Evaluation of a Targeted Prodrug Strategy to Enhance Oral Absorption of Poorly Water-Soluble Compounds

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Purpose. The purpose of this research was to examine a targeted prodrug strategy to increase the absorption of a poorly water-soluble lipophilic compound.

Methods. Three water-soluble prodrugs of Cam-4451 were synthesized. The amino acid (Cam-4562, Cam-4580) or phosphate (Cam-5223) ester prodrugs introduced moieties ionized at physiological pH and targeted intestinal brush-border membrane enzymes for reconversion to the parent. Selectivity for reconversion of the three prodrugs was examined in rat intestinal perfusate and brush-border membrane suspensions. Bioavailability of Cam-4451 in rats was evaluated after administering orally as the parent or as prodrugs in a cosolvent vehicle or in methylcellulose.

Results. Cam-5223 was highly selective for reconversion at the brush-border, but was rapidly reconverted in intestinal perfusate. Cam-4562 was not as selective but was more stable in the perfusate, whereas Cam-4580 was neither selective nor stable. Oral bioavailability of Cam-4451 was 14% after dosing as the parent in the cosolvent vehicle, 39% and 46%, respectively, as Cam-4562 and Cam-5223. Oral bioavailability was only 3.6% when the parent was dosed in methylcellulose, whereas the bioavailability was 7-fold higher when dosed as the phosphate prodrug.

Conclusions. Water-soluble prodrugs that target brush-border membrane enzymes for reconversion can be useful in improving drug oral bioavailability.

KEY WORDS: absorption; bioavailability; neurokinin; solubility; tachykinin; targeted prodrug.

INTRODUCTION

For orally administered compounds, drug dissolution generally precedes gastrointestinal absorption and systemic availability. The rate of drug dissolution often determines the rate and extent of drug absorption. In recent years, with the development of combinatorial chemistry and high throughput biological screening (HTS), new chemical leads tend to be more lipophilic and less water-soluble (1). Compounds examined in HTS are frequently dissolved in dimethyl sulfoxide (DMSO) stocks, rendering very water-insoluble compounds for testing. In addition, chemistry efforts at the early discovery stage have concentrated on optimizing *in vitro* activity of the chemical leads instead of physicochemical properties of the compounds. As a result, many discovery compounds are poorly water-soluble and exhibit low oral bioavailability.

The purpose of this research was to examine a targeted prodrug strategy to increase the absorption, hence the oral bioavailability of a poorly water-soluble lipophilic compound. The prodrug strategy (2) involved synthesizing water-soluble prodrugs by introducing functional groups that ionized at physiological pH. The prodrugs were targeted to intestinal brush-border membrane enzymes for reconversion to the lipophilic parent compound, which was readily absorbed into the adjacent enterocytes. The increased solubility of the prodrug and high membrane permeability of the parent compound provided the driving force for increased flux of the drug, with the reconversion reaction maintaining sink conditions at the membrane.

The prototype parent compound, Cam-4451, is a selective, high-affinity NK_1 neurokinin receptor antagonist with an IC_{50} of 1.2 nM in human IM-9 lymphoma cells. It is a nonpeptide, α-methyltryptophan derivative (Fig. 1). The compound is lipophilic with a calculated log partition coefficient (ClogP) of 4.4 and has a molecular weight of 512. It has a melting point of 79–80°C and a hydrogen bonding potential of 5.5, estimated using an adaptation of the method of Stein (3). It is neutral at physiological pH and exhibits very low aqueous solubility (< 2 μg/mL). In this study, Cam-4451 was found to have poor oral bioavailability in rats. Earlier work with Cam-2445 (Fig. 1), an analog of Cam-4451 with similar physicochemical properties, indicated that GI dissolution and/or precipitation from the dosing vehicle was limiting to the oral bioavailability of the compound (4). Cam-4451 was likely plagued with the same problem as Cam-2445.

Three prodrugs were evaluated in this study and the structures are depicted in Figure 1. Cam-4562 and Cam-4580, the amino acid prodrugs, consist of a dimethylglycine group and a leucine moiety, whereas Cam-5223 is a monophosphate ester.

MATERIALS AND METHODS

Cam-4451 and prodrugs were synthesized at Parke-Davis Neuroscience Research Centre (Cambridge, UK). [^{14}C]PEG 4000 was obtained from New England Nuclear/Dupont (Boston, MA). PEG 400, 2-(N-morpholino)ethane-sulfonic acid (MES), α-chymotrypsin, L-leucine-p-nitroanilide, and 4-nitrophenylphosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Methylcellulose was manufactured by Dow Chemical (Midland, MI) and was premium grade. Solvents for HPLC use were of HPLC grade. All other chemicals were of analytical grade or better. Male white Wistar rats weighing 0.252–0.355 kg were obtained from Charles River Lab (Wilmington, MA). All animal research adhered to the "Principles of Laboratory Animal Care" as outlined in NIH publication #85-23. Data analyses were performed using Lotus 1-2-3, release 3.1.

Incubations in Biological Matrices

To evaluate the stability of the parent compound in the gastrointestinal lumen and against enzymes in plasma, Cam-4451 was incubated in hydrochloric acid (0.1N), α-chymotrypsin (5 μM), rat intestinal perfusate, brush-border membrane (BBM; 1:5 or 1:500 dilution) suspension, and rat plasma. The

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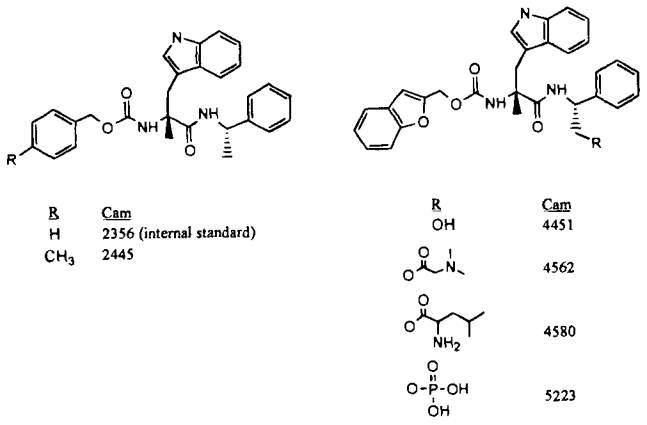

Fig. 1. Chemical structures of Cam-2445, Cam-4451, prodrugs, and internal standard.

prodrugs were also incubated in rat intestinal perfusate and in BBM suspension to compare reconversion to the parent compound in the intestinal lumen *vs* at the brush-border.

Blank intestinal perfusate was prepared as described previously (4) and used the same day it was generated. Rat jejunal BBM was prepared as reported by Tsuji et al. (5). The BBM preparation was frozen at −70°C until use. Aminopeptidase and alkaline phosphatase activity of the biological preparations were determined using model substrates, leu-p-nitroanilide and 4-nitrophenylphosphate, respectively. Enzyme activities of the various batches of the preparations were found comparable to each other; thus reconversion half-lives of different prodrugs can be compared.

The matrices were spiked with Cam-4451 (1 μg/mL) or the prodrugs (4–50 μg/mL) and aliquoted into 200-μL fractions. The preparations were incubated in a 37°C shaking water bath (Precision Scientific, Model 50, Chicago, IL). At selected time points up to 4 h, the fractions were removed from the water bath in duplicates and quenched immediately with acetonitrile (ACN), which also removed the compounds from potential nonspecific binding sites. Protein was precipitated and the supernatant was injected onto the HPLC for analysis. Log fraction of drug remaining with time was examined.

In Situ Intestinal Perfusion

Intestinal permeability of the parent compound, Cam-4451, was evaluated in a single-pass rat intestinal perfusion model. Rats (n = 3) were surgically prepared as previously reported (6). Drug solutions (1. 3 μg/mL) were prepared in MES buffer (10 mM MES, 135 mM NaCl, 5 mM KCl, pH 6.5) and [^{14}C] PEG 4000 was added to allow correction of effluent concentrations for water flux in the intestine. Cam-4451 was perfused into jejunal segments of 9–10.3 cm at a constant flow rate of 0.35 mL/min. Effluent perfusate was collected at 10-min interval for 90 min in tared containers. To correct influent concentrations for adsorption to tubing, a sham was set up where no intestine was cannulated. Perfusate samples were weighed and equal volumes of ACN were added, using a perfusate density of 1 g/mL. Protein was precipitated, and the supernatant was injected onto the HPLC. The concentration of [^{14}C] PEG 4000 was measured by liquid scintillation counting (Packard Tri-Carb, Downers Grove, IL).

In Vivo Studies

Rats were randomly assigned to groups of four. The day prior to the experiment, the animals were surgically prepared and then fasted overnight with water *ad libitum*. Doses of Cam-4451, Cam-4562, or Cam-5223 were freshly prepared in a cosolvent of 15% ethanol, 40% PEG 400, and 45% water or in 0.5% methylcellulose immediately before dosing. Cam-4451 was administered as a slow bolus intravenously into the jugular vein (IV; 5.14 mg/kg), intraduodenally via a duodenal cannula (ID; 17 mg/kg), or orally as a gastric gavage (PO; 20–26 mg/kg). The prodrugs were dosed orally by gavage (12–16 mg/kg dose equivalent of parent drug). Systemic blood was sampled via a jugular vein cannula predose and at selected times up to 24 h after dosing. Blood samples (0.5 mL) were collected on ice, heparinized and immediately centrifuged, then plasma was harvested and stored at −20°C until analysis by HPLC.

The plasma samples were analyzed by validated HPLC assays for Cam-4451 and simultaneously for Cam-4562, when dosed. The chromatographic peak of Cam-5223 could not be adequately resolved from the plasma solvent front and was not monitored. Plasma (200 μL) was spiked with 10 μL of internal standard (Cam-2356; 1.35 μg/mL in ACN) and 425 μL ACN to precipitate protein. The supernatant was transferred to a clean test tube and evaporated to dryness. The residue was reconstituted in 50:50 ACN:water and injected onto the HPLC.

HPLC Analysis

A Hewlett Packard Model 1090 HPLC (Palo Alto, CA) was used for plasma sample analysis. Separation was achieved with a Spherisorb C_{18} column (5 μm, 150 × 4.6 mm) protected by a C_{18} guard column (5 μm, 30 × 4.6 mm). The mobile phase consisted of 54% ACN and 46% water (with 0.1% triethylamine, pH 3.0 with phosphoric acid). The column was maintained at a flow rate of 1 mL/min and at ambient temperature. Cam-4451, Cam-4562, and the internal standard were monitored using a Perkin Elmer Model LS 40 fluorescence detector (Norwalk, CT) with excitation at 280 nm and emission at 340 nm. Chromatographic peaks were integrated with an electronic integrator (Spectra-Physics ChromJet; San Jose, CA). The limit of quantitation was 2.5 ng/mL for Cam-4451 and 15 ng/mL for Cam-4562. Standard curves were linear up to 2 μg/mL. Precision CV was <10% and accuracy was within ±10% error at all concentrations.

Samples from *in vitro* incubations and intestinal perfusion were analyzed using similar methods modified from the plasma assay. Both the parent compound and prodrug (Cam-4562, Cam-4580, or Cam-5223) were monitored in the samples.

Data Analysis

In the single-pass rat intestinal perfusion, the effective permeability (P_{eff}) at steady state was calculated from (7):

$$P_{eff} = (1 - \{C_{out}/C_{in}\}_{ss}) * Q/(2\pi r\ell)$$

where C_{in} is the influent drug concentration corrected for adsorption to tubing; C_{out} is the effluent drug concentration corrected for water flux; r is radius of rat intestinal segment, assumed to be 0.2 cm; ℓ is the intestinal segment length; and Q is the perfusion flow rate.

Systemic plasma clearance of Cam-4451 was determined after intravenous dosing of the parent compound

$$Cl = Dose_{IV}/AUC_{IV}$$

Oral bioavailability of Cam-4451, was calculated after administration of the parent compound or the prodrugs by:

$$F = (AUC_{PO}/Dose_{PO}) * Cl$$

where AUC = area under the plasma concentration time curve of Cam-4451

Dose = dose equivalent of the parent compound

RESULTS AND DISCUSSION

Stability and Permeability of the Parent Compound, Cam-4451

Cam-4451 was stable in incubations with hydrochloric acid (pH = 1), with the pancreatic enzyme, α-chymotrypsin, in rat intestinal perfusate, BBM suspension, and in rat plasma, with >90% remaining at 4 h. The diprotected design of Cam-4451 contains an N-terminal carbamate and an α-methylated Trp group that apparently conveyed stability of the compound in the GI tract (4).

In the single-pass perfusions of Cam-4451 in rat intestine, steady state was achieved at about 30 min. Water flux across the intestinal mucosa was minimal and was comparable using either PEG 4000 or gravimetric approach. Cam-4451 exhibited high permeation in rat jejunum with a permeability of $91 \pm 28 * 10^{-6}$ cm/s, higher than the $30 \pm 16 * 10^{-6}$ cm/s of prednisolone determined under similar experimental conditions (log P = 1.58, >70% absorbed in human) (6). Although the permeability parameter was estimated by disappearance from the mucosal side at steady state instead of appearance of drug in the serosal side of the barrier, the fact that Cam-4451 was stable in the enriched enzyme preparations suggested that the permeability measurement was reasonably representative of absorption of the dissolved drug.

The high permeability and stability of Cam-4451 in the biological matrices made the compound a good candidate for the parent drug in the targeted prodrug strategy. In addition, the hydroxyl functionality in the chemical structure provided an attachment point for the pro-groups.

Reconversion of Prodrugs to Parent Compound

The amino acid or phosphate pro-groups examined in this study introduced either a cation or anion to the parent molecule. At physiological pH, the prodrugs were ionized and had much greater water solubility compared to the parent. The aqueous solubilities of the prodrugs, Cam-4562 (sulfate salt), Cam-4580 (hydrochloride salt), and Cam-5223 (sodium salt) at pH 6.5 were 3, 0.1, and > 61 mg/mL, respectively, in contrast to the < 2 μg/mL of the parent compound. The prodrugs were targeted for reconversion to the parent compound by brush-border enzymes, aminopeptidases for the amino acid prodrugs and alkaline phosphatase for the phosphate prodrug. Premature reconversion of the prodrugs due to instability in the lumen would result in the parent compound precipitating out in the intestine, instead of being absorbed at the gut wall (2,8).

The relative reconversion rates of the three prodrugs in the intestinal lumen *vs* at the brush-border membrane were compared. The reconversions were evaluated in incubations with intestinal perfusate and BBM, which were enriched with intestinal enzymes (9). Hence, the reconversion half-lives would not represent the actual rate of reconversion *in vivo* but a measure of the specificity for reconversion at the brush-border. This information provided insight for selection of compounds for further evaluation in the *in vivo* model. The results are presented in Table 1. Since the phosphate prodrug was reconverted very rapidly in the 1:5 dilution of BBM used for the amino acid prodrugs, the reconversion was evaluated in a 1:500 dilution and corrected to allow comparison to the other two prodrugs, assuming the reconversion rate constant to be directly proportional to the enzyme concentration. A selectivity ratio, defined as the ratio of the reconversion half-life in perfusate to that in BBM, provided estimates of selectivity of the prodrug for reconversion at the brush-border to that in the lumen. An ideal prodrug for this application would have a slow reconversion in the lumen and a fast reconversion at the brush-border, resulting in a high selectivity ratio. As shown in Table 1, Cam-5223, the phosphate prodrug, was highly selective for reconversion at the brush-border; however, the short reconversion half-life in the intestinal perfusate suggested the caveat of reconversion and precipitation in the lumen. Conversely, the dimethylglycine ester prodrug, Cam-4562, was not as selective for reconversion at the brush-border, but was more stable in the perfusate compared to the phosphate prodrug. The leucine ester prodrug, Cam-4580, had the lowest selectivity ratio and a short reconversion half-life in the perfusate, and therefore was not evaluated further in the *in vivo* model.

In Vivo Results

Mean plasma concentration profiles of Cam-4451 after administration of the parent or prodrugs are shown in Fig. 2 and pharmacokinetic parameters are presented in Table 2. In order to dose the parent compound as a solution, the drug was dissolved in a cosolvent vehicle consisting of 15% ethanol and 40% PEG 400 in water. After intravenous dosing of Cam-4451, plasma concentrations decreased polyexponentially. The apparent elimination half-life was 4.3 h and systemic plasma clearance was 37.1 ± 2.3 mL/min-kg. With the assumptions that blood clearance did not differ very much from the plasma clearance, that systemic clearance was mainly hepatic, and liver blood flow was 55.2–69 mL/min-kg in the rat (10,11), hepatic extraction of Cam-4451 was calculated to be 54–67%. Thus, if the oral dose was totally absorbed from the GI tract and not metabolized during absorption into the portal system, the oral bioavailability could reach 46%.

Consistent with high permeability of the parent drug, absorption was rapid after ID or PO administration of Cam-4451 in the ethanol/PEG 400 cosolvent vehicle. Plasma concentrations peaked at 15–30 min after either route of dosing (Fig. 2a), suggesting that Cam-4451 was absorbed from the cosolvent in the stomach. The dose-normalized plasma profiles almost overlapped and bioavailability was the same at 14% after the ID or PO doses, implying that the ethanol/PEG 400 formulation was stable in the stomach after PO dosing. These results indicated that membrane penetration by dissolved drug was expedi-

Table 1. Reconversion Half-Lives and Selectivity Ratio of Prodrugs

Prodrug	Pro-group	$t_{1/2}$ in perfusate (min)	$t_{1/2}$ in BBM (min)	Selectivity ratio
Cam-4580	Leucine	5.34 ± 0.98	148 ± 38.4	0.036
Cam-4562	Dimethylglycine	17.3 ± 5.3	70.5 ± 6.5	0.245
Cam-5223	Phosphate	5.62 ± 2.5	0.0668 ± 0.0062	84

Note: Selectivity ratio = $t_{1/2}$ in perfusate / $t_{1/2}$ in BBM.

tious, which was in agreement with the high permeability observed in the intestinal perfusions.

Although the aqueous solubilities of the prodrugs were sufficient for oral dosing, Cam-4562 and Cam-5223 were administered in the same cosolvent vehicle as the parent compound to allow direct comparison. The HPLC assay allowed simultaneous quantitation of Cam-4451 and Cam-4562. However, the prodrug was not detected in plasma except for one or two samples in 2 out of the 4 rats. Consequently, Cam-5223 was not assayed since the chromatographic peak could not be readily separated from the plasma solvent front. Reconversion of the prodrugs to the parent compound was rapid. Cam-4451 was detected in the first plasma samples at 5 min, although plasma concentrations did not peak until 30 min after dosing the phosphate or 67 min after administering the dimethylglycine prodrug. The dose-normalized peak concentration for parent drug was similar after dosing either prodrug, but was significantly higher than after administering the parent compound (Fig. 2a). Bioavailability of Cam-4451 was 39% and 43%, respectively, after Cam-4562 and Cam-5223 administration, which represented a 3-fold increase compared to dosing of the parent. The result also approximated the maximum bioavailability calculated from the systemic clearance, which may explain why Cam-5223 was not more effective than Cam-4562, considering its high selectivity ratio for reconversion at the brush-border.

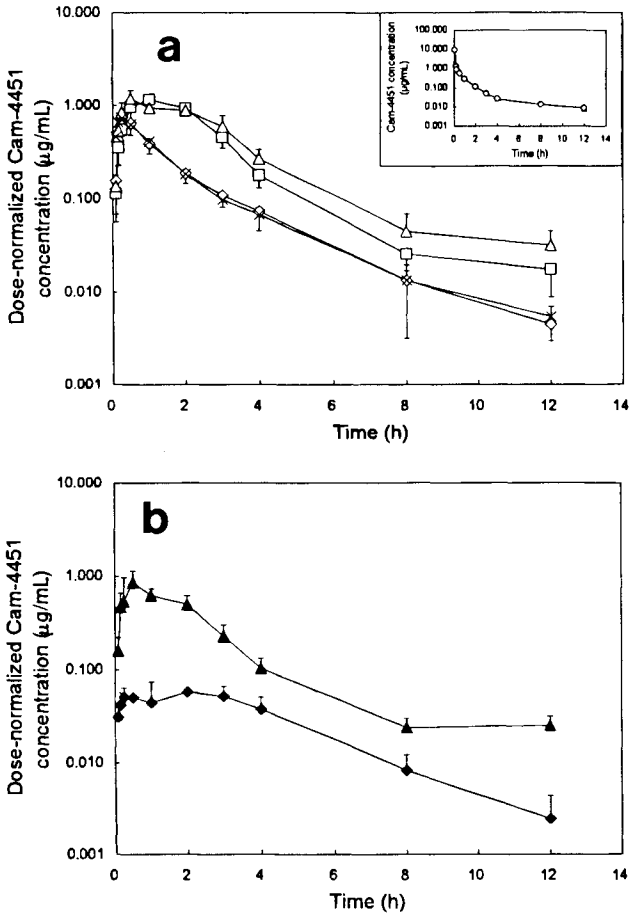

Fig. 2. Mean (± SD) plasma Cam-4451 concentrations in male Wistar rats after administration of a single dose, (a) Cam-4451 PO (◇), Cam-4451 ID (x), Cam-4562 PO (□), or Cam-5223 PO (△) in ethanol/PEG 400 cosolvent (Inset: Cam-4451 IV), (b) Cam-4451 PO (◆) or Cam-5223 PO (▲) in methylcellulose. Cam-4451 concentrations were normalized to a 20 mg/kg dose-equivalent of parent compound PO or ID.

While the bioavailability of 14% for Cam-4451 was adequate for oral activity, the dosing vehicle of ethanol/PEG 400 could not be used routinely, particularly in the clinic. Thus, Cam-4451 and Cam-5223 were also dosed to rats orally in methylcellulose (0.5%), which was an acceptable dosing vehicle in chronic toxicological studies. The parent compound was in suspension, whereas the phosphate prodrug was in solution. Absorption of Cam-4451 after dosing of the parent drug was slow and continued for at least 3 hours. The plasma profile did not peak distinctively but was flat from 15 min to 3 h (Fig. 2b). The dose-normalized maximum concentration was an order-of-magnitude lower than when Cam-4451 was administered in the cosolvent vehicle and oral bioavailability was only 3.6%. The result indicated that solubility or dissolution was limiting to the oral absorption of Cam-4451. After dosing of the phosphate ester in methylcellulose, the prodrug was promptly reconverted to the parent compound. Cam-4451 was detected in the first plasma samples at 5 min, and plasma concentrations peaked at 30 min, as after dosing Cam-5223 in the cosolvent. Bioavailability of the parent compound was 24% after administration of the phosphate prodrug. This result was lower than that obtained from dosing the phosphate ester in the cosolvent vehicle. Since the reconversion half-life of the phosphate prodrug was short in intestinal perfusate, it was possible that the prodrug partially reconverted back to the parent compound in the intestinal lumen. In the presence of the cosolvent vehicle, the parent drug was considerably more soluble than in methylcellulose, resulting in a higher oral bioavailability of the parent from dosing the prodrug in the cosolvent than in methylcellulose. Nevertheless, the bioavailability of 24% from administering the prodrug represented a 7-fold increase compared to dosing the parent itself. The targeted prodrug strategy for Cam-4451 can be improved by designing prodrugs that are as highly selective for reconver-

Table 2. Pharmacokinetics and Bioavailability of Cam-4451 After Administration in Male Wistar Rats as the Parent Compound or as Prodrug (Mean ± SD)

Compound route	Vehicle[a]	Dose equivalent to parent (mg/kg)	C_{max} (μg/mL)	t_{max} (min)	λ_Z (h^{-1})	$t_{1/2}$[b] (h)	AUC (μg-min/mL)	F (%)
Cam-4451 IV	ethanol/PEG 400	5.14	10.0 ± 0.976 [c]	5[c]	0.161 ± 0.0211	4.32	139 ± 8.80	
Cam-4451 ID	ethanol/PEG 400	17.0	0.621 ± 0.0516	18.7 ± 7.5	0.317 ± 0.0227	2.19	66.0 ± 3.89	14.2 ± 0.7
Cam-4451 PO	ethanol/PEG 400	26.3	0.883 ± 0.283	22.5 ± 8.7	0.347 ± 0.00578	2.00	100 ± 19.7	14.1 ± 2.6
Cam-4451 PO	methylcellulose	19.9	0.0597 ± 0.0124	63.8 ± 43.1	0.359 ± 0.0497	1.93	19.4 ± 4.99	3.6 ± 0.9
Cam-4562 PO	ethanol/PEG 400	12.3	0.769 ± 0.102	67.5 ± 37.7	0.303 ± 0.0591	2.29	128 ± 24.8	38.6 ± 7.5
Cam-5223 PO	ethanol/PEG 400	12.1	0.725 ± 0.169	30	0.276 ± 0.0341	2.51	141 ± 33.3	43.0 ± 9.9
Cam-5223 PO	methylcellulose	16.0	0.687 ± 0.219	30	0.241 ± 0.0162	2.88	104 ± 25.8	24.1 ± 5.9

[a] Ethanol/PEG 400 = 15% ethanol, 40% PEG 400, 45% water.
[b] Harmonic mean.
[c] First sample point.

sion at the brush-border as Cam-5223, but are more stable in the intestinal lumen, such as Cam-4562.

Until the mid-80's, there were few clinically relevant examples of prodrug use to increase oral absorption of poorly water-soluble compounds, partly because of the earlier drug screening process (12). Pharmacological responses of new drug candidates were typically examined after oral or subcutaneous administration to animals. Thus, insoluble compounds would often be eliminated in the screening process. More recently, orally administered prodrugs were successful in improving absorption by reducing intermolecular hydrogen bonding to increase aqueous solubility or by coupling increased solubility to carrier-mediated transport (see references 8 and 13 for examples). However, the targeted prodrug strategy met with limited results. As illustrated by this study, the stability of the prodrugs in the GI lumen as well as the kinetics of prodrug reconversion at the brush-border membrane and subsequent permeation of the parent compound are critical to improving the delivery of the parent drug. Selection of the appropriate candidate for the parent compound is also very important for the targeted prodrug strategy to be successful. The hepatic first-pass of the parent drug establishes the upper limit of oral bioavailability of the compound. Thus, a compound like renin inhibitors (14) which are extensively extracted by the liver would not demonstrate a significant increase in oral bioavailability. Drugs which are used in low oral dose, like hydrocortisone (15), that completely dissolve in the GI absorption window would likely not benefit from the targeted prodrug strategy.

CONCLUSIONS

Cam-4451, which was practically insoluble in water, stable in the GI tract, highly permeable, and not extensively extracted by the liver, was a good candidate for the targeted prodrug strategy. The phosphate prodrug, which was selective for reconversion at the intestinal brush-border membrane, and the dimethylglycine prodrug, which was relatively stable in the intestinal lumen, were successful in increasing oral bioavailability of the parent compound. In conclusion, water-soluble prodrugs that target brush-border membrane enzymes for reconversion can be useful in improving oral drug absorption of sparingly water-soluble compounds.

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APPENDIX

Synthesis and Identification of Compounds

Melting points were determined with a Reichert Thermovar hotstage apparatus. Proton and phosphorous NMR spectra were recorded on a Varian Unity +400 spectrometer; chemical shifts were recorded in parts per million (ppm) downfield from tetramethylsilane or 85% phosphoric acid. IR spectra were recorded with the compound neat on a sodium chloride disc with a Perkin-Elmer System 2000 Fourier Transform spectrometer. Optical rotations were determined with a Perkin-Elmer 241 polarimeter. Mass spectra were recorded with a Fisons VG Trio-2A or a Finnigan MAT TSQ70 triple quadruple mass spectrometer. Elemental analyses were determined by Medac Ltd., Uxbridge, U.K.. Normal phase silica gel used for chromatography was Merck No. 9385 (230–400 mesh); reverse phase silica gel used was Lichroprep RP-18 (230–400 mesh).

Cam-4451, Cam-2356

The parent compound, Cam-4451, and the internal standard, Cam-2356, were prepared using methods analogous to those reported in *Bioorg. Med. Chem.* **2**:357–370 (1994).

Cam-4562 (sulfate salt)

A slurry of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (17.65 g, 92.1 mmol), *N,N*-dimethylglycine (3.79 g, 36.8 mmol) and 4-dimethylaminopyridine (2.25 g, 18.4 mmol) in dichloromethane (250 mL) was stirred for 5 minutes at room temperature before adding Cam-4451 (9.41 g, 18.4 mmol). After stirring for 2 hours the reaction mixture was washed with water (100 mL), 10% sodium bicarbonate (3 × 100 mL), brine (50 mL), dried over magnesium sulfate, filtered and the solvent removed under reduced pressure to give a yellow oil. Crystallization from ethyl acetate gave Cam-4562 (4.95 g, 45%): mp 151–152°C.

A solution of sulfuric acid (47 μL, 0.84 mmol) in methanol (250 μL) was added to a solution of Cam-4562 (0.50 g, 0.84 mmol) in dichloromethane (5 mL). The solvent was removed under reduced pressure, the product precipitated from dichloromethane (5 mL) and then recrystallized from acetonitrile (60 mL) to give Cam-4562 (sulfate salt) (284 mg, 49 %): mp 180.5–184°C; $[\alpha]_D$ − 5.17° (c = 0.174, MeOH, 20 °C); IR (film) 2918, 1732, 1652, 1539, 1455, 1249, 1138, 745cm^{-1}; ^{1}H NMR (DMSO−d_6) δ 1.30 (3H, s), 2.82 (6H, s), 3.17 (1H, d, 14.4 Hz), 3.43 (1H, d, 14.2 Hz), 4.11 (1H, d, 17.1 Hz), 4.16 (1H, d, 16.8 Hz), 4.33–4.49 (2H, m), 5.15–5.30 (3H, m), 6.82–7.02 (4H, m), 7.20–7.45 (9H, m), 7.57 (1H, d, 8.1 Hz), 7.66 (1H, d, 7.6 Hz), 8.30 (1H, d, 8.5 Hz), 9.92 (1H, s), 10.86 (1H, s); MS *m/e* (APCI) 597.5 (M^+ + H, 45), 494.3 (100). Anal. ($C_{34}H_{36}N_4O_6H_2SO_4$) C, H, N, S.

Cam-4580 (hydrochloride salt)

To a solution of Boc-(S)Leu-OH (316 mg, 1.37 mmol) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (519 mg, 1.37 mmol) in *N,N*-dimethylformamide (10 mL) was added *N,N*-diisopropylethylamine (177 mg, 1.37 mmol) and stirred for 10 minutes before adding Cam-4451 (700 mg, 1.37 mmol), *N,N*- diisopropylethylamine (177 mg, 1.37 mmol) and 4-dimethylaminopyridine (167 mg, 1.37 mmol). After 1 hour the solvent was removed under reduced pressure. The residue was taken up in ethyl acetate (100 mL) and washed with 10% citric acid (3 × 30 mL), water (30 mL), sodium bicarbonate (2 × 30 mL), brine (30 mL), dried over magnesium sulphate, filtered and the solvent removed under reduced pressure. Chromatography on normal phase silica using dichloromethane gave the ester as a foam (695 mg, 70%).

This ester intermediate (630 mg, 0.87 mmol) was added to a mixture of formic acid (20 mL), anisole (1 mL) and water (1 mL) and stirred for 2 hours before removing the solvent under reduced pressure. The residue was taken up in ethyl acetate (100 mL) and washed with sodium bicarbonate (2 × 50 mL), brine (50 mL), dried over magnesium sulphate, filtered and solvent removed under reduced pressure. Chromatography on normal phase silica using ethyl acetate gave Cam-4580 (434 mg, 80%).

To a stirred solution of Cam-4580 (434 mg, 0.695 mmol) in anhydrous ether (70 mL) was added 4M hydrogen chloride in dioxan (0.2 mL, 0.8 mmol) and filtration gave Cam-4580 (hydrochloride salt) as a white solid (418 mg, 91%): mp 110–115°C; $[\alpha]_D$ + 2.3° (c = 0.5, MeOH, 19°C); IR (film) 3500–3100 (br), 2961, 1732, 1661, 1505, 1455, 1250, 1135, 1070, 742cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.70-0.80 (6H, m), 1.50–1.80 (6H, m), 3.31 (1H, d, 14.9 Hz), 3.48 (1H, d, 14.4 Hz), 3.80–3.90 (1H, m), 3.98–4.18 (1H, m), 4.37–4.43 (1H, m), 5.10–5.25 (3H, m), 5.80–6.00 (1H, br. s), 6.72 (1H, s), 6.80–7.30 (12H, m), 7.42 (1H, d, 8.3Hz), 7.48–7.54 (2H, m), 8.55 (1H, s), 8.50–8.80 (3H, br.s); MS *m/e* (CI^+) 625 (M^+ + H, 4). Anal. ($C_{36}H_{40}N_4O_6$.HCl) C, H, N, Cl.

Cam-5223 (sodium salt)

A stirred solution of Cam-4451 (5.12 g, 10 mmol) and di-*t*-butyl diethylphosphoramidite (4.02 g, 15 mmol, 93% pure) in anhydrous tetrahydrofuran (30 mL) was cooled to 0°C and 1*H*-tetrazole (2.10 g, 30 mmol) was added in one portion. After 5 minutes at 0°C the reaction was stirred at room temperature for 90 minutes. The reaction mixture was then cooled to −40°C and a solution of 3-chloroperoxybenzoic acid (2.72 g, 15 mmol, 95% pure) in anhydrous tetrahydrofuran (10mL) was added dropwise, maintaining the temperature below 0°C. After 20 minutes at room temperature a 10% solution of sodium bisulphite (50 mL) was added and the reaction stirred for a further 15 minutes before diluting with ethyl acetate (50 mL). The organic layer was washed successively with 10% sodium bisulphite (2 × 50 mL), 10% sodium bicarbonate (2 × 50 mL), brine (50 mL), dried over magnesium sulphate, filtered and the solvent removed under reduced pressure to give a yellow oil. Chromatography on normal phase silica using 0.5% pyridine/ 2.5% methanol/ 97% dichloromethane gave the phosphate ester as an oil (3.51 g). This was stirred for 3 hours in formic acid (20 mL) before removing the solvent under reduced pressure. The residue was purified by chromatography on reverse phase silica using a solvent gradient of 0–40% acetonitrile in water and freeze drying the pure fractions gave the phosphate as an off white solid (1.64 g, 27%).

To a stirred solution of the phosphate (296 mg, 0.5 mmol) in acetone (150 mL) was added a 0.05N sodium hydroxide solution (10 mL, 0.5 mmol) dropwise over 10 minutes. After 20 minutes at room temperature the acetone was removed under reduced pressure and then diluted with water (40 mL) before freeze drying to give Cam-5223 (monosodium salt) as an off white solid (272 mg, 89%): mp 147–151°C; IR (film) 3391, 3060, 2938, 1705, 1652, 1495, 1455, 1251, 1071, 743 cm^{-1}; ^{1}H NMR (D_2O) δ 1.38 (3H, s), 3.10–3.22 (1H, m), 3.38–3.50 (1H, m), 3.92–4.06 (2H, m), 4.98–5.10 (3H, m), 6.60–7.50 (18H, m); ^{31}P NMR (D_2O) δ 1.60 (br.s); MS *m/e* (FAB) 614 (M^+ + H, 30). Anal. ($C_{30}H_{29}N_3NaO_8P.H_2O$) C, H, N, Na, P.

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