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Research Paper

Permeability of a novel β-lactamase inhibitor LK-157 and its ester prodrugs across rat jejunum *in vitro*

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Abstract

Objectives LK-157 is a novel 10-ethylidene tricyclic carbapenem that resembles the structure of the broad-spectrum antibiotic sanfetrinem and acts as a potent inactivator of β-lactamases of classes A, C and D. LK-157 is a highly soluble but poorly permeable drug. Since most of the β-lactams are poorly absorbed, ester prodrugs LK-159, LK-157E1 and LK-157E2 were designed to enhance membrane permeability. This study investigated the permeability of LK-157 and the three ester prodrugs across rat intestine *in vitro*. The morpholinoethyl ester of sanfetrinem was also investigated.

Method Permeability across rat jejunum was determined using EasyMount side-by-side diffusion chambers.

Key findings The solubility and permeability of morpholinoethyl ester LK-157E2 were superior to those of LK-159 and LK-157E1. The morpholinoethyl ester of sanfetrinem LK-176E1 had the highest observed permeability coefficient and consequently the highest predicted absorption in humans.

Conclusions These results suggest that the morpholinoethyl esters of LK-157 and sanfetrinem could be further investigated to assess bioavailability *in vivo*.

Keywords β-lactamase inhibitor; drug permeability *in vitro*; morpholinoethyl ester prodrug; LK-157

Introduction

The widespread emergence of resistance is increasingly limiting the effectiveness of current antimicrobial agents. The β-lactamase superfamily has more than 700 members, many of which differ by only a single amino acid (www.lahey.org). The commercially available inhibitors potassium clavulanate, sulbactam and tazobactam inhibit most class A and some class D β-lactamases, but generally have poor activity against class C types. It is therefore crucial to design a novel β-lactamase inhibitor that has broad activity against both class A and class C β-lactamases that can be co-administered with current β-lactam antibiotics, prolonging their life and extending the spectrum of action. Several β-lactamase inhibitors have been reported in the literature, such as alkylidene penems, 2β-substituted penam sulfones, oxapenems, cephalosporin-derived compounds and cyclic acyl phosphonates; however, only NXL-104 has reached phase I clinical development.[1–5]

LK-157 is a promising broad-spectrum β-lactamase inhibitor, recently selected as the lead compound from a series of new 10-ethylidene derivatives of tricyclic carbapenems.[6] LK-157 inhibits TEM-1, SHV-1 and AmpC β-lactamase enzymes at nanomolar concentrations. Its activity against several clinically important strains has been further confirmed by the reduction in minimum inhibitory concentration when used in combination with various β-lactam antibiotics. Its spectrum of action includes class C enzymes, which is a clear advantage over clavulanic acid and tazobactam. The stability profile and pharmacokinetic parameters determined in rats and Beagle dogs support further development of this compound.[7,8]

The most convenient method for drug delivery is oral administration. However, most β-lactams possess low membrane permeability and are therefore poorly absorbed.[9] Ester prodrugs are commonly used to enhance the membrane permeability and transepithelial transport of hydrophilic drugs by increasing the lipophilicity of the parent compound, resulting in enhanced transmembrane transport by passive diffusion.[10] The β-lactam

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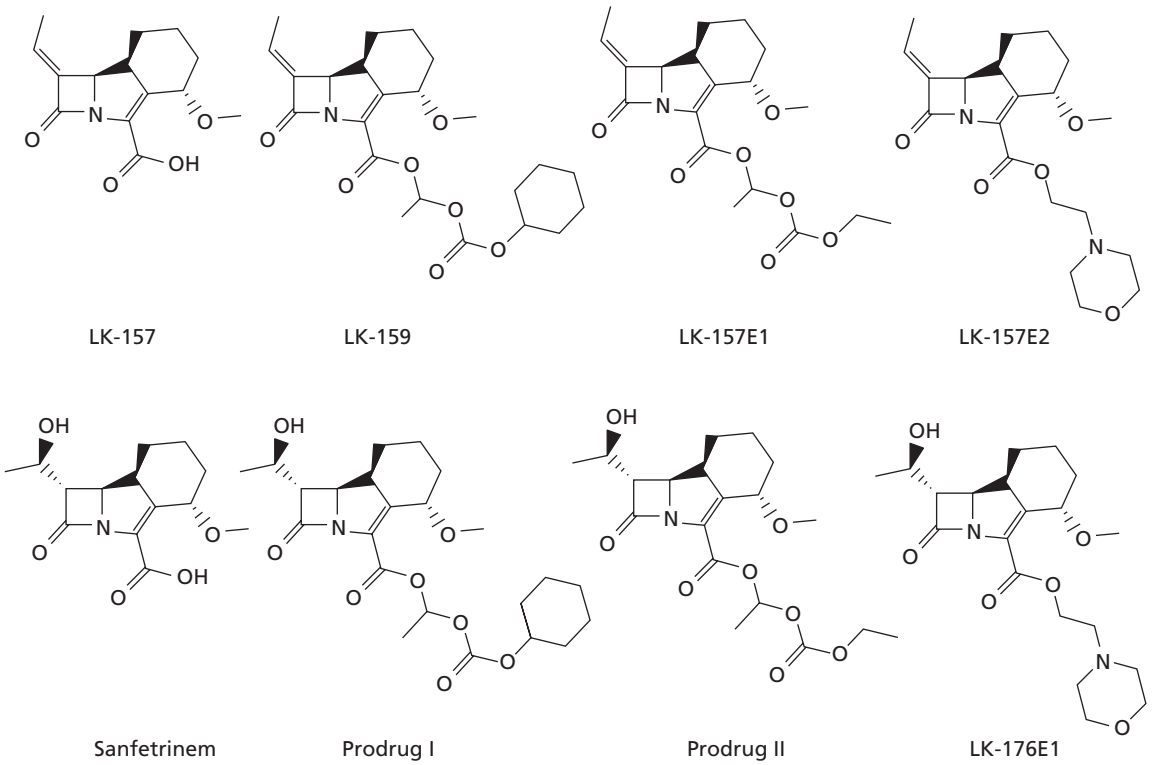

Figure 1 Chemical structures of LK-157, LK-159 (1-(cyclohexyloxycarbonyloxy)ethyl (8S,9R)-10-(E)-ethylidene-4-(S)-methoxy-11-oxo-azatricyclo[7.2.0.0^{3,8}]undec-2-ene-2-carboxylate), LK-157E1 (ethyloxycarbonyloxyethyl (8S,9R)-10-(E)-ethylidene-4-(S)-methoxy-11-oxo-azatricyclo[7.2.0.0^{3,8}]undec-2-ene-2-carboxylate), LK-157E2 (morpholinoethyl (8S,9R)-10-(E)-ethylidene-4-(S)-methoxy-11-oxo-azatricyclo[7.2.0.0^{3,8}] undec-2-ene-2-carboxylate), sanfetrinem, prodrug I (cilexetil ester of sanfetrinem), prodrug II (1-(ethyloxycarbonyloxy)ethyl ester of sanfetrinem) and LK-176E1 (morpholinoethyl (8R,9R)-10-(S)-[1-(R)-hydroxyethyl]-4-(R)-methoxy-11-oxo-azatricyclo[7.2.0.0^{3,8}]undec-2-encarboxylate)

antibiotic sanfetrinem, which closely resembles the structural formula of LK-157, is reported to poorly absorbed after oral administration (bioavailability in rat < 5%). Two ester prodrugs were therefore synthesised to improve its permeability. Bioavailability following oral administration of cilexetil and 1-(ethyloxycarbonyloxy)ethyl ester prodrugs in rats was 32% and 47%, respectively.[11]

Here we report the synthesis, solubility and in-vitro permeability across rat jejunum for three ester prodrugs of LK-157: LK-159, LK-157E1 and LK-157E2. In addition, a morpholinoethyl ester of sanfetrinem, LK-176E1, has been prepared and evaluated to assess its promising permeability characteristics.[12,13] The structures of these compounds are shown in Figure 1.

Materials and Methods

Chemicals

Ammonium acetate, acetonitrile and acetic acid were obtained from Merck (Darmstadt, Germany). All reagents used were at least of analytical grade, except acetonitrile, which was HPLC grade. HPLC-grade water was obtained using a Milli-Q purification system (Millipore Corp. Milford, MA, USA).

Tween 80 and Synperonic PE/L101 were from Uniqema (Chocques, France). Cremophor RH 40, Cremophor EL and propylene glycol were obtained from BASF (Ludwigshafen, Germany). Gelucir 50/13 was purchased from Gattefosse (St Priest, Cedex, France), sodium taurocholate from Fluka (Deisenhofen, Germany), lecithin from Calbiochem (Darmstadt, Germany) and PEG 400 and PEG 6000 from Clariant GmbH (Sulzbach, Germany).

Synthesis of prodrugs

Synthesis of LK-159

A solution of 156 mg (0.700 mmol, 1.02 EQ) triethylbenzyl ammonium chloride and 702 mg (3.396 mmol, 4.96 EQ) chloroethyl cyclohexyl carbonate in 17 ml DMF was heated to 68°C. A hot solution of 195 mg (0.684 mmol, 1.00 EQ) LK-157 in 3.4 ml DMSO was then added and the reaction mixture mixed at 65–70°C for 40 min. After that, the mixture was purified by extraction with diethyl ether and ice-cold water, followed by gradient column chromatography (cyclohexane : diethyl ether 4 : 1 → 1 : 1) to give LK-159 in 56% yield.

^1H NMR (300 MHz, $CDCl_3$): (δ, ppm) 1.20–2.10 (16H, m, 5,6,7,cyclohexyl), 1.61 (3H, d, J = 5.4, $CHCH_3''$), 1.64 (3H, d, J = 5.4, $CHCH_3'$), 1.81 (3H, dd, J = 7.2, 0.9, $=CHCH_3''$), 1.82 (3H, dd, J = 7.2, 0.9, $=CHCH_3'$), 3.25 (3H, s, OMe'), 3.27 (3H, s, OMe''), 3.29 (1H, m, 8), 4.65 (1H, m, $-(CH_2)-CH-(CH_2)-$), 4.79 (1H, br d, J = 10.5, 9), 4.92 (1H, t, J = 2.9, 4), 6.50 (1H, dq, J = 7.2, 1.8, $=CHCH_3$), 6.93 (1H, m, J = 5.4, $CHCH_3$).

^{13}C NMR (75 MHz, $CDCl_3$): (δ, ppm) 15.2, 19.6, 20.0, 23.5, 23.6, 25.1, 25.2, 30.7, 30.8, 31.3, 32.4, 45.0, 45.1, 56.1, 56.2, 60.8, 60.9, 72.3, 72.4, 77.6, 91.6, 91.8, 126.7, 126.8, 129.6, 140.1, 140.2, 148.6, 149.2, 152.4, 152.5, 159.3, 159.4, 171.0, 171.3.

Synthesis of LK-157E1

Chloroethyl ethyl carbonate (168.6 mg, 1.11 mmol, 1.2 EQ) and triethylbenzyl ammonium chloride (245.4 mg, 1.08 mmol, 1.2 EQ) were added to a suspension of LK-157 (256 mg, 0.898 mmol, 1.0 EQ) in DMF (12 ml) and the mixture heated for 70 min at 65°C in a microwave reactor. The raw product obtained was purified by extraction followed by flash chromatography (cyclohexane : diethyl ether 4 : 1 → 0 : 1) to afford pure LK-157E1 in 58% yield.

^{1}H NMR (300 MHz, $CDCl_3$): (δ, ppm) 1.32 (t, J = 7.1, 3H, OCH_2CH_3''), 1.33 (t, J = 7.1, 3H, $OCH2CH_3'$), 1.37–2.05 (m, 2 × 6H, 5′, 6′, 7′, 5″, 6″, 7″), 1.61 (d, 3H, J = 5.5, $CHCH_3''$), 1.64 (d, 3H, J = 5.5, $CHCH_3'$), 1.82 (d, 3H, J = 7.3, $=CHCH_3''$), 1.83 (d, 3H, J = 7.3, $=CHCH_3'$), 3.25 (s, 3H, OCH_3''), 3.26 (s, 3H, OCH_3'), 3.30 (m, 2 × 1H, 8), 4.23 (q, J = 7.3, 2H, $OCH_2''CH_3$), 4.25 (q, J = 7.3, 2H, $OCH_2''CH_3$), 4.80 (d, J = 10.5, 2 x 1H, 9), 4.92 (t, J = 3.0, 1H, 4″), 4.94 (t, J = 3.0, 1H, 4′), 6.50 (m, 2 × 1H, $=CHCH_3$), 6.92 (m, 2 × 1H, $CHCH_3$).

^{13}C NMR (75 MHz, $CDCl_3$): (δ, ppm) 14.1, 15.2, 19.49, 19.52, 19.9, 25.2, 30.7, 30.8, 32.4, 45.0, 45.1, 56.05, 56.12, 60.8, 60.9, 64.4, 64.5, 64.9, 72.3, 72.4, 84.4, 91.6, 91.8, 126.6, 126.7, 129.6, 140.1, 140.2, 148.9, 149.3, 152.8, 152.9, 153.0, 159.30, 159.37, 171.03, 171.26.

Synthesis of LK-157E2

Potassium fluoride (55.8 mg, 1.0 EQ) and 4-(2-chloroethyl) morpholine (173 mg, 1.16 mmol, 1.2 EQ) were added to a suspension of LK-157 (275 mg, 0.964 mmol, 1 EQ) in DMF (13.5 ml) and the mixture heated for 55 min at 100°C in a microwave reactor. The raw product obtained was purified by extraction followed by flash chromatography (dichloromethane : ethylacetate 1 : 0 → 1 : 3) to afford pure LK-157E2 in 26% yield.

^{1}H NMR (300 MHz, $CDCl_3$): (δ, ppm): 1.00–2.07 (m, 6H, 5, 6, 7), 1.83 (dd, J = 7.2, 3H, $CHCH_3$), 2.54 (t, J = 4.6, 4H, $-N(CH_2)_2$), 2.72 (t, J = 6.0, 2H, $-COOCH_2CH_2-$), 3.24–3.29 (m, 1H, 8), 3.27 (s, 3H, OCH_3) 3.69 (t, J = 4.6, 4H, $O(CH_2)_2$), 4.28–4.49 (m, 2H, $-COOCH_2CH_2-$), 4.77–4.81 (d, J = 10.5, 1H, 9), 4.98 (t, J = 3.0, 1H, 4), 6.51 (qd, J = 1.7, 7.2, 1H, $CHCH_3$).

^{13}C NMR (300 MHz, $CDCl_3$): (δ, ppm): 15.2, 20.0, 30.8, 32.4, 44.8, 53.7, 56.1, 56.7, 60.9, 62.2, 66.9, 72.4, 127.3, 129.5, 140.0, 147.7, 161.4, 171.4.

Synthesis of LK-176E1

4-(2-Chloroethyl)morpholine (755.6 mg, 5.05 mmol, 1.25 EQ) was added to a suspension of LK-176 (1.224 g, 4.04 mmol, 1 EQ) in DMF (20 ml) and the mixture heated for 32 min at 100°C in a microwave reactor. The raw product obtained was purified by extraction (300 ml DCM, 2 × 250 ml water). The organic phase was dried over $MgSO_4$, filtered, then evaporated, followed by flash column chromatography using hexane : ethylacetate : ethanol (1 : 1 : 0 → 0 : 1 : 1, v/v/v) as an eluent to obtain pure LK-176E1 in 46% yield.

^{1}H NMR (400 MHz, $CDCl_3$): (δ, ppm): 1.20–2.20 (9H, m, 5, 6, 7, $CH(OH)CH_3$), 2.45–2.55 (4H, m, $N(CH_2)_2$), 2.68 (2H, t, J = 6.0 Hz, $COOCH_2CH_2$), 3.20–3.30 (5H, m, OCH_3, 8, 10), 3.65–3.75 (4H, m, $O(CH_2)_2$), 4.19 (1H, m, 9), 4.23 (1H, m, CH(OH)), 4.25–4.45 (2H, m, $COOCH_2CH_2$), 4.98 (1H, t, J = 3.0 Hz, 4).

^{13}C NMR (400 MHz, $CDCl_3$): (δ, ppm): 20.2, 21.6, 30.6, 32.5, 44.0, 53.7, 54.8, 56.1, 56.7, 60.3, 62.1, 65.5, 66.9, 72.3, 126.2, 149.3, 161.0, 175.6.

Solubility studies

The ester prodrugs (0.5 mg) were added to 1.5 ml of the appropriate surfactant solution (in phosphate buffer, pH 6.8) and vortex mixed (1400/min) for 60 min at room temperature (22 ± 1°C). The solution was then passed through a 0.45 μm filter. Solubility was also studied at 37°C, where 3.3 mg LK-157E1 or 4.0 mg LK-157E2 were added to 5 ml of the appropriate solution and mixed vigorously on the magnetic stirrer for 60 min. The concentrations of ester prodrugs in the filtrate were determined by HPLC. All experiments were performed in duplicate.

In-vitro intestinal transport studies

The research complied with national legislation and with the company policy on the care of use of animals and related codes of practice. All animals received care in compliance with the European Convention on Animal Care. The study protocol was approved by the Veterinary Administration of the Republic of Slovenia with regard to the care and use of laboratory animals.

Male Wistar rats (250–320 g) had free access to standard laboratory food and tap water until 18 h before the experiment. Rats were decapitated and the small intestine excised immediately and placed into ice-cold 10 mmol/l D-glucose in Ringer buffer, bubbled with carbogen (95 : 5 O_2/CO_2), for no longer than 30 min. The jejunum located 25 cm distal from the caecum was used for experiments. The tissue was rinsed with ice-cold standard Ringer buffer to remove luminal contents and cut into 3 cm long segments. Care was taken to avoid visible Peyer's patches. The intestinal segments were opened along the mesenteric border, mounted onto a special insert and placed between two EasyMount side-by-side diffusion chambers with an exposed tissue area of 1 cm^2 (Physiologic Instruments, San Diego, CA, USA).

During the experiment the tissue was incubated on both sides with the appropriate Ringer buffer (standard Ringer buffer, pH 7.50, or modified Ringer buffer, pH 6.85) containing 10 mmol/l D-glucose at the serosal side and 10 mol/l D-mannitol at the mucosal side. Modified Ringer buffer was prepared by changing the amount of $NaHCO_3$, NaH_2PO_4 and Na_2HPO_4 in the original Ringer buffer recapture. The buffers were gassed continuously with carbogen at 37°C. The pH of the buffers was stable during the experiments and the osmolality was 307 mOsm/kg.

After 25 min' equilibration, the substance under investigation was added to the mucosal side when studying mucosal-to-serosal (m-to-s) transport, or to the serosal side when studying serosal-to-mucosal (s-to-m) transport. The

final volume of the solution in each compartment was 2.5 ml. The concentrations of LK-157 and LK-157E2 in the donor compartment were 1.9 and 1.3 mol/l, respectively. For the less-soluble LK-159 and LK-157E1, excess compound was first dispersed in the appropriate Ringer buffer then mixed for 1 h on the magnetic stirrer. The filtered (filter 0.45 μm) clear solution was added to the donor compartment. The concentrations of LK-157 and LK-157E1 in the donor compartment depended on the conditions and on average were 0.1 and 0.3 mol/l, respectively. The concentration in the donor compartment was determined by HPLC.

Samples of 250 μl were withdrawn from the acceptor or donor compartments at certain time points and replaced with fresh Ringer buffer containing 10 mol/l D-glucose (m-to-s transport) at the serosal or 10 mol/l D-mannitol (s-to-m transport) at the mucosal side.

The chambers were equipped with two pairs of Ag/AgCl electrodes connected to the chambers via 3 mol/l KCl / 3.5% agar bridges; one pair of electrodes was used for measuring the transepithelial potential difference (PD) and one pair for passing current. The tissue viability and integrity were checked by monitoring PD, short-circuit current (Isc) and transepithelial electrical resistance (TEER). The increase in I_{sc} and PD after the addition of D-glucose (25 mol/l) to the mucosal compartment at the end of experiments was recorded, which reflected the activity of sodium/D-glucose co-transporters.

For the determination of tissue resistance, a bipolar pulse (amplitude 50 μA) was applied every 120 s for 200 ms by the computer controlled voltage–current clamp apparatus (Scientific Instruments, Aachen, Germany).

The background PD (asymmetry of the electrodes and liquid junction potential) was compensated before mounting the tissue in the diffusion chamber system. I_{sc} and TEER were corrected for fluid resistance. TEER was determined according to Ohm's law.

Data analysis

An apparent permeability coefficient (P_{app}) was calculated according to the equation:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A \cdot C_o} (cm/s) \quad (1)$$

where $\frac{dQ}{dt}$ is the steady-state appearance rate of LK-157 for the experiments with LK-157, with LK-159 or LK-157E1, or a cumulative amount of LK-157 and LK-157E2 for the experiments with LK-157E2 or a cumulative amount of sanfetrinem and LK-176E1 for the experiments with LK-176E1 on the acceptor side of the tissue; A is the exposed area of the tissue; and C_o is the concentration of LK-157, (LK-157 + LK-159), (LK-157 + LK-157E1), (LK-157 + LK-157E2) or (sanfetrinem + LK-176E1) measured in the donor compartment at 120 min. Results are given as means ± SD (n = 2–6).

Chromatography conditions

The concentrations of LK-157, LK-159, LK-157E1, LK-157E2, sanfetrinem or LK-176E1 in the samples from the transport experiments were analysed by HPLC with UV detection. The HPLC system consisted of a 2695 Separation Module and a 2996 PDA detector (Waters Corporation, MA, USA); UV detection was at 260 nm. The system control and integration was performed using Empower software (Waters Corp.). We used an XTerra RP-18 analytical column (150 × 4.6 mm ID, 3.5 μm particle size, Waters, Milford, MA, USA) at 30°C. Mobile phase A consisted of 25 mol/l ammonium acetate (adjusted to pH 6.0 using 0.1 mol/l acetic acid), and acetonitrile (95 : 5 v/v); mobile phase B was 25 mol/l ammonium acetate, pH 6.0, and acetonitrile (10 / 90 v/v). Starting with 85% mobile phase A, an isocratic elution was performed for 3 min, then the composition was changed to 100% mobile phase B over the next 0.5 min; it was then constant for 3.5 min, after which the composition was changed over 0.5 min to 85% mobile phase A and then equilibrated for the next 2.5 min. The flow rate was 1.2 ml/min and the injection volume was 10 μl.

Calibration curves were linear over the range 80–120% of expected values, with correlation coefficients of 0.999. Using analysis of variance, statistical significance was checked by an F-test of the overall fit, followed by t-tests of individual parameters. The results demonstrated acceptable accuracy and reproducibility. The limit of detection was determined at a signal-to-noise ratio of 3 and was 0.03, 0.03, 0.1, 0.08, 0.04 and 0.07 μmol/l for LK-157, LK-159, LK-157E1, LK-157E2, sanfetrinem and LK-176E1, respectively. The lower limits of quantitation were 0.1, 0.1, 0.3, 0.3, 0.2 and 0.3 μmol/l, respectively.

Statistical analysis

The prodrugs (LK-159, LK-157E1 and LK-157E2) were tested in three different media. A non-parametric Kruskal–Wallis test was performed to test the significance of differences between the median permeability coefficients in different media at a 6.7% level. To determine which groups differed from one another, the Nemenyi test for multiple comparisons was used. Values for $P \leq 0.05$ were considered significant.

To evaluate the effect of pH on the permeability of LK-157 and to evaluate the difference in the permeability of LK-157 in the m-to-s and s-to-m directions, two-group comparisons were analysed by unpaired two-tailed t-test and first applying the F-test for variance. If the variances were equal, the standard Student's t-test was performed; otherwise, the Behrens–Fisher test was used. The difference was considered significant at $P < 0.05$.

Results

Influence of surfactant/co-solvent solution on solubility of LK-159 and LK-157E1

LK-159 was practically insoluble in the standard buffer solutions used in permeability studies. We therefore tested different surfactants and/or co-solvents in order to increase the solubility of tested compounds to concentrations high enough for permeability measurements. The solubility enhancement of the compounds was determined at concentrations of surfactants and co-solvents that were previously shown not to affect the viability of the excised rat intestine,

Table 1 Effect of different surfactants and co-solvents on the solubility of LK-159 and LK-157E1 in a phosphate buffer (pH 6.85)

	Solubility (mmol/l)	
	LK-159	LK-157E1
Pure buffer solution	< 0.0005	0.148 ± 0.018
Tween 80 (5%)	0.030 ± 0.007	0.749 ± 0.071
Cremophor RH40 (5%)	0.042 ± 0.005	0.409 ± 0.090
Synperonic PE/L101 (5%)	0.041 ± 0.007	0.406
Gelucir 50/13 (1%)	0.021 ± 0.012	0.300 ± 0.063
Cremophor EL (1%)	0.013 ± 0.001	0.274 ± 0.050
PEG 6000 (2%)	< 0.0005	0.137 ± 0.032
DMSO (2%)	< 0.0005	0.108 ± 0.011
PEG 400 (2%)	< 0.0005	0.153 ± 0.024
Propylene glycol (2%)	< 0.0005	0.092 ± 0.042
Tween 80 (1%) + propylene glycol (1%)	0.014 ± 0.003	0.422 ± 0.098
Tween 80 (1%) + PEG 400 (1%)	0.018 ± 0.001	0.364 ± 0.042
Tween 80 (1%) + Cremophor RH40 (1%) + Synperonic PE/L101 (1%)	0.044 ± 0.005	0.804 ± 0.113
Sodium taurocholate (5 mmol/l) + lecithin (1.5 mmol/l)	0.001 ± 0.000	0.208 ± 0.026

Values are concentrations of dissolved prodrug in the medium; mean ± SD (n = 2).

as determined by measurement of the intestinal electrophysiological parameters (data not shown). The co-solvents PEG 6000, DMSO, PEG 400 and propylene glycol had practically no effect on the solubility of LK-159 and LK-157E1 (Table 1). On the other hand, surfactants markedly increased the solubility, with the most pronounced effect observed for the combination of Tween 80, Cremophor RH40 and Synperonic PE/L101 (Table 1). We also tested the effect of endogenous surfactants (sodium taurocholate and lecithin) at concentrations about two-fold higher than those used in fasted-state simulated intestinal fluid.[14] The solubility of LK-159 in this medium was too low to perform permeability studies (Table 1).

As shown in Table 1, LK-157E1 was markedly more soluble than LK-159 in all media tested. LK-157E2 was even more soluble (data not shown). Permeability experiments with LK-157E1 and LK-157E2 could therefore be performed without the addition of surfactants in the donor compartment. Surfactants increased the solubility of LK-157E1 and LK-157E2, with a more pronounced effect observed for the less soluble LK-157E1.

Permeability experiments

Transport of LK-157

Transport of LK-157 across the rat jejunum in the m-to-s direction was higher at a lower pH than at a higher pH (P_{app} = $1.21 \times 10^{-6} \pm 0.37 \times 10^{-6}$ cm/s (n = 3) for pH 6.85 vs $0.77 \times 10^{-6} \pm 0.11 \times 10^{-6}$ cm/s (n = 3) at pH 7.50) (P = 0.08). Although the difference is not significant, higher permeability at lower pH is in accordance with the acidic nature of the substance and could be explained by the mechanism previously suggested for monocarboxylic-acid-type drugs.[11,15] These results also suggest that LK-157 would be much better absorbed from the more proximal regions of the intestinal tract (duodenum/proximal jejunum), where the pH is lower, than from the more distal regions, where the pH is higher.

Transport of LK-157 in the m-to-s direction ($P_{app} = 1.11 \times 10^{-6} \pm 0.64 \times 10^{-6}$ cm/s; n = 3) was similar to that in the s-to-m direction ($P_{app} = 1.03 \times 10^{-6} \pm 0.26 \times 10^{-6}$ cm/s; n = 3), suggesting that LK-157 was not actively transported across the rat jejunum.

Transport of ester prodrugs

When ester prodrug was added to the mucosal side of the tissue there were no significant differences (at P = 0.067) between permeability coefficients in different media determined using the Kruskal–Wallis non-parametric test followed by Nemenyi test. However, because we had doubts about homogeneity of variance we performed an additional analysis of variance F-test, which showed differences at a significance level of 0.01.

The results in Table 2 show that the concentration of ester prodrug decreased with time, while the concentration of LK-157 increased. Under the same conditions in the donor compartment (5% Cremophor RH40), LK-157E1 was most extensively converted to LK-157, followed by LK-159, while LK-157E2 disintegrated much less.

In the case of LK-159 and LK-157E1, only LK-157 was determined in the acceptor compartment, demonstrating that LK-159 and LK-157E1 were completely metabolised during transport across the rat jejunum *in vitro*. Because LK-159 and LK-157E1 were not degraded in solubility experiments, we concluded that both prodrugs are stable in aqueous media and in the absence of the hydrolytic enzymes but highly susceptible to enzymatic hydrolysis. In contrast, LK-157E2 was only partially hydrolysed during transport across the rat intestine. LK-157E2 disintegrated to LK-157 most probably non-enzymatically, because similar kinetics of degradation could be observed in the solutions without the biological material.

In the case of LK-159, similar degradation to LK-157 was observed with Synperonic PE/L101 and Cremophor RH40, while there was less disintegration of LK-159 when a combination of Synperonic PE/L101, Cremophor RH40 and Tween 80 was applied to the donor side (Table 2).

In the case of LK-157E1, the most pronounced degradation was observed in the Ringer buffer without surfactants,

Table 2 Metabolic conversions at the mucosal side of the rat intestine during permeability studies at pH 6.85

	Time (min)		
	5	120	210
LK-159 to LK-157			
5% Synperonic PE/L101	9 ± 1	42 ± 7	88 ± 9
5% Cremophor RH40	3 ± 2	39 ± 16	81 ± 8
2% Tween 80 + 2% Synperonic PE/L101 + 2% Cremophor RH40	2 ± 0	24 ± 8	58 ± 31
LK-157E1 to LK-157			
Ringer buffer	14 ± 6	96 ± 0	100 ± 0
5% Cremophor RH40	2 ± 0	62 ± 2	92 ± 2
2% Tween 80 + 2% Synperonic PE/L101 + 2% Cremophor RH40	2 ± 0	42 ± 0	64 ± 5
LK-157E2 to LK-157			
Ringer buffer	19 ± 0	33 ± 0	46 ± 0
5% Cremophor RH40	17 ± 1	26 ± 0	38 ± 1
Sodium taurocholate (1.5 mmol/l) + lecithin (0.5 mmol/l)	19 ± 0	33 ± 1	51 ± 3
LK-176E1 to sanfetrinem			
Ringer buffer	6 ± 1	31 ± 1	44 ± 2 (180 min)

Values are % metabolic conversion (prodrug to LK-157 or sanfetrinem); mean ± SD (n = 2).

which reflects in-vivo conditions. The presence of surfactants in the donor solution appeared to attenuate the degradation of LK-157E1. This could be attributed to inhibition of esterases by the surfactants, or to the entrapment of LK-157E1 in the micelles formed by the surfactants, which protected LK-157E1 from the action of esterases (Table 2).

The P_{app} of LK-159, LK-157E1 and LK-157E2 obtained in the different conditions in the donor compartment of the rat jejunum *in vitro* are given in Table 3.

In the case of LK-159, the highest permeability was observed in the experiment with Cremophor RH40, while similar permeabilities were observed in experiments with Synperonic PE/L101 and a combination of Tween 80, Synperonic PE/L101 and Cremophor RH40. This effect of Cremophor RH40 was surprising because Cremophor RH40 in combination with Tween 80 and Synperonic PE/L101 had no such effect (Table 3). Additionally, LK-159 disintegrated similarly to LK-157 in experiments with Synperonic PE/L101 and Cremophor RH40 (Table 2); thus, the increased permeability observed in the experiment with Cremophor RH40 could not be explained by different degradation. It seems that the addition of Synperonic PE/L101 decreased the permeability of LK-159, most probably because of the entrapment of LK-159 in the micelles formed by this surfactant. This entrapment decreases the diffusion coefficient of LK-159 by increasing its 'size'.

In the case of LK-157E1, the highest permeability was observed in an experiment with the Ringer buffer without the addition of surfactants, while similar permeabilities were observed in experiments with Cremophor RH40 and a combination of Tween 80, Synperonic PE/L101 and Cremophor RH40 (Table 3). This could be caused by the entrapment and/or binding of LK-157E1 in/to the micelles formed by the surfactants, which reduced the diffusion coefficient of LK-157E1.

In the case of LK-157E2, a lower permeability was observed in the experiment with Cremophor RH40, most probably due to micelle formation, while similar permeabilities were observed in the experiment with Ringer buffer and sodium taurocholate + lecithin.

The decreased permeability due to surfactant addition was in agreement with results obtained by Nerurkar *et al*., who reported that the addition of Tween 80 and Cremophor EL

Table 3 Effect of different incubation media on the permeability coefficient (P_{app}) of LK-157 across the rat jejunum after the addition of the ester prodrugs to the mucosal side, and of sanfetrinem after the addition of LK-176E1 to the mucosal side

Prodrug	Medium	P_{app} ($\times 10^{-6}$ cm/s)
LK-159	5% Synperonic PE/L101	0.54 ± 0.24
	5% Cremophor RH40	1.43 ± 0.11
	2% Tween 80 + 2% Synperonic PE/L101 + 2% Cremophor RH40	0.39 ± 0.02
LK-157E1	Ringer buffer	1.88 ± 0.13
	5% Cremophor RH40	0.76 ± 0.32
	2% Tween 80 + 2% Synperonic PE/L101 + 2% Cremophor RH40	0.66 ± 0.00
LK-157E2	Ringer buffer	3.06 ± 0.03
	5% Cremophor RH40	1.76 ± 0.62
	Sodium taurocholate (1.5 mmol/l) + lecithin (0.5 mmol/l)	3.91 ± 3.70
LK-176E1	Ringer buffer	8.49 ± 3.71

Values are means ± SD (n = 2–6).

decreased permeability of the model drug because of the association of the drug with micelles.[16]

Our primary aim for the development of ester prodrugs was to increase the absorption of the active drug. The P_{app} of LK-157E1 obtained without the addition of surfactants ($1.88 \times 10^{-6} \pm 0.13 \times 10^{-6}$ cm/s) was higher, although not significantly, than the P_{app} of LK-157 ($1.16 \times 10^{-6} \pm 0.25 \times 10^{-6}$ cm/s), indicating that the absorption of LK-157 could be increased by application of the prodrug LK-157E1. Furthermore, the P_{app} of LK-157E2 ($3.06 \times 10^{-6} \pm 0.03 \times 10^{-6}$ cm/s) was higher than the corresponding values for LK-159 and LK-157E1 (Table 3).

Transport of sanfetrinem

The solubility of LK-176E1 was high enough to perform permeability experiments without the addition of surfactants in the donor compartment. Similarly to LK-157E2, LK-176E1 was only partially hydrolysed during transport across the rat intestine. The amount of LK-176E1 in the acceptor compartment was lower than in the donor compartment. The permeability of LK-176E1 would be expected to be higher than that of sanfetrinem. For this reason, some enzyme-catalysed hydrolysis of LK-176E1 during the transport was possible, as was similarly observed with LK-157E2. The permeability of LK-176E1 across the jejunum was the highest among all tested compounds ($P_{app} = 8.49 \times 10^{-6} \pm 3.71 \times 10^{-6}$ cm/s), also suggesting the highest absorption from the intestinal tract.

Discussion

LK-157 could be classified as a drug with a very low permeability, as its P_{app} ($1.11 \times 10^{-6} \pm 0.64 \times 10^{-6}$ cm/s) is much lower than that of enalaprilat, a model for a drug with low permeability,[17,18] which makes this drug unsuitable for oral administration. Poor permeability is probably due to unfavourable physicochemical properties (i.e. high hydrophilicity) rather than active secretion of the drug across the intestinal epithelium.

Prodrug esters with increased lipophilicity and known safety were therefore designed. LK-159 could be classified as a drug with very low solubility – solubility in physiological medium (pH 6.8, sodium taurocholate/lecithin) was only 0.3 μg/ml. LK-159, which was in contact with the mucosal side of the rat jejunum *in vitro*, partially disintegrated to LK-157 (50–65% in 2 h) before being transported to the intestinal epithelial cells. LK-159 also completely disintegrated to LK-157 during transport across the rat jejunum *in vitro*. Therefore, the P_{app} of LK-159 (or more precisely the P_{app} of LK-157 after the application of LK-159) was low (much less than the P_{app} for enalaprilat), with very low absorption predicted in humans.[17,18] The low solubility and low permeability of LK-159 were the main reasons to discontinue evaluation.

The solubility of LK-157E1 was much higher (about 260-times in the artificial intestinal fluid) than that of LK-159, which makes LK-157E1 a much better prodrug for LK-157 than LK-159. The permeability of LK-157E1 was higher than that of LK-157, although still very low compared with the model low-permeable drug enalaprilat.[17,18] The low permeability could be partially attributed to the rapid pre-absorptive degradation of LK-157E1 into LK-157. The pre-absorptive degradation can be attenuated by various surfactants (i.e. Cremophor RH40 or combination of Cremophor RH40, Tween 80 and Synperonic PE/L101), but the addition of these surfactants to the mucosal compartment at a very high concentration (i.e. 5%) also decreased the P_{app} of LK-157E1. In addition, LK-157E1 was almost completely disintegrated to LK-157 during absorption across the rat jejunum.

The P_{app} of LK-157E2 is the highest among the LK-157 prodrugs tested (Table 3) and is comparable to the permeability of the low-permeable model drug enalaprilat, suggesting a similar extent of absorption in humans (about 25%).[17,18] This prodrug is only partially metabolised to LK-157 during absorption across the intestinal epithelium, which is probably the main reason for its higher permeability.

The permeability of LK-176E1 was the highest among all tested prodrugs, demonstrating that this prodrug had the most favourable physicochemical properties. Based on the comparison with the permeabilities of model drugs,[17–19], 70–100% absorption is expected in humans.

It is reasonable to assume that the bioavailability of LK-176E1 would be superior to that of cilexetil sanfetrinem (Figure 1), development of which was stopped in phase II. Head-to-head comparisons of bioavailability in rats, dogs and humans would be needed to evaluate the excellent in-vitro characteristics of the novel morpholinoethyl ester prodrug LK-176E1.

Conclusions

This study suggest that LK-157, which is a promising broad-spectrum tricyclic carbapenem inhibitor of class A, C, and D β-lactamases to be co-administered with a selected cephalosporin antibiotic, is a highly soluble but poorly permeable drug. The synthesised ester prodrugs improved the permeability of LK-157 across the rat jejunum. Interestingly, the permeabilities of LK-157E2, the morpholinoethyl ester of LK-157, and LK-176E1, the morpholinoethyl ester of sanfetrinem, were superior to those of the others. LK-176E1 could be investigated further to assess this promising bioavailability.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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