The Use of Transporter Probe Drug Cocktails for the Assessment of Transporter-Based Drug–Drug Interactions in a Clinical Setting—Proposal of a Four Component Transporter Cocktail

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Received 24 February 2015; revised 10 April 2015; accepted 10 April 2015

Published online 15 May 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24489

ABSTRACT: Probe drug cocktails are used clinically to assess the potential for drug–drug interactions (DDIs), and in particular, DDIs resulting from coadministration of substrates and inhibitors of cytochrome P450 enzymes. However, a probe drug cocktail has not been identified to assess DDIs involving inhibition of drug transporters. We propose a cocktail consisting of the following substrates to explore the potential for DDIs caused by inhibition of key transporters: digoxin (P-glycoprotein, P-gp), rosuvastatin (breast cancer resistance protein, BCRP; organic anion transporting polypeptides, OATP), metformin (organic cation transporter, OCT; multidrug and toxin extrusion transporters, MATE), and furosemide (organic anion transporter, OAT). Furosemide was evaluated *in vitro*, and is a substrate of OAT1 and OAT3, with K_m values of 38.9 and 21.5 μM, respectively. Furosemide was also identified as a substrate of BCRP, OATP1B1, and OATP1B3. Furosemide inhibited BCRP (50% inhibition of drug transport: 170 μM), but did not inhibit OATP1B1, OATP1B3, OCT2, MATE1, and MATE2-K at concentrations below 300 μM, and P-gp at concentrations below 2000 μM. Conservative approaches for the estimation of the likelihood of *in vivo* DDIs indicate a remote chance of *in vivo* transporter inhibition by these probe drugs when administered at low single oral doses. This four component probe drug cocktail is therefore proposed for clinical evaluation. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:3220–3228, 2015

Keywords: ABC transporters; drug interaction; efflux pumps; inhibition; kinetics; organic anion transporters; organic anion-transporting polypeptide transporters; organic cation transporters; P-glycoprotein

INTRODUCTION

Transporters play a critical role in facilitating the absorption, distribution, and elimination of many drugs. It is recognized that nearly all drugs and metabolites of drugs are likely to interact to a certain extent with membrane transporters.[1,2] Therefore, determining the potential for interaction of new drug candidates with drug transporters including ATP-binding cassette (ABC) and solute carrier (SLC) transporters has become an indispensable part of nonclinical and clinical drug development. The important role of transporter-based drug–drug interactions (DDIs) has consequently resulted in the inclusion of this topic in recent regulatory guidelines.[3–5] Generally, such investigations start with *in vitro* studies and result in data that, when considered together with pharmacokinetic parameters for a drug candidate, can be used to make an informed decision regarding the assessment of transporter-based DDIs in dedicated clinical trials.

Although drugs in development are routinely evaluated for their potential interactions with a variety of transporters, the results of such *in vitro* transporter investigations have to be interpreted carefully because of the lack of highly specific tools and the use of complex experimental systems.[1] In addition, drug transporters generally exhibit broad substrate specificity, and it is frequently observed that drugs interacting with one transporter will most likely interact with many other transporters.[2,6] Contributing to this challenging situation is the fact that results generated using different experimental systems in different laboratories are highly variable.[7] The lack of definitive and standardized *in vitro* methodologies for reliably predicting clinical transporter-mediated DDIs has been recognized by regulatory agencies, who have in response taken a cautious position when evaluating *in vitro* transporter data within the context of patient safety. As a consequence, the need for an increased number of clinical drug transporter-based DDI trials may be triggered by this cautious interpretation of *in vitro* data. In addition, because of the broad substrate specificity of drug transporters, a single clinical DDI trial may not be sufficient to explore the entire "interaction potential" between a drug and a transporter.

In order to effectively explore whether a drug candidate will act as an inhibitor of several drug transporters *in vivo*, a probe drug cocktail approach may be applied to investigate potential clinical DDIs. Such an approach is based on the simultaneous dosing of a group of drugs as probe substrates for the most important transporters from a DDI perspective, together with the drug candidate being investigated, followed by an assessment of effects of the drug candidate on the pharmacokinetic profiles of the probe drugs. Various probe drug cocktails, such as the "Pittsburgh Cocktail" or "Cooperstown Cocktail" have been utilized in drug development for the assessment of DDIs that are based on interactions with drug metabolizing

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Journal of Pharmaceutical Sciences, Vol. 104, 3220–3228 (2015)

enzymes, specifically cytochrome P450 (CYP450) enzymes.[8–10] On the basis of this precedent and their successful use over the last years, guidelines established by the European Medicines Agency (EMA), US Food and Drug Administration (FDA), and Pharmaceuticals Medical Devices Agency (PMDA) explicitly make reference to the use of probe drug cocktails to perform clinical DDI trials.

Presently, there is no published literature describing the use of dedicated transporter probe drug cocktails, and according to currently available information in the public domain, no such trials have been performed. To explore the possibility of developing a cocktail-based approach to evaluate transporter-mediated DDIs, in this paper, we propose the use of a four-component probe drug cocktail and support our concept with *in vitro* data that indicate the suitability of the chosen probe drugs. Additionally, as adequate literature data for one of the drugs we propose to include in the probe drug cocktail, furosemide, was not available, we conducted a series of *in vitro* studies to characterize the potential for furosemide to interact with key ABC and SLC transporters.

MATERIALS AND METHODS

Chemicals

Acylovir, cimetidine, digoxin, estrone 3-sulfate (E-sul), fumitremorgin C (FTC), furosemide, 1-methyl-4-phenylpyridinium (MPP^+), and rifampicin were obtained from Sigma–Aldrich (St. Louis, MO, USA) . Metformin and probenecid were obtained from Wako (Osaka, Japan). The multidrug resistance-associated protein (MRP) inhibitor MK-571, the MATE inhibitor pyrimethamine, and furosemide d_5 (furfuryl-d_5) were obtained from Alexis Biochemicals (Lausen, Switzerland), MP Biomedicals (Santa Ana, CA, USA), and CDN Isotope (Quebec, Canada), respectively. [^{3}H]Digoxin and [^{3}H]E-sul were obtained from PerkinElmer (Waltham, MA, USA). [^{3}H]Acylovir and [^{14}C]mannitol were obtained from Moravek Biochemicals, Inc. (Brea, CA, USA). [^{3}H]Rosuvastain, [^{14}C]metformin, [^{3}H]MPP^+, and [^{3}H]metoprolol were from obtained American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Zosuquidar (ZSQ) was prepared by custom synthesis. All other chemicals were of the highest reagent grade available from commercial sources.

Plasmids

Plasmids (pcDNA5/FRT-DEST; Invitrogen) were used that contain cDNA of organic anion transporter (OAT) 1, OAT3, organic anion transporting polypeptide (OATP) 1B1, OATP1B3, or organic cation transporter (OCT) 2.[11,12]

Cells

Caco-2 cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Parental HEK293 cells were purchased from Health Science Research Resource Bank (Japan). HEK293 cells stably transfected with either empty vector, multidrug toxin extrusion (MATE) 1, or MATE2-K were obtained from GenoMembrane, Inc. (Japan) under license agreement.

Cell Culture

Caco-2 cells were maintained as described in a previous report.[13] About 1×10^6 cells were seeded per flask. The culture medium was changed three times each week. Caco-2 cells were seeded at a density of 1.5×10^5 cells/cm^2 on Transwell filter inserts. The cells were cultured at 37°C, 8% CO_2, and 90% relative humidity in Dulbecco's modified Eagle medium (DMEM) culture medium for 15–16 days. Parental HEK293 cells were maintained in DMEM culture medium [low-glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 g/mL amphotericin B] at 37°C, 5% CO_2, and 95% relative humidity. HEK293 cells stably transfected with empty vector; MATE1 or MATE2-K were maintained in DMEM culture medium supplement with 10% FBS, 50 U penicillin, 50 μg/mL streptomycin, and 500 μg/mL G418 (Invitrogen). The transport activity of each cell line was confirmed by examining the uptake of probe substrates.

Cellular Uptake

For expression of OAT, OATP, and OCT transporter isoforms, parental HEK293 cells were seeded onto lysine-coated 24-well plates at a density of 0.75×10^5 cells/well. On the next day, transfection was conducted according to manufacturer's protocol. Approximately 24 h after transfection, culture medium was changed to DMEM culture medium supplemented with 5 mM sodium butyrate, and incubated in culture medium for an additional 24 h to allow for plasmid transporter gene expression. For MATE1 and MATE2-K, HEK293 cells stably expressing MATE1 or MATE2-K, or stably transfected with empty vector, were seeded onto lysine-coated 24-well plates at a density of 1.5×10^5 cells/well and were cultured for 3 days. On the day of experiment, cells expressing transporter isoform transiently were rinsed twice and preincubated in transport buffer (modified Krebs–Henseleit buffer supplemented with 25 mM HEPES, 1.5 mM calcium chloride) at 37°C for 15–60 min. Stably transporter-expressing cells were rinsed twice with MATE transport buffer (130 mM KCl, 2 mM KH_2PO_4, 1.2 mM $MgSO_4$ $7H_2O$, 1 mM $CaCl_2$ $2H_2O$, 20 mM HEPES, 5 mM D-(+)-glucose, pH 7.4) and the cells were preacidified with MATE transport buffer supplemented with NH_4Cl (final 20 mM) for 10 min at 37°C. The MATE transport buffer with NH_4Cl was replaced with normal MATE transport buffer and cells were incubated for additional 5 min. For both transient and stable systems, the transport buffer was removed and replaced with fresh transport buffer containing radiolabeled drug and varying concentrations of inhibitor specific for each transporter. Initial drug concentration was determined at the initiation of the incubation ($t0$). The concentration of the solvent in which the drug was initially dissolved did not exceed 0.5% by volume. The incubation was stopped by aspirating the buffer and replacing it with ice-cold transport buffer. The cells were rinsed three times with ice-cold transport buffer, and the buffer was aspirated after the final rinse. For radioactivity measurement, the cells were lysed with NaOH for 1 h at 37°C. The reaction was neutralized with HCl. Radioactivity in the cells was determined for 3 min in a liquid scintillation analyzer (TRI-CARB 3100TR and 3110TR; Packard). Protein concentrations were measured using the Lowry method. For furosemide measurement, cells were collected using a rubber scraper after the addition of 200 μL water. Adequate amounts of cell suspension were transferred to test tubes and the volume was adjusted to 180 μL with blank matrix that was prepared using untreated cells on the same day of experiment.