THE ROLE OF INTESTINAL UDP-GLUCURONOSYLTRANSFERASES IN 7-ETHYL-10-HYDROXY-CAMPTOTHECIN (SN-38) CONJUGATION AND TOXICITY

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ABSTRACT

Melanie Nicole Tallman: The Role of Intestinal UDP-Glucuronosyltransferases in 7-Ethyl-10-Hydroxy-Camptothecin (SN-38) Conjugation and Toxicity
(Under the direction of Philip C. Smith, Ph.D.)

A significant number of cancer patients treated with the topoisomerase inhibitor, irinotecan, experience severe, delayed-onset diarrhea. Clinicians will typically reduce subsequent doses of irinotecan in affected patients, which may result in diminished effectiveness of the anti-cancer agent. The active metabolite of irinotecan, 7-ethyl-10-hydroxy-camptothecin (SN-38), has been implicated as the colon-toxic agent, by causing direct injury to colonic epithelial cells. SN-38 is consequently detoxified to SN-38 glucuronide (SN-38G) by UDP-glucuronosyltransferase (UGT) isozymes expressed not only in the liver, but also in the gastrointestinal tract. Although luminal SN-38 has been identified as an important mediator of diarrhea, the influence of intestinal UGTs at the site of toxicity has not been addressed. Thus, intestinal SN-38 glucuronidation was studied to address the hypothesis that UGT activity in the intestine is a critical factor in modulating SN-38 toxicity, as enteric cells have the ability to prevent SN-38 toxicity locally through UGT expression.

The primary human and rat isoforms that glucuronidate SN-38 were identified, many of which are localized in the intestine. SN-38 carboxylate was found to be a suitable, more soluble surrogate for the active lactone form for *in vitro* glucuronidation, as the rank order of UGT isoform catalysis was similar for the two forms, despite different rates of conjugation. Intersubject SN-38 glucuronidation in human intestinal tissue from duodenal, jejunal, and colonic regions was variable, and was highly correlated with both UGT1A1 and 1A9 probe

substrate glucuronidation, indicating a primary role for these isoforms in intestinal SN-38G formation. The hypothesis that intestinal UGTs modulate SN-38-induced diarrhea was directly tested in a Gunn rat model with reconstituted hepatic UGT activity via adenoviral gene delivery. These rats, devoid of intestinal UGT1A activity, were highly susceptible to intestinal damage and diarrhea after irinotecan administration, compared to control heterozygote rats, which possessed both hepatic and intestinal UGTs. Collectively, this research advanced the knowledge of the metabolic detoxification of SN-38 in the intestine by establishment and identification of appropriate *in vitro* reaction conditions, isoform specificity and expression, and variability in glucuronidation. These studies have also supported the hypothesis that intestinal UGT expression modulates the incidence of gastrointestinal toxicity to SN-38.

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LIST OF ABBREVIATIONS

Abs	Absorbance
APC	7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin
AUC	Area under the plasma concentration versus time curve
AZT	Azidothymidine
Cmax	Maximal plasma concentration
CPT-11	Irinotecan
DMSO	Dimethyl sulfoxide
EHC	Enterohepatic recirculation
Etoposide-G	Etoposide glucuronide
HPLC	High performance liquid chromatography
IC_{50}	Concentration of an inhibitor that elicits 50% inhibition
j/j	Gunn rat
j/jAV	Gunn rat infected with adenoviral vector
j+	Heterozygote Gunn rat
j+AV	Heterozygote Gunn rat infected with adenoviral vector
Km	Concentration of substrate that will provide an *in vitro* glucuronidation rate half of what is maximally possible
LC-MS	Liquid chromatography with mass spectrometry detection
MPA	Mycophenolic acid
MTT	Methylthiazolyldiphenyl-tetrazolium assay
NPC	7-ethyl-10-(4-amino-1-piperidino)carbonyl-oxycamptothecin

PAH	Polyhalogenated arylhydrocarbons
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
Propofol-G	Propofol glucuronide
SN-38	7-ethyl-10-hydroxy-camptothecin
SN-38G	SN-38 glucuronide
SNP	Single nucleotide polymorphism
TBS-T	Tris buffered saline with tween
UDP	Uridine diphosphate
UDPGA	Uridine diphosphoglucuronic acid
UDP-Glucuronosyltransferase	Uridine diphosphate glucuronosyltransferase
UGT	Uridine diphosphate glucuronosyltransferase
Vmax	Maximal *in vitro* glucuronidation rate

CHAPTER 1

INTRODUCTION

A. INTRODUCTION

Drug metabolizing enzymes often play a critical role in mediating drug effect and drug toxicity. In most cases, these enzymes chemically modify their xenobiotic substrates, such that pharmacological activity is lost. Highly efficient drug metabolism may compromise a compound's efficacy, while inefficient metabolism may predispose patients to adverse effects due to accumulation of drug with chronic dosing. Keeping these considerations in mind, rational drug design and development would ideally include knowledge of a compound's therapeutic and toxic windows, but also the influence that variability in drug metabolizing enzymes have on the dosing regimen to provide concentrations that fall within these windows.

Without downplaying the importance of drug metabolism, other factors can also play a role in mediating xenobiotic concentrations at sites of pharmacologic effect and toxicity, such as transporters or protein binding. Additionally, multiple drug metabolizing enzymes may recognize the drug substrate, obscuring the importance of any one factor in mediating drug disposition.

These complexities related to efficacy, toxicity, metabolism, and systemic disposition are highlighted with a clinical example involving 7-ethyl-10-hydroxy-camptothecin (SN-38), the active metabolite of the anti-cancer agent, irinotecan, and the UDP-Glucuronosyltransferase (UGT) family of drug metabolizing enzymes (Figure 1.1). Irinotecan and SN-38 are substrates for numerous enzyme systems and transporters in the liver. Upon biliary excretion, both are substrates for intestinal metabolic enzymes and transporters, which may be similar or different from those responsible for the disposition of these compounds systemically. To further complicate this scenario, each compound can exist

in two chemically different forms that take on distinct biological and physicochemical properties. It is ultimately the UGTs that detoxify SN-38 by forming its glucuronide, SN-38G, which is not stable when in the intestinal lumen. Irinotecan, through the actions of SN-38, has a narrow therapeutic window, with a large subset of patients experiencing life-threatening neutropenia or diarrhea. Thus far, despite an enigmatic disposition for irinotecan/SN-38, a clear role for low UGT activity has been linked to neutropenia, however, primary mechanisms for mediating diarrhea are still ambiguous.

In this review, an extensive background regarding the UGT1A family will be given, highlighting tissue-specific differences in expression and glucuronidation, as well as environmental and genetic factors that can alter expression and function. Irinotecan pharmacology will also be discussed. The hypotheses and methodologies explored thus far to predict and prevent irinotecan-induced toxicity will be detailed. Ultimately, it is hoped that the reader will understand that research is lacking with respect to elucidation of specific mechanisms behind the incidence of diarrhea and protective enzymes at the site of toxicity, the intestinal UGTs.

B. URIDINE DIPHOSPHATE GLUCURONOSYLTRANSFERASES (UGT)

B.1. FUNCTION

Uridine Diphosphate Glucuronosyltransferases (UGTs or UDP-Glucuronosyltransferases) are a superfamily of enzymes that catalyze the transfer of a glucuronic acid moiety from the nucleotide sugar UDP-glucuronic acid (UDPGA) to a lipid-soluble molecule (Mulder, 1992; Burchell, 1999). UGTs are anchored in the membrane of the endoplasmic reticulum and following conjugation, the more hydrophilic conjugates are

expelled by transporters into the lumen of the ER. In an S_N2 reaction, a nucleophilic attack by the substrate on the C1 pryanose acid carbon of UDPGA results in the formation of a β-D glucuronic acid product (Figure 1.1) (Clarke and Burchell, 1994). The nucleophilic moiety of the substrate might be one or more functional groups, including carboxylic acids, hydroxyl, thiol, amino, or activated carbon centers (Burchell, 1999). The addition of a polar glucuronic acid group often reduces the volume of distribution and increases elimination through urinary and biliary excretion relative to the aglycone. The latter processes are facilitated by either an increase in hydrophilicity (urinary excretion), an increase in molecular weight or charge state (biliary excretion), or affinity for transporters (both excretory processes) (Fisher et al., 2000; Johnson and Klaassen, 2002).

The common role of the UGTs is detoxification of both endogenous and exogenous compounds. Conjugation reduces the likelihood that nucleophilic sites will be converted to electrophilic species that may otherwise cause protein damage (Bock and Lilienblum, 1994). The hepatotoxin acetaminophen has several metabolic pathways and can be metabolized to a glucuronide or a reactive quinoneimine. The latter compound can chemically modify numerous cellular proteins and induce apoptotic events at higher doses. In rats deficient in UGTs, a higher percentage of quinoneimine is formed than in rats with conjugating ability. This deficiency significantly predisposes rats to hepatic damage, underscoring the importance of UGTs in abating toxicity (de Morais and Wells, 1988; Park et al., 2005). Electrophilic compounds can also react with DNA, a process that is a well-known precursor to carcinogenesis. Recent evidence suggests that having a mutation that results in less efficient UGT catalysis may predispose people to developing gastrointestinal or digestive system cancers (Strassburg et al., 2002; Ockenga et al., 2003). UGTs can react with such

compounds and reduce their potential for toxicity, in addition to providing an alternative metabolic pathway that decreases the percentage of a toxin that can undergo conversion to an electrophile. In its detoxifying or protective role, UGTs prevent genotoxic and cytotoxic substances from accumulating in the cell and causing irreversible damage.

B.2. GENOMIC STRUCTURE AND PHYLOGENY OF UGTs

In mammals, there have been 47 UGT genes isolated, which belong to one of three families. Within a family, each member has greater than 45% sequence homology with other members. Genes within two families, UGT1 and UGT2, are divided into four subfamilies, with each gene in a subfamily having at least 60% homology. Following proper nomenclature, the family is denoted with an Arabic number, followed by the subfamily denoted with a letter, and finally each gene with an Arabic number (Mackenzie et al., 1997). The UGT1A and UGT2B family contain the isoforms most responsible for glucuronidation in humans. UGT2B transcripts are formed from individual genes encoded on chromosome 4q13, encoded by six exons. There are seven functional UGT2B proteins: UGT2B4, UGT 2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28 (Tukey and Strassburg, 2000; Guillemette, 2003). Although encoded by individual genes, all members of this family are at least 78% homologous with each other at the protein level. The homology may be as much as 94%, as between UGT2B15 and UGT2B17 and UGT2B11 and UGT2B28 (Guillemette, 2003). UGT1A isozymes are transcribed from one gene on chromosome 2q37. The UGT1A gene has thirteen 5' first exons with 12 unique promoters and four 3'exons. The DNA polymerase complex recognizes the promoter sequence of one of the 5'exons and transcription is initiated. The primary transcript will contain the 5'exon, all other downstream 5' exons, as well as the four 3'exons. Differential splicing then occurs, with the downstream

5'exons becoming intronic. Each final UGT1A transcript possesses one 5' exon sequence and the four 3' exons. Therefore, each UGT1A protein contains the same 245 carboxy terminal sequence (the four shared 3' exons) and an isoform-specific N-terminal comprised of 280-289 amino acids (first 5' exon) (Ritter et al., 1992; Owens and Ritter, 1995; Tukey and Strassburg, 2000). Substrate binding occurs at the N-terminal region, whereas the co-substrate UDP-glucuronic acid binds to the conserved region (Mackenzie, 1990). Despite 13 possible UGT1A isoforms that could exist due to the presence of 13 promoter regions and 13 distinct exons 1, four encode UGT1A pseudogenes in humans. The nine functional UGT1A proteins are: UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10 (Guillemette, 2003). The rat UGT1A locus is arranged and transcribed similarly, although there are only nine divergent first exons reported to date, and rat UGTs 1A4 and 1A9 are pseudogenes (Emi et al., 1995). Like the UGT2B family, the UGT1A family is also highly homologous. UGT1A7-10 are 93% homologous in the first exon, while UGT1A3 and 1A4 share 94% of the same amino acids (Tukey and Strassburg, 2001; Guillemette, 2003). A consequence of highly conserved protein sequences within the UGT1A family is the difficulty in generating specific antibodies to individual isoforms. As UGT1A1 and 1A6 have the most unique amino acid sequences among the UGT1A isoforms, laboratory and commercially prepared antibodies are available to UGT1A1 and 1A6 (Tukey and Strassburg, 2001). A UGT1A antibody, which recognizes all UGT1A isoforms and is generated against a sequence in the conserved C-terminal region, is also commonly used to assess UGT1A expression.

B.3. SUBSTRATES

Due to the high level of homology between the UGT isoforms, there is understandably a broad overlap in substrate specificity. Thus, there are very few isoform-specific probe substrates that have been identified to date. Investigators have used morphine (3' glucuronide, UGT2B7), azidothymidine (UGT2B7), propofol (UGT1A9), flavopiridol (UGT1A9), serotonin (UGT1A6), naphthol (UGT1A6), bilirubin (UGT1A1) and estradiol (3' glucuronide, UGT1A1) as probe substrates for the isoforms in parenthesis; however, most of these compounds are glucuronidated from a small to a substantial degree by other isoforms, (Bosma et al., 1994; Ramirez et al., 2002; Court et al., 2003; Krishnaswamy et al., 2003; Soars et al., 2004). Elucidating the substrates of the UGTs has been made possible by the generation of cDNA-expressed cellular systems of individual isoforms. UGT substrate specificity and or capacity has recently been attributed to two factors beyond enzyme-substrate recognition: the extent of UGT phosphorylation and the association of the UGTs into dimers or tetramers (Ouzzine et al., 2003; Basu et al., 2005).

The UGT family recognizes numerous endogenous and exogenous compounds as substrates. Major classes of endogenous compounds that are substrates for the UGTs include bile acids, steroids, thyroid hormones, and retinoic acids (Clarke and Burchell, 1994). As a general rule in humans (albeit with several exceptions), the UGT2B family typically glucuronidates more endogenous substrates, whereas the UGT1A family normally glucuronidates more xenobiotics and exogenous compounds (Ethell et al., 2003). UGTs are responsible for the conjugation reactions of more xenobiotics than any other conjugating enzyme, accounting for over a third of all conjugation reactions that occur (Evans and Relling, 1999). There are multiple UGT substrates where high levels of unmetabolized or

unconjugated drug may lead to toxicity, thus glucuronidation rates may be important in modulating exposure and toxicity. These include the analgesics acetaminophen (UGT1A1, 1A6, 1A9 substrate) and morphine (multiple UGT1A and 2B7), the anti-cancer agents SN-38 (UGT1A1, 1A7, 1A9) and etoposide (UGT1A1), the immunosuppressive drugs mycophenolic acid (MPA) (UGT1A8, 1A9), tacrolimus and cyclosporine (UGT2B7), the anti-convulsant valproic acid (UGT1A6, 1A9, and 2B7), the anti-diabetic troglitazone (UGT1A1, 1A8-10) and the tri-cyclic antidepressants imipramine and amitriptyline (UGT1A4) (Breyer-Pfaff et al., 1997; Court et al., 2001; Strassburg et al., 2001; Cummings et al., 2002; Gagne et al., 2002; Nakajima et al., 2002; Watanabe et al., 2002; Ethell et al., 2003; Stone et al., 2003; Watanabe et al., 2003; Bernard and Guillemette, 2004). Despite the potential for UGT substrates to be co-administered to humans, which may lead to drug interactions and toxicity, this rarely occurs. Most UGT substrates have high Km values (a measure of affinity) and are glucuronidated by multiple UGT isoforms (Williams et al., 2004).

B.4. HEPATIC AND INTESTINAL GLUCURONIDATION

Given the existence of unique promoter regions for each UGT1A exon, tissue-specific expression of the UGT1A isoforms was predicted to occur. This has indeed been observed by a number of investigators (see Table 1.1). Due to the lack of specific antibodies as discussed above, UGT isoform localization has been characterized by the presence or absence of its transcript mRNA, which may or may not reflect protein expression. Certain isoforms are expressed exclusively in extra-hepatic tissues (UGT1A7, 1A8, and 1A10), while others are found in most organs of elimination (UGT1A1). Little is known about why this occurs, however, several transcription factors have been found to play a role in the

constitutive expression of the UGTs in particular tissues. A binding site for hepatocyte nuclear factor 4α in the UGT1A9 promoter seems crucial for 1A9 expression in the liver. The lack of such a binding site in the promoters of UGT1A7, 1A8, and 1A10 may be a factor in the absence of these isoforms from the liver (Barbier et al., 2005). The intestinal expression of UGT1A8 and UGT1A10 (and to a certain degree 1A9) is thought to be governed by a transcription factor complex composed in part by hepatocyte nuclear factor 1, Sp1, and the caudal homeodomain 2 protein. The latter transcription factor is expressed exclusively in the intestine (Gregory et al., 2004). Regionality in the expression of the UGT1A family is also found in the rat (Table 1.2).

Since the capacity of a tissue to glucuronidate a substrate depends on the UGT isoforms present in a tissue and their abundance, it is inevitable that tissue differences in catalytic activity will exist, despite overlapping substrate specificities of the UGT1A isoforms (Radominska-Pandya et al., 1999). It is generally noted that liver tissue possesses greater glucuronosyltransferase activity in comparison with the intestine or colon for a wide range of compounds, due in part, to greater hepatic UGT1A levels as measured through mRNA (Strassburg et al., 1998; Strassburg et al., 1999a). However, large differences in protein levels of the UGT1A family are not observed, and hepatic and extrahepatic tissues have been shown to have similar catalytic rates (normalized to the amount of tissue protein) for a number of substrates, including carcinogens, tertiary amines and steroids (Strassburg et al., 1999a; Strassburg et al., 2000). Considering that intestinal and colonic UGTs may be exposed to high levels of orally administered substrates or glucuronides excreted into the bile and deconjugated by β-glucuronidase, their relative contribution to drug disposition may be greater than anticipated (Tukey et al., 2002).

The importance of intestinal UGTs towards modulating the bioavailability of substrates has been shown in early studies with substrates such as ethinyl estradiol, morphine, 1-naphthol, and salicylamide (Goon and Klaassen, 1992). By comparing arterial and portal vein concentrations in the rat, intestinal glucuronidation of an intravenous dose of 4-methylumbelliferone accounted for 40% of the clearance of the drug (Mulder et al., 1984). Recently, it was estimated that intestinal glucuronidation accounted for up to two-thirds of the metabolism of the flavonoid apigenen in the perfused rat intestine, which would largely explain its poor bioavailability (Chen et al., 2003). The idea of intestinal UGTs functioning as a barrier is further substantiated by their predominant location in the villi, the physiological region of the intestinal mucosa most likely to come in contact with foreign substances introduced into the body (Strassburg et al., 2000).

B.5. ENTEROHEPATIC RECIRCULATION

After oral administration of a UGT substrate, the aglycone can be glucuronidated in the intestine or absorbed as unconjugated drug. In the liver, if the substrate is glucuronidated, it then may become a substrate for a canalicular transporter, namely by the organic anion transporter MRP2 (Gerk and Vore, 2002; Roberts et al., 2002). MRP3, on the sinusoidal membrane, can also transport glucuronides from the hepatocyte into blood (Hirohashi et al., 1999; Gerk and Vore, 2002). Glucuronides extruded into the bile can be subject to intestinal β-glucuronidase, a bacterial enzyme which cleaves the glucuronic acid resulting in the regeneration of the aglycone. The aglycone can then be reabsorbed in a process termed enterohepatic recirculation (EHC). This phenomenon may manifest in a characteristic plasma concentration versus time profile including one or more peaks in species with gallbladders (Roberts et al., 2002). Compounds administered intravenously can

also undergo EHC. Often, EHC can significantly enhance drug exposure, with this process accounting for 40% of the area under the concentration versus time curve (AUC) for MPA (Naderer et al., 2005). Antibiotic therapy can eliminate the bacteria that produce β-glucuronidase, introducing the potential for decreased efficacy of substrates like MPA and ethinylestradiol (Shenfield and Griffin, 1991; Naderer et al., 2005).

B.6. VARIABILITY IN UGT EXPRESSION AND FUNCTION

B.6.a. GENETIC POLYMORPHISMS OF UGTs

Interindividual variability in glucuronidation rates can be conferred through polymorphic expression of the UGT isoforms. Flavopiridol glucuronidation, catalyzed by UGT1A9 and 1A4, varied 12-fold in a sample of 62 liver microsomes (Ramirez et al., 2002). Fisher et al. (2000) found that glucuronidation rates for the probes morphine (2B7 substrate) and acetaminophen (1A6 and 1A9 substrate) varied 3 and 7-fold respectively, in a sample of microsomes prepared from 20 livers (Fisher et al., 2000). When a probe for UGT1A1 was examined (estradiol), 30-fold variations in glucuronidation rates were observed, largely due to low rates in patients with Gilbert's syndrome (Fisher et al., 2000). Gilbert's syndrome arises most often arises with an inherited extra TA repeat in the UGT1A1 promoter sequence $(TA)_6TAA$ to form $(TA)_7TAA$ (known as UGT1A1*28 allele). Patients homozygous for $(TA)_7TAA$ typically glucuronidate bilirubin at rates that are approximately 30% of normal. Gilbert's patients (those homozygous for UGT1A1*28) often will have elevated bilirubin levels, however, due to the large influence of environmental factors, a large number of patients will have no overt signs of the syndrome. Thus, glucuronidation rates of drug substrates for UGT1A1 are often significantly lower in Gilbert's patients, predisposing these patients to toxicities from elevated drug or bilirubin levels (Burchell and Hume, 1999).

Although a diagnosis of Gilbert's syndrome necessitates homozygosity for increased TA repeats, patients heterozygous for a $(TA)_7TAA$ repeat may have higher bilirubin levels and lower glucuronidating capacity (Miners et al., 2002). The incidence of the UGT1A1*28 allele is anywhere from 7 to 55% of the population. Racial differences in glucuronidation rates may also occur due to unequal distribution of the mutation throughout different populations. One-fourth of the African population is found to be homozygous, whereas only 3% of Asians have Gilbert's syndrome (Burchell and Hume, 1999).

Crigler-Najjar Type I and II are more rare and serious diseases that are associated with one or more of 30 mutations documented in either the promoter or exons 1-5 of the UGT1A1 gene. Type I patients have no UGT1A1 activity and cannot survive without liver transplantation, whereas Type II patients possess about 10% activity toward bilirubin. The hyperbilirubinemia observed in these patients is often treated with inducing agents such as phenobarbital (Ritter et al., 1999; Tukey and Strassburg, 2000). An animal model of Crigler-Najjar syndrome Type I, the Gunn rat, has led to a significant understanding of the role that UGTs play in homeostasis, drug disposition, and detoxification. A mutant strain derived from the Wistar rat, the Gunn rat has no functional UGT1A protein, as a result of a single base pair deletion in exon four of the UGT1A gene. These animals predictably have much of the same pathophysiology as human Crigler Najjar patients: jaundice, low survival rates, and encephalopathy (Iyanagi et al., 1998).

There is literature to support the presence of numerous polymorphisms or gene mutations in virtually all other UGT1A genes or promoters. In most cases, after the mutation is discovered, a typical experimental approach is where cells expressing the mutated UGT are incubated with substrates for the isoform and glucuronidation rates are compared with those

from wild-type expressed enzyme. Although a number of the mutations impart altered UGT activity *in vitro*, their functional significance *in vivo* is unclear. For instance, a UGT1A7 allele with three missense mutations (UGT1A7*3) glucuronidated SN-38 at a much lower efficiency relative to wild-type alleles, as evidenced by a 59% loss of activity (Gagne et al., 2002). However, patients with one or more UGT1A7*3 alleles were at no greater risk for developing toxicity when treated with SN-38 (Ando et al., 2002a). The UGT1A7*3 allele has been shown to be strongly associated with the occurrence of lung, hepatic, and gastrointestinal tract cancer, although the basis for this is not understood, as UGT1A7 appears to have limited distribution *in vivo* (Strassburg et al., 2002; Araki et al., 2005; Tseng et al., 2005). Propofol and MPA glucuronidation rates in human microsomes were significantly higher in those containing two single nucleotide polymorphisms (T-275A and C-2152T) in the promoter of UGT1A9 relative to those from wild-type individuals (Girard et al., 2004). In renal transplant patients with these polymorphisms who received high-dose mycophenolate mofetil (the prodrug of MPA), exposure to MPA was lower as characterized by a lower area under the concentration curve (AUC), less MPA was estimated to undergo enterohepatic recirculation, and higher MPA clearance values were calculated. These altered pharmacokinetic parameters could be responsible for graft rejection, or lower incidences of MPA adverse effects, including diarrhea and leukopenia, however these outcomes are unknown (Kuypers et al., 2005). In contrast to the above examples where the incidence of UGT1A7*3, UGT1A1*28, and the UGT1A9 promoter mutations all have an allelic frequency of at least five percent, numerous polymorphisms reported in the literature are either isolated mutations found in a single subject or exist in a small fraction of the population (Saeki et al., 2005a; Saeki et al., 2005b). Thus, small sample sizes preclude an *in*

vivo investigation of the effect of the genetic mutation. The following are additional polymorphisms identified in a significant proportion of the population and that demonstrate alterations in *in vitro* glucuronidation activity of substrates tested: UGT1A6*2 (higher activity versus wild-type, 30% allelic frequency), UGT1A3, 4 different amino acid changes (frequency of 5-13%, activity 70-370% of wild-type activity, and UGT1A8*3 (frequency of 3%, lower activity) (Huang et al., 2002; Iwai et al., 2004; Krishnaswamy et al., 2005). Other polymorphisms, such as UGT1A9*3 (4% allelic frequency) and UGT 1A4 L48V (16%), show altered conjugating ability for some substrates, while the glucuronidation of other aglycones are unaffected (Villeneuve et al., 2003; Mori et al., 2005).

B.6.b. INDUCTION/INHIBITION OF UGTs

Environmental exposures can also contribute to interindividual variability in glucuronidation rates, namely through induction or inhibition. Compounds capable of induction are traditionally classified as belonging to either the monofunctional or bifunctional class of inducers. Bifunctional inducers have been shown to upregulate both Phase I (i.e., cytochrome P450s) and II enzymes (i.e., conjugating enzymes, such as UGTs and glutathione-S transferases) through the aryl hydrocarbon receptor (Prochaska and Talalay, 1988). These compounds include polyhalogenated arylhydrocarbons (PAHs) such as 3-methylcolanthrene, 2,3,7,8-tetrachlorodibenzo-p-dioxin, and β-napthoflavone. Monofunctional inducers, such as 1,7-phenanthroline, butylated hydroxyanisole, butylated hydroquinone, and the isothiocyanates more selectively stimulate the production of Phase II enzymes. The precise mechanism for monofunctional induction is largely unknown, however, electrophilic stress is thought to trigger an intracellular phosphorylation cascade that results in nuclear transcription factors binding to UGT promoters and facilitates

transcription (Wasserman and Fahl, 1997; Rushmore and Kong, 2002). There can be significant interplay between the monofunctional and bifunctional pathways. For example, certain PAHs (such as β-napthoflavone) are metabolized to strong electrophiles, which might then activate the monofunctional pathway (Bock et al., 1998; Munzel et al., 1999). Recent data has suggested that certain UGTs are also responsive to pregnane X receptor (UGT1A1), constitutive androstane receptor (UGT1A1), glucocorticoid receptor (UGT1A1) and peroxisome proliferator-activated receptor ligands (UGT1A1 and 1A9) (Sugatani et al., 2001; Barbier et al., 2003; Xie et al., 2003; Sugatani et al., 2005). Numerous drugs, food additives, and environmental compounds have the ability to alter UGT expression which is anticipated, given the variety of mechanisms by which UGT1A1 protein can be upregulated.

Inducers have been noted to elicit different effects on intestinal and hepatic glucuronidation, which may be a function of inducer exposure or isoform-specific regulation. For instance, van der Logt et al. (2003) treated rats orally with a series of anticarcinogenic compounds and assessed their effects on UGT expression. Ellagic acid and brussel sprouts induced only hepatic UGTs; curcumin and tannic acid induced only intestinal UGTs; and coumarin and quercetin induced UGTs in both organs (Van Der Logt et al., 2003). The inducibility of an isoform in a given tissue has also been noted to be inversely related to its constitutive expression (Munzel et al., 1994; Munzel et al., 1996).

In vitro reactions have identified a number of UGT inhibitors, the coadministration of which may influence glucuronidation rates. There are very few potent *in vitro* UGT inhibitors, however, vinorelbine, chrysin, tangeretin, and silybin are among a small number of compounds with inhibitor concentrations which elicit a 50% reduction in substrate turnover (IC_{50}) values less than 10 μM as assessed in liver microsomes or expressed isoforms

(Said et al., 1992; Grancharov et al., 2001; Charasson et al., 2002; Williams et al., 2002; Sridar et al., 2004). Coupled with the fact that most inhibitors have low systemic concentrations relative to their IC_{50} concentrations and the reasons given previously, there are very few clinically significant drug interactions with UGT inhibitors and substrates (Williams et al., 2004). *In vivo*, valproic acid and indinavir have been shown to inhibit glucuronidation (Gupta et al., 1997b; Samara et al., 1997; Zucker et al., 2001). Only with the latter compound was any phenotypic interaction observed. Indinavir was shown to increase bilirubin levels relative to pretreatment values in patients, especially in patients hetero- or homozygous for UGT1A1*28 (Zucker et al., 2001).

C. IRINOTECAN

C.1. CLINICAL USES

The anticancer agent irinotecan (or CPT-11) is approved in the US for use, either alone or with fluorouracil and leucovorin, in the treatment of metastatic colorectal cancer (Saltz et al., 2000). Regimens containing irinotecan are not currently used as first-line therapy, since the combination of oxaliplatin, flurouracil, and leucovorin proved to be superior in terms of survival rates and side effect profiles (Goldberg et al., 2004). Nevertheless, irinotecan is used to treat thousands of cancer patients each year, as it is also being included in chemotherapy regimens for small cell and non-small cell lung cancer, refractory lymphoma, malignant gliomas, and gynecological cancers (Langer, 2003; Reardon et al., 2003). In the most common colon cancer regimen, irinotecan is dosed at 125 mg/m^2 to be given intravenously over 90 minutes once weekly for 4 weeks in 6 week cycles. Subsequent cycles are given based upon patient response and tolerability, with a 20-40%

reduction in dose for grade 3-4 adverse reactions (namely diarrhea, vomiting, or neutropenia) (Saltz et al., 2000).

C.2. CHEMOTHERAPEUTIC ACTIVITY/METABOLISM

Members of the camptothecin family, including irinotecan, function in the S-phase of the cell cycle, by forming a stable complex with DNA and topoisomerase I, an enzyme that relieves supercoiling strain through transient nicking of the template DNA, ahead of the DNA replication fork. When the fork collides with the complex, cell division arrests. The DNA breaks become irreversible and cell death then ensues through the apoptotic pathway (Rivory et al., 1997). The cytotoxicity of irinotecan is achieved largely through its primary metabolite, SN-38, which is at least 100-fold more potent in inducing cell death *in vitro*. Irinotecan is administered as a prodrug for SN-38, as the latter compound cannot be directly administered to patients due to its poor aqueous solubility (Kawato et al., 1991). SN-38 is formed by the metabolism of irinotecan by carboxylesterases in human liver, blood, intestinal, and tumor tissue (Khanna et al., 2000; Mathijssen et al., 2001). SN-38 is then glucuronidated by UGT1A1, UGT1A7, and UGT1A9, with similar reported catalytic efficiencies, to form SN-38 glucuronide (SN-38G) (Gagne et al., 2002) (Figure 1.1). Cytochrome P-450 3A4 mediated metabolism of irinotecan does occur, resulting in the formation of two inactive metabolites, APC and NPC (Figure 1.2). Roughly 11% of an irinotecan dose is accounted for as APC (Slatter et al., 2000). This metabolite is not a potent topoisomerase inhibitor and is not metabolized further (Rivory et al., 1996). NPC, accounting for 1.5% of an irinotecan dose in bodily fluid, is not an active metabolite, but is subject to esterase cleavage by carboxylesterases to form SN-38 (Dodds et al., 1998; Slatter

et al., 2000). Irinotecan circulates through the body predominantly as unchanged drug, with 55% of a dose being eliminated as irinotecan (Slatter et al., 2000).

C.3. PHARMACOKINETICS

Peak plasma concentrations of irinotecan typically occur at the end of a 90-minute infusion, and disposition then follows a two-compartment model. The half-life of distribution is approximately 2 hours, and the elimination half-life varies, but averages 6-15 hours. Irinotecan extensively distributes to tissue, as demonstrated by the average distribution volume of 136 L/m^2 in humans. Irinotecan displays linear kinetics in adults over a wide range of doses (50-750 mg/m^2), with a clearance value of roughly 430 mL/min. Peak plasma levels of SN-38 occur approximately 30 minutes after those of irinotecan, and are roughly 60-100 fold lower than those of irinotecan on a molar basis. Most studies have found that the half-life of SN-38 is longer than that of the parent, however, large differences in the measurement exist (reports of 7 to 29 hr). SN-38G levels are highest approximately one hour after the infusion of irinotecan. After an infusion, SN-38G levels are 3-7 fold higher than those of SN-38. The half-life of SN-38G is reported to be 12-35 hours (de Forni et al., 1994; Gupta et al., 1997a; Rivory et al., 1997; Sparreboom et al., 1998b; Slatter et al., 2000; Mathijssen et al., 2001). Large interpatient variability in the glucuronidation of SN-38 is frequently seen, as AUC ratios of SN-38G to SN-38 can vary 3 to 18-fold within treatment groups administered commonly used dosages of irinotecan (Rivory et al., 1997). The disposition of irinotecan and its metabolites generally display similar trends in humans and rats, however the half-life of irinotecan is much shorter in rats at only 1.3 hours (Kaneda and Yokokura, 1990). Although the half-life of SN-38 in rats is reported to be 2.8 hours after irinotecan administration, an intravenous dose of SN-38 to rats has a half-life of seven

minutes, indicating formation-rate limited kinetics of the metabolite (Atsumi et al., 1995). Plasma protein binding estimates are 92-99% for SN-38. Irinotecan is mainly localized in erythrocytes with a blood to plasma typically greater than one, but is highly dependent upon concentration and subject smoking status (Mathijssen et al., 2001; Dumez et al., 2005). Irinotecan is protein bound in plasma at 30-65% (Mathijssen et al., 2001; Garcia-Carbonero and Supko, 2002; Dumez et al., 2005).

Biliary secretion, and urinary and fecal excretion data for irinotecan and its metabolites are summarized in Table 1.3. In humans, SN-38 has shown to be much less efficiently cleared into bile than its parent or glucuronide (Lokiec et al., 1995; Slatter et al., 2000). The high ratio of SN-38 to SN-38G in feces is likely due to β-glucuronidase activity in the colon, which cleaves SN-38G to SN-38. The contribution of enterohepatic recycling (EHC) to the disposition of SN-38 is unclear. Coadministration of antibiotics with irinotecan has no effect on SN-38 AUC (Kehrer et al., 2001). In support of this study, intestinal β-glucuronidase activity, which was found to vary one hundred fold between subjects, was not correlated to any SN-38 pharmacokinetic parameter (Kehrer et al., 2000). Taken together, these results indicate that any SN-38 liberated via β-glucuronidase does not contribute appreciably to the systemic disposition of SN-38. Further, almost no published studies show the characteristic multiple peak phenomenon linked to EHC for SN-38 (Mathijssen et al., 2001). However, in a study where a secondary SN-38 peak is apparent in some patients, EHC may contribute up to 12% to the total SN-38 AUC (Gupta et al., 1994). Additionally, although the bioavailability for irinotecan is estimated to be just 12-25% after an oral dose, appreciable levels of SN-38 are detected in the plasma (Stewart et al., 1997; Stewart et al., 2004). Presumably, this is due to the relatively high concentrations of irinotecan achieved in

the intestine or portal circulation cleaved by esterases (Drengler et al., 1999; Soepenberg et al., 2005). This same phenomenon may also occur after gall bladder contraction, facilitating a contribution to SN-38 EHC. Furthermore, upon analyzing plasma samples 500 hours after an intravenous dose of irinotecan, SN-38 half-life and AUC were increased by two-fold compared with estimates from a more abbreviated sampling schedule. A shorter sampling schedule may not capture the recirculation phase, which would manifest in a lower AUC and quicker half-life (Kehrer et al., 2000). The presence of a secondary plasma concentration peak for irinotecan is noted in the plasma profiles of some studies, which can at least partially be explained by its uptake into erythrocytes after an intravenous infusion, followed by its rapid release (Mathijssen et al., 2001).

C.4. LACTONE HYDROLYSIS/RECYCLIZATION OF IRINOTECAN/SN-38

The lactone rings of all camptothecins, including irinotecan and its metabolites, can undergo a reversible pH-dependent hydrolysis to form a carboxylate group both *in vitro* and *in vivo* (lactone moieties shown in Figure 1.1). The lactone and carboxylate may essentially be considered as two distinct compounds with only the lactone form of SN-38 active in binding to topoisomerase. Additionally, the lactone and carboxylate forms express different affinities for transporters and exhibit differences in pharmacokinetics in animals and humans. The passive and overall uptake rate of SN-38 and irinotecan lactone into HT-29 cells was higher than the corresponding carboxylates, which were transported actively (Kobayashi et al., 1999). In rats devoid of MRP2, SN-38 and irinotecan carboxylate biliary clearance values were reduced whereas lactone forms were unchanged, suggesting a role of MRP2 in the hepatic transport of the carboxylate forms only (Chu et al., 1997). These differences in transport may stem from the fact that the lactone form of SN-38 is not charged at

physiological pH. Additionally, the negative charge imparted by the carboxylate moiety of SN-38 and CPT-11 is typical of the substrates for MRP2.

The *in vitro* half-life of SN-38 lactone (for conversion to the carboxylate) is 32 minutes in Tris buffer at physiological pH, and at equilibrium the lactone represents 18% of total SN-38 concentrations (Chourpa et al., 1998). After an infusion of irinotecan, irinotecan carboxylate becomes the predominate form in plasma, comprising 64% of the total AUC (Rivory et al., 1994). This may be due to the above-mentioned pH-dependent conversion, but is also at least partially due to higher clearance of irinotecan lactone in humans (Xie et al., 2002b). The higher clearance of irinotecan lactone may also contribute to the higher levels of SN-38 lactone, which comprises 63% of the total SN-38 AUC. SN-38 plasma lactone levels range from 70% of total SN-38 equivalents at the end of the infusion to 50% in 24 hours (Rivory et al., 1994). Based upon *in vitro* measurements, this ratio would not be predicted, however, the preferential binding of SN-38 lactone to albumin stabilizes this form and modifies the equilibrium (Mathijssen et al., 2001; Garcia-Carbonero and Supko, 2002).

C.5. TRANSPORT OF IRINOTECAN/SN-38

As previously mentioned, transport of SN-38 and irinotecan carboxylate from the liver to the bile is mediated by MRP2. As indicated by bile studies in MRP2 competent and deficient rats, the majority of SN-38 excreted in the bile is in the ionic, carboxylate form, and any SN-38 lactone that is excreted is not due to MRP2 (Chu et al., 1997). Both SN-38G lactone and carboxylate are also predictably transported by MRP2 (Chu et al., 1997; Sugiyama et al., 1998). For irinotecan carboxylate, MRP2 represents the low affinity transport pathway into bile, while the high affinity pathway is mediated through MDR1, or P-gp (Chu et al., 1999a; Iyer et al., 2002). SN-38 and its glucuronide are not substrates for P-

gp, as demonstrated both *in vitro* and in mouse P-gp knockout biliary excretion studies (Chu et al., 1999b; Iyer et al., 2002). Numerous *in vitro* studies have found that both SN-38 and SN-38G are substrates for the canalicular efflux transporter BCRP (Schellens et al., 2000; Nakatomi et al., 2001). In mice *in vivo*, BCRP is found to modulate the bioavailability of irinotecan and systemic levels of SN-38, through the inhibition of intestinal BCRP (Stewart et al., 2004). Currently, it is unknown whether MRP3 may transport SN-38G from the liver to the blood, however, it does not recognize SN-38 as a substrate (Cummings et al., 2004). The accumulation of irinotecan and SN-38 in cells transfected with the sinusoidal transporter MRP1 was dramatically decreased and imparted chemotherapeutic resistance to these cells, indicating a role for MRP1 in irinotecan and SN-38 transport (Chen et al., 1999). SN-38G is also a substrate of MRP1, as shown by high uptake rates in the presence of ATP and membrane vesicles made from an MRP1 overexpressing cell line (Chu et al., 1999b). The contribution of MRP1 to the systemic disposition to irinotecan or metabolites is unknown, since all data has been generated in *in vitro* studies. Recently, another sinusoidal transporter, MRP4, has been shown to confer resistance to irinotecan and SN-38 in a cell line overexpressing this protein (Tian et al., 2005b).

Little is known about active uptake with regards to irinotecan and metabolites. OATP-C facilitates the uptake of SN-38 into hepatocytes, but is not involved in the transport of SN-38G or irinotecan (Nozawa et al., 2005). In intestinal cells isolated from hamsters, the lactone forms of SN-38 and irinotecan were taken up at a much faster rate when compared with the carboxylate forms. The carboxylate forms also exhibited saturable uptake into these cells, indicating active uptake (Kobayashi et al., 1999). Each transporter with affinity for irinotecan, SN-38, or SN-38G, with the exception of OATP-C, is also expressed in human

intestine (Tirona and Kim, 2002). Thus, intestinal transporters also play a role in systemic disposition and enterocyte intracellular levels, which may influence therapeutic efficacy or toxicity.

C.6. ADVERSE EFFECTS OF IRINOTECAN/SN-38

The major dose-limiting toxicities of irinotecan are neutropenia and diarrhea (Iyer et al., 1998; Saliba et al., 1998). Approximately 23-31% of treated patients experience severe grade 3-4 diarrhea, characterized by incontinence and/or greater than seven stools per day (grade 3) or life threatening systemic sequelae, such as hypotension or electrolyte disturbances (grade 4) (Saltz et al., 2000). Pathophysiologically, irinotecan-induced diarrhea is mainly secretory, with exudative components. Stools have increased weight and sodium content (secretory) yet also have increased levels of protein, resulting from a decrease in mucosal integrity (exudative) (Saliba et al., 1998). The onset of diarrhea typically occurs on the 5th-12th day after therapy and lasts for 5-7 days. Loperamide and octreotide are used to reduce diarrheal episodes, but are of mediocre efficacy (Barbounis et al., 2001). Patients experiencing this toxicity will receive lower doses on any subsequent cycles, possibly compromising the full therapeutic potential of their anti-cancer regimen. Accumulation of SN-38 in enterocytes is thought by most to be the causative agent behind the incidence of diarrhea (Araki et al., 1993; Iyer et al., 1998).

C.6.a. PREDICTION OF ADVERSE EFFECTS

Investigators have tried to elucidate predictive factors that might identify patients susceptible to irinotecan-induced diarrhea. Early studies focused on pharmacokinetic indicators linked to toxicity, the most common modality of therapeutic drug monitoring. Several studies found a significant correlation between SN-38 AUC or maximal plasma

concentration (Cmax) with the incidence of diarrhea. However, this is not a reliable finding, even in studies with similar administered doses and regimens (Kudoh et al., 1995; Sasaki et al., 1995a; Mathijssen et al., 2001; Xie et al., 2002a). An early hypothesis was that low rates of hepatic glucuronidation may result in elevated bile concentrations of SN-38, leading to an increase of unconjugated SN-38 in the intestine, and thus increased toxicity. To examine this hypothesis, Gupta et al. predicted a correlation of diarrhea with the 'biliary index,' defined as the ratio of SN-38 to SN-38G AUCs multiplied by the AUC of CPT-11. In this study, patients with a higher 'biliary index' were more likely to experience diarrhea (Gupta et al., 1994; Gupta et al., 1997a). Another large study found that the 'biliary index' did not predict toxicity, albeit different CPT-11 regimens were administered (Xie et al., 2002a). Using the 'biliary index' to anticipate diarrhea may be potentially flawed on a physiological level. Since it has been consistently demonstrated that essentially no SN-38G is found in the feces with correspondingly high levels of SN-38 (Table 1.3), β-glucuronidase is efficient at cleaving SN-38G to SN-38. Thus, patients with a high $AUC_{SN\text{-}38G}$, and lower biliary index score, may still be expected to have high intestinal levels of SN-38 (Tukey et al., 2002).

Patients having poor UGT expression, as might occur through polymorphisms in UGT1A1, 1A7, or 1A9, may predispose patients to intestinal toxicity due to relatively high levels of the aglycone, SN-38. As previously mentioned, the presence of UGT1A7*3 or any other known 1A7 polymorphism does not seem to increase the likelihood for diarrhea, and in fact, may decrease the possibility (Ando et al., 2002a; Carlini et al., 2005). The presence of one or two UGT1A9*22 alleles, a single nucleotide polymorphism (SNP) that confers a thymidine insertion into the UGT1A9 promoter, may protect patients from irinotecan-

mediated toxicity. This mutation is associated with greater gene transcription, but has not yet been shown to impart high SN-38 conjugation (Yamanaka et al., 2004; Carlini et al., 2005).

Mutations in the UGT1A1 gene have been explored and appear much more frequently to predict patients with toxicity. Patients with UGT1A1*28 alleles typically have a lower $AUC_{SN\text{-}38G}/AUC_{SN\text{-}38}$ ratio, but the use of this ratio does not always correlate with toxicity (Gupta et al., 1994; Ando et al., 2002b; de Jong et al., 2005). Nevertheless, significant differences in SN-38 glucuronidation rates in human liver microsomes between UGT1A1 genotypes were observed, such that patients hetero- and homozygous for UGT1A1*28 exhibited lower catalytic rates. Patients with 2 UGT1A1*28 alleles may have up to a 17-fold lower activity than a person with 2 wild-type alleles (Iyer et al., 1999). With respect to gastrointestinal toxicity, in a study of 118 Japanese patients, those with at least one UGT1A1*28 allele were 7 times as likely to experience severe diarrhea and/or neutropenia (Ando et al., 2000). Considerable differences in the occurrence of diarrhea that correlates with genotype was also substantiated in one additional trial with a large number of patients (Marcuello et al., 2004). Numerous studies link the incidence of neutropenia to UGT1A1*28 genotype (de Jong et al., 2005). Due to the high correlation between neutropenia and UGT1A1*28, an FDA advisory panel has recommended changes to the package insert for irinotecan, recommending lower initial doses for patients homozygous for UGT1A1*28, and kits for widespread UGT1A1*28 genotyping have recently become commercially available (Invader®, UGT1A1 molecular assay, Third Wave Technologies). Genotyping is necessary to determine patients with Gilbert's syndrome, as baseline serum bilirubin levels do not always predict toxicity or the presence of mutant TATA box promoters (Meyerhardt et al., 2004). Recent work in limited clinical studies suggests that mutations in the UGT1A1

promoter may also predict toxicity independently or through high linkage disequilibrium with UGT1A1*28 (Innocenti et al., 2004a; Kitagawa et al., 2005). Haplotype analysis of mutations within genes (i.e., UGT 1A1) or between genes (i.e., UGT1A1 and UGT1A9) is quickly introducing groups of mutations that may predict toxicity in patients treated with irinotecan (Sai et al., 2004; Innocenti et al., 2005). Obviously, the analysis of thousands of possible gene permutations is difficult to sort out, and is only in its infancy.

Although polymorphisms in transporters have not been linked to the incidence of adverse effects in patients treated with irinotecan, there are reports where transporter SNPs are associated with pharmacokinetic alterations. These reports however, are not always consistent. In an analysis of the effect of single SNPs on irinotecan pharmacokinetics, patients homozygous for the P-gp mutation 1236C>T were found to have higher irinotecan and SN-38 AUC values (Mathijssen et al., 2003). The presence of this SNP, along with two other P-gp mutations in high linkage disequilibrium, was associated with a decrease in renal clearance of irinotecan and SN-38, but did not influence AUC values of these two compounds (Sai et al., 2003). The presence of the 3972C>T variant in MRP2 in preliminary findings is reported to increase irinotecan and SN-38G AUCs (de Jong et al., 2005; Smith et al., 2006). Although BCRP is thought to significantly mediate the transport of SN-38 and SN-38G within tumors, thus far, there have been no links to SNPs within the BCRP gene and the systemic disposition of irinotecan and its metabolites (de Jong et al., 2004).

C.6.b. PREVENTION OF ADVERSE EFFECTS

In addition to prediction of which patients will be susceptible to irinotecan gastrointestinal toxicity, a significant effort has been put forth to prevent it. In doing so, investigators have uncovered mechanisms crucial to the disposition of irinotecan, SN-38, and

SN-38G. Hypotheses put forth to prevent SN-38 mediated diarrhea with only preliminary data include the inhibition of cyclooxygenase-2 and intestinal carboxylesterases which can generate SN-38 from irinotecan at the site of toxicity (Trifan et al., 2002; Wadkins et al., 2004).

As a means to exploit the poor cytotoxicity profile and slow uptake of SN-38 carboxylate into intestinal cells, it was hypothesized that by shifting the equilibrium in the intestine to predominantly the carboxylate form, diarrhea might be evaded (Kobayashi et al., 1999). This was accomplished by raising the intestinal pH by giving sodium bicarbonate in drinking water of both hamsters and humans (Takeda et al., 2001; Ikegami et al., 2002). It is currently unknown whether this intervention altered either efficacy or the pharmacokinetic profiles of irinotecan, SN-38, or SN-38G.

Little SN-38G is found in the feces relative to that found in bile, indicating a role for β-glucuronidase cleavage of the conjugate. An inhibitor of β-glucuronidase, saccharic acid 1,4-lactone, protected rats from pathological changes common in the intestine after irinotecan treatment (Fittkau et al., 2004). By the administration of antiobitics that ameliorate the bacteria that form β-glucuronidase, intestinal toxicity was avoided, both in rats and humans (Takasuna et al., 1996; Kehrer et al., 2001). Coupled with positive pharmacodynamic outcomes, antibiotics reduce the SN-38 AUC in the large intestine by 85%, indicating that β-glucuronidase liberation of SN-38 is often sufficient to drive a patient from tolerating therapy to succumbing to its adverse effects (Takasuna et al., 1998). However, whether such perturbations of EHC would extend to other drugs or endogenous bile acids is not clear.

As a related hypothesis, Horikawa et al. (2002) sought to test whether or not inhibiting the primary biliary transport system of SN-38 and SN-38G would protect an

animal from gastrointestinal toxicity, as intraluminal concentrations of SN-38 would be reduced. In this study, probenecid, an inhibitor of MRP2, was found to reduce the biliary clearance of irinotecan, SN-38, and SN-38G in rats after a 5 mg/kg dose of irinotecan given intravenously, which concomitantly reduced intestinal concentrations and diarrhea scores. As a result of this inhibition, plasma concentrations were higher, such that a reduced dose could be given in the presence of probenecid. Because probenecid is a non-specific inhibitor of organic anion transporters that are also present in the kidney, renal clearance of SN-38 also was reduced (Horikawa et al., 2002). Although reduced biliary excretion of irinotecan and metabolites seems to protect against gastrointestinal toxicity, higher plasma levels of SN-38 may predispose patients to neutropenia or alter SN-38 disposition in tumor tissues.

Investigators from the University of Chicago have explored the coadministration of cyclosporine with irinotecan in humans to test a similar hypothesis to the one proposed previously, that only SN-38 excreted in the bile is responsible for gastrointestinal adverse effects and is independent of systemic SN-38. Cyclosporine, as both an MRP2 and P-gp inhibitor, increased the AUC of SN-38 and irinotecan and appeared to have a low incidence of diarrhea in a small number of patients (Innocenti et al., 2004b; Desai et al., 2005). Phenobarbital, a UGT inducer found to reduce the SN-38 AUC in rodents, was then added to the regimen (Gupta et al., 1997b). By doing so, it was hoped that inducing hepatic formation of SN-38G would reduce the amount of SN-38 available for biliary excretion, hence reducing the incidence of diarrhea. The AUC of SN-38 was reduced in these patients, allowing an increase in the maximum tolerated dose, and the incidence of diarrhea was similar to or slightly less than historical controls. However, the authors do not mention the potential for phenobarbital induction on intestinal UGTs and the possible protective consequences that

may have. Also, as the SN-38G/SN-38 ratio is increased with phenobarbital treatment, the authors fail to recognize the importance of excreted SN-38G, in that this compound is the primary source for SN-38 in the intestine, which was shown directly in biliary excretion studies and in indirectly in those involving β-glucuronidase inhibition. Since cyclosporine and phenobarbital also inhibit and induce transporters, respectively, it is suspected that if a protective effect of these drugs is observed in subsequent studies, it is likely due to a much more complex interplay than that simply stated by the authors' hypotheses (Innocenti et al., 2004b).

In a recent publication, investigators gave chrysin, a UGT inducer with poor bioavailability, concomitantly with irinotecan to patients and evaluated the pharmacokinetics and tolerability of the chemotherapeutic against historical controls. The pharmacokinetics of irinotecan and metabolites were unchanged with chrysin. The incidence of diarrhea in the chrysin study was lower than the controls (10% versus 19%), albeit there were no Gilbert's patients in the former study. There was no critical data that came from this publication, although it is among the first to suggest that alterations in the SN-38G/SN-38 ratio via intestinal UGTs may be a prime factor in mediating diarrhea (Tobin et al., 2006).

The role of intestinal UGTs in mediating SN-38 toxicity has been demonstrated *in vitro*. Kobayashi et al. (1999) reported that higher intracellular SN-38 concentrations resulted in a greater incidence of cell death in the HT-29 colon carcinoma cell line (Kobayashi et al., 1999). In this same cell line, propofol, a UGT1A9 substrate was found to lower the IC_{50} of SN-38 in this UGT-expressing cell line. In HCT-116 cells, which not do have any functional UGTs, propofol had no effect upon the SN-38 IC_{50} (Cummings et al., 2003). Although this manuscript does focus primarily upon glucuronidation as a resistance

mechanism in tumors, they do highlight that in the intestine, glucuronidation may have a role in modulating toxicity, as resistance and reduced toxicity both should result from lower intracellular SN-38 concentrations.

D. RATIONALE AND OVERVIEW OF PROPOSED RESEARCH

Currently, there is no pharmacokinetic or genetic indicator to predict or prevent diarrhea in susceptible patients. Despite all of the research devoted to finding such a "magic bullet" to spare vulnerable patients, data from current clinical studies indicate that patients are still experiencing grade 3 or 4 diarrhea at rates upwards of 20% (Ziotopoulos et al., 2005). The change in dosing irinotecan to Gilbert's patients discussed above comes in response to the data supporting the high incidence of neutropenia in these patients when treated with standard doses. In fact, every voting member of the FDA Clinical Pharmacology Subcommittee responsible for recommending this dosage change agreed that there was not sufficient evidence linking the incidence of diarrhea in patients with Gilbert's syndrome (Scharen, 2004). The lack of a connection between diarrhea and the UGT1A1*28 genotype may be due to the following: a patient with Gilbert's syndrome will excrete less SN-38G due to inefficient production of this metabolite, but should excrete SN-38 at the same low rate as a person without Gilbert's genotype. This suggests that in a patient with wild-type UGT1A1 alleles, more SN-38 will exist in the intestinal lumen than a patient with UGT1A1*28, as SN-38G is efficiently cleaved in the intestine by β-glucuronidase. Few dispute that direct enterocyte injury due to high intestinal levels of SN-38 is ultimately the preceding cause of toxicity, as demonstrated by studies with both β-glucuronidase and SN-38 biliary excretion inhibition, so patients with normal UGT1A1 genes may still get diarrhea. However,

intestinal UGTs are presumably a local protective mechanism that can reduce the amount of intracellular SN-38, and may be a key factor to modulating toxicity. This would explain why some patients with UGT1A1*28 ultimately get diarrhea, which is not due to a lack of hepatic glucuronidation, but because the intestine cannot effectively glucuronidate SN-38 through UGT1A1. Diarrhea may be avoided in some Gilbert's patients for a number of reasons, including higher intestinal expression of UGT1A1, or from the glucuronidation of SN-38 by other individual isoforms. Since UGT1A9 catalyzes SN-38 glucuronidation at a similar rate to 1A1, high intestinal expression or the presence of common extensive-metabolizer polymorphisms such as T-275A and C-2152T in UGT1A9 may help spare patients from gastrointestinal toxicity. These are all factors that are largely unknown, due to the limited availability of human intestinal tissue, or UGT isoform probe substrates and antibodies. Although proposed in the literature by Tukey et al. (2002), no publications have surfaced to address the contribution of intestinal UGTs to SN-38 gastrointestinal toxicity, which may be due in part to the difficulty in designing experiments to separate the contribution of intestinal UGTs from those of the liver in a toxicological model that takes days to develop (Tukey et al., 2002). As discussed earlier, hepatic glucuronidation may not be a surrogate for, or highly correlated with, intestinal glucuronidation capacity, due to the presence of tissue-specific transcriptional proteins, differential isoform composition, levels of expression, and exposure to dietary and environmental compounds that can induce the expression of UGTs. Ultimately, the amount of residual intestinal UGT activity needed to prevent diarrhea is unknown, and avoiding diarrhea is likely a balance between the efficiency of UGT catalysis and intracellular levels of SN-38. It is recognized that intracellular exposure of SN-38 involves more than the UGTs, and the overall prediction of diarrhea is probably a

combination of all these factors, but one of the most important seems to be glucuronidation for the reasons outlined above.

The major hypothesis governing the experiments in this thesis proposal is that **intestinal UGTs are a critical factor in mediating SN-38 induced diarrhea**. In order for this hypothesis to be directly addressed, it is crucial to first investigate intestinal UGTs and whether this hypothesis is physiologically feasible through the following specific objectives:

1. **Identify differences in SN-38 lactone and carboxylate glucuronidation for hepatic and intestinal UGT isoforms.** Almost all data about isoform specificity and UGT catalysis is for the inactive carboxylate form of SN-38 due to its better solubility. Should intestinal UGT isoforms preferentially glucuronidate the carboxylate form, these UGTs may do little in sparing the enterocyte from SN-38-induced cytotoxicity. Also, understanding the catalysis of specific isoforms will allow interpretation of *in vitro* data obtained with human and animal tissues, such as liver and intestine. The following experiments were conducted to assess possible differences:
   (a) Establish a high performance liquid chromatography (HPLC) method for simultaneous detection of SN-38 lactone and carboxylate.
   (b) Determine rates of hydrolysis or lactonization for SN-38 lactone and carboxylate in the incubation mixtures.
   (c) Measure glucuronidation rates of each form with recombinant isoforms and microsomes with SN-38 lactone and carboxylate, using rates above in (b) to determine incubation time minimizing interconversion between the forms.

(d) Utilize pharmacokinetic modeling to recover glucuronidation rates of pure SN-38 lactone and carboxylate.

2. **Assess the variability of SN-38 glucuronidation in the human intestine and correlate the glucuronidation with probe substrates of active UGT isoforms.** Prior to substantiating the hypothesis that intestinal UGTs mediate SN-38 diarrhea, it is important to determine whether there was significant interpatient variability in the population with respect to intestinal SN-38G formation. These studies are among the first to characterize intestinal UGT1A isoform expression:

   (a) Establish *in vitro* glucuronidation assays, HPLC, and liquid chromatography with mass spectrometry detection (LC-MS) methods for SN-38, and propofol (UGT1A9) probe.

   (b) Validate etoposide as a UGT1A1 probe and establish an in vitro glucuronidation assay and LC-MS method for etoposide-glucuronide detection.

   (c) Perform genotype analysis for highly polymorphic allele UGT1A1*28, which has been shown to modulate the pharmacokinetics of substrates and the incidence of SN-38-induced neutropenia. The presence of this genotype could potentially explain high or low SN-38 glucuronidation rates.

Finally, the hypothesis will be directly tested in Specific Objective #3:

3. **The gastrointestinal toxicity and pharmacokinetic profile of irinotecan, SN-38, and SN-38G will be compared in rats with and without intestinal UGT1As.** In order to evaluate the contribution of the intestinal UGTs to diarrhea, systemic exposure to these compounds must remain similar. In order to do this, hepatic

glucuronidation will be restored in the Gunn rat through gene transfer of rat UGTs using an adenoviral vector. The series of experiments to test the above stated hypothesis are as follows:

(a) Establish a model for irinotecan gastrointestinal toxicity in the UGT-transfected Gunn rat and compare it to the toxicity profile of a heterozygote Gunn/Wistar rat.

(b) Determine the systemic disposition of irinotecan, SN-38, and SN-38G in both groups of rats.

(c) Compare the *in vitro* expression and glucuronidation capacity of intestines and livers from UGT-transfected Gunn rats and the Gunn/Wistar heterozygotes.

(d) Assess the intestinal exposure of irinotecan and metabolites through biliary excretion studies in both groups of rats.

In addition to the above studies, preliminary experiments in the HT-29 colon carcinoma cell line are described in Chapter 5. In these experiments, the co-incubation of SN-38 and piperine, a UGT inhibitor, was intended to predispose cells to SN-38-mediated apoptosis and cell death. Finally, potential clinical implications, future studies, and the conclusions drawn from the research presented within this dissertation project will be addressed in Chapter 6.

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Table 1.1 Localization of UGT1A mRNA in human tissues (Strassburg et al., 1997; Strassburg et al., 1998; Strassburg et al., 1999b; Strassburg et al., 2000).

Enzyme	Localization
UGT1A1	Biliary and liver tissue, colon, most duodenal samples, a few jejunal and ileal samples
UGT1A3	Liver, biliary, gastric, colon, most duodenal, jejunal, ileal samples
UGT1A4	Liver, biliary, colon, most duodenal, jejunal, ileal samples
UGT1A6	Liver, biliary, gastric, colon, most duodenal, ileal, and a few jejunal samples
UGT1A7	Gastric, esophagus
UGT1A8	Colon, esophagus
UGT1A9	Liver, colon, esophagus
UGT1A10	Biliary, gastric, colon, esophagus, duodenum, jejunum, ileum

** UGT1A2, 1A11, and 1A12 are pseudogenes and do not encode functional proteins (Emi et al., 1995; Owens and Ritter, 1995).

Table 1.2 Localization of UGT1A mRNA in rat tissues (Emi et al., 1995; Grove et al., 1997; Grams et al., 2000; Shelby et al., 2003).

Enzyme	Localization
UGT1A1	Liver, kidney, stomach, small and large intestine, brain, lung, testis
UGT1A2	Small and large intestine, stomach
UGT1A3	Small and large intestine
UGT1A5	Liver
UGT1A6	Small and large intestine, stomach, kidney, liver, lung, brain, esophagus
UGT1A7	Small and large intestine, stomach, kidney, lung, spleen, ovary
UGT1A8	Low expression in liver and kidney

**Rat UGT1A4 and 1A9 are pseudogenes and do not encode functional proteins (Emi et al., 1995).

Table 1.3 Cumulative biliary, urinary, and fecal excretion in humans expressed as percentage of dose (Lokiec et al., 1995; Sparreboom et al., 1998b; Slatter et al., 2000).

	Irinotecan	**SN-38**	**SN-38G**	**Total**
Bile	3-22%	0.1-0.9%	1.2-3%	3.6-30%
Urine	11-22%	0.1-0.4%	3-3.4%	21-30%
Feces	15-32%	2.5-8.2%	0.3%	24-62%

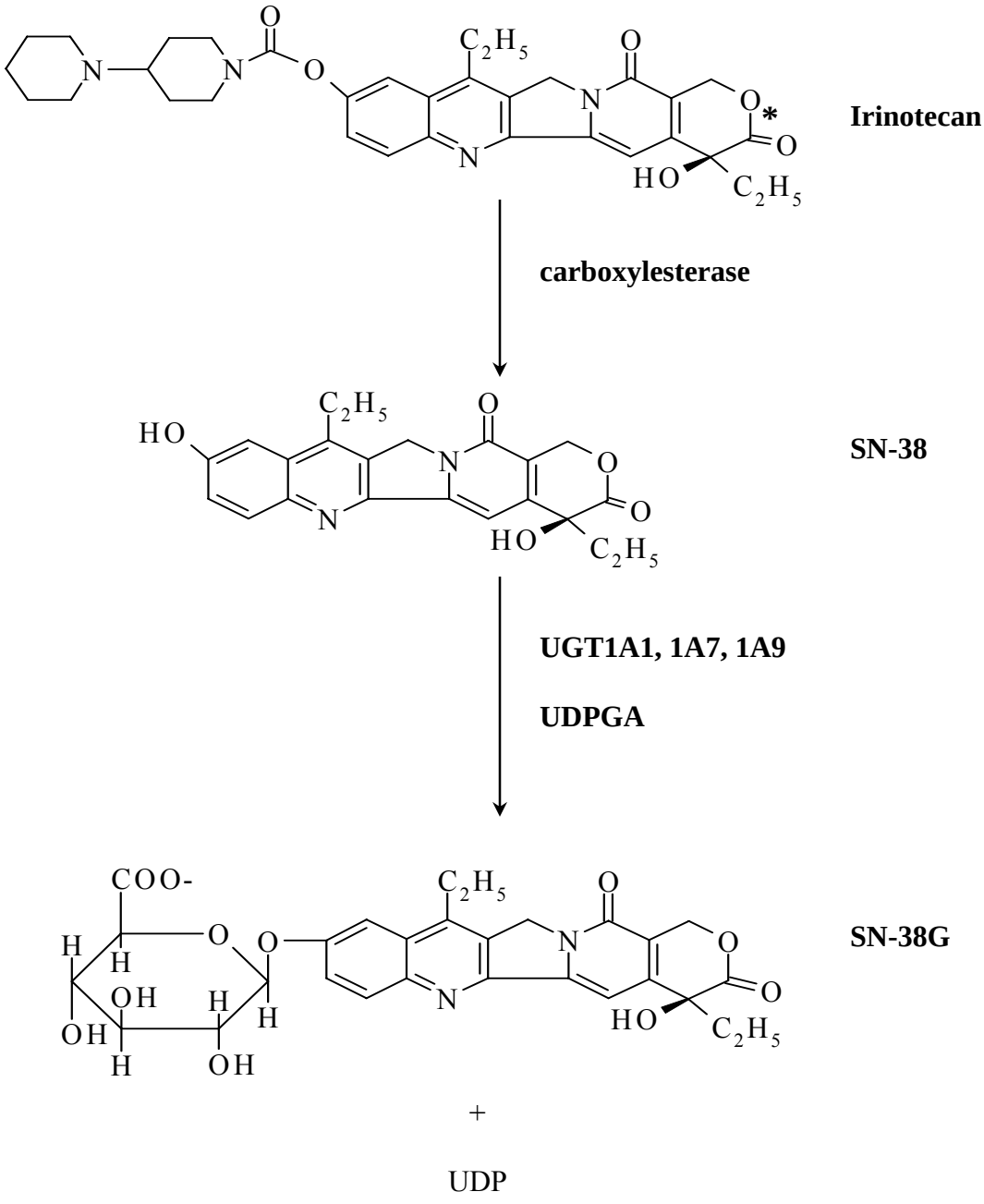

Figure 1.1 Glucuronidation of SN-38 after carboxylesterase cleavage of irinotecan. UGT1A1, 1A7, and 1A9 facilitate the transfer of glucuronic acid from UDPGA to the substrate, generating the β-glucuronide of the substrate and uridine diphosphate (UDP). The pH-labile lactone moiety is indicated with a (*) on irinotecan. An equilibrium exists between the lactone form and its hydrolysis product, the carboxylate form, for all three compounds.

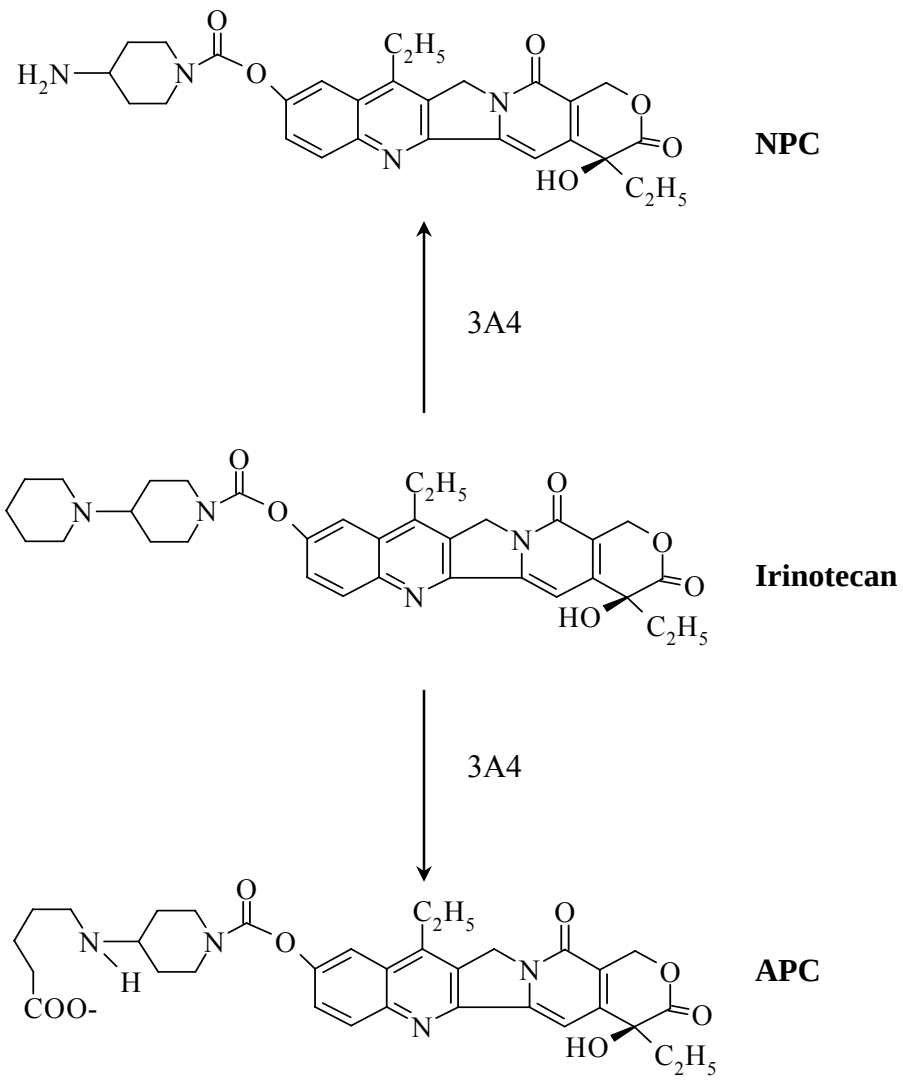

Figure 1.2 Cytochrome P450 3A4-mediated metabolism of irinotecan.

CHAPTER 2

DIFFERENTIAL RATES OF GLUCURONIDATION FOR 7-ETHYL-10-HYDROXY-CAMPTOTHECIN (SN-38) LACTONE AND CARBOXYLATE IN HUMAN AND RAT MICROSOMES AND RECOMBINANT UGT ISOFORMS

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A. ABSTRACT

SN-38, the active metabolite of the anti-cancer agent irinotecan, contains a lactone ring that equilibrates with a carboxylate form. Since SN-38 lactone is the active and toxic form, it is prudent to examine whether the more soluble carboxylate is a surrogate for SN-38 lactone conjugation. Therefore, relative rates of glucuronidation and isoform specificity of SN-38 lactone and carboxylate were characterized. The stability of SN-38 lactone and carboxylate in incubation mixtures of microsomes and UGT isoforms was used to determine optimal incubation times. Microsomal incubations were conducted using rat and human intestinal and hepatic microsomes and human and rat recombinant UGT 1A isoforms. Where estimates of lactone and carboxylate glucuronidation rates could not be established due to short incubation times and detection limits, kinetic modeling was used to recover these rate constants. The stability experiments revealed that the lactone was stabilized by rat microsomes, however, the opposite was observed in human microsomes and recombinant isoforms. For all tissues and most UGT isoforms examined, the lactone consistently had catalytic rates up to 6-fold greater than the carboxylate. The rank order of glucuronidation for both SN-38 lactone and carboxylate was 1A7 > 1A1 > 1A9 > 1A8 and 1A7> 1A8 > 1A1 for human and rat isoforms, respectively. This study provides further support that SN-38 lactone and carboxylate may be considered pharmacokinetically distinct agents. The *in vivo* impact of this conjugation difference is unknown, since variations in protein binding and transport proteins may affect intracellular concentrations of the lactone or carboxylate.

B. INTRODUCTION

The topoisomerase-I inhibitor, irinotecan (manufactured as irinotecan hydrochloride trihydrate [Camptosar®, Pfizer Inc.]), in combination with fluorouracil and leucovorin is approved in the treatment of metastatic colorectal cancer. Irinotecan is increasingly being utilized in chemotherapy regimens for many other malignancies, including pancreatic and lung cancers (Rothenberg, 2001). The cytotoxicity of irinotecan is achieved largely through a metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38), which is at least 100-fold more potent in inducing cell death *in vitro* (Kawato et al., 1991). Irinotecan is a prodrug, as SN-38 is not directly administered to patients due to its poor aqueous solubility and unacceptable toxicity (Rajkumar and Adjei, 1998). SN-38 is formed by the metabolism of irinotecan by carboxylesterases in various tissues (Khanna et al., 2000; Mathijssen et al., 2001). SN-38 is then glucuronidated by UDP-glucuronosyltransferase (UGT) 1A1, UGT1A7, and UGT1A9, with similar reported catalytic efficiencies, to form SN-38 glucuronide (SN-38G) (Gagne et al., 2002) (Figure 2.1).

The lactone ring of SN-38, like all camptothecin derivatives, can undergo reversible, pH-dependent hydrolysis to yield the corresponding carboxylate (Figure 2.1). The equilibrium favors the poorly soluble lactone form at lower pH values, and at neutral and basic pH values, the more soluble carboxylate form predominants. Both forms exist considerably *in vivo*, with the lactone accounting for 54-64% of the total SN-38 area under the plasma concentration versus time curve (Rivory et al., 1994; Sasaki et al., 1995b). The lactone and carboxylate are considered two pharmacologically distinct compounds, as the intact lactone is necessary for topoisomerase binding and carboxylate is only weakly cytotoxic (Hertzberg et al., 1990). Additionally, SN-38 lactone and carboxlyate possess

different affinities for transporters and exhibit differences in pharmacokinetics in animals and humans. In EHBR rats (rats deficient in MRP2), SN-38 carboxylate biliary clearance was reduced whereas for the lactone form it was unchanged, suggesting a role of mrp2 in the hepatic transport of only the carboxylate (Chu et al., 1997). SN-38 lactone has a greater volume of distribution and binds more tightly to albumin when compared with SN-38 carboxylate (Burke and Mi, 1993; Kaneda et al., 1997; Xie et al., 2002b). An intact lactone may also be favored for substrate-enzyme binding or catalysis, as irinotecan lactone is metabolized to SN-38 at a greater rate than irinotecan carboxylate (Haaz et al., 1997b). It is unknown whether or not this phenomenon occurs for SN-38 with the UGTs, and there is contradictory data suggesting metabolic differences between SN-38 lactone and carboxylate. Haaz et al. (1997a) assessed rates of lactone and carboxylate glucuronidation from a 15 minute microsomal incubation of SN-38 in human liver microsomes. The authors estimated that by 15 minutes, 70% of the lactone form was hydrolyzed to the carboxylate, and no significant differences in glucuronidation were noted. However, it did appear that the lactone form was metabolized more quickly (Haaz et al., 1997a). SN-38 lactone administered by intravenous bolus to rats was cleared 2-fold faster by metabolism and excretion when compared with administration of SN-38 carboxylate (Kaneda et al., 1997). In contrast, a population pharmacokinetic study in humans predicted via extensive modeling that SN-38 lactone and carboxylate had similar total clearance values (Xie et al., 2002b).

The purpose of these studies was to assess whether SN-38 lactone and carboxylate are glucuronidated by human and rat tissues and recombinant isoforms at different rates and whether the lactone and carboxylate differed in their specificity for individual UGT isoforms. Deciphering the latter is important, as the more soluble carboxylate is often used as a

surrogate for the active lactone form in microsomal metabolism studies (Ciotti et al., 1999; Hanioka et al., 2001a; Hanioka et al., 2001b; Gagne et al., 2002). Knowledge of differences in glucuronidation rates or isoform specificity between SN-38 lactone and carboxylate may be instrumental in understanding cellular toxicity or resistance to the chemotherapeutic agent.

C. MATERIALS AND METHODS

Chemicals and reagents. Irinotecan and SN-38G methyl ester were a gift from Dr. Robert Kelly at Pfizer (Kalamazoo, MI), previously Pharmacia. SN-38 was cleaved from irinotecan by hydrolysis with base according to a procedure provided by Dr. Kelly. The purity of SN-38 obtained was confirmed by high performance liquid chromatography (HPLC) with UV detection and by liquid chromatography-mass spectrometry. SN-38G methyl ester was also hydrolyzed by base to produce SN-38G. The concentration of SN-38G diluted in methanol was determined by β-glucuronidase treatment and the resultant SN-38 generated was analyzed by HPLC using SN-38 standards. Chemicals used in the microsomal preparations and reactions were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents used were of reagent grade and obtained through commercial vendors.

Microsome and recombinant isoform preparation. Human recombinant UGT isoforms and human liver microsomes were obtained commercially from Gentest (Woburn, MA). Human intestinal microsomes were purchased from Xenotech LLC (Lenexa, KS). Rat recombinant UGT isoforms were prepared from HEK cell membranes as previously described (Kessler et al., 2002). Rat hepatic and intestinal microsomes were prepared and pooled from four male Sprague-Dawley rats, weighing 250 g (Charles River, Raleigh, NC). Mucosal scrapings of the enterocytes or portions of the liver were subject to homogenization in 250 mM sucrose with ethylenediaminetetraacetic acid (1mM), dithiothreitol (0.1mM), and phenylmethylsulfonyl fluoride (PMSF) (0.25mM) and centrifuged at 10,000g x20 min. The supernatant was then spun at 100,000g x1 hr. The resulting pellet was reconstituted in 250 mM sucrose containing PMSF (0.25 mM) and leupeptin (10 mM). Protein concentrations were determined by the Bradford method, using albumin as a standard.

HPLC Analysis. The HPLC method to analyze SN-38 and SN-38G was modified from a published method (Sparreboom et al., 1998a). The HPLC system consisted of an isocratic pump (LC-600, Shimazdu, Tokyo, Japan), autosampler (AS-100; Biorad, Hercules, CA), and fluorescence detector (HP1046A, Hewlett Packard, Palo Alto, CA). A Hypersil BDS C-18 column (5 uM, 150x4.6 mm) was used to separate analytes (Thermo Hypersil, Woodstock, GA). For the SN-38G glucuronidation assay, the mobile phase consisted of methanol: tetrabutylammonium sulfate (10mM) and ammonium acetate (100 mM), pH 4.9 (37%: 63%). For the purposes of quantitating SN-38G in microsomal reactions, 200-300 μL microsomal aliquots were precipitated with acetonitirile (800 μL) containing irinotecan (70 ng) as internal standard and perchloric acid (5%, 5 μL). SN-38 (30 μg) was added to prevent adsorption of irinotecan and SN-38G to the reaction tubes. The acetonitrile was evaporated under a stream of nitrogen. The residue was then reconstituted in 120 μL weak mobile phase, pH 4.9 (30% methanol). The pH of this solution ensures that all SN-38G is in the lactone form. SN-38G was detected by fluorescence using excitation and emission wavelengths of 229 and 420 nm, respectively, which provided optimal sensitivity for SN-38G, but not SN-38. The chromatographic separation of SN-38G (3.5 minute retention time), irinotecan (5 minutes), and SN-38 (17 minutes) is shown in Figure 2.2. Standard curves for SN-38G were linear between 1 ng/mL - 1000 ng/mL.

When assessing SN-38 lactone and carboxylate stability, the mobile phase pH was not adjusted, and a pH of approximately 5.6 was employed. Also, to elute SN-38 carboxylate and lactone more quickly, the mobile phase strength was increased to 42% methanol. SN-38 lactone and carboxylate were detected using optimized excitation and emission wavelengths of 229 and 543 nm, respectively. Under these conditions, the retention times were

approximately 3.0 and 6.0 minutes for the carboxylate and lactone, respectively. To assess whether a similar response was obtained when SN-38 carboxlyate and lactone were injected onto the HPLC in equal amounts, the relative response ratio was determined. Aliquots (20 μL) from three separate solutions of SN-38 carboxylate and lactone (5 μM) were analyzed and SN-38 lactone consistently had a 1.78-fold greater response than the carboxylate. Therefore, when relating the peak areas of SN-38 lactone to carboxylate and vice versa in assessing their stability, the area of the carboxylate was multiplied by 1.78 to account for the lower fluorescence response.

Stability Studies. Solutions of SN-38 lactone and carboxylate were made by dissolving SN-38 in DMSO/ 0.1M sodium acetate (50:50, pH 4.5) and DMSO/ 1 x 10^{-5} M sodium hydroxide (50:50, pH 9.5), respectively, and incubated for 24 hours at 37°C. This procedure produced lactone or carboxylate, respectively, at approximately 99% content, as verified by HPLC. SN-38 lactone or carboxylate (5 μM, final concentration in solution) was added to either buffer or the reaction mixture containing recombinant isoforms or microsomes described below at 37°C without uridine diphosphate glucuronic acid (UDPGA). At various times, 20 μL of the buffer or incubation mixture was directly injected onto HPLC. First-order rate constants for conversion ($k_{L\rightarrow C}$ or $k_{C\rightarrow L}$) were generated by using non-linear regression analysis software (Winnonlin, Pharsight; Palo Alto, CA). These rate constants were used to determine the length of incubation where <5% conversion from the lactone to carboxylate (and vice versa) occurred or were used as constants in kinetic modeling.

Microsomal Reaction Conditions. Incubation mixtures contained Brij 35 (0.5 mg/mg protein), D-Saccharic acid 1,4-lactone (10mM), magnesium chloride (10 mM), UDPGA (2 mM), and microsomal or recombinant protein (0.1 mg/mL for human recombinant UGTs,

0.25 mg/mL for rat recombinant UGTs, or 0.5 mg/mL for human and rat hepatic and intestinal microsomes) in a final volume of 500-1600 μL of 0.1M Tris, pH 7.0. The protein concentrations for each matrix were fixed and used previously to determine the stability of SN-38 lactone and carboxylate. The reaction was initiated by spiking in SN-38 lactone or carboxylate (5 μM, final concentration in solution), and terminated at times predetermined by the stability studies to minimize lactone and carboxylate interconversion (<5%). The concentration of dimethyl sulfoxide in the incubation was 1%.For incubations where quantitation of SN-38G from relatively pure SN-38 lactone or carboxylate was not possible due to rapid conversion, SN-38G concentrations from at least five time points from 5-60 minutes were analyzed to allow glucuronidation rate constants for SN-38 lactone and carboxylate to be recovered from kinetic modeling.

Kinetic Modeling. Compartmental modeling using Winnonlin provided estimates of the glucuronidation rate constants of SN-38 lactone and carboxylate by various microsomes and isoforms. Data used in this model (Figure 2.3) to generate the glucuronidation rate constants, $k_{gluc,\ lactone}$ and $k_{gluc,\ carboxylate}$, was expressed as cumulative formation of SN-38G over time. Both SN-38 lactone and carboxylate are depicted to form SN-38 lactone due to the acidified solution injected onto the HPLC, which converts any SN-38G carboxylate to SN-38G lactone (see HPLC analysis under MATERIALS AND METHODS). Simultaneous modeling of the cumulative SN-38G formed when the carboxylate and lactone were spiked ($X_{o,\ carboxylate}$ or $X_{o,\ lactone}$) in separate experiments allowed the rate constants to be recovered. Interconversion rate constants between SN-38 lactone and carboxylate ($k_{L\rightarrow C}$ or $k_{C\rightarrow L}$) found in the stability studies were held as constants. Initial iterative values for the glucuronidation rate constants were set as the slope of the percent of SN-38 remaining versus time. An assumption was

made that glucuronidation proceeded under non-saturable conditions. It was also assumed that the initial conversion rates found in the stability studies were uni-directional conversion rates, since the stability profiles (Figure 2.4) were log-linear, reflective of a first-order process.

D. RESULTS

The stability of SN-38 lactone and carboxylate in various buffers is shown in Figure 2.4. The experimentally-derived rate constants were similar to what was expected and previously reported, as the half-life of SN-38 lactone at pH 7.0 and 37°C is 95 minutes versus 96 minutes as calculated from rate constants published by Akimoto et al (1994). These values were also in rough agreement with Chorupa et al (1998), who reported a half – life of approximately 32 minutes at a higher pH (7.3), were lactonolysis is increasingly favored (Akimoto et al., 1994; Chourpa et al., 1998).Consistently, the lactone remained intact at low pH values, and the carboxylate was more stable in neutral and basic aqueous buffers. These findings gave confidence that the HPLC method used to determine the first-order rate constants for hydrolysis and lactonization in microsomes and recombinant isoforms was accurate with no conversion on the column during the analysis (Table 2.1). The stability of SN-38 lactone and carboxylate was initially assessed in buffers with recombinant human UGT1A1 and 1A8, with virtually identical hydrolysis and lactonization rates. Therefore, these values were employed for these and other human UGT isoforms. The same was observed for recombinant rat UGT1A1, 1A8, and 2B2. The data in Table 2.1 indicate that hydrolysis (i.e. $k_{L\rightarrow C}$) is facilitated by the addition of human microsomes or human or rat recombinant isoforms to Tris buffer. Conversely, the presence of rat microsomes stabilized the lactone moiety. SN-38 carboxylate was cyclized to the lactone (i.e. $k_{C\rightarrow L}$) at a faster rate in rat microsomes relative to Tris buffer. However, both SN-38 lactone and carboxylate were sufficiently stable in rat hepatic and intestinal microsomes to allow SN-38G quantitation from a microsomal reaction with little conversion to the other form (less than 3%).

Once the stability of the lactone/carboxylate forms was established, catalytic rates of formation of SN-38G from each form was estimated *in vitro* with the presence of UDPGA. In both rat intestinal and hepatic microsomes, SN-38 lactone was glucuronidated more quickly compared with the carboxylate (Table 2.2), where these reactions could be conducted in 6.0-15.3 minutes with insignificant conversion to the carboxylate (<2.5%). However, a 1.5 minute or less reaction time would have been required to estimate such values for the lactone in human microsomes and recombinant isoforms due to rapid hydrolysis in these matrices (Table 2.1). Since these constraints were experimentally unfeasible for the lactone, kinetic modeling was used to recover glucuronidation rates for SN-38 carboxylate and lactone in human microsomes and human and rat recombinant isoforms (Figure 2.5 and Table 2.3). Although glucuronidation rates (nmol/min/mg protein) could be recovered for pure carboxylate without modeling, modeling was used so that a comparison could be made between SN-38 lactone and carboxylate by using first-order glucuronidation rate constants. SN-38 lactone and carboxylate had glucuronidation rates in the same rank order, with UGT1A7 being the most active, followed by UGT1A1, 1A9, and 1A8. Barely quantifiable levels of SN-38G were produced with UGT1A6 and UGT1A10 at 60 minutes, and UGTs 1A3, 1A4, 2B4, 2B7, 2B15, and 2B17 were inactive towards both forms of SN-38. Rat UGT1A7 glucuronidated SN-38 carboxylate and lactone at the fastest rate, with rat UGT1A8 and 1A1 also having activity. Rat UGT1A2, 1A3, 1A5, 1A6, 2B2, 2B3, and 2B18 did not glucuronidate SN-38. Virtually every isoform or tissue that had measurable catalysis glucuronidated SN-38 lactone at a greater rate compared to the carboxylate. This difference was typically a 3.6-fold increase in glucuronidation, however, up to a 6-fold difference was

observed with UGT1A1. UGT1A8 was the lone exception, with the carboxylate being metabolized approximately twice as quickly compared to the lactone.

E. DISCUSSION

It is well established that SN-38 lactone and carboxylate exhibit pharmacodynamic and pharmacokinetic differences. Here, we have verified that these differences also extend to rates of glucuronidation. For all the rat and human isoforms that appreciably catalyze SN-38G formation, the lactone is almost always conjugated at a faster rate compared to the carboxylate. The faster SN-38 lactone turnover relative to the carboxylate by individual isoforms should translate into higher glucuronidation rates for the lactone by tissues, which was found for microsomes prepared from human and rat liver and intestine. A potential explanation for this difference may be an increased ability of the lactone to gain access to the catalytic site of the UGT in the relatively lipophilic environment of the microsome. Although a detergent (Brij 35) was used to expose the active site, the carboxylate may still not partition well due to its negative charge. It is likely that *in vivo*, the lactone would more easily access the active site of the UGT if passive processes are operative, as the enzyme exists in the inner lumen of the endoplasmic reticulum (Yokota et al., 1992; Meech and Mackenzie, 1997). Alternatively, the UGTs may simply preferentially recognize and have a higher binding affinity (discussed below) for the intact SN-38 lactone compared to the acid functional group.

The extrahepatic isoform UGT1A8 does metabolize SN-38 carboxylate faster than the lactone. An *in vivo* effect of this unusual behavior is not likely, as the rate of glucuronidation by this isoform is approximately 150, 40, and 4 fold less than UGT1A7, 1A1, and 1A9 respectively (Table 2.2). It is possible that a preference for SN-38 carboxylate conjugation may be observed in tissues where UGT1A8 is highly expressed compared to UGT 1A7, 1A1,

and 1A9. 1A8 has not been reported to be in the liver and small intestine but is found in the colon and esophagus (Strassburg et al., 1998).

Although intrinsic clearance determinations may be a more relevant *in vivo* value to obtain compared with a velocity at a certain concentration, the latter approach was used to rank active isoforms and compare the efficiency of lactone and carboxylate glucuronidation since solubility limitations of SN-38 lactone preclude estimation of Michaelis-Menten enzyme kinetics. The solubility of SN-38 lactone in aqueous buffers published by Zhang et al. (2004) is approximately 18 μM (7.2 μg/mL), which is a value similar to that obtained in our laboratory. Michaelis-Menten studies were initially conducted using SN-38 lactone and did yield the characteristic concentration versus velocity curve, and the apparent Vmax was obtained at approximately the solubility limit (data not shown). Therefore, it is not clear whether a true Vmax was reached or that the plateau was due to solubility. For this reason, we conducted studies at 5 μM, a concentration at which both SN-38 lactone and carboxylate are soluble and does not saturate the enzyme (Haaz et al., 1997a) (data not shown). Elucidation of a Km for SN-38 in various tissues and UGT isoforms was not attempted in this study due to the solubility limitations of the lactone as previously discussed. Although Km values are helpful for *in vitro-in vivo* predictions, knowledge of these values for SN-38 lactone or carboxylate in tissues or UGT isoforms would not be expected to be helpful. Peak plasma concentrations of SN-38 are in the nanomolar range after a standard irinotecan dose, which is significantly below any literature-reported Km in the micromolar range (Haaz et al., 1997a; Hanioka et al., 2001b; Gagne et al., 2002). Hence, the rank-order of active isoforms and the relative differences observed between SN-38 lactone and carboxylate that are reported here should mimic the true *in vivo* situation. Since SN-38 concentrations *in vivo* and

in the experiments presented here are below the Km, Michaelis-Menten kinetics are operative and the rates of glucuronidation both *in vivo* and *in vitro* are proportional to SN-38 concentrations.

The results of this study substantiate those of previous publications identifying UGT1A7, 1A1, and 1A9 as having the highest relative glucuronidation activity towards SN-38 (Ciotti et al., 1999; Hanioka et al., 2001b; Gagne et al., 2002). In these studies, either the authors state that SN-38 carboxylate was utilized as the substrate (Hanioka et al., 2001b), or the upper concentrations of SN-38 used in the microsomal reactions implies that the carboxylate was used due to solubility limitations with the lactone form, allowing a larger concentration range over which to conduct Km and Vmax studies (Ciotti et al., 1999; Gagne et al., 2002). Should SN-38 lactone and carboxylate be preferentially glucuronidated by different UGT isoforms, SN-38 carboxylate would not be a suitable surrogate for assessing the glucuronidation profiles of the more cytotoxic lactone form. From this study, it is clear that investigators may continue to compare relative glucuronidation patterns of cells and tissues using SN-38 carboxylate in *in vitro* reactions. However, investigators should not rely upon Vmax and intrinsic clearance values generated for SN-38 carboxylate, as they may underestimate the true values for the active species, the lactone.

Interestingly, rat intestinal and hepatic microsomes were the only matrices in which the lactone was relatively stable to hydrolysis, and were the only matrices prepared with the protease inhibitors PMSF and leupeptin. It was then theorized that proteases in the recombinant isoforms and human microsomes were catalyzing SN-38 lactonolysis. To test this theory, human hepatic microsomes were treated with PMSF and leupeptin in concentrations equal to that in rat microsomes prior to the stability study with SN-38 lactone.

The addition of these protease inhibitors did not have an effect on the conversion rate of SN-38 lactone to carboxylate (data not shown). Therefore, it is unknown why lactonolysis proceeds so rapidly in recombinant isoforms and human microsomes compared with rat microsomes or buffer alone.

The *in vivo* impact of the differential catalysis of SN-38 lactone and carboxylate is unknown. The predominance of SN-38 lactone observed in the plasma of patients may not be observed intracellularly at the site of action, if SN-38 lactone is more rapidly glucuronidated. Since cellular damage is presumably mediated by intracellular SN-38 lactone, one might propose that reducing intracellular levels of SN-38 lactone by glucuronidation may increase tumor resistance or reduce the toxicity profile of irinotecan/SN-38 (Gupta et al., 1994; Tukey et al., 2002). Although glucuronidation may reduce SN-38 lactone relative to carboxylate, other factors may exist that would impact intracellular levels, such as pH or differential protein binding, uptake or efflux.

To summarize, SN-38 lactone and carboxylate are glucuronidated in the same rank order by both rat and human UGT1A isoforms. However, the lactone was almost consistently glucuronidated at a faster rate by these isoforms, which translated into faster overall catalysis of SN-38 lactone in rat and human hepatic and intestinal microsomes. Due to the solubility limitations of SN-38 lactone, investigators are unable to elucidate Km values for this form in various matrices. Therefore, utilizing SN-38 carboxylate as a more soluble surrogate will allow investigators to obtain an *in vitro* Km, Vmax, and intrinsic clearance. However, these values should be cautiously interpreted, as the Vmax is likely to be underestimated and the Km values for the two forms are not necessarily equivalent.

F. ACKNOWLEDGEMENTS

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Table 2.1 First-order rate constants for lactonization and hydrolysis of SN-38 carboxylate and lactone, respectively, in Tris buffer and biological matrices/Tris buffer at 37°C, pH 7.0. Incubations contained 0.5 mg protein/mL for human and rat hepatic and intestinal microsomes, 0.1 mg/mL for human recombinant UGTs, and 0.25 mg/mL for rat recombinant UGTs and were conducted without cofactor, UDPGA. Subsequent glucuronidation experiments were conducted using these fixed protein concentrations.

Matrix	$\mathbf{k_{C \rightarrow L}}$ **(min^{-1})**	$\mathbf{k_{L \rightarrow C}}$ **(min^{-1})**
0.1 M Tris, pH 7.0	5.49×10^{-4}	71.1×10^{-4}
Human intestinal microsomes	11.2×10^{-4}	103×10^{-4}
Human hepatic microsomes	2.30×10^{-4}	138×10^{-4}
Human recombinant UGTs	3.74×10^{-4}	161×10^{-4}
Rat intestinal microsomes	37.9×10^{-4}	16.6×10^{-4}
Rat hepatic microsomes	42.4×10^{-4}	16.4×10^{-4}
Rat recombinant UGTs	3.13×10^{-4}	108×10^{-4}

Table 2.2 SN-38 lactone and carboxylate glucuronidation rates[a] in rat hepatic and intestinal microsomes. Values in parentheses indicate the standard deviation between the averages of two different experiments (each with three replicates).

Matrix	**Lactone (pmol/min/mg protein)**	**Carboxylate (pmol/min/mg protein)**
Rat intestinal microsomes	18.5	6.5
	(1.6)	(0.6)
Rat hepatic microsomes	5.7	3.0
	(0.6)	(0.4

[a]: Study performed with an incubation time that only permitted 2.5% conversion of lactone to carboxylate or carboxylate to lactone

Table 2.3 First-order rate constants for glucuronidation of SN-38 lactone and carboxylate in microsomes and recombinant isoforms. Numbers in parentheses indicate the parameter coefficient of variance (%).

Matrix	$\mathbf{k_{gluc,\ lactone}}$ **($\mathbf{min^{-1}}$)**	$\mathbf{k_{gluc,\ carboxylate}}$ **($\mathbf{min^{-1}}$)**
Human UGT 1A1	5.17×10^{-4} (3.51)	0.855×10^{-4} (11.5)
Human UGT 1A7	8.52×10^{-4} (4.62)	2.73×10^{-4} (12.4)
Human UGT 1A8	0.0526×10^{-4} (10.3)	0.0940×10^{-4} (4.11)
Human UGT 1A9	0.427×10^{-4} (4.13)	0.121×10^{-4} (3.51)
Human hepatic microsomes	68.0×10^{-4} (4.23)	15.4×10^{-4} (12.5)
Human intestinal microsomes	9.54×10^{-4} (3.51)	2.73×10^{-4} (11.1)
Rat UGT 1A1	8.23×10^{-4} (8.05)	4.49×10^{-4} (12.7)
Rat UGT 1A7	81.2×10^{-4} (10.0)	23.6×10^{-4} (20.1)
Rat UGT 1A8	27.9×10^{-4} (3.51)	5.06×10^{-4} (30.6)

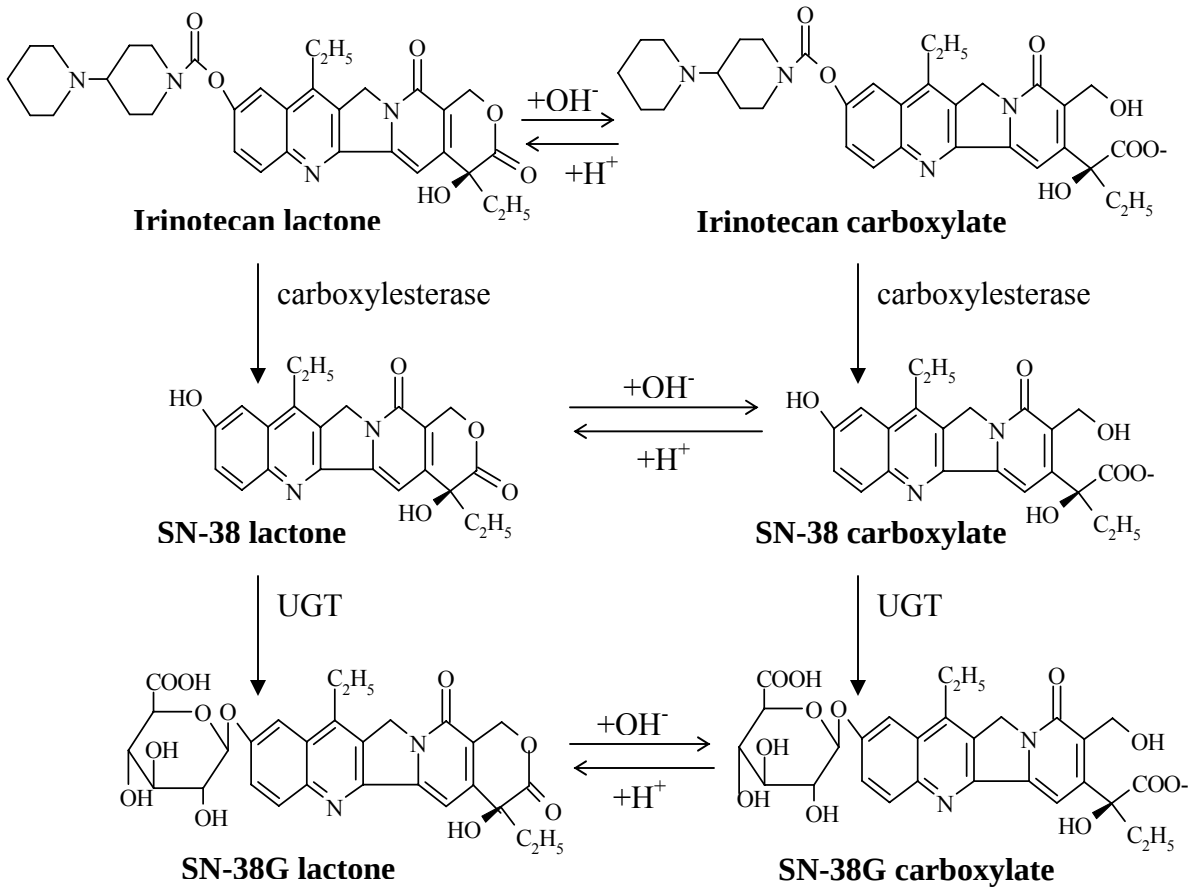

Figure 2.1 Glucuronidation of SN-38 lactone and carboxylate after esterase cleavage of irinotecan. The scheme depicts the lactone and carboxylate species of each compound. Loss of water is not shown with lactone cyclization.

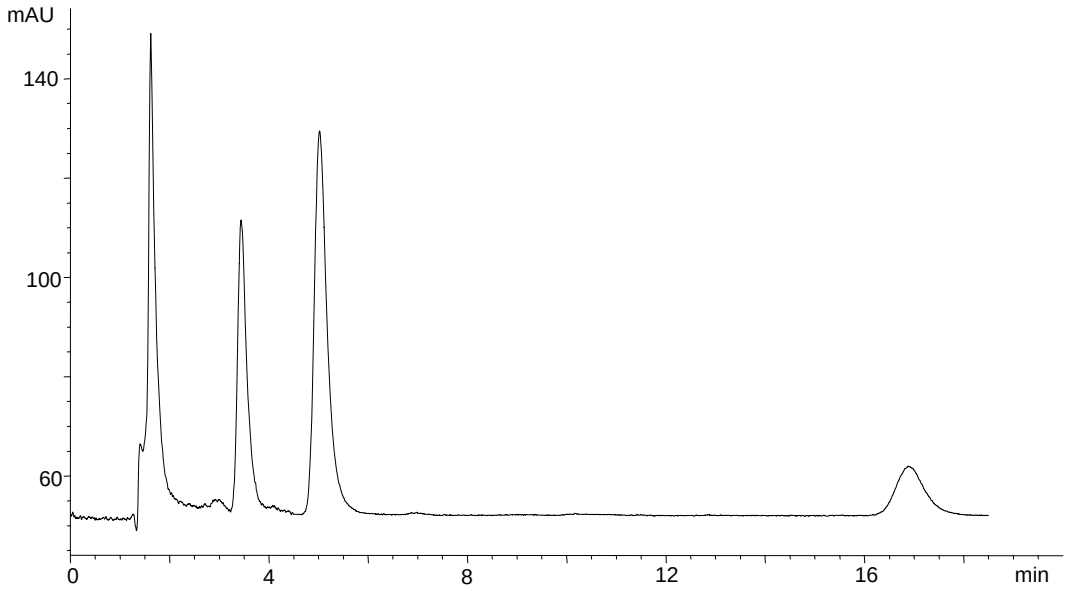

Figure 2.2 Sample chromatogram of rat recombinant 1A1 glucuronidation of SN-38 lactone over a 10 minute incubation period. SN-38G has a retention time of 3.5 minutes, irinotecan (internal standard) elutes at 5 minutes, and SN-38 is detected at 17 minutes (although poorly fluorescent at excitation 229 nm and emission 420 nm).

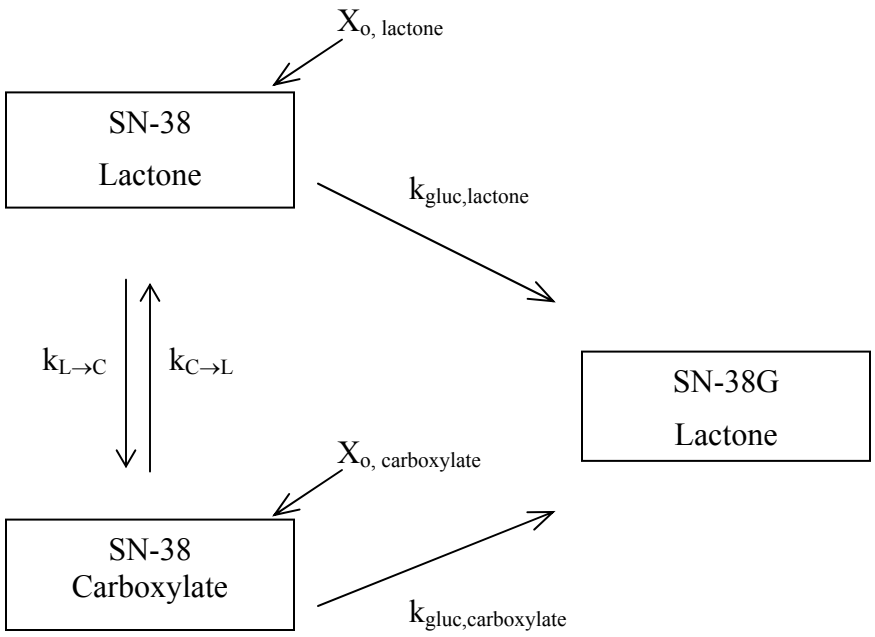

Figure 2.3 Kinetic model used to determine glucuronidation rate constants of SN-38 lactone or carboxylate ($k_{gluc,\ lactone}$ and $k_{gluc,\ carboxylate}$). This model utilizes SN-38G lactone concentrations generated from spiking either SN-38 lactone or carboxylate ($X_{o,\ lactone}$, or $X_{o,\ carboxylate}$) in two separate experiments. Any SN-38G carboxylate formed is converted to SN-38G lactone in the sample workup, as an acidified solution was used to reconstituted each sample (see HPLC Analysis under MATERIALS AND METHODS). Hence, both SN-38 lactone and carboxylate are depicted to form SN-38G lactone. The hydrolysis and lactonization rate constants, $k_{L\rightarrow C}$ or $k_{C\rightarrow L}$, respectively, were generated from studies assessing the stability of each form in microsomal or recombinant isoform matrix.

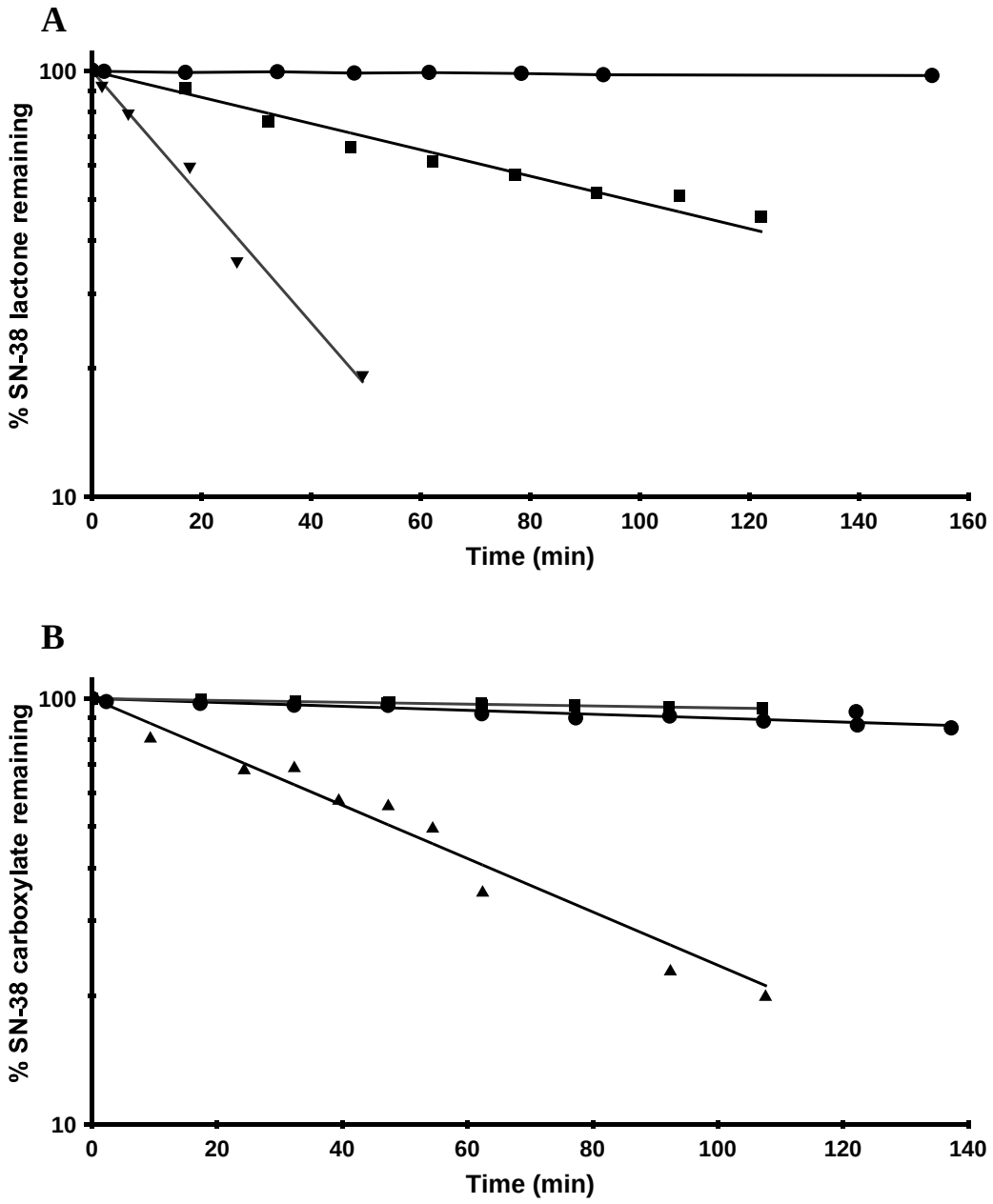

Figure 2.4 Representative plots of the stability of SN-38 lactone (A) or carboxylate (B) as determined by HPLC in 0.1 M sodium acetate, pH 4.5 (▲), 0.1 M sodium phosphate, pH 6.0 (●), 0.1 M Tris, pH 7.0 (■), or 1 x10^{-5} M sodium hydroxide, pH 9.5 (▼) over time at 37° C. Data shown are from up to three incubations per pH studied.

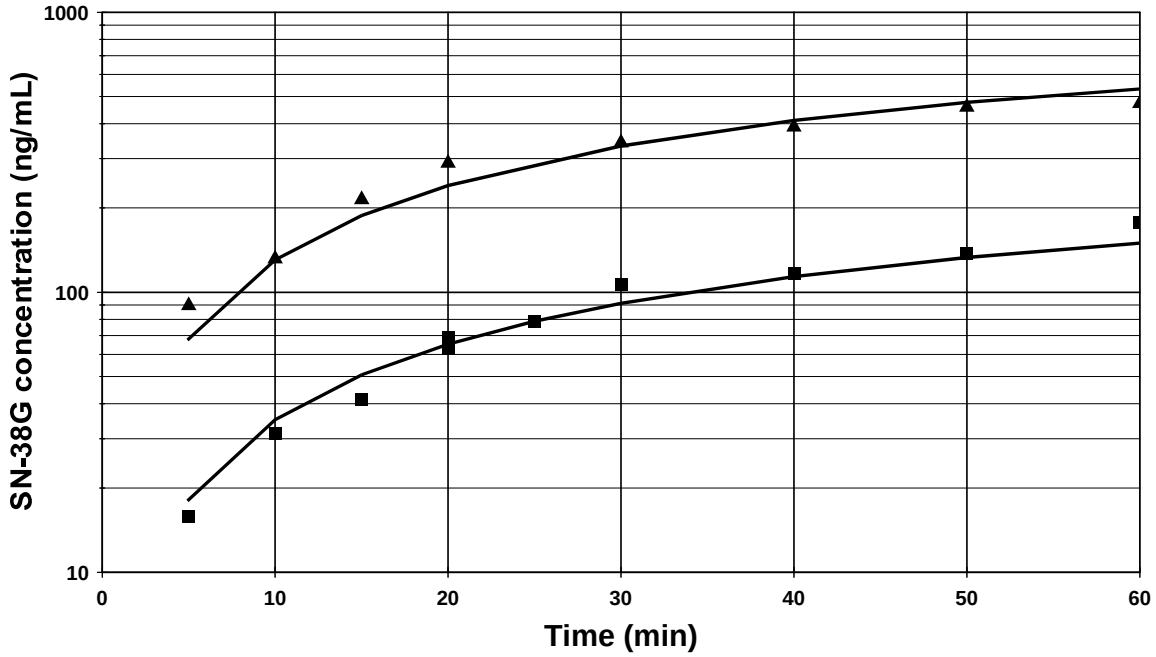

Figure 2.5 Representative plots of the formation of SN-38G in human hepatic microsomes when SN-38 carboxylate (-■-) or SN-38 lactone (-▲-) was the substrate. SN-38G concentration is represented in SN-38 equivalents in ng/mL. The points on each line shown represent data from three different incubations, as more than one incubation was needed to provide this number of data points.

CHAPTER 3

VARIABILITY OF SN-38 GLUCURONIDATION IN HUMAN INTESTINE: THE INFLUENCE OF ISOFORMS AND GENOTYPE

A. ABSTRACT

SN-38 is the active metabolite of irinotecan, a drug included in numerous regimens to treat solid tumors. Diarrhea is a primary dose-limiting toxicity which is believed to be caused by the accumulation of SN-38 in rapidly dividing enterocytes. As SN-38 can be detoxified by UDP-glucuronosyltransferases (UGTs), intestinal UGT activity may be a major factor in determining which patients experience diarrhea. In order for this hypothesis to be supported, significant variability in glucuronidation must exist across the population. Thus, a bank of tissue and biopsy samples from human duodenum, jejunum, and colon were screened for variability in SN-38 glucuronidation. These samples were also used to assess UGT1A1 and UGT1A9 activity through glucuronidation of the probe substrates, etoposide and propofol, respectively. Probe substrate experiments confirmed the presence of UGT1A1 and 1A9 protein throughout the small and large intestine. SN-38, propofol, and etoposide glucuronidation was highly variable throughout the intestine, with up to a 37-fold difference between subjects for SN-38 glucuronidation in the jejunum. Average SN-38G formation rates were similar in the duodenum and jejunum, but colon had about one-half of these rates. SN-38 conjugation exhibited significant correlation with propofol and etoposide conjugation in all regions of the intestine, supporting the role of UGT1A1 and 1A9 as primary detoxifying isoforms in the gastrointestinal tract. Interestingly, the presence of one or more UGT1A1*28 alleles did not appear to reduce SN-38 glucuronidation in the intestinal regions analyzed. Results indicate that in addition to UGT1A1, polymorphisms of UGT1A9 and its expression should be examined for potential effects on SN-38 mediated toxicity.

B. INTRODUCTION

UDP-glucuronosyltransferases (UGT) are a superfamily of enzymes that catalyze the transfer of UDP-glucuronic acid to a number of xenobiotic and endobiotic substrates (Figure 1.1). Glucuronidation typically renders a substrate pharmacologically inactive, and makes it more readily excretable from the body (Mulder, 1992; Guillemette, 2003). Although hepatic UGTs have been more extensively studied and thought to be the primary site of systemic glucuronidation, intestinal glucuronidation may contribute significantly to first-pass due to the initial contact that the intestinal mucosa has with orally administered drugs (Strassburg et al., 1999a; Tukey and Strassburg, 2000). Additionally, intestinal UGTs play a primary role in enteric and enterohepatic cycling, which may limit or prolong systemic exposure of substrates, respectively (Chen et al., 2003). UGT1A family expression is regionally regulated, which may lead to striking differences between overall glucurondiation rates between two tissues (Strassburg et al., 1999a; Gregory et al., 2004). For instance, UGT1A8 and 1A10 message is confined to the gastrointestinal tract, whereas UGT1A1 and 1A6 mRNA is more ubiquitously expressed in various organs (Tukey and Strassburg, 2001). Despite differences in localization, UGT1A isoforms often have considerable overlap in substrates, which is a consequence of highly conserved amino acid sequences (Guillemette, 2003). Because of this homology, generation of antibodies that recognize specific isoforms in the 1A family have been limited to the most divergent members, UGT1A1 and 1A6 (Ritter et al., 1999; Tukey and Strassburg, 2001). Thus, probe substrate glucuronidation is a valuable surrogate for quantitative isoform expression and activity in a given tissue.

Irinotecan is a topoisomerase I inhibitor approved as a staple of several chemotherapy regimens treating colon cancer, but is widely being used in other solid tumor malignancies.

Its pharmacological effect is achieved through SN-38, the metabolite formed by carboxylesterase-mediated hydrolysis. SN-38 is rendered inactive by glucuronidation, forming SN-38G primarily by UGT1A1, UGT1A7, and UGT1A9 (Gagne et al., 2002; Tallman et al., 2005). Irinotecan causes severe diarrhea in roughly 20% of patients, which is purportedly due to accumulation of SN-38 within the enterocyte (Araki et al., 1993; Iyer et al., 1998; Saltz et al., 2000). This has been indirectly supported by studies in both humans and rats showing that reduced intraluminal concentrations of SN-38 will diminish the incidence of diarrhea (Takasuna et al., 1996; Kehrer et al., 2001; Horikawa et al., 2002; Ikegami et al., 2002). In addition to experiencing a poor quality of life, patients that suffer from irinotecan-induced diarrhea will receive lower subsequent doses, which may adversely influence the efficacy of the oncology drug regimen (de Jong et al., 2005).

Intestinal glucuronidation of SN-38 may be an important, though seldom studied, factor in the incidence of diarrhea. Low activity may predispose patients to diarrhea, as cells would have limited defense in the detoxification of SN-38. Intestinal UGT levels are likely a function of numerous factors, among them, genetic polymorphisms or dietary components that may increase or decrease SN-38 conjugation.

In this study, the variability of SN-38 glucuronidation in human duodenum, jejeunum, and colon was assessed. Variability in intestinal SN-38 glucuronidation across patients is a requisite of the hypothesis that low UGT activity increases the likelihood of SN-38 toxicity. SN-38G formation will also be examined for correlations with the expression of UGT1A1 and UGT1A9, through glucuronidation of the novel probe, etoposide (1A1) and propofol (1A9) (Figure 3.1) (Watanabe et al., 2003; Soars et al., 2004). Finally, the effect of UGT1A1*28 genotype on SN-38 glucuronidation will be evaluated. This genotype imparts a

clinical condition known as Gilbert's syndrome, and is genotypically characterized by an extra TA repeat in the TATA box promoter of UGT1A1 (seven or more repeats versus the wild-type six repeats). Patients with Gilbert's syndrome possess reduced UGT1A1 glucuronidation, retaining approximately 30% activity of this isoform (Miners et al., 2002). The Gilbert's genotype has been found to significantly increase the incidence of irinotecan-associated neutropenia, but thus far, its correlation with the incidence of diarrhea has not been fully substantiated (Bosma et al., 1995; Innocenti et al., 2004a; Marcuello et al., 2004; Carlini et al., 2005)

C. MATERIALS AND METHODS

Materials. SN-38G methyl ester and irinotecan were gifts from Dr. Robert Kelly of Pfizer (Kalamazoo, MI), previously Pharmacia. Propofol, etoposide, and chemicals used in biopsy/tissue preparation and incubations were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant UGT isoforms were purchased from BD Biosciences (Franklin Lakes, NJ). Human liver and intestinal microsomes were obtained from Xenotech (Lenexa, KS). Monoclonal anti-villin antibody and anti-mouse immune globulin G (IgG) were purchased from Chemicon (Temecula, CA). All Western blotting apparatuses and reagents were obtained from Biorad (Hercules, CA) unless otherwise noted.

Patient Treatment and Samples. Jejunal sections were obtained from hepatic transplant recipients at the University of North Carolina at Chapel Hill. During the transplant, reconnection of the bile duct of the new liver into the intestine required the excision of jejunal tissue, which was preserved on dry ice. Duodenal and colonic biopsies were obtained from two separate studies conducted at UNC Hospitals, both of which were conducted under protocols approved from the University's Institutional Review Board. In the first, colon cancer patients underwent both endoscopy and flexible sigmoidoscopy. Enrolled patients did not have immunodeficiencies, renal, liver, or other bowel disease, as assessed by neutrophil count, creatinine, transaminases, and bilirubin levels. Additionally, patients could not be receiving medications for anticoagulation or that may affect glucuronidation, including, but not limited to, phenobarbital and valproic acid. During these procedures, six pinch biopsies were obtained from normal duodenum and colon tissue, respectively. Samples were immediately stored on dry ice and later transferred to -80° C. Patients later received irinotecan (125 mg/m^2), followed by leucovorin and fluorouracil in most cases. Irinotecan

was subsequently administered in six week cycles, consisting of once weekly dosing for four weeks. Patients were asked on a weekly basis to report diarrheal episodes. In the second study, patients undergoing routine or diagnostic endoscopy, colonoscopy, or flexible sigmoidoscopy at UNC Hospitals were approached to consent to biopsy excision during their procedure. Upon consent, six biopsies were obtained and stored as described above. Exclusion criteria included overt renal, liver, or bowel disease, age below 18 or older than 70, obesity, anticoagulants or other contraindications to biopsy, and glucuronidation-modulating medications. Biopsies and tissues from all studies were stored at -80°C until use.

Tissue Preparation. Biopsies (n = 4 per patient) and tissue samples were homogenized on ice in 20% glycerol/50 mM Tris (600 μL), pH 7.4, containing EDTA (2 mM), phenylmethylsulfonylfluoride (1 mM), and leupeptin (100 μM). Protein concentration was determined by the Bradford assay and based upon standard curves of bovine serum albumin. Aliquots of homogenate (100 μL) were stored at -80 until use. Separate aliquots were used for each glucuronidation assay such that freeze-thaw effects were avoided. One biopsy or small piece of tissue was devoted to the isolation of genomic DNA using a DNeasy Tissue kit (Qiagen, Valencia, CA).

SN-38 Glucuronidation and Analysis. SN-38 was synthesized by irinotecan hydrolysis as previously described (Chapter 2). Reactions to determine the maximal glucuronidation rate (Vmax) and the substrate affinity (Km) for SN-38 were conducted in rat hepatic microsomes and contained the following: Brij 35 (0.5 mg/mg protein), D-saccharic acid 1,4 lactone (10 mM), magnesium chloride (10 mM), SN-38 carboxylate (5-400 μM), and microsomal protein (0.5 mg/mL) in a final volume of 200 μL of 0.1 M Tris, pH 7.0. Reactions were initiated with UDP-glucuronic acid (UDPGA) (2 mM) and quenched after 30 minutes at 37° C with

four volumes of acetonitrile containing internal standard (25 ng irinotecan). Incubations for intestinal biopsies and microsomes were similar, except for differing protein concentrations (biopsy, 0.2 mg/mL or jejunal tissue, 0.5 mg/mL), a fixed SN-38 concentration (300 μM), and an incubation time of one hour. After centrifugation at 15,000g for 10 minutes, the supernatant of all samples was evaporated under nitrogen and reconstituted in 30% methanol/ 100 mM ammonium acetate (200 μL). Aliquots of 15 μL were injected onto the high performance liquid chromatography (HPLC) column for analysis of SN-38G formed.

The SN-38G standards were prepared from SN-38G methyl ester as previously described (Chapter 2). Standards were spiked into incubation matrix from 0.75 ng/mL to 200 ng/mL. After workup, 6-25 μL of standards were injected onto the HPLC column, providing a range of 19-1200 pg of SN-38G injected on-column. The HPLC system used to separate and detect SN-38G was similar to that used previously (Chapter 2), with an SIL10A autosampler used for sample injection (Shimadzu, Tokyo, Japan). Standard curves were linear throughout the range and were reproducible (less than 15% coefficient of variance in peak area ratio at high and low concentrations). Interday variability was assessed, as samples were prepared and analyzed on two separate occasions. SN-38 glucuronidation rates in human liver microsome controls were within 15% of each other.

Propofol Glucuronidation and Analysis. The *in vitro* reaction to generate propofol glucuronide (propofol-G) in human intestinal biopsies and tissues contained the following: Brij 35 (0.5 mg/mg protein), D-saccharic acid 1,4-lactone (10 mM), magnesium chloride (10 mM), propofol (1 mM), and biopsy/tissue (0.2 mg/mL) in a final volume of 250 μL of 0.1 M Tris, pH 7.0. Reactions were initiated with UDPGA (2 mM) and quenched after 40 minutes at 37°C with two volumes of acetonitrile containing internal standard (2 μg ibuprofen).

Samples were centrifuged at 15,000g for 10 minutes and the acetonitrile supernatant was evaporated under nitrogen. Samples were reconstituted with 20% acetonitrile/ 10 mM ammonium acetate (100 μL). Prior to this experiment, incubations were performed in human intestinal microsomes (0.2 mg/mL) with propofol concentrations ranging from 0.025 mM-5 mM in order to obtain Michaelis-Menten parameters.

Propofol-G standards were synthesized via a large-scale *in vitro* incubation of propofol with human liver microsomes and prepared as described above, which was then separated using preparative HPLC. The fractions containing propofol-G were verified by liquid chromatography with mass spectrometry detection (LC-MS), pooled, lyophilized, and reconstituted with 50% acetonitrile. As the glucuronide of propofol is not liable to β-glucuronidase cleavage, strong acid was used to generate propofol from an aliquot of propofol-G stock. The liberated propofol was then quantified, which yielded the concentration of propofol-G standard stock solution in propofol equivalents.

Propofol and propofol-G were quantified using LC-MS using ibuprofen as an internal standard. The liquid chromatography system used consisted of an HP1050 quaternary pump, mobile phase degasser and autosampler (Agilent, Palo Alto, CA) and a Zorbax RX-C8 column (150 x 2.1 mm, 5 μM) (Agilent). Compounds were eluted in 15 minute runs with ammonium acetate (10 mM, A) and acetonitrile in a gradient elution (0.3 mL/min): at 0 minutes, 90% A; at 9 minutes, 10% A; at 10 minutes, 10% A; at 11 minutes, 90% A. A single quadrupole mass spectrometer (Sciex API 100; MDS Sciex, Concord, CA) coupled with an electrospray interface in negative ion mode detected the compounds. Ion source conditions include a voltage of -3300V, temperature of 400° C, and a dwell time of 300 ms. Selected ion monitoring was used to detect propofol-G (m/z 353), ibuprofen (m/z 205), and

propofol (m/z 177) at 3.2, 6.4, and 7.8 minutes, respectively. Calibration curves of propofol-G were linear from 0.05 μg/mL to 4 μg/mL, with an injection volume of 15 μL for all samples and standards. Human liver microsome control samples (0.1 mg/mL) conducted on seperate analysis days had propofol glucuronidation rates within 25% of each other. A representative chromatogram is given in Figure 3.2.

Etoposide Glucuronidation and Analysis. In order to assess the selectivity of etoposide for UGT1A1, incubations (2 hours) of etoposide in all commercially available UGT isoforms were performed as follows: alamethacin (50 μg/mg protein), D-saccharic acid 1,4-lactone (10 mM), magnesium chloride (10 mM), etoposide (400 μM), recombinant isoform (0.5 mg/mL), and UDPGA (2 mM) in a final volume of 250 μL of 0.1M Tris, pH 7.0. Incubations conducted with human microsomes (0.5 mg/mL) for determination of etoposide Km and Vmax were similar to above, but utilized an etoposide concentration range of 5 μM to 2,000 μM in a final volume of 200 μL over 30 minutes. Due to limited amounts of tissue, etoposide glucuronidation was not conducted in jejunal tissue or several distal and proximal intestinal biopsies. In the final set of etoposide incubations, intestinal biopsies (0.2 mg/mL) were incubated with conditions outlined in the Km/Vmax experiment, except a fixed etoposide concentration of 1 mM was used. After each incubation, three volumes of acetonitrile containing internal standard (azidothymidine (AZT), 6.25 μg) was used to quench the reaction. The supernatant was then blown to dryness under nitrogen and reconstituted with 125 μL of 0.1% formic acid/ acetonitrile (80% : 20%).

Etoposide glucuronide (etoposide-G) standards were also synthesized in an *in vitro* reaction with human liver microsomes. After the final reconstitution of a 20 mL reaction, preparative HPLC with UV detection at 254 nm was used to separate analytes. Fractions

containing etoposide-G (as assessed by mass spectrometry) were pooled, lyophilized, and reconsitituted in 50% acetonitrile. A small aliquot of this solution was treated with β-glucuronidase and the liberated etoposide was quantitated against a standard curve. This allowed the concentration of etoposide-G (in etoposide equivalents) to be calculated in the standard stock solution.

The chromatography system, column, and mass spectrometer used for quantitation of etoposide-G was described in the propofol-G section. The turbo ionspray source in negative ion mode was also set with the same parameters as the propofol-G assay: Etoposide, etoposide-G, and AZT were eluted via a gradient over 18 minutes using 0.1% formic acid (A) and acetonitrile (B) (0.3 mL/min) following the following time course: at 0 minutes, 90% A; from 10-12 minutes, 50% A; and at 14-18 minutes, 90% A. Using selective ion monitoring, AZT (m/z 266), etoposide-G (m/z 763.2), and etoposide (m/z 587.2) were detected at 6.1, 8, and 10.6 minutes, respectively. Etoposide-G standard curves were linear and reproducible after 20 μL injections of concentrations ranging from 2.5 to 1000 ng/mL. All samples within the three described experimental procedures were prepared and analyzed on the same day, negating the need for interday variability assessment. A representative chromatogram is given in Figure 3.2.

Villin Western Blot Analysis. Intestinal biopies and tissues may contain different levels of enterocytes per weighed amount of tissue for numerous reasons, including the biopsy technique of the physician. As such, simply normalizing glucuronidation rates to the amount of total protein in the preparation is erroneous, as only enterocytes contain UGTs (Strassburg et al., 2000). Thus, all glucuronidation rates were normalized for the enterocyte content of the respective sample, as measured by villin expression (Lown et al., 1997).

Biopsy or tissue proteins (40 μg) that underwent one freeze-thaw cycle were seperated by electrophoresis on 4-15% gradient Tris gels (Ready Gel, Bio-Rad). Proteins were then transferred for one hour at 110 volts to nitrocellulose membranes. After the transfer, membranes were blocked in 5% milk/0.1% Tween in Tris-Buffered Saline (TBS-T) for one hour to prevent non-specific antigen-antibody binding. Blots were probed with anti-villin antibody (in 0.1% TBS-T/0.5% milk) overnight, followed by the secondary antibody, anti-mouse IgG in (0.1% TBS-T/0.5% milk), for one hour. After the blocking, primary, and secondary antibody step, the blots were washed three times for five minutes each with 0.1% TBS-T. Following the final wash, chemiluminescent reagent (SuperSignal, Pierce, Rockford, IL) was pipetted over the blot. Protein bands were then imaged via a Versadoc Imaging System (Biorad). A molecular weight marker was added to each gel to identify the villin protein (95 kDa). Densities of the protein bands were determined using Quantity One software (Biorad). Protein content and image time were both validated for linearity. As electrophoresis was conducted in multiple gels, an aliquot of concentrated intestinal sample was added to each blot as an internal standard. Densities were reported as the ratio of the densities of the protein of interest relative to the internal standard of the respetive blot. To ensure the accuracy of this method of quantification, seven samples were run on two seperate days. The interday variability of the normalized band densities of proteins was no higher than 12%.

UGT1A1 Genotyping. Upon isolation of DNA from the biopsies and tissue, DNA concentration was spectrophotometrically determined. Genotypes for the UGT 1A1 $[TA]_n$ promoter region (GenBank Accession Number AF297093) were ascertained by direct sequencing. Forward (5'-TCC CTG CTA CCT TTG TGG AC-3') and reverse (5'-AGA GGT

TCG CCC TCT CCT AC-3') primers were designed using Primer3 (primer3_www.cgi v 0.2). DNA (30 ng) was amplified in a 50 μl reaction volume containing 800 nM of each primer, 200 μM dNTPs (Amersham Biosciences Inc., Piscataway, NJ), 1.5 mM $MgCL_2$, 1x PCR Buffer (Invitrogen, Carlsbad, CA), and 0.25U Platinum *Taq* (Invitrogen). Polymerase chain reaction (PCR) cycling conditions including an initial denaturation at 95°C for 5 min, 35 cycles of 94°C for 30 s, 54°C for 40 s, 72°C for 40 sec, and a final extension at 72°C for 10 min. The PCR product was resolved by gel electrophoresis on a 3% agarose gel and the 121 bp fragment was excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The sequencing reaction, containing 2 μL of purified product and 10 pmoles of forward primer, was done using the ABI PRISM BigDye Version 1.1 Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Foster City, CA). Samples were sequenced at the UNC Genome Analysis Facility on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Data Analysis. Vmax and Km values for propofol and etoposide were determined using nonlinear regression software (Winnonlin version 5.0, Pharsight, Mountain View, CA). Due to the small amount of available sample, data from incubations with intestinal biopsies and tissues are presented as the average of duplicate incubations from one experiment. All glucuronidation rates are normalized for the villin content in the biopsy or tissue. Comparisons between substrate glucuronidation in duodenum, jejunum, and colon or between UGT1A1 genotype groups were made with the Kruskal-Wallis test, followed by the Dunn's post-hoc test. In the case of etoposide, the comparison of duodenal and colonic glucuronidation was made using the Mann Whitney Rank sum test. Correlations between UGT1A1 and UGT1A9 probe substrates and SN-38 were compared using Spearman rank

order test. All statistical tests were performed using Sigma Stat software (version 2.0, Systat, Point Richmond, CA) and p values ≤ 0.05 were considered significant.

D. RESULTS

Propofol had previously been identified as a UGT1A9 probe by Soars et al. (2004), and is widely used as such (Girard et al., 2004; Soars et al., 2004; Court, 2005). While UGT1A8 does retain approximately six percent of the activity of UGT1A9 based on commercially available recombinant isoforms, its use a probe is relatively free from the contribution of other isoforms (Soars et al., 2004). Watanabe et al. (2003) identified etoposide as an exclusive substrate for UGT1A1, although not all UGT isoforms were tested (Watanabe et al., 2003). Results from experiments in our lab with all commercially available isoforms identify UGT1A1 as the main enzyme responsible for glucuronidation, with UGT1A8 retaining only five percent of the activity that UGT1A1 possesses (Figure 3.3). Because of this data, etoposide was used to quantitatively evaluate relative protein levels of UGT1A1 in the intestinal tissue samples.

Michaelis-Menten kinetic parameters were determined for SN-38, propofol, and etoposide in various microsomal matrices (Figure 3.4-Figure 3.6). A concentration two to four times greater than the Km estimate would be used to assess interpatient differences in the intestinal glucuronidation for each aglycone. As the biopsies and tissues provided less than 1 mg of homogenate protein, a single measure of glucuronidation for each substrate was attained in these samples, rather than a full kinetic profile. Incubations in rat liver microsomes showed that SN-38 glucuronidation reached a maximal velocity at approximately 300 μM SN-38 (Figure 3.4). At 500 μM, SN-38 visibly precipitated out of the reaction. Since 300 μM SN-38 was soluble in the incubation matrix, this concentration was used in further incubations. Likewise, high concentrations of propofol were also not soluble, so 1 mM was used in the reaction (Figure 3.5). Based on the Km data for both human

intestine and human liver, 1 mM etoposide was used in additional *in vitro* reactions (Figure 3.6, Table 3.1).

Overall, 53 normal intestinal samples were collected from patients or subjects (n = 18 duodenal biopsies, n = 16 jejeunal tissue pieces, n = 19 colon biopsies). Eight paired duodenum and colon biopsy sets were obtained from colon cancer patients prior to receiving irinotecan. Demographic information available on these patients is given in Table 3.2. Although different comorbidities segregated amongst the intestinal area excised (i.e., more liver transplant patients donated jejunum), it appears that coexisting conditions did not affect glucuronidation. Likewise, age, race, and sex were not mitigating factors in glucuronidation rates in this study. Roughly 0.6 mg of total protein was derived upon the homogenization of the biopsies and jejunal segments.

The average duodenal, jejunal, and colonic glucuronidation rates for SN-38, etoposide, and propofol are given in Table 3.3. Considerable variability between subjects was evident in all intestinal regions for both SN-38 and the probe substrates. For SN-38, there was an 8.1, 37, and 36-fold range of SN-38G formation for the duodenum, jejunum, and colon, respectively. Despite the high intersubject variability in the glucuronidation of the substrates, SN-38 and etoposide glucuronidation was significantly lower in the colon. To add support to this finding, the proximal intestine consistently displayed higher catalytic rates for SN-38 and etoposide in paired duodenum and colon samples (Figure 3.7). In contrast, propofol glucuronidation was highest in the distal intestine, although the broad range of propofol-G formation rates prohibited any statistical difference (Table 3.3). In the paired samples, propofol activity was higher in the colon versus the duodenum, although this was not a consistent outcome from the eight matched pairs of tissue biopsies (Figure 3.7). In

order to gain insight into isoform-specific glucuronidation of SN-38, etoposide and propofol glucuronidation was correlated with that of SN-38 (Figures 3.8 and 3.9). In all areas of the intestine, SN-38G formation was highly associated with that of UGT1A9 and UGT1A1 activity as determined by propofol and etoposide glucuronidation, respectively.

Because UGT1A1*28 genotype has been associated with low hepatic SN-38 glucuronidation, subjects in this study were genotyped in an attempt to explain the variability in intestinal SN-38 glucuronidation. Roughly 44% of the population sampled here was homozygous for wild-type UGT1A1, 33% was heterozygous, and 20% were homozygous for UGT1A1*28, characterized by having two alleles with 7 or 8 TA repeats in the TATA box promoter of the gene (Miners et al., 2002) (Table 3.4). Interestingly, the presence of one or more UGT1A1*28 alleles does not appear to appreciably influence intestinal SN-38 glucuronidation rates, though the sample size is not large (Table 3.5). Two out of the eight patients who received irinotecan experienced Grade 3 diarrhea, characterized by incontinence or an increase of at least seven stools per day. Neither patient had Gilbert's syndrome and neither had the lowest duodenal or colonic SN-38 glucuronidation rate of the patients who received irinotecan (Table 3.6).

E. DISCUSSION

The capacity of the intestine to glucuronidate substrates no doubt arises from the fact that it physically contacts potentially toxic, orally administered compounds or those excreted through the bile, necessitating a detoxification pathway (Tukey and Strassburg, 2000). This has been demonstrated in the proximal gastrointestinal tract with tobacco containing carcinogens and UGT1A7. Several polymorphisms in the UGT1A7 isoform impart low glucuronidation rates of benzo-α-pyrene and derivatives and also correlate with a patient's predisposition to develop to orolaryngeal cancers (Zheng et al., 2001; Strassburg et al., 2002). UGT1A7 transcripts are localized exclusively to upper gastrointestinal tissues, suggesting a potential role for the importance of UGT isoforms expressed in the gastrointestinal tract in mediating toxicity (Tukey and Strassburg, 2000; Zheng et al., 2002). A similar phenomenon may exist with SN-38 and intestinal UGTs. As the intestine is the site of SN-38 toxicity and contains local detoxification mechanisms in the expression of UGTs, polymorphisms or low UGT activity may predispose patients to experiencing SN-38 related diarrhea. In contrast to the benzo-α-pyrene and UGT1A7 example, the specific role of the intestine in mediating diarrhea is less clear, as UGT1A1 and UGT1A9, the enzymes purportedly responsible for intestinal SN-38 catalysis, are also hepatically expressed (Strassburg et al., 1997; Gagne et al., 2002; Tallman et al., 2005). Although SN-38 is also cleared by UGT1A7 in *in vitro* reactions, the location of this isoform in the proximal gastrointestinal tract likely excludes a role for it in the intestinal glucuronidation of SN-38 (Tukey and Strassburg, 2000; Gagne et al., 2002; Tallman et al., 2005).

The variability of SN-38 glucuronidation observed in the intestinal samples in this study is similar to the interpatient variability in glucuronidation reported previously for SN-

38 and other UGT substrates. When SN-38 glucuronidation was assessed in normal colon biopsies from eight patients, an approximate seven-fold difference in glucuronidation was observed between patients, excluding data from two patients with barely quantifiable SN-38G levels (Cummings et al., 2003). The glucuronidation of estrone in colonic microsomes made from four female patients ranged from roughly 100-2500 pmol/min/mg protein (Czernik et al., 2000). Strassburg et al. (1999) report a 2-5 fold difference in glucuronidation rates of various flavones, bile acids, steroids, and phenolic compounds in colon samples from five subjects (Strassburg et al., 1999a). Although SN-38 glucuronidation rates did vary to a greater degree in the present study (up to 37-fold in jejunal samples), the number of patients sampled was much greater compared to these earlier studies, allowing increased likelihood that intestinal samples from subjects with extremely low or high SN-38 conjugation would be analyzed. The formation of SN-38G was also highly variable in the colon, albeit slower than the proximal intestine. The large intestine did, however, retain significant UGT activity to SN-38. Glucuronidation in this portion of the intestine may be an especially important determinant of diarrhea, as this is the specific site of pathophysiological changes induced by SN-38. Additionally, enterocytes in this region may come in contact with more SN-38 relative to other intestinal segments, as β-glucuronidase-facilitated cleavage of SN-38G to SN-38 is highest in the distal intestine (Ilett et al., 1990).

The use of probe substrates as a measure of UGT activity has several advantages over other methods to assess UGT1A isoform expression. The first is that probe substrates allow a quantitative measure of relative isoform levels, whereas the band densities generated from western blots are only semi-quantitative. Unfortunately, for accurate glucuronidation rates in these intestinal samples, quantitative measures of UGT1A9 and UGT1A1 activity were

normalized for villin expression as determined by Western blot. However, a second advantage to utilizing probe substrates is that there is a lack of antibodies that specifically recognize most UGT1A isoforms, including UGT1A9. We and others have prepared a UGT1A9 antibody, however, cross-reactivity with UGT1A7, 1A8, and 1A10 exists, as these isoforms are highly homologous (Girard et al., 2004; Miles et al., 2005). Both flavopiridol and propofol have proven useful UGT1A9 probe substrates, however the latter compound is more widely used and is commercially available (Ramirez et al., 2002; Soars et al., 2004; Court, 2005). Although suitable UGT1A1 antibodies have been produced and could have been used in this study, the amount of available intestinal tissue was limiting. Various intestinal segments have been previously probed for UGT1A1 protein via western blots in several studies, albeit with small numbers of patients (Paine and Fisher, 2000; Strassburg et al., 2000). There is only one known report of human intestinal UGT1A1 expression as assessed by probe substrate activity. Fisher et al. (2000) assessed estradiol-3 glucuronide formation in three human intestinal microsome preparations (Fisher et al., 2000). However, UGT1A3, 1A8, and 1A10 have been found to have significant or higher catalytic activity relative to UGT1A1 for this compound, limiting use of estradiol as a UGT1A1 probe (Soars et al., 2004). Besides estradiol, bilirubin has been extensively used as a UGT1A1 probe, although this compound is highly photosensitive. Although Watanabe et al. (2003) identified etoposide as a UGT1A1 probe, not all isoforms were tested for specificity and, until the current investigation, has not been utilized in the reported literature (Watanabe et al., 2003).

The UGT1A phenotyping with etoposide and propofol provides some interesting insight into the intestinal expression of these two isoforms. In studies detecting RNA, UGT1A9 and 1A8 were not detected in any small intestinal segment (Strassburg et al., 2000).

Interestingly, although the colon overall had a higher rate of propofol glucuronidation than the other two segments on average, the duodenum and colon retained significant propofol catalytic activity. With respect to UGT1A1, both transcript and protein analysis confirmed the presence of UGT1A1 throughout the intestine (McDonnell et al., 1996; Strassburg et al., 1998; Strassburg et al., 2000). The current study confirms anecdotal evidence that UGT1A1 activity declines in the distal regions of the intestine (Peters et al., 1989; McDonnell et al., 1996). One disadvantage to using probe substrate glucuronidation is that glucuronidation rates for specific probes cannot be used to compare the expression levels between UGT isoforms. Along with intrinsic clearance rates for a given substrate and isoform, this information would allow investigators to determine which isoforms contribute most significantly to glucuronidation within a given tissue. From these results, both UGT1A1 and 1A9 seem to contribute considerably to the *in vitro* formation of SN-38G throughout the intestine as assessed through correlation analyses, and it is only speculative to support the *in vivo* involvement of one isoform over another.

Genetic polymorphisms and environmental exposures are the two factors likely contributing to the variability in glucuronidation observed with the aglycones studied. The latter may greatly influence rates in the intestine relative to other organs, due to its exposed location and direct physical contact with ingested substances. In fact, normalized UGT1A transcript levels extracted from the liver showed little variability, whereas those derived across the small intestine exhibited marked differences in abundance (Strassburg et al., 2000). Should this finding be translated into UGT1A protein expression, it may be expected that the variability in the intestinal glucuronidation might be higher than from the liver.

To date, only the UGT1A1*28 polymorphism in these two genes has been found to affect both glucuronidation and toxicity of SN-38. In numerous studies, homozygosity for this isoform predisposes patients to neutropenia (de Jong et al., 2005). Additionally, SN-38 glucuronidation in liver microsomes from patients hetero- and homozygous for UGT1A1*28 were lower than patients with two wild-type alleles (Iyer et al., 1999). As reported by Carlini et al. (2005), the association of the UGT1A1*28 allele and diarrhea is not always substantiated, which may suggest that more than one isoform may be responsible for SN-38 catalysis and detoxification (Carlini et al., 2005). In the current study, there was no clear association between the Gilbert's genotype and glucuronidation. However, there were very few samples analyzed within each genotype group and intestinal region, so the results should be cautiously interpreted. Hepatic microsomal experiments conducted with the probe substrates bilirubin and estradiol revealed that glucuronidation rates varied tremendously between Gilbert's patients, with coefficients of variance of 113 and 60%, respectively (Iyer et al., 1999; Fisher et al., 2000). This illustrates that even within a genotype group, tremendous variability can occur and with small numbers of patients, differences based upon genotype may be difficult to detect. The results of the present study indicate also that relative UGT1A1 protein expression as measured by the probe substrate, etoposide, is clearly a better predictor of SN-38 glucuronidation than genotype.

In conclusion, the small and large intestine exhibit large interpatient variability in the glucuronidation of SN-38. This variability is highly linked to both UGT1A1 and UGT1A9 protein expression, as assessed through probe substrate glucuronidation, and not UGT1A1*28 genotype, as preliminary results indicate. Although the influence of UGT1A1 genotype on SN-38 glucuronidation and toxicity has been extensively studied, the effects of

UGT1A9 polymorphisms have received scant attention. Poor and extensive metabolizer phenotypes have been established for several genotypes *in vitro*, using SN-38 and other 1A9 substrates, one of which has been shown affect tumor response and toxicity (Villeneuve et al., 2003; Girard et al., 2004; Yamanaka et al., 2004; Carlini et al., 2005). Coupled with the results of this study, the previous reports provide support that UGT1A9 may play a major *in vivo* role in the conjugation of SN-38. Although not directly addressed in this study, the results suggest that the substantial differences in intersubject intestinal SN-38 glucuronidation may be a major factor in the development of SN-38-mediated diarrhea.

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Table 3.1 Vmax and Km estimates for etoposide glucuronidation in pooled human intestinal and human liver microsomes. Values were obtained through nonlinear regression software, and the associated coefficients of variance of the parameters are given in parenthesis.

	Vmax (pmol/min/mg protein)	**Km** (μM)
Intestine	34.0 (3.0%)	435 (8.6%)
Liver	82.2 (5.1%)	157 (19%)

Table 3.2 Demographic information available on enrolled patients. This list is not exhaustive, as information on all patients was not available and all demographic factors are not listed.

	Duodenum	**Jejunum**	**Colon**
Age			
20-30	2		1
30-40	2	1	1
40-50	1	6	2
50-60	7	2	11
60-70	4	3	3
Sex			
Male	7	9	10
Female	11	5	9
Race			
Lumbee Indian		1	
Black	4	0	6
White	13	12	13
Coexisting Conditions			
Diverticulitis	1		2
Colon cancer	8		8
Liver transplant	3	16	
Hepatitis B		2	
Hepatitis C	1	4	
Medications			
Mycophenolate mofetil	1	2	
Tacrolimus	3	3	
Interferon		2	
Cyclosporine		1	

Table 3.3 Average *in vitro* glucuronidation rates of SN-38, propofol, and etoposide in human duodenum, jejunum, and colon. Substrate concentrations employed in assays were 300 μM for SN-38, and 1 mM for both propofol and etoposide. Each sample contributing to the mean ± SD is an average of duplicate incubations. All rates are given in pmol/min/mg protein normalized to villin density. Statistical tests on raw data were conducted using Kruskal-Wallis test, followed by the Dunn's post-hoc test, except with etoposide rates from the colon and duodenum, which was conducted with the Mann Whitney Rank sum test.

	Rate of Glucuronidation (pmol/min/mg protein)		
	Duodenum (n = 18)	**Jejunum** (n = 16)	**Colon** (n = 19)
SN-38	12.4 ± 7.95	13.0 ± 7.99	6.60 ± 5.90*
Propofol	426 ± 371	693 ± 361	1150 ± 1490
Etoposide	12.3 ± 5.24^{a}	N/A	2.41 ± 0.85$^{***, b}$

* $p < 0.05$, *** $p < 0.001$
^{a} n = 15, ^{b} n = 12

Table 3.4 UGT1A1 genotype distribution in study subjects. Each box contains the number of subjects with the genotype and are also segregated based upon intestinal segments. Biopsies are from 45 subjects (53 samples total), with 18 providing duodenal biopsies, 16 providing jejeunal tissue, and 19 providing colon samples. Genotypes are as given as the number of TA repeats per allele: 6/6 refers to a patient homozygous for wild-type UGT1A1 alleles, 6/7 is heterozygous, and 7/7 is homozygous for Gilbert's syndrome (UGT1A1*28). TA repeats lower then 6 impart normal UGT1A1 function, whereas TA repeats higher than 7 impart poor function (Miners et al., 2002).

	Genotype				
	5/7	**6/6**	**6/7**	**7/7**	**7/8**
Duodenum	1	9	6	2	0
Jejunum	0	5	6	4	1
Colon	1	11	5	2	0
Total number of subjects with genotype	**1**	**20**	**15**	**8**	**1**

Table 3.5 SN-38 glucuronidation rates (pmol/min/mg protein) as a function of genotype. Each sample contributing to the mean ± SD is an average of duplicate incubations. Rates are normalized for villin content of the sample.

	Duodenum		**Jejunum**		**Colon**	
	Rate (pmol/min/mg)	n	Rate (pmol/min/mg)	n	Rate (pmol/min/mg)	n
6/6	8.86 ± 2.9	9	10.9 ± 5.7	5	6.72 ± 6.9	11
6/7	12.5 ± 6.2	6	12.3 ± 9.1	6	6.30 ± 4.2	5
7/7	15.4 ± 0.28	2	15.9 ± 9.3	5*	3.30 ± 3.3	2

*Glucuronidation rate from subject with 7/8 genotype was included

Table 3.6 Intestinal glucuronidation, genotype, and toxicity profiles of eight patients receiving irinotecan 125 mg/m^2 plus leucovorin and 5-flurouracil. Glucuronidation rates are the average of two incubations and are normalized for biopsy villin content. To assess the incidence of diarrhea, patients were phone weekly and medical charts were examined for dosage reduction or notes on toxicity.

				SN-38 glucuronidation (pmol/min/mg protein)			
Patient	**Age**	**Sex**	**Race**	**Duodenum**	**Colon**	**Grade 3/4 diarrhea**	**Genotype**
1	47	Female	Black	20.6	8.22	No	6/7
2	63	Female	White	17.7	11.8	**Yes**	6/7
3	24	Male	White	10.3	3.6	No	6/6
4	55	Male	Black	38.6	13.3	No	5/7
5	59	Female	White	6.04	4.33	**Yes**	6/6
6	59	Female	White	12.6	2.3	No	6/6
7	68	Male	Black	11.1	3.58	No	6/6
8	38	Female	Black	4.74	3.53	Unknown	6/6

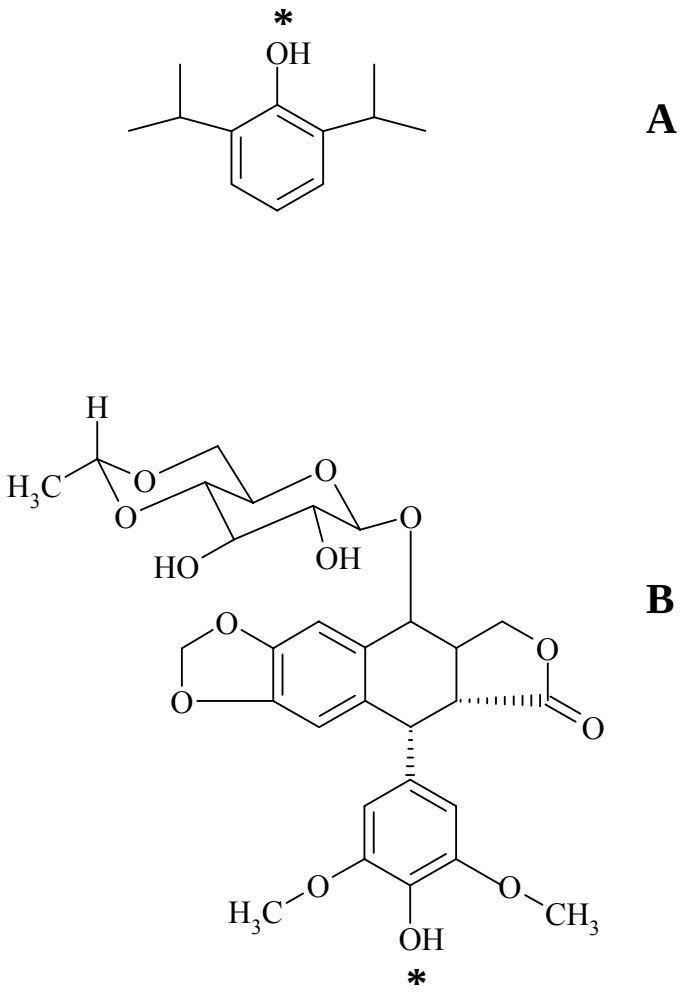

Figure 3.1 Chemical structures of propofol (A) and etoposide (B). Site of propofol glucuronidation and proported site of etoposide glucuronidation is indicated with *.

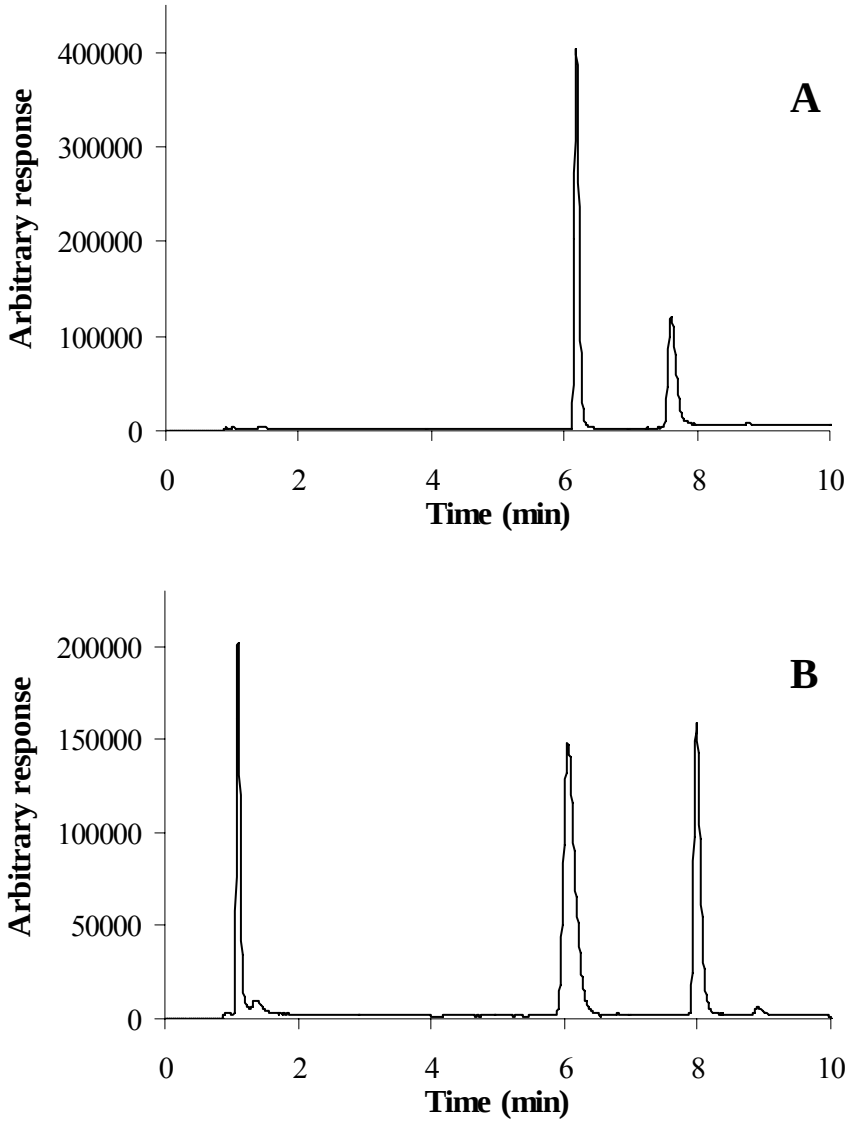

Figure 3.2 LC-MS chromatograms for propofol-G (A) and etoposide-G (B). Elution times for **A** are 6.2 minutes for propofol-G (0.2 μg/mL) and 7.6 minutes for ibuprofen (internal standard) and for **B** are 6.1 minutes for AZT (internal standard) and 8 minutes etoposide-G (0.25 μg/mL). Compounds were detected with electrospray interface using negative ion mode. Selected ion monitoring was utilized in both assays. Assay conditions are provided in METHODS.

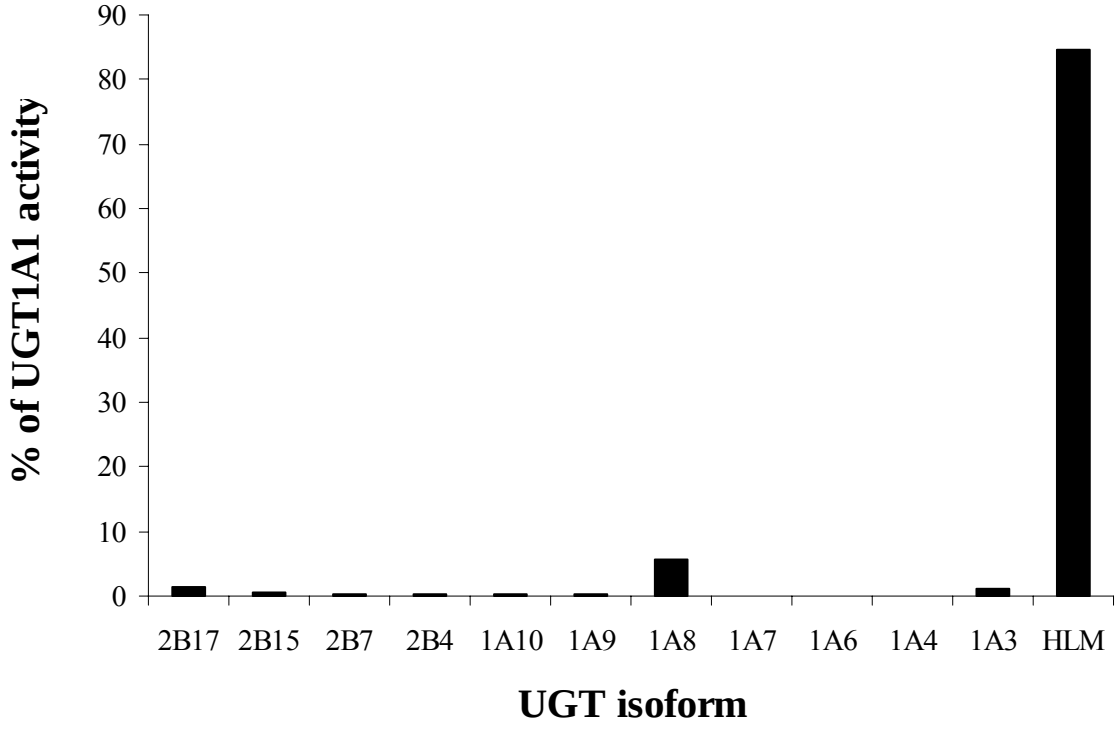

Figure 3.3 Etoposide glucuronidation by recombinant isoforms. Reactions contained 0.5 mg/mL of recombinant isoform or human liver microsome and 400 μM etoposide, and proceeded for 2 hours at 37°C. Each bar is the average peak area of etoposide-G yielded from duplicate incubations relative to the average of that for UGT1A1, multiplied by 100%.

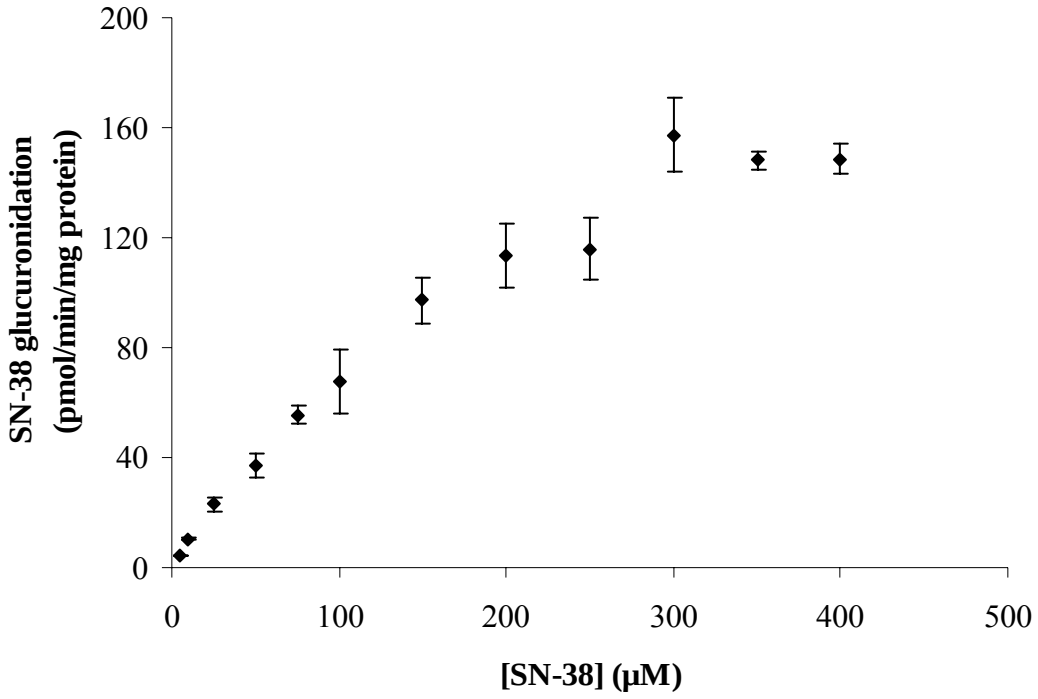

Figure 3.4 SN-38 glucuronidation in rat hepatic microsomes. Incubations proceeded at 37°C for 30 minutes with 0.5 mg/mL protein. Data points are mean ± SD (n = 3-6 replicates per concentration).

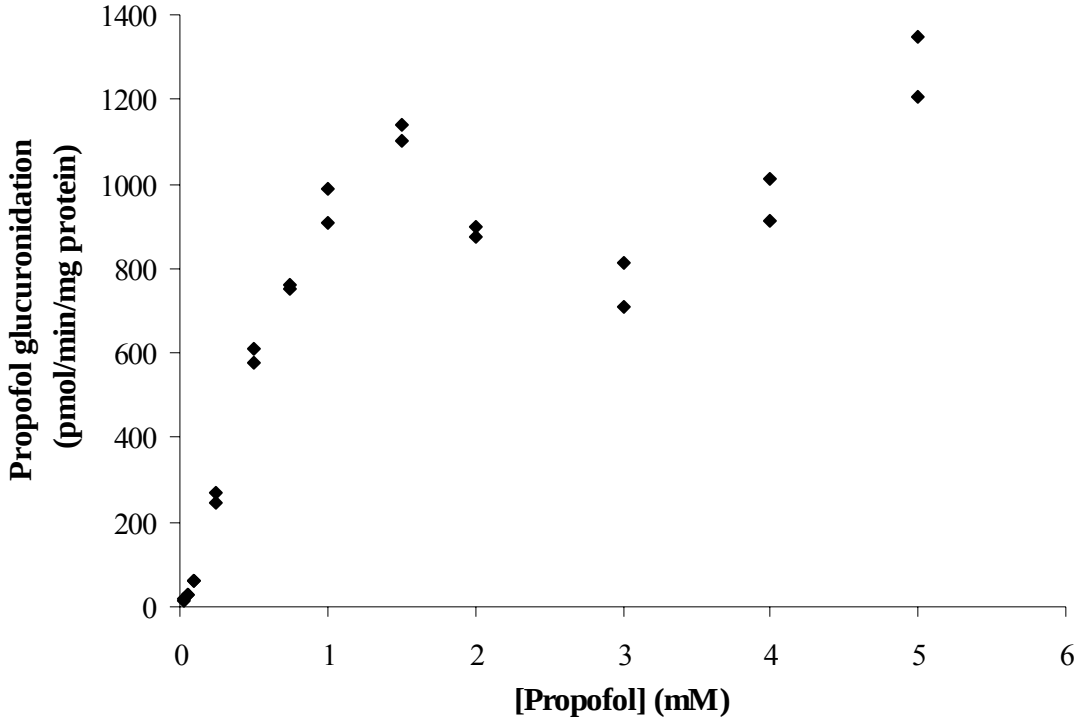

Figure 3.5 Propofol glucuronidation in human intestinal microsomes. Incubations were conducted over 40 minutes at 37°C with 0.2 mg/mL microsomal protein. Points are glucuronidation rates for individual samples (duplicate incubations per concentration).

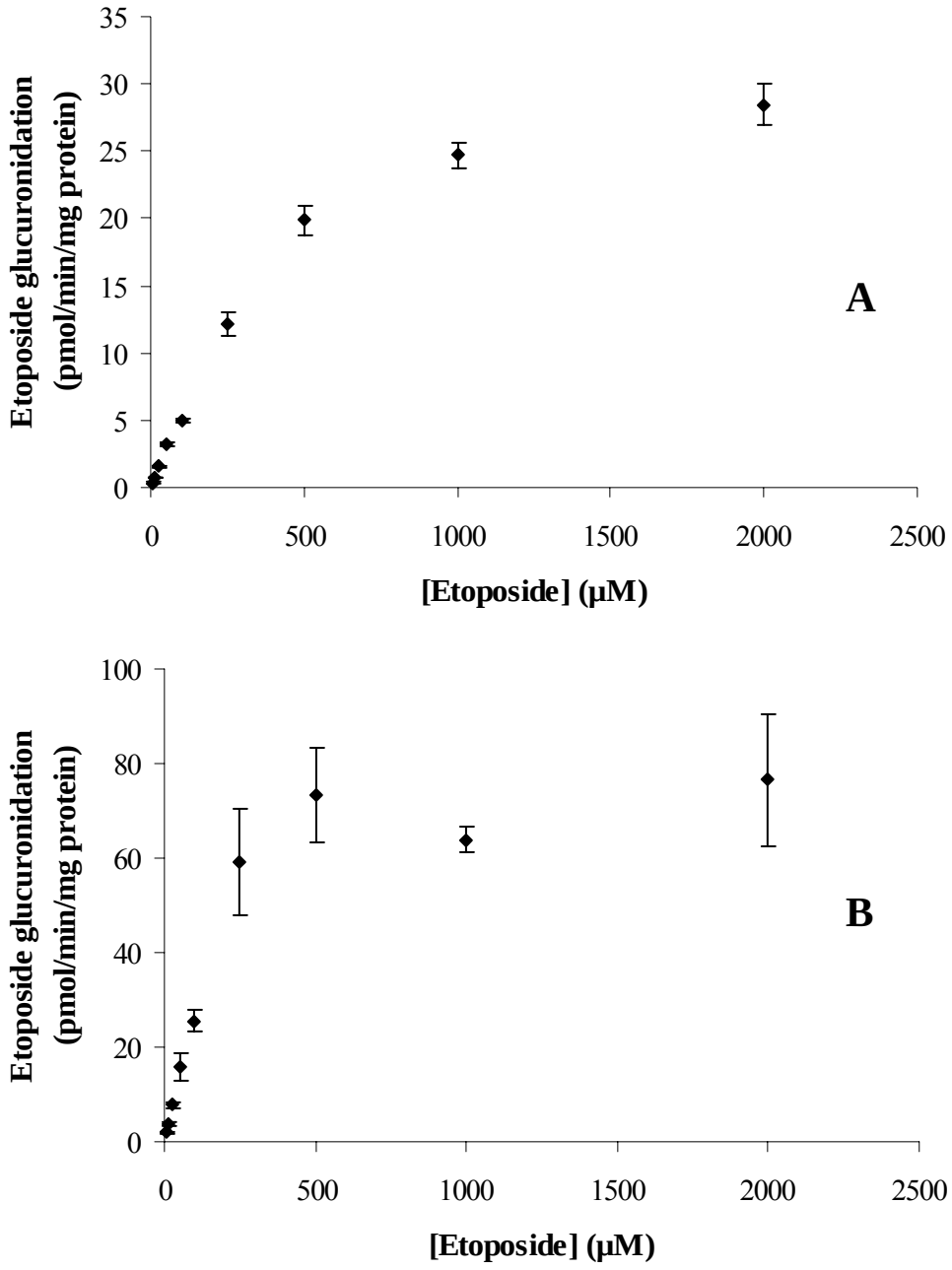

Figure 3.6 Kinetic profiles of etoposide in human intestinal (A) and human liver microsomes (B). Reactions contained 0.5 mg/mL of microsomal protein and proceeded for 30 minutes at 37°C. Data are mean ± SD (n = 3 replicates).

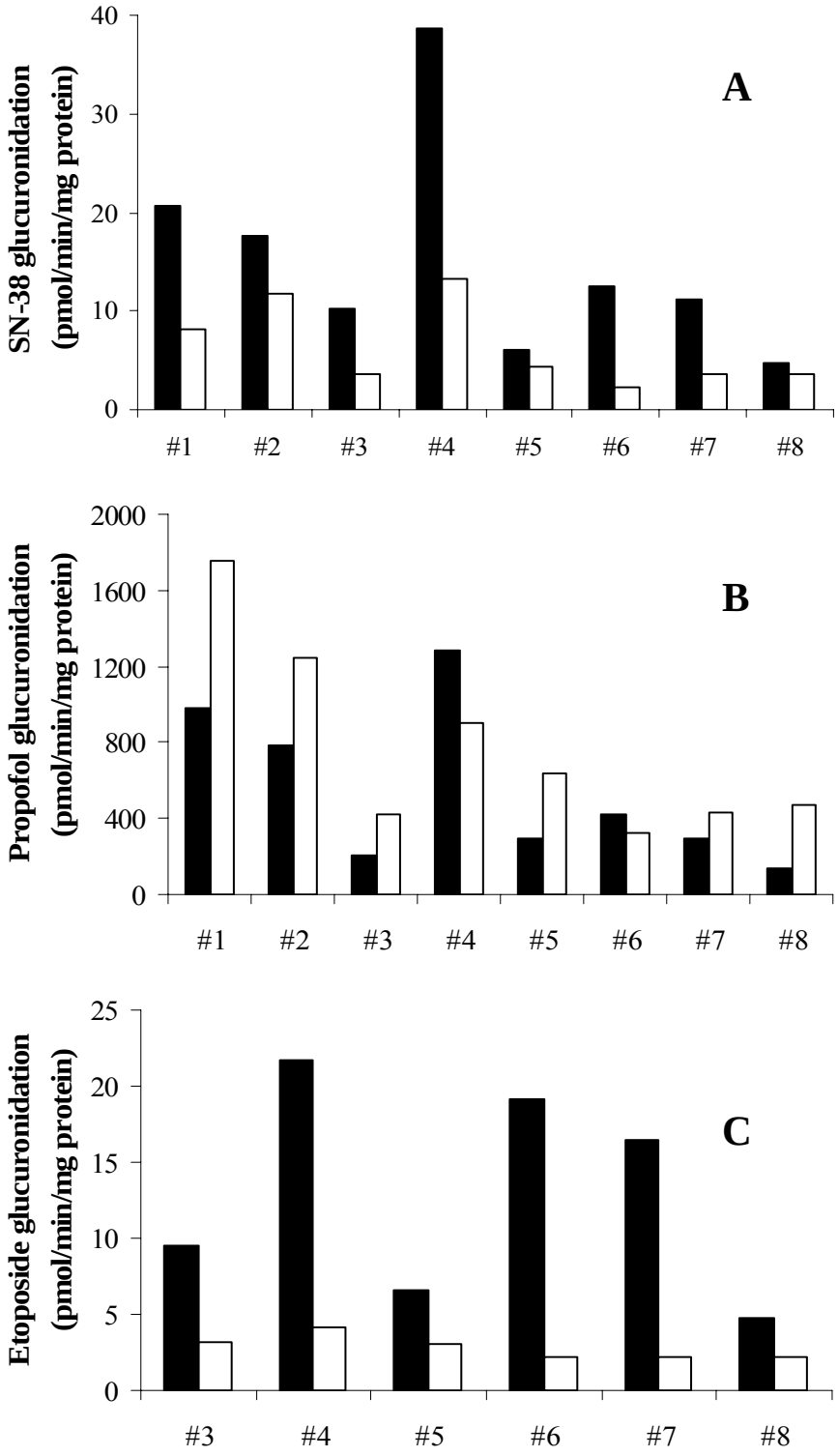

Figure 3.7 SN-38 (A), propofol (B), and etoposide (C) glucuronidation in patients providing both duodenal (filled bars) and colonic (open bars) biopsies. Bars are an average of two incubations. Numbers along horizontal axis correspond to a patient identifier.

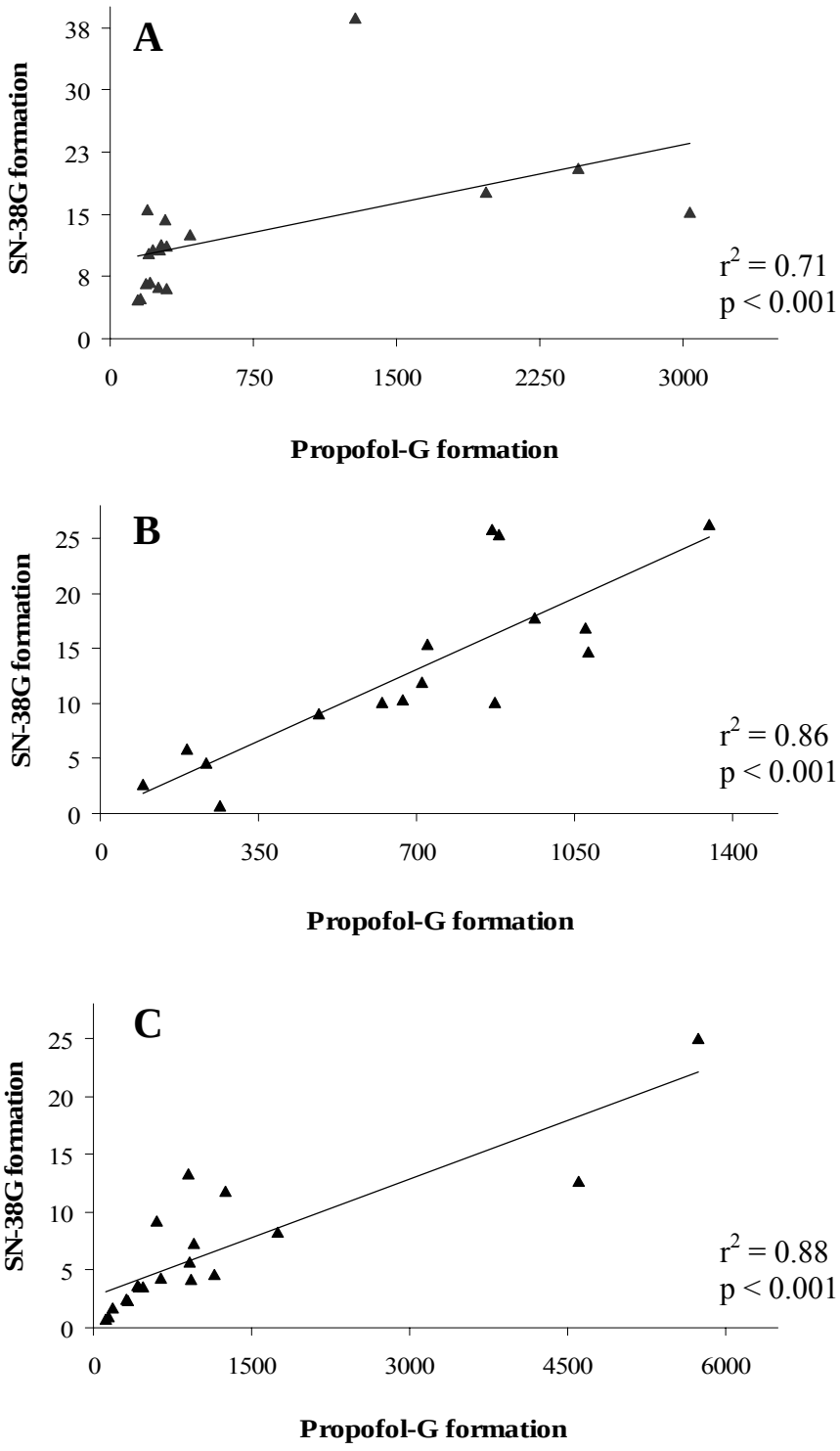

Figure 3.8 Correlation analyses of SN-38 and propofol glucuronidation (pmol/min/mg protein) in human duodenum (A), jejunum (B), and colon (C). The best-fit regression line, Spearman correlation coefficient, and associated p value are given for each plot.

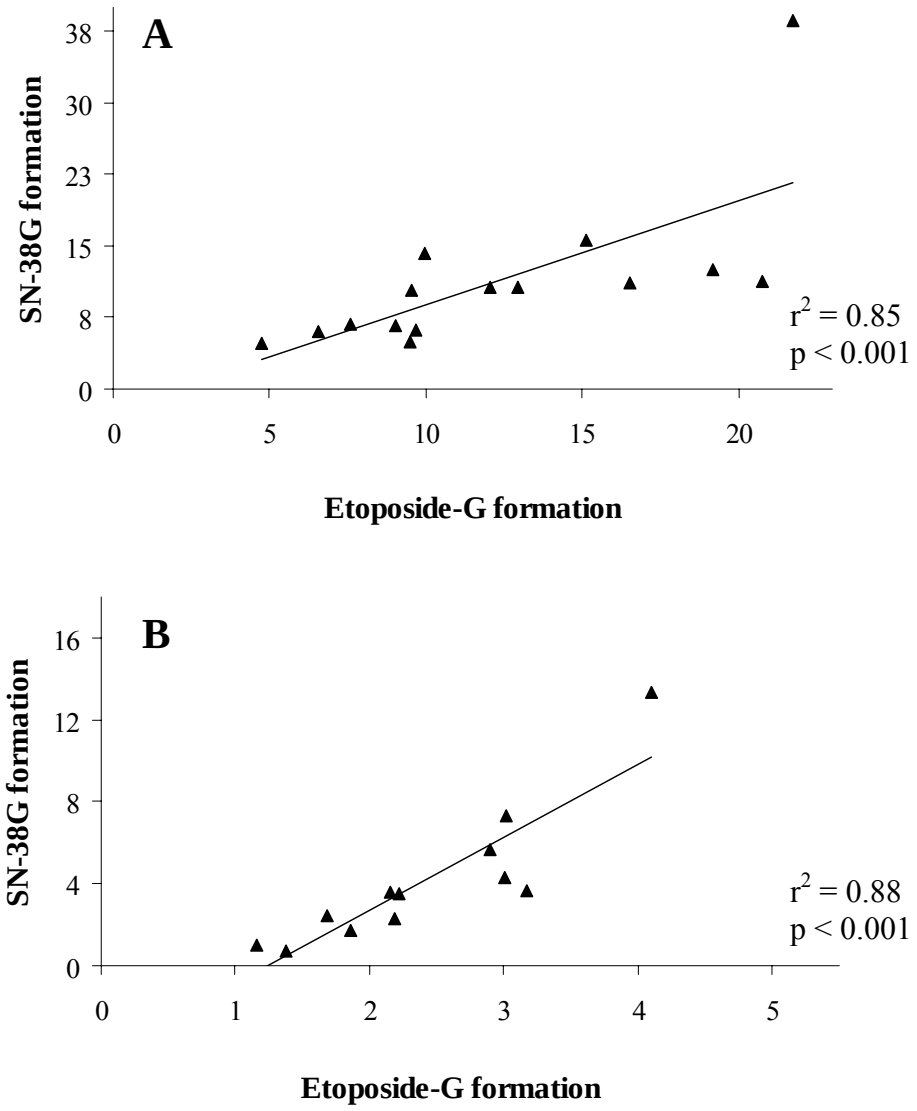

Figure 3.9 Correlation analyses of SN-38 and etoposide glucuronidation in human duodenum (A) and colon (B). Rates are pmol/min/mg protein. The best-fit regression line, Spearman correlation coefficient, and associated p value are given for each plot.

CHAPTER 4

THE CONTRIBUTION OF INTESTINAL UDP-GLUCURONOSYLTRASFERASES IN THE MODULATION OF SN-38 INDUCED GASTROINTESTINAL TOXICITY IN RATS

A. ABSTRACT

Life-threatening diarrhea afflicts a considerable percentage of patients treated with irinotecan, an anti-cancer agent with effects elicited through its active metabolite, SN-38. The primary detoxification pathway for SN-38 is glucuronidation. The purpose of this study was to evaluate the role that intestinal UDP-glucuronosyltransferases (UGTs) have from hepatic UGTs in modulating this diarrhea. To investigate this, Gunn rats devoid of UGT1A activity were injected with recombinant adenoviral vectors expressing UGT1A1, 1A6, and 1A7, resulting in reconstituted hepatic UGT expression comparable to a heterozygote. Hepatic microsome studies indicated that four to seven days after adenoviral injection, transfected Gunn rats (j/jAV) had SN-38 glucuronide (SN-38G) formation rates three times higher than control heterozygote rats (j+AV). The adenovirus did not impart any glucuronidating capacity to the intestine in j/jAV rats, while j+AV rats possess intestinal UGT function. After the administration of 20 mg/kg/day irinotecan i.p. to j/jAV rats four days after adenovirus injection, diarrhea ensued before the fourth irinotecan dose. j+AV rats were spared the diarrhea, and the toxicity was mild compared to the j/jAV rats, as measured by diarrhea scores, weight loss, and histological assessments of the cecum and colon. The pharmacokinetics of irinotecan, SN-38, and SN-38G indicate that the systemic exposure of SN-38 and SN-38G was higher and lower, respectively, in j/jAV rats. However, the excretion of irinotecan and metabolites were similar between the two groups. As intestinal UGTs are the main discriminating factor between j/jAV and j+AV rats, their presence may be responsible for the gastrointestinal protection observed in j+AV rats.

B. INTRODUCTION

The UDP-glucuronosyltransferases (UGT) are a superfamily of metabolic enzymes that catalyze the transfer of UDP-glucuronic acid to endogenous and xenobiotic substrates. Glucuronidation is a detoxification mechanism from several standpoints. It decreases the apparent volume of distribution, increases the molecular weight, and increases substrate specificity for active transport by imparting a negative charge, which are all processes that facilitate substrate elimination from the body. Also, in most cases, glucuronidation renders the substrate inactive with respect to its pharmacological or physiological target. A Wistar-derived rat model of UGT1A subfamily deficiency, the Gunn rat, has allowed tremendous insight into the importance of the UGT1A family in the metabolism and toxicity of substrates (Wells et al., 2004). The abolition of UGT1A glucuronidation in these rats stems from a frameshift mutation that yields a truncated, nonfunctional protein unable to bind UDP-glucuronic acid (Iyanagi et al., 1989).

Although the primary organ of glucuronidation is historically thought to be the liver, research on intestinal UGTs has shown their importance. Large differences in protein levels of the UGT1A family are not observed between these organs, and hepatic and intestinal tissues have been shown to have similar catalytic rates for a number of substrates, including carcinogens, tertiary amines, and steroids (Strassburg et al., 1999a; Strassburg et al., 2000). Differences in the tissue glucuronidation profile for a given substrate is likely due to tissue-specific UGT isoform expression evident in both humans and rodents (Strassburg et al., 1999a; Shelby et al., 2003; Gregory et al., 2004). Due to their localization, intestinal UGTs have been shown to contribute significantly to first-pass metabolism after oral administration

of substrates and may limit the systemic exposure of substrates undergoing enterohepatic recirculation (Sfakianos et al., 1997; Chen et al., 2003).

SN-38, the active metabolite of the chemotherapeutic drug irinotecan (Camptosar®, Pfizer, Inc), is a substrate for hepatic and intestinal UGT1A isoforms in both rats and humans (Hanioka et al., 2001b; Gagne et al., 2002; Tallman et al., 2005). SN-38 glucuronide (SN-38G), the resulting metabolite, is inactive and non-toxic (Figure 1.1). By directly injuring enteric cells, SN-38 causes a dose-limiting diarrhea in approximately 20% of patients (Araki et al., 1993; Iyer et al., 1998; Saltz et al., 2000). Several strategies have been successfully employed to prevent this diarrhea. These have focused mostly upon reducing intraluminal SN-38 concentrations, and include the inhibition of SN-38 and SN-38G biliary excretion and the inhibition of β-glucuronidase, an intestinal enzyme that will hydrolyze SN-38G to form SN-38 (Takasuna et al., 1996; Kehrer et al., 2001; Horikawa et al., 2002; Desai et al., 2005). Similarly, it was proposed by Gupta et al. (1994) that patients with poor hepatic SN-38 glucuronidation would have high biliary SN-38 concentrations and likely experience gastrointestinal toxicity. They devised a mathematical equation to describe this scenario, given as the ratio of the AUC values of SN-38 to SN-38G multipled by irinotecan AUC. In this study, the values, termed 'bilary index', were significantly higher in patients with diarrhea (Gupta et al., 1994). However, the biliary index has not been reproducibly validated by other investigators, probably because SN-38G excreted in the bile also contributes to toxicity after cleavage with β-glucuronidase to form SN-38 (Canal et al., 1996; Xie et al., 2002a).

Thus far, the approaches to both predict and prevent SN-38 mediated diarrhea have neglected the capacity for inherent enterocyte protection through intestinal UGT expression.

This concept of intestinal UGT protection in irinotecan gastrointestinal toxicity was originally proposed by Tukey et al. in 2002, yet no one has investigated it, likely due to the difficulty in isolating their contribution from hepatic UGTs in a toxicity model that takes several days to develop (Tukey et al., 2002). To address the hypothesis that intestinal UGTs play a major role in mediating SN-38-induced diarrhea, we utilize a Gunn rat model with reconstituted hepatic UGT activity, achieved by adenoviral infection. As described previously, intravenous injection of adenoviral vectors carrying rat UGT1A1, 1A6, and 1A7 will exclusively infect the liver, and in specific doses, have been shown to elicit a hepatic glucuronidation pattern similar to a Wistar/Gunn heterozygote (Amalfitano, 2004; Miles et al., 2006b). By comparing the pharmacokinetic and toxicity profiles from these transfected Gunn rats, possessing only hepatic UGTs, to those of heterozygous Gunn rats, possessing both hepatic and intestinal UGTs, we show that intestinal conjugation of SN-38 is critical in reducing the incidence of gastrointestinal toxicity.

C. MATERIALS AND METHODS

Materials. For calibration curves, irinotecan and SN-38G methyl ester were kindly provided by Dr. Robert Kelly of Pfizer (Kalamazoo, MI), previously Pharmacia. The procedure to hydrolyze SN-38 from irinotecan is published (Ni, 2001). SN-38G cleavage from SN-38G methyl ester was detailed earlier (Chapter 2). After β-glucuronidase hydrolysis of an SN-38G stock solution aliquot to form SN-38, SN-38G stocks were quantitated based upon SN-38 standard curves (Chapter 2). Reagents for microsomal experiments were purchased through Sigma-Aldrich (St. Louis, MO). Electrophoresis, gel/membrane transfer boxes, and western blot reagents were obtained through Bio-Rad (Hercules, CA) unless stated. All other chemicals and solvents were purchased through commercial sources.

Generation and Propagation of Recombinant Adenoviruses. First generation adenoviral vectors carrying rat UGT1A1, 1A6, 1A7, or 1A10 isoforms were produced using the homologous recombination method in *E. coli* by the Massey Cancer Center Virus Vector Shared Resource Facility at Virginia Commonwealth University. The vector plasmid, pTG-CMV, contained the E1 and E3 deleted adenoviral strain H5d1324. The shuttle plasmid, pZero-TG-CMV-rUGT1A, contained a cloned rat UGT isoform within the E1 deleted region. After the vector plasmid was cut with restriction enzyme ClaI, both plasmids were co-transformed into *E.coli*. Selected recombinant plasmids were transfected into human embryonic kidney 293B cells, which provide the deleted viral E1 proteins necessary for reproduction, and were used to propagate the viruses (American Type Culture Collection, Manassas VA). Replication-deficient viruses were then isolated from cellular components through freeze-thaw and centrifugation cycles, then purified and concentrated through cesium chloride gradients and dialysis (Miles et al., 2006b). By lysing and measuring the

absorbance of an aliquot of diluted virus at 260 nm, the final viral concentration was determined and given in optical density units (OD), where 1 OD_{260} is 1 x 10^{12} viral particles per mL of dialysate. Adenoviral stocks were stored at -80° C.

Animals. Male Gunn (j/j) and heterozygous (j+) Gunn rats (weighing 200-275 g) used in this study were colony-bred from j/j males and j+ females at Virginia Commonwealth University. Gunn rats were selected from j+ littermates by jaundice evident on their feet and ears. During the study, animals were housed two per cage in a temperature and humidity-regulated room with a 12 hour light-dark cycle. Standard rat chow and water were made freely available. All care and conducted procedures were approved by the University of North Carolina at Chapel Hill and Virginia Commonwealth University Institutional Animal Care and Use Committees.

Animal Treatment and Toxicity. On day 1, j/j rats (n = 5) received 0.15 mL adenovirus as a bolus through the tail vein. The total dose of 0.13 OD of adenovirus consisted of 0.1 OD of adenovirus encoding rat UGT1A1, 0.025 OD of 1A6, and 0.005 OD of 1A7. To control for any effects adenoviral injection may have in the j/j rats, j+ rats (n = 5) received 0.13 OD units of adenovirus carrying rat UGT1A10, an isoform with no activity towards SN-38 (data not shown). Hepatic UGT expression in the infected Gunn (j/jAV) animals was deemed adequate four days after adenoviral infection, as determined by visually inspecting the plasma for the resolution of hyperbilirubenemia. Thus, four days after infection, j/jAV and infected (j+AV) rats received 20 mg/kg of commercially available irinotecan (Camptosar, Pfizer, 20 mg/mL) in a final volume of 0.4 mL in their intraperitoneal space. Blood (0.1 mL) was then taken at 0.25, 0.5, 1, 2, 4, 8, and 12 hours after this first dose of irinotecan. The blood was centrifuged and plasma was stored at -20° C until analysis. Irinotecan was

administered daily to j/jAV and j+AV for three days. Prior to each dose of irinotecan, the rats were weighed and assessed for the extent of diarrhea on a scoring system: 0, firm stool; 1, malformed stool; 2, watery stool with perianal staining; 3, severe perianal staining (Stern et al., 2006). On the seventh day after adenovirus administration, the animals were euthanized. Livers from each rat were placed at -80°C. Colons and cecums were rinsed and placed in 10% formalin (Fisher Scientific, Hampton, NH).

In a separate study, j/jAV (n= 4) and j+AV (n= 2) rats were anesthetized and biliary catheters were placed four days after adenovirus injection with the isoforms and doses as described above for the respective genotype. Biliary cannulas were placed, and bile was collected over the following intervals: 0-0.25 hr, 0.25-0.5 hr, 0.5-1 hr, 1-2 hr, 2-4 hr, and 4-6 hr. After the final collection interval was completed, rats were euthanized. Livers from each rat were placed at -80° C.

Microsomal preparation and glucuronidation assay. Livers from all rats in the biliary excretion study and the toxicity/pharmacokinetic study were used to make hepatic microsomes. Intestines and colons from irinotecan-naiive j/jAV and j+AV (n = 2 in each group) rats were extracted seven days after adenovirus injection and used to make microsomes. The procedures followed to make microsomes from these organs were described previously (Chapter 2). Microsomal protein concentrations were determined by the Bradford method, using albumin as a standard.

In vitro conjugation reactions of SN-38 with the hepatic and intestinal microsomes contained the following components: magnesium chloride (10 mM assay concentration), Brij 35 (0.5 mg/ mg protein), D-saccharic acid 1,4-lactone (10 mM), SN-38 carboxylate (300 μM), and microsomal protein (0.25 mg/mL) in a final volume of 200 μL 0.1 M Tris, pH 7.0.

The reaction was initiated by the addition of UDP-glucuronic acid (2mM final concentration) and proceeded at 37° C for 40 minutes and quenched with acetonitrile.

Western blotting. Hepatic, intestinal, and colonic microsomal proteins (50 μg) were subjected to electrophoresis through a 4-15% gradient Tris gel (Ready Gel, Bio-Rad). Upon separation, proteins were transferred to nitrocellulose membranes using 105 volts for one hour. Membranes were then washed in 5% milk/0.2% Tween in Tris-Buffered Saline (TBS-T) for one hour to prevent non-specific antigen-antibody binding. After two washes in 0.5% TBS-T, blots with hepatic microsomes were incubated with either α-rat UGT1A1 (diluted 1:1000 in 0.5% milk/0.2% TBS-T) or α-rat UGT1A7 (1:1500 in 0.5%milk/0.2% TBS-T) for one hour each. Membranes with intestinal and colonic microsomes were probed with α-UGT1A antiserum (1:1000 in 0.5% milk/0.2%TBS-T) overnight. Information on specificity and production of antiserum reacting with rat UGT1A1, 1A7, or with the UGT1A common region on all rat UGT1A proteins was formerly published (Kessler et al., 2002; Webb et al., 2005). Antiserum was washed off twice with 0.5% TBS-T, and a horseradish peroxidase conjugated α-mouse IgG (1:10,000, Chemicon) was incubated with all blots for one hour. After two washes with 0.5% TBS-T, chemiluminescent reagent (ECL, Amersham Biosciences) was applied to hepatic microsomal membranes, exposed to film (Biomax, Kodak) for four minutes, and developed. For intestinal protein blots, chemiluminescent reagent (SuperSignal, Pierce) was applied and a phosphoimager (Versadoc, Biorad) was used to visualize theresulting bands. In all cases, a molecular weight marker was added to the gel to verify the presence of the UGT band (approximately 55 kDa).

Chromatographic Analysis. High performance liquid chromatography (HPLC) with fluorescence detection was used to quantify irinotecan, SN-38, and SN-38G concentrations

from plasma, bile, and *in vitro* microsomal incubations. Acetontrile (300 µL), internal standard (camptothecin, 200 ng) and perchloric acid (5 µL, 5% solution) were added to each plasma sample (25µL). Bile was first diluted 1 to 200 in Tris, and 200 µL were subject to acetonitrile (800 µL) and perchloric acid precipitation and the addition of camptothecin (100 ng). For microsomal samples, acetonitrile (800 µL) containing perchloric acid and irinotecan as an internal standard (50 ng) was added. All samples were then centrifuged at 15,000 g for 10min. Next, the aceontrile layer was extracted and evaporated under a stream of nitrogen. Samples were then reconstituted with 200 µL of 30% methanol/ 100 mM ammonium acetate. Standards were prepared as above for each matrix and contained blank bile, blank plasma, or microsomal protein.

The HPLC system used to inject, separate, and detect analytes was as described in Chapter 2, however an SIL10A autosampler was used (Shimadzu, Tokyo, Japan). Mobile phase consisted of 37% methanol/100 mM ammonium acetate and 5 mM tetrabutylammonium sulfate, pH 4.9 run at 1.5 mL/min for microsomal samples. For plasma and bile samples, 39% methanol/100 mM ammonium acetate and 15 mM tetrabutylammonium sulfate, pH 4.9 was run as mobile phase at 1.3 mL/min. Standard curves in each matrix were linear and validated if the lower limit of quantitation coefficient of variance was ≤ 15% and the analyte to internal standard ratio normalized to concentration was constant. Calibration curve ranges were as follows: for plasma, 2.5-200 ng/mL SN-38G, 100-10,000 ng/mL irinotecan, 2.5-50 ng/mL SN-38; for bile, 1.25 ng -100 ng/mL SN-38G, 12.5 ng-1000 ng/mL irinotecan, 2.5-75 ng/mL SN-38; and for microsomes, 2-300 ng/mL SN-38G. Excitation and emission wavelengths were 229 and 420 nm, respectively, for SN-38G, irinotecan, and camptothecin. The emission wavelength used to detect SN-38 was changed

to 543 nm during each chromatographic run. To avoid inter-day variability, all samples in a given matrix were processed and run on the same day. A representative chromatograph for bile, plasma, and microsomes is given in Figure 4.1.

Histology. Colon and cecum samples were removed from formalin, rinsed, and stored in 70% ethanol. Samples were then emedded in paraffin, sectioned, mounted on slides, and stained with hematoxylin and eosin by the Histopathology Core Lab in the Lineberger Comprehensive Center at UNC.

Data Analysis. Pharmacokinetic parameters for irinotecan, SN-38, and SN-38G were determined by noncompartmental analysis using WinNonlin software (version 5.0, Pharsight, Mountain View, CA). Statistical tests were perfomed using Sigma Stat software (version 2.0, Systat, Point Richmond, CA). A t-test was used to assess differences between j/jAV and j+AV rats given irinotecan, as the data were found to be normally distributed with equal variance. The Mann-Whitney rank sum test was used to analyze differences between the groups with respect to diarrhea scores. To assess biliary excretion, the amounts of irinotecan and metabolites excreted over the collections intervals were summed. Statistical significance was reached at $p \leq 0.05$.

D. RESULTS

All rats injected with adenovirus tolerated the dose well, with no immunological response or behavorial or pathophysiological changes evident after administration. Gunn rats receiving 0.13 OD of adenovirus expressed rat UGT1A1 and 1A7, the isoforms relevant to SN-38 glucuronidation, in the liver (Figure 4.2). By visual inspection of the blots and glucuronidation profiles in individual rats, it appears that rat UGT1A7 expression is more highly associated with SN-38 glucuronidation when compared with the expression of rat UGT1A1 (Figure 4.2). In the j/jAV rats, hepatic SN-38 glucuronidation reached its maximum expression four days after adenoviral infection (average of 38.8 pmol SN-38G formed/min/mg protein), and was sustained over the experimental period to seven days post-infection (average of 34.8 SN-38G formed/min/mg protein). Over this time frame, the j/jAV rats have an average of three to four times greater SN-38 hepatic glucuronidation capacity versus the j+AV rats (Figures 4.2 and 4.3). The difference in activity between j/jAV and j+AV rats was significant at seven days post-infection ($p=0.003$) (Figure 4.2). Hepatic glucuronidation of SN-38 in the j/jAV rats was solely due to the adenovirus delivery, as uninfected Gunn rats possess no intrinisic SN-38 catalytic activity (data not shown). Figure 4.4 reveals that an intravenous injection of adenovirus does not impart any UGT1A expression or function to the intestine or colon of a j/jAV rat.

The pharmacokinetic profile and parameters of irinotecan and its metabolites are shown in Figure 4.5 and Table 4.1. The pharmacokinetics of irinotecan were similar between j/jAV and j+AV rats. SN-38 exposure, as represented by AUC, was significantly higher in j/jAV rats. The elimination half-life of SN-38G was not calculated due to the lack of a reproducible terminal concentration/time slope in the profiles of most rats. Despite higher *in*

vitro SN-38 glucuronidation rates, the $AUC_{SN\text{-}38G}$ in j/jAV rats was approximately two-fold lower than j+AV rats on average.

In a separate experiment to determine whether intestinal exposure to irinotecan, SN-38, and SN-38G was similar between j/jAV and j+AV rats, bile was collected from rats from both groups after a single dose of irinotecan 20 mg/kg i.p. four days after adenovirus injection. Although statistical analysis is not applicable (n=2 j+AV rats), it appears that irinotecan is excreted to a greater degree in j+AV rats relative to j/jAV rats over a six hour period (Figure 4.6). Little or no difference in the excretion of SN-38 and SN-38G between the two groups is apparent.

The two groups of rats studied tolerated repeated doses of irinotecan with dissimilar results. Twenty-four hours after the second and third dose of irinotecan (20 mg/kg/day i.p.), j/jAV were found to have lost a greater percent of their body weight relative to baseline (Figure 4.7). After finding that several j/jAV rats had reached the predetermined weight loss limit of 20% the morning after the third irinotecan dose was administered, the study was ended and all animals were euthanized. At this time, every j/jAV had at least grade 2 diarrhea, characterized by loose stools and peri-anal staining. This outcome was significantly different from all j+AV rats, which did not exhibit any diarrheal symptoms (Table 4.2) Since toxicity differences between the groups were strikingly evident on a macroscopic basis, microscopic differences in colon and cecum integrity and composition were compared (Figure 4.8). In the colon and cecum of j/jAV rats, there was a significant collapse of crypts. Inflammatory cell debris and mucoid material filled the crypts. Infiltrating neutrophils were also evident. Goblet cells were widely depleted. Epithelial cells lining the crypt were fused or severely necrosed. In patchy areas, low cuboidal epithelial cells with thin cytoplasm and

large nuclei lined the crypt, indicative of regeneration. In cecum and colon samples from j+AV rats, tall columnar epithelial cells lined intact villi. Goblet cells containing mucous filled the crypts. Overall, colon and cecal mucosa in j+AV rats was normal in appearance. In several j/jAV and j+AV cecum and colon samples, moderate submucosal edema was present.

E. DISCUSSION

In this study, we examine the importance of intestinal UGTs in modulating irinotecan-induced diarrhea. This has been accomplished by using a rat model essentially engineered to differ from its control in intestinal UGT expression only. By utilizing an adenoviral vector containing rat UGT1A isoforms constitutively expressed in other Wistar-derived rat strains, Gunn rats were found to have systemic SN-38 conjugation activity. After intravenous injection of adenoviruses, any virions not sequestered by Kupffer cells will be exclusively taken up by surrounding hepatocytes, precluding intestinal infection (Amalfitano, 2004). The lack of intestinal UGT expression and function in j/jAV rats was indeed shown in this study. Therefore, by j/jAV rats gaining hepatic UGT activity and j+AV rats naturally possessing both hepatic and intestinal UGTs, we could test the hypothesis that intestinal UGTs are local, protective mechanisms inherent in enteric cells, the site of SN-38 cytotoxicity, and their expression is critical in the prevention of diarrhea. As striking differences in the incidence of diarrhea and lower intestinal pathology were noted after both groups of rats were challenged with irinotecan, further experiments were conducted to ensure that the toxicity that ensued was due to a lack of intestinal SN-38 conjugation and not due to differences in systemic or intestinal irinotecan, SN-38, or SN-38G exposure.

Ideally, hepatic glucuronidation and the systemic disposition of irinotecan, SN-38, and SN-38G in the j/jAV rats would precisely mimic that in the j+AV rats. Over the entire irinotecan-dosing interval, microsomal reactions demonstrated that j/jAV rats possessed significantly higher SN-38 conjugation rates. This is most likely due to higher expression of UGT1A7 in j/jAV animals, as glucuronidation rates mimic rat 1A7 levels. Additionally, UGT1A7 is the major rat isoform responsible for SN-38 catalysis as determined by

recombinant isoform studies (Tallman et al., 2005). Despite higher *in vitro* SN-38 glucuronidation rates, the exposure to SN-38G in the plasma of j/jAV rats was two-fold lower than the j+AV rats. It is only speculative to compare data from these two different measures of glucuronidation. However, one explanation for this apparent discrepancy may be more efficient SN-38G basolateral transport mechanisms in the j+AV rat, which would manifest in higher plasma glucuronide levels despite lower intracellular glucuronidation rates. In these rats, there is a physiological need for basolateral transporters that recognize glucuronides, most notably MRP3. Conversely, in an uninfected j/j rat, levels of these transporters may be low due to the lack of glucuronide substrates present intracellularly. Similar theories have been proposed, where MRP3 expression is regulated by accumulating intracellular substrates (Donner and Keppler, 2001; Johnson et al., 2005). In j/jAV rats, induction of MRP3 may lag behind UGT expression, which would explain why less SN-38G was measured in plasma. To address this, Western blots of hepatic membrane proteins from j+AV, j/jAV, and j/j rats were run. From this data, there is no observable difference in the expression of MRP3 between these groups (data not shown). Thus, factors influencing the discordance between *in vitro* and *in vivo* measures of SN-38 glucuronidation are currently unknown.

SN-38 levels are significantly higher in j/jAV rats. This may be a function of less SN-38 consumed, as reflected in lower SN-38G levels, differences in transport of SN-38 from the hepatocyte to blood, or more efficient reabsorption of SN-38 in j/jAV animals. In a rat without functional intestinal glucuronidation, enteric cycling of SN-38 would be nonexistent and more SN-38 could be reabsorbed into the blood. This is not very likely, as apical secretion of SN-38 was several fold more efficient than basolateral flux as determined

in Caco-2 cells (Kehrer et al., 2000). Additionally, the inhibition of β-glucuronidase does not reduce plasma AUC_{SN-38} relative to control-treated rats, indicating little enterohepatic circulation (Takasuna et al., 1998).

With the knowledge that high intestinal SN-38 concentrations may be a predisposing factor in the development of diarrhea, differences in irinotecan, SN-38, and SN-38G biliary excretion in j/jAV and j+AV rats were studied. In the intestinal lumen, irinotecan may be converted to SN-38 by carboxylesterases and SN-38G excreted into bile is almost completely hydrolyzed to SN-38 by fecal β-glucuronidases (Takasuna et al., 1998; Khanna et al., 2000; Slatter et al., 2000). Differences in excretion of these compounds between the two groups of rats may be a confounding factor in describing the precise role that intestinal UGTs have in mediating toxicity. Although the data were derived from a small number of rats, the amounts of SN-38 and SN-38G extruded into bile were similar. The excretion of irinotecan was approximately double in the j+AV animals. From this data, j+AV rats may then have a slightly greater intestinal SN-38 burden by iriniotecan hydrolysis. Overall, *in vitro*, biliary excretion, and pharmacokinetic data indicate that the disposition of irinotecan and its metabolites are similar in j/jAV and j+AV rats. The small differences observed between the groups of rats with regards to these parameters likely do not explain the extraordinary degree of intestinal toxicity experienced only by the j/jAV animals.

The *in vivo* data presented here support published *in vitro* data relating to UGT protection in intestinal cells. Propofol, a competitive substrate for UGT1A9, reduced the concentration of SN-38 needed to elicit death in 50% of HT-29 colon carcinoma cells in culture. In contrast, propofol did not affect this value in HCT-116 colon cancer cells. As the former cells have demonstrated UGT activity while the latter cells do not, the results from

this study also indicate toxicity is inversely related to SN-38G formation (Cummings et al., 2003).

Currently, it is unknown what level of constitutive intestinal UGT function will spare an animal from toxicity to SN-38. Heterozygote Gunn rats have only intermediate intestinal UGT1A expression relative to a Wistar rat, yet the residual activity in the former animals was sufficient to protect them from diarrhea. As with rats, patients with the lowest intestinal SN-38G formation may be those experiencing diarrhea. In humans, large differences in intestinal SN-38 conjugation are noted, and may result from numerous factors (Chapter 3). Various components of food, such as chrysin or octylgallate, have been found to selectively induce or potently inhibit intestinal SN-38G formation, respectively (Cummings et al., 2003; Tobin et al., 2006). As UGT1A1 and UGT1A9 are found in the lower intestine and recognize SN-38 as a substrate, polymorphisms in these isoforms may impart low or high catalytic turnover and effect gastrointestinal adverse events (Strassburg et al., 1998; Tallman et al., 2005). For instance, SN-38G formation was lower in hepatic microsomes from patients with Gilbert's syndrome, caused by a mutation in the UGT1A1 promoter, relative to the glucuronidation in patients with a wild-type promoter (Iyer et al., 1999). The presence of this polymorphism was found to be predictive of patients developing diarrhea in one clinical study (Marcuello et al., 2004).

To date, low hepatic SN-38 glucuronidation has most commonly been linked to the development of diarrhea, as evident through the establishment of the biliary index (Gupta et al., 1994). However, our data indicate that intestinal toxicity ensues regardless of hepatic UGT expression and supports the following scenario. Patients with inefficient hepatic glucuronidation (i.e., Gilbert's patients) will excrete less SN-38G and similar levels of SN-

38. In contrast to a patient with efficient hepatic activity, these patients will have less total intestinal SN-38 for several reasons. SN-38G is more efficiently transported into bile and all SN-38G in the intestine is cleave to SN-38 by β-glucuronidase (Chu et al., 1997; Slatter et al., 2000). Despite this, Gilbert's patients may still experience diarrhea, because of low enteric SN-38G formation. Thus, the biliary index proposed by Gupta et al. (1994) may correlate with the incidence of diarrhea in these patients, although toxicity has little to do with systemic glucuronidation (Gupta et al., 1994). Patients with a low biliary index may also suffer adverse effects, resulting from poor intestinal turnover of a high SN-38 burden. This scenario, similarly proposed by others, may explain the poor correlation between the biliary index calculation and diarrhea (Tukey et al., 2002).

The j/jAV and j+AV model is being utilized in our laboratories to study the influence of intestinal UGTs on local toxicity. This model should be applicable to a wide range of toxic substrates which are highly glucuronidated by intestinal isoforms to inactive conjugates, such as mycophenolic acid (MPA). MPA is the active metabolite of the immunosuppressant mycophenolic acid mofetile (MMF) which induces gastrointestinal toxicity in a higher percentage of treated patients. Indeed, despite similar pharmacokinetic profiles, j/jAV rats treated with 45 mg MMF/kg/day i.p. for three days exhibited severe diarrhea in constrast to the j+AV rats that were spared this adverse effect (Miles et al., 2006a). In summary, the studies with MPA and SN-38 in j/jAV and j+AV rats demonstrate that intestinal UGTs function in a primary role to protect the integrity of the gastrointestinal mucosa against cytotoxic agents.

F. ACKNOWLEDGEMENTS

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Table 4.1 Pharmacokinetic parameters after a single dose of irinotecan, 20 mg/kg i.p., in j/jAV and j+ AV rats (n = 5 each) as determined by noncompartmental analysis. AUC refers to that determined over the blood collection interval, from 0-12 hrs. ** $p<0.01$ or *** $p < 0.001$ compared with parameter average j+AV rats. Values are given as mean ± SE.

Parameter	**j/j AV**	**j+AV**
Irinotecan AUC (min*μg/mL)	602 ± 120	411 ± 71
Irinotecan V/F (mL)	1103 ± 190	1540 ± 170
Irinotecan Cl/F (mL/min)	7.10 ± 1.5	12.7 ± 2.2
Irinotecan $t_{1/2}$ (min)	127 ± 35	88.6 ± 9.1
SN-38 AUC (min*μg/mL)	8.64 ± 0.83**	5.37 ± 0.45
SN-38 $t_{1/2}$ (min)	651 ± 98	478 ± 39
SN-38G AUC (min*μg/mL)	21.3 ± 1.3***	45.5 ± 2.6

Table 4.2 Diarrhea score in j/jAV and j+AV rats (n = 5 in each group) as determined 24 hours after each daily dose of irinotecan, 20 mg/kg i.p. Scoring criteria are given under MATERIALS and METHODS. ** indicates $p<0.01$ compared with scores in j+AV rats after the same dose.

Group/irinotecan dose number	**Diarrhea Score**				
	0	1	2	3	Mean Score
j/jAV/Dose 1	5[a]	0	0	0	0
j+AV/Dose 1	5	0	0	0	0
j/jAV/Dose 2	5	0	0	0	0
j+AV/Dose 2	5	0	0	0	0
j/jAV/Dose 3	0	0	1	4	2.8**
j+AV/Dose 3	5	0	0	0	0

[a]number of rats experiencing the grade of diarrhea the specified group

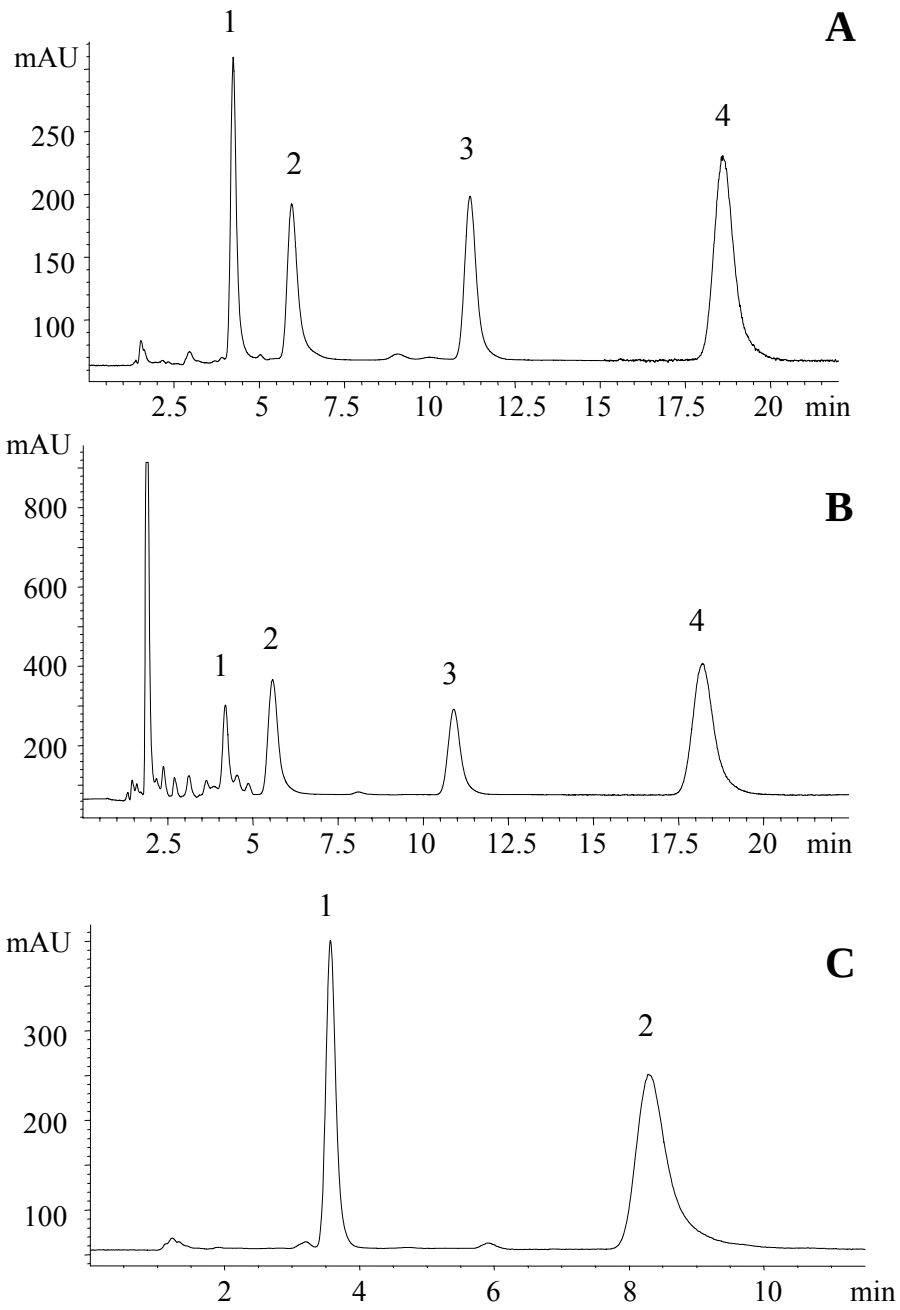

Figure 4.1 Chromatographs of SN-38G (1), irinotecan (2), camptothecin (3), and SN-38 (4) standards in bile, diluted 1:200, 200 µL (A); plasma, 25 µL (B); or microsomal reaction, 200 µL (C). Concentrations are: A) 1, 25 ng/mL; 2, 250 ng/mL; 4, 50 ng/mL. B) 1, 100 ng/mL; 2, 10 µg/mL; 4, 100 ng/mL. C) 1, 125 ng/mL.

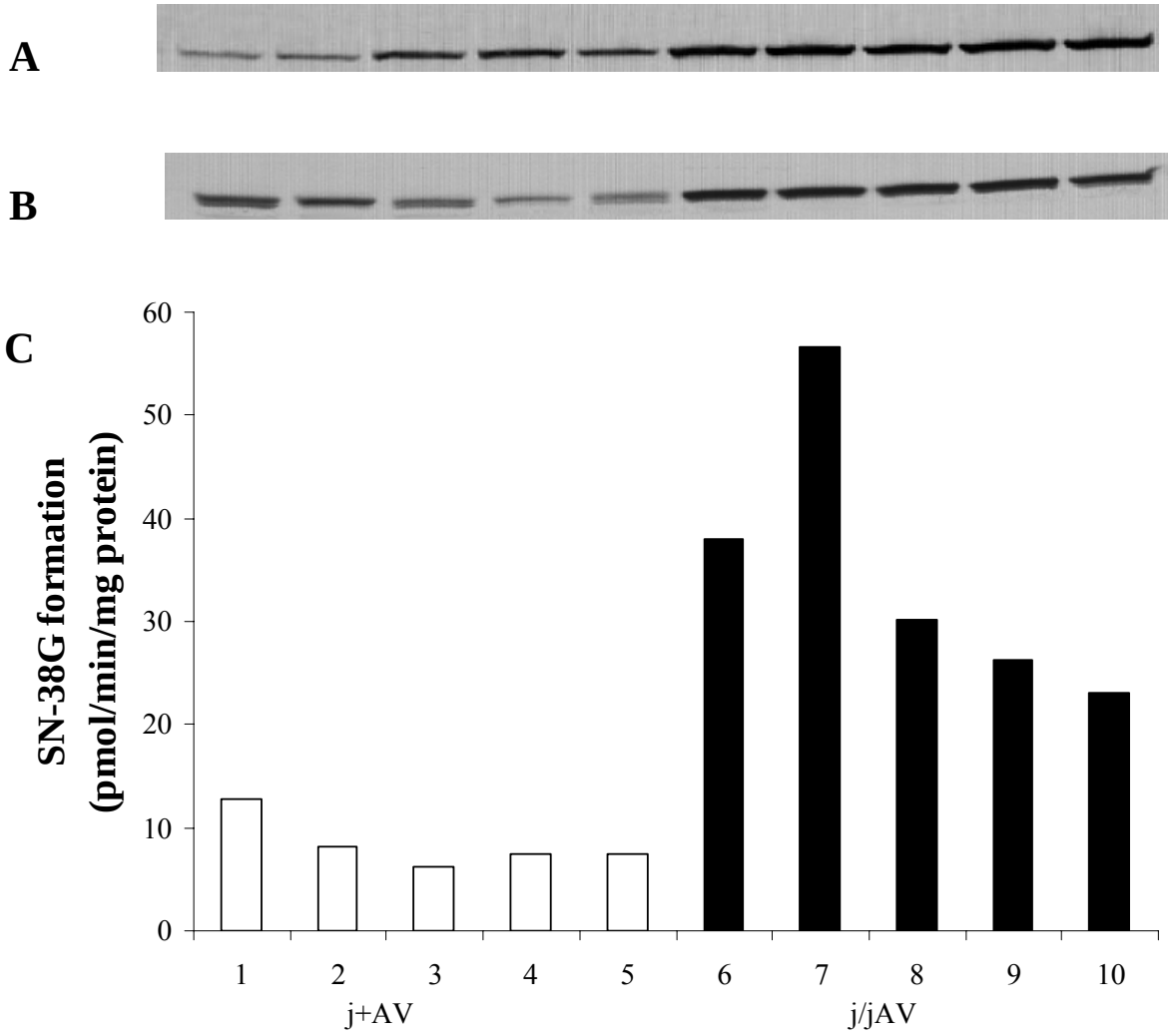

Figure 4.2 Hepatic expression of rat UGT1A1 (A) and rat UGT1A7 (B) in j+ AV rats (lanes 1-5) and j/jAV rats (lanes 6-10) seven days after adenoviral injection of rat UGT1A10 to j+ rats or UGT1A1, 1A6, and 1A7 to j/j rats. Corresponding *in vitro* SN-38 glucuronidation rates for j+ AV (□, bars 1-5) and j/jAV (■, bars 6-10) (C). Bars represent data from a single rat and are an average of duplicate activity measurements. Hepatic microsomes were prepared from rats involved in the pharmacokinetic/toxicity study.

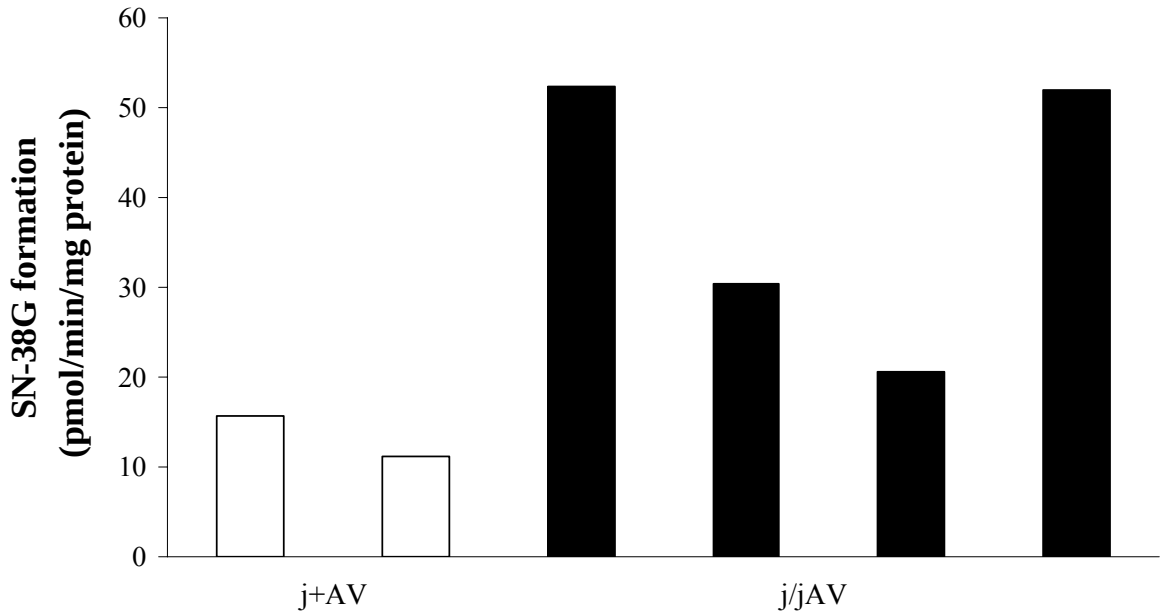

Figure 4.3 Hepatic SN-38 glucuronidation rates for j+ AV (□) and j/jAV (■) four days after adenoviral injection of 0.13 OD rat UGT1A10 or 1A1, 1A6, and 1A7, respectively. Bars represent data from a single rat and are an average of duplicate activity measurements. Hepatic microsomes were prepared from rats used for the biliary excretion study. Rats received one dose of irinotecan 20 mg/kg/day i.p.

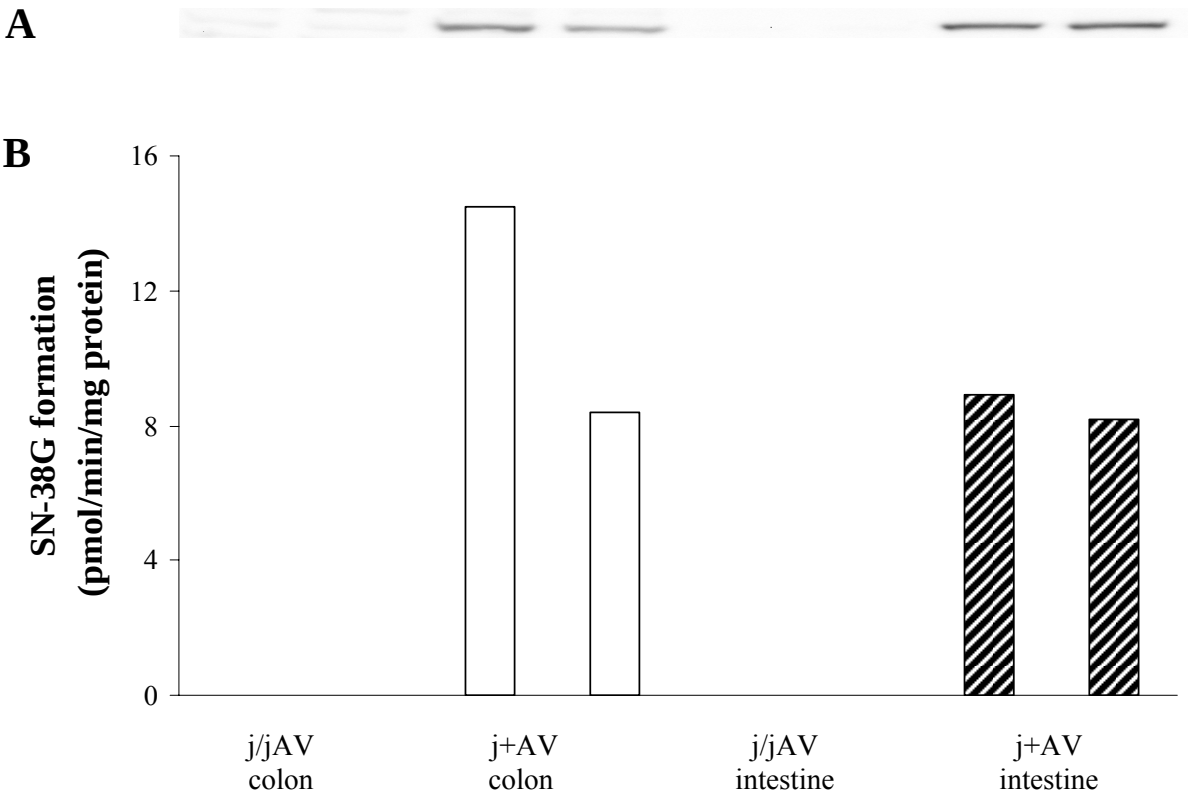

Figure 4.4 Intestinal and colonic rat UGT1A expression (A) and SN-38 glucuronidation rates (B) for j/jAV and j+AV colon (open bars) and intestinal (thatched bars) microsomes seven days after adenoviral injection of 0.13 OD rat UGT1A10 or 1A1, 1A6, and 1A7, respectively. Bars represent data from a single rat and are an average of duplicate activity measurements. Rats did not receive irinotecan.

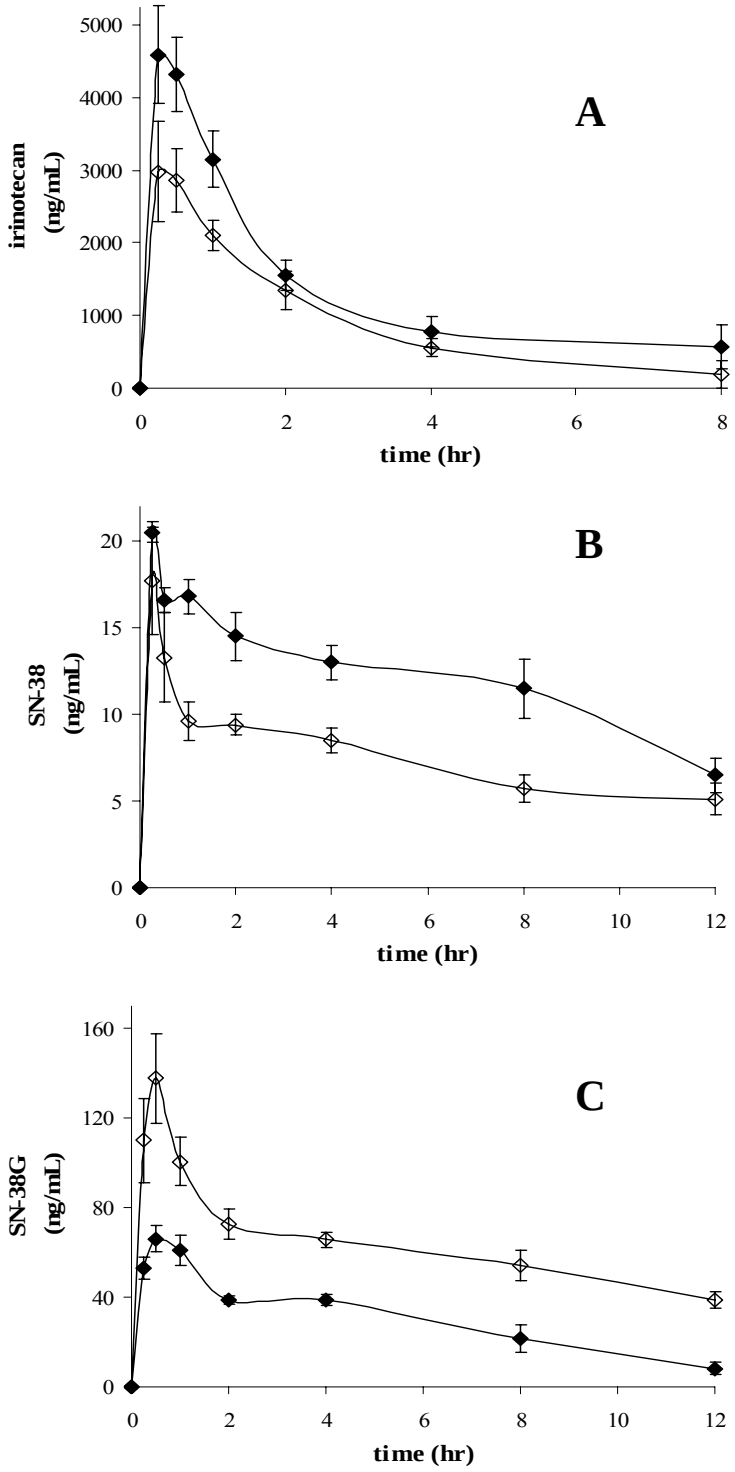

Figure 4.5 Plasma profiles of irinotecan (A), SN-38 (B), and SN-38G (C) in j/jAV (-♦-) and j+AV (-◊-) rats after a 20 mg/kg dose of irinotecan i.p. (n = 5 rats per group). Each data point is the mean concentration in rats of that group with associated standard error bars.

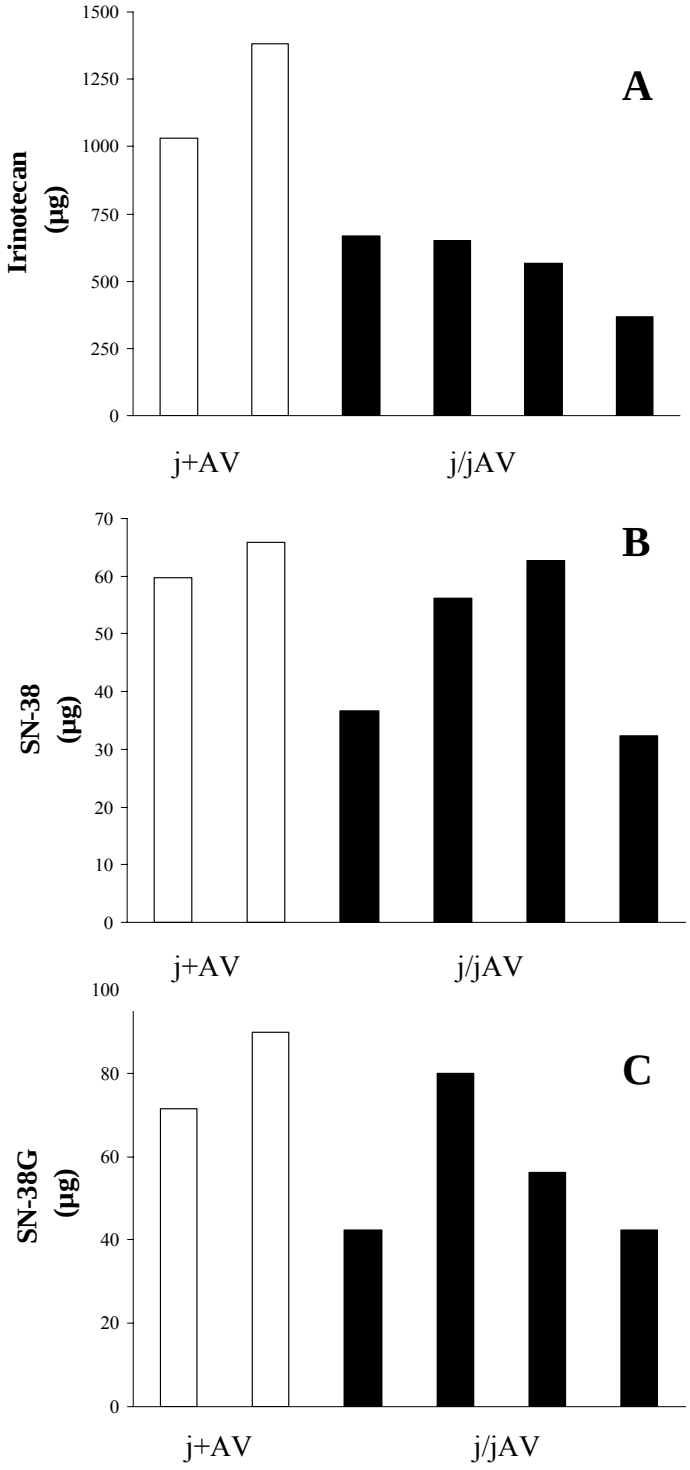

Figure 4.6 Biliary excretion (0-6 hr collection) of irinotecan (A), SN-38 (B), and SN-38G (C) in j+AV (-□-) and j/jAV (-■-) rats after a single 20 mg/kg i.p. dose of irinotecan. Bars represent data from a single rat and are an average of duplicate activity measurements.

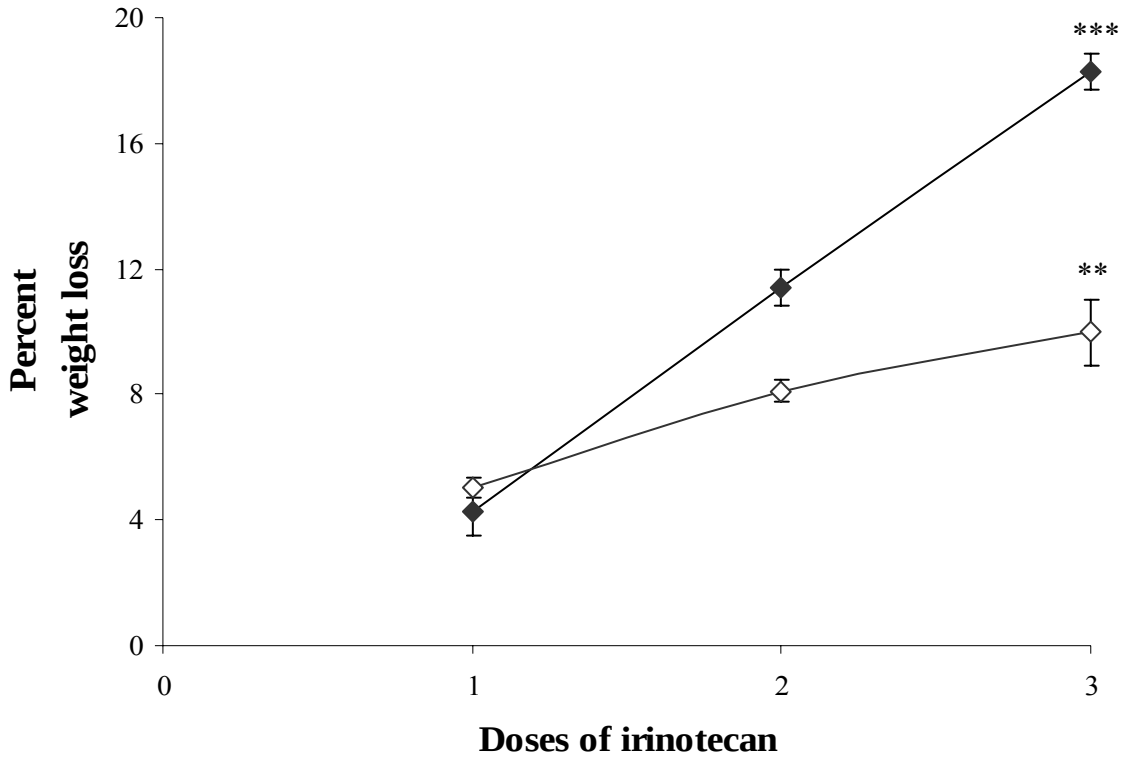

Figure 4.7 Percentage weight loss relative to baseline over time in j/jAV (-♦-) and j+AV (-◊-) rats (n = 5 in each group) treated with irinotecan 20 mg/kg/day i.p. x3 doses. Each weight measurement was taken 24 hours after the dose of irinotecan was administered. Points represent mean ± S.E. **p<0.01, ***p<0.001.

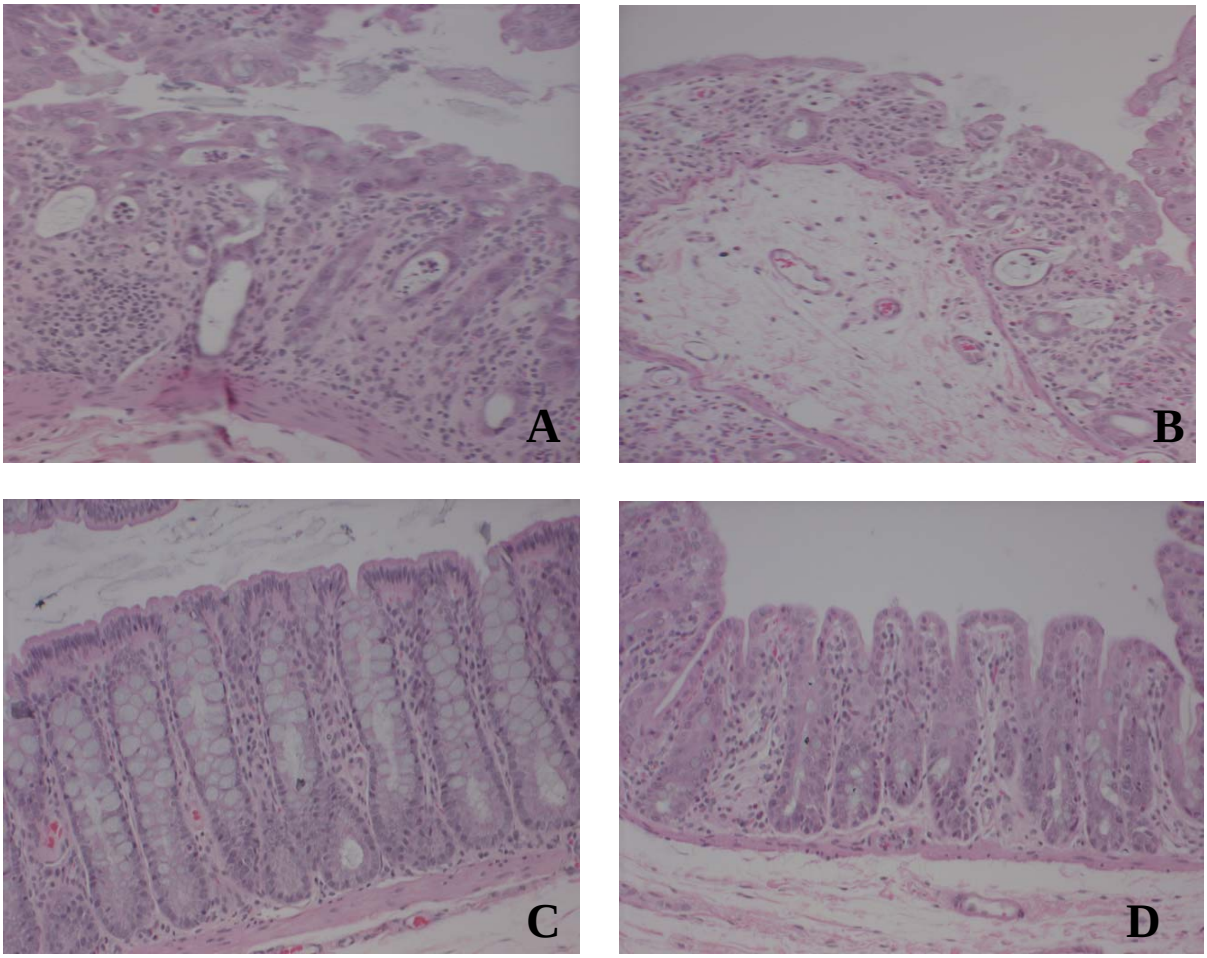

Figure 4.8 Representative micrographs (10x magnification) of j/j AV colon (A) and cecum (B) and j+AV colon (C) and cecum (D). Tissues were extracted upon euthanizing the rats, 24 hours after the third dose of irinotecan, 20 mg/kg/day i.p. A description of the observed toxicity on each group of rats is given under RESULTS.

CHAPTER 5

THE INFLUENCE OF PIPERINE ON SN-38 GLUCURONIDATION AND CYTOTOXICITY IN HT-29 COLON CARCINOMA CELLS

A. ABSTRACT

Diarrhea is a main, dose-limiting adverse effect experienced by some patients treated with the chemotherapeutic agent, irinotecan. Poor intestinal UDP-glucuronosyltransferase (UGT) activity may be the antecedent factor to the development of diarrhea in patients, as SN-38, the active metabolite of irinotecan, is detoxified through glucuronidation. The purpose of these experiments was to assess whether lower SN-38 glucuronidation rates, via UGT inhibition, corresponded to higher rates of cytotoxicity *in vitro* in a colon cancer cell line. Several substrates were screened for their ability to inhibit SN-38G formation in HT-29 cells. Borneol did not inhibit SN-38 glucuronidation, and the inhibition achieved with valproic acid was not consistent over time or concentration. Piperine, a component of black pepper, exhibited a concentration-dependent, competitive inhibition of SN-38 glucuronidation. However, piperine did not increase the cytotoxicity of SN-38 in HT-29 cells, and actually may have prevented it, as determined by a slight increase in SN-38 IC_{50} in cells treated with piperine. Potential reasons for this apparent discrepancy may be that the residual catalytic activity after piperine treatment is sufficient to protect the cells from harm or that piperine may prevent uptake or facilitate efflux of SN-38 from the cells. Alternative research avenues in *in vitro* systems should be explored to support or confirm the intestinal SN-38 protection theory, such as transfection of UGTs into a cell line naturally devoid of them or inducing UGTs in a cell line with poor expression.

B. INTRODUCTION

Irinotecan is an anti-cancer agent given to patients suffering from a multitude of solid tumors. Its chemotherapeutic effects are mediated through the active metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38), which is then detoxified by glucuronidation (Kawato et al., 1991). SN-38G, the resultant glucuronide, is formed in humans by UDP-Glucuronosyltransferases (UGT) 1A1, 1A7, and 1A9 (Figure 1.1) (Gagne et al., 2002; Tallman et al., 2005). All three compounds are excreted into the bile, where irinotecan may be hydrolyzed to SN-38, and all SN-38G is converted back to SN-38 via lower intestinal β-glucuronidase (Slatter et al., 2000; Wadkins et al., 2004). High intestinal concentrations of SN-38 have shown to be a predisposing factor for the development of diarrhea, a major dose-limiting side effect of irinotecan (Gupta et al., 1994; Kehrer et al., 2001; Horikawa et al., 2002). However, enteric cells possess UGT1A1 and 1A9 and gut epithelium has demonstrated catalytic turnover of SN-38 (Strassburg et al., 1998) (Chapter 2). Thus, intestinal UGT activity may be an important predictor in gastrointestinal toxicity, as proposed by us and others (Tukey et al., 2002).

Testing this hypothesis with a complex *in vivo* model may preclude mechanistic insight. *In vitro* models have demonstrated the protective effects of UGTs for SN-38 and other toxic substances. HT-29 colon carcinoma cells required much higher concentrations of mycophenolic acid (MPA) to reduce their cloning efficiency relative to EMT6 cells. The former cells efficiently glucuronidated MPA, whereas the latter cells did not (Franklin et al., 1995; Franklin et al., 1996). A subculture of a lung cancer cell line (PC-7/CPT) continuously exposed to irinotecan was found to be resistant to SN-38 and to have upregulated levels of UGTs relative to parent cells. The resistance of PC-7/CPT was abolished when a

competitive UGT inhibitor was co-incubated (Takahashi et al., 1997). The simultaneous administration of high-dose propofol and SN-38 resulted in a 50% reduction in the concentration of SN-38 needed to inhibit HT-29 proliferation. After further investigation, the increase in SN-38 toxicity with propofol was attributed to competitive inhibition for glucuronidation, as propofol glucuronides were detected in media and propofol significantly raised intracellular SN-38 concentrations (Cummings et al., 2003). Similary, in another investigation, intracellular SN-38 levels correlated with toxicity in HT-29 cells (Kobayashi et al., 1999). Overall, mounting evidence suggests that *in vitro*, conjugation can reduce intracellular levels of SN-38 and spare the cell from death.

Thus, the goal of these experiments was to modulate glucuronidation in a colon-derived immortal cell line to lend support to the theory that intestinal UGTs are an important factor in the incidence of toxicity. To do this, several compounds with notable UGT inhibitory capacity (valproic acid, piperine, and borneol) were screened for their effect on SN-38 glucuronidation in HT-29 cells (Gupta et al., 1997b; Lambert et al., 2004; Dong et al., 2006). Although significant inhibition was achieved by piperine, a component of black pepper, it did not facilitate SN-38 induced cytotoxicity. Mitigating considerations for these observations are discussed.

C. MATERIALS AND METHODS

Materials. HT-29 cells were obtained through the Lineberger Comprehensive Cancer Center Tissue Culture Facility at UNC. Cell culture media, trypsin, and antibiotics were obtained through Gibco (Invitrogen, Carlsbad, CA). Charcoal/dextran treated fetal bovine serum was purchased from Hyclone (Logan, UT). SN-38 was hydrolyzed from irinotecan, which was graciously provided by Dr. Robert Kelly (Pfizer, Kalamazoo, MI). A description of the hydrolysis process is provided in another text (Ni, 2001). Procedures detailing SN-38G cleavage from SN-38G methyl ester (also provided by Dr. Kelly) and quantitation are given in Chapters 2 and 3. Piperine, valproic acid, borneol, and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals involved in high performance liquid chromatography (HPLC) analysis were reagent-grade and purchased through commercial vendors.

Cell Culture. HT-29 cells were grown at 37° C in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal serum albumin, 100 u/mL penicillin, and 100 µg/mL streptomycin at 5% CO_2. Cells were passaged at 90% confluence, with passage numbers 121-149 used in all experiments.

Inhibition Screen. Dimethyl sulfoxide (DMSO), valproic acid, borneol, or piperine stocks were added to media at to yield the following concentrations: 0.1% DMSO; 20, 50, 100 µM piperine; 1 mM borneol; or 1, 5, or 10 mM valproic acid (Figure 5.1). Media (3 mL) was then added to HT-29 cells (80% confluent) plated in 60 mm dishes (in duplicate for each inhibitor concentration). After a pre-incubation period of 30 min, SN-38 (10 µM) was added to each dish. Aliquots (300 µL) of media were removed from one plate at 0.5, 2, and 8 hours after the addition of SN-38 and at 1, 4, and 24 hours in another plate. After the 8 and 24 hour

time points, cells were scraped into 1 mL phosphate-buffered saline (PBS) and sonicated. An aliquot of cell sonicate was used to assess the total protein content of the plate, as determined by the Bradford assay using bovine serum albumin as a standard.

Acetonitrile (900 μL) and perchloric acid (5%, 5 μL) containing irinotecan (30 ng) as an internal standard were added to each tube of media. After centrifugation at 15,000g for 10 minutes, the aceonitrile layer was saved and blown down under a stream of nitrogen. The residue was then reconstituted in 30% methanol/ 100 mM ammonium acetate (200 μL), and 30 μL was injected onto HPLC to measure SN-38G media concentrations.

Additional experiments with different piperine concentrations were performed as above, with some procedural differences. In the first experiment, piperine (0, 75, 100, or 150 μM) was incubated with cells for up to 48 hours in an attempt to discern whether or not piperine was a competitive UGT inhibitor (Figure 5.2). At 3, 7, 23, and 47 hours after adding piperine, SN-38 (5 μM) was added to each dish for 1 hour. At 4, 8, and 24 hours, an aliquot of media was removed for analysis from all plates, and fresh media was replaced, with or without piperine. Plates with 0 and 75 μM piperine were treated for the full 48 hours with piperine; plates with 100 μM piperine were treated from 0-8 hrs, and plates with 150 μM were treated from 0-4 hrs. For these high concentrations of piperine, after 4 and 8 hours, media without piperine was replaced for the remaining 44 and 40 hour experimental period. At 48 hours, an aliquot of media was removed, and the cells were scraped into PBS, sonicated, and analyzed for protein content. A portion of the cells (300 μL) were then analyzed similarly to media.

In a second experiment, piperine-containing media (0, 20, 50, or 75 μM) was added to dishes thirty minutes prior to SN-38 (5 μM). Two aliquots each of media and cell

sonicates were saved (300 μL per aliquot) after a four-hour SN-38 incubation. After centrifugation, the acetonitrile from the duplicate samples was pooled and evaporated.

HPLC Analysis. The HPLC system used to inject, separate, and detect analytes was as described in Chapter 2, however an SIL10A autosampler was used (Shimadzu, Tokyo, Japan). Mobile phase consisted of 37% methanol/100 mM ammonium acetate and 5 mM tetrabutylammonium sulfate, pH 4.9 run at 1.5 mL/min. Excitation and emission wavelengths were held constant at 229 and 420 nm, respectively, and SN-38 was not monitored. Standards were prepared in media and prepared similarly to samples. Standard curves were linear and reproducible (coefficient of variance ≤ 15% for replicates) from 1.4 ng SN-38G/mL (0.13 ng on column) to 125 ng SN-38G/mL (5.6 ng on column). A sample chromatogram is given in Figure 5.3.

Cytotoxicity assay. HT-29 viability after the addition of SN-38, piperine, or a combination of the two was determined by the methylthiazolyldiphenyl-tetrazolium (MTT) assay. Formazan, a purple product of the reduction of MTT by mitochondrial dehydrogenases, will accumulate in viable cells. After lysing these cells, formazan can be solubilized and quantified (Marshall et al., 1995). HT-29 cells were seeded in 96 well plates (5,000 cells/well) and were cultured for 2 days. On the third day, media (100 μL) containing piperine (0-75 μM) or SN-38 (0-13 μM) or a combination of both was added for 8-48 hours. When piperine and SN-38 were co-incubated, piperine containing media was initially added to all wells, followed by the addition of SN-38 stock in thirty minutes. After these incubations, media was removed and replaced with fresh media containing MTT (0.5 mg/mL). Following a three hour incubation, the media was removed, and cells were lysed with 75% isopropyl alcohol/25% 0.04 N hydrochloric acid (200 μL). Absorbance (abs) was

determined at 595 nm with a 96-well plate reading spectrophotometer (Biorad). Background absorbance (wells with no MTT) was subtracted from all values, and the percent viability in the presence of each drug concentration was normalized to the viability attained with no piperine, SN-38 or combination given as:

$$\text{Viability (\%)} = 100 \times \frac{\text{Abs}_{595,\text{ treated}} - \text{Abs}_{595,\text{ no MTT reagent}}}{\text{Average } (\text{Abs}_{595,\text{ no treatment}} - \text{Abs}_{595,\text{ no MTT reagent}})}$$

The percent viability at each SN-38 concentration (with or without piperine) was input data for an inhibitory effect model using nonlinear regression analysis software (Winnonlin; Pharsight, Mountain View, CA), and SN-38 IC_{50} values (concentration that induces 50% of cell death) were found by iteration:

$$\text{Inhibition (\%)} = \text{Maximal Inhibition (\%)} \times \left(1 - \frac{C}{C + IC_{50}}\right)$$

In a similar set of experiments designed to assess the influence of treatment protocol upon the S-phase specific toxicity of SN-38, piperine (0 or 50 μM) was coadministered with SN-38 for 24 hours. In some plates, piperine (0 or 50 μM) was incubated for an additional 24 hours after SN-38 was removed. After piperine removal, all cells were subjected to a drug-free period of 24-48 hours prior to MTT analysis.

D. RESULTS

UGT catalysis of SN-38 was detected in HT-29 cells, with roughly 5% of SN-38 (10 μM) glucuronidated in 24 hours. The effect of various UGT inhibitors on SN-38 glucuronidation is given in Figure 5.4. Valproic acid generally inhibited SN-38 glucuronidation, however, inhibition was variable over time and the strength of inhibition was modest (average of 25% inhibition) despite high concentrations of valproic acid (10 mM). Co-incubation of SN-38 with borneol (1 mM) did not reduce the glucuronidation rate of SN-38 relative to controls over this same period. Piperine consistently inhibited SN-38G formation, with 20 μM piperine reducing SN-38G formation by an average of 43%, 50 μM reducing the rate by 54%, and 75 μM by 75%. Thus, piperine was chosen to modulate glucuronidation and to test its effects on SN-38 cytotoxicity.

In order to attain a satisfactory concentration-response curve for SN-38 toxicity in HT-29 cells, a 48-hour incubation with the drug was necessary. A one-day incubation did not achieve sufficient cell killing at high SN-38 concentrations, which precluded estimation of a reliable IC_{50} value (data not shown). Accordingly, the cytotoxicity of piperine in HT-29 cells was assessed at 48 hours (Table 5.1). Concentrations of piperine greater than 75 μM elicited significant cell death at 48 hours, but not at 4 or 8 hours. Because higher piperine concentrations also inhibited more SN-38G formation (Figure 5.4), short-term, high-dose piperine exposure was evaluated for its inhibitory capabilities over 48 hours (Figure 5.5). After removal of 150 μM piperine at 4 hours or 100 μM at 8 hours, SN-38 glucuronidation rates increased, indicating that piperine must be present in the media for inhibition (i.e. likely acts through competitive inhibition). By observing the increase in glucuronidation rate over time in the 100 and 150 μM piperine treated cells relative to control, piperine treatment may

have induced UGT1A expression (Figure 5.5). Continual exposure to piperine ensured inhibition over a 48 hour period, as evident in the studies with 75 μM piperine. As high concentrations of piperine were too cytotoxic for prolonged exposure to 48 hours, concentrations greater than 75 μM piperine were not utilized in further inhibition or cytotoxicity assays.

The effect of piperine at non-toxic concentrations was then assessed for its inhibition in HT-29 cells (Table 5.2). A stepwise increase in SN-38 glucuronidation inhibition was seen from 20-75 μM, similar to that observed in the pilot inhibitor screen study (Figure 5.4). By analyzing cell content, SN-38G was shown to be efficiently extruded into media and total glucuronide formed (sum of media and cells) was less in piperine-treated cells. Subsequently, the cytotoxicity of SN-38 was measured in the presence and absence of piperine, 20-75 μM (Table 5.3, Figure 5.6). Highest toxicity (i.e. lowest IC_{50}) was observed in cells treated with SN-38 alone. A 2.3-fold higher SN-38 concentration was needed to kill 50% of cells treated with 50 μM piperine versus 0 μM piperine. The additional experiment comparing IC_{50} values for SN-38 in cells treated with 0 or 50 μM piperine with a washout period showed that piperine had no effect on toxicity (data not shown).

E. DISCUSSION

In this study, several compounds were examined for their capability to inhibit SN-38 glucuronidation *in vitro*. In rat hepatocytes co-incubated with borneol (1 mM), virtually all glucuronidation of ibuprofen, benoxaprofen, and flunoxaprofen was abolished (Dong et al., 2006). Most findings of UGT inhibition with borneol are with UGT2B family substrates, for which it is also a substrate (Tian et al., 2005a). Previous studies report that borneol depletes UDP-glucuronic acid (UDPGA), the cofactor necessary for glucuronidation, presumably by efficient borneol glucuronidation (Watkins and Klaassen, 1982). Although this is an effect that should reduce glucuronidation of all compounds, borneol had no effect upon SN-38, a UGT1A substrate. The lack of an effect by borneol may be attributed to low expression of UGT2B isoforms in HT-29 cells, as glucuronides for UGT2B4, 2B7, and 2B15 probes were negligible after cellular incubations (Cummings et al., 2003). Valproic acid is a substrate for UGT1A6 and 1A9 and UGT2B7, albeit with poor affinity and turnover (i.e., mM Km values and high pmol/min turnover of conjugate) (Ethell et al., 2003). Valproic acid has been found to inhibit both UGT1A and 2B substrates, both competitively and non-competitively (Ethell et al., 2003). In rats, valproic acid raised systemic exposure to SN-38 by three-fold and lowered exposure to SN-38G by 99% (Gupta et al., 1997b). Despite the *in vivo* inhibition of SN-38G formation, the strength of the inhibition was not reciprocated in this cellular system. Piperine has demonstrated UGT inhibitory effects in microsomes, cells, and *in vivo*. It is glucuronidated, within several metabolic pathways (after oxidative metabolism), and has been found to deplete UDPGA (Bhat and Chandrasekhara, 1986; Singh et al., 1986). Piperine inhibits first-pass metabolism, concomitantly increasing bioavailability of some aglycones, and in HT-29 cells has demonstrated similar potent inhibitory effects upon UGT

substrates in this and other studies (Shoba et al., 1998; Lambert et al., 2004). The potency of inhibition of SN-38 glucuronidation and another UGT1A substrate, epigallocatechin-3-gallate, by piperine in HT-29 cells was similar in that piperine concentrations slightly greater than 20 μM reduced glucuronidation by 50% (Lambert et al., 2004). In this cell line, piperine concentrations of 100 μM or greater for extended periods caused toxicity, which is likely because of generation of free radicals and lipid peroxidation (Unchern et al., 1998). Although these concentrations more strongly inhibited glucuronidation, it was difficult to generate SN-38 concentration-response curves due to low viability in the control cells exposed to piperine (i.e. piperine with no SN-38).

The objective of the work described here was to support data showing that UGT inhibition by propofol in HT-29 cells predisposes cells to SN-38 toxicity (Cummings et al., 2003). The outcomes from these two studies were completely different, with the current study not supporting the intestinal UGT protection hypothesis. This may have arisen due to one or more differences between the experimental procedures employed. The IC_{50} for SN-38 in this study was 10-fold higher than that reported in another study in HT-29 cells (161 nM v. 16.5nM) (Cummings et al., 2003). SN-38 cytotoxicity is highly dependent on the dosing/recovery regimen employed, as the drug is S-phase specific in its effects. For example, treating cells with SN-38 for one day and waiting two days before performing the MTT assay lowered the IC_{50} to 15 nM (data not shown). The schedule employed for the MTT assay described in this work was chosen because of decreased variability at high SN-38 concentrations. In this experiment, regardless of the regimen used, piperine did not increase SN-38 mediated cytotoxicity in cells. Additionally, cytotoxicity measurements were different between the two studies. Selection of a poorly sensitive cytotoxicity assay may

yield completely different results from those of a more sensitive assay, and the degree of assay sensitivity is often drug/cell line dependent (Petty et al., 1995; Fotakis and Timbrell, 2006). However, it is likely that if a robust difference in SN-38 toxicity between piperine and control treated cells did exist, the MTT assay could discriminate this.

Another difference between this and a similar study showing that UGT inhibition in HT-29 cells induces toxicity is the choice of inhibitor (piperine versus propofol). As mentioned, the toxicity inflicted upon cells by piperine may be a complicating factor. Additionally, little is known about its effects upon the disposition of compounds, namely through its potential interaction with transport proteins. Should piperine inhibit the uptake of SN-38, glucuronidation of the compound might decrease (as shown in Table 5.2), yet because intracellular levels of the aglycone are low, toxicity would be reduced. The increase in IC_{50} for SN-38 toxicity shown in Table 5.3 may also be substantiated if piperine was found to facilitate the efflux of SN-38. Piperine has been found to inhibit P-glycoprotein transport of digoxin and verapamil in the Caco-2 system with IC_{50} values in the μM range (Bhardwaj et al., 2002). Although P-glycoprotein is not found in HT-29 cells and inhibition of efflux would predispose cells to toxicity, the interaction with other uptake/efflux transporters is highly conceivable (Guichard et al., 2005). Unfortunately, intra-and extra-cellular levels of SN-38 were not monitored in the current study due to modifications necessary for its detection via HPLC.

Overall, the reasons why this series of experiments with piperine did not support several other studies substantiating the intestinal UGT protection hypothesis is unknown. As the focus of these experiments was not to uncover why piperine did not increase SN-38 toxicity, but was to prove that intestinal glucuronidation modulates toxicity *in vitro*, it is

likely the outcome of this experiment will not be mechanistically explained. Instead, new methods to simplify the testing of this hypothesis should be explored. It may be possible to upregulate intestinal UGTs in a cell line possessing constitutively low levels. Inducing UGTs should increase IC_{50} values, in contrast to reducing them with an inhibitor. In Caco-2 cells treated with 50 μM chrysin, another phytochemical, for four days, SN-38G formation was increased by 16-fold relative to control cells in microsomes (data not shown). However, chrysin has been found to be a BCRP inhibitor, which may lead to the accumulation of intracellular SN-38 (Zhang et al., 2004). Alternative ways to increase UGTs *in vitro* would be through transfection of plasmids or adenoviral-mediated UGT gene expression. These approaches are more likely to be a more conclusive system, as less confounding factors are introduced versus the myriad of possible effects a foreign chemical may have on cellular function and homeostasis.

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Table 5.1 Percent of viable HT-29 cells as a function of piperine concentration (no SN-38). Cells were exposed to piperine for 4, 8, or 48 continuous hours. Viability was assessed by the MTT assay and normalized relative to viability measured in cells without piperine treatment. Data is presented as mean ± SE (6 replicates per concentration).

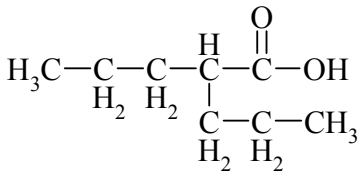
[Piperine] (μM)	Percent Viability at 4 hours	Percent Viability at 8 hours	Percent Viability at 48 hours
0	100 ± 2.0	100 ± 1.9	100 ± 2.7
10	101 ± 1.8	97.5 ± 1.9	118 ± 2.4
20	93.9 ± 2.3	97.1 ± 1.1	130 ± 1.2
50	110 ± 3.7	92.0 ± 2.1	100 ± 4.1
75	95.0 ± 3.7	83.9 ± 1.4	79.3 ± 2.8
100	97.9 ± 6.4	74.7 ± 2.8	58.9 ± 4.6
150	93.2 ± 3.1	56.9 ± 3.0	29.6 ± 0.79

Table 5.2 SN-38G formation in the presence of piperine, 0- 75 μM, in HT-29 cells over four hours. A total of 5.9 μg SN-38 was added to each dish at a concentration of 5 μM. Data are an average amount of SN-38G formed from three 60 mm dishes ± SE.

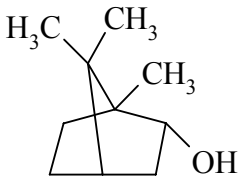
[Piperine] (μM)	SN-38G in media (ng)	SN-38G in cells (ng)
0	43.7 ± 6	10.8 ± 0.3
20	33.3 ± 6	3.5 ± 0.3
50	17.3 ± 2	1.4 ± 0.1
75	7.52 ± 0.3	0.81 ± 0.05

Table 5.3 IC_{50} values of SN-38 in the presence or absence of piperine. The SN-38 concentration ranged from 0-13 μM, and viability was determined in six wells for each concentration. Values are determined by non-linear regression software and the associated coefficient of variance is given in parenthesis.

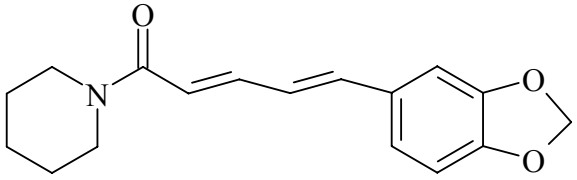
	0 μM piperine	20 μM piperine	50 μM piperine	75 μM piperine
IC_{50} (nM)	161 (15)	198 (15)	371 (16)	285 (15)



A



B



C

Figure 5.1 Chemical structures of the UGT inhibitors valproic acid (A), borneol (B), and piperine (C).

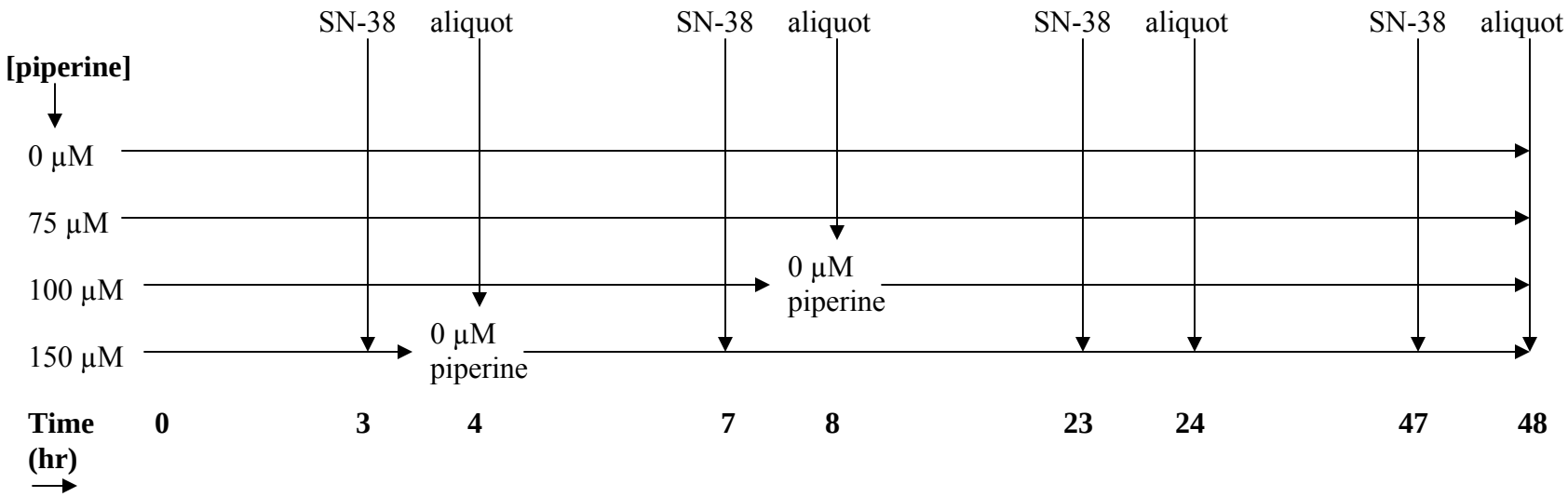


Figure 5.2 Experimental design to ascertain whether piperine is a competitive or noncompetitive inhibitor. Piperine (0, 75, 100, or 150 μM) was incubated with cells for up to 48 hours. At 3, 7, 23, and 47 hours after adding piperine, SN-38 (5 μM) was added to each 60 mm dish for 1 hour. At 4, 8, and 24 hours, an aliquot of media was removed for analysis from all plates, and fresh media was replaced, with or without piperine. Plates with 0 and 75 μM piperine were treated for the full 48 hours with piperine; plates with 100 μM piperine were treated from 0-8 hrs, and plates with 150 μM were treated from 0-4 hrs. For high concentrations of piperine, after 4 and 8 hours, media without piperine was replaced for the remaining 44 and 40 hour experimental period.

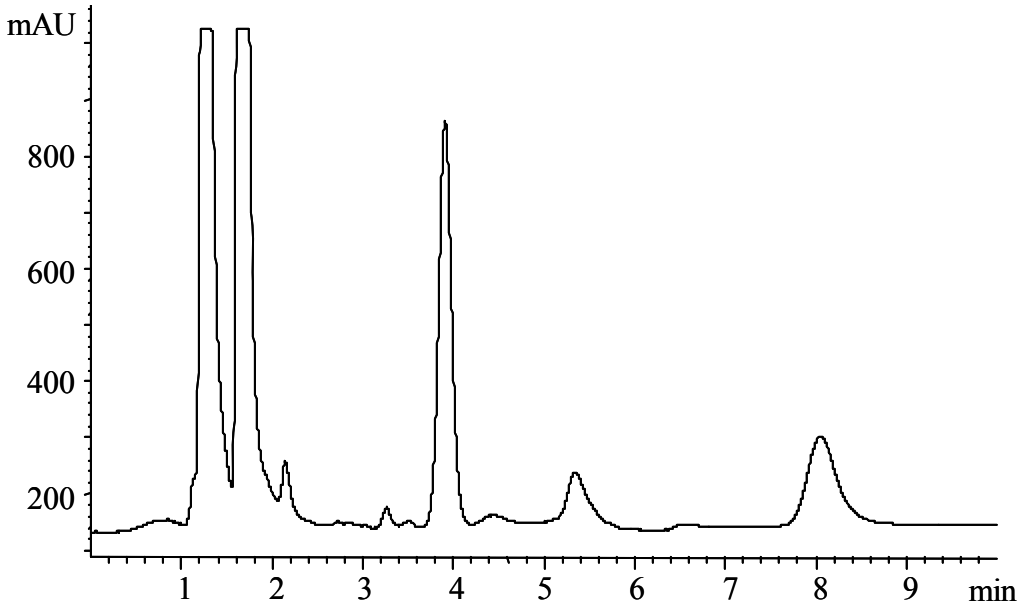


Figure 5.3 Representative SN-38G (125 ng/mL, 3.9 min) and irinotecan (internal standard, 30 ng, 8.1 min) chromatogram in cell culture media. Compounds were extracted from media and analyzed via HPLC with fluorescence detection.

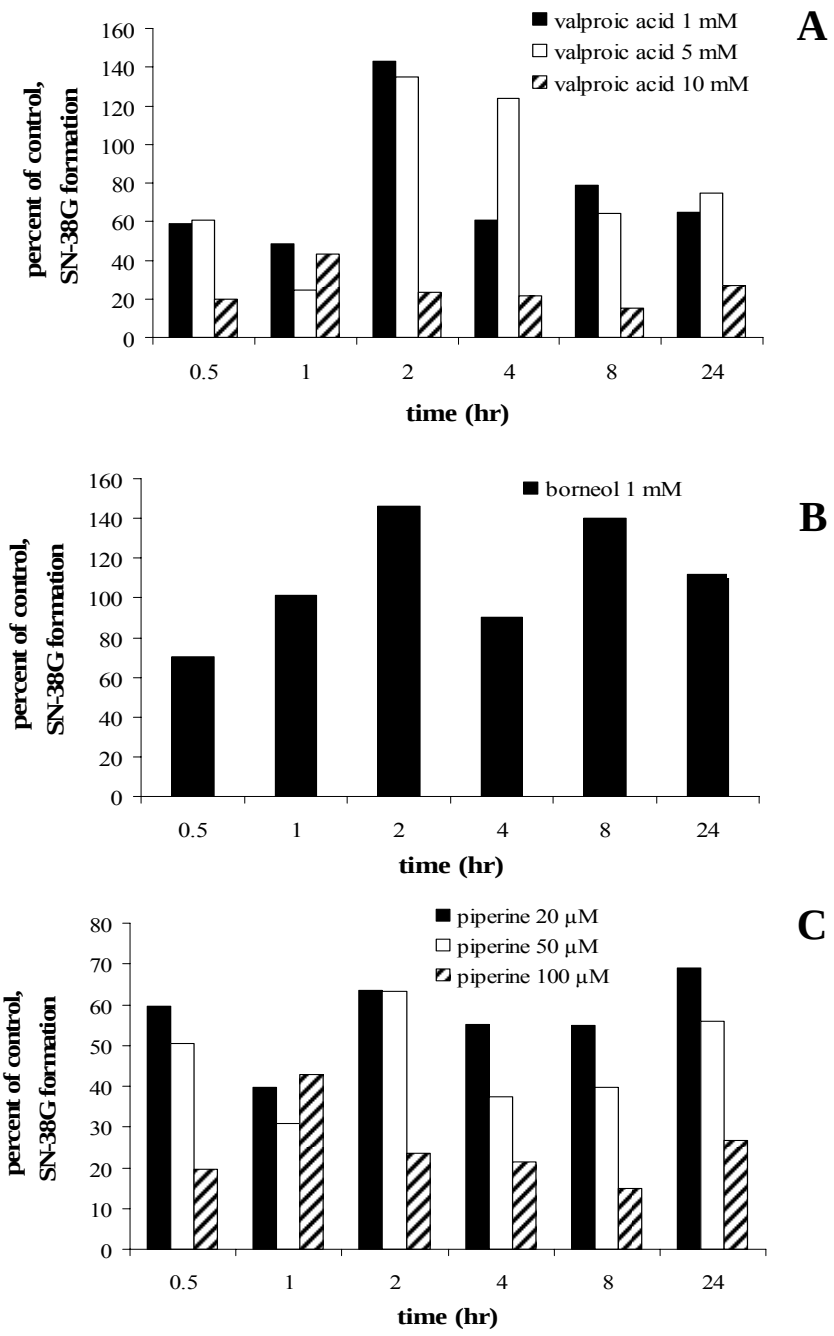


Figure 5.4 Time-dependence of valproic acid (A), borneol (B), and piperine (C) inhibition of SN-38 glucuronidation in HT-29 cells relative to control (DMSO 0.1%, 100% formation) as assessed by SN-38G release into media from 0.5-24 hour exposure to both SN-38 and piperine. Inhibitor concentrations are given in each legend. Bars indicate analysis of one aliquot/time point.

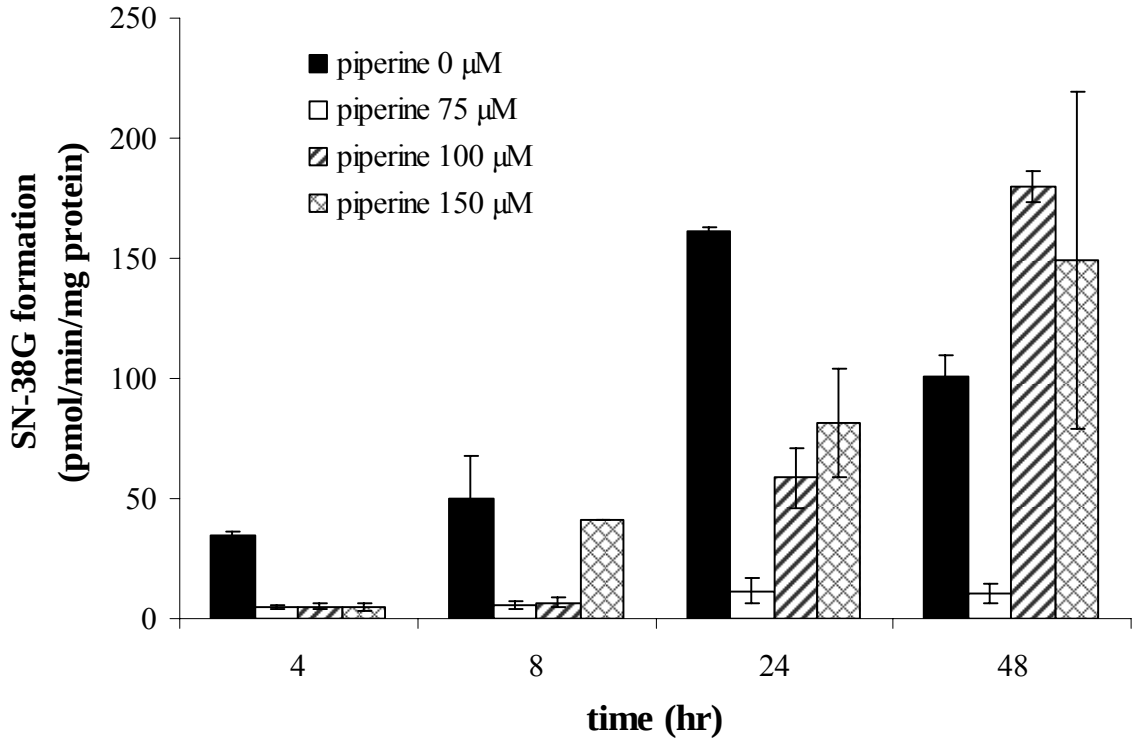


Figure 5.5 Effect of piperine on SN-38G formation in HT-29 cells. SN-38 (5 μM) was incubated for one hour at 3, 7, 23, and 47 hours following prior exposure to piperine. Plates contained either piperine 0 μM (0-48 hrs), 75 μM (0-48 hours), 100 μM (0-8 hrs, then 0 μM piperine from 8-48 hrs) or 150 μM (0-4 hrs, then 0 μM from 4-48 hrs). At 4, 8, 24, and 48 hours, an aliquot of media was saved for SN-38G analysis from all plates. Bars are mean velocity ± SE and corresponding concentrations are identified in the legend (n = 3 plates per concentration).

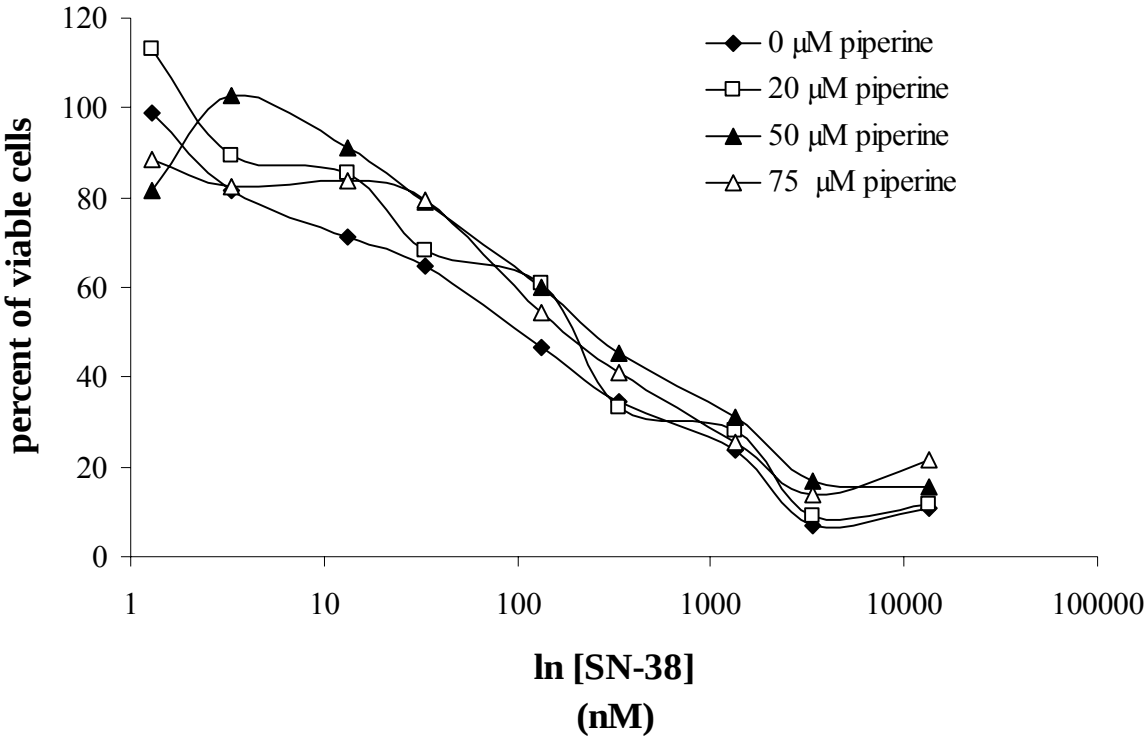


Figure 5.6 Concentration response curves for SN-38 in the presence and absence of piperine in HT-29 cells over 48 hours. Viability is relative to baseline (i.e., 100% viability at 0 ng/mL SN-38). Treatment is given in the legend, and each point represents the average viability from six replicates.

CHAPTER 6

CONCLUSIONS

The experiments conducted in this dissertation have advanced the understanding of intestinal glucuronidation and the role these metabolic enzymes play in the disposition and toxicity of a selected compound, 7-ethyl-10-hydroxy-camptothecin, or SN-38. SN-38 is the active metabolite of the anti-cancer topoisomerase I inhibitor, irinotecan, given to thousands of patients yearly as treatment for solid tumor carcinoma. SN-38 is metabolically detoxified by UDP-Glucuronosyltransferases (UGTs), which exist primarily in the liver, kidney, and intestine. Irinotecan/SN-38 has been extensively studied, and it is one of the first compounds where a dose-limiting adverse event (neutropenia) can be predicted in part by pharmacogenomic analysis (UGT1A1*28 homozygosity) (de Jong et al., 2005). A mechanistic basis for the incidence of the other main dose-limiting toxicity of irinotecan/SN-38, diarrhea, is much less clear. Investigations in elucidating the causative mechanisms behind the incidence of diarrhea have focused broadly upon the reduction of luminal SN-38 available to the enterocytes (Takasuna et al., 1996; Kehrer et al., 2001; Horikawa et al., 2002; Innocenti et al., 2004b). In the literature, intestinal UGT capacity has been largely ignored as a crucial factor in protecting or predisposing patients to SN-38-induced diarrhea. These enzymes afford a local means of SN-38 detoxification, which may be particularly important in the colon, due to the high level of unconjugated SN-38 in the intestinal lumen via effective β-glucuronidase cleavage of all luminal SN-38 glucuronide (SN-38G). Thus, we hypothesized that intestinal UGT expression may mediate diarrhea, which then guided the experiments in this text.

Prior to this dissertation research, most investigators utilized the ionized, carboxylate form of SN-38 for *in vitro* glucuronidation experiments, due to its increased solubility, relative to the closed, lactone form. SN-38 carboxylate is not active and displays differing

pharmacokinetics and affinities for transport proteins from the lactone form. As such, using the carboxylate as a surrogate for the pharmacologically and physicochemically distinct lactone form of SN-38 was a considerable assumption. Thus, studies to decipher differences in UGT isoform specificity and tissue glucuronidation for these two forms were formulated and conducted. Experiments involved assessing the stability of the forms in various *in vitro* matrices, conducting glucuronidation assays, and using pharmacokinetic modeling to recover pure rates of elimination for each form. This study also identified the rat UGT isoforms responsible for SN-38 conjugation. The very high rate of SN-38G formation by the intestinally-expressed UGT1A7 supports the finding that the greatest rates of SN-38 glucuronidation occur in the rat intestine (Shelby et al., 2003). Additionally, intestinal rat UGT1A7 expression may explain why Sprague-Dawley rats were refractory to diarrhea when treated with extremely large doses of irinotecan (200 mg/kg/day) (data not shown). Results from this study also indicated that SN-38 lactone conjugation proceeded at a much faster rate relative to the carboxylate in virtually all matrices, so using carboxylate-derived intrinsic clearance values or *in vitro-in vivo* scaling with such data is not accurate. Both forms were found to be glucuronidated by the same UGT isoforms in a similar rank order. Given the regional expression of several UGT1A isoforms, this is important, and indicates that when comparing SN-38G formation rates between tissues or individuals with differing levels of UGT expression, the carboxylate is a suitable surrogate for the poorly-soluble and toxic SN-38 lactone (Chapter 2).

As an extension of the hypothesis that intestinal UGTs mediate diarrhea in patients, we surmised that patients with low intestinal UGT expression might be susceptible to diarrhea while those with higher expression might be spared this adverse effect. For this to

be feasible, significant variability in intersubject intestinal SN-38G formation must exist. Therefore, a bank of normal duodenal, jejunal, and colon samples was screened, with variability in SN-38 glucuronidation that ranged 8, 37, and 36-fold in the intestinal regions, respectively. This variability is on par with that observed with SN-38 in the liver (Iyer et al., 1998). These rates were also highly correlated with UGT1A1 and UGT1A9 expression, as assessed through probe substrate glucuronidation. Preliminary evidence from this study suggested that homozygosity for UGT1A1*28 did not predict low SN-38 glucuronidation rates, indicating that analysis at the protein level is a better predictor of SN-38 conjugating ability in the intestine relative to UGT1A1 genetic analysis. Currently, UGT1A1*28 has not reliably been associated with intestinal toxicity status, while there is only one study assessing UGT1A9 promoter genotypes, which found an association between one polymorphism and diarrhea (Marcuello et al., 2004; Carlini et al., 2005). The results of the current study indicate that UGT1A9 may play a primary role in the intestinal detoxification of SN-38. In fact, there have been no *in vitro* studies assessing a possible correlation between hepatic UGT1A9 expression and SN-38 glucuronidation. In addition to the variability found in intestinal conjugation of SN-38, this work indicates that efforts should be directed towards determining the influence of UGT1A9 polymorphisms and expression upon systemic and intestinal SN-38 disposition and toxicity (Chapter 3). Further, a large study whereby the colonic glucuronidation of SN-38 is correlated with the incidence diarrhea in patients receiving irinotecan is obviously needed to ultimately test the influence of intestinal UGTs on regional toxicity.

Due to a lack of access to patients for the above-mentioned clinical study, we tested the hypothesis that intestinal UGTs mediate irinotecan/SN-38-induced diarrhea utilizing a

unique, genetically-modified Gunn rat model. Hepatic glucuronidation was reconsitituted in the UGT1A-deficient Gunn rat via adenoviral delivery of UGT isoforms typically expressed in a heterozygote Wistar (j+AV). This reconstitution slightly "overshot" UGT levels found in the livers of j+AV rats, although systemic exposure of SN-38 and SN-38G was higher and lower in the transfected Gunn rats (j/jAV), respectively, after a dose of irinotecan. Regardless of this, similar levels of irinotecan and its metabolites were found in bile of both groups of rats. After repeated administration of irinotecan, j/jAV rats had high rates of weight loss, gross histopathological changes in the colon and cecum, and severe diarrhea. Following a similar irinotecan regimen, j+AV rates had modest rates of weight loss, and had no evidence of intestinal tissue damage, manifest by normal cecum and colon tissue structure and no diarrhea. Overall, these findings indicate that intestinal glucuronidation is critical to preventing SN-38-induced intestinal damage and diarrhea (Chapter 4). Future studies with this animal model should focus upon the influence of UGT expression upon transporter protein levels. Members of the multidrug resistance-associated protein family appear to be regulated, in part, by intracellular concentrations of glucuronides in rats, as shown in studies with bile-duct ligated and MRP2-decifient rats (Ogawa et al., 2000). Enterocytes from the j/jAV and j+AV rats should contain different levels of glucuronide conjugates, which may cause subsequent alteration of transporter expression. As SN-38 and SN-38G are both substrates for similar transporters, altered transporter expression may also influence toxicity profiles.

The influence of UGT inhibition on SN-38 cytotoxicity was studied in HT-29 colon carcinoma cells. To validate the hypothesis, UGT inhibition via the phytochemical piperine, would increase toxicity relative to control-treated cells. Although an 85% decrease in SN-38

glucuronidation at the highest concentration of piperine was achieved, these cells were not predisposed to toxicity. Several reasons for this lack of relationship were previously outlined, and include possible transport modulation by piperine or sufficient residual glucuronidation to protect cells despite significant inhibition. In hindsight, this study is frought with too many unidentified variables to discount the hypothesis. Future *in vitro* studies should include well-studied inhibitors and inducers, or cleaner, less complex systems such as single interfering RNA or UGT transfection of cells, which may simplify analysis (Chapter 5).

Intestinal UGT expression may be crucial in mediating both toxicity and efficacy for a number of compounds, though only one compound, SN-38, was explored in this dissertation research. For instance, mycophenolic acid (MPA), the prodrug of the immunosuppressive agents mycophenolate mofetil and mycophenolate sodium, is eliminated primarily through glucuronidation and causes severe diarrhea in significant numbers of patients (Wang et al., 2004). In j/jAV rats, MPA systemic exposure was similar to that in control rats with both hepatic and intestinal conjugation (data not shown). However, only j/jAV rats experienced diarrhea, indicating that intestinal UGT capacity is a better indicator of toxicity relative to pharmacokinetic parameters (Miles et al., 2006a). In addition to toxicity, intestinal glucuronidation may also influence the efficacy of certain medications. For example, ezetimibe is an anti-hyperlipidemic medication that binds to cholestrol transporters in the gastrointestinal tract, and is glucuronidated by UGT1A1 and 1A3. In a recent study, rifampin-mediated induction of intestinal UGT1A1, evaluated via pinch biopsies, reduced the chlosterol-lowering effect of ezetimibe (Oswald et al., 2006). Although hepatic UGTs are also likely influenced by rifampin, the idea that intestinal UGTs can shift

the equilibrium from active aglycone to purportedly inactive glucuronide and mediate the pharmacologic efficacy is highly feasible with ezetimibe (Oswald et al., 2006). Another example where intestinal UGTs may alter intestinal drug levels necessary for effect is with tegaserod, a serotonin agonist used to treat irritable bowel syndrome (Vickers et al., 2001). As substrates for glucuronidation are rarely subject to drug interactions in constrast to those metabolized by cytochromes P450, the former route of metabolism is desirable from a drug development standpoint (Williams et al., 2004). Thus, it is likely that in the future, intestinal UGTs will play a role in the detoxification and efficacy profiles to a wider range of new drug candidates.

Although significant effort was devoted to evaluating the role of intestinal UGTs in SN-38 metabolism and toxicity, we cannot discount that a considerable number of factors probably contribute to the occurance of this toxicity. As previously mentioned, transport proteins influence luminal concentrations of irinotecan and metabolites through biliary excretion, as well as the initial exposure of enterocytes to these compounds (Horikawa et al., 2002). Hepatic glucuronidation likely plays an indirect role in intestinal toxicity. High levels of hepatic glucuronidation may lead to increased intestinal levels of SN-38, as SN-38G is transported into bile and all SN-38G is hydrolyzed to SN-38 in the intestinal lumen (Chapter 4) (Chu et al., 1997; Slatter et al., 2000). A patient's diet may also greatly influence toxicity. For instance, ingestion of high amounts of basic antacids favor the existence of SN-38 carboxylate, which is poorly taken up by enterocytes relative to the lactone form (Takeda et al., 2001). Thus, multiple mechanisms are likely to influence toxicity, with intestinal UGT catalysis being one such mechanism.

In summary, the work presented here has advanced the understanding of intestinal UGT catalysis of SN-38, as well as the regional glucuronidation by specific UGT1A isoforms. Most importantly, through studies with a unique Gunn rat model, this dissertation supports the hypothesis that intestinal UGTs are an important mediator of SN-38-induced diarrhea. Should this hypothesis be conclusively proven in humans, steps could be taken to proactively exploit intestinal UGT expression. For instance, poorly absorbed UGT inducers may locally provide the increase in SN-38 glucuronidation needed to spare susceptible patients from diarrhea. Currently, chrysin, a phyochemical that we found would increase SN-38 turnover in Caco-2 cells by 15-fold, is being explored as such an agent in clinical trials (data not shown) (Tobin et al., 2006). Alternatively, physicians could excise intestinal biopsies and assess *in vitro* SN-38 glucuronidation similar to that in Chapter 3, prior to determining a patient's dose of irinotecan. Since most patients undergo diagnostic colonoscopy for colon carcinoma, the primary cancer treated with irinotecan, this procedure would not be unusual or intrusive. Ultimately, these strategies, based upon work performed in this thesis, may lead to drug treatment which every physician and patient desires—optimal pharmacological efficacy with minimal debilitating adverse effects.

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