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RESEARCH PAPER

Function and expression of the proton-coupled amino acid transporter PAT1 along the rat gastrointestinal tract: implications for intestinal absorption of gaboxadol

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BACKGROUND AND PURPOSE

Intestinal absorption via membrane transporters may determine the pharmacokinetics of drug compounds. The hypothesis is that oral absorption of gaboxadol (4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridine-3-ol) in rats occurs via the proton-coupled amino acid transporter, rPAT1 (encoded by the gene *rSlc36a1*). Consequently, we aimed to elucidate the *in vivo* role of rPAT1 in the absorption of gaboxadol from various intestinal segments obtained from Sprague-Dawley rats.

EXPERIMENTAL APPROACH

The absorption of gaboxadol was investigated following its administration into four different intestinal segments. The intestinal expression of *rSlc36a1* mRNA was measured by quantitative real-time PCR. Furthermore, the hPAT1-/rPAT1-mediated transport of gaboxadol or L-proline was studied in hPAT1-expressing *Xenopus laevis* oocytes, Caco-2 cell monolayers and excised segments of the rat intestine.

KEY RESULTS

The absorption fraction of gaboxadol was high (81.3–91.3%) following its administration into the stomach, duodenum and jejunum, but low (4.2%) after administration into the colon. The pharmacokinetics of gaboxadol were modified by the co-administration of L-tryptophan (an hPAT1 inhibitor) and L-proline (an hPAT1 substrate). The *in vitro* carrier-mediated uptake rate of L-proline in the excised intestinal segments was highest in the mid jejunum and lowest in the colon. The *in vitro* uptake and the *in vivo* absorption correlated with the expression of *rSlc36a1* mRNA along the rat intestine.

CONCLUSIONS AND IMPLICATIONS

These results suggest that PAT1 mediates the intestinal absorption of gaboxadol and therefore determines its oral bioavailability. This has implications for the *in vivo* role of PAT1 and may have an influence on the design of pharmaceutical formulations of PAT1 substrates.

Abbreviations

C_{max}, maximal plasma concentration; C_T, cycle threshold; F_a, fraction of absorption; Gbx, gaboxadol; GIT, gastrointestinal tract; hPAT1, human proton-coupled amino acid transporter 1; hPEPT1, human di-/tripeptide transporter 1; k_e, elimination rate constant, MES, 2-(*N*-morpholino)ethanesulfonic acid; rGapdh, rat glyceraldehyde 3-phosphate dehydrogenase; rPAT1, rat proton-coupled amino acid transporter 1; rPEPT1, rat di-/tripeptide transporter 1; *rSlc15a1*, rat solute carrier family 15, subfamily a, member 1; *rSlc36a1*, rat solute carrier family 36, subfamily a, member 1; *SLC36A1*, human solute carrier family 36, subfamily a, member 1; TEVC, two-electrode voltage clamp; T_m, melting temperature; t_{max}, time of maximal plasma concentration

Introduction

Membrane transporters in the gastrointestinal tract (GIT) are increasingly being recognized as important for drug absorption and hence for the overall pharmacokinetic profile of drug substances (Dobson and Kell, 2008; Giacomini *et al.*, 2010; Sugano *et al.*, 2010). Moreover, investigations into the role of transporters in drug–drug and drug–food interactions in relation to the pharmacokinetic profile of new chemical entities are thus being outlined in draft guidelines of the regulatory authorities (Center for Drug Evaluation and Research, 2006; European Medicines Agency, 2010; Giacomini *et al.*, 2010). Consequently, increased knowledge on drug transporter function and expression *in vivo* is essential to improve drug safety, efficacy and to prevent unfavourable drug–drug or drug–food interactions (Naruhashi *et al.*, 2002; Sai, 2005; Choudhuri and Klaassen, 2006). However, *in vivo* investigations on the role of transporters are hugely under-investigated compared to *in vitro* investigations. Moreover, understanding the effect of a transporter on the pharmacokinetics of a drug substance *in vivo* is complex and requires information on both the expression and function of the candidate transporter.

In this study, we have investigated the intestinal expression and function of the intestinal amino acid transporter rPAT1 (*Slc36a1*). PAT1 is a proton-coupled amino acid transporter for proline and glycine present in the luminal membrane of enterocytes (Thwaites *et al.*, 1993; Anderson *et al.*, 2004). In *in vitro* settings, PAT1 has been shown to transport amino acid mimetics such as GABA (Thwaites *et al.*, 1995; 2000), vigabatrin (Abbot *et al.*, 2006), δ-aminolevulinic acid (Frolund *et al.*, 2010b) and gaboxadol (Gbx) (Larsen *et al.*, 2009). Whether this indicates that PAT1 is instrumental for intestinal absorption and bioavailability of these compounds *in vivo* remains unclear. In the case of Gbx, *in vivo* studies in dogs and rats have provided some evidence for the *in vivo* relevance of drug transport via PAT1, as oral co-administration of inhibitors of PAT1 such as L-tryptophan and 5-hydroxy-L-tryptophan reduced the absorption rate of Gbx (Larsen *et al.*, 2009; 2010).

The objectives of the present study were to investigate the absorption of Gbx from different regions of the rat GIT *in vitro* and *in vivo*, and to compare the absorption pattern to the relative expression levels of *rSlc36a1* mRNA along the GIT. We found that the absorption fraction of Gbx depends on the intestinal site of administration, with high bioavailability after administration in the proximal parts of the intestine and practically no bioavailability after colonic administration. The expression of *rSlc36a1* mRNA was highest in the middle parts of the intestine and absent in the colon. The *in vitro* uptake of L-proline into intestinal sheets showed a similar pattern. Collectively, our results suggest that PAT1 is the molecular correlate to the high oral bioavailability of Gbx, thereby illustrating an *in vivo* role of PAT1 in intestinal drug absorption.

Methods

Chemicals and reagents

The nomenclature used for the transporters in the present study follows that presented in the *BJP* Guide to Receptors and Channels (GRAC), 5th Edition (Alexander *et al.*, 2011). The chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Gaboxadol 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) used as internal standard were synthesized by H. Lundbeck A/S (Valby, Denmark). [2,3,4,5-^{3}H]-L-Proline (80 Ci·mmol^{-1}) was purchased from Larodan (Malmö, Sweden). All chemicals were of analytical reagent grade.

Rat husbandry

Male Spraque-Dawley rats were obtained from Charles River (Sülzfeld, Germany). The animals were acclimatized to the laboratory conditions for at least 5 days prior to the experiments and the animals had access to food and water *ad libitum*. The animals were housed, with 2-4 animals per cage, on a wooden bedding (Tapvei, Kortteinen, Finland) in plastic cages, 595x380x200 mm, with a stainless-steel grid (Scanbur, Sollentuna, Sweden) in an air-conditioned building with controlled environmental parameters (relative humidity 50 ± 10%, temperature 20 ± 18°C, light 06:00-18:00 h). The total number of animals used in the study was 74 of which 64 were used for the pharmacokinetic evaluation, 6 for mRNA expression and 4 for uptake into intestinal sheets. Experimental protocols were approved by the Animal Welfare Committee, appointed by the Danish Ministry of Justice, and all animal procedures were carried out in compliance with EC Directive 86/609/EEC, with the Danish law regulating animal experiments and the NIH guidelines. The weight of the animals at the day of administration or tissue sample isolation was 260–320g. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Operation of animals

A 20 cm BPU-T20 tubing (Instech Laboratories, Plymouth Meeting, PA, USA) was used as intestinal cannula. Two stop-

pers, of 2 mm 20G feeding tube (Instech Laboratories), were placed 1 and 17 cm from the intestinal end and sealed by cyanoacrylat (Dana lim, Køge, Denmark). Furthermore, a silicone stopper was placed 6 cm from the intestinal end of the cannula. The animals were anaesthetized with ~4% isoflurane/oxygen and the abdominal skin shaved, followed by disinfection with 96% ethanol and 1% iodine. The animals were placed on a heating mat under ~2% isoflurane/oxygen anaesthesia and covered with sterile drape. Buprenorphine 0.05 mg·kg^{-1} (Temgesic, Schering-Plough, Hull, UK), carprofen 5.0 mg·kg^{-1} (Rimadyl, Pfizer, Dundee, UK) and enrofloxacin 10.0 mg·kg^{-1} (Baytril, Bayer, Kiel, Germany) in sterile isotonic NaCl were administered s.c. pre-operatively to the animals. The level of general anaesthesia and body temperature was monitored continuously during the procedure; anaesthesia was monitored by the assessment of respiratory frequency, depth of respiration and signs of pain reaction after pinching between the toes. A 5 cm incision was made in the ventral abdominal midline of the skin. The intestines were cannulated: (i) 1 cm from pylorus in the duodenum; (ii) in jejunum approximately 28 cm from pylorus or (iii) in the colon 1 cm below the caecum. For the jejunal cannula, the large artery to the caecum was located and five large mesenteric arterial branches were counted in the oral direction (ie proximal or towards the stomach). The catheter was placed anti-mesenterically (opposite side of the mesentery border) just after the fifth arterial branch. At each position, the intestinal wall was punctured by a 23G needle and the cannula fastened to the intestine with a purse string suture. The intestines were gently placed *in situ* in the abdominal cavity and the catheter sutured to the muscle next to the xiphoid process. Peritoneum and muscle layers were closed with a running suture (Vicryl 5-0, Ethicon, Cornelia, GA, USA) and skin by a running everted subcuticular technique (Prolene 5-0, Ethicon). A pean was tunnelled s.c. from the xiphoid process towards a 1.5 cm incision in the neck and guided out. The second stopper was anchored to the subcutis in the neck and the skin closed by interrupted knots (individual sutures). The catheter was flushed with sterile NaCl and closed by melting. The animal's well-being and the efficacy of the s.c. carprofen (5.0 mg·kg^{-1}) pain relief were monitored twice daily and additional analgesics given if needed. The recovery period after the surgical procedure was 2.5–3 days.

Pharmacokinetic study

Before entry to the pharmacokinetic experiment, both the operated and the non-operated animals were deprived of food for approximately 16–20 h and randomly assigned to receive one of the treatments. All animals received Gbx 10.0 mg·kg^{-1}. Half of the animals were dosed with the Gbx in a solution that also contained 100.0 mg·kg^{-1} L-tryptophan and 100.0 mg·kg^{-1} L-proline as a co-administration. The Gbx solutions for administration in the GIT contained 1.0 mg·mL^{-1} (7.1 mM), whereas the i.v. solutions contained 2.0 mg·mL^{-1}. The gastrointestinal concentrations of L-tryptophan and L-proline were 49.0 and 86.9 mM, respectively. The nine dose groups consisted of four to seven animals (Figure 1A–D and Table 1). In a further study, six rats were dosed i.v. with Gbx and four rats with both Gbx and 100.0 mg·kg^{-1} L-tryptophan and 100.0 mg·kg^{-1} L-proline i.v. (Figure 1E). All solutions were adjusted to pH 5.2 before the osmolarity was checked on a Vapro vapor pressure osmometer (model 552O, Wescor Inc., Logan, UT, USA) and adjusted with NaCl to iso-osmolarity. The oral solutions were administered into the stomach by gavage. The i.v. bolus was injected in a tail vein, whereas the duodenal, jejunal and colonic bolus were administered via the neck. The blood samples were obtained by individual vein puncture and collected into 0.2 mL Eppendorf tubes with 20 I.E. heparin. Ten samples were collected in the time period of 5 min to 8 h after administration. The samples were handled and stored as described previously (Larsen *et al.*, 2010). The animals were killed by administration of CO_2 after 8 h.

Intestinal tissue preparation

Rats were anaesthetized and operated on as described above. Approximately 1 cm of the intestine was removed for every 5 cm (for the mRNA expression analysis) or 10 cm (for the transport function assay) along the proximal-distal axis of the intestine between the duodenum and the colon. From the colon, three segments were sampled: one from 1 cm after the caecum, one from the middle and finally one from the rectum. During the isolation of intestinal segments, the remaining part of the intestine was kept alive by keeping it moistured and vascularized. The ectomized segments were transferred to ice-cold Ringer buffer and cleaned by carefully removing the fat and veins with a pair of tweezers. Thereafter, the segments were cut open lengthwise and shaken until food content became detached. For the *in vitro* transport assay, the open segment was cut into smaller samples of 50–150 mg and quickly transferred to incubation medium (see next section). For the RNA analysis, samples of mucosal scrapings were obtained from intestinal sheets of approximately 1 cm with a scalpel. The epithelium samples were handled and stored as described in the section *RNA isolation and reverse transcription*. At the end of the experiment, the animals were killed by decapitation.

In vitro *uptake in rat intestinal sheets*

The incubation media were HBSS buffer solutions with 10.0 mM MES/HEPES, adjusted to pH 6.0/7.4, hereafter denoted as HBSS$^+$. The buffers also contained 0.1–30.0 mM L-proline with or without 50.0 mM GABA. The osmolality of the solutions ranged from 285 to 353 mOsmol·kg^{-1}. The intestinal sheets were transferred to a 4 mL incubation chamber containing 2 mL of HBSS$^+$ buffer with 1.0 µCi·mL^{-1} [2,3,4,5-^{3}H]-L-proline. 1.0 µCi·mL^{-1} [1-^{14}C]-D-mannitol was used to correct for extracellular volume. The chamber was placed on a rotamixer at approximately 60 r.p.m. at room temperature (20–21°C) for 15 or 20 min before the tissue was washed three times with 3 mL of ice-cold HBSS$^+$. The intestinal sheets were then homogenized with zirconium oxide beads and 1 mL of HBSS, for 2 × 20 s. Radioactivity of 400 µL of homogenate was counted in scintillation vials with 4 mL of Optiphase Supermix scintillation liquid (PerkinElmer, Boston, MA, USA) in a Tri-Carb 2100 TR Liquid Scintillation Analyzer (Packard, Ramsey, MN, USA).

RNA isolation and reverse transcription

The rat mucosal scrapings were homogenized with 2.8 mm zirconium oxide beads on a Precellys 24 (Bertin Technologies, Montigny, France). The NucleoSpin® RNA/Protein kit

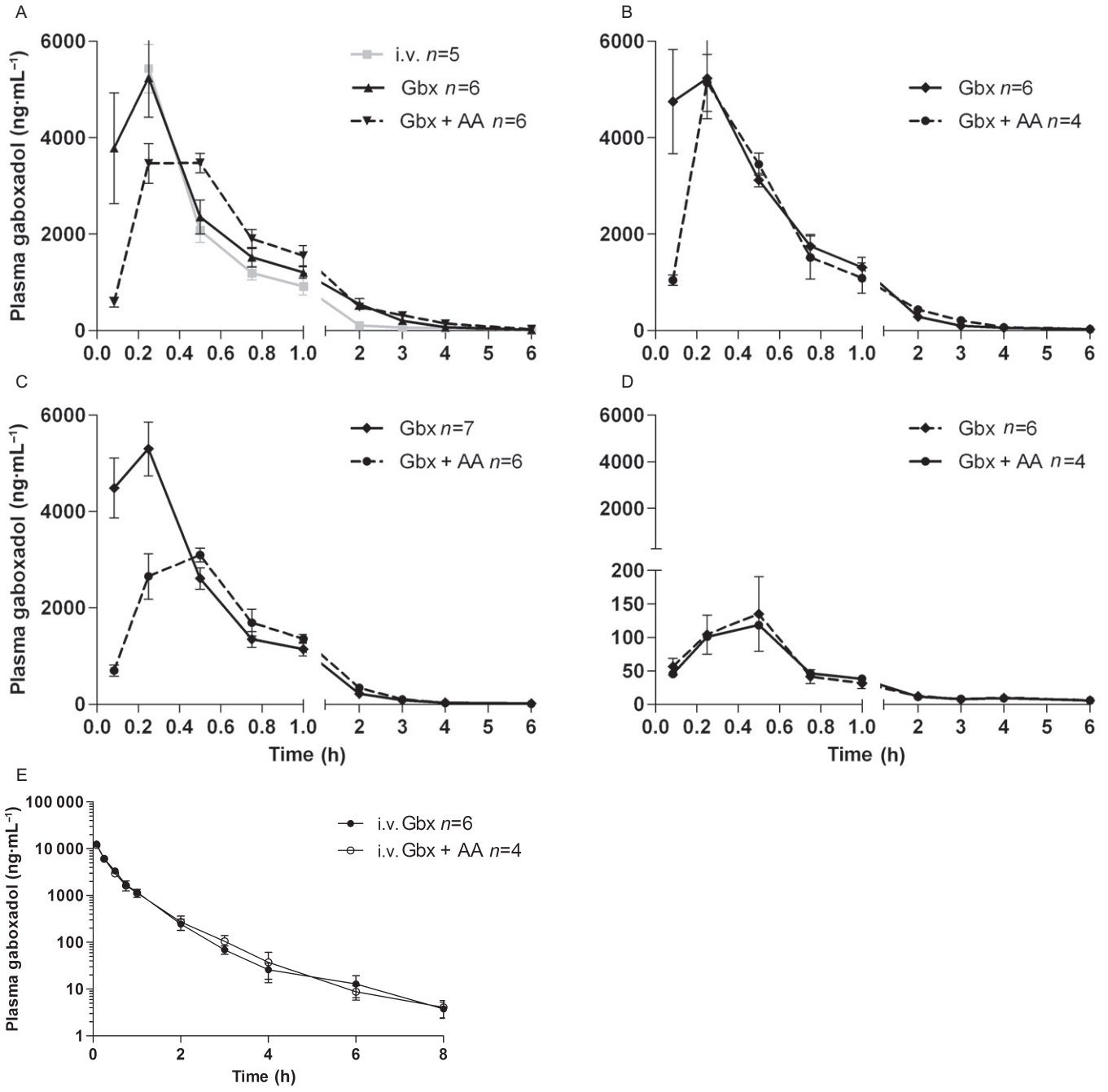

Figure 1

The plasma concentration–time profile of gaboxadol (Gbx) in male Sprague-Dawley rats after administration in different segments of the intestine. Gbx (10 mg·kg^{-1}) was in the stomach (A), duodenum (B), jejunum (C) or colon (D) in the absence and the presence of 100 mg·kg^{-1} L-tryptophan and 100 mg·kg^{-1} L-proline [amino acid (AA)]. (E) Intravenous Gbx (10 mg·kg^{-1}) administration in the presence or absence of 100.0 mg·kg^{-1} L-tryptophan and 100.0 mg·kg^{-1} L-proline. Data points represent the mean ± SEM of four to seven animals as indicated.

(Macherey-Nagel, Düren, Germany) was used to isolate the RNA according to the manufacturer's instructions. One microgram RNA was used for reverse transcription with a TaqMan® Reverse Transcription Reagents kit from Applied Biosystems (Roche Molecular Systems Inc., Branchburg, NJ, USA) according to the manufacturer's manual. First-strand synthesis was performed on 1 µg total RNA using 2.5 µM random hexamers by incubating a 100 µL sample for 10 min at 25°C, 30 min at 48°C and finally 5 min at 95°C (Christensen *et al.*, 2010).

Analysis of mRNA expression by quantitative real-time (RT)-PCR

The *rSlc36a1*, *rSlc15a1* and the rat glyceraldehyde 3-phosphate dehydrogenase (*rGapdh*) mRNA reference

Table 1

Pharmacokinetic parameters of gaboxadol (10 mg·kg^{-1}) in rats after i.v., oral (dosed in the stomach by gavage), duodenal (Duo), jejunal (Jeju) and colonic administration. Gaboxadol was given in the absence and presence of amino acids (AA); L-tryptophan and L-proline (each 100 mg·kg^{-1})

Mean ± SEM	i.v.	Oral	Oral + AA	Duo	Duo + AA	Jeju	Jeju + AA	Colon	Colon + AA
AUC (h·ng·mL^{-1})	4669 ± 385	4180 ± 342	4219 ± 227	4265 ± 310	3935 ± 252	3797 ± 207	3185 ± 170	###196 ± 18	###158 ± 30
C_{max} (ng·mL^{-1})	–	5807 ± 727	*3922 ± 250	5788 ± 888	5140 ± 589	5636 ± 533	**3337 ± 97	###137 ± 25	###139 ± 52
t_{max} (h)	–	0.19 ± 0.04	**0.42 ± 0.05	0.19 ± 0.04	0.25 ± 0.00	0.20 ± 0.03	*0.38 ± 0.06	#0.35 ± 0.06	#0.38 ± 0.07
F_a	100.0 ± 8.2	89.5 ± 7.3	90.4 ± 0.0	91.3 ± 6.6	84.3 ± 5.4	81.3 ± 4.4	a68.2 ± 3.6	a###4.2 ± 0.4	a###3.4 ± 0.6
k_e (h^{-1})	0.95 ± 0.09	0.97 ± 0.07	0.92 ± 0.06	0.93 ± 0.10	0.90 ± 0.09	0.90 ± 0.06	1.03 ± 0.06	0.76 ± 0.11	0.68 ± 0.07

Data shown are mean ± SEM of four to seven animals.
[a]Significantly different from F_a of oral dosing, $P < 0.05$ (Jeju + AA) and $P < 0.001$ (Colon and Colon + AA).
and #significantly different from dose group Oral, Duo and Jeju; $P < 0.001$ and $P < 0.05$ respectively.
* and **significantly different from the administration in the absence of AA $P < 0.05$ and 0.01 in a Student's *t*-test.

sequences for rat were identified from the NCBI's RefSeq database and gene specific primers were designed using NCBI Primer-BLAST (Christensen *et al.*, 2010). The forward and reverse primers were: *Slc36a1* [5′-TGGTTG TACCAGTCGGTGAA] and [5′-GGCCAGAACACATGTCAC AC]; *Slc15a1* [5′- CCCTGATAGCCCTTGGTACA] and [5′-ACA CAATTAGGGCAACAGCC]; and *rGapdh* [5′-AGACAGCC GCATCTTCTTGT] and [5′-CTTGCCGTGGGTAGAGTCAT]. Quantitative RT-PCR was performed on a CFX96 (Bio-Rad) using reagents from Bio-Rad Laboratories (Copenhagen, Denmark) according to the manufacturer's instructions. Quantitative PCR measurements were analysed by applying the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen, 2001; Schmittgen *et al.*, 2000). In short, the relative expression level of each cDNA was calculated by normalizing it to the expression levels of *rGapdh* cDNA in the sample, and set relative to the mean normalized expression levels of the control sample; that is, the first sample of duodenum. Each sample was quantified from triplicate determinations. The qPCR data presented are the mean ± SEM of four to six animals. The expression level of *rGapdh* mRNA, measured as C_T, was found not to vary along the intestine in the animals ($P > 0.05$). However, the expression level of both *rSlc36a1* and *rSlc15a1* mRNA varied significantly along the rat intestine ($P < 0.01$), data not shown.

In vitro *uptake of L-proline and Gly-Sar in Caco-2 cell monolayers*

The Caco-2 cell monolayers were cultured and handled as previously described (Larsen *et al.*, 2008). Cells of passages 26–28 were used on days 25–28 after seeding on Transwell™ inserts. The apical uptake of 13.3 nM [^{3}H]-L-proline or 17.8 μM [^{14}C]-glysylsarcosine (Gly-Sar), 1.0 μCi·mL^{-1} each, into Caco-2 cell monolayers was measured for 5 min in HBSS$^+$ buffer solutions. On the apical side of the cells, buffers of pH 6.0 were added, whereas buffers of pH 7.4 were kept on the basolateral side. The uptake rate of L-proline was measured in the absence and presence of 7.1 mM Gbx, 20.0 mM GABA and 5.8 mM Gly-Pro, and was quantified, as described previously (Larsen *et al.*, 2008), in three individual cell passages with two replications.

Two-electrode voltage clamp measurements

TEVC measurements were performed on *Xenopus laevis* oocytes as described previously (Frolund *et al.*, 2010a). The oocytes were injected with 9–23 nL of diluted human *SLC36A1* cRNA (2.5 μg·μL^{-1}) or water (served as negative control) and TEVC measurements were performed 4–6 days post-injection. The oocytes were voltage clamped at –60 mV and continuously perfused with Ringer's solution, pH 6.0. The compounds were dissolved in Ringer's solution and added by full bath application, while the change in membrane current was monitored.

Analytical methods

The quantification of plasma Gbx was carried out by extraction of plasma samples as described in Larsen *et al.* (2009) and a hydrophilic interaction chromatography followed by

MS/MS detection using a protocol modified from Kall *et al.* (2007).

Data analysis

The pharmacokinetic parameters were determined for 60 out of 64 animals (60/64) using Phoenix™ WinNonlin® version 6.0.0.6148 (Pharsight Corporation, Mountain View, CA, USA). The plasma concentration data from the animals given i.v. doses of the compounds were fitted to a two-compartment model, whereas a non-compartment model was used to analyse the data obtained following administration of compounds into the GIT (Larsen *et al.*, 2010). C_{max} and t_{max} were found as mean values of the animals' plasma profiles within each group. AUC was calculated by the linear trapezoidal method, where the last measured plasma concentration was extrapolated to infinity. Linear regression of 4–9 (with a mean of 6.7) data points was used to obtain the elimination rate constant, k_e. In the present study, the fraction absorbed, F_a, of Gbx was calculated for the individual animal using the equation:

$$\text{Fraction absorbed, } F_a = \left[\frac{\text{AUC}(x)}{\text{AUC(i.v.)}}\right]\left[\frac{\text{Dose(i.v.)}}{\text{Dose}(x)}\right]\times 100\%$$

where AUC(i.v.) and AUC(x) are the area under the curve for the i.v. and gastrointestinal administration of Gbx, respectively. Results are expressed as mean ± SEM of six animals.

The K_M value was estimated from the concentration-dependent inward currents measured by TEVC or the uptake into segments of rat's intestine *in vitro* after application of Gbx/L-proline. The data were fitted to the Michaelis–Menten equation:

$$I = \frac{I_{max}[S]}{K_M + [S]}$$

where I is the inward current measured relative to the current induced by 20 mM proline, I_{max} is the maximal inward current, K_M is the Michaelis–Menten constant in mM and [S] is the concentration of substrate in mM (Larsen *et al.*, 2008; Frolund *et al.*, 2010a).

Statistical analysis of data

Statistical analysis was performed in Sigma Stat version 3.5 from Systat Software Inc. (Richmond, CA, USA) or in GraphPad version 4.0.2 (La Jolla, CA, USA) using Student's unpaired *t*-test, an unpaired Welch's test or a one-way ANOVA, followed by multiple comparisons versus a control group (Holm–Sidak method) or a Tukey's multiple comparison test. $P < 0.05$ was considered significant.

Results

The absorption of Gbx depends on the site of administration in the GIT

In order to evaluate the absorption of Gbx along the rat intestine, solutions of Gbx (10 mg·kg^{-1}) were administered to Sprague-Dawley rats directly into four different segments of the GIT, as well as i.v. (Figure 1A–D). The absorption of Gbx following administration in the stomach (oral), duodenum and jejunum happened fast as the mean plasma concentrations after 5 min were above 4000 ng·mL^{-1} and the mean peak plasma concentrations of Gbx (approximately 5600–5800 ng·mL^{-1}) were reached before 15 min. The administration of Gbx in the stomach (oral), duodenum and jejunum resulted in similar plasma concentration profiles with high absorption fractions, F_a, of 81.3–91.3%. The pharmacokinetic parameters C_{max}, t_{max} and AUC were not significantly different between the groups (Table 1). Administration of Gbx in the colon, however, resulted in a strikingly different plasma concentration profile, with a mean peak plasma concentration of Gbx of only approximately 137 ng·mL^{-1}. The AUC and C_{max} were significantly decreased (both $P < 0.001$) and the F_a of Gbx when administered in the colon was only 4.2% ($P < 0.001$). The elimination rate constant, k_e, was not different between any of these dose groups. In total, this indicates that the absorption of Gbx is dependent on the intestinal segment of administration.

Co-administration of L-proline and L-tryptophan decreases the initial Gbx absorption

So as to estimate the carrier-mediated component of the intestinal absorption, Gbx was co-administered with 100 mg·kg^{-1} L-proline and 100 mg·kg^{-1} L-tryptophan. The presence of the amino acid reduced the initial absorption of Gbx after administration in the stomach; for example, 5 min after administration, the plasma concentrations were reduced to one-fifth as compared with plasma concentrations of Gbx administered in the absence of amino acids ($P < 0.05$). This was also observed after administration in the duodenum ($P < 0.05$) and the jejunum ($P < 0.001$). However, 15 min after administration, the mean plasma concentrations were still lower, 50.0 and 66.1% for the animals dosed in the jejunum ($P < 0.001$) or the stomach, respectively (Figure 1A–D), whereas plasma concentrations were similar in the absence and presence of amino acid after administration in the duodenum. The C_{max} and t_{max} of Gbx absorption changed markedly with the co-administration of amino acids (Table 1); for example, C_{max} of Gbx was reduced to 67.5 ($P < 0.05$), 88.8 and 59.2% ($P < 0.01$) of the C_{max} in the absence of amino acids for oral, duodenal and jejunal administration respectively. Furthermore, t_{max} of Gbx was approximately doubled in the presence of amino acids after oral and jejunal administration ($P < 0.05$ and $P < 0.01$). In the colon, however, the presence of amino acids did not influence the absorption of Gbx or the pharmacokinetic parameters measured. As shown in Figure 1E, the Gbx plasma profile was investigated after i.v. administration in the absence or presence of co-administration of the amino acid mixture. In these experiments, the AUC was 5874 ± 524 and 5963 ± 430 h·ng·mL^{-1} in the absence or presence of co-administered amino acids, respectively, and the corresponding elimination rate constants were 1.04 ± 0.27 and 1.10 ± 0.30 h^{-1}. This shows that the amino acid mixture does not itself affect the pharmacokinetic profile of Gbx. Moreover, co-administration of amino acids did not change the AUC or k_e observed after administration of Gbx in any of the intestinal segments. These results suggest a delay in the absorption of Gbx from the small

intestine when the rats were co-administered with L-proline and L-tryptophan.

L-Tryptophan abolish the hPAT1-mediated Gbx transport in oocytes

To evaluate whether Gbx interacts with other transporters, for example, hPEPT1, the uptake of [^{14}C]-Gly-Sar as well as [^{3}H]-L-proline was measured in Caco-2 cell monolayers. As expected, 7.1 mM Gbx and 20 mM GABA decreased the uptake of L-proline by 30.8 and 54.3%, respectively ($P < 0.0001$, Figure 2A). Gbx had no significant influence on the Gly-Sar uptake rate (Figure 2B), whereas Gly-Pro was able to inhibit the uptake of Gly-Sar ($P < 0.0001$). That Gbx is a translocated substrate of hPAT1 was shown directly for the first time by the ability of Gbx to induce an inward current in

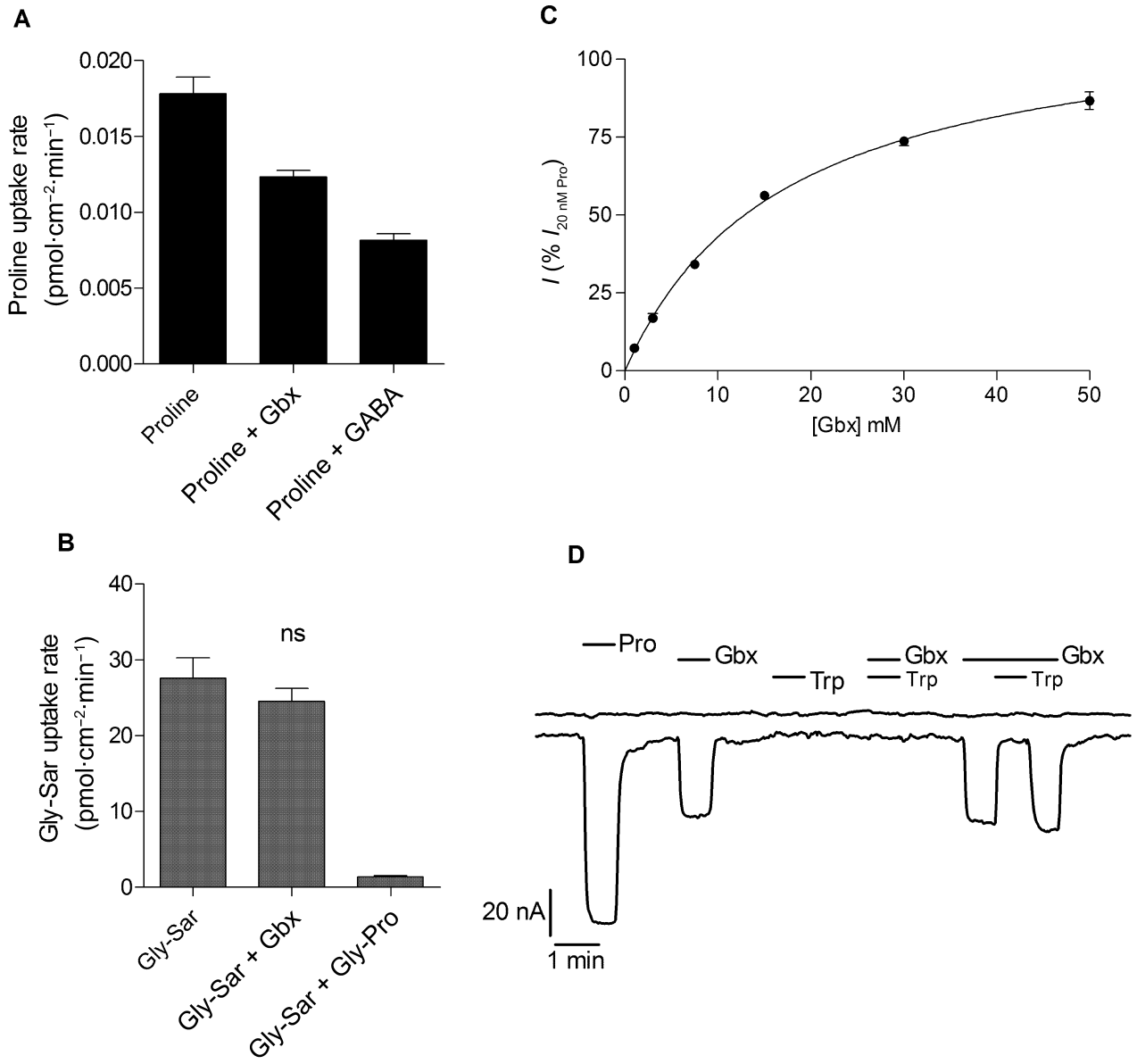

Figure 2

The uptake rate of (A) [^{3}H]-L-proline and (B) [^{14}C]glysylsarcosine (Gly-Sar) into Caco-2 cell monolayers was studied in the absence or presence of 7.1 mM gaboxadol (Gbx), 20.0 mM GABA and 5.8 mM glycyl-L-proline (Gly-Pro). The uptake rate of L-proline was significantly decreased by gaboxadol and GABA, $P < 0.0001$. The uptake rate of Gly-Sar was decreased by Gly-Pro, $P < 0.0001$, but not by gaboxadol (ns). The bars show mean ± SEM of 2 repetitions in three cell passages. (C) Concentration-dependent hPAT1-mediated current induced by gaboxadol in *Xenopus laevis* oocytes. The current was measured relative to the current induced by 20.0 mM proline. Each data point represents the mean ± SEM (n = 7). (D) Representative traces showing the inward current obtained after application of 8.7 mM L-proline (Pro), 7.1 mM gaboxadol, 49.0 mM L-tryptophan or gaboxadol and L-tryptophan. Upper trace: water injected *X. laevis* oocyte; lower trace: human *SLC36A1* cRNA injected *X. laevis* oocyte. The pH was maintained at 6.0. The traces from at least six different oocytes showed similar results.

human *SLC36A1* cRNA-injected *X. laevis* oocytes (Figure 2C and D). A Michaelis–Menten fit of the concentration-dependent hPAT1-mediated current provided a K_m value of 17.1 ± 1.4 mM (mean ± SEM). A representative trace of the hPAT1-mediated current is shown for both L-proline and Gbx (Figure 2D). To confirm that L-tryptophan is an efficient inhibitor of PAT1-meditated transport, L-tryptophan was applied alone or together with Gbx. More interestingly, complete inhibition of the Gbx-induced current was observed when L-tryptophan was co-administered. This demonstrates that 49.0 mM L-tryptophan, corresponding to the concentrations used in the *in vivo* study, is sufficient to completely abolish the hPAT1-mediated translocation of 7.1 mM Gbx.

In vitro *uptake of L-proline varies along the length of the rat intestine*

To evaluate the absorptive function of rPAT1 along the length of the rat intestine, a specific combination of PAT1-substrates, L-proline and GABA, was chosen. To our knowledge, no other intestinal luminal transporter accepts both these compounds as substrates. The uptake of 2.5 mM [^{3}H]-L-proline into rat intestinal sheets was investigated in the absence or presence of GABA (50.0 mM). The uptake rate of L-proline in the absence of GABA increased from the duodenum to the middle of the jejunum (Figure 3A). From the middle of the jejunum towards the ileum and the middle of colon, the uptake rate of L-proline decreased from a mean of 74.2 nmol·g^{-1}·min^{-1} to approximately zero. Surprisingly, a measurable L-proline uptake was observed in the rectum, which could be inhibited with GABA. In the presence of GABA, the mean uptake rate of L-proline into sheets of the duodenum and most of the jejunum decreased significantly from 28.3 to 74.2 nmol·g^{-1}·min^{-1} to a mean of 18.0 nmol·g^{-1}·min^{-1}, $P < 0.05$–0.001. The resulting rPAT1-mediated jejunal uptake of L-proline was therefore estimated to contribute to between 34 to 79% of the total uptake (Figure 3B). In the last part of jejunum, in the ileum and beginning of the colon, GABA had no significant effect on the uptake rate of L-proline. However, in the rectum, the uptake of L-proline seemed to be mainly rPAT1-mediated. To further investigate the carrier-mediated uptake, the concentration dependency of L-proline uptake into jejunal sheets was investigated (Figure 3C). The uptake rate versus L-proline concentration was fitted to the Michaelis–Menten equation and a K_M of 2.7 ± 0.8 mM and a V_{max} of 103.8 ± 9.4 nmol·g^{-1}·min^{-1} were obtained.

The expression of rSlc36a1 *and* rSlc15a1 *mRNA varies along the rat intestine*

The expression level of *rSlc36a1* mRNA along the intestine was investigated in order to compare the *rSlc36a1* expression along the intestine with the *in vitro* and *in vivo* absorption of Gbx and L-proline. The expression of *rSlc15a1* mRNA (encoding rPEPT1) was included as a control. The relative expression of mRNA of both *rSlc36a1* and *rSlc15a1* significantly varied along the length of the rat intestine, $P < 0.001$. The expression of *rSlc36a1* mRNA in the colonic segments 24 and 25 was different from that of the jejunal segments 12 and 15, $P < 0.05$ (Figure 4A). The expression level of *rSlc15a1* mRNA in colonic segments 24, 25 and 26 was low compared with

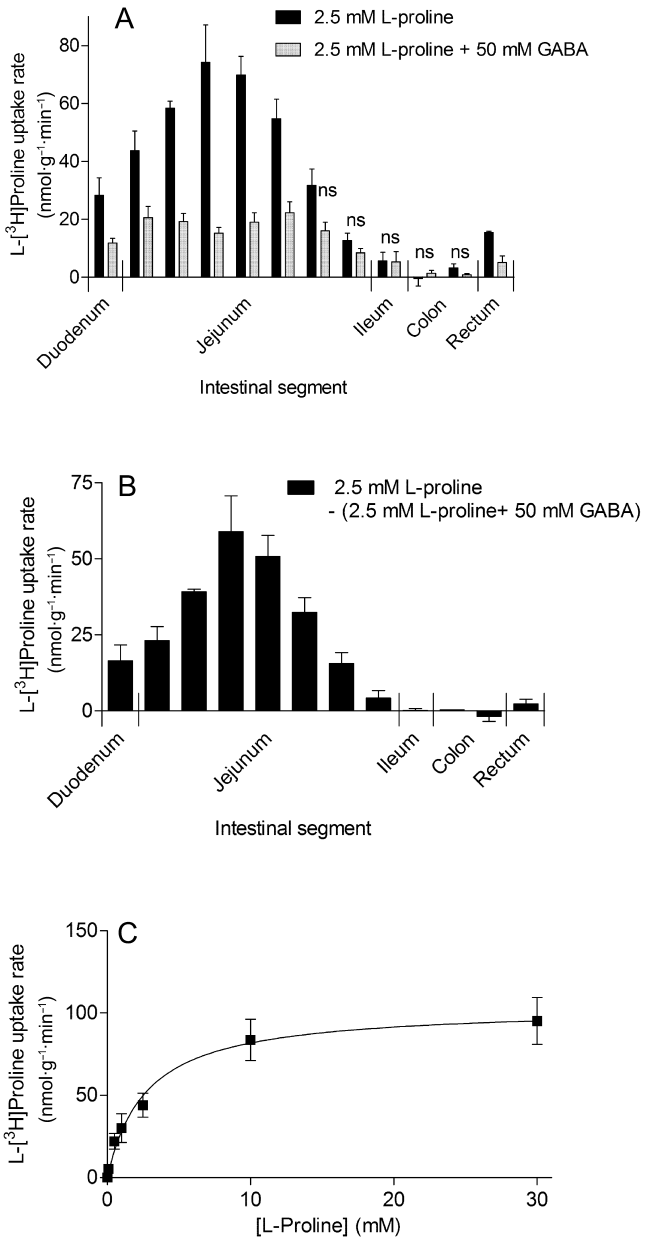

Figure 3

(A) Uptake of 2.5 mM [^{3}H]-L-proline into rat intestinal sheets in the absence or presence of 50.0 mM GABA. The uptake rate was measured for 20 min. The presence of GABA significantly decreased the uptake of [^{3}H]-L-proline, $P < 0.05$. ns, denotes not significant different. (B) The difference between the uptake of [^{3}H]-L-proline and the uptake of [^{3}H]-L-proline in the presence of GABA. (C) Uptake of [^{3}H]-L-proline into sheets from the mid-jejunum. The uptake rate was measured for 15 min. All data shown are mean ± SEM of intestinal sheets from four animals.

the level in the last part of the jejunum and in the ileum; segments 15–17, 19, 20 and 23 ($P < 0.05$), and the expression in the end of the jejunum and ileum (segments 19, 20 and 23) was different from the expression in the duodenum and proximal jejunum (segments 1–14, $P < 0.05$, Figure 4B). Overall, these results show that the mRNA expression of

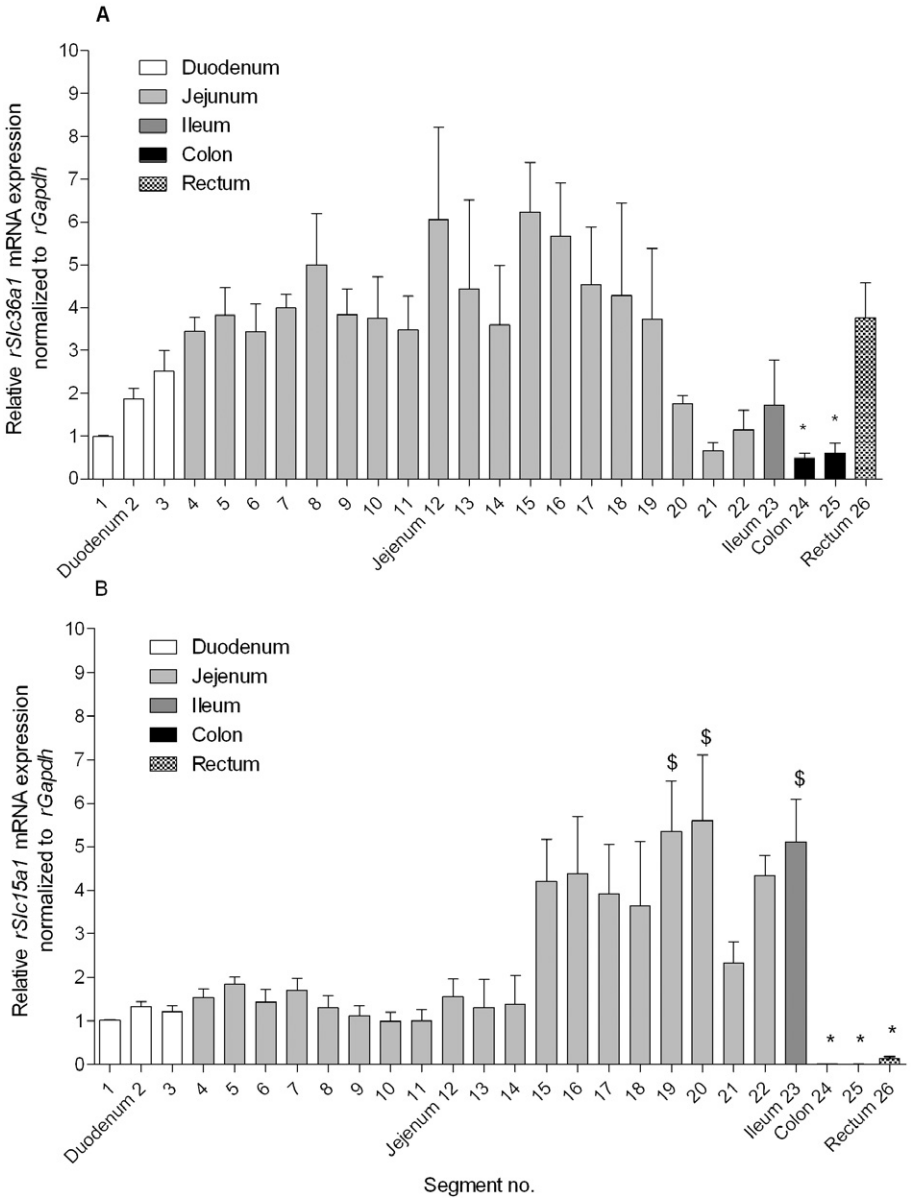

Figure 4

The relative expression of (A) *rSlc36a1* mRNA (encoding rPAT1) and (B) *rSlc15a1* mRNA (encoding rPEPT1) along the rat intestine. The *rSlc36a1*/*rSlc15a1* mRNA expression is normalized to *rGapdh* mRNA and compared with the expression in the first segment of duodenum. Each bar represents the mean ± SEM of four animals, $n = 4–6$. (A) *$P < 0.05$, significantly different from segments 12 and 15. (B) *$P < 0.05$, significantly different from segments 15–17, 19–20 and 23. \$$P < 0.05$, significantly different from segments 1–14.

rSlc36a1 increased along the intestine from the duodenum until its maximum in the middle of jejunum. From the middle of jejunum towards the ileum, the mean relative expression of *rSlc36a1* mRNA decreased, and in the colon, the expression was significantly lower, approximately 10% of that in jejunum. In the rectum, however, the expression of *rSlc36a1* mRNA increased to the same level of expression as in the beginning of jejunum. The intestinal mRNA expression of *rSlc15a1* relative to *rGapdh* was constantly between 1 and 2 from duodenum until the second half of jejunum and the ileum where the level increased four to five times (Figure 4B). The relative expression of *rSlc15a1* mRNA in the colon was minimal as compared with the duodenum and jejunum.

Discussion and conclusions

The absorption of Gbx from different segments of the rat intestine was correlated with mRNA expression and rPAT1-mediated uptake in intestinal sheets. It was found that intestinal absorption of Gbx was dependent on the site of administration. Expression of *rSlc36a1* and carrier-mediated

uptake of L-proline also varied with the location of the intestinal segment. In segments with high *rSlc36a1* mRNA expression and L-proline uptake, the absorption of Gbx was almost complete and could be inhibited by L-tryptophan and L-proline. In segments with low *rSlc36a1* mRNA expression and no carrier-mediated L-proline uptake, that is, the colon, absorption of Gbx was very low, with no effect of co-administered amino acids. Despite the large amount of *in vitro* data showing drug interactions with intestinal transporters, only a few studies have assessed their *in vivo* relevance. Our study provides reasonable evidence for a significant role of carrier-mediated uptake by rPAT1 in the *in vivo* absorption of Gbx.

Almost the entire dose of Gbx was absorbed immediately after dosing directly into the stomach, the duodenum or the jejunum. Only 4% of the administered Gbx was absorbed after direct dosing into the colon. The differences in Gbx absorption along the rat intestine suggest the involvement of a transporter present in the small intestine, but absent or not functional in the colonic epithelium. Differences in absorption could also be the result of the shorter length of the colon, reduced surface area or lower solubility in the intestinal fluid compared with the small intestine. However, the colonic transit time in rats is quite long, estimated to be 300 min (Chen *et al.*, 2010), while the surface area of the rat colon is approximately 25–35% of the small intestine (Yuasa, 2008), and the aqueous solubility of Gbx is high. Generally, drugs with a good human intestinal permeability due to sufficient passive diffusion have a high absorption fraction from the colon, if solubility is not limiting (Tannergren *et al.*, 2009). Drugs such as budesonide, metoprolol and theophylline have complete absorption from the small intestine and the colon in humans (Tannergren *et al.*, 2009). The combination of transit time, surface area and solubility should be sufficient to allow Gbx absorption from the colon.

Evidence that the responsible carrier is rPAT1 comes from the *rSlc36a1* mRNA expression pattern, which showed a relatively high level of *rSlc36a1* mRNA in most of the jejunum, and low levels in the colon. *SLC36A1* mRNA has been detected in the human GIT by PCR/RT-PCR (Chen *et al.*, 2003; Anderson *et al.*, 2004; 2009). These studies observed relatively high *SLC36A1* mRNA levels in the jejunum, the ileum and the colon. In one study, colonic expression was approximately equal to that in the jejunum (Chen *et al.*, 2003). This discrepancy may be caused by species differences or differences in methodology. A fourth study found that the *rSlc36a1* mRNA expression was almost equal in six samples from along the rat small intestine (duodenum-ileum), except for a significantly decreased expression in the ileum (Howard *et al.*, 2004), which was comparable to the present study. Surprisingly, we found that *rSlc36a1* mRNA was also expressed at relatively high levels in the rectum. The levels of *rSlc15a1* mRNA (encoding rPEPT1) along the rat intestine in the present study were similar to previous findings, where mRNA was found in the small intestine and not in the colon under physiological conditions (Herrera-Ruiz *et al.*, 2001; Naruhashi *et al.*, 2002; Ziegler *et al.*, 2002; Englund *et al.*, 2006). The level of *rSlc15a1* mRNA in the small intestine has previously been shown to correlate with the extent of intestinal absorption of the PEPT1 substrate cefadroxil (Naruhashi *et al.*, 2002).

Further support for the hypothesis that rPAT1 is responsible for Gbx absorption comes from the *in vitro* uptake rate of L-proline along the rat intestine. The contribution of rPAT1-mediated uptake to overall L-proline uptake was measured as the difference between the uptake rate in the absence and presence of 50.0 mM GABA at pH 6.0. Under these conditions, we consider carrier-mediated uptake to be due to rPAT1 alone because L-proline and GABA, to our knowledge, are not shared substrates of other intestinal luminal transporters. Additionally, the concentration-dependent uptake rate of L-proline into segments of jejunum was characterized by an affinity value of 2.3 mM, which is comparable to previous affinity values obtained for L-proline uptake in hPAT1-expressing oocytes or cells (Boll *et al.*, 2002; Chen *et al.*, 2003). rPAT1 mediated L-proline uptake was evident in the duodenum and had its maximum rate in the jejunum, whereas no rPAT1-mediated uptake could be measured in the distal parts of the ileum and the colon. The rPAT1-mediated uptake of L-proline observed in the rectum was consistent with the level of *rSlc36a1* mRNA. Rectal PAT1 expression may provide a new strategy for exploiting carrier-mediated absorption of drugs targeting PAT1 following rectal administration. In another study, we found that rPAT1 protein is expressed in the rectal epithelium (Holm *et al.*, 2012). We attempted to exploit the expression of rPAT1 in the rectum to formulate vigabatrin for rectal administration, which could be attractive for paediatric use of vigabatrin. However, in rats, it was not possible to achieve significant vigabatrin plasma concentrations following rectal vigabatrin administration (Holm *et al.*, 2012).

If rPAT1 is the major transporter causing intestinal absorption of Gbx, it should be possible to decrease the absorption rate of rPAT1 by co-administration of other substrates or inhibitors of rPAT1. The most obvious would be L-proline, which is the standard PAT1 substrate (Thwaites *et al.*, 1993; Boll *et al.*, 2002). Alternatively, 5-hydroxytryptophan (5-HTP) or L-tryptophan, which have been identified as PAT1 inhibitors (Metzner *et al.*, 2005), could be used. Due to adverse effects observed with 5-HTP (Larsen *et al.*, 2010), a combination of high concentrations of L-proline and L-tryptophan was chosen. It was confirmed in *X. laevis* oocytes expressing hPAT1 that Gbx is a substrate of hPAT1 and that L-tryptophan inhibits carrier-mediated translocation of Gbx at relevant concentrations. The K_M value of hPAT1-mediated transport of Gbx was 17.1 mM, which is comparable to other indirectly measured affinity values of Gbx for hPAT1 (Larsen *et al.*, 2009; Frolund *et al.*, 2011). Gbx is thus a medium affinity hPAT1 substrate, such as glycine (Thwaites *et al.*, 1995; Foltz *et al.*, 2005) and δ-aminolevulinic acid (Frolund *et al.*, 2010b). Recently, substrate overlap between hPAT1 and hPEPT1 has been shown (Frolund *et al.*, 2010a,b), and we therefore investigated whether Gbx interacted with hPEPT1. In Caco-2 cells, Gbx is not able to inhibit apical Gly-Sar uptake, suggesting that PEPT1 is not relevant for the absorption of Gbx.

After administration of Gbx and amino acids orally and directly into the duodenum and jejunum, a marked decrease in the Gbx plasma concentration was observed in the first plasma samples. Oral and jejunal co-administration of L-tryptophan and L-proline significantly changed the plasma C_{max} and t_{max}. After administration in the duodenum, the presence of amino acids had no effect on the C_{max} and t_{max} of

Gbx. However, the plasma concentrations in the presence or absence of amino acids are comparable to those observed after oral and jejunal administration at the time points investigated, with the exception of the second sampling time in the duodenum. The higher plasma concentration observed at this time point may be due to biological variation. As the absorption of Gbx happens fast, the second time point is important for estimating C_{max} and t_{max}. The presence of the amino acids was not able to alter the AUC significantly, which was probably the result of intestinal transit, dilution, absorption and redundancy of transporters along the length of the proximal intestine.

In conclusion, the level of *rSlc36a1* mRNA and the uptake of L-proline suggest that rPAT1 is present and functional in the duodenum and small intestine of the rat, but not in the large intestine, except for the rectum. The mRNA levels and the *in vitro* studies complemented the regional differences in Gbx absorption in rats, which strongly suggests that the absorption of Gbx is defined by the transport function of rPAT1. This suggests that rPAT1 has a major influence on the pharmacokinetics of Gbx, especially on the initial absorption, as well as on plasma C_{max} and t_{max} values. These findings have direct pharmacological implications for optimizing the therapeutic use of Gbx and potentially other PAT1 substrates, and may have an influence on the design of pharmaceutical formulations of PAT1 substrates, especially oral controlled-release products. Overall, this study provides evidence for the importance of an amino acid transporter in mediating *in vivo* intestinal drug absorption.

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Conflicts of interest

None.

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