Evaluation of in Vitro Models for Screening Alkaline Phosphatase-Mediated Bioconversion of Phosphate Ester Prodrugs

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ABSTRACT:

Generating a phosphate prodrug is one of the common approaches for circumventing poor solubility issues of a parent drug. Alkaline phosphatase (ALP) level was determined in rat intestine mucosa scraps, human colon carcinoma (Caco-2) cells, and Madin-Darby canine kidney (MDCK) cells to characterize in vitro models for ALP-mediated phosphate prodrug conversion. In addition, fosphenytoin and fosfucnozole were used as probe prodrugs to evaluate the models. The highest amount of ALP was detected in rat intestinal mucosa scraps, whereas ALP in 5-day cultured MDCK cells was minimal. As anticipated, ALP levels correlated with the parent drug conversion; the shortest cleavage half-life (t½) was observed in rat mucosa scraps; and MDCK cells showed the slowest conversion. Furthermore, the polarized conversion for the prodrugs was observed in Caco-2 monolayer cells, suggesting the polarized localization of alkaline in differentiated Caco-2 cells. The rate of ALP-mediated conversion was prodrug concentration-dependent with Michaelis-Menten constants of 1160 and 351 µM for fosphenytoin and fosfucnozole, respectively, determined in Caco-2 cells. The results revealed that whereas the intestinal mucosa scraps reserved the highest ALP activities and were shown as a promising in vitro tool for screening the bioconversion of phosphate prodrug, Caco-2 monolayers could provide the predictive information of bioconversion and further offer the capability in characterizing the permeability of prodrug and parent drug.

Poor aqueous solubility could be a serious barrier for the successful delivery of a therapeutic candidate for both oral and parenteral administrations. For oral delivery, drugs with poor aqueous solubility often show low or erratic bioavailability, such as large pill size, or/and certain food and water restrictions are required. In parenteral use, the drug candidates with poor water solubility often require a large injection volume for dissolving the drug mass and longer injection time, which may cause side effects, such as local irritation at the injection site or erratic systemic delivery as a result of the possible crystallization at the local administrative site. Phosphate prodrugs are one of the effective and most commonly used approaches used to overcome these issues in drug delivery (Fleisher et al., 1996). By introducing an ionizable phosphate group to the parent drug molecule, phosphate prodrugs are usually highly water-soluble. Most importantly, phosphate prodrugs are readily cleaved by alkaline phosphatase (ALP), an enzyme widely distributed in plasma and a variety of tissues, to their parent drugs (Fleisher et al., 1985, 1996). This approach has been successfully used for a number of oral and parenterally administered drug candidates on the market (de Jong et al., 1997).

Because of the introduction of ionic and polar features, a phosphate prodrug usually has inherent low permeability than its parent drug molecule. There is abundant ALP expressed on intestine epithelial membrane cells; therefore, when orally administered, a phosphate prodrug is rapidly cleaved to its parent drug before the parent drug is absorbed into the systemic circulation (Fleisher et al., 1985; Amidon et al., 1995; Kasim et al., 2004). To achieve the maximal absorption, it is ideally required that the poorly soluble parent drug possesses high permeability, known as class II compounds in the biopharmaceutical classification system (Amidon et al., 1995; Fleisher et al., 1996; Kasim et al., 2004). The antiviral drug fosamprenavir serves as an example of a phosphate prodrug converted to its parent drug, amprenavir, by ALP located in intestine epithelial membranes. The highly permeable parent drug amprenavir is subsequently rapidly absorbed (Becker and Thornton, 2004; Furfine et al., 2004). However, there are many phosphate prodrugs that fail to achieve an acceptable oral profile; thus, the phosphate prodrug approach still remains a challenge for providing an oral delivery option. Therefore, efficient in vitro screening tools for phosphate prodrug bioconversion and absorption assessment are needed at an early stage of drug discovery in the pharmaceutical industry.

In vivo, ALP is broadly distributed in plasma, the brush-broader membrane of the gastrointestinal tract, and other soft tissues. ALP is also used as a marker of cell differentiation and polarity in in vitro cell cultures because epithelial cells have shown an asymmetric distribution of ALP on their luminal surface (Lai et al., 2002). Human colon carcinoma cell line (Caco-2) is spontaneously differentiated and forms a monolayer structure that highly mimics the intestine epithelium when cultured in vitro. ALP levels in Caco-2 cells are considered to
be similar to that in human small intestine (Pinto et al., 1983). Therefore, Caco-2 cell monolayer has been used for the evaluation of phosphate prodrug bioconversion and permeation (Heimbach et al., 2003). However, long-time culture of Caco-2 cell to achieve the differentiated characteristics limited the application of this model as a high throughput tool for the drug screening. More recently, Madin-Darby canine kidney (MDCK) cells have become an alternative to Caco-2 cells for high-throughput screening of cell permeability of new chemical entities because they require less time to differentiate to form the monolayer morphology. Although ALP has been detected as a marker enzyme for differentiation of MDCK cells as well (Lai et al., 2002), the efficiency of ALP-mediated bioconversion of phosphate prodrugs in this cell model remains unknown. In this study, we investigated ALP-mediated conversion of probe phosphate prodrugs, fosphenytoin and fosfluconazole, in rat intestine mucosa scraps, Caco-2 cells, and MDCK cells. The results will provide the information for the in vitro model selection in phosphate drug screening.

Materials and Methods

Chemicals and Reagents. Fosfluconazole and fosphenytoin (Fig. 1) were synthesized internally by Pfizer Global Research and Development (St. Louis, MO). High-performance liquid chromatography-grade acetonitrile and water were purchased from Honeywell Burdick & Jackson (Muskegon, MI) and EMD Chemicals, Inc. (Gibbstown, NJ), respectively. The ALP quantification kit was purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM), minimum essential medium, fetal bovine serum (FBS), nonessential amino acids, GlutaMAX, 1 mM sodium pyruvate, and 0.06 mg/ml gentamicin. The cells were maintained in DMEM with 10% FBS, 1% nonessential amino acids, 1% GlutaMAX, 1 mM sodium pyruvate, and 0.06 mg/ml gentamicin. The cells were seeded at a density of 1 × 10^5 cells/cm^2 in 24-well Transwell plates (Millipore Corporation, Billerica, MA) and cultured for 21 to 25 days before assay. Transepithelial electrical resistance values were measured to ensure that tight junctions are formed (≥600 ohms/cm^2). 25-μl aliquot was collected from both the apical (A) and basal (B) sides at indicated time points, and compound concentrations were determined by LC/MS/MS.

Bioconversion Rate of Phosphate Prodrugs in Rat Intestine Mucosa Scraps. An aliquot of 200 μl of mucosa scrap lysis solution was mixed with 100 mM phosphate buffer, pH 7.4, to a final volume at 1 ml. The concentration of the test compounds (fosphenytoin and fosfluconazole) was 10 μM. The incubation medium was prewarmed at 37°C before the reaction was initiated. An aliquot of 100 μl was collected from the incubation vial at the time points 0, 5, 10, 20, 30, 45, and 60 min and transferred to a 96-well plate, in which 100 μl of acetonitrile was prefilled to terminate the reaction. The samples were diluted 5-fold with acetonitrile containing 1 μM toluene as an analytical internal standard. The samples were centrifuged at 4000 rpm for 5 min to precipitate protein. The supernatant was transferred to a new 96-well plate for concentration analysis by liquid chromatography/tandem mass spectrometry (LC/MS/MS).

Bioconversion and Transwell Transport of Prodrugs in Caco-2 Cells. Caco-2 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM with 10% FBS, 1% nonessential amino acids, 1% GlutaMAX, 1 mM sodium pyruvate, and 0.06 mg/ml gentamicin. The cells were seeded at a density of 1 × 10^5 cells/cm^2 in 24-well Transwell plates (Millipore Corporation, Billerica, MA) and cultured for 21 to 25 days before assay. Transepithelial electrical resistance values were measured to ensure that tight junctions are formed (≥600 ohms/cm^2). After the Transwell filter was washed three times with Hanks’ balanced salt solution, pH 7.4, each prodrug (10 μM) was applied to the donor side (either apical or basal chamber) to initiate the bioconversion and subsequent transport in Caco-2 monolayer. The incubation was maintained at 37°C for 2 h under gentle shaking (Precision Scientific, Winchester, VA). A 25-μl aliquot was collected from both the apical (A) and basal (B) sides at indicated time points, and compound concentrations were determined by LC/MS/MS. The extent of permeation was generated for both A→B and B→A transport. Both parental
compounds and prodrugs were monitored. The concentration-dependent bioconversions mediated by ALP were also conducted to investigate the kinetics of bioconversion.

**ALP-Mediated Prodrug Bioconversion in MDCK Cells.** MDCK cells were maintained in MEM-α medium supplemented with 10% FBS, 100 units of penicillin, 100 μg/ml streptomycin, 1% l-glutamine, and 1% nonessential amino acids. The cells were grown on Millipore 24-well Transwell plates for 4 to 5 days before transport assay. Similar to Caco-2 cells, the transport and bioconversion assays were conducted in live cells and initiated by applying the testing compounds to the donor chamber (either apical or basal chamber). The following procedures were similar to Caco-2 assays described above.

**LC/MS/MS Analysis.** An API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) with a turbo ion-spray interface operated in positive ionization mode was used for the multiple reaction monitoring LC/MS/MS analysis. An AD20 quaternary micropump (Shimadzu, Columbia, MD) and an Agilent Technologies (Santa Clara, CA) Zorbax Extend C18 (50 × 2.1 mm, 3.5 μm particle size) column were used for the chromatographic separation. The autosampler was an HTS-PAL from Leap Technologies (Carrboro, NC). The mobile phases were water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). Chromatographic separation was achieved by maintaining 90% A for 0.5 min isocratically and ramped to 90% B in 3.5 min, holding for 0.5 min followed by decreasing to 10% B in 0.2 min, and holding at 10% B another 1.3 min before the next injection. The total run time was 6 min. The flow rate was maintained at 0.4 ml/min. The mass spectrometric conditions were optimized for fosphenytoin, phenytoin, fosfluconazole, fluconazole, and tolbutamide (internal standard). The following precursor→product ion transitions were used for multiple reaction monitoring: fosfluconazole, m/z 385→79; fosphenytoin, m/z 363→237; fluconazole, m/z 307→220; phenytoin, m/z 253→182; and tolbutamide, m/z 272→155, respectively.

**Data Analysis.** The concentration-time profiles were fitted to a one-compartment model using equation $V = \frac{V_{max}[S]}{K_m + [S]}$, where $V_0$ and $A_0$ represent the prodrugs level at time 0 and at time $t$. $k_t$ is the first-order elimination rate constant, and $t_{1/2}$ was calculated as 0.693/k. Data were representative of a minimum of two experiments performed on different days with different batches of cells or scrap preparations. The Michaelis-Menten constant for prodrug conversions was estimated using the equation:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

where $V$ is the apparent linear initial rate, $[S]$ is the initial substrate concentration, $V_{max}$ is the maximum bioconversion rate, and $K_m$ is the Michaelis-Menten constant.

**Results**

**ALP in Intestinal Mucosa Scraps and MDCK and Caco-2 Cells.** In the present study, we have evaluated the ALP amount in the in vitro models that might potentially be used for the screening of ALP-mediated phosphate prodrug bioconversion. As described above, the ALP levels in rat intestinal mucosa scraps and Caco-2 and MDCK cells were quantified by an ALP fluorescence detection kit. The results are shown in Fig. 2. The ALP in rat intestine scraps exhibited the highest level among the tested models. The ALP level detected in Caco-2 monolayers cultured for 21 days was approximately 10-fold less compared with the rat intestinal mucosa scraps. The 5-day cultured MDCK monolayers expressed the least amount of ALP compared with other models.

**Bioconversion of Fosphenytoin and Fosfluconazole in Intestinal Mucosa Scraps and MDCK and Caco-2 Cells.** Fosphenytoin and fosfluconazole were used as probe substrates to assess the bioconversion of phosphate prodrugs. Initially, the disappearance of the phosphate prodrugs was monitored during the incubation. As anticipated based on ALP levels, the intestinal mucosa scraps showed the shortest half-lives for the cleavage of fosphenytoin and fosfluconazole compared with MDCK and Caco-2 monolayers. The overall clearance rate of both probe substrates was correlated with the amount of ALP detected in the individual model. In addition, we observed that the disappearance of fosphenytoin was faster than fosfluconazole in all the tested models (Table 1). The apparent half-life for fosphenytoin bioconversion in rat mucosa scraps was 1.2 min (Table 1). In contrast, a flat disappearance slope was found for fosfluconazole when incubating with intestinal mucosa scraps compared with bioconversion of fosphenytoin (Fig. 3a). More flat disappearance slopes were observed in MDCK and Caco-2 monolayers for the cleavage of both probe substrates (Fig. 3, b and c). The apparent half-life for fosfluconazole bioconversion in intestinal mucosa scraps was 10 min (Table 1), whereas it was stable in Caco-2 and MDCK cell monolayers with half-lives of 31 and 83 min, respectively. The results revealed that there are at least two factors, chemical structure/properties and ALP expression, determining the bioconversion of phosphate prodrugs in in vitro systems.

**Phosphate Prodrug Bioconversion and Transport in Caco-2 Monolayer.** Caco-2 cells are spontaneously differentiated after reaching confluence when cultured on a Transwell membrane. The cells form polarized monolayers and express ALP activity at their apical membranes in levels similar to those found in the human intestine (Pinto et al., 1983). In the present study, the ALP-dependent bioconversions of fosphenytoin and fosfluconazole in Caco-2 cells were also examined. To investigate the polarized bioconversion and the Transwell transport of phosphate prodrugs in Caco-2 monolayer, 10 μM fosfluconazole or fosphenytoin was dosed either in the apical or basal compartment in Transwell plates. As shown in Fig. 4, both prodrugs were efficiently cleaved in the apical compartment after a 2-h incubation. The converted parent drugs, phenytoin and fluconazole, were detected in the dosing chamber and in the receiver chamber (Fig. 4). In contrast, the bioconversions for both prodrugs were minimal or undetectable when dosing in the basal chamber. The results suggested the ALP-mediated bioconversion occurred on the apical side of Caco-2 monolayers. However, the prodrugs were not detectable in the receiver compartment irrespective of dosing in the apical or basal chamber, corroborating the poor permeability of phosphate prodrugs. To further investigate the kinetics of ALP-mediated bioconversion, the concentration-dependent ALP-mediated bioconversions were conducted to determine the Michaelis-Menten constant ($K_m$) of prodrug bioconversion in Caco-2 monolayers. As shown in Fig. 5, the saturation curves of fosphenytoin and fosfluconazole with the concentration increase were found. The estimated $K_m$ values of fosphenytoin and fosfluconazole were 1160 and 357 μM, respectively.

**Discussion**

ALPs are phosphatidylinositol-linked membrane hydrolase enzymes responsible for removing phosphate moiety from many type of...
molecules. At least four genes that encode distinct ALP proteins occur in different tissues: intestinal, placental, placental-like, and liver/bone/kidney ALPs (Henthorn et al., 1988). The ALP level in blood represents the total amount of ALPs released from these tissues. Based on the concept of in vivo bioconversion of ALPs, phosphate ester prodrugs are designed to successfully overcome drug delivery problems that may compromise the therapeutic utility of a potential drug (Boucher, 1996; Becker and Thornton, 2004). Through a direct incorporation of a phosphate moiety into the hydroxyl or amine functionalities of a parent drug, for example, to form a phosphonomoester or attaching it to the parent drug via a chemical linker phosphate prodrug gains sufficient aqueous solubility and adequate chemical stability and undergoes quantitative in vivo bioconversion to the pharmacologically active drug. The chemical linkers were basically used to enhance the enzymatic conversion because of the steric hindrance. The linkage was made through the nitrogen on the indole moiety of the parent drug, such as the prodrug of phenytoin, fosphenytoin (Luer, 1998). A methylene spacer is also a very common linker that is used for the prodrug design. Such prodrugs release the parent via a two-step conversion process, an ALP-mediated dephosphorylation followed by a fast spontaneous chemical breakdown of the hemiacetal intermediate (Fig. 1). However, in terms of bioconversion mediated by ALPs, not all the phosphate prodrugs undergo the desired rapid bioconversion to release the parent drug. For example, reports have shown that phosphomonoesters of secondary and tertiary alcohols undergo slower rates of enzymatic conversion in vitro, whereas phosphononoesters are better substrates for ALP than phosphoramidates (Kearney and Stella, 1992; Safadi et al., 1993; Vyas et al., 1993). Therefore, in vitro/in vivo models for evaluating bioconversion are potentially of great value at the early stage of drug discovery.

In the present study, two probe compounds were selected as model compounds to study the in vitro bioconversion. One is the fosphenytoin (Fig. 1) (Diflucan from Pfizer), in which the phosphoryl group is linked on the tertiary alcohol of fluconazole. Unlike the parent compound, fosfluconazole is more highly water-soluble (>300 versus 1 mg/ml) and chemically stable in the solid state and in aqueous solution (Yamreudeewong et al., 1993; Bentley et al., 2002). Another substrate used in present study is fosphenytoin, in which the phosphoryl group is attached to NH group on the indole ring of phenytoin through a self-cleavable linker, the hydroxymethyl group (Fig. 1). With the hydroxymethyl linker, fosphenytoin conversion is fast and efficient in vivo (8.1 min), whereas fosfluconazole is hydrolyzed by ALPs in blood and tissues with the t\(_{1/2}\) of 1.5 to 2.5 h (Boucher, 1996; Sobue et al., 2004). Coupled with the confirmation of ALP level, we concluded that clearance rate of phosphate prodrugs in an in vitro model can reflect the chemical structure/properties. Rat intestinal mucosa scraps reserved ALP activities and thus is a promising model for in vivo phosphate prodrug screening. In addition, because the preparation was isolated directly from tissues and could be pooled and stored in a freezer for repeated use, the bioconversion data obtained from the model could offer predictive information in an in vivo situation.

Because of the similarity of the differentiated monolayer formed by Caco-2 cells with human intestinal mucosa, Caco-2 cells have been widely used to predict drug absorption in humans. In the present study, Caco-2 cells showed the efficient bioconversion for probe phosphate prodrugs (Fig. 4). Simultaneously, the permeability of prodrug and parent drug molecules could also be determined. More recently, because of labor savings on cell maintenance, MDCK cells show promise as an alternative approach for permeability screening by the pharmaceutical industry. However, the 5-day grown MDCK cells produced only minimal amount of ALP; therefore, MDCK monolayer was not able to distinguish bioconversion rates related to diversified chemical spaces and might not be a suitable tool for phosphate prodrug screening.

In conclusion, the intestinal mucosa scraps reserved the highest ALP activities and are a promising in vitro tool for screening the bioconversion of phosphate prodrug. MDCK cells show promising labor savings as a predictive tool for drug absorption; however, they might not replace Caco-2 cells for the investigation of phosphate prodrugs because of lack of efficient ALP-mediated bioconversion. The special value of the Caco-2 cell model is that Caco-2 cells might not replace Caco-2 cells for the investigation of phosphate prodrug.
be able to characterize the permeability of the prodrug and the parent drug.

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References


