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*Symposium Report*

# "Target-Site" Drug Metabolism and Transport

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## ABSTRACT

**The recent symposium on "Target-Site" Drug Metabolism and Transport that was sponsored by the American Society for Pharmacology and Experimental Therapeutics at the 2014 Experimental Biology meeting in San Diego is summarized in this report. Emerging evidence has demonstrated that drug-metabolizing enzyme and transporter activity at the site of therapeutic action can affect the efficacy, safety, and metabolic properties of a given drug, with potential outcomes including altered dosing regimens, stricter exclusion criteria, or even the failure of a new chemical entity in clinical trials. Drug metabolism within the brain, for example, can contribute to metabolic activation of therapeutic drugs such as codeine as well as the elimination of potential neurotoxins in the brain. Similarly, the activity of oxidative and conjugative drug-metabolizing enzymes in the lung can have an effect on the efficacy of compounds such as resveratrol. In addition to metabolism, the active transport of compounds into or away from the site of action can also influence the outcome of a given therapeutic regimen or disease progression. For example, organic anion transporter 3 is involved in the initiation of pancreatic $\beta$-cell dysfunction and may have a role in how uremic toxins enter pancreatic $\beta$-cells and ultimately contribute to the pathogenesis of gestational diabetes. Finally, it is likely that a combination of target-specific metabolism and cellular internalization may have a significant role in determining the pharmacokinetics and efficacy of antibody-drug conjugates, a finding which has resulted in the development of a host of new analytical methods that are now used for characterizing the metabolism and disposition of antibody-drug conjugates. Taken together, the research summarized herein can provide for an increased understanding of potential barriers to drug efficacy and allow for a more rational approach for developing safe and effective therapeutics.**

## Introduction

Although drug metabolism is most often thought of as a mechanism to eliminate toxic substances and therapeutic entities from systemic circulation, emerging evidence has highlighted the importance of drug-metabolizing enzymes and transporters at the site of therapeutic action. These enzymes and transporters are widely expressed throughout the body and often serve as detoxification mechanisms at a cellular level. In an attempt to maintain a homeostatic environment, however, the enzymes and transporters may also serve to decrease the amount of drug available to reach a given target, ultimately attenuating the desired pharmacodynamic response of the drug (Urquhart et al., 2007; Chen et al., 2012). In many cases, their expression is sensitive to physiologic and pathologic changes, with altered regulation of drug-metabolizing enzymes and transporters observed in both oncology and inflammatory diseases (Renton, 2004, 2005; Morgan et al., 2008).

In general, the liver, intestine, and kidney are believed to be the major organs involved in drug elimination, with the first two often having a significant role in the first-pass metabolism of orally administered xenobiotics (Pond and Tozer, 1984). However, the expression of drug-metabolizing enzymes such as cytochrome P450s and UDP-glucuronosyltransferases is not limited to these organs, but rather occurs in organs throughout the body (Guengerich, 2005; Remmel and Zhou, 2009; Foti and Fisher, 2012). Significant expression levels of drug-metabolizing enzymes have been found in the brain, lung, nasal mucosa, trachea, esophagus, stomach, and colon (Ding and Kaminsky, 2003). Although these additional sites of

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**ABBREVIATIONS:** ABC, ATP binding cassette; ADC, antibody-drug conjugate; AUC, area under the curve; BCRP, breast cancer resistance protein; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanopropanoic acid; CNS, central nervous system; DDI, drug-drug interaction; DM1, mertansine; EMB, ethambutol dihydrochloride; HIV, human immunodeficiency virus; hOAT, human organic anion transporter; hOCT, human organic cation transporter; ICV, intracerebroventricular; MBI, mechanism-based inhibitor; MPP+, 1-methyl-4-phenylpyridinium; MRP, multidrug resistance–associated protein; OAT, organic anion transporter; OCT, organic cation transporter; P450, cytochrome P450; P-gp, P-glycoprotein; RES, trans-resveratrol; R3G, trans-resveratrol-3-glucuronide; R3S, trans-resveratrol-3-sulfate; SLC, solute carrier; SULT, sulfotransferase; TB, tuberculosis; UGT, UDP-glucuronosyltransferase; XME, xenobiotic-metabolizing enzyme.

expression may not always represent significant clearance pathways, they can play a role in affecting local tissue concentrations of a given drug and, in turn, the efficacy of the drug against a target located in that tissue. For example, many drugs against neurologic targets are also metabolized by cytochrome P450s that are located in the brain (Ferguson and Tyndale, 2011; Miksys and Tyndale, 2013; Ravindranath and Strobel, 2013). Similarly, the activity of inhaled drugs such as ciclesonide or 17-beclomethasone dipropionate is dependent upon metabolic activation by drug-metabolizing enzymes located within the lungs (Nave et al., 2007, 2010). Conversely, intestinal and hepatic metabolism and transport play an important role in limiting the systemic exposure of drugs such as loperamide, budesonide, or sulfasalazine which bind to therapeutic targets located in the intestine (Peppercorn and Goldman, 1972; Schinkel et al., 1996; Kim et al., 2004; Acharya et al., 2006; O'Donnell and O'Morain, 2010).

The role of drug transporters in affecting the efficacy of clinical therapeutics has also received a great deal of interest. The effect of transporter-mediated multidrug resistance on the outcome of therapeutic treatments is perhaps the quintessential example of absorption, distribution, metabolism, excretion and toxicity factors that influence the efficacy of a drug at the site of action (Gottesman et al., 2002). Efflux transporters such as P-glycoprotein (P-gp), the breast cancer resistance protein (BCRP), and the multidrug resistance–associated proteins (MRPs) have been implicated in the therapeutic limitations of drugs such as doxorubicin, vinblastine, and topotecan (Awasthi et al., 1994; Kruijtzer et al., 2002). Similarly, activity of uptake transporters, including members of the solute carrier 22 (SLC22) family, influence the therapeutic activity of antibiotics (penicillin G), diuretics (furosemide), and antineoplastic agents (cisplatin) (Sweet, 2010). As a testament to the importance of overcoming the ramifications of transporter-mediated drug resistance, multiple clinical evaluations aimed at identifying inhibitors of these transporters have been undertaken (Morrissey et al., 2013; Videira et al., 2014). While representing a key research focus, drug resistance or altered efficacy due to transporters at the site of therapeutic action is not limited to oncology indications. For example, the expression of P-gp on proinflammatory Th17 cells is a determining factor in the resistance of these cells to glucocorticoid treatment in autoimmune diseases (Ramesh et al., 2014). In vitro results suggest that MRP1 and BCRP expression in synovial tissue of rheumatic patients may impact the clinical efficacy of disease-modifying antirheumatic drugs such as methotrexate and leflunomide (van der Heijden et al., 2009). Finally, some patients taking pravastatin and gemfibrozil in combination experienced increased pravastatin plasma levels and decreased renal clearance (altered pharmacokinetics) resulting in increased pravastatin-associated adverse events, and preliminary evidence suggests this drug-drug interaction may involve inhibition of human organic anion transporter 3 (hOAT3)–mediated pravastatin transport by gemfibrozil (Kyrklund et al., 2003; Nakagomi-Hagihara et al., 2007).

The effects of metabolism and transport at the site of therapeutic action are not limited to small molecules but also play a role in determining the pharmacokinetics and efficacy of protein therapeutics. In the case of antibody-drug conjugates, for example, the mechanism of action begins with binding and internalization of the conjugate through receptor-mediated endocytosis (Ghose and Blair, 1987; Chari, 2008). Lysosomal metabolism and release of the cytotoxic agent result in the observed pharmacodynamic effects of the antibody-drug conjugate (Erickson et al., 2006). As such, optimization efforts for antibody-drug conjugates are often focused on factors which influence the internalization and subsequent degradation at the site of action (Alley et al., 2010; Perez et al., 2014).

The 2014 Experimental Biology symposium entitled "Target-Site" Drug Metabolism and Transport was sponsored by the American Society for Pharmacology and Experimental Therapeutics and brought together experts whose research focused on the localized effects of drug-metabolizing enzymes and transporters. This report serves to summarize the research and overarching themes which were presented and discussed during the symposium.

## Drug Metabolism within the Brain Changes Drug Response In Vivo (K.L.P.G. and R.F.T.)

Drug metabolism by cytochrome P450 enzymes (P450s) occurs mainly in the liver, and it is this hepatic expression that is a major contributor to pharmacokinetics and resulting systemic drug and metabolite levels. However, drug-metabolizing P450s are also expressed extrahepatically and may play a role in local metabolism within these specific organs. These P450s are expressed in the brain (Miksys et al., 2000; Ferguson and Tyndale, 2011), where protein levels in certain cell types can be as high as in hepatocytes (Miksys et al., 2000). They are functionally active ex vivo, but more importantly in vivo, indicating that sufficient levels of cofactors and coenzymes are colocalized within the brain (Miksys and Tyndale, 2009). This suggests that metabolism of drugs that act on the central nervous system (CNS) could occur in the brain independent of liver metabolism. This is particularly relevant since the clinical response to CNS-acting drugs does not always correlate with plasma drug levels (Michels and Marzuk, 1993). As examples, we will describe two well known drug-metabolizing enzymes which are expressed in the brain, CYP2B and CYP2D, both of which can metabolize several CNS-acting drugs and neurotoxins. By selectively manipulating these enzymes in the brain through induction and inhibition, the CNS activity and subsequent responses to CYP2B and CYP2D substrates can be altered. Thus, the expression of P450s in the brain may play a role in drug response, and as they are highly variable, may also contribute to interindividual variation in drug response to CNS-acting drugs.

CYP2B can metabolize many CNS-acting drugs, such as bupropion (Hesse et al., 2000), methadone (Gerber et al., 2004), and nicotine (Yamazaki et al., 1999; Yamanaka et al., 2005), the primary psychoactive ingredient in cigarettes. Large variations in CYP2B isoform levels have been found in the brain (Miksys et al., 2003), in part due to induction by common drugs such as nicotine. Brain CYP2B expression can be increased in primates and rodents: higher CYP2B6 and CYP2B1 levels are found after chronic nicotine treatment in monkeys (Lee et al., 2006) and rats (Miksys et al., 2000), respectively, consistent with higher levels in brains of smokers (Miksys et al., 2003). The human *CYP2B6* gene is highly polymorphic (Lang et al., 2001); one common decreased expression variant, *CYP2B6*6*, was associated with reduced protein levels in the human brain (Miksys et al., 2003). Thus, variation in CYP2B expressed in the brain may influence the levels of its substrates and their resulting drug and toxic responses.

CYP2B metabolically inactivates the anesthetic propofol. In humans, brain propofol levels were a better predictor of the behavioral response (anesthesia or sleep time) when compared with plasma propofol levels (Liu et al., 2009), providing a rationale for examining the role of brain CYP2B in metabolizing propofol and the behavioral response to this drug. To do this, mechanism-based inhibitors (MBIs), substrates that are metabolized to a reactive intermediate that subsequently inactivates the enzyme, were used to inhibit CYP2B activity specifically in the brain (Fig. 1). C8-xanthate and 8-methoxypsoralen are two structurally distinct MBIs that can selectively inhibit CYP2B

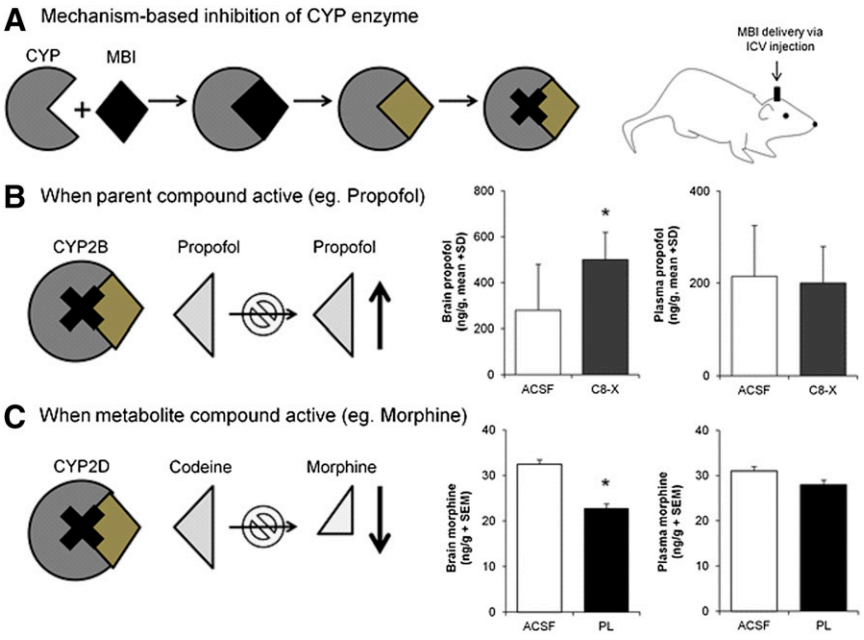


**Fig. 1.** Inhibition of CYP2 activity in the brain. Inhibiting activity of CYP2 enzymes specifically in the brain via MBI pretreatment can alter substrate and metabolite brain levels. (A) MBIs are substrates for their target enzymes, which are metabolized into intermediate compounds that bind irreversibly to the enzyme, thus inactivating it. MBIs are injected into the brain via i.c.v. injections. (B) Brain P450 inhibition increases the parent drug, illustrated for the active CYP2B substrate propofol: brain CYP2B inhibition by the i.c.v. MBI C8-xanthate (C8-X) increased propofol brain levels relative to vehicle [artificial cerebrospinal fluid (ACSF)] pretreatment; there was no difference in plasma propofol levels. (C) Brain P450 inhibition reduces metabolite levels, illustrated for the metabolically activated metabolite morphine from CYP2D substrate codeine: brain CYP2D inhibition by MBI propranolol (PL) pretreatment decreased morphine levels in the brain; there was no difference in plasma morphine levels ($*P < 0.05$). Data compiled from previously published reports (Khokhar and Tyndale, 2011; Zhou et al., 2013).

(Koenigs and Trager, 1998; Yanev et al., 1999). Delivery of either of these CYP2B MBIs into rat brain via i.c.v. or regional injection was used to demonstrate that P450s within the brain were functional in vivo (Miksys and Tyndale, 2009). Intracerebroventricular injections of CYP2B MBIs increased rat brain propofol levels (Fig. 1B) and prolonged sleep time (Fig. 2A); this was found with both MBIs, supporting the selectivity of CYP2B inhibition (Khokhar and Tyndale, 2011). The effect on brain propofol levels and response increased with increasing doses of one of the MBIs. Plasma propofol levels in vivo and hepatic CYP2B activity ex vivo were not influenced by MBI i.c.v. treatment, supporting the contention that CYP2B inhibition via i.c.v. administration was selectively achieved within the brain and had little/no impact on hepatic metabolism. CYP2B induction within the brain and not in the liver increased brain CYP2B enzyme levels and propofol inactivation. CYP2B can be induced in the brain, but not the liver, by a week of nicotine treatment (Miksys et al., 2000; Khokhar et al., 2010); the treatment resulted in lower propofol levels and reduced sleep times, which could be reversed by an i.c.v. injection of a MBI (Fig. 2B). Thus, having increased CYP2B enzyme in the brain resulted in lower brain propofol levels and shorter sleep times, whereas having inhibited enzyme in the brain resulted in increased brain propofol levels and longer sleep times. Together this suggests that variable CYP2B activity within the brain can alter the rate of inactivation of propofol and influence drug response. Recently, it has also been shown that inhibiting CYP2B in rat brain can also alter nicotine levels in the brain, measured by microdialysis, and resulting nicotine self-administration (Garcia et al., 2015).

P450s also metabolize substrates from inactive parent compounds into active metabolites. For example, CYP2B metabolically activates the pesticide chlorpyrifos to chlorpyrifos-oxon (Tang et al., 2001), a toxic

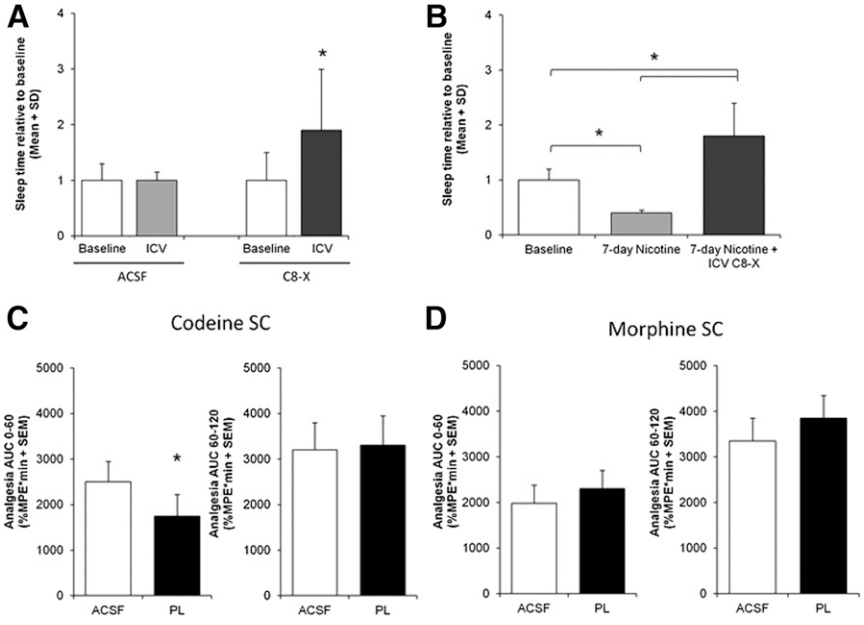


**Fig. 2.** Effect of CYP2 inhibition on the efficacy of CNS-acting substrates. Inhibiting the specific activity of CYP2 enzymes in the brain can influence the behavioral response to CNS-acting substrates. (A) CYP2B inhibition by the CYP2B MBI C8-xanthate (C8-X) in the brain increased propofol-induced sleep times in rats. (B) Inducing CYP2B in the brain by 7 days of nicotine treatment reduced propofol-induced sleep times, and this effect was reversed when rats were given C8-X. (C) CYP2D inhibition by the CYP2D MBI propranolol (PL) in the brain reduced analgesia at early time points after administration of its substrate codeine, but had no effect at later time points when morphine made peripherally enters the brain. (D) CYP2D inhibition by propranolol in the brain after a morphine injection did not have an effect on analgesia at any time; morphine is not a substrate for CYP2D. Data compiled from previously published reports (Khokhar and Tyndale, 2011; Zhou et al., 2013). $*P < 0.05$. ACSF, artificial cerebrospinal fluid; %MPE, percent maximal possible effect; SC, subcutaneous.

metabolite that inhibits acetylcholinesterase activity (Sultatos, 1994). Excess acetylcholine from reduced acetylcholinesterase activity leads to cholinergic system overstimulation, resulting in neurotoxicity and cognitive deficits, indicating the brain is one target of this toxicity (Steenland et al., 2000). There is negligible oxon detectable in the plasma after chlorpyrifos exposure due to its rapid metabolism to 3,5,6-trichloro-2-pyridinol in the blood by esterases (Costa et al., 1990; Drevenkar et al., 1993); therefore, the oxon formed from the liver might not reach the brain. In vitro brain homogenates can metabolize chlorpyrifos to the oxon (Chambers and Chambers, 1989), suggesting that brain-specific formation of this metabolite may be important in producing chlorpyrifos-induced neurotoxicity. This hypothesis was tested by administration of CYP2B MBI in the brain: rats were treated i.c.v. with MBI or vehicle and given a peripheral injection of chlorpyrifos (Khokhar and Tyndale, 2012). With MBI CNS treatment, brain chlorpyrifos levels increased while chlorpyrifos-oxon levels decreased, indicating that the MBI blocked CYP2B activation of the oxon metabolite. Remaining brain acetylcholine esterase activity was higher in MBI-treated rats, demonstrating that reducing CYP2B enzyme activity in the brain lowered the metabolite-mediated inhibition of the esterase. In rats, chlorpyrifos-oxon toxicity causes acute reductions in body temperature (hypothermia) and lasting behavioral impairments (e.g., reduced gait and aerial righting reflexes). MBI i.c.v. pretreatment reduced both the hypothermia and the behavioral impairments in animals given chlorpyrifos while having no impact in the absence of chlorpyrifos and no effect on systemic chlorpyrifos levels. Initially, MBI i.c.v. treatment had been given prior to chlorpyrifos to determine whether inhibiting brain CYP2B can prevent or reduce neurotoxicity. Subsequently, it was shown that, even following chlorpyrifos exposure, MBIs given i.c.v. could ameliorate toxicity days after chlorpyrifos treatment due to the long duration of chlorpyrifos in the body (Khokhar and Tyndale, 2014). This work demonstrated that MBI delivered directly to the brain can reduce the behavioral impairments, suggesting that in humans, inhibition of CYP2B6 might be a useful adjunct for treating chlorpyrifos toxicity. These results, together with the findings for propofol, demonstrate that brain-specific metabolism by CYP2B can significantly influence the response to CNS-acting substrates.

CYP2D can metabolize a variety of CNS-acting compounds including opiates, amphetamines, and antidepressants, as well as endogenous substances including numerous neurotransmitters (Zanger et al., 2004). Similar to *CYP2B6*, the gene that encodes human *CYP2D6* is highly polymorphic, and genetic variants have been associated with lower or higher metabolism of CYP2D6 substrates (Gaedigk et al., 2008). CYP2D6 has been identified in the brain; individuals with variants which disrupt the gene have lower CYP2D6 protein levels in the brain as well as in the liver (Miksys et al., 2002). Behavioral traits have been associated with *CYP2D6* genetic variation where genetically slower metabolizers score higher on anxiety and socialization indexes, suggesting a possible role for the enzyme within the brain (Bertilsson et al., 1989; Llerena et al., 1989, 1993; Gan et al., 2004; Roberts et al., 2004). CYP2D6 genetic variation is also associated with different levels of human resting cerebral brain perfusion, suggesting that fast versus slow metabolizers have an altered response to environmental stimuli (Kirchheiner et al., 2011). Thus, in addition to a potential role in response to drugs and toxins within the brain, CYP2D activity may influence endogenous brain processes.

CYP2D metabolizes codeine to morphine, which is the active compound that elicits analgesia (Adler et al., 1955). *CYP2D6* genetic variation can alter codeine-induced analgesia: genetically poor metabolizers do not exhibit an analgesic response to codeine due to their inability to convert codeine to morphine (Sindrup et al., 1990, 1992; Chen et al., 1991). This metabolic activation is mediated mainly by CYP2D6 in the liver, and morphine subsequently crosses the blood-brain barrier and elicits analgesia. However, codeine is more lipophilic than morphine and crosses the blood-brain barrier faster than morphine, and morphine is also actively transported out of the brain (Oldendorf et al., 1972; Bouw et al., 2000); therefore, the early time points of analgesia following codeine administration could be due to codeine entering the brain and being converted to morphine within the brain by CYP2D. This was tested by measuring analgesia after i.c.v. injection of competitive (propafenone) or mechanism-based (propranolol) inhibitors of CYP2D directly into the brain, as described earlier for CYP2B (Zhou et al., 2013). Intracerebroventricular injection of either inhibitor resulted in a reduction of codeine-mediated analgesia in rats. This reduction occurred for the first 30 minutes after codeine administration, whereas at later time points there was no effect of inhibition, suggesting that morphine from the periphery had sufficient time to enter the brain (Fig. 2C). Intracerebroventricular inhibitor pretreatment reduced brain morphine levels, whereas plasma morphine levels were unchanged (Fig. 1C), providing evidence that the inhibitor did not influence hepatic CYP2D activity. The inhibitor had no effect on either morphine's ability to enter the brain or morphine-induced analgesia, which supports the selectivity of the inhibitor's effect on codeine metabolism and resulting analgesia (Fig. 2D). Smokers have higher CYP2D6 protein in the basal ganglia and cerebellar regions, and chronic nicotine treatment can increase CYP2D6 expression in monkeys (Mann et al., 2008) and CYP2D1 expression in rats (Yue et al., 2008). Thus, it is likely that induced levels of CYP2D within the brain will increase the formation rate of morphine and subsequently increase analgesia—which was recently confirmed (McMillan and Tyndale, 2015).

CYP2D can metabolically inactivate the neurotoxin 1-methyl-4-phenylpyridinium ($MPP^+$), the active metabolite from the precursor 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. $MPP^+$ interferes with oxidative phosphorylation in mitochondria, resulting in cell death (Ramsay et al., 1991), and exposure to this toxin can elicit neurodegenerative effects similar to the symptoms observed in Parkinson's disease (Deng et al., 2004). Consistent with this metabolic inactivation, *CYP2D6* genetic poor metabolizers have an increased risk of developing Parkinson's disease (McCann et al., 1997). Thus, it is possible that CYP2D6 inactivation of toxins in the brain could be important in protecting against neurotoxicity and neurodegeneration. The influence of CYP2D6 in $MPP^+$ toxicity in neuronal tissue has been evinced in vitro: applying $MPP^+$ to human brain cell lines dose dependently increased cell death, whereas overexpression of CYP2D6 in these cell lines protected them from $MPP^+$ toxicity (Matoh et al., 2003). Conversely, inhibiting CYP2D6 in the presence of $MPP^+$ increased this cytotoxic effect likely by reducing its inactivation, analogous to the increased risk for Parkinson's disease seen in individuals without this enzyme (Mann and Tyndale, 2010). Consistent with this, brain CYP2D6 protein levels are lower in Parkinson's disease patients compared with age-matched healthy controls (Mann et al., 2012). The ex vivo and postmortem brain results suggest that having lower CYP2D6 activity in the brain may increase vulnerability to neurochemical insults by reducing the ability to inactivate certain classes of neurotoxins. Thus, CYP2D6 expression in the brain may play a role in protecting the CNS from toxins as well as mediating drug response.

Altering brain activity of either CYP2B or CYP2D can influence the CNS levels of drugs and toxins and make a significant impact on their subsequent behavioral effects. These proof-of-concept findings suggest that CNS expression of P450s contributes to drug and toxin metabolism within the brain and the resulting response. This not only demonstrates the potential importance of extrahepatic expression of

enzymes in drug response but also provides some understanding of the sources of variation in drug response, including genetic and environmental alterations of brain P450 activity. Thus, although early in our understanding of the role of P450 enzymes within the brain, these studies provide evidence that under some circumstances this may produce important variation in drug and toxin response.

### The SLC22 Transporter Family: Impact on Drug Efficacy, Drug-Drug Interactions, and Pathophysiology (D.H.S.)

In the kidney, the proximal tubule region of the mature nephron is tasked with performing much of the secretion and reabsorption of charged organic solutes. The limited passive diffusibility of such compounds necessitates utilization of dozens of membrane-bound transporters for efficient renal transcellular organic solute flux. Two major transporter superfamilies overseeing this transcellular flux are the ATP binding cassette (ABC) transporters (directly bind and hydrolyze ATP as an energy source) and the SLCs (driven by membrane potential and/or energy stored in concentration gradients) (Sweet, 2005, 2010). The human SLC superfamily is composed of ~55 unique gene families with ~400 confirmed or putative transporters (He et al., 2009). The SLC22 family (organic cation/anion/zwitterion transporter family) encompasses ~30 identified and putative members and includes the organic cation transporters (OCTs and organic cation transporters-novel) and the organic anion transporters (OATs) (Sweet, 2005, 2010; VanWert et al., 2010). Results from a variety of comparative models, e.g., intestinal sacs, renal and hepatic tissue slices and membrane vesicles, intact isolated renal tubules, liver, choroid plexus, and brain capillaries, demonstrated that the "physiological footprint" of mediated organic cation and anion flux across each of these tissues was similar (Pritchard and Miller, 1993), and it has been established that "renal" OCTs and OATs are expressed and function in barrier tissues throughout the body (Pritchard et al., 1999; Sweet et al., 2001; Sweet, 2010; VanWert et al., 2010; Farthing and Sweet, 2014). SLC22 transporters interact with a plethora of structurally diverse endogenous and foreign organic solutes such as metabolic byproducts, hormones, mycotoxins, pesticides, and a number of the "top 200" prescribed drugs, including diuretics, antibiotics, antivirals, and antidiabetic and chemotherapeutic agents; thus, SLC22 transporter activity is a major determinant of the pharmacokinetic, pharmacodynamic, and toxicity profiles of these compounds. Clearly, a fundamental understanding of SLC22 transporters is required to properly evaluate their impact on drug efficacy and exposure, drug-drug interactions (DDIs), and the pathophysiology of some disease states.

Review of the clinical literature revealed a number of apparent DDIs between probenecid (OAT inhibitor) or cimetidine (OCT inhibitor) and numerous therapeutics such as ciprofloxacin, cefaclor, famotidine, acyclovir, cidofovir, furosemide, and metformin, suggesting SLC22 transporter function impacts their clinical profiles (Morrissey et al., 2013). Indeed, a 4- to 5-fold decrease in in vivo efficacy of furosemide was observed in organic anion transporter 1 knockout mice (Eraly et al., 2006). Thus, if renal proximal tubule cells and/or the urinary space is the intended therapeutic target, loss of OAT1 function (via DDI or genetics) might be associated with decreased efficacy. When probenecid was coadministered with mesna, an agent that reduces the renal toxicity of some chemotherapeutic agents, mesna excretion was decreased ~70% and total renal clearance was decreased ~55% (Cutler et al., 2012). Follow-up in vitro studies with cloned hOAT1, hOAT3, and hOAT4 demonstrated probenecid-sensitive transport of mesna with inhibitory constants ($K_i$) below the clinical concentration, strongly implicating OATs as having a role in this DDI (Cutler et al., 2012). Similarly, the pharmacological action and associated toxicity of platinum antineoplastic agents (e.g., cisplatin and oxaliplatin) involves human OCT (hOCT) transport activity, and cotreatment with hOCT inhibitors (e.g., cimetidine) blunted accumulation and toxicity of cisplatin in renal proximal tubule cells and stably transfected transporter-expressing cells (Nelson et al., 1984; Pan et al., 1999; Ciarimboli et al., 2005; Yokoo et al., 2007, 2008). Recently, regulatory and scientific experts proposed guidelines for assessing the potential clinical relevance of such DDIs for about a dozen transporters, including the SLC22 family members OCT2, OAT1, and OAT3. As part of these recommendations, a DDI index was defined as the maximal unbound plasma concentration ($C_{max}$ or $C_{ss}$) of a compound after administering the highest clinical dose divided by the compound's in vitro inhibition potency ($K_i$ or $IC_{50}$) on the transporter of interest (Giacomini et al., 2010). A resultant DDI index value $\geq$0.1 (~9% inhibition) was conservatively selected as indicating the need for follow-up clinical DDI investigations.

Such analysis was used to assess the DDI potential of the antituberculosis drug ethambutol dihydrochloride (EMB) on hOCTs (Pan et al., 2013). The World Health Organization recommends EMB, along with pyrazinamide, isoniazid, and rifampin, as a front-line antituberculosis drug. Treatment of multidrug-resistant tuberculosis (TB; estimated to be 3.7% of new cases and 20% of previously treated cases) includes EMB to reduce the emergence of isoniazid- or rifampin-resistant mycobacteria (http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502_eng.pdf). In vitro characterization demonstrated that EMB inhibits hOCT1-, hOCT2-, and hOCT3-mediated transport (Pan et al., 2013). Using an estimated EMB concentration of 67 $\mu$M in the portal system after oral dosing, DDI index analysis indicated a rank order of DDI potencies on hOCT1 as enterocyte (0.6) = hepatocyte (0.6) > proximal tubule cell (0.2), all above the 0.1 threshold value (Pan et al., 2013). These values are indicative of high potential for EMB-mediated DDIs on hOCT1 affecting disposition and clearance. The largest DDI index (8.3, ~90% inhibition) was predicted for hOCT3 in enterocytes, suggesting orally dosed EMB could negatively impact cationic drug and/or nutrient absorption. DDIs involving EMB on hOCT2 expressed in the renal proximal tubule were predicted to be of low likelihood (DDI index = 0.1).

Further, many antidiabetic (metformin) and anti–human immunodeficiency virus (HIV; lamivudine, raltegravir, tenofovir, and zalcitabine) therapeutics are known substrates and/or inhibitors of SLC22s (Koepsell et al., 2007; Sweet, 2010; VanWert et al., 2010; Wang and Sweet, 2013). Thus, TB therapy is further complicated by the rise in clinical association of TB with diabetes or HIV, with TB now listed as a leading cause of mortality among HIV-infected patients (in 2011: 430,000 out of the 1.4 million TB deaths were HIV-associated) (Faurholt-Jepsen et al., 2012; Pawlowski et al., 2012). Overall, TB, TB-diabetes, and TB-HIV patients are subjected to polypharmacy to minimize development of drug resistance or treat these comorbidities, greatly increasing the risk of experiencing SLC22 transporter–mediated DDIs. For example, a patient population expressing a mutant form of hOCT1 with reduced transport function exhibited significantly altered metformin pharmacokinetics and experienced poorer glucose-lowering effects, i.e., loss of efficacy (Shu et al., 2008). The DDI index of 0.6 for hOCT1 in enterocytes and hepatocytes predicts as much as a 38% loss of hOCT1 transport activity in these cell types during routine EMB therapy (Pan et al., 2013). Together, these observations indicate a strong potential for EMB-metformin interactions on hOCT1 that result in altered drug efficacy in TB-diabetes patients.

In addition to altered xenobiotic disposition, renal toxicity and/or insufficiency is often clinically associated with a systemic increase in

endogenous molecules, including the so-called uremic retention solutes or uremic toxins. More than 150 compounds that are elevated in the biofluids of uremic patients have been identified, and their urinary excretion has been demonstrated to be largely a consequence of active tubular secretion as opposed to glomerular filtration (Masereeuw et al., 2014). Therefore, the pathophysiological progression of chronic kidney disease often includes the simultaneous increase in the systemic concentration of a variety of uremic toxins that interact with the organic cation and anion (i.e., SLC22-mediated) transport pathways, potentially leading to competitive interactions for OCT- and OAT-mediated transporter function.

In human kidneys, OCT2 appears to be the major OCT expressed; however, its role in uremic toxin accumulation/clearance is largely unknown. A number of cationic uremic toxins are known to effectively inhibit OCT2-mediated transport in vitro, and transporter expression was reduced in in vivo rodent models of acute and chronic kidney failure, supporting a link between transporter function and disease progression (Masereeuw et al., 2014). Similarly, it was observed decades ago that exposing rat kidney slices in vitro to the serum of uremic patients blocked accumulation of *p*-aminohippurate (prototypical organic anion substrate), and now dozens of uremic toxins are known substrates and/or inhibitors of OAT1, OAT2, OAT3, OAT4, and urate transporter 1 (White, 1966; VanWert et al., 2010; Wang and Sweet, 2013). Moreover, loss of renal OAT1 transport function was linked to elevated serum levels of several uremic toxins, i.e., decreased uremic retention solute elimination, via metabolomic profiling of organic anion transporter 1 knockout mice (Wikoff et al., 2011).

In humans, the uremic toxin hippuric acid is predicted to have high DDI potential on hOAT1 (DDI index = 12.3, ~92% inhibition) and on hOAT3 (DDI index = 7.5, ~88% inhibition) (Masereeuw et al., 2014). Indoxyl sulfate, strongly suspected as a causal factor in chronic renal failure, has a 10-fold stronger affinity for hOAT1 (~18 $\mu$M) than for hOAT3 (VanWert et al., 2010; Wang and Sweet, 2013). Given the 15 $\mu$M unbound plasma concentration in uremic patients, indoxyl sulfate could produce ~50% inhibition of hOAT1, correlating with the predicted DDI index of 0.8 or ~44% inhibition (Masereeuw et al., 2014). Finally, affinity of 3-carboxy-4-methyl-5-propyl-2-furanopropanoic acid (CMPF) for hOAT1 ($K_m$ = ~140 $\mu$M) appears too low to warrant any DDI concerns (DDI index = 0); however, the DDI index on hOAT3 ($K_m$ = ~27 $\mu$M) is 0.9, suggesting potential for marked inhibition of hOAT3 (47%) during chronic kidney disease (VanWert et al., 2010; Wang and Sweet, 2013; Masereeuw et al., 2014). As indicated, dozens of uremic toxins increase in concentration simultaneously, most likely leading to additive or synergistic effects that are greater than those produced by any single compound and indicating significant potential for SLC22-mediated drug–uremic toxin interactions in renal insufficiency patients.

Interestingly, elevated CMPF levels were recently observed in plasma from gestational diabetes mellitus, type 2 diabetes, and prediabetic patients versus individuals exhibiting normal glucose tolerance (Prentice et al., 2014). This suggested that CMPF might be linked to both gestational diabetes mellitus and the progression to type 2 diabetes. Supporting this connection, artificially elevating plasma CMPF in mice in vivo to human diabetic levels (150–300 $\mu$M) induced glucose intolerance, impaired glucose-stimulated insulin secretion, and decreased glucose utilization (Prentice et al., 2014). Additionally, in vitro treatment of isolated murine or human islet cells with 200 $\mu$M CMPF for 24 hours significantly decreased insulin secretion. Taken together with the fact that hOAT3′s affinity for CMPF is 7- to 10-fold higher than human diabetic CMPF plasma levels, it was hypothesized that OAT3 expressed in islet cells might mediate the accumulation of CMPF and, thus, directly impact its $\beta$-cell toxicity and the development of gestational diabetes, as well as subsequent progression to type 2 diabetes (Prentice et al., 2014).

In support of this contention, $\beta$-cell–specific expression of hOAT1, hOAT3, and hOAT4 was detected by microarray, quantitative polymerase chain reaction, immunoblot, and immunohistochemistry (Prentice et al., 2014). Moreover, treatment with probenecid (pan OAT inhibitor) or penicillin G (OAT3 substrate), but not *p*-aminohippurate (OAT1 substrate), prevented CMPF-induced impairment of insulin secretion (200 $\mu$M CMPF for 24 hours) in isolated murine islets. Importantly, islets isolated from organic anion transporter 3 knockout mice were insensitive to CMPF-induced $\beta$-cell dysfunction, retaining glucose-stimulated insulin secretion and normal total insulin content despite 24-hour exposure to 200 $\mu$M CMPF (Prentice et al., 2014). Thus, OAT3 mediates $\beta$-cell entry of CMPF, and inhibition of this function prevents the deleterious effects of CMPF, suggesting CMPF and hOAT3 provide a link between $\beta$-cell dysfunction and gestational diabetes mellitus/type 2 diabetes that could be targeted therapeutically.

In conclusion, it is well established that members of the SLC22 transporter family represent major transport pathways for the absorption, tissue distribution, elimination, and reabsorption of a variety of xenobiotic and endogenous compounds. The same SLC22 transporters often have functional roles in multiple barrier tissues. OCTs and OATs are emerging as sites for many clinically relevant DDIs, and as such, activity of these transporters markedly influences the pharmacokinetic profiles of many therapeutics, significantly impacting dosing regimen, pharmacodynamics, efficacy, and toxicity. Loss of specific SLC22 transporter function leads to alterations in drug pharmacokinetics and is associated with accumulation of uremic retention solutes. Subsequent SLC22 interactions with uremic toxins may provide a link between renal insufficiency/failure and progression of uremic syndrome, development of associated uremic encephalopathies, etc. In particular, recent evidence strongly implicates a link between the uremic toxin, CMPF, hOAT3 function in $\beta$-cells, and manifestation of gestational diabetes mellitus and progression to type 2 diabetes. Clearly, the SLC22 transporter family markedly impacts drug efficacy, drug-drug interactions, and pathophysiology.

### Pulmonary Metabolism of Resveratrol: In Vitro and In Vivo Evidence (S.S. and S.N.)

Prediction of drug and xenobiotic absorption, distribution, metabolism and excretion requires knowledge of disposition in major organs of absorption, distribution, and metabolism. Although the liver, intestine, kidney, and brain are commonly evaluated for xenobiotic distribution and metabolism, additional organs may play a major role in transport as well as metabolism of compounds. Organ-specific disposition is especially important for specific organs that are the target site for the compound. The human lung is one such organ with respect to disposition of drugs (e.g., lung cancer chemotherapy), xenobiotics (e.g., tobacco-specific carcinogens), and endobiotics (e.g., steroids) (Hecht, 2008; Gundert-Remy et al., 2014).

Expression and activity of xenobiotic-metabolizing enzymes (XMEs) in the lung have been evaluated extensively. The expression of various P450 isozymes has been reported in the lung (Fig. 3). P450 expression in various pulmonary cell lines representing the complex heterogenous cell layers of the lung has been reported, and variable P450 isozyme levels are thought to be expressed across the pulmonary cell layers (Gundert-Remy et al., 2014). Thus, Somers et al. (2007) reported modest human lung parenchymal levels of CYP1A1, 2J2, and 2A5, but lower expression levels compared with the liver. Numerous studies have shown modest expression of CYP1A1 in the bronchus and lung, with highly induced levels of the enzyme in smokers (Anttila et al., 2011).

| CYTOCHROME P450 | UGT |
|---|---|
| CYP1A1<br>CYP2F1<br>CYP2J2<br>CYP2S1<br>CYP3A5 | UGT1A6<br>UGT2A1<br>UGT2B4, UGT2B7, UGT2B10,<br>UGT2B11, UGT2B15, UGT2B15 |
| **SULT** | **TRANSPORTERS** |
| SULT1A1, SULT1A2, SULT1A3<br>SULT1B1<br>SULT1C2<br>SULT1E1<br>SULT2A1<br>SULT2B1. SULT2B2 | ABC<br>P-gp, MRP1-9, BCRP<br>SLC<br>OCTN1, OCTN2<br>PEPT1, PEPT2<br>SLCO<br>OATP1B3, OATP2B1,<br>OATP4A1, OATP4C1 |

**Fig. 3.** XME and transporter expression in human lung. The key P450, UGT, SULT, and drug transporters known to be expressed in the human lung are depicted (PEPT, peptide transporter; SLCO, solute carrier organic anion transporter; OATP, organic anion–transporting polypeptide). Data compiled from previously published reports (Gallagher et al., 2007; Somers et al., 2007; Bosquillon, 2010; Anttila et al., 2011; Sharan and Nagar, 2013; Jones and Lazarus, 2014).

P450 expression in the lung is highly relevant in the context of endogenous steroid homeostasis as well as activation of tobacco-specific lung carcinogens (Hecht, 2008; Gundert-Remy et al., 2014).

The human lung additionally expresses "phase II" enzymes, among which the XMEs uridine diphosphoglucuronosyltransferases (UGTs) and sulfotransferases (SULTs) are important enzyme superfamilies. These enzymes play important roles in steroid homeostasis as well as in the detoxification and elimination of procarcinogens and carcinogens (Dalhoff et al., 2005; Nagar and Remmel, 2006). Nakamura et al. (2008) detected UGT2B4 and 2B7 in normal human lung samples, whereas UGT1A1 and 1A3 were additionally reported in human lung adenocarcinoma A549 cell lines (Zhang et al., 2013). In another study, relatively high UGT1A6 and 2A1 were reported in human lung parenchyma, comparable to human liver expression (Somers et al., 2007). A recent study evaluated UGT2B expression in the upper respiratory tract, and reported low expression of various UGT2B isozymes (2B4, 2B7, 2B10, 2B11, 2B15, and 2B17) in the human lung (Jones and Lazarus, 2014). SULT pulmonary expression has similarly been reported, with low expression overall; SULT1E1 levels in the lung were the highest, followed by SULT1A1, 1A3, 1B1, and 2A1 (Riches et al., 2009). Somers et al. (2007) reported robust expression of several SULT isozymes in human lung parenchyma relative to human liver. Pharmacogenetic variability in XMEs can impact their role in the overall disposition of drugs and xenobiotics, and may predict the clinical outcome. An example is the *UGT2B17* gene deletion, which was found to correlate with increased incidence of lung adenocarcinoma in women, possibly due to decreased glucuronidation (detoxification) of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (Gallagher et al., 2007).

Trans-resveratrol (RES) is a polyphenolic compound present in various dietary sources, and has been shown to have numerous health effects via different mechanisms (Baur and Sinclair, 2006). RES is extensively metabolized into its sulfated and glucuronidated metabolites, with trans-resveratrol-3-sulfate (R3S) and trans-resveratrol-3-glucuronide (R3G) as major metabolites (Juan et al., 2010; Iwuchukwu et al., 2012; Sharan et al., 2012). RES is sulfated mainly by SULT1A1 with minor contribution from SULT1E1 (Ung and Nagar, 2007). RES is glucuronidated at its 3-OH position via UGT1A1, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, and UGT2B7 (Brill et al., 2006). Metabolism of RES is dependent on the site of metabolism. Human intestinal microsomes have a higher capacity for glucuronidation of RES (Iwuchukwu and Nagar, 2008) as compared with human liver microsomes. Systemic clearance of RES in mouse is much higher than the hepatic blood flow, indicating extrahepatic conjugation in the mouse (Sharan et al., 2012). Liver and gut as sites of RES metabolism have been well documented (Brill et al., 2006; Iwuchukwu and Nagar, 2008; van de Wetering et al., 2009), but the role of lungs in the metabolism of RES was not previously known. For a compound to be absorbed when given orally, it has to cross gut, liver, and lungs before it is available to the entire body. Metabolism by lungs in addition to liver and gut can significantly influence drug disposition, as the lung receives the entire cardiac output. Phenols are known to have higher phase II metabolism in lungs as compared with liver (Cassidy and Houston, 1980). In comparison with human cryopreserved hepatocytes, human lung parenchymal cells have higher sulfation activity (Somers et al., 2007). SULT1A1, SULT1A3, and SULT1E1 are the major SULT enzymes present in human lungs (80% of all SULTs expressed in human lungs) (Riches et al., 2009), which are also involved in the sulfation of RES (Miksits et al., 2005). There are conflicting reports about UGT expression in human lungs, showing low to no UGT activity in normal lung tissue (Zheng et al., 2002; Somers et al., 2007; Nakamura et al., 2008). Mouse lungs have been shown to express Ugt1a6 (Buckley and Klaassen, 2007), which is also involved in glucuronidation of RES. The aforementioned information about expression of metabolizing enzymes and their involvement in RES metabolism suggests the possibility of pulmonary contribution in the metabolism of RES.

Pulmonary metabolism of RES in vivo can be identified using multiple sites of administration and a single site of sampling approach (Cassidy and Houston, 1980). To evaluate the pulmonary contribution of RES, the right carotid artery and jugular vein of male C57BL/6 mice were cannulated under anesthesia with 1.5% isoflurane and 2 l/min oxygen. RES solubilized in 20% 2-hydroxylpropyl-$\beta$-cyclodextrin in saline was administered by i.v. or i.a. route at a dose of 15 mg/kg (Sharan et al., 2012; Sharan and Nagar, 2013). A carotid artery cannula was used for blood sampling. Blood samples were centrifuged at 14,000 rpm for 2 minutes, and plasma was stored at −80°C until analysis. Plasma samples were analyzed using electrospray ionization liquid chromatography–tandem mass spectrometry set in negative ion scan mode as per a published protocol (Iwuchukwu et al., 2012). Phoenix WinNonlin (version 6.1; Pharsight Corporation, Palo Alto, CA) was used for noncompartmental pharmacokinetic analysis of RES, R3G, and R3S. RES area under the curve exposure (AUC) and its half-life ($t_{1/2}$) were significantly lower (AUC: $P = 0.01$, $t_{1/2}$: $P = 0.04$) when administered by venous route (294.98 ± 137.87 minutes*$\mu$M, 101.30 ± 43.41 minutes) as compared with arterial route (591.08 ± 167.29 minutes*$\mu$M, 190.58 ± 69.65 minutes). The bioavailability of RES after i.v. administration, defined as a ratio of mean $AUC_{0\text{-inf}}$ after i.v. and i.a. route of administration, was 0.49. Exposure of R3G (2268.35 ± 517.00 minutes*$\mu$M) after RES i.v. administration was significantly higher ($P = 0.004$) as compared with R3G exposure (921.23 ± 457.07 minutes*$\mu$M) after RES i.a. administration. In vivo results were further confirmed by in vitro experiments in both mouse and human lung fractions. Mouse lungs showed both sulfation and glucuronidation with a partial substrate inhibition profile. Glucuronidation ($V_{max}$: 324.40 ± 13.05 pmol/min/mg, $K_m$: 7.34 ± 1.60 $\mu$M, $K_i$: 6632 ± 1198 $\mu$M) was more pronounced in mouse than sulfation ($V_{max}$: 7.05 ± 0.28 pmol/min/mg, $K_m$: 2.69 ± 0.45 $\mu$M, $K_i$: 2021 ± 717.6 $\mu$M). Interestingly, in experiments with human lung fractions, sulfation ($V_{max}$: 16.15 ± 0.48 pmol/min/mg, $K_m$: 4.45 ± 0.79 $\mu$M, $K_i$: 23,238 ± 7305 $\mu$M) was observed to be the major metabolic process. No R3G was observed above the limit of quantification (10 ng/ml) at the end of 60-minute incubations, indicating minimal contribution of pulmonary glucuronidation of

RES in humans. The differences in pulmonary metabolism of RES in mice and humans can be a possible explanation for the observed differences in the disposition of RES in rodents and humans. R3G has been reported as the major metabolite in rodents (Juan et al., 2010; Iwuchukwu et al., 2012; Sharan et al., 2012) in terms of exposure as compared with R3S in humans (Boocock et al., 2007; Brown et al., 2010). This also reaffirms the differences in the way drugs are handled among species and emphasizes the need for proper understanding of interspecies differences to effectively use preclinical data for extrapolation to humans.

The importance of drug transporters in the disposition of compounds and their metabolites is increasingly recognized (Hillgren et al., 2013; Zamek-Gliszczynski et al., 2014). RES has been reported to be a substrate for BCRP, whereas its conjugated metabolites are effluxed by BCRP, MRP2, and MRP3 (van de Wetering et al., 2009; Planas et al., 2012). Interestingly, these and many other transporters have been shown to be expressed in the lung (Fig. 3) (Bosquillon, 2010; Gumbleton et al., 2011; Doring and Petzinger, 2014). Thus, various family members of the ABC transporter family as well as SLC and solute carrier organic anion transporters are expressed in the lung (Bosquillon, 2010). The solute carrier organic anion transporter OATP1B3 is expressed in normal lung as well as in solid lung tumors, and was shown to correlate with disposition and toxicity of platinum cancer chemotherapy drugs (Lancaster et al., 2013).

The interplay between XMEs and transporters is of great interest, especially in the context of metabolism with subsequent efflux of metabolites. This interplay has been evaluated at the level of common regulatory pathways for XMEs and transporters, and in regard to the pharmacokinetics of compounds that are substrates for both XMEs and transporters (Nies et al., 2008; Pang et al., 2009; Zhang et al., 2009; Fan et al., 2010). XMEs as well as drug transporters share common regulatory pathways; for example, pregnane X receptor, constitutive androstane receptor, AhR/ARNT, and Nrf2-Keap mediated regulation of protein expression. Overlapping roles of metabolism and transport impact the overall disposition of RES and its conjugates (Maier-Salamon et al., 2013). It therefore becomes important to integrate metabolism and transport of RES across all eliminating organs to understand the target tissue concentrations of the active compound. Figure 4 depicts proposed pathways for the disposition of RES and its major metabolites in the mouse lung, liver, and gut.

Several XMEs and drug transporters are expressed in the lung. Their relative expression and activity across various pulmonary cells and experimental pulmonary cell lines are not completely understood. Pulmonary XME expression is inducible, with common regulatory pathways between XMEs and transporters. The present case study used RES as a model substrate, which was shown to be conjugated in the mouse as well as human lung in vitro, with clear species differences in the kinetics of RES conjugation. The reversible conjugation of RES along with its transport is tissue-dependent. It is clear that, in addition to hepatic metabolism and transport, the extrahepatic disposition of compounds and their relevant metabolites must be evaluated in an integrated manner before target-site drug concentrations and pharmacodynamics can be better predicted.

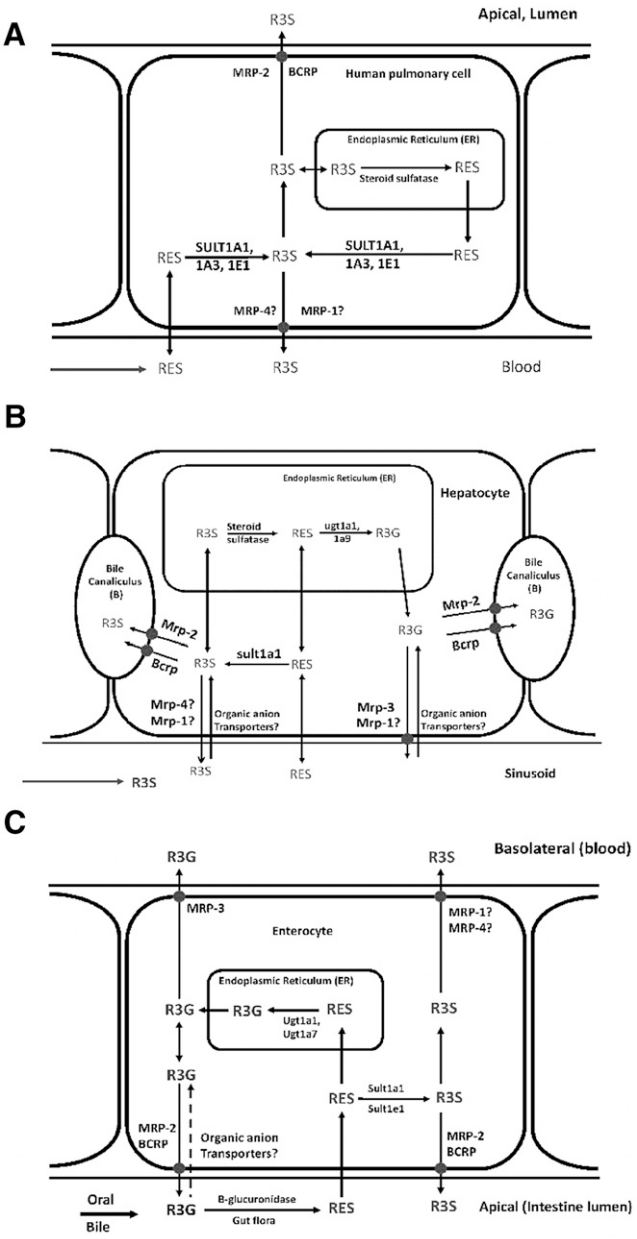


**Fig. 4.** Proposed scheme of transporter and metabolizing enzyme interaction mediating the disposition of RES and its metabolites in mouse lung cells (A), hepatocytes (B), and gut (C). Solid arrows represent pathways based on published reports for metabolism or transport of RES, R3S, and R3G. Dashed line represents pathways based on reports of similar substrates and transporters. Transporters followed by "?" indicate that the role of these transporters has not been established for transport of R3S and R3G but is hypothesized based on literature for other glucuronidated and sulfated metabolites.

### Tumor Metabolism of Antibody-Drug Conjugates: Effect on Pharmacokinetics and Efficacy (D.R.)

Therapeutics based upon antibody-drug conjugates (ADCs) are being pursued with a renewed vigor based largely upon advances in the next generation of linkers and payloads that have increased selectivity and potency, respectively, in this class of molecules. This is evidenced with the recent approval of Adcetris (brentuximab vedotin) to treat Hodgkin lymphoma and Kadcyla (ado-trastuzumab emtansine) to treat patients with human epidermal growth factor receptor 2-positive, late-stage breast cancer (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm268781.htm; http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm340704.htm). Of particular interest is the prediction of released payload concentrations at the site of therapeutic action, which may be significantly higher than the concentration observed in plasma (Alley et al., 2009). As direct clinical measurement of released payload concentrations through the use of tumor distribution studies is often logistically challenging, a significant effort has recently been placed on the development of in

vitro or in silico techniques to predict the effect of therapeutic site-specific catabolism on the observed target-site concentrations of ADC payloads (Shah et al., 2014). The final aspect of this symposium focused on the use of in vitro cellular assays to determine the effect of ADC catabolism at the site of therapeutic action on the observed pharmacodynamics of the ADC.

The stability of the cytotoxin conjugated to the antibody is paramount to achieving the therapeutic window required for the success of novel ADCs. Due to the long half-life of antibodies, stability testing with ADCs requires evaluation over the course of days as opposed to hours as is typical for small molecules. This represents a significant challenge in testing ADC stability in preclinical systems. For example, plasma may not be a suitable matrix for assessing stability in vitro beyond 48 hours due to changes in pH, altered reduction potential, and protein aggregation (Rossi et al., 2002). This poses a particular challenge for noncleavable ADCs where the linker is nonreducibly attached to the antibody with a covalent bond that is designed to be highly stable. Moreover, the size of ADCs (>150 kDa) precludes catabolism by drug-metabolizing enzymes found in hepatic liver microsomes.

Cell-based assays may be more suitable for stability and catabolism assessment needs for ADCs given the ability to culture cells for days. However, even in vitro uptake of antibodies in non–target-expressing cells via endocytosis or phagocytosis is slow and can limit the detection of antibody or ADC catabolism. Therefore, cells expressing the antigen, such as tumor cells or engineered cells transfected with tumor antigen, could be considered to interrogate ADC stability (Erickson et al., 2006). Furthermore, the cell-based assay format can mimic the in vivo conditions in which the ADC is expected to bind and undergo receptor-mediated endocytosis en route to lysosomal processing (Fig. 5).

Prior to conducting cell-based experiments with ADCs, the integrity of the physical properties of the ADC should be assessed. That is, the conjugated antibody should retain similar physical and biosphysical properties (binding to the antigen and to the neonatal Fc receptor) as compared with the unconjugated antibody. ADC conjugation to the antibody most commonly occurs through the $\varepsilon$-amine of the lysine side chain or via cysteine residues, either from reduction of the interchain disulfides or engineered cysteine residues. The resultant conjugated antibody material is composed of a heterogeneous population of species, each with varying conjugation stoichiometry. Each species could behave uniquely relative to the unconjugated antibody (Hamblett et al., 2004). Although any biophysical measurement of a heterogeneous population cannot read distinct properties of binding interactions within a mixture, the composite binding isotherm may indicate the presence of a strong outlier within the distribution (typically >2-fold change from unconjugated antibody). These results provide further confidence that subsequent cellular experiments are not selecting a subset of the ADC population for cellular catabolism, which may limit the accurate interpretation of the stability results. This should also be factored into the cellular experimental design wherein saturating the antigen-expressing cells with excess ADC to antigen creates a preference for the higher affinity species (unconjugated antibody) to limit binding of the lower affinity (high drug to antibody-derived material), thus preventing unbiased ruling on the in vitro stability. This can be overcome by the high antigen-to-antibody ratios possible in the test system and under conditions which consume >50% of dosed ADC to help ensure greater sampling of the mixture.

Cell-based stability experiments with antigen-positive cells can be used with ADCs to facilitate the uptake of the ADC into the cell, where it is exposed to endosome lysosome sorting pathways similar to those which will be encountered in vivo. There is a time component to the experiment findings given the need for antibody binding, internalization, and proteolytic processing. Incubations require a balance in concentrations used due to cellular toxicity upon ADC

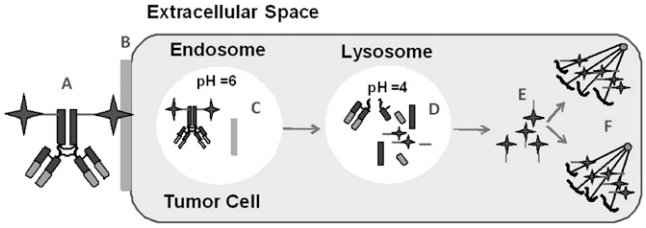


**Fig. 5.** Schematic of ADC catabolism. The catabolic pathway includes ADC (A) binding to tumor antigen (B); internalization of the ADC-tumor antigen complex into the endosome (noncleavable linker is stable) (C); a portion of the endosome is sorted to the lysosome where ADC is catabolized (D); and the catabolite exits the lysosome (E) and binds to microtubulin (F).

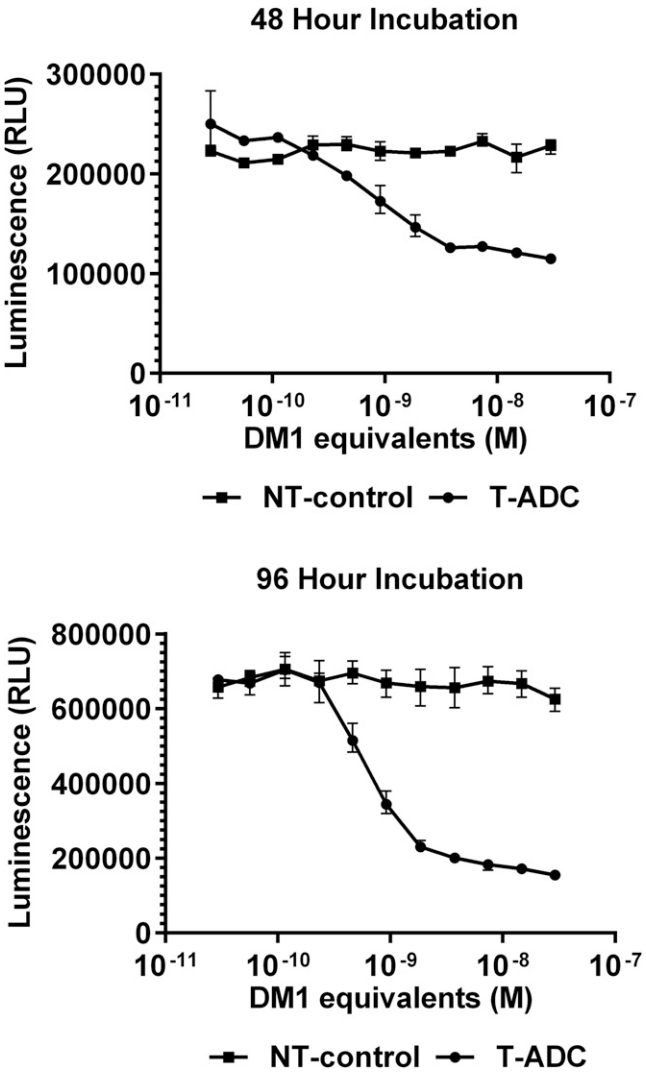


**Fig. 6.** Effect of tumor antigen on the cytotoxicity profile of ADCs. Example ADC incubated in cells transfected with the tumor antigen (T), whereas the same ADC incubated with control cells without tumor antigen (NT). Time-dependent cellular cytotoxicity at 48 hours (top) and 96 hours (bottom) illustrating the selectivity of toxicity toward tumor antigen–expressing cells.

degradation. Some noncleavable ADCs are more stable to proteolysis depending upon the site of conjugation (Shen et al., 2012). Furthermore, the compartment of degradation can be tuned for protease-cleavable linkers. Okeley et al. (2014) designed a series of linkers with distinct processing within the cell leading to increased cellular cytotoxicity. This is another advantage to the cell-based stability evaluation in that it allows for catabolism to be investigated in different cellular compartments. However, most noncleavable linker systems require lysosomal processing. To demonstrate the time dependence and importance of target expression in the design of in vitro stability assays, varying concentrations of a mertansine (DM1)-conjugated monoclonal antibody were incubated for 48 or 96 hours in antigen-positive target cells (T-ADC) and compared with incubations with cells devoid of the target antigen (NT-control). Cells were plated (96-well) and allowed to adhere overnight, after which new culture medium containing the DM1 conjugate was added to the cells. Cellular survival was determined using a luciferase-based ATP quantification assay and visualized as luminescence output versus DM1 equivalents. The time course for the degradation process is shown in Fig. 6 by tracking cell viability for this antigen-positive cell. The nontargeting control provides corresponding control for the lack of activity with antibody without cell surface antigen conjugated with the cytotoxin through the same linker.

The cellular supernatant and cell lysis can be evaluated for ADC catabolites. As most cell types share overlapping proteases in their lysosome, it is assumed the catabolism would represent that detected from a variety of cell types upon in vivo processing. To test this hypothesis, an antibody linked via succinimidyl 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate to DM1 was incubated for 24 or 48 hours in isolated lysosomal fractions from HepG2 cells, cellular lysate, non-adherent HepG2 cells, and control cell media. Lysosomes were isolated from HepG2 cells with the use of a lysosome isolation kit (Thermo Scientific, Waltham, MA) and resuspended in 100 mM sodium acetate buffer (pH 5.5) containing sodium chloride (100 mM), diethyldithiocarbamate (2 mM), EDTA (1 mM), and Triton X-100 (0.05%). Formation of lysine-[N-maleimidomethyl]-cyclohexane-1-carboxylate-DM1 (lysine-MCC-DM1) and other potential metabolites was assessed using a liquid chromatography–tandem mass spectrometry approach as previously described (Davis et al., 2012). Incubations resulted in the identification of a sole detectable catabolite of lysine-MCC-DM1. More detailed processing of the cells to isolate lysosome was conducted to isolate the subcellular compartment thought to be responsible for catabolite formation. As shown in Fig. 7, upon lysosome isolation, there is clear detection of lysine-MCC-DM1 in this compartment, consistent with this organelle as the origin of the catabolite. Not only is it present, but it is found in excess when compared with the cell supernatant and half of that detected in the lysate of the cells.

The absence of lysine-MCC-DM1 in the supernatant of cells may be due to the low passive permeability of lysine-MCC-DM1 which was determined in LLC-PK1 cells. Furthermore, as this is expected to be the primary ADC catabolite from succinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate linked DM1 to antibodies the efflux due to P-gp was also assessed in LLC-PK1 cells transfected with the human MDR1 gene (P-gp, *ABCB1*). In brief, cell culture medium containing L-glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 $\mu$g/ml), and fetal bovine serum (10%, v/v) was used to culture and plate cells in 96-well plates (90,000 cells per 150 $\mu$l) as previously described (Schinkel et al., 1995; Booth-Genthe et al., 2006). After 5 days of plating, cell medium was replaced with 150 $\mu$l of Hanks' buffered saline solution containing 10 mM Hepes and various concentrations of $^{14}$C-lysine-MCC-DM1. $^{14}$C-mannitol was used as a marker of monolayer integrity, $^{14}$C-cyclosporin A as a positive control for P-gp–mediated efflux, and elacridar as an inhibitor of P-gp activity in vitro. Plates were incubated for 2 hours, after which sample radioactivity was measured from the apical and basolateral compartments using liquid scintillation counting. Calculation of efflux ratios [ratio of apparent permeability coefficient ($P_{app}$) of basolateral to apical compartment divided by the $P_{app}$ of the apical to basolateral compartment] suggests lysine-MCC-DM1 exhibits low passive permeability and a low potential for the catabolite to serve as a substrate for P-gp (Fig. 8).

The data presented support the use of cellular systems to evaluate ADC catabolism and aid in the characterization of ADC absorption, distribution, metabolism and excretion in preclinical testing.

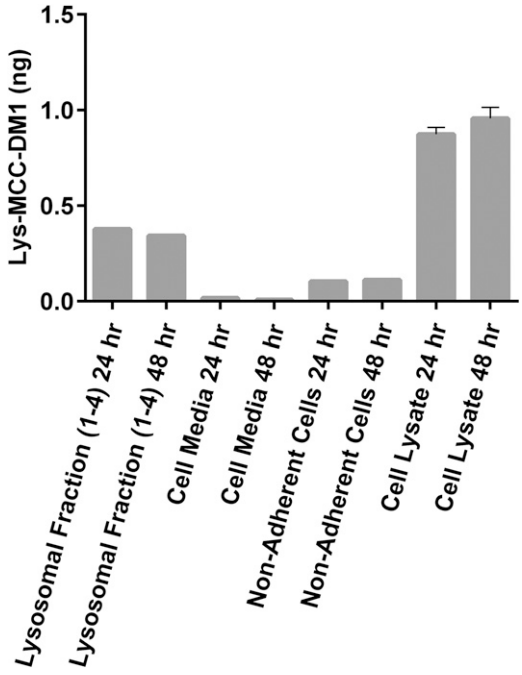


**Fig. 7.** Effect of tumor antigen on the distribution of ADCs in vitro. Cell lysate from cells transfected with tumor antigen contains the majority of catabolite, with lysosome harboring approximately half of all cell-generated catabolite. No catabolite was detected in cell media.

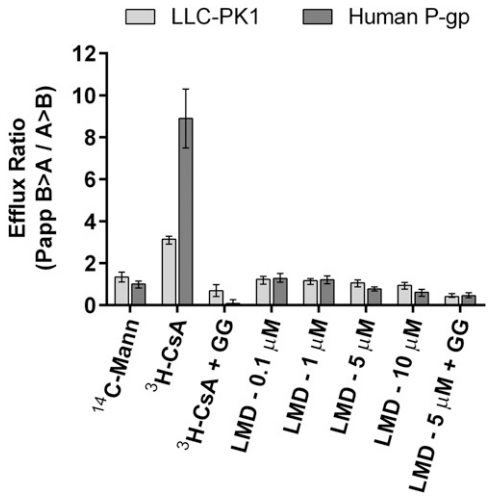


**Fig. 8.** Lysine-MCC-DM1 catabolite permeability at 0.1, 1.0, 5.0, and 10 $\mu$M. Data reveal low passive permeability of lysine-MCC-DM1. There is no evidence implicating lysine-MCC-DM1 as a P-gp substrate. CsA, cyclosporin A; GG, elacridar; LMD, lysine-MCC-DM1; Mann, mannitol.

## Summary

The research which was presented at the "Target-Site" Drug Metabolism and Transport symposium at the 2014 Experimental Biology meeting and which is summarized in this report highlights the importance of drug-metabolizing enzymes and transporters at the therapeutic site of action for a given drug. An increased understanding of such phenomena should allow for a more rational approach to drug optimization while increasing our knowledge of the potential barriers to drug efficacy. Ultimately, such research should serve to increase our ability to provide more effective clinical therapies in a safe and timely manner.


### Authorship Contributions

*Wrote or contributed to the writing of the manuscript:* Foti, Tyndale, Garcia, Sweet, Nagar, Sharan, Rock.

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