UNDERSTANDING DRUG-DRUG INTERACTION DUE TO ENZYMATIC INHIBITION USING *IN VITRO* APPROACHES

A thesis

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Abstract

Drug-drug interaction due to enzymatic inhibition is the focus of this research. Two different projects to address separate questions of drug-drug interaction were carried out applying the *in vitro* approaches.

In the project 1, we generated a fuller picture on the *in vitro* inhibitory effects of the selected anti-tuberculosis drugs on common human hepatic CYP450 and UGT enzymes in hope that these data may provide an *in vitro* basis for understanding some clinical DDI observations and practice. Briefly, the comorbidities of tuberculosis and diseases such as HIV require long-term treatment with multiple medications. Despite extensively available in vitro and in vivo information on effects of rifampicin and isoniazid on human CYPs, there is limited published data regarding the inhibitory effects of other anti-TB drugs on human CYPs and UGTs. The inhibitory effects of 5 first-line anti-TB drugs (isoniazid, rifampicin, pyrazinamide, ethambutol, and rifabutin), and the newly approved bedaquiline, were evaluated for 6 common human hepatic UGT enzymes (UGT1A1, 1A4, 1A6, 1A9, 2B7 and 2B15) *in vitro* using HLMs. Pyrazinamide, ethambutol, rifabutin and bedaquiline were also studied for their inhibitory effects on 8 of the most common human CYP enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A). Rifabutin was observed *in vitro* to inhibit different CYPs to varying degrees, but with all IC_{50} values exceeding 25 μM. Rifabutin and rifampicin also inhibited several tested human UGTs. The Ki value for rifabutin on human hepatic UGT1A4 was 2 μM. In addition, the 6 selected anti-TB drugs produced minimal inhibition of acetaminophen glucuronidation suggesting that DDI between APAP and the selected drugs is unlikely.

In the project 2, the mechanism of the protective effects of two flavonoids, namely luteolin and quercetin, on APAP induced hepatotoxicity was investigated in the *in vitro* settings using HLMs. Acetaminophen is a common over-the-counter analgesic and antipyretic. When overdosed, APAP can cause acute hepatic necrosis. The key mechanism in APAP induced hepatotoxicity is the CYP450 catalyzed formation of the reactive metabolite, N-acetyl-p-benzoquinone imine which depletes hepatic glutathione and accumulates to cause excessive cellular oxidative stress. In this study, we observed that luteolin and quercetin inhibited *in vitro* most of the hepatic CYP450 enzyme isoforms including several key isoforms which are responsible to the formation of NAPQI; both luteolin and quercetin strongly inhibited APAP sulfation. In addition, although both luteolin and quercetin inhibited individually several UGT isoforms *in vitro*, they didn't inhibit the overall APAP glucuronidation. Thus, the beneficial effects of luteolin and quercetin against APAP induced hepatotoxicity possibly result from their properties of being able to partially block the CYP-mediated oxidation and to drive the reaction via APAP glucuronidation.

"..., God is love." – 1 JOHN 4:8

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List of Abbreviations

ACN	Acetonitrile
APAP	Acetaminophen
3-AAP	3-Acetaminophenol
CO	Carbon monoxide
CYP450	Cytochrome P450
DDI	Drug-drug interactions
DMSO	Dimethyl sulfoxide
FA	Formic acid
FDA	U.S. Food and Drug Administration
HLM	Human liver microsome
MEOH	Methanol
$MgCl_2$	Magnesium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NAPQI	*N*-acetyl-*p*-benzoquinone imine
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAPS	3'-phosphoadenosine-5'-phosphosulfate
S9	Human liver S9 fraction
SULTs	Sulfotransferases
TFA	Trifluoroacetic acid
TB	Tuberculosis
UDPGA	Uridine 5'-diphosphoglucuronic acid
UGT	UDP-glucuronosyltransferase

Introduction

1.1 Cytochrome P450

CYP450s belong to the superfamily of hemeproteins which contain a heme cofactor. The term P450 depicts the fact that when the enzyme is in its reduced state and complexed with CO, the absorption wavelength is 450 nm [(Axelrod, 1955, Brodie et al., 1955)]. CYP enzymes are widely present in animals, plants, fungi, protists, bacteria, archaea, and viruses [(Lamb et al., 2009, Sigel et al., 2007, Danielson, 2002)].

The Human Genome Project has identified 57 human genes coding for the various human CYP450 enzymes [(Guengerich, 2005)]. The majority of these enzymes are in families CYP1 to CYP4. Families CYP1, CYP2 and CYP3 are primarily associated with the phase 1 metabolism of exogenous compounds, while the others play a role in the biosynthesis of steroids, fatty acids and bile acids [(Lewis, 2004)]. Among the CYP450s which catalyze xenobiotics (about 15 CYPs), a selected list of 7 CYPs are recommended by FDA for routine assessment of metabolism-mediated drug-drug interaction, namely CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6,and CYP3A [(FDA Draft, 2012)].

CYP450 enzymes can be found throughout the body, particularly at interfaces such as the liver, intestine, nasal epithelia, skin, lung and kidney. The liver and the intestinal epithelia are the predominant sites for CYP450-mediated drug elimination. The relative distributions of each major human CYP450 at the sites of liver and intestines have been

reported previously [Evans and Relling, 1999, Paine et al., 2006, Shimada et al., 1994]. The activity of individual CYPs is not strictly proportional to their protein expression. For an example, according to a published survey in 2002 which involved the top 200 prescribed drugs marketed in the US, CYP2D6, even though is only about less than 5% of the total hepatic CYP450s, has a disproportional share in metabolizing about 25% of marketed pharmaceutics metabolized by P450s [Guengerich, 2005, Williams et al., 2004, Evans and Relling, 1999].

1.2 UDP-glucuronosyltransferases

Glucuronidation is an important phase II metabolism which detoxifies many xenobiotics including many drugs, dietary chemicals, environmental pollutants and endogenous compounds [Miners et al., 2002]. The corresponding UGT enzymes are classified into families and further into subfamilies as UGT1A, UGT2A and UGT2B [Mackenzie et al., 1997]. The major hepatic UGTs include UGT1A1, 1A4, 1A6, 1A9, 2B7 and 2B15 [Court, 2010]. There are also extrahepatic UGTs. For an example, UGT1A7, 1A8 and 1A10 are exclusively expressed in extrahepatic tissues and found in the gastrointestinal (GI) tract [Strassburg et al., 1997, Strassburg et al., 1998]. It has been reported that 1 in 13 drugs of the top 200 prescribed drugs in 2002 in the United States is metabolized by glucuronidation and that UGT2B7, UGT1A4 and UGT1A1 are among the most common UGT enzymes to metabolize the top 200 drugs [Williams et al., 2004].

1.3 *In vitro* inhibition studies of metabolic enzymes

Drug metabolism in the body is generally divided into two groups, phase I and phase II reactions. Phase I reactions involve hydrolysis, reduction and oxidation. Phase II reactions include various conjugation reactions, such as glucuronidation, sulfation,

acetylation, methylation, glutathione conjugation, and amino acid conjugation. *In vitro* models using human liver microsomes, liver S9 fractions, liver slices, intact hepatocytes or recombinant CYP enzymes are cost-effective ways to study drug metabolism compared to clinical studies. The *in vitro* methods on drug metabolism include but not limited to studying biotransformation of a drug of interest, identifying the involved metabolic enzymes by reaction phenotyping and assessing potential drug-drug interaction by enzymatic inhibition or induction. Drug-drug interaction due to enzymatic inhibition is the focus of this research.

Inhibition of metabolic enzymes by co-administered medications causes clinical relevant sometimes serious outcomes. It is recognized by FDA and other regulatory agencies as an important aspect relating to drug safety when developing a drug (FDA , 2012, European Medicines Agency, 2012). *In vitro* studies are cost-efficient and provide useful information for necessary clinical trials on drug-drug interaction. The inhibition mechanisms of some metabolic enzymes are reversible, while others are time-dependent (TDI) (White, 2000) that is also termed as mechanism-based inhibition.

1.3.1 Reversible inhibition

Reversible inhibition involves rapid association and dissociation of drugs and enzymes and may be competitive (Equation 2), noncompetitive (Equation 3), uncompetitive (Equation 4) or mixed inhibition of competitive and noncompetitive (Equation 5). The simplest enzyme kinetic model which is with no inhibition can be described as Michaelis Menten model (Equation 1).

Equation 1 No inhibition

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

Equation 2 Competitive

$$v = \frac{V_{max} \cdot [S]}{K_m \cdot (1 + \frac{[I]}{K_i}) + [S]}$$

Equation 3 Noncompetitive

$$v = \frac{V_{max} \cdot [S]}{K_m \cdot (1 + \frac{[I]}{K_i}) + (1 + \frac{[I]}{K_i}) \cdot [S]}$$

Equation 4 Uncompetitive

$$v = \frac{V_{max} \cdot [S]}{K_m + (1 + \frac{[I]}{K_i}) \cdot [S]}$$

Equation 5 Mixed competitive and noncompetitive inhibition

$$v = \frac{V_{max} \cdot [S]}{K_m \cdot (1 + \frac{[I]}{K_i}) + (1 + \frac{[I]}{\alpha K_i}) \cdot [S]}$$

v is the initial rate of the reaction; [S] is the substrate concentration; [I] is the inhibitor concentration; V_{max} and K_m are the kinetic constants for a given enzyme; V_{max}, the maximum reaction velocity and K_m, the Michaelis-Menten constant; K_i is the inhibitor constant; α is a number derived mathematically (Greenblatt et al., 2011).

IC_{50} in general is greater than or equal to K_i. α equals to 'infinity' for pure competitive inhibition (Equation 6) and equals to 1.0 for pure noncompetitive inhibition (Equation 7) (Greenblatt et al., 2011).

Equation 6

$$IC_{50} = K_i(1 + \frac{[S]}{K_m})$$

Equation 7

$$IC_{50} = K_i$$

1.3.2 Time dependent inhibition (TDI)

Time dependent inhibition (TDI) is characterized by an increase on inhibitory potency as compounds turnover. TDI often results in more significant drug interactions, as the restoration of enzyme activity generally requires *de novo* synthesis of the enzyme. TDI could result from irreversible covalent binding which is also called mechanism based inhibition or quasi-irreversible noncovalent tight binding of a chemically reactive intermediate to the enzyme or reversible inhibition from a metabolite(s) generated *in situ* (Grimm et al., 2009; Kalgutkar et al., 2007). A typical initial test for TDI is an "IC_{50} shift" assay. The

Pharmaceutical Research and Manufacturers of America (PhRMA) survey participated by 17 companies in 2009 indicated that 47% of companies used the model of "IC_{50} shift" as the initial TDI assessment [(Grimm et al., 2009)]. A decrease of 1.5 to 2 fold for the initial IC_{50} value of the enzyme of interest with a 30 min pre-incubation with the compound of interest represents a signal to trigger further definitive time-dependent inhibition studies to obtain TDI parameters (i.e., k_{inact} and K_I) [(Grimm et al., 2009, Berry and Zhao, 2008)]. k_{inact} is the maximum inactivation rate which is a theoretical value and cannot be experimentally observed, and K_I is the inactivator concentration when the rate of inactivation reaches half of the k_{inact} value [(Silverman, 1996)]. A series of *in vitro in vivo* extrapolation mathematical models have been developed using the *in vitro* TDI parameters to predict the AUCR value, which is the ratio of the AUCs with and without inhibition [(Grimm et al., 2009)]. When AUCR is greater than 1.25, drug-drug interaction is possible and a clinical study using an appropriate probe substrate is recommended [(FDA, 2012, Zhang et al., 2010)].

1.4 Project 1: Inhibitory effects of anti-TB drugs on human CYPs and UGTs

1.4.1 Tuberculosis

Tuberculosis is a leading cause of morbidity and mortality worldwide. The World Health Organization estimated that in 2013 there were around 9.0 million people that developed TB, and 1.5 million deaths from TB, including 360,000 deaths associated with co-infection with HIV [(WHO, 2014)]. The comorbidity of TB and other diseases requires treatment with multiple medications. Understanding of potential drug-drug interactions (DDIs) is of importance in planning safe and effective combination therapies.

1.4.2 Anti-TB drugs

Isoniazid, rifampicin (or rifampin), pyrazinamide, ethambutol, rifabutin and rifapentine are the recommended first-line anti-TB drugs to treat drug-susceptible tuberculosis [Zumla et al., 2013] (Table 1, and 2). Bedaquiline is a novel anti-mycobacterial agent, which was newly approved by FDA to treat multidrug resistant tuberculosis [Chahine et al., 2014, Worley et al., 2014]. Among those, rifampicin is a potent inducer of the CYPs, UGTs, as well as the P-glycoprotein transport system both *in vitro* [Rae et al., 2001, van de Kerkhof et al., 2008, Soars et al., 2004] and clinically [Baciewicz et al., 2013]. Rifampicin is also reported to be an inhibitor of some human CYPs *in vitro* [Kajosaari et al., 2005]. Overall, rifampicin clinically reduces serum concentrations of many drugs [Ochs et al., 1981]. Compared to rifampicin, rifabutin has less potency as a CYP3A inducer and is used as a substitute for rifampicin in patients receiving protease inhibitor-based antiretroviral therapy [Zumla et al., 2013, Baciewicz et al., 2013, WHO, 2010]. Isoniazid is known as an inhibitor of many human CYPs *in vitro* [Wen et al., 2002, Desta et al., 2001, Polasek et al., 2004] and clinically [Ochs et al., 1981, Ochs et al., 1987].

Both the induction effects of rifampicin and inhibitory effects of isoniazid on human CYPs have been extensively reported *in vitro* and *in vivo*. However, the data of their inhibitory effects on human UGTs is limited. The information for other anti-TB drugs is also limited. In this work, inhibitory properties of the selected anti-TB drugs, including pyrazinamide, ethambutol, rifabutin and bedaquiline were studied with both common CYP and UGT enzymes; Inhibitory effects of isoniazid and rifampicin on human hepatic UGTs were also studied. Since acetaminophen is so widely used as an analgisic agent, we also evaluated the effects of the anti-TB drugs on acetaminophen glucuronidation.

Table 1 Main tuberculosis drugs in clinical use and their pharmacological mechanisms

Drug (year of discovery)	Mechanism of Action
Isoniazid (1952)	Inhibits mycolic acid synthesis
Rifampicin (1963)	Inhibits transcription
Pyrazinamide (1954)	Inhibits translation and trans-translation, acidifies cytoplasm
Ethambutol (1961)	Inhibits arabinosyl transferases
Rifabutin (1975)	blocks the DNA-dependent RNA-polymerase of the bacteria
Bedaquilline (2012 FDA approval))	inhibits the c subunit of ATP synthase, thereby decreasing intracellular ATP levels

Table 2 Plasma concentrations of the drugs (Peloquin et. al., 2002)

	Usual adult dosage	Plasma Cmax (μg/mL)	Plasma Cmax (μM)
Isoniazid	300mg qd	3.0-6.0	21.9-43.8
	900mg biwk	9-18 mg/ml	65.6-131.3
Rifampin	600mg qd	8.0-24	9.7-29.2
Rifabutin	300mg qd	0.3-0.9	0.4-1.1
Pyrazinamide	25mg/kg qd	20-50	162.5-406.1
	50mg/kg biwk	40-100	324.9-812.3
Ethambutol	25mg/kg qd	2.0-6.0	9.8-29.4
	50mg/kg biwk	4.0-12	19.6-58.7
Bedaquiline	400mg qd for a week	5.5	9.9

1.5 Project 2: Metabolic interactions between APAP and two flavonoids (luteolin and quercetin)

1.5.1 Acetaminophen and its metabolism

Acetaminophen is a common over-the-counter pain reliever and fever reducer. When overdosed, APAP can cause acute hepatic necrosis. APAP induced hepatotoxicity is the

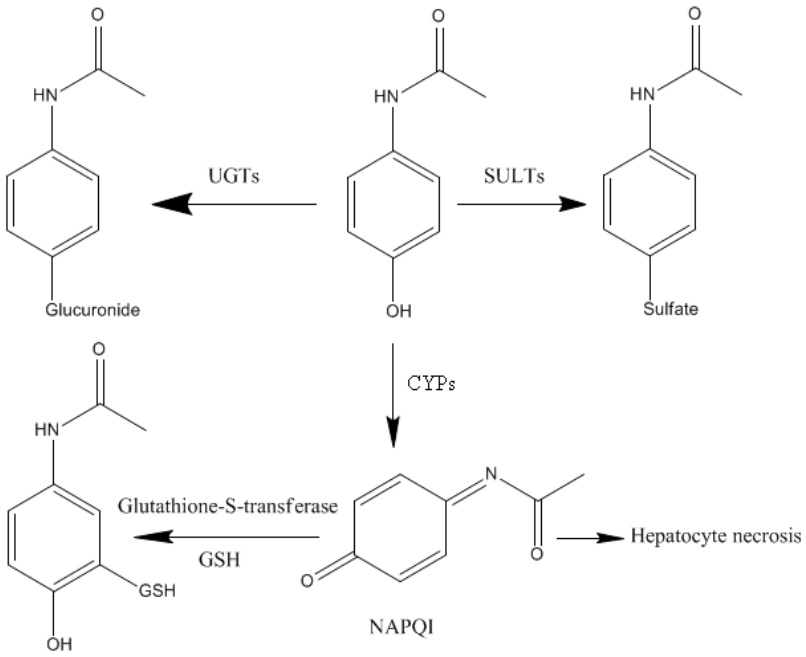
leading cause of acute liver failure in the US [Larson et al., 2005]. The key mechanism in APAP induced hepatotoxicity is the CYP450-catalyzed formation of the reactive metabolite, NAPQI, which depletes hepatic glutathione and accumulates to cause excessive cellular oxidative stress [Miner and Kissinger, 1979] (Fig 1). Approximately 5% to 9% of an orally administered APAP is metabolized by the CYP450-dependent oxidation pathways. CYP3A4 [Laine et al., 2009, Thummel et al., 1993, Wolf et al., 2005], CYP2E1 [Laine et al., 2009, Wolf et al., 2007, Manyike et al., 2000], CYP1A2 [Laine et al., 2009, Tonge et al., 1998, Zaher et al., 1998], CYP2D6 [Laine et al., 2009, Zhou et al., 1997] and CYP2A6 [Chen et al., 1998; 1998] are involved in the formation of NAPQI. Parallel to the CYP450-dependent oxidation, other major metabolic pathways of APAP are glucuronidation (about two-thirds of an orally administered dose) and sulfation (about one-third) which produce the nontoxic metabolites [Benson et al., 2005]. UGT1A1, 1A6, 1A9 and 2B15 are important human UGTs involved in the glucuronidation of APAP [Court and Greenblatt, 1997, Court and Greenblatt, 2000, Court et al., 2001, Krishnaswamy et al., 2005, Fisher et al., 2000, Mutlib et al., 2006]. SULT1A1, SULT1A3/4, SULT1E1, and SULT2A1 have been reported to be major contributors to APAP sulfation [Adjei et al., 2008].

1.5.2 Cimetidine with its protective effects on APAP induced hepatotoxicity

Various chemicals and therapeutics have been studied for their protective effects on APAP induced hepatotoxicity in animal models as well as clinically [Tran et al., 2001, Slattery et al., 1989]. For an example, in a mouse study, a simultaneous treatment of cimetidine at 75 mg/kg with APAP significantly increased the LD_{50} of APAP [Abernethy et al., 1983]. The protective effects of cimetidine may be due to its greater inhibition to CYP450-depedent oxidation than the glucuronidation pathway. It has been reported that cimetidine

significantly inhibited CYP1A2, 2C9, 2D6, and 3A4 both *in vitro* and clinically [Martinez et al., 1999]; And that it also inhibited APAP glucuronidation *in vitro*, but with a Ki value 10 fold higher than required for CYP450-depedent oxidation [Mitchell et al., 1984]. Clinically, cimetidine didn't protect against APAP induced hepatotoxicity, as the required effective dose could not be reached by the normal therapeutical ranges [Slattery et al., 1989].

Figure 1 Acetaminophen metabolism



1.5.3 Quercetin and luteolin with their protective effects on APAP induced hepatotoxicity

Phytochemicals such as certain flavonoids in herbal extracts have been reported to have protective effects on liver in experimental animals [Aycan et al., 2014, Kiran et al., 2012, Sakeran et al., 2014, Gilani et al., 1997, Shivashri et al., 2013, Yousef et al., 2010]. Although the effective mechanism is usually identified as antioxidant effects, the mechanism of protection remains elusive for

the following reasons. The diversity of phytochemicals in various herbs makes it difficult to pinpoint the effective chemical components. Furthermore, the properties of the relevant phytochemicals are not well studied.

Flavonoids are widely distributed in edible plants such as fruits and vegetables. Many studies have reported that diets high in flavonoids may be associated with possible preventive effects of diseases such as cancer, cardiovascular and neurodegenerative diseases [Manach et al., 2004, Williamson and Manach, 2005, Hertog et al., 1994, Hertog et al., 1993]. Previous studies have shown that quercetin (a flavonoid) is beneficial in alleviating APAP induced hepatotoxicity [Gilani et al., 1997, Yousef et al., 2010]. A study using a freshwater fish has also demonstrated that the abnormalities associated with APAP exposure were reversed on the treatment with celery extract which is abundant in flavonoids such as rutein, quercetin and luteolin [Shivashri et al., 2013].

The objective of this study is to provide *in vitro* evidence on the protective mechanism of the selected flavonoids (luteolin and quercetin) on APAP induced hepatotoxicity. We observed that luteolin and quercetin inhibited most of the hepatic CYP450 enzymes including several key isoforms which are responsible to the formation of NAPQI in APAP metabolism; both luteolin and quercetin strongly inhibited APAP sulfation. However, neither luteolin nor quercetin inhibited the overall APAP glucuronidation. The *in vitro* data collectively shed light onto the explanation of the protective effects of luteolin and quercetin against APAP induced hepatotoxicity.

Material and Methods

2.1 Materials

Chemicals and solvents were purchased from Sigma-Aldrich Corp (St. Louis, MO) and Fisher Scientific (Pittsburg, PA). Water was purified with a Mili-Q system (Milford, MA).

2.2 Inhibition studies on CYP-mediated oxidation using HLMs

HLMs were prepared as previously described [Greenblatt et al., 2011, von Moltke et al., 1993a]. Fifty-three individual liver microsomes were combined to make a batch of pooled HLMs. The previously published incubation procedures for *in vitro* inhibition studies using HLMs [Greenblatt et al., 2011, von Moltke et al., 2001] were applied with modifications. Briefly, appropriate substrates and positive controls (Table 3) were added to incubation tubes. The anti-TB drugs or luteolin or quercetin were individually added in a series of concentrations to separate incubation tubes. The solvent (methanol) was evaporated to dryness at 40°C under mild vacuum conditions. Due to their poor solubility in methanol, propofol (the UGT1A9 substrate) and bedaquiline were prepared in DMSO and added directly to incubation tubes (1% DMSO v/v). Methanol at 1% (v/v) in the final incubation mixture was added to reconstitute the anti-TB compounds after dryness. The incubation mixtures for CYP-mediated oxidation contained 50 mM phosphate buffer (pH 7.5), 5mM $MgCl_2$, 0.5mM NADP, isocitrate and an isocitric dehydrogenase regenerating system, and appropriate amounts of the pooled HLMs. The anti-TB drugs were pre-incubated with HLMs (without the index substrates) for 20 minutes at 37°C, and then followed by

another timed incubation with the substrates (250μL). 100 μL of acetonitrile (or acidified acetonitrile adjusted with 85% H_3PO_4 for CYP2B6 and 2C9) with internal standards was used to stop the reactions. All incubations were performed in duplicate. Initial tests for detecting IC_{50} shifts were carried out by comparing incubations with 20 minutes' preincubation to incubations without preincubations. The supernatant was transferred to HPLC vials for HPLC-UV or HPLC-fluorescence analysis.

2.3 Solvent effects of methanol and DMSO on human hepatic CYPs

The inhibitory effects of methanol and DMSO on some selected human CYP enzymes were investigated. Briefly, various percentages of methanol or DMSO, namely 0, 0.05, 0.1, 0.5, 1 and 2% v/v, were introduced in the incubation mixtures containing the index substrates. The incubations were conducted without pre-incubation, and all incubations were performed in duplicate with the pooled HLMs. Samples were incubated at 37°C for appropriate duration and stopped by addition of 100 μL cold acetonitrile (in 250 μL of the incubation mixture) with internal standards.

2.4 K_i value for reversible enzymatic inhibition

Inhibition of UGT1A4 by rifabutin was observed to have a low IC_{50} value around 11 μM which is low enough to trigger a DDI concern. As there was no IC_{50} shift between with and without pre-incubation, the experimental design for reversible enzymatic inhibition was applied to determine the K_i value for rifabutin versus human UGT1A4 using the pooled HLMs (Greenblatt et al., 2011). Varied concentrations of the index substrate (trifluoperazine) at 2, 5, 10, 20, 34.2, 72.4, 144.9 and 336.6 μM were incubated at 37°C

with the pooled HLMs in presence of varied concentrations of the tested inhibitor (rifabutin), at 0, 1.25, 5, 10, 30, and 60 µM respectively. Probenecid at 2.4 mM was used as the positive inhibitory control. After 30 minutes' incubation, the reactions were stopped with 40 µL of acetonitrile (in the incubation mixtures of 100 µL) with the internal standard (phenacetin). The supernatant was transferred to HPLC vials for HPLC-UV analysis.

2.5 Inhibition studies on glucuronidation using HLMs

Previously described incubation procedures were used with modifications [von Moltke et al., 1993b, Court, 2010, Court, 2005]. The incubation mixtures for glucuronidation was prepared with 50 mM phosphate buffer (pH 7.5), 5mM $MgCl_2$, alamethicin (50 µg per mg protein) and appropriate amounts of the pooled HLMs and kept on ice for 5 minutes. UDPGA was prepared separately and fresh in the phosphate buffer. The reactions were initiated by addition of the UDPGA solution (a final concentration of 10 mM) in the incubation mixtures (100uL). All incubations were performed in duplicate. The incubations were conducted without pre-incubation except for those with β-estradiol (UGT1A1), trifluoperazine (UGT1A4), and APAP, for which the incubations with 20 minutes' pre-incubation were also conducted. The reactions were stopped by addition of 40 µL of acetonitrile (or acidified acetonitrile adjusted with 85% H_3PO_4 for UGT2B7 and APAP glucuronidation) with internal standards. The supernatant was transferred to HPLC vials for HPLC-UV analysis.

Table 3 *In vitro* systems using HLMs for evaluating inhibitory activities of the selected anti-TB drugs or flavonoids on human CYPs and UGTs

Enzyme Isoform	***Substrate (Concentration)***	***Internal standard***	***Metabolite assayed***	***Inhibitor***
CYP1A2	Phenacetin (100uM)	2-acetaminophenol	Acetaminophen	α-Naphthoflavone
CYP3A	Triazolam (250uM)	Phenacetin	α-Hydroxytriazolam	Ketoconazole
CYP2B6	Bupropion (80uM)	2-acetaminophenol	Hydroxybupropion	Clopidogrel
CYP2C8	Taxol (25uM)	Phenacetin	6-Hydroxytaxol	Quercetin
CYP2C9	Flurbiprofen (5uM)	Naproxen	4'-Hydroxyflurbiprofen	Sulfaphenazole
CYP2C19	S-mephenytoin (25uM)	Phenacetin	4'-Hydroxymephenytoin	Ticlopidine
CYP2D6	Dextromethorphan (25uM)	Pronethalol	Dextrorphan	Quinidine
CYP2E1	Chlorzoxazone (50uM)	Phenacetin	6-Hydroxychlorozoxazone	Diethydithiocarbamate
UGT1A1	β-Estradiol (100uM)	Phenacetin	Estradiol-3-glucuronide	Probenecid
UGT1A4	Trifluoperazine (200uM)	Phenacetin	Trifluoperazine-glucuronide	Probenecid
UGT1A6	Serotonin (4 mM)	Phenacetin	Serotonin-glucuronide	Probenecid
UGT1A9	Propofol (100uM)	3-acetaminophenol	Propofol-glucuronide	Niflumic acid
UGT2B7	3'-azidothymidine (AZT) (500uM)	3-acetaminophenol	AZT-glucuronide	Probenecid
UGT2B15	Oxazepam (100uM)	Phenacetin	S-oxazepam-glucuronide	Niflumic acid
APAP Glucuronidation	Acetaminophen (0.6mM)	3-acetaminophenol	APAP-glucuronide	Probenecid

2.6 Inhibition studies on APAP sulfation

The incubation procedures for APAP sulfation was based on previously published methods with modifications [(Miksits et al., 2009, Yang et al., 2011)]. Briefly, APAP as the substrate

was added to incubation tubes and the solvent (methanol) was evaporated to dryness at 40°C under mild vacuum conditions. The amount of APAP was 0.6 mM in the final volume of 100 uL. Luteolin (or quercetin) in a series of concentrations was added and the solvent (methanol) was evaporated to dryness. 1% (v/v) of methanol was then added to reconstitute luteolin (or quercetin). The incubation mixture for APAP sulfation was prepared with 50 mM phosphate buffer (pH 7.5), 5 mM $MgCl_2$, and 800 μg/ml pooled human S9. The PAPS cofactor solution (PAPS at 0.2 mM) was prepared in water separately and fresh before the incubation experiments. The reactions were started with addition of the PAPS solution into the incubation mixture (100uL). After 2 hours' incubation at 37°C, the reaction was stopped with 40 μL of acidified acetonitrile 0.5% (v/v) with 85% H_3PO_4 and 3-AAP (internal standard). The supernatant after centrifugation was transferred to HPLC vials for HPLC-UV.

2.7 Analytical methods

Previously described methods, with modifications were used for analysis of the *in vitro* samples [(von Moltke et al., 2001, Court, 2005)]. The HPLC conditions and detection methods are summarized in Table 4 and Figure 2-16. APAP glucuronide generated from *in vitro* incubation was analyzed using the previously described method, with modifications [(Zhao et al., 2015)]. Briefly, the HPLC analysis was carried out on a Hydro-RP column (4 μm, 250x4.6 mm, Synergi Hydro-RP, Phenomenex, Torrance, CA), with a flow rate of 1.2 mL/min. The injection volume was 30 μL, and the UV detection wavelength was 254 nm. A multistep gradient for HPLC separation was started at 96.5% mobile phase A (20 mM potassium phosphate buffer, pH 2.2) and 3.5% mobile phase B (methanol) for 5

minutes, increased to 16% B during the next 5 minutes, and reached to 20% B at 15 minutes, then to 40% B at 30 minutes, followed by a 9 minutes' isocratic run at 100% mobile phase C (50% H_2O, 50% methanol) , followed by another 10 minutes' isocratic run at 3.5% B. The integration and quantitation were done with the software Chemistation (Agilent, Santa Clara, California).

Since 1% organic solvent was introduced in the incubation mixture to improve solubility, he studies on the effects of MEOH and DMSO were also conducted in this study. In order to minimize the solvent effects, controls which contain the same percentages of methanol or DMSO as the incubation samples were used to normalize for each sample.

Table 4 Analytical methods used in this study

Index reactions	***Metabolite assayed***	***Column***	***Mobile phase***	***HPLC conditions***	***Detection***
CYP1A2	Acetaminophen	*a*	50mM potassium phosphate buffer in mixture (H_2O:ACN, 88:12)	isocratic separation	UV 254nm
CYP3A	α-Hydroxytriazolam	*a*	10mM potassium phosphate buffer in mixture (H_2O:ACN:MEOH, 67.5:22.5:10)	isocratic separation	UV 220nm
CYP2B6	Hydroxybupropion	*b*	50mM potassium phosphate buffer in mixture (H_2O:ACN,	isocratic separation	UV 214nm

			85:15); pH=3		
CYP2C8	6-Hydroxytaxol	*c*	H2O:MEOH, 40.5:59.5	isocratic separation	UV 230nm
CYP2C9	4'-Hydroxyflurbiprofen	*a*	20mM potassium phosphate buffer in mixture (H_2O:ACN, 65:35); pH=2.5	isocratic separation	fluorescence excitation 260nm; emission 320nm
CYP2C19	4'-Hydroxymephenytoin	*b*	50mM potassium phosphate buffer in mixture (H_2O:ACN, 80:20)	isocratic separation	UV 204nm
CYP2D6	Dextrorphan	*b*	50mM potassium phosphate buffer in mixture (H_2O:ACN, 75:25); pH=6	isocratic separation	fluorescence excitation 280nm; emission 310nm
CYP2E1	6-Hydroxychlorozoxazone	*a*	50mM potassium phosphate buffer in mixture (H_2O:ACN, 75:25)	isocratic separation	UV 295nm
UGT1A1	Estradiol-3-glucuronide	*d*	Mobile phase A: 20mM potassium phosphate buffer, pH=4.5; Mobile phase B: MEOH	Court, 2005	UV 280nm
UGT1A4	Trifluoperazine-glucuronide	*d*	Mobile phase A: 0.1% TFA in H_2O; Mobile phase B: MEOH	Court, 2005	UV 254nm
UGT1A6	Serotonin-glucuronide	*d*	Mobile phase A: 20mM potassium phosphate	Court, 2005	UV 270nm

			buffer, pH=4.5; Mobile phase B: MEOH		
UGT1A9	Propofol-glucuronide	*a*	Mobile phase A: 20mM potassium phosphate buffer, pH=4.5; Mobile phase B: MEOH	Court, 2005	UV 214nm
UGT2B7	AZT-glucuronide	*d*	Mobile phase A: 20mM potassium phosphate buffer, pH=2.2; Mobile phase B: MEOH	Court, 2005	UV 266nm
UGT2B15	S-oxazepam-glucuronide	*d*	Mobile phase A: 20mM potassium phosphate buffer, pH=4.5; Mobile phase B: MEOH	Court, 2005	UV 214nm
APAP Glucuronidation[e]	APAP-glucuronide	*d*	Mobile phase A: 20mM potassium phosphate buffer, pH=2.2; Mobile phase B: MEOH	Zhao, 2014	UV 254nm
APAP sulfation	APAP-sulfate	*d*			

a: C_{18} reversed phase 150 x 3.9 mm Nova-pak (Waters); *b*: C_{18} reversed phase 300 x 3.9 mm uBondapak (Waters); *c*: ODS reversed phase 250 x 4.6mm (Alltech); *d*: Synergi 4μm Hydro-RP 80A 250 x 4.6mm (Phenominex); [e] APAP glucuronidation involves several UGT isoforms, such as UGT1A1, 1A6, 1A9 and 2B15.

Figure 2, HPLC chromatogram of the CYP1A2 specific incubation sample using HLMs a) acetaminophen as the phenacetin metabolite formed by HLMs, b) 2-acetaminophenol as the IS and c) phenacetin.

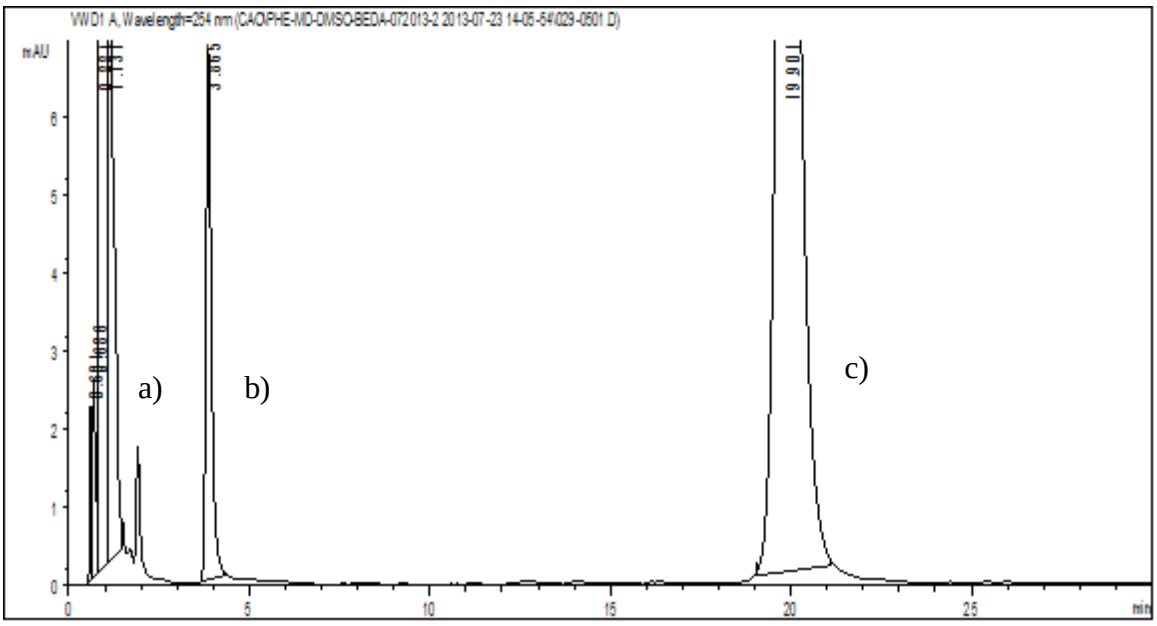


Figure 3, HPLC chromatogram of the CYP3A specific incubation sample using HLMs a, b) α-hydroxytriazolam and 4-hydroxytriazolam as the triazolam metabolites formed by HLMs, c) triazolam.

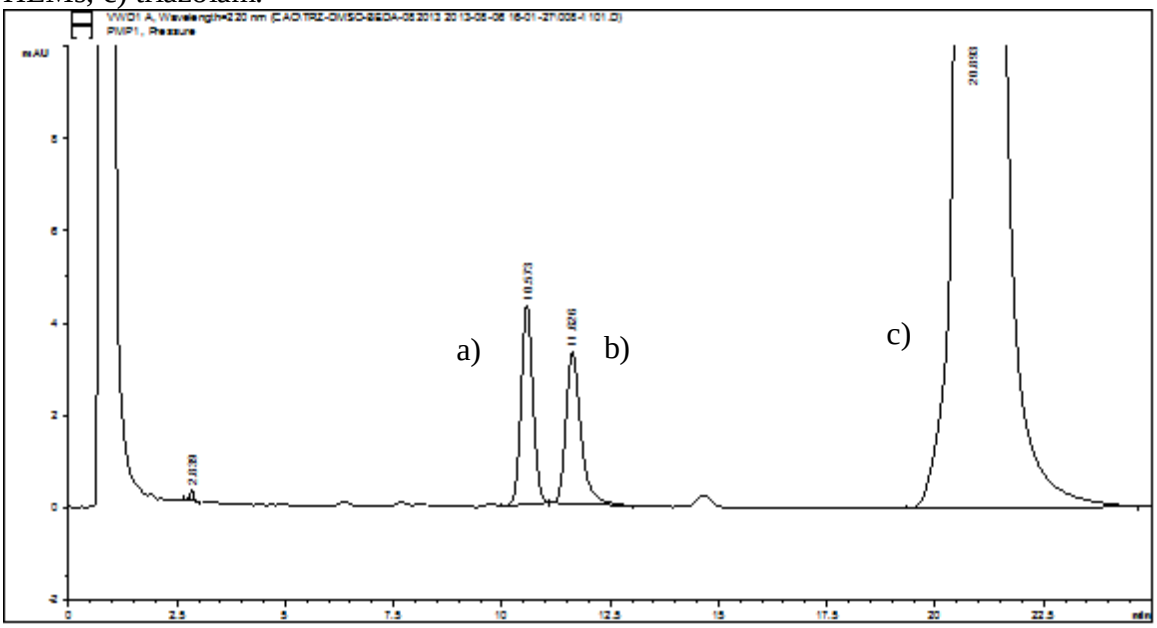


Figure 4, HPLC chromatogram of the CYP2B6 specific incubation sample using HLMs a) 2-acetaminophenol as the IS, b) hydroxybupropion as the bupropion metabolite formed by HLMs and c) bupropion.

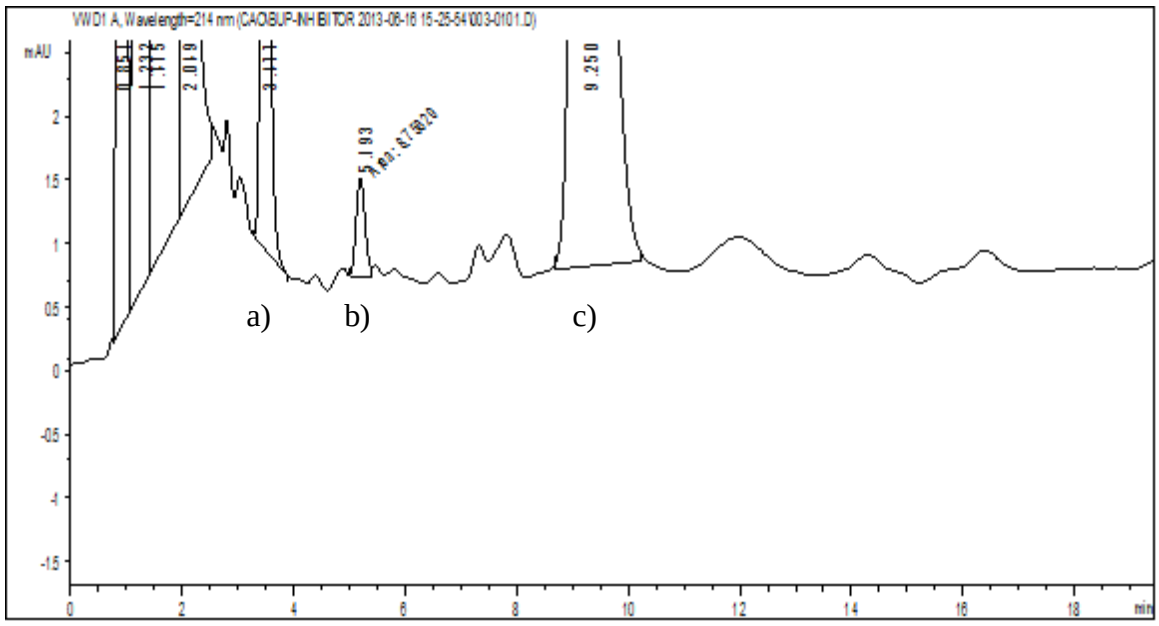


Figure 5, HPLC chromatogram of the CYP2C8 specific incubation sample using HLMs a) 6-hydroxytaxol as the taxol metabolite formed by HLMs, b) taxol and c) phenacetin as the IS.

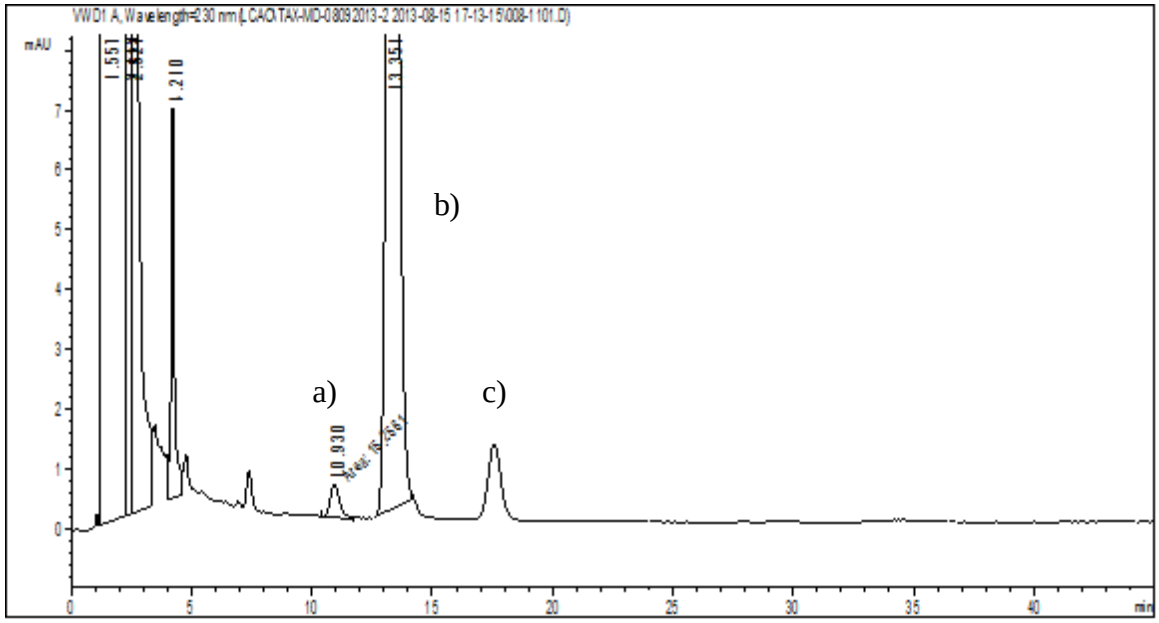


Figure 6, HPLC chromatogram of the CYP2C9 specific incubation sample using HLMs a) 4'-hydroxyflurbiprofen as the flurbiprofen metabolite formed by HLMs, b) naproxen as the IS and c) flurbiprofen.

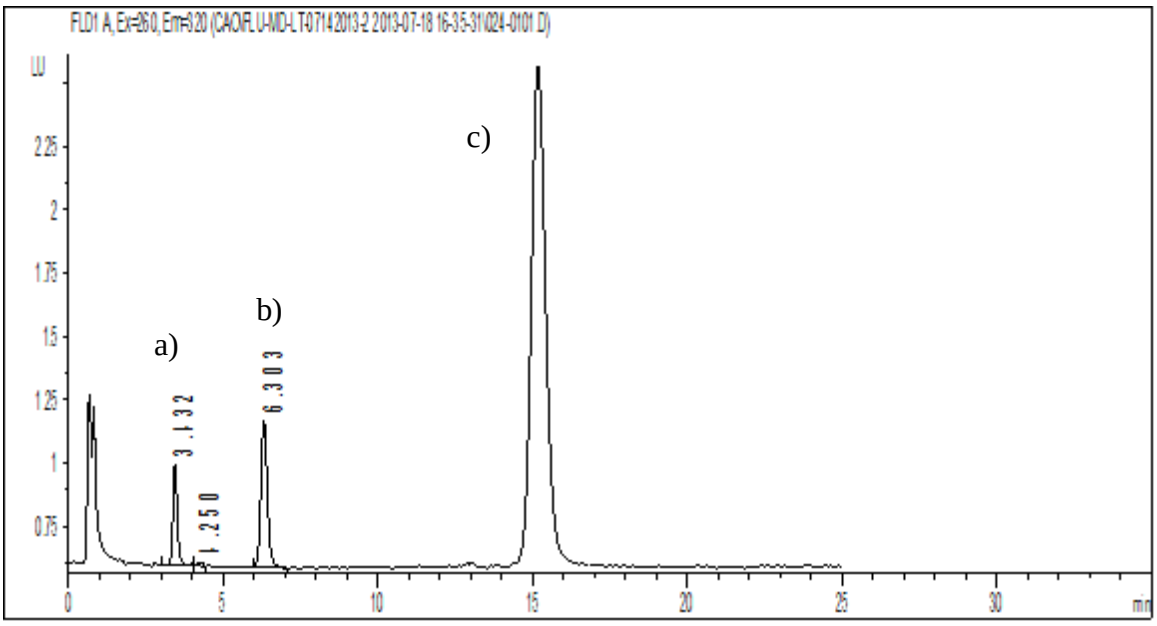


Figure 7, HPLC chromatogram of the CYP2C19 specific incubation sample using HLMs a) 4'-hydroxymephenytoin as the s-mephenytoin metabolite formed by HLMs, b) phenacetin as the IS, c) s-mephenytoin and d) a case-specific peak of quercetin washout in this particular study.

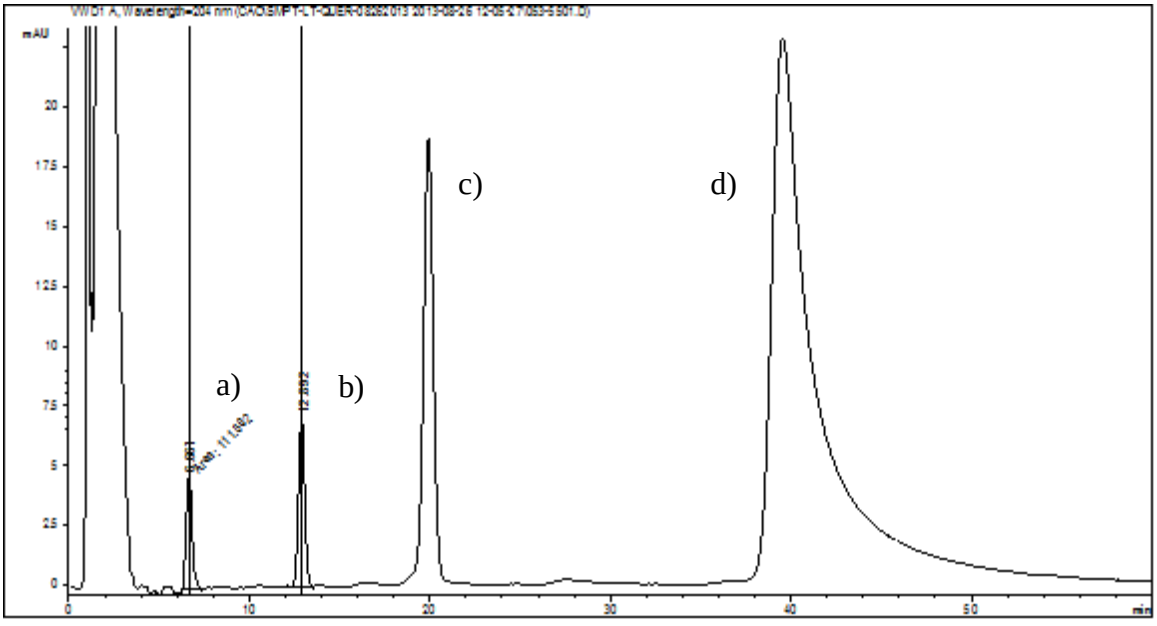


Figure 8, HPLC chromatogram of the CYP2D6 specific incubation sample using HLMs a) dextrorphan as the dextromethorphan metabolite formed by HLMs, b) pronethalol as the IS, and c) dextromethorphan.

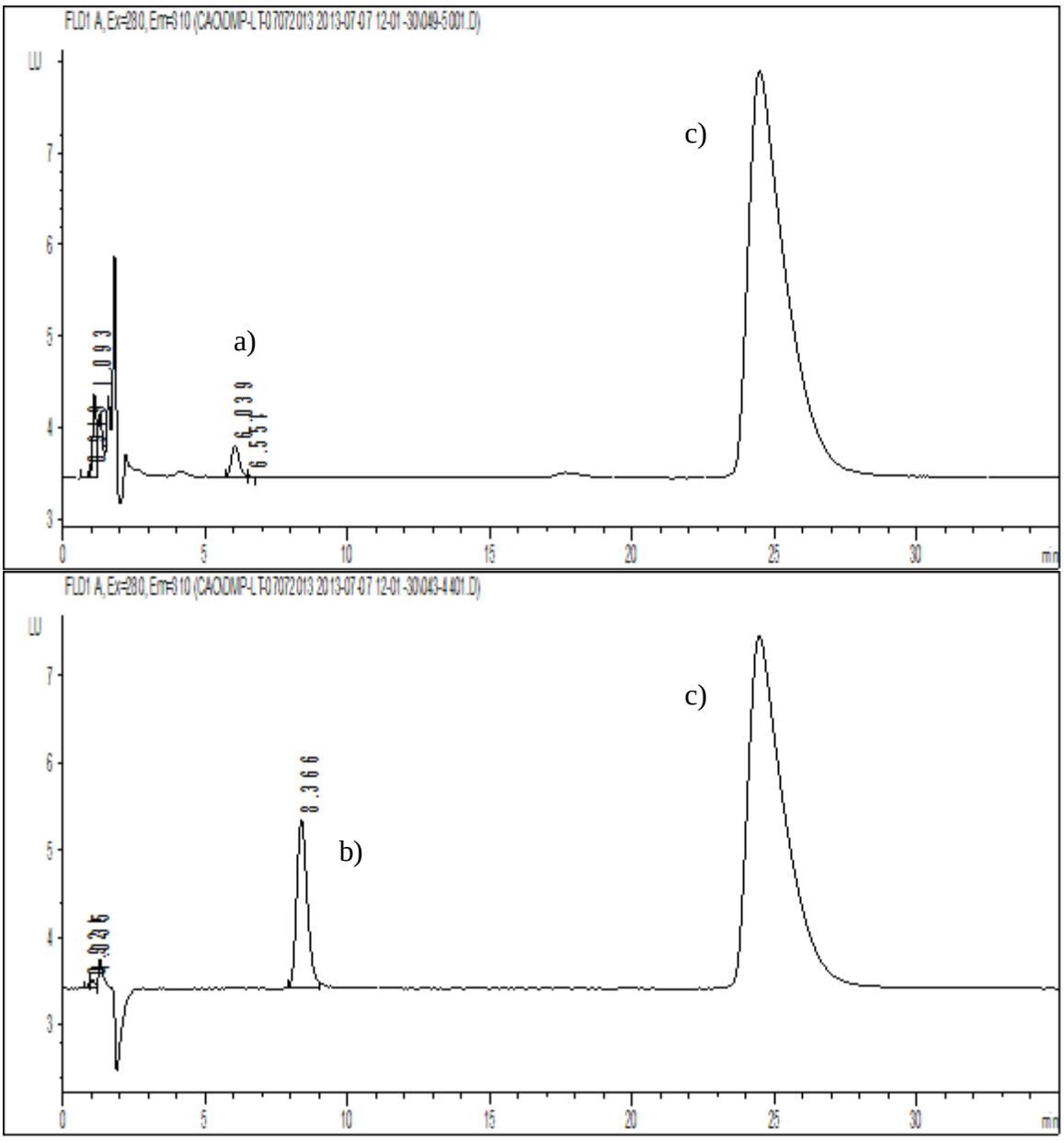


Figure 9, HPLC chromatogram of the CYP2E1 specific incubation sample using HLMs a) 6-hydroxychlorozoxazone as the chlorzoxazone metabolite formed by HLMs, b) phenacetin as the IS, and c) chlorzoxazone.

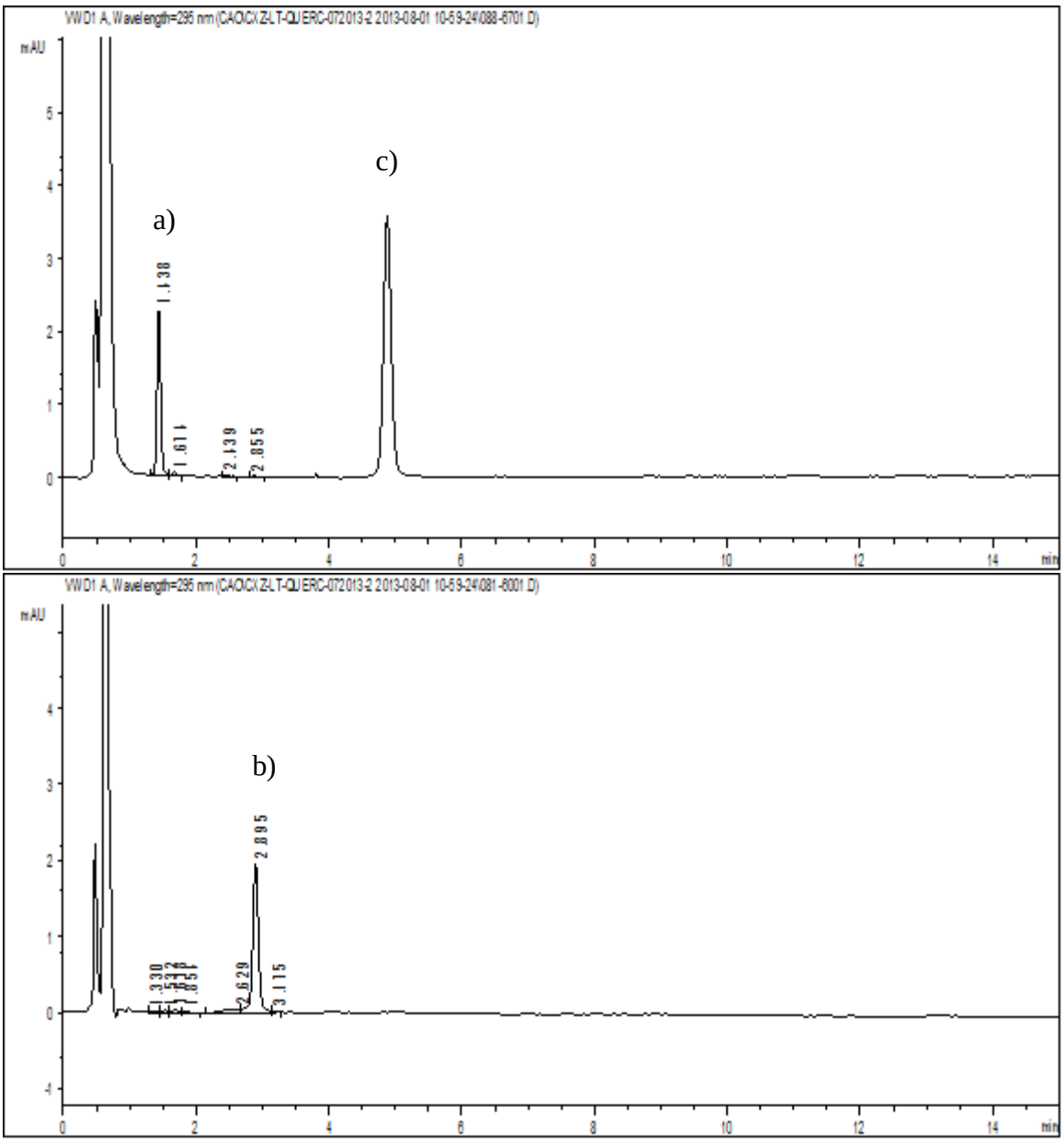


Figure 10, HPLC chromatogram of the UGT1A1 specific incubation sample using HLMs a, c) estradiol-3-glucuronide and estradiol-17-glucuronide as the β-Estradiol metabolite formed by HLMs, and b) phenacetin as the IS.

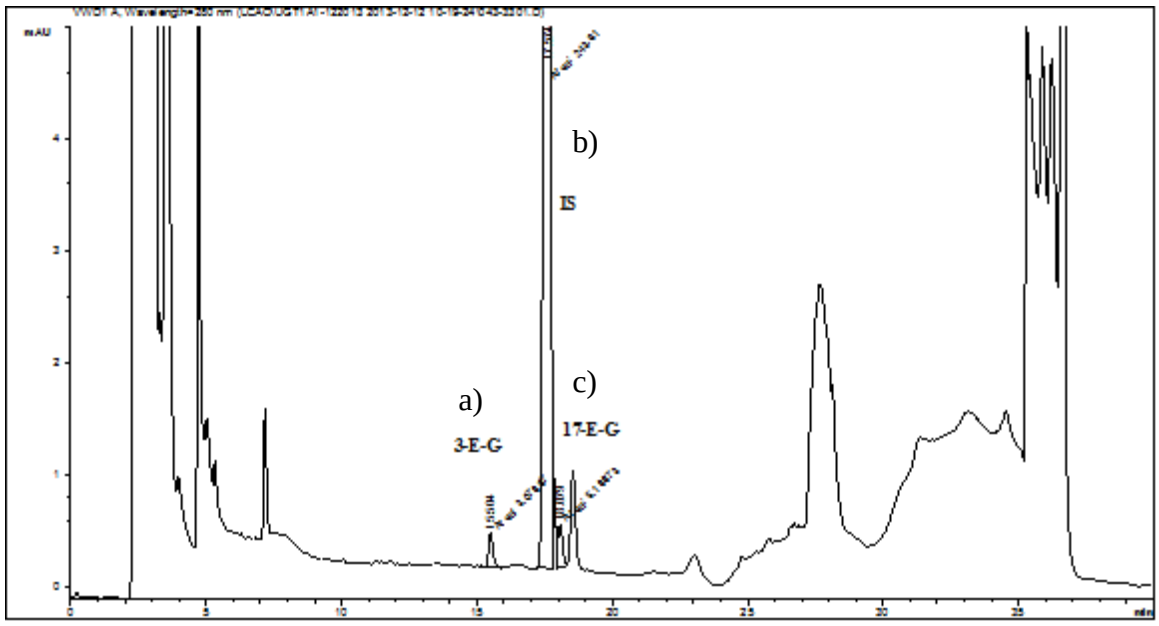


Figure 11, HPLC chromatogram of the UGT1A4 specific incubation sample using HLMs a) phenacetin as the IS, b) trifluoperazine-glucuronide as the trifluoperazine metabolite formed by HLMs, and c) trifluoperazine.

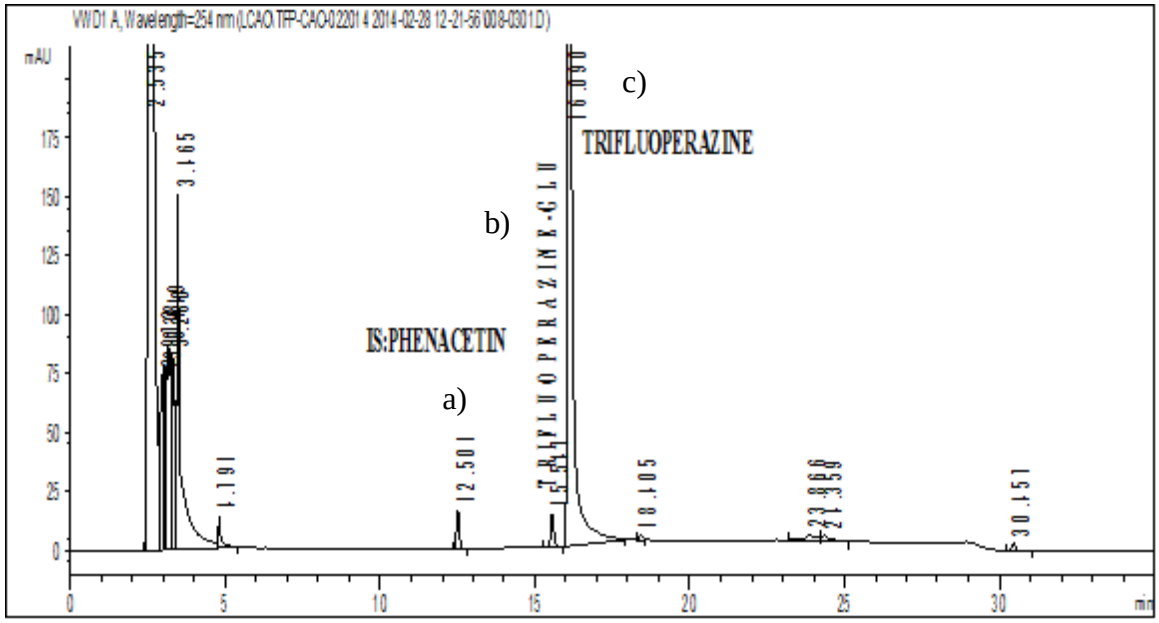


Figure 12, HPLC chromatogram of the UGT1A6 specific incubation sample using HLMs a) serotonin-glucuronide as the serotonin metabolite formed by HLMs, b) serotonin, and c) phenacetin as the IS.

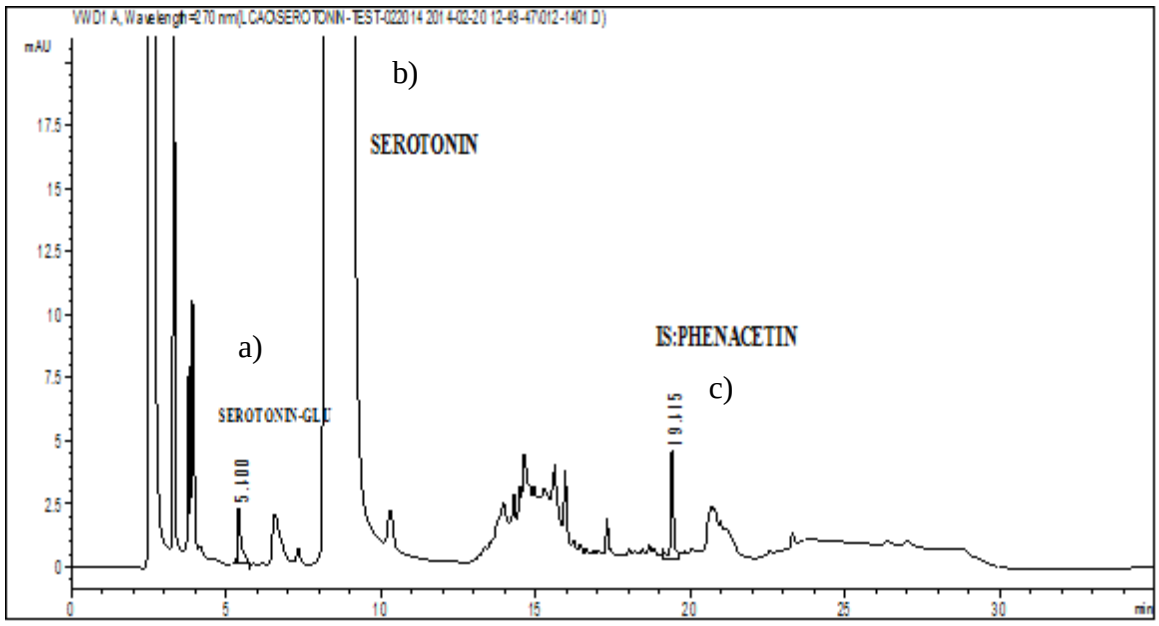


Figure 13, HPLC chromatogram of the UGT1A9 specific incubation sample using HLMs a) propofol, b) 3-acetaminophenol as the IS, and c) propofol-glucuronide as the propofol metabolite formed by HLMs.

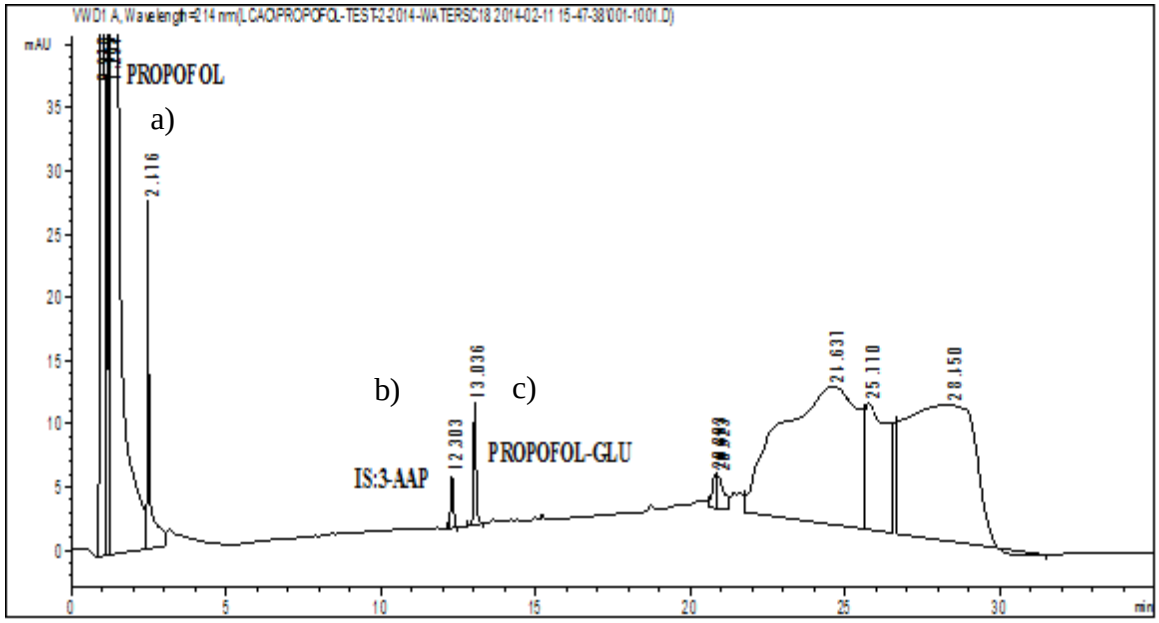


Figure 14, HPLC chromatogram of the UGT2B7 specific incubation sample using HLMs AZT-glucuronide as the 3'-azidothymidine (AZT) metabolite formed by HLMs, 3-acetaminophenol as the IS

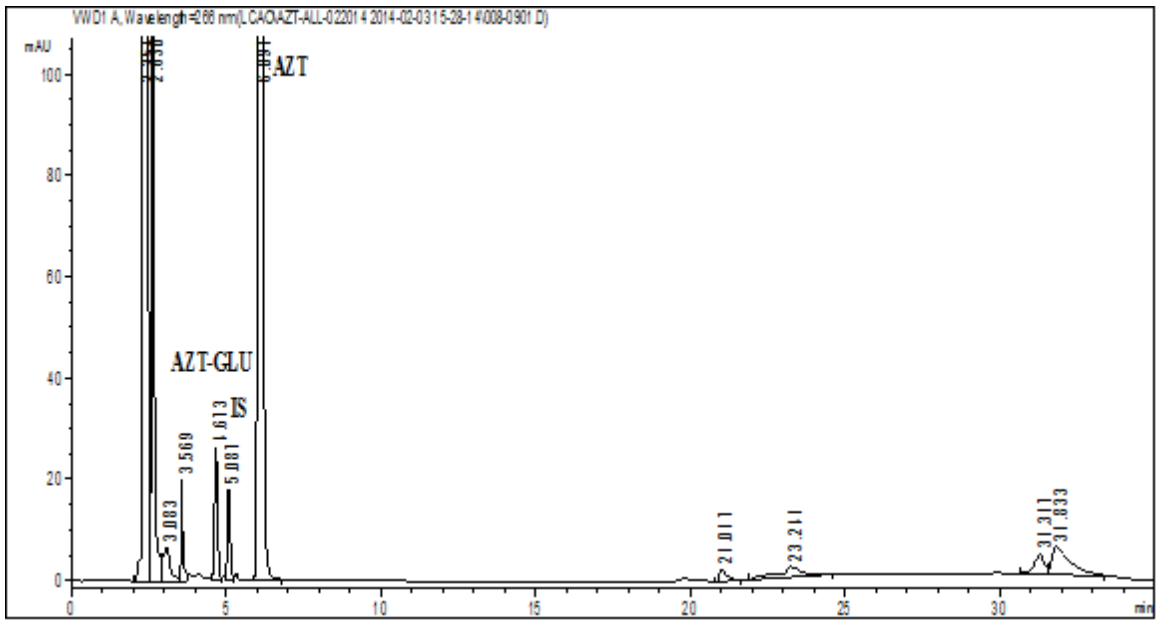


Figure 15, HPLC chromatogram of the UGT2B15 specific incubation sample using HLMs R-oxazepam glucuronide and S-oxazepam glucuronide as the metabolites of oxazepam formed by HLMs, 3-acetaminophenol as the IS

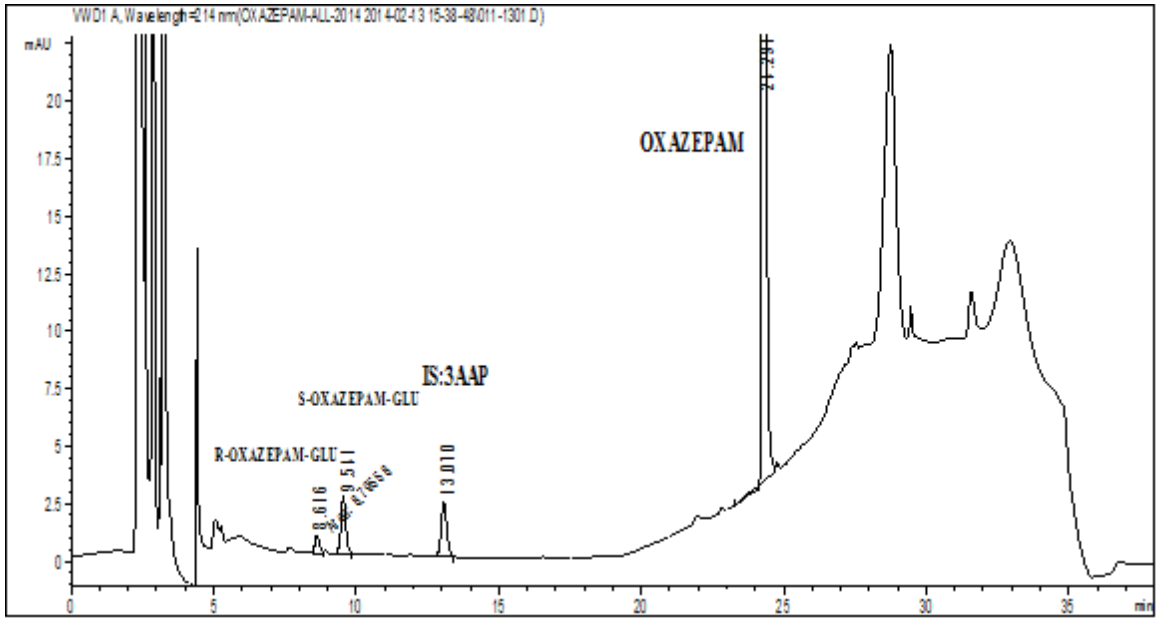
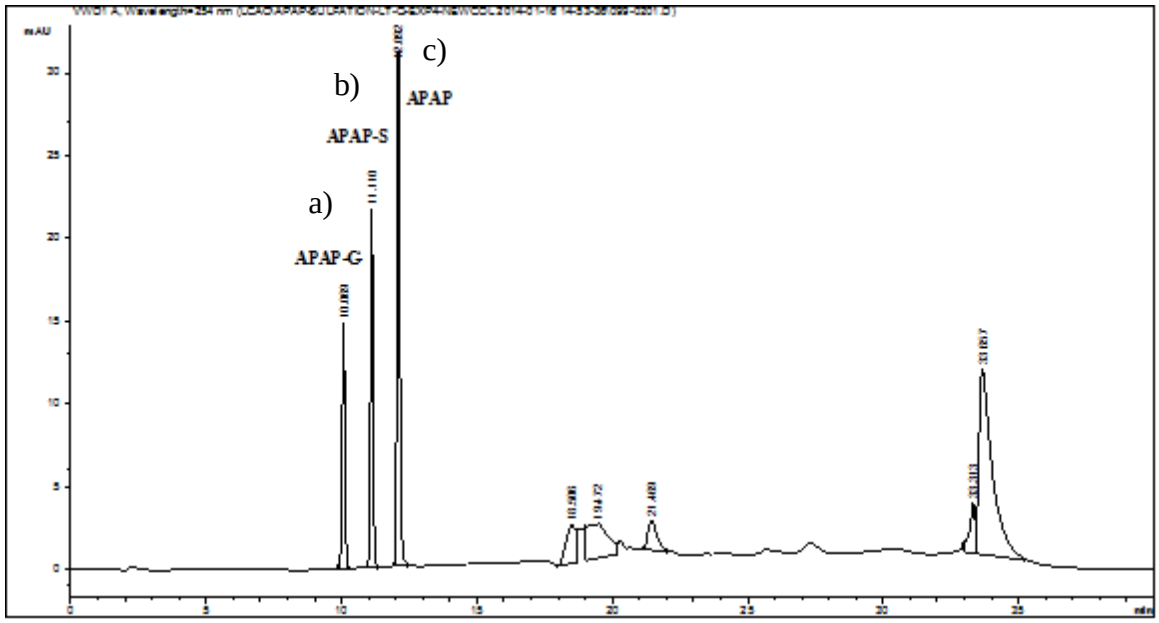
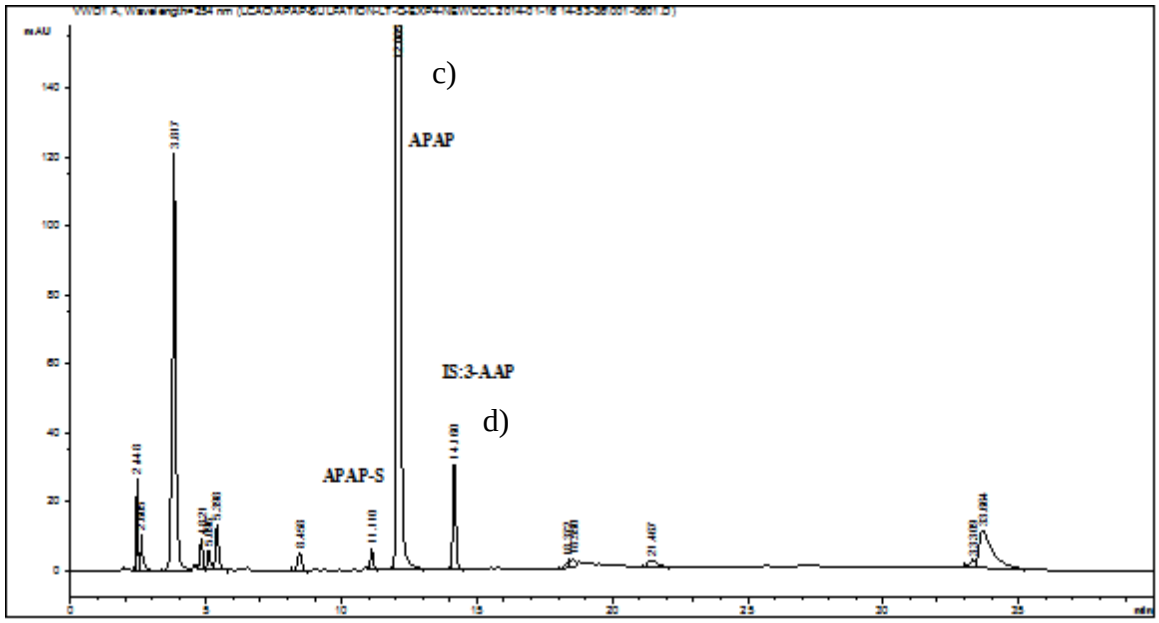


Figure 16, HPLC chromatogram of APAP glucuronidation using HLMs a) APAP-glucuronide, b) APAP-sulfate, c) APAP and d) 3-acetaminophenol (3AAP) as the IS.





2.8 Data analysis

2.8.1 IC_{50} calculation

IC_{50} values were determined using nonlinear regression based on Equation 8 [Greenblatt et al., 2011, von Moltke et al., 2001]. Sigmaplot 11.0 was applied for the curve fitting. The IC_{50} values were generated from the IC values using Equation 9.

$$R = 100\left(1 - \frac{E_{\max}[I]^b}{[I]^b + IC^b}\right) \qquad \text{Equation 8}$$

$$IC_{50} = \frac{IC}{(2E_{\max} - 1)^{1/b}} \qquad \text{Equation 9}$$

R is the reaction velocity expressed as the percentage of the control velocity with no inhibitor present for metabolites generated with co-addition of the compounds of interest as inhibitors; E_{max}, the maximum degree of inhibition; [I], the concentration of the anti-TB drugs; b, an exponent; IC, the inhibitor concentration at 50% inhibition of absolute 100% activity; $IC_{50,}$ the inhibitor concentration at 50% inhibition of maximal activity which is related to E_{max} and its value is calculated from IC using Equation 9.

2.8.2 K_i value for reversible enzymatic inhibition

K_i is the normalized concentration of inhibitor which gives half the maximal rate of inactivation. The K_i value of rifabutin on human UGT1A4 was fitted using the reversible inhibition model of full competitive inhibition on Sigmaplot 13.0. Significant substrate

inhibition of trifluoperazine was observed at the highest tested concentration (336.6 μM) in our study, as reported previously [Uchaipichat et al., 2006].

Results

3.1 Project 1: Inhibitory effects of anti-TB drugs on human CYPs and UGTs

3.1.1 IC_{50} values for rifabutin on human hepatic CYPs

Rifabutin was observed to inhibit human CYP3A, 2B6, 2D6, 1A2 and 2C9 to varying degrees *in vitro* using the pooled HLMs (Table 5, Figure 17). At the highest tested concentration (600 μM), no inhibition of human CYP2E1, 2C19 or 2C8 was observed with rifabutin (Figure 17).

3.1.2 IC_{50} values for rifabutin on human hepatic UGTs, and K_i value for rifabutin on UGT1A4

Rifabutin inhibited UGT1A1, 1A4 and 2B15 at varied levels (Table 5, Figure 18, 19), and partially inhibited human UGT1A9 and 2B7 (Table 5, Figure 18) at a high concentration of 600 μM. The IC_{50} values for rifabutin on human hepatic UGT1A4 were 10.8 and 11.3 μM respectively, for the incubations with and without pre-incubation. The K_i value of rifabutin on UGT1A4 using trifluoperazine as the index substrate was 2 μM with the pattern of inhibition consistent with reversible competitive inhibition (Figure 20).

Table 5 IC_{50} values of the selected anti-TB drugs on common human hepatic CYPs and UGTs

	Bedaquiline		***Rifabutin***		***Pyrazinamide***		***Ethambutol***		***Rifampicin***		***Isoniazid***	
	W[a] μM	W/O[b] μM	W μM	W/O μM	W μM	W/O μM	W μM	W/O μM	W μM	W/O μM	W μM	W/O μM
CYP1A2	NC[c]	NC	**183.3**	NC	NC	NC	NC	NC	-	-	-	-
CYP3A	NC	NC	**27.9**	**31.5**	NC	NC	NC	NC	-	-	-	-
CYP2B6	NC	NC	**120.8**	**165.3**	NC	NC	NC	NC	-	-	-	-
CYP2C8	NC	NC	NC	NC	NC	NC	NC	NC	-	-	-	-
CYP2C9	NC	NC	**75**	**150.9**	NC	NC	NC	NC	-	-	-	-
CYP2C19	NC	NC	NC	NC	NC	NC	NC	NC	-	-	-	-
CYP2D6	NC	NC	**147.7**	**166.5**	NC	NC	NC	NC	-	-	-	-
CYP2E1	NC	NC	NC	NC	NC	NC	NC	NC	-	-	-	-
UGT1A1	NC	NC	**35**	**44**	NC	NC	NC	NC	**70**	**63**	NC	NC
UGT1A4	-[d]	NC	**10.8**	**11.3**	-	NC	-	NC	-	**230**	-	NC
UGT1A6	-	NC	-	NC	-	NC	-	NC	-	[f]	-	NC
UGT1A9	-	NC	-	NC	-	NC	-	NC	-	NC	-	NC
UGT2B7	-	NC	-	[e]	-	NC	-	NC	-	NC	-	NC
UGT2B15	-	NC	-	**81.3**	-	NC	-	NC	-	**357**	-	NC
APAP-Glucuronida-tion	NC	NC	**237.2**	**422.2**	NC	NC	NC	NC	**860**	**397**	NC	NC

[a]: Incubations with pre-incubation; [b]: Incubations without pre-incubation; [c]: Not calculated (No IC_{50} values were calculated due to less than 50% inhibition at the highest tested concentrations where the highest concentrations for rifabutin, pyrazinamide, ethambutol, and isoniazid were 1000 μM, rifampicin 600 μM, bedaquiline 25 μM); [d] : not tested; [e]: 57% inhibition at 600 μM; [f]: 58% inhibition at 1000 μM

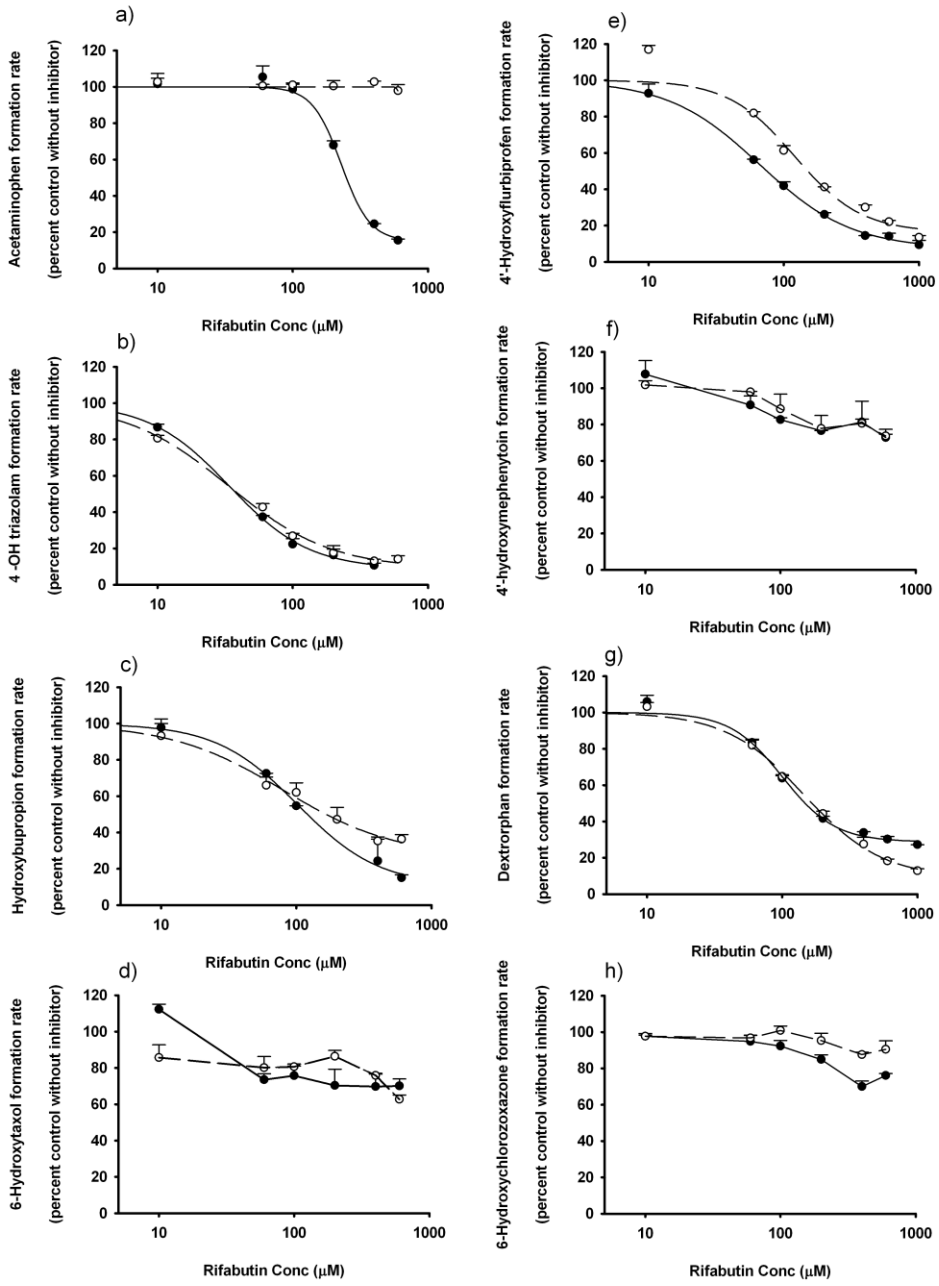


Figure 17 *In vitro* inhibitory effects of rifabutin on **a)** CYP1A2, **b)** CYP3A, **c)** CYP2B6, **d)** CYP2C8, **e)** CYP2C9, **f)** CYP2C19, **g)** CYP2D6 and **h)** CYP2E1; Data points represent the means ± standard errors (SEM) of each concentration of rifabutin that was tested in duplicate. IC_{50} values were determined by non-linear regression and summarized in Table 5.

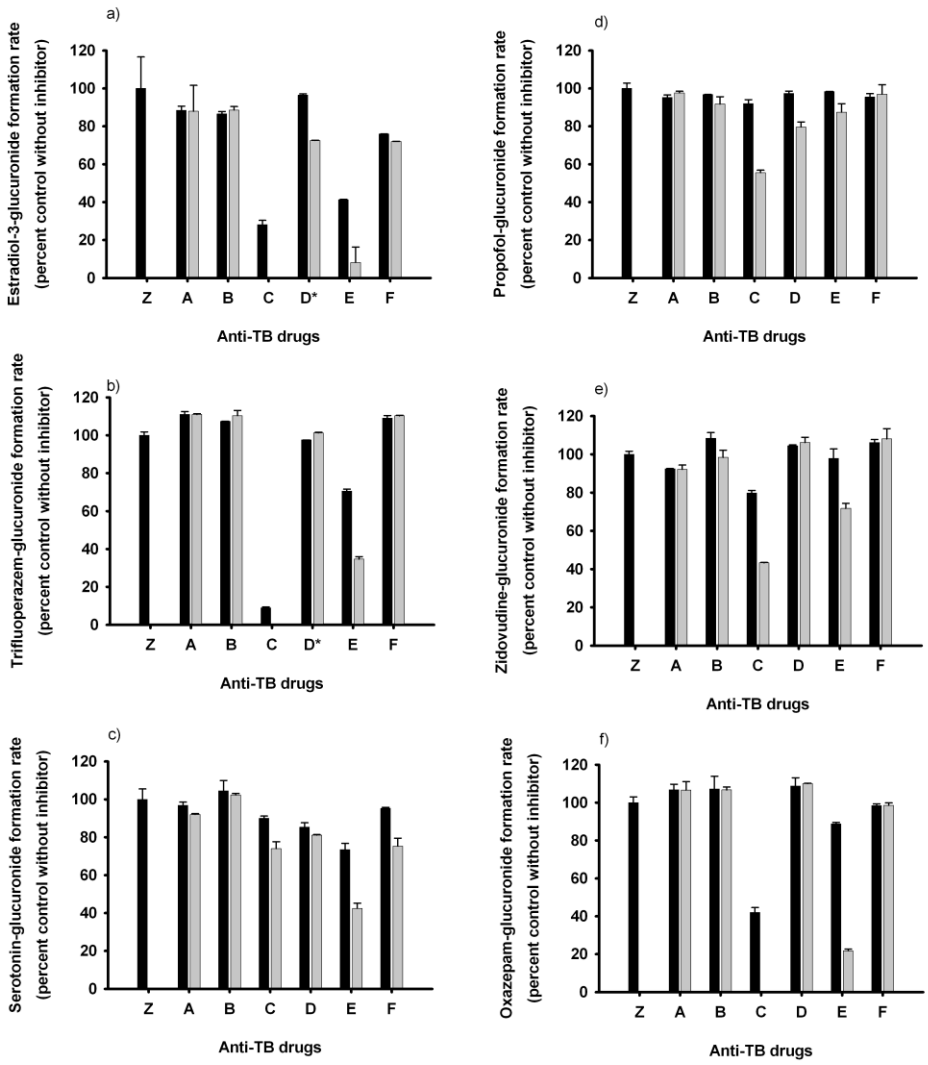


Figure 18 *In vitro* inhibitory effects of the selected anti-TB drugs on human hepatic **a)** UGT1A1, **b)** UGT1A4, **c)** UGT1A6, **d)** UGT1A9, **e)** UGT2B7, and **f)** UGT2B15; Two concentrations of each compound were tested. **Z: Control** without inhibition; **A: Pyrazinamide** (black:100 μM, gray:1000 μM); **B: Ethambutol** (black:100 μM, gray:1000 μM); **C: Rifabutin** (black:100 μM, gray:600 μM); **D*: Bedaquiline** (black: 26 μM, gray: 52 μM); **D: Bedaquiline** (black:12.5 μM, gray: 25 μM); **E: Rifampicin** (black:100 μM, gray:1000 μM); **F: Isoniazid** (black:100 μM, gray:1000 μM)

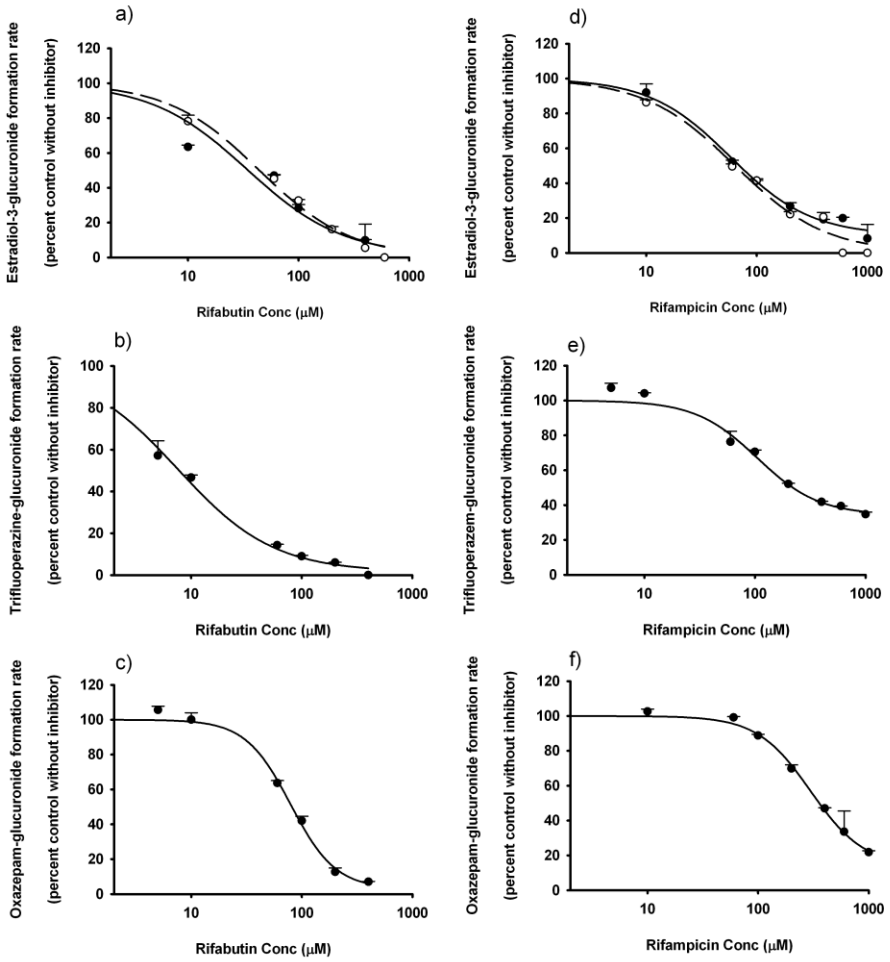


Figure 19 *In vitro* inhibitory effects of rifabutin and rifampicin on human UGT1A1, 1A4 and 2B15 **a, b, c)** rifabutin, **d, e, f)** rifampicin, **a, d)** UGT1A1, **b, e)** UGT1A4 and **c, f)** UGT2B15; The incubations were with pre-incubation (**closed circle**) and without pre-incubation (**open circle**); Data points represent the means ± standard errors (SEM) of each drug concentration that was tested in duplicate. IC_{50} values were determined by non-linear regression and summarized in Table 5.

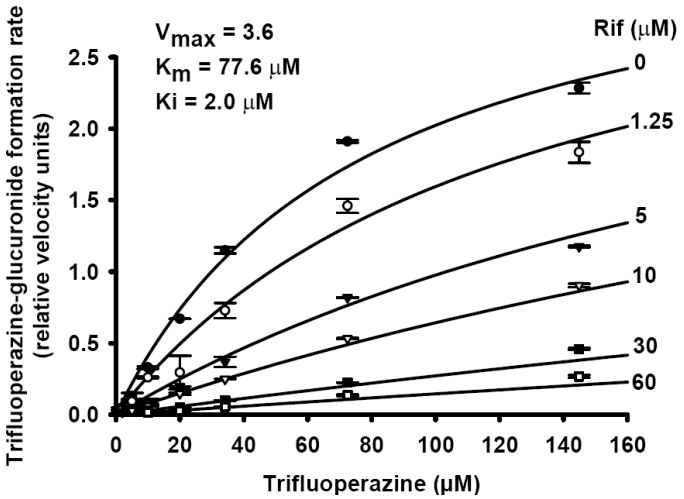


Figure 20 Rates of formation of trifluoperazine glucuronide in presence of inhibitory rifabutin (Rif) in a series of concentrations (0, 1.25, 5, 10, 30 and 60 μM). The K_i value for rifabutin on human hepatic UGT1A4 using trifluoperazine as the index substrate is 2 μM (K_m = 77.6 μM, V_{max} = 3.6). Data points represent the means ± standard errors (SEM) of duplicate incubations.

3.1.3 IC_{50} values for rifampicin and isoniazid on human hepatic UGTs

Rifampicin had inhibitory effects on UGT1A1, 1A4 and 2B15 with varied IC_{50} values (Figure 19); Partial inhibition of human UGT1A6 was observed at the highest tested concentration (1000 μM) (Figure 18). Up to the highest tested concentration (1000 μM), no inhibitory effects of isoniazid were observed (Figure 18).

3.1.4 Inhibitory effects of pyrazinamide, ethambutol and bedaquiline on human hepatic CYPs and UGTs

Up to the highest tested concentration (1000 μM), no inhibitory effects of pyrazinamide or ethambutol were observed on the 8 screened CYPs (Table 5) or 6 UGTs (Figure 18). At the highest tested concentration (25 μM), bedaquiline partially inhibited human hepatic CYP3A, 2B6, 2C8, 2C19 and 2D6 at varied levels, but inhibition did not exceed 50% of metabolite formation (Figure 18).

3.1.5 Effects of anti-TB drugs on APAP glucuronidation

The IC_{50} values on the inhibition of APAP glucuronidation for rifabutin with or without 20 minutes' pre-incubation were 237.2 μM and 422.2 μM respectively, and 860 μM and 397 μM respectively for rifampicin. Isoniazid, pyrazinamide, ethambutol, and bedaquiline provided minimal inhibition of APAP glucuronidation (Table 5, Figure 21). The positive control (probenecid, 0.5mM) produeced approximately 50% inhibition of APAP glucuronidation.

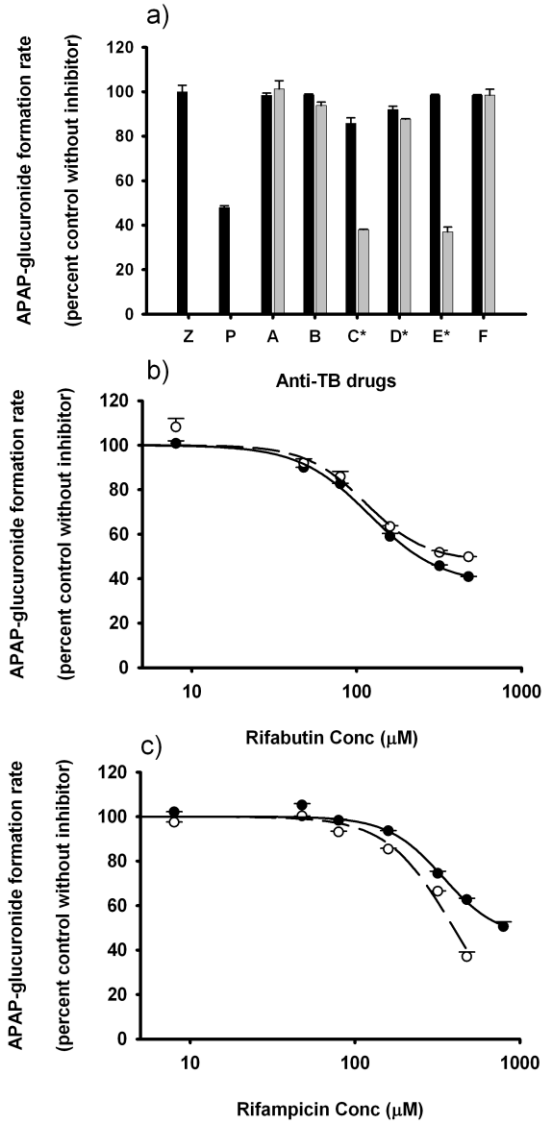


Figure 21 a) *In vitro* inhibitory effects of the selected anti-TB drugs on APAP glucuronidation using HLMs; Two concentrations of each compound were tested. **Z: Control** without inhibitor, **P: Probenecid** as the positive control 0.5mM, **A: Pyrazinamide** (black:100 μM, gray:1000 μM), **B: Ethambutol** (black:100 μM, gray:1000 μM), **C*: Rifabutin** (black:80 μM, gray:600 μM), **D*: Bedaquiline** (black:26 μM, gray:52 μM), **E*: Rifampicin** (black:80 μM, gray:1000 μM), **F: Isoniazid** (black:100 μM, gray:1000 μM);

b) *In vitro* inhibitory effects of rifabutin and **c)** rifampicin on APAP glucuronidation Incubations with pre-incubation (closed circle) or without pre-incubation (open circle); Data points represent the means ± standard errors (SEM) of each drug concentration that was tested in duplicate. IC_{50} values were determined by non-linear regression and summarized in Table 5.

3.2 Project 2: Metabolic interactions between APAP and two flavonoids (luteolin and quercetin)

3.2.1 IC_{50} values for luteolin and quercetin

The *in vitro* IC_{50} values for luteolin and quercetin for individual CYP and UGT enzymes using the pooled HLMs are in Table 6. Both luteolin and quercetin were observed to inhibit human hepatic CYP1A2, 3A, 2B6, 2C8, 2C9, 2C19, 2D6 and 2E1 as well as UGT1A1 and 1A4 at different levels. Left shifts of IC_{50}s between incubations without and with 20 minutes' pre-incubation were noticed (Figure 23, 24, and 26), indicating possibilities of mechanism based inhibition [(Grimm et al., 2009, Bertelsen et al., 2003)]. In addition, up to the highest tested concentrations of luteolin and quercetin (600 μM for the CYP systems and 250 μM for the UGT systems), no IC_{50}s for luteolin and quercetin were obtained for UGT1A6, UGT1A9, UGT2B7 and UGT2B15 due to less than 50% inhibition (Table 6, Figure 25).

Table 6 IC_{50} values of luteolin, quercetin, probenecid, MEOH and DMSO on human hepatic CYPs, and UGTs

	Luteolin		*Quercetin*		*Probenecid*		*MEOH*	*DMSO*
	W[a] (μM)	W/O[b] (μM)	W (μM)	W/O (μM)	W (mM)	W/O (mM)	W/O (%)	W/O (%)
CYP1A2	**1.6**	**3.5**	**4.5**	**5.2**	-	-	NC	NC
CYP3A	**6.1**	**12.4**	**7.5**	**10.4**	-	-	NC	NC
CYP2B6	**37.8**	**103.4**	**41.7**	**89.2**	-	-	NC	NC
CYP2C8	**4**	**2.1**	**7.1**	**2.4**	-	-	NC	NC
CYP2C9	**13.8**	**28.9**	**21.7**	**72.1**	-	-	NC	**2.4**
CYP2C19	**47.9**	NC[c]	**31.9**	**65.1**	-	-	NC	NC
CYP2D6	**132.6**	**152.7**	**99.4**	**233.6**	-	-	NC	NC
CYP2E1	**81.8**	**89.3**	**58**	**66.2**	-	-	**0.63**	**0.06**
UGT1A1	**93**	**44**	**86**	**54**	-	-	-	-
UGT1A4	-[d]	**48.1**	-	**50.2**	-	-	-	-
UGT1A6	-	NC	-	NC	-	-	-	-
UGT1A9	-	NC	-	NC	-	-	-	-
UGT2B7	-	NC	-	[g]	-	-	-	-
UGT2B15	-	NC	-	NC	-	-	-	-
APAP-Glucuronidation[e]	NC	NC	**286.5**	NC	**0.42**	**0.5**	-	-
APAP-sulfation[f]	-	**0.87**	-	**3.5**	-	-	-	-

[a]: Incubations with pre-incubation; [b]: Incubations without pre-incubation; [c]: Not calculated (No IC_{50} values were calculated due to less than 50% inhibition at the highest tested concentrations where luteolin and quercetin were 600 μM for the CYP systems and 250 μM for the UGT systems, and the highest tested percentage for methanol and DMSO was 2%); [d] : not tested; [e]: APAP glucuronidation is a mixed effect of several UGT isoforms such as UGT1A6, 1A1,1A9 and 2B15; [f]: APAP sulfation is a mixed effect of several SULT isoforms; [g]: 52% inhibition at 250 μM

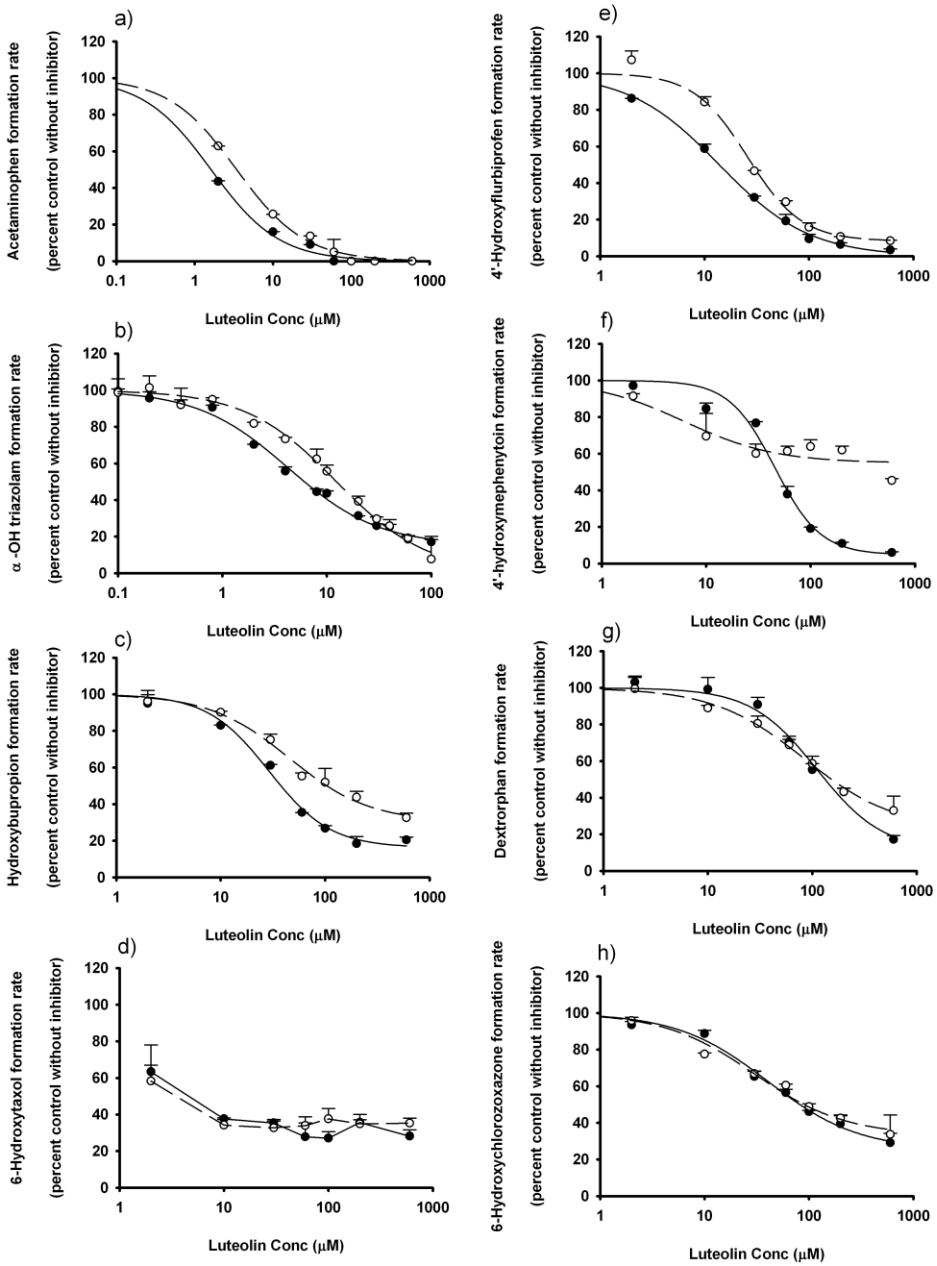


Figure 22 *In vitro* inhibitory effects of luteolin on **a)** CYP1A2, **b)** CYP3A, **c)** CYP2B6, **d)** CYP2C8, **e)** CYP2C9, **f)** CYP2C19, **g)** CYP2D6 and **h)** CYP2E1; Data points represent the means ± standard errors (SEM) of each concentration that was tested in duplicate. IC_{50} values were determined by non-linear regression and summarized in Table 6.

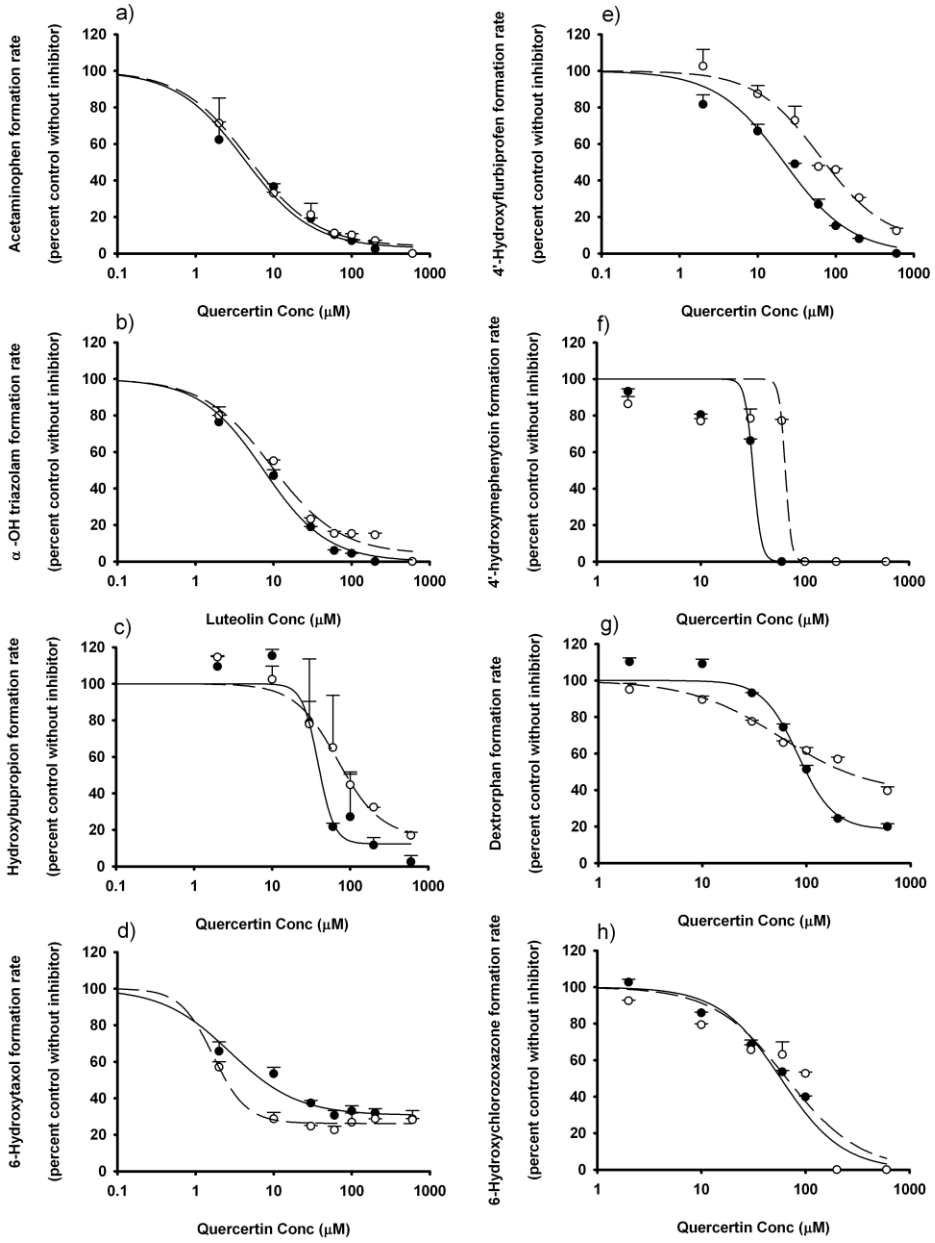


Figure 23 *In vitro* inhibitory effects of quercetin on **a)** CYP1A2, **b)** CYP3A, **c)** CYP2B6, **d)** CYP2C8, **e)** CYP2C9, **f)** CYP2C19, **g)** CYP2D6 and **h)** CYP2E1; Data points represent the means ± standard errors (SEM) of each concentration that was tested in duplicate. IC_{50} values were determined by non-linear regression and summarized in Table 6.

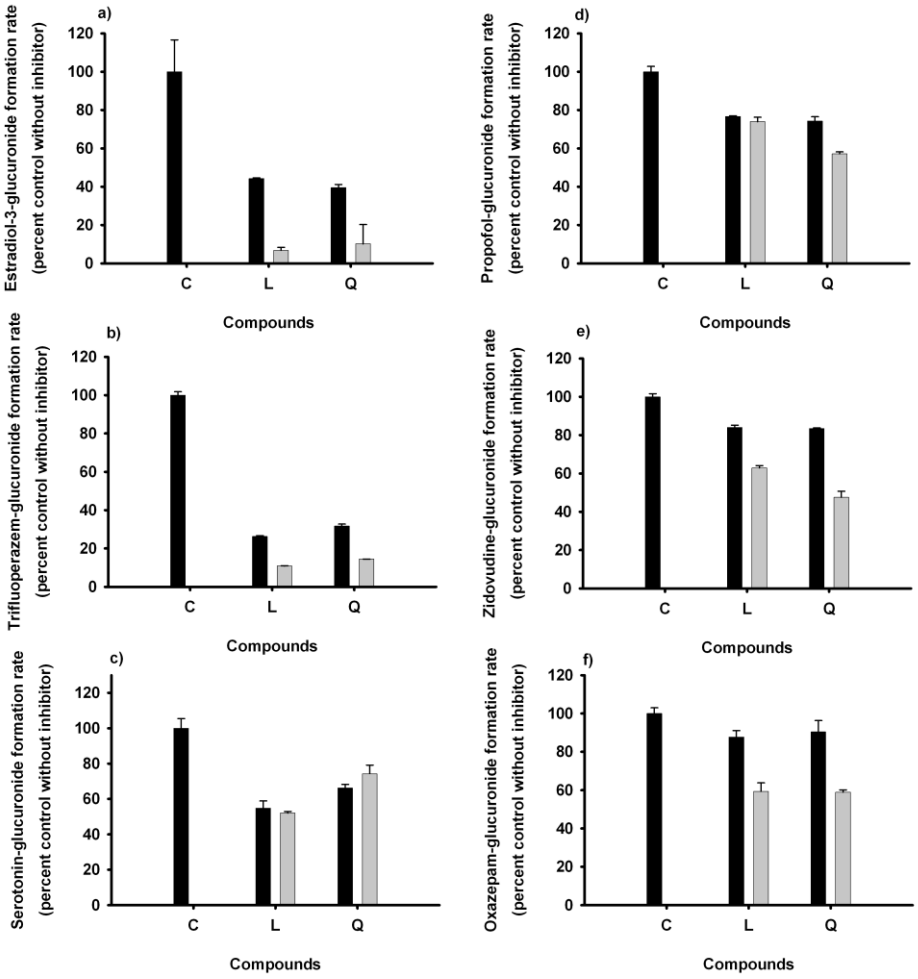


Figure 24 *In vitro* inhibitory effects of luteolin and quercetin on human hepatic **a)** UGT1A1, **b)** UGT1A4, **c)** UGT1A6, **d)** UGT1A9, **e)** UGT2B7 and **f)** UGT2B15; **C: Control** without inhibition (black: 0 μM); **L: Luteolin** (black:100 μM, gray:250 μM); **Q: Quercetin** (black:100 μM, gray:250 μM)

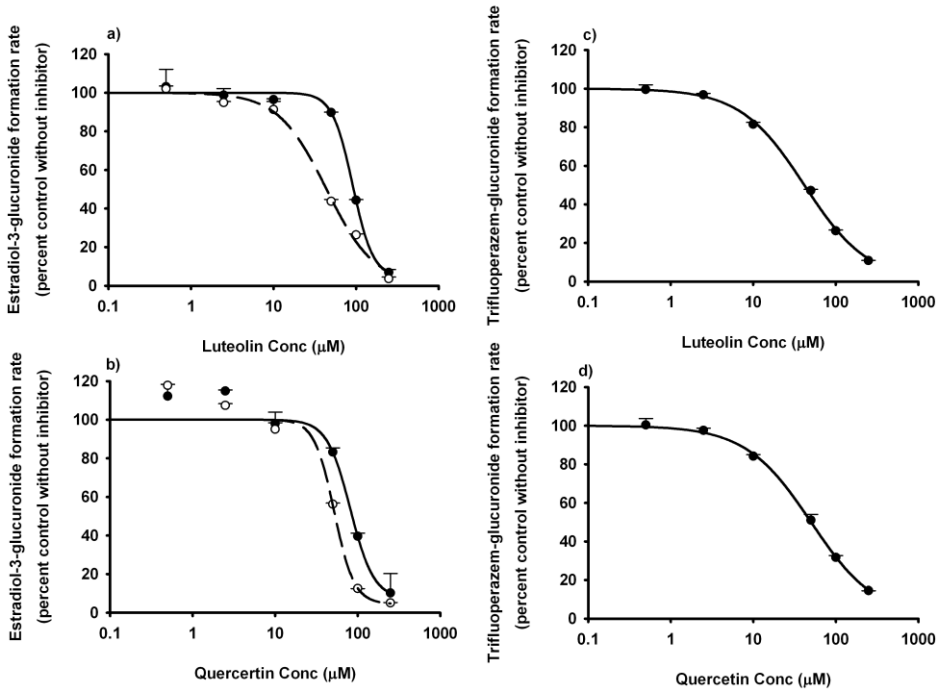


Figure 25 *In vitro* inhibitory effects of luteolin and quercertin on **a, b)** UGT1A1 or **c, d)** UGT1A4; Data points represent the means ± standard errors (SEM) of each concentration that was tested in duplicate. IC_{50} values were determined by non-linear regression and summarized in Table 6.

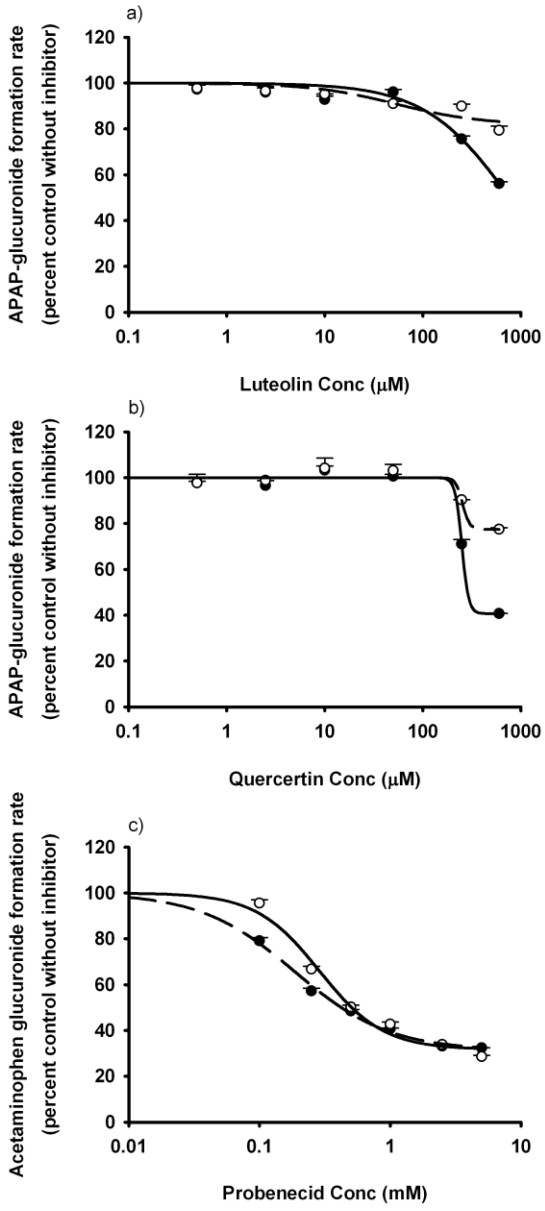


Figure 26 *In vitro* inhibitory effects of **a)** luteolin, **b)** quercertin and **c)** probenecid on APAP glucuronidation with pooled HLMs; Data points represent the means ± standard errors (SEM) of each drug concentration that was tested in duplicate. IC_{50} values were determined by non-linear regression and summarized in Table 6.

Furthermore, the inhibitory effects of luteolin and quercetin on overall APAP glucuronidation were also tested using APAP as the substrate. While probenecid, a known inhibitor for APAP glucucronidation as the positive control, yielded an IC_{50} value of 0.5 mM, we didn't observe luteolin or quercetin to inhibit overall APAP glucuronidation (Figure 26).

APAP sulfation is another major detoxifying pathway to clear APAP safely from the body. In our *in vitro* experiments, we observed that both luteolin and quercetin were strong inhibitors toward APAP sulfation with IC_{50} values of 0.87 and 3.5 μM respectively (Figure 27).

3.2.2 Solvent effects of methanol and DMSO on human hepatic CYPs

CYP2E1 was most sensitive for both methanol and DMSO. The IC_{50} values for methanol and DMSO on CYP2E1 were 0.63% and 0.06% respectively. The IC_{50} value for DMSO on CYP2C9 was 2.4%. CYP2B6 was also partially inhibited by 1% methanol. (Table 6, Figure 28, 29)

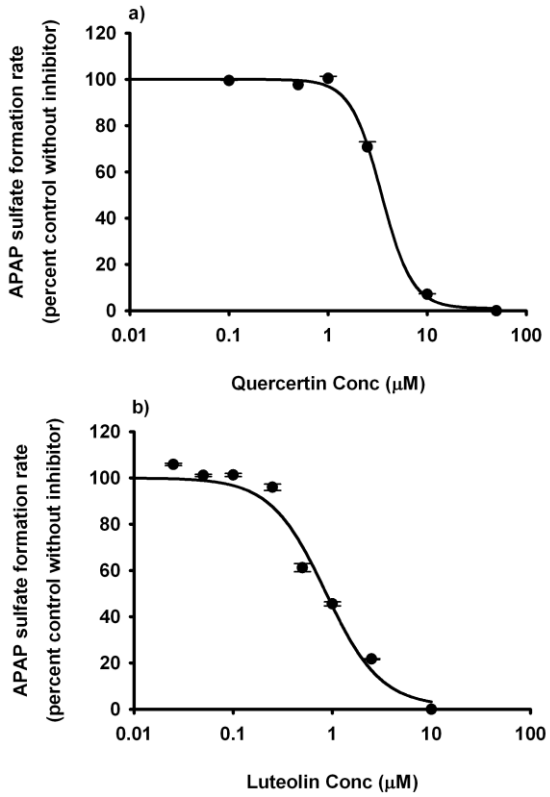


Figure 27 *In vitro* inhibitory effects of **a)** luteolin and **b)** quercertin on acetaminophen sulfation; Data points represent the means ± standard errors (SEM) of each drug concentration that was tested in duplicate. IC_{50} values were determined by non-linear regression and summarized in Table 6.

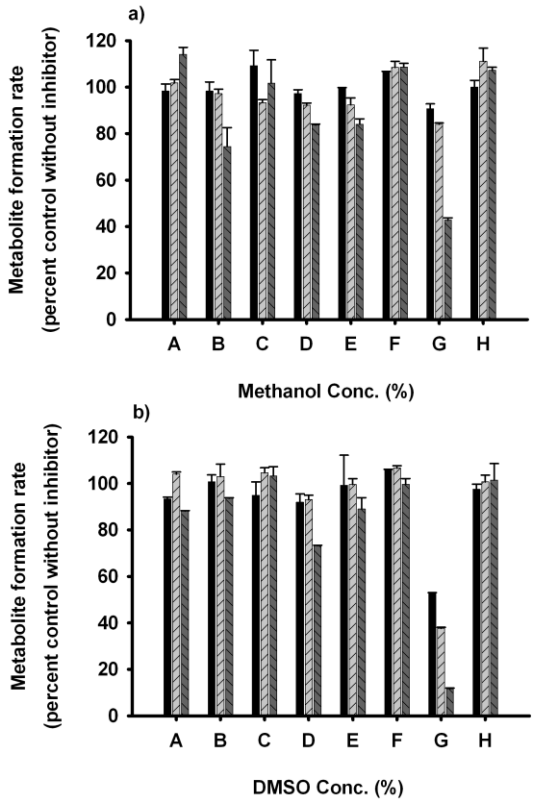


Figure 28 Inhibitory effects of **a)** methanol or **b)** DMSO on human hepatic CYP enzymes with pooled HLMs; Methanol percentages were at 0.05% (black), 0.1% (gray), or 1% (dark gray); **A**: CYP1A2; **B**: CYP2B6; **C**: CYP2C8; **D**: CYP2C9; **E**: CYP2C19; **F**: CYP2D6; **G**: CYP2E1; **H**: CYP3A.

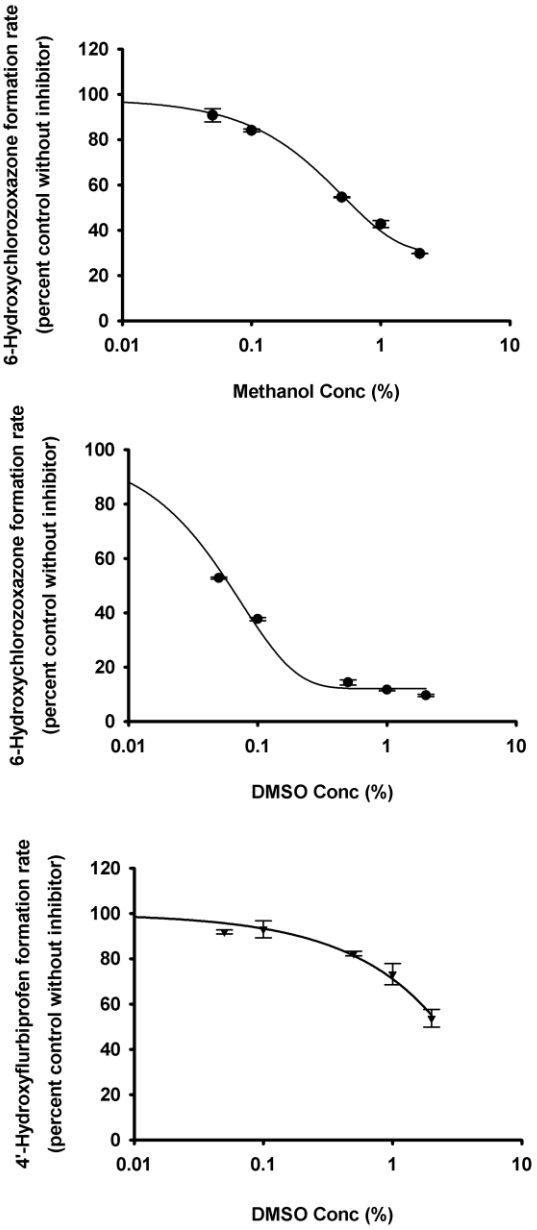


Figure 29 *In vitro* inhibition of CYP2E1 in **a)** methanol**,** in **b**) DMSO, or **c**) inhibition of CYP2C9 in DMSO; Data points represent the means ± standard errors (SEM) of each solvent concentration that was tested in duplicate. IC_{50} values were determined by non-linear regression and summarized in Table 6.

Discussion

4.1 Project 1: Inhibitory effects of anti-TB drugs on human CYPs and UGTs

Rifabutin inhibited human CYP3A, 2B6, 2D6, 1A2, 2C9, UGT1A1, 2B15, UGT1A9 and 2B7 in HLMs, with varying inhibitory potency. However, most of those inhibitory effects observed *in vitro* are not likely to be of clinical importance since the IC_{50} values were much higher than typical clinical plasma concentrations of rifabutin, which are usually less than 1.1 μM [(Peloquin, 2002)].

UGT1A4 is an important drug-metabolizing enzyme [(Williams et al., 2004)], and the IC_{50} value for rifabutin on human hepatic UGT1A4 was low enough to be of potential clinical relevance. A K_i value for rifabutin on UGT1A4 was determined using the pooled HLMs and the result was 2 μM. Based on FDA guidance [(CDER, 2012)], an approximate estimation of the produced DDI was calculated using the ratio of $[I]/K_i$ where [I] is the maximum *in vivo* plasma concentration of rifabutin (1.1 μM) [(Peloquin, 2002)] and K_i was 2 μM. The ratio of 0.55 indicates a possibility that rifabutin may increase the systemic exposure of some drugs metabolized mainly by human UGT1A4. On the other hand, it has been widely reported that rifabutin induces human CYPs and UGTs [(Baciewicz et al., 2013)]. Thus the prediction of the overall drug-drug interaction of rifabutin needs to take into account of both its inhibitory and possible inductive properties.

Human UGT1A1 is the main metabolizing enzyme for several anti-HIV drugs such as raltegravir [(Kassahun et al., 2007)] and dolutegravir [(Castellino et al., 2013)]. The IC_{50} values of rifabutin and rifampicin on UGT1A1 were around 35 μM and 70 μM respectively (Figure 18). The inhibitory effects of rifabutin and rifampicin are not clinically relevant, as their

overall effects show prominent inductive properties. Rifampicin significantly decreased the systemic exposure of dolutegravir [Dooley et al., 2013]. Co-administration of rifabutin, on the other hand, did not alter the pharmacokinetics of raltegravir [Brainard et al., 2011] or dolutegravir [Dooley et al., 2013].

The IC_{50} values for rifabutin on CYP1A2 shifted leftward between the incubations without and with pre-incubation (Figure 17). However, a value of K_i was not determined. Nevertheless, no clinically meaningful DDIs due to inhibition of CYP1A2 by rifabutin have been reported.

Since high concentrations of the anti-TB drugs were used in the incubations, 1% methanol was introduced to improve solubility. Bedaquiline was prepared in DMSO for better solubility and added directly to the incubation mixtures. Because methanol and DMSO may themselves inhibit metabolic activities of CYPs and UGTs, inhibitor-free controls were included using the same amounts of these solutions to normalize the solvent effects.

Glucuronidation is one of the major metabolizing pathways in the clearance of acetaminophen in parallel with the CYP mediated oxidation pathway. Therefore, APAP glucuronidation diverts APAP clearance away from generation of the toxic intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI) [Miner and Kissinger, 1979], known to be responsible for acetaminophen hepatotoxicity. APAP glucuronidation is likely to be mediated by several UGTs [Court et al., 2001, Court and Greenblatt, 2000, Court and Greenblatt, 1997, Krishnaswamy et al., 2005, Mutlib et al., 2006] including UGT1A1, 1A6, 1A9 and 2B15. In this study, none of the selected

anti-TB drugs significantly inhibited glucuronidation of APAP (Figure 21), suggesting that DDIs which involve increasing APAP toxicity are unlikely.

In conclusion, this study provided a relatively complete survey on *in vitro* inhibitory effects of the common anti-TB drugs on CYPs and UGTs using HLMs. The findings from this study do not raise substantial new concerns about DDIs involving anti-TB drugs.

4.2 Project 2: Metabolic interactions between APAP and two flavonoids (luteolin and quercetin)

Flavonoids are important dietary constituents consumed daily. In the US, a study indicated that normal daily intake of 5 flavonoids including quercetin, kaempferol, myricetin, apigenin and luteolin was 20-22 mg/d (Chun et al., 2007). In another study among a Chinese university-campus population, the daily consumption of luteolin was reported as 7.96 ± 5.64 mg/d, with the plasma exposure at 100. 63 ± 98.51 nmol/L (Zhang et al., 2010). In addition to the daily intake of flavonoids, consumption of dietary supplements containing flavonoids in some populations can dramatically increase the systemic exposure of flavonoids. In a study of daily supplementation of quercetin, the median maximum plasma concentrations of quercetin including its glucuronidated and sulfated metabolites were observed as 431 nmol/L at 3 hours after intake of 150 mg quercetin (Egert et al., 2008). When we apply the reported *in vivo* plasma concentrations to understand the *in vitro* data, we need to take into account of many factors, including but not limited to the following considerations. First, the plasma concentrations are not equivalent to the concentrations in the tissues of interest. The exposure of the flavonoids such as luteolin and quercetin

may be more concentrated in liver than in plasma [Shimoi, 2001]. Thus, in this study, an arbitrary multiplication, for an example, 10 fold of those reported plasma values *in vivo*, was applied to roughly estimate the upper limits of the exposure of the flavonoids in liver, that is 1 μM for luteolin and 4 μM for quercetin. Second, only aglycone luteolin and quercetin were studied in this study. But in the *in vivo* systems, flavonoids such as luteolin and quercetin are known for having extensive glucuronide and sulfate conjugates as well as methylated metabolites [Spencer, 2003], and those major metabolites could have their inhibitory effects on the liver metabolic enzymes, which have not yet been investigated. Third, the reported *in vivo* plasma concentrations of luteolin or quercetin usually include the aglycone forms and their metabolites due to the enzymatic hydrolysis steps commonly used in the sample preparation.

CYP3A4, CYP2E1, CYP1A2, CYP2D6 and CYP2A6 have previously been reported to be responsible for the production of NAPQI. Both luteolin and quercetin were observed to inhibit CYP1A2 and CYP3A at low concentrations with their IC_{50} values less than 10 μM (Table 6). It is likely that both luteolin and quercetin could inhibit part of the CYP-mediated oxidation of APAP by inhibiting CYP1A2 and 3A.

Because APAP glucuronidation and sulfation are also important pathways to detoxify APAP in the body parallel to the CYP-mediated oxidation, we also tested whether luteolin and quercetin inhibited the human hepatic UGT systems as well as APAP sulfation *in vitro*. Six human hepatic UGT isoforms were individually tested (Figure 24). We observed that both luteolin and quercetin inhibited UGT1A1 and 1A4 with IC_{50} values around 50 μM in the *in vitro* systems without pre-incubation (Figure 25). In

addition, at 100 μM which is a much higher concentration than the possible physiological exposure, luteolin and quercetin only partially inhibited UGT1A6 (less than 50%) and UGT1A9 (around 20%). The inhibitory effects of luteolin and quercetin on overall APAP glucuronidation, which is the combined effect from all of the individual hepatic UGTs, were also studied using APAP as the substrate. Luteolin or quercetin didn't inhibit overall APAP glucuronidation, while probenecid, a positive inhibitor of APAP glucucronidation as the positive control, had an IC_{50} value of 0.5 mM **(**Figure 24**)**. Therefore, based on our *in vitro* data, APAP glucuronidation is not likely to be inhibited by luteolin or quercetin. As for APAP sulfation, we observed that both luteolin and quercetin were strong inhibitors of APAP sulfation (Figure 27).

In conclusion, our observed *in vitro* data suggest that both luteolin and quercetin may inhibit some of the CYP450 isoforms which are responsible for the NAPQI formation, such as CYP1A2 and CYP3A, with the IC_{50} values less than 10 μM. Luteolin or quercetin is not likely inhibitory to APAP glucuronidation. Luteolin and quercetin however may strongly inhibit APAP sulfation. Therefore, the beneficial effects of luteolin and quercetin against APAP induced hepatotoxicity possibly result from their properties of being able to block partially the CYP-mediated oxidation and to drive the reaction via APAP glucuronidation.

References

Abernethy DR, Greenblatt DJ, Divoll M, Ameer B, and Shader RI. (1983) Differential effect of cimetidine on drug oxidation (antipyrine and diazepam) vs. conjugation (acetaminophen and lorazepam): prevention of acetaminophen toxicity by cimetidine. *J Pharmacol Exp Ther* **224**:508-513.

Adjei AA, Gaedigk A, Simon SD, Weinshilboum RM, and Leeder JS. (2008) Interindividual variability in acetaminophen sulfation by human fetal liver: implications for pharmacogenetic investigations of drug-induced birth defects. *Birth Defects Res A Clin Mol Teratol* **82**:155-165.

Axelrod J. (1955) The enzymatic demethylation of ephedrine. J Pharmacol Exp Ther 114:430-438.

Aycan IO, Tufek A, Tokgoz O, Evliyaoglu O, Firat U, Kavak GO, Turgut H, and Yuksel MU. (2014) Thymoquinone treatment against acetaminophen-induced hepatotoxicity in rats. *Int J Surg* **12**:213-218.

Baciewicz AM, Chrisman CR, Finch CK, and Self TH. (2013) Update on rifampin, rifabutin, and rifapentine drug interactions. *Curr Med Res Opin* **29**:1-12.

Benson GD, Koff RS, and Tolman KG. (2005) The therapeutic use of acetaminophen in patients with liver disease. *Am J Ther* **12**:133-141.

Berry LM and Zhao Z. (2008) An examination of IC50 and IC50-shift experiments in assessing time-dependent inhibition of CYP3A4, CYP2D6 and CYP2C9 in human liver microsomes. Drug Metab Lett 2:51-59.

Bertelsen KM, Venkatakrishnan K, von Moltke LL, Obach RS, and Greenblatt DJ. (2003) Apparent mechanism-based inhibition of human CYP2D6 in vitro by

paroxetine: comparison with fluoxetine and quinidine. *Drug Metab Dispos* **31**:289-293.

Brainard DM, Kassahun K, Wenning LA, Petry AS, Liu C, Lunceford J, Hariparsad N, Eisenhandler R, Norcross A, DeNoia EP, Stone JA, Wagner JA, and Iwamoto M. (2011) Lack of a clinically meaningful pharmacokinetic effect of rifabutin on raltegravir: in vitro/in vivo correlation. *J Clin Pharmacol* **51**:943-50.

Brodie BB, Axelrod J, Cooper JR, Gaudette L, La Du Bn, Mitoma C, and Udenfriend S. (1955) Detoxication of drugs and other foreign compounds by liver microsomes. Science 121:603-604.

Castellino S, Moss L, Wagner D, Borland J, Song I, Chen S, Lou Y, Min SS, Goljer I, Culp A, Piscitelli SC, and Savina PM. (2013) Metabolism, excretion, and mass balance of the HIV-1 integrase inhibitor dolutegravir in humans. *Antimicrob Agents Chemother* **57**:3536-3546.

Chahine EB, Karaoui LR, Mansour H. (2014) Bedaquiline: a novel diaryquinoline for multidrug-resistant tuberculosis. *Annals of Pharmacotherapy* **48**:107-115

Chen W, Koenigs LL, Thompson SJ, Peter RM, Rettie AE, Trager WF, and Nelson SD. (1998; 1998) Oxidation of Acetaminophen to Its Toxic Quinone Imine and Nontoxic Catechol Metabolites by Baculovirus-Expressed and Purified Human Cytochromes P450 2E1 and 2A6. *Chem Res Toxicol* **11**:295-301.

Chun OK, Chung SJ, and Song WO. (2007) Estimated Dietary Flavonoid Intake and Major Food Sources of U.S. Adults. *J Nutr* **137**:1244-1252.

Court MH. (2010) Interindividual variability in hepatic drug glucuronidation: studies into the role of age, sex, enzyme inducers, and genetic polymorphism using the human liver bank as a model system. *Drug Metab Rev* **42**:209-224.

Court MH. (2005) Isoform-selective probe substrates for in vitro studies of human UDP-glucuronosyltransferases. *Methods Enzymol* **400**:104-116.

Court MH and Greenblatt DJ. (2000) Molecular genetic basis for deficient acetaminophen glucuronidation by cats: UGT1A6 is a pseudogene, and evidence for reduced diversity of expressed hepatic UGT1A isoforms. *Pharmacogenetics* **10**:355-369.

Court MH and Greenblatt DJ. (1997) Molecular basis for deficient acetaminophen glucuronidation in cats. An interspecies comparison of enzyme kinetics in liver microsomes. *Biochem Pharmacol* **53**:1041-1047.

Court MH, Duan SX, von Moltke LL, Greenblatt DJ, Patten CJ, Miners JO, and Mackenzie PI. (2001) Interindividual variability in acetaminophen glucuronidation by human liver microsomes: identification of relevant acetaminophen UDP-glucuronosyltransferase isoforms. *J Pharmacol Exp Ther* **299**:998-1006.

Danielson PB. (2002) The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. Curr Drug Metab 3:561-597.

Desta Z, Soukhova NV, and Flockhart DA. (2001) Inhibition of cytochrome P450 (CYP450) isoforms by isoniazid: potent inhibition of CYP2C19 and CYP3A. *Antimicrob Agents Chemother* **45**:382-392.

Dooley KE, Sayre P, Borland J, Purdy E, Chen S, Song I, Peppercorn A, Everts S, Piscitelli S, Flexner C. (2013) Safety, Tolerability, and Pharmacokinetics of the HIV

Integrase Inhibitor Dolutegravir Given Twice Daily With Rifampin or Once Daily With Rifabutin: Results of a Phase 1 Study Among Healthy Subjects. *JAIDS* **62**:21-27

Egert S, Wolffram S, Bosy-Westphal A, Boesch-Saadatmandi C, Wagner AE, Frank J, Rimbach G, and Mueller MJ. (2008) Daily quercetin supplementation dose-dependently increases plasma quercetin concentrations in healthy humans. *J Nutr* **138**:1615-21.

European Medicines Agency. (2012) Guideline on the Investigation of Drug Interactions.

Evans WE and Relling MV. (1999) Pharmacogenomics: translating functional genomics into rational therapeutics. Science 286:487-491.

FDA. Guidance for Industry, Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations. (Draft 2012)

Fisher MB, Campanale K, Ackermann BL, VandenBranden M, and Wrighton SA. (2000) In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. *Drug Metab Dispos* **28**:560-566.

Gilani AH, Janbaz KH, and Shah BH. (1997) Quercetin exhibits hepatoprotective activity in rats. *Biochem Soc Trans* **25**:S619.

Graham GG, Davies MJ, Day RO, Mohamudally A, and Scott KF. (2013) The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. *Inflammopharmacology* **21**:201-232.

Greenblatt DJ, Zhao Y, Venkatakrishnan K, Duan SX, Harmatz JS, Parent SJ, Court MH, and von Moltke LL. (2011) Mechanism of cytochrome P450-3A inhibition by ketoconazole. *J Pharm Pharmacol* **63**:214-221.

Grimm SW, Einolf HJ, Hall SD, He K, Lim HK, Ling KH, Lu C, Nomeir AA, Seibert E, Skordos KW, Tonn GR, Van Horn R, Wang RW, Wong YN, Yang TJ, and Obach RS. (2009) The conduct of in vitro studies to address time-dependent inhibition of drug-metabolizing enzymes: a perspective of the pharmaceutical research and manufacturers of America. *Drug Metab Dispos* **37**:1355-1370.

Guengerich RP. (2005) Human Cytochrome P450 Enzymes, in Cytochrome P450: Structure, Mechanism, and Biochemistry, 3rd ed. (Paul R and Montellano O eds.), Kluwer Academic / Plenum Publishers, New York.

Hertog MG, Feskens EJ, Hollman PC, Katan MB, and Kromhout D. (1994) Dietary flavonoids and cancer risk in the Zutphen Elderly Study. *Nutr Cancer* **22**:175-184.

Hertog MG, Feskens EJ, Hollman PC, Katan MB, and Kromhout D. (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **342**:1007-1011.

Hjelle JJ and Klaassen CD. (1984) Glucuronidation and biliary excretion of acetaminophen in rats. *J Pharmacol Exp Ther* **228**:407-413.

Kalgutkar AS, Obach RS, Maurer TS. Mechanism-based inactivation of cytochrome P450 enzymes: Chemical mechanisms, structure-activity relationships and relationship to clinical drug-drug interactions and idiosyncratic adverse drug reactions. Curr Drug Metab. 2007 Jun;8(5):407-47.

Kajosaari LI, Laitila J, Neuvonen PJ, and Backman JT. (2005) Metabolism of repaglinide by CYP2C8 and CYP3A4 in vitro: effect of fibrates and rifampicin. *Basic Clin Pharmacol Toxicol* **97**:249-256.

Kassahun K, McIntosh I, Cui D, Hreniuk D, Merschman S, Lasseter K, Azrolan N, Iwamoto M, Wagner JA, and Wenning LA. (2007) Metabolism and disposition in humans of raltegravir (MK-0518), an anti-AIDS drug targeting the human immunodeficiency virus 1 integrase enzyme. *Drug Metab Dispos* **35**:1657-1663.

Kiran PM, Raju AV, and Rao BG. (2012) Investigation of hepatoprotective activity of Cyathea gigantea (Wall. ex. Hook.) leaves against paracetamol-induced hepatotoxicity in rats. *Asian Pac J Trop Biomed* **2**:352-356.

Krishnaswamy S, Hao Q, Al-Rohaimi A, Hesse LM, von Moltke LL, Greenblatt DJ, and Court MH. (2005) UDP glucuronosyltransferase (UGT) 1A6 pharmacogenetics: II. Functional impact of the three most common nonsynonymous UGT1A6 polymorphisms (S7A, T181A, and R184S). *J Pharmacol Exp Ther* **313**:1340-1346.

Laine JE, Auriola S, Pasanen M, and Juvonen RO. (2009) Acetaminophen bioactivation by human cytochrome P450 enzymes and animal microsomes. *Xenobiotica* **39**:11-21.

Lamb DC, Lei L, Warrilow AG, Lepesheva GI, Mullins JG, Waterman MR, and Kelly SL. (2009) The first virally encoded cytochrome p450. J Virol 83:8266-8269.

Larson AM, Polson J, Fontana RJ, Davern TJ, Lalani E, Hynan LS, Reisch JS, Schiodt FV, Ostapowicz G, Shakil AO, Lee WM, and Acute Liver Failure Study Group. (2005) Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* **42**:1364-1372.

Lewis DF. (2004) 57 varieties: the human cytochromes P450. Pharmacogenomics 5:305-318.

Mackenzie PI, Owens IS, Burchell B, Bock KW, Bairoch A, Belanger A, Fournel-Gigleux S, Green M, Hum DW, Iyanagi T, Lancet D, Louisot P, Magdalou J,

Chowdhury JR, Ritter JK, Schachter H, Tephly TR, Tipton KF, and Nebert DW. (1997) The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. Pharmacogenetics 7:255-269.

Manach C, Scalbert A, Morand C, Remesy C, and Jimenez L. (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**:727-747.

Manyike PT, Kharasch ED, Kalhorn TF, and Slattery JT. (2000) Contribution of CYP2E1 and CYP3A to acetaminophen reactive metabolite formation. *Clin Pharmacol Ther* **67**:275-282.

Martinez C, Albet C, Agundez JA, Herrero E, Carrillo JA, Marquez M, Benitez J, and Ortiz JA. (1999) Comparative in vitro and in vivo inhibition of cytochrome P450 CYP1A2, CYP2D6, and CYP3A by H2-receptor antagonists. *Clin Pharmacol Ther* **65**:369-376.

Miksits M, Sulyok M, Schuhmacher R, Szekeres T, and Jager W. (2009) In-vitro sulfation of piceatannol by human liver cytosol and recombinant sulfotransferases. *J Pharm Pharmacol* **61**:185-191.

Miner DJ and Kissinger PT. (1979) Evidence for the involvement of N-acetyl-p-quinoneimine in acetaminophen metabolism. *Biochem Pharmacol* **28**:3285-3290.

Miners JO, McKinnon RA, and Mackenzie PI. (2002) Genetic polymorphisms of UDP-glucuronosyltransferases and their functional significance. Toxicology 181-182:453-456.

Mitchell MC, Schenker S, and Speeg KV,Jr. (1984) Selective inhibition of acetaminophen oxidation and toxicity by cimetidine and other histamine H2-receptor antagonists in vivo and in vitro in the rat and in man. *J Clin Invest* **73**:383-391.

Mutlib AE, Goosen TC, Bauman JN, Williams JA, Kulkarni S, and Kostrubsky S. (2006) Kinetics of acetaminophen glucuronidation by UDP-glucuronosyltransferases 1A1, 1A6, 1A9 and 2B15. Potential implications in acetaminophen-induced hepatotoxicity. *Chem Res Toxicol* **19**:701-709.

Ochs HR, Greenblatt DJ, Roberts GM and Dengler HJ. (1981) Diazepam interaction with antituberculosis drugs. *Clin Pharmacol and Therap* **29**:671-678.

Ochs HR, Greenblatt DJ and Knüchel M. (1983) Differential effect of isoniazid on triazolam oxidation and oxazepam conjugation. *Br J Clin Pharmacol* **16**:743-746

Oesch F, Arand M, Benedetti MS, Castelli MG, and Dostert P. (1996) Inducing properties of rifampicin and rifabutin for selected enzyme activities of the cytochrome P-450 and UDP-glucuronosyltransferase superfamilies in female rat liver. *J Antimicrob Chemother* **37**:1111-1119.

Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, and Zeldin DC. (2006) The human intestinal cytochrome P450 "pie". Drug Metab Dispos 34:880-886.

Peloquin CA. (2002) Therapeutic drug monitoring in the treatment of tuberculosis. *Drugs* **62**:2169-2183.

Polasek TM, Elliot DJ, Lewis BC, and Miners JO. (2004) Mechanism-based inactivation of human cytochrome P4502C8 by drugs in vitro. *J Pharmacol Exp Ther* **311**:996-1007.

Rae JM, Johnson MD, Lippman ME, and Flockhart DA. (2001) Rifampin is a selective, pleiotropic inducer of drug metabolism genes in human hepatocytes: studies with cDNA and oligonucleotide expression arrays. *J Pharmacol Exp Ther* **299**:849-857.

Sakeran MI, Zidan N, Rehman H, Aziz AT, and Saggu S. (2014) Abrogation by Trifolium alexandrinum root extract on hepatotoxicity induced by acetaminophen in rats. *Redox Rep* **19**:26-33.

Shimada T, Yamazaki H, Mimura M, Inui Y, and Guengerich FP. (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 270:414-423.

Shimoi K, Saka N, Nozawa R, Sato M, Amano I, Nakayama T, Kinae N. (2001) Deglucuronidation of a flavonoid, luteolin monoglucuronide, during inflammation. *Drug Metab Dispos* **29**:1521-4.

Shivashri C, Rajarajeshwari T, and Rajasekar P. (2013) Hepatoprotective action of celery (Apium graveolens) leaves in acetaminophen-fed freshwater fish (Pangasius sutchi). *Fish Physiol Biochem* **39**:1057-1069.

Sigel R, Sigel A, and Sigel H. (2007) The Ubiquitous Roles of Cytochrome P450 Proteins: Metal Ions in Life Sciences, Volume 3Wiley, New York.

Silverman RB. (1996) Mechanism-based enzyme inactivation, in Contemporary Enzyme Kinetics and Mechanisms, 2nd ed. (Purich D ed.), Academic Press, San Diego.

Slattery JT, McRorie TI, Reynolds R, Kalhorn TF, Kharasch ED, and Eddy AC. (1989) Lack of effect of cimetidine on acetaminophen disposition in humans. *Clin Pharmacol Ther* **46**:591-597.

Soars MG, Petullo DM, Eckstein JA, Kasper SC, and Wrighton SA. (2004) An assessment of UDP-glucuronosyltransferase induction using primary human hepatocytes. *Drug Metab Dispos* **32**:140-148.

Spencer JP. (2003) Metabolism of tea flavonoids in the gastrointestinal tract. *J Nutr* **133**:3255S-3261S.

Strassburg CP, Manns MP, and Tukey RH. (1998) Expression of the UDP-glucuronosyltransferase 1A locus in human colon. Identification and characterization of the novel extrahepatic UGT1A8. J Biol Chem 273:8719-8726.

Strassburg CP, Oldhafer K, Manns MP, and Tukey RH. (1997) Differential expression of the UGT1A locus in human liver, biliary, and gastric tissue: identification of UGT1A7 and UGT1A10 transcripts in extrahepatic tissue. Mol Pharmacol 52:212-220.

Thummel KE, Lee CA, Kunze KL, Nelson SD, and Slattery JT. (1993) Oxidation of acetaminophen to N-acetyl-p-aminobenzoquinone imine by human CYP3A4. *Biochem Pharmacol* **45**:1563-1569.

Tonge RP, Kelly EJ, Bruschi SA, Kalhorn T, Eaton DL, Nebert DW, and Nelson SD. (1998) Role of CYP1A2 in the hepatotoxicity of acetaminophen: investigations using Cyp1a2 null mice. *Toxicol Appl Pharmacol* **153**:102-108.

Tran A, Treluyer JM, Rey E, Barbet J, Ferracci G, d'Athis P, Vincent J, and Pons G. (2001) Protective effect of stiripentol on acetaminophen-induced hepatotoxicity in rat. *Toxicol Appl Pharmacol* **170**:145-152.

U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER). (2012) Guidance for Industry Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations (DRAFT GUIDANCE).

Uchaipichat V, Mackenzie PI, Elliot DJ, and Miners JO. (2006) Selectivity of substrate (trifluoperazine) and inhibitor (amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfinpyrazone) "probes" for human UDP-glucuronosyltransferases. *Drug Metab Dispos* **34**:449-456.

van de Kerkhof EG, de Graaf IA, Ungell AL, and Groothuis GM. (2008) Induction of metabolism and transport in human intestine: validation of precision-cut slices as a tool to study induction of drug metabolism in human intestine in vitro. *Drug Metab Dispos* **36**:604-613.

von Moltke LL, Greenblatt DJ, Harmatz JS, and Shader RI. (1993a) Alprazolam metabolism in vitro: studies of human, monkey, mouse, and rat liver microsomes. *Pharmacology* **47**:268-276.

von Moltke LL, Manis M, Harmatz JS, Poorman R, and Greenblatt DJ. (1993b) Inhibition of acetaminophen and lorazepam glucuronidation *in vitro* by probenecid. *Biopharma and Drug Dispos* **14**:119-130.

von Moltke LL, Greenblatt DJ, Giancarlo GM, Granda BW, Harmatz JS, and Shader RI. (2001) Escitalopram (S-citalopram) and its metabolites in vitro: cytochromes mediating biotransformation, inhibitory effects, and comparison to R-citalopram. *Drug Metab Dispos* **29**:1102-1109.

Wen X, Wang JS, Neuvonen PJ, and Backman JT. (2002) Isoniazid is a mechanism-based inhibitor of cytochrome P450 1A2, 2A6, 2C19 and 3A4 isoforms in human liver microsomes. *Eur J Clin Pharmacol* **57**:799-804.

White RE. (2000) High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery. Annu Rev Pharmacol Toxicol 40:133-157.

Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, Peterkin V, Koup JR, and Ball SE. (2004) Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios. *Drug Metab Dispos* **32**:1201-1208.

Williamson G and Manach C. (2005) Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* **81**:243S-255S.

Worley MV, and Estrada SJ. (2014) Bedaquiline: A novel antitubercular agent for the treatment of multidrug-resistant tuberculosis. *Pharmacotherapy* **34**:1187-1197

World Health Organization (WHO). (2014) Global Tuberculosis Report 2014.

World Health Organization (WHO). (2010) Guidelines for treatment of tuberculosis.

Wolf KK, Wood SG, Allard JL, Hunt JA, Gorman N, Walton-Strong BW, Szakacs JG, Duan SX, Hao Q, Court MH, von Moltke LL, Greenblatt DJ, Kostrubsky V, Jeffery EH, Wrighton SA, Gonzalez FJ, Sinclair PR, and Sinclair JF. (2007) Role of CYP3A and CYP2E1 in alcohol-mediated increases in acetaminophen hepatotoxicity: comparison of wild-type and Cyp2e1(-/-) mice. *Drug Metab Dispos* **35**:1223-1231.

Wolf KK, Wood SG, Hunt JA, Walton-Strong BW, Yasuda K, Lan L, Duan SX, Hao Q, Wrighton SA, Jeffery EH, Evans RM, Szakacs JG, von Moltke LL, Greenblatt DJ, Court MH, Schuetz EG, Sinclair PR, and Sinclair JF. (2005) Role of the nuclear receptor pregnane X receptor in acetaminophen hepatotoxicity. *Drug Metab Dispos* **33**:1827-1836.

Yang CH, Tang L, Lv C, Ye L, Xia BJ, Hu M, and Liu ZQ. (2011) Sulfation of selected mono-hydroxyflavones by sulfotransferases in vitro: a species and gender comparison. *J Pharm Pharmacol* **63**:967-970.

Yousef MI, Omar SA, El-Guendi MI, and Abdelmegid LA. (2010) Potential protective effects of quercetin and curcumin on paracetamol-induced histological changes, oxidative stress, impaired liver and kidney functions and haematotoxicity in rat. *Food Chem Toxicol* **48**:3246-3261.

Zaher H, Buters JT, Ward JM, Bruno MK, Lucas AM, Stern ST, Cohen SD, and Gonzalez FJ. (1998) Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice. *Toxicol Appl Pharmacol* **152**:193-199.

Zhang L, Reynolds KS, Zhao P, and Huang SM. (2010) Drug interactions evaluation: an integrated part of risk assessment of therapeutics. Toxicol Appl Pharmacol 243:134-145.

Zhang Y, Cao J, Chen W, Yang J, Hao D, Zhang Y, Chang P, and Zhao X. (2010) Reproducibility and relative validity of a food frequency questionnaire to assess intake of dietary flavonol and flavone in Chinese university campus population. *Nutr Res* **30**:520-526.

Zhao Y, Harmatz JS, Epstein CR, Nakagawa Y, Kurosaki C, Nakamura T, Kadota T, Giesing D, Court MH and Greenblatt DJ. (2015) Favipiravir inhibits acetaminophen sulfate formation but minimally affects systemic pharmacokinetics of acetaminophen. *Br J Clin Pharmacol* 80:1076-1085

Zhou L, Erickson RR, Hardwick JP, Park SS, Wrighton SA, and Holtzman JL. (1997) Catalysis of the cysteine conjugation and protein binding of acetaminophen by microsomes from a human lymphoblast line transfected with the cDNAs of various forms of human cytochrome P450. *J Pharmacol Exp Ther* **281**:785-790.

Zumla A, Nahid P, and Cole ST. (2013) Advances in the development of new tuberculosis drugs and treatment regimens. *Nat Rev Drug Discov* **12**:388-404.