A Novel Strategy for ADME Screening of Prodrugs: Combined Use of Serum and Hepatocytes to Integrate Bioactivation and Clearance, and Predict Exposure to Both Active and Prodrug to the Systemic Circulation

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ABSTRACT: Common strategies to optimize prodrugs use either *in vitro* or rodent *in vivo* approaches, which do not consider elimination pathways that do not result in the generation of the desired product or might be misleading because of species differences, respectively. As a step forward, we have incorporated a novel application of hepatocytes into our prodrug optimization strategy to increase the bioavailability of a poorly soluble drug candidate by attaching a charged ester linker. The model involves the incubation of hepatocytes from multiple species in serum-containing medium to mimic formation as well as simultaneous metabolism of both prodrug and active drug. Using this strategy, a correlation between the *in vitro* AUC and the AUC after intravenous administration was obtained for active drug formation in several species. Moreover, hepatocytes correctly predicted the likelihood of undesired exposure with nonhydrolyzed prodrug. This novel approach enabled us to identify several prodrugs, which showed improved exposure over a wide dose range. Furthermore, a strategy was developed resulting in a decision tree that can be used to determine the applicability of the hepatocyte model in the screening process. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:1504–1514, 2014
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INTRODUCTION

Prodrugs are drug derivatives with improved physicochemical properties that ideally reveal no pharmacological activity on their own but undergo biotransformation to therapeutically active metabolites or active drugs.[1] Prodrugs that are activated by metabolism represent a significant group of drugs used today, for example, approximately 7% of the active drugs marketed in Germany in 2002 were prodrugs.[2] Approximately 50% of all prodrugs are activated by ester hydrolysis. Prodrug design therefore offers a versatile and powerful method that can be applied to a wide range of parent drug molecules, administration routes, and formulations. The aim of exploiting a prodrug approach is to produce a drug with improved pharmaceutical, pharmacokinetic (PK), or pharmacodynamic profiles (reviewed by Testa).[3] Such improvements include increasing solubility in aqueous media to facilitate dissolution or to allow parenteral formulation, increasing permeability to aid absorption, and improving therapeutic index by targeting a drug to a specific tissue or cell type.[4] Prodrugs may enhance oral (p.o.) availability of poorly permeable drugs, for example, by conversion of a carboxylate to an ester. Examples for such prodrugs where a charged acid is "masked" by an unstable ester include ramipril, Oseltamivir, or simvastatin. Some prodrugs increase the solubility of intravenous(ly) (i.v.) or p.o. drugs by adding water-soluble promoieties with biodegradable linkers, for example, rolitetracycline and fosphenytoin. A third class of prodrug is the bioprecursors, which do not contain linker or masking groups but are activated by oxidation, reduction, or hydrolysis (active metabolites, e.g., levodopa or omeprazole).

There are numerous reviews describing the ideal features of different classes of prodrugs, how modifications lead to altered bioavailability and general strategies for optimization of drugs for this purpose but few actually put forward suggested strategies of combining *in vitro* tests by which potential prodrugs can be tested and screened in early drug discovery. Of the few reports available, most describe only a single assay that was used to optimize or select prodrugs for better PK properties (Table 1). In the case of prodrugs with low permeability, Caco-2 cells are commonly used to screen for improved permeability.[5,6] Interestingly, Caco-2 cells although of human origin seem to express esterases that are more similar to rodent, which makes predictions for the human situation difficult.[7] Furthermore, the hydrolysis of ester prodrugs is frequently studied in serum or tissue fraction such as intestinal or hepatic S9 fraction by measuring the half-life of the conversion of prodrug to active

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Table 1. *In Vitro* Methods used for Ester Prodrug Screening

Prodrug(s)	Screening Tool	
	Properties	*In Vitro* Model
Selected commercial drugs with an amide or ester functional groups,[10] including tenofer (a double ester prodrug)[11]	Rate of bioconversion of functional group	Rat serum stability (3 h)
VLA-4 antagonists (anti-inflammatory agent)[12]	Solubility in simulated physiological buffers	Caco-2 absorption
	pK_a at pH 11.0 and pH 2.9	Rat serum stability (24 h)
	Log D_{oct}	
Prodrugs of perzinfotel (NMDA receptor antagonist)[13]	Stability in buffers between pH 1 and pH 9 (24 h)	Pharmacological efficacy
		Stability SGF, SIF, and SIBLM
		Stability of the prodrug and measurement of production and stability of the active drug in rat serum (up to 24 h)
Prodrugs of nipecotic acid[14]	Stability in pH 7.4 buffer (7 days)	Stability in porcine liver esterase (16 h)
Prodrugs of 20(S)-camptothecin (antitumor agent)[15]	Stability in pH7.4 phosphate buffer (24 h)	Stability in human serum and mouse serum (24 h)
		Stability in porcine liver esterase (80 min)
		Cytotoxicity in cancer cell lines
Prednisolone acetate, prednisolone hemisuccinate[5]	Permeability through Caco-2 cells	Caco-2 cells and homogenates of scraped intestinal mucosa
	Ester hydrolysis	
ME3229, an ester-type prodrug of a hydrophilic glycoprotein	Metabolism in S9 fraction of the small intestinal mucosa and liver.	Stability in serum and rat liver and small intestinal mucosa S9
IIb/IIIa antagonist[16]	Permeability though Caco-2 cells	Caco-2 cells
	Efflux of active drug into intestinal lumen	(*Ex vivo*) single-pass perfused rat small intestine experiment. Absorption into enterocytes and the mesenteric vein, and degradation in the gut lumen evaluated separately
O-acyl propranolol (PL) prodrugs[17]	Stability in pH 7.4 buffer	pH 7.4 Tris–HCl buffer
	Stability in liver fractions	Rat and dog serum and liver microsomes and cytosol

NMDA antagonism was assessed using a glutamate-stimulated TCP binding assay.
SGF, simulated gastric fluid; SIBLM, simulated intestinal bile fluid; SIF, simulated intestinal fluid.

drug. However, all these methods are focusing on the rate of formation of active drug by ester hydrolysis and are not considering nonproductive metabolism. A prodrug approach can only be successful if the rate of the formation of active drug is higher than the degradation rate of the active drug.[8] However, there are no approaches that integrate the clearance of active drug (and prodrug) and formation of active drug.[9]

Reports in the literature on the use of hepatocytes for metabolism studies have continued to increase over the past two decades; however, the publications involving the use of microsomes for the same purpose have dropped by nearly 50% in the same time frame (data gathered from PubMed: http://www.ncbi.nlm.nih.gov/pubmed). Intact hepatocytes provide a more relevant model for predicting *in vivo* metabolism because they retain many phase 1 and 2 enzyme activities that are lacking in microsomes.[18] Even subcellular fractions or homogenates with supplemented cofactors have been shown to lack activation pathways of a prodrug, for example, 9-3 hydroxypropoxy guanine; however, hepatocytes were capable of both the oxidation and dealkylation pathways involved in this prodrug's bioactivation.[19] Because of the fact that many prodrugs are mainly bioactivated in serum or in the liver, hepatocytes suspended in serum should be an ideal tool to investigate the formation of active drug under conditions of simultaneous elimination of both prodrug and active drug by xenobiotic metabolizing enzymes such as cytochrome P450s (CYPs). Thus, this modified hepatocyte model should be capable of integrating productive and nonproductive pathways and increase the correct prediction of *in vivo* exposure. Therefore, we have incorporated this test system into our screening protocols to investigate the production with simultaneous elimination of the active drug in more quantitative terms. Although limited in its application because of the expression of extrahepatic esterases, we have investigated whether there was a correlation between *in vitro* data with *in vivo* animal data with respect to the exposure (AUC) of the active drug formed from ester prodrugs in several species. Obviously, this model is only applicable in cases where the liver plays a major role in the bioactivation of the prodrug. If the liver is not the main site of conversion and the active drug is formed in the gut lumen or in enterocytes of the intestine, then this screening strategy will not be applicable. Therefore, it is important to determine the sites of bioactivation in the different species and for this purpose a decision tree was designed to put the hepatocyte data into context with the stability of the prodrugs in buffer, intestinal S9 fraction, and serum. It should be emphasized that the described