In Vitro Studies of the Effects of HAART Drugs and Excipients on Activity of Digestive Enzymes

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Purpose. Side effects of diarrhea and steatorrhea diminish the therapeutic value of highly active antiretroviral therapy (HAART). We report *in vitro* studies of the effect of HAART drugs on the activity of pancrelipase, trypsin, and enterokinase and restoration of activity by subsequent addition of excess pancrelipase or colipase.

Methods. Commercial formulations of sixteen HAART drug formulations with solvent and four excipients were mixed with substrate. Activity of pancrelipase was recorded after addition of the enzyme; restoration of activity was monitored after addition of excess pancrelipase or colipase to the reaction mixture.

Results. Five protease inhibitors (Agenerase solution, Agenerase capsules, Norvir, Viracept, Kaletra, and Fortovase) and the excipient TPGS (d-α-tocopheryl polyethylene glycol 1000 succinate) inhibited lipase significantly at or below physiological concentrations. Neither nucleoside reverse transcriptase inhibitors nor non-nucleoside reverse transcriptase inhibitors showed significant lipase inhibition at physiological levels. Addition of excess pancrelipase to the medium completely reversed inhibition by Agenerase, Fortovase, Norvir, and TPGS and reactivated lipase; it diminished inhibition by Kaletra and Viracept but did not completely restore activity. Addition of colipase reversed inhibition by Agenerase solution, Agenerase capsules, and TPGS; inhibition by Kaletra and Fortovase recovered slightly. No compounds tested inhibited trypsin or enterokinase.

Conclusions. These results justify evaluating protocols involving coadministration of buffered pancrelipase with protease inhibitors to reduce or eliminate diarrhea and steatorrhea in individuals being treated for HIV.

KEY WORDS: diarrhea; excipients; highly active antiretroviral therapy; pancrelipase; side effects.

INTRODUCTION

Currently, the most effective treatment of individuals infected with HIV is a combination of drugs known as highly active antiretroviral therapy (HAART) (1,2). The active agents administered in HAART influence the mechanism of reproduction of HIV. A recent study monitoring HIV-positive patients from 1995 through 1997 indicated a decrease in opportunistic infections from 53% to 13% but also showed an increase in noninfectious diarrhea from 32% to 70% (3). The mild to severe diarrhea experienced by these patients causes maldigestion of food and excretion of undigested fat (steatorrhea) (4), reduces the absorption of exogenously administered drugs, and leads to reduced immunocompetence and loss of muscle mass. When HAART treatment is discontinued, diarrhea ceases, fat digestion normalizes, and the bowel movements of patients return to normal. Because the quality of their life is reduced due to the side effects of HAART, patients have difficulties complying with the therapy. Over-the-counter treatments available for diarrhea (oat bran, psyllium, SP-303, and others) are only marginally effective in HIV-positive individuals (5).

More than 95% of dietary fats ingested by the average adult in a day are triglycerides, and if these nutrients remain undigested, diarrhea can result. The digestion of triglycerides is chemically complicated and involves two fundamentally different but closely interrelated processes: the activation of several inactive proenzymes (zymogens), and the emulsification of nutrient lipids with bile salts. In the first part of the process, trypsin converts the zymogen procolipase, secreted by the pancreas, into the 12-kDa protein colipase. In the second part of the process, colipase anchors the complex formed between lipase and a micellar bile acid to its triglyceride substrate, thereby stabilizing the complex and activating it enzymatically; the triglyceride may now be hydrolyzed to free fatty acids and monoacyl glycerol. The trypsin needed in the first part of the process is produced by the action of enterokinase, secreted by the Brunner's gland in the duodenum, on the zymogen trypsinogen, secreted by the pancreas (6). The inhibition of pancreatic lipase or any enzyme in the zymogen-activating cascade leads to undigested fats that become hydroxylated by the intestinal bacterial flora. Hydroxylated fatty acids are well-known diarrhetics.

The mechanism by which HAART drugs induce diarrhea and steatorrhea is unknown. However, deficiencies in fat and lipid digestion in other medical conditions such as cystic fibrosis and alcohol-induced pancreatitis and the disruption of digestive processes by lipase inhibitors like orlistat are known to result in steatorrhea (7,8). If it can be determined that specific drugs influence or inhibit pancreatic digestive enzymes, lipase–bile salt–colipase complexation, or the zymogen-activating cascade, then the use of exogenous pancrelipase as part of a therapeutic protocol to reduce or eliminate diarrhea and steatorrhea in HIV-positive individuals undergoing HAART should be investigated.

HAART drugs fall into two categories: protease inhibitors (PIs) or inhibitors of reverse transcriptase. The latter category is further subdivided into nucleoside reverse transcriptase inhibitors (NRTIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs). The *in vitro* study described herein assesses the inhibitory effect of PI, NRTI and NNRTI antiretroviral drugs and excipients present in their formulations on the activity of pancreatic lipase, trypsin, and enterokinase. In addition, the possibility of overcoming inhibitory activity of PIs by the addition of excess pancrelipase or colipase to the reaction media is evaluated.

MATERIALS AND METHODS

Materials

United States Pharmacopoeia (USP) reference standards for pancrelipase (24.8 U/mg) and crystallized trypsin (3200 BAEE U/mg) were used in the studies along with colipase

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and enterokinase (670 U/mg). Appropriate concentrations of enzymes as described in USP or reference standard procedures were prepared in cold distilled water. Quantities of HAART drug formulations were weighed to give appropriate amounts of active ingredients. The HAART drug formulations were prepared in cold distilled water, reagent-grade methanol, DMSO, or olive oil. HAART drug formulations were prepared in solvents selected to maximize the solubility of the active ingredient in the formulations as provided by the manufacturers of the drugs. Kaletra was prepared in olive oil in order to maintain consistent results in dose–response studies. The following commercially available preparations of HAART drugs (gift from I. D. Care, Inc., Hillsborough, NJ, USA) were used in this study: Agenerase solution, Agenerase capsules, Combivir, Epivir, Retrovir, and Ziagen (registered trademarks of GlaxoSmithKline, Pittsburgh, PA, USA); Norvir and Kaletra (Abbott Laboratories, Inc., Abbott Park, IL, USA); Viracept and Rescriptor (Agouron Pharmaceuticals, Inc., New York, NY, USA); Fortovase, Invirase, and Hivid (Roche Laboratories, Inc., Nutley, NJ, USA); Crixivan (Merck & Co., West Point, PA, USA); Sustiva, Videx, and Zerit (Bristol-Myers Squibb Co., Princeton, NJ, USA); and Viramune (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, USA). Orlistat was a gift from Roche Labs (Switzerland); d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) was provided by Eastman Chemical Co. (Kingsport, TN, USA); vitamin E was purchased from Eckerd Drugs (Bethlehem, PA, USA); povidone was provided by ISP Technologies (Calvert City, KY, USA). Solutions of excipients were prepared in distilled water or reagent-grade methanol (Aldrich, Milwaukee, WI, USA).

Methods

For those drugs and excipients that exhibited inhibition, the concentration of inhibitor that resulted in 50% of the initial pancrelipase activity (the IC-50) was determined by plotting the log of the drug or excipient concentration against percent lipase activity.

Inhibition of Pancrelipase

Pancrelipase activity was determined according to the procedure described in the *United States Pharmacopoeia*, Volume XXIV (9). In brief, the substrate for these evaluations consisted of 10% (vol/vol) olive oil in acacia solution [10% (wt/vol) aqueous], 40 mg/ml sodium taurocholate, 0.075 M calcium chloride, 0.05 M Tris in 3.0 M NaCl aqueous buffer (pH 8.5), and distilled water. The substrate was emulsified, incubated at 37°C, and brought to a pH of 9.0 by the addition of 0.02 N NaOH. At time zero, 1.0 ml of pancrelipase solution (8–16 U/ml) was added to the substrate. A Radiometer (Copenhagen, Denmark) PHM 290 pH stat autotitrator maintained the pH at 9.0, and the volume of 0.02 N NaOH dispensed per unit time was recorded for 5 min. Lipase activity was calculated from the initial slope of the plot of volume of NaOH dispensed as a function of time.

For the evaluation of inhibition of pancrelipase activity, HAART drug formulations with solvent were prepared daily and were mixed with the substrate prior to incubation and addition of the enzyme. Orlistat was used as a positive reference control at concentrations determined from recommended doses (10).

Recovery of Pancrelipase Activity After Inhibition

The method to assay for the inhibition of pancrelipase was also used to monitor the restoration of activity, except that drug formulations, in concentrations to give 40–60% inhibition, were not preincubated with the substrate. At time $t = 0$ min, 1.0 ml of enzyme was added to initiate the reaction. At $t = 5$ min, 1.0 ml of a HAART drug formulation with solvent was added to the reaction mixture to induce inhibition. At $t = 12$ min, an additional 1.0 ml of enzyme was added to the reaction mixture to overcome inhibition. The reaction was then monitored for 20 min.

Junge *et al.* (11) described a procedure for evaluating the reactivation of lipase by colipase. The lipase assay again was used, except aqueous colipase solution replaced the distilled water so that the concentration of colipase in the final reaction mixture was 5 μg/ml. HAART drug formulations with solvent, in concentrations to give 60–90% inhibition, were preincubated with substrate containing colipase.

Inhibition of Trypsin

Trypsin activity (U/mg) was determined by a trypsin-specific spectrophotometric assay described by Bergmeyer (12). In brief, 0.75 ml triethanolamine (TEA) solution (0.2 M TEA, 0.020 M $CaCl_2$, pH 7.8) was mixed with 0.075 ml of HAART drug formulation with solvent (DMSO or water). After mixing, 0.025 ml of trypsin solution (0.32 mg/ml in 1.0 mM HCl) was added, and this mixture was incubated at 25°C for 1 min. At time $t = 0$, 0.20 ml benzoyl-L-arginine-4-nitroanilide substrate (0.8 mM in 40% DMSO, 60% TEA solution) was added to give an assay volume of 1.05 ml. The change in absorbance at 405 nm was monitored over time for 5 min to check for linearity and product inhibition. Each subsequent run was determined over a 1-min span. HAART drug formulations with solvent were mixed with the TEA solution prior to incubation. Aprotinin and soy bean trypsin were used as positive reference controls.

Inhibition of Enterokinase

The activity of enterokinase was determined by a coupled (enterokinase-trypsin) enzyme assay provided by Biozyme (San Diego, CA, USA) (13). In brief, the substrate consisted of trypsinogen (1.2 mg/ml in 1.0 mM HCl/5.0 mM $CaCl_2$). A series of test tubes was labeled for each enzyme preparation and enzyme blank. Each tube contained substrate (trypsinogen) equilibrated to 25°C with 0.07 M sodium succinate buffer (pH 5.6) and distilled water. Enterokinase solution was added to each reaction tube, and distilled water was added to blank tubes. The total contributions of each component to the volume were 1.0 ml succinate buffer, 0.8 ml distilled water, 0.1 ml enterokinase, 0.5 ml trypsinogen, and 0.2 ml of either HAART drug formulation with solvent (DMSO or water) or DMSO/water as a control to yield a total reaction volume of 2.6 ml. All tubes were incubated for 30 min for the enterokinase-catalyzed reactions. After incubation, the reaction was quenched with 2.0 M HCl. The test and blank solutions were added separately to *N*-benzoyl-L-arginine ethyl ester hydrochloride (0.25 mM in 0.067 M potassium phosphate, pH 7.6) and observed spectrophotometrically for an increase in absorbance at 253 nm for 5 min to monitor the trypsin catalyzed reaction.

RESULTS

The HAART drugs, their recommended dose, aqueous solubility, physiological concentration, quantities tested, percent inhibition of pancrelipase as a function of concentration, and percentage of patients experiencing diarrhea on the HAART drugs are listed in Table I. An observed inhibition equal to or greater than 30% is considered physiologically significant.

Among the HAART drugs, only the PIs caused a significant inhibition of pancrelipase at or below physiological concentrations as calculated from the plots to determine IC-50 values. Among the PIs, Agenerase solution, Agenerase capsules, Norvir, Viracept, Kaletra, and Fortovase caused greater than 70% inhibition at physiological concentrations; Invirase and Crixivan caused no significant inhibition under the conditions of the assay.

The NRTIs showed no significant pancrelipase inhibition; only Combivir and Ziagen demonstrated any measurable inhibition at physiological concentrations. The NNRTIs showed no significant inhibition of pancrelipase at physiological levels. However, above the physiological concentration, pancrelipase inhibition by Rescriptor increased to 25% and by Sustiva to 41%. None of the HAART drugs evaluated produced a measurable inhibition of either trypsin or enterokinase under the assay conditions. The IC-50 of the known trypsin inhibitor aprotinin was determined in this assay to be 0.67 μg/ml.

Because the PIs were the only formulations with significant pancrelipase inhibition at physiological levels, the excipients PEG-400, povidone, vitamin E, and TPGS used in their formulations were also tested in the assay for inhibition of lipase activity. The data from these evaluations are listed in Table II. Of the four excipients tested, only TPGS caused physiologically significant inhibition of pancrelipase. Concentrations of TPGS ranging from 20% to 2% (wt/vol) in the test media resulted in greater than 90% inhibition of lipase; concentrations of TPGS as low as 0.5% (wt/vol) exhibited greater than 30% inhibition.

The inhibition of pancrelipase by the PIs and the excipient TPGS was reversed by the addition of excess pancrelipase to the test solutions (Figs. 1 and 2). Total activity (100%) was restored in the test media containing Agenerase solution, Norvir, Fortovase, and TPGS, and approximately 88% and 52% activity was restored in the test media containing Kaletra and Viracept, respectively.

Pancrelipase was also reactivated when excess colipase was added to reaction mixtures containing Agenerase solution, Agenerase capsules, and TPGS. The percentage of pancrelipase activity increased from 20% to 60% for Agenerase solution, from 27% to 74% for Agenerase capsules, and from 24% to 83% for TPGS. Addition of excess colipase increased the pancrelipase activity from 50% to 71% in reaction mixtures containing Kaletra and from 15% to 26% for Fortovase. Addition of excess colipase had no effect on reaction mixtures containing USP lipase reference standard, Norvir, Viracept, or the reference control orlistat (Fig. 3).

The IC-50s for the PIs and the excipient TPGS were determined and are listed in Table III. The IC-50 of the known pancrelipase inhibitor orlistat was also evaluated in this assay and is listed in the table for reference. Orlistat was the most potent inhibitor of pancrelipase by a factor of over 400 compared to the Agenerase solution and the excipient TPGS, which were essentially equivalent in their potency. Agenerase solution was over four times more potent than Agenerase capsules.

DISCUSSION

Diarrhea and steatorrhea caused by unmetabolized fats are common side effects associated with HAART. The percentage of patients that experience diarrhea while on HAART drugs is given in Table I. The mechanism by which HAART drugs induce these side effects is unknown; however, pancreatic insufficiency manifested in other medical conditions such as cystic fibrosis and alcohol-induced pancreatitis is characterized by the induction of steatorrhea (7,8). Furthermore, the inhibition of digestive processes caused by the lipase inhibitor orlistat also causes this side effect (14). Indeed, unmetabolized fats may be a consequence of the inhibition of pancrelipase or any enzyme in the zymogen activation cascade. The fate of these undigested fats is hydroxylation by the intestinal bacterial flora; once converted to hydroxylated fatty acids, they then cause diarrhea. There is a relationship between the percentage of patients experiencing diarrhea on HAART drugs and the degree of inhibition of pancrelipase by protease inhibitors. A higher percentage of patients on HAART drugs experience diarrhea with protease inhibitors that have a significant inhibition of pancrelipase at physiological concentrations.

The hydrophobic PIs in this study have been shown to inhibit specifically pancrelipase but not trypsin or enterokinase in the zymogen-activating cascade. This result points to the possible interaction of these drugs with the pancrelipase–colipase–micelle (PL–CL–M) ternary complex that has been suggested as the active unit that binds to and then hydrolyzes lipids in the duodenum. Hermoso *et al.* (15–18) have recently discussed that the active "open flap" conformation of pancrelipase is stabilized when the PL–CL complex associates with a micelle of appropriate size. This conformation exposes an extensive hydrophobic region on the PL to the aqueous environment, in which the binding of the lipid phase of the substrate to this hydrophobic region should be favorable. Opening the flap, which is a large amphiphilic loop from the N-terminal domain of PL that covers the active site, requires both CL and a micelle within the assemblage. Exposing the active site by repositioning the flap is a reversible process (17).

Hermoso *et al.* (15) have reported the crystal structure of a complex of porcine lipase–colipase–tetraethylene glycol monooctyl ether micelle [PL–CL–$M_{(TGME)}$]. When the TGME is present in submicellar concentrations, one molecule of monomeric TGME occupies the substrate-binding region of the complex. The authors speculate that the binding of the TGME to the active site may be responsible for its observed inhibitory effect on triglyceride hydrolysis by the enzyme. Modeling suggests that the aliphatic region of TGME binds to the hydrophobic substrate binding site on PL whereas the polar region of the detergent associates with either the Ser of the Ser^{153}-His^{264}-Asp^{177} catalytic triad or with Gly^{77} and Trp^{86}. Because octyl-β-D-glycopyranoside (βoG) also inhibits enzymic hydrolysis at submicellar concentrations, the authors speculate that nonionic detergents, in submicellar concentrations, may be a general class of lipase inhibitors. Inhibition by

Table I. Inhibition of Pancrelipase by HAART Drugs at pH 9.0

Trade name (active ingredient)	Recommended dose (mg)	Aqueous solubility (mg/ml)	Physiological concentration* (mg/ml)	Drug tested (mg/ml)†	Inhibition of pancrelipase (%)	Patients on drug that experience diarrhea (%)‡
Protease inhibitors (PIs)						
Agenerase (amprenavir) solution	1400	0.04	5.60	2.50	99	39§
				0.50	99	
				0.10	79	
				0.050	33	
				0.010	4	
Agenerase (amprenavir) capsules	1200	0.04	4.80	2.50	91	39§
				0.50	65	
				0.25	49	
Norvir (ritonavir)	600	Insoluble	2.40	2.67	62	15
				1.30	66	
				0.67	27	
				0.07	6	
Viracept (nelfinavir)	750	Insoluble	3.00	3.33	91	20§
				1.67	62	
				0.42	43	
				0.10	12	
Kaletra‖ (lopinavir/ritonavir)	670	Insoluble	2.60	2.20	81	24§
				1.33	52	
				1.10	36	
				0.89	36	
				0.44	29	
Fortovase (saquinavir)	1200	Insoluble	4.80	6.67	87	20§
				4.00	61	
				2.33	34	
				1.33	23	
				0.66	3	
Invirase (saquinavir mesylate)	600	2.2	2.40	1.61	21	4.9
				1.29	24	
				0.16	24	
Crixivan (indinavir sulfate)	800	Soluble	3.20	4.27	6	3.3
				1.60	0	
Nucleoside reverse transcriptase inhibitors (NRTIs)						
Combivir‖ (lamivudine/zidovudine)	450	20	1.80	2.99	9	18
				1.20	15	
Epivir (lamivudine)	150	70	0.60	1.31	9	18
				0.26	0	
Hivid (zalcitabine)	0.75	76	0.003	0.005	0	2.5
Retrovir (zidovudine)	300	20	0.120	1.33	0	8.0
Videx (didanosine)	400	27	1.60	2.67	5	57¶
				0.80	1	
Zerit (stavudine)	40	83	0.160	0.08	0	50
Ziagen (abacavir)	300	77	0.120	1.32	7	12
				0.65	13	
				0.38	4	

Non-nucleoside reverse transcriptase inhibitors (NNRTIs)						
Rescriptor (delavirdine)	400	Insoluble	0.160	1.49	25	4.2§
				0.90	9	
Sustiva (efavirenz)	600	Insoluble	2.40	3.33	41	6.0§
				1.33	3	
Viramune (nevirapine)	200	Insoluble	0.80	1.33	4	4.2§
				0.40	0	

* Physiological concentration is calculated as follows: recommended dose (mg)/approximate volume in one 8 ounce (250 mL) glass of water.
† Mass of drug based on label claims: Agenerase solution was used neat; Agenerase capsules, Fortovasel, Norvir, Rescriptor, and Viramune were dissolved in 50% methanol; Sustiva was dissolved in 70% methanol; Viracept was dissolved in 100% methanol; Kaletra was dissolved in olive oil. All NRTIs were dissolved in distilled water. Values shown have been corrected for solvent effects based on control experiments in our laboratory.
‡ The percentage of patients that experience diarrhea on a given drug was obtained from the prescribing information provided by the drug manufacturer.
§ In combination with NRTIs or NNRTIs.
‖ Kaletra is a mixture of the active PIs lopinavir and ritonavir. Combivir is a mixture of the active NRTIs lamivudine and zidovudine.
¶ In combination with PI.

Fig. 1. Recovery of pancrelipase activity at pH 9.0 after inhibition by Agenerase solution (all PIs exhibiting inhibition resulted in a similar curve). (A) $t = 0$; 1.0 ml pancrelipase added. (B) $t = 5$ min; 1.0 ml of PI added. (C) $t = 12$ min; 1.0 ml pancrelipase added.

βoG and the TGME monomer is not reversed by the addition of colipase.

Bile salts are known lipase inhibitors as well, but the mechanism of their inhibition is different than that of the nonionic detergents; bile salts inhibit lipase by coating the water–lipid interface. Inhibition by bile salts is reversed *in vivo* by colipase, which binds to lipase via a polar domain and to the interface via a nonpolar tyrosine-rich domain to produce the active PL–CL–M complex.

The inhibition of pancrelipase caused by the hydrophobic PIs and TPGS is largely reversed by the addition of excess pancrelipase to the test media (Figs. 1 and 2). Because only the hydrophobic PIs caused significant inhibition of pancrelipase, this may indicate a possible interaction of the PIs with the water–lipid interface, the hydrophobic binding site, PL/CL binding, or PL–CL–M complexation, all of which require

Fig. 2. Recovery of pancrelipase activity after drug inhibition followed by addition of excess pancrelipase. (*Agenerase solution. Agenerase capsule was not available for testing.)

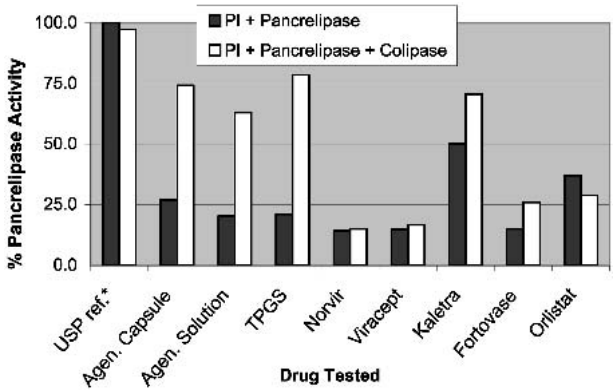

Fig. 3. Recovery of pancrelipase activity after drug inhibition followed by addition of colipase. (*No drug added to this sample.)

hydrophobic interactions. Further study is required to determine the mechanism of inhibition of the PIs. Furthermore, inhibition caused by Agenerase solution, Agenerase capsules, and TPGS can also be reversed by the addition of excess colipase to the medium (Fig. 3). It is unclear from this study if the inhibition by Agenerase solution and Agenerase capsules is due to the active protease inhibitor amprenavir or to the excipient TPGS, which is present only in formulations of Agenerase and not in those of the other PIs. A quantitative observation suggests that inhibition by the two different formulations of Agenerase (i.e., solution and capsules) may be due to the TPGS: the solution contains about four times more TPGS than does the capsule per mg of active drug, and the IC-50 of the capsule is about four times greater than the IC-50 of the solution (Table III).

The restoration of activity by the addition of either excess pancrelipase or excess colipase to the test media in the assays involving TPGS may also suggest a possible mechanism for inhibition by the excipient. The TPGS monomer is similar to colipase, in that it has a hydrophobic section (vitamin E) that corresponds to the hydrophobic tyrosine-rich section of colipase; this subunit of colipase binds to the bile salt–coated water–lipid interface ([1] in Fig. 4). The hydrophobic subunit of TPGS is bound via a succinate linker to a 21–24 residue-long hydrophilic oligomer of ethylene glycol that corresponds to the polar region of colipase. The polar region of colipase binds to the C-terminal domain of lipase by

Table III. IC-50 Values for Inhibitors of Pancrelipase at pH 9.0

Compound tested	IC-50 (μg/ml)	IC-50 (μM)
Agenerase solution	88.9	176
Agenerase capsules	326	620
Norvir	331	603
Viracept	619	1100
Kaletra	1138	843
Fortovase	2810	4205
TPGS	275	182
Orlistat	0.217	0.438

ion pairing between a glutamic acid residue (Glu^{45}) on CL and a lysine residue (Lys^{400}) on PL. Colipase also has a secondary binding site that makes polar and van der Waals contacts with the lipase flap (17). The sites are connected by a hinge region and are represented by [3] in Fig. 4.

The lengths of the three parts of the TPGS molecule were determined from energy minimizations run in Spartan (Wavefunction, Inc., Irvine, CA, USA) and were compared to values reported in the literature (15,16,18) for the colipase binding sites. The hydrophilic flap on pancrelipase is approximately 50 A° long; the distance from the Lys^{400} in the C-terminal domain of pancrelipase to the open flap is approximately 25 A°. For comparison, the hydrophobic vitamin E moiety in TPGS is approximately 22 A° long; the succinate linker, 5–6 A°, and the linear PEG 23-mer between 43 and 55 A° long. These linear dimensions correspond reasonably with the size of the sites of interaction of colipase with pancrelipase (Fig. 4). Consequently, one can envision TPGS effectively competing with colipase and blocking colipase from binding with pancrelipase and forming the active complex.

It is reasonable to suggest that under the conditions of the assay, the long hydrophilic PEG chain of TPGS binds to either the polar C-terminal domain in PL, or to the flap, or to both (sections of [3] in Fig. 4). The vitamin E portion of TPGS could then bind to the hydrophobic interface ([1] in Fig. 4). In the first case with the PEG moiety covering the site on the polar C-terminal domain, the binding of PL to CL would be blocked; in addition, the vitamin E moiety could bind to the hydrophobic interface and block CL from binding to the micelle ([1] in Fig. 4). In the second case with the PEG moiety

Table II. Inhibition of Pancrelipase at pH 9.0 by Excipients

Excipient	Drugs containing excipient	Aqueous solubility (mg/ml)	Maximum amount in preparation (wt%)	Wt% excipient in source used (100% = pure)	Percent inhibition of pancrelipase
PEG-400	Agenerase*	Soluble	30	100	0
	Norvir		30	100	0
Povidone	Invirase	Soluble	25	40	17
TPGS	Agenerase*	200		20	96
	Capsule: per cap		10	4	95
	Solution: per ml		3	2	93
				1	79
				0.5	43
				0.2	6
				0.02	0
Vitamin E	Fortovase	Insoluble	0.005	50	20

* Both solution and capsules contain PEG-400 and TPGS.

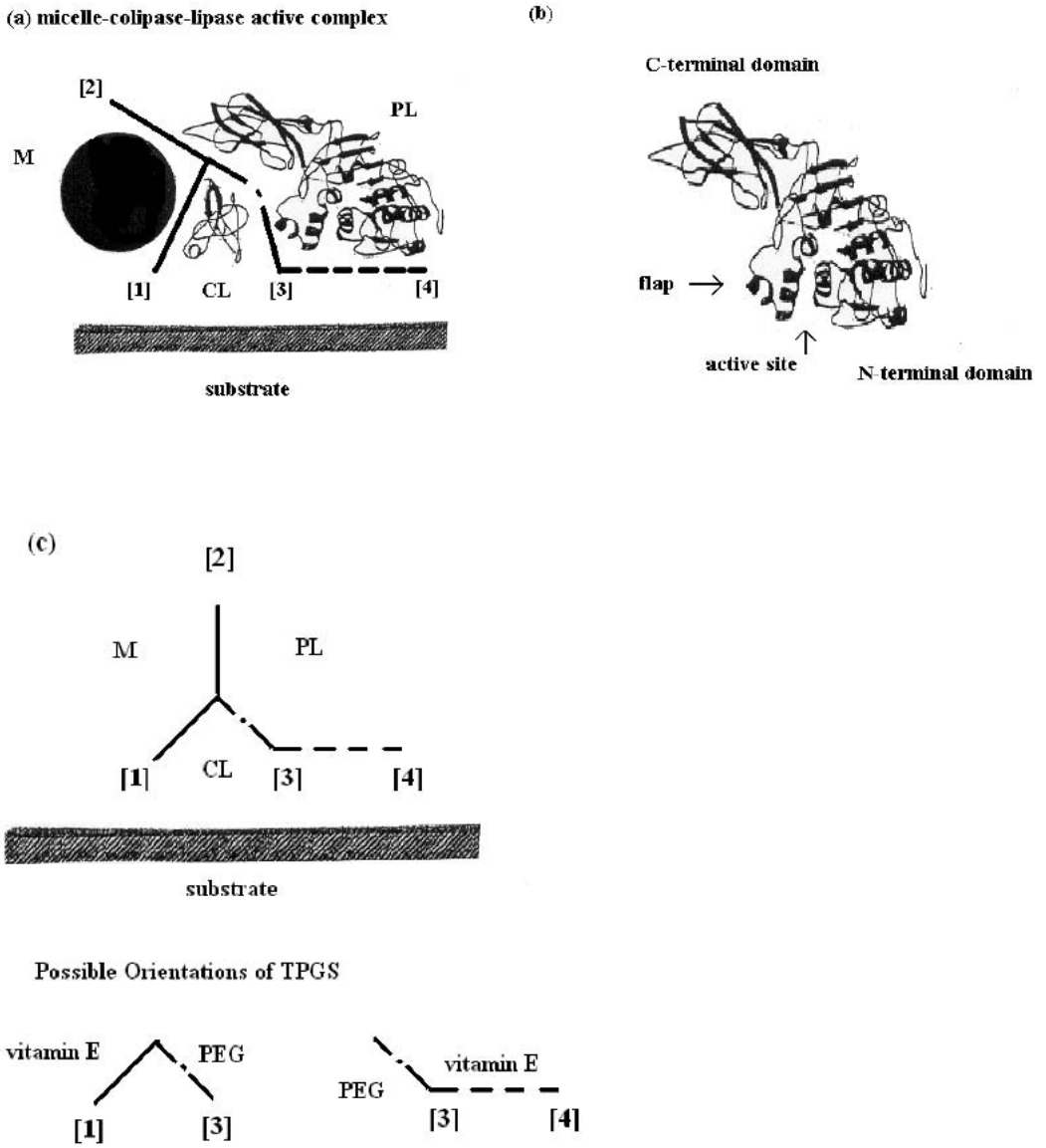

Fig. 4. Cartoon of possible orientations for d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) at binding sites on the pancrelipase–colipase–micelle (PL–CL–M) complex. (a) Relative orientation of components of active complex and substrate; solid lines indicate binding sites between CL and M ([1]), M and PL ([2]), CL and PL ([3]), and dashed line between PL and substrate ([4]) [after Hermoso (16) with permission of the author]. (b) Conformation of PL in active complex [after Hermoso (16) with permission of the author]. (c) Cartoon of binding sites showing possible orientations for TPGS.

covering the flap, two scenarios are possible. The PEG chain may bind to but not open the flap and block the interaction of PL and CL. If the flap were opened by binding to the oligomer, the PL–CL interaction would still be blocked, while the vitamin E moiety could position itself favorably with respect to the hydrophobic substrate binding site and thereby block access of the complex to the substrate ([4] in Fig. 4). Excess CL could displace the TPGS and re-establish the active complex in any of these scenarios. It should also be noted that vitamin E alone causes a measurable but not physiologically significant inhibition of pancrelipase (Table II).

In summary, we suggest that TPGS mimics the binding of colipase and thereby blocks the association of colipase with pancrelipase. Excess colipase added to the mixture displaces the TPGS and re-forms the active complex.

CONCLUSIONS

The protease inhibitor Agenerase formulated as a solution or a capsule exhibits complete inhibition of pancrelipase at physiological concentration. Regarding the other protease inhibitors, Viracept produces 89% inhibition at physiological concentration; Kaletra^{T}, 83% inhibition; Norvir, 72% inhibition, and Fortovase, 75% inhibition as calculated from the plots to determine IC-50 values. The inhibition caused by Agenerase, Norvir, Fortovase, and TPGS is completely overcome by the addition of pancrelipase to the test solutions; activity in the Kaletra- and Viracept-containing solutions is partly restored.

The addition of colipase to reaction mixtures inhibited by Agenerase and/or TPGS also restores pancrelipase ac-

tivity. Addition of colipase to test solutions containing Norvir and Viracept has no effect. Only a slight recovery of activity is observed with addition of colipase to Kaletra and Fortovase.

In contrast, the NRTIs and NNRTIs exhibit no specific inhibition of pancrelipase. Neither the PIs, nor the NRTIs, nor the NNRTIs inhibit trypsin or enterokinase.

These *in vitro* results provide insight into the causes of HAART-induced diarrhea and steatorrhea. More importantly, they establish a clear justification for the evaluation of protocols to reduce or eliminate diarrhea and steatorrhea in patients by the coadministration of buffered pancrelipase (pH 9.0) with protease inhibitors.

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