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RESEARCH PAPER

The proton-coupled oligopeptide transporter 1 plays a major role in the intestinal permeability and absorption of 5-aminolevulinic acid

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BACKGROUND AND PURPOSE

5-Aminolevulinic acid (5-ALA) has been widely used in photodynamic therapy and immunofluorescence of tumours. In the present study, the intestinal permeability and oral pharmacokinetics of 5-ALA were evaluated to probe the contribution of the proton-coupled oligopeptide transporter 1 (PEPT1) to the oral absorption and systemic exposure of this substrate.

EXPERIMENTAL APPROACH

In situ single-pass intestinal perfusions and *in vivo* oral pharmacokinetic studies were performed in wildtype and *Pept1* knockout mice. Perfusion studies were performed as a function of concentration dependence, specificity and permeability of 5-ALA in different intestinal segments. Pharmacokinetic studies were performed after 0.2 and 2.0 μmoL·g^{-1} doses of 5-ALA.

KEY RESULTS

The permeability of 5-ALA was substantial in duodenal, jejunal and ileal regions of wildtype mice, but the residual permeability of 5-ALA in the small intestine from *Pept1* knockout mice was only about 10% of that in wildtype animals. The permeability of 5-ALA in jejunum was specific for PEPT1 with no apparent contribution of other transporters, including the proton-coupled amino acid transporter 1 (PAT1). After oral dosing, the systemic exposure of 5-ALA was reduced by about twofold during PEPT1 ablation, and the pharmacokinetics were dose-proportional after the 0.2 and 2.0 μmol·g^{-1} doses. PEPT1 had a minor effect on the disposition and peripheral tissue distribution of 5-ALA.

CONCLUSION AND IMPLICATIONS

Our findings suggested a major role of PEPT1 in the intestinal permeability and oral absorption of 5-ALA. In contrast, another proton-coupled transporter, PAT1, appeared to play a limited role, at best.

Abbreviations

5-ALA, 5-aminolevulinic acid; AUC, area under the plasma concentration-time curve; C_{max}, maximum plasma concentration; CL/F, oral clearance; CL, total plasma clearance; GlySar, glycylsarcosine; OATs, organic anion transporters; OCTs, organic cation transporters; PAH, para-aminohippuric acid; PAT1, proton-coupled amino acid transporter 1; PEPT1, proton-coupled oligopeptide transporter 1; PHT1/2, peptide-histidine transporters 1/2; TEA, tetraethylammonium; T_{max}, time at which maximum plasma concentration is reached; V_{max}, maximum transport rate

Tables of Links

TARGETS
Transporters
PEPT1, proton-coupled oligopeptide transporter 1 (SLC15A1)
PAT1, proton-coupled amino acid transporter 1 (SLC36A1)
PHT1, peptide-histidine transporter 1 (SLC15A4)
PHT2, peptide-histidine transporter 2 (SLC15A3)

LIGANDS
5-ALA, 5-aminolevulinic acid
Cefadroxil
PAH, *p*-aminohippuric acid
TEA, tetraethylammonium

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

5-Aminolevulinic acid (5-ALA), a naturally occurring intermediate in the haem biosynthesis pathway, has been widely used in photodynamic therapy and fluorescence detection for the last 20 years (Peng *et al.*, 1997a,b; Kelty *et al.*, 2002; Krammer and Plaetzer 2008). Currently, 5-ALA and its esters have been approved by the FDA as a promising treatment of several malignant and premalignant conditions such as actinic keratosis, basal cell carcinoma, Bowen's disease and bladder cancer. In Europe, 5-ALA has been approved for intraoperative photodynamic diagnosis of residual malignant glioma, while the methyl ester and hexyl ester derivatives of 5-ALA have been approved for the treatment of basal cell carcinoma and actinic keratosis and for the diagnostic application of bladder cancer respectively (Dolmans *et al.*, 2003; Fotinos *et al.*, 2006; Krammer and Plaetzer 2008; Nokes *et al.*, 2013).

Oral dosing is an administration route that is commonly used in the clinical application of 5-ALA. In practice, high accumulation of 5-ALA has been reported in normal enterocytes after oral administration (Loh *et al.*, 1993; Regula *et al.*, 1995). Moreover, good oral bioavailability of 5-ALA was observed in several experimental and clinical studies (van den Boogert *et al.*, 1998; Dalton *et al.*, 1999; Dalton *et al.*, 2002) despite the fact that 5-ALA is a hydrophilic and polar molecule. In 1998, Döring *et al.* provided direct evidence for the translocation of 5-ALA in *Xenopus laevis* oocytes and *Pichia pastoris* yeast cells expressing the rabbit proton-coupled oligopeptide transporter 1 (PEPT1; SLC15A1). Given this finding, and the abundant expression of PEPT1 mRNA in rabbit small intestine, these authors suggested that intestinal PEPT1 could explain the good bioavailability of 5-ALA when administered orally. However, subsequent studies by Frølund *et al.* (2010) and Anderson *et al.* (2010) in Xenopus-expressing oocytes, transfected cell lines and Caco-2 cells suggested that the proton-coupled amino acid transporter 1 (PAT1; SLC36A1), along with PEPT1, contributed significantly to the *in vivo* oral absorption of 5-ALA. The former authors (Frølund *et al.*, 2010), using transfected MDCK/*hPEPT1*, COS-7/*hPAT1* and Caco-2 cells, further suggested that PAT1 may be the intestinal transporter responsible for the majority of 5-ALA's oral absorption during clinical dosing.

While *in vitro* experimental systems, such as oocytes, transfected cells and cell lines, are valuable for mechanistic studies, they have several disadvantages including the absence of an intact blood supply, physiological residence times at the site of action and transit along the various segments of the small and large intestines. Moreover, they study transport proteins in isolation without the complement of other possible intestinal transporters being present. Thus, it is still unclear as to the role and relevance of PAT1, compared with PEPT1, in the intestinal uptake and oral absorption of 5-ALA. With this in mind, we proposed the following specific aims: (i) to determine *in situ* the quantitative contribution of PEPT1 and PAT1 on the effective permeability of 5-ALA during single-pass intestinal perfusions of wildtype and *Pept1* knockout mice; and (ii) to characterize *in vivo* the oral absorption, tissue distribution and pharmacokinetics of 5-ALA in PEPT1-competent and PEPT1-deficient mice.

Methods

Animals

All animal care and experimental procedures complied with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health and were approved by the Animal Use Committee of the University of Michigan. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Gender-matched wildtype and *Pept1* knockout mice (>99% C57BL/6 genetic background, 8–10 weeks old) were bred in-house at the University of Michigan (Hu *et al.*, 2008). The mice were kept under a 12 h light/dark cycle and in an ambient temperature-controlled environment, fed with standard diet and water *ad libitum* in the Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI, USA).

Stability of 5-ALA in intestinal segments

Single-pass perfusions were performed *in situ* on different intestinal segments in which the inlet and the outlet perfusate samples were collected and subsequently analysed by HPLC. The HPLC system consisted of a Waters 515 pump (Waters Inc., Milford, MA, USA) and a β-RAM 5 radiochemical

detector and Laura (Version 4.1.8) data acquisition software (LabLogic Systems, Brandon, FL, USA). A reversed phase 250 × 4.6 mm C18 column (Discovery; Supelco, Bellefonte, PA, USA) was used for chromatographic separation. The mobile phase consisted of 5% acetonitrile plus 0.1% trifluoroacetic acid and pumped isocratically at 1.0 mL·min^{-1} under ambient conditions. All perfusate samples were centrifuged at 10 000× *g* for 10 min, and 20 μL aliquots of supernatant were inserted manually into the loop injector. Under these conditions, the retention time of [^{14}C]5-ALA was 4.1 min.

In situ single-pass intestinal perfusion study

Gender-matched wildtype and *Pept1* knockout mice were fasted overnight (~16 h) with free access to water. Perfusion experiments were performed according to methods described previously (Adachi *et al.*, 2003; Jappar *et al.*, 2010). Briefly, the mice were anaesthetized with sodium pentobarbital (40–60 mg·kg^{-1} i.p.) and placed on a heated pad to maintain normal body temperature. Prior to surgery, isopropyl alcohol was applied to sterilize the abdominal area, and the abdomen was opened through a 1.5 cm midline incision longitudinally to expose the small intestine. During jejunal perfusions, an 8 cm segment of proximal jejunum was isolated (i.e. ~2 cm distal to the ligament of Treitz) with incisions made at both the proximal and distal ends. Glass cannulas (2 mm outer diameter) were then inserted into both ends of the jejunum and secured in place with silk suture. Following cannulation, the isolated segment was rinsed with isotonic saline solution and covered with saline-wetted gauze and parafilm to prevent dehydration. After the surgical procedure, the mice were transferred to a temperature-controlled chamber (31°C) to maintain the body temperature during experimentation. The inlet cannula was connected to a 30 mL syringe placed on a perfusion pump (Model 22; Harvard Apparatus, South Natick, MA, USA), and the outlet tubing was placed in a collection vial.

The buffer (pH 6.5), containing 135 mM NaCl, 5 mM KCl, 10 mM 2-(N-morpholinoethanesulfonic acid, 0.01% (w/v) [^{3}H]inulin and [^{14}C]5-ALA, was perfused through the jejunum at rate of 0.1 mL·min^{-1} for 90 min. The exiting perfusate was then collected every 10 min for 90 min. A 100 μL aliquot from each 10 min collection was added to a vial containing scintillation fluid (CytoScint®; MP Biomedicals, Solon, OH, USA) and the sample measured by a dual-channel liquid scintillation counter (Beckman LS 6000 SC; Beckman Coulter, Inc., Fullerton, CA, USA). The jejunal permeability of 5-ALA was determined at steady state, which was achieved 30 min after initiating the perfusion. Water flux was corrected by the nonpermeable marker [^{3}H]inulin. At the end of each experiment, the length of intestinal segment was directly measured.

For concentration-dependent studies, perfusate concentrations of 5-ALA were ranged from 0.01 to 50 mM in order to assess the transport kinetics [i.e. maximum transport rate (V_{max}), K_M] of 5-ALA in wildtype jejunum. For specificity studies, jejunal perfusions were performed with 10 μM 5-ALA in the absence and presence of 25 mM potential inhibitors such as glycylsarcosine (GlySar), cefadroxil, tetraethylammonium (TEA), *p*-aminohippuric acid (PAH), L-histidine, L-proline or β-alanine. For region-dependent studies, the duodenum, jejunum, ileum and colon were perfused simultaneously in wildtype and *Pept1* knockout mice with 10 μM of 5-ALA in the perfusate. In addition to the jejunal segment (as described before), a 2 cm segment of duodenum (i.e. ~0.25 cm distal to the pyloric sphincter), a 6 cm segment of ileum (i.e. ~1 cm proximal to the caecum) and a 3 cm segment of colon (i.e. ~0.5 cm distal to the caecum) were isolated and perfused as described previously.

Real-time PCR

Quantitation of *Pat1* gene expression was performed in the small intestine, large intestine and kidney of wildtype and *Pept1* knockout mice using a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) as described previously (Hu *et al.*, 2014).

Pharmacokinetic study of 5-ALA after oral administration

Gender-matched wildtype and *Pept1* knockout mice were fasted overnight, after which radiolabeled [^{14}C]5-ALA and cold 5-ALA were dissolved in normal saline (0.04 μCi·μL^{-1}) and the 5-ALA drug solution (200 μL or 8.0 μCi·mouse^{-1}) administered orally by gavage at single doses of 0.2 or 2 μmol·g^{-1} body weight. Serial blood samples were then collected (~20 μL) via tail nicks in 0.2 mL microcentrifuge tubes containing 7.5% potassium EDTA at 5, 10, 20, 30, 45, 60, 90, 120, 150 and 180 min after the oral dose. Plasma samples were obtained after centrifuging the blood at 3000 g × 3 min, and a 10 μL aliquot was transferred to a glass scintillation vial. Scintillation fluid (6 mL) was added to each sample and the plasma radioactivity measured by a dual-channel liquid scintillation counter. For each experiment, mice were given i.p. injections of 0.3 mL warm saline, 45 min after the oral dose, to prevent dehydration.

Tissue distribution study of 5-ALA after oral administration

At the end of sampling for the 0.2 μmol·g^{-1} oral dose study of [^{14}C]5-ALA (at 180 min; see previous text), tissue samples were also collected. Several tissues including the eye, spleen, duodenum, jejunum, ileum, colon, stomach, kidney, liver, lung, heart and skeletal muscle were harvested, blotted dry, weighed and then solubilized overnight in 0.33 mL of 1 M hyamine hydroxide at 37°C. Whole blood samples (10 μL) were also collected at this same time. After incubation with hyamine hydroxide, a 40 μL aliquot of hydrogen peroxide (30% w/w) was added to each sample for decolorization. A 6 mL aliquot of scintillation fluid was then added to the tissue samples, and radioactivity was measured by a dual-channel liquid scintillation counter. To determine the tissue vascular space, 100 μL [^{3}H]dextran 70 000 (0.2 μCi·mouse^{-1}) was administered via a tail vein injection 5 min before harvesting the tissues. The corrected tissue concentrations of 5-ALA were then calculated as (Ocheltree *et al.*, 2005; Shen *et al.*, 2007): Ctiss.corr = Ctiss − V × Cb, where Ctiss.corr and Ctiss are the corrected and uncorrected tissue concentrations of 5-ALA (nmol·g^{-1}), V is the blood vascular space as determined by dextran in the tissue (mL·g^{-1}) and Cb is the 5-ALA blood concentration (nmol·mL^{-1}).

Pharmacokinetic study of 5-ALA after i.v. administration

Radiolabeled [^{14}C]5-ALA and cold 5-ALA were dissolved in normal saline (0.025 μCi·μL^{-1} and 2 nmol·μL^{-1}) prior to the experiment. Following sodium pentobarbital anaesthesia (~40–60 mg·kg^{-1} i.p.), wildtype and *Pept1* knockout mice received [^{14}C]5-ALA (10 nmol·g^{-1} body weight) through a tail vein injection. Serial blood samples were collected (~20 μL·sample^{-1}) via tail nicks in 0.2 mL microcentrifuge tubes containing 7.5% potassium EDTA at 0.25, 1, 2, 5, 10, 15, 20, 30, 45 and 60 min after the i.v. bolus dose. For each sample, a 10 μL aliquot of plasma was obtained after centrifugation of blood at 3000 g × 3 min and then transferred to a glass scintillation vial. A 6 mL aliquot of scintillation fluid was added to each sample, and radioactivity was measured by a dual-channel liquid scintillation counter.

Data analysis

The permeability of 5-ALA was calculated at steady state by the equation (Johnson and Amidon, 1988; Sinko and Amidon, 1988): $P_{eff} = -Q \bullet \ln(C_{out}/C_{in})/(2\pi RL)$, where P_{eff} is the effective permeability, C_{out} and C_{in} are the outlet (corrected for water flux) and inlet concentrations of 5-ALA in perfusate, Q is the perfusate flow rate, R is the radius of intestinal segment and L is the length of intestinal segment. The steady-state flux (J) across the intestinal membrane was calculated as follows: $J = P_{eff} \bullet C_{in}$. Those 5-ALA flux values were then used to determine the transport parameters (V_{max}' and K_M') when referenced to inlet drug concentrations as shown by $J = V_{max}' \bullet C_{in}/(K_M' + C_{in})$. V_{max} and K_M, determined after factoring out resistance across the unstirred water layer, were also determined by relating the 5-ALA flux values to intestinal wall concentrations (C_w) as shown by $J = V_{max} \bullet C_w/(K_M + C_w)$. The relationship between intestinal wall and inlet drug concentrations is $C_w = C_{in} \bullet (1 - P_{eff}/Paq)$, where Paq is the unstirred aqueous layer permeability. $Paq = (A \bullet R \bullet Gz^{1/3}/D)^{-1}$, where D is the aqueous diffusion coefficient (6.596×10^{-4} cm^{-2}·min^{-1}), calculated according to the Hayduk–Laudie expression, Gz is the Graetz number (0.0829), and A is a unitless constant (1.332) estimated by: $A = 2.5Gz + 1.125$.

The pharmacokinetics of 5-ALA, after oral and i.v. administrations, were calculated by noncompartmental analyses (Phoenix WinNonlin v1.3; Certara, St. Louis, MO, USA). Parameters after oral dosing included the maximum plasma concentration (C_{max}) and time to reach the maximum plasma concentration (T_{max}), the area under the plasma concentration-time curve from time zero to the last experimental time point ($AUC_{0\text{-}t}$) and from time zero to infinity ($AUC_{0\text{-}\infty}$), the oral clearance (CL/F), the oral volume of distribution, and the log-linear terminal rate-constant and $t_{1/2}$. Parameters after i.v. dosing included the $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$, the total plasma clearance (CL), the volume of distribution steady-state, and the mean residence time after i.v. dosing and $t_{1/2}$.

Statistical Analysis

Data were reported as mean ± SEM. Unpaired two-tailed student's *t*-tests were used to evaluate statistical differences between two groups. One-way ANOVA followed by Dunnett's or Tukey's *post hoc* test were performed to evaluate statistical differences between multiple groups (Prism v5.0; GraphPad Software Inc., La Jolla, CA, USA). The quality of fits for nonlinear regression was assessed by coefficient of determination (r^2), by variation of parameter estimates and by visual inspection of the residuals. For all statistical tests, $P \leq 0.05$ was considered significant.

Materials

[^{14}C]5-Aminolevulinic acid (55 mCi·mmol^{-1}), [^{3}H]inulin (201 mCi·g^{-1}) and [^{3}H]dextran (MW 70 000; 100 mCi·g^{-1}) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Hyamine hydroxide was obtained from ICN Pharmaceuticals (Costa Mesa, CA, USA). Unlabeled 5-ALA hydrochloride and other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Results

Concentration-dependent uptake in perfusion studies

To evaluate whether the jejunal uptake of 5-ALA was saturable, *in situ* perfusion experiments were conducted in wildtype mice over a wide range of 5-ALA concentrations in perfusate (i.e. 0.01–50 mM). As shown in Figure 1A, 5-ALA exhibited saturable kinetics that was best described by a single Michaelis–Menten term in which the transport parameters were estimated as $V_{max}' = 2.30 \pm 0.16$ nmol^{-1}·cm^{-2}·s^{-1} and $K_M' = 13.4 \pm 2.4$ mM when referenced to inlet perfusate concentrations ($r^2 = 0.937$). When referenced to intestinal wall concentrations (Figure 1B), the intrinsic transport parameters of 5-ALA were estimated as $V_{max} = 1.89 \pm 0.12$ nmol^{-1}·cm^{-2}·s^{-1} and $K_M = 3.74 \pm 0.95$ mM ($r^2 = 0.857$). Based on these values, subsequent studies were performed under linear conditions with 10 μM of 5-ALA in the perfusate.

Substrate specificity in perfusion studies

To probe the specificity of PEPT1-mediated transport of 5-ALA in jejunum, the effective permeability of 10 μM 5-ALA was evaluated in wildtype mice by co-perfusing this substrate with a variety of potential inhibitors (25 mM). As shown in Figure 2, the jejunal permeability of 5-ALA was substantially reduced in the presence of the PEPT1 substrates GlySar (residual of 31%) and cefadroxil (residual of 16%). In contrast, 5-ALA permeability was not altered when co-perfused with L-histidine (substrate of the peptide-histidine transporters, PHT1/2), L-proline or β-alanine (substrates of PAT1), TEA (substrate of the organic cation transporters, OCTs) or PAH (substrate of organic anion transporters, OATs). These results demonstrated that the jejunal permeability of 5-ALA was primarily, if not exclusively, mediated by PEPT1. The contribution of other transporters, including PAT1, in mediating the intestinal permeability of 5-ALA is unlikely or minor at best.

Region-dependent uptake in perfusion studies

To investigate whether the uptake of 5-ALA was different along the length of the small and large intestines, the permeability of 10 μM 5-ALA was determined in four intestinal segments in both genotypes. As portrayed in Figure 3A, the

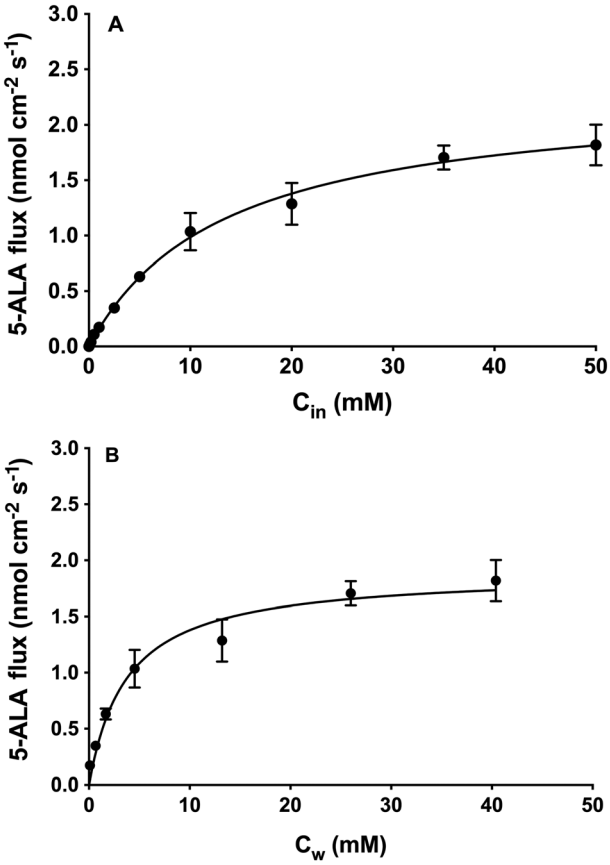

Figure 1

Concentration-dependent flux of [^{14}C]5-ALA (0.01–50 mM total substrate) during jejunal perfusions of wildtype mice. Studies were performed in pH 6.5 perfusion buffer. Data are expressed as mean ± SEM (n = 4). C_{in} represents the perfusate concentration of 5-ALA (A), and C_w represents the estimated concentration of 5-ALA at the intestinal wall (B).

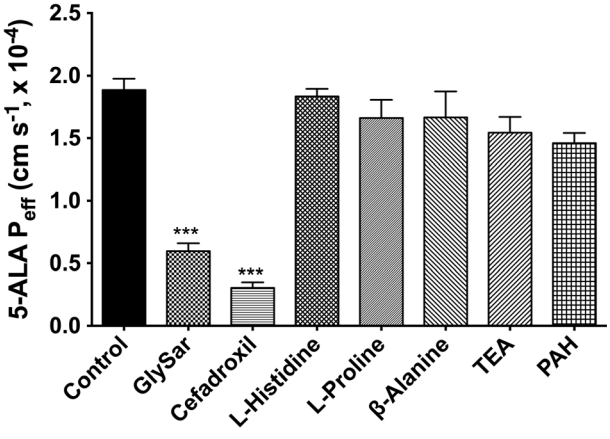

Figure 2

Effect of potential inhibitors (25 mM) on the permeability of 10 μM [^{14}C]5-ALA during jejunal perfusions of wildtype mice. Studies were performed in pH 6.5 perfusion buffer. Data are expressed as mean ± SEM (n = 4). $^{***}P \leq 0.001$, significantly different from control; one-way ANOVA and Dunnett's test.

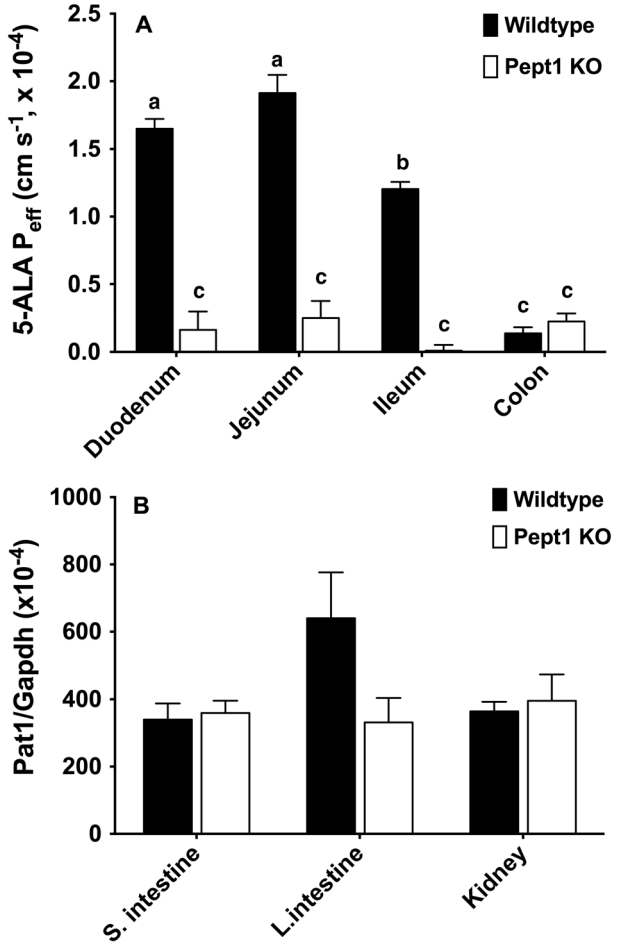

Figure 3

Permeability of 10 μM [^{14}C]5-ALA in the duodenum, jejunum, ileum and colon of wildtype and *Pept1* knockout (KO) mice (A). Studies were performed in pH 6.5 perfusion buffer. Data are expressed as mean ± SEM (n = 4). Treatment groups with the same letters (a, b, c) are not different from each other but statistically different from groups with a different letter; one-way ANOVA and Tukey's test. Real-time PCR of *Pat1*, corrected for *Gapdh*, in the small intestine, large intestine and kidney of wildtype and *Pept1* KO mice (B). No statistical differences were observed in *Pat1* gene expression between the two genotypes. Data are expressed as mean ± SEM (n = 6).

effective permeability of 5-ALA in wildtype mice was calculated as 1.65×10^{-4} cm·s^{-1} in duodenum, as 1.91×10^{-4} cm·s^{-1} in jejunum, as 1.20×10^{-4} cm·s^{-1} in ileum and as 0.14×10^{-4} cm·s^{-1} in colon. In contrast, the permeability of 5-ALA in *Pept1* knockout mice was only about 10% of that in wildtype animals for duodenum, jejunum and ileum. The colonic permeability of 5-ALAwas very small and did not differ between the two genotypes. No statistical differences were observed in *Pept1* knockout mice between any of the intestinal segments evaluated. Moreover, the gene expression of *Pat1* did not differ between genotypes in the small intestine, large intestine or kidney (Figure 3B).

Stability of 5-ALA in perfusion studies

It should be noted that 5-ALA was not metabolized or degraded in any of the samples obtained from wildtype and

Pept1 knockout mice during the perfusion of small and large intestinal segments (data not shown). Thus, the permeability calculations of 5-ALA were not confounded by substrate stability during these experiments.

Pharmacokinetics of 5-ALA after oral administration

The plasma concentration-time curves of 5-ALA, in both wildtype and *Pept1* knockout mice, are depicted in Figure 4A and B, respectively, following low (0.2 μmol·g^{-1}) and high (2.0 μmol·g^{-1}) dose oral administrations of drug. As shown in this figure, the maximum plasma concentration and systemic exposure (i.e. AUC) was much lower in *Pept1* knockout mice as compared with wildtype animals. In general, the oral absorption of 5-ALA appeared to be rapid in both genotypes with peak plasma concentrations being reached in about 10 min. As shown in Tables 1 and 2, significant differences were observed in C_{max}, AUC and CL/F. These differences reflected a twofold reduction in the extent of oral absorption during PEPT1 ablation. It does not appear that absorption rate was different between wildtype and *Pept1* knockout mice, however, because no statistically significant differences were observed between genotypes in either T_{max} or the $t_{1/2}$ of 5-ALA. Moreover, there was no evidence for dose-dependent kinetics of 5-ALA. In comparing the C_{max} and AUC of 5-ALA after oral doses of 0.2 and 2.0 μmol·g^{-1}, the dose-normalized values were close to one another and showed no statistical difference in both genotypes, indicating an 'apparent linearity' over this oral dose range.

Table 1

Non-compartmental analysis of 5-ALA after oral administration of 0.2 μmol·g^{-1} [^{14}C]5-ALA in WT and *Pept1* KO mice

Parameter (unit)	WT	KO	Ratio (KO·WT^{-1})
C_{max} (μM)	188 (18)	81 (22)**	0.46
T_{max} (min)	8.8 (1.3)	22.0 (9.4)	2.51
$AUC_{0\text{-}180}$ (μM·min)	7659 (1127)	3444 (466)*	0.45
$AUC_{0\text{-}\infty}$ (μM·min)	8883 (1400)	3696 (498)*	0.42
CL/F (mL·min^{-1})	0.49 (0.08)	1.15 (0.13)**	2.37
V/F (mL)	57.9 (9.3)	79.0 (16.5)	1.36
λz (min^{-1})	0.0086 (0.0009)	0.0163 (0.0029)	1.89
$t_{1/2}$ (min)	83.3 (10.1)	48.3 (8.4)	0.58

λz, log-linear terminal rate-constant; CL/F, oral clearance; C_{max}, maximum plasma concentration; KO, knockout; T_{max}, time at which maximum plasma concentration is reached; V/F, oral volume of distribution; WT, wildtype.
Data are expressed as mean ± SEM (n = 4–5). *$P < 0.05$, **$P < 0.01$, significantly different from WT mice.

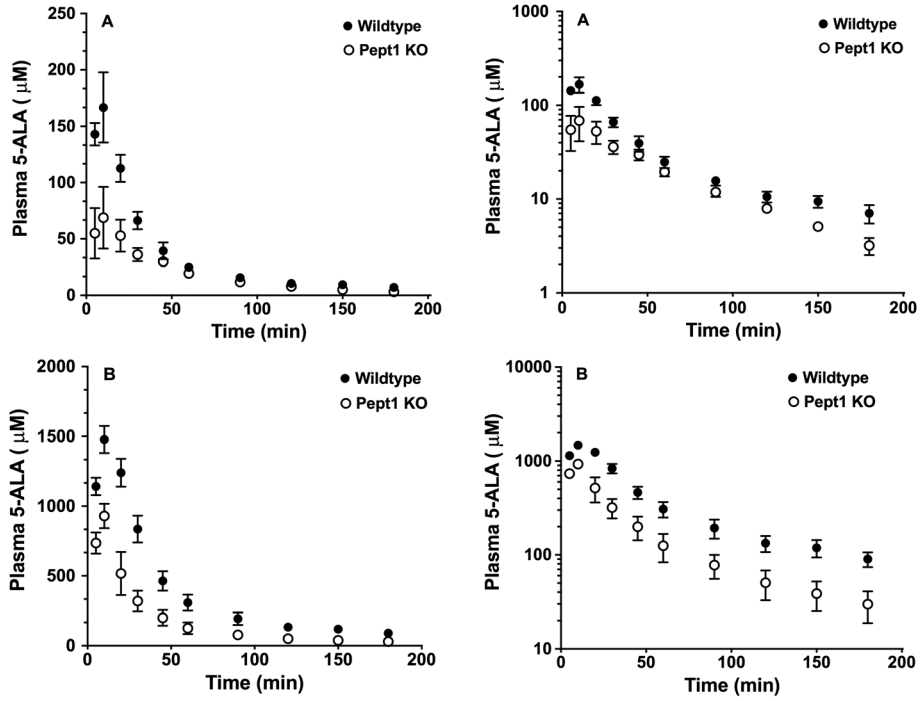

Figure 4

Plasma concentration-time profiles of [^{14}C]5-ALA in wildtype and *Pept1* knockout (KO) mice after a 0.2 μmoL·g^{-1} oral dose (A) and after a 2.0 μmoL·g^{-1} oral dose (B). Data are expressed as mean ± SEM (n = 4–5), where the Y-axis is displayed on a linear scale (left panels) and on a logarithmic scale (right panels).

Table 2

Non-compartmental analysis of 5-ALA after oral administration of 2.0 μmol·g^{-1} [^{14}C]5-ALA in WT and *Pept1* KO mice

Parameter (unit)	WT	KO	Ratio (KO·WT^{-1})
C_{max} (μM)	1478 (97)	930 (88)**	0.63
T_{max} (min)	10 (0)	10 (0)	1.00
$AUC_{0\text{-}180}$ (μM·min)	68212 (6884)	31056 (6338)**	0.46
$AUC_{0\text{-}\infty}$ (μM·min)	82207 (7925)	33807 (7689)**	0.41
CL/F (mL·min^{-1})	0.50 (0.05)	1.36 (0.26)*	2.71
V/F (mL)	79.8 (16.0)	107.5 (24.4)	1.35
λz (min^{-1})	0.0072 (0.0017)	0.0135 (0.0025)	1.87
$t_{1/2}$ (min)	109 (18)	57 (10)	0.52

λz, log-linear terminal rate-constant; CL/F, oral clearance; C_{max}, maximum plasma concentration; KO, knockout; T_{max}, time at which maximum plasma concentration is reached; V/F, oral volume of distribution; WT, wildtype.
Data are expressed as mean ± SEM (n = 4). *$P < 0.05$, **$P < 0.01$, significantly different from WT mice.

Tissue distribution of 5-ALA after oral administration

Given the application of 5-ALA for photodynamic therapy and immunofluorescence of tumours, investigating the role of PEPT1 on the *in vivo* distribution of 5-ALA was of interest. Several tissues were collected 180 min after oral dosing of 0.2 μmol·g^{-1} 5-ALA and, to rule out differences caused by varying blood concentrations, the results presented as tissue-to-blood concentration ratios. As shown in Figure 5, no statistically significant differences were observed between the two genotypes for 5-ALA concentration ratios in all systemic tissues tested. The same was true for gastrointestinal tissues with one exception. Here, a significant reduction in the ileal-to-blood concentration ratio was observed in *Pept1* knockout mice as compared with wildtype animals. These results indicated that PEPT1 does not play an important role in affecting the *in vivo* systemic tissue distribution of 5-ALA.

Pharmacokinetics of 5-ALA after i.v. administration

The lower AUC of 5-ALA in *Pept1* knockout mice, as compared with wildtype mice, may reflect a reduction in the extent of intestinal absorption (F) and/or an increase in systemic clearance (CL) after oral dosing (i.e. $AUC_{oral} = F \bullet D_{oral}/CL$). To rule out the latter, studies were performed after i.v. bolus administrations of 5-ALA in both genotypes. A low dose (0.01 μmol·g^{-1}) was chosen for the study to assure linear pharmacokinetics. As depicted in Figure 6, the plasma concentration-time curves of 5-ALA were nearly superimposable between the two genotypes. Pharmacokinetic parameters, shown in Table 3, were not significantly different between wildtype and *Pept1* knockout mice, differing by <15%. These results indicated that differences observed between the genotypes in AUC after oral dosing were reflective of a reduced extent of intestinal absorption during PEPT1 ablation.

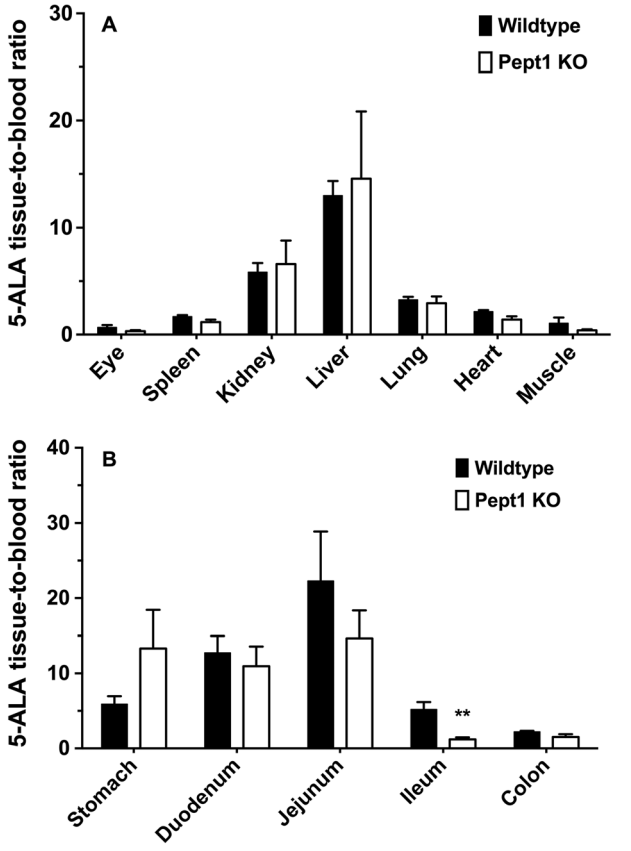

Figure 5

Tissue-to-blood concentration ratios of [^{14}C]5-ALA in systemic tissues (A) and gastrointestinal segments (B) of wildtype and *Pept1* knockout (KO) mice, 180 min after a 0.2 μmoL·g^{-1} oral dose. Data are expressed as mean ± SEM ($n = 4$–5). **$P \leq 0.01$, significantly different from wildtype mice.

Discussion and conclusions

Several novel findings were revealed by studying the *in situ* intestinal permeability and *in vivo* oral pharmacokinetics of 5-ALA in wildtype and *Pept1* knockout mice, such that: (i) the permeability of 5-ALA was substantial in the duodenal, jejunal and ileal regions of wildtype mice; (ii) the residual permeability of 5-ALA in *Pept1* knockout small intestine was only about 10% of that observed in wildtype animals; (iii) the permeability of 5-ALA in jejunum was specific for PEPT1 with no apparent contribution of other transporters, including that of PAT1; (iv) the systemic exposure of 5-ALA in *Pept1* knockout mice was 40–45% of that in wildtype animals after oral dosing; (v) the pharmacokinetics of 5-ALA was dose-proportional after 0.2 and 2.0 μmol·g^{-1} oral doses; and (vi) PEPT1 ablation had a minor effect on the disposition and

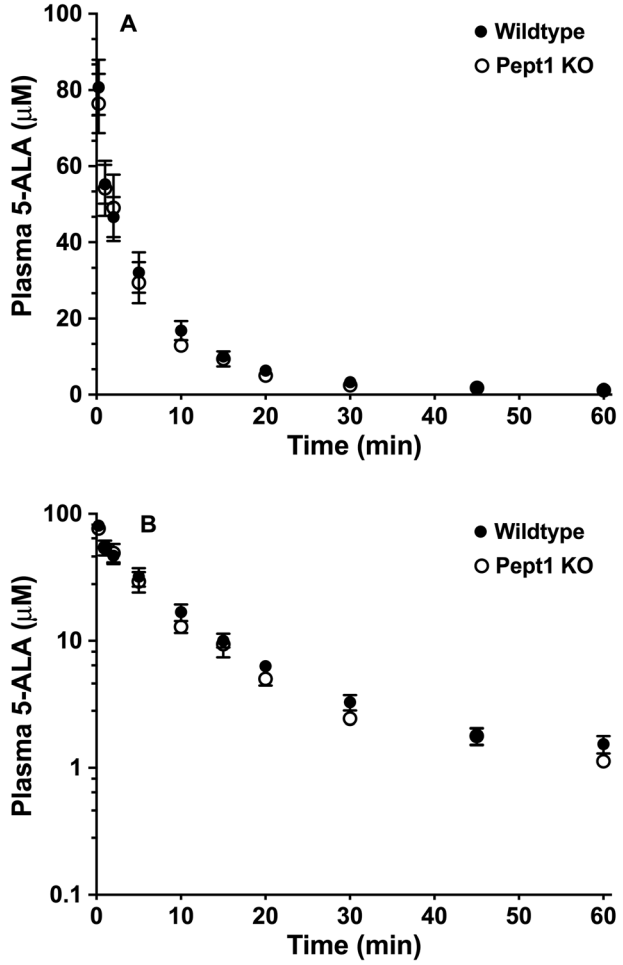

Figure 6

Plasma concentration-time profiles of [^{14}C]5-ALA in wildtype and *Pept1* knockout (KO) mice after a 0.01 μmoL·g^{-1} i.v. bolus dose. Data are expressed as mean ± SEM (n = 7), where the Y-axis is displayed on a linear scale (A) and on a logarithmic scale (B).

peripheral tissue distribution of 5-ALA. Collectively, our findings suggested a major role of PEPT1 in the intestinal permeability and oral absorption of 5-ALA. In contrast, PAT1 appeared to play a limited role, at best.

The contribution of other transporters, in addition to that of PEPT1, was examined during *in situ* jejunal perfusion studies in which 5-ALA was co-perfused with excess concentrations (25 mM) of potential inhibitors. The dipeptide GlySar and the β-lactam antibiotic cefadroxil served as positive controls and significantly reduced the intestinal permeability of 5-ALA. In contrast, the PHT1/2 substrate L-histidine, the PAT1 substrates L-proline and β-alanine, the OCT substrate TEA, and the OAT substrate PAH all failed to exhibit inhibitory activity when co-administered with 5-ALA. These results support the dominant, and perhaps exclusive, role of PEPT1 in the small intestinal uptake of 5-ALA. The extremely low permeability of 5-ALA (i.e. in both small and large intestines) after *Pept1* gene ablation also argues against a significant role of other transporters in the uptake of 5-ALA. It is worth noting that this finding was contradictory to other studies regarding the role of PAT1 in transporting 5-ALA. In this regard, Frølund *et al.* (2010)

Table 3

Non-compartmental analysis of 5-ALA after i.v. bolus administration of 0.01 μmol·g^{-1} [^{14}C]5-ALA in WT and *Pept1* KO mice

Parameter (unit)	Wildtype	KO	Ratio (KO·WT^{-1})
$AUC_{0\text{-}60}$ (μM·min)	583 (61)	527 (71)	0.90
$AUC_{0\text{-}\infty}$ (μM·min)	647 (65)	579 (71)	0.89
CL (mL·min^{-1})	0.34 (0.05)	0.38 (0.045)	1.12
Vdss (mL)	6.7 (0.9)	7.6 (1.1)	1.14
MRTiv (min)	20.2 (2.4)	20.2 (2.4)	1.00
$t_{1/2}$ (min)	27.8 (2.0)	30.4 (4.6)	1.11

5-ALA, 5-aminolevulinic acid; CL, total plasma clearance; KO, knockout; MRTiv, mean residence time after intravenous dosing; Vdss, volume of distribution steady-state; WT, wildtype.
Data are expressed as mean ± SEM (n = 7). No statistical differences were observed between WT and KO mice.

reported the saturable uptake of 5-ALA in MDCK cells stably transfected with *hPEPT1* (K_M = 6.4 mM) and in COS-7 cells transiently transfected with *hPAT1* (K_M = 3.8–6.8 mM). In the study by Anderson *et al.* (2010), saturable uptake of 5-ALAwas reported in *Xenopus* oocytes expressing *hPEPT1* (K_M = 1.6 mM) and mouse *Pat1* (K_M = 10.4 mM). In our perfusion study, 5-ALA had an intrinsic K_M = 3.7 mM, which was comparable with literature values and consistent with the low-affinity properties of PEPT1-mediated transport.

In wildtype mice, the permeability of 5-ALA was 1.65 × 10^{-4} cm·s^{-1} in duodenum, 1.91 × 10^{-4} cm·s^{-1} in jejunum and 1.20 × 10^{-4} cm·s^{-1} in ileum, whereas the 5-ALA permeability in colon was only 0.14 × 10^{-4} cm·s^{-1}, a value substantially lower than those observed in small intestinal segments. These results, which reflected PEPT1 functional activity, were similar to the PEPT1 protein expression pattern observed in mice (i.e. abundant in small intestine but negligible in colon) (Jappar *et al.*, 2010). In *Pept1* knockout mice, the residual permeability of 5-ALAwas only about 10% of that in wildtype animals but similar to that observed for 5-ALA in the colon of both genotypes. Thus, during *in situ* perfusions, PEPT1 was responsible for about 90% of the 5-ALA uptake in mouse small intestine, a finding consistent with other PEPT1 substrates (GlySar, cefadroxil and valacyclovir) studied by our laboratory (Jappar *et al.*, 2010; Posada and Smith, 2013a; Yang and Smith, 2013).

Following the oral administration of 5-ALA, significant differences were observed in the plasma concentration-time profiles between wildtype and *Pept1* knockout mice. In particular, PEPT1 ablation leads to twofold reductions in the peak plasma concentration and systemic exposure of 5-ALA at both dose levels. This finding confirmed the quantitative importance of PEPT1 in the *in vivo* oral absorption of 5-ALA and supported our previous *in situ* perfusion results that PEPT1 played a pivotal role in the intestinal uptake of 5-ALA. However, the magnitude of differences between genotypes in the *in situ* (i.e. 10-fold) and *in vivo* (twofold) experiments was pronounced, although

similar discrepancies were reported by our laboratory for GlySar (Jappar *et al.*, 2011) and valacyclovir (Yang *et al.*, 2013). One possible explanation for the discrepancy is that, given the small size of 5-ALA (MW 131), passive mechanisms (e.g. diffusion or paracellular uptake) may play a bigger role than previously believed during the *in vivo* oral absorption of 5-ALA. It is also possible that the long residence times of 5-ALA, as it transits from the proximal small intestine to distal large intestine, may allow for greater than expected oral absorption of substrate. These differences demonstrate that, during our wildtype versus *Pept1* knockout mouse studies, the *in situ* method overpredicts the contribution of PEPT1 to *in vivo* oral absorption. While both methods support the important role of PEPT1 in 5-ALA intestinal permeability and oral absorption, the *in situ* method is more mechanistically valid than quantitatively predictive of the *in vivo* oral drug performance. Nevertheless, up-regulation of renal PEPT2 did not occur in *Pept1* knockout mice (Hu *et al.*, 2014) and therefore cannot explain the 'apparent' discrepancy in the magnitude of change during these *in situ* and *in vivo* studies. Interestingly, dose proportionality was observed for both genotypes after the 0.2 and 2.0 $\mu mol \cdot g^{-1}$ oral doses of 5-ALA, including the peak plasma concentration (C_{max}), systemic exposure (AUC) and CL/F. The oral doses of 5-ALA chosen in our mouse studies were based on allometric scaling (i.e. by surface area) of the doses typically used (10–60 $mg \cdot kg^{-1}$) for photodynamic therapy and immunofluorescence of tumours in patients (Regula *et al.*, 1995; Webber *et al.*, 1997; Ackroyd *et al.*, 2000; Hinnen *et al.*, 2000; Perez *et al.*, 2013). As a result, similar concentrations of 5-ALAwould be expected initially in the gastrointestinal tract for both species, being on the order of 10–100 mM.

Finally, these biodistribution studies with 5-ALA agree with other studies (Jappar *et al.*, 2011; Posada and Smith 2013b; Yang *et al.*, 2013) demonstrating that PEPT1 protein expression, although critically important in the small intestine, has little effect on the systemic tissue distribution of peptides/mimetics and peptide-like drugs. This finding reflects the fact that PEPT1 is only weakly expressed in kidney and, although expressed in the pancreas, bile duct and liver, its role and relevance in these tissues remain to be elucidated (Brandsch *et al.*, 2008; Rubio-Aliaga and Daniel, 2008; Smith *et al.*, 2013). In contrast, PEPT2 is more widely expressed in peripheral tissues, especially in the kidney, brain and lung. PHT1 is abundantly expressed in the brain and eye, and PHT2 is expressed in the lung, spleen, thymus and, faintly, in the brain. Still, the functional importance of PHT1/2 in these tissues is unknown.

In conclusion, the present study shows for the first time that PEPT1 accounts for about 90% of the *in situ* permeability of 5-ALA in mouse small intestine and that PEPT1 ablation substantially reduces the *in vivo* extent of oral absorption for 5-ALA. Our findings further cast doubt on PAT1 having an important role in the intestinal absorption of 5-ALA, although, definitive evidence will have to await studies being performed in *Pat1* knockout models.

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Author contributions

Y. X. contributed to the design of the study, carried out all experiments, performed data collection and analysis, and drafted the manuscript. Y. H. contributed to the design of the study and data interpretation. D. E. S. contributed to the design of the study, data interpretation and drafting of the manuscript.

Conflict of interest

There are no competing interests to declare.

References

Ackroyd R, Brown NJ, Davis MF, Stephenson TJ, Marcus SL, Stoddard CJ *et al.* (2000). Photodynamic therapy for dysplastic Barrett's oesophagus: a prospective, double blind, randomised, placebo controlled trial. Gut 47: 612–617.

Adachi Y, Suzuki H, Sugiyama Y (2003). Quantitative evaluation of the function of small intestinal P-glycoprotein: comparative studies between *in situ* and *in vitro*. Pharm Res 20: 1163–1169.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Transporters. Br J Pharmacol 170: 1706–1796.

Anderson CM, Jevons M, Thangaraju M, Edwards N, Conlon NJ, Woods S *et al.* (2010). Transport of the photodynamic therapy agent 5-aminolevulinic acid by distinct H^+-coupled nutrient carriers coexpressed in the small intestine. J Pharmacol Exp Ther 332: 220–228.

Brandsch M, Knutter I, Bosse-Doenecke E (2008). Pharmaceutical and pharmacological importance of peptide transporters. J Pharm Pharmacol 60: 543–585.

Dalton JT, Meyer MC, Golub AL (1999). Pharmacokinetics of aminolevulinic acid after oral and intravenous administration in dogs. Drug Metab Dispos 27: 432–435.

Dalton JT, Yates CR, Yin D, Straughn A, Marcus SL, Golub AL *et al.* (2002). Clinical pharmacokinetics of 5-aminolevulinic acid in healthy volunteers and patients at high risk for recurrent bladder cancer. J Pharmacol Exp Ther 301: 507–512.

Dolmans DE, Fukumura D, Jain RK (2003). Photodynamic therapy for cancer. Nat Rev Cancer 3: 380–387.

Doring F, Walter J, Will J, Focking M, Boll M, Amasheh S *et al.* (1998). Delta-aminolevulinic acid transport by intestinal and renal peptide transporters and its physiological and clinical implications. J Clin Invest 101: 2761–2767.

Fotinos N, Campo MA, Popowycz F, Gurny R, Lange N (2006). 5-aminolevulinic acid derivatives in photomedicine: characteristics, application and perspectives. Photochem Photobiol 82: 994–1015.

Frølund S, Marquez OC, Larsen M, Brodin B, Nielsen CU (2010). Delta-aminolevulinic acid is a substrate for the amino acid transporter SLC36A1 (hPAT1). Br J Pharmacol 159: 1339–1353.

Hinnen P, Rooij FWM, Terlouw EM, Edixhoven A, Dekken H, Hillegersberg R *et al.* (2000). Porphyrin biosynthesis in human Barrett's oesophagus and adenocarcinoma after ingestion of 5-aminolaevulinic acid. Br J Cancer 83: 539–543.

Hu Y, Smith DE, Ma K, Jappar D, Thomas W, Hillgren KM (2008). Targeted disruption of peptide transporter Pept1 gene in mice significantly reduces dipeptide absorption in intestine. Mol Pharm 5: 1122–1130.

Hu Y, Xie Y, Keep RF, Smith DE (2014). Divergent developmental expression and function of the proton-coupled oligopeptide transporters PepT2 and PhT1 in regional brain slices of mouse and rat. J Neurochem 129: 955–965.

Jappar D, Wu SP, Hu Y, Smith DE (2010). Significance and regional dependency of peptide transporter (PEPT) 1 in the intestinal permeability of glycylsarcosine: in situ single-pass perfusion studies in wild-type and Pept1 knockout mice. Drug Metab Dispos 38: 1740–1746.

Jappar D, Hu Y, Smith DE (2011). Effect of dose escalation on the in vivo oral absorption and disposition of glycylsarcosine in wild-type and Pept1 knockout mice. Drug Metab Dispos 39: 2250–2257.

Johnson DA, Amidon GL (1988). Determination of intrinsic membrane transport parameters from perfused intestine experiments: a boundary layer approach to estimating the aqueous and unbiased membrane permeabilities. J Theor Biol 131: 93–106.

Kelty CJ, Brown NJ, Reed MWR, Ackroyd R (2002). The use of 5-aminolaevulinic acid as a photosensitiser in photodynamic therapy and photodiagnosis. Photochem Photobiol Sci 1: 158–168.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. Br J Pharmacol 160: 1577–1579.

Krammer B, Plaetzer K (2008). ALA and its clinical impact, from bench to bedside. Photochem Photobiol Sci 7: 283–289.

Loh CS, MacRobert AJ, Bedwell J, Regula J, Krasner N, Bown SG (1993). Oral versus intravenous administration of 5-aminolaevulinic acid for photodynamic therapy. Br J Cancer 68: 41–51.

McGrath J, Drummond G, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Nokes B, Apel M, Jones C, Brown G, Lang JE (2013). Aminolevulinic acid (ALA): photodynamic detection and potential therapeutic applications. J Surg Res 181: 262–271.

Ocheltree SM, Shen H, Hu Y, Keep RF, Smith DE (2005). Role and relevance of peptide transporter 2 (PEPT2) in the kidney and choroid plexus: in vivo studies with glycylsarcosine in wild-type and PEPT2 knockout mice. J Pharmacol Exp Ther 315: 240–247.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al.* (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucl. Acids Res. 42 (Database Issue): D1098–1106.

Peng Q, Berg K, Moan J, Kongshaug M, Nesland JM (1997a). 5-Aminolevulinic acid-based photodynamic therapy: principles and experimental research. Photochem Photobiol 65: 235–251.

Peng Q, Warloe T, Berg K, Moan J, Kongshaug M, Giercksky KE *et al.* (1997b). 5-Aminolevulinic acid-based photodynamic therapy: clinical research and future challenges. Cancer 79: 2282–2308.

Perez MH, Rodriguez BL, Shintani TT, Watanabe K, Miyanari S, Harrigan RC (2013). 5-Aminolevulinic acid (5-ALA): analysis of preclinical and safety literature. Food and Nutrition Sciences 4: 1009.

Posada MM, Smith DE (2013a). Relevance of PepT1 in the intestinal permeability and oral absorption of cefadroxil. Pharm Res 30: 1017–1025.

Posada MM, Smith DE (2013b). In vivo absorption and disposition of cefadroxil after escalating oral doses in wild-type and PepT1 knockout mice. Pharm Res 30: 2931–2939.

Regula J, MacRobert AJ, Gorchein A, Buonaccorsi GA, Thorpe SM, Spencer GM *et al.* (1995). Photosensitisation and photodynamic therapy of oesophageal, duodenal, and colorectal tumours using 5 aminolaevulinic acid induced protoporphyrin IX–a pilot study. Gut 36: 67–75.

Rubio-Aliaga I, Daniel H (2008). Peptide transporters and their roles in physiological processes and drug disposition. Xenobiotica 38: 1022–1042.

Shen H, Ocheltree SM, Hu Y, Keep RF, Smith DE (2007). Impact of genetic knockout of PEPT2 on cefadroxil pharmacokinetics, renal tubular reabsorption, and brain penetration in mice. Drug Metab Dispos 35: 1209–1216.

Sinko PJ, Amidon GL (1988). Characterization of the oral absorption of beta-lactam antibiotics. I Cephalosporins: determination of intrinsic membrane absorption parameters in the rat intestine in situ. Pharm Res 5: 645–650.

Smith DE, Clemencon B, Hediger MA (2013). Proton-coupled oligopeptide transporter family SLC15: physiological, pharmacological and pathological implications. Mol Aspects Med 34: 323–336.

van den Boogert J, van Hillegersberg R, de Rooij FW, de Bruin RW, Edixhoven-Bosdijk A, Houtsmuller AB *et al.* (1998). 5-Aminolaevulinic acid-induced protoporphyrin IX accumulation in tissues: pharmacokinetics after oral or intravenous administration. J Photochem Photobiol B 44: 29–38.

Webber J, Kessel D, Fromm D (1997). Plasma levels of protoporphyrin IX in humans after oral administration of 5-aminolevulinic acid. J Photochem Photobiol B 37: 151–153.

Yang B, Smith DE (2013). Significance of peptide transporter 1 in the intestinal permeability of valacyclovir in wild-type and PepT1 knockout mice. Drug Metab Dispos 41: 608–614.

Yang B, Hu Y, Smith DE (2013). Impact of peptide transporter 1 on the intestinal absorption and pharmacokinetics of valacyclovir after oral dose escalation in wild-type and PepT1 knockout mice. Drug Metab Dispos 41: 1867–1874.