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RESEARCH PAPER

In vivo and *In vitro* Evaluations of Intestinal Gabapentin Absorption: Effect of Dose and Inhibitors on Carrier-Mediated Transport

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ABSTRACT

Purpose Gabapentin exhibits saturable absorption kinetics, however, it remains unclear which transporters that are involved in the intestinal transport of gabapentin. Thus, the aim of the current study was to explore the mechanistic influence of transporters on the intestinal absorption of gabapentin by both *in vivo* and *in vitro* investigations

Methods Pharmacokinetic parameters were determined following a range of intravenous (5–100 mg/kg) and oral doses (10–200 mg/kg) in rats. Transepithelial transport (50 μM–50 mM) and apical uptake of gabapentin (0.01–50 mM) were investigated in Caco-2 cells. The effect of co-application of the LAT-inhibitor, BCH, and the $b^{0,+}$-substrate, L-lysine, on intestinal transport of gabapentin was evaluated *in vivo* and *in vitro*.

Results Gabapentin showed dose-dependent oral absorption kinetics and dose-independent disposition kinetics. Co-application of BCH inhibited intestinal absorption *in vivo* and apical uptake *in vitro*, whereas no effect was observed following co-application of L-lysine.

Conclusions The present study shows for the first time that BCH was capable of inhibiting intestinal absorption of gabapentin *in vivo*. Furthermore, in Caco-2 cell experiments BCH inhibited apical uptake of gabapentin. These findings may imply that a BCH-sensitive transport-system was involved in the apical and possibly the basolateral transport of gabapentin across the intestinal wall.

KEY WORDS BCH · gabapentin · LAT · L-lysine · rat

ABBREVIATIONS

GBP	Gabapentin
BBB	Blood–brain barrier
Leu	L-Leucine
Phe	L-Phenylalanine
Lys	L-Lysine
Arg	L-Arginine
CssC	L-Cystine
BCH	2-amino-2-norbornanecarboxylic acid
(A-B)	Apical to basolateral
(B-A)	Basolateral to apical

INTRODUCTION

Gabapentin was originally developed as an inhibitory analogue of the neurotransmitter γ-aminobutyric acid (GABA) with improved ability to permeate the blood–brain barrier (BBB) [1]. Presently, gabapentin is approved for treatment of seizures and neuropathic pain, but also applied for anxiety disorders [2–4]. Gabapentin is a zwitterion at physiological pH (pK_a=3.68 and pK_a=10.70 [5]) and has an apparent distribution coefficient below unity (log $D_{7.4}$=−2 [6]) implying that its ability to

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permeate biomembranes passively should be modest. In spite of this, gabapentin is capable of crossing both the intestinal wall and the BBB. These observations suggest the involvement of carrier-mediated transport. In support of this, previous studies have shown that gabapentin's pharmacokinetics was subjected to saturable transport processes. *In situ* [7, 8] and clinical [9, 10] investigations have revealed that intestinal absorption of gabapentin is dose-dependent. Moreover, Luer *et al.* found that the ratio of AUC_{ECF}/AUC_{plasma} decreased with increasing dose, suggesting the presence of a saturable transport component in the BBB [11]. Gabapentin's pharmacokinetics has been investigated, however, only at a single or a couple of doses [12, 13]. One multiple dose study has been reported, however, the study design did not allow for determination of the gabapentin pharmacokinetics [14]. Consequently, only limited knowledge is available with respect to potentially saturable transport processes for gabapentin within the small intestine, BBB and kidneys [7, 11, 15]. Thus, in order to obtain a better understanding of potentially saturable transport processes, this work systematically investigated the effect of dose-escalation on intravenous and oral gabapentin pharmacokinetics in rats.

The role of carrier-mediated transport in the intestinal absorption of gabapentin has been the subject of several *in vitro* and *in situ* investigations [7, 8, 16–19]. It has been reported that intestinal absorption of gabapentin is both sodium- and proton-independent and that gabapentin is mainly absorbed in the small intestine. Furthermore, amino acids have been applied as transport inhibitors *in vitro* and *in situ* to identify the transport systems involved in the uptake of gabapentin. These studies showed that gabapentin exhibits cross-inhibition with both neutral amino acids, *e.g.* L-leucine (Leu) and L-phenylalanine (Phe), and cationic amino acids, *e.g.* L-lysine (Lys) and L-arginine (Arg) [7, 16–19]. Two transport systems, hence, seem most likely to be involved in the intestinal absorption of gabapentin, the $b^{0,+}$-transport system and the LAT-system [18]. In the intestine the rBAT/$b^{0,+}$AT is primarily expressed in the apical membrane, whereas the most abundant LAT-isoform in the intestine (4F2hc/LAT-2) is expressed primarily in the basolateral membrane. Both transporters are exchangers and belong to the solute carrier families 3 and 7 (SLC3 and SLC7) [20–22]. Despite the extensive work devoted to identification of the transporters involved in intestinal absorption of gabapentin, no consensus has yet been reached [16, 18, 19]. Moreover, previous inhibition studies have all been carried out *in vitro* or *in situ*, which may not necessarily represent what transpires *in vivo*. Hence, the aim of the current study was to explore the mechanistic influence of transporters on the intestinal absorption of gabapentin by both *in vivo* and *in vitro* investigations.

MATERIALS AND METHODS

Materials

Sodium chloride and formic acid (>98% pure) were obtained from Merck (Darmstadt, Germany), acetonitrile (LC-MS grade) from Fluka (St. Gallen, CH). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2-(*N*-morpholino)ethanesulfonic acid (MES) monohydrate were from AppliChem GmbH (Darmstadt, Germany). 1-(Amino-[^{3}H]-methyl)-[2,3,5,6-^{3}H]-cyclohexaneacetic acid ([^{3}H]-gabapentin) was purchased at American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). D-[1-^{14}C]-mannitol and Ultima Gold scintillation liquid was purchased at PerkinElmer (Boston, MA, USA). All remaining chemicals and media were obtained from Sigma-Aldrich (St. Louis, MO, USA). Purified water was obtained from an Elga Purelab Ultra Analytic purification system (Elga, High Wycombe, UK). All other chemicals used were of analytical grade or higher.

In vivo study in rats

The protocol used was approved by the institutional animal ethics committee in accordance with Danish law regulating experiments on animals and in compliance with EC directive 2010/63/EU, and the NIH guidelines on animal welfare. Gabapentin was dissolved in Elga water in concentrations from 1 to 20 mg/mL. Other compounds used were dissolved in a similar manner. Solutions for intravenous administration were adjusted to isotonicity with sodium chloride. Osmolalities were measured using a vapour pressure osmometer by Wescor Vapro model 5520 (Wescor, Inc, Logan, Utah, USA).

Male Sprague Dawley rats weighing approximately 300 g (286-338 g on the day of gabapentin administration) were obtained from Charles River (Sulzfeld, Germany) and used for the pharmacokinetic studies ($n=6$ per group unless otherwise stated). The animals were acclimatized minimum 5 days in groups of 2 on wooden bedding (Tapvei, Kortteinen, Finland) in plastic cages, $595\times380\times200$ mm, with a stainless-steel grid (Scanbur, Sollentuna, Sweden) in an air-conditioned building with controlled environmental parameters (relative humidity 40–60%, temperature 20 ± 1°C, light from 06:00 to 18:00 h). Prior to dosing the rats were fasted for 16–20 h with free access to water. During the experiment, water was restrained until 3 h after dosing.

The animals were dosed either orally by gavage (10–200 mg/kg) or intravenously (5–100 mg/kg) by injection into the tail vein. For the study investigating the effect of co-administration of transport inhibitors on gabapentin pharmacokinetics, solutions containing gabapentin and inhibitors (BCH and L-lysine) were dosed orally by gavage. An overview of *in vivo* investigations is presented in Table I. Blood samples

Table I Groups and gabapentin doses included in the *in vivo* study (n is the number of animals)

	n	Dose (mg/kg)	Dose of amino acid (mg/kg)
Intravenous	5–6	5, 10, 25 and 100	
Oral	6–12	10, 25, 50, 100 and 200	
Oral co-administration	6–12	10	200 (BCH)
		10	200 (L-lysine)
		10	200 (BCH) and 200 (L-lysine)

of 200 μL were obtained from the lateral tail vein by individual vein puncture and collected into potassium-EDTA tubes (Microvette 500 K3E, Sarstedt, Nümbrecht, Germany) at 0 to 8 h after administration. Pre-dose blood samples were obtained from the sublingual vein by individual vein puncture approximately 30 min before dosing. Detailed description of the sample design is provided in the supplementary materials. Plasma was harvested immediately by 10 min of centrifugation at 4°C, 2,765 × g (Multifuge 1 S-R, Heraeus, Hanau, Germany) and stored at −80°C until analyzed. At the end of the experiment, the animals were sacrificed by spinal dislocation using a guillotine.

Analysis of Gabapentin Plasma Samples

Plasma samples were analyzed using LC-MS/MS. The plasma samples were precipitated by spiking 15 μL of plasma with 210 μL of the internal standard (20 ng/mL gabapentin-D_{10} in acetonitrile with 0.1% v/v formic acid) and centrifuged for 20 min at 4°C, 6,189 × g (Sigma 4 k15, Osterode, Germany). 15 μL of supernatant was transferred to vials, added 285 μL of Elga water with 0.1% v/v of formic acid and analyzed by reverse phase UPLC coupled with MS/MS. The standard curve was linear in the range 50 to 50,000 ng/mL. Preparation of the standard curve, protein precipitation of the samples and transfer of supernatant to vials was done using the automated liquid handling station Microlab Star (Hamilton, Kista, Sweden). A Waters Acquity ultra-performance liquid chromatograph separation module (Waters, Milford, MA) equipped with a degasser, a column heater, binary pump, and a sample organizer was used. A C-8 BEH column (Waters 50 mm × 2.1 mm, 1.7 μm, Milford, MA) was used for separation, column temperature was maintained at 40°C. The mobile phase consisted of Elga water (A) and acetonitrile (B) both containing 0.1% v/v formic acid. The gradient applied for separation was, 0-1.5 minutes: 2% B; 1.5-2.2 minutes: 95% B; and 2.2-3 minutes: 2% B. The flow rate was 0.6 mL/min and injection volume was 10 uL. An AB Sciex API 4,000 mass spectrometric detector (MDS SCIEX, Ontario, Canada) equipped with an electrospray ionization ion source, operating in the positive mode at a temperature of 600°C, was used for detection. The ions were detected by multiple reaction monitoring (MRM), monitoring the $[M + H]^+$ transition of the m/z of the precursor ion to the m/z of the product ion for gabapentin and gabapentin-D_{10}. The MS/MS transitions utilized for analysis were m/z 172.114 → 154.1 for gabapentin and m/z 182.057–>164.1 for gabapentin-D_{10}.

Caco-2 Cell Culturing

Protocols for culturing of Caco-2 cells were as previously described [23]. Caco-2 cells (passage 6–10, DSMZ, Braunschweig, Germany) for transepithelial transport were seeded onto Transwell polycarbonate filters (area, 1.12 cm^2; pore size, 0.4 μm; cell density, 10^5 cells per insert; Corning Life Sciences, Tewksbury, MA, USA), whereas Caco-2 cells (passage 3–10, DSMZ, Braunschweig, Germany) for initial apical uptake were seeded onto Costar 24 well plates (area per well, 1.9 cm^2; cell density, 1.7×10^5 cell per well; Corning Life Sciences, Tewksbury, MA, USA). Experiments were conducted on day 19–20 or day 13 after seeding for transepithelial transport and apical uptake studies, respectively. Before initiation of the transepithelial transport studies the barrier properties of the Caco-2 monolayers were assessed by transepithelial electrical resistance (TEER) measured at room temperature (20°C) in a tissue resistance measurement chamber (Endohm-12) with an Epithelial Voltammeter (EVOM 2), both of which were from World Precision Instruments (Sarasota, FL, USA). TEER values were 399–642 Ω cm^2.

Transepithelial Transport Across Caco-2 Cell Monolayers

Transepithelial transport studies across Caco-2 cell monolayers were performed essentially as previously described [23, 24]. Compounds were dissolved in i) Hank's Balanced Salt Solution buffer (HBSS, in mM: $CaCl_2$, 1.3; MgCl, 0.49; $MgSO_4$, 0.41; KCl, 5.4; KH_2PO_4, 0.44; NaCl, 138; Na_2HPO_4, 0.34; $NaHCO_3$, 4.5; D-glucose, 5.6; containing 0.05% bovine serum albumin (BSA)) supplemented with 10 mM HEPES, pH 6.8 or 7.4, osmolality of 285–332 mOsm/kg or ii) modified HBSS buffer (mHBSS, as HBSS except for 55 mM NaCl; used in cases were the concentration of amino acids or amino acid mimetics exceeded 10 mM) supplemented with 10 mM HEPES, pH 6.8 or 7.4, and 0–35 mM mannitol to a total osmolality of 287–321 mOsm/kg. The apical to basolateral (A-B) and basolateral to apical (B-A) transport of 50 μM (8.6 μg/mL),

5.8 mM (1 mg/mL) and 50 mM (8.6 mg/mL) of gabapentin ([^{3}H]-gabapentin (1–2 μCi/mL)) in the presence or absence of the Lys (10 and 68.4 mM, corresponding to 1.5 and 10 mg/mL) or BCH (10 and 64.4 mM, corresponding to 1.6 and 10 mg/mL) were measured for 150 min. Gabapentin concentrations was chosen in a range relevant for the intestinal concentrations in both the nonclinical and clinical use of the compound. The transport experiments were initiated by adding the appropriate solutions to the apical (0.5 mL, pH 6.8 or 7.4) and basolateral (1.0 mL, pH 7.4) chambers. The cells were placed on an orbital shaker (90 rpm) while the temperature was maintained at 37°C. 100 or 50 μL samples were taken from the basolateral or apical receiver chamber, respectively, after 30, 60, 90, 120 and 150 min. The samples were replaced by similar volumes of the appropriate HBSS solutions. Donor samples of 20 μL were taken before the solutions were added to the donor chamber (0 min) and after 150 min. The transport was in all cases linear with time for up to 150 min (data not shown) and the resulting apparent permeability coefficient, P_{app}, was calculated from Eq. (1).

In order to continuously assess the integrity of the barrier properties of the cell monolayer the transepithelial transport of the paracellular marker mannitol (17 μM, ([^{14}C]-mannitol 1 μCi/mL)) was measured simultaneously. The experiments were terminated by washing the monolayers three times with ice-cold HBSS. The polycarbonate filters were cut from the Transwell supports and the radioactivity in the filters were analyzed to achieve end-point cellular accumulation (EPA) of gabapentin. To each sample or filter 2 mL Ultima Gold scintillation liquid was added and the radioactivity was quantified by scintillation counting on a Packard TriCard liquid scintillation counter (Meriden, CT, USA). Intracellular end-point concentrations (EPC) of gabapentin were calculated based on an estimated intracellular volume of 2.24 μL, as the growth area of the Transwell filters were 1.12 cm^2 and previous studies have shown that the thickness of Caco-2 cell monolayers are approximately 20 μm [25].

Initial Apical Uptake in Caco-2 Cell Monolayers

The initial apical uptake was measured essentially as described by Frølund *et al.* [26]. The uptake of gabapentin (0.01–50 mM, ([^{3}H]-gabapentin 1 μCi/mL)) in Caco-2 cell monolayers was measured in the absence or presence of Lys (10 or 68.4 mM), BCH (0.025–10 or 64.4 mM) or L-cystine (CssC, 1 mM). Compounds were dissolved in HBSS buffer supplemented with 10 mM HEPES, pH 6.8, and 0.5 mL was added per well. The cells were placed on an orbital shaker (90 rpm) at 37°C. The uptake was measured for 5 min and terminated by washing the cell-layers three times with ice-cold HBSS buffer. The cells were detached from the well by incubating with 150 μl 0.1% Triton-X in water for 10 min at 37°C. The detached cell suspension was transferred to scintillation vials containing 2 mL Ultima Gold scintillation liquid and the amount of radioactivity was quantified by scintillation counting on a Packard TriCard liquid scintillation counter (Meriden, CT, USA). The uptake of [^{3}H]-gabapentin was corrected for the amount of gabapentin present in the extracellular fluid using [^{14}C]-mannitol (1 μCi/mL) as a marker of the extracellular volume, whereas the uptake of 0.01–5.8 mM [^{3}H]-gabapentin was also corrected for unspecific-binding of gabapentin using the scintillation counts measured at 50 mM gabapentin as a measure of the non-displaceable counts. The gabapentin (50 μM, ([^{3}H]-gabapentin 1 μCi/mL)) uptake measured at pH 7.4 and 6.0 and in the absence of sodium was measured as described above using HBSS buffer supplemented with 10 mM HEPES or 10 mM MES, pH 7.4 and 6.0, respectively, or Na^+-free HBSS buffer (in mM: $CaCl_2$, 1.3; MgCl, 0.49; $MgSO_4$, 0.41; KCl, 5.4; KH_2PO_4, 0.44; $C_5H_{14}ClNO$, 138; K_2HPO_4, 0.34; D-glucose, 5.6; containing 0.05% BSA) supplemented with 10 mM HEPES, pH 6.8.

Data and Statistical Analyses

Pharmacokinetic parameters were determined by non-compartmental analysis using Phoenix WinNonLin version 6.3 (Pharsight Corporation, Mountain View, CA). The following primary parameters, terminal elimination rate constant (λ_z), maximum plasma concentration (C_{max}), time to maximum plasma concentration (t_{max}) and area under the curve from time 0 to infinity ($AUC_{0\text{-inf}}$) and the following secondary parameters, terminal half-life ($t_{1/2}$), clearance (CL) and volume of distribution (V) were determined. $AUC_{0\text{-inf}}$ was determined by the linear up-log down trapezoidal method with 1/(y*y) weighting, and was extrapolated to infinity. Oral bioavailability (F) was estimated relative to the mean clearance value determined following intravenous administration of 5, 10, 25 and 100 mg/kg gabapentin.

The transepithelial flux, J, of gabapentin (mass/time/area) across the Caco-2 cell monolayer was calculated as the steady-state amount of gabapentin, Q, accumulating in the receptor compartment per time, t, and area, A:

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

where C is the initial donor concentration (mM) of gabapentin and P_{app} is the apparent permeability coefficient (cm/s) of gabapentin across the cell monolayer. The initial uptake rate of gabapentin as a function of the substrate concentration was fitted to a Michaelis-Menten type equation:

$$V = \frac{V_{max} \cdot [S]}{[S] + K_m} \quad (2)$$

where V is the uptake rate (pmol/cm^2/min), V_{max} is the maximal uptake rate (pmol/cm^2/min), K_m is the Michaelis

constant (mM), and [S] is the concentration of gabapentin (mM). The IC_{50} value of BCH was determined as the concentration of BCH, [I] (mM), at which the initial uptake rate of 50 μM gabapentin, V (pmol/cm^2/min), was reduced to 50% of the uptake rate measured in the absence of BCH. The IC_{50} value (mM) was determined by fitting the data to Eq. 3 giving a sigmoidal dose-inhibition curve:

$$V = \frac{100\%}{1 + 10^{(\log[I] - \log IC_{50})}} \quad (3)$$

All data and statistical analyses were performed using GraphPad Prism (version 6.03; San Diego, CA, USA). Data are presented as mean ± SEM. Differences between means were analyzed by one-way ANOVA followed by a multiple comparisons test. Tukey's post hoc test for comparison of the individual groups, the Sidak's post hoc test for comparison of a specific set of means and the Dunnett's post hoc test for comparison with a control group. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Intravenous Gabapentin Pharmacokinetics

The plasma concentration-time profiles following intravenous administration of 5, 25 and 100 mg/kg gabapentin are depicted in Fig. 1 and the associated pharmacokinetic parameters of 5, 10, 25 and 100 mg/kg gabapentin are presented in Table II. A proportional increase in exposure with dose was observed following intravenous administration of gabapentin in the range of 5 to 100 mg/kg as the mean clearance did not differ significantly between the groups (CL 0.27 to 0.37 L/h/kg). Volume of distribution was not significantly affected by dose and ranged from 0.86 to 1.26 L/kg. Correspondingly, the terminal half-life, $t_{1/2}$, obtained from the log-linear slope of the plasma concentration-time profiles was found to be in the range of 2.3 to 2.7 h, without any significant effect of dose. These results show linear disposition kinetics of gabapentin in the range of 5 to 100 mg/kg, though a tendency for an increase in clearance and decrease in $AUC_{0\text{-inf}}$ was observed at 100 mg/kg.

Table II Pharmacokinetic parameters of gabapentin in rats obtained following intravenous administration. $t_{1/2}$ is the terminal elimination half-life, V is the volume of distribution determined from the terminal phase, CL is clearance and $AUC_{0\text{-inf}}$ is the area under the plasma concentration-time curve from time 0 and extrapolated to infinity. $n = 5$–6. Data presented as mean ± SEM

Dose (mg/kg)	$t_{1/2}$ (h)	V (L/kg)	CL (L/h/kg)	$AUC_{0\text{-inf}}$ (μg·h/L)
5	2.47 ± 0.18	0.95 ± 0.10	0.27 ± 0.02	19,394 ± 1,732
10	2.29 ± 0.30	0.86 ± 0.15	0.27 ± 0.05	43,263 ± 7,821
25	2.65 ± 0.27	1.13 ± 0.14	0.29 ± 0.01	85,785 ± 4,086
100	2.36 ± 0.27	1.26 ± 0.15	0.37 ± 0.01	269,248 ± 5,713

Oral Gabapentin Pharmacokinetics

The plasma concentration-time profiles obtained after oral administration of 10 mg/kg gabapentin are presented in Fig. 2, and the associated pharmacokinetic parameters of 10, 25, 50, 100 and 200 mg/kg gabapentin are summarized in Table III. Plasma concentration-time profiles for the other doses are presented in the supplementary materials. Maximum gabapentin plasma concentrations, C_{max}, occurred after 1.04 to 2.03 h, with t_{max} of the 200 mg/kg dose being significantly delayed relative to that of the 10 mg/kg dose ($p < 0.01$). As apparent from Fig. 3a significant decrease in oral bioavailability, F, from 95 to 30% was found when dose was increased from 10 to 200 mg/kg ($p < 0.0001$). Also no dose-proportional

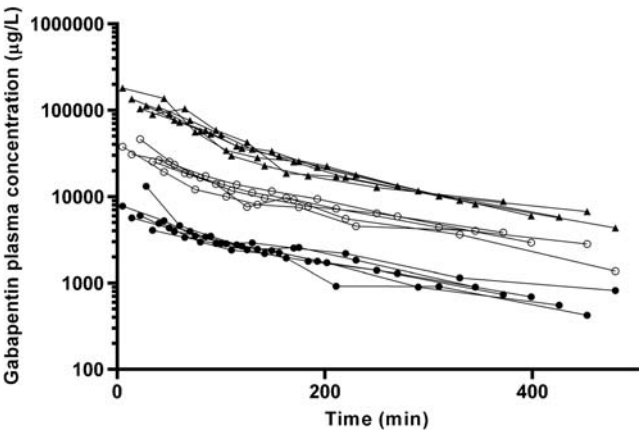

Fig. 1 Individual plasma concentration-time profiles of gabapentin in rats following intravenous administration of 5 mg/kg gabapentin (●), 25 mg/kg gabapentin (○) or 100 mg/kg gabapentin (▲) ($p = 5$–6).

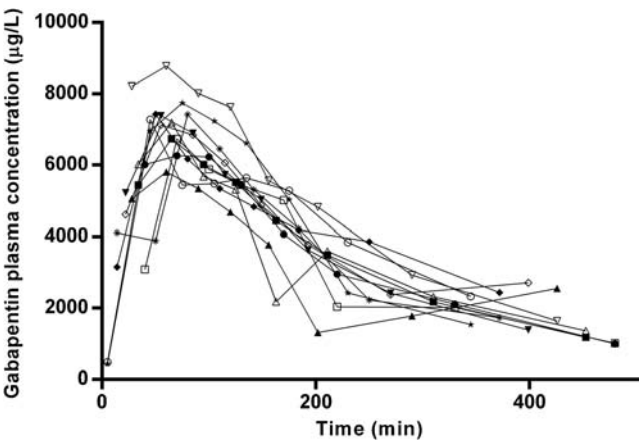

Fig. 2 Oral plasma concentration-time profiles of gabapentin following oral administration of 10 mg/kg. ($n = 12$).

Table III Pharmacokinetic parameters determined after oral administration of gabapentin to rats. $t_{1/2}$ is the terminal elimination half-life, t_{max} is the time of C_{max} in plasma, V/F is the volume of distribution determined from the terminal phase divided by F, CL/F is clearance divided by F, $AUC_{0\text{-}inf}$ is the area under the plasma concentration-time curve from time 0 and extrapolated to infinity and F is the absolute oral bioavailability. Dose-corrected $AUC_{0\text{-}inf}$ is corrected relative to 5 mg/kg. $n = 6$, except for 10 mg/kg where $n = 12$. Data presented as mean ± SEM

Dose (mg/kg)	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (μg/L)	V/F (L/kg)	CL/F (L/h/kg)	$AUC_{0\text{-}inf}$ (μg·h/L)	Dose-corrected $AUC_{0\text{-}inf}$ (μg·h/L)	F (%)
10	2.55 ± 0.14	1.04 ± 0.05	7,160 ± 217	1.17 ± 0.05	0.32 ± 0.01	31,639 ± 1,202	$15{,}819 \pm 601^{A}$	95.3 ± 3.6^{A}
25	2.70 ± 0.36	1.21 ± 0.25	13,133 ± 475	1.55 ± 0.13	0.41 ± 0.02	62,176 ± 3,411	$12{,}435 \pm 682^{A}$	74.9 ± 4.1^{A}
50	2.46 ± 0.06	1.57 ± 0.29	17,400 ± 1,011	2.11 ± 0.11	0.59 ± 0.03	85,085 ± 3,363	$8{,}509 \pm 336^{B,C,E}$	$51.2 \pm 2.0^{B,C,E}$
100	2.52 ± 0.13	1.46 ± 0.13	23,983 ± 2,589	3.09 ± 0.21	0.86 ± 0.07	119,774 ± 9,129	$5{,}989 \pm 456^{B,C}$	$36.1 \pm 2.7^{B,C}$
200	2.96 ± 0.33	2.03 ± 0.37^{B}	33,100 ± 3,037	4.75 ± 0.99	1.13 ± 0.21	200,962 ± 26,877	$5{,}024 \pm 672^{B,C,D}$	$30.3 \pm 4.0^{B,C,D}$

Significant different from: A = all other groups, B = 10, C = 25, D = 50, and E = 200 mg/kg

increase in C_{max} was observed, illustrated by a significant decrease in the dose-corrected C_{max} at 200 mg/kg relative to 10 mg/kg ($p < 0.0001$).

Oral Gabapentin Pharmacokinetics Following Co-Administration of Transport Inhibitors

The effect of concurrent oral administration of BCH and Lys on gabapentin pharmacokinetics was investigated, aiming at inhibiting the LAT- and the $b^{0,+}$-system, respectively. The pharmacokinetic parameters determined are given in Table IV and the plasma concentration-time profiles can be found in the supplementary materials. No significant delay of t_{max} was found following concomitant administration of inhibitors, a trend *versus* longer t_{max} was observed following co-administration of BCH. However, a significant delay in t_{max} was observed on simultaneous administration of both inhibitors ($p < 0.05$). $t_{1/2}$ was not significantly affected by the presence of inhibitors, though there was a trend towards a shorter half-life following co-administration of BCH. Co-administration of BCH led to a significant increase in both V/F ($p < 0.05$) and CL/F ($p < 0.0001$). However, the relative increases of both parameters were similar, indicating that the increase is likely to be caused by a decrease in F rather than an increase in volume of distribution or clearance. Co-administration of BCH significantly decreased the oral bioavailability of gabapentin from 95 to 65% ($p < 0.0001$). Likewise, a significant drop in C_{max} was observed ($p < 0.01$). Co-administration of Lys did not have any effect on absorption or disposition of gabapentin.

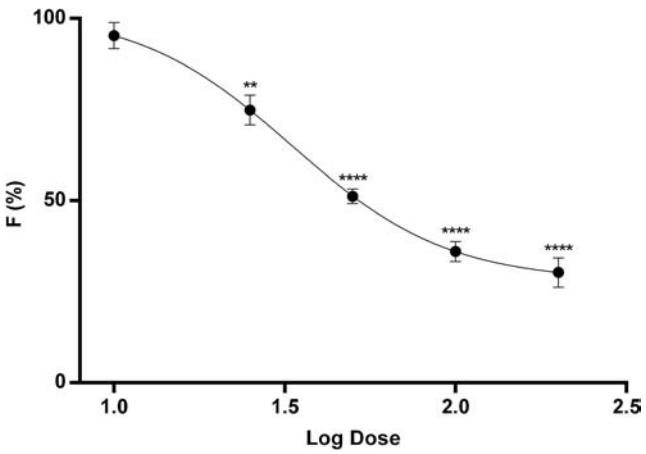

Fig. 3 Fraction of dose absorbed (±SEM) following oral doses (log-scale) of gabapentin in rats. A multiple comparison between groups was performed with 10 mg/kg as control. Data was fitted to a Michaelis-Menten type equation and the following parameters were estimated: $K_m = 33.3 \pm 0.20$ mg/kg, Hill slope $= -1.91 \pm 0.74$ and $R^2 = 0.89$. $^{**}p < 0.01$, $^{****}p < 0.0001$. (n = 6–12).

Transepithelial Transport Across Caco-2 Cell Monolayers

Gabapentin permeability across Caco-2 cell monolayers was generally low ranging from $6.3–10.9 \times 10^{-7}$ cm/s (Fig. 4a.), which is only approximately twice of the mannitol permeability ($3.0–5.1 \times 10^{-7}$ cm/s). A-B permeability was concentration-independent in the concentration range investigated, and no polarization of the transport was observed at 5.8 mM gabapentin, whereas the permeability at 50 μM gabapentin was slightly higher in the B-A than in the A-B direction (efflux ratio of 1.3). Co-application of 68.4 mM Lys or 64.4 mM BCH with 50 or 5.8 mM gabapentin did not affect permeability. In contrast, co-application of 10 mM BCH with 50 μM gabapentin resulted in a 26% decrease in permeability. End-point accumulation (EPA) of gabapentin was determined (Fig. 4b-d) and no effect on EPA was observed when either 68.4 mM Lys or 64.4 mM BCH was co-applied apically with 50 or 5.8 mM gabapentin. However, 10 mM BCH caused a 60% decrease in EPA when co-applied apically with 50 μM gabapentin ($p < 0.05$). Surprisingly, after basolateral application of 5.8 mM and 50 μM gabapentin EPA was 5 and 2.5 times greater than after apical application, respectively. Importantly, this was not due to retention in the filter support. The estimated intracellular end-point

Table IV Pharmacokinetic parameters of gabapentin following concurrent oral administration of 10 mg/kg gabapentin and inhibitor(s) to rats. $t_{1/2}$ is the terminal elimination half-life, t_{max} is the time of C_{max} in plasma, V/F is the volume of distribution divided by F and CL/F is clearance divided by F, $AUC_{0\text{-}inf}$ is the area under the plasma concentration-time curve from time zero and extrapolated to infinity and F is the absolute oral bioavailability. $n = 6$, except for the control group and the group co-administered with Lys where $n = 12$. Data presented as mean ± SEM

Dose, gabapentin (mg/kg)	Dose, inhibitor (mg/kg)	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (μg/L)	V/F (L/kg)	CL/F (L/h/kg)	$AUC_{0\text{-}inf}$ (μg·h/L)	F (%)
10	Control	2.55 ± 0.14	1.04 ± 0.05	7,160 ± 217	1.17 ± 0.05	0.32 ± 0.01	31,639 ± 1,202	95.3 ± 3.6
10	200, BCH	2.15 ± 0.18	1.29 ± 0.06	5,577 ± 323 *	1.49 ± 0.21 *	0.47 ± 0.03 *	21,419 ± 1,200 *	64.5 ± 3.6 *
10	200, Lys	2.37 ± 0.08	1.17 ± 0.08	6,732 ± 219	1.19 ± 0.04	0.35 ± 0.01	28,866 ± 1,054	86.9 ± 3.1
10	200, BCH and 200, Lys	2.21 ± 0.11	1.38 ± 0.18 *	6,098 ± 397*	1.44 ± 0.10	0.45 ± 0.03 *	22,514 ± 1,504 *	67.8 ± 4.5 *

A Dunnett's multiple comparison test was performed with 10 mg/kg gabapentin as control. *$p < 0.05$

concentrations (EPC) are presented in Table V together with the end-point concentrations of gabapentin in the apical and basolateral chambers. After apical gabapentin application EPC only reached 10–30% of the donor concentration. However, transport across the apical membrane seemed concentration-dependent as the relative amount of gabapentin crossing the apical membrane increased from 10 to 30% when the apical concentration was decreased from 50 to 50 μM. In contrast, the exit of gabapentin across the basolateral membrane seemed low and independent of EPC of gabapentin as the resulting basolateral concentration in all cases only reached 3–5% of the EPC. After basolateral application EPCs were 71–76% of the donor concentration, indicative of a higher permeability across the basolateral

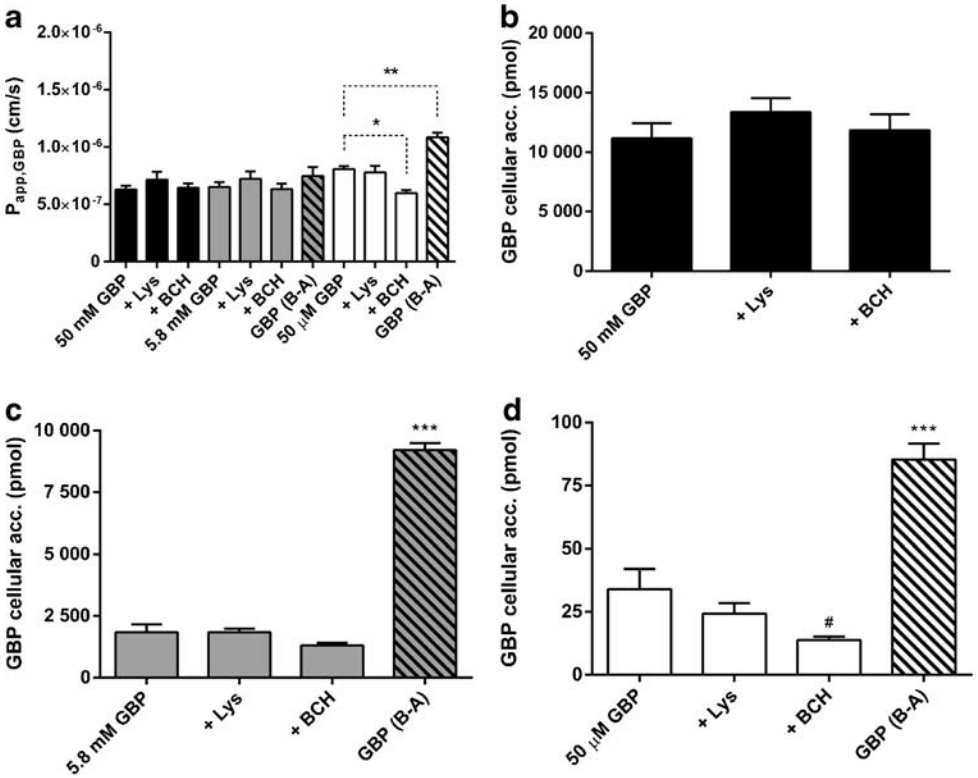

Fig. 4 The apparent transepithelial permeability, P_{app}, (**a**) and end-point (150 min) intracellular gabapentin (GBP) accumulation (**b**-**d**) across Caco-2 cell monolayers. The transepithelial transport was measured for 150 min and the permeability was calculated from Eq. 1 and expressed as mean ± SEM of measurements performed in three independent cell passages. Apical and basolateral pH was in all cases 6.8 and 7.4, respectively. Black bars indicate 50 mM gabapentin, + 68.4 mM Lys, and + 64.4 mM BCH; gray bars indicate 5.8 mM gabapentin, + 68.4 mM Lys, and + 64.4 mM BCH; white bars indicate 50 μM gabapentin, + 10 mM Lys, and + 10 mM BCH; and scratched bars indicate transport in the basolateral to apical direction (B-A). Significant difference in gabapentin permeability was observed for 50 μM gabapentin *versus* 50 μM gabapentin + 10 mM BCH and for 50 μM gabapentin *versus* 50 μM gabapentin (B-A); * $p < 0.05$ and ** $p < 0.01$ (Sidak's multiple comparisons test). Significant differences in end-point intracellular gabapentin accumulation was observed for 5.8 mM and 50 μM gabapentin when transport was measured in the apical to basolateral (A-B) direction *versus* the basolateral to apical (B-A) direction; *** $p < 0.001$ (Dunnett's multiple comparisons test), and when 10 mM BCH was co-applied with 50 μM gabapentin (Student's *t*-test) # $p < 0.05$. No significant differences in mannitol permeability or end-point mannitol accumulation were observed among the different conditions.

Table V Estimated intracellular gabapentin concentrations. The concentrations were estimated at the end of the transepithelial transport study (after 150 min) as end-point concentrations (EPC). The cellular concentrations were estimated based on the intracellular accumulation data shown in Fig. 4b-d and an estimated intracellular volume of 2.24 μl (1.12 $cm^2 \times 20\,\mu m$)

Apical	→	Cellular	→	Basolateral	Basolateral	→	Cellular	→	Apical
50 mM	10%	5.0 mM	5%	270 μM					
5.8 mM	14%	0.8 mM	4%	35 μM	5.8 mM	71%	4.1 mM	2%	92 μM
50 μM	30%	15 μM	3%	0.38 μM	50 μM	76%	38 μM	3%	1.0 μM
50 μM + BCH	12%	6.0 μM	4%	0.26 μM					

membrane than across the apical membrane. Apical co-application of 10 mM BCH with 50 μM gabapentin caused a 60% reduction in EPC, while exit across the basolateral membrane seemed unaffected. Based on the observed low cellular efflux of gabapentin and the fact that the proposed intestinal transporters $b^{0,+}$ and LAT2 are exchangers, attempts to increase efflux *via* trans-stimulation using the $b^{0,+}$-substrate Lys and the LAT-substrate Leu were made. The resulting permeability and EPA of gabapentin are shown in Fig. 5. The A-B gabapentin permeability was increased by 40% when Leu was present in the basolateral chamber. Apical co-application of BCH and basolateral Leu application reduced the gabapentin permeability to the control level, while a non-significant reduction in EPA was observed. BCH in the basolateral receiver chamber caused a non-significant increase in gabapentin permeability. The B-A gabapentin permeability was unaffected by apical Lys application. However, Lys caused a significant increase in gabapentin EPA. Basolateral co-application of BCH in the donor chamber reduced gabapentin EPA by 48–50%. This inhibition was, however, associated with a significant reduction in EPA of mannitol, without a reduction in neither gabapentin nor mannitol transepithelial permeability. BCH present in the apical receiver chamber led to a significant decrease in the EPA of gabapentin, however without affecting the transepithelial permeability of gabapentin.

Initial Apical Uptake in Caco-2 Cells

To specifically investigate the apical uptake of gabapentin initial apical uptake experiments in Caco-2 cells were performed (Fig. 6). The apical uptake of gabapentin was pH- and sodium-independent, but significantly reduced by BCH (n= 3–4, p<0.05–0.001). The $b^{0,+}$-substrates Lys and L-cystine (CssC) did not cause any significant reduction in the initial uptake rate of gabapentin. BCH concentration-dependently inhibited the initial apical uptake of 50 μM gabapentin with an IC_{50}-value of 252 μM ($LogIC_{50}$ of −0.6 ± 0.08) (Fig. 6d). As expected the initial apical uptake rate of gabapentin was concentration-dependent (Fig. 6e), and described by a K_m of

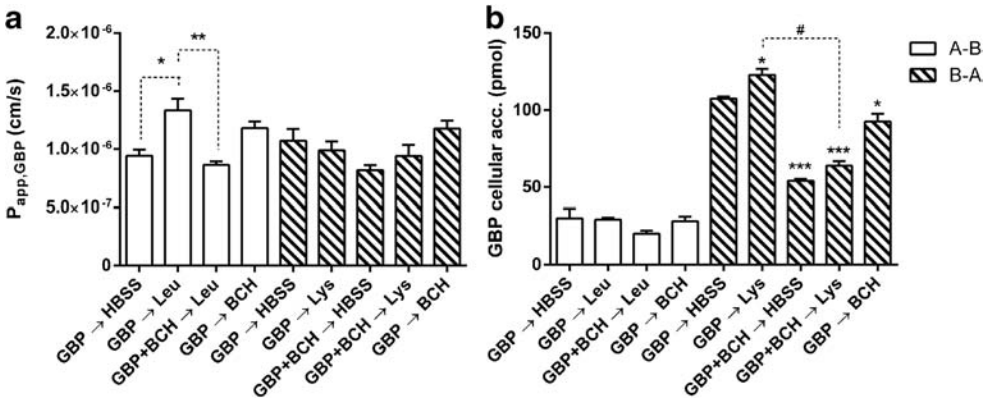

Fig. 5 The apparent transepithelial permeability, P_{app}, (**a**) and end-point (150 min) intracellular gabapentin (GBP) accumulation (**b**) across Caco-2 cell monolayers. The donor concentration of gabapentin was 50 μM and the concentration of Lys, BCH and Leu 10 mM. The transepithelial transport was measured for 150 min and the permeability was calculated from Eq. 1 and expressed as mean ± SEM of measurements performed in three independent cell passages. Apical and basolateral pH was maintained at pH 7.4. Scratched bars indicate transport in the basolateral to apical direction (B-A). Significant difference in the apical to basolateral gabapentin permeability was observed when Leu was applied on the basolateral side, and when BCH was co-applied on the apical side * $p<0.05$ and ** $p<0.01$ (Sidak's multiple comparisons test). Significant difference in end-point intracellular accumulation of gabapentin from the basolateral chamber was observed in all cases when compared to the GBP → HBSS column * $p<0.05$, *** $p<0.001$; and when Lys was present on the apical side and BCH co-applied on the basolateral side $^{\#}$ $p<0.001$ (Sidak's multiple comparisons test). No significant differences in mannitol permeability were observed among the different conditions, whereas the end-point mannitol accumulation from the basolateral chamber was inhibited to a significant degree in the presence of BCH (data not shown).

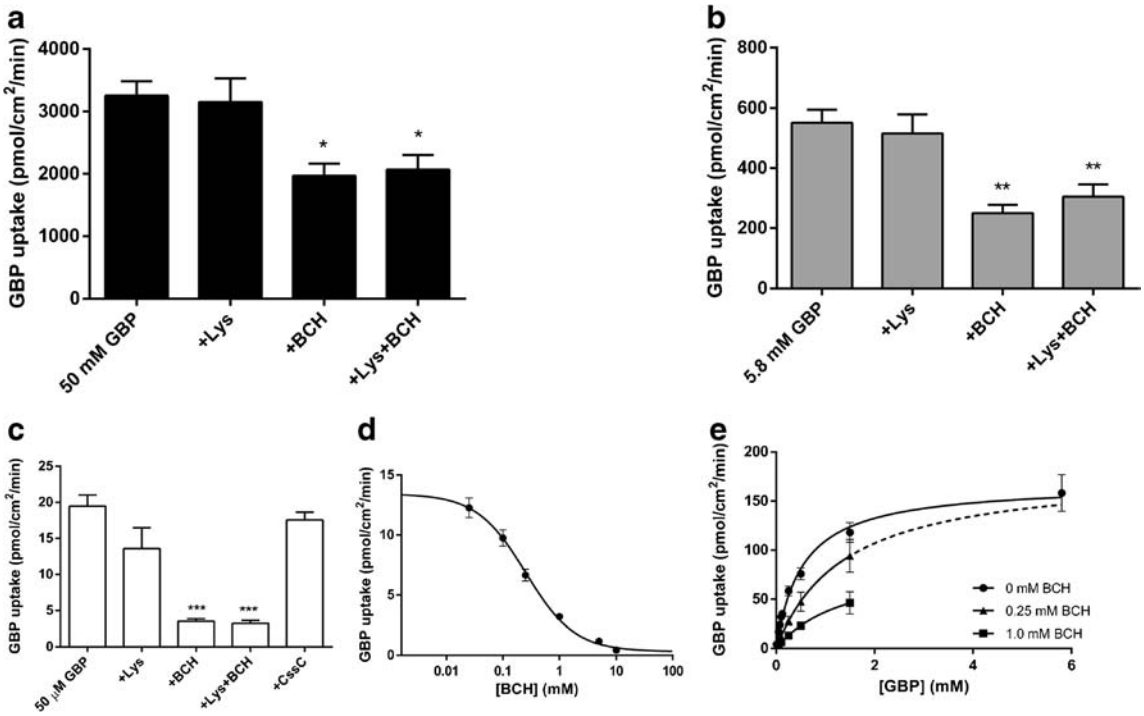

Fig. 6 Initial apical uptake of gabapentin (GBP) in Caco-2 cells. The uptake was measured for 5 min in Caco-2 cells grown on the bottom of multiwell plates. The apical pH was maintained at pH 6.8 unless stated otherwise. All data are shown as mean ± SEM of measurements performed in 3–4 independent passages. (**a**-**c**) The uptake of gabapentin (50 mM, 5.8 mM and 50 μM) measured in the absence or presence of Lys (68.4 mM in B-C and 10 mM in D), BCH (64.4 mM in B-C and 10 mM in D) or CssC (1 mM in D). (**d**) BCH-mediated concentration-dependent inhibition of the uptake of 50 μM gabapentin. (E) Concentration-dependent uptake of gabapentin measured in the absence (circle) or presence of 0.25 mM (triangle) or 1.0 mM (square) BCH. Significant difference from uptake measured in the absence of inhibitor: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Dunnett's multiple comparisons test).

536 ± 89 μM and a V_{max} of 168 ± 9 pmol/cm^2/min. 0.25 mM BCH significantly increased K_m to 1.4 ± 0.4 mM ($p < 0.05$) whereas V_{max} remained unaffected (181 ± 29 pmol/cm^2/min), indicative of competitive inhibition. However, co-application of 1.0 mM BCH caused not only an increase in K_m to 1.4 ± 0.5 mM, but also a decrease in V_{max} to 90.7 ± 18 pmol/cm^2/min, complicating the identification of the inhibitory kinetics. Regardless, BCH and gabapentin interact at an apical located transporter, resulting in a reduced gabapentin transport in the presence of BCH.

DISCUSSION

To investigate the effect of dose escalation on gabapentin distribution and elimination a series of intravenous doses, ranging from 5 to 100 mg/kg, were administered to rats in the present study. A proportional increase in $AUC_{0\text{-inf}}$ was observed in the range 5 to 100 mg/kg, in agreement with a previous study in rats showing a dose-proportional increase in exposure following intravenous administration of gabapentin in the range 4–500 mg/kg [13]. Similarly, gabapentin exhibited dose-independent distribution and elimination kinetics, as no significant change in neither volume of distribution nor clearance was observed following dose-escalation. However, at the highest dose a tendency towards non-linear elimination kinetics was observed as illustrated by a slight increase in clearance and a decrease in $AUC_{0\text{-inf}}$. The maximum dose of gabapentin administered to humans is 4,800 mg/day, resulting in a C_{max} around 12,000 μg/L [9]. In accordance with the present study, clinical trials have shown that gabapentin exhibits dose-dependent absorption kinetics, as bioavailability is decreased from 73.8 to 35.7% when dose was increased from 100 to 1,600 mg [7]. It is known that suggested gabapentin transporters, $b^{0,+}$ and LAT2, are involved in reabsorption of amino acids in the kidneys [21, 27, 28]. Thus, a possible explanation for this non-linearity could, therefore, be saturation of reabsorption transporters in the kidneys.

A tendency towards an increase in t_{max} with increasing gabapentin dose was observed and a significant delay in t_{max} was observed following oral administration of 200 mg/kg relative to 10 mg/kg. Given that gabapentin is absorbed by a transporter [7], this delay might result from a decrease in the absorption rate due to saturation of the intestinal transporter. The estimated areas under the curves determined in this study are slightly higher than those previously reported [12, 14], but in the same range. In agreement with prior reports a significant drop in bioavailability, from 95 to 30%, was observed

when dose was increased from 10 to 200 mg/kg [13]. Further, Radulovic *et al.* [11] showed that the urinary excretion of the dosed gabapentin decreased from 99.8 to 79.0% when the dose was increased from 10 to 50 mg/kg in rats. These findings suggest that gabapentin was subjected to saturable absorption kinetics based on the fact that (i) gabapentin distribution and elimination apparently were unaffected/affected to a limited degree by dose-escalation, (ii) metabolism and protein-binding was negligible in rats [11], and (iii) gabapentin was readily soluble in water [29].

The uptake experiments in Caco-2 cells revealed a dose-dependent uptake across the apical membrane, supporting the hypothesis that intestinal gabapentin absorption is transporter mediated. In contrast, the transepithelial transport rate across Caco-2 cells (A-B) seemed concentration-independent and was only twice the rate of the paracellular marker mannitol. Similar results have been reported in previous studies [30, 31]. This may suggest that (i) carrier mediated transport was not the primary mechanism responsible for the observed decrease in bioavailability with increasing dose, or (ii) Caco-2 cells were not suited for predictions of intestinal transport of gabapentin.

Prior to this study, others have sought to identify and characterize the transport systems responsible for the saturable absorption kinetics of gabapentin through *in vitro* and *in situ* studies [7, 16, 18]. In these studies gabapentin exhibited cross-inhibition with cationic amino acids, such as Arg and Lys, and neutral amino acids/amino acid analogues, such as Leu and BCH [7, 8, 16, 18, 19, 30]. Based on these results and considering the proton- and sodium-independency of gabapentin absorption, the two transport systems $b^{0,+}$ and LAT have been suggested to be involved in the intestinal absorption of gabapentin [17, 18]. According to immunohistochemistry studies performed in murine intestines, $b^{0,+}$ is located in the apical membrane, while LAT2 is located in the basolateral membrane [22].

Contrary to a previously reported *in situ* study [18], no significant inhibition of gabapentin transport was observed following oral co-administration of Lys *in vivo* or co-application *in vitro* in the present study. These apparent discrepancies may arise from the fact that a higher Lys to gabapentin concentration ratio was applied in the previous *in situ* study (2000:1) relative to the present *in vivo* and *in vitro* studies (20:1 and 200:1, respectively). In a similar manner, co-application of another $b^{0,+}$-substrate, CssC, had no effect on the initial apical uptake of gabapentin. Moreover, co-application of Lys in the apical receiver chamber showed no effect on trans-stimulation. In summary, these findings suggest that $b^{0,+}$, in contrary to previous studies [17, 18], has no or only limited involvement in the intestinal absorption of gabapentin in rats and Caco-2 cells.

Our *in vivo* study shows a significant decrease in the absorption of gabapentin following co-administration of BCH, indicating the involvement of a BCH-sensitive gabapentin transporter in the intestinal wall. Furthermore, our *in vitro* experiments in Caco-2 cells suggest the presence of BCH-sensitive gabapentin transporters in both the apical and basolateral membrane. BCH has shown some affinity for apical transport systems, such as B^0 and $B^{0,+}$. However, involvement of these transport systems in gabapentin transport in Caco-2 cells seems unlikely since the present study along with previous reports have shown that intestinal absorption of gabapentin is sodium-independent [16, 18]. As regards to $b^{0,+}$, an uptake study has shown that BCH do not inhibit uptake of Arg, a typical $b^{0,+}$-substrate, in Caco-2 cells, indicating that $b^{0,+}$ is not BCH-sensitive [16]. Gabapentin and BCH are both neutral and bulky/branched amino acids, fitting the characteristics of a LAT-substrate [32] and BCH is a known LAT-substrate/inhibitor [33], however, the major LAT-isoform in the small intestine, LAT2, is located in the basolateral membrane [21]. As regards to LAT1 conflicting results have been reported. Dave *et al.* reported no expression of LAT1 in murine intestines [22], while others have shown expression of LAT1 in Caco-2 cells, IEC-6 cells and rat intestinal mucosa and trans-stimulation experiments have identified a Leu-sensitive exchanger in the apical membrane of Caco-2 and IEC-6 cells, possibly LAT [34, 35]. Our apical uptake studies show similar values of k_m/ IC_{50} for gabapentin (536 μM) and BCH (252 μM), while others have found similar K_m- and V_{max}-values for Leu and gabapentin [16]. Hence, gabapentin seems to share transport characteristics (high affinity and low capacity) with typical LAT-substrates/inhibitors in Caco-2 cells. However, it's generally believed that LAT is expressed in the basolateral membrane and as there is currently no evidence for the expression of LAT in the apical membrane of fully differentiated Caco-2 cells (around 20 days) no firm conclusions can be drawn [16, 34, 35].

In accordance with previous suggestions [17, 18], co-application of Leu in the basolateral receiver chamber caused an increase in the apparent permeability of gabapentin, presumably *via* trans-stimulation of a shared basolateral exchanger. Furthermore, basolateral influx of gabapentin was inhibited by co-application of BCH in the basolateral donor chamber. These transport characteristics points towards the involvement of a BCH-sensitive exchanger in the basolateral transport of gabapentin in Caco-2 cells. In this context, LAT2 appears a plausible candidate considering its known expression in the basolateral membrane of Caco-2 cells and the intestinal wall [21, 34]. To further investigate this theory, the ion-dependency of basolateral gabapentin transport needs to be examined.

The generated knowledge of gabapentine and transporter dependency is important when gabapentine is used as a reference compound when developing new molecules for the treatment of pain. Further, from a clinical perspective it's known that in addition to the dose-dependent absorption kinetics the oral bioavailability have been demonstrated to

be subjected to large inter-individual variation [36]. Hence, dose-determination poses a major challenge to the physician and may at least partially explain the high incidence of non-responders in the treatment of neuropathic pain [10]. Unfortunately, progress within the field of optimization of personal dosage scheduling is impeded by gabapentin's complex pharmacokinetics, which studies like this can help explain.

In conclusion, this study have for the first time shown that gabapentin transport across the intestinal wall was mediated by a BCH-sensitive transporter *in vivo*. Also, in Caco-2 cell experiments BCH inhibited apical uptake of gabapentin. In contrast, no effect on intestinal transport was observed following co-application of Lys *in vivo* or *in vitro*. The data obtained in the present study may therefore imply that a BCH-sensitive transport-system was involved in the apical and possibly the basolateral transport of gabapentin across the intestinal wall.

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