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Metabolism of a neurotensin (8–13) analog by intestinal and nasal enzymes, and approaches to stabilize this peptide at these absorption sites

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Abstract

Adamantoyl-Lys-Pro-Tyr-Ile-Leu (Ada-KPYIL) is a neurotensin analog which has analgesic activity when dosed parenterally, but not when dosed orally. In vitro studies were carried out to determine whether metabolism was responsible for oral inactivity. Ada-KPYIL was stable in simulated gastric fluid containing pepsin at acidic pH. Ada-KPYIL was immediately metabolized in simulated intestinal fluid containing pancreatin, a mixture of pancreatic enzymes, and also in dilute solutions containing this enzyme. Chymotrypsin, trypsin, elastase, and carboxypeptidase were each capable of metabolizing Ada-KPYIL, and chymotrypsin was active at the lowest concentration. Chymostatin effectively inhibited Ada-KPYIL metabolism by chymotrypsin. Formulation of Ada-KPYIL in mixed micelles or in an oil-in-water emulsion also inhibited metabolism by chymotrypsin. However, there was no inhibition of metabolism in a solution containing only the bile salt, suggesting that partitioning of Ada-KPYIL into the oil phase is the protective mechanism. Ada-KPYIL was stable when perfused through a rat nasal cavity. These oral formulation approaches or nasal administration may be useful for improving the delivery of Ada-KPYIL or other peptides with similar delivery problems.

Keywords: Neurotensin; Peptide; Metabolism; Peptidase; Absorption; Bioavailability

1. Introduction

Neurotensin (NT) is a 13-amino-acid peptide endogenous to the central nervous system and digestive tract. When injected, NT produces an array of neuromodulatory and gastrointestinal effects, one of which is analgesia. The C-terminal hexapeptide portion of NT (NT_{8-13} or Arg-Arg-Pro-Tyr-Ile-Leu) has all the structural features required for receptor binding and pharmacologic activity (Kitabgi et al., 1980). Substitution at the N-terminus of this neurotensin fragment with bulky, lipophilic groups led to NT_{8-13} analogs with improved binding to the NT receptor, and with potent and long acting analgesic effects when injected i.v. (Cain et al., 1993; Christos et al., 1993). One of these analogs was N^{α}-(1-

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adamantoyl)-Lys-Pro-Tyr-Ile-Leu (Ada-KPYIL). This compound had potent analgesic and neuroleptic activity in mice and rats when injected i.v., but was inactive when administered orally (Christos et al., 1993). Thus, this peptide analog represents a lead in a new series of analgesic compounds, but oral inactivity is a property requiring improvement.

Our goals were to develop an understanding of the causes of poor oral bioavailability of Ada-KPYIL and to investigate possible ways to improve bioavailability. In the first of these studies the stability of Ada-KPYIL in simulated gastric and intestinal fluids was evaluated. Since Ada-KPYIL was unstable in simulated intestinal fluid, various pancreatic enzymes were then individually tested for their capability to metabolize Ada-KPYIL. Then several approaches for inhibiting metabolism by the intestinal luminal enzymes were examined. Strategies that have been successful in reducing the presystemic metabolism of other peptides include analogs and prodrugs, use of enzyme inhibitors, and formulations such as liposomes, that shield the peptide from enzymatic attack. One approach we tested was to use specific enzyme inhibitors to reduce metabolism. Another approach was to incorporate Ada-KPYIL into mixed micelle and emulsion vehicles. Entrapment of this peptide analog into the oil phase of these vehicles seemed likely, due to the hydrophobicity that the adamantoyl substituent adds.

For peptide drugs that are not orally bioavailable, another approach to attain bioavailability is to administer them by other routes, such as via the nasal mucosa. These alternative routes may offer the advantages of less extensive presystemic metabolism and increased permeability of the membrane, relative to the oral route (Lee and Yamamoto, 1990). We therefore also tested whether Ada-KPYIL was subject to metabolism at the nasal mucosal absorption site.

Metabolism at the absorption site or prior to reaching the absorption site represents only some of the possible barriers to attaining bioavailability of Ada-KPYIL or other peptides. Membrane permeability could represent another significant barrier which would have to be addressed separately.

2. Methods

2.1. Materials

Ada-KPYIL was prepared at the DuPont Merck Research Laboratories as previously described (Christos et al., 1993). It was isolated as an acetate salt. The following enzymes were obtained from Sigma Chemical Co.: pancreatin, from porcine pancreas, activity 4 times the USP limits for amylase, lipase, and protease activities; pepsin, from porcine stomach mucosa, 2200 U/mg protein; trypsin, from bovine pancreas, 12 200 BAEE U/mg protein, L-1-tosylamide-2-phenylethyl chloromethyl ketone treated; α-chymotrypsin, from bovine pancreas, 54 U/mg protein, N^{α}-p-tosyl-L-lysine chloromethyl ketone treated; pancreatic elastase, from bovine pancreas, 89 U/mg protein; carboxypeptidase A, from bovine pancreas, 54 U/mg protein, diisopropyl fluorophosphate treated; and α-chymotrypsin, insoluble enzyme attached to beaded agarose, from bovine pancreas, 560 U/g solid. Chymostatin (from microbial source) was also obtained from Sigma Chemical Co.

Simulated gastric fluid and simulated intestinal fluid were prepared according to USP specifications. Simulated gastric fluid contained 2 g/l sodium chloride and 3.2 g/l pepsin in 0.7% (v/v) HCl, at pH 1–2. Simulated intestinal fluid contained 10 g/l pancreatin in a pH 7.5 phosphate buffer. Studies were also performed using solutions with lower pancreatin concentrations.

2.2. In vitro stability studies

Stability studies were performed in simulated gastric fluid, simulated intestinal fluid, and solutions of various enzymes in 0.1 M phosphate buffer, pH 7.4. All studies were carried out using 100 μM Ada-KPYIL as the substrate concentration. Enzyme kinetic constants were not determined because the intent of these studies was to make a preliminary assessment of which enzymes metabolize Ada-KPYIL, and because we had only a small amount of Ada-KPYIL available. For these reasons also, some experiments were not

duplicated, and the reported data represent single determinations.

A stock solution of 10 mM Ada-KPYIL in dimethyl sulfoxide was prepared and metabolism was initiated upon addition of 0.1 ml of this solution to 10 ml of the enzyme solution in a 37°C shaking water bath. For studies using Ada-KPYIL in mixed micelle or emulsion vehicles, the reaction was initiated upon addition of the enzyme. After various incubation times, 0.5 ml samples were removed and quenched with 0.5 ml acetonitrile. Samples were typically taken at 0, 1, 2, 5, 10, 20, and 30 min. The sample times may have been modified in repeat experiments depending on whether metabolism was faster or slower than this time frame. Ada-KPYIL concentrations in the samples were determined using HPLC. A Zorbax C8 column and a mobile phase consisting of 0.1% trifluoroacetate/acetonitrile/tetrahydrofuran (53/37/10) were used. The retention time of Ada-KPYIL was 6.5–7 min at a 1.1 ml/min flow rate. Detection was by UV absorbance at 226 nm. No attempt was made to identify or quantitate metabolites formed.

Ada-KPYIL percentage remaining vs time plots were made. Enzyme concentrations were varied in search of a concentration between the extremes of immediate Ada-KPYIL disappearance and complete stability. Under those conditions at which Ada-KPYIL disappearance was gradual over 30 min, the disappearance profiles fit first-order kinetics, and half-lives were determined.

2.3. Stability to nasal mucosal enzymes

The potential metabolism of Ada-KPYIL by nasal mucosal enzymes was examined using a recirculating nasal perfusion technique (Hirai et al., 1981). Male Lewis rats weighing approx. 300 g were anesthetized with pentobarbital. The trachea was cannulated for breathing, and another cannula was inserted via the esophagus caudally to abut the posterior portion of the nasal cavity. Rats remained anesthetized throughout the study, lying on a heated pad. A perfusion solution containing 100 μM Ada-KPYIL in 0.1 M phosphate buffer at pH 7.4 was circulated from a reservoir, through the esophageal cannula into the nasal cavity, and out the nares. This was collected and recirculated. The reservoir volume was 10 ml, and it was kept at 37°C. The flow rate was 1.5 ml/min. Samples (0.1 ml) were removed from the reservoir and quenched and assayed as in the other experiments.

3. Results

The first barriers to absorption that an orally administered peptide must overcome are the peptidases and proteases within the lumen of the gastrointestinal tract. Ada-KPYIL stability in simulated gastric and intestinal fluids was studied to determine the significance of these barriers. Ada-KPYIL concentrations did not change over a 24 h incubation in simulated gastric fluid, containing pepsin (1.65×106 U/l) at pH 1–2. However, in simulated intestinal fluid containing 10 g/l pancreatin, a crude mixture of pancreatic enzymes, Ada-KPYIL was not measurable in the zero time sample. Ada-KPYIL was also almost immediately metabolized in a 100-fold dilution of simulated intestinal fluid. A study was performed in which the lumen of the jejunum from a fasted rat was rinsed with 10 ml saline; this medium also immediately metabolized Ada-KPYIL. These results indicate that pancreatic peptidases represent a formidable barrier to Ada-KPYIL oral absorption.

The next series of studies was to see which pancreatic enzymes were responsible for Ada-KPYIL degradation. Stability studies were performed with 100 μM Ada-KPYIL and varying concentrations of individual pancreatic enzymes. The results are summarized in Table 1. At 100 μg/ml concentrations, trypsin, elastase, and carboxypeptidase A each metabolized Ada-KPYIL with half-lives of 5–13 min. Ada-KPYIL was stable in the presence of 10 μg/ml and lower concentrations of elastase and carboxypeptidase A. Chymotrypsin was effective in metabolizing Ada-KPYIL at lower concentrations. Ada-KPYIL metabolism was immediate with 10 and 100 μg/ml chymotrypsin, and metabolism half-lives could be measured only at concentrations ≤ 2

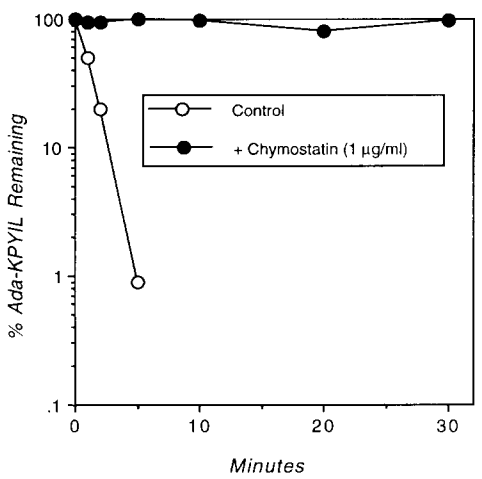

Fig. 1. Degradation of Ada-KPYIL (0.1 mM) in the presence of 2 μg/ml chymotrypsin, and the effects of 1 μg/ml chymostatin.

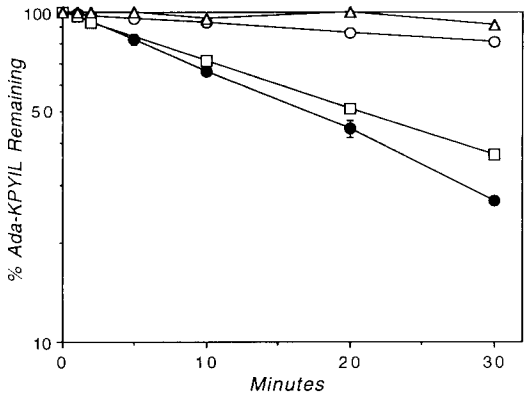

Fig. 2. Degradation of Ada-KPYIL (0.1 mM) in the presence of 0.1 U/ml immobilized chymotrypsin in control ((●), mean ± S.D., $N = 5$)), mixed micelle (○), sodium glycocholate (□), and emulsion (△) media in vitro.

μg/ml. There was considerable variability in duplicate measures of these half-lives, which could be related to differences in the chymotrypsin preparations and the extreme sensitivity of Ada-KPYIL to metabolism by chymotrypsin.

Metabolism of Ada-KPYIL by chymotrypsin was inhibited by chymostatin, an inhibitor isolated from a microbial source. While Ada-KPYIL was metabolized with a half life < 5 min with 2 μg/ml chymotrypsin alone, the addition of chymostatin at 0.1 or 1 μg/ml completely inhibited metabolism. The effects of 1 μg/ml chymostatin are illustrated in Fig. 1. At 0.01 μg/ml, chymostatin also inhibited the immediate metabolism of Ada-KPYIL when the chymotrypsin concentration was 2 μg/ml, but had no effect at 100 μg/ml chymotrypsin (results not shown).

Another approach to inhibit metabolism that was tested was to entrap Ada-KPYIL in the internal phase of mixed micelle or emulsion vehicles. Immobilized chymotrypsin was used for these studies, to ensure that the enzyme and substrate would not be coentrapped within the internal lipid phase. A mixed micelle composition of 40 mM oleic acid and 40 mM sodium glycocholate, with pH 7.4 buffer as the aqueous phase, was used. In the control medium (immobilized enzyme, buffer and substrate only), Ada-KPYIL was metabolized with a half-life of 15.9 ± 0.5 min (mean ± S.D., $N = 5$). However, when Ada-KPYIL was in the mixed micelle composition its metabolism was inhibited, and the estimated half-life was 103.6 min ($N = 2$). This comparison is shown in Fig. 2. Sodium glycocholate (40 mM) alone had no effect on Ada-KPYIL degradation.

Table 1
Degradation (half-lives in min, if measurable) of Ada-KPYIL when exposed to various concentrations of proteolytic enzymes in vitro, using initial Ada-KPYIL concentrations of 100 μM

Enzyme	Concentration (μg/ml)				
	100	10	5	2	1
Pepsin	Stable				
Trypsin	8.8				
Chymotrypsin	immediate	immediate		0.7, 3.5	4.4, 20.6
Elastase	7.9, 13.1	stable	stable		stable
Carboxypeptidase	8.5, 5.5	stable	stable		stable

Individual half-life values or results are reported.

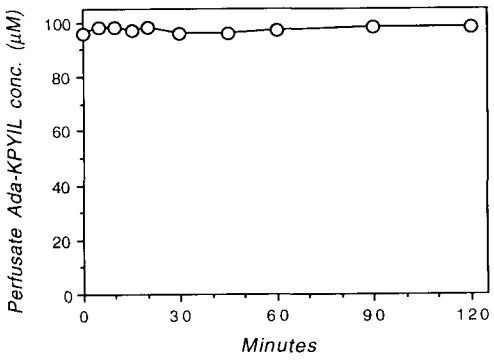

Fig. 3. Stability of Ada-KPYIL when perfused through the nasal cavity of rats ($N = 3$).

Similarly, when Ada-KPYIL was incorporated into an oil-in-water emulsion composition (8% oleic acid, 0.8% polysorbate 20 in buffer), its metabolism by immobilized chymotrypsin was also effectively prevented (Fig. 2).

A third approach to reducing Ada-KPYIL degradation at the absorption site is to utilize a non-oral absorption site where these proteolytic enzymes are less active or absent. This approach was examined by evaluating Ada-KPYIL stability in the rat nasal cavity, using a nasal perfusion technique. Other peptides, such as leucine enkephalin (Hussain et al., 1989) and thymopentin (Hussain et al., 1990), have been shown to be rapidly metabolized when examined using this technique. However, Ada-KPYIL was completely stable (Fig. 3). This suggests that mucosal metabolism may not be a barrier to the nasal absorption of this peptide.

4. Discussion

Ada-KPYIL is a peptide analog with potential therapeutic benefits, which has been shown to be active by the intravenous but not the oral route (Cain et al., 1993; Christos et al., 1993). In this report, we have shown that Ada-KPYIL is rapidly metabolized in vitro by the pancreatic proteases normally present within the gastrointestinal tract, and is especially susceptible to chymotrypsin. Thus, metabolism is the first barrier to oral absorption that must be overcome. It was further shown that the use of an enzyme inhibitor, chymostatin, or the incorporation of Ada-KPYIL in a mixed micelle or emulsion vehicle greatly improved its stability in the presence of chymotrypsin. Since Ada-KPYIL has the lipophilic adamantoyl substituent on the N-terminus, it is presumed to partition into the internal lipid phases of these vehicles, and that this affords protection from metabolism. Sodium glycocholate alone had no stabilizing effect, which supports the conclusion that Ada-KPYIL must partition into the lipid phase for stabilization.

Similar approaches have been explored for other peptides or proteins susceptible to metabolism by pancreatic proteases. Coadministration of insulin with a chymotrypsin inhibitor reportedly increased its oral absorption in rats and dogs (Fujii et al., 1985). Various encapsulation methods have also been utilized in attempts to stabilize insulin to proteolytic degradation, but these efforts have not always been successful. For example, insulin incorporated into polymeric microspheres was protected from metabolism by trypsin and chymotrypsin, but only when enzyme inhibitors were also included (Morishita et al., 1992). In the presence of 40 mM sodium glycocholate, insulin metabolism by chymotrypsin was accelerated, and this appeared to be due to dissociation of insulin oligomers to monomers (Li et al., 1992). The incorporation of insulin in linoleic acid: sodium glycocholate mixed micelles further increased its rate of metabolism by chymotrypsin (Li et al., 1993). Entrapment of 9-desglycinamide 8-arginine vasopressin in niosomes, nonionic surfactant vesicles, delayed its metabolism when exposed to rat intestinal mucosa in vitro (Yoshida et al., 1992).

Another possible approach for delivering this peptide analog would be to use the nasal route. Ada-KPYIL was not metabolized when perfused through the rat nasal cavity. This nasal perfusion method has been used in previous studies which showed rapid metabolism of other peptides by aminopeptidases (Hussain et al., 1989,1990). The major peptide metabolizing enzymes of the nasal cavity are aminopeptidases (Kashi and Lee, 1986). With the adamantoyl N-terminus, Ada-KPYIL

was not a good substrate for nasal aminopeptidases.

The next steps in testing Ada-KPYIL would involve evaluation of intestinal brush border and cellular metabolism and intestinal permeation. It has been shown that neurotensin and acetylneurotensin$_{8-13}$ are metabolized by angiotensin converting enzyme and endopeptidase 24.11 on the rat intestinal brush border (Bai, 1993). Intestinal membrane permeation might also present a significant barrier to the absorption of Ada-KPYIL. The studies reported here therefore only identified the initial barrier to absorption, and tested various approaches to solve this problem.

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