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Oral administration of peptides: study of a glycerolipidic prodrug

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Abstract

In order to explore the possibility of administering a peptide by the oral route, a diglyceride prodrug of a pentapeptide has been evaluated in vitro and in vivo. Different formulations of the prodrug have been studied: an aqueous suspension, different o/w emulsions, an oily solution and different aqueous solutions in which the prodrug was solubilized with the aid of taurocholate in the presence of palmitic acid or calcium chloride. The stability of the prodrug in a lipase medium was correlated with its solubility in the aqueous phase. The lymphatic kinetics after oral administration of the peptide prodrug have been determined after mesenteric lymphatic cannulation in the rat. The lymphatic uptake of the prodrug has been found to be somewhat erratic and finally, the prodrug does not seem to cross intact the enteral barrier. Different hypotheses are proposed to explain these results.

Keywords: Peptide; Oral delivery; Prodrug; Lymphatic system; Lymphatic absorption; Pancreatic lipase

1. Introduction

Among the approaches attempted to enhance the oral bioavailability of peptide drugs (Lee, 1986; Verhoef et al., 1990; Bundgaard, 1992), an original strategy consists in employing diglyceride prodrugs in which a peptide is covalently bound to the 2-position of a 1,3-diglyceride. Such a 'triglyceride-like' molecule is expected to follow the intestinal metabolism pathway of natural triglycerides protecting the peptide from gastrointestinal proteases hydrolysis and promoting its enteral and lymphatic absorption. Lymphatic resorption is a determinant factor because it involves the peptide avoiding the portal circulation and, in addition, the hepatic first-pass effect.

In a recent paper, the synthesis of such a prodrug was designed with a pentapeptide renin inhibitor coupled with 1,3-dipalmitoylglycerol as diglyceride moiety (Delie et al., 1994). This prodrug was able to protect efficiently the peptide from gastric and intestinal proteases hydrolysis and particularly from α-chymotrypsin degrada-

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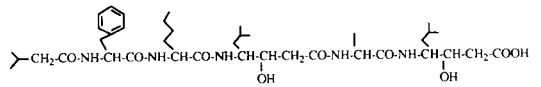
tion. In vitro, studies revealed that the prodrug could be cleaved by pancreatic lipase only when it was solubilized with the aid of dimethyl sulfoxide (DMSO). In the intestinal process of natural triglycerides, pancreatic lipase hydrolysis is required before absorption by enterocytes takes place (Tso, 1985). Therefore, the hydrolysis of the triglyceride-like prodrug in the intestinal lumen is clearly a determinant step to initiate the absorption by enteral cells. In this paper, the sensitivity of the prodrug to hydrolysis by pancreatic lipase has been assessed using different formulations. Thereafter, the ability of the prodrug to cross over the intestinal wall has been evaluated by measuring lymphatic concentrations after oral administration to the rat.

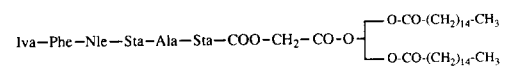
2. Materials and methods

2.1. Materials

The prodrug derived from a Sanofi Laboratories pentapeptide renin inhibitor, SR42128 (Guégan et al., 1986). 1,3-Dihexadecanoyl-2-[isovaleryl-L-phenylalanyl-L-norleucyl-L-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-L-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoyloxyacetyl]-glycerol, denoted as 1,3-DP-SR42128, was synthesized as described elsewhere (Delie et al., 1994). The chemical structures of both peptide and prodrug are represented in Fig. 1. Sodium taurocholate, palmitic acid, linoleic acid and calcium chloride were supplied by Sigma (France) and Methocel A15 LV Prem EP by Dow (France). Solvents (chloroform, DMSO, HPLC grade) were purchased from Prolabo (France).

I 

SR42128: Iva-Phe-Nle-Sta-Ala-Sta

II 

1,3-DP-SR42128

Fig. 1. Chemical structures of the peptide, SR42128 (I) and its prodrug, 1,3-DP-SR42128 (II).

2.2. Sample preparation

Different formulations were used as follows:

(a) The prodrug was dissolved in DMSO at a concentration of 0.2 mmol/ml.

(b) Solubilization of the prodrug in different aqueous media was achieved as follows: the prodrug (2.7 mg) was solubilized in 1 ml of chloroform, then 10 ml of a taurocholate solution (40 mM) or of a taurocholate (40 mM) and calcium chloride (2 mM) solution were added under stirring before solvent was evaporated at 50°C. The same procedure was used to obtain formulations with palmitic acid. In this case, palmitic acid was added to the chloroform solution at a concentration of 1.025 mg/ml.

(c) A chloroform solution (1 ml) of the prodrug (2.70 mg) was suspended in 10 ml of an aqueous solution of methylcellulose (0.5% w/v). The mixture was stirred and the organic solvent was evaporated at 50°C to yield the aqueous supension.

(d) The oily solution was obtained by dissolution of the prodrug (33 mg) in 1.5 ml of linoleic acid.

(e) Different emulsions were obtained by stirring the oily solution of the prodrug (33 mg/0.5 ml linoleic acid) with 1 ml of an aqueous phase made of water or of an aqueous taurocholate solution (40 mM).

2.3. Analytical methods

High-performance liquid chromatography was performed on a Waters apparatus (Waters-Millipore, France) equipped with a 501 pump, a WISP 712 injector, a Lambda Max 481 spectrophotometer and a 745 recorder. The chromatographic conditions were as follows: 150 × 3.9 mm μ-Bondapack column (Waters-Millipore, France) eluted by a mixture of triethylammonium phosphate (pH 3.5) (TEAP) and acetonitrile (55:45) for the peptide or by TEAP and methanol (2:98)

for the prodrug. The flow rate was 0.8 ml/min, the column was warmed to 40 ± 0.5°C and the detector was set at 215 nm.

2.4. Stability in lipase medium

0.6 mg (78 U) of pig pancreatic lipase (130 U/mg, Merck, France) was dissolved in 1 ml of buffer (50 mM Tris, 0.17 mM NaCl, pH 7.8). 50 μl of the prodrug sample were incubated with 100 μl of the pancreatic lipase solution at 37 ± 0.2°C. Samples were withdrawn at different time intervals from 15 to 240 min. The reaction was stopped by freezing (−20°C), samples were freeze-dried and then redissolved in 100 μl of CH_3Cl before HPLC analysis. The concentrations of the remaining compounds were determined by HPLC as described above.

2.5. In vivo studies

2.5.1. Animals

Male Sprague-Dawley rats (280–320 g) were purchased from IFFA-CREDO (Arbresles, France) and maintained on commercial chow (UAR; Villemoisson, France) and water until experiments.

2.5.2. Surgical procedures

Rats, fasted or not (see below), were anesthetized by intraperitoneal injection (0.2 ml/100 g body weight) of a mixture (20/80) of acepromazin 100 mg/ml (Vetranquil®, Sanofi, France) and ketamine 50 mg/ml (Ketalar®, Substantia, France).

The mesenteric lymph duct cannulation was adapted from the method described by Warshaw (1972). Briefly, the collateral lymph duct was ligatured and a PE 10 polyethylene tube (Intramedic PE 10, RUA, France) was introduced into the major lymphatic duct. The cannula was secured by a drop of biological adhesive (Histo-acryl®, Bruneau; France) and externalized through the skin, near the right kidney. The abdomen was finally sutured with cotton. Before awakening, animals were placed into a restraining jacket and the lymph was collected into polyethylene tubes.

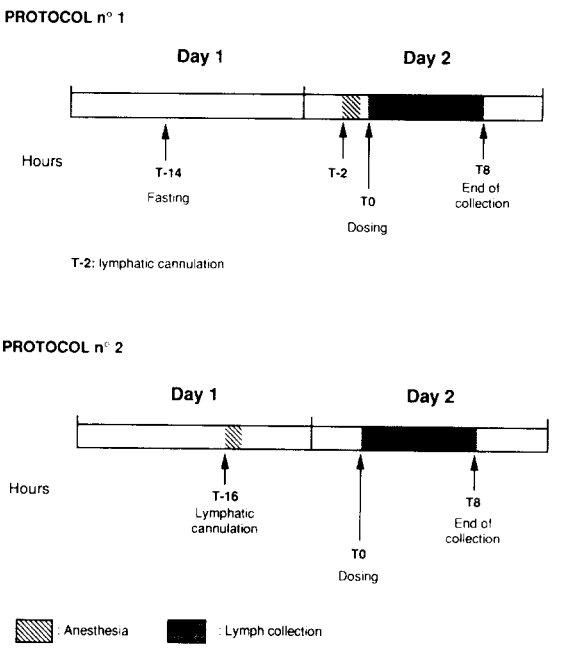


Fig. 2. Schedule for animal handling and prodrug administration.

After experiments, rats were killed by chloroform inhalation, and autopsied to verify the position of the catheter.

2.5.3. Sample analysis

After collection, lymphatic samples were quickly frozen (−20°C) and freeze-dried. For the determination of the prodrug concentration, the residue was resuspended in 100 μl of chloroform before HPLC analysis. For the determination of peptide concentration, samples were resuspended in 50 μl of distilled water before analysis. In order to limit interference due to different lymphatic compounds, the conditions of HPLC assay were slightly modified in adjusting the wavelength to 225 nm.

2.5.4. Experimental procedures

Two different protocols were followed as depicted in Fig. 2. In the first one, the surgical procedure occurred in fasted animals, and the prodrug was administered intragastrically just af-

ter they had woken. Lymph was collected every hour from 1 to 8 h after dosing. In the second protocol, the surgical procedure took place on non-fasted animals and prodrug was administered intragastrically the day after. Lymph was collected under the same conditions as in first protocol.

Experiments were performed in 21 animals, divided into six groups, each being dosed with different formulations of the prodrug. The distribution is listed in Table 1.

2.6. Statistical analysis

Results were compared by analysis of variance (ANOVA) followed by Fisher's test.

3. Results

3.1. Incubation with lipase

The degradation curves of the prodrug incubated under different conditions with lipase are presented in Fig. 3. Absolutely no degradation occurred for the case where the prodrug was suspended in water. When solubilized with taurocholate in the absence or presence of palmitic acid, the prodrug underwent slow hydrolysis. The presence of calcium chloride slowed down the rate of hydrolysis of the prodrug.

A linear correlation was found between the sensitivity of the prodrug toward lipase and its solubility in the incubation medium (Fig. 4).

Table 1
Administration of the prodrug

Groups	Formulation	Dose (μmol/kg)	*n*	Protocol
A	aqueous suspension	73	4	1
B	linoleic acid	73	3	1
C	emulsion: linoleic acid/water	73	5	1
D	emulsion: linoleic acid/taurocholate	73	3	2
E	taurocholate (40 mM)	47	3	2
F	taurocholate (40 mM) + palmitic acid (20 mM)	29	3	2

n, number of animals per group.

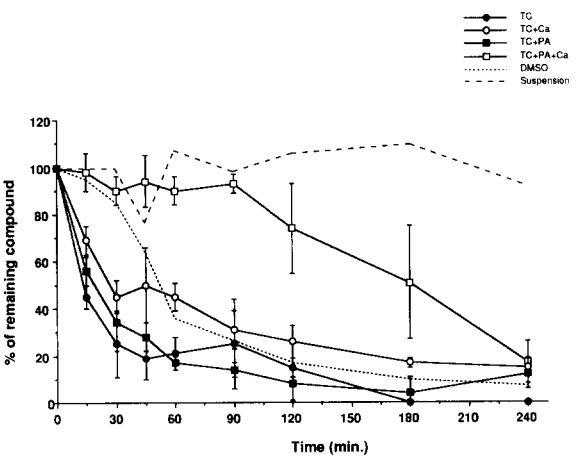


Fig. 3. Prodrug stability when solubilized or suspended in the presence of pancreatic lipase (TC, taurocholate; TC+Ca, taurocholate and calcium chloride; TC+PA, taurocholate and palmitic acid; TC+PA+Ca, taurocholate, palmitic acid and calcium chloride).

3.2. Lymphatic kinetics

The lymph flows summarized in Table 2 showed very important inter-individual and inter-group variations. Comparison of the results obtained with the two protocols revealed a significant difference between each method (Table 3). Thus, when the animals were operated the day before administration, higher lymph flow was observed.

HPLC chromatograms of lymph samples showed a peak corresponding to the prodrug. Sometimes, another unexpected peak was observed with a longer retention time. Although the exact nature of this compound remained uniden-

Table 2
Lymphatic flow in rats after cannulation of the lymphatic mesenteric duct

Group (n)	Lymphatic flow (μl/h) (mean ± SE)
A (4)	144.7 ± 24.60
B (3)	103.1 ± 25.46
C (5)	91.1 ± 18.87
D (3)	93.2 ± 52.50
E (3)	488.9 ± 307.67
F (3)	299.2 ± 266.51

n, number of animals per group.

Table 3
Comparison of lymphatic flows obtained with protocols 1 and 2

Protocol [a] (n) [b]	Lymphatic flow (μl/h) (mean ± SE)
1 (12)	112 ± 32.46
2 (9)	293.8 ± 267.37 [c]

[a] Refer to Fig. 2.
[b] Number of animals per group.
[c] Significant at $p < 0.05$ confidence level.

tified, its HPLC retention time suggested that the product was different from the peptide and from the adjuvants administered together with the prodrug to the animals. Nevertheless, the progressive appearance of this product in the lymph led us to suppose that it might be related to feeding with the prodrug and could be ascribed to a metabolite, probably resulting from either the hydrolysis or the transesterification of the triglyceride-like molecule. However, the presence of these two compounds (prodrug or its metabolite) was not observed with all the animals tested. In fact, in the groups dosed with the prodrug solubilized with the aid of taurocholate (group E), taurocholate and palmitic acid (group F), linoleic acid (group B) or emulsified with taurocholate and linoleic acid (group D), no traces of any compound could be detected in lymph samples.

Table 4
Summary of the lymphatic kinetic data

Group	A	B	C	D	E	F
n	4	3	5	3	3	3
Dose (μmol/kg)	73	73	73	73	47	29
Q (nmol)	226.5 (1) [a]	0	5.66 (1) [a]	0	0	0
Presence of a metabolite	–	–	+(2) [a]	–	–	–

n, animals per group. Q, total quantity of the prodrug recovered in lymph.
[a] Number of animals where the appearance of a metabolite was observed.

Erratic absorption of the prodrug was noted when animals were fed with the aqueous suspension of prodrug (group A). Similarly, in group C (o/w emulsion), the presence of the prodrug was demonstrated in only one animal. In this group, the presence of the metabolite was observed with two animals. The results of lymphatic uptake are summarized in Table 4.

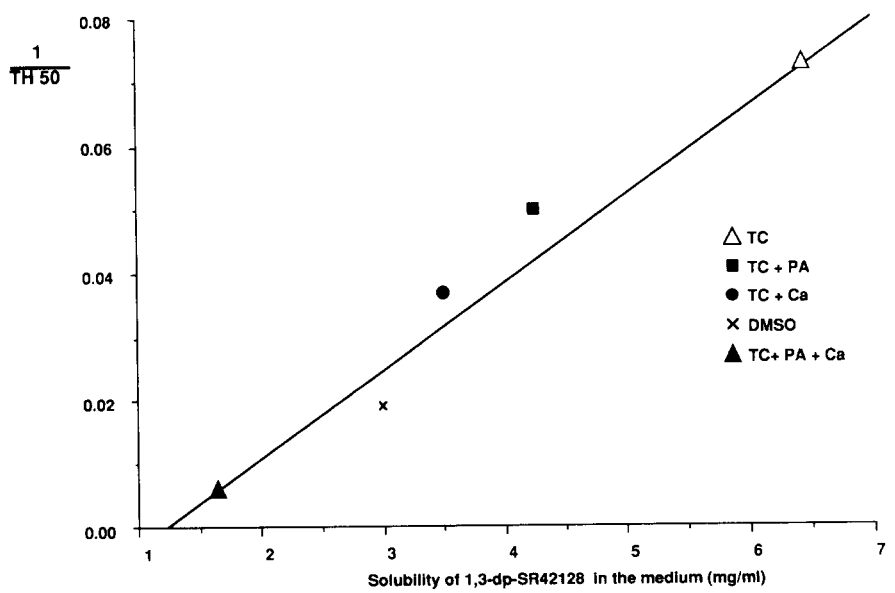


Fig. 4. Prodrug sensitivity to the hydrolysis by pancreatic lipase as a function of its solubility in the incubation medium (TC, taurocholate; TC + Ca, taurocholate and calcium chloride; TC + PA, taurocholate and palmitic acid; TC + PA + Ca, taurocholate, palmitic acid and calcium chloride). TH_{50} represents the time needed to degrade 50% of the prodrug. Correlation coefficient: $r = 0.960$.

In any of the analysed samples, the peptide was identified.

4. Discussion

4.1. Incubation with lipase

The intestinal absorption of natural triglycerides involves the hydrolysis of the ester bound in positions 1 and 3 of the molecule leading to the formation of the corresponding monoglyceride and fatty acids. Subsequently, these compounds are absorbed by enterocytes through a passive diffusion process (Tso, 1985). Once in the cell, a new triglyceride is re-synthesized in order to be incorporated in chylomicrons which are specifically released in lymphatic vessels (Rao and Johnston, 1966). Thus, the hydrolysis by the lipase of the diglyceride prodrug is the key event for the formation of a 'pseudo-monoglyceride' (i.e., the 1,3-dihydro-2-acetyl-SR42128) which could be absorbed by the enterocytes. Therefore, the sensitivity to the hydrolysis in the presence of pancreatic lipase may be considered as a good criterion for the in vitro evaluation of different produg formulations designed for oral administration.

DMSO was first used to solubilize SR42128 prodrug. However, this solvent is potentially toxic due to membrane alterations (Brown et al., 1963) and was therefore avoided for in vivo experiments. Thus, solubilization agents, like sodium taurocholate and palmitic acid, were chosen among substances known to enhance enteral absorption or to increase lymphatic resorption (Cheema et al., 1987; Swenson and Curatolo, 1992). The effect of the presence of calcium salts was also assessed, since this cation is known to increase the resorption of triglycerides (Saunders and Sillery, 1979).

The results showed that the prodrug was always hydrolyzed by pancreatic lipase irrespective of the formulation used, however, it was observed that the hydrolysis kinetics varied from one formulation to another (Fig. 3). A relationship between the solubility of the compound and its sensitivity to enzymatic hydrolysis was found (Fig. 4). The more soluble the prodrug, the greater was the rate of hydrolysis. Free calcium salt preparations seemed to be more sensitive to hydrolysis by pancreatic lipase. Consequently, solubilization of the prodrug with the aid of taurocholate or taurocholate and palmitic acid was retained for in vivo experiments.

4.2. Lymphatic kinetics

The aim of the lymphatic kinetic study was to demonstrate the uptake of prodrug by lymphatic circulation. Increasing the lymphatic flow was of great interest since it allowed the use of a more comfortable analytical range. The increase in lymphatic flow as observed on rats treated according to protocol 2 was the result of withdrawal from anesthesia and free access to water, facilitating the rehydration of the animals. These two factors are well known to influence considerably lymph flow (Charman et al., 1986; Takeshita et al., 1988; McHale and Thornbury, 1989).

The choice of the formulations to be tested was made in the light of the sensitivity to the pancreatic lipase as explained before. Due to the highly lipophilic nature of the prodrug implying a low aqueous solubility, it was not even possible to administer more than 47 mmol/kg for the prodrug aqueous solutions. Consequently, formulations allowing higher doses were considered, such as oily solution, emulsions or an aqueous suspension which is currently used for diglyceride prodrugs (Garzon-Aburbeh et al., 1983, 1986).

In contrast to different studies performed previously with glycerolipidic prodrugs, such as acetylsalicylic acid, L-dopa or chlorambucil derivatives (Paris et al., 1979; Garzon-Aburbeh et al., 1983, 1986), the lymphatic kinetics after oral administration of the SR42128 prodrug solubilized with taurocholate (group E), with taurocholate and palmitic acid (group F), with linoleic acid (group B) or emulsified with taurocholate and linoleic acid (group D) made it evident that this compound did not present any significant lymphotropism. These results could be related to the peptidic nature of the active substance. Indeed, after lipase hydrolysis, the metabolite (1,3-dihydro-2-acetyl-SR42128) was likely recognized

as a peptide rather than a lipidic compound. As a consequence, it could be then hydrolyzed by the peptidases located in the intestinal lumen, in the brush border barrier or intracellularly.

When dosed in the form of a suspension or emulsified in linoleic acid and water, the prodrug was detected in the lymph samples of two animals. Such an erratic response in lymphatic absorption could be related to the influence of gastric emptying which is the limiting factor for oral absorption of drugs. In our experiments, no individual parameters could explain why the prodrug was only detected in lymphatic samples of the rats dosed with suspension or oil/water emulsion. In vitro studies revealed that the prodrug in an aqueous suspension was not hydrolyzed in the presence of pancreatic lipase. As confirmation of the stability of the prodrug in intestinal juice, an experiment on a rat dosed with the suspension showed that at least 20% of the administered dose was excreted intact in the feces (data not shown). The most appropriate explanation for the presence of the prodrug in the lymphatic samples of the animals administered with the suspension is that this compound is absorbed intact via either the intra- or paracellular route. The presence of the prodrug in lymphatic samples of one animal dosed with the linoleic acid/water emulsion could result from the absorption of the intact prodrug or, more likely, from the resorption of a product which has followed the natural triglyceride metabolism pathway.

Finally, the appearance of an unexpected metabolite has already been described by Garzon-Aburbeh et al. (1986) with an L-dopa prodrug. As proposed by these authors, such a compound could result from the neo-synthesis of a triglyceride derivative with a fatty acid other than palmitic acid.

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