Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 261 (2003) 81–92
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Enzyme-mediated precipitation of parent drugs from their phosphate prodrugs

Tycho Heimbach [a], Doo-Man Oh [b], Lilian Y. Li [a], Naír Rodríguez-Hornedo [a], George Garcia [a], David Fleisher [a,*]

[a] *College of Pharmacy, The University of Michigan, 428 Church Street, Ann Arbor, MI 48109-1065, USA*
[b] *Aventis Pharmaceuticals, 1041 Route 202-206 North, P.O. Box 6800, Bridgewater, NJ 08807-0800, USA*

Received 23 December 2002; received in revised form 1 May 2003; accepted 2 May 2003

Abstract

Many oral phosphate prodrugs have failed to improve the rate or extent of absorption compared to their insoluble parent drugs. Rapid parent drug generation via intestinal alkaline phosphatase can result in supersaturated solutions, leading to parent drug precipitation. The purpose was to (1) investigate whether parent drugs can precipitate from prodrug solutions in presence of alkaline phosphatase; (2) determine whether induction times are influenced by (a) dephosphorylation rate, (b) parent drug supersaturation level, and (c) parent drug solubility. Induction times were determined from increases in optical densities after enzyme addition to prodrug solutions of TAT-59, fosphenytoin and estramustine phosphate. Apparent supersaturation ratios (σ) were calculated from parent drug solubility at intestinal pH. Precipitation could be generated for all three prodrugs. Induction times decreased with increased enzyme activity and supersaturation level and were within gastrointestinal residence times for TAT-59 concentration $\geq$ 21 μM ($\sigma \geq 210$). Induction times for fosphenytoin were less than the GI residence time (199 min) for concentrations of approximately 352 μM ($\sigma = 4.0$). At approximately 475 μM ($\sigma = 5.3$) the induction times were less than 90 min. For estramustine-phosphate, no precipitation was observed within GI residence times. Enzyme-mediated precipitation will depend on apparent supersaturation ratios, parent drug dose, solubility and solubilization by the prodrug.

Keywords: Induction time; Phosphate prodrug; Precipitation; TAT-59; Supersaturation; Enzyme

1. Introduction

Most phosphate ester prodrugs marketed to date are soluble prodrugs of poorly water-soluble parent drugs that are used in parenteral formulations (Fleisher et al., 1996), such as fosphenytoin (Cerebryx®, Pfizer) and hydrocortisone-phosphate (Hydrocortone-Phosphate®, Merck). In contrast, very few oral phosphate prodrugs have shared this success, as they often fail to show improvements in the rate or extent of absorption compared to their parent drugs (de Jong et al., 1997). In theory, increasing drug solution concentration through a soluble prodrug should increase absorptive flux. The fact that this is not observed for a number of oral phosphate prodrugs prompted an investigation for the causes of failure, which focused on rate-limiting

* Corresponding author. Tel.: +1-734-764-2070; fax: +1-734-763-2022.
E-mail address: fleisher@umich.edu (D. Fleisher).

0378-5173/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0378-5173(03)00287-4

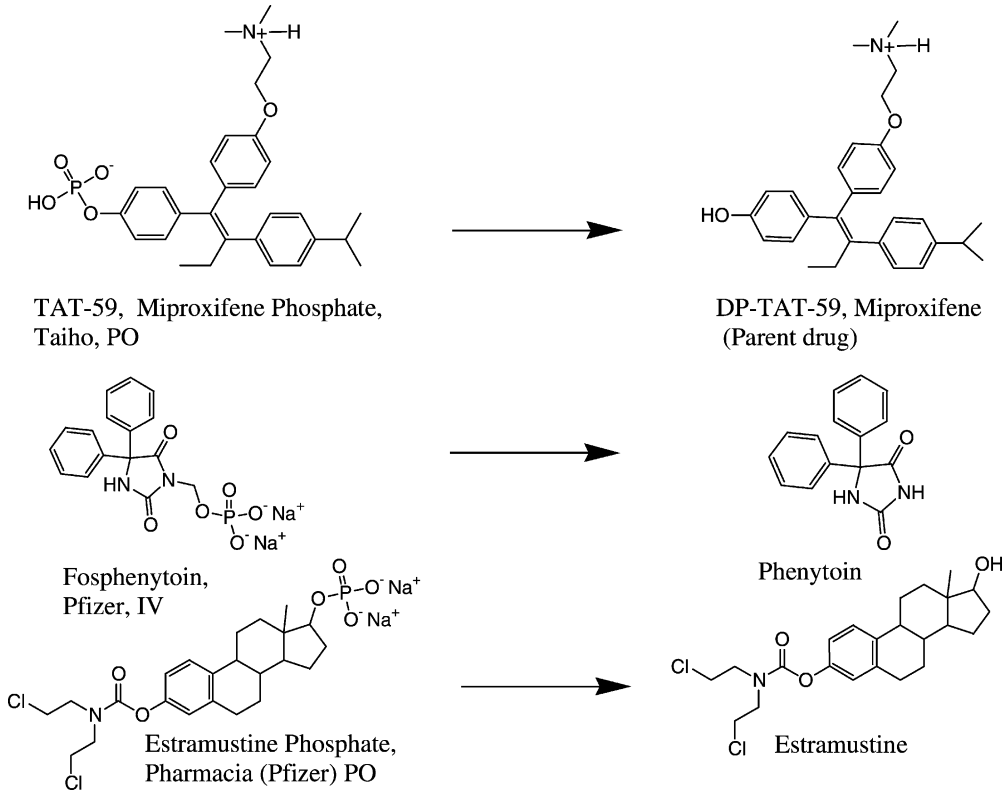

Fig. 1. Structure of phosphate ester prodrugs. TAT-59 is provided as the free acid, while the other prodrugs are disodium salts. In presence of intestinal alkaline phosphatase the phosphate ester is hydrolyzed to yield the corresponding alcohol or parent drug.

steps for the process of drug absorption from phosphate prodrug administration. Ideally this process involves rapid prodrug dephosphorylation by intestinal membrane-bound alkaline phosphatase yielding high solution concentrations of the poorly soluble parent drug at the apical membrane. The regenerated lipophilic parent drugs should be well absorbed compared to their polar, ionized prodrugs (Amidon et al., 1985), provided that the parent drug does not precipitate prior to absorption across the intestinal epithelia. Thus, absorption of phosphate esters can be potentially compromised by: (a) dissolution of the prodrug which is rarely a limitation for a solubility-enhancing prodrug (Heimbach, 2002), (b) poor enzymatic bioconversion, (c) precipitation of the parent drug, and (d) poor permeability of the parent drug (Heimbach et al., 2003). This report details an investigation into the potential of parent drug precipitation from phosphate prodrug solutions in the presence of alkaline phosphatase. TAT-59 (Miproxifene-phosphate), fosphenytoin and estramustine phosphate were used as model prodrugs (Fig. 1), which are hydrolyzed to their corresponding parent drugs DP-TAT-59, phenytoin and estramustine, respectively. The prodrugs differ in their parent drug's solubility and targeted oral dose (Table 1). TAT-59 (Miproxifene Phosphate) is a unique, practically insoluble zwitterionic phosphate ester ($s \sim 52\,\mu g/ml$ at pH 7.4, 23 °C) (Heimbach, 2002; Matsunaga et al., 1993) of the even more insoluble DP-TAT-59 ($s \sim 50\,ng/ml$ at pH 7.4, 23 °C) (Fig. 1) that was under development by Taiho Pharmaceutical Co. Ltd. for the treatment of breast cancer (Nomura et al., 1998), with a recommended dose of 20 mg. Fosphenytoin is a successful parenteral prodrug of phenytoin (Dilantin®) to treat epilepsy, which exhibits a high aqueous solubility of 140 mg/ml (Stella, 1996) compared to the 24 μg/ml (pH 7.4, 23 °C) of the practically insoluble phenytoin. Oral phenytoin

Table 1
Effect of apparent maximum supersaturation (c/s) on induction times for the precipitation of prodrugs fosphenytoin, TAT-59, and estramustine phosphate in the presence of pure calf intestinal alkaline phosphatase-mediated dephosphorylation at 23 °C in pH 7.4 buffer

Prodrug	Targeted oral prodrug dose (mg)	Parent drug s[b] (μM) [mg/ml]	Theoretical c/s of parent drug after oral prodrug dose	Alkaline phosphatase activity (μmol 4-NP/min)	Induction times τ (min)[a]										
Fosphenytoin	155[c]	89 [0.0244]	16		1–3.3[d]	4.0	5.3	6.7	50	56	100	125	150	210	210
				9.5	>720	514 (110)	88 (9)	71 (13)	37 (11)	41 (5)	30 (2)	31	34 (2)	27 (2)	27 (2)
				6.2	>720	455 (109)	87 (8.5)	64 (10)	ND	27 (6)	28 (2)	ND	ND	36 (5)	36 (5)
				1.1	>720	ND	ND	266 (117)	129 (9)	ND	103 (11)	120 (2)	100(11)	144 (11)	144 (11)
TAT-59	5–80	0.1 [0.00005]	392–6275		50–150[d]	210	250	300	400	500	510	700	1070		
				9.5	>720	58 (18)	33 (6)	20 (4)	19 (1)	17 (3)	13 (1)	10 (1)	8.4 (0.4)		
				6.2	>720	75 (16)	36	ND	22 (1)	ND	18 (2)	17 (1)	8.4 (1)		
				1.1	>720	438 (54)	205	133 (6)	136 (13)	168 (4)	111 (5)	83 (11)	31 (3)		
Estramustine-phosphate	400–1000	2.3 [0.001]	1249–3122		8[d]	38	113	322							
				9.5	>720	>720	>720	689[e]							
				6.2	>720	>720	>720	>720							
				1.1	>720	>720	>720	>720							

ND: not determined; induction times are expressed as average ($N = 3$–8), standard deviations are shown in parentheses, except for $N = 2$.

[a] Measurements were done at pH 7.4 and 23 °C, values of >720 indicate that no precipitation was observed after 12 h. In control experiments devoid of alkaline phosphatase, no increase in absorbance was observed and induction times were not detected.

[b] Equilibrium solubility at pH 7 and 23 °C.

[c] Unlike TAT-59, and estramustine phosphate, fosphenytoin was not developed for an oral dosage form. Fosphenytoin's oral dose was calculated assuming a dose equivalent 100 mg phenytoin parent drug.

[d] c/s is the apparent dimensionless supersaturation ratio calculated by dividing the initial prodrug concentration by the parent equilibrium solubility, s.

[e] $n = 2$ of 8, for 6-well induction times were >720 mm.

doses are typically in the range of 100–300 mg for adult patients. Estramustine-phosphate, is a high dose prodrug (400 mg–1 g) of estramustine and this parent drug has a low aqueous solubility near 1 μg/ml (pH 7.4, 23 °C). Unlike most other phosphate prodrugs estramustine-phosphate is successfully marketed as an oral formulation in Europe and the United States as Emcyt® (Pharmacia) for the treatment of prostate cancer (Perry and McTavish, 1995).

2. Materials and methods

TAT-59 and its parent drug, DP-TAT-59, were gifts of Taiho Pharmaceutical Co. Ltd. (Tokushima, Japan). Estramustine-phosphate was a gift of Pharmacia (Milano, Italy). Fosphenytoin was donated by Pfizer (formerly Parke-Davis, Ann Arbor, MI). Hank's balanced salt solution (HBSS) was purchased from Gibco BRL Life Technology (Grand Island, NY). Calf intestinal alkaline phosphatase (5TU) was obtained from Calbiochem (San Diego, CA). Phenytoin and all other reagents were purchased from Sigma–Aldrich (St. Louis, MO).

2.1. Induction time measurements in presence of alkaline phosphatase

Induction times for the precipitation of parent drugs were measured as a function of prodrug and alkaline phosphatase concentration at 23 °C. Due to TAT-59's poor aqueous solubility at pH 6.5 near 1 μM (Heimbach, 2002; Matsunaga et al., 1993), experiments were only carried out at pH 7.4, where the prodrug is soluble at 100 μM. Prodrugs were dissolved in sterile HEPES-HBSS pH 7.4 buffer (5.37 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 136.89 mM NaCl, 4.17 mM $NaHCO_3$, 3.38 mM Na_2HPO_4, 5.55 mM D-glucose, and 5 mM HEPES). An attempt was made to simulate alkaline phosphatase activities observed in Caco-2 cells, which reportedly mimic those found in the human intestine (Pinto et al., 1983). Ten microliters of calf intestinal alkaline phosphatase were added to 200 μl freshly prepared prodrug solution. The alkaline phosphatase activity was measured at 23 °C in control wells using 4-nitrophenylphosphate (4-NPP) as a reference substrate and the measured dephosphorylation rates were 1.1 ± 0.2 μmol, 6.2 ± 0.3 μmol and 9.5 ± 0.5 μmol 4-nitrophenol (4-NP) generated per minute. The lowest rate was comparable to the dephosphorylation rate, i.e. the alkaline phosphatase activity measured in our Caco-2 cells (Section 2.2). A Spectramax 250 UV-Vis 96-well plate reader (Molecular Devices Corp., Sunnyvale, CA) was used to measure changes in optical densities, which were monitored at 23 °C post enzyme addition in kinetic intervals of 30 s. The experiments were conducted for up to 12 h, since typical small intestinal residence time (3–4 h) may be impacted by increased upper GI residence time under certain conditions, such as the presence of food (Crison, 2000). Onset of nucleation was detected by measuring changes in optical density at 405 and 650 nm, where an increase in 0.02 absorbance units was visually associated with the formation of a solid. Induction time was defined as the time interval measured from enzyme addition to the time of detection of a 0.02 absorbance unit increase.

Prodrug solutions were chemically stable over the duration of the experiments (Kearney and Stella, 1993; Matsunaga et al., 1996) and no increase in optical density was detected after 16 h in solutions devoid of the enzyme. Parent drug formation in the presence of alkaline phosphatase was confirmed for all three prodrugs by using liquid chromatography–mass spectrometry (Heimbach, 2003; Heimbach et al., 2003). With TAT-59 experiments, the precipitate readily dissolved when the pH was lowered to pH 4. This indicates that the precipitate is the parent drug, DP-TAT-59, since the solubility of this weak base increases with decreasing pH. This is not the case for the weak acid prodrug, TAT-59, and its solubility decreases with increasing pH (1 μg/ml at pH < 5).

Apparent maximum supersaturation was defined as $\sigma = c/s$, where c is the initial prodrug concentration from the solution in pH 7.4 buffer and s is the equilibrium solubility of the corresponding parent drug at pH 7.4 and 23 °C (Heimbach, 2002) (Table 1). In these experiments σ represents the maximum theoretically achievable supersaturation ratio which to occur (a) requires complete enzymatic conversion from the prodrug to the parent drug, and (b) assumes that the

parent drug solubility, *s*, is not altered by the presence of the prodrug.

Prodrug solutions with parent drug equivalent concentrations above the parent drug's solubilities were prepared such that σ values with a range of greater than 200 and less than or equal to 50 were achieved (Table 1). This range did not always bracket theoretically achievable in vivo supersaturation ratios (Table 1, column 4), where these ratios were calculated from the oral doses taken with 250 ml fluid, divided by the parent drug solubility, assuming complete conversion of the prodrug to the parent drug. For TAT-59 the upper limit of achievable σ was limited by the prodrug's equilibrium solubility (≈100 μM at pH 7.4) (Heimbach, 2003; Heimbach et al., 2003), which limited our induction studies to supersaturation ratios up to 1070 (Table 1). TAT-59's lower limit of achievable σ was near supersaturation ratios of 50, due to long induction times (>12 h), associated with low σ (Table 1, column 6). Similarly, the upper limit of σ for estramustine-phosphate was limited by the prodrugs solubility (≈300 μM at pH 7.4) (Heimbach, 2003).

2.2. Dephosphorylation-supersaturation time profiles in apical Caco-2 chambers

The potential for phosphate prodrugs, fosphenytoin and TAT-59, to create supersaturated solutions of their corresponding parent drugs, phenytoin and DP-TAT-59, respectively was investigated in Caco-2 cells. These cells form polarized monolayers that show alkaline phosphatase activity at their apical membranes in levels similar to those found in the human gut (19) and these cells are frequently used in drug transport studies. Caco-2 cells were maintained and studies were carried out as described previously (Stilgenbauer et al., 2000; Wu et al., 2000). The alkaline phosphatase activity for the hydrolysis of 100 μM 4-NPP at pH 7.4 in apical Caco-2 chambers was near 1 μM 4-NP generated per minute. TAT-59 and fosphenytoin solutions were dosed to apical chambers at 40 and 300 μM, respectively. These concentrations were chosen to simulate the minimal theoretical σ following a low oral dose, of 5 and 20 mg, respectively, while maintaining cell viability. These studies were carried out at 37 °C and 5–10 μl aliquots were taken over time up to only 180 min (Heimbach, 2003; Heimbach et al., 2003), since longer experiment times can reduce monolayer integrity. Prodrug and parent drug concentrations were monitored simultaneously using liquid chromatography–mass spectrometry. Induction times were also determined using a SpectrofluorPlus^{TM} reader (Tecan, Raleigh, NC), since this reader is suitable for obtaining measurements in 24-well Caco-2 donor plates. The analytical wavelength for measuring changes in optical density was 590 nm instead of 405 and 650 nm, since a filter with the latter two wavelengths was not available for this instrument. As before, an increase in 0.02 absorbance units was visually associated with the formation of a solid. In these studies, supersaturation ratios were defined as $\sigma_m = c_m/s$, where c_m is the highest experimentally measured parent drug solution concentration in apical donor cells from the solution in pH 7.4 buffer and *s* is the equilibrium solubility of the corresponding parent drug.

3. Results

The effect of enzyme activity and level of apparent supersaturation on induction times of prodrug solutions was determined. The induction time, τ was longest under conditions of low σ and low enzyme activity as shown in Table 1 and Fig. 2. For example, τ was 438 ± 54 min at $c/s = 210$ for TAT-59 at the lowest enzyme activity which decreased to 58 ± 13 min at the highest enzyme activity. At higher apparent supersaturation e.g. $c/s = 1070$, τ is reduced to 31 ± 3 min at to lowest enzyme activity and is only 8.4 ± 0.4 min at the highest enzyme activity. Fig. 3 shows a plot of τ as a function of σ for phenytoin and DP-TAT-59 from their corresponding prodrugs fosphenytoin and TAT-59. The induction time data are plotted in Fig. 4A and B according to the induction time dependence on supersaturation (Mullin, 1993) as predicted by the nucleation and induction time equations Eqs. (1) and (2). Fig. 4A and B shows plots of $\ln \tau$ versus $(\ln \sigma)^{-2}$ for prodrugs TAT-59 and fosphenytoin, respectively, at varying enzyme activity and constant temperature. The plots show that precipitation is more rapid with increased enzyme activity and at higher apparent supersaturation ratios. For both TAT-59 and fosphenytoin, the induction times were longer for the lowest enzyme activity (1.1 μmol 4-NP/min) when compared

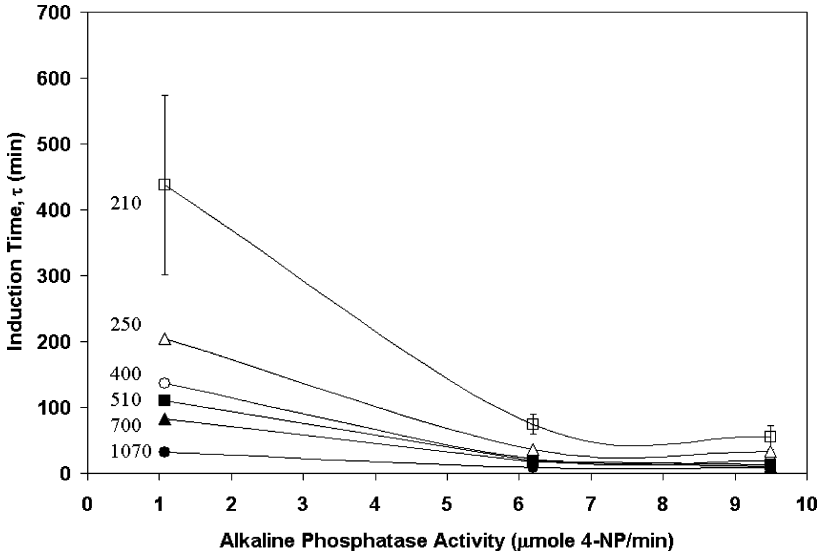

Fig. 2. Induction times for the precipitation of DP-TAT-59 from TAT-59 solutions at 23 °C in pH 7.4 buffer as a function of calf intestinal alkaline phosphatase activity and apparent supersaturation ratio, σ (inserted in the left side of graph). Each data point represents the mean ± S.D. of 4–8 determinations. The percent relative standard deviation was <20% for most measurements.

to the two higher activities (9.5 and 6.2 μmol 4-NP/min), which yielded practically identical induction times. Under constant supersaturation conditions between $c/s = 50$ to 210, induction times were shorter for fosphenytoin solutions, compared to TAT-59 solutions (Table 1, Fig. 4) and TAT-59 did not yield precipitation below a *c/s* of 210 during the course of the experiment (12 h). At *c/s* of 100 and at the lowest enzyme activity,

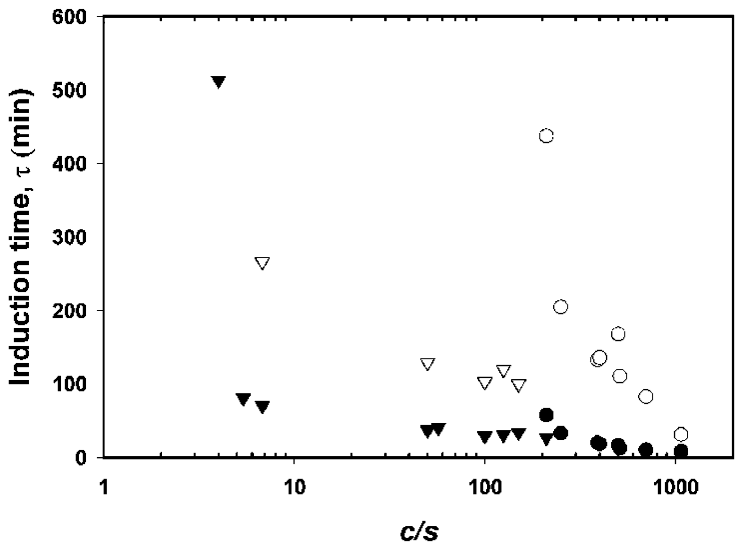

Fig. 3. The effect of apparent parent drug supersaturation ($\sigma = c/s$) obtained by prodrug dephosphorylation on the induction time (τ) required to form detectable precipitation from supersaturated solutions at pH 7.4 and 23 °C. DP-TAT-59 with alkaline phosphatase activity: (●) 9.5 μmol 4-NP/min, (○) 1.1 μmol 4-NP/min, respectively. Phenytoin with alkaline phosphatase activity: (▼) 9.5 μmol 4-NP/min and (▽) 1.1 μmol 4-NP/min, respectively. Each data point represents the mean ± S.D. of 3–8 determinations.

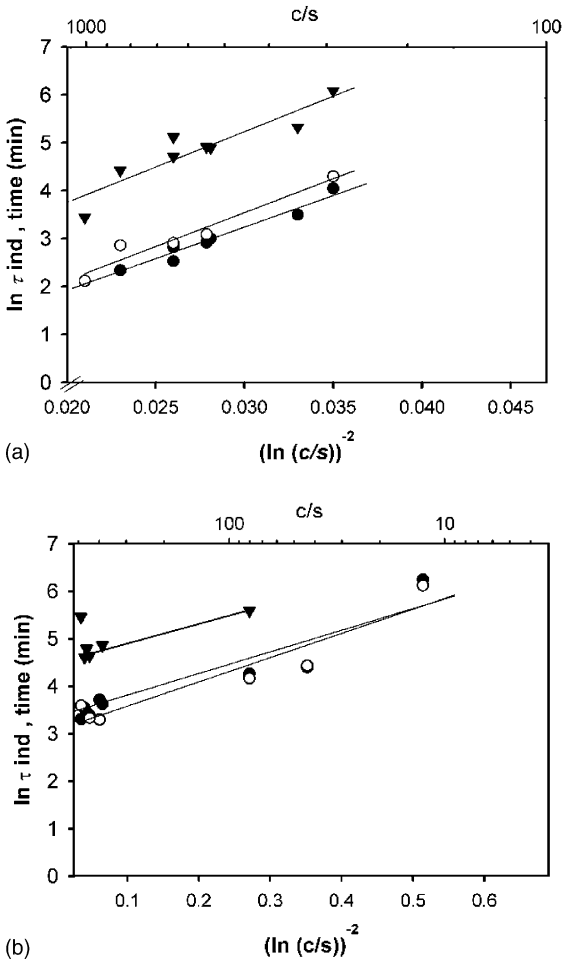

Fig. 4. (A) The effect of DP-TAT-59 apparent supersaturation ($\sigma = c/s$) obtained by TAT-59 dephosphorylation on the induction time (τ) required to form detectable DP-TAT-59 precipitation from supersaturated solutions at pH 7.4 and 23 °C. Alkaline phosphatase activity: (●) 9.5 μmol 4-NP/min, (○) 6.2 μmol 4-NP/min, and (▼) 1.1 μmol 4-NP/min, respectively. Each data point represents the mean ± S.D. of 3–8 determinations. (B) The effect of phenytoin apparent supersaturation ($\sigma = c/s$) obtained by fosphenytoin dephosphorylation on the induction time (τ) required to form detectable phenytoin precipitation from supersaturated solutions at pH 7.4 and 23 °C. Alkaline phosphatase activity: (●) 9.5 μmol 4-NP/min, (○) 6.2 μmol 4-NP/min, and (▼) 1.1 μmol 4-NP/min, respectively. Each data point represents the mean ± S.D. of 3–8 determinations.

the induction time for fosphenytoin was 103 ± 11 min, while for TAT-59 it was >720 min. Similarly, at $c/s = 210$ it was 144 ± 11 min for fosphenytoin, while it was 438 ± 54 min for TAT-59 (Table 1).

Induction times for TAT-59 were within gastrointestinal residence times, (near 3–4 h) (Yu, 1999), at relatively low concentrations of 21 μM and above ($\sigma > 210$). Precipitation from fosphenytoin required much higher prodrug concentrations of at least 475 μM ($\sigma > 5.4$) to occur in that time frame. For estramustine-phosphate no precipitation within 12 h was observed, except at the highest enzyme activity and prodrug concentration at 740 μM ($\sigma = 322$) (Table 1).

Supersaturation conditions of parent drugs phenytoin and DP-TAT-59 after dosing their prodrug solutions at concentrations that simulate a minimal theoretical σ following a low oral drug dose, were confirmed in Caco-2 apical donor compartments. Prodrug dephosphorylation is mediated by alkaline phosphatase which is located on the apical side of the Caco-2 cell monolayers (Amidon et al., 1985; TenHoor and Stewart, 1995). After dosing 300 μM fosphenytoin, the measured phenytoin solution concentrations achieved were approximately three times those ($\sigma_m \approx 2.9$) of phenytoin's equilibrium solubility near 89 μM (Fig. 5). No visible precipitation was observed during that experiment. Induction times were measured in separate Caco-2 cells and were longer than 180 min (not shown). These results mirror those observed with pure alkaline phosphatase, where no precipitation was observed within 12 h for the range of σ between 1 and 3.3 at a similar alkaline phosphatase activity at 1.1 μmol 4-NP generated per minute (Table 1, column 6). However, at high fosphenytoin concentrations near 2.4 mM ($\sigma = 272$) phenytoin crystals could be observed in Caco-2 donor chambers. Phenytoin crystals grown in presence of Caco-2 cells tended to aggregate, while individual needle-shaped crystals were obtained in the presence of pure calf intestinal alkaline phosphatase solutions lacking Caco-2 cells. By comparison, after dosing 40 μM TAT-59, the measured DP-TAT-59 solution concentrations achieved were approximately 68 times ($\sigma_m \cong 68$) those of DP-TAT-59's equilibrium solubility near 0.1 μM (Fig. 6). Turbidity was observed in the apical donor cells and measured induction times were less than 90 min. DP-TAT-59 solution concentrations were much lower than predicted from prodrug loss (Fig. 6), which are likely caused by visible precipitation in the donor chamber and at the apical membrane.

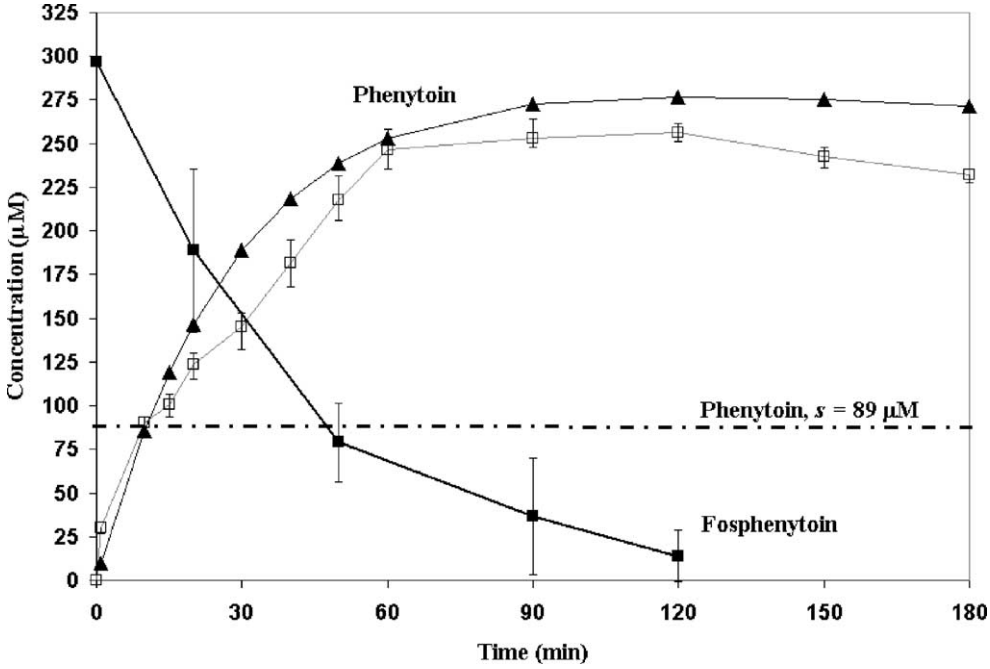

Fig. 5. Fosphenytoin prodrug loss (■) in apical Caco-2 cell compartments and generation of its parent drug phenytoin (□) after dosing with a 300 μM prodrug solution; (▲) represents the predicted apical concentration of the parent drug, phenytoin, corrected for basolateral transport (Heimbach, 2003; Heimbach et al., 2003); the dotted line represents the equilibrium solubility of phenytoin at pH 7.4. Each data point represents the mean ± S.D. of four determinations.

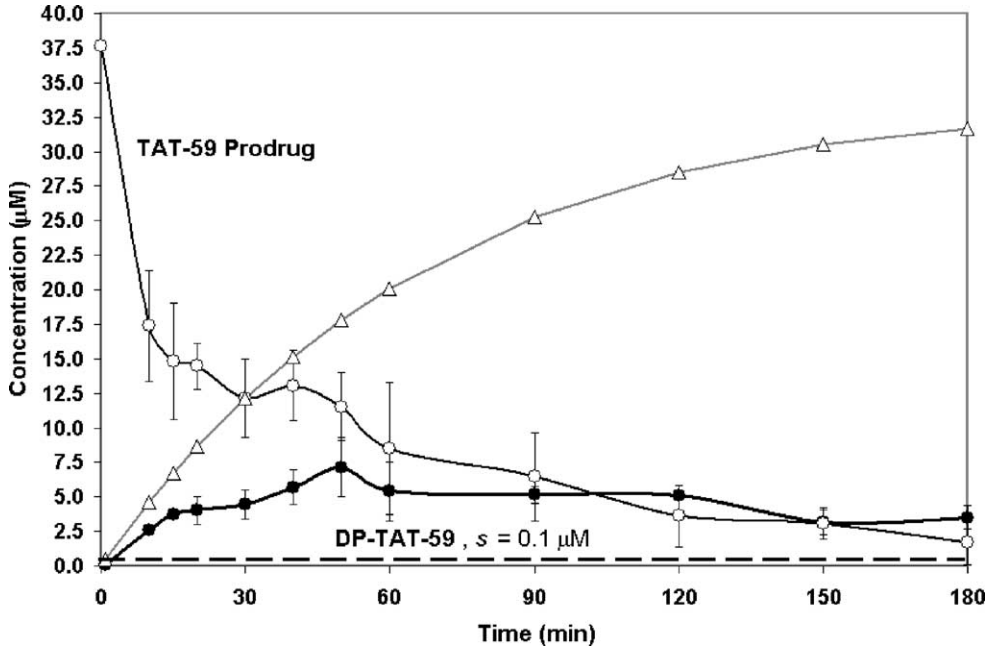

Fig. 6. TAT-59 prodrug loss (○) in apical Caco-2 cell compartments and measured concentrations of its parent drug, DP-TAT-59 (●), after dosing with a 40 μM prodrug solution. (△) Predicted solution concentration of the parent drug, DP-TAT-59, corrected for basolateral transport (Heimbach, 2003; Heimbach et al., 2003); the dotted line represents the equilibrium solubility of DP-TAT-59. Each data point represents the mean ± S.D. of four determinations.

4. Discussion

For a successful oral phosphate prodrug strategy several potentially rate-limiting factors to absorption have to be overcome (Fleisher et al., 1996) which may include precipitation of the parent drug. An ideal phosphate prodrug will be dephosphorylated by mucosal membrane-bound alkaline phosphatase producing local supersaturated concentrations of the parent drug at the membrane without precipitation. In an attempt to project the potential of parent drug precipitation in vivo, the precipitation of three parent drugs from their phosphate prodrugs was studied in solutions containing alkaline phosphatase as well as in Caco-2 cells with alkaline phosphatase activity at the mucosal membrane.

Precipitation of drug compounds usually occurs in a sequence of three steps: (a) achievement of supersaturation, (b) formation of crystal nuclei, and (c) subsequent growth of crystals. Supersaturation is commonly created by methods that control solute solubility. This can be achieved by changing solution conditions such as pH, temperature or addition of a cosolvent, or by dissolving a more soluble solid state form, such as an amorphous or polymorphic solid form (Rodriguez-Hornedo and Murphy, 1999). Supersaturation and subsequent precipitation from prodrug solutions could also occur when parent drug generation is rapid, as is the case for enzyme-mediated hydrolysis (McComb et al., 1979), such that solution concentrations of parent drug formed are above the parent drug's solubility. Reports of enzyme-mediated precipitation reactions are rare (Macaskie et al., 2000) and have not been investigated for pharmaceuticals. In this study, precipitation of parent drugs by alkaline phosphatase-mediated hydrolysis of phosphate prodrugs is demonstrated. Alkaline phosphatase has a broad substrate specificity (Walsh, 1979) and the catalytic mechanism for this enzyme involves the hydrolysis of a phosphoryl-enzyme intermediate as the rate-limiting step for most substrates. Given this, it is to be expected that at prodrug concentrations greater than the Michaelis constant, K_m (~5-fold or more) the dephosphorylation rates will be similar. It should be noted that these studies correlate in vitro experiments with calf intestinal alkaline phosphatase with results involving Caco-2 cells. Given the broad substrate specificity of alkaline phosphatase and the common rate-limiting step, it is reasonable to assume that the species differences will be negligible.

Nucleation can occur after some time from a supersaturated solution and increases in supersaturation (σ) will lead to an increase in nucleation rate (J) (Mullin, 1993; Rodriguez-Hornedo and Murphy, 1999). The nucleation rate is a function of temperature, solid–liquid interfacial tension, solubility and supersaturation and is given by

$$J = N_0 \nu \exp\left(\frac{-16\pi \upsilon^2 \gamma_{12}^3}{3(k_b T)^3 \ln(\sigma)^2}\right) \quad (1)$$

where N_0 is the number of molecules of the crystallizing phase in a unit volume, ν is the frequency of molecular transport at the nucleus-liquid interface, υ is the molecular volume of the crystallizing parent drug, k_b is the Boltzman constant, γ_{12} is the interfacial energy between the medium 1 and the nucleating cluster 2, and T is the temperature. The induction time (τ), i.e. the time between achievement of supersaturation and the onset in crystal growth is often used as a measure for nucleation rate, since τ can be defined as $\tau = N_0 J^{-1}$ and therefore:

$$\tau = \nu^{-1} \exp\left(\frac{16\pi \upsilon^2 \gamma_{12}^3}{3(k_b T)^3 \ln(\sigma)^2}\right). \quad (2)$$

Thus, induction times should decrease with an increase in supersaturation, where $\sigma = c/s$. Under constant supersaturation conditions and with all other variables constant, induction times will decrease with an increase in parent drug solubility, i.e. nucleation is faster in solutions where solubility is high. This is expected as long as solute solvent or solute-additive interactions do not interfere with the formation of molecular clusters that precede nucleation or with the integration of growth units into the crystal lattice (Gu et al., 2001; Rodriguez-Hornedo and Murphy, 1999). Indeed precipitation occurred earlier for fosphenytoin, compared to TAT-59 at similar supersaturation ratios (Table 1) and the reduction in induction time is likely due to phenytoin's higher aqueous solubility (89 μM at pH 7.4), compared to that of DP-TAT-59 (0.1 μM at pH 7.4). This is easily understood, since an increase in solubility increases N_0 and ν, i.e. the number of molecules in solution and the probability of intermolecular interactions, respectively.

However, estramustine-phosphate did not show significant precipitation under the experimental conditions studied. Since estramustine parent drug solubility is higher than that of DP-TAT-59 (Table 1), lower induction times were to be expected at similar supersaturation levels. The reasons for these unexpected results can include: (1) low prodrug to parent drug conversion rates, (2) prodrug inhibition of the precipitation of the parent drug, and (3) prodrug increasing the solubility of the parent drug. While the catalytic efficiencies of alkaline phosphatase were slightly higher for TAT-59 (6-fold) and fosphenytoin (3.7-fold) compared to estramustine phosphate, suggesting that estramustine-phosphate is slightly more slowly dephosphorylated than TAT-59 and fosphenytoin (Heimbach et al., 2000). However, a more likely reason for the lack of nucleation is the strong solubilizing effect of estramustine-phosphate on its parent drug, estramustine. The changes in molecular structure between prodrug and parent drug are akin to the strategy used in designing "tailor-made" additives to control nucleation and growth of molecular crystals from solution (Addadi et al., 1985). These additives have molecular structures similar to those of the crystallizing solute (e.g. replacing only a functional group such as an amide with a carboxylic acid) and have shown to interfere with the growth of pre-nucleation clusters and crystal faces. This approach has been successfully applied to control crystallization of amino acids, carboxylic acids, amides, sugars and steroids (Garnier et al., 2002; Weissbuch et al., 1991). In the case of phosphate prodrugs, self-association of the parent drug that leads to crystallization can be disturbed by the part of the prodrug that differs from the parent drug. The crystal structures of estramustine (hydrate and anhydrous) (Punzi et al., 1992), similar to other steroid derivatives, are characterized by chains of hydrogen bonded molecules in a head to tail fashion with hydrophobic and hydrophilic domains between the layers. A search of the Cambridge Structural Database (version 5.23, 2002) reveals the propensity of steroids to bind to other molecules through hydrogen bonding and form a large variety of co-crystals or solvates. Strong binding between prodrug and parent drug also leads to increased solubility as shown by the strong solubilizing effect of estramustine-phosphate on its parent drug, estramustine. Our results show that the solubility of estramustine in pH 7.4 buffer is increased from 0.001 mg/ml (2.3 μM) to 0.04 mg/ml (92 μM) in the presence of the prodrug (≈200 μM). This represents a 40-fold increase in solubility. By comparison, no parent drug solubility increase was observed for TAT-59 and fosphenytoin under conditions studied.

Higher enzyme activities lead to shortened induction periods at constant supersaturation level for both TAT-59 and fosphenytoin (Table 1, Fig. 2). This is expected, since an increase in enzyme activity increases the overall rate of dephosphorylation (Michaelis and Menten, 1913). This then results in an increased rate of production of the parent drug. Presumably, this increased rate will lead to higher levels of supersaturation that will yield an increased nucleation rate and thus result in shorter induction times. Fig. 4A and B shows such plots of induction time versus supersaturation for prodrugs TAT-59 and fosphenytoin, respectively, at varying enzyme activities. The plots indicate that precipitation is more rapid with increased enzyme activity and nucleation is more rapid at higher supersaturation ratios, or lower $(\ln(c/s))^{-2}$. Since the slopes are not changed with increased enzyme activity, the data suggest that the interfacial energy is not affected by the presence of the enzyme.

In addition to precipitation potential, targeted oral dose, parent drug solubility and permeability are additional key factors that determine whether a phosphate prodrug will be successful as an oral formulation (Fleisher et al., 1996). If the parent drug has poor membrane permeability, increasing solubility through a prodrug approach will not improve absorption (Heimbach, 2003; Heimbach et al., 2003). The magnitude of parent drug solubility is an important prodrug consideration for two reasons. As shown here, since induction periods are shorter for higher supersaturation ratios (Table 1, Figs. 2–4A and B), parent drugs with low solubility require higher c/s for nucleation to occur from prodrug solutions. In addition, a high parent drug dose-to-solubility ratio, which results a high prodrug number (P_N) (Heimbach, 2002) is an important consideration in deciding whether a soluble prodrug strategy will potentially increase the extent of intestinal absorption compared to a carefully formulated parent drug (Heimbach, 2002). DP-TAT-59 has a high P_N on the basis of moderate dose and very low aqueous drug solubility. Estramustine-phosphate has a high P_N on the basis of moderate drug solubility and a very high dose. High P_N suggests that both

estramustine and DP-TAT-59 are good candidates for an oral phosphate prodrug approach. Phenytoin has a moderate solubility and is typically administered at a moderate dose. Thus, the P_N of fosphenytoin is low and the prodrug would not be projected to provide much improvement in the extent of phenytoin absorption. While fosphenytoin was developed to be used for parenteral administration only (Stella, 1996), oral administration of the phosphate prodrug could be justified when higher phenytoin doses are desired.

DP-TAT-59 is an example of a parent drug where high theoretical supersaturation ratios can be achieved based on its low aqueous solubility (0.1 μM) and a targeted oral dose of 5–80 mg (Tominaga, 1995). Given this dose, when the prodrug is orally administered with 8 oz of water, theoretical solution concentrations over 39 μM could be obtained in the gastrointestinal tract. This corresponds to apparent supersaturation ratios of over 390 (Table 1). Such high supersaturation ratios led to induction times shorter than the mean small intestinal residence times (T_{Si}), $\tau < T_{Si} = 199$ min (Crison, 2000; Yu, 1999), for TAT-59 solutions concentrations between 21 μM ($\sigma = 210$) to 107 μM ($\sigma = 1070$). In spite of parent drug precipitation, reflected by apical solution turbidity in this study, TAT-59 was shown to increase parent drug flux across Caco-2 cells by 10-fold (Heimbach, 2003; Heimbach et al., 2003). While parent drug precipitation can be observed in this system, parent drug solution concentration above the parent drug's equilibrium solubility may be achieved from hydrolysis by membrane-bound alkaline phosphatase to enhance parent drug flux (Fig. 6). In addition, this prodrug is unusually lipophilic and may also permeate lipophilic membranes intact.

Fosphenytoin is an example of a parent drug with low theoretical supersaturation ($\sigma = 16$, Table 1, column 4) based on its a targeted oral dose (155 mg prodrug, is equivalent to ∼100 mg phenytoin) and moderate aqueous solubility (89 μM) and therefore is less prone to precipitation. In the presence of alkaline phosphatase, induction times for fosphenytoin were less than the GI transit time (3–4 h) for concentrations of approximately 352 μM ($\sigma = 4.0$). At 475 μM ($\sigma = 5.3$) the induction times were less than 90 min (Table 1). In the Caco-2 cell model solution concentrations of phenytoin, three-fold above its equilibrium solubility were achieved without precipitation (Fig. 5) and drug flux was increased nearly four-fold (Heimbach, 2003; Heimbach et al., 2003).

For estramustine-phosphate no precipitation within gastrointestinal residence times was seen even at the highest apparent supersaturation ratios. Estramustine is a parent drug with a low water solubility and as expected achieves higher theoretical supersaturation ratios before the onset of nucleation than the more soluble phenytoin, however, these supersaturation ratios are lower than those of TAT-59 the least soluble parent drug we studied. The supersaturation threshold for nucleation increases in the order estramustine phosphate > TAT-59 > fosphenytoin, with the least soluble drugs experiencing supersaturation ratios 50–100 higher than those of fosphenytoin. These results show that the onset of nucleation is dependent on drug solubility, prodrug–parent drug interactions, and solubilization. For estramustine-phosphate the dose of the prodrug is three to seven times that of fosphenytoin, the most soluble parent drug considered in this study, yet no precipitation within gastrointestinal residence times was observed even at the highest supersaturation ratios ($\sigma = 322$), which may be one of the reasons for the success of this marketed prodrug.

5. Conclusions

In summary, precipitation of parent drugs from phosphate prodrug solutions can be enzyme-mediated. Precipitation of parent drugs can also be observed for certain prodrugs in the Caco-2 cell model. Since induction times decrease and nucleation rates increase with high supersaturation ratios, parent drugs can precipitate when targeted prodrugs concentration are much higher than the parent drug's solubility, i.e. for parent drugs with high supersaturation ratios. The extent to which a parent drug precipitates during conversion of the prodrug is dependent on the prodrug to parent drug conversion rates, prodrug effect on the precipitation of the parent drug, and prodrug solubilization of the parent drug.

References

Addadi, L., Berkovitchyellin, Z., Weissbuch, I., Vanmil, J., Shimon, L.J.W., Lahav, M., Leiserowitz, L., 1985. Growth and dissolution of organic-crystals with tailor-made inhibitors—

implications in stereochemistry and materials science. Angewandte Chemie-International Edition in English 24, 466–485.
Amidon, G.L., Stewart, B.H., Pogany, S., 1985. Improving the intestinal mucosal cell uptake of water insoluble compounds. J. Control. Release 2, 13–26.
Crison, J.R., 2000. Biopharmaceutical aspects of water-insoluble drugs for oral drug delivery. Water-Insoluble Drug Formation, 97–110.
de Jong, R.S., Mulder, N.H., Uges, D.R., Kaul, S., Winograd, B., Sleijfer, D., Groen, H.J., Willemse, P.H., van der Graaf, W.T., de Vries, E.G., 1997. Randomized comparison of etoposide pharmacokinetics after oral etoposide phosphate and oral etoposide. Br. J. Cancer 75, 1660–1666.
Fleisher, D., Bong, R., Stewart, B.H., 1996. Improved oral drug delivery: solubility limitations overcome by the use of prodrugs. Adv. Drug Del. Rev. 19, 115–130.
Garnier, S., Petit, S., Coquerel, G., 2002. Influence of supersaturation and structurally related additives on the crystal growth of alpha-lactose monohydrate. J. Crystal Growth 234, 207–219.
Gu, C.-H., Young Jr., V., Grant, D.J.W., 2001. Polymorph screening: influence of solvents on the rate of solvent-mediated polymorphic transformation. J. Pharm. Sci. 90, 1878–1890.
Heimbach, T., 2002. Drug Candidates for an Oral Phosphate Prodrug Strategy: Screening Experiments and Identification of Rate Limiting Factors to Absorption. Poster presented at the AAPS Annual Meeting, Toronto, Canada.
Heimbach, T., 2003. Oral Phosphate Prodrugs: Absorption Rate Limit Considerations. Ph.D. Thesis, University of Michigan.
Heimbach, T., Li, L., Kang, J., Laliberte, K., Flynn, G., Fleisher, D., 2000. In Vitro Enzymatic Hydrolysis and Caco-2 Permeability of Selected Phosphate Ester Prodrugs. Poster presented at the AAPS Annual Meeting, Indianapolis, IN, USA.
Heimbach, T., Oh, D.M., Li, L.Y., Forsberg, M., Leppänen, J.Y.M., Flynn, G., Fleisher, D., 2003. Absorption rate limit considerations for oral phosphate prodrugs. Pharm. Res. 20, 848–856.
Kearney, A.S., Stella, V.J., 1993. Hydrolysis of pharmaceutically relevant phosphate monoester monoanions: correlation to an established structure-reactivity relationship. J. Pharm. Sci. 82, 69–72.
Macaskie, L.E., Bonthrone, K.M., Yong, P., Goddard, D.T., 2000. Enzymically mediated bioprecipitation of uranium by a *Citrobacter* sp.: a concerted role for exocellular lipopolysaccharide and associated phosphatase in biomineral formation. Microbiology 146, 1855–1867.
Matsunaga, Y., Bando, N., Yuasa, H., Kanaya, Y., 1996. Effects of grinding and tableting on physicochemical stability of an anticancer drug, TAT-59. Chem. Pharm. Bull. (Tokyo) 44, 1931–1934.
Matsunaga, Y., Ohta, R., Bando, N., Yamada, H., Yuasa, H., Kanaya, Y., 1993. Effects of water content on physical and chemical stability of tablets containing an anticancer drug TAT-59. Chem. Pharm. Bull. (Tokyo) 41, 720–724.
McComb, R.B., Bowers, G.N.J., Posen, S., 1979. Alkaline Phosphatase. Plenum Press, New York.
Michaelis, L., Menten, M.L., 1913. Die Kinetik der Invertinwirkung. Biochem. Z. 49, 333–369.
Mullin, J.W., 1993. Crystallization. Butterworth-Heinemann, Ltd., Oxford.
Nomura, Y., Nakajima, M., Tominaga, T., Abe, O., 1998. Late phase II study of TAT-59 (miproxifene phosphate) in advanced or recurrent breast cancer patients (a double-blind comparative study with tamoxifen citrate). Gan to Kagaku Ryoho (Jpn. J. Cancer Chemother.) 25, 1045–1063.
Perry, C.M., McTavish, D., 1995. Estramustine phosphate sodium. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in prostate cancer. Drugs Aging 7, 49–74.
Pinto, M., Robine Leon, S., Appay, M.D., 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell 47, 323–330.
Punzi, J.S., Duax, W.L., Strong, P., Griffin, J.F., Flocco, M.M., Zacharias, D.E., Carrell, H.L., Tew, K.D., Glusker, J.P., 1992. Molecular conformation of estramustine and two analogues. Mol. Pharmacol. 41, 569–576.
Rodriguez-Hornedo, N., Murphy, D., 1999. Significance of controlling crystallization mechanisms and kinetics in pharmaceutical systems. J. Pharm. Sci. 88, 651–660.
Stella, V.J., 1996. A case for prodrugs: Fosphenytoin. Adv. Drug Del. Rev. 19, 311–330.
Stilgenbauer, L.A., Surendran, N., Reddy, A., Michael, S., Liu, H., Freiwald, S., Bobrowski, W.C.S., Heimbach, T., 2000. Validation of 24-Well Plate Format For High Throughput Screening Of Permeability In Caco-2 Cells. Poster presented at the AAPS, Indianapolis.
TenHoor, C.N., Stewart, B.H., 1995. Reconversion of fosphenytoin in the presence of intestinal alkaline phosphatase. Pharm. Res. 12, 1806–1809.
Tominaga, T., 1995. Early Phase II clinical study of TAT-59 (new antiestrogen) in patients with breast cancer. In: Proceedings of the 19th International Chemotherapy Congress (Montreal) 397C (Abs 3045).
Walsh, C., 1979. Enzymatic Reaction Mechanism. W.H. Freeman and Company, San Francisco.
Weissbuch, I., Addadi, L., Lahav, M., Leiserowitz, L., 1991. Molecular recognition at crystal interfaces. Science 253, 637–645.
Wu, X., Whitfield, L.R., Stewart, B.H., 2000. Atorvastatin transport in the Caco-2 cell model: contributions of P-glycoprotein and the proton-monocarboxylic acid co-transporter. Pharm. Res. 17, 209–215.
Yu, L.X., 1999. An integrated model for determining causes of poor oral drug absorption. Pharm. Res. 16, 1883–1887.