} in Caco-2 cells. The IC_{50} values are surprisingly close to each other considering that tryptophan inhibition is measured for two different compounds (proline and gaboxadol), and that the *in vivo* transport is composed of not only luminal transport but appearance in the systemic circulation, which encounters the transfer of gaboxadol across several membranes. hPAT1 substrates are characterized by having affinities in the millimolar range, and the transporter, which is expressed along the entire intestinal tract, has a high capacity (Chen *et al.*, 2003). The molar dosing ratio between gaboxadol and tryptophan was up to 1:41, and as they both have comparable affinities for hPAT1, the reduced C_{max} and k_a may be due to the competitive interaction between gaboxadol and tryptophan at the site of absorption, that is, at the PAT1 protein in the luminal membrane of the small intestinal enterocytes. The maximal plasma concentration thus occurs with a prolonged T_{max}, but due to the excessive capacity and the intestinal expression of PAT1, the absorption fraction remains unchanged as the absorption proceeds along the length of the intestine. The peak plasma concentration of gaboxadol may thus be reduced by modifying the absorption process as illustrated here, or through a

more classical sustained release formulation approach as suggested earlier (Kjaer and Nielsen, 1983).

In conclusion, the present study shows for the first time that the high permeability of gaboxadol across Caco-2 cell monolayers is most likely due to PAT1-mediated transport across the luminal membrane resulting in a high transepithelial transport. The *in vitro* gaboxadol transport kinetics and the pharmacokinetics observed in dogs support the conclusion that PAT1 mediates transport of gaboxadol across the mucosal membrane both *in vitro* as well as *in vivo*. In addition, the present study indicates that it is possible to exploit transporter activity in order to modify or control the intestinal absorption of drug substances. The formulation design provides a simple basis for decreasing peak plasma concentration of gaboxadol, while maintaining a high bioavailability. This may aid in reducing side effects related to high plasma peak concentrations.

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Conflict of interest

The authors state no conflict of interest.

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