Synthesis and *in Vitro* Study of a Diglyceride Prodrug of a Peptide

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A diglyceride derivative of a pentapeptide renin inhibitor, the 1,3-dipalmitoyl-[Iva-Phe-Nle-Sta-Ala-Sta-acetyl]-glycerol was synthesized and tested *in vitro* as a potential prodrug for oral administration. The ability of the diglyceride analog to inhibit the renin activity was equivalent to that of the parent peptide after predigestion with pancreatic lipase. Furthermore, the presence of the palmitoyl groups was found to induce, *in vitro*, an efficient protection of the peptide from gastric and intestinal hydrolysis. During incubation with intestinal and gastric fluids, and with α-chymotrypsin and pancreatic lipase, the glycerolipidic derivative was more stable than the peptide alone. These results support the use of glycerolipidic prodrug for oral administration of peptides.

KEY WORDS: peptides; oral delivery; glycerolipidic prodrugs; gastric juice; intestinal juice; α-chymotrypsin.

INTRODUCTION

Peptide and protein drugs derived from endogenous compounds can be potent pharmacological agents with low toxicities. However, because of extensive hydrolysis in the gastrointestinal tract (1), their oral bioavailability is low, and administration is limited to the parenteral route.

The development of prodrugs represents a strategy capable of circumventing these drawbacks (2). Diglyceride derivatives are attractive prodrugs for the oral administration of peptides. This concept is based on the covalent linking of a drug to the 2-position of a diglyceride to yield a structural analog of natural triglycerides. These are absorbed in the intestinal tract after specific hydrolysis of the 1- and 3-positions to yield a monoglyceride and two free acids that are absorbed by enterocytes through a passive diffusion process (3). In the enterocytes, a new triglyceride is resynthesized, incorporated in chylomicrons (4) and excreted into the lymphatic circulation to reach the blood vessels through the subclavian artery. A diglyceride prodrug, also called "triglyceride-like" prodrug, is thus expected 1) to prevent enzymatic degradation of the linked peptide, 2) to increase its enteral absorption and 3) to circumvent the hepatic first pass metabolism.

Renin inhibitors represent a new class of potentially therapeutic agents for the treatment of hypertension and congestive heart failure. Owing to their peptidic structure, the development of these compounds has been hampered by their lack of sufficient oral absorption (5,6). To demonstrate improved oral bioavailability of a peptide as a glycerolipidic derivative, we have chosen a pseudo-peptide, SR42128 (Fig. 1), which is a potent renin inhibitor (7). This paper describes the synthesis of a peptide derivative of 1,3-dipalmitoyl-glycerol and the protection of the peptide by prodrug formation against degradation by gastrointestinal enzymes.

MATERIALS AND METHODS

Chemicals

The peptide, SR42128 (Iva-Phe-Nle-Sta-Ala-Sta), was a gift from Sanofi Recherche (Montpellier, France). All chemicals were purchased from Aldrich (Strasbourg, France). Solvents were removed by evaporation. Thin-layer chromatographies (TLC) were performed on silica gel plates (Merck 60- F-254, Merck, Nogent/Marne; France) eluted with the following systems: (A): n-hexane/CH_2Cl_2/MeOH: 6/3/1 and (B): $CHCl_3$/MeOH/acetic acid: 90/10/1. The spots were detected with H_2SO_4 followed by calcination. Preparative liquid chromatography was performed on silica column (40-63 μm, Merck, Nogent/Marne; France) eluted by a gradient of ethyl acetate in CH_2Cl_2. The intermediate and final products have been identified by ^1H NMR, mass spectrometry and melting point. ^1H NMR was performed at 500 MHz with a Bruker WM 500 apparatus in DMSO as solvent. Mass spectra were recorded on a VG-Analytical ZAB-2E using the direct route of sample introduction. Melting points (uncorrected) were determined on a Köffler banc.

Methods

Synthesis of the prodrug: 1,3-dipalmitoyl-SR42128-acetyl-glycerol: 1,3-dihexadecanoyl-2-[isovaleryl-L-phenylalanyl-L-norleucyl-L-(3S,4S)-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-L-(3S,4S)-4-amino-3-hydroxy-6-methylheptanoyl-oxy-acetyl]-glycerol

The synthesis, as depicted in the figure 2, involved three steps.

(I) Preparation of the 1,3-dipalmitoyl-2-bromoacetyl-glycerol (c). The 1,3-dipalmitoyl-2-glycerol (**b**) was obtained by the method described by Bentley and Mc Crae (8). Compound **b** (7g, 12.3 mmol) and pyridine (37 mmol) were dissolved in 5 ml of CH_2Cl_2. Bromoacetylchloride (20 mmol) was added to this solution and the mixture was stirred, protected from light, at room temperature, during 14 hours. The mixture was then washed with 100 ml of 1N HCl, then with distilled water and dried with anhydrous Na_2SO_4. The organic layer was evaporated under vacuum and the residue was dissolved in CH_2Cl_2. Cold ethanol was added and the crystallization occurred at room temperature to afford 7.45 g (88%) of **c**.

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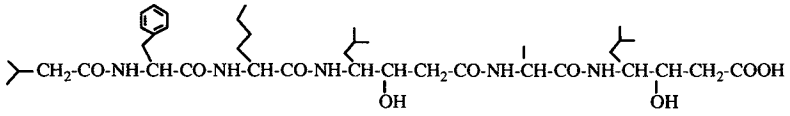

SR42128

Figure 1: Structure of compound SR42128.

(II) Preparation of the cesium salt of the peptide. An aqueous solution of Cs_2CO_3 (0.211 g/ml, 0.65 M) was added to an ethanolic solution of the peptide (1 g/50 ml, 26 M). The mixture was stirred during 30 minutes and the solvent was evaporated under vacuum.

(III) Preparation of 1,3-dipalmitoyl-2-(Iva-Phe-Nle-Sta-Ala-Sta-acétyl)-glycérol (f). The peptide was esterified according to a modified protocol (9). Instead of reacting at room temperature in dimethylformamide (DMF), the reaction occurred at 40°C in order to obtain complete dissolution of c. In addition, higher yields were obtained when DMF was replaced by dimethylsulfoxide (DMSO). Compounds e (1 g, 1.1 mmol) and c (1 g, 1.4 mmol) were dissolved in 2 ml of DMSO at 40°C and stirred at this temperature, protected from light during 14 hours. The reaction was stopped by cold water and a precipitate containing compound f appeared. The precipitate was filtered and redissolved in 50 ml of CH_2Cl_2. The organic phase was washed with water and dried with anhydrous Na_2SO_4 before being evaporated under vacuum. Compound f was purified by liquid chromatography on silica to yield 1.15 g of f (77%).

Renin inhibition studies

A pool of human plasma rich in renin (50 µl) was incubated one hour at 37 ± 0.2°C in a pH 5.7, 0.1 M phosphate buffer (220 µl) with increasing concentrations of the peptide or the prodrug (20 µl) and in presence of human angiotensinogen (200 µl) and phenylmethylsulfonyl-fluoride (10 µl). The reaction was stopped by chilling the tubes in an ice bath. Then angiotensin I was measured by radioimmunoassay (10).

To determine the influence of lipase hydrolysis on the anti-renin activity, the compounds (20 µl) were submitted to a pre-incubation at 37 ± 0.2°C, during 5 min with 20 µl of a pancreatic lipase solution (78 U/ml). Then, 20 µl of the mixture were withdrawn and the inhibitory activity was determined as described above.

The results were expressed as IC_{50} values which represent the molar concentration of the compound inducing a 50% renin inhibition.

HPLC assay for SR42128 and the prodrug

The dosages of the two compounds were assayed by reverse phase high performance liquid chromatography. HPLC 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 pH 3.5 triethylammonium phosphate buffer (TEAP) and acetonitrile (55/45) for the peptide, or TEAP and methanol (2/98) for the prodrug. The flow

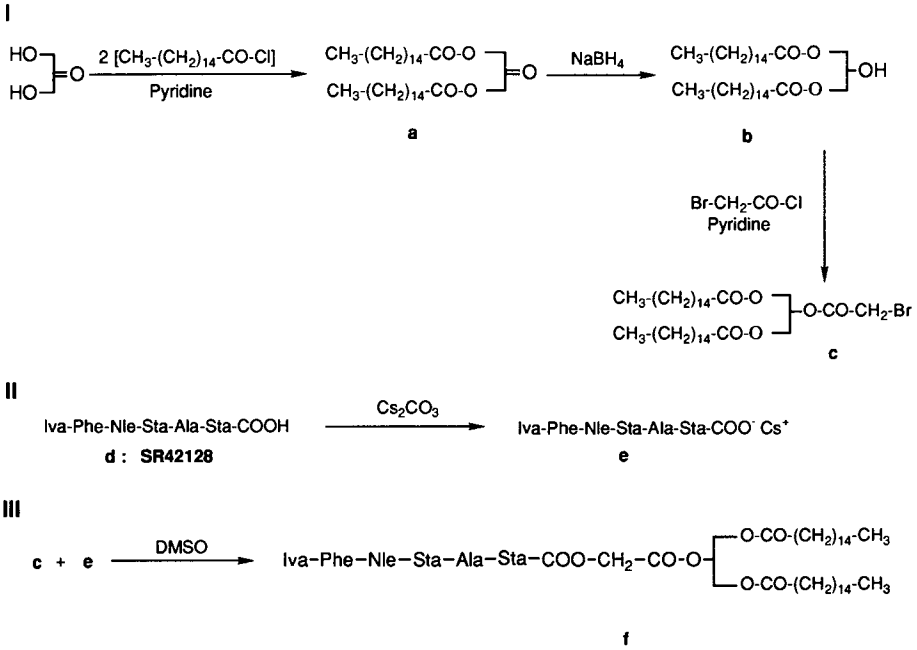

Figure 2: Synthesis of the glycerolipidic derivative.

rate was of 0.8 ml/min, the column was warmed up at 40°C ± 0.5°C and the detector set at 215 nm.

Determination of partition coefficients

The partition coefficients of the compounds were determined in an octanol/water system. SR42128 was solubilized in 1 ml of distilled water and the prodrug in 1 ml of octanol. The second solvent (1 ml) was added and the two phases were shaken for one hour at room temperature. The compound concentrations in each phase were determined by HPLC analysis. The partition coefficient was expressed as log (P), where $P = C_{Oct}/C_{Wat}$, and C_{Oct} was the concentration in the octanol phase and C_{Wat} the concentration in the aqueous phase.

Sample preparation

The peptide was dissolved in distilled water and the prodrug was dissolved in DMSO at the same concentration of 0.2 mmol/ml.

An aqueous dispersion of the prodrug was obtained as follows: a chloroform solution of the lipidic compound (2.70 mg/ml) was added to 10 ml of an aqueous solution of methylcellulose (0.5% w/v; Methocel A15 LV Prem EP, Dow, Boulogne-Billancourt; France). The mixture was stirred, sonicated, and the organic solvent evaporated at 50°C.

Hydrolysis in buffer solutions

The stability of the two compounds was studied in aqueous phosphate-citrate-borate buffer at pH 2, pH 7.4 and pH 12 (11). 1.5 ml of the sample preparation were incubated at 37°C ± 0.2°C with 5 ml of the buffer. The final concentration of DMSO when it was used was 8%. A sample of 200 µl was withdrawn at different time intervals from 15 to 240 minutes. The concentration of the compound remaining intact was determined by HPLC analysis as described above.

In vitro *stability in enzymatic solutions*

Gastric and intestinal fluids (Fluka, Switzerland) were prepared as described in US Pharmacopea (XXIInd ed., 1990, p. 1788 and 1789). For the lipase incubation 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). Fifty microliters of the sample preparation were incubated with 100 µl of the enzymatic solution at 37°C ± 0.2°C in polyethylene tubes. The final concentration in DMSO when it was used was 11%. Samples were withdrawn at different time intervals from 15 to 240 min. For the peptide samples, the reaction was stopped by addition of 50 µl of 2N $HClO_4$; after centrifugation (3000 rpm, 15 min), the peptide was dosed in the supernatant. For the prodrug, the reaction was stopped by congelation (−25°C), the samples were freeze-dried and then redissolved in 100 µl of CH_3Cl before HPLC analysis.

For the α-chymotrypsin incubation medium, bovine α-chymotrypsin (1 mg, 38 U) (Sigma, France) was dissolved in 1 ml of buffer (0.1 M Tris, 0.01 M $CaCl_2$, pH 7.4). Samples made of 600 µl of the compound preparation were incubated with 100 µl of the enzymatic solution. The final concentration in DMSO when it was used was 28%. Samples were collected at different time intervals from 10 to 120 min. The reaction was stopped and the samples were prepared as mentioned above before HPLC analysis.

Statistical analysis

For all the studies, results were expressed as the mean of three experiments and statistical differences between the peptide and the prodrug were established by analysis of variance (ANOVA) followed by Fisher's test.

RESULTS

Synthesis

The glycerolipidic derivative, 1,3-dipalmitoyl-SR42128-acetyl-glycerol was synthesized with high yields in three steps (Fig. 2). First, the diglyceride (b) was condensed with bromoacetylchloride to afford 1,3-dipalmitoyl-2-bromoacetyl-glycerol (c). Meanwhile, the carboxylic function of the renin inhibitor (d) was converted into its cesium salt (e) by titration to neutrality with aqueous Cs_2CO_3. In the last step, c was esterified by e, in the conditions adapted from Wang et al. (9) to give the expected compound (f). The spectral data (NMR and MS) were in agreement with the expected product. The found analytical data ($C_{76}H_{133}N_5O_{15}$) were within ±0.4% of theoretical values.

Renin inhibitory activity

Table I shows the anti-renin activity of the peptide and the prodrug. When it was not preceded by pancreatic lipase hydrolysis, the potency of the prodrug was not detectable. In contrast, after pancreatic lipase pre-incubation, renin inhibition was equivalent for both compounds.

Partition coefficient

As expected, the diglyceride derivative was highly lipophilic. The octanol/water partition coefficient, expressed as log (P), was of 3 instead of −0.78 for the peptide.

Stability in buffer solutions

The stability of the peptide in aqueous solution and of the prodrug in aqueous suspension or solubilized in DMSO was tested at pH 2, pH 7.4 and pH 12. No significant degradation of the peptide or the prodrug could be observed whatever the pH was.

Stability in enzymatic medium

The stability of the peptide and the prodrug in enzymatic media was evaluated by measuring the percentage of com-

Table I. Renin Inhibitory Activity of SR42128 and the Prodrug.

Compounds	IC_{50} at pH 5.7 (M)	
	Without lipase incubation	With lipase incubation
SR42128	4.5×10^{-9}	4.5×10^{-9}
Prodrug	$>1^{-5}$	3.0×10^{-9}

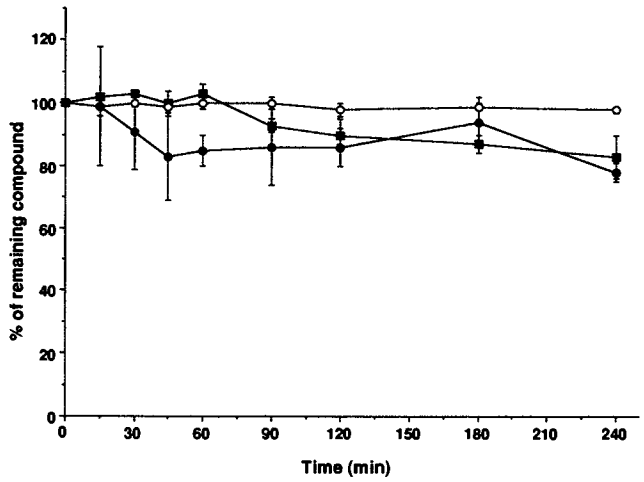

Figure 3: Stability of SR42128 (■) and the prodrug suspended (●) or solubilized (○) incubated with gastric fluid (37°C).

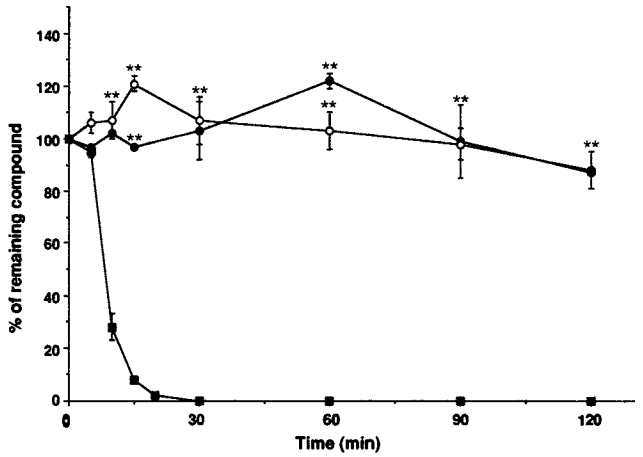

Figure 5: Stability of SR42128 (■) and the prodrug suspended (●) or solubilized (○) incubated with α-chymotrypsin (37°C). **: significant at 0.99% confidence level.

pound remaining intact as a function of time. The curves are presented in figures 3, 4, 5, and 6. To assess the enzyme activity in DMSO solution, the hydrolysis of the peptide dissolved in this solvent was tested. The curves are not presented here but the degradation profiles demonstrated that the enzymatic activities were preserved in the presence of DMSO.

In standard gastric juice, both peptide and prodrug derivative were stable (Fig. 3). In intestinal juice (Fig. 4), whatever formulation used, the prodrug was always more stable than the peptide. When incubated in suspension form, 40% of the prodrug remained after 4 hours whereas, when it was solubilized in DMSO, 20% remained intact after the same time. After incubation with α-chymotrypsin, the peptide was quickly hydrolyzed (Fig. 5). On the contrary, diglyceride prodrug, either suspended or solubilized in DMSO, was not degraded at all.

Figure 6 shows that the peptide was quickly hydrolyzed in the presence of pancreatic lipase whereas, under the same conditions, the prodrug was still intact after 4 hours when incubated as a suspension form. When solubilized with DMSO, the prodrug was slowly degraded.

DISCUSSION

The glycerolipic prodrug, 1,3-dipalmitoyl-SR42128-acetyl-glycerol, was synthesized and evaluated as a mean of improving the oral bioavailability of a peptide. The anti-renin activity of the prodrug was equivalent to that of the parent peptide after hydrolysis by pancreatic lipase. The renin inhibitory potency of pepstatin analogues is mediated by the establishment of a strong non covalent interaction between the peptide and the renin active site (12). This binding involves the first statine of the peptide and requires a close interaction between the active site of the enzyme and its inhibitor. Thus, our results revealed that such an interaction was not possible when the palmitic groups of the prodrug were still present. After hydrolysis of the 1- and 3-positions

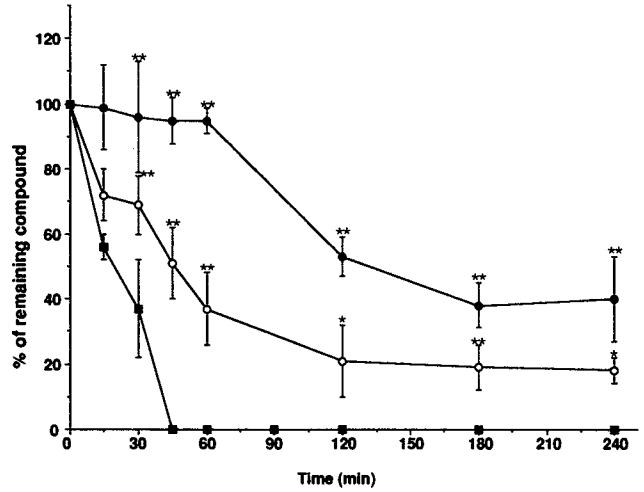

Figure 4: Stability of SR42128 (■) and the prodrug suspended (●) or solubilized (○) incubated with intestinal fluid (37°C). *: significant at 0.95% confidence level; **: significant at 0.99% confidence level.

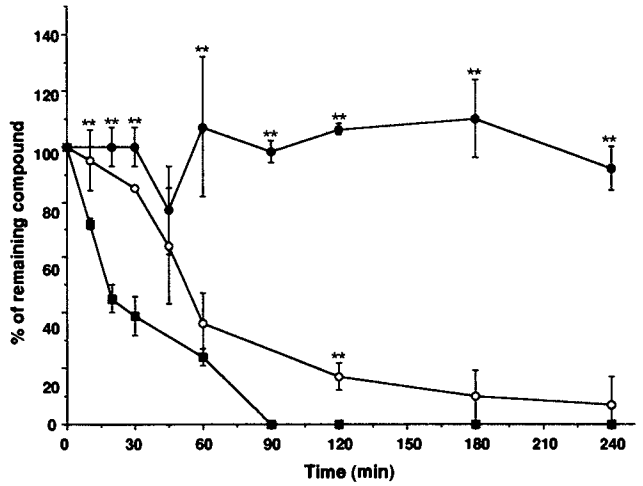

Figure 6: Stability of SR42128 (■) and the prodrug suspended (●) or solubilized (○) incubated with pancreatic lipase (37°C). **: significant at 0.99% confidence level.

in the presence of pancreatic lipase, the inhibitory site of the SR42128 is, again, accessible and the IC_{50} of the compound is equal to that of the parent peptide.

In the different enzymatic systems tested, the prodrug was always more stable than the peptide drug, especially in the presence of α-chymotrypsin. The major enzymatic activity of the standard gastric fluid is pepsin (E.C. 3.1.1.3.). The stability of the peptide incubated in gastric juice was related to its acidic protease inhibitory activity and the IC_{50} of the peptide against pepsin is 2.8×10^{-8} M (13). In the experimental conditions used, the pepsin activity was thus already inhibited.

In intestinal juice, which is a more complex medium constituted of a mixture of lipase, proteases and amylase, the peptide was rapidly degraded (Fig. 4). Thus, the prodrug partially protected the peptide against enzymatic degradation in the intestinal medium. On the other hand, the hydrolysis rate of the prodrug was slower when the prodrug was in suspension form. These results suggest that solubilization of the prodrug may influence its stability in the intestinal medium. In order to test whether the stability of the prodrug was due to its insolubility in aqueous solution or to a molecular protection of the peptide from the enzymes, the stability of the prodrug was tested in medium containing only one enzyme, either α-chymotrypsin (E.C. 3.4.21.1.) or pancreatic lipase (E.C. 3.4.23.1). These enzymes are specific for peptides or triglycerides respectively (14,15).

α-Chymotrypsin is an endopeptidase (serine-protease) and hydrolyses preferentially the peptide bonds involving carbonyl groups of aromatic amino acids such as tryptophan, tyrosine and phenylalanine, and into a lesser extent methionine and leucine (14). The catalytic activity of α-chymotrypsin involves a positional site and a catalytic site (16). The positional site includes a hydrophobic binding pocket in which the phenyl ring of Phe fits very well. The protection generated by the prodrug appeared to be due to the presence of palmitic residues which interfere with the phenyl binding in this positional site. Many authors have attempted to protect peptides from hydrolysis by α-chymotrypsin with chemical derivatizations near the bond which has to be cleaved (17–20). The results demonstrated that if the substitution was localized on a different bond from the one directly involved in the hydrolysis, there was no efficient protection. In this study, the prodrug was able to prevent enzymatic degradation of a peptide although the protecting group was bound far from the phenyl moiety.

The degradation of the peptide in the lipase medium could be mainly related to the animal origin of the enzyme and subsequent contamination by proteases or also to the esterase activity of the lipase itself. The fact that the prodrug in aqueous suspension was not sensitive to the lipolytic activity of the lipase, was correlated to its insolubility in the aqueous medium. Upon solubilization in DMSO, hydrolysis did occur.

This study revealed that a diglyceride derivative of a renin inhibitor peptide behaved *in vitro* as a prodrug: its renin inhibitory activity is not expressed if it is not activated by lipase. After lipase activation, the inhibitory potency of the glycerolipidic derivative is already the same than that of the parent peptide.

In vitro stability studies in intestinal media demonstrated the ability of this derivative to protect a peptide from gastrointestinal enzymatic hydrolysis. Thus, protection of a peptide against proteolysis by using glycerolipidic prodrugs is compatible with the preservation of pharmacological activity. Further, solubility of the prodrug was shown to affect hydrolysis by pancreatic lipase. Since the lipase hydrolysis is a required step for penetration of triglycerides in the enterocyte, the optimal form for administering the prodrug to animals should be a solution. DMSO is a poor candidate for *in vivo* administration because it induces membrane alterations (21). On the contrary, micellar solubilization with the aid of bile salts could be considered for *in vivo* administration of the prodrug. Addition of calcium salts is also suggested because this ion is known to favor enteral absorption of natural triglycerides (22).

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