Vitamin E-TPGS Increases Absorption Flux of an HIV Protease Inhibitor by Enhancing Its Solubility and Permeability[1]

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Purpose. To investigate the effect of vitamin E-TPGS, d-α-tocopheryl polyethylene glycol 1000 succinate, on the solubility and permeability of amprenavir, a potent HIV protease inhibitor.

Methods. The aqueous solubility of amprenavir was measured as a function of vitamin E-TPGS concentration. Directional transport through Caco-2 cell monolayers was determined in the presence and absence of vitamin E-TPGS and P-glycoprotein inhibitors. Absorption flux was estimated from Caco-2 cell permeability and aqueous solubility.

Results. The solubility of amprenavir in a pH 7 buffer at 37°C was 0.036 ± 0.007 mg/mL. The solubility linearly increased with increasing vitamin E-TPGS concentration (above 0.2 mg/mL). Polarized transport was demonstrated in the basolateral to apical direction, exceeding apical to basolateral transport by a factor of 6. The active efflux system was inhibited by vitamin E-TPGS and known P-glycoprotein inhibitors verapamil and GF120918.

Conclusions. The solubility of amprenavir was improved in the presence of vitamin E-TPGS through micelle solubilization. Vitamin E-TPGS inhibits the efflux system and enhances the permeability of amprenavir. Overall, vitamin E-TPGS enhanced the absorption flux of amprenavir by increasing its solubility and permeability. The enhancement is essential to the development of the novel soft gelatin capsule formulation of amprenavir for use in the clinic.

KEY WORDS: amprenavir; vitamin E-TPGS; solubility; permeability; absorption flux.

INTRODUCTION

Amprenavir (alternatively known as141W94 or VX-478) is a potent HIV protease inhibitor recently approved by the US Food and Drug Administration (1). Its oral bioavailability in the powder in capsule and conventional tablet formulations is low (undetected blood levels). A novel formulation, containing vitamin E-TPGS, d-α-tocopheryl polyethylene glycol 1000 succinate, was developed with sufficient oral bioavailability for use in the clinic (2).

Vitamin E-TPGS is a water-soluble derivative of a natural-source vitamin E (3). It was clinically demonstrated that vitamin E-TPGS improved the oral bioavailability of vitamin E (4) and cyclosporin (5). The enhancement of cyclosporin bioavailability is suspected to be due to enhanced solubility, improved permeability, or reduced intestinal metabolism (5–7).

It was shown, recently, that HIV protease inhibitors are substrates of P-glycoprotein (9–11). It was observed in *mdr1a* knockout mice that the oral absorption and the blood-brain barrier penetration are limited by the action of P-glycoprotein (9). Surfactants, such as Cremophor EL and Polysorbate, were found to be potent inhibitors of P-glycoprotein (11–13). However, no in vivo data or actual example supports these in vitro findings. The present study was undertaken to investigate the effect of vitamin E-TPGS on both solubility and Caco-2 cell permeability of amprenavir. We established a relationship between absorption flux and vitamin E-TPGS concentration. It was shown that the absorption flux correlated to dog bioavailability better than either solubility or permeability alone.

MATERIALS AND METHODS

Materials

Amprenavir, ^{14}C-labeled amprenavir and GF120918 hydrochloride were synthesized at GlaxoWellcome, Inc. Vitamin E-TPGS was from Eastman Chemical Co. Sodium phosphate, monobasic and sodium hydroxide were purchased from J. T. Baker. (+/−)Verapamil hydrochloride and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co. Acetonitrile was HPLC grade and purchased from E. M. Sciences.

Cellgro Minimum Essential Medium Eagle (Mod.) 1X was obtained from Mediatech, Fetal Bovine Serum was from Gibco BRL, Nonessential Amino Acid Solution (100X), Hank's Balanced Salt Solution (modified; with $NaHCO_3$, without phenol red) and HEPES buffer (1M) were obtained from Sigma Chemical Co. Caco-2 cells were obtained from American Tissue Culture Collection, and were used between passages 34 and 45.

Methods

Solubility Studies

Solubility of amprenavir was measured in aqueous solutions of vitamin E-TPGS. The appropriate amount of vitamin E-TPGS was dissolved in a pH 7 phosphate buffer with ionic strength 0.15 M and solutions of 0.005%, 0.01%, 0.02%, 0.04%, 0.08%, 0.2%, 0.5%, 1% and 2% (w/v) vitamin E-TPGS were prepared. Experiments were performed in a shaking water bath (Blue M Electric Co.) at 37°C. Excess amprenavir was added to each of the vitamin E-TPGS buffer solutions. The mixtures were agitated for 3 days. At the end of this period, the mixtures were filtered with 0.2-μm nylon syringe filter. The concentrations of the filtrates were assayed by HPLC.

HPLC assays were performed on a Hewlett Packard HP1100 using a Zorbax SB-Phenyl column, a UV detector at 268 nm and a linear gradient of 100% A (5% acetonitrile and 0.1% TFA in water) to 100% B (95% acetonitrile and 0.1% TFA in water) in 15 minutes. The flow rate was set at 1 mL/min and the column was kept at ambient temperature. The injection volume was 50 μL.

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Permeability Studies

Permeability of amprenavir was measured using Caco-2 cell monolayers. Caco-2 cells were cultured in minimum essential medium containing 10% fetal bovine serum and 1% nonessential amino acids on 1.0 cm^2 polycarbonate membranes of Costar Transwells™ seeded at a density of 60,000 cells/well to late confluency (20–25 days) or were cultured using the Biocoat™ (three-day) system. Culture medium was changed every two days after seeding.

Monolayers were preincubated in transport medium (Hank's balanced salt solution containing 25 mM HEPES buffer) at 37°C one hour prior to the transport experiment. Known p-gp inhibitors or Vitamin E TPGS were added to the apical (AP) compartment transport buffer during preincubation to pre-expose the monolayers to the inhibitors. Both AP and basolateral (BL) solutions were replaced with fresh transport medium, containing inhibitors and/or [^{14}C]-amprenavir (4–5 μM), where appropriate, to begin the transport experiments. Transport rates were monitored by scintillation counting of the radioactivity present in the receiver chamber after one hour. For the mannitol test of monolayer integrity in the presence of Vitamin E TPGS, transport of [^{14}C]-mannitol (2–3 μM) was monitored for two hours to simulate the timecourse for both preincubation and transport. Transport experiments were carried out under sink conditions (less than 10% transported over the duration of the experiment) and three determinations were made. Ratios of total radioactivity recovered in the receiver solution to total radioactivity added to the donor compartment were used to calculate apparent permeability coefficients (P_{app}) from the expression:

$$P_{app} = \frac{1}{AC_0}\frac{dQ}{dt} \quad (1)$$

where C_0 is the donor drug concentration at time 0, Q is the amount of drug found in the receiver solution at time t, and A is the surface area of the membrane.

RESULTS

Effect of Vitamin E-TPGS on Solubility of Amprenavir

Amprenavir is practically insoluble in a pH 7 buffer at 37°C; 0.036 ± 0.007 mg/mL (mean ± standard deviation, n = 3). The solubility at this pH and temperature remains virtually unchanged, 0.037 ± 0.004 mg/mL (mean ± standard deviation, n = 12), up to a vitamin E-TPGS concentration of 0.2 mg/mL as shown in the inset of Fig. 1. Above 0.2 mg/mL (which is the cmc of vitamin E TPGS in water (15)), the solubility of amprenavir is directly proportional to the vitamin E-TPGS concentration. At a vitamin E-TPGS concentration of 20 mg/mL, the solubility of amprenavir reaches 0.72 mg/mL, a 20-fold increase over its solubility in the plain pH 7 buffer.

The total amprenavir concentration (S_{total}) above the cmc of vitamin E-TPGS is the sum of free (S_{free}) and micelle-bound (S_{bound}) amprenavir:

$$S_{total} = S_{free} + S_{bound} \quad (2)$$

The following free solute–micelle-solubilized solute equilibrium is assumed to hold (16):

$$k_a = \frac{S_{bound}}{S_{free} \cdot (SAA)_m} \quad (3)$$

where k_a is the equilibrium distribution coefficient and $(SAA)_m$ is the concentration of vitamin E-TPGS in micelle form, which is equal to the difference between total vitamin E-TPGS concentration and the critical micelle concentration. From Eq. (2) and Eq. (3), we have

$$S_{total} = S_{free}[1 + k_a(SAA)_m] \quad (4)$$

Fitting Eq. (4) to the amprenavir solubility produces a straight line in the vitamin E-TPGS from 0.2 to 20 mg/mL. The slope of the line gives an equilibrium distribution coefficient, k_a, of 1.1 mM^{-1}.

Effect of Vitamin E-TPGS on Permeability of Amprenavir

Figure 2 shows the results of Caco-2 transport of amprenavir in the AP > BL and the BL > AP directions, in the presence and absence of P-glycoprotein (P-gp) inhibitors. Polarized transport was demonstrated in the BL > AP direction (secretory pathway), exceeding AP > BL transport (absorption pathway) by a factor of 6 in the absence of inhibitors. In the presence of 20 μM verapamil, the rate of transport in the BL > AP direction was only two-fold greater than that in the AP > BL direction, and in the presence of 0.5 μM GF120918, it is not significantly greater than one.

Figure 3 shows the effect of vitamin E-TPGS on the transport of amprenavir. Increasing vitamin E-TPGS concentration resulted in increasing AP > BL permeability and decreasing BL > AP permeability. The limiting permeability of amprenavir was achieved between 0.02 mg/mL and the cmc of vitamin E-TPGS (0.2 mg/mL). At the cmc, the apparent permeability in the AP > BL direction was 12.2 ± 1.4 × 10^{-6} cm/sec, a 3-fold increase over the permeability in the absence of P-gp inhibitors. Further increase in vitamin E-TPGS concentration above the cmc resulted in a decrease in apparent permeability of amprenavir.

Table 1 shows the effect of vitamin E-TPGS on the AP > BL transport of [^{14}C]-mannitol permeability. In the interest of time, the experiment was carried out with Biocoat™-grown three-day monolayers. The Biocoat™-grown monolayers were validated in house and gave the same results as the 21-day culture in inhibition experiments except that P-gp expression was not as great in the three-day culture. Table 1 shows that, even at higher concentrations of TPGS (2mg/mL), mannitol permeability was not different from control, suggesting that vitamin E-TPGS in the range of 0.0002–2 mg/mL do no functional damage to the cell monolayers.

The reduction in AP > BL apparent permeability above the cmc (Fig. 3) can be explained by thermodynamic activity (12). Below the cmc of vitamin E-TPGS, the apical compartment consists entirely of free drug molecules and free vitamin E TPGS molecules. Above the cmc there is significant interaction between vitamin E TPGS molecules and amprenavir, which decreases the concentration of free amprenavir molecules in the apical solution. At 2 mg/mL vitamin E-TPGS, the concentration of free amprenavir was about 2.2 μM compared to the total initial concentration of 5 μM. Since apparent permeability is calculated from the total initial concentration (Eq. (1)), the

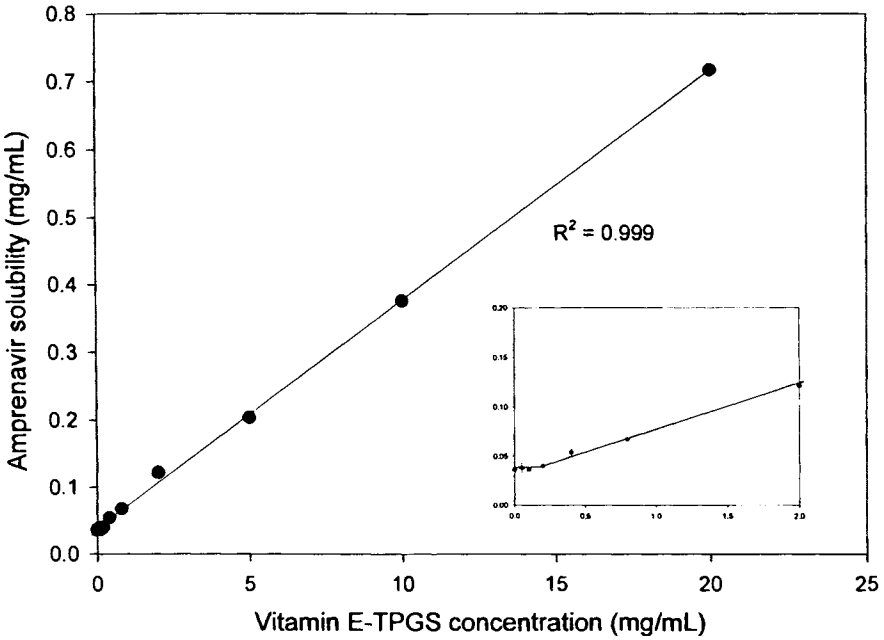

Fig. 1. Amprenavir solubility as a function of vitamin E-TPGS concentration in pH 7 phosphate buffer, ionic strength 0.15M. The cmc of vitamin E-TPGS was determined to be 0.2 mg/mL from the inset figure. The solubility of amprenavir was unchanged below the cmc and increased linearly with increasing vitamin E-TPGS concentration above the cmc. The data point and error bars represent the mean ± SD of three replicates.

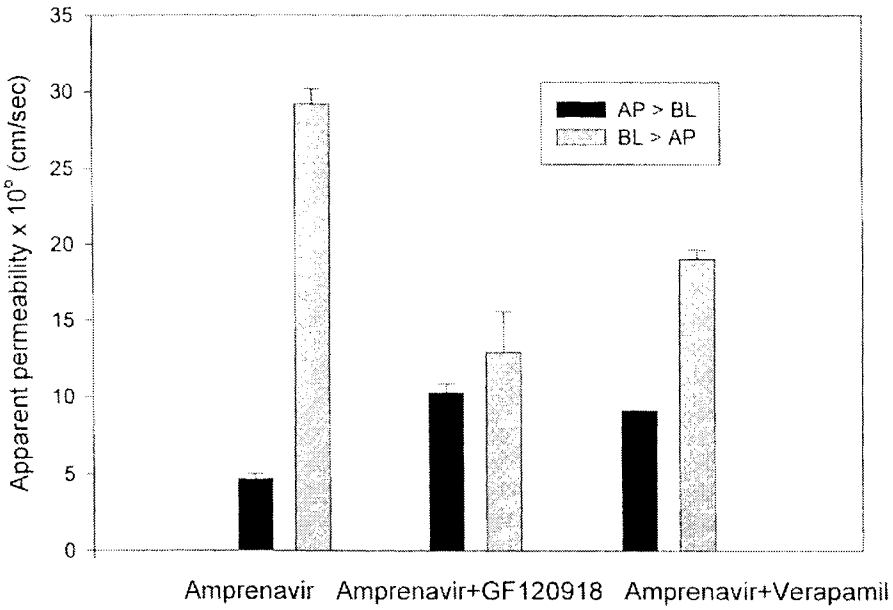

Fig. 2. Amprenavir permeability across Caco-2 cell monolayers in the presence and absence of P-gp inhibitors GF120918 (0.5 μM) and verapamil (20 μM). BL > AP transport of amprenavir was, on average, 6-fold higher than AP > BL transport in the absence of inhibitors, suggesting that amprenavir is a substrate of an efflux system. The effux system was inhibited by GF120918 and verapamil. The data point and error bars represent the mean ± SD of three replicates.

reduction in apparent permeability can be explained by the reduced concentration of free amprenavir. For instance, when the concentration of vitamin E-TPGS was 2 mg/mL, the apparent permeability was reduced to 6.5 ± 0.4 × 10^{-6} cm/sec. From Eq. (4), we have

$$P_{int} = [1 + k_a(SAA)_m]P_{app} = 15.0 \pm 0.9 \times 10^{-6}\ cm/sec$$

which is slightly higher than the apparent permeability at the cmc of vitamin E TPGS, 12.2 ± 1.4 × 10^{-6} cm/sec. Thus, the intrinsic permeability, P_{int}, remains unchanged.

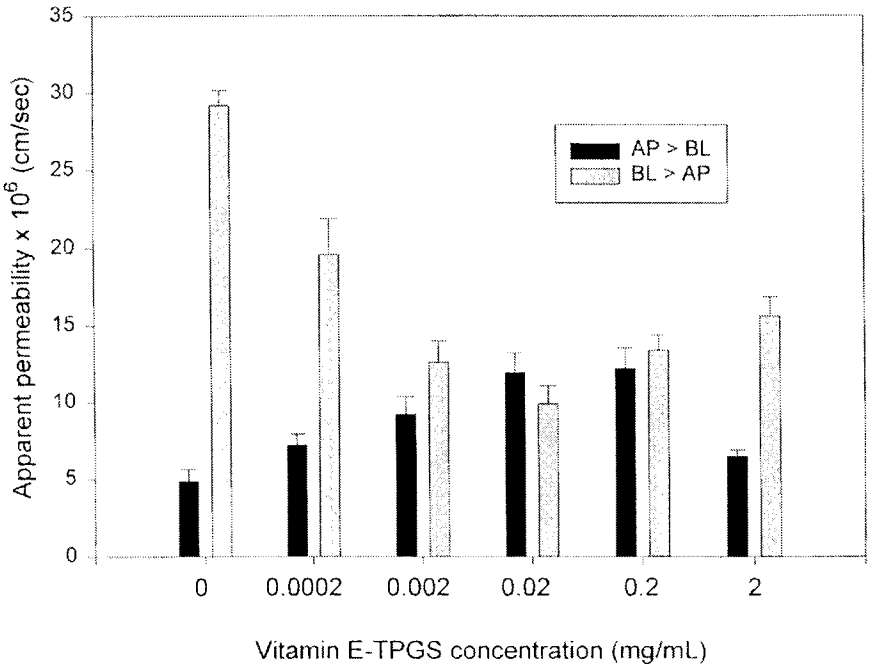

Fig. 3. Effect of vitamin E-TPGS on transport of amprenavir. The data point and error bars represent the mean ± SD.

ıble 1. Effect of Vitamin E-TPGS on the AP > BL Transport of Mannitol

Vitamin E-TPGS concentration (mg/mL)	$P_{app} \times 10^6$ (cm/sec) of mannitol*
0	1.22 (0.08)
0.0002	1.23 (0.14)
0.002	0.89 (0.04)
0.02	1.19 (0.47)
0.2	1.08 (0.3)
2	1.15 (0.38)

The values in parentheses represent standard deviation from three experiments.

ffect of Vitamin E-TPGS on Absorption Flux of mprenavir

The amount of drug absorbed can be expressed in terms absorption flux, a product of permeability and intestinal ıncentration (17):

$$J = P_{eff} \cdot C_{lumen} \quad (5)$$

here J is the absorption flux, P_{eff} is the effective permeability, ıd C_{lumen} is the concentration of the drug in the intestinal men. Total drug absorbed is then the integral of absorption ux over intestinal surface area and absorption time. Eq. (5) ıggests that for poorly soluble compounds, neither solubility ır permeability alone seems sufficient to explain in vivo phe- ımena. Unfortunately, the literature has yet given a full atten- ın to this important parameter. For a poorly soluble compound, e intestinal lumen concentration may be replaced by its solu- lity as a rough estimate.

Figure 4 shows the increase in absorption flux of amprenavir with increase in vitamin E-TPGS concentration. Absorption flux increases rapidly at low vitamin E-TPGS concentrations but begins to level off at higher concentrations. At 0.05 mg/mL vitamin E-TPGS, the absorption flux is increased from 0.17 $\times 10^{-6}$ mg/cm^2/sec to 0.38 $\times 10^{-6}$ mg/cm^2/sec, about a 2-fold increase; and at 2 mg/mL, the absorption flux is 0.79 $\times 10^{-6}$ mg/cm^2/sec, about a 4 to 5-fold increase.

DISCUSSION

From the solubility measurement, the cmc of vitamin E-TPGS at 37°C was determined to be approximately 0.2 mg/mL which is quite similar to the literature values of 0.2 mg/mL (4) and 0.2–0.4 mg/mL (14) from surface tension measurements. Below the cmc, vitamin E-TPGS has no effect on the solubility of amprenavir but above the cmc, the solubility linearly increases with vitamin E-TPGS concentration. This is in agreement with literature results for cyclosporin (14) and the classical micelle solubilization theory (15). From the linear regression in Fig. 1, approximately 10 molecules of vitamin E-TPGS are needed to solubilize one amprenavir molecule.

Amprenavir showed polarized transport across Caco-2 cells; BL > AP transport rates were approximately 6-fold higher than AP > BL rates. Two other protease inhibitors, saquinavir and ritonavir, have exhibited polarized apical efflux (9,10). It was also demonstrated that P-gp limits oral drug absorption and brain entry of indinavir, nelfinavir, and saquinavir (8). Secretory transport through Caco-2 cells was shown to be highly temperature-dependent, implicating the involvement of an active energy-dependent transport system. Furthermore, both verapamil and GF120918, two known inhibitors of P-gp, increased the net absorption of amprenavir by decreasing efflux.

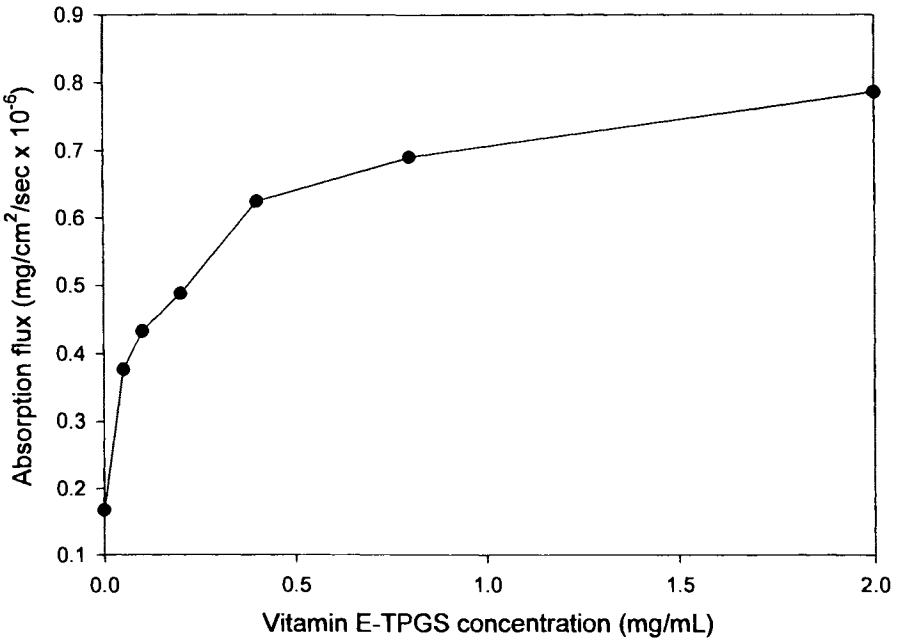

Fig. 4. Amprenavir absorption flux as a function of vitamin E-TPGS concentration. At low vitamin E-TPGS concentrations, absorption flux increases rapidly with increase in vitamin E-TPGS; above 0.5 mg/mL vitamin E-TPGS the rate of increase levels off.

These results indicate that amprenavir is a substrate of an active efflux system present in Caco-2 cells, most likely P-gp (9,10).

Despite the fact that vitamin E-TPGS increases the bioavailability of cyclosporin, no literature reports have disclosed the effect of vitamin E-TPGS on permeability. Our results show that vitamin E-TPGS is an effective efflux inhibitor. Nearly complete inhibition was achieved at a concentration 10-fold below the cmc, suggesting that vitamin E-TPGS is a potent inhibitor. These findings are similar to those reported for polysorbate 80 and cremophore EL for which the convergence concentrations are well below or close to their respective cmcs (11–13). Above the cmc, the apparent permeability of amprenavir tends to decrease with increasing concentration of vitamin E-TPGS, but intrinsic permeability does not. Overall, the vitamin E-TPGS data support the conclusion that only monomer surfactant molecules are involved in the inhibition of the polarized efflux pump (12).

Vitamin E-TPGS has been used as a vitamin E supplement for treatment of vitamin E deficiency in children (5). The acute oral LD50 of vitamin E-TPGS is over 7000 mg/kg for young adult rats (4). In clinical studies, vitamin E-TPGS is used at 2256 mg twice daily. Assuming a stomach volume of 250 mL, the vitamin E-TPGS concentration *in vivo* is as high as 9 mg/mL. Thus, the concentrations in our Caco-2 cell studies were relatively low and should not cause cell damage. The in vitro results with BioCoat-grown Caco-2 cell monolayers further support the in vivo observation.

Absorption flux increases with increasing vitamin E-TPGS concentration although the improvement at high concentration is diminished compared to that at low concentration. The improvement is very significant since the absolute bioavailability of amprenavir in conventional capsule or tablet formulations is zero (3). The softgel formulation containing 20% vitamin E-TPGS gives 69 ± 8% absolute bioavailability in beagle dogs after a 25 mg/kg dose (S. Studenbery et al. unpublished results). Increasing vitamin E-TPGS in the formulation from 20% to 50%, improved the absolute bioavailability from 69 ± 8% to 80 ± 16%. Thus, these observations in vivo are in agreement with in vitro results, as shown in Fig. 4. It seems that the absorption flux (Fig. 4) is better correlated to the in vivo results than solubility (Fig. 1) or permeability (Fig. 3).

CONCLUSIONS

Vitamin E-TPGS significantly improved the solubility of an HIV protease inhibitor, amprenavir, through micelle solubilization. At a concentration of 20 mg/mL vitamin E-TPGS, the enhancement is about 20-fold. The cmc from the solubility measurement is 0.2 mg/mL. The transport results in Caco-2 cells suggest that amprenavir is a substrate of an efflux mechanism, most likely P-glycoprotein, which acts as a counter-transporter. Vitamin E-TPGS inhibits the efflux system and enhances the permeability of amprenavir. As a result, vitamin E-TPGS enhances the absorption flux of amprenavir by increasing its solubility and permeability. The enhancement is essential to the development of a novel soft gelatin capsule formulation of amprenavir for use in the clinic.

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