Characterization of recombinantly expressed rat and monkey intestinal alkaline phosphatases: in vitro studies and in vivo correlations


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Recombinant intestinal alkaline phosphatase characterization

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Abbreviations:

rIALPs: recombinant intestinal alkaline phosphatases, IALPs: intestinal alkaline phosphatases, ALP: alkaline phosphatase; IVIVC: in-vitro in vivo correlation, RIMS: rat intestinal mucosal scrap; UPLC-MS/MS: ultra-performance liquid chromatography combined with tandem mass spectrometry; PNPP: p-nitrophenolphosphate; PNP p-nitrophenol, in vitro t\(_{1/2}\): incubation time rendering 50% consumption of phosphate prodrug.
Abstract

Intestinal alkaline phosphatases (IALPs) are widely expressed in the brush border of epithelial cells of the intestinal mucosa. Although their physiological role is unclear, they are very significant when it comes to the release of bioactive parent from orally dosed phosphate prodrugs. Such prodrugs can be resistant to cleavage by IALP, or alternatively undergo rapid cleavage leading to the release and precipitation of the less soluble parent. Because purified IALPs from pre-clinical species are not commercially available, and species differences have not been investigated to date, an effort was made to recombinantly express, purify and characterize rat and cynomolgus monkey IALP (rIALP). Specifically, rIALP-catalyzed cleavage of five prodrugs (fosphenytoin, clindamycin phosphate, dexamethasone phosphate, ritonavir phosphate and ritonavir oxymethyl phosphate) was tested in vitro and parent exposure was assessed in vivo (rat only) following an oral dose of each prodrug. It was determined that the rate of phosphate cleavage in vitro varied widely; direct phosphates were more resistant to bioconversion, whereas faster conversion was observed with oxymethyl linked prodrugs. Overall, the rat rIALP-derived data were qualitatively consistent with in-vivo data; prodrugs that were readily cleaved in vitro rendered higher parent drug exposure in vivo. Of the five prodrugs tested, one (ritonavir phosphate) showed no conversion in vitro and minimal parent exposure in vivo. Finally, the apparent $K_m$ values obtained for fosphenytoin and clindamycin phosphate in vitro suggest that IALP is not likely to be saturated at therapeutic doses.
DMD#51987

Introduction:

Poor aqueous solubility could be a serious barrier for the successful delivery of a therapeutic candidate. Therefore, a soluble prodrug approach is generally considered for the purpose of enhancing exposures. Phosphate prodrugs are an effective and commonly used approach to overcome solubility issues in drug delivery and have successfully been used for a number of oral and parenterally administered marketed drugs (de Jong et al., 1997; Fleisher et al., 1996; Heimbach et al., 2003a; Huttunen et al., 2011; Jana et al., 2010; Rautio et al., 2008; Stella, 2010). The advantages of phosphate prodrugs include their chemical stability, several orders of magnitude of improved solubility as compared to parent, and relative ease of synthesis in the presence of a suitable handle (Heimbach et al., 2003a). Importantly, phosphate prodrugs are cleaved in the intestine and generate a super-saturated parent solution that enhances the absorption flux (Heimbach et al., 2003a). On the other hand, poor phosphate cleavage can give rise to an unacceptable oral profile, low parent drug exposure, and a lack of an improvement in clinical efficacy (de Jong et al., 1997; Heimbach et al., 2003a; Stella, 2010). Poorly soluble parents with sufficient solubility in the formulation vehicle, and dissolution times less than their gastrointestinal transit times will exhibit slow yet complete absorption and in these cases a phosphate prodrug may not be beneficial (Heimbach et al., 2003a). Therefore, the successful identification of ideal candidates for an oral phosphate prodrug strategy requires a careful analysis of the properties of the parent drug and promoiety. These include drug and prodrug solubility, dissolution, rate of bioconversion, active and passive transport characteristics, as well as the likelihood for precipitation. Multiple authoritative reviews have elaborated these properties in great detail (Heimbach et al., 2003a; Huttunen et al., 2011; Jana et al., 2010; Rautio et al., 2008; Stella, 2010; Stella and Nti-Addae, 2007).
Following oral dosing, phosphate prodrug cleavage is mediated by intestinal alkaline phosphates (IALPs). The physiological role of IALP is unclear since knock-out mice do not display an overt phenotype but it has been speculated that they play a role in regulating fat absorption, and in preventing the duodenum from damage due to bursts of acid from the stomach (Akiba et al., 2007; Fernandez and Kidney, 2007; Mizumori et al., 2009). IALPs are catalytically promiscuous and are abundantly expressed on intestinal epithelial membrane cells. An orally administered phosphate prodrug must be cleaved to its parent drug, before the parent drug is absorbed into the systemic circulation since the charged phosphate promoiety has very poor intrinsic passive permeability (Fleisher et al., 1996; Heimbach et al., 2003a; Rautio et al., 2008). Given the key role that IALPs play in the success of a phosphate prodrug, it is important to understand their kinetic characteristics, species differences, and in vitro to in vivo correlations (IVIVCs). Calf intestinal, bovine intestinal and kidney, porcine kidney and intestine, rabbit intestine, and human placental, liver and leucocyte alkaline phosphatases (ALPs) are available commercially; however, rat and monkey IALPs, two common preclinical species in drug discovery, are not commercially available. Hence, in the present study, we have characterised rat and monkey in-house expressed recombinant IALP (rIALP). Probe phosphate prodrugs, either directly attached to a molecule or incorporated via linkers, were selected for this study. Fosphenytoin (Pubmed CID 56339), dexamethasone phosphate (Pubmed CID 9400), clindamycin phosphate (Pubmed CID 443385), ritonavir oxymethyl phosphate (DeGoey et al., 2009) and ritonavir phosphate (DeGoey et al., 2009) were selected as model prodrugs, which are hydrolyzed to their corresponding highly permeable and poorly soluble parent drugs phenytoin (Pubmed CID 657302), dexamethasone (Pubmed CID 5743), clindamycin (Pubmed CID 29029), and ritonavir.
DMD#51987

(Pubmed CID 392622), respectively (Figure 1). It was also possible to study the bioconversion rates of the probe substrates in the presence of rat intestinal mucosal scraps (RIMS). Furthermore, the kinetic parameters describing fosphenytoin and clindamycin phosphate cleavage by rat and monkey rIALP, as well as RIMS, were determined. Finally, in vivo oral pharmacokinetic studies in rats were also conducted to understand the impact of in vitro bioconversion rate of phosphate prodrugs on in vivo exposure of their respective parent compounds. Based on the IVIVC in rat (and monkey in vitro data), and the availability of a well-characterized recombinant human IALP enzyme (subject of a separate manuscript), it may be possible to predict whether a new phosphate prodrug will show improved exposures over parent in humans. This information can be used in support of decision-making in an early drug discovery setting.

Materials and methods

Chemicals

Human placental ALP, bovine ALP, fosphenytoin, sodium phenytoin, clindamycin phosphate, clindamycin hydrochloride, sodium chloride, glycerol, protease inhibitor cocktail, IGEPAL (octylphenoxypolyethoxyethanol), 2-hydroxypropyl-beta-cyclodextrin, EDTA, tris-HCl and phenylmethylsulphonyl fluoride were purchased from Sigma-Aldrich (St. Louis, USA). Ritonavir phosphate and ritonavir oxymethyl phosphate were synthesized (>98% pure) by the Discovery Chemical Synthesis department at Biocon-Bristol Myers Squibb Research and Development centre (BBRC), Bangalore, India. QuantiChrom™ Alkaline Phosphatase Assay Kit was purchased from BioAssay Systems (Hayward, CA). The bioanalytical internal standard was
an in-house proprietary compound. Liquid chromatography grade acetonitrile, dimethylsulfoxide and ethanol were purchased from E Merck Limited (Mumbai, India). MultiScreen Solvinert filter plates (0.45 µM, low binding hydrophilic PTFE) were purchased from Millipore (Carriigtwohill, Ireland). All aqueous solutions were prepared in Mili-Q water (Millipore, MA). All other chemicals were of analytical grade.

**Expression and purification of IALP**

The cloning, expression, purification and biochemical characterization of rat and cynomolgus rIALP are described elsewhere (Ghosh et al., submitted). Briefly, the IALP genes were cloned from intestinal RNA (Biochain, CA) using standard molecular biology techniques. The active forms of these enzymes were expressed in Sf9 insect cell and purified from the media after secretion of the post-translationally modified protein. The original secretion signal sequence at the N-terminus was retained and the extreme C-terminal region was replaced by a hexa-histidine tag to facilitate purification. These recombinant enzymes were found to be glycosylated and active under standard reaction conditions.

**Preparation of RIMS**

Immediately after excising intestines from male SD rats, a segment of approximately 1 m of small intestine was placed in an ice-cold Petri dish and washed with phosphate-buffered saline (pH 7.4), containing phenylmethylsulphonyl fluoride (1 mM). Subsequently, the mucosa was scraped off with a glass slide, minced and homogenized with ice cold lysis buffer using a homogenizer. The lysis buffer was composed of 50 mM, tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% IGEPAL, 5% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitor cocktail. This homogenate was used for ALP activity determination. The total
protein concentration was determined by the bicinchoninic acid protein assay kit (Sigma Aldrich).

**Evaluation of ALP activity**

ALP activity of RIMS and rIALP was determined using QuantiChrom™ Alkaline Phosphatase Assay Kit according to manufacturer suggested protocol. The measurement of ALP activity was based on the hydrolysis of p-nitrophenolphosphate (PNPP) to p-nitrophenol (PNP), a yellow colored product which shows absorbance at 405 nm. The absorbance was measured using UV spectrophotometer (Tecan, Männedorf, Switzerland). Activities were expressed as IU/mL (1 IU = 1 µmole PNNP metabolized/minute at 37ºC, pH 9.8), and determined in triplicate.

**Determination of prodrug cleavage rate in vitro**

To assess the potential for generation of parent drug from the phosphate prodrug, 200 µL of rIALP or RIMS dissolved in 100 mM Tris-HCl buffer (pH 7.4) was added to 200 µL of freshly prepared prodrug solution (10 µM) prepared in the same buffer. The ALP activites ranged from 0.004 to 0.03 IU/mL. Incubations were performed in triplicate using 96 well plates (Hamburg, Germany) at 37°C in a shaking water bath (Julabo Scientific, Germany). The prodrug and enzyme solution were pre-warmed at 37°C before the reaction was initiated by addition of substrate. The incubation was performed for 120 minutes with 25 µl aliquots taken at 0, 5, 10, 15, 30, 60, 90 and 120 minutes. The assays were terminated at designated time points by the addition of 200 µL of ice-cold acetonitrile containing internal standard followed by centrifugation at 4000 x g for 4 min. The supernatant was transferred to a new 96-well plate for analysis by ultra-performance liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS). RIMS, rat rIALP, monkey rIALP, human placental ALP and bovine ALP were
DMD#51987

tested in this assay. The t1/2 value for each prodrug/protein combination was determined from the
natural logarithm of % remaining versus time plots.

Determination of kinetic parameters

Incubations were performed in triplicate using 96 well plates (Hamburg, Germany). The reaction
mixture (100 µL) contained 100 mM tris buffer (pH 7.4), rIALP protein (0.4 ng/mL protein for
fosphenytoin; 12.5 ng/mL protein for clindamycin phosphate) or RIMS (500 ng/mL protein for
fosphenytoin and 1250 ng/mL protein for clindamycin phosphate) and varying concentration of
fosphenytoin (0.15 to 1000 µM) and clindamycin phosphate (19 to 5000 µM). Prior to reaction
initiation by the addition of substrate, the incubation medium containing protein was incubated at
37°C for 10 min. Concentration of the organic solvent used (dimethyl sulfoxide) was < 0.5% of
the total incubation volume. Control incubations were performed for each substrate with no
protein present to account for any potential protein independent loss of the prodrug over the
incubation time. Incubations were performed at 37°C in a shaking water bath for 10 minutes and
terminated by the addition of 200 µL of ice-cold acetonitrile containing internal standard,
followed by centrifugation at 4000 x g for 4 min. The supernatant was transferred to a new 96-
well plate for analysis by UPLC-MS/MS. The protein concentrations and incubation time for the
kinetic reactions were selected based on preliminary assays to ensure linearity of product
formation, and that the substrate consumption during the reaction was less than 20%.

Pharmacokinetic study

Animals and formulation. SD rats weighing 260-280 g (8-10 weeks age) were obtained from
Harlan Laboratories Inc. (Netherlands). All the animal experiments were conducted in the
animal research facility of BBRC (Bangalore, India). Rats were housed in well ventilated cages at room temperature (24 ± 2°C) and 40-60 % relative humidity while on a regular 12 h light-dark cycle. Animal study protocols were approved by the organizational Animal Ethics Committee. Phenytoin (sodium salt), fosphenytoin, dexamethasone and dexamethasone phosphate formulation were prepared in water, whereas clindamycin (hydrochloride salt) and clindamycin phosphate were prepared in phosphate buffer, pH 7.4. Ritonavir, ritonavir oxymethyl phosphate and ritonavir phosphate formulation were prepared in 10% ethanol, 36 % of 2-hydroxypropyl-beta-cyclodextrin and 54 % water. All the formulations were filtered through 0.22 μm filter before administration.

**Oral pharmacokinetic study.** Male SD rats were fasted overnight (12-14 h) before dosing and had free access to water throughout the experimental period. All the parent drugs and their corresponding phosphate prodrugs were administered orally as solution (5 mg/mL) to a group of three rats. All prodrugs were administered at the dose equivalent to their respective parent dose. The doses are summarized in Table 5. Animals were provided with their standard diet 4 h after dosing, and blood samples (approximately 0.2 mL) were collected from the jugular vein into microfuge tubes containing EDTA at predetermined time points (10 min to 24h). Plasma was harvested by centrifuging the blood at 4000 x g for 3 min on Eppendorf 5810 R (Hamburg, Germany) and stored frozen at -70 ± 10°C until analysis.

**Sample Preparation.** Plasma (50 μL) and internal standard in acetonitrile (200 μL) were mixed in 96-well hydrophilic solvinert plates. The acetonitrile mixtures were vortexed and centrifuged
at 4000 x g for 4 min. Filtrates were collected in a 96-well * 1 mL round collection plate and analysed by UPLC-MS/MS.

**UPLC-MS/MS analysis**

Standard curves for prodrug and parent were prepared separately by spiking a known amount of compound into blank matrix and processed according to the procedures described above. Analysis was carried out using UPLC-MS/MS consisting of a Waters Acquity Integrated System (Milliford, USA) and API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode. A 4 µL aliquot of each processed sample was injected into a reverse phase column (BEH C18, 1.7 μm, 2.1 × 50 mm). The MS/MS conditions are summarized in Table 1. Chromatographic resolution of prodrugs and parents were achieved using a gradient UPLC method at a flow rate of 0.7 ml/min. Mobile phase A consisted of 0.1% ammonia for negative mode, or 0.1% Formic Acid in water for positive mode while mobile phase B consisted of 0.1% formic acid in acetonitrile for positive mode, or acetonitrile for negative mode MS/MS. The gradient UPLC method began with 5% B maintained for 0.8 min, ramped up to 95% from 0.8 to 1.2 min, maintained at 95% B up to 1.5 min, ramped down to 5% B from 1.5 to 1.8 min, and maintained at 5% B until 2.2 minutes, the total run time per injection. The lower limit of quantitation for all the analytes was 0.61 nM. The standard curves were linear (r ≥ 0.995) over the concentration of 0.61 to 5000 nM for all the analytes. The assay accuracy was found to be between 85 and 115%. The parent and prodrug were separated chromatographically and no in-source conversion was observed. Ex-vivo stability in buffer was ensured prior to conduct of the all in-vitro experiments.
**Data analysis:**

The rates of formation of the parent compound versus the prodrug concentrations were fit to the Michaelis-Menten equation (Equation 1) using GraphPad Prism 3.03 software (GraphPad Inc., San Diego, CA, USA). Estimated kinetic parameters were: $V_{\text{max}}$, the maximal velocity of uninhibited reaction, $K_m$, substrate concentration at which the rate of reaction is half of $V_{\text{max}}$.

\[
v = \frac{V_{\text{max}} \cdot [S]}{K_m + S} \quad \text{Equation 1}
\]

In some cases, the data were not adequately described by Equation 1 and had to be fit to an equation which assumes two binding sites (Equation 2); one with a low $V_{\text{max}}$ and $K_m$, and the other with a high $V_{\text{max}}$ and $K_m$.

\[
v = \frac{(V_{\text{max}1} \cdot [S]) + (C_{\text{int}} \cdot S^2)}{K_{m1} + S} \quad \text{Equation 2}
\]

Pharmacokinetic parameters were determined based on a non-compartmental approach using Kinetica software (version 4.4.1, Thermo Electron Corporation). The area under the plasma concentration versus time curve up to the last quantifiable time point (AUC$_{0-t}$) was computed using the mixed log linear method.

**Results**

**PNNP Cleavage Rate Determination**

The rate of cleavage of PNNP to PNP was determined in the presence of rat and monkey rIALP and RIMS (Table 2). RIMS exhibited lower activity on a per mg total protein basis, whereas activity was greatest with monkey rIALP versus rat rIALP. The lower activity of RIMS is
unsurprising since that preparation contains multiple mucosal proteins and the result is similar to that reported previously (Wada et al., 2001).

Prodrug cleavage rate in vitro:

Fosphenytoin, dexamethasone phosphate, clindamycin phosphate, ritonavir oxymethyl phosphate and ritonavir phosphate (10 µM) were used as probe substrates to assess the bioconversion of phosphate prodrugs using RIMS, rat rIALP and monkey rIALP. The disappearance of the phosphate prodrugs and appearance of parent were monitored during the incubation. A standard 0.03 IU/ml protein concentration was used, except for RIMS and monkey rIALP wherein lower protein concentrations were utilized. This eliminates the need for any protein optimization and provides a consistent starting concentration for all t_{1/2} experiments. Since, the starting protein concentration will have a significant impact on the t_{1/2} of the prodrug, the cleavage rates normalized to protein concentrations were also calculated (summarized in Table 3). While ritonavir phosphate showed no turnover in all of the test systems, dexamethasone phosphate and fosphenytoin showed rapid cleavage, and ritonavir oxymethyl phosphate and clindamycin phosphate showed intermediate cleavage rates. RIMS and rat rIALP showed similar rates of cleavage when the protein concentrations were expressed in IU units. For 3 of 4 substrates, monkey rIALP cleavage rates were higher than rat rIALP cleavage rates (Table 3). Human placental ALP and bovine IALP were also investigated for the rate of turnover of the probe substrates. Both these enzymes showed rapid conversion of all probe substrates, including those that showed relatively slower cleavage in rIALP (clindamycin phosphate and ritonavir oxymethyl phosphate).
Kinetics of prodrug cleavage in vitro:

The kinetic parameters describing clindamycin phosphate and fosphenytoin cleavage are presented in Table 4. The optimal protein concentrations and incubation times are also summarized in the table. In addition, representative kinetic plots for fosphenytoin and clindamycin are presented in Figures 2 and 3, respectively. In general, the $K_m$ values were high suggesting a low possibility of kinetic saturation at therapeutic doses. However, high gut concentrations may still predispose saturation in cases of high doses. For fosphenytoin, monkey rIALP had a much higher $K_m$ when compared to rat, while for clindamycin phosphate the opposite trend was noted. The $V_{max}$ values (expressed in IU units) for both substrates were similar between the two species. The similarity in $V_{max}$ values were consistent with the normalized cleavage rates obtained from the half life ($t_{1/2}$) experiment. Additionally, the $V_{max}$ for fosphenytoin was higher than clindamycin, consistent with the $t_{1/2}$ data. In the presence of RIMS, both fosphenytoin and clindamycin phosphate exhibited biphasic kinetics. Both $V_{max}$ and $K_m$ values for the low-$K_m$ binding site are reported when biphasic kinetics was observed. The rate for high-$K_m$ phosphate cleavage was higher for fosphenytoin versus clindamycin (2.4 $\mu$L/min/ng-protein versus 0.38 $\mu$L/min/ng-protein).

Pharmacokinetic study:

The results from the in vivo studies are summarized in Table 5. In the case of fosphenytoin, dexamethasone phosphate and ritonavir oxymethyl phosphate, dosing a prodrug gave a clear improvement in oral exposure (AUC increased by 2.1 - 2.5 fold, $C_{max}$ increased by 1.9 - 13.5 fold). The changes in $C_{max}$ were statistically significant for phenytoin, clindamycin and ritonavir,
while the AUC changes were statistically significant for ritonavir. Clindamycin phosphate cleavage was slowest in the presence of RIMS, compared to other substrates and that could explain why no C_{max} improvement was observed. All prodrugs, except ritonavir phosphate, cleaved in vitro in rIALP and RIMS; hence the improved exposure is consistent with in vitro data. In the case of ritonavir phosphate no cleavage was observed in-vitro and no exposure of ritonavir was observed upon dosing of the prodrug. The IVIVC for each prodrug is summarized in Table 6 and discussed further in the following section.

Discussion:

To date, no previous study has characterized expressed recombinant rat and monkey IALP. Here, we report detailed kinetic characterization of rIALP from these two species, and correlation of in vitro systems to in vivo systemic exposure in rats. A previous study evaluated a rat intestinal preparation of ALP and attempted to quantify the amount of protein and its kinetics (Yuan et al., 2009). However, there were multiple issues with this study starting from the fact that a secreted human placental ALP was used to calibrate rat IALP which assumes that rat IALP and human placental ALP have identical activities and hence the activity of human placental ALP can be used to determine the quantity of rat IALP. Additionally, the activity assays in that study employed phosphate buffer as the medium, and it has been reported (and confirmed in the author’s laboratory, data not shown) that phosphate inhibits IALP activity (Coburn et al., 1998). Finally, the kinetic parameters determined from the intestinal preparation reported neither incubation time nor protein concentration, and there was no confirmation of linear conditions maintained during the kinetic characterization.
The pH values to be utilized during in vitro incubations also require special consideration since the incubation pH of 7.4 represents neither the intestinal pH (pH range of 4 to 6.5), nor the optimum pH of these enzymes (pH range of 8 to 11). While, IALP is present in the slightly acidic intestinal environment, it is reported that duodenal secretion of bicarbonate may provide an alkaline microclimate, via a disequilibrium unstirred boundary layer, to boost its activity (Akiba et al., 2007; Mizumori et al., 2009). The bicarbonate secretion is a part of an ecto-purinergic pH regulatory system to protect the duodenum against damage from periodic bursts of concentrated gastric acid and may also be one of the key physiological functions of IALP (Akiba et al., 2007; Mizumori et al., 2009). It has also been suggested that the pH optimum varies with substrate concentration and at physiological concentrations of phosphate esters the optimum pH is 7.4 whereas at higher substrate concentrations, the optimal pH is higher (Warnes, 1972). Hence, using a pH of 7.4 for in vitro incubations may be a reasonable compromise.

The rIALP mediated cleavage rates were found to be in the range of ~ 0.5 to 70 mL/min/IU for all probe phosphate prodrugs indicating the important role of the linker connecting the phosphate group to the parent (Table 3). Other authors have also reported different dephosphorylation rates depending on the prodrug (DeGoey et al., 2009; Heimbach et al., 2003a). Fosphenytoin has a methylene linker and shows the fastest turnover. Dexamethasone phosphate, although a direct prodrug, has the phosphate moiety on a flexible primary alcohol, so the enzyme is probably able to access the enzyme active site with ease and shows rapid conversion. Clindamycin is a direct prodrug and shows relatively slower turnover. Amongst the two ritonavir prodrugs, the candidate with a flexible oxymethyl linker cleaves while the direct phosphate shows no turnover, which could be due to the poor accessibility of the phosphate ester into the enzyme active site (DeGoey et al., 2009). The rank order (from slowest to fastest turnover) in case of rat rIALP was ritonavir
oxymethyl phosphate, clindamycin phosphate, dexamethasone phosphate and fosphenytoin, whereas in RIMS the rank order was clindamycin phosphate, ritonavir oxymethyl phosphate, dexamethasone phosphate and fosphenytoin. Only the two slowest substrate rank ordered differently, probably due to the fact that both these prodrugs had similarly slow cleavage rates. A scaling factor, calculated as the ratio of rat rIALP normalized cleavage rate divided by RIMS normalized cleavage rate, was between 1.1 and 3.6 suggesting that the protein concentration used in the rat rIALP assay was not too different from what could be encountered in vivo.

Complete kinetic characterization of fosphenytoin and clindamycin phosphate cleavage was carried out after compound incubation with rat and monkey rIALP and RIMS (Table 4, Figures 1 and 2). Fosphenytoin and clindamycin phosphate were chosen for further kinetic characterization since they represented the fastest and amongst the slowest turned over prodrugs. The V_max values were found to be lower for clindamycin formation across the species in comparison to phenytoin formation. However, for a particular substrate there was not much difference between rat and monkey in V_max values, although the K_m values differed considerably between rat and monkey rIALP. Fosphenytoin had a lower K_m in rat as compared to monkey whereas clindamycin phosphate showed the opposite trend. RIMS for both substrates showed biphasic kinetics with a low- and high-K_m component. Given the K_m values, it appears unlikely that saturation could occur at therapeutic doses of these prodrugs. It should be noted that solubility limitations of the parent upon cleavage from the prodrug could influence the kinetic parameters.

In vivo studies were performed for 4 pairs of parents and prodrugs to verify if in vitro cleavage could predict in vivo exposure improvement (Table 5). It was not possible to provide quantitative estimates of improvement in AUC based on in vitro cleavage rates (Table 6). Both the rapidly turned over fosphenytoin and slowly turned over clindamycin phosphate showed roughly the
same increase in AUC (~2-fold). However prodrugs that cleaved in vitro provided 2-fold or higher in vivo exposures of their respective parents, whereas the one prodrug that did not cleave in vitro did not show any in vivo exposure of its parent. A better correlation was observed in fold-changes in C_max versus cleavage rates. For example, the two prodrugs with the slowest cleavage rates (clindamycin phosphate and ritonavir oxymethyl phosphate) showed the lowest fold increases in C_max (<1.9) whereas the rapidly turned over fosphenytoin and dexamethasone phosphate showed 3.7 and 13.5 fold improvements in C_max, respectively. An examination of parent solubilities and permeabilities could not provide an explanation for the quantitative differences in C_max and AUC ratio changes. Suspension dosing of parent or prodrug may have provided some information into building an IVIVC but in these studies the parents possessed sufficient solubility for solution dosing in aqueous vehicles (except for ritonavir and its prodrugs). While it is assumed that IALPs are responsible for all dephosphorylation activity, one report suggests that phytases (which releases usable inorganic phosphate and essential metals from phytic acid) can also perform this reaction, at acidic pH values (Nakano et al., 2001). This could also lead to a potential disconnect between in vitro rIALP activity and in vivo exposure. As previously mentioned, a number of phosphate prodrugs do not show improved in vivo exposure due to a number of reasons since very rapid cleavage by IALP may result in supersaturated solutions of the parent and its subsequent precipitation, whereas very slow cleavage may result in insufficient parent being available for absorption. In one study, wherein multiple prodrugs with different parent solubilities and cleavage rates were synthesized, it was found that while good prodrug solubility is a pre-requisite, only those prodrugs with a cleavage t_1/2 around a narrow window of 35 min offered much better exposure of parent (Sohma et al., 2003). Rapid
DMD#51987

drop-offs in exposure were observed with minor deviations in the t_{1/2} away from 35 min. This study captures the challenge associated in balancing solubility with cleavage rate.

The rIALP in vitro experiment occurs in a static system wherein involvement of active or passive absorption, and oxidative and conjugative metabolism are absent. As such, it represents only one of several dimensions of a complex in vivo system. Reports have shown that including an absorptive component can alter the dynamic equilibrium between bioconversion, supersaturation and absorption (Bevernage et al., 2012; Brouwers et al., 2007; Heimbach et al., 2003b). While this is a typical shortcoming of all fractional in vitro systems, the advantage of the rIALP turnover assay is that when the right protein concentration is used, it provides a strong indication if cleavage is going to be insufficient or potentially very rapid in vivo. While, in isolation, the rate of metabolism of prodrug to parent is probably of limited value, it shows promise when correlated with increased in vivo exposure. Once a correlation between in vitro cleavage rate and in vivo exposure is established, in vitro experiments may replace more resource intensive in vivo experiments as the cleavage rate is optimized. Another utility of rIALP is in assessing species differences/similarities. For example, if a rat IVIVC is obtained and confirmed with monkey in vitro and in-vivo experiments, then it may be possible to predict if human exposures will improve with a phosphate prodrug. This has great value in a discovery setting when a number of prodrug candidates may be under consideration.

In summary, we have elucidated the cleavage rates of 5 prodrugs in three matrices, determined detailed kinetics for two prodrugs and correlated in vitro data with in vivo exposures in rats. While the activity assay showed that direct linked phosphate prodrugs are resistant to cleavage, the detailed kinetics showed in general high K_m values. The in-vivo improvement in AUC and C_{max} were consistent with in vitro cleavage of phosphate, although not in a quantitative sense.
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References:


conversion time for higher gastrointestinal absorption of prodrugs based on spontaneous chemical cleavage. *J Med Chem* **46**:4124-4135.


Figure Legends

Figure 1: Prodrug and parent structures of direct (clindamycin phosphate, ritonavir phosphate, dexamethasone phosphate) and linker based (fosphenytoin and ritonavir oxymethyl phosphate) prodrugs.

Figure 2: Kinetics of formation of phenytoin (parent drug) from fosphenytoin (phosphate prodrug) in the presence of rIALP (A) and RIMS (B). The incubation time was 10 min, while the IALP (IU/ml) concentrations were 74*10^{-6}, 121.2*10^{-6} and 185*10^{-6} for monkey, rat rIALP and RIMS, respectively.

Figure 3: Kinetics of formation of clindamycin (parent drug) from clindamycin phosphate (phosphate prodrug) in the presence of rIALP (A) and RIMS (B). The incubation time was 10 min, while the IALP (IU/ml) concentrations were 2300*10^{-6}, 3787*10^{-6} and 1000*10^{-6} for monkey, rat rIALP and RIMS, respectively.
Table 1: UPLC-MS/MS conditions for the detection of prodrugs and parent.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM transition</th>
<th>DP</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenytoin</td>
<td>251/102</td>
<td>-72</td>
<td>-33</td>
</tr>
<tr>
<td>Fosphenytoin</td>
<td>361/331</td>
<td>-60</td>
<td>-15</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>425.2/126.2</td>
<td>100</td>
<td>43</td>
</tr>
<tr>
<td>Clindamycin phosphate</td>
<td>503.2/467</td>
<td>-110</td>
<td>-30</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>721/296.1</td>
<td>72</td>
<td>28</td>
</tr>
<tr>
<td>Ritonavir phosphate</td>
<td>799.2/629.3</td>
<td>-90</td>
<td>-27</td>
</tr>
<tr>
<td>Ritonavir oxymethyl phosphate</td>
<td>831/733.2</td>
<td>60</td>
<td>19</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>393.2/373</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>Dexamethasone phosphate</td>
<td>471.2/139</td>
<td>-100</td>
<td>-38</td>
</tr>
</tbody>
</table>

DP: declustering potential; CE: collision energy
Table 2: Alkaline phosphatase (ALP) specific activity of rIALP and RIMS

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein conc. (mg/mL)</th>
<th>ALP specific activity (IU/mL)</th>
<th>ALP specific activity (IU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkey-rIALP</td>
<td>0.67</td>
<td>203</td>
<td>303</td>
</tr>
<tr>
<td>Rat-rIALP</td>
<td>1.12</td>
<td>206</td>
<td>183.9</td>
</tr>
<tr>
<td>RIMS</td>
<td>13.93</td>
<td>5.21</td>
<td>0.37</td>
</tr>
</tbody>
</table>

rIALP: recombinant intestinal alkaline phosphatase

RIMS: rat intestinal mucosal scraps

IU = 1 µmole PNNP metabolized/min at 37°C, pH 9.8

Data are reported as the mean of triplicates (CV <10%)
Table 3: In vitro alkaline phosphatase (ALP) mediated half-life and normalized rate of metabolism rate of various phosphate prodrugs

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme conc (IU/mL)</th>
<th>Dexamethsone Phosphate</th>
<th>Fosphenytoin Clindamycin phosphate</th>
<th>Ritonavir Oxy-methyl Phosphate</th>
<th>Ritonavir Phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat rIALP</td>
<td>0.03</td>
<td>1.7 (13.6)</td>
<td>0.7 (33.0)</td>
<td>11.5 (2.0)</td>
<td>13.1 (1.8)</td>
</tr>
<tr>
<td>RIMS</td>
<td>0.0185</td>
<td>3.2 (11.7)</td>
<td>1.3 (28.8)</td>
<td>66.3 (0.56)</td>
<td>36.9 (1.0)</td>
</tr>
<tr>
<td>Monkey rIALP</td>
<td>0.0045</td>
<td>6.6 (23.3)</td>
<td>2.2 (70.0)</td>
<td>120 (1.3)</td>
<td>11.6 (13.3)</td>
</tr>
<tr>
<td>Human placental ALP</td>
<td>0.03</td>
<td>1.5 (15.4)</td>
<td>1.4 (16.5)</td>
<td>9.6 (2.4)</td>
<td>14.6 (1.6)</td>
</tr>
<tr>
<td>Bovine ALP</td>
<td>0.03</td>
<td>8.3 (2.8)</td>
<td>6.2 (3.7)</td>
<td>17.1 (1.4)</td>
<td>5.0 (4.6)</td>
</tr>
</tbody>
</table>

$t_{1/2}$ unit: min

Normalized rate of metabolism units (rate/protein concentration): ml/IU/min

Data are reported as the mean of triplicates (CV <10%)
Table 4: Kinetic parameters for the alkaline phosphatase-catalysed hydrolysis of various phosphate prodrugs.

<table>
<thead>
<tr>
<th>Preps</th>
<th>$K_m$ (µM)$^a$</th>
<th>$V_{max}$ (nmol/min/ng-protein)$^b$</th>
<th>$V_{max}$ (µmol/min/IU)</th>
<th>$V_{max}/K_m$ (µL/min/ng)</th>
<th>$V_{max}/K_m$ (mL/min/IU)</th>
<th>Protein Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fosphenytoin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey-rIALP</td>
<td>768.0 ± 221.9</td>
<td>2.69 ± 0.40</td>
<td>8.88</td>
<td>3.50</td>
<td>11.55</td>
<td>0.4 ng/mL, 74 *10^-6 IU/mL</td>
</tr>
<tr>
<td>Rat-rIALP</td>
<td>25.8 ± 9.1</td>
<td>1.56 ± 0.12</td>
<td>8.48</td>
<td>60.40</td>
<td>328.44</td>
<td>0.4 ng/mL, 121.2 *10^-6 IU/mL</td>
</tr>
<tr>
<td>RIMS</td>
<td>23.1 ± 3.1</td>
<td>0.002 ± 0.00</td>
<td>Biphasic 5.40</td>
<td></td>
<td></td>
<td>500 ng/mL, 185 *10^-6 IU/mL</td>
</tr>
<tr>
<td><strong>Clindamycin-Phosphate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey-rIALP</td>
<td>86.4 ± 11.6</td>
<td>0.08 ± 0.002</td>
<td>0.264</td>
<td>0.92</td>
<td>3.036</td>
<td>12.5 ng/mL, 2300 *10^-6 IU/mL</td>
</tr>
<tr>
<td>Rat-rIALP</td>
<td>460.8 ± 52.5</td>
<td>0.16 ± 0.005</td>
<td>0.870</td>
<td>0.34</td>
<td>1.849</td>
<td>12.5 ng/mL, 3787 *10^-6</td>
</tr>
</tbody>
</table>

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| RIMS   | 5.2 ± 18.8 | 0.001 ± 0.004 | Biphasic 2.70 | 2703 ng/mL, $1000 \times 10^{-6}$ IU/mL |

Incubation time: 10 min
Table 5: Pharmacokinetic parameters after oral administration of parent and their corresponding phosphate prodrugs to rats (n = 3; mean ± S.D).

<table>
<thead>
<tr>
<th>Compound Administered</th>
<th>PO Dose, mg/kg</th>
<th>AUC_{inf} (nM*h)</th>
<th>C_{max} (nM)</th>
<th>T_{max} (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenytoin</td>
<td>5</td>
<td>1738 ± 633</td>
<td>1193 ± 777</td>
<td>0.25</td>
</tr>
<tr>
<td>Fosphenytoin</td>
<td>6.5</td>
<td>3639 ± 1032</td>
<td>4439 ± 1000*</td>
<td>0.25</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>50</td>
<td>13741 ± 1329</td>
<td>9371 ± 985</td>
<td>0.33 ± 0.1</td>
</tr>
<tr>
<td>Clindamycin phosphate</td>
<td>56.5</td>
<td>32968 ± 12197</td>
<td>9069 ± 1703</td>
<td>0.67 ± 0.1</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>5</td>
<td>109 ± 38</td>
<td>40 ± 19</td>
<td>1.25 ± 0.8</td>
</tr>
<tr>
<td>Ritonavir Phosphate</td>
<td>5.6</td>
<td>BLQ (&lt;0.6nM)*</td>
<td>BLQ (&lt;0.6nM)*</td>
<td>BLQ (&lt;0.6nM)</td>
</tr>
<tr>
<td>Ritonavir oxymethyl phosphate</td>
<td>5.8</td>
<td>242 ± 97</td>
<td>77 ± 35</td>
<td>2.0</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>5</td>
<td>1529 ± 219</td>
<td>642 ± 162</td>
<td>0.25</td>
</tr>
<tr>
<td>Dexamethasone phosphate</td>
<td>6</td>
<td>3213 ± 2718</td>
<td>8670 ± 660*</td>
<td>0.25</td>
</tr>
</tbody>
</table>

- p<0.05 in a one-way analysis of variation test.
Table 6: In vitro cleavage rates correlated with observed in vivo changes in exposure.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Normalized rate of metabolism, (ml/IU/min)</th>
<th>Fold-increase in $C_{\text{max}}$</th>
<th>Fold-increase in AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fosphenytoin to Phenytoin</td>
<td>33.00</td>
<td>3.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Clindamycin phosphate to Clindamycin</td>
<td>2.01</td>
<td>1.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Ritonavir Phosphate to Ritonavir</td>
<td>&lt;0.19</td>
<td>No exposure</td>
<td></td>
</tr>
<tr>
<td>Ritonavir oxymethylphosphate to Ritonavir</td>
<td>1.76</td>
<td>1.9</td>
<td>2.5</td>
</tr>
<tr>
<td>Dexamethasone Phosphate to Dexamethasone</td>
<td>13.59</td>
<td>13.5</td>
<td>2.1</td>
</tr>
</tbody>
</table>
Figure 1

Fosphenytoin → Phenytoin

Clindamycin Phosphate → Clindamycin

Dexamethasone phosphate → Dexamethasone

Ritonavir Phosphate → Ritonavir
Figure 2A

Phenytoin Rate (nmols/min/ng-protein) vs. Fosphenytoin Conc. (mM)

- ■ monkey
- ◯ rat

Figure 2B

Phenytoin Rate (nmols/min/ng-protein) vs. Fosphenytoin conc (mM)