Title

Evaluating the Application of the BDDCS to Assess the Risk of Skin and Liver Toxicity Potential in Small Molecules Using In Vitro and Human Clinical Data

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Publication Date

2017

Peer reviewed|Thesis/dissertation

Evaluating the Application of the BDDCS to Assess the Risk of Skin and Liver Toxicity Potential in Small Molecules Using *In Vitro* and Human Clinical Data

by

Rosa Chan

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Sciences and Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

[redacted]

DEDICATION

my mom and dad,

my big sister, my little brother,

and all of my family and friends,

whose unconditional love, support, and unwavering faith

has always been my source of inspiration.

ACKNOWLEDGEMENTS

Chapter 2 was modified from "Use of the biopharmaceutics drug disposition classification system (BDDCS) to help predict the occurrence of idiosyncratic cutaneous adverse drug reactions associated with antiepileptic drug usage" as it was published in the AAPS Journal in May 2016. Rosa Chan, and Leslie Benet contributed to the study design. Chun-yu Wei was responsible for the experimental data collection. Rosa Chan carried out statistical analyses and wrote the manuscript. Rosa Chan, Chun-yu Wei, Yuan-tsong Chen, and Leslie Benet assisted with manuscript preparation.

Chapter 3 was modified from "Evaluation of DILI predictive hypotheses in early drug development" as it was published in the Chemical Research in Toxicology Journal in April 2017. Rosa Chan and Leslie Benet contributed to study design, which was executed and analyzed by Rosa Chan. Rosa Chan wrote the manuscript with contributions from Leslie Benet.

I am deeply grateful to my advisor, Professor Leslie Benet, who was also my mentor, cheerleader, and friend. He guided me on this journey with his vision, humor, boundless enthusiasm, and endless patience. His kindness, humility, and humanity has touched the lives of more people than one can imagine. It has been a true honor and privilege to be part of the Benet "student" family.

During my time as a graduate student in the lab, I met some wonderful people including Hsin-Fang Wu, Frances Peterson, Andre Dezani, Thaisa Dezani, Yi Zheng,
Ji Li, Chelsea Hosey, Marc Yago, Alan Wolfe, Hideaki Okochi, Fabio Broccatelli, Xin Huang, Shufang Liu, Christine Bowman and Jasleen Sodhi. I have had opportunities to travel all over the world with them during my graduate work, including Australia, China, Taiwan and Sweden. I will be forever grateful for their friendship, support, good times, insight, sanity and insanity as needed. All of the members of the Benet group have contributed immensely to my personal and professional development at UCSF.

I would also like to thank Drs. Chris Vulpe and Dale Johnson from the Molecular Toxicology program at UC Berkeley, my calculus teacher Dr. Dan Smith, and my former boss at the FDA, Dr. Kirk Arvidson for their support and encouragement during my undergraduate years. The Biology Scholars Program and Ronald McNair Scholars Program have contributed greatly to creating a community with invaluable experiences that have allowed me to thrive in sciences and achieve my goals.

For this dissertation I would like to thank my committee members: Drs. Matt Jacobson and Alan Wu who were more than generous with their expertise and precious time. I would also like to thank the other two members of my oral defense committee, Drs. Nadav Ahituv and Raja

Rajalingam, for their time and insightful questions. Their excitement and willingness to provide feedback made the completion of this project an enjoyable experience.

Last but not least, I am thankful to my parents, Foon and Yao, for teaching me about the importance of an education. My sister, Beleza and my brother, Oscar for always being there for me even when we were miles away. And to a group of special people in my life Pablo Moreira, Angel Rodriguez, Julie Hanh, Jenny Lee, Jessica Camacho, Ed Lee, and Leo Dionisi. Thank you for all your love and support.

ABSTRACT

Evaluating the Application of the BDDCS to Assess the Risk of Skin and Liver Toxicity Potential in Small Molecules Using In Vitro and Human Clinical Data

Rosa Chan

Drug hypersensitivity can be defined as a serious adverse drug reaction (ADR) often with an immunological etiology to an otherwise safe and effective therapeutic agent. The frequency and severity of drug hypersensitivity are variable, increasing with disease and dose. Hence, it is important to understand the biology of the patient/immune system, the pathophysiology of the disease in question, and the chemistry of the drug antigen.

The objective of this research project is to advance the understanding of drug toxicities associated with the liver and the skin, the two organs most commonly involved in serious adverse drug reactions by investigating the potential of the Biopharmaceutics Drug Disposition Classification System (BDDCS) as a methodology for evaluating toxicological outcome of therapeutic agents.

One of the key gaps moving forward is our understanding of and ability to predict the contribution of immune activation in idiosyncratic adverse drug reactions. This work will focus on immune mediated idiosyncratic adverse drug reactions associated with HLA-B*15:02 and on the presently proposed/hypothesized *in vitro* mechanism based toxicity mechanisms for drug-induced liver injury (DILI). The advances being made in microphysiological systems have a great potential to transform our ability to risk assess reactive metabolites, and an overview of the key components of these systems are presented.

Our published analyses suggest that comparison of drug hypersensitivity prediction methodologies with BDDCS classification is a useful tool to evaluate the potential reliability of newly proposed algorithms. This is true since almost all of these predictive metrics do no better than just avoiding BDDCS Class 2 drugs.

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CHAPTER 1: Challenges in the Prediction of Drug Hypersensitivity Reactions

Drug hypersensitivity can be defined as a serious adverse drug reaction (ADR) often with an immunological etiology to an otherwise safe and effective therapeutic agent. Of note, the definition is used to describe reactions targeting skin and internal organs that manifest in typically a very small percentage of individuals exposed to a therapeutic agent. The frequency and severity of drug hypersensitivity are variable, increasing with disease and dose. Hence, it is important to understand the biology of the patient/immune system, the pathophysiology of the disease in question, and the chemistry of the drug antigen (1).

There are multiple mechanisms by which a drug may act as an antigen or immunogen to activate the immune system and induce targeted tissue damage. The hapten, the pharmacological interaction with immune receptors, and the altered self-peptide hypotheses go some way to explain the molecular pathomechanisms underlying drug hypersensitivity reactions (DHRs) (See Figure 1.1). These running hypotheses define and characterize the different aspects of the drug antigen such as haptenicity, antigenicity, immunogenicity, and hypersensitivity. Haptens are defined as low molecular weight chemicals with the propensity to bind covalently to protein. In the context of this perspective, the term antigen is used to describe a drug-related substance that interacts specifically with immunological receptors such as antibodies or T-cell receptors. Finally, immunogens are molecules capable of stimulating a cellular and/or humoral immune response (See Figure 1.1A) (2).

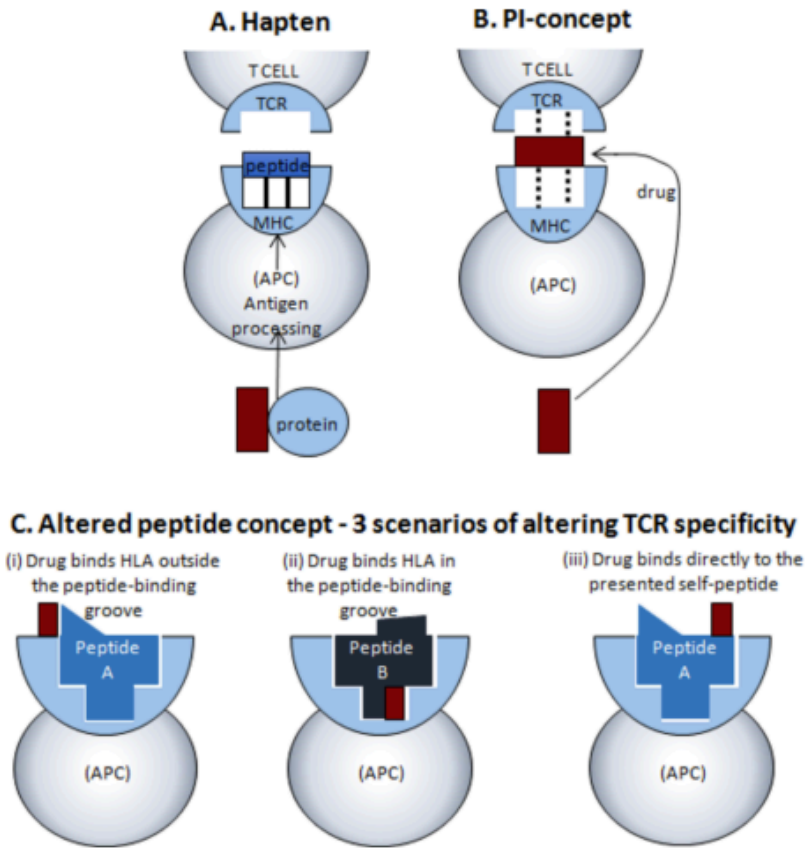

Figure 1.1 Models of T-cell receptor (TCR) activation by drug or chemical antigens.

(A) The hapten hypothesis states that a drug binds to protein to form a hapten and become recognizably immunogenic. The hapten is then internalized and processed by antigen presenting cells to form antigenic peptide fragments that are subsequently loaded onto major histocompatibility complex (MHC) molecules (covalent binding) and presented at the cell surface to passing T-cells. (B) The pharmacological interaction (PI) concept states that chemically inert parent drugs or chemicals can interact (noncovalently) directly with the MHC-TCR without the need for protein binding or antigen processing. (C) The altered peptide concept states that a drug may bind to the MHC-peptide complex in such a way that altered self-peptides represent an antigenic signal. This may refer to binding of the drug (i) to human leukocyte antigen (HLA) outside the peptide binding groove, (ii) to HLA in the peptide binding groove, or (iii) directly to the self-presented peptide. Peptide A = normal self-peptide; peptide B = altered self-peptide. Reproduced from Ogese et al. (1).

Characterization of the molecular pathophysiological mechanism(s) of drug hypersensitivity using a combination of *in vitro* assays and animal models is a critical step toward designing assays that will accurately predict which new drug will cause these reactions before they become widely used as therapeutics (1). However, prediction in this area is still very limited. The HLA

has been associated with a number of drug hypersensitivity reactions, however due to its polymorphic nature the risk allele does not develop reactions when exposed to a candidate drug, genetic, environmental, or disease. Risk factors must impact on patient susceptibility. The objective of this research project is to advance the understanding of drug toxicities associated with the liver and the skin, the two organs most commonly involved in serious adverse drug reactions by investigating the potential of the Biopharmaceutics Drug Disposition Classification System (BDDCS) as a methodology for evaluating toxicological outcome of therapeutic agents.

Biopharmaceutics Drug Disposition Classification System (BDDCS)

In 2005, Wu and Benet, proposed the BDDCS (3). BDDCS provides a useful tool in drug discovery for predicting routes of elimination, oral drug disposition, food effects on drug absorption, transporter effects on drug absorption, and potentially clinically significant drug interactions that may arise in the intestine, liver and brain (4). BDDCS recognizes that drugs exhibiting a high passive intestinal permeability rate (BDDCS Class 1 and 2) are also extensively metabolized, while low passive permeability rate drugs (BDDCS Class 3 and 4) are primarily eliminated as unchanged drug in the bile or the urine. Thus, BDDCS classifies drugs according to the extent of metabolism, aqueous solubility and membrane permeability rate. While these relationships have been uncovered in terms of drug disposition, it would be useful exercise to understand how these physicochemical properties can be related to the toxicity risk of compounds during early drug development.

Reactive metabolites are widely accepted as playing a pivotal role in the pathogenesis of idiosyncratic adverse drug reactions (See Figure 1.2). While there are today well-established strategies for the risk assessment of stable metabolites within the pharmaceutical industry, there is still no consensus on reactive metabolite risk assessment strategies. This is due to the

complexity of the mechanisms of these toxicities as well as the difficulty in identifying and quantifying short-lived reactive intermediates such as reactive metabolites. In this work, we apply the BDDCS to evaluate reactive metabolite risk, hazard assessment approaches are discussed, and the strengths and weaknesses are highlighted.

One of the key gaps moving forward is our understanding of and ability to predict the contribution of immune activation in idiosyncratic adverse drug reactions. Sections in this thesis will focus on immune mediated idiosyncratic adverse drug reactions associated with HLA-B*15:02 and on the presently proposed/hypothesized *in vitro* mechanism based toxicity mechanisms for the understanding of immune activation by reactive metabolites. The advances being made in microphysiological systems have a great potential to transform our ability to risk assess reactive metabolites, and an overview of the key components of these systems is presented. Finally, the potential impact of systems pharmacology approaches in reactive metabolite risk assessments is highlighted.

Drug metabolites have also been implicated in a number of DHRs; it is arguable that the expression of polymorphic drug metabolizing enzymes may expose individuals within a population to varied quantities of antigenic moieties. Indeed, this may affect both phase I and II metabolism pathways, where an individual may be more susceptible to the formation of active products but also be less susceptible to their subsequent detoxification. Despite this, genetic variation in drug metabolism may rarely be a simple susceptibility to DHRs, but instead, metabolic rate may be a factor for the rate of onset of a DHR. Impaired renal clearance and comorbidity are other important factors to consider. Therefore, individuals are still exposed to potentially immunogenic metabolites independent of metabolic rate, and thus, it is unclear how metabolic variation translates to a predisposition to hypersensitivity. This may be explained by

danger signaling, as certain individuals would be exposed to higher and thus more toxic concentrations of certain compounds, and would therefore be subject to enhanced danger signaling and an enhanced likelihood of T-cell activation (1).

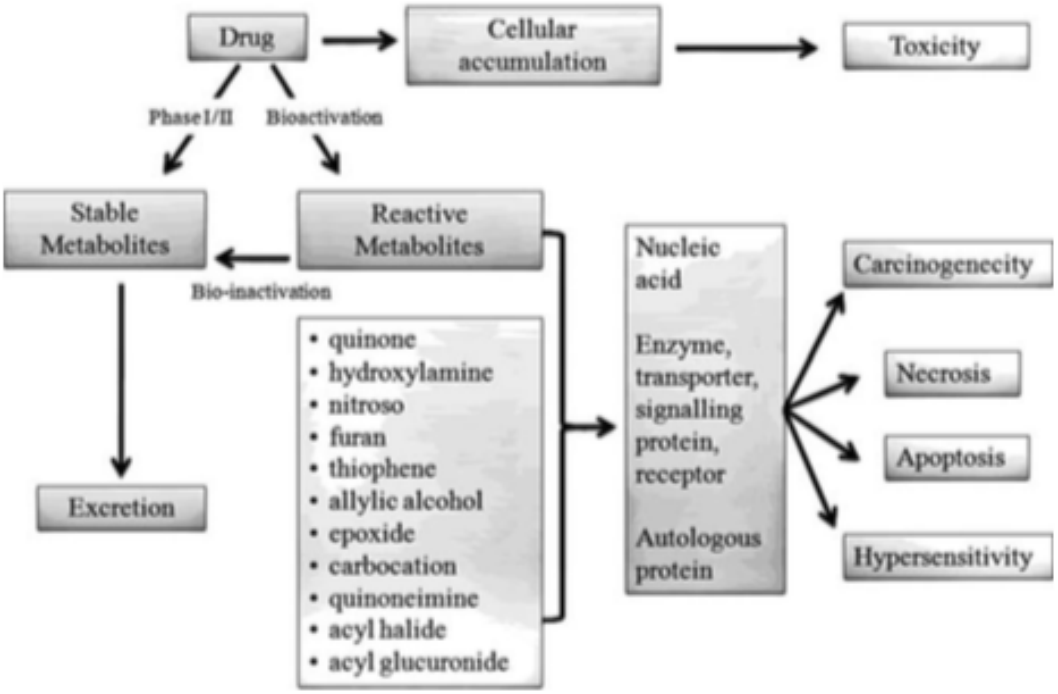

Figure 1.2. Relationship between drug metabolism and toxicity.

Toxicity may accrue through accumulation of parent drug or, via metabolic activation, through formation of a chemically reactive metabolite, which, if not detoxified, can effect covalent modification of biological macromolecules. The identity of the target macromolecule and the functional consequence of its modification will dictate the resulting toxicological response. Reproduced from Srivastava et al. (5).

Because the characteristics of small molecules that are capable of initiating DHRs remains unknown and the possibility of reactive metabolites contributing to the risk of DHR, here we will investigate the potential of using the BDDCS as a tool to assess the predictability of drug hypersensitivity reactions.

Cutaneous Adverse Reactions (CARs)

Drug reactions in the skin are more common than at any other site; adult-onset neurologic disorders affect almost 7 % of the US population, and their therapies cause a spectrum of skin reactions, with morbilliform and urticarial eruptions seen most often. Anticonvulsants and disease-modifying multiple sclerosis therapies can precipitate emergent cutaneous drug reactions, including serious infection, Stevens–Johnson syndrome, toxic epidermal necrolysis, and drug reaction with eosinophilia and systemic symptoms, which require immediate drug withdrawal and supportive measures in an intensive care unit with specialist consult (5).

Toxic epidermal necrolysis and Stevens–Johnson syndrome are two of the most acute life-threatening drug hypersensitivity reactions. Epidermal necrosis causes erosion of the mucous membranes, extensive detachment of the epidermis, and severe constitutional symptoms. The physiopathologic mechanisms of these conditions are not established. When there is very extensive skin detachment and a poor prognosis (death rates of 30 to 40 percent), the condition is usually called toxic epidermal necrolysis. Milder forms are known as Stevens–Johnson syndrome or overlapping Stevens–Johnson syndrome and toxic epidermal necrolysis. Toxic epidermal necrolysis is usually drug-related. Drugs are an important cause of Stevens–Johnson syndrome, but infections or a combination of infections and drugs have also been implicated. In case reports and studies, more than 100 drugs have been implicated as causes of Stevens–Johnson syndrome or toxic epidermal necrolysis. A limited number of drugs, including sulfonamides, anticonvulsant agents, and allopurinol, are the most consistently associated with the conditions (6).

Cutaneous adverse reactions (CARs) from antiepileptic drugs (AEDs) are common, ranging from mild to life-threatening, including Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). The identification of subjects carrying the HLA-B*15:02, an inherited allelic

variant of the HLA-B gene, and the avoidance of carbamazepine (CBZ) therapy in these subjects is strongly associated with a decrease in the incidence of carbamazepine-induced SJS/TEN. In spite of the strong genetic associations, the initiation of hypersensitivity for AEDs is still not very well characterized. Predicting the potential for other AEDs to cause adverse reactions will be undoubtedly beneficial to avoid CARs. Here, we will explore the use of the Biopharmaceutics Drug Disposition Classification System (BDDCS) to distinguish AEDs associated with and without CARs by examining the binding relationship of AEDs to HLA-B*15:02 and data from extensive reviews of medical records. We will evaluate the lack of benefit from a Hong Kong population policy on the effects of screening for HLA-B*15:02 and previous incorrect structure-activity hypotheses (7). We will follow up on the potential of using the HLA-B *in vitro* assay on these apparent determinant properties in predicting toxicity potential. We will examine how these general characteristics described by BDDCS class can allow for the prediction of these two types of idiosyncratic adverse drug reactions. We will examine other antiepileptic drugs and their binding interaction with HLA-B*15:02 and other HLA-B alleles.

However, prediction of DHRs in the clinic, based solely on HLA-genotype, remains very limited. This is because the majority of individuals who carry known HLA risk alleles do not develop immunological reactions when exposed to a culprit drug. We must therefore assume that immunological parameters, other than HLA genotype, may also contribute to the development of a drug-specific T-cell response. Since susceptibility to drug hypersensitivity is a function of the patient's individual biology, the prediction of drug hypersensitivity will involve capturing the patient's biology and variability during the early stages of drug development within preclinical test systems.

Drug-induced liver injury (DILI) is a leading cause of drug failure in clinical trials and a major reason for drug withdrawals from the market (8). Idiosyncratic DILI has been shown to be dependent on both daily dose and extent of hepatic metabolism of a drug (9, 10). Here we will perform a comprehensive analysis to examine the clinical impact of BDDCS in evaluating the severity of DILI warning in drug labels approved by the Food and Drug Administration (FDA), the withdrawal status due to ADRs, the role of BSEP inhibition and daily dosages prescribed. We will also evaluate the use of BDDCS in differentiating DILI potential (11). Furthermore, we will explore the extent in which we can considered BSEP inhibitors and the hypothesis that BSEP inhibition is a driving force for DILI potential of therapeutic agents.

This work highlights the correlation between BDDCS determinant properties and reports of serious adverse events. We conclude that overall the BDDCS Classification may be useful as a comparison tool for evaluating the usefulness of *in vitro* assays and animal models in the prediction of which new drug will cause these adverse drug reactions.

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CHAPTER 2: Use of the Biopharmaceutics Drug Disposition Classification System (BDDCS) to Help Predict the Occurrence of Idiosyncratic Cutaneous Adverse Drug Reactions Associated with Antiepileptic Drug Usage

ABSTRACT*

Cutaneous adverse reactions (CARs) from antiepileptic drugs (AEDs) are common, ranging from mild to life-threatening, including Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). The identification of subjects carrying the HLA-B*15:02, an inherited allelic variant of the HLA-B gene, and the avoidance of carbamazepine (CBZ) therapy in these subjects is strongly associated with a decrease in the incidence of carbamazepine-induced SJS/TEN. In spite of the strong genetic associations, the initiation of hypersensitivity for AEDs is still not very well characterized. Predicting the potential for other AEDs to cause adverse reactions will be undoubtedly beneficial to avoid CARs, which is the focus of this report. Here we explore the use of the Biopharmaceutics Drug Disposition Classification System (BDDCS) to distinguish AEDs associated with and without CARs by examining the binding relationship of AEDs to HLA-B*15:02 and data from extensive reviews of medical records. We also evaluate the lack of benefit from a Hong Kong population policy on the effects of screening for HLA-B*15:02 and previous incorrect structure-activity hypotheses. Our analysis concludes that BDDCS Class 2 AEDs are more prone to cause adverse cutaneous reactions than certain BDDCS Class 1 AEDs and that BDDCS Class 3 drugs have the lowest levels of cutaneous adverse reactions. We propose that BDDCS Class 3 AEDs should be preferentially used for patients with Asian

* Modified from publication: Chan R, Wei C, Chen Y, Benet LZ. 2016. Use of the biopharmaceutics drug disposition classification system (BDDCS) to help predict the occurrence of idiosyncratic cutaneous adverse drug reactions associated with antiepileptic drug usage. *AAPS J.* 18(3):757–66

backgrounds (i.e., Han Chinese, Thai and Malaysian populations) if possible and in patients predisposed to skin rashes.

INTRODUCTION

Cutaneous adverse reactions (CARs) from antiepileptic drugs (AEDs) are common, ranging from mild to life-threatening, including maculopapular eruption, drug reaction with eosinophilia and systemic symptoms (DRESS), Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) (1, 2). The mortality rates are approximately 10-15% in SJS, 30% in overlapping SJS/TEN and up to 50% in TEN (3). For years, the pathological determinants of SJS/TEN remained elusive. The identification of subjects carrying the HLA-B*15:02, an inherited allelic variant of the HLA B gene, and the avoidance of carbamazepine (CBZ) therapy in these subjects is strongly associated with a decrease in the incidence of carbamazepine-induced SJS/TEN (4–9). HLA-B*15:02 screening policies have been implemented in a number of countries with respect to CBZ dosing, including the U.S. when in 2007 the FDA published an alert (10) stating that "Patients with ancestry from areas in which HLA-B*1502 is present should be screened for the HLA-B*1502 allele before starting treatment with carbamazepine." In a research setting, screening in Taiwan was associated with a reduced incidence of CBZ-induced SJS/TEN (11). Recently, however, the results of a routine clinical service policy at a system-wide level in Hong Kong implemented in 2008 was reported to be associated with prevention of CBZ-induced SJS/TEN without reducing the overall burden of AED-induced SJS/TEN in more than 110,000 epilepsy patients (12). Attempts to predict the potential for various AEDs to cause cutaneous hypersensitivity through structure-activity relationships, suggesting that CARs occur with aromatic AEDS, but not with non-aromatic AEDs (13, 14), have ignored data for aromatic AEDs exhibiting low CARs incidence such as clobazam and clonazepam. Thus, in spite of the strong genetic associations and some structure-activity success, the initiation of hypersensitivity

for AEDs is still not very well characterized. Predicting the potential for other AEDs to cause adverse reactions will be beneficial to avoid CARs, which is the focus of this report.

In 2005, Wu and Benet proposed the Biopharmaceutics Drug Disposition Classification System (BDDCS) (15). BDDCS provides a useful tool in early drug discovery for predicting routes of elimination, oral drug disposition, food effects on drug absorption, transporter effects on drug absorption, and potentially clinically significant drug interactions that may arise in the intestine, liver, and brain (15, 16). BDDCS recognizes that drugs exhibiting a high passive intestinal permeability rate (BDDCS Class 1 and BDDCS Class 2) are extensively metabolized in humans, while low passive permeability rate drugs (BDDCS Class 3 and BDDCS Class 4) are primarily eliminated as unchanged drug in the bile or the urine (Figure 2.1).

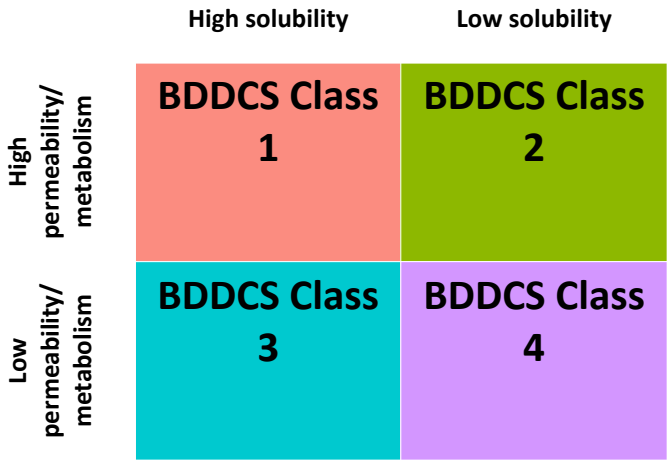

Figure 2.1. Biopharmaceutics Drug Disposition Classification System (BDDCS).

Because the specific drug characteristics linking to adverse events remain controversial, here we expand the use of BDDCS in assisting the prediction of AED drug hypersensitivity reactions, conducted a systematic review to appraise the strength of BDDCS in the association of the incidence of CARs induced by AEDs, and performed in vitro studies to identify specific

HLA/drug interaction patterns. In addition to exploring the use of BDDCS in the pathogenesis of CARs, the results of this work may help identify other AEDs or drugs in other therapeutic categories that can elicit SJS/TEN.

METHODS

HLA-B *In Vitro* Assay

We used the Biacore T200 SPR biosensor for analyzing the interaction between HLA-B proteins and drugs according to the manufacturer's protocol (GE). We immobilized the purified soluble HLA-B proteins (acting as ligands) on the chips by an amine coupling reaction, and the immobilized levels of sHLA-Bs were 9373-9812 response units (R.U). PBS was used as running buffer and the flow rate was 10 mg/min. The compounds (10 AEDs, 2 active metabolites and 1 non-active backbone structure) dissolved in PBS with 5% DMSO were evaluated and flowed through the solid phase with the running buffer PBS with 5% DMSO. Responses of the interaction were reference subtracted and corrected with a standard curve for the DMSO effects. We used BIA evaluation Version 3.1 for data analysis (17).

Compilation of AED-Related Adverse Cutaneous Reactions Studies

Data were extracted from four systematic published reviews of medical records of patients with epilepsy for documentation of CARs from AEDs. AED-related skin reactions studies were found in three main populations: American, Chinese and Norwegian patients. We also used DailyMed (http://dailymed.nlm.nih.gov/dailymed/) to review rash and more serious dermatologic conditions reported in FDA package inserts, in addition to literature reports/reviews.

American Retrospective Study

The study in America was carried out at the Columbia Comprehensive Epilepsy Center between January 1, 2000 and January 1, 2005. A total of 1,875 patients were included with altogether 5,050 exposures to 15 different AEDs (18). The attribution of rash was based on the patient's description of the rash or on the medical examination, if the physician concluded it was most likely due to the AED. Overall 14.3% (269/1875) of patients experienced a skin reactions to at least one AED.

Chinese Retrospective Studies

Although two Chinese studies were available in the literature and were carried out around the same time, we have analyzed them independently. The studies were carried out at the Epilepsy Center of the Chinese PLA General Hospital in Beijing, China. The first study period was from February 1999 to April 2010. A total of 3,793 patients were included with altogether 7,353 exposures to 11 different AEDs (19). Overall 3.61% (137/3793) of patients experienced a skin reaction to at least one AED. The second study period was between February 1999 and September 2010. A total of 4,037 patients were included with altogether 5,355 exposures to 9 different AEDs (14). Overall 4.06% (164/4037) of patients experienced a skin reaction to at least one AED. A CAR was defined as any type of rash (erythematous, maculopapular, papular, pustular or unspecified) that had no other obvious cause apart from an AED that resulted in contacting a physician.

Norwegian Retrospective Study

The study in Norway was carried out in three specialist outpatients clinics in Middle Norway served by neurologists from Trondheim University Hospital. A total of 663 patients were included with altogether 2,567 exposures to 15 different AEDs (20). A skin reaction was

defined as a diffuse rash (including MPE, DRESS, urticaria, erythema nodosum and SJS) that was reported in the medical records and had no other obvious reason than a drug. As initial symptoms of hypersensitivity most frequently occur up to 8 weeks after starting a drug, treatments lasting less than 3 months and stopped for any other reason than a rash were not included as an exposure. Overall 14% (93/663) of patients experienced a skin reactions to at least one AED.

Determining the Changes in AED Prescribing Practice with HLA-B*15:02 and the incidence of SJS/TEN.

Data were extracted from the Hong Kong Hospital Authority Clinical Data Repository to determine changes in AED prescribing practice in all patients, in AED-naïve patients, and in patients with newly treated epilepsy and the incidence of AED-induced SJS/TEN, following implementation of the HLA-B*15:02 screening policy (12). The study period covered 3 years before the implementation date (prepolicy: September 16, 2005 to September 15, 2008) and 3 years after (postpolicy: September 16, 2008 to September 15, 2011). Patients of interest were those who had at least one AED newly commenced and/or underwent testing for HLA-B*15:02 in the study period. An AED was defined as newly commenced if there was no record of its prescription in at least the previous 12 months. A total of 111,242 patients were included and 4,149 were tested for HLA-B*15:02. SJS/TEN was attributed to an AED if the patient was hospitalized for SJS/TEN within 90 days of commencing an AED, and the patient's allergy histories did not suggest other pharmaceutical products (12).

Compilation of BDDCS properties, **Correlation and Statistical Analyses**

Data are expressed as percentages of cutaneous incidence rate given the number of patients affected divided by the number of exposures associated with each AED together with the

BDDCS class. The BDDCS class assignment and properties were obtained from the BDDCS applied to over 900 drugs paper (21). Missing data were complemented by literature searches. Data with absolute values of each AED exposure along with BDDCS were also included.

The BDDCS class prescription pattern across the three different groups: all patients, AED-naïve patients, and patients with newly treated epilepsy in the AED prescribing practice for HLA-B*15:02 was also analyzed. Data are expressed as the percent of each AED prescription in the prepolicy along with absolute values of each AED exposure and BDDCS class. Differences in the proportions of BDDCS classes associated with CARs and prescription patterns were determined using chi-squared tests. The differences of SJS/TEN incidence between the prepolicy and postpolicy were calculated using the Fisher's Exact test.

The 12 AED related compounds were evaluated using the *in vitro* assay relative response binding to HLA-B*15:02 versus the incidence of cutaneous adverse drug reactions reported with the Spearman rank correlation coefficient (ρ) and Spearman correlation test. For statistical tests, a p-value less than 0·05 was considered significant. Analyses and plots were carried out using R (http://cran.r-project.org) and GraphPad Prism software version 6·0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Incidence of Cutaneous Adverse Reactions and BDDCS class

Using the BDDCS classification, the drugs associated with the highest incidence of cutaneous adverse reactions fall in BDDCS Class 2 in four retrospective studies (18–20, 22), with the lowest incidence for BDDCS Class 3 AEDs as depicted in Figure 2.1. BDDCS Class 2 drugs (lamotrigine, oxcarbazepine, carbamazepine and phenytoin) showed the highest rate of

cutaneous adverse drug reactions across all retrospective studies. Gabapentin, felbamate, clobazam, clonazepam, valproate, topiramate, levetiracetam and vigabatrin had consistently the lowest rates of CARs. Hence, it appears that BDDCS Class 2 AEDs exhibit the highest trend of causing cutaneous adverse reactions followed by certain BDDCS Class 1 drugs, in particular zonisamide, phenobarbital and tiagabine. Valproic acid, a widely used AED, clonazepam and clobazam are BDDCS Class 1 presenting lower levels of adverse cutaneous reactions than the other aforementioned BDDCS Class 1 drugs. Levetiracetam, a BDDCS Class 3 drug, shows a high efficacy in vulnerable populations, e.g. elderly (23) and children (24), and low levels of CARs. Felbamate is the only BDDCS Class 4 AED and it shows a low rate of CARs.

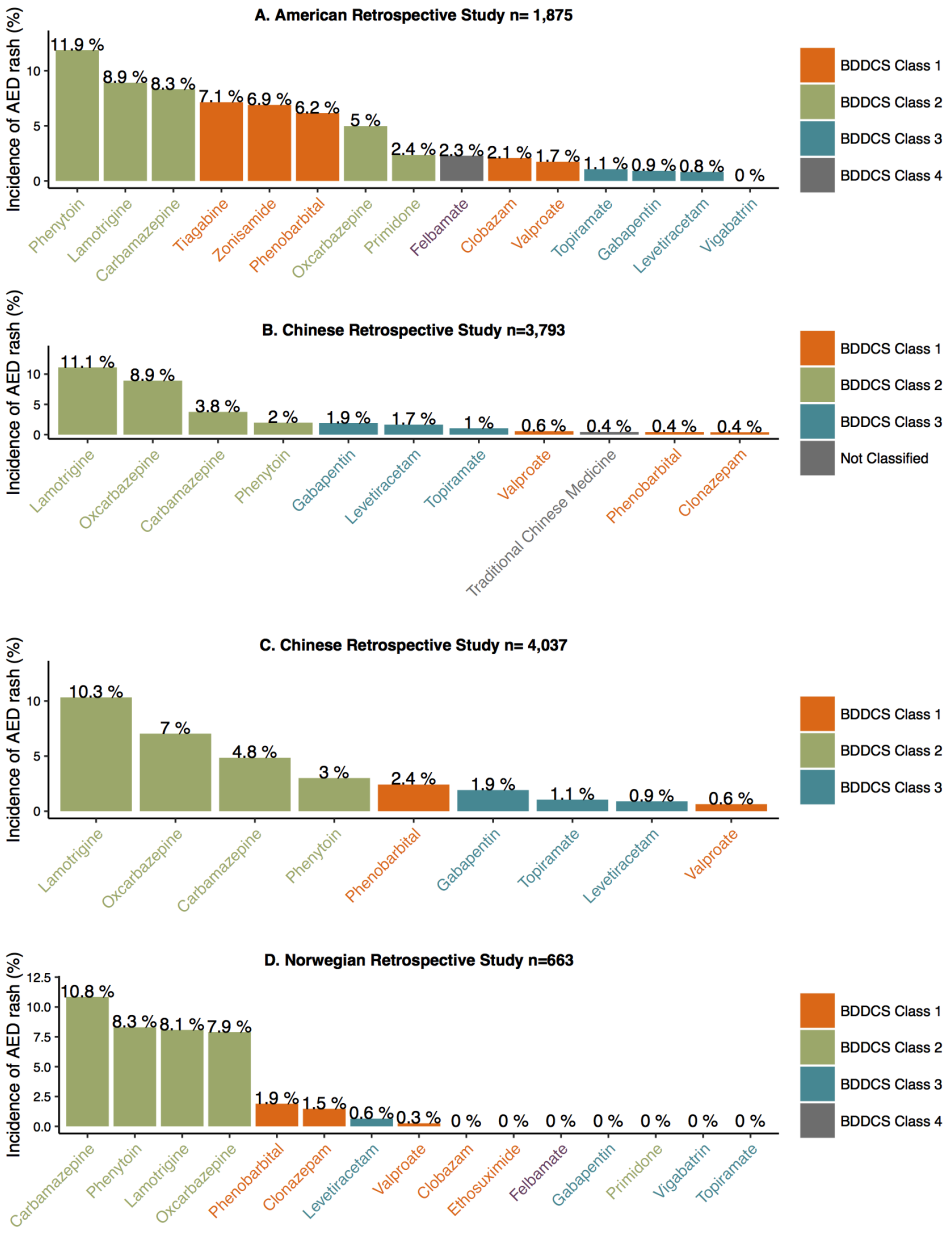

Figure 2.2 Incidence of AED-related skin rash (%) and BDDCS Classification in Americans, Chinese and Norwegians.

[A] BDDCS Class 2 drugs accounted for 55.6% incidence rates of AED-related skin rashes, followed by 36.6% for BDDCS Class 1, 4.3% for BDDCS Class 3 and 3.5% BDDCS Class 4 in the American retrospective study. *

[B] BDDCS Class 2 drugs accounted for 80.0% incidence rates of AED-related skin rashes, followed by 4.3% for BDDCS Class 1, 14.4% for BDDCS Class 3 and 1.3% for the not classified compounds in the Chinese retrospective study. *

[C] BDDCS Class 2 drugs accounted for 78.5% incidence rates of AED-related skin rashes, followed by 9.5% for BDDCS Class 1, 12.0% for BDDCS Class 3 in the Chinese retrospective study. *

[D] BDDCS Class 2 drugs accounted for 89.2% incidence rates of AED-related skin rashes, followed

by 9.2% for BDDCS Class 1, 1.6% for BDDCS Class 3 and 0% BDDCS Class 4 in the Norwegian retrospective study. *
*For all studies, p-values were < 0.05 (using the chi-squared test), providing evidence that rates of AED-related skin rashes differed significantly between BDDCS classes.

Numbers of AED Exposure and BDDCS Classification

When examining AED exposure, the drugs associated with the highest exposure number are BDDCS Class 2 in each of the four studies, followed by Class 1. Figure 2.3 depicts the numbers of exposure for each AED across the four retrospective studies. Carbamazepine, phenytoin and valproate are among the highest prescribed AEDs across all studies. Although, BDDCS Class 2 and 1 have the highest rates of cutaneous adverse reactions, they are three times more likely to be prescribed than BDDCS Class 3 and 4 AEDs, which show the lowest rate of cutaneous adverse reactions.

It is interesting to note that the same general pattern of CARs outcome is found in the American and Norwegian studies in Fig. 2.2 as seen for the Chinese studies, suggesting that CARs potential occurs for populations not exhibiting the HLA-B*15:02 to a significant extent. We plan to examine this finding in our future studies.

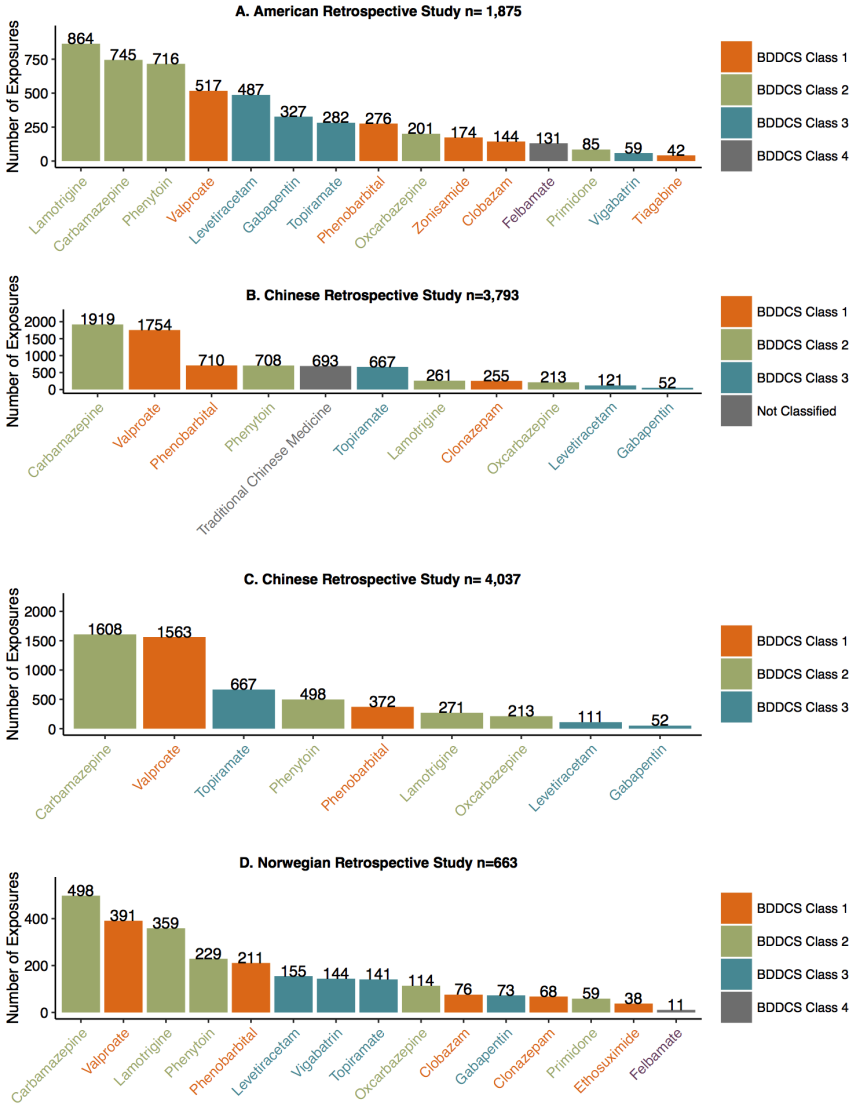

Figure 2.3 Relationship between the exposure to each AED and BDDCS Classification in Americans, Chinese and Norwegians

[A] BDDCS Class 2 drugs accounted for 51.7% AED exposures, followed by 22.9% for BDDCS Class 3, 22.8% for BDDCS Class 1 and 2.6 % BDDCS Class 4 in the American retrospective study. *

[B] BDDCS Class 2 drugs accounted for 42.2% AED exposures, followed by 37.0% for BDDCS Class 1, 11.4% for BDDCS Class 3 and 9.4% for the not classified compounds in the Chinese retrospective study. *

[C] BDDCS Class 2 drugs accounted for 48.4% AED exposures, followed by 36.1% for BDDCS Class 1 and 15.5% for BDDCS Class 3 in the Chinese retrospective study. *

[D] BDDCS Class 2 drugs accounted for 49.0% AED exposures, followed by 30.5% for BDDCS Class 1, 20.0% for BDDCS Class 3 and 0.4% BDDCS Class 4 in the Norwegian retrospective study. *

*For all studies, p-values were < 0.05 (using the chi-squared test), providing evidence that AED exposure is significantly different between BDDCS classes.

HLA-B*15:02 Binding to AEDs

Figure 2.4B depicts the differential BDDCS response in binding observed among 10 AEDs, 2 active metabolites and 1 non-active backbone structure (5HB) when analyzed using an HLA *in vitro* binding assay. The results are depicted as the mean ± standard error of the mean (SEM) for 6 independent experiments with each compound. The HLA *in vitro* binding data depict that the drugs associated with the strongest binding to HLA-B*15:02 are BDDCS Class 2 (See Table 2.1 and Figure 2.4A). Carbamazepine, oxcarbazine, eslicarbazepine acetate, phenytoin and lamotrigine demonstrate a strong binding interaction with HLA-B*15:02, but not with other HLA-B alleles. AEDs presenting a weak binding interaction with HLA-B*15:02 were levetiracetam, topiramate, gabapentin, ethosuximide and valproic acid, as well as the non-active structural backbone of some AEDs, iminostilbene (5-HB). That is, BDDCS Class 3 drugs and the Class 1 drugs ethosuximide and valproic acid interact poorly with HLA-B*15:02. Class 2 carbamazepine-10,11-epoxide, a carbamazepine metabolite, also presented a strong binding affinity to HLA-B*15:02. The primary metabolite and active entity of oxcarbazepine, licarbazepine had three times lower binding affinity to HLA-B*15:02 than the stereospecific eslicarbazepine acetate and other strong binding AEDs.

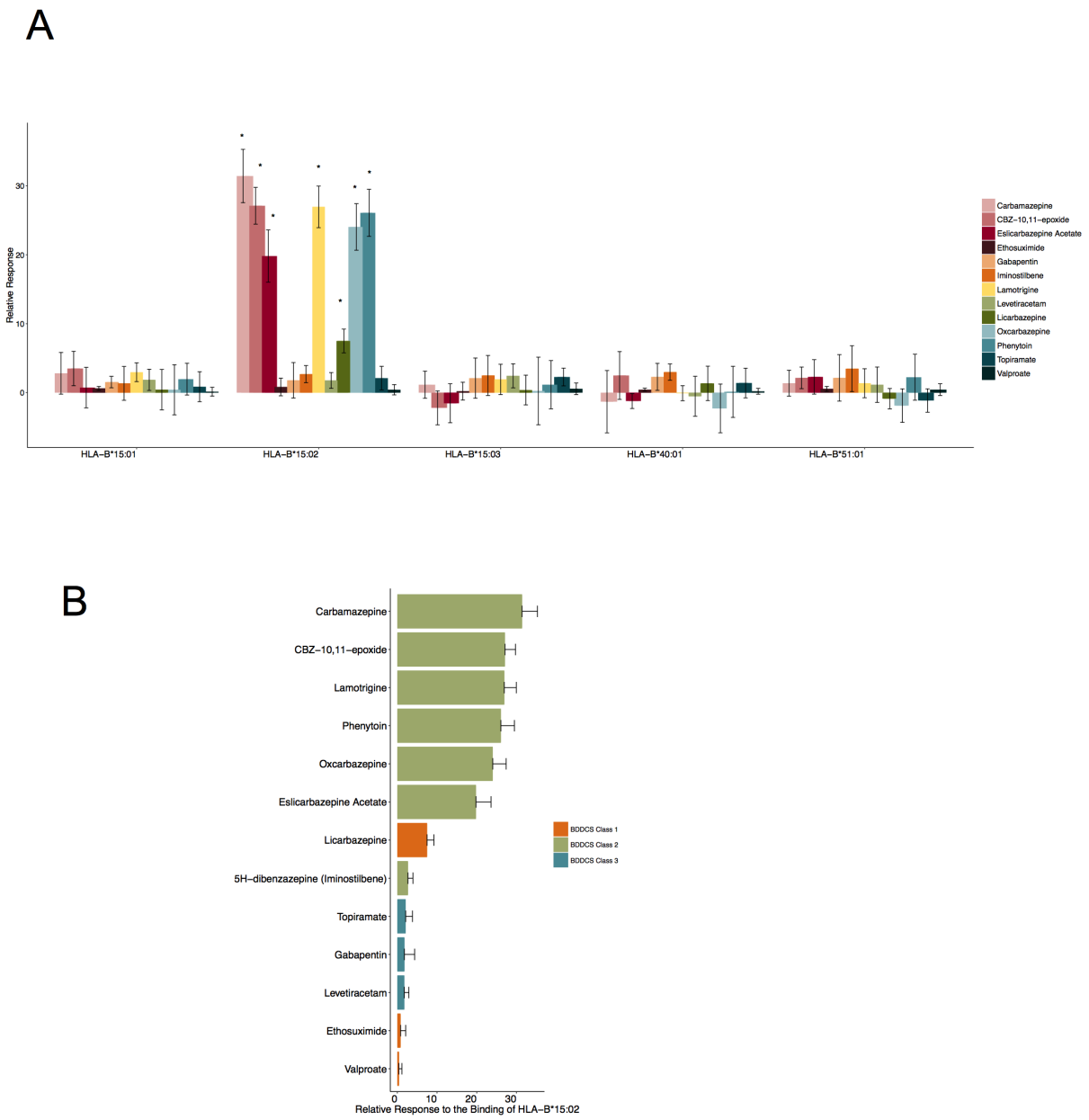

Figure 2.4A. Surface Plasmon Resonance (SPR) data.
Surface Plasmon Resonance (SPR) data demonstrating the specific interactions of 10 AEDs, 2 metabolites, and 1 non-active structural backbone (1mM) to HLA-B*15:01, HLA-B*15:02, HLA-B*15:03, HLA-B*40:01, and HLA-B*51:01

Figure 2.4B. BDDCS Classification of the SPR results with the AEDs.
*P<0.05 show compounds with a significant difference from the response of vehicle. All p-values were calculated with the two-tailed Student's t-test. Results are representative of 6 independent experiments (mean ± SEM).

Comparison of Cutaneous Adverse Reactions and the HLA-B *In Vitro* Assay

Table 2.1 illustrates the relationship between the incidence of cutaneous adverse reactions and the HLA-B binding assay. The 14 drugs in Table 2.1 are ordered based on the mean % incidence of AED rash for the 4 studies presented in Fig. 2.2, highest to lowest, when an AED was reported in two or more evaluations. We arbitrarily classified the rash incidence as high when the mean for a drug in the four evaluations was ≥ 5%, intermediate when mean rash incidence was between 2 and 5% and low when the mean incidence was < 2%. For the 8 drugs where *in vitro* binding to HLA-B*15:02 was available the strength of binding was also included. For each of the retrospective studies correlation between incidence of AED and the strength of HLA-B*15:02 binding for 8 AEDs is very high and significant as presented in Figure 2.5 (American study (n=1,875): ρ =0.762, p-value = 0.028; Chinese study (n = 3,793): ρ =0.810, p-value = 0.015, Chinese study (n=4,037): ρ =0.857, p-value = 0.007; Norwegian study (n=663): ρ =0.763, p-value = 0.017). These data reflect the BDDCS Class 2 vs. Class 3 differentiation. Hence, these strong correlations show a high concordance between the available clinical data and the potential of the HLA-B *in vitro* assay to predict these cutaneous adverse reactions.

Table 2.1. Relationship Between the Incidence of AED Rash.
Note: From Fig. 2.2 for drugs investigated in at least two of the four retrospective studies and relative response to the *in vitro* binding of HLA-B*15:02 from Figure 2.4[a]

Generic Name	BDDCS Class	Comments
Lamotrigine	2	High rash incidence and strong *in vitro* binding
Oxcarbazepine	2	High rash incidence and strong *in vitro* binding
Carbamazepine	2	High rash incidence and strong *in vitro* binding
Phenytoin	2	High rash incidence and strong *in vitro* binding
Phenobarbital	1	Intermediate rash incidence
Primidone	2	Low/no rash incidence
Gabapentin	3	Low/no rash incidence and weak *in vitro* binding
Felbamate	4	Low/no rash incidence
Clobazam	1	Low/no rash incidence
Clonazepam	1	Low rash incidence
Valproate	1	Low rash incidence and weak *in vitro* binding
Topiramate	3	Low/no rash incidence and weak *in vitro* binding
Levetiracetam	3	Low rash incidence and weak *in vitro* binding
Vigabatrin	3	No reported rash incidence

[a.] Two further BDDCS Class 1 drugs (tiagabine, zonisamide) reported in only one study exhibited rash incidence, which would classify as high.

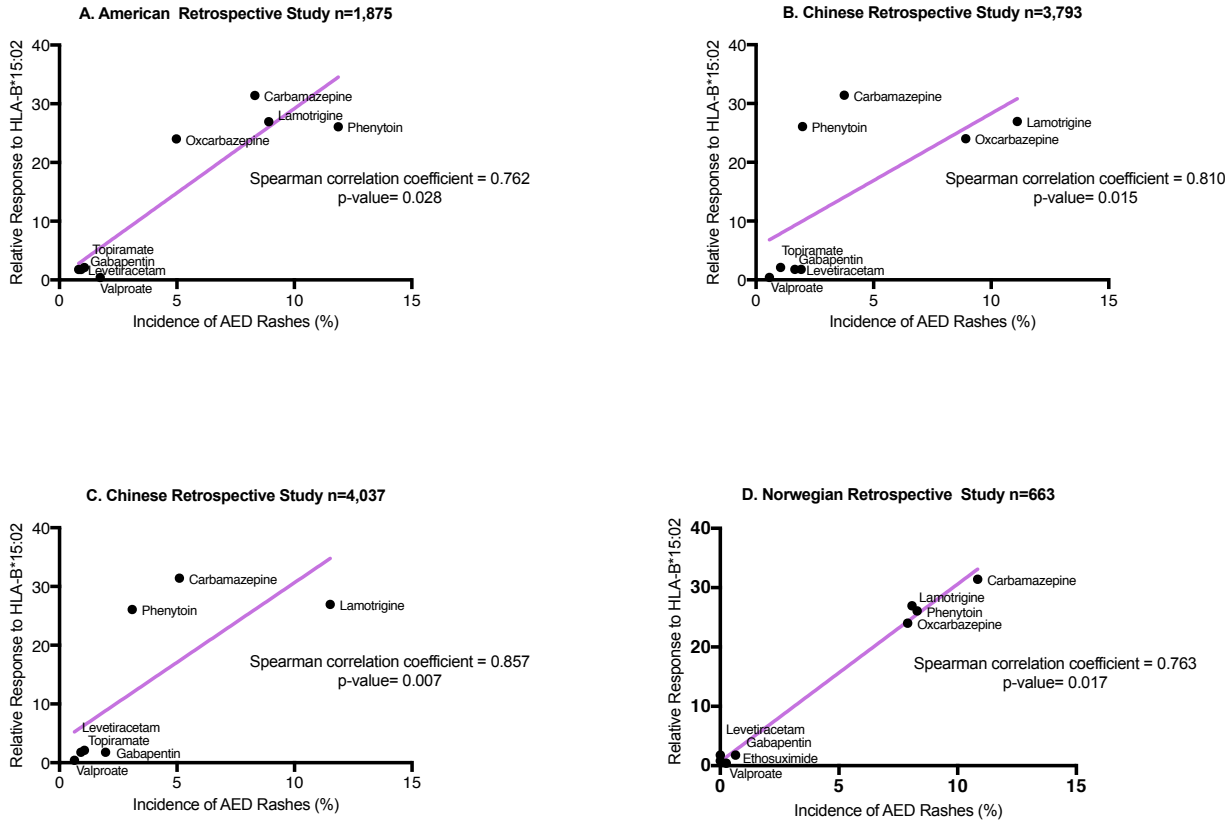

Figure 2.5. Spearman correlation between the relative response to the binding of HLA-B*15:02 and incidence of AED rash.

Changes of AED Prescription Pattern, HLA-B*15:02 Screening and BDDCS Classification

Figure 2.6, using BDDCS, depicts the change of AED prescription pattern from prior to post HLA-B*15:02 policy implementation in Hong Kong. Prior to policy implementation phenytoin, valproic acid and carbamazepine had the highest usage numbers in the total population. Following policy implementation, gabapentin, valproic acid, phenytoin and clonazepam had the highest prescription numbers. Although there was a significant increase in the percent of BDDCS Class 3 drugs (pregabalin, gabapentin and levetiracetam) in the entire population, BDDCS Class 2 drugs still represented 24.3% of prescribed AEDs. Similar trends

were also observed in the subset of patients receiving their first ever AED where postpolicy 25.3% of prescribed AEDs were BDDCS Class 2 drugs (Figure 2.7). In the newly treated epilepsy subset post-policy the decrease in carbamazepine prescriptions from prepolicy numbers was almost matched by the increase in Class 2 phenytoin dosing (Figure 2.8). Thus, the high presence of BDDCS Class 2 AEDs potentially hinders the lowering of CAR incidence in this population.

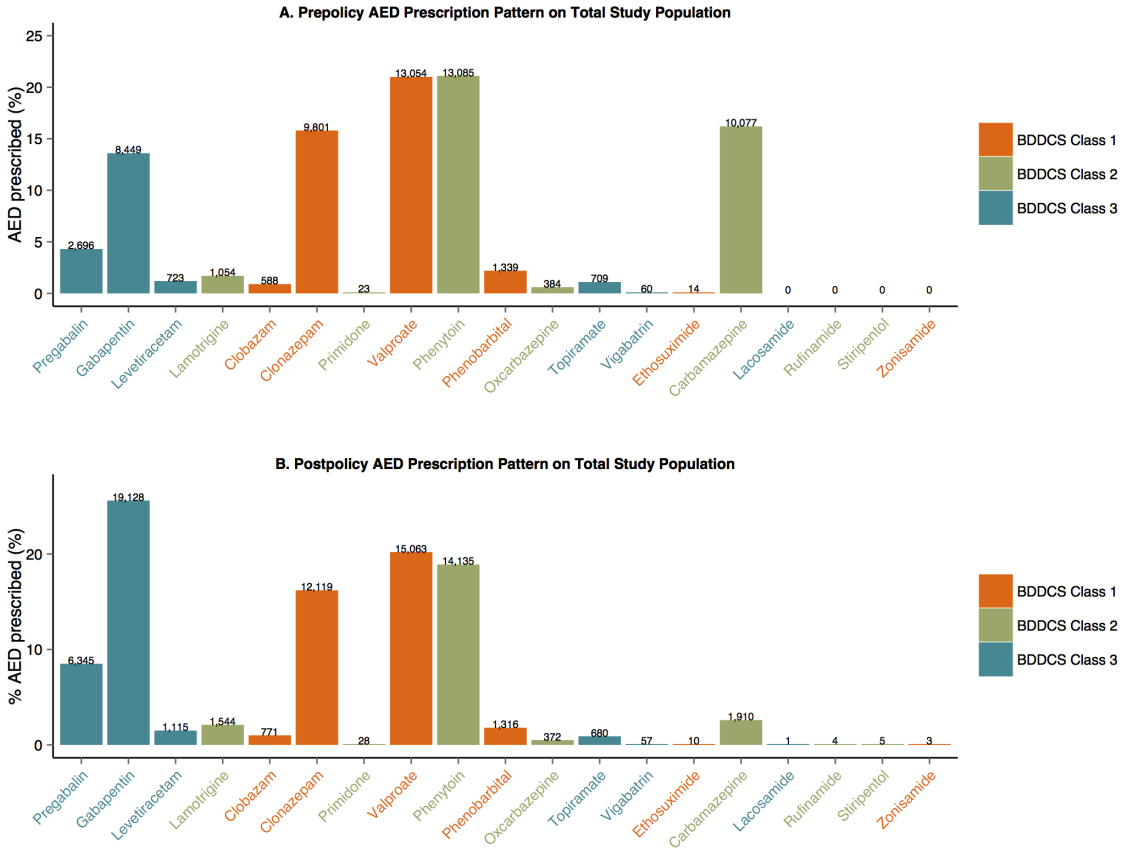

Figure 2.6. AED prescription patterns prior and post HLA-B*15:02 screening implementation in the total Hong Kong population.
[A] Prior to the policy implementation, BDDCS Class 1 drugs accounted for 40.0% of all prescriptions, followed by 39.7% for BDDCS Class 2 and 20.3% for BDDCS Class 3. [B] In the postpolicy, BDDCS Class 1 accounted for 39.2% of all prescriptions, followed by 36.5% for BDDCS Class 3 and 24.3% for BDDCS Class

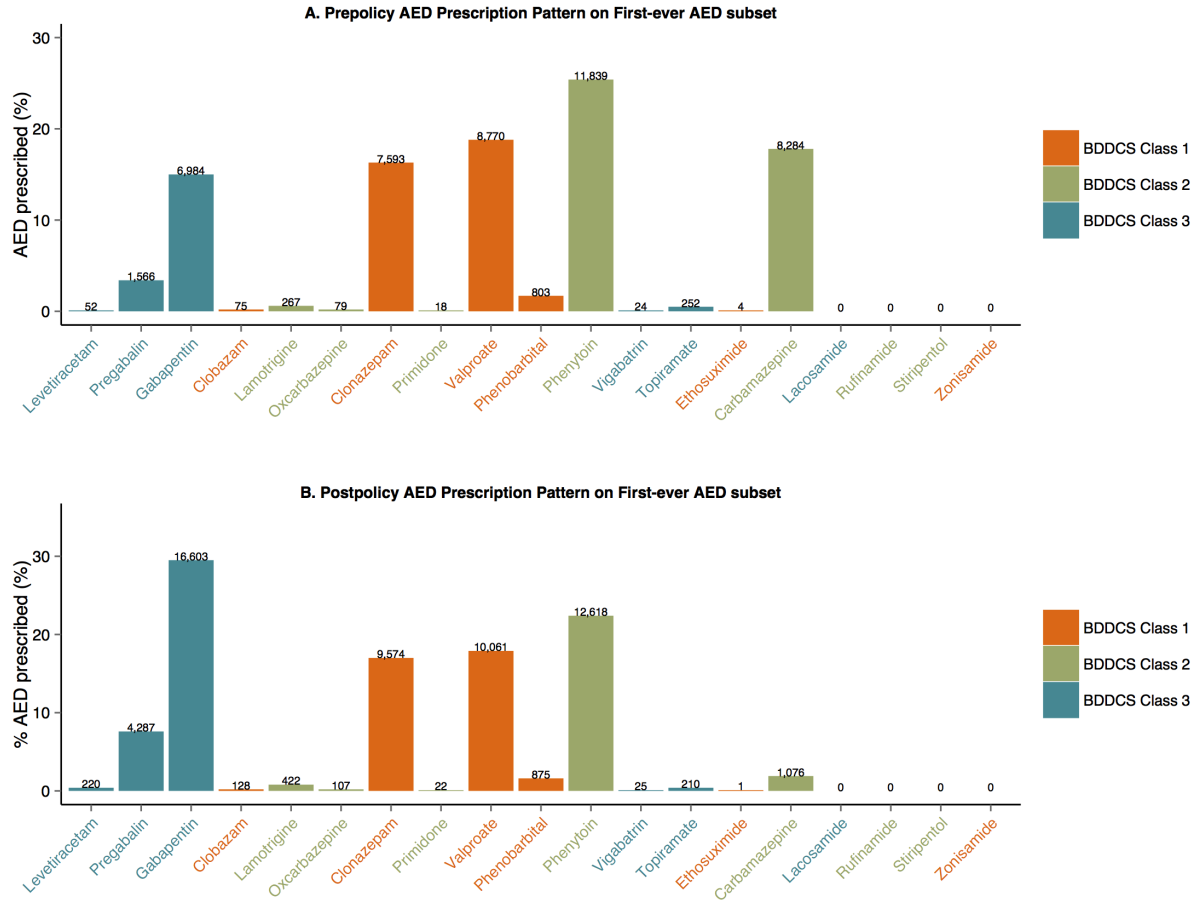

Figure 2.7. AED prescription patterns prior and post HLA-B*15:02 screening implementation in the first ever AED subset in the Hong Kong population.
[A] Prior to the policy implementation, BDDCS Class 2 drugs accounted for 44.0% of all prescriptions, followed by 37.0% for BDDCS Class 1 and 19% for BDDCS Class 3. [B] In the postpolicy, BDDCS Class 3 accounted for 37.9% of all prescriptions, followed by 36.7% for BDDCS Class 1 and 25.3% for BDDCS Class 2.

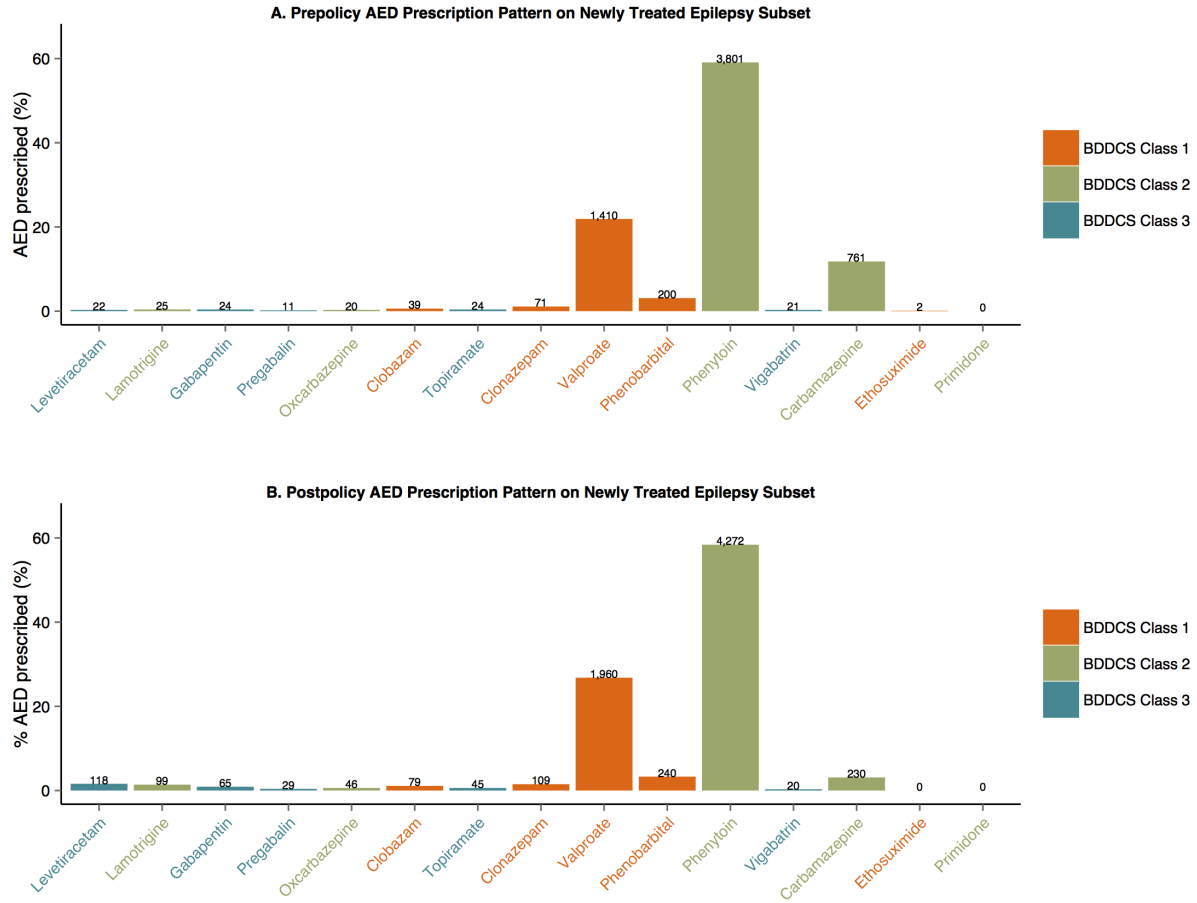

Figure 2.8. AED prescription patterns prior and post HLA-B*15:02 screening implementation in the newly treated epilepsy subset in the Hong Kong population.
[A] Prior to the policy implementation, BDDCS Class 2 drugs accounted for 71.6% of all prescriptions, followed by 26.8% for BDDCS Class 1 and 1.6% for BDDCS Class 3. [B] In the postpolicy, BDDCS Class 2 accounted for 63.5% of all prescriptions, followed by 32.7% for BDDCS Class 1, 3.8% for BDDCS Class 3.

DISCUSSION

We observed a high concordance between the HLA-B*15:02 *in vitro* assay and the incidence of cutaneous adverse reactions associated across all retrospective studies. Phenytoin, lamotrigine, carbamazepine and oxcarbazepine showed high levels of cutaneous adverse reactions. These drugs are also the major causative AEDs for CARs (2, 22). Our BDDCS analysis shows that these AEDs share common properties of being highly metabolized and having low solubility, i.e., BDDCS Class 2. In contrast, AEDs showing a high solubility and poor extent of metabolism (gabapentin, levetiracetam and topiramate) showed a poor interaction for the HLA-B *in vitro* assay. In agreement with this, gabapentin, levetiracetam and topiramate are also AEDs showing minimal levels of CARS (See Figure 2.2, Table 2.1). Iminostilbene, the carbamazepine structural backbone, had a lower binding affinity. We speculate that this low binding affinity is due to the lack of polar groups thereby not allowing the formation of H-bonds with the HLA-B pocket. However, iminostilbene also exhibits low, if any, antiepileptic potency. On the other hand, carbamazepine -10, 11-epoxide presented a strong interaction. According to the results from the HLA-B *in vitro* test and the incidence of cutaneous adverse reactions, we observe that compounds that are extensively metabolized and have low solubility are more susceptible to interacting with HLA-B*15:02 *in vitro*, and have higher incidences of cutaneous adverse reactions. Thus, we recommend that to minimize CARs, epileptic patients be placed on BDDCS Class 3 AEDs if possible, and that for patients exhibiting the HLA-B*15:02 allele all BDDCS Class 2 AEDS may be expected to exhibit the same toxicity potential as carbamazepine. It is more difficult to extrapolate these findings to BDDCS Class 1 AEDs, where some of these drugs (e.g., zonisamide and phenobarbital) cause significant CARs, while others (e.g., valproic

acid, clobazam, clonazepam and ethosuximide) exhibit similar adverse reaction profiles to the BDDCS Class 3 drugs.

It has been previously hypothesized that "idiosyncratic" hypersensitive reactions occur with AEDs containing an aromatic ring in their chemical structure that can form an arene-oxide intermediate (13). This chemically reactive product may become immunogenic through interactions with proteins or cellular macromolecules in accordance with the hapten hypothesis (25). Apart from the hapten formation hypothesis, another immune mechanism might be involved. In this alternate hypothesis, there is a direct, non-convalent binding of the drug to the T-cell receptor to specific T-cell clones. Drug-specific T cells have been identified for lamotrigine and carbamazepine (26, 27). Handoko and coworkers have also confirmed that the association for T-cell mediated reactions was strongest in cutaneous reactions (13). Although, aromatic vs. non-aromatic AED studies have demonstrated that cutaneous hypersensitive reactions can be partly explained by a commonality in chemical structures (13, 14), these studies did not consider and failed to explain why clobazam and clonazepam, which are AEDs with aromatic rings, do not show a significant number of hypersensitive reactions as observed in our analysis. The strong association of hypersensitivity reactions with BDDCS Class 2 drugs, certain BDDCS Class 1 drugs and our *in vitro* results suggest that parent or a combination of parent/metabolite interactions are responsible for the drug hypersensitivity event. One might expect that measures of lipophilicity might differentiate reactive vs. nonreactive AEDs with respect to CARs. However, examination of measured Log P, measured Log D7.4 and calculated Clog P, as tabulated by Benet et al. (21), do not reveal a consistent pattern. (See Table 2.2).

Table 1.2 Parameters for the 17 Most Commonly Prescribed AEDs.

Generic Name	% Excreted Unchanged	Oral Bioavailability	Measured Solubility (mg/mL)	Measured LogP	Measured LogD7.4	Calculated cLogP	Maximum Strength Dose (mg)	Dose Number	BDDCS Class	BCS Class
Clobazam	2	90	0.188	2.12	1.9	2.44	10	0.21	1	2[A]
Clonazepam	2	90	0.03	2.41	2.41	2.38	2	0.27	1	1
Ethosuximide	25	100	39.2	0.38	1.13	0.4	250	0.03	1	1
Phenobarbital	24	100	1.11	1.47	1.34	1.37	60	0.22	1	1
Tiagabine	2	100	24.4	-0.7	NA	0.04	16	0	1	1
Valproate	1.8	100	1.27	-2.16	-2.6	-2.22	250	0.79	1	1
Zonisamide	22	100	0.8	0.91	-0.84	0.88	100	0.5	1	1
Carbamazepine	0.5	85	0.256	2.45	2.45	2.38	300	4.69	2	2
Lamotrigine	10	100	0.17	2.5	-0.19	2.53	200	4.71	2	2[B]
Oxcarbazepine	27	100	0.085	1.5	1.25	1.21	600	28.24	2	2
Phenytoin	2	95	0.02	2.47	2.47	2.08	300	60	2	2
Primidone	35	100	0.6	2.6	NA	2.78	250	1.67	2	2
Gabapentin	95	65	10	-1.1	-1.31	-0.66	800	0.32	3	3
Levetiracetam	66	100	1040	-0.6	NA	-0.34	1000	0	3	1
Topiramate	70	100	9.8	2.75	0.13	2.76	200	0.08	3	1
Vigabatrin	95	100	55.1	0.5	-0.1	-0.36	500	0.04	3	1
Felbamate	45	100	0.7	0.3	-0.29	0.5	600	3.43	4	4

BCS, Biopharmaceutics Classification System.
[A] Although the FDA reports that clobazam is likely to belong to BCS Class 2, the published solubility data for clobazam is 0.08 mg/mL (28), hence it should rather be BCS Class 1.
[B] The published solubility data for lamotrigine are 0.17 mg/mL or 42.5 mg/250 L at 25°C, and 0.18 mg/mL (29) at 37°C. A previous article by Anderson (30) has reported lamotrigine to be BCS Class 1. The FDA reports lamotrigine as being BCS 2.

Although many studies have observed intermediate levels of CARs with phenobarbital, limited or no cases of rash were attributed to primidone in the retrospective studies analyzed here, which is surprising because primidone is metabolized to phenobarbital. It appears that patients tend to be given phenobarbital much more frequently than primidone, from its higher numbers of exposure across all retrospective studies, and those patients with previous rash to phenobarbital are unlikely to be given primidone subsequently; this would result in a low-risk group of patients being given primidone, as proposed by Arif and coworkers (31). Primidone is a BDDCS Class 2 drug and therefore shares reactive properties that we hypothesize would cause a drug hypersensitivity event, as observed in the American retrospective study (Figure 2.2).

Carbamazepine induced SJS/TEN is strongly associated with HLA-B*15:02 across broad Asian populations (4–9). Screening for HLA-B*15:02 in individuals of such ethnic descent before commencing carbamazepine, with avoidance of the drug in individuals testing positive, is recommended by regulatory agencies. Upon examination of the correlation between the HLA-B*15:02 binding affinity and AED SJS/TEN incidence in the Hong Kong population prior to the policy implementation, we found a strong correlation with carbamazepine and phenytoin showing high rates of SJS, and levetiracetam and gabapentin showing low rates of SJS (See Table 2.3). Here again, we observe the BDDCS Class 2 and Class 3 separation. However, the lack of the exact AED SJS/TEN incidence data among the other ethnic groups limits our analysis. Analysis of the AED prescription practice changes on the whole-population of Hong Kong shows a marked reduction in carbamazepine use after the implementation of HLA-B*15:02 screening policy. Although carbamazepine-induced SJS/TEN was prevented, the

incidence of SJS/TEN induced by AEDs overall was not significantly changed (12). The increase of non-carbamazepine BDDCS Class 2 AEDs may have led to an increase in the incidence of SJS/TEN induced by other AEDs, particularly phenytoin. Under the Hong Kong Hospital Authority's drug formulary, one of the older AEDs (carbamazepine, phenobarbital, phenytoin, valproic acid) should be used as first-line treatment for epilepsy. This explains the corresponding increases in phenytoin and valproic acid prescriptions among this patient group. The shift from carbamazepine to phenytoin and valproate induced by the screening policy, such as the risk of teratogenicity (32), which is higher for valproate compared with carbamazepine may have exerted a negative effect on population health. Our analysis shows that there was no major shift in the BDDCS Class 2 and 1 prescription pattern, and this potentially explains the lack of reduction in SJS incidence.

The Food and Drug Administration (FDA) currently recommends that phenytoin, fosphenytoin and lamotrigine should be avoided as an alternative for carbamazepine patients positive for HLA-B*15:02 (10, 33). HLA-B*15:02 is largely absent in individuals not of Asian origin (e.g., Caucasians, African-Americans, Hispanics, and Native Americans); nonetheless we observe a strong correlation between the drugs associated with cutaneous adverse reactions across different populations. Other HLA-B alleles such as HLA-A*31:01 (34) and HLA-B*15:11 (35) have been associated with carbamazepine associated SJS but no *in vitro* assay have been performed as yet with these other alleles. BDDCS class 2 AEDs appear to be more reactive than other BDDCS classes.

Table 2.1. SJS Incidence in the Hong Kong Population and BDDCS Classification.

		Prepolicy			Postpolicy			
Culprit AED	**BDDCS Class**	**Patients (n)**	**SJS/TEN (n)**	**SJS/TEN (%)**	**Patients (n)**	**SJS/TEN (n)**	**SJS/TEN (%)**	**p-value[a]**
Phenobarbital	1	803	1	0.12	875	0	0	0.48
Valproic acid	1	8,770	1	0.01	10,061	1	0.01	NS
Carbamazepine	2	8,284	20	0.24	1,076	0	0	0.16
Phenytoin	2	11,839	18	0.15	12,618	33	0.26	0.07
Gabapentin	3	6,984	1	0.01	16,603	2	0.01	NS
Levetiracetam	3	52	0	0	220	1	0.45	NS
Pregabalin	3	1,566	0	0	4,287	1	0.02	NS
Multiple AEDs[b]	2 and 1		1			1		
Total[c]		45,832	42	0.09	55,326	39	0.07	0.26

NS, not significant

[a] Fisher's Exact Test comparing the incidence of SJS/TEN in the prepolicy and postpolicy periods.

[b]Two patients developed SJS/TEN while commenced on phenytoin and valproic acid concurrently.

[c] Total incidences of first AED-induced SJS/TEN were calculated based on total patient numbers.

Through a review of FDA package labels, in contrast to the 2% or less incidence of SJS/TEN for the BDDCS Class 3 drugs listed in Table 2.4, the values for the BDDCS Class 2 drugs phenytoin (5-10%), lamotrigine (10%), carbamazepine (4-11%) and oxcarbazepine (2.5%) are often much higher (See Table 2.1). As seen in the data presented here, patient exposure to BDDCS Class 2 and 1 AEDs is much higher (See Figure 2.3). For clinicians to be able to reduce the number of patient suffering from drug hypersensitivity reactions, they should understand that continual high prescription exposure of BDDCS Class 2 and certain Class 1 drugs may contribute to the reported adverse cutaneous reactions in patients who are at risk.

Table 2.4. Rash and More Serious Dermatologic Conditions From the FDA Package Insert and Literature Reports.

Generic Drug Name	Rash Incidence	BDDCS Class
Clobazam	Package Insert: • Rash listed under Warnings and Precautions and Adverse Reactions (36) SJS/TEN: • Listed under Warnings and Precautions and Adverse Reactions (36) Other sources: •Approximately 2% (31)	1
Clonazepam	Package Insert: •Rash listed under Adverse Reactions (36) SJS/TEN: •not mentioned Other Sources: •not available	1
Ethosuximide	Package Insert: •Rash listed under Warnings; Precautions and Adverse Reactions sections (36) SJS/TEN: •Listed under Warnings (36) Other Sources: •not available	1
Phenobarbital	Package Insert: •Rash listed under Adverse Reactions (36) SJS/TEN: •not mentioned Other Sources: • 1-2% (37) • 8.1/10,000[43]	1
Tiagabine	Package Insert: • Rash Rate: Adults: 5% (36) • Rash listed under Precautions and Adverse Reactions (36) Other Sources: • 2.5% (31)	1
Valproate	Package Insert: • Rash: >1% but less than 5% in both epilepsy and migraine trials (36) • Rash listed under Warning and Precautions and Adverse Reactions sections (36) SJS/TEN: •"Rare" (36) Other Sources: • Approximately 1% (31) • 0.5/10,000 (38)	1

Zonisamide	Package Insert: • Rash: Adults = 1.4-2.2% (36) • Rash listed under Warnings; Precautions and Adverse Reactions sections (36) SJS/TEN: •46 per 1,000,000 (36) • Listed under Warnings (36) Other Sources: •4% (31)	1
Carbamazepine	Package Insert: • Rash: 1/10,000-6/10,000 (36) • Rash listed under Warnings and Precautions and Adverse Reactions (36) SJS/TEN: •Listed under Boxed Warning; Warnings and Adverse Reactions (36) Other Sources: • SJS/TEN: 1.4/10,000 (38) • Rash: 4-11% (31)	2
Lamotrigine	Package Insert: • Rash: Epilepsy Trials = 4.5-10% in adults, 4.4-14% in pediatric cases; Bipolar Trials: adults =7-11% (36) • Rash listed under Boxed Warning; Warnings and Precautions; Adverse Reactions (36) SJS/TEN: • 0.3% adults with epilepsy; 0.8% in pediatric patients with epilepsy (< 16 years); 0.08% adults with bipolar disorder (using current titration schedules) (36) •Listed under Boxed Warning; Warnings and Precautions;Adverse Reactions (36) Other Sources: • 2.5/10,000 (38) • 10% (31)	2
Oxcarbazepine	Package Insert: • Rash: Adults =1.4- 4%; Pediatrics = 1.3- 5.3% (36) • Rash listed under Warnings and Precautions and Adverse Reactions (36) SJS/TEN: "Rare" (36) • Listed under Warnings and Precautions (36) Other Sources: • 2.5% (31)	2

Phenytoin	Package Insert: • Rash: Rate not given (36) • Rash listed under Warnings and Precautions and Adverse Reactions (36) SJS/TEN: •Rate not given • Listed under Warnings (36) Other Sources: • 5-10% (37)	2
Primidone	Package Insert: •Rash listed as a possible side effect (36) SJS/TEN: •not mentioned Other Sources: •Contraindications: patients who are hypersensitive to phenobarbital (39)	2
Gabapentin	Package Insert: • Rash: Adults = 1.2-1.3% (36)• Listed under Adverse Reactions (36) SJS/TEN: •not mentioned Other Sources: • 1% (31)	3
Levetiracetam	Package Insert: • Rash: Adults: 0% (36) SJS/TEN: •not mentioned Other Sources: •not available	3
Topiramate	Package Insert: • Rash: Adults = 1%; 2-4% in migraine; Pediatrics = 2% (36)• Listed under Adverse Reactions (36) SJS/TEN: •not mentioned Other Sources: • 1% (31)	3
Vigabatrin	Package Insert: • Rash: Adults: 0% (36) • Rash listed under Adverse Reactions (36) SJS/TEN: • Listed under Adverse Reactions (36) Other Sources: •not available	3
Felbamate	Package Insert: •Rash: (1.2%) (36) • Rash listed under Adverse Reactions (36) SJS/TEN: •not mentioned	4

Use of BDDCS in the FDA Guidance for Drug Hypersensitivity Reactions

The previous discussion of BDDCS and AEDs in the literature was related to generic equivalence and interchangeability of AEDs. In that work, Bialer and Midha (40) contrasted the aspects of the FDA Guidance of waiver of bioequivalence studies based on the Biopharmaceutics Classification System (BCS) (41) and the clinician's interchangeability of brand versus generic AED prescriptions. It is important to understand the distinction between BCS, which is based on the extent of drug permeability/absorption, versus BDDCS, which is based on the rate of drug permeability/absorption. In the BCS system, levetiracetam, gabapentin, and vigabatrin are classified as BCS Class 1 drugs (42). These compounds are completely absorbed with the exception of gabapentin which is about 70% absorbed in humans (43), although quite slowly. These three drugs, in contrast, are classified as BDDCS Class 3 (See Table 2.2). Thus, the predictability of hypersensitivity reactions for AEDs is based on BDDCS, not BCS, classification, since BCS does not predict whether drugs will be extensively metabolized or not.

Conclusion:

Drug-induced CARs constitute the most frequent idiosyncratic reactions confronting clinicians treating patients with epilepsy. Unfortunately, there is no reliable way to determine early in the clinical course of a rash if it is going to remain as a benign maculopapular rash or evolve into a severe skin reaction. Therefore, the drug should be discontinued as soon as possible in most cases. Our analysis concludes that BDDCS Class 2 and 1 AEDs are more prone to cutaneous toxicity and BDDCS Class 3 AEDs have the lowest cutaneous rash incidence across the studied ethnic groups. We propose that, if possible, BDDCS Class 3 AEDs should be preferentially dosed to patients of East Asian ancestry who most predominantly exhibit the HLA-B*15:02 allele (i.e. Han Chinese, Thai, and Malaysian populations), where an association

between HLA-B*15:02 and carbamazepine-induced SJS and TEN has been demonstrated (4–9). We believe that categorizing drugs by BDDCS classification adds to the understanding of idiosyncratic reactions. We plan to further test other AEDs in the HLA-B *in vitro* assay. Other toxicity models using BDDCS such as the Torsade de Pointes (44) and Drug Induced Liver Injury (DILI) (45) are starting to emerge. BDDCS may help characterize and predict drugs having the potential for greater toxicity.

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CHAPTER 3: Evaluation of DILI Predictive Hypotheses in Early Drug Development

ABSTRACT[1]

Drug-induced liver injury (DILI) is a leading cause of drug failure in clinical trials and a major reason for drug withdrawals. DILI has been shown to be dependent on both daily dose and extent of hepatic metabolism. Yet, early in drug development daily dose is unknown. Here, we perform a comprehensive analysis of the published hypotheses that attempt to predict DILI, including a new analysis of the Biopharmaceutics Drug Disposition Classification System (BDDCS) in evaluating the severity of DILI warning in drug labels approved by the FDA and the withdrawal status due to ADRs. Our analysis confirms that higher doses ≥ 50mg/day lead to increased DILI potential but this property alone is not sufficient to predict DILI potential. We evaluate prior attempts to categorize DILI such as Rule of 2, BSEP inhibition, and measures of key mechanisms of toxicity compared to BDDCS classification. Our results show that BDDCS Class 2 drugs exhibit the highest DILI severity, and that all of the published methodologies evaluated here, except when daily dose is known, do not yield markedly better prediction than BDDCS. The assertion that extensive metabolized compounds are at higher risk of developing DILI is confirmed, but can be enhanced by differentiating BDDCS Class 2 from Class 1 drugs. We do not propose that BDDCS classification, which does not require knowledge of the clinical dose, is sufficiently predictive/accurate of DILI potential for new molecular entities, but suggest that comparison of proposed DILI prediction methodologies with BDDCS classification is a useful tool to evaluate the potential reliability of newly proposed algorithms. **Conclusion:** The most successful approaches to predict DILI potential all include a measure of dose, yet there is a

[1] Modified from publication:Chan R, Benet LZ. 2017. Evaluation of DILI predictive hypotheses in early drug development. *Chem. Res. Toxicol.* 30:1017–29

quantifiable uncertainty associated with the predicted dose early in drug development. Here we compare the possibility of predicting DILI potential using BDDCS classification versus previously published methods, and suggest that comparison of predictive metrics versus the outcome by just avoiding BDDCS Class 2 drugs may serve as a useful baseline in evaluating these metrics.

INTRODUCTION

Drug-induced liver injury (DILI) is a leading cause of drug failure in clinical trials and a major reason for drug withdrawals from the market. Idiosyncratic DILI (IDILI) is very complex. Most IDILI appears to be immune mediated, and reactive metabolites appear to be involved in most, but not all IDILI. In addition, there are probably several mechanisms by which a drug or reactive metabolite can induce an immune response. Numerous compound- and/or patient-specific risk factors can contribute to the susceptibility to DILI. IDILI has been shown to be dependent on both daily dose and extent of hepatic metabolism of a drug (1–4).

Dose appears to be a key component in the risk assessment of toxicity. While there is not a clear dose-response relationship for idiosyncratic adverse drug reactions, epidemiological DILI studies have shown that dose of a compound is an important parameter in determining the likelihood that an individual drug will cause an idiosyncratic adverse drug reaction in the human population(2). At the same time, numerous studies have shown that dose alone is not an adequate discriminator between high and low risk compounds(5). There are a number of preclinical strategies where dose has been combined with other parameters directly or indirectly related to key measures of toxicity endpoints to help assess the potential DILI risk such as the formation of reactive metabolites, inhibition of the bile salt export pump, BSEP, resulting in the intracellular accumulation of bile salts and high covalent body burden(6, 7), mitochondrial dysfunction (resulting in the depletion of cellular energy supply and the generation of damaging reactive oxygen species), cell damage from oxidative stress (caused by reactive oxygen or reactive nitrogen species), and local inflammatory effects(8). All of these mechanisms are often interconnected and have, under various circumstances, been associated with the formation of chemically reactive metabolites. Recently, Chen et al. reported that high lipophilicity in

combination with high daily dose increases DILI risk potential in humans(9). However, one would like to have a predictive DILI methodology early in drug development, long before the clinical dose is known.

Here we consider the possibility of using the Biopharmaceutics Drug Disposition Classification System (BDDCS), which can be determined prior to dosing a drug to humans or animals, as a potential baseline tool to be compared with presently proposed predictive procedures in evaluating DILI toxicity. The BDDCS was developed in 2005 after Wu and Benet recognized that highly permeable compounds, as outlined by the Biopharmaceutics Classification System (BCS), were extensively metabolized, while poorly permeable drugs were primarily eliminated unchanged in the urine or bile(10). Furthermore, BDDCS demonstrated that simple passive membrane permeability measures were highly selective in differentiating extensively vs. poorly metabolized drugs in humans. Drugs in the BDDCS are classified according to the membrane permeability rate and aqueous solubility. These characteristics have helped BDDCS define whether metabolic enzymes and/or transporters are clinically important. BDDCS features are demarcated by high and low values, classifying drugs into four categories. These classes are each associated with specific predictions regarding route of elimination and which interactions may be a clinical concern.

Since its inception, the BDDCS has been useful in drug discovery for predicting routes of elimination, oral drug disposition, food effects on drug absorption, transporter effects on drug absorption, and potentially clinically significant drug interactions that may arise in the intestine, liver and brain(11). Most recently we have shown in Chapter 2 that the BDDCS can be useful in predicting the potential for antiepileptic drugs to cause cutaneous adverse reactions(12). A goal of this work was to explore the extent to which BDDCS defining characteristics, independent of

knowing actual drug pharmacokinetics/pharmacodynamics and dose can be used as a comparison baseline matrix of potential DILI adverse events with prior published predictive proposals(9, 13–16).

Here, we perform a comprehensive analysis to examine the clinical impact of BDDCS in evaluating the severity of DILI warning in drug labels approved by the Food and Drug Administration (FDA)(17), the withdrawal status due to ADRs, the role of BSEP inhibition, and proposed models including: the Rule of 2 (Ro2), Ro2 and reactive metabolite formation, maximum daily dosages prescribed, and assays applied to cover various mechanisms and endpoints associated with human DILI. (These assays included the generation of reactive metabolites, namely time-dependent inhibition (TDI) of Cytochrome P450 3A4 and glutathione (GSH) adduct formation, inhibition of the human bile salt export pump (BSEP), mitochondrial toxicity and cytotoxicity)(14). Recently, Zhang et al.(16) evaluated specific metabolic pathways predictive of DILI and Chen et al.(13) added the measurement of known reactive metabolites, both reporting a marked improvement in the previous methodologies employed to predict DILI; we have also included these studies in our analysis.

Because one of the strongest determinant hypotheses with respect to DILI is reactive metabolite formation, we expect that drugs that are extensively metabolized/highly permeable (BDDCS Class 1 and 2) will have heightened susceptibility to DILI. Conversely, drugs that are poorly metabolized/poorly permeable (BDDCS Class 3 and 4) will be at a lower risk for causing DILI because they are primarily eliminated unchanged into the urine and bile. The strong relationship between dose, metabolic susceptibility, solubility and idiosyncratic DILI highlights the potential benefits of BDDCS as a comparison matrix for DILI prediction.

Relationship between FDA Drug Label Section, DILI Assessment and BDDCS Classification

In our current comparative analysis, we leveraged the unique information contained in FDA drug labels and DILI severity assessment with respect to the BDDCS classification system. The DILI potential of the drugs in the data set was classified on the basis of the information on hepatic ADRs extracted from FDA drug labels; we note that only drugs that have been on the market for a minimum of ten years were chosen for review(18). Briefly, depending on the ADR severity, off market status and FDA drug labels, ADRs may be classified in different categories ("Discontinued", "Withdrawn", "Boxed Warning", "Warning and Precautions", "Adverse Reactions" and "No mention", ordered by decreasing severity)(19–21). The DILI severity assessment is categorized as follows: "Severe DILI", "Moderate DILI", "Mild DILI", and "No DILI", ordered by decreasing severity as described by Chen et al.(18). However, with the recent publication of prediction based on metabolic pathways(16), "Moderate DILI" and "Mild DILI" were combined into a category designated "Non-severe DILI," which we have utilized here.

BDDCS Classification

The BDDCS Class of each drug was initially evaluated based on the available solubility data, maximum dose strength (mg), and extent of metabolism(22). Recently we expanded the list of BDDCS drug classification to more than 1100 drugs, including many drugs that have been removed from the market as a result of toxic manifestations(23). Expansion of the BDDCS classification list was particularly challenging since for many drugs that came onto the market a number of years ago, and then removed because of toxicity, little reliable information both in terms of metabolism and solubility can be found in the literature. Therefore, when a drug is on the border of two classes, the BDDCS class is selected based on expected or known drug interactions.

There is a marked distinction between extensively and poorly metabolized compounds and this can be well predicted based on an *in vitro* measure of drug permeability(24). Recently, Dave and Morris showed that the solubility classification could be evaluated using a 0.3 mg/mL cut-off (25), thus not requiring knowledge of the clinical dose, thereby allowing BDDCS classification to be made without knowledge of clinical dose (See Figure 3.1).

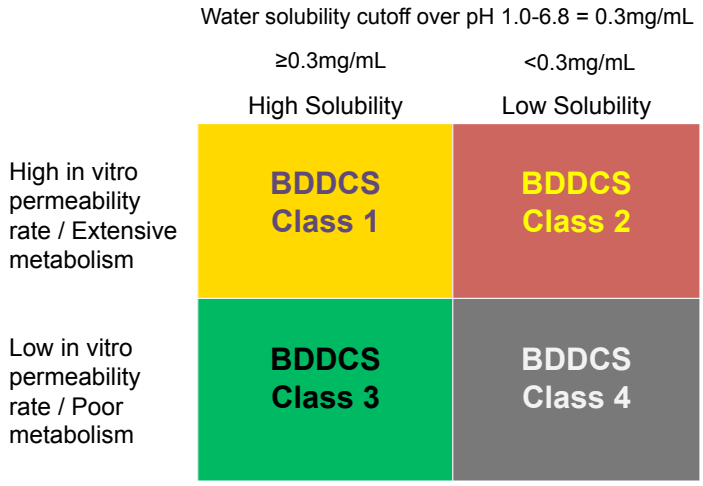


Figure 3.1. Biopharmaceutics Drug Disposition Classification System (BDDCS).

We examined the BDDCS class relationship of hepatotoxicity between the different ADR categories by calculating the proportion of drugs in each FDA hepatic liability category, and DILI severity category. Categorical variables were tested for statistically significant differences using the chi-square tests (test for trends in proportions and test of equal or given proportions), $p < 0.05$ was considered statistically significant. Analyses and plots were carried out using R (http://cran.r-project.org)(26, 27) and GraphPad Prism software version 7.0 (GraphPad Software, Inc., San Diego, CA). Furthermore, The p-values for evaluating BDDCS class trends of FDA hepatic liability category and DILI severity are computed by the implemented functions in R for testing for trends in proportions. The test of equal or given proportions was used for testing the null hypothesis that the proportions in several groups are the same as the "No mention" or "No DILI" where applicable. Out of 287 eligible compounds from the NCTR dataset, 19 compounds could not be classified due to limited available data (See legend of Figure 3.2).

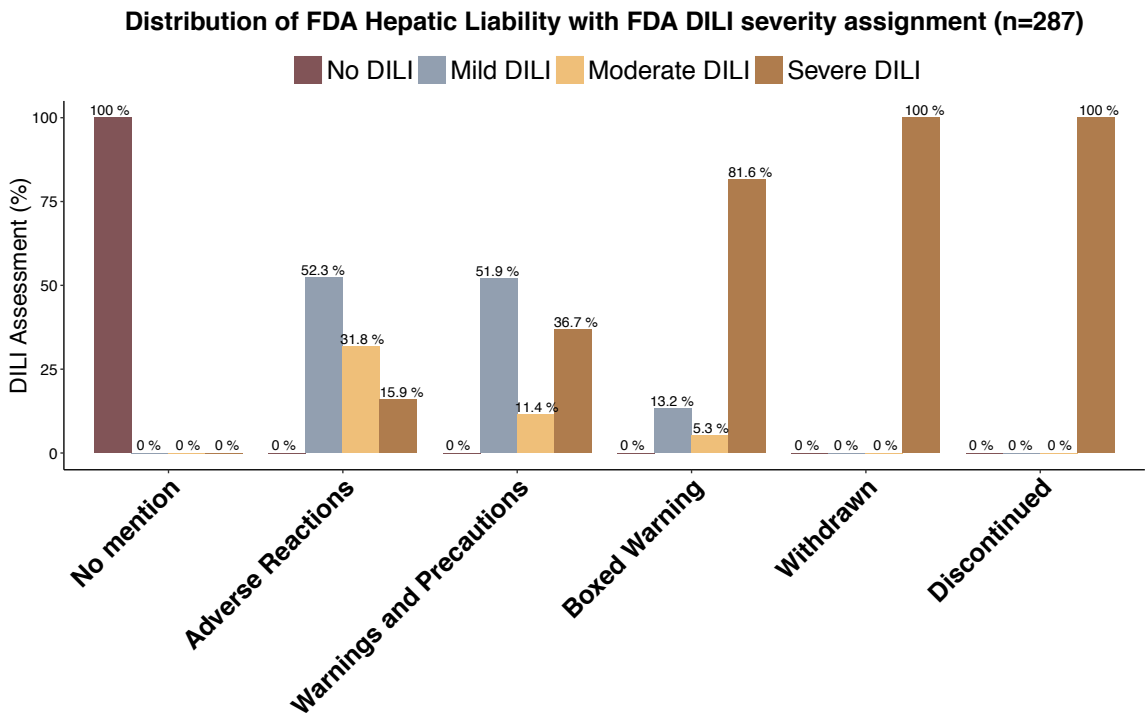


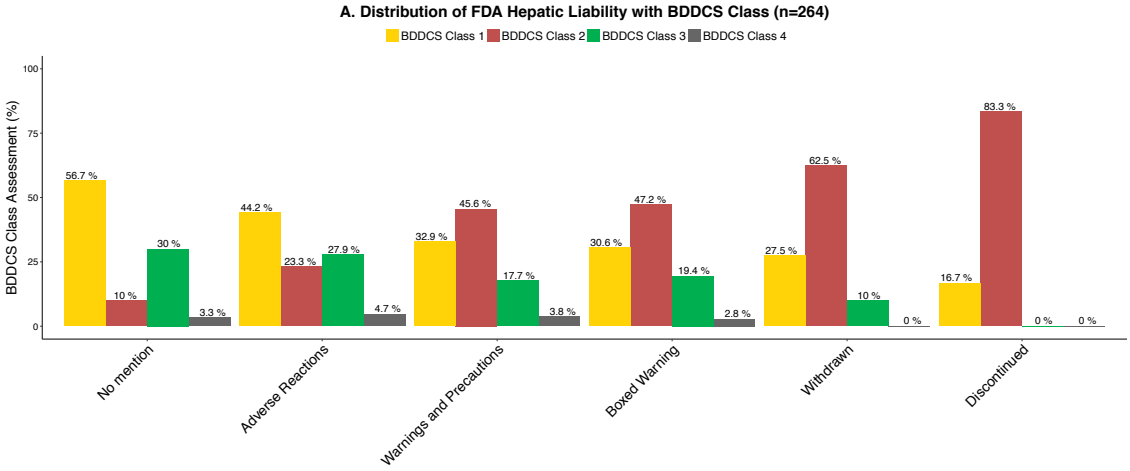
Figure 3.2. Distribution of FDA Hepatic Liability with FDA DILI Severity assignment.

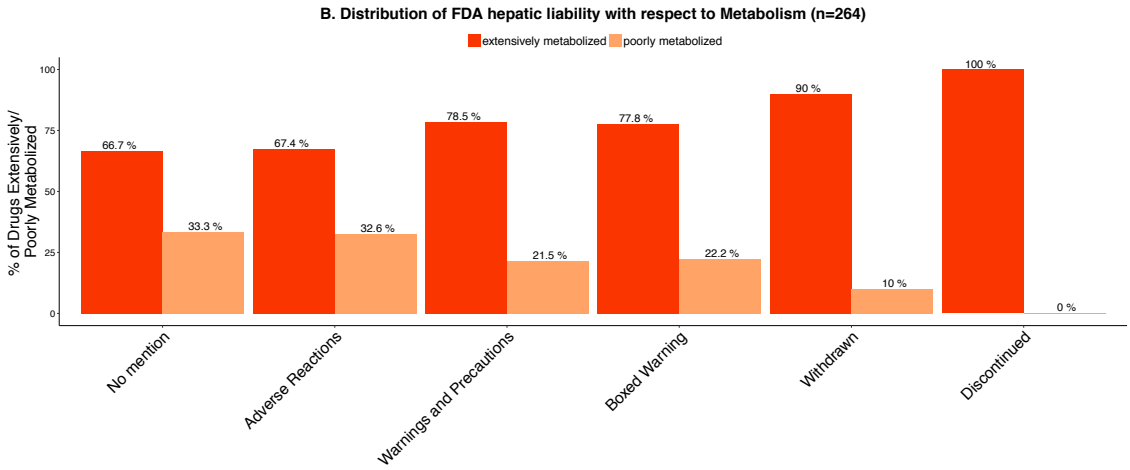
Confirming this classification, keywords that define severe DILI (e.g., acute liver failure and liver necrosis) were more often reported in the "Boxed Warning" or "Warnings and Precautions" sections than in the "Adverse Reactions" section. By contrast, milder DILI (e.g., increased liver aminotransferases and liver steatosis) were more frequently reported in the "Adverse Reactions" section. This indicates that classifying DILI severity according to the FDA drug label sections was applicable for the purpose of our study.

The "Black Box Warning" for moderate DILI was 5.3% (2/38) and 13.2% (5/38) for mild DILI. All of the discontinued (n=7) and withdrawn drugs (n=54) were labeled with severe DILI. We note that under the FDA DILI severity assignment scale there are more compounds assigned to the "Moderate DILI" category in the "Adverse Reactions" section 31.8% (14/44) than the "Warning and Precautions " section (11.4%, 9/79).

We observe that as the hepatic warning severity increases, the proportion of BDDCS Class 2 drugs increases and the proportions of both BDDCS Class 1 and 3 drug decrease as depicted in Figure 3.3A, all with highly significant trends. The "No mention" category is significantly different from all other categories, except for "Adverse Reactions." BDDCS Class 2 drugs were incriminated with the highest proportions in the following drug label sections: "Warning and Precautions" (45.6%, 36/79), "Boxed Warning" (47.2%, 17/36),

"Withdrawn" (62.5%, 25/40) and "Discontinued" (83.3%, 5/6). Obviously, the number of drugs designated as exhibiting severe DILI drugs increases as the ADR severity increases. That is, 15.9% (7/44) in the "Adverse Reactions" category, 36.7% (29/79) in the "Warning and Precautions" and 81.6% (31/38) of the drugs in the "Black Box Warning" are assessed to exhibit severe DILI (See Figure 3.2**).** In Figure 3.3B and 3.3C the two BDDCS determinants (extent of metabolism and solubility) are examined. The percentage of poorly metabolized and of highly soluble drugs decrease, while low solubility drugs increase with hepatic liability. The percent of extensively metabolized drugs also increases with hepatic liability, but since almost 2/3 of "No mention" drugs are metabolized, it is apparent that extent of metabolism itself is not a discriminating parameter. Although greater extent of metabolism has been reported to significantly increase the potential of a compound to cause DILI (1), this property alone is not able to distinguish compounds that are "No mention" of hepatic liability from those compounds exhibiting hepatic liability (See Figure 3.3B).


A. Distribution of FDA Hepatic Liability with BDDCS Class (n=264)
BDDCS Class 1
BDDCS Class 2
BDDCS Class 3
BDDCS Class 4
BDDCS Class Assessment (%)
100
75
50
25
0
56.7 %
10 %
30 %
3.3 %
44.2 %
23.3 %
27.9 %
4.7 %
32.9 %
45.6 %
17.7 %
3.8 %
30.6 %
47.2 %
19.4 %
2.8 %
27.5 %
62.5 %
10 %
0 %
16.7 %
83.3 %
0 %
0 %
No mention
Adverse Reactions
Warnings and Precautions
Boxed Warning
Withdrawn
Discontinued


B. Distribution of FDA hepatic liability with respect to Metabolism (n=264)
extensively metabolized
poorly metabolized
% of Drugs Extensively/ Poorly Metabolized
100
75
50
25
0
66.7 %
33.3 %
67.4 %
32.6 %
78.5 %
21.5 %
77.8 %
22.2 %
90 %
10 %
100 %
0 %
No mention
Adverse Reactions
Warnings and Precautions
Boxed Warning
Withdrawn
Discontinued

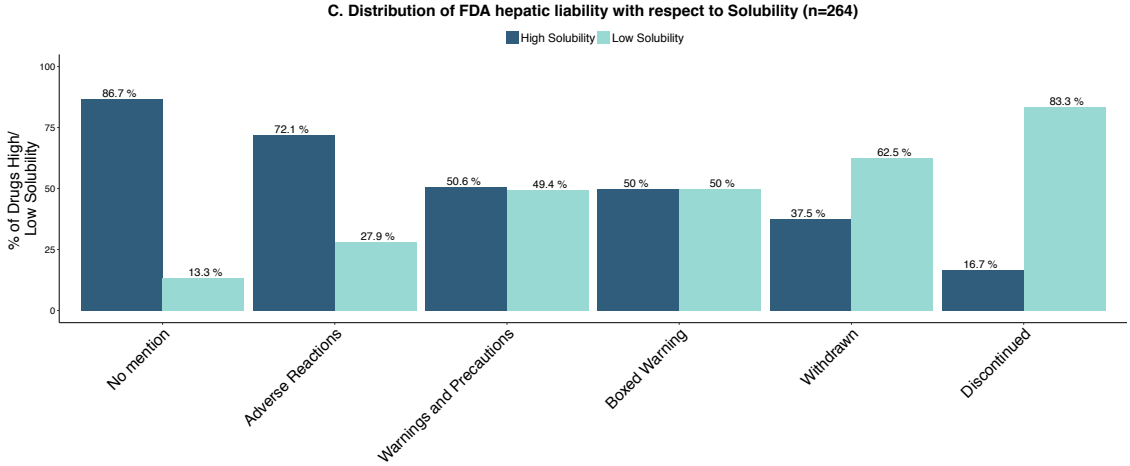

C. Distribution of FDA hepatic liability with respect to Solubility (n=264)
High Solubility
Low Solubility
% of Drugs High/ Low Solubility
100
75
50
25
0
86.7 %
13.3 %
72.1 %
27.9 %
50.6 %
49.4 %
50 %
50 %
37.5 %
62.5 %
16.7 %
83.3 %
No mention
Adverse Reactions
Warnings and Precautions
Boxed Warning
Withdrawn
Discontinued

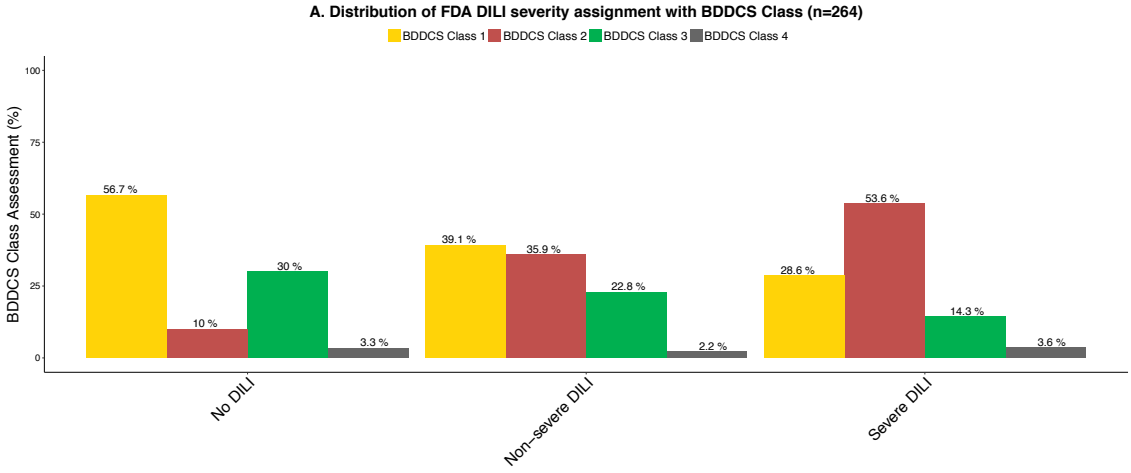
Figure 3.3A. Distribution of FDA Hepatic Liability with BDDCS class.
Drugs were assigned according to the most severe drug label section reporting a hepatic ADR or withdrawn and discontinued, and to the "No mention" class if no hepatic ADRs were reported. Bars show the percentage of all compounds in the same category that are associated with each FDA hepatic liability. BDDCS Class 2 drugs are shown to significantly increase the frequency of hepatic ADRs reported in the "Boxed Warning", "Warning and Precautions", "Withdrawn" and "Discontinued" categories. There was a significant difference between BDDCS Classes when the proportionality trend test was calculated: BDDCS Class 1 trend p-value = 0.0003842; BDDCS Class 2 trend p-value = 2.014e-10; BDDCS Class 3 trend p-value = 0.003928; BDDCS Class 4 trend NS, p-value = 0.2963. Differences in the BDDCS Class distributions were evaluated among the following groups: "No mention" vs. "Adverse Reactions", NS, p-value = 0.2908; "No mention" vs. "Warning and Precautions", p-value=0.0001058; "No mention" vs. "Boxed Warning", p-value=0.0006222; "No mention" vs. "Withdrawn", p-value= 6.439e-07"; No mention" vs. "Discontinued", p-value= 9.36e-05.

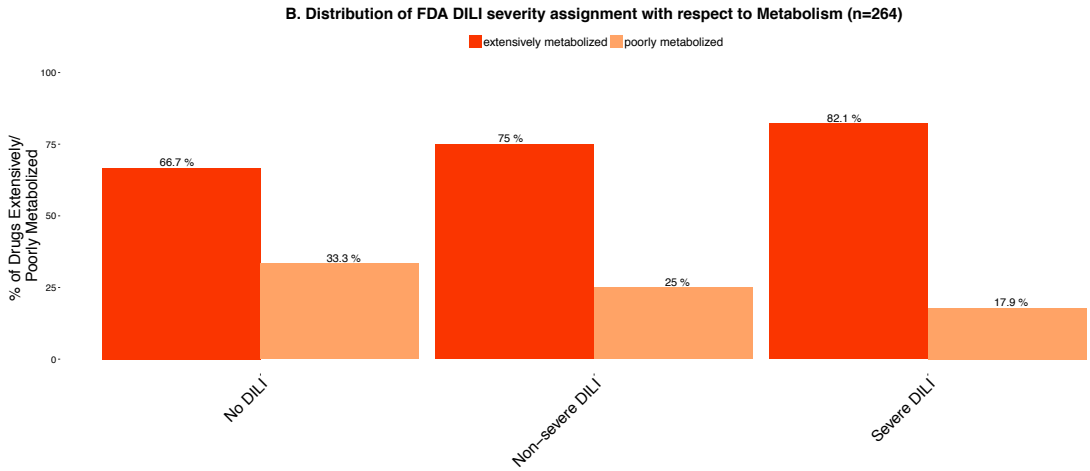
Figure 3.3B. Distribution of FDA Hepatic Liability with respect to extensively metabolized vs. poorly metabolized drugs.
The extensive hepatic metabolism group consisted of 102 BDDCS Class 1 and 99 BDDCS Class 2 drugs; the poor hepatic metabolism group consisted of 55 BDDCS Class 3 and 8 BDDCS Class 4 drugs. There was a significant difference between extensively metabolized vs. poorly metabolized drugs when the proportionality test was calculated p-value = 0.001536).

Figure 3.3C. Distribution of FDA Hepatic Liability with respect to high solubility vs. low solubility drugs.
The high solubility group consisted of 102 BDDCS Class 1 and 55 BDDCS Class 3 drugs; the low solubility group consisted of 99 BDDCS Class 2 and 8 BDDCS Class 4 drugs. There was a significant difference between high solubility vs. low solubility drugs when the proportionality test was calculated p-value = 3.481e-09.

When assessing DILI severity using the FDA DILI severity assignment (but combining "Mild" and "Moderate" DILI as "Non-severe DILI") with BDDCS Class (See Figure 3.4A), we also observe statistically significant trends for the increase in BDDCS Class 2 and decreases for BDDCS Classes 1 and 3. BDDCS Class 2 represents 53.6% (60/112) of the drugs in the "Severe DILI" category vs. 10% (6/60) in the "No DILI" category. BDDCS Class 1 represents 28.6% (32/112) of drugs in the "Severe DILI" vs. 56.7% (34/60) in the "No DILI" category. BDDCS Class 3 represents 14.3% (16/112) of drugs in the "Severe DILI" vs. 30% (18/60) in the "No DILI" category. The "Severe DILI" category comprises the following endpoints: acute liver

failure, fatal hepatotoxicity, and “Discontinued and Withdrawn” drugs as defined by Chen et al. (18). The “Non-severe DILI” category comprises compounds exhibiting hyperbilirubinemia, jaundice, and/or liver necrosis (“Moderate DILI”) and compounds exhibiting liver aminotransferases increase (“Mild DILI”). In the “No DILI” category, we observe that most drugs are BDDCS Classes 1 (56.7%, 34/60) and 3 (30%, 18/60). Here again, we observe a significant trend of high DILI liability for extensively metabolized compounds (Figure 3.4B). Drugs exhibiting high solubility (Figure 3.4C) show a trend of lower DILI severity.


A. Distribution of FDA DILI severity assignment with BDDCS Class (n=264)
BDDCS Class 1
BDDCS Class 2
BDDCS Class 3
BDDCS Class 4
BDDCS Class Assessment (%)
100
75
50
25
0
56.7 %
10 %
30 %
3.3 %
39.1 %
35.9 %
22.8 %
2.2 %
28.6 %
53.6 %
14.3 %
3.6 %
No DILI
Non-severe DILI
Severe DILI


B. Distribution of FDA DILI severity assignment with respect to Metabolism (n=264)
extensively metabolized
poorly metabolized
% of Drugs Extensively/ Poorly Metabolized
100
75
50
25
0
66.7 %
33.3 %
75 %
25 %
82.1 %
17.9 %
No DILI
Non-severe DILI
Severe DILI

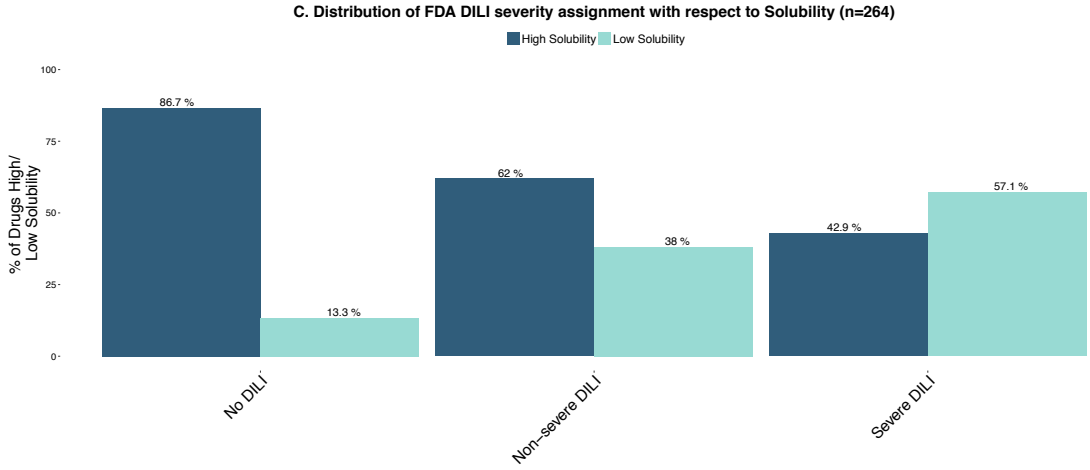

C. Distribution of FDA DILI severity assignment with respect to Solubility (n=264)
High Solubility
Low Solubility
% of Drugs High/ Low Solubility
100
75
50
25
0
86.7 %
13.3 %
62 %
38 %
42.9 %
57.1 %
No DILI
Non-severe DILI
Severe DILI

Figure 3.4A. Distribution of FDA DILI severity assignment with BDDCS Class.
FDA Drugs Labels were assigned to a DILI severity class according to the most severe reporting of a hepatic ADR or to the No DILI class if no hepatic ADRs were reported (6). Bars show the percentage of all compounds in the same DILI severity class ("No DILI", "Non-severe DILI", and "Severe DILI"). BDDCS Class 2 shows the highest frequency in the "Severe DILI" class assessment. BDDCS Class 1 and 3 drugs show the highest frequency in the "No DILI" class assessment. There was a significant difference between BDDCS classes when the proportionality trend test was calculated: BDDCS Class 1 trend p-value = 0.0003608; BDDCS Class 2 trend p-value = 2.105e-08; BDDCS Class 3 trend NS, p-value = 0.01297; BDDCS Class 4 trend NS, p-value = 0.8457. There was also significant differences in the BDDCS Class distributions among the following groups: "No DILI" vs. "Non-severe DILI", p-value = 0.005063; "No DILI" vs. "Severe DILI", p-value = 4.627e-07.

Figure 3.4B. Distribution of FDA DILI severity assignment with respect to extensively metabolized vs. poorly metabolized drugs.
There was a significant difference between extensively metabolized vs. poorly metabolized drugs when the proportionality test was calculated p-value = 0.02208.

Figure 3.4C. Distribution of FDA DILI severity assignment with respect to high vs. low solubility drugs.
There was a significant different between high vs. low solubility drugs when the proportionality test was calculated p-value = 2.23e-08.

Our examination of the relationship between the BDDCS's determinant properties: solubility and extent of metabolism led to some novel observations. Drugs belonging to BDDCS Class 1 and 3 exhibited a lower proportion of DILI severity. Drugs that are extensively metabolized and have low aqueous solubility, i.e., BDDCS Class 2 drugs have the highest rates of DILI risk. BDDCS Class 2 drugs exhibited the highest proportions among the "Warning and Precautions", "Black Box Warning", "Withdrawn" and "Discontinued" categories. These are notably considered the most serious DILI risk categories (See Figure 3.3A). These findings demonstrate the importance of the intrinsic drug properties as a potential factor for the development of a DILI event.

Relationship between Daily Dosage, FDA Drug Label and DILI Assessment Score

Lammert and coworkers (1, 2) have attributed hepatic adverse events to compounds with significant hepatic metabolism and daily dose ≥ 50mg. We have also evaluated the relationship between daily dosages ≥ 50mg against the already assessed FDA hepatic liability categories and DILI severity assessment (9). Our analysis concurs with the association of drugs being given at dosages ≥ 50mg/day having more adverse hepatic events. We have further evaluated this observation by examining the FDA hepatic liability distribution and DILI severity assessment. Drugs with a daily dose ≥ 50mg had a much higher frequency of toxicity as evidenced by the higher percentages in the "Warning and Precautions", "Boxed Warning" and "Withdrawn" label sections. For the DILI assessment in Figure 3.5B we also observe a higher frequency in DILI severity for compounds that are dosed at ≥ 50mg/day.

Although, there is strong evidence that dosages ≥ 50mg/day are associated with increased risk for hepatotoxicity, many drugs are safe at such dosages. For instance, the 50mg/day dosage cut off would predict that 44% of "No mention" and/or "No DILI" drugs (See Figure 3.5A and 3.5B) exhibit "Not Safe" potential in terms of hepatotoxicity. Thus, supporting that daily dosage alone is not a reliable means of guiding the drug development process, regulatory application, and clinical practice.

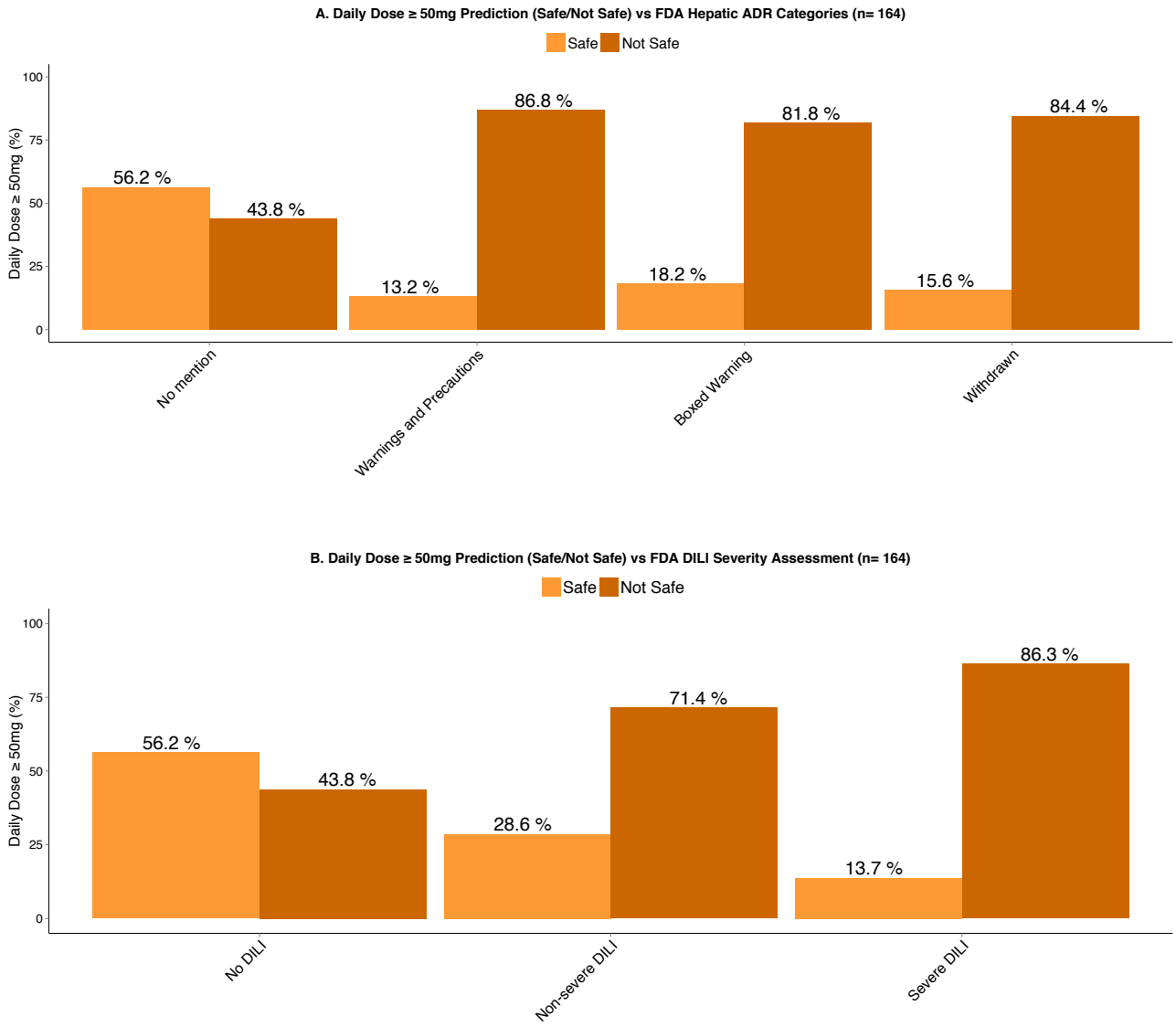


Figure 3.5A. Daily Dose ≥ 50mg prediction (Safe/ Not Safe) vs. FDA Hepatic ADR Categories. There is a marked increase in the proportion of compounds that are dosed at greater than 50mg/day and have FDA drug label warnings associated with DILI adverse effects as illustrated in the "Warning and Precautions", "Boxed Warning" and "Withdrawn" categories.

Figure 3.5B. Daily Dose ≥ 50mg prediction (Safe/ Not Safe) vs. FDA DILI severity assessment. Similarly, there is a marked increase in the proportion of compounds that are dosed at greater than 50mg/day and have some type of DILI toxicity as illustrated in the "Non-severe DILI" and "Severe DILI" categories.

Comparison of DILI – No DILI Predictive Metrics

In our comparative analysis we believe that positive predictive value (PPV, i.e., those drugs predicted to cause DILI that actually do so, or the true positive rate) is the most important value, since a high percentage will indicate the ability of the method to identify drugs that cause DILI. We believe that the false negative rate (FNR, i.e. those drugs causing DILI that are not identified by the metric, the type 2 error of the metric) is the second most important criteria, since a low number indicates that we do not incorrectly predict DILI when it occurs. The third parameter that we list, accuracy of the metric (ACC, i.e., the true positive and true negative predictions of the metric divided by the total number of compounds evaluated), represents the total % predicted correctly. Many other predictive metrics can be calculated, as has been done. However, we believe that PPV and FNR are the most relevant in evaluating an analysis of toxicity potential.

Chen and coworkers(9) have proposed that drugs with high lipophilicity (LogP) given at high doses likely become hepatotoxic as expressed in the Ro2. Using the same data set and the same annotations that Chen and coworkers(9) used for the proposed Ro2 (log P $\geq$ 3 and daily dosages $\geq$ 100mg (n=164)), we have reviewed the relationship between DILI hepatic adverse events and daily dose $\geq$ 50mg, daily dose $\geq$ 100mg, BDDCS Class, cLogP $\geq$ 3 and combinations of these characteristics. The data set was classified into two categories: “Most DILI Concern” and “No DILI Concern.” We believe that the use of these standardized annotations allows for a more direct comparison of the models. Of the 164 drugs, BDDCS classification could be assigned for 151 drugs. Our evaluation includes a comparison of the different predictive metrics against the first proposed Ro2 Chen et al. data set(9) and the most recent report adding generation of reactive metabolites to the Ro2(13).

Chen and coworkers(9) claim that the Ro2 is the best method for identifying drugs that cause DILI (PPV=95.3%), but we maintain that it is not a good method in terms of its FNR (61.7%), and therefore its ACC is low (55.0%). We can see in Table 3.1 that comparing the Ro2 with cLogP ≥ 3 or cLogP ≥ 3 + Dose ≥ 50mg (rather than 100mg), that similar ACC values are achieved, but PPV for cLogP ≥ 3 alone is markedly decreased. BDDCS Class 2 identification yields a slightly higher ACC than Ro2 due to the bigger decrease in FNR vs. PPV. We observe that accuracy of DILI is best predicted by "Dose ≥ 50mg", followed by "Dose ≥ 100mg". The next best predictive model was "Metabolism (BDDCS Class 1 and 2) + Dose ≥ 50mg" together. Additionally, when we compared the Ro2 with "cLogP ≥ 3" or "cLogP ≥ 3 + Dose ≥ 50mg" (rather than 100mg), we observe similar ACC values, but PPV for cLogP ≥ 3 alone is markedly decreased. Thus showing that dose alone is a stronger contributing factor to DILI risk than cLogP. Although BDDCS Class 2 and Ro2 show relatively high PPV, their ACC is decreased due to FNR outcomes. We also found that poor solubility "(BDDCS Class 2 + 4)" has a correlation with DILI toxicity, but this characteristic alone was not able to distinguish accurately DILI vs. No DILI events. Our comparison in Table 3.1 suggests that BDDCS classification alone is not sufficiently predictive of DILI potential, but that the Ro2, which includes a dose parameter may be no better a predictor and possibly even poorer than just looking at BDDCS Class 2, which does not require knowledge of dose.

Table 3.1. Comparison of Different Predictive Metrics for the First Published Chen et al. Data Set (9).

Criteria	% Correct (Positive Predictive Value, PPV)	% DILI Missing (False Negative Rate, FNR)	% Accuracy (ACC) (True Positive + True Negative)/151
Rule of Two	95.3%	61.7%	55.0%
BDDCS Class 2	90.2%	48.6%	61.6%
BDDCS Class 2 + Dose ≥ 50mg	94.1%	55.1%	58.9%
BDDCS Class 2+ Dose ≥ 100 mg	93.8%	57.9%	57.0%
Dose ≥ 50mg	83.5%	15.0%	77.5%
Dose ≥ 100mg	85.6%	22.4%	74.8%
BDDCS Class (1 + 2)	74.8%	16.8%	68.2%
BDDCS Class (1 + 2) + Dose ≥ 50mg	87.4%	29.0%	72.2%
BDDCS Class (1 + 2) + Dose ≥ 100mg	90.8%	35.5%	70.2%
BDDCS Class (2 + 4)	89.4%	44.9%	63.6%
BDDCS Class (2 + 4) + Dose ≥ 50mg	92.9%	51.4%	60.9%
BDDCS Class (2 + 4) + Dose ≥ 100mg	92.5%	54.2%	58.9%
CLogP ≥ 3	76.1%	52.3%	52.3%
CLogP ≥ 3 +Dose ≥50mg	91.7%	58.9%	55.6%
BDDCS Class 1	58.6%	68.2%	35.8%
BDDCS Class 3	51.9%	86.9%	29.8%
BDDCS Class 4	80.0%	96.3%	31.1%

A more recent report on the Ro2(13) includes the addition of reactive metabolites formation(13). Of the 192 drugs used in their follow up analysis, BDDCS classification could be assigned to 166 drugs. This comparative analysis is depicted in Table 3.2. This "Ro2 + Reactive Metabolites" shows an increase in PPV, but only has a marginal improvement in the overall ACC and, in fact, appears to be less useful than "BDDCS Class 2 + Reactive Metabolites". BDDCS Class 2 compounds show higher DILI predictability as compared to the other BDDCS classes. Furthermore, BDDCS Class 2 alone in this dataset performed better in terms of ACC than the first proposed "Ro2" and most recently proposed model for "Ro2 + Reactive Metabolite formation". We also observe that reactive metabolite formation alone, followed by "Dose ≥ 50mg + Reactive Metabolite Formation" and "Dose ≥ 100mg + Reactive Metabolite Formation" had the best performance in terms of ACC and PPV. However, these conditions also have an increase in FNR. The next best predictive model was "Metabolism (BDDCS Class 1 and 2) + Reactive Metabolite Formation" together, which does not require any knowledge of the dose taken, performed similarly to "Metabolism (BDDCS Class 1 and 2) + Dose ≥ 50mg or Dose ≥ 100mg", which has showed the best predictability in our initial analysis. Moreover, taking into account reactive metabolite formation or having better methods to account for reactive metabolite formation together with high permeability compounds can potentially lead to an improvement in DILI prediction without the need to rely on dose.

Table 3.2. Comparison of Different Predictive Metrics for the Chen et al. Data Set (13) (Filtered for only BDDCS Classifiable Drugs).

Criteria	% Correct (Positive Predictive Value, PPV)	% DILI Missing (False Negative Rate, FNR)	% Accuracy (ACC) (True Positive + True Negative)/166
Rule of Two	92.2%	58.0%	58.4%
Rule of Two + Reactive Metabolite Formation	100.0%	60.7%	59.0%
BDDCS Class 2	92.4%	45.5%	66.3%
BDDCS Class 2 + Reactive Metabolite Formation	98.2%	50.9%	65.1%
BDDCS Class (1 + 2)	71.4%	15.2%	66.9%
BDDCS Class (1 + 2) + Reactive Metabolite Formation	91.2%	25.9%	77.7%
Dose ≥50	78.3%	9.8%	76.5%
Dose ≥100	82.6%	15.2%	77.7%
Dose ≥50 + Reactive Metabolite Formation	93.4%	24.1%	80.1%
Dose ≥100 + Reactive Metabolite Formation	95.2%	28.6%	78.3%
Reactive Metabolite Formation	88.7%	16.1%	81.9%

Lammert and coworkers(1) attributed hepatic adverse events to compounds exhibiting extensive metabolism. This attribute is represented by "BDDCS Class (1+ 2)" in Table 3.1 and Table 3.2 that shows better ACC than Ro2 and "BDDCS Class 2" because of the marked decrease in FNR, but the lower PPV value is probably higher than is acceptable for DILI predictions. Lammert et al.(2) had previously suggested that significant hepatic metabolism and daily dose ≥ 50mg was potentially predictive of hepatic adverse events. However, addition of dose to BDDCS Class (1 + 2) shows an increase in PPV, but with a corresponding increase in FNR, yielding negligible ACC changes. It is noteworthy that the best ACC in Table 3.1 is achieved with dose alone (i.e. Dose ≥ 50mg and Dose ≥ 100mg) with slightly lower PPV but the lowest FNRs as compared to Ro2 and BDDCS Class 2. In Table 3.2 what we find illuminating for this data set is that BDDCS Class 2 by itself performed better than "Ro2" and "Ro2 and reactive metabolite formation." We also observe a comparable performance of "BDDCS Class (1+2) + reactive metabolite formation" vs. dose alone. But we also note that just considering only reactive metabolite formation yields the highest ACC of all the other methodologies, even when dose is added to reactive metabolite formation. (In Supplementary Tables 3.3A and 3.3B we show that selection of only BDDCS Class drugs vs. all drugs in the new Chen et al. (13) data set does not bias the outcome.)

Table 3.3A. Comparison of Different Predictive Metrics for the Most Recent Chen et al. Data Set (13).

Criteria	% Correct (Positive Predictive Value, PPV)	% DILI Missing (False Negative Rate, FNR)	% Accuracy (ACC) (True Positive + True Negative)/192
Rule of Two	92.7%	58.9%	59.9%
Rule of Two + Reactive Metabolite Formation	100.0%	61.3%	60.4%
Dose ≥ 100mg+ 1 ≤ CLogP < 3	81.1%	75.8%	47.4%
Dose ≥ 100mg+ 1 ≤ CLogP < 3 + Reactive Metabolite Formation	92.3%	80.6%	46.9%
Dose ≥ 100mg	80.8%	15.3%	77.1%
CLogP ≥ 3	77.2%	50.8%	57.8%
Reactive Metabolite Formation	88.0%	16.9%	81.8%

Table 3.3B. Comparison of Different Predictive Metrics for the Most Recent Chen et al. Data Set (13) (Filtered for only BDDCS Classifiable Drugs).

Criteria	% Correct (Positive Predictive Value, PPV)	% DILI Missing (False Negative Rate, FNR)	% Accuracy (ACC) (True Positive + True Negative)/166
Rule of Two	92.2%	58.0%	58.4%
Rule of Two + Reactive Metabolite Formation	100.0%	60.7%	59.0%
Dose ≥ 100mg+ 1 ≤ CLogP < 3	83.3%	77.7%	44.6%
Dose ≥ 100mg+ 1 ≤ CLogP < 3 + Reactive Metabolite Formation	90.9%	82.1%	43.4%
Dose ≥ 100mg	82.6%	15.2%	77.7%
CLogP ≥ 3	79.2%	49.1%	57.8%
Reactive Metabolite Formation	88.7%	16.1%	81.9%

Relationship between BSEP Inhibition and BDDCS Classification

Another model we used to evaluate DILI toxicity has been the supposition that BSEP inhibitors lead to DILI causation. FDA drug labels for 182 registered drugs have been evaluated for their BSEP inhibition by Pedersen et al.(15). Assignment to BSEP inhibition categories was based on the ATP dependent taurocholate transport rate. Compounds that inhibited BSEP more than 50% at concentration of 50uM were considered "BSEP Inhibitors"; compounds in the 50%-72.5% range were considered "Weak BSEP Inhibitors"; compounds that inhibited less that 27.5% were considered "BSEP Non-Inhibitors". All compounds but L-carnitine ("No mention", "No DILI") could be classified. For BDDCS classification, only active species were considered. The distribution of BSEP inhibition in each FDA hepatic liability category and BDDCS class were evaluated. 73/181 drugs were assigned to the "Adverse Reactions" category, 61/181 to the "Warning and Precautions", 12/181 to the "Boxed Warning", 2/181 in the "Withdrawn" category and 33/181 to the "No mention" category.

When BSEP inhibition data were correlated with FDA drug labels of registered drugs(15), we observed no discernible pattern between BSEP inhibition and ADR categories (See Figure 3.6A). For the BDDCS classification, we observe that the great majority of strong BSEP inhibitors are BDDCS Class 2 drugs, with concomitant decreases in the percentages of BDDCS class 1 and 3 drugs as BSEP inhibition increases, as depicted by Figure 3.6B. Here we point out that because we are able to make similar predictions just based on simple physicochemical parameters, this leads us to dismiss the predictive ability of the mechanistic association of BSEP and DILI. We suspect that previous analyses predicting that BSEP inhibition leads to DILI may have been confounded by the observation that most BSEP inhibitors are BDDCS Class 2 drugs, which show a high prevalence for DILI. In Table 3.4, we observe that

in the condition of a positive GSH + BDDCS Class 2 or BSEP +BDDCS Class 2 we observe a marked improvement in the PPV. However, the predictability of these assays is still very limited as noted by their high FNR outcomes. Consideration of Cmax of drugs in relation to IC50 of BSEP inhibition could possibly improve the prediction of DILI based on BSEP inhibition.

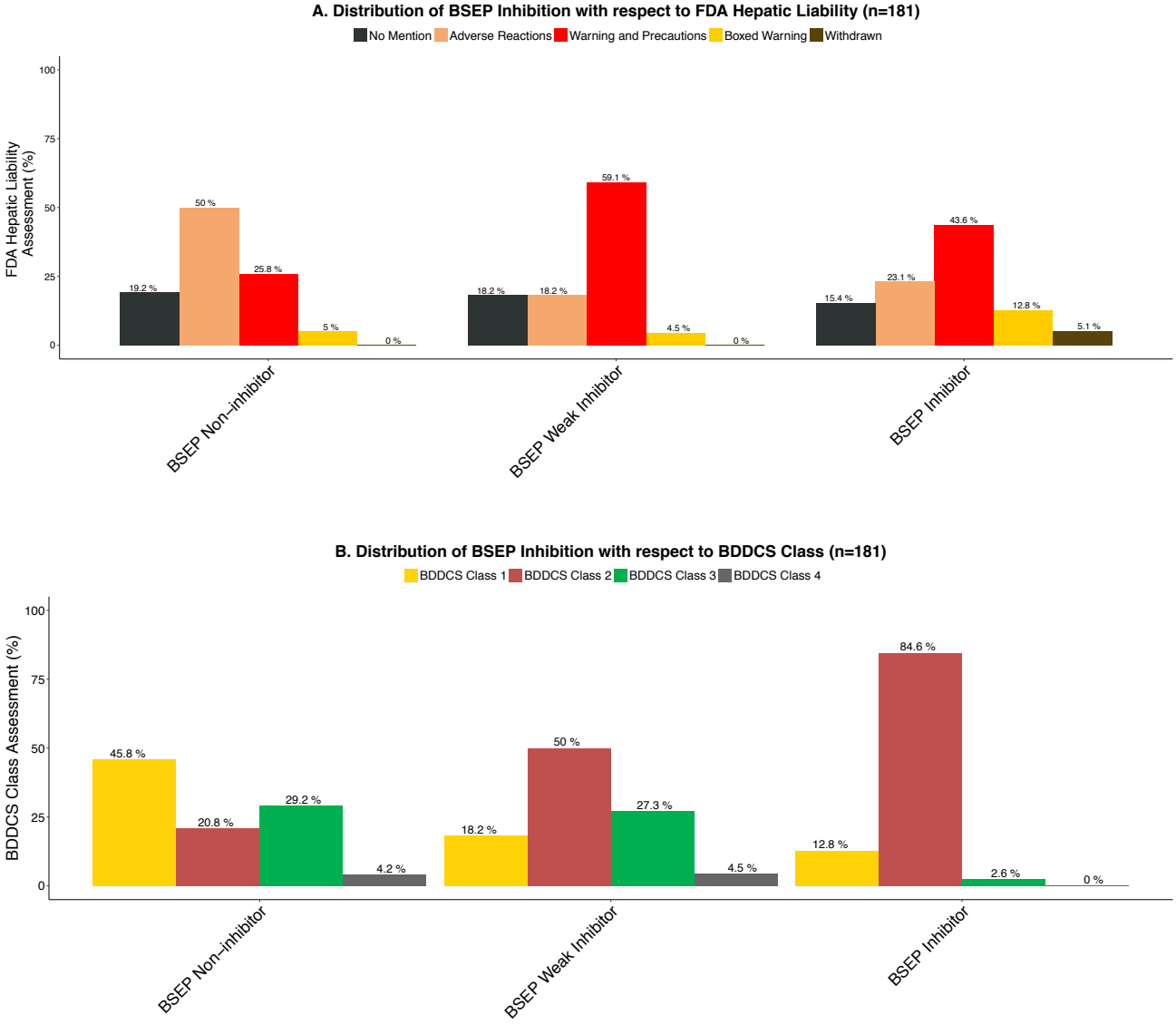


Figure 3.6A: Distribution of BSEP inhibition with respect to FDA Hepatic Liability assignment. Assignment to BSEP inhibition categories was based on the ATP dependent taurocholate (TC) transport rate. Compounds that inhibited TC transport more than 50% were considered BSEP Inhibitors; compounds inhibiting TC transport rate in the 27.5%-50.0% range were considered Weak BSEP Inhibitors; compounds inhibiting TC transport less than 27.5% were considered BSEP Non-inhibitors. Bars show the percentage of all compounds in the same BSEP inhibition class (BSEP Non-Inhibitor, BSEP Weak Inhibitor, BSEP Inhibitor). There was a significant difference between the FDA hepatic liability categories when the proportionality trend test was calculated: "No mention" trend, NS, p-value = 0.6018, "Adverse Reactions" trend, p-value =

0.0007441, “Warning and Precautions” trend, p-value = 0.01111, “Boxed Warning” trend, NS, p-value = 0.1129, “Withdrawn” trend, p-value = 0.01243.

Figure 3.6B. Distribution of BSEP inhibition assignment with respect to BDDCS Class.
BSEP inhibitors are overwhelmingly composed of BDDCS Class 2 drugs (84.6%). There was a significant difference between BDDCS Classes when the proportionality trend test was calculated: BDDCS Class 1 trend, p-value = 5.521e-05; BDDCS Class 2 trend, p-value = 5.009e-13; BDDCS Class 3 trend, p-value = 0.00115; BDDCS Class 4 trend, NS, p-value = 0.2432. There was also significant differences in the BDDCS Class distributions among the following BSEP inhibition groups: “BSEP Non-inhibitor” vs. “BSEP Weak Inhibitor”, p-value = 0.0214; “BSEP Non-inhibitor” vs. “BSEP Inhibitor”, p-value = 2.78e-11.

Comparison of Mechanism Based Toxicity Endpoints

Although, a number of compound–specific liability factors have been linked with DILI susceptibility, it is difficult to understand which risk factors are more important in patient-specific responses and/or environmental stimuli. One approach followed by many research groups to assess and reduce some of the more common, drug-specific factors in a set of targeted *in vitro* assays. The most common mechanisms covered in such *in vitro* panels or hazard matrices include formation of reactive metabolites, inhibitions of drug transporters involved in hepatobiliary elimination of bile acids and other metabolic endogenous products (BSEP, MRPs), mitochondrial toxicity and different cellular toxicity assays covering the formation of drug-metabolites(28, 29). Various approaches are used in the pharmaceutical industry for hazard identification and risk assessment of reactive metabolites and more integrated strategies that include measures of the initial mechanism of toxicity have been highlighted in our analysis.

Zhang et al.(16) evaluated the *in vitro* hepatic toxicity of 152 drugs from the Chen and coworkers(9) data set using four mechanistically relevant endpoints. They reported that the ratio of the measured reactive oxygen species to cellular ATP depletion (ROS/ATP) was able to not only differentiate compounds exhibiting severe DILI (65 compounds) from no DILI (35 compounds) but also severe DILI from non-severe DILI (52 compounds). Of the 152 drugs,

streptozocin could not be BDDCS classified and chlorpropamide as a class 0 drug (extent of metabolism highly dependent upon urinary pH) was not included in our analysis. For the 152 drugs from the Chen dataset evaluated by Zhang et al.(16), 134 drugs were BDDCS Class known. When we carried out an analysis for this data set in terms of BDDCS Class as was done for the entire Chen et al. (9) data set as shown in Fig. 3.4 a similar trend was observed (data not shown) but the trends were not to the same degree of significance. What would be more illuminating would be individual drug results with respect to the specific mechanisms based outcomes, which were not presented. However, the accuracy of this characterization remains in question. In the manuscript by Zhang et al.(16) ibuprofen and atorvastatin were characterized as "severe DILI", felbamate, methimazole, and pyrazinamide were characterized as "non-severe DILI" and streptozocin and penicillamine were characterized as "no DILI", which we believe are inaccurate classifications. In addition, Zhang et al. include in their study directly cytotoxic anticancer drugs such as cisplatin, dacarbazine, bleomycin, etc., which should be evaluated separately from other drugs.

We also include here a comparison of the different predictive metrics in the various assays measuring key mechanisms of toxicity endpoints associated with DILI from the Schadt et al. data set(14). Schadt et al.(14) evaluated 120 marketed or withdrawn drugs, which were analyzed independent of FDA classification. These workers categorized severe and moderate DILI as "high DILI concern" and mild and no DILI as "low DILI concern." The "high DILI concern" category was a merger of moderate and severe risk compounds based on the FDA categorization. Generation of reactive metabolites was tested via GSH adduct formation and P450 3A4 time-dependent inhibition (TDI). Further key measures of initial mechanism of toxicity were monitored in a panel consisting of assays assessing BSEP inhibition, mitochondrial

toxicity and cytotoxicity. In the Schadt et al. data set of 120 compounds, 14 compounds had not been BDDCS classified. As depicted in Table 3.4 we evaluated 106/120 drugs that were screened based on different in vitro mechanism endpoints and BDDCS class, which has been previously published. The assays that performed the best were GSH and BSEP assays; this was determined based on the balance of the lowest FNR and highest PPV. However the FNR rates of these two assays are also very high, and the accuracy of these tests is comparable to the analysis of BDDCS class 2 alone. When GSH or BSEP measurements are added to BDDCS Class 2 PPV and FNR both increase but accuracy is no better. The highest ACC is obtained when all of the mechanisms of toxicity endpoints are confirmed, due to the low FNR. However, having a PPV of only 65.1% does not give much confidence.

Table 3.4 Comparison of Various Assays Measuring Key Mechanisms of Toxicity Endpoints Associated with DILI (14).

Criteria	% Correct (Positive Predictive Value, PPV)	% DILI Missing (False Negative Rate, FNR)	% Accuracy (ACC) (True Positive + True Negative)/106
GSH	71.9%	52.1%	69.1%
TDI	75.0%	81.3%	61.8%
Cytotoxicity (3T3 cells)	48.3%	70.8%	55.5%
Mitotox	71.4%	79.2%	61.8%
BSEP	69.2%	62.5%	65.5%
All assays	65.1%	14.6%	73.6%
BDDCS Class 1	33.3%	75.0%	45.5%
BDDCS Class 2	64.6%	35.4%	69.1%
BDDCS Class 1 and 2	51.2%	10.4%	58.2%
GSH and BDDCS Class 1	46.2%	87.5%	55.5%
GSH and BDDCS Class 2	89.5%	64.6%	70.0%
GSH and BDDCS Class 1 and 2	71.9%	52.1%	69.1%
BSEP and BDDCS Class 1	37.5%	93.8%	54.5%
BSEP and BDDCS Class 2	87.5%	70.8%	67.3%
BSEP and BDDCS Class 1 and 2	70.8%	64.6%	65.5%

Some of these risk factors can be mitigated during the drug design/development process to identify drugs with better chemical attributes with reduced potential to cause human DILI. The strengths and weaknesses have been highlighted in our analysis

Although there may be some general trends between simple physical parameters, it is unlikely that such considerations could accurately predict risk. This problem could potentially be alleviated by the new *in vitro* approaches and utilization of state of the art instrumentation currently being evaluated. The development of improved physiological test systems based on information gained from studies with model hepatotoxins are required to encompass both chemical and biological factors associated with hepatotoxicity to try to screen for rare but often fatal idiosyncratic hepatotoxicities earlier in drug development.

BDDCS Classification Prior to Dosing in Humans

Although the finding of Uetrecht shows that idiosyncratic drug reactions were rare among individuals given drug doses <10mg/day and more likely among individuals given drug doses ≥ 1000mg/day(30), the dose relationships can only be determined for a new molecular entity after the drug has been administered to human subjects/patients. In contrast, BDDCS Class can be predicted prior to ever dosing the compounds to humans as we have proposed previously(31). Hosey and Benet(24) showed that based on *in vitro* permeability measurements, PPV for prediction of extensive metabolism were all 90% or greater. And most recently Dave and Morris(25) showed that they were able to correctly predict highly soluble vs. poorly soluble drugs using measured solubility parameters with greater than 85% probability. Thus as seen in Table 3.1, just knowing if a compound is BDDCS Class 2 prior to drug dosing has the ability to identify DILI potential with 90.2% PPV and 61.6% ACC. And in Table 3.2, the incorporation of

better assays to the assignment of BDDCS Class and reactive metabolite formation can perform as well as dose without knowing the actual dose. As noted above, negligible improvements in PPV and decrements in ACC are observed when dose size is added to BDDCS categorization. BDDCS Class (2 + 4) gave comparable results for BDDCS Class 2 alone, but since there are so few Class 4 drugs, it is difficult to conclude if this is relevant.

Relationships between BDDCS and Toxicity

The hypothesis that compounds with significant hepatic metabolism may potentially be more hepatotoxic due to the generation of reactive intermediates and subsequent metabolic idiosyncrasies was first uncovered in an epidemiological survey by Lammert and coworkers(1) who reported in their analysis that compounds exhibiting a significant hepatic metabolism resulted in ALT > 3 times ULN, liver failure, liver transplantation, and fatal DILI versus compounds with lesser degrees of hepatic metabolism. Our results show that DILI toxicity is most apparent in BDDCS Class 2 drugs, exhibiting the highest proportions among the ”Warning and Precautions”, “Black Box Warning”, “Withdrawn” and “Discontinued” categories. The great majority of approved drugs that cause acute liver failure, fatal hepatotoxicity, discontinued and or withdrawn are BDDCS Class 2 drugs. BDDCS Class 3 and 4 drugs show little risk of liver aminotransferases increase and hyperbilirubinemia. Lammert’s assertion that extensively metabolized compounds are at an increased risk to develop DILI is limited since we show in our data analysis that BDDCS Class 1 compounds, which are extensively metabolized, represent the majority of the compounds in the “No mention” and “No DILI” groups (See Figure 3.3A and Figure 3.4A). The compounds that show the most toxicity are the extensively metabolized, low

solubility compounds, i.e. BDDCS Class 2. Overall BDDCS classification appears to have an association with drug toxicity potential to lead to DILI adverse events.

Drugs belonging to BDDCS Class 3 and 4 exhibited much lower proportions in the FDA hepatic liability and DILI severity assessment categories (See Figures 3.3 and 3.4). However, we note the underrepresentation of BDDCS Class 4 drugs in the overall scheme of marketed approved drugs. Compounds with poor hepatic metabolism had been previously noted to be significantly less likely to cause hepatotoxicity(1). In support of this observation, we also observe the increasing trend of BDDCS Class 3 drugs as the DILI severity decreases as depicted by Figure 3.4. Although a lack of hepatic metabolism does not assure total lack of hepatotoxicity, it indeed appears that BDDCS Class 3 and 4 drugs lead to a lower DILI severity.

We are not the first to investigate the BDDCS Class relationship and DILI. Previously Vuppalanchi et al.(32) have analyzed 383 cases of DILI caused by a single orally administered prescription agent from the DILI Network Prospective Study. The relationship of daily dosage (≥ 50 mg vs. ≤ 49mg), preponderance of hepatic metabolism (≥50% vs. <50%), and BDDCS class were compared with clinical characteristics and outcomes. A total of 99 drugs belonging to BDDCS Classes 1 through 4 were responsible for the DILI episodes. In concordance with daily dosage relationship previously reported, there are a much smaller number of cases of DILI in the ≤ 49 mg/day group (n=50) than those with daily dosages ≥ 50mg/day (n=324). There is also a higher number of cases of DILI from drugs that underwent significant hepatic metabolism (n=305) compared to those without hepatic metabolism (n=71). However, in their BDDCS case analysis breakdown, they report 118 cases with BDDCS Class 1, 96 cases with BDDCS Class 2, 112 cases with BDDCS Class 3, and 38 cases with BDDCS Class 4, which shows that the actual number of extensively metabolized drugs is 214, while it is 150 for poorly metabolized drugs.

Vuppalanchi et al. concludes that there is no DILI difference between BDDCS Class 1 and BDDCS Class 2 drugs. Patients with DILI caused by medications with or without preponderant hepatic metabolism did not differ in clinical characteristics or outcomes. There was also no significant difference between BDDCS 1, 2, 3 classes in terms of DILI cases. BDDCS Class 1 compounds were reported to have a longer latency and exhibit a greater proportion of hepatocellular injury. However, in our current analysis we observe that the majority of drugs in the "No DILI" group are composed of BDDCS Class 1 and BDDCS Class 3 and there is a much greater risk of BDDCS Class 2 leading to idiosyncratic DILI than BDDCS Class 1 or 3 compounds.

In this work and in our previous study reported in Chapter 2, predicting the prevalence of cutaneous adverse reactions with antiepileptic drug (12), BDDCS Class 2 drugs appear to present the most toxic liability. Why should this be true? A major finding in the development of the BDDCS classification system was the recognition that extensively metabolized, high permeability, high solubility Class 1 drugs may be shown in vitro to be substrates of both uptake and efflux transporters, but that effects of transporters on BDDCS Class 1 drugs are essentially clinically insignificant in the liver and intestine, as well as the brain. Thus, for BDDCS Class 1 drugs unbound concentrations in the systemic circulation will reflect unbound concentrations in the liver as well as in the rest of the body. However, this will not be true for BDDCS Classes 2, 3 and 4 drugs where transporter effects will lead to different unbound concentrations in the liver and throughout the body. That is, Class 1 drugs will follow the long held assumption in deriving pharmacologic/toxicologic relationships that free drug concentrations are the same throughout the body. But this assumption in pharmacology was made prior to any recognition of the importance of drug transporters in controlling permeability. Thus, according to BDDCS

classification(22, 33) approximately 40% of marketed drugs (i.e., those that are Class 1) will still follow the equivalent free drug concentration hypothesis. It is important to recognize that the compounds evaluated here are drugs that reach the market where sponsors were able to convince the regulatory agencies based on *in vitro* and preclinical animal studies that toxicity potential, particularly DILI, would be manageable or at least acceptable when the drugs reached the market and were taken by large patient populations as compared to those limited number of patients studied during drug development. Thus, according to our hypothesis, drug company sponsors in their preclinical and clinical studies of Class 1 drugs would be able to reasonably predict drug concentrations in the liver and throughout the body. In contrast, for BDDCS Class 2 drugs, where metabolism is the significant process of elimination, drug concentration measurements in the systemic circulation for these compounds both in the preclinical and clinical studies would poorly predict what occurs in the liver and in other organs of the body. And since it is obvious that DILI occurs more frequently with metabolized drugs, studies in drug development with Class 2 drugs would be poorer predictors of toxicity potential due to the challenges to estimate intracellular concentrations and metabolic processes. Thus, the prevalence of DILI with BDDCS Class 2 drugs could just be circumstantial in that sponsors would be unable to properly evaluate hepatic toxicity for these compounds in designing their clinical studies. This problem could potentially be alleviated by new *in vitro* approaches and utilization of state of the art instrumentation currently being evaluated.

Conclusion

In our analysis we confirm previous reports that the best predictor of DILI requires knowledge of the daily dose, an unknown quantity early in drug development. We show here that the BDDCS methodology, where assignment can be made prior to ever dosing a drug to animals

or man, yields similar and in a number of cases better than the DILI predictive potential of other methodologies such as Ro2. Although we observe strong trends of BDDCS Class 2 increasing toxicity as DILI severity increases, overall, BDDCS Classification only marginally improves the prediction of DILI toxicity potential. However, we observe that "BDDCS Class 2" alone have performed better than "Ro2" and "Ro2 + reactive metabolite formation." As seen in Figs. 3.3 and 3.4, the BDDCS Class 2 versus Class 1 differentiation only becomes evident with the most severe hepatic toxicities, and then only a 2:1 differentiation between BDDCS Class 2 versus Class 1 is found.

Similarly, we demonstrate that those previous proposed models to predict DILI potential such as the "Ro2" and "Ro2 + reactive metabolite formation", daily dosage ≥ 50mg, and the supposition that BSEP inhibitors lead to DILI causation are still not sufficiently predictive. Lammert et al.'s(1) assertion that extensive metabolized compounds are at higher risk of developing DILI can be much improved by differentiating BDDCS Class 2 from BDDCS Class 1 drugs. Ro2 shows a high FNR missing significant cases of DILI assignment when "DILI" occurs and that the daily dosage ≥ 50mg alone can only depict a clear relationship with dose with compounds that have been previous associated with DILI, but very limited predictability in differentiating compounds with "No DILI" assignment. We also suspect that previous analyses predicting that BSEP inhibition leads to DILI may be confounded by our finding that most BSEP inhibitors are BDDCS Class 2 drugs. Thus, our BDDCS analysis and previous DILI toxicity potential are not sufficiently accurate in allowing early identification of new molecular entities that will be DILI free. But we believe that comparison of proposed DILI predictive methodology with BDDCS assignment offers a useful tool by which new DILI predictive hypotheses can be evaluated. Hopefully, development of mechanism based toxicity endpoints, such as those

proposed by Chen et al.(13), Zhang et al.(16) and Schadt et al.(14), as discussed above, will greatly improve future predictability.

Toxicologists, medicinal chemists and drug development scientists will most likely conclude that no one in the drug development process will discontinue a drug candidate based on the predictive DILI potential using BDDCS class. We agree. The purpose of this analysis was to point out that many of the published "predictive DILI" hypotheses do no better than just avoiding BDDCS Class 2 drugs. We propose that comparison of predictive DILI hypotheses with BDDCS class assignment is a useful exercise in determining the relevance of predictive metrics.

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CHAPTER 4: Measures of BSEP Inhibition *In Vitro* Are Not Usefully DILI Predictive

ABSTRACT*

Inhibition of the bile salt export pump (BSEP) by a drug has been implicated as a risk factor for a drug's potential to cause DILI and is thought to be an important mechanism leading to DILI. For a wide variety of drugs a correlation has been observed between the potency of *in vitro* BSEP inhibition and its propensity to cause DILI in humans. These findings were interpreted to suggest that BSEP inhibition could be an important mechanism to help explain how some drugs initiate DILI. Recently, the International Transporter Consortium has highlighted BSEP as one of the emerging transporters that need to be considered when evaluating drug safety. However, the practical utility of this approach is still in its infancy and needs to be further evaluated. Because BDDCS can be useful in characterizing and predicting some important transporter effects in terms of drug-drug interactions, we evaluated the information provided by BDDCS in order to understand the inhibition propensity of BSEP. Here we analyze the relationship between a compound's ability to inhibit BSEP function and cause liver injury in humans using a compilation of published DILI datasets that have screened for BSEP inhibitors, other hepatic transporters and other mechanism based toxicity endpoints such as the mitochondrial toxicity assay. Our results demonstrate that there is little support for BSEP inhibition being universally DILI predictive. Rather we show that most potent BSEP inhibitors are BDDCS Class 2 drugs, which we have demonstrated previously is the BDDCS class most likely to be DILI related.

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* Modified from the manuscript submitted:Chan R, Benet LZ. 2017. Measures of BSEP inhibition in vitro are not usefully DILI predictive. *Toxicol. Sci.*

INTRODUCTION

Drug-induced liver injury (DILI) encompasses a spectrum from mild biochemical abnormalities to acute liver failure. DILI is often difficult to distinguish from natural causes of liver injury such as viral hepatitis or autoimmune conditions (1, 2). DILI often exhibits delayed onset (5 to >100 days) during continuous therapy and even may occur after cessation of therapy. Although, the underlying pathophysiological mechanism of DILI is still poorly understood, there is increasing evidence that cholestatic forms of DILI result from a drug- or metabolite-mediated inhibition of hepatobiliary transporter systems (3). Inhibition of the bile salt export pump (BSEP) by a drug has been implicated as a risk factor for the drug's potential to cause DILI and is thought to be an important mechanism that leads to DILI (3–5).

Many drugs that cause infrequent but clinically severe liver injury in humans have been found to inhibit BSEP activity *in vitro* using a variety of different experimental model systems, and *in vivo* in experimental animals (5, 6). For a wide variety of drugs a correlation has also been observed between propensity to cause DILI in humans, potency of *in vitro* BSEP inhibition and their therapeutic plasma drug concentrations (7). These findings suggest that BSEP inhibition could be an important mechanism that helps explain how some drugs initiate DILI. Recently, BSEP has also been highlighted by the International Transporter Consortium as one of the emerging transporters that need to be considered when evaluating drug safety (8). However, the practical utility of this approach is still in its infancy and needs to be further evaluated. BSEP inhibition is just one of many possible mechanisms that can initiate idiosyncratic DILI, therefore it has been suggested that screening for *in vitro* BSEP inhibition is likely to be of greatest value if undertaken together with screening for other relevant adverse effects (e.g. mitochondrial injury, cell cytotoxicity, metabolic bioactivation) and understanding its inhibition predisposition along

with some basic physicochemical properties (9, 10). Recent research suggests that bile acids affect the mitochondria and potentially lead to mitochondrial membrane permeability transition (11).

We have recently compared as presented in Chapter 3 the possibility of predicting DILI potential using the Biopharmaceutics Drug Disposition Classification System (BDDCS) versus previously proposed published methods (12). Because BDDCS can be useful in characterizing and predicting some important transporter effects in terms of drug-drug interactions (13), we believe it would be useful to apply BSEP as a potential biomarker and evaluate the information provided by BDDCS in order to understand the inhibition propensity of BSEP. Our previous analyses suggest that just avoiding BDDCS Class 2 drugs may serve as a useful baseline in evaluating these metrics (12). We suggest in Chapter 3 that if a correlation of a particular measure with toxicity (e.g., the ability of BSEP inhibition to predict DILI) is not better than the correlation of the toxicity measure with BDDCS Class 2 assignment, then the field can have no confidence that the measurement will usefully serve as a mechanistic predictor (12).

Several groups of researchers have proposed that pro-active *in vitro* screening for BSEP during drug discovery may aid in early flagging and de-selection of compounds that exhibit a high propensity to cause idiosyncratic DILI (4, 5, 14). Therefore, our present goal is to evaluate the potential of *in vitro* BSEP inhibition screening in aiding the prediction of DILI. Here we analyze the relationship between a compound's ability to inhibit BSEP function and cause liver injury in humans using a compilation of published DILI datasets that have screened for BSEP inhibitors, other hepatic transporters and other mechanism based toxicity endpoints such as the mitochondrial toxicity assay.

For each pharmaceutical drug, we applied BDDCS classification. Our analysis only includes drugs that have been BDDCS classified. We previously used the BDDCS classification as presented in Chapter 3 as a preliminary baseline tool to assess the relationship of *in vitro* BSEP screening with a drug's DILI predictability (12). Here we provide a more in depth evaluation of the relationship between BSEP inhibition and screening using other *in vitro* platforms (e.g. mitochondrial toxicity, cell cytotoxicity, MRP3 and MRP4 inhibition) (4, 9, 14–17).

MATERIALS AND METHODS

Compilation of BSEP Datasets

Classifying BSEP Inhibition

FDA drug labels for 182 registered drugs have been evaluated by Pedersen *et al*. (14) for BSEP inhibition using an *in vitro* membrane vesicle BSEP inhibition assay. Assignment to BSEP inhibition categories was based on the ATP dependent taurocholate (TC) transport rate when co-incubated with 50 μM of test compound. Pedersen *et al*. (14) defined compounds as: BSEP Inhibitors when they decreased TC transport by more than 50%; BSEP Weak Inhibitors when TC transport was decreased by 27.5 to 50%; BSEP Non-Inhibitors showed a minimal decrease of TC transport by less than 27.5%. All compounds but L-carnitine ("No mention", "No DILI") could be classified. For BDDCS Classification, only active species (e.g., drug but not prodrug) were considered. In cases where DILI knowledge is limited by FDA drug labels, we have used annotations of human DILI concern collected by Chen *et al.* (18). All compounds except glyburide ("Adverse Reactions"), lopinavir ("Warning and Precautions") and sulfamethoxale ("Warning and Precautions") were assigned a DILI concern by Chen *et al.* (18). We also reviewed the Dawson *et al.* (4) data set that investigated the relationship between human BSEP

inhibition for 85 pharmaceuticals *in vitro*. As defined by Dawson *et al.* (4), IC50 <300 μM gave an optimal separation between drugs that causes cholestatic/mixed DILI and drugs that caused hepatocellular or no DILI. Drugs with IC50< 300 μM were considered as BSEP Inhibitors, while all others were considered BSEP Non-Inhibitors (this includes BSEP Weak Inhibitors where 300 μM < IC50 >1000 μM). All compounds except clobetasol propionate ("No DILI") and picotamide ("No DILI") could be BDDCS classified. Chlorpropamide was also removed from the analysis because it is a BDDCS Class 0 compound (i.e. BDDCS class changes as a function of urine pH).

Classifying BSEP Inhibition and Mitochondrial Toxicity

Aleo *et al.* (9) selected 72 compounds from the 287 compounds reported by Chen *et al*. (19) to test the hypothesis of a synergistic relationship between BSEP inhibition and mitochondrial toxicity. However, since they were testing a BSEP inhibition hypothesis, they ignored any "Most-DILI concern" molecules that did not exhibit BSEP inhibition. In our analysis here we evaluated 42 drugs in the Aleo data set, 24 drugs that exhibited "Most DILI concern" and 18 drugs that exhibited "No DILI concern" for which BDDCS classification was available. That is, we ignored drugs classified as "Less DILI" concern. Categorization of DILI concern were derived by examining the currently approved label in the Chen *et al.* (19) data set (and thus the Aleo *et al.* (9) data set). In this data set, compounds with IC50 > 100 μM were defined as BSEP Non-Inhibitors and Mitotox IC50 <100 nmol/mg were defined as mitochondrial toxic compounds. We have also collected data from the 120 compounds investigated by Schadt *et al.* (17) for a number of assays that covered various mechanisms and endpoints associated with human DILI. In that data set 106 drugs were BDDCS classified. For the purpose of this study we chose to focus only on the results of BSEP, mitochondrial toxicity, and cytoxicity assays. As

defined by Schadt *et al.* (17) drugs with BSEP IC50 >250 μM were considered BSEP Non-Inhibitors all others were considered BSEP Inhibitors. For the mitochondrial toxicity assay a ratio of IC50glucose/IC50galactose ≥3 was considered a mitochondrial toxicity flag. Compounds with TC50 <100 μM were considered positive for cellular toxicity.

Classifying BSEP, MRP3 and MRP4 *in vitro* Transport Inhibition

The inhibitory effect of 88 drugs (100 mM) on MRP3- and MRP4- mediated substrate transport was measured in membrane vesicles by Köck *et al.* (16). Drugs selected for investigation included 50 BSEP non-inhibitors (24 non-cholestatic; 26 cholestatic) and 38 BSEP inhibitors (16 non- cholestatic; 22 cholestatic). All compounds but clobetasol propionate ("No DILI"), fluorescein ("No DILI") and valinomycin ("No DILI") could be BDDCS classified. Chlorpropamide was also removed because it is a BDDCS Class 0 compound. Vinblastine ("Hepatocellular") was also omitted from the data set because no BSEP inhibition information was reported. Drugs were also categorized as cholestatic or hepatocellular, according to the DILI type reported in the literature. As defined by Köck *et al.* (16) the compounds were further classified as active for the specified transporter if they had an IC50 ≤ 135 μM for BSEP or a percent inhibition ≥ 21% compared with control at 100 μM for MRP3 and MRP4; otherwise, they were classified as inactive against that transporter. The MRP4 classifications are based on findings by Köck *et al.* (16) that compounds that inhibit MRP4 by at least 21% have a 50% chance of being cholestatic and the rationale for the BSEP classifications is to identify inhibitor compounds with both potent and moderate cholestatic risk, similar to Morgan *et al.* (3).

We also investigated 125 pharmaceuticals (70 of Most DILI Concern and 55 of No DILI Concern) that were screened for MRP3 inhibition (15). For each compound, the IC50 value was also considered in terms of its *in vitro* BSEP inhibition potential. All compounds but triprolidine

hydrochloride (No DILI concern), brompheniramine (No DILI concern), doxylamine (No DILI concern), carbetapentane citrate (No DILI concern), zimeldine (Most DILI concern) and pamabrom (No DILI concern) could be BDDCS classified. BSEP Inhibitors were defined as having IC50> 100 μM (which included the BSEP Weak Inhibitors); MRP3 Inhibitors were defined as having IC50> 300 μM (which included the MRP3 Weak Inhibitors).

BDDCS Classification

As we previously reported in Chapter 3 (12), the assignment of BDDCS Class of each drug was performed by evaluating the available solubility data, maximum dose strength (mg), and extent of metabolism (20). There was a recent expansion on the list of BDDCS drug classification to more than 1100 drugs, including many drugs that have been removed from the market as a result of toxic manifestations (21). This BDDCS classification list was particularly challenging since for many drugs that came onto the market a number of years ago, and then removed because of toxicity, little reliable information both in terms of metabolism and solubility can be found in the literature. Therefore, when a drug is on the border of two classes, the BDDCS class is selected based on expected or known drug interactions.

Hosey and Benet (2015) noted a marked distinction between extensively and poorly metabolized compounds and this can be well predicted based on an *in vitro* measure of drug permeability. Recently, Dave and Morris (2016) showed that the solubility classification could be evaluated using a 0.3 mg/mL cut-off, thus not requiring knowledge of the clinical dose.

Classifying DILI FDA Drug Labels and DILI Severity of Drugs in the Data Set

The DILI potential of the drugs in the data set was classified on the basis of the information on hepatic ADRs extracted from FDA drug labels (19). Briefly, depending on the ADR severity, off market status and FDA drug labels, ADRs may be classified in different

categories as “Discontinued”, “Withdrawn”, “Boxed Warning”, “Warning and Precautions”, “Adverse Reactions” and “No Mention”, ordered by decreasing severity. The DILI severity assessment was categorized as follows: “Most DILI Concern”, “Less DILI Concern” and “No DILI Concern”, ordered by decreasing severity as described by Chen *et al.* (18, 19).

Data Analysis

The distribution of BSEP inhibition in each FDA hepatic liability category, the Chen DILI assessment and the BDDCS class were evaluated. Proportions of each of the assays: hepatobiliary transporters, cell cytotoxicity, or mitochondrial toxicity were tabulated. Positive Predictive Value (PPV), False Negative Rate (FNR), and Accuracy (ACC) were calculated in order to analyze the ability of these *in vitro* assays to predict DILI.

RESULTS

Relationship between BSEP Inhibition and FDA Drug Labels and FDA DILI Severity Assignment

Using the FDA DILI severity assessment, we observe in Figure 4.1A that among the BSEP inhibitors only 29.7% were characterized in the “Most DILI concern” category, while BSEP weak inhibitors show an even higher proportion of 42.9% for “Most DILI concern.” In addition, when we look at the distribution between BSEP Non-Inhibitors vs. BSEP Inhibitors in terms of DILI severity assessed, we observe 14.2% among BSEP Non-Inhibitors vs. 18.9 % among BSEP Inhibitors in the “No DILI” group. Similarly to this point, 29.7% of BSEP Inhibitors vs. 19.2% of BSEP Non-Inhibitors are associated with “Most DILI Concern.” When BSEP inhibition data are correlated with FDA drug labels of registered drugs as shown in Figure 4.1B, we observe no discernible pattern between BSEP inhibition and FDA hepatic liability categories. The distribution of BSEP Inhibitors with higher toxic liability is given as follows:

43.2% of “Warning and Precautions”, 13.5% “Boxed Warning” and 5.4% “Withdrawn” drugs. We note that there is no toxicity differentiation between BSEP Weak Inhibitors and BSEP Inhibitors, with 57.1% of BSEP Weak Inhibitors being in “Warning and Precautions” and 4.8% in the “Boxed Warning.” These results show that BSEP Weak Inhibitors and BSEP Inhibitors are both equally likely to cause hepatotoxicity. Therefore, using BSEP inhibition alone is not an adequate biomarker given the poor differentiation that we observe in the analysis of this dataset.

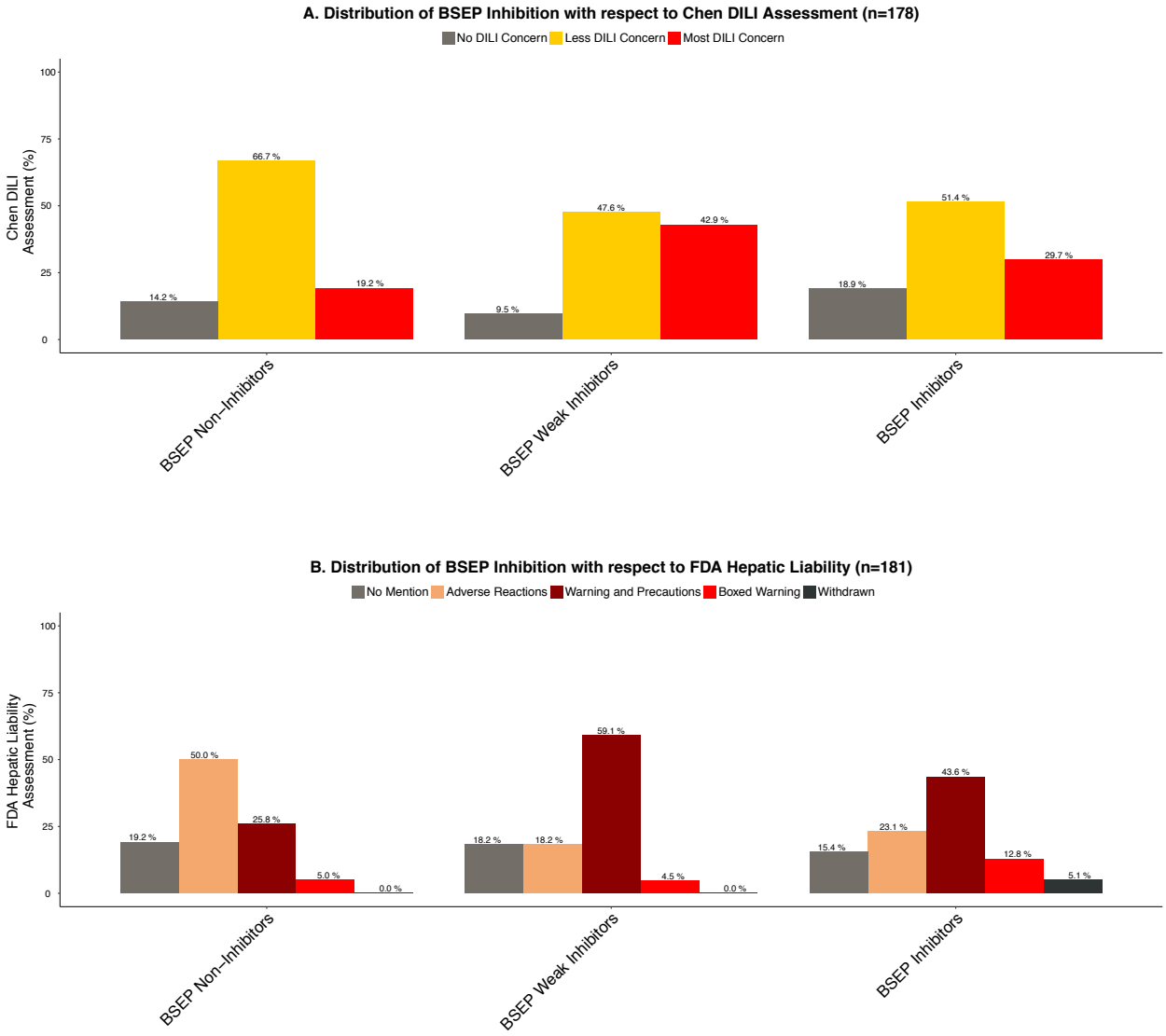


Figure 4.1A. Distribution of BSEP inhibition with respect to the Chen DILI assessment.
(120 drugs BSEP Non-Inhibitors, 21 BSEP Weak Inhibitors, and 37 BSEP Inhibitors)

Figure 4.1B. Distribution of BSEP inhibition with respect to FDA Hepatic Liability.
Drugs assigned according to the most severe drug label section.
(120 drugs BSEP Non-Inhibitors, 22 BSEP Weak Inhibitors, and 39 BSEP Inhibitors)

Relationship between BSEP Inhibition and BDDCS Class

When BSEP inhibition was correlated with BDDCS Class a highly significant result (p-value <0.05) was found. BSEP inhibition was most significant among BDDCS Class 2 compounds (84.6%, n=33/39) (See Figure 4.2A). Our data also depict concomitant decreases in the percentages of BDDCS Class 1 and 3 compounds as the strength of BSEP inhibition increases. We have previously observed as reported in Chapter 3 that as hepatic warning severity increases, the proportion of BDDCS Class 2 drugs increase and the proportions of both BDDCS Class 1 and 3 decrease (12). BDDCS Class 2 drugs were incriminated with the highest proportions in the following drug label sections: "Warning and Precautions" (45.6%, 36/79), "Black Box Warnings"(47.2%, 17/36), "Withdrawn" (62.5%, 25/40) and "Discontinued" (83.3%, 5/6) (See Figure 4.2B). The most potent BSEP inhibitors are BDDCS Class 2 drugs, which we have demonstrated previously is the BDDCS class most likely to be DILI related.

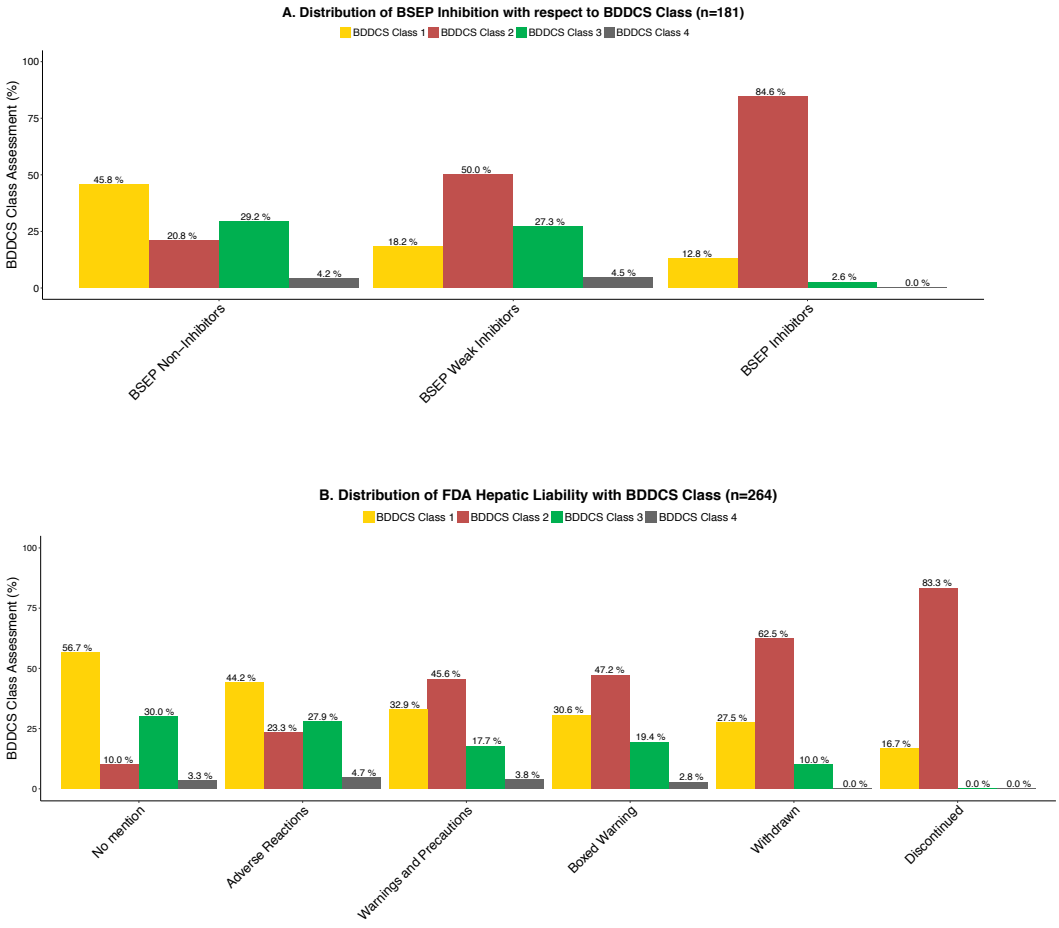


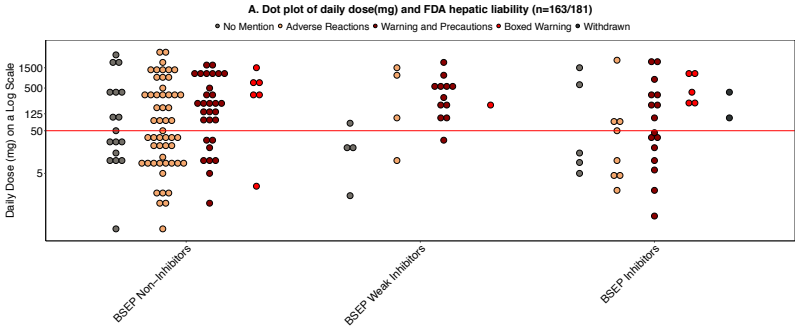
Figure 4.2A. Distribution of BSEP inhibition with respect to BDDCS Class.
(120 drugs BSEP Non-Inhibitors, 22 BSEP Weak Inhibitors, and 39 BSEP Inhibitors)

Figure 4.2B. Distribution of FDA Hepatic Liability with BDDCS Class.
(60 No mention, 43 Adverse Reactions, 79 Warning and Precautions, 36 Boxed Warning, 40 Withdrawn, 6 Discontinued)

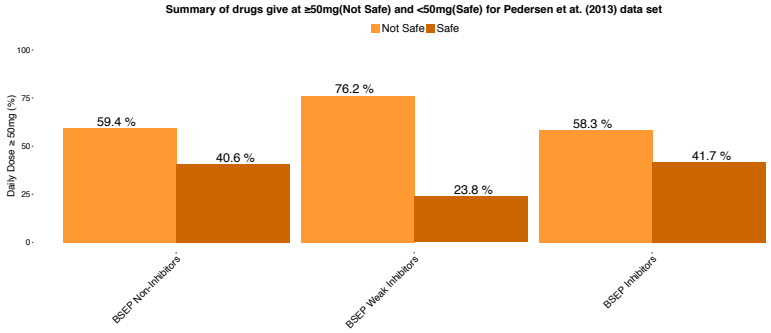
Relationship between BSEP Inhibition and Daily Dosage

Lammert and co-workers have attributed hepatic adverse events to compounds with significant hepatic metabolism and daily dose ≥50mg. We confirmed in Chapter 3 that daily dose provided the best DILI predictability (12). Here we have examined the relationship between

daily dosages against the FDA hepatic liability categories according to the three BSEP inhibition groups for the Pedersen *et al*. (14) data set (See Figure 4.3A). As seen in the dot plot, we observe no difference in the spread of the drugs and the distribution of BSEP inhibition group. We would expect BSEP inhibitors to exhibit a differentiation at dose ≥50mg, but no shift is observed. In Figure 4.3B, we see no difference in terms of dose distribution between BSEP Non-Inhibitors (59.4%) and for BSEP Inhibitors (58.3%) given at "Safe" doses of <50mg for the Pedersen *et al*. (14) data set. However a different conclusion is seen with the Aleo *et al.* (15) data as depicted in Figure 4.3C. The daily dose distribution for BSEP Non-Inhibitors and Weak Inhibitors is almost identical to that observed by Pedersen *et al*. (14). However, a marked distinction for BSEP Inhibitors is seen in the Aleo *et al.* (15) data set, where 75% of BSEP Inhibitors are given at daily doses ≥50mg (Figure 4.3C).



B



C

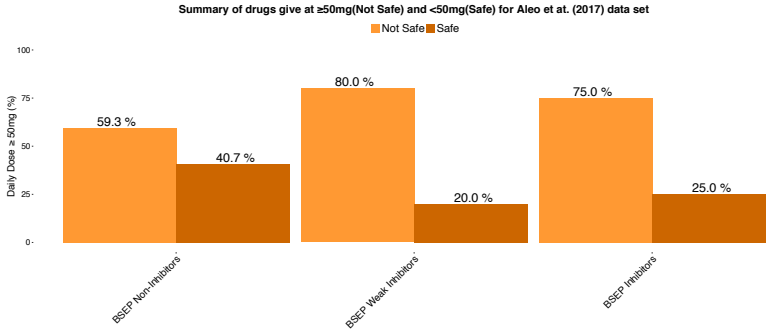


Figure 4.3A. Dot plot of daily dose (mg) and FDA Hepatic Liability from Pedersen *et al*. (14) data set (n=163/181).
(106 drugs BSEP Non-Inhibitors, 21 BSEP Weak Inhibitors, and 36 BSEP Inhibitors)

Figure 4.3B. Summary of drugs given at ≥50mg (Not Safe) and <50mg (Safe) from Pedersen *et al*. (14) data set (n=163/181).
(106 drugs BSEP Non-Inhibitors, 21 BSEP Weak Inhibitors, and 36 BSEP Inhibitors)

Figure 4.3C. Summary of drugs given at ≥50mg (Not Safe) and <50mg (Safe) from Aleo *et al.* (15) data set (n=125).

(91 drugs BSEP Non-Inhibitors, 10 BSEP Weak Inhibitors, and 24 BSEP Inhibitors)

Relationship between Type of Liver Toxicity and MRP3, MRP4 and BSEP Inhibition

With respect to type of liver toxicity, looking at the relationship between BSEP inhibitors vs. BSEP non-inhibitors in Figure 4.4, we observe the least differentiation between cholestatic type of injury (60.5% of BSEP Inhibitors vs. 53.3% of BSEP Non-Inhibitors). When we looked at the relationship between MRP3 Inhibitors vs. MRP3 Non-Inhibitors we observe an increase in MRP3 inhibitors being associated with cholestatic type of injury (64.4% vs. 47.4%). However, examining the relationship for MRP4, we observe that MRP4 had the highest differentiation in terms of cholestatic type of liver injury between MRP4 Inhibitors (72.5%) vs. MRP4 Non-Inhibitors (31.2%) (See Figure 4.4).

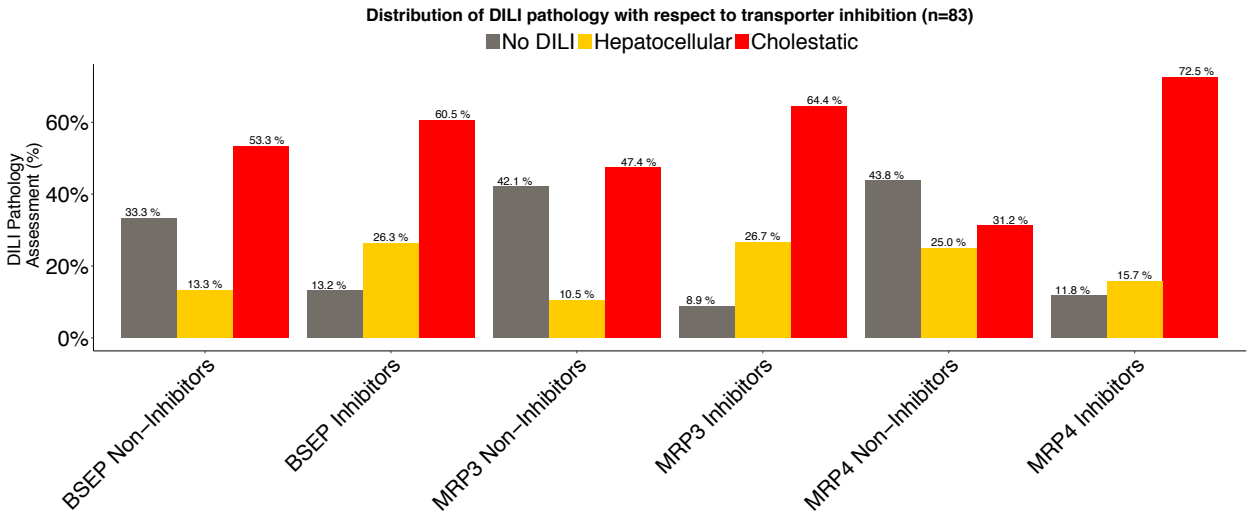


Figure 4.4. Distribution of DILI pathology with respect to transporter inhibition.
(45 BSEP Non-Inhibitors, 38 BSEP Inhibitors)
(38 MRP3 Non-Inhibitors, 45 MRP4 Inhibitors)
(32 MRP3 Non-Inhibitors, 32 MRP4 Inhibitors)

For extent of hepatocellular injury in Figure 4.4, we note a 2-fold increase in BSEP inhibitors that are associated with hepatocellular injury vs. BSEP non-inhibitors. An even greater differentiation is seen for MRP3 Non-Inhibitors 10.5% vs. 26.7% for MRP3 Inhibitors. This differentiation in hepatocellular injury goes the opposite way for MRP4, 25% MRP4 Non-Inhibitors vs. 15.7% MRP4 Inhibitors.

As seen in Figure 4.5, there is marked difference in BDDCS distribution of BSEP. That is, 68.4% of BSEP Inhibitors are BDDCS Class 2 drugs vs. 15.6% of BSEP Non-Inhibitors. In contrast the percentages of Class 1 and Class 3 drugs decrease markedly. For MRP3 and MRP4 Inhibitors, we observe that the distribution of BDDCS Class 1 and 2 is very similar, and we do not observe as much of a decrease between non-inhibitors and inhibitors for BDDCS Class 1 drugs as seen for BSEP. However for all three transporters BDDCS Class 2 compounds constitute the majority of inhibitors.

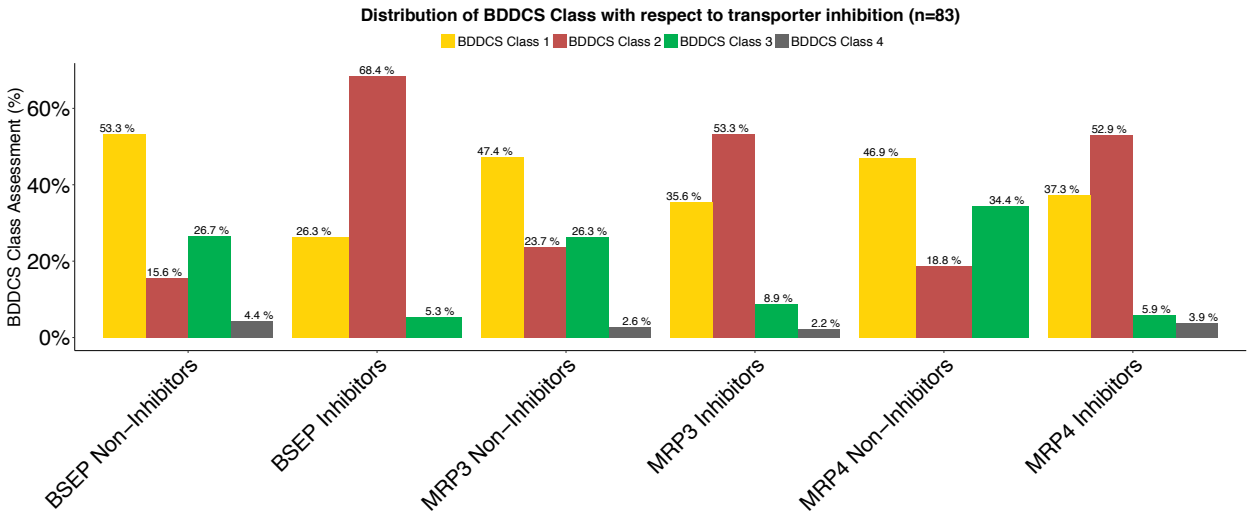


Figure 4.5. Distribution of BDDCS Class with respect to transporter inhibition.
(45 BSEP Non-Inhibitors, 38 BSEP Inhibitors)
(38 MRP3 Non-Inhibitors, 45 MRP4 Inhibitors)
(32 MRP3 Non-Inhibitors, 32 MRP4 Inhibitors)

We note that the highest groups associated with "No DILI" were MRP3 Non-Inhibitors and MRP4 Non-Inhibitors (Figure 4.4), they also have the highest percentage of BDDCS Class 3 drugs (Figure 4.5). In terms of the BDDCS Assessment, we observe a trend that BDDCS Class 3 drugs are much less likely to cause transporter inhibition for BSEP, MRP3 and MRP4.

Comparative Analysis of Mitochondrial Toxicity and BSEP Inhibition Assay

Aleo *et al.* (9) have proposed a synergetic relationship between BSEP and mitochondrial toxicity. They suggest that the involvement of mitochondrial dysfunction appears to be an additional mechanistic liability for DILI. Mitochondrial dysfunction can de-energize a cell and lead to oxidative stress, apoptosis, and hepatocellular injury. Moreover, the accumulation of cytotoxic bile acids within hepatocytes, has been long known to disrupt mitochondrial function. It has been hypothesized that the combination of these attributes of potent inhibition of mitochondrial function and BSEP transport may be more frequently associated with drugs that cause more severe forms of human DILI. It should also be noted that in certain disease states, like type 2 diabetes and nonalcoholic steatohepatitis, there are significant deficits in normal mitochondrial function, which in turn may further predispose individual patients to DILI through this mechanism.

To study this effect we analyzed the Aleo *et al.* (9) data set (Table 4.1A) together with the Schadt *et al.* (17) data set (Table 4.1B) to see if there is indeed a strong correlation between mitochondria mitotoxicity and BSEP inhibition acting synergistically. When comparing the correlation between BSEP inhibition and DILI comparable PPV values are observed for the Aleo and Schadt data sets. The FNR for the Aleo data set is zero because as noted above, Aleo

eliminated any drug showing Most-DILI concern that was not a BSEP inhibitor. Thus, the ACC of the Aleo data set for BSEP inhibition is greater than that for BSEP inhibition in the Schadt data set. For the mitotoxicity assay, Aleo report a higher PPV, lower FNR and higher ACC than Schadt. However, in both Tables 4.1A and 4.1B, BDDCS class 2 characterization shows comparable results to both BSEP and mitotoxicity. Thus, we believe there is no support for either of these measures being useful predictors of DILI potential. As seen in Table 4.1B, Schadt *et al.* (17) also investigated the relationship with cellular toxicity yielding even poorer correlations. Both Aleo *et al.* (9) and Dawson *et al.* (4) differentiated BSEP Inhibitors, as noted earlier, as Weak and Strong Inhibitors. In the analyses here we combined both Weak and Strong as BSEP Inhibitors. However, we tested each relationship reported using the data for only Strong Inhibitors. No marked differences from the reported results were seen between the sets of analyses as we show here in Table 4.1C for the Dawson *et al.* (4) data set.

Table 4.1A. Comparison of BSEP and Mitochondrial Toxicity Assays Associated with DILI (Aleo *et al.* (9) data set).

Criteria	**% Correct (Positive Predictive Value, PPV)**	**% DILI Missing (False Negative Rate, FNR**	**% Accuracy (ACC (True Positive + True Negative/42**
BSEP	72.7%	0.0%	78.6%
Mitotox	94.1%	33.3%	78.6%
BDDCS Class 2	80.0%	16.7%	78.6%

Table 4.1B. Comparison of BSEP and Mitochondrial Toxicity Assays Associated with DILI (Schadt *et al.* (17) data set).

Criteria	% Correct (Positive Predictive Value, PPV)	% DILI Missing (False Negative Rate, FNR	% Accuracy (ACC (True Positive + True Negative/110
BSEP	69.2%	62.5%	65.5%
Mitotox	71.4%	79.2%	61.8%
Cellular Toxicity	48.3%	70.8%	55.5%
BDDCS Class 2	64.6%	35.4%	69.1%

Table 4.1C. BSEP Inhibition Assay Associated with DILI (Dawson *et al.* (4) data set).

WEAK INHIBITORS INCLUDED AS BSEP-INHIBITORS

Criteria	% of Drugs with DILI Predicted Correctly, PPV	% of DILI Missing in the Prediction, FNR	% of DILI Predicted Accurately, ACC (n=83)
BSEP (Strong and Weak Inhibitors)	84.2%	49.2%	54.9%
BDDCS Class 2	88.2%	52.4%	54.9%

WEAK INHIBITORS EXCLUDED

Criteria	% of Drugs with DILI Predicted Correctly, PPV	% of DILI Missing in the Prediction, FNR	% of DILI Predicted Accurately, ACC (n=77)
BSEP (Strong Inhibitors)	87.5%	52.5%	53.9%
BDDCS Class 2	89.7%	55.9%	52.6%

Comparison of BSEP, MRP3 and MRP4 *in vitro* transport inhibition

In Table 4.2, we report the results of our analysis on the effect of transporter inhibition in the prediction of DILI using the Köck *et al.* (16) compilation defined above. Here again for this data set, comparable results are obtained for BSEP inhibition and BDDCS class 2 categorization. However, better predictability values are seen for the correlation with MRP3 and MRP4 inhibition, with MRP3 or MRP4 Inhibitors giving the best predictability. Adding BSEP inhibition to these measures decreases predictability back to BDDCS Class 2 values.

In Table 4.2B we carry out the same assessment with the Aleo *et al.* (15) data set. Here we see no differentiation for MRP3 inhibition with BDDCS Class 2 drugs always giving the best predictability. Our analysis of these data do not support the Aleo *et al.* (15) contention that avoiding dual BSEP and MRP3 inhibitors could lead to less likelihood of causing clinical DILI.

Table 4.2A. Summary of BSEP, MRP3 and MRP4 In Vitro Transport Inhibition and DILI Assessment for the Köck *et al.* (16) Data Set.

Criteria	% of Drugs with DILI Predicted Correctly, PPV	% of DILI Missing in the Prediction, FNR	% of DILI Predicted Accurately, ACC
BSEP Inhibitors	86.8%	47.6%	57.8%
MRP3 Inhibitors	91.1%	34.9%	68.7%
MRP4 Inhibitors	88.2%	28.6%	71.1%
MRP3 or MRP4 Inhibitors	88.3%	15.9%	79.5%
BDDCS Class 2	87.9%	54.0%	54.2%
BSEP and MRP3 Inhibitors	92.6%	60.3%	51.8%
BSEP and MRP4 Inhibitors	90.3%	55.6%	54.2%
BSEP and MRP3 or MRP4 Inhibitors	91.7%	47.6%	60.2%

Table 4.2B. Summary of BSEP and MRP3 In Vitro Transport Inhibition and DILI Assessment for the Aleo *et al.* (15) Data Set.

Criteria	% of Drugs with DILI Predicted Correctly, PPV	% of DILI Missing in the Prediction, FNR	% of DILI Predicted Accurately, ACC
BSEP Inhibitor	80.0%	70.4%	56.3%
MRP3 Inhibitor	55.6%	53.7%	49.0%
BDDCS Class 2	85.2%	57.4%	63.5%
BSEP and MRP3 Inhibitor	77.8%	74.1%	54.2%

DISCUSSION

The accumulation of bile acids within hepatocytes is thought to be a primary mechanism for the development of DILI. However, few reports indicate that drug-induced BSEP dysfunction actually leads to hepatotoxicity, and the relationship between drug-induced BSEP dysfunction and liver injury risk is yet to be determined. Here we show that pharmacological BSEP interference by small molecules is not a strong susceptibility factor. BSEP inhibition alone cannot accurately predict hepatotoxic potential of drugs as depicted by Figure 4.1B. It is unclear as to what extent BSEP inhibition is functionally significant *in vivo*. We observe that the great majority of compounds that have been associated with DILI and are BSEP inhibitors are also BDDCS Class 2. Because we are able to make similar predictions based on BDDCS determinant characteristics, this leads us to discount the predictive ability of mechanistic association of BSEP and DILI. We have previously in Chapter 3 observed that as hepatic warning severity increases, the proportion of BDDCS Class 2 drugs increases and the proportions of both BDDCS Class 1 and 3 drugs decrease (12).

The translation of in vitro potency of a small molecule on inhibiting BSEP to human risk of liver injury is problematic for many reasons. Drug concentrations within human hepatocytes *in vivo* are unknown. It is likely that they are much higher than plasma concentrations. The apparent IC50 values assume all added drug is available in solution. True values are likely to be much lower, due to binding to proteins and lipids. BSEP inhibition by drug metabolites not evaluated in the assay also may be markedly more potent than parent drug.

Aleo *et al.* (9) suggest that mitochondrial toxicity together with BSEP inhibition may provide improved DILI predictability. When we analyzed the predictability of BSEP inhibition together with mitochondrial toxicity, we observe that BDDCS class 2 characterization shows comparable results. Thus, we believe that neither BSEP inhibition nor mitochondrial toxicity are useful independent predictors of DILI.

The activities of a compound on other related transporters, such as the multidrug resistance-associated proteins MRP3, MRP4, and potentially others, may show a greater affect on overall liver injury. Köck *et al.* (16) demonstrated that inhibition of MRP4, in addition to BSEP, may be a risk factor for the development of cholestatic DILI. In Table 4.2A, we report comparable results for BSEP inhibition and BDDCS class 2 categorization. However, MRP4 inhibition gives the best performance amongst MRP3, MRP4, and BSEP inhibition. Our data analysis suggests that screening for MRP4 or MRP3 (although only data from Köck *et al.* (16) and not that of Aleo *et al.* (15) was positive for MRP3) could lead to higher accuracy than BSEP, but that addition of BSEP inhibition to measures of MRP4 and MRP3 inhibition gives less predictability, back to values similar to BDDCS Class 2 only.

Idiosyncratic DILI presents with an array of clinical symptoms and can vary in severity from a mild increase in liver enzymes (alanine aminotransferase (ALT), bilirubin, and alkaline phosphatase (ALP)) to acute liver failure and death. Assessment is based on clinical and biochemical findings, and accurate diagnosis with drug causality requires detailed case patient records reviewed by multiple expert hepatologists. On the basis of biochemical measures, three types of DILI can occur “hepatocellular” caused by damage predominantly to hepatocytes, where serum ALT at the time of maximum elevation is

greater than ALP, “cholestatic” caused by disruption in biliary excretion of bilirubin, where serum bilirubin is elevated and ALP at the time of maximum elevation is greater than ALT, and “mixed”, where ALT, ALP, and bilirubin are elevated. A concomitant rise in ALT (>3× the upper limit of normal range, ULN) and bilirubin (>2× ULN) is suggestive of severe liver injury where hepatocyte damage is coupled with disrupted biliary excretion, increased serum bilirubin, and jaundice. With respect to liver type of toxicity, when we looked at the relationship between BSEP inhibitors and non-inhibitors, we observe that there is no significant difference between cholestatic type of injury, although there was an increase in hepatocellular injury (Figure 4.4). However, the BSEP inhibitor and non-inhibitor from the Köck *et al.* (16) data in Figure 4.4 were compiled from previous reports of Dawson *et al.* (4) and Morgan *et al.* (3). In Dawson *et al.* (4), one can determine that cholestatic DILI was caused by 68.4% of strong BSEP Inhibitors while hepatocellular DILI was caused by only 15.8% of strong BSEP Inhibitors. Such as analysis from the Morgan *et al.* (3) paper is not readily available, but was carried out by Köck *et al.* (16). Yet the data for BSEP inhibition in Figure 4.4 would suggest that the Morgan *et al.* (3) results contradict the Dawson *et al.* (4) report. Thus, we view with skepticism any utility of BSEP inhibition screening for predicting DILI since such predictions as indicated in Tables 4.1 and 4.2 show no differentiation with drugs being BDDCS Class 2.

DILI is multifactorial; inhibition of multiple hepatic efflux transporters could confer additional risk. DILI for many drugs involves cholestasis and accumulation of bile acids within hepatocytes. The adaptive response by the liver is an important component in predicting the potential for cholestatic hepatotoxicity. Bile acid disposition is tightly

regulated by the Farnesoid X Receptor (FXR). FXR activation leads to increased fibroblast growth factor 19 (FGF19), suppression of cytochrome P450 7A1 (CYP7A1), induction of BSEP, MRP3, and organic solute transporter alpha/beta (OSTα/β). Chenodeoxycholic acid (CDCA), an endogenous FXR agonist, upregulates BSEP in human sandwich culture hepatocytes (24). Increased function of basolateral efflux transportes can be an important "safety valve" if BSEP-mediated efflux is compromised. Chenodeoxycholic acid (CDCA) upregulates OSTα/β. Adaptation to the harmful effects of such accumulation can mean the difference between hepatocyte death and survival (25). Basolateral and canalicular efflux transporters play a critical role in hepatic and systemic exposure for some drugs, endogenous compounds, and metabolites. Inhibition of hepatic efflux transporters may increase hepatocyte exposure and cause toxicity. Induction of basolateral efflux transporters may decrease intracellular concentrations and increase systemic exposure. At this stage our analysis suggests that BSEP inhibition itself is not an adequate or useful predictor of DILI potential.

Although mutations in BSEP have been associated with liver disease in a univariate manner, (5) it is not yet fully understood how pharmacological inhibition of BSEP in humans in vivo relates to the familial dysfunction of this transporter. The case examples where autoantibodies to BSEP led to posttransplant liver failure in patients with PFIC2 (26, 27) offer a glimpse at how complete shutdown of BSEP might manifest when exposed to an unlimited challenge. However, this is an example of extreme pharmacology and not necessarily representative of what occurs with small molecules.

There is a general acceptance that inhibitors of BSEP are a source of toxicity. However, according to our analysis of DILI this is not true. What we find is

that most DILI occurs with BDDCS Class 2 compounds and almost all BSEP inhibitors are Class 2 compounds, but we do not see a relationship with the strength of BSEP inhibition and toxicity, which makes us believe that the generally held hypothesis is incorrect.

For the purposes of early screening, binning compounds based on their relative BSEP mediated inhibition does not limit the possibility of liver liabilities in humans. Our data suggest that compounds that are BDDCS Class 2 are as likely as BSEP inhibitors to lead to DILI. As we noted earlier, if potential drug characteristics, such as BSEP inhibition *in vitro* (or mitochondrial toxicity) provides no better prediction than BDDCS Class 2 categorization, one cannot have faith in the proposed toxicology screen.

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CHAPTER 5: Further Examination of the HLA-B*15:02 *In Vitro* Assay and BDDCS Against Other AEDs and Clinical Data on Cutaneous Adverse Reactions

ABSTRACT

Recent advances in pharmacogenetic studies reveal strong genetic associations between human leukocyte antigen (HLA) alleles and their susceptibility to drug hypersensitivity reactions (DHRs). In particular, HLA-B*15:02 has been associated with carbamazepine-induced SJS/TEN. Previously, we have been able to show a strong correlation between the HLA-B *in vitro* assay binding and the toxic responses from cutaneous adverse reactions (cADRs) of antiepileptic drugs (AEDs). The goal of the present study was to further examine the relationship of the BDDCS and the utility of the HLA-B in vitro assay by analyzing the binding/interaction response of other AEDs in this assay. Here we observe that several BDDCS Class 1 drugs are capable of a strong interaction with the HLA-B *in vitro* assay, and that the excellent correlation that we observed in Chapter 2 may not be consistent when expanding the AEDs investigated. Our data show that it is not possible to define which BDDCS Class 1 drugs will predict drug hypersensitivity reactions, some of them are very reactive and bind very strongly in the HLA-B *in vitro* assay (e.g. tiagabine, clonazepam clobazam) and have deleterious effects, while others are not reactive but have deleterious effects (e.g. phenobarbital), and others are not reactive and do not have deleterious effects (e.g. valproic acid and ethosuximide). However, it still appears that good predictions can be made with BDDCS Class 2 and Class 3 drugs.

INTRODUCTION

Recent advances in pharmacogenetic studies revealed strong genetic associations between human leukocyte antigen (HLA) alleles and their susceptibility to drug hypersensitivity reactions. T-cell mediated drug hypersensitivity reactions may range from mild rash to severe fatal reactions. Among them, drug reaction with eosinophilia and systemic symptoms (DRESS) or drug-induced hypersensitivity syndrome (DIHS), Stevens-Johnson syndrome/ toxic epidermal necrolysis (SJS/TEN), are some of the most life-threatening severe cutaneous adverse reactions (SCARs). Very strong associations between HLA-B*15:02 and carbamazepine-induced SJS/TEN have been found among the Han Chinese in Taiwan, which were confirmed by various case-control studies of Southeastern Asian patients (1–10). HLA-B*15:02 is a member of the serotype HLA-B75. Carbamazepine-induced SJS/TEN have also been detected in carriers of some HLA-B75 members, including HLA-B*15:08, HLA-B*15:11 and HLA-B*15:21 in Asian countries, including India, Thailand, Korea and Japan (7, 8, 11).

Considerable effort has been devoted to developing assays that diagnose immunological drug reactions in drug hypersensitive patients. A study by Wei et al. demonstrated involvement of HLA-B75 members in the development of SJS/TEN (12). The rationale is that these HLA alleles share high amino acid sequence homology that may resemble structural features of HLA-B*15:02 and thus may be able to trigger a similar cutaneous adverse reaction to carbamazepine (CBZ) (12). Thus, HLA-B75 can be said to be a risk factor for carbamazepine-induced SJS/TEN in Asian individuals.

Our previous work evaluated the use of the Biopharmaceutics Drug Disposition Classification System (BDDCS) in assisting the prediction of AED drug hypersensitivity reactions and we performed *in vitro* studies to identify specific HLA/drug interactions patterns

(13). Briefly, the BDDCS is a simple classification system that recognizes that drugs exhibiting a high passive permeability rate (BDDCS Class 1 and 2) are extensively metabolized in humans while low passive permeability rate drugs (BDDCS Class 3 and 4) are primarily eliminated as unchanged drug in the bile or the urine (14). We were able to demonstrate a strong correlation between the HLA-B *in vitro* assay and the toxic responses from cutaneous adverse reactions for BDDCS Class 2 AEDs. The current studies follow up on these apparent determinant properties in predicting toxicity potential using BDDCS. For instance, our data on the incidence of cutaneous adverse reactions reveal that toxicity is not limited to just HLA-B*15:02, since Americans and Norwegians, which are populations that do not have this allele present, show the same pattern of toxicity. Because of this finding, here we examine how these general characteristics described by BDDCS class could potentially shed some insights in predicting drug hypersensitivity reactions.

In this work we further examine the relationship of the BDDCS and the utility of the HLA-B *in vitro* assay by analyzing the binding/interaction response of other AEDs examined in the American and Norwegian retrospective studies that have not been tested by us previously.

METHODS

HLA-B *In Vitro* Assay

Surface plasmon resonance (SPR) has previously been used to examine the molecular interactions between soluble major histocompatibility complex (MHC) molecules and peptides, and the specificity and advantages of the method has been established (15). Using this methodology as reported in Chapter 2, we have tested the applicability of this tool as an HLA-B in vitro assay in which we examine the molecular interaction between the soluble MHCs and

small molecules. We used the Biacore T200 SPR biosensor for analyzing the interaction between HLA-B proteins and drugs according to the manufacturer's protocol (GE). We immobilized the purified soluble HLA-B proteins (acting as ligands) on the chips by an amine coupling reaction. The immobilized level for HLA-B*15:02 was 9311 response units (RU). We have specifically selected to test: HLA-B*15:02. HLA-B*81:02 and HLA-B*40:01 were used as our negative controls. PBS was used as running buffer and the flow rate was 10 mL/min. The compounds (7 marketed drugs to complement our previous findings and analysis) dissolved in PBS with 5% DMSO were evaluated and flowed through the solid phase with the running buffer PBS with 5% DMSO. Responses of the interaction were reference subtracted and corrected with a standard curve for the DMSO effects. We used BIA evaluation Version 3.1 for data analysis. Assays were performed in triplicate.

BDDCS Classification

The BDDCS class assignments for the compounds were obtained from the "BDDCS applied to over 900 drugs" paper (16). The 7 additional drugs tested here were: clobazam, clonazepam, felbamate, phenobarbital, primidone, tiagabine, and vigabatrin of which 4 were BDDCS Class 1 drugs, 1 BDDCS Class 2 drug, 1 BDDCS Class 3 drug, and 1 BDDCS Class 4 drug.

Data Analysis

To complement our literature search for validating the finding of the SPR HLA-B *in vitro* assay, we have reviewed the literature for any information available for the cutaneous adverse reactions associated with the compounds tested.

RESULTS

Analysis of AEDs tested in the HLA-B*15:02 *in vitro* assay and their BDDCS Classification

In Figure 5.1, which includes results from the present investigation and our previously published results (13) reviewed in Chapter 2, we observe that many of the BDDCS Class 1 drugs that we have tested bind very strongly to HLA-B*15:02, even more strongly than we previously reported with BDDCS Class 2 drugs. We also present data for one BDDCS Class 4 drug, felbamate, which shows a poor binding interaction to HLA-B*15:02. We observe weak binding interactions for all the BDDCS Class 3 drugs tested. Combining our previous results with the data here, we observe that the following BDDCS Class 1 drugs are strong binders to HLA-B*15:02: tiagabine, clonazepam, and clobazam. The following BDDCS Class 2 drugs: carbamazepine, lamotrigine, phenytoin and oxcarbazepine interacted strongly with HLA-B*15:02. Primidone is the only BDDCS Class 2 drug that showed poor binding to HLA-B*15:02. All of the BDDCS Class 3 drugs: topiramate, gabapentin, levetiracetam, and vigabatrin showed a poor interaction with HLA-B*15:02. The following BDDCS Class 1 drugs showed a poor interaction with HLA-B*15:02: ethosuximide, valproate and phenobarbital. Felbamate a BDDCS Class 4 drug also showed a poor binding interaction with HLA-B*15:02.

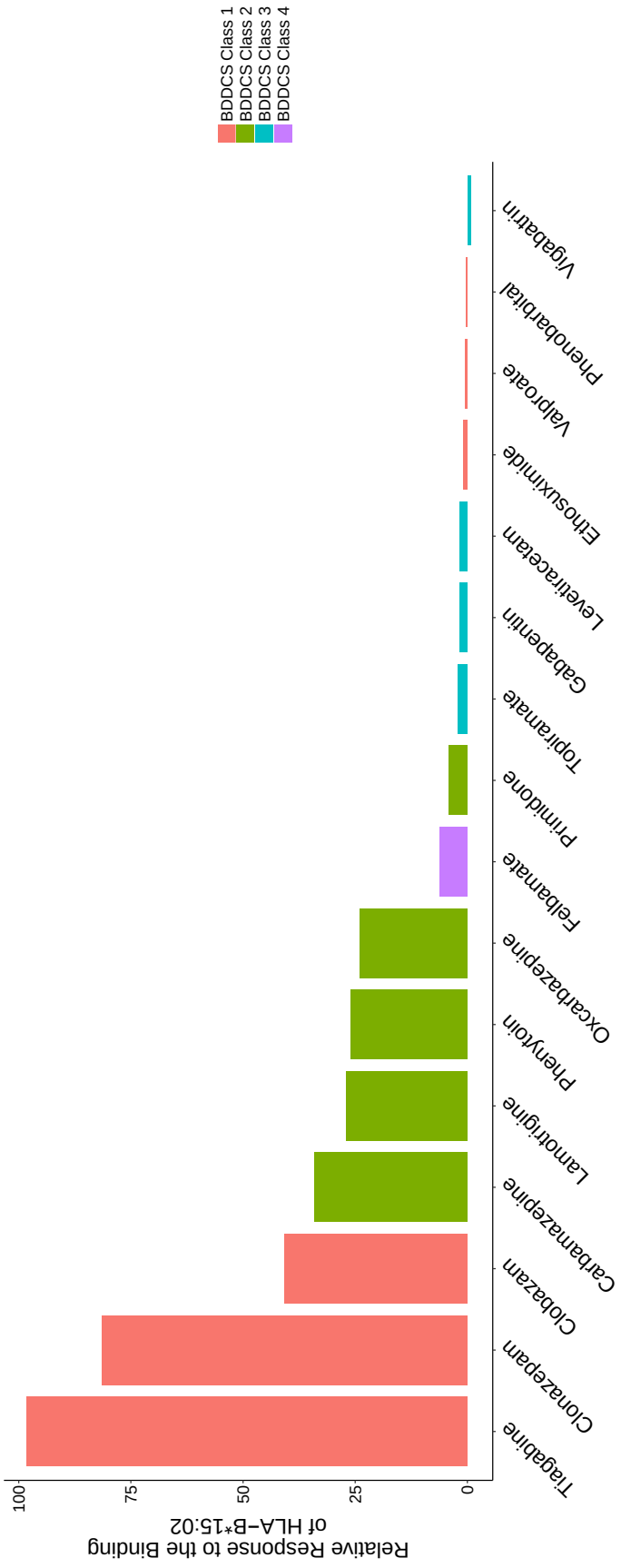

Figure 5.1: Surface Plasmon Resonance (SPR) data demonstrating the specific interactions of 16 molecules binding responses to HLA-B*15:02.
The HLA-B *in vitro* assay was performed at 500 µM for clobazam, clonazepam, felbamate, phenobarbital, primidone, tiagabine, and vigabatrin. All other drugs were tested at 1mM.

Clinical Data of AEDs and its Correlation with HLA-B*15:02 Assay

In the American retrospective study by Arif and coworkers (17), we observe that BDDCS class 2 drugs (phenytoin, lamotrigine, and carbamazepine) showed the highest rates of cutaneous adverse reactions. We observe that BDDCS class 1 drugs (tiagabine, zonisamide, and phenobarbital) also showed a high rate of cutaneous adverse reactions (See Figure 5.2A). Primidone is the BDDCS class 2 that showed the lowest rate of cutaneous adverse reactions and it also showed poor binding in the HLA-B*15:02 assay.

In the Norwegian retrospective study by Alvestad and coworkers (18), we observe that BDDCS class 2 drugs (carbamazepine, phenytoin, lamotrigine, oxcarbazepine) show the highest rates of cutaneous adverse reactions. BDDCS Class 1 drugs (phenobarbital, clonazepam, valproate, clobazam, ethosuximide) show a much lower rate of cutaneous adverse reactions (See Figure 5.2B).

Our results show that phenobarbital and primidone, two antiepileptic drugs that have been on the market for quite some time, do not bind to HLA-B*15:02. Primidone gets converted to phenobarbital when metabolized. We observe that phenobarbital, a BDDCS class 1 drug, has a high rash rate in the American study but a much lower rash rate in the Norwegian study. In the HLA-B *in vitro* assay, phenobarbital shows a poor binding interaction with HLA-B*15:02. When primidone is given, sufficient doses are usually administered to produce therapeutic concentrations of both phenobarbital and primidone. At present, concentrations of the other possible active metabolite of primidone, PEMA, are not routinely measured. While animal experiments indicate that primidone has inherent antiseizure activity, some clinicians believe that phenobarbital is the predominant species responsible for the therapeutic effect of primidone in

humans (19). Because phenobarbital and PEMA are produced via hepatic metabolism of primidone, it is very difficult to study the antiepileptic activity of primidone alone in patients. In contrast, primidone a BDDCS Class 2 showed a low rash rate in the Norwegian study and a poor binding interaction in the HLA-B *in vitro* assay.

BDDCS class 2 drugs (lamotrigine, oxcarbazepine, carbamazepine, and phenytoin) showed the highest rate of cutaneous adverse drug reactions across both studies. Gabapentin, felbamate, clobazam, clonazepam, valproate, topiramate, levetiracetam, and vigabatrin had the lowest rates of CARs. Hence, it appears that BDDCS class 2 AEDs exhibit the highest trend of causing cutaneous adverse reactions followed by certain BDDCS class 1 drugs, in particular zonisamide, phenobarbital, and tiagabine. Valproic acid, a widely used AED, clonazepam, and clobazam are BDDCS class 1 presenting lower levels of adverse cutaneous reactions than the other aforementioned BDDCS class 1 drugs.

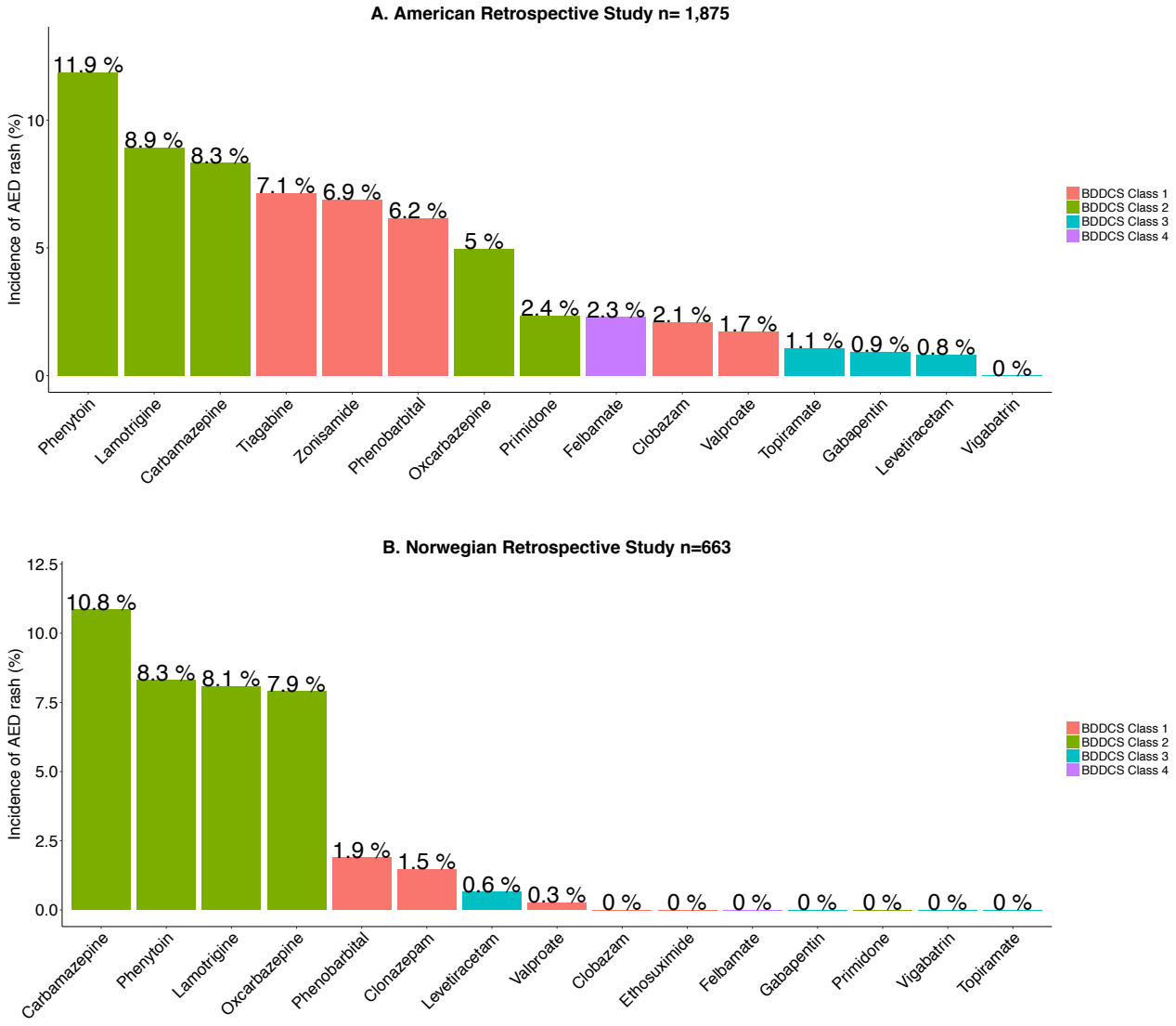

Figure 5.2A. American retrospective study of cutaneous rash rates.
Figure 5.2B. Norwegian retrospective of cutaneous rash rates.

DISCUSSION

Several studies have revealed that HLA-B*15:02 is associated with an increased risk of SJS/TEN upon exposure to phenytoin, oxcarbazepine, and potentially lamotrigine in a Taiwanese population although the strength of these associations was weaker than that of CBZ (20). Small case studies in Thailand (4 cases of phenytoin induced SJS) and Hong Kong (single cases of phenytoin and lamotrigine induced SJS) also showed the presence of HLA-B*15:02 in all SJS patients (1, 2). Recently, a meta-analysis of the relationship between aromatic amine anticonvulsants-induced SJS/TEN and HLA-B*15:02 in Han Chinese populations showed a strong association of HLA-B*15:02 with phenytoin (OR 4.26; 95% CI 1.93–9.93;) and with lamotrigine (OR 3.59; 95% CI 1.15–11.22; = 0.03) (21). These studies confirmed a clinically relevant association between the HLA-B*15:02 allele and phenytoin-induced SJS/TEN, supporting the US Food and Drug Administration recommendation that health care providers should consider avoiding phenytoin and its prodrug, fosphenytoin, as alternatives for CBZ in HLA-B*15:02 carriers (22). Reports in the literature also present a statistical association between HLA-B*15:02 and lamotrigine-induced SJS/TEN in Han Chinese subjects (23). Because of these studies and the positive results of our *in vitro* HLA-B assay with carbamazepine, phenytoin, oxcarbazepine, and lamotrigine, we believe that there is value in further working on this assay for industry use in the screening of drugs. Previously, we did not observe a strong interaction for the two BDDCS Class 1 investigated drugs (ethosuximide and valproic acid) in the HLA-B *in vitro* assay. We had seen that the majority of the binders were BDDCS Class 2 drugs (13).

Our HLA-B *in vitro* data show that BDDCS Class 1 drugs: tiagabine, clonazepam and clobazam have a high risk of leading to drug hypersensitivity reactions. Although, we observe a great consensus for the prediction of toxicity for BDDCS Class 2 drugs, this is not true for

BDDCS Class 1 drugs. Moreover, we cannot trust our results for BDDCS Class 1 drugs because there is a high variability between the *in vitro* results and the clinical data reported. For instance, valproate is an AED that shows no interaction in the *in vitro* assay and no toxicity, but tiagabine, clonazepam, clobazam show very strong interaction and in some cases high toxicity and phenobabrbital, no interaction yet in some cases high toxicity.

We have previously reported a trend of BDDCS class 2 drugs (lamotrigine, oxcarbazepine, carbamazepine, and phenytoin) having the highest rate of cutaneous adverse drug reactions. In the current study, we show that primidone, a BDDCS Class 2 compound, does not interact with HLA-B*15:02 in the HLA-B *in vitro* SPR binding assay. However, high rates of phenobarbital-induced SJS/TEN has been reported according to the US Food and Drug Administration and our literature review.

In patients with a history of drug-induced skin rash, BDDCS Class 3 AEDs such as gabapentin, topiramate, levetiracetam and vigabatrin appear to carry a lower risk of skin rash and other cross-reactivities. A few BDDCS class 1 drugs such as valproate and ethosuximide also appear to be at low risk, results confirmed in our HLA-B *in vitro* assay. However, other BDDCS class 1 drugs (tiagabine, clonazepam, and clobazam) show a strong binding to HLA-B*15:02, and in some cases clinical data demonstrating high rates of cutaneous adverse reactions.

Apart from HLA alleles association with drug hypersensitivity, contributions of genetic variants of metabolic enzymes in cutaneous adverse drug reactions had been proposed as well. For example, primidone converts to phenobarbital and PEMA; it is still unknown which exact cytochrome P450 enzymes are responsible for this metabolism.

Overall, after we further examined additional BDDCS Class 1 drugs (tiagabine, clonazepam, clobazam, and phenobarbital), and an additional BDDCS Class 2 drug, we observe

that the HLA-B *in vitro* assay does not predict as well as we have shown previously. It looks like we can trust the prediction for BDDCS Class 2, except for primidone. But we cannot trust our results for BDDCS Class 1 drugs because there is a high variability between the in vitro results and the clinical data reported. For instance, valproate is an AED that shows no interaction in the in vitro assay and no toxicity, but tiagabine, clonazepam, clobazam show very strong interaction and in some cases high toxicity and phenobarbital, no interaction yet in some cases high toxicity (See Figure 5.1 and Figure 5.2).

In patients who are positive for HLA-B**15:02*, we believe that alternative medications should be used as first-line therapy. Consideration in the choice of alternative medications should be given to the possibility of cross-reactivity with structurally similar AEDs (lamotrigine, phenytoin, oxcarbazepine) according to our HLA-B *in vitro* assay results.

The risk factors for drug hypersensitivity reactions (DHRs) include high mass dose, route of administration, sex, viral infections and genetic factors. There appear to be other HLA alleles that can link to carbamazepine (CBZ)-induced SJS/TEN. Some of the cases of CBZ-induced SJS/TEN did not carry the HLA-B*15:02 allele, suggesting that other genetic variants may play a role. HLA-A*31:01 (24) is a risk factor for various types of carbamazepine-induced cADRs, ranging from mild ones such as maculopapular exanthema (MPE) to severe ones, including SJS/TEN and DIHS, in both Asian and white patients. The pathogenesis of HLA-A*31:01 involvement in the development of cADRs remains to be elucidated, which could help discriminate rashes that are likely to progress from those that are likely to resolve. To date, approximately 25 HLA-associated ADRs have been identified, most of them reaching genome wide significance (25). The discovery of strong associations between DHRs and particular HLA

alleles further implicates MHC-restricted T-cell responses in disease etiology as the primary role of the proteins encoded by HLA is to effectively present antigenic peptides to passing T-cells.

However, prediction of DHRs in the clinic, based solely on HLA-genotype, remains very limited. This is because the majority of individuals who carry known HLA risk alleles do not develop immunological reactions when exposed to a culprit drug. We must therefore assume that immunological parameters, other than HLA genotype, may also contribute to the development of a drug-specific T-cell response (26). Therefore the utility of the BDDCS drug disposition characteristics come into play. Since susceptibility to drug hypersensitivity is a function of the patient's individual biology, the prediction of drug hypersensitivity will involve capturing the patient's biology and variability during the early stages of drug development within preclinical test systems. Characterization of the molecular pathophysiological mechanism(s) of drug hypersensitivity using a combination of *in vitro* assays and animal models is a critical step toward designing assays that will accurately predict which new drug will cause these reactions before they become widely used as therapeutics.

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CHAPTER 6: Review of the Use of the BDDCS to Evaluate the Relevance of DILI Predictive Hypotheses in Early Drug Development

ABSTRACT*

Severe drug-induced liver injury (DILI) remains a major safety concern due to its frequency of occurrence, idiosyncratic nature, poor prognosis, and diverse underlying mechanisms. Numerous experimental approaches have been published to improve human DILI prediction with modest success. A number of *in vitro* screening assays have been developed to help assess the potential DILI risk, such as inhibition of mitochondrial function, hepatobiliary transporter inhibition, reactive metabolite formation and covalent binding, and cellular health. Several studies have also shown a correlation of total administered dose alone or in combination with drug lipophilicity with higher risk of DILI. However, it would be best to have a predictive DILI methodology early in drug development, long before the clinical dose is known. Here we discuss the extent to which BDDCS defining characteristics, independent of knowing actual drug pharmacokinetics/pharmacodynamics and dose, can be used as a comparison baseline matrix of potential DILI adverse events for prior published predictive proposals. Our results show that BDDCS Class 2 drugs exhibit the highest DILI severity, and that all of the published methodologies evaluated here, except when daily dose is known, do not yield markedly better predictions than BDDCS. The assertion that extensively metabolized compounds are at higher risk of developing DILI is confirmed, but can be enhanced by differentiating BDDCS Class 2

* Modified from manuscript submitted: Chan R, Benet LZ. 2017. Review of the use of the BDDCS to evaluate the relevance of DILI predictive hypotheses in early drug development. *Toxicol. Res. (Camb).*

from Class 1 drugs. Conclusion: Our published analyses suggest that comparison of proposed DILI prediction methodologies with BDDCS classification is a useful tool to evaluate the potential reliability of newly proposed algorithms. This is true since almost all of these predictive DILI metrics do no better than just avoiding BDDCS Class 2 drugs.

INTRODUCTION

Severe drug-induced liver injury (DILI) remains a major safety concern due to its frequency of occurrence, idiosyncratic nature, poor prognosis, and diverse underlying mechanisms. Numerous experimental approaches have been published to improve human DILI prediction with modest success. Idiosyncratic DILI (IDILI) is very complex. Most IDILI appears to be immune mediated, and reactive metabolites appear to be involved in most, but not all IDILI. Reactive metabolites are widely accepted as playing a pivotal role in the pathogenesis of idiosyncratic adverse drug reactions. While there are today well-established strategies for the risk assessment of stable metabolites within the pharmaceutical industry, there is still no consensus on reactive metabolite risk assessment strategies. This is due to the complexity of the mechanisms of these toxicities as well as the difficulty in identifying and quantifying short-lived reactive intermediates such as reactive metabolites. In addition, there are probably several mechanisms by which a drug or reactive metabolite can induce an immune response.

Across the pharmaceutical industry, systems of screening drug candidates have emerged that include transcriptomic profiling of animals in addition to animal pathology, assessment of covalent binding and glutathione (GSH) adducts in microsomal test systems and *in vivo*, inhibition of bile salt export pump (BSEP) *in vitro*, impairment of function of isolated animal mitochondria, and cell stress responses and viability in human hepatoma and hepatocyte culture systems. Several common themes emerge in all these test systems especially involving oxidative stress, mitochondrial impairment, covalent binding, and endoplasmic reticulum stress. It has been proposed that test systems have moderately strong predictive value for IDILI (1–3), which we evaluate here. Others have examined combinations of mechanistic assays to better predict hepatotoxicity potential (4, 5), also evaluated here. Several studies have shown a correlation of

total administered dose alone (6) or in combination with drug lipophilicity (7) with higher risk of DILI. However, it would be best to have a predictive DILI methodology early in drug development, long before the clinical dose is known.

Since liver injury has been reported with a large number of drugs, efforts have been undertaken to compile human hepatotoxicity data, including the National Institute of Health LiverTox Database(8) and the FDA Liver Toxicity Knowledge Base (LTKB) (9). These publicly available datasets have enabled development of new structure activity relationships for hepatotoxicity endpoints or triggered the development of knowledge-based and quantitative structure activity relationships (QSAR) models (10–12).

We have reviewed the applicability of the Biopharmaceutics Drug Disposition Classification System (BDDCS) to be compared with presently proposed predictive procedures in evaluating DILI toxicity. Since its inception, the BDDCS has been useful in drug discovery for predicting routes of elimination, oral drug disposition, food effects on drug absorption, transporter effects on drug absorption, and potentially clinically significant drug interactions that may arise in the intestine, liver and brain (13). In Chapter 2 and 3 we have shown that the BDDCS can be useful in predicting the potential for antiepileptic drugs to cause cutaneous adverse reactions and DILI (14, 15). BDDCS's strong relationship between dose, metabolic susceptibility, solubility and idiosyncratic DILI highlights the potential benefits of BDDCS as a comparison matrix for DILI prediction.

The BDDCS was developed in 2005 after Wu and Benet recognized that highly permeable compounds, as outlined by the Biopharmaceutics Classification System (BCS), were extensively metabolized, while poorly permeable drugs were primarily eliminated unchanged in the urine or bile (16). BDDCS demonstrated that simple passive membrane permeability

measures were highly selective in differentiating extensively vs. poorly metabolized drugs in humans. Drugs in the BDDCS are classified according to the membrane permeability rate and aqueous solubility. These characteristics have helped BDDCS define whether metabolic enzymes and/or transporters are clinically important. BDDCS features are demarcated by high and low values, classifying drugs into four categories. These classes are each associated with specific predictions regarding route of elimination and which interactions may be a clinical concern.

Here we provide a review on the extent to which BDDCS defining characteristics, independent of knowing actual drug pharmacokinetics/pharmacodynamics and dose, can be used as a comparison baseline matrix of potential DILI adverse events with prior published predictive proposals (13, 15). We review the clinical impact of BDDCS in evaluating the severity of DILI warnings in drug labels approved by the Food and Drug Administration (FDA) (17), the withdrawal status due to adverse drug reactions (ADRs), the role of BSEP inhibition, maximum daily dosages prescribed, and *in vitro* toxicology assays applied to cover various mechanisms and toxicity endpoints associated with human DILI (15).

Assessment of the BDDCS Classification on FDA Drug Labels Associated with DILI Hepatic Liability

In Chapter 3, we reported the BDDCS class relationship of hepatotoxicity between the different ADR categories by calculating the proportion of drugs in each FDA hepatic liability category, and each DILI severity category (15). As depicted in Figure 3.3A, we observe that as the hepatic warning severity increases, the proportion of BDDCS Class 2 drugs increases and the proportions of both BDDCS Class 1 and 3 drug decrease, all with highly significant trends. The "No mention" category is significantly different from all other categories, except for "Adverse

Reactions." BDDCS Class 2 drugs were incriminated with the highest proportions in the following drug label sections: "Warning and Precautions" (45.6%, 36/79), "Boxed Warning" (47.2%, 17/36), "Withdrawn" (62.5%, 25/40) and "Discontinued" (83.3%, 5/6). Obviously, the number of drugs designated as exhibiting severe DILI increases as the ADR severity increases. That is, 15.9% (7/44) in the "Adverse Reactions" category, 36.7% (29/79) in the "Warning and Precautions" and 81.6% (31/38) of the drugs in the "Black Box Warning" are assessed to exhibit severe DILI (See Figure 3.2). In Figure 3.3B and 3.3C the two BDDCS determinants (extent of metabolism and solubility) are examined. The percentages of poorly metabolized (Figure 3.3B) and of highly soluble (Figure 3.3C) drugs show statistically significant decreases with hepatic liability, while low solubility drugs increase significantly (Figure 3.3C) with hepatic liability. The percent of extensively metabolized drugs also increases with hepatic liability, but since almost 2/3 of "No mention" drugs are metabolized, it is apparent that extent of metabolism itself is not a discriminating parameter. Although greater extent of metabolism has been reported to significantly increase the potential of a compound to cause DILI (18), this property alone is not able to distinguish compounds that are "No mention" of hepatic liability from those compounds exhibiting hepatic liability (See Figure 3.3B).

Our examination of the relationship between the BDDCS's determinant properties: solubility and extent of metabolism, led to some novel observations. Drugs belonging to BDDCS Class 1 and 3 exhibited a lower proportion of DILI severity. Drugs that are extensively metabolized and have low aqueous solubility, i.e., BDDCS Class 2 drugs have the highest rates of DILI risk. BDDCS Class 2 drugs exhibited the highest proportions among the "Warning and Precautions", "Black Box Warning", "Withdrawn" and "Discontinued" categories. These are notably considered the most serious DILI risk categories (See Figure 3.3A). These findings

demonstrate the importance of intrinsic drug properties as a potential factor for the development of a DILI event.

Drugs belonging to BDDCS Class 3 and 4 exhibited much lower proportions in the FDA hepatic liability (See Figure 3.3A). Moreover, BDDCS Class 3 and 4 drugs show little risk of liver aminotransferases increase and hyperbilirubinemia. However, we note the underrepresentation of BDDCS Class 4 drugs in the overall scheme of marketed approved drugs. Compounds with poor hepatic metabolism had been previously noted to be significantly less likely to cause hepatotoxicity (18). Although a lack of hepatic metabolism does not assure total lack of hepatotoxicity, it indeed appears that BDDCS Class 3 and 4 drugs lead to a lower DILI severity.

Barton and co-workers (19) have previously discussed a new paradigm for navigating compound properties related to drug attrition. Optimizing the exposure of potent compounds at the desired site of action and in tissues associated with toxicity is fundamental to addressing attrition via efficacy and safety. Traditional oral drug space is well defined with respect to physicochemical properties and absorption, distribution, metabolism, excretion and toxicity (ADMET) risks but increased focus on ligand-lipophilicity efficiency, maximizing enthalpy contributions and new target classes challenge this paradigm. Barton et al. (19) propose that BDDCS Class 3 compounds should be significantly more associated with drug attrition because they tend to be transporter substrates or inhibitors. Furthermore, they suggest that compounds that are substrates for transporters as being a toxicity liability. We completely disagree with this suggestion based on our analysis of DILI potential (15) and antiepileptic drugs' cutaneous adverse events (14). Our analysis suggests that BDDCS Class 3 compounds exhibit less toxicity potential.

Assessment of Daily Dosage on FDA Drug Labels and DILI Severity

Numerous compound- and/or patient-specific risk factors can contribute to the susceptibility to DILI. IDILI has been shown to be dependent on both daily dose and extent of hepatic metabolism of a drug (6, 18, 20, 21). Lammert and coworkers (6, 18) have attributed hepatic adverse events to compounds with significant hepatic metabolism and daily dose ≥ 50mg. Formation of reactive metabolites, high covalent body burden (22, 23), mitochondrial dysfunction (resulting in the depletion of cellular energy supply and the generation of damaging reactive oxygen species), cell damage from oxidative stress (caused by reactive oxygen or reactive nitrogen species), and local inflammatory effects (24). All of these mechanisms are often interconnected and have, under various circumstances, been associated with the formation of chemically reactive metabolites.

In Chapter 3 evaluated the relationship between daily dosages ≥ 50mg against the already assessed FDA hepatic liability categories and DILI severity assessment (15). Our analysis concurs with the association of drugs being given at dosages ≥ 50mg/day having more adverse hepatic events. We have further evaluated this observation by examining the FDA hepatic liability distribution and DILI severity assessment. Drugs with a daily dose ≥ 50mg had a much higher frequency of toxicity as evidenced by the higher percentages in the "Warning and Precautions", "Boxed Warning" and "Withdrawn" label sections (Figure 3.5A). For the DILI assessment in Figure 3.5B we also observe a higher frequency in DILI severity for compounds that are dosed at ≥ 50mg/day.

Although, there is strong evidence that dosages ≥ 50mg/day are associated with increased risk for hepatotoxicity, many drugs are safe at such dosages. For instance, the 50mg/day dosage cut off would predict that 44% of "No mention" and/or "No DILI" drugs (See Figure 3.5) exhibit

"Not Safe" potential in terms of hepatotoxicity. Thus, supporting that daily dosage alone is not a reliable means of guiding the drug development process, regulatory application, and clinical practice.

BDDCS Classification Prior to Dosing in Humans

Although the finding of Uetrecht shows that idiosyncratic drug reactions were rare among individuals given drug doses <10mg/day and more likely among individuals given drug doses ≥ 1000mg/day (25), the dose relationships can only be determined for a new molecular entity after the drug has been administered to human subjects/patients. In contrast, BDDCS class can be predicted prior to ever dosing the compounds to animals and humans as we have proposed previously (26). Hosey and Benet (27) showed that based on *in vitro* permeability measurements, the positive predictive value (PPV) for prediction of extensive metabolism were all 90% or greater. And most recently Dave and Morris (28) showed that they were able to correctly predict highly soluble vs. poorly soluble drugs using measured solubility parameters with greater than 85% probability.

Drug Metabolism and Propagation of Drug Hypersensitivity Reactions

Drug metabolism also plays an important role in the initiation and propagation of drug hypersensitivity through the generation of neoantigens that are recognized by the cellular and humoral immune systems (5). Although the majority of drug biotransformations occur in the liver, there is overwhelming evidence to suggest that localized drug metabolism by immune cells is critical for organ-specific reactions such as cutaneous adverse drug reactions (5, 29, 30). These reactions are usually rare and are not typically present in animal species, but they can be serious

and even fatal in humans (31, 32) and may lead to the withdrawal of otherwise effective therapeutic agents. At present, during preclinical drug evaluation there are no widely accepted methods for the identification of drugs that may cause hypersensitivity or idiosyncratic reactions. It has been demonstrated that HLA-B*1502 is not only a genetic marker but also a key determinant in the pathophysiology of carbamazepine related Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis (SJS/TEN). In Chapter 2 we assessed the use of the Biopharmaceutics Drug Disposition Classification System (BDDCS) to distinguish antiepileptic drugs (AEDs) associated with and without cutaneous adverse events by examining the binding relationship of AEDs to HLA-B*15:02 and data from extensive reviews of medical records. We also evaluated the lack of benefit from a Hong Kong population policy on the effects of screening for HLA-B*15:02 and previous incorrect structure-activity hypotheses. Our analysis concludes that BDDCS Class 2 AEDs are more prone to cause adverse cutaneous reactions than certain BDDCS Class 1 AEDs and that BDDCS Class 3 drugs have the lowest levels of cutaneous adverse reactions. We propose that BDDCS Class 3 AEDs should be preferentially used for patients with Asian backgrounds (i.e., Han Chinese, Thai and Malaysian populations) if possible and in patients predisposed to skin rashes (14).

Alfirevic and Pirmohamed (33) and Urban et al. (34) have summarized the current state of pharmacogenomics and suggested that although certain HLA and other differences are related to a higher susceptibility of DILI from a number of agents, the actual number of drugs identified as having these genetic risks is still quite small, and the accuracy of most polymorphisms is limited. Although significant advances in our hepatotoxicity knowledge base have been made by the DILI Network and others (35), when it comes to identifying the specific components of DILI

risk, it appears to be much more complicated than just being a matter of daily dose or drug disposition (20).

Comparison of *In Vitro* Mechanism Based Toxicity Endpoints

Although, a number of compound–specific liability factors have been linked with DILI susceptibility, it is difficult to understand which risk factors are more important in patient-specific responses and/or environmental stimuli. One approach followed by many research groups is to assess and reduce some of the more common, drug-specific factors in a set of targeted *in vitro* assays. The most common mechanisms covered in *in vitro* high throughput screening assays include reactive metabolite formation and covalent binding (36, 37), inhibition of drug transporters involved in hepatobiliary elimination of bile acids and other metabolic endogenous products (BSEP, MRPs) (3, 38), mitochondrial toxicity (39) and different cellular toxicity assays covering the formation of drug-metabolites (4, 40–43). Various approaches are used in the pharmaceutical industry for hazard identification and risk assessment of reactive metabolites and more integrated strategies that include measures of the initial mechanism of toxicity have been highlighted in our analysis.

In Chapter 3 we performed a comparison of the different predictive metrics in the various assays measuring key mechanisms of toxicity endpoints associated with DILI from the Schadt et al. data set (42). The toxicity endpoints were monitored in a panel consisting of assays assessing the generation of reactive metabolites tested via GSH adduct formation, P450 3A4 time-dependent inhibition (TDI), BSEP inhibition, mitochondrial toxicity and cytotoxicity. In the Schadt et al. data set of 120 compounds marketed compounds, 14 compounds had not been BDDCS classified. Our analysis is depicted in Table 6.1. The assays that performed the best were GSH adduct formation and BSEP inhibition. We noted that although the positive predictive

value (PPV) for these measurements were somewhat better than for BDDCS Class 2 classification, the false negative rate (FNR) for these measures was much greater than BDDCS Class 2, so that in terms of accuracy (ACC), the GSH and BSEP assays were no better than just avoiding BDDCS Class 2 drugs. When GSH and BSEP assays were combined with BDDCS Class 2, again higher PPV values are obtained, but because FNR also increased, ACC is not better than just avoiding BDDCS Class 2 drugs. A slightly higher ACC is obtained when all of the mechanisms of toxicity endpoints are confirmed, due to the low FNR. However, having a PPV of only 65.1% does not give much confidence.

Table 6.1. Comparison of Various Assays Measuring Key Mechanisms of Toxicity Endpoints Associated with DILI.

Criteria	% Correct (Positive Predictive Value, PPV)	% DILI Missing (False Negative Rate, FNR)	% Accuracy (ACC) (True Positive + True Negative)/106
GSH	71.9%	52.1%	69.1%
TDI	75.0%	81.3%	61.8%
Cytotoxicity (3T3 cells)	48.3%	70.8%	55.5%
Mitotox	71.4%	79.2%	61.8%
BSEP	69.2%	62.5%	65.5%
All assays	65.1%	14.6%	73.6%
BDDCS Class 1	33.3%	75.0%	45.5%
BDDCS Class 2	64.6%	35.4%	69.1%
GSH and BDDCS Class 2	89.5%	64.6%	70.0%
BSEP and BDDCS Class 2	87.5%	70.8%	67.3%
BSEP and Mitotox	50.0%	95.8%	56.4%

Although there may be some general trends between simple physical parameters, it is unlikely that such considerations could accurately predict risk. This problem could potentially be alleviated by the new *in vitro* approaches in physiological test systems with model hepatotoxins and utilization of state of the art instrumentation currently being evaluated encompassing chemical and biological factors associated with hepatotoxicity earlier in drug development (15, 44).

Assessment of BDDCS Classification on BSEP Inhibition and DILI risk

The accumulation of bile acids within hepatocytes is thought to be a primary mechanism for the development of DILI, although as we show with the Schadt et al. data, this is not confirmed. Inhibition of the bile salt export pump (BSEP) by a drug has been implicated as a risk factor for a drug's potential to cause DILI. However, few reports indicate that drug-induced BSEP dysfunction actually leads to hepatotoxicity, and the relationship between drug-induced BSEP dysfunction and liver injury risk is yet to be determined. Recently, the International Transporter Consortium has highlighted BSEP as one of the emerging transporters that need to be considered when evaluating drug safety. However, the practical utility of this approach still needs to be further evaluated. We analyzed further data encompassing the relationship between a compound's ability to inhibit BSEP function and cause liver injury in humans using a compilation of published DILI datasets that have screened for BSEP inhibitors, other hepatic transporters and other mechanism based toxicity endpoints such as the mitochondrial toxicity assay (4, 42, 45, 46). We evaluated the information provided by using BDDCS in order to understand the inhibition propensity of BSEP. Our results presented in Chapter 4 demonstrate that there is little support for BSEP inhibition being usefully DILI predictive. Rather we show

that most potent BSEP inhibitors are BDDCS Class 2 drugs, which we have demonstrated previously is the BDDCS class most likely to be DILI related (47).

When BSEP inhibition data by Pedersen et al. (48) were correlated with Chen DILI Assessment and FDA drug labels of registered drugs, we observed no discernible pattern between BSEP inhibition and ADR categories (47) (See Figure 4.1A and 4.1B). For the BDDCS classification, we observe that the great majority of strong BSEP inhibitors are BDDCS Class 2 drugs, with concomitant decreases in the percentages of BDDCS class 1 and 3 drugs as BSEP inhibition increases, as depicted by Figure 4.2A. Here we show that pharmacological BSEP interference by small molecules is not a strong susceptibility factor. BSEP inhibition alone cannot accurately predict hepatotoxic potential of drugs as depicted by Figure 4.1B. It is unclear as to what extent BSEP inhibition is functionally significant *in vivo*. We observe that the great majority of compounds that have been associated with DILI and are BSEP inhibitors are also BDDCS Class 2. Because we are able to make similar predictions based on BDDCS determinant characteristics, this leads us to discount the predictive ability of mechanistic association of BSEP and DILI. We have previously in Chapter 3 observed that as hepatic warning severity increases, the proportion of BDDCS Class 2 drugs increases and the proportions of both BDDCS Class 1 and 3 drugs decrease (15). We conclude that previous analyses predicting that BSEP inhibition leads to DILI may have been confounded by the observation that most BSEP inhibitors are BDDCS Class 2 drugs, which show a high prevalence for DILI.

Aleo et al. (4) suggest that mitochondrial toxicity together with BSEP inhibition may provide improved DILI predictability. When we analyzed the predictability of BSEP inhibition together with mitochondrial toxicity, we observe that BDDCS class 2 characterization shows comparable results (47). This is further confirmed in the Schadt et al. data set where we show in

Table 6.1 that combining the BSEP inhibition and mitotoxicity yields a very high FNR and no improvement in ACC. Thus, we believe that neither BSEP inhibition nor mitochondrial toxicity are useful independent or combined predictors of DILI.

Why are BDDCS Class 2 drugs more toxic than BDDCS Class 1 drugs?

Several studies have also shown a correlation of total administered dose alone or in combination with drug lipophilicity with higher risk of DILI. However, as we show in Fig. 2 and in our discussion above, dose alone is not able to accurately discriminate all drugs causing DILI. Chen et al. (7) proposed a Rule of 2 where PPV for DILI was very high when considering drugs with cLogP ≥3.0 (calculated lipid water partition coefficient) and dose > 100mg. However, we have shown that the Rule of 2 was slight less accurate than just BDDCS Class 2 assignment (15).

Highly lipophilic drugs are cleared by the liver and generally require biotransformation to be eliminated. The parameter clogP may simply be a surrogate for extensive biotransformation and hepatic exposure to a reactive metabolite. If cLogP that could differentiate DILI potential, we would see equal chances of BDDCS Class 1 and 2 drugs leading to DILI toxicity. As seen in Figs. 3.1, BDDCS Class 2 compounds predominate among the most severe hepatic toxicities. Furthermore, in our previous analysis (15), we have observed that PPV for cLogP ≥ 3 alone is fairly low (76.1% and ACC is 52.3%). So we do not believe extensive metabolism is an adequate DILI predictor.

A major finding in the development of the BDDCS was the recognition that BDDCS Class 1 drugs, i.e. extensively metabolized, high permeable, high soluble, may be shown in vitro to be substrates of both uptake and efflux transporters, but that effects of transporters on BDDCS Class 1 drugs are essentially clinically insignificant in the liver and intestine, as well as the brain.

Thus, the unbound concentrations of BDDCS Class 1 drugs in the systemic circulation will reflect unbound concentrations in the liver as well as in the rest of the body, since it is transporters that lead to differences in unbound concentrations in different organs. According to BDDCS (49, 50) approximately 40% of marketed drugs (i.e., those that are Class 1) will still follow the equivalent free drug concentration hypothesis. However, this will not be true for BDDCS Classes 2, 3 and 4 drugs where transporter effects will lead to different unbound concentrations in the liver and throughout the body. That is, Class 1 drugs will follow the long held assumption in deriving pharmacologic/toxicologic relationships that free drug concentrations are the same throughout the body. But this assumption in pharmacology was made prior to any recognition of the importance of drug transporters in controlling permeability.

It is important to recognize that the compounds evaluated here are drugs that reach the market where sponsors were able to convince the regulatory agencies based on *in vitro* and preclinical animal studies that toxicity potential, particularly DILI, would be manageable or at least acceptable when the drugs reached the market and were taken by large patient populations as compared to those limited number of patients studied during drug development. Thus, according to our hypothesis, drug company sponsors in their preclinical and clinical studies of Class 1 drugs would be able to reasonably predict drug concentrations in the liver and throughout the body. In contrast, for BDDCS Class 2 drugs, where metabolism is the significant process of elimination, drug concentration measurements in the systemic circulation for these compounds both in the preclinical and clinical studies would poorly predict what concentrations are present in the liver and in other organs of the body. And since it is obvious that DILI occurs more frequently with metabolized drugs, studies in drug development with Class 2 drugs would be poorer predictors of toxicity potential due to the challenges to estimate intracellular

concentrations and metabolic processes. Thus, the prevalence of DILI with BDDCS Class 2 drugs could just be circumstantial in that sponsors would be unable to properly evaluate hepatic toxicity for these compounds in designing their clinical studies. This problem could potentially be alleviated by new *in vitro* approaches and utilization of state of the art instrumentation currently being evaluated.

Conclusion

The application of the BDDCS methodology can help evaluate the potential validity of risk assessment hypotheses. The BDDCS Class 2 susceptibility factor yields similar and in a number of cases better accuracy than the DILI predictive potential biomarkers of other methodologies. Since there is no mechanistic basis for BDDCS Class 2 drugs being most DILI related, if an alternate hypothesis is no more predictive than BDDCS Class assignment, we maintain that the alternate hypothesis is not sufficiently predictive, nor a mechanistic valid hypothesis. As seen in Fig. 3.1, the BDDCS Class 2 versus Class 1 differentiation only becomes evident with the most severe hepatic toxicities, and then only a 2:1 differentiation between BDDCS Class 2 versus Class 1 is found. Lammert et al.'s (18) assertion that extensive metabolized compounds are at higher risk of developing DILI can be much improved by differentiating BDDCS Class 2 from BDDCS Class 1 drugs. Daily dosage ≥ 50mg alone can only depict a clear relationship with dose with compounds that have been previously associated with DILI, but very limited predictability in differentiating compounds with "No DILI" assignment. There is a general acceptance of BSEP inhibitor as being a source of toxicity. However, according to our analysis of DILI this is not true. What we find is that most DILI occurs with BDDCS Class 2 compounds and almost all BSEP substrates and inhibitors are

Class 2 compounds, but we do not see a relationship with the strength of BSEP inhibition and toxicity, which makes us believe that the generally held hypothesis is incorrect.

Thus, our review of the BDDCS analysis alongside other DILI toxicity potential biomarkers show that none of the current *in vitro* methodologies are sufficiently accurate and effective in allowing early identification of new molecular entities that will be DILI free. The comparison of proposed DILI predictive methodology with BDDCS assignment offers a useful tool by which new DILI predictive hypotheses can be evaluated. Furthermore, using BDDCS classification and finding that a compound is Class 2, one would recognize that priority should be given to more aggressively investigate its DILI potential in mechanistic DILI assays. Some DILI risk factors can be mitigated during the drug design/development process to identify drugs with better chemical attributes with reduced potential to cause human DILI. Hopefully, development of mechanism based toxicity endpoints, such as those proposed by Chen et al. (51), and Schadt et al. (42), as discussed above, will greatly improve future predictability.

Our review of this work has clearly pointed out that many of the published "predictive DILI" hypotheses do no better than just avoiding BDDCS Class 2 drugs. We propose that comparison of predictive DILI hypotheses with BDDCS class assignment is a useful exercise in determining the relevance of predictive metrics. The results presented herein illustrate how BDDCS can be applied to better understand clinically observed hepatotoxicity and aid in the DILI risk assessment of new molecular entities.

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