

Absorption Rate Limit Considerations for Oral Phosphate Prodrugs

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Purpose. To evaluate the potential of phosphate ester prodrugs to significantly improve the absorptive flux of poorly soluble parent drugs.

Methods. Absorptive transport studies of parent drugs and their prodrugs were carried out in Caco-2 cells. Prodrugs of parent drugs with variable aqueous solubilities were tested: Hydrocortisone-phosphate/Hydrocortisone, Fosphenytoin/phenytoin, TAT-59/DP-TAT-59, and Entacapone phosphate/Entacapone. Additional absorption studies were carried out in rats.

Results. Absorptive fluxes of DP-TAT-59 and phenytoin increased 9.8 or 3.3-fold after dosing TAT-59 and 500 μ M fosphenytoin, respectively. Hydrocortisone's flux did not increase with hydrocortisone-phosphate at 100 μ M. Permeability of the highly lipophilic and protein bound compound, DP-TAT-59, was significantly increased with serosal albumin. No permeability increase was observed for the other drugs with albumin. Entacapone phosphate failed to improve the flux of entacapone compared to an entacapone solution, but the prodrug solution did yield higher entacapone plasma levels in rats when compared with an entacapone suspension.

Conclusion. Ideal phosphate prodrug candidates are characterized by high permeability and low solubility (BCS Class II drugs). For low dose BCS Class II drug candidates, however, no biopharmaceutical advantage may be gained. Phosphate prodrugs of parent drugs with limited permeability may fail. When screening highly lipophilic parent drugs transport studies should be done with albumin.

KEY WORDS: absorption; caco-2; permeability; phosphate ester prodrug; protein effects.

INTRODUCTION

Phosphate prodrugs are often used to develop injectable formulations of poorly soluble parent drugs and many are

marketed as injectable dosage forms (1). Following parenteral administration, the prodrugs are enzymatically cleaved by endogenous alkaline phosphatases yielding the parent drug (1,2). Examples for these "solubility-prodrugs" include Fosphenytoin (Cerebryx®, Pfizer) (3), for the treatment of status epilepticus, prodrugs of glucocorticoids such as dexamethasone (Decadron-Phosphate®) (4), and Etoposide-phosphate (Etopophos®, Bristol-Myers Squibb) for the treatment of lung cancer (5).

However, very few oral phosphate prodrugs have made it to the market place in spite of favorable dissolution characteristics and their inherent chemical stability (6). Estramustine phosphate (EMP), which is available in both intravenous and oral formulations (7), has been on the market in Europe and the United States as Emcyt® (Pharmacia) for the treatment of prostate cancer. Prednisolone phosphate is available as a liquid formulation (Pediapred®, Celltech). Monofluorophosphate (Monocal®, Mericon) is not a "solubility-prodrug" but provides an oral dosage that shows less gastrointestinal toxicity than sodium fluoride (8). An antiviral agent and prodrug of fludarabine, fludarabine phosphate (Fludara®, Berlex, Schering) (9), was recently approved in Europe as an oral dosage form and Fosamprenavir (VX-175/GW433908), a phosphate ester prodrug of the HIV protease inhibitor amprenavir, is being developed by GlaxoSmithKline in an oral dosage form to treat HIV infection in adults and children (10).

Some oral prodrugs fail because of "lack of offering a clinically relevant benefit" over the parent drug, such as oral etoposide (11). Others fail because they do not improve rate or extent of absorption of the parent drug in animal models, such as LY307853 (12), or fail due to poor enzymatic bioconversion, such as phosphate esters of taxol (13). The surprising inability to use phosphate prodrugs by the oral route prompted a study in a system being used to screen drug candidates for absorption potential. In Caco-2 intestinal absorption screening systems the drug flux (i.e., the amount of drug passively absorbed per time per area (14)) is a product of drug permeability and mucosal solution concentration. Since this monolayer provides mucosal alkaline phosphatase activity similar to that of normal human intestine, limited absorption caused by low parent drug permeability can be clearly demonstrated.

The purpose of this study is to determine whether phosphate prodrugs can significantly improve parent drug absorption flux across Caco-2 cell monolayers. The findings suggest which drugs are the most ideal candidates for a phosphate prodrug strategy. Phosphate prodrugs of parent drugs with varying physico-chemical parameters were chosen for evaluation in this study (Fig. 1). TAT-59 is an unusually lipophilic, zwitterionic, phosphate prodrug of the extremely insoluble DP-TAT-59 (BCS Class II) (15). Fosphenytoin is a conventional dianionic prodrug of the poorly water-soluble phenytoin (BCS Class II), a lipophilic compound with slow yet complete absorption. Entacapone phosphate is a prodrug of the weak acid entacapone, a hydrophilic parent drug with intermediate solubility, which is soluble at intestinal pH, but is insoluble at gastric pH (BCS Class IV). Hydrocortisone phosphate was chosen as a prodrug model compound with no advantages from an oral delivery standpoint, since it is the

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ABBREVIATIONS: BCS, Biopharmaceutical Classification Scheme; DP-TAT-59, dephosphorylated TAT-59; DPH 5, 5-diphenylhydantoin phenytoin; DMEM, Dulbecco's modified Eagles medium; FBS, fetal bovine serum; FDPH, Fosphenytoin; HBSS, Hank's balanced salt solution; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); HSA, Human serum albumin; MES, 2-(N-morpholino)ethanesulfonic acid; Tam, tamoxifen; TAT-59, miproxi-fene phosphate ester prodrug.

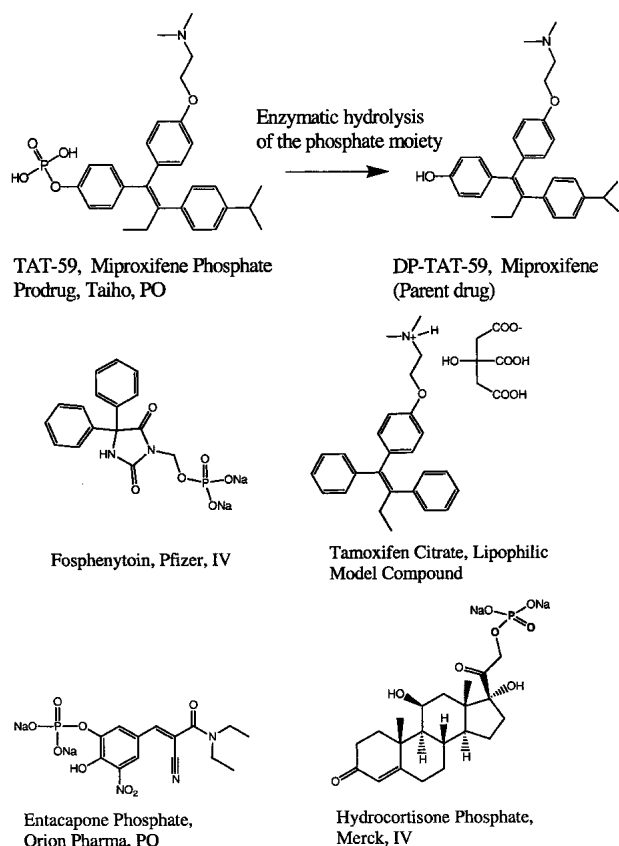


Fig. 1. Structure of phosphate ester prodrugs. TAT-59 is provided as the free acid, while the other prodrugs are disodium salts. In presence of alkaline phosphatase the phosphate group is hydrolyzed to yield the corresponding alcohol, or parent drug. Tamoxifen was used as a reference compound.

prodrug of the soluble and permeable parent drug hydrocortisone (BCS Class I, at low doses). In addition, since the permeability of lipophilic parent drugs can be underestimated in Caco-2 cells because of cell retention and non-specific binding, the influence of basolaterally added albumin was investigated (16). *In vivo* studies assessing absorption of the triphenylethylene phosphate prodrug, TAT-59, were also conducted in the rat intestinal perfusion model. Finally, the utility of a phosphate ester prodrug strategy for entacapone to increase that drugs systemic levels was evaluated in rats.

MATERIALS AND METHODS

Materials

TAT-59 and its parent drug, DP-TAT-59, were gifts of Taiho Pharmaceutical Co., Ltd. (Tokushima, Japan). Entacapone phosphate and its parent drug, entacapone, were gifts of Orion Pharma (Turku, Finland). Fosphenytoin was donated by Pfizer (formerly Parke-Davis, Ann Arbor, MI, USA). Hydrocortisone, hydrocortisone-phosphate, phenytoin and tamoxifen were obtained from Sigma-Aldrich (St. Louis, MO, USA).

^3H -metoprolol (90 Ci/mmol) was obtained from Moravек Biochemicals (Brea, CA, USA). ^3H -tamoxifen (76 Ci/mmol), ^{14}C -phenytoin (50 mCi/mmol), and ^{14}C -mannitol (51.5 mCi/mmol) were obtained from NEN Life Science

Products (Boston, MA, USA). Dulbecco's modified Eagles medium (DMEM, 4.5 g/L glucose) was purchased from CELOX Laboratories (St. Paul, MN, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals, Inc. (Norcross, GA, USA). MEM nonessential amino acids (10 mM), glutamine (200 mM), and Hank's balanced salt solution (HBSS) were purchased from Gibco BRL Life Technology (Grand Island, NY, USA). Trypsin-versene mixture containing trypsin (0.5 mg/ml) and EDTA (0.2 mg/ml) was obtained from BioWhittaker, Inc. (Walkersville, MD, USA). The Caco-2 cell line was obtained from American Type Culture Collection at passage 17 (Rockville, MD, USA). Other chemicals were obtained from either Sigma-Aldrich or Gibco BRL Life Technology.

Cell Culture

The Caco-2 cell model was chosen since they are derived from human adenocarcinoma cells and form polarized monolayers that show alkaline phosphatase levels similar to levels found in the human gut (17). Caco-2 cells were maintained in T125 flasks and seeded at 70–80% confluency on 24-well Multiwell Plates®, BD BioSciences (Bedford, MA, USA) or Costar® 24-well plates (Cambridge, MA, USA) at the seeding density of 75000 cells per 0.3 cm² transwell. The cell plates were maintained at 37°C with 5% CO₂ and 95% humidity in DMEM supplemented with 10% Fetal bovine serum, 1% non-essential amino acids and 1% L-glutamine. Media was changed every 48–56 h and cells used in this study were at passages 22–49. Cell plates used were between 15 and 25 days post seeding. Caco-2 monolayers formed under these conditions typically yielded electrical resistance values of 600–1200 Ω·cm².

Caco-2 Transport Experiments

The apparent permeability of radiolabeled compounds across Caco-2 cell monolayers was determined using 24-well Caco-2 plates. In permeability experiments for all drugs except TAT-59 and DP-TAT-59, HEPES-HBSS buffer (1.8 mM CaCl₂, 5.37 mM KCl, 0.44 mM KH₂PO₄, 0.49 mM MgCl₂, 0.41 mM MgSO₄, 136.89 mM NaCl, 4.17 mM NaHCO₃, 3.38 mM Na₂HPO₄, 5.55 mM D-glucose, and 5 mM HEPES, pH 7.4) was used in both mucosal and serosal chambers. Due to TAT-59's limited solubility in the presence of free calcium, pH 7.4 HEPES buffer devoid of calcium was used on the apical side. Since DP-TAT-59 is poorly soluble at pH 7.4, permeability experiments were carried out at pH 6 using MES buffer (1.8 mM CaCl₂, 5 mM KCl, 120 mM NaCl, 10 mM D-glucose, and 10 mM MES) and HEPES buffer (1.8 mM CaCl₂, 5 mM KCl, 120 mM NaCl, 10 mM D-glucose, and 10 mM HEPES, pH 7.4) in the apical and basolateral sides, respectively. Prior to experiments, culture medium was replaced with the respective buffers that had been warmed at 37°C, and the TEER values were measured. The experiment was initiated by adding 0.2 mL drug solution to the apical donor chamber and 1 mL respective buffer to the receiver chamber. ^{14}C -Mannitol (1.85 μM) or ^3H -metoprolol (4 nM) was used as cell monolayer integrity and passive permeability markers, respectively. Transport experiments were carried out at 37°C in either the apical-to-basolateral (A-to-B) or the B-to-A direction (unless otherwise stated). For DP-TAT-59 studies, ethanol or DMSO

was used as a co-solvent at a final concentration of less than 2% (v/v). One hundred fifty microliter aliquots were sampled and replenished from the receiver chambers at several time points (30, 60, 90, or 120 min; or 0, 30, 60, 120, 150, 180 min). Twenty microliter donor aliquots were minimally taken at 0 and 120 or 0 and 150 min. The radioactivity associated with the samples was determined by scintillation counting using a Packard Topcount counter.

The apparent permeability coefficient (P_{app}) of parent drugs was calculated using the following equation:

$$P_{app} = \frac{1}{A \cdot C_D(0)} \cdot \frac{dM_R}{dt}$$

where A is the surface area of the cell monolayer, $C_D(0)$ is the initial concentration of parent drug in the donor chamber, and dM_R/dt is the linear appearance rate of parent drug mass in the receiver chamber.

To compare parent drugs and prodrugs, the absorptive (A-to-B) flux of the parent drug instead of P_{app} was calculated, since $C_D(0)$ for the permeable parent drug is not constant during the experiment. Since no significant amount of intact phosphate prodrugs can be detected in the basolateral receiver chambers the transepithelial flux of parent drugs (J_{A-B}) expressed as $\text{nmol}/\text{cm}^2/\text{min}$, was calculated according to the following equation:

$$J_{A-B} = \frac{1}{A} \frac{dM_R}{dt}$$

J_{A-B} was determined as a function of the basolateral receiver's protein concentration ranging from 0 to 4% HSA at pH 7.4. Furthermore, the dosing concentration of parent drug was varied by either dosing the parent drug directly or by prodrug dosing. Cellular retention studies were carried out as described by Aungst (16).

Rat Jejunal Perfusion Experiments of TAT-59, DP-TAT-59 and Hydrocortisone

Animal studies were carried out consistent with the guidelines set by the National Institutes of Health (Publication 85-23, revised 1985). The perfusion studies were carried out as previously reported (18,19) in fasted male Sprague-Dawley rats weighing approximately 300–400 g. Propranolol was added to drug solutions as a high permeability marker. Relative prodrug loss from the perfusate was measured by a reverse phase HPLC assay that utilizes an ion-pairing reagent that allowed detection of both parent and prodrug. The effective permeability (P_{eff}) measured by intestinal perfusion was based on the loss of drug from the perfusate

$$P_{eff} = \left(-\frac{Q}{2\pi rL} \right) \left(\ln \frac{C'_{out}}{C_{in}} \right)$$

where Q is the perfusate flow rate through the segment (0.14 ml/min), r is the radius of the segment (0.2 cm), L is the length of the perfused segment (10 cm), and C_{in} is the drug or prodrug concentration of the perfusate entering the intestinal segment. C'_{out} is the remaining prodrug and parent drug concentration in the exiting perfusate, (C_{out}), corrected for water transport by using the gravimetry method.

Entacapone and Entacapone Phosphate Pharmacokinetics in Rats

Male Han/Wistar rats (National Laboratory Animal Center, Kuopio, Finland), weighing 180–300 g ($n = 44$) were fasted 24 h before the experiment. Tap water was available *ad libitum*. Animals were decapitated 0, 0.5, 1, 2, 4 or 6 h after oral administration of 1.867×10^{-5} mol/kg entacapone parent drug (equivalent to 5.7 mg/kg) as a clear solution (pH 7.4) or as its phosphate prodrug solution (pH 7.4). All procedures with animals were reviewed and approved by the Animal Ethics Committee at the University of Kuopio.

Blood was collected into tubes containing disodium-EDTA. Plasma was separated by centrifugation for 10 min at 1500 g at +4°C and samples were stored at -70°C until analyzed. Plasma entacapone concentrations were determined according to Savolainen *et al.* (20). Area under concentration-time curve values ($\text{ng}/\text{ml} \times \text{h}$) from 0 to 2 h and from 0 to 6 h were calculated by Graph Pad Prism 3.02 software (Graph Pad Software, San Diego, California, USA) using the trapezoid rule. The relative bioavailabilities (F_{rel}) of entacapone phosphate (pH 7.4) and entacapone suspension (pH 3.0) were calculated by equation

$$F_{rel} = \left(\frac{AUC_A}{AUC_B} \right) \left(\frac{Dose_B}{Dose_A} \right)$$

where A refers to entacapone phosphate solution or entacapone suspension and B to entacapone solution. The AUC_{0-2h} value of entacapone suspension was calculated from earlier published data (20). In the case of entacapone phosphate, only plasma entacapone concentrations were used for calculations (intact entacapone phosphate was detected only in three samples).

RESULTS

TAT-59 and DP-TAT-59 Transport across Caco-2 Cell Monolayers

The flux of DP-TAT-59 was found to be dependent on basolateral protein concentration as well as on parent and prodrug dosing concentrations. At 20 μM concentration of DP-TAT-59, the P_{app} was below that of the non-permeable marker mannitol in the absence of albumin [$0.096 (\pm 0.03) \times 10^{-6}$ cm/s], while this triphenylethylene derivative showed a higher permeability than the high permeability marker metoprolol in the presence of 4% albumin [$32.6 (\pm 6.5) \times 10^{-6}$ cm/s] (Table I). P_{app} also increased in the presence of lower levels of albumin and was $2.6 (\pm 0.7) \times 10^{-6}$ cm/s and $14 (\pm 2.6) \times 10^{-6}$ cm/s, in presence of 0.5% and 1% HSA, respectively. The absorptive flux of DP-TAT-59 is lower than that of TAT-59 at equimolar dosing of prodrug and parent drug (Fig. 2A). At 100 μM , dosing TAT-59 increased the absorptive flux of DP-TAT-59 [$9.09 (\pm 3.9)$ nM/cm²/min] by nearly 10-fold, which is characteristic of the increased solubility achieved by the prodrug approach [$89.39 (\pm 36.4)$ nM/cm²/min] (Table II). On the contrary, at 10 μM dosing, no pronounced difference in flux was observed. The likely reason for this observation is that poor aqueous solubility of DP-TAT-59 limits its absorptive flux. DP-TAT-59 is extremely insoluble in pH 7.4 buffer (0.1 μM) and is still insoluble even at pH 6.0 (9.3 μM) (15). Thus, when DP-TAT-59 is dosed as a suspension at 100 μM ,

Table I. Effect of Protein Added to Basolateral Receiver Chambers on A-B Permeability of Parent Drugs and Reference Compounds

Parent drug	C _S (mg/ml)	P _{app, A-B} × 10 ⁶ cm/s, 0% HSA	P _{app, A-B} × 10 ⁶ cm/s, 4% HSA
DP-TAT-59 ^a	0.00005	0.096 ± 0.03 ^b	32.6 ± 6.5 ^b
Phenytoin	0.02	33.49 ± 1.18	29.9 ± 2.92
Entacapone	1.75	1.1 ± 0.05	0.32 ± 0.5 ^c
Hydrocortisone	0.28	17.49 ± 4.13	16.7 ± 1.2
LY303366	0.005	3.5 ± 1.10 ^d	NA
Metoprolol (Reference)	NA	22.9 ± 2.75	20.6 ± 3.8
Mannitol (Reference)	NA	1.1 ± 0.3	0.9 ± 0.26

Note: Data are presented as the mean ± SD, of 3–6 determinations. C_S: Minimal aqueous solubility at neutral pH from references (3,15,29). NA: not available, compounds are freely soluble.

^a This experiment was performed at pH 6.0 and 7.4 in the apical donor and basolateral receiver chambers, respectively.

^b Significantly different from the respective control ($p < 0.001$).

^c 1% HSA.

^d From Li et al. (37).

its solution concentration is still limited by its solubility near 10 μM, and therefore the observed flux remains low. The prodrug TAT-59 however, is soluble up to 100 μM when dosed at pH 7.4, which on apical dephosphorylation may yield supersaturated DP-TAT-59 solutions and supersaturation ratios near 60 have been observed (15).

Polarized permeation of TAT-59 across Caco-2 cell monolayers was observed at 100 μM concentration with a J_{A-B} of 89.39 ± 36.4 compared to J_{B-A} 8.18 ± 0.92 nmole/cm²/min (Table II). This 11-fold higher A-to-B permeability of TAT-59 suggests that the prodrug is rapidly dephosphorylated by apical alkaline phosphatase to yield the non-charged DP-TAT-59, which is then absorbed. The unexpected, high J_{B-A} flux after basolateral prodrug dosing is apparently the result of TAT-59's physicochemical properties. TAT-59 is an unusually lipophilic phosphate ester, and its anionic charge is masked, since it possesses a basic alkylamino side chain. At intestinal pH, TAT-59 can exist up to nearly 50% as a neutral zwitterion with the remaining fraction being the net-monoanion. Even though the net-monoanionic form predominates at basolateral pH, the prodrug exists in significant amounts as a zwitterion. The zwitterionic TAT-59 is responsible for the unusually high log D when compared to other phosphates, of 1.2 at pH 6.5 and 0.08 at pH 7.4 (Table III), respectively (15). For DP-TAT-59 a smaller asymmetry with J_{B-A}/J_{A-B} near 3.0 and 4.3 was observed for 10 μM and 100 μM, respectively (Table II). This effect can be explained by the pH asymmetry often observed with weak bases when the apical pH used in the transport studies is lower than that of the basolateral pH (21) or by involvement of an efflux system.

Entacapone Phosphate and Entacapone Flux across Caco-2 Cell Monolayers

Entacapone was found to be poorly absorbed in Caco-2 cells [$1.1 (\pm 0.3) \times 10^{-6}$ cm/s] and its P_{app} was not increased in the presence of basolateral albumin (Table I). Furthermore, the prodrug of entacapone did not improve the absorptive flux over that of entacapone, which was low compared to

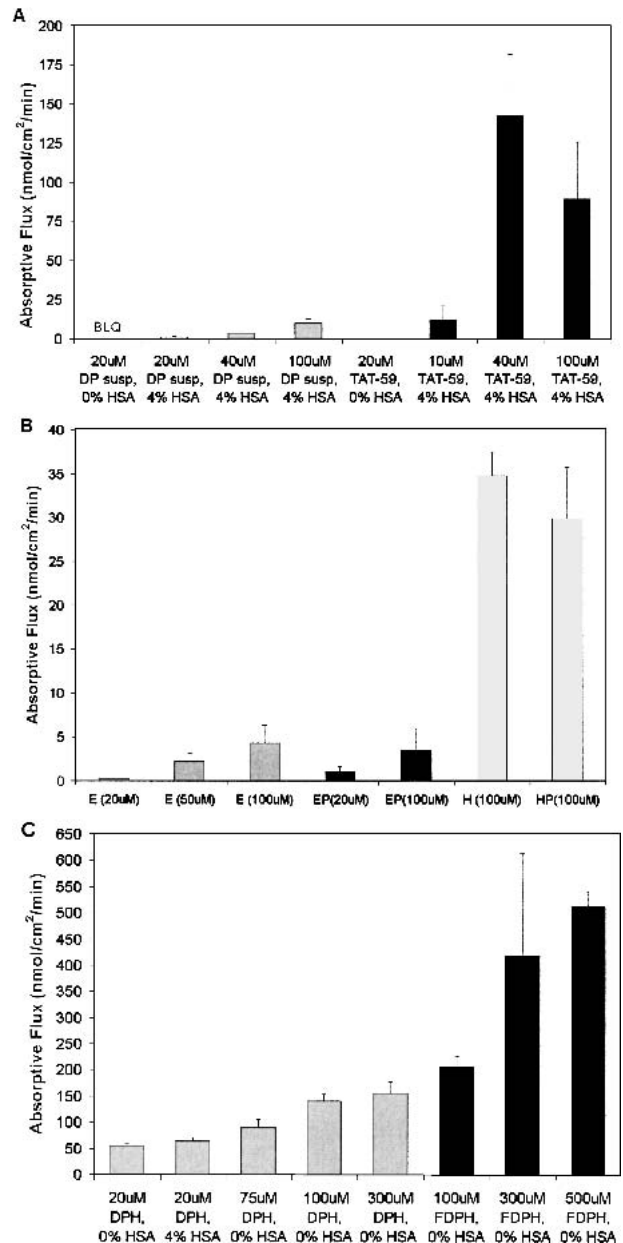


Fig. 2. Absorptive fluxes of parent drugs across Caco-2 cells measured after dosing parent drugs and their corresponding phosphate prodrugs. The A-to-B parent drug fluxes were determined as a function of basolateral human serum albumin [HSA] concentration and drug concentration. (A) Parent Drug DP-TAT-59, dosed as a suspension, and Prodrug TAT-59; (B) Parent drugs Entacapone [E] and Hydrocortisone [H], and their corresponding prodrugs, Entacapone phosphate [EP], Hydrocortisone-Phosphate [HP], respectively; (C) Parent drug phenytoin [DPH] and prodrug fosphenytoin [FDPH]. Each point represents the mean (\pm SD.) of 3–8 determinations.

those of the other parent drugs such as hydrocortisone (Fig. 2B).

Hydrocortisone Phosphate and Hydrocortisone Flux across Caco-2 Cell Monolayers

No significant differences in hydrocortisone flux were seen for hydrocortisone-phosphate and hydrocortisone when the compounds were dosed below the solubility of the parent

Table II. Absorptive and Exsorptive Flux for TAT-59 and DP-TAT-50^a

Compound	J _{app, A-B} , nmol/cm ² /min	J _{app, B-A} , nmol/cm ² /min
TAT-59 (10 μM)	12.3 ± 8.79	5.15 ± 3.64
DP-TAT-59 (10 μM) ^b	7.94 ± 3.45	23.55 ± 5.7 ^d
TAT-59 (100 μM) ^c	89.39 ± 36.4 ^d	8.18 ± 0.92
DP-TAT-59 (100 μM) ^b	9.09 ± 3.9	39.09 ± 16.36 ^d

^a This experiment was performed at pH 7.4 in both the apical and basolateral chambers for TAT-59 due to the prodrug's low solubility at pH 6.5. For DP-TAT-59, apical donor solutions with pH 6.0 were employed, due to DP-TAT-59's low solubility at pH 7.4 in absence of HSA. Basolateral DP-TAT-59 solutions were at pH 7.4 and contained 4% HSA. Data are presented as the mean ± S.D. of 3–6 determinations with Caco-2 monolayers.

^b Concentrations are not solution concentrations but are those of the dosed suspensions due to the very low solubility of DP-TAT-59 at neutral pH.

^c The flux is determined by the appearance of the permeable parent drug, DP-TAT-59 on the receiver side; TAT-59 concentration in receiver side is negligible.

^d Significantly different from the respective parent control and B-A control ($p < 0.01$).

drug (near 100 μM) and absorptive fluxes were consistent with high intestinal permeability (Fig. 2B). An increase in membrane permeability with the phosphate prodrug had been previously reported in rat jejunum and while a higher C_{max} and earlier t_{max} was observed with oral administration of the prodrug in dogs, oral bioavailability was lower with the prodrug as compared to the parent steroid (22).

Fosphenytoin and Phenytoin Fluxes across Caco-2 Cell Monolayers

Phenytoin's flux increased 2.7-fold to 3.3-fold after high dosing concentrations of fosphenytoin of 300 and 500 μM, respectively (Fig. 2C). The parent drug is regenerated rapidly, yet variably in apical donor cells, with an apparent half-life $t_{1/2}$ of 27.7 ± 13.8 min. (data not shown) suggesting that dephosphorylation is complete within typical human small intestinal residence times of 3–4 h. No precipitation or turbidity of donor solutions was observed during the duration of transport experiments.

TAT-59 and DP-TAT-59 Rat Jejunal Permeability

In the rat model DP-TAT-59 is well absorbed after jejunal dosing of TAT-59 and DP-TAT-59, compared to high permeability markers hydrocortisone and propranolol (Fig. 3). The results are in agreement with previous work (23). After prodrug dosing, the dephosphorylation is catalyzed by alkaline phosphatase and DP-TAT-59 is reportedly absorbed (23). However, after 100 μM dosing of TAT-59 the P_{eff} is not significantly reduced in the presence of L-phenylalanine (L-Phe) an alkaline phosphatase inhibitor, suggesting possible absorption of TAT-59 or inefficient inhibition.

Entacapone and Entacapone Phosphate Pharmacokinetics in Rats

Plasma entacapone levels were slightly lower after oral administration of entacapone phosphate solution (pH 7.4)

than after entacapone solution (pH 7.4) but higher than after entacapone suspension (pH 3.0), as is shown in Fig. 4. The AUC_{0-2h} values of entacapone after administration of entacapone solution, entacapone phosphate solution and entacapone suspension were 1728, 1149, and 475 ng/ml × h, respectively. Correspondingly, the plasma entacapone concentrations at 0.5 h after dosing were 2017 ± 207, 1111 ± 298, and 500 ± 75 ng/ml (mean ± SEM; $n = 4 \times 15$). The AUC_{0-6h} values of entacapone solution and entacapone phosphate were 2616 ng/ml × h and 1586 ng/ml × h, respectively. The relative bioavailabilities of entacapone phosphate and entacapone suspension were 66% and 28% (entacapone solution as reference dosage).

Effect of Lipophilicity and Protein Binding on the Apparent Permeability of Parent Drugs in Caco-2 Cells

The drug flux or permeability of lipophilic compounds can be underestimated in Caco-2 cells for highly lipophilic or protein bound compounds due to cellular retention or non-specific binding (16,24). This has been previously demonstrated for tamoxifen which is in the same chemical and therapeutic class as TAT-59. P_{app} (A-B) of highly lipophilic and protein bound compounds, DP-TAT-59, TAT-59 and tamoxifen were significantly increased in the presence of basolateral albumin (Table I). This was not the case for hydrocortisone, phenytoin, and entacapone which are less lipophilic and not as highly protein bound compared to TAT-59 (Tables I, III). Furthermore, 4% albumin significantly increased the release of DP-TAT-59 and tamoxifen from Caco-2 cells, compared to control, but this effect was not observed for hydrocortisone and phenytoin (Fig. 5).

DISCUSSION

A phosphate prodrug approach offers several advantages when developing or formulating a poorly soluble compound for which conventional formulation approaches, such as salt selection, particle size reduction and use of solubilizing agents have failed or are impractical. Phosphate prodrugs are chemically stable, their synthesis is usually straightforward in the presence of a hydroxyl moiety (6) and the increase in solubility imparted by the dianionic phosphate group is often 2–3 orders in magnitude (3). Less use of solubilizing excipients can lead to cost reduction and a higher drug load in capsules (10), allowing for a more convenient dosage regimen. Despite these advantages very few phosphate prodrugs are developed beyond the discovery stage.

Failure of phosphate prodrugs to increase drug absorption can be attributed to several potential rate-limiting factors in the drug absorption process (25). After oral administration, phosphate ester prodrugs are dephosphorylated in the GI tract by membrane-bound alkaline phosphatase (AP). In theory, the lipophilic parent drugs generated are well absorbed compared to the polar prodrugs and drug flux is increased. The maximum flux, or amount of parent drug passively absorbed per time per area (14), can be expressed as:

$$J = P_{eff} C_s$$

where J is the absorption flux, P_{eff} is the effective permeability, and C_s is the mucosal parent drug concentration which is maximum at the drug's solubility. Maximum driving force

Table III. Albumin Effect on the A-B Permeability of Drugs across Caco-2 Cells^a

Parent or prodrug	HSA effect on A-B P_{app} or absorptive flux	$\log D_{O/B}$ ^b	Plasma protein binding ^c (%)	Bioavailability ^d (%)
Tamoxifen (Reference)	22.3-fold	5.7 ^e	>98	NA ^h
DP-TAT-59	>25-fold	>3.0 (pH 6.5)	>99.9	NA ^h
		0.08 (pH 7.4)		28.8 (rats)
TAT-59 ^f	>25-fold	1.2 (pH 6.5)	>99.9	23.8 (dogs)
Hydrocortisone	not significant	1.6	75–95, 89	96 (human)
Phenytoin	not significant	2.5	89	90 (human)
Entacapone ^g	not significant	0.18	98	29–46
Metoprolol (Marker)	not significant	1.97	10	38
Mannitol (Marker)	not significant	-3.1	10	NA ^h

^a Experiments were done with 4% HSA.

^b Distribution coefficient in octanol/water.

^{c,d} Data were compiled from the literature (15,16).

^e Calculated using ACD Lab®.

^f P_{app} is based on parent drug, since prodrug is not detected in basolateral receivers.

^g 1% HSA.

^h NA: no literature bioavailability data available.

requires a sink condition assumption that is not unreasonable for most drugs given the small amount of drug entering the basolateral compartment in the Caco-2 system. For drugs, like DP-TAT-59, for which cellular accumulation may diminish the transepithelial driving force, basolateral albumin serves to maintain sink conditions in line with *in vivo* absorption.

Phosphate ester prodrugs often exhibit an aqueous solubility that is several orders higher than that of the corresponding parent drug compounds (1,15). Following enzymatic conversion of the prodrug solution, parent drug solution concentration above the parent drug's equilibrium solubility may be achieved leading to parent drug supersaturation, or the parent drug may be solubilized in the presence of the dianionic prodrug (15) increasing the driving force for absorption. Thus, the transepithelial concentration gradient C_s , driving drug flux, can be enhanced if P_{eff} is not rate limiting to absorption.

If the prodrug hydrolysis rate is first order, the surface reaction kinetics can be lumped into the effective permeability that takes the form of a hyperbolic trigonometric function

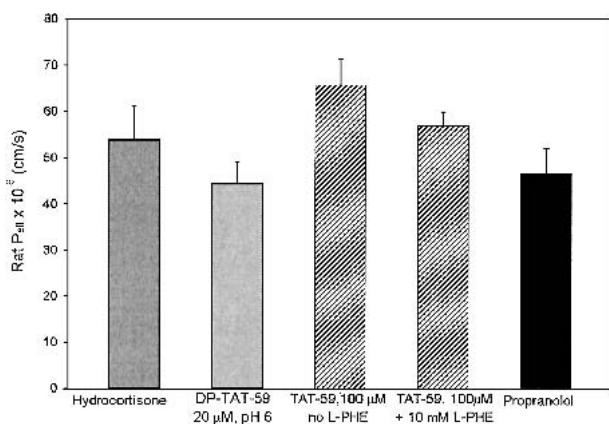


Fig. 3. DP-TAT-59 and TAT-59's Intestinal Permeability in the Rat Jejunum. The effective permeabilities P_{eff} of Parent Drugs determined in phosphate buffer in the rat intestinal perfusion model. The calculated P_{eff} after prodrug dosing is based on the loss of parent drug. Results represent mean \pm SEM from at least 4 animals.

of hydrolysis and membrane permeation parameters (26). Further, enzymatic prodrug hydrolysis may follow Michaelis-Menten kinetics as a function of prodrug concentration. If enzymatic hydrolysis controls permeation rate, enzyme kinetic parameters can be used as a transport membrane boundary condition similar to membrane permeation that is subject to carrier-mediated saturation (27).

Potential rate limits to parent drug absorption from phosphate prodrugs thus include prodrug dissolution and potential parent drug precipitation, enzymatic bioconversion to the parent drug by membrane-bound alkaline phosphatase (28) and limited intestinal permeability of the parent drug.

This study indicates that intestinal permeability of parent drugs can limit the success of a phosphate prodrug strategy. While other rate limitations are possible, phosphate esters usually increase solubility and dissolution rates and alkaline phosphatase is a non-specific enzyme that efficiently cleaves a wide range of esters including thioesters (28). This is illus-

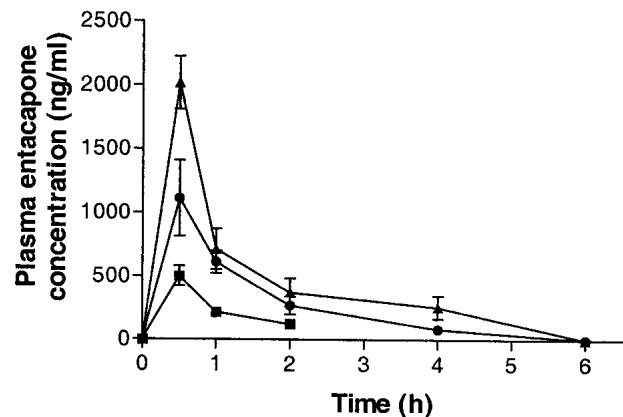


Fig. 4. Entacapone plasma levels in rats after oral administration. (▲) Entacapone dosed as a solution at pH 7.4; (●) Entacapone dosed as its prodrug, entacapone-phosphate, in solution at pH 7.4; and (■) Entacapone dosed as a suspension at pH 3. Entacapone dose was kept constant at 1.867×10^{-5} mol/kg. Each data point represents the mean \pm SEM of 4-15 determinations. Entacapone suspension data is from Savolainen *et al.* (20).

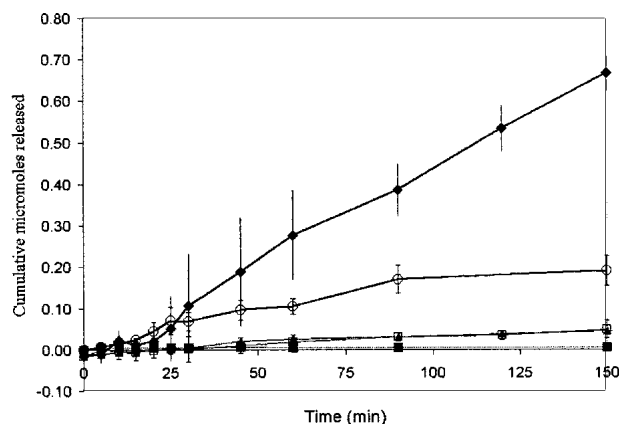


Fig. 5. Release Profiles of Drugs from Caco-2 Cell Monolayers. The release of Tamoxifen (◆) and DP-TAT-59 (○) from 24-well Caco-2 monolayers significantly increased ($p < 0.01$) in the presence of 4% HSA compared to pure buffer lacking HSA (■). No significant increase compared to a control was observed for hydrocortisone (□) and phenytoin (▲). Each data point represents the mean \pm SD of 4 determinations.

trated utilizing several phosphate ester prodrugs with varying physico-chemical properties and targeted doses, namely hydrocortisone-phosphate, fosphenytoin, TAT-59, and entacapone phosphate. While these prodrugs are rapidly dephosphorylated in the presence of alkaline phosphatase (15,23,29) their parent drugs differ in their degree of permeability-lipophilicity.

Hydrocortisone-phosphate (Hydrocortone-Phosphate®, Merck) is only available as the prodrug for parenteral use, while for oral administration the parent drug is marketed. Hydrocortisone for oral delivery (Hydrocortone®, Merck) is a low dose compound (5–20 mg) and this polar steroid has a relatively high solubility near 0.3 mg/ml at intestinal pH (15). Combined with its high P_{eff} in rats (22) (Fig. 3) and its bioavailability of 96% it can be concluded that hydrocortisone is completely absorbed at the low doses typically administered. While a prodrug would likely be bioequivalent as is the case with the steroid drug prednisolone (30), the hydrocortisone-phosphate prodrug would not offer any biopharmaceutical advantage for drug delivery at usual oral doses. In our Caco-2 experiments hydrocortisone and hydrocortisone-phosphate both were both well absorbed and had similar high absorptive fluxes (Fig. 2B). In the case of this highly soluble parent drug, the prodrug approach did not significantly increase the parent drug flux. The success of a phosphate prodrug for a parent drug with sufficient solubility to completely dissolve within small intestinal residence time should be a function of the targeted dose. In the case of glucocorticoids, it is possible that a phosphate prodrug would improve absorption at some of the very high oral doses used in chemotherapy.

Fosphenytoin is a successful parenteral prodrug of phenytoin (Dilantin®), which exhibits a high aqueous solubility of 140 mg/ml compared to the 20 $\mu\text{g}/\text{ml}$ of the practically insoluble phenytoin (3). Phenytoin is a BCS Class II drug (i.e., a drug with low solubility and high permeability) (14). Its absorption is nearly complete in humans when dosed as its soluble sodium salt, but erratic at doses of 100 mg (31). For that reason the prodrug would only offer a flux advantage in humans if high doses of phenytoin were to be administered. At high doses phenytoin's absorption will be solubility-

dissolution rate limited. With the prodrug fosphenytoin, solution concentrations of phenytoin above its equilibrium solubility can be achieved without precipitation, likely due to supersaturation. Thus, fosphenytoin and phenytoin represent a case where the flux can be increased for poorly to moderately soluble parent drugs when high doses are required. In fact, oral dosing of fosphenytoin has been found to yield an earlier t_{max} and higher C_{max} in rats (32) and dogs (3) and has also lead to an increased relative bioavailability in that species (3).

TAT-59 (Miproxifene Phosphate) was under development and in clinical trials by Taiho Pharmaceutical Co. Ltd. for the treatment of breast cancer (33), with a recommended dose of 20 mg. TAT-59 is a practical insoluble phosphate ester (34) of DP-TAT-59 (Fig. 1). The prodrug approach had been chosen, since unlike tamoxifen, no stable citrate or other salt of DP-TAT-59 could be synthesized. Despite TAT-59's low solubility in water (34), its solubility is higher than that of DP-TAT-59. TAT-59 through its metabolite DP-TAT-59 was previously shown to be well absorbed in rats (23), yet in the Caco-2 model the measured permeability of DP-TAT-59 was low when no albumin was added [$0.096 (\pm 0.3) \times 10^{-6} \text{ cm/s}$]. The permeability was significantly increased in the presence of albumin [$32.6 (\pm 6.5) \times 10^{-6} \text{ cm/s}$] (Table I), implying high intestinal permeability *in vivo*. Due to its low solubility and high permeability, DP-TAT-59 is a BCS Class II drug. TAT-59 is a successful phosphate prodrug since it increased drug flux of DP-TAT-59 by nearly 10-fold (Table II) at a concentration relevant for human dosing. This lipophilic parent drug is an example where the prodrug approach can increase the parent drug flux through an increase in aqueous solubility, since the low solubility of the parent drug is a rate-limiting step for absorption.

Entacapone phosphate is an investigative phosphate ester prodrug of entacapone (Comtan®). Entacapone is rapidly absorbed but has a low and variable bioavailability of 29–46% in humans (35). One of the reasons for the low bioavailability was thought to be entacapone's pH dependent solubility, which is that of a typical weak acid (20,36). At pH 1.2, entacapone's solubility is near to 0.017 mg/ml, but it rises to 1.75 mg/ml at pH 7.4 (20,29). The rationale for synthesizing a phosphate prodrug of entacapone was to increase the aqueous solubility and dissolution rate of entacapone (29) since the improved solubility could lead to higher plasma entacapone levels and improved bioavailability. The aqueous solubility of entacapone phosphate was found to be significantly higher than that of entacapone, both at pH 1.2 and 7.4 (29). Present results also suggest that bioavailability of entacapone can be improved with the prodrug. The $\text{AUC}_{0-2 \text{ h}}$ of entacapone was higher after administration of entacapone phosphate than after administration of entacapone suspension and the relative bioavailability of entacapone phosphate was over 2-fold better than that of entacapone suspension. Thus, because of its increased solubility, the phosphate prodrug seems to overcome the dissolution problem related to the poor bioavailability of parent entacapone. In this study, the administration of a solid dosage form was simulated by giving entacapone as a suspension. However, the prodrug did not yield plasma levels as high as those achieved with entacapone solution. The fact that entacapone flux was not significantly increased in Caco-2 cells (Fig. 2B), is not surprising since the parent drug has moderate solubility and its absorption is likely permeability rate-limited. Since the plasma entacapone

levels and the relative bioavailability were lower after dosing entacapone as a phosphate prodrug solution than as the parent-drug solution, the phosphate pro-moiety may hinder absorption of entacapone, or its enzymatic hydrolysis may become rate limiting.

Another example where drug permeability limits a prodrug approach to improve absorption is the echinocandin B analog LY303366. LY303366 has a poor intestinal permeability, as measured in the Caco-2 model (37) (Table I), in spite of a favorable log octanol/water partition coefficient of 2.0. Limited solubility of 0.005 mg/ml led to the development of the phosphate prodrug, LY307853. While the prodrug greatly increased aqueous solubility, it failed to improve LY303366 plasma levels in dogs (12). In contrast, an example of a phosphate prodrug with good parent drug permeability is the recently approved oral dosage form of fludarabine-phosphate (Fludara®, Schering) (9), where the IV formulation was marketed first.

For all phosphate esters studied, no significant amount of intact prodrug was detected in receiver chambers. Apical prodrug and parent drug concentrations are time dependent, as was shown for fosphenytoin and phenytoin, suggesting rapid dephosphorylation by apical alkaline phosphatase followed by absorption of the permeable parent drug, while the highly charged phosphate is not absorbed in significant amounts. For that reason, the success of a phosphate prodrug strategy is dependent on the permeability of the parent drug.

Moreover, the drug flux or permeability of lipophilic or highly plasma bound compounds, which are ideal phosphate prodrug candidates can be underestimated in the Caco-2 model (24). For phenytoin and tamoxifen the results are consistent with a previous study that showed a 1.4-fold increase for phenytoin, while tamoxifen could not be detected in the absence of albumin and P_{app} could not be determined (16). The use of albumin in Caco-2 transport studies apparently reduces intracellular accumulation of highly protein bound drugs and mimics more physiologic sink conditions, as the albumin concentration *in vivo* is near 4% (16). To avoid an underprediction of absorption following both parent and prodrug dosing, it is therefore suggested to add albumin to the basolateral chambers.

In conclusion, this study demonstrated that, when targeting phosphate prodrugs for oral delivery, the permeability of the parent drugs could rate limit the absorptive flux. For that reason ideal phosphate prodrug candidates have a high permeability or lipophilicity and low solubility (i.e., are BCS Class II drugs, such as DP-TAT-59). However, for BCS Class II drugs administered at sufficiently low doses to permit complete dissolution within intestinal residence time, such as hydrocortisone and phenytoin, no biopharmaceutical advantage is gained. Phosphate prodrugs are not likely to optimize the absorptive drug flux of parent drugs with low permeability and intermediate to high solubility, such as entacapone. Furthermore, when using the Caco-2 model to evaluate phosphate ester prodrugs, albumin should be added to basolateral receivers for highly lipophilic parent drugs, since failure to consider protein binding can result in the underestimation of absorption.

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REFERENCES

1. D. Fleisher, R. Bong, and B. H. Stewart. Improved oral drug delivery: solubility limitations overcome by the use of prodrugs. *Adv. Drug Deliv. Rev.* **19**:115–130 (1996).
2. A. S. Kearney and V. J. Stella. The *in vitro* enzymic liabilities of chemically distinct phosphomonoester prodrugs. *Pharm. Res.* **9**: 497–503 (1992).
3. V. J. Stella. A case for prodrugs: Fosphenytoin. *Adv. Drug Deliv. Rev.* **19**:311–330 (1996).
4. P. Rohdewald, H. Möllmann, J. Barth, J. Rehder, and H. Derendorf. Pharmacokinetics of dexamethasone and its phosphate ester. *Biopharm. Drug Dispos.* **8**:205–212 (1987).
5. K. R. Hande. Etoposide: four decades of development of a topoisomerase II inhibitor. *Eur. J. Cancer* **34**:1514–1521 (1998).
6. A. S. Kearney and V. J. Stella. Hydrolysis of pharmaceutically relevant phosphate monoester monoanions: correlation to an established structure-reactivity relationship. *J. Pharm. Sci.* **82**:69–72 (1993).
7. P. O. Gunnarsson, S. B. Andersson, S. A. Johansson, T. Nilsson, and G. Plym-Forsell. Pharmacokinetics of estramustine phosphate (Estracyt) in prostatic cancer patients. *Eur. J. Clin. Pharmacol.* **26**:113–119 (1984).
8. P. van Asten, S. A. Duursma, J. H. Glerum, F. F. Ververs, H. J. van Rijn, and A. van Dijk. Absolute bioavailability of fluoride from disodium monofluorophosphate and enteric-coated sodium fluoride tablets. *Eur. J. Clin. Pharmacol.* **50**:321–326 (1996).
9. M. A. Boogaerts, A. Van Hoof, D. Catovsky, M. Kovacs, M. Montillo, P. L. Zinzani, J. L. Binet, W. Feremans, R. Marcus, F. Bosch, G. Verhoef, and M. Klein. Activity of oral fludarabine phosphate in previously treated chronic lymphocytic leukemia. *J. Clin. Oncol.* **19**:4252–4258 (2001).
10. L. A. Sorbera, L. Martin, J. Castaner, and R. M. Castaner. Fosamprenavir. *Drugs of the Future* **26**:224–231 (2001).
11. R. S. de Jong, N. H. Mulder, D. R. Uges, S. Kaul, B. Winograd, D. Sleijfer, H. J. Groen, P. H. Willemsse, W. T. van der Graaf, and E. G. de Vries. Randomized comparison of etoposide pharmacokinetics after oral etoposide phosphate and oral etoposide. *Br. J. Cancer* **75**:1660–1666 (1997).
12. S. Hawser and R. F. Hoechst Marion Roussel. LY-303366 (Eli Lilly & Co). *Curr. Opin. Anti-Infect. Invest. Drugs* **1**:353–360 (1999).
13. Y. Ueda, A. B. Mikkilineni, J. O. Knipe, W. C. Rose, A. M. Casazza, and D. M. Vyas. Novel water soluble phosphate prodrugs of taxol possessing *in vivo* antitumor activity. *Bioorg. Med. Chem. Lett.* **3**:1761–1766 (1993).
14. G. L. Amidon, H. Lennernaes, V. P. Shah, and J. R. Crison. A theoretical basis for a biopharmaceutic drug classification: the correlation of *in vitro* drug product dissolution and *in vivo* bioavailability. *Pharm. Res.* **12**:413–420 (1995).
15. T. Heimbach. Oral Phosphate Prodrugs: Absorption Rate Limit Considerations. *Ph.D. Thesis*: University of Michigan (2003).
16. B. J. Aungst, N. H. Nguyen, J. P. Bulgarelli, and K. Oates-Lenz. The influence of donor and reservoir additives on Caco-2 permeability and secretory transport of HIV protease inhibitors and other lipophilic compounds. *Pharm. Res.* **17**:1175–1180 (2000).
17. M. Pinto, S. Robine Leon, and M. D. Appay. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* **47**:323–330 (1983).
18. D. Fleisher, N. Sheth, H. Griffin, M. McFadden, and G. Aspacher. Nutrient influences on rat intestinal phenytoin uptake. *Pharm. Res.* **6**:332–337 (1989).
19. L. Y. Li, G. L. Amidon, J. S. Kim, T. Heimbach, F. Kesiosoglou, J. T. Topliss, and D. Fleisher. Intestinal metabolism promotes regional differences in apical uptake of indinavir: Coupled effect of P-glycoprotein and cytochrome P450 3A on indinavir membrane permeability in rat. *J. Pharmacol. Exp. Ther.* **301**:586–593 (2002).
20. J. Savolainen, M. Forsberg, H. Taipale, P. T. Mannisto, K. Jarvinen, J. Gynther, P. Jarho, and T. Jarvinen. Effects of aqueous

- solubility and dissolution characteristics on oral bioavailability of entacapone. *Drug Dev. Res.* **49**:238–244 (2000).
21. K. Palm, K. Luthman, J. Ros, J. Grasjo, and P. Artursson. Effect of molecular charge on intestinal epithelial drug transport: pH-dependent transport of cationic drugs. *J. Pharmacol. Exp. Ther.* **291**:435–443 (1999).
 22. D. Fleisher, K. C. Johnson, B. H. Stewart, and G. L. Amidon. Oral absorption of 21-corticosteroid esters: a function of aqueous stability and intestinal enzyme activity and distribution. *J. Pharm. Sci.* **75**:934–939 (1986).
 23. K. Ikeda, M. Nagamachi, H. Masuda, S. Nagayama, and Y. Kawaguchi. Metabolic fate of TAT-59. (3rd report). Absorption and metabolism of TAT-59. *Yakuri to Chiryō* **26**:797–807 (1998).
 24. P. Wils, A. Warnery, V. Phung-Ba, S. Legrain, and D. Scherman. High lipophilicity decreases drug transport across intestinal epithelial cells. *J. Pharmacol. Exp. Ther.* **269**:654–658 (1994).
 25. G. L. Amidon, B. H. Stewart, and S. Pogany. Improving the intestinal mucosal cell uptake of water insoluble compounds. *J. Control. Release* **2**:13–26 (1985).
 26. G. L. Amidon, G. D. Leesman, and R. L. Elliott. Improving intestinal absorption of water-insoluble compounds: a membrane metabolism strategy. *J. Pharm. Sci.* **69**:1363–1368 (1980).
 27. D. A. Johnson and G. L. Amidon. Determination of intrinsic membrane transport parameters from perfused intestine experiments: a boundary layer approach to estimating the aqueous and unbiased membrane permeabilities. *J. Theor. Biol.* **131**:93–106 (1988).
 28. R. B. McComb, G. N. J. Bowers, and S. Posen. *Alkaline Phosphatase*, Plenum Press, New York and London, 1979.
 29. J. Leppänen, J. Huuskonen, J. Savolainen, T. Nevalainen, H. Taipale, J. Vepsäläinen, J. Gynther, and T. Jarvinen. Synthesis of a water-soluble prodrug of entacapone. *Bioorg. Med. Chem. Lett.* **10**:1967–1969 (2000).
 30. A. Olivsei. Oral Prednisolone-21-phosphate is absorbed at the same rate and to the same extent as oral prednisolone in normal adults. *Therapie* **40**:1–4 (1985).
 31. W. K. Sietsema. The absolute oral bioavailability of selected drugs. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **27**:179–211 (1989).
 32. A. H. Burstein, D. Cox, B. Mistry, and N. Eddington. Phenytoin pharmacokinetics following oral administration of phenytoin suspension and fosphenytoin solution to rats. *Epilepsy Res.* **34**:129–133 (1999).
 33. Y. Nomura, O. Abe, K. Enomoto, K. Fujiwara, T. Tominaga, K. Hayashi, J. Uchino, M. Takahashi, A. Hayasaka, K. Asaishi, M. Okazaki, R. Abe, I. Kimishima, T. Kajiwara, S. Haga, T. Shimizu, I. Miyazaki, M. Noguchi, M. Yoshida, S. Miura, T. Taguchi, J. Oota, K. Sakai, H. Kinoshita, and H. Tashiro. Phase I study of TAT-59 (a new antiestrogen) in breast cancer. *Gan To Kagaku Ryoho* **25**:553–561 (1998).
 34. Y. Matsunaga, R. Ohta, N. Bando, H. Yamada, H. Yuasa, and Y. Kanaya. Effects of water content on physical and chemical stability of tablets containing an anticancer drug TAT-59. *Chem. Pharm. Bull. (Tokyo)* **41**:720–724 (1993).
 35. B. S. Chong and T. L. Mersfelder. Entacapone. *Ann. Pharmacother.* **34**:1056–1065 (2000).
 36. J. Savolainen, J. Leppänen, M. Forsberg, H. Taipale, T. Nevalainen, J. Huuskonen, J. Gynther, P. T. Mannisto, and T. Jarvinen. Synthesis and in vitro/in vivo evaluation of novel oral N-alkyl- and N,N-dialkyl-carbamate esters of entacapone. *Life Sci.* **67**:205–216 (2000).
 37. C. Li, D. Fleisher, L. Li, J. R. Schvier, S. A. Sweetana, V. Vasudevan, L. L. Zornes, L. H. Pao, S. Y. Zhou, and R. E. Stratford. Regional-dependent intestinal absorption and meal composition effects on systemic availability of LY303366, a lipopeptide antifungal agent, in dogs. *J. Pharm. Sci.* **90**:47–57 (2001).