BIOPHARMACEUTICS & DRUG DISPOSITION
Biopharm. Drug Dispos. **23**: 131–141 (2002)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1002/bdd.302

The Effect of Enzyme Inhibitor and Absorption Site Following [D-ala^2, D-leu^5]Enkephalin Oral Administration in Rats

Hye J. Lee[a,*] and Gordon L. Amidon[b]
[a] *College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, San 56-1, Shinlim-Dong, Kwanak-Gu, Seoul 151-742, South Korea*
[b] *College of Pharmacy, The University of Michigan, Ann Arbor, MI 48109, USA*

ABSTRACT: The effects of enzyme inhibitor, amastatin, and absorption site following intravenous (i.v.) oral (p.o.), jejunal and ileal administration of [D-ala^2, D-leu^5]enkephalin (YdAGFdL) were investigated in rats. Model dependent and independent pharmacokinetic parameters were obtained and compared. Linear pharmacokinetics of YdAGFdL were evaluated at 0.28 and 500 μg doses for i.v. and at 1, 500, and 1000 μg for p.o. and ileal routes. Plasma samples were collected and assayed for intact YdAGFdL using a radiometric thin layer chromatography. The clearance (CL) and half lives of the distribution and elimination phases following the 0.28 μg ($n = 6$) i.v. dose were 42.7 ± 26.2 (S.D.) ml/min, 0.48 ± 0.17 min, and 3.98 ± 0.92 min, while those of the 500 μg dose ($n = 6$) were 48.0 ± 23.3 ml/min, 0.59 ± 0.25, and 6.81 ± 3.12 min, respectively, suggesting apparent linear kinetics. The CL values were close to the cardiac output of rats (50 ml/min) indicating very rapid elimination from the body. Mean bioavailability (F) values following p.o. ($n = 15$), jejunal ($n = 4$), and ileal ($n = 16$) administration were 0.40 ± 0.24% (S.E.), 1.25 ± 0.39, and 1.78 ± 0.40, respectively, and were not significantly different ($p < 0.05$) among three doses (1, 1000, 5000 μg). The F value of YdAGFdL following ileal administration in the presence of amastatin was 8.76 ± 4.47% ($n = 6$), a 22 fold increase over po administration and a five fold increase over ileal administration without an inhibitor. These results indicate that 'effective' oral delivery of small peptides may be achievable. Copyright © 2002 John Wiley & Sons, Ltd.

Key words: YdAGdL; pharmacokinetics; oral delivery; site specific; enzyme inhibitor

Introduction

With the rapid development of recombinant DNA technology, proteins, peptides and other macromolecules are receiving increased interest as potential drugs. During the last several decades, many investigators have attempted to develop strategies for peptide delivery. In clinic, peptide and protein-based drugs are typically administered by frequent injection because of negligible oral bioavailability and short half lives resulting in poor patient convenience and compliance [1,2]. Enzymatic instability of these drugs in gastrointestinal tract has greatly limited the development and evaluation of oral formulations [2,3]. In addition, a very large first pass metabolism and or hepatic extraction which could be in part due to the enzymatic instability [3,4], low membrane permeability and or low solubility are also major obstacles for peptide oral delivery [5,6]. Various alternate routes for systemic administration of peptide drugs have also been investigated [2,7]; buccal [8], vaginal [9], rectal [8], dermal [10], ocular [11], and nasal [12]. Stabilizers, enhancers, and enzyme inhibitors

*Correspondence to: College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, San 56-1, Shinlim-Dong, Kwanak-Gu, Seoul 151-742, Korea.
E-mail: hjlee@smu.ac.kr

Received 18 October 2001
Revised 28 January 2002
Accepted 8 February 2002

have also been employed to improve the systemic availability of peptide drugs [7,13].

Among the various routes of administration for peptide delivery, the advantages of oral route are so great that repeated attempts have been made to improve systemic availability [13-15]. However, the peptide transport and metabolism mechanisms in the intestine especially for tetrapeptides and larger has not been systemically explored. Consequently, very little definitive data on peptides or peptide drugs are available concerning *in vivo* absorption and disposition kinetics. This is probably due to the lack of a specific and reliable analytical method to quantify intact peptides in blood and in part the limited availability of the drug itself.

Although many inhibitors of the metabolism of peptides have been studied [13–16], oral delivery systems for peptides using this approach have not yet been successful. Recently, in our laboratory, selective enzyme inhibitors have been investigated in order to reduce the intestinal component of the metabolism using single-pass rat intestinal perfusion and rat/rabbit intestinal brush-border membrane vesicles [13,17]. This is similar to the delivery strategy for levodopa, i.e., the concurrent administration of carbidopa, a metabolic inhibitor of aromatic L-amino acid (dopa) decarboxylase that is unable to penetrate into the CNS but readily diminishes the decarboxylation of levodopa in peripheral tissues [18]. In addition to the employment of an enzyme inhibitor(s), the most optimal site for peptide absorption in the gut needs to be investigated [19] since the participating enzymes for the peptide metabolism in the gut are brush-border membrane and cytosolic enzymes and the distribution of these enzymes are reported to be site dependent [20,22]. Therefore, using an enzyme inhibitor combined with controlling the absorption site may be the most effective strategy for peptide oral delivery.

In this report [D-ala, D-leu]-enkephalin (YdAGFdL) was chosen as a relatively stable model peptide to investigate oral peptide delivery strategies. In previous studies, the principal route of metabolism was brush-border aminopeptidase mediated [17] while other studies indicated that the membrane permeability was not the principal limitation to effective oral delivery [23]. In this paper we report the results of pharmacokinetic and bioavailibility studies of YdAGFdL following intravenous and oral administration to rats. In addition, we also investigated the effect of site of administration, jejunum vs ileum, and the co-administration of an aminopeptidase metabolic inhibitor, amastatin, on the systemic availability of YdAGFdL. The results indicate that effective oral delivery of peptides can be achieved through a combined strategy of chemical stabilization, metabolic inhibition and site specific delivery.

Methods

Materials and animals

[^{3}H]-YdAGFdL (specific activity–$\sim$40 Ci/mmol), [3H]-tyrosine (specific activity–100 Ci/mmol), and aqueous scintillation cocktail were purchased from Amersham (ACS®, Arlington Heights, IL). All peptides and amino acids used in our study were obtained from Bachem (Torrance, CA), and amastatin was from Sigma Co. (St. Louis, MO). Reverse phase KC-18 TLC plates (5×20 cm, Whatman Inc., Princeton, NJ) were used for an assay. All solvents were obtained from Fisher Scientific Co. (Fair Lane, NJ). High purity water from Milli-Q water system (Millipore, Bedford, MA) was used for all solution preparations. Other chemicals were a reagent grade. Male Sprague–Dawley rats, weighting 300–350 g, were used for animal experiments.

Dosing solution preparation

Two dosing levels, 0.28 and 500 μg, for i.v. administration and three dosing levels, 1.1, 1000, and 5000 μg for p.o. and ileal administration were studied. For jejunal administration, only a 1.1 μg dose was studied. For the inhibitor study, 1 mg of amastatin with 1 mg of YdAGFdL was administered ilealy. The solutions of labeled YdAGFdL were evaporated under a gentle stream of N_2 at room temperature before each experiment. Each dosing solution was freshly prepared in 0.3 ml of 0.1 M phosphate buffer (pH 7.4). Amastatin was added to the dosing solution

of 0.3 ml of 0.1 M phosphate buffer. For the preparation of various dosing solutions, the amount of radiolabeled YdAGFdL was fixed; e.g., 0.28 μg for an i.v. study and 1.1 μg for other studies. The amount of unlabeled YdAGFdL was increased to make up the total dose.

Intravenous and oral administration

Rats implanted with a cannula in the left or right jugular vein, or tail vein were used and fasted overnight for the p.o. administration. Each dose level was tested in six rats in a cross-over fashion with a 1 week washout period. Each rat was used two times for i.v. and three times for p.o. administration. About 0.22 ml of blood samples were collected from the cannula at 0, 0.5 (for i.v. only), 1, 3, 5, 8, 10, 15, 20, 25, 30, 40, and 50 min. An equal volume of heparinized saline (10 IU/ml) was replaced after each sampling. One hundred microliters of the blood samples were vortexed with 200 μl of ice-cold acetonitrile and centrifuged for 1 min at 3000 rpm. These samples were stored in a refrigerator for the assay of intact YdAGFdL by the thin layer chromatography (TLC) method. Another set of samples was prepared as above for total radioactivity counting using a liquid scintillation counter (LS 5000TD, Beckman Inc., Fullerton, CA).

Jejunal and ileal administration

Four to eight rats were prepared with a chronic jejunum or ileum fistula according to the method of Poelma and Tukker [24], resulting in a loop of about 10 cm. A 7–10 day recovery period was allowed. Solid mucus secretion from the isolated loop and weight gain were used as an indication of recovery and viability of the brush-border membranes. During the recovery period 10,000 units of penicillin were administered daily via intraperitoneal administration. The isolated loop was flushed with saline solution once a day. The jugular or tail vein cannula was implanted about 1 h prior to each dosing for blood sampling. Dosing solution was administered to the isolated jejunal or ileal loop after flushing with saline solution. For ileal administration, among the three dose levels (1.1, 1000, and 5000 μg), 1.1 and 5000 μg doses were studied in a cross-over fashion with a 1-week washout period. The 1000 μg dose group was served as a control group for a cross-over study of the amastatin group. Only one dose (1.1 μg) was used for jejunal administration. Blood sampling points were identical to those in the p.o. study.

The effect of enzyme inhibitor on the bioavailability of YdAGFdL

Amastatin, which is known as an inhibitor for aminopeptidase [16,23], was chosen for the study of the effect of an enzyme inhibitor. Previous studies have shown that amastatin significantly inhibited YdAGFdL *in vitro* metabolism by rabbit intestinal brush-border membrane [17]. The ileal route of administration was selected to test the enzyme inhibitor since the systemic availability of YdAGFdL following ileal administration was greater than that of jejunal administration. This site dependence was also consistent with the results from salmon calcitonin and cholecystokinin octapeptide sulfated which were also shown to be metabolized by aminopeptidase [25,26]. The ileal vs jejunal bioavailabilities of salmon calcitonin and cholecystokinin octapeptide sulfated were 5% vs 0.4% and 2.1% vs $<0.5\%$, respectively.

Assay procedure

One set of blood samples was used for total radioactivity counting and the other set for the YdAGFdL TLC assay. TLC assay method was adapted from the method of Ziring *et al.* [27] with a slight modification. Total radioactivity in blood was counted with an LS 5000TD after treating the supernatant of centrifuged blood samples with 10 ml of scintillation cocktail solution. The blood precipitate after centrifuge was found to contain less than 5% of the total radioactivity. For YdAGFdL quantitation, the resulting supernatant from the centrifuged blood samples after adding acetonitrile was applied to a TLC plate about 5 cm from the bottom. Phosphate buffer solution was prepared using 0.1 M sodium phosphate dibasic and adjusted to pH 4.1 with orthophosphoric acid. One hundred milliliter of freshly prepared solvent (propanolol-1:phosphate buffer, 30:70) was added to a standard rectangular-glass TLC tank and allowed to equilibrate to room temperature for at least 30 min. Plates were then developed in the tank

approximately up to 3 cm from the top. The band of the response factor (RF) of 0.1–0.3 for YdAGFdL was scraped into a 12 ml cocktail solution and counted with an LS 5000TD.

RF determination of [^{3}H]-YdAGFdL, [^{3}H]-YdAG and [^{3}H]-Y

Individual RF values for [^{3}H]-YdAGFdL and possible metabolites which contain radiolabeled tyrosine; i.e., [^{3}H]-YdAG and [^{3}H]-Y, were determined by coapplying 1 mg/10 µl solutions of unlabeled YdAGFdL, YdAG, and Y to a TLC plate. The unlabeled YdAGFdL and its metabolites from the developed plate were visualized by spraying the plate with 0.01% fluorescamine in acetone, followed immediately by spraying with 0.5% pyridine in acetone. The resulting fluorescent spots or bands were viewed with an ultraviolet lamp (366 nm) and used to determine the RF values of the intact [^{3}H]-YdAGFdL in the blood. The corresponding bands for [^{3}H]-YdAGFdL and its metabolites were scraped into 12 ml scintillation cocktail solution. These samples were stored in the refrigerator for 24 h to quench the chemiluminescence and counted.

Standard curve preparation

The standard solutions of [^{3}H]-YdAGFdL in blood were prepared by spiking a 10 µl of appropriate diluted solutions of [^{3}H]-YdAGFdL to the 90 µl of blood yielding 2.2×10^2, 6.6×10^2, 2.2×10^3, 6.6×10^3, 2.2×10^4, 2.2×10^5, and 2.2×10^6 dpm/ml, respectively. These were prepared in triplicate.

Stability of YdAGFdL in blood

Radiolabeled YdAGFdL in an amount resulting in a final concentration of 10,000 dpm/ml was incubated and gently rotated for 0, 30s, 1, 5, 10, and 30 min at room temperature. Intact YdAGFdL was then measured by the TLC method.

Pharmacokinetic (PK) analysis of data

For (PK) analysis, radioactivity was converted to the blood YdAGFdL concentration based on the specific activity of each batch; e.g., 1 dpm/ml = 6.345 fg/ml, and the concentration of intact YdAGFdL in blood (C_T) = concentration of labeled YdAGFdL (C_H)+concentration of unlabeled YdAGFdL; i.e., $C_H \times$ unlabeled/labeled by weight ratio in dosing solutions (C_C). Since the PK analysis of peptide drugs is not readily available in the literature, both model-dependent and model-independent statistical moment analyses were attempted and the values were compared [28].

Intravenous blood data were fitted into one, two, and three-compartmental models using PCNONLIN (SCI Software, Lexington, KE). Intravenous blood data were best described by the two-compartment open model based on their r^2 (r^2 being 0.999 in most cases at both doses) and the Akaike Information Criterion and Schwarz Criterion (values being all <60). Clearance (CL), volume of distribution (V_d), half lives of distribution (t_α) and elimination phase (t_β), distribution (α) and elimination phase (β) rate constants were estimated. For p.o., jejunal and ileal data analysis, PK parameters of mean absorption time (MAT) and absolute bioavailability (F) were estimated based on the model-independent analysis [28] assuming linear PK. The maximum concentration (C_{max}) and the time for a maximum concentration (T_{max}) were read directly from the blood concentration profiles.

Student T-test was used to evaluate statistical difference of PK parameters among dosing groups and routes, and p value being <0.05 was considered significant.

Results

TLC assay method

The radiometric TLC assay method of Zyring *et al.* [27] was found to be adequate to quantitate the intact [D-ala^2, D-leu^5]-enkephalin (YdAGFdL) in rat plasma. The [^{3}H]-YdAGFdL was well separated from the other possible tyrosine-containing metabolites; e.g., YdAG, and Y. The values of RF for YdAGFdL, YdAG, and Y were 0.17, 0.73 and 0.89, respectively. The standard curve of YdAGFdL was linear with an r^2 value of 0.999 and a slope of 0.79. The intra-day assay variability was negligible but the slope was decreased about 20% over 1 month indicating

instability of radiolabeled YdAGFdL during the storage. The detection limit of YdAGFdL in blood with a CV of 15% was 2200 dpm/ml which was equivalent to 14 pg/ml. In the YdAGFdL absorption studies, no radiolabeled metabolites other than tyrosine were observed.

Stability of YdAGFdL

Degradation of YdAGFdL in blood was less than 0.7% min^{-1} at room temperature. All blood samples were treated within 0.5 min to prevent metabolism subsequent to sampling.

Intravenous pharmacokinetics of YdAGFdL after dosing of 0.28 and 500 μg

Figure 1 shows blood concentration–time data of YdAGFdL, averaged from six rats after i.v. administration of 0.28 and 500 μg doses. Mean blood concentrations for each sampling point were obtained from two different doses after normalized to the dose of 0.28 μg. The two blood profiles are very similar except for the terminal phase. The significance of this difference in terminal phase is uncertain due to the limited assay sensitivity at these low concentrations. As expected for a peptide, YdAGFdL was rapidly eliminated from the blood. The individual blood profile of YdAGFdL after i.v. administration was well described by a biexponential equation. Intravenous PK parameters of YdAGFdL obtained by fitting into the two-compartmental open model are shown in Table 1. The pharmacokinetic parameters including CL and Vd were not significantly different between two different doses and between two different analyses. The CL for YdAGFdL at the doses of 0.28 and 500 μg were 42.7 ± 26.2 and 48.0 ± 23.3 (S.D.) ml/min, respectively. These estimates are somewhat similar to the cardiac output of a rat, which ranges from 15 to 80 ml/min [29]. These results suggest that YdAGFdL may be rapidly metabolized in blood, possibly by membrane bound endothelial cell aminopeptidases (see below). Among the three possible radiolabeled-tyrosine-containing metabolites, only tyrosine was seen in blood after various routes of administration including i.v. The profiles of the YdAGFdL metabolite, tyrosine, were quite predictable, i.e., it reached to plateau within 15 min for i.v. administration and within 30 min for p.o., jejunal, and ileal administration. The plateau levels were then maintained during the study period indicating rapid metabolizing process to tyrosine and circulation of tyrosine in blood until its utilization to other metabolism cycle(s). Mean residence time of YdAGFdL ranged from 2.67 to 4.45 min. The half lives of YdAGFdL in the distribution phase at low and high doses were 0.48 and 0.59 min while those in the elimination phase were 3.98 and 6.81 min, respectively. These values were consistent with the values of 4.08 and 7.97 min from the statistical moment analysis. The CL estimates were slightly higher than those from the statistical analysis. This is probably due to the underestimation of AUC from zero to the first sampling

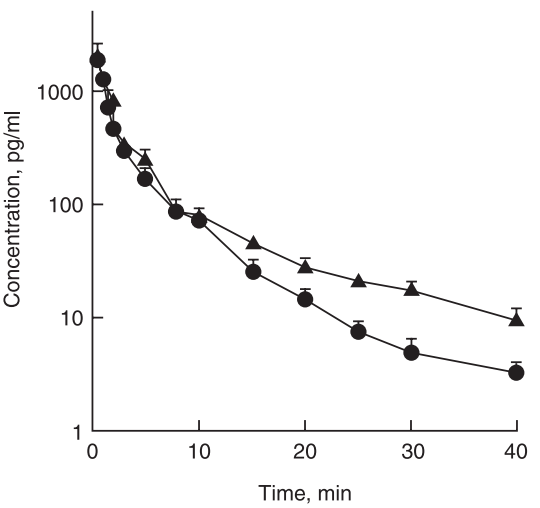

Figure 1. Mean ($n = 6$) ± S.E. blood concentration-time data of YdAGFdL following intravenous dosing of 0.28 (●) and 500 μg (▲) to rats in a cross-over fashion; mean blood concentrations for each sampling point were obtained from two different doses after normalized to the dose of 0.28 μg

Table 1. Pharmacokinetic parameters of YdAGFdL obtained from the two-compartmental analysis of i.v. and p.o. data at two doses (0.28 and 500 μg) in six rats[a]

Parameters	500 μg	0.28 μg
CL (ml/min)	48.0 ± 23.3	42.7 ± 26.2
V_d (ml)	71.9 ± 48.5	76.9 ± 49.0
t_α (min)	0.59 ± 0.25	0.48 ± 0.17
t_β (min)	6.81 ± 3.12	3.98 ± 0.92
α (1/min)	1.38 ± 0.68	1.71 ± 0.79
β (1/min)	0.13 ± 0.07	0.18 ± 0.04

[a] Values are mean ± S.D.

point (0.5 min) in the model-independent statistical moment analysis.

Pharmacokinetics of YdAGFdL following various oral, jejunal and ileal doses in rats

The pharmacokinetics of YdAGFdL were characterized after oral and ileal dosing of 1.1, 1000, and 5000 μg. The mean YdAGFdL blood profiles following various p.o. and ileal dosing in six fistulated rats are shown in Figure 2a and b, respectively. All blood concentrations shown are based on the radiolabeled YdAGFdL. Each sampling point represents mean values from six rats except for the 5000 μg dose for p.o. and ileal studies, while error bars indicate standard error of the mean. In Figure 2a, there was apparently no clear difference in blood concentrations among different doses indicating dose-independent PK in oral administration within the range of dose tested. However, T_{max} after oral dosing appears to vary greatly. This could be due to variation in gastric emptying, enzyme distribution, blood flow rate, gastrointestinal transit time and/or intestinal contents in the GI tract. The blood concentrations from 10 out of 17 p.o. studies were below the detection limit indicating negligible absorption of YdAGFdL over the 50 min experimental period. One rat died due to the cannula mislocation. The rats with the below detection limit were included to calculate mean concentrations and other PK parameters in Tables 2 and 3, which resulted in the mean values of each time point and C_{max} values below the detection limit of 14 pg/ml. No significant dose dependency in the PK is observed from ileal administration (Figure 2b). The mean MAT and T_{max} values in ileal studies seem to be shorter than those from the p.o. studies for the 1.1 and 1000 μg dose levels (Table 2) but the trend was reversed for the 5000 μg dose. A more sensitive and specific assay method may be necessary to clarify this finding. In contrast to the p.o. studies, the blood YdAGFdL was not detectable in only one out of 16 ileal studies. This implies better and more reproducible absorption of YdAGFdL following the ileal administration than that of the oral administration.

PK parameters of YdAGFdL after various doses of po and ileal administration are summarized in Table 2. The F values of YdAGFdL after po administration of 1.12, 1000, and 5000 μg of YdAGFdL were 0.39 ± 0.33 (S.E.), 0.42 ± 0.46, and $0.38 \pm 0.46\%$, respectively, while those after ileal administration were 1.27 ± 0.71, 2.14 ± 0.70, and 1.94 ± 0.86. The F values were not signifi-

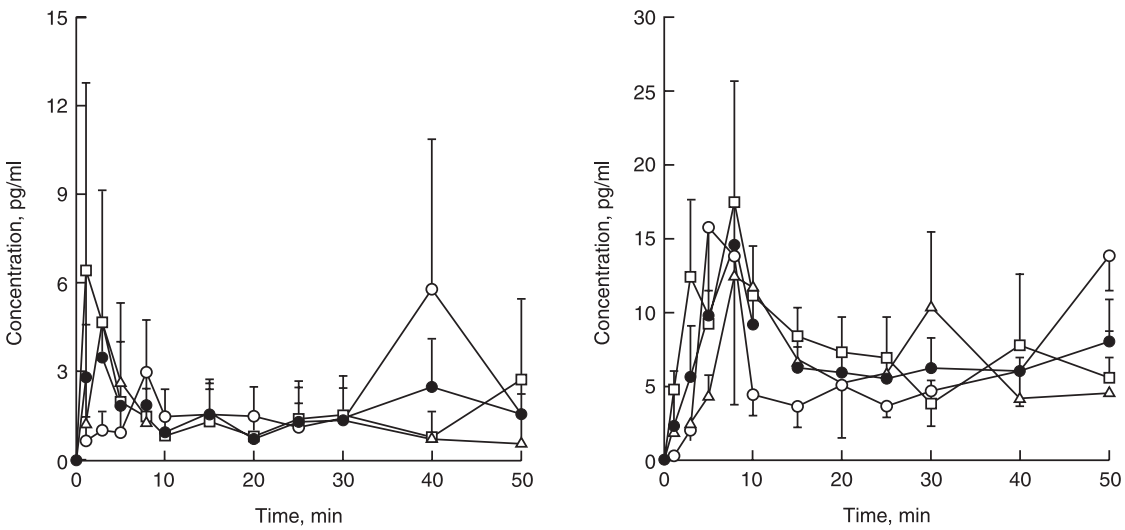

Figure 2. Mean ($n = 6$) ± S.E. blood concentration–time data of YdAGFdL following oral (a) and ileal (b) administration of 1.1 (○), 1000 (□), and 5000 μg (▲), respectively: closed circles (●) are for the pooled mean values of three dose groups after normalized to the 1.1 μg of dosing level

cantly different among doses in either p.o. or ileal administration. On average the F of YdAGFdL was improved about five fold in the ileal study when compared to the p.o. The MAT from p.o. studies varied from 5.2 to 48 min and T_{max} from 3 to 50 min.

Blood YdAGFdL profiles following jejunal administration are shown in Figure 3 with the mean blood profiles after i.v. p.o. and ileal administration. It shows that the average blood concentrations of YdAGFdL over 50 min were in the order: ileal >jejunal >p.o. administration. The comparison of absolute bioavailabilities among p.o., jejunal, and ileal administrations is shown in Figure 4. Since no significant dose-dependent absorption was observed from p.o. and ileal studies, mean bioavailabilities were averaged from the values of the various dosing levels.

Table 2. P.O. and ileal pharmacokinetics at various doses of YdAGFdL

Dose (μg)	1.12	1001.12	5001.12
p.o.			
N	6 (4)[a]	6 (4)[a]	5 (2)[a]
MAT (min)[b]	28.7 ± 14.0	21.3 ± 25.0	9.89 ± 8.3
T_{max} (min)	29.0 ± 10.1	25.8 ± 24.5	9.12 ± 6.32
C_{max} (pg/ml)[c]	5.85 ± 5.12	6.41 ± 6.41	4.73 ± 4.22
F (%)[b]	0.39 ± 0.33	0.42 ± 0.46	0.38 ± 0.46
Ileal			
N	6 (1)[a]	6	4
MAT (min)	20.6 ± 14.2	17.1 ± 7.49	22.3 ± 8.00
T_{max} (min)	20.8 ± 10.3	19.3 ± 10.3	15.3 ± 8.85
C_{max} (pg/ml)	21.1 ± 4.91	28.5 ± 11.1	16.4 ± 3.85
F (%)	1.27 ± 0.71	2.14 ± 0.70	1.94 ± 0.86

[a] The number(s) denotes the number of rats with no detectable absorption of YdAGFdL.
[b] The values for MRTi.v. and AUCi.v./DOSEi.v. were taken from the means of i.v. data.
[c] The concentrations were normalized to the dose of 1.12 μg.

The overall mean F values are 0.38, 1.25 and 1.78% for p.o., jejunal, and ileal administration, respectively (Table 3). The F value increased about 40% following ileal administration compared to the F value of the jejunal administration.

The effect of amastatin on the ileal absorption of YdAGFdL

The comparison of YdAGFdL blood profiles after coadministered with amastatin to those without amastatin in individual experiments is shown in Figure 5. Amastatin (1 mg) was observed to increase the F of YdAGFdL to 8.76 ± 4.47% (Table 3). In separate experiments, simultaneous i.v. administration of YdAGFdL and po administration of the inhibitor were performed in two rats (not shown). No significant changes in the i.v. pharmacokinetic parameters was observed. These results suggest that the inhibitor, when administered orally, does not effect the systemic clearance and is consistent with its effect being on local GI metabolism.

Discussion

The results of our studies on the YdAGFdL pharmacokinetics and the effect of inhibitor and the absorption site show that YdAGFdL is poorly absorbed ($F < 0.4\%$) after p.o. administration and rapidly cleared following absorption. The absolute F of YdAGFdL was improved about five fold when delivered to the ileum and about five fold from the ileum in the presence of amastatin. The strategy for p.o. peptide delivery employing a selective enzyme inhibitor and a specific absorption site was successful. These results demonstrated that a peptide with five amino acids can be absorbed through the gastrointestinal

Table 3. Absolute bioavailabilities of YdAGFdL following various routes of administration and ileal coadministration of amastatin[a]

	p.o.	Jejunal	Ileal	Ileal/inhibitor
N	17	4	16	6
F ± S.E. (%)	0.40 ± 0.24	1.25 ± 0.39	1.78 ± 0.40	8.76 ± 4.47
$F_{increase}$ (%)	100 (control)	213	345	2090

[a] $F_{increase}(\%) = \frac{F_{inhibitor} - F_{control}}{F_{control}} \times 100.$

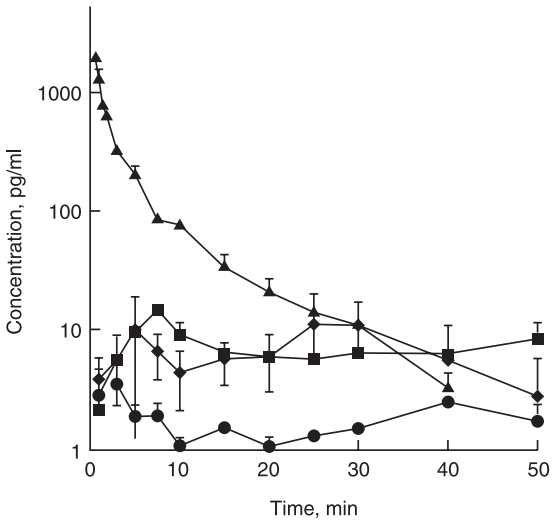

Figure 3. Mean ± S.E. blood concentration-time data of YdAGFdL following intravenous (▲), p.o. (●), jejunal (◆), and ileal administration (■) to rats: Values are mean of $n = 17$ or 16 for oral and ileal, 4 for jejunal, and 12 for intravenous administration; normalized doses are based on 0.28 μg for intravenous and 1.12 μg for oral, jejunal, and ileal administration

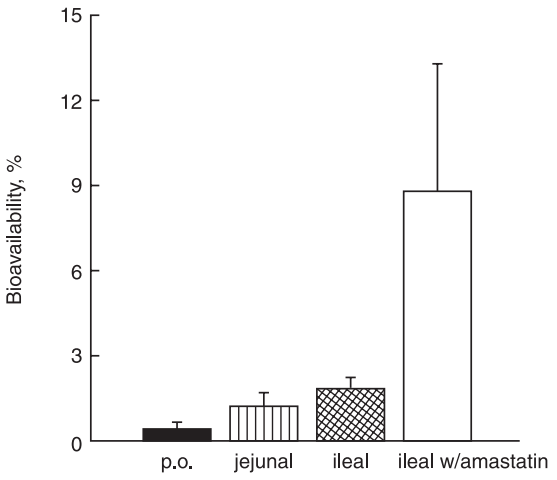

Figure 4. Absolute bioavailabilities of YdAGFdL after oral, jejunal, ileal and ileal with amastatin administration with the standard error of mean bars

membrane in a site-dependent manner. Moreover, the present study demonstrates that the systemic availabilities of peptides can be significantly increased by preventing metabolic degradation of peptides by the brush-border enzymes.

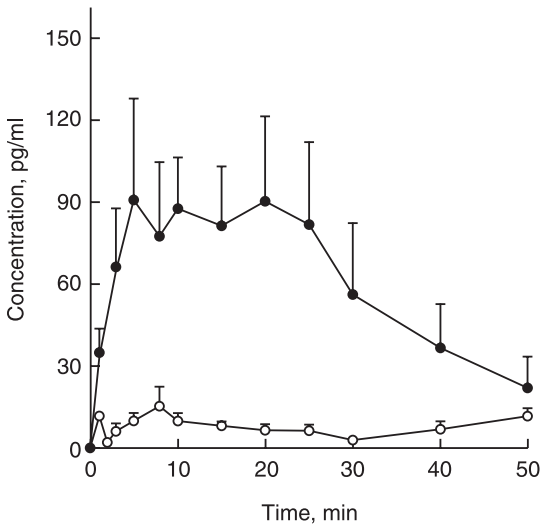

Figure 5. Mean ± S.E. blood concentration–time data of YdAGFdL after ileal administration in the presence of amastatin (●) compared to those in the absence of amastatin (○) in chronically fistulated rats

The significant increase in F after ileal administration of YdAGFdL (F value being 1.78 ± 0.40) compared to the jejunal (1.25 ± 0.39) and especially p.o. administration (0.40 ± 0.24) may be explained as follows. First, there may be less of the participating enzymes in the ileum, especially aminopeptidase (see below). Comparing the isolated loops between the jejunum and the ileum in our animal model, the major difference is the distribution of microvilli. Most of the luminal contents in the isolated loops were washed out with saline solution before the dosing. Garrido et al. [30] have found that hydrolytic activity for all enzymes (including the subcellular fractions of the aminopeptidase activities) and absorption rates from both the free amino acid and peptide solutions were reduced in bypassed jejunal segments. In a previous study [23], it was shown that the permeability of YGGFL and YdAGFL in the single-pass jejunal perfusion method was 1.83. In the recent report, the permeability of YdAGFdL in the jejunum [31] was estimated as 1.5. Since compounds with permeability greater than 1 are generally well absorbed [4], the membrane permeability is not the limiting factor in the p.o. availability of YdAGFdL. Secondly, YdAGFdL may be hydrolyzed by the pH of the gastric juice and/or other

luminal enzymes resulting in zero bioavailability in 10 out of 17 rats and 0.4% in 17 rats following p.o. administration. Tritiated tyrosine appeared in blood within 1 min after p.o. dosing and the concentration of tyrosine was maximum between 3 and 5 min in most cases after p.o. administration, indicating rapid degradation and absorption into the blood stream. In four out of 17, the T_{max} for tyrosine was delayed to 40–50 min. This is probably due to the variation in the gastric emptying time and the gastro-intestinal transit time [32]. These findings suggest that the site-dependent absorption phenomena may not be observed in the presence of luminal contents. A broad spectrum of enzyme inhibitors might be then necessary to improve the systemic availability of peptides. Third, there might be a specific absorption site for YdAGFdL toward the ileum. The major contribution to the poor p.o. bioavailability is probably the longer transit time for YdAGFdL in the gut to reach this absorption site and the longer contact time with luminal enzymes.

The 22 fold increase in bioavailability (compared to p.o.) with the coadministration of amastatin with ileal dosing of YdAGFdL suggests that the knowledge of the potency and or efficacy of participating enzymes for metabolic pathways of peptides may be required for the successful delivery of peptides. Our choice of an inhibitor and its concentration in these studies was based primarily on data arising from metabolism studies of enkephalin analogs in rabbit brush-border membranes [17]. The metabolizing enzymes for the analogs, leu-enkephalin (YGGFL) and [D-ala^2, leu^5]-enkephalin (YdAGFL) in the rabbit intestinal brush-border membranes were found to be aminopeptidase, endopeptidase, and angiotensin converting enzyme [17]. For example, 100 μM of amastatin effectively inhibit metabolism of aminopeptidase mediated cleavage; i.e., Y–G or Y–dA bonds. However, thiorphan apparently showed no inhibition of the hydrolysis of the F–dL bond of YdAGFdL, implying that aminopeptidase plays the more important role in the metabolism of YdAGFdL. For this reason, amastatin was chosen as the selective enzyme inhibitor for our study. Interestingly, in the *in vitro* metabolism study of YdAGFdL in rabbit intestinal brush-border membranes and in rat intestinal perfusate, YdAGFdL was found to remain almost intact for over 60 min of incubation or single-pass perfusion period [17, 23]. This is quite in contrast to the less than 0.4% of bioavailability in our p.o. studies. This indicates factors present in gastrointestinal tract *in vivo* for the degradation of peptides that are absent in the *in vitro* vesicle metabolism. However, the kinetic constant for Y–dA bond cleavage of YdAGFdL during 60 min of incubation period at 25°C was about 0.4 min^{-1}/mg protein but not zero [17]. This rate constant was 50 times slower than Y–dA bond cleavage rate constant in YdAGFL and about 400 times slower than the rate constant of Y–G bond cleavage of YGGFL [17]. These rate constants were reduced about 80 fold for YGGFL and more than 60 fold for YdAGFdL in the presence of amastatin [17]. In view of the above discussion, one could strongly suggest that amastatin was effective to inhibit the metabolism of YdAGFdL in brush-border membrane vesicles by preventing the cleavage of Y–dA bond. Consequently the *in vivo* effect of amastatin on the increase of bioavailability following the ileal administration of YdAGFdL may be mainly due to the inhibitory activity toward aminopeptidase.

In the pharmacokinetic studies of YdAGFdL, there was no evidence for dose–dependent absorption, distribution, or metabolism even though the dose levels were nearly 5000 and 2000 fold different for the p.o. and ileal and the i.v. administration, respectively. This is consistent with other reports in the literature [33] showing that the AUC after 3 and 30 μg/kg intraperitoneal injection of YGGFL to rats was dose proportional. On the other hand, saturable metabolism of YGGFL in the rat nasal cavity has been reported [34]. Unlikely to the gastrointestinal tract, limitation in absorption site of the nasal cavity could be attributed to this finding.

Half lives of YdAGFdL in distribution and elimination phases were very short, about 0.5 and 5 min, respectively. In *in vitro* plasma, YdAGFdL is 16.2% metabolized in 2.5 h, while in *in vivo* plasma 64% is metabolized in 15 min after intraperitoneal administration [33, 35]. This difference between *in vivo* and *in vitro* metabolism

also suggests that further metabolism is mediated by non-circulating enzymes in peripheral tissues. It is of interest to note that the absorption seems to be much slower than the elimination in p.o., jejunal, and ileal blood profiles (Figure 3), resulting in a slower elimination phase than from the i.v. study. Similar results were reported from an intraperitoneal study of YGGFL and YdAGFdL [33] and ocular administration of YdAGFM [36]. The observed 'flip-flop' kinetics are probably due to the rapid elimination kinetics of these compounds relative to the slower transmembrane absorption of YdAGFdL.

In summary, the present study demonstrates that the aminopeptidase inhibitor, amastatin, significantly improved the absorption of the pentapeptide YdAGFdL when coadministered in the ileum and suggests that the aminopeptidase enzymes in brush-border membrane play a significant role in limiting the oral absorption of these peptides. The 20 fold improvement in the absolute bioavailability indicates that 'effective' oral therapy for peptide type drugs can be achieved through a combination of inhibition of luminal and brush border enzymes and site specific delivery. Future studies should focus on the application of this strategy to various peptide drugs.

Acknowledgements

The authors wish to thank John Wlodyga for his excellent technical assistance.

References

1. Johnson O, Cleland J, Lee HJ, Charnis M, Duenas E, Jaworowicz W, Shepard D, Shahzamani A, Jones AJS, Putney SD. A month-long effect from a single injection of microencapsulated human growth hormone. *Nature Med*, 1996; **2**: 795–799.
2. Amidon GL, Lee HJ. Absorption of peptide and peptidomimetic drugs. *Annu Rev Pharmacol Toxicol* 1994; **34**: 321–341.
3. Taki Y, Sakane T, Nadai T, Sezaki H, Amidon GL, Langguth P, Yamashita S. First-pass metabolism of peptide drugs in rat perfused liver. *J Pharm Pharmacol* 1998; **50**: 1013–1018.
4. Amidon GL, Sinko PJ, Fleisher D. Estimating human oral fraction dose absorbed: a correlation using rat intestinal membrane permeability for passive and carrier-mediated compounds. *Pharm Res* 1988; **5**: 651–654.
5. Amidon GL, Sinko PJ, Hu M, Leesman GD. Absorption of difficult drug molecules: carrier-mediated transport of peptides and peptide analogues. In *New Drug Delivery and Its Therapeutic Application*, Prescott LV, Nimmo WS (ed). Wiley: New York, 1989; 46–56.
6. Amidon GL, Leesman GD, Sinko PJ, Hu M. Design of prodrugs for oral drug delivery. In *Xenobiotic Metabolism and Disposition*. Kato R, Estabrook RW, Cayen MN (eds). Proceedings of the second international ISSX Meeting, Kobe, Japan 1989; 117–124.
7. Lee HJ. Biopharmaceutical properties and pharmacokinetics of peptide and protein drugs. In *Peptide-based Drug Design*. Taylor MD, Amidon GL (eds). American Chemical Society: Washington, DC, 1995; 69–97.
8. Veuillez F, Kalia YN, Jacques Y, Deshusses J, Buri P. Factors and strategies for improving buccal absorption of peptides. *Eur J Pharm Biopharm* 2001; **51**: 93–109.
9. Torres-Lugo M, Peppas NA. Transmucosal delivery systems for calcitonin: a review. *Biomaterial*s. 2000; **21**: 1191–1196.
10. Choi HK, Flynn GL, Amidon GL. Percutaneous absorption and dermal delivery of cyclosporin A. *J Pharm Sci* 1995; **84**: 581–583.
11. Robert PY, Leconte V, Olive C, Ratsimbazafy V, Javerliat M, Adenis JP. Cyclosporin A eyedrops: manufacturing, toxicity, pharmacokinetics and indications in 2000. *J Fr Ophtalmol* 2001; **24**: 527–535.
12. Hussain AA. Intranasal drug delivery. *Adv Drug Deliv Rev* 1998; **29**: 39–49.
13. Lee HJ, Amidon GL. Oral peptide delivery: improving the systemic availability of small peptides and enkephalin analogs. *NIDA Res Monogr* 1995; **154**: 86–106.
14. Aungst BJ, Blake JA, Hussain MA. An in vitro evaluation of metabolism and poor membrane permeation impeding intestinal absorption of leucine enkephalin, and methods to increase absorption. *J Pharmacol Exp Ther* 1991; **259**: 139–145.
15. Crane CW, Luntz GR. Absorption of insulin from the human small intestine. *Diabetes* 1968; **17**: 625–627.
16. Bunnet NW. Release and breakdown: postsecretory metabolism of peptides. *Am Rev Respir Dis* 1987; **136**: S27–S34.
17. Tamai I, Lee HJ, Stewart B, Amidon GL. Kinetic analysis of the metabolism of leucine enkephalin and analogues by rabbit intestinal brush-border enzymes: assessment of the potential for effective oral delivery. *Pharm Res* 1991; **8**: S270.
18. Nutt JG, Woodward WR, Anderson JL. The effect of carbidopa on the pharmacokinetics of intravenously administered levodopa: the mechanism of action in the treatment of parkinsonism. *Ann Neurol*. 1985; **18**: 537–543.
19. Kidron M, Bar-On H, Berry EM, Ziv E. The absorption of insulin from various regions of the rat intestine. *Life Sci* 1982; **31**: 2837–2841.

20. Garvey TQ, Hyman PE, Isselbacher KJ. Gamma-glutamyl transpeptidase of rat intestine: localization and possible role in amino acid transport. *Gastroenterology* 1976; **71**: 778–785.
21. Miura S, Song IS, Morita RH, Erickson RH, Kim YS. Distribution and biosynthesis of aminopeptidase N and dipeptidyl aminopeptidase IV in rat small intestine. *Biochim Biophys Acta* 1983;**761**: 66–75.
22. Sterchi EE, Woodley J. Peptide hydrolases of the human intestinal mucosa: distribution of activities between brush-border and cytosol. *Clinica Chemica Acta*. 1980; **102**: 49–56.
23. Friedman DI, Amidon GL. Oral absorption of peptides: influence of pH and inhibitors on the intestinal hydrolysis of leu-enkephalin and analogues. *Pharm Res* 1991; **8**:93–96.
24. Poelma FGJ, Tukker JJ. Evaluation of a chronically isolated intestinal loop in the rat for the study of drug absorption kinetics. *J Pharm Sci* 1987; **76**: 433–436.
25. Lee HJ, Kim JS, Amidon GL, Chandrasekharan R, Weiner ND. Effective delivery of salmon calcitonin employing a surfactant, liposome and a specific absorption site in rats. *Pharm Res* 1993; **10**: 291S.
26. Su SF, Lee HJ, Amidone GL. A systemic bioavailability study of cholescystokinin octapeptide in rats: characterization targeted delivery. *Pharm Res* 1993; **10**: 291S.
27. Ziring B, Sheppard S, Kreek MJ. Reversed phase thin-layer chromatography for the separation of β-lipotropin and enkephalins. *Int J Pep Pro Res* 1983; **22**: 32–38.
28. Gibaldi M, Perrier D. Noncompartmental analysis based on statistical moment theory. In *Pharmacokinetics*, Marcel Dekker Inc: New York and Basel, 1982; 409–417.
29. van Dongen JJ, Remie R, Rensema JW, van Wunnik GHJ. Manual of microsurgery on the laboratory rat. In *Techniques in the Behavioral and Neural Sciences*, vol. 4, Huston JP (ed). Elsevier: Amsterdam, 1990; 287–289.
30. Garrido AB Jr, Freeman HJ, Kim YS. Amino acid and peptide absorption in bypassed jejunum following jejunoileal bypass in rats. *Dig Dis Sci* 1981; **26**: 107–112.
31. Sherman J, Amidon GL. Intestinal/hepatic transport and metabolism issues for enkephalin analogues: model pentapeptide compound with potential for oral delivery. *Pharm Res* 1993; **10**: 291S
32. Yu LX, Lipka E, Crison JR, Amidon GL. Transport approaches to the biopharmaceutical design of oral drug delivery systems: prediction of intestinal absorption. *Adv Drug Deliv Rev.* 1996; **19**:359–376.
33. Schulteis G, Weinberger SB, Martinez JL Jr. Plasma uptake and in vivo metabolism of [leu]enkephalin following its intraperitoneal administration to rats. *Peptides* 1989; **10**: 913–919.
34. Faraj JA, Hussain AA, Aramaki Y, Iseki K, Kagoshima M, Dittert LW. Mechanism of nasal absorption of drugs. IV: plasma levels of radioactivity following intranasal administration of [^{3}H] leucine enkephalin. *J Pharm Sci* 1990; **79**: 768–770.
35. Weinberger SB, Martinez JL Jr. Characterization of hydrolysis of [leu]enkephalin and D-ala2-[L-leu]enkephalin in rat plasma. *J Pharm Exp Ther* 1988; **247**: 129–248.
36. Straford RE Jr, Carson LW, Dodda-Kashi S, Lee VHL. Systemic absorption of ocularly administered enkephalinamide and inulin in the albino rabbit: extent, pathways, and vehicle effects. *J Pharm Sci* 1988; **77**: 838–842.

