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The Role of Bacteria Beta Glucuronidase Activity in Irinotecan-Induced Diarrhea

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THE ROLE OF BACTERIAL BETA GLUCURONIDASE ACTIVITY IN IRINOTECAN-INDUCED DIARRHEA

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Chemotherapy-induced diarrhea is a common side effect but is an understudied area in cancer management. This problem is especially significant with irinotecan hydrochloride (CPT-11), a prodrug of SN-38 (7-ethyl-10-hydroxy camptothecin) used in treating metastatic colon cancer as well as other types of cancers (e.g., lung, pancreatic). It is reported that more than 80% of patients treated with irinotecan experienced diarrhea, with up to 40% experiencing severe (grade 3 and 4) diarrhea. Different anti-diarrhea medications (e.g., loperamide, octreotide, tincture of opium) have been recommended, but diarrhea is still a major concern in many patients treated with irinotecan as they do not respond well to these treatments. Disposition of irinotecan has been well studied. After being administered through intravenous infusion, irinotecan is mainly activated to SN-38 by carboxylesterase (CE) and then detoxified to SN-38 glucuronide (SN-38G) by UDP-glucuronosyltransferase (UGT) in the liver. Irinotecan and its metabolites are secreted into the intestine through biliary excretion, where SN-38G can be hydrolyzed back to SN-38 through the action of β-glucuronidase (β-GUS) produced by the intestinal bacteria. Accumulation of SN-38 in the intestinal tract then causes intestinal mucosal injury,

resulting in delayed-onset diarrhea. Therefore, the purpose of this study is to determine the role of intestinal bacterial *β*-glucuronidase (*β*-GUS) in irinotecan-induced diarrhea.

Glucuronide hydrolysis by bacterial *β*-glucuronidase (*β*-GUS) is a well-known reaction. Typically, substrates will be incubated with fecal enzymes prepared from feces to determine bacterial *β*-glucuronidase (*β*-GUS) activity. Different methods have been reported for fecal enzyme preparation and different conditions have been used in incubating substrates with fecal enzymes. However, the method for enzyme preparation and the reaction condition were not standardized and different conditions may affect the GUS activity. Therefore, we first used a standard GUS substrate pNPG and a natural glucuronide wogonoside as the substrate to determine how enzyme preparation procedure and reaction conditions will affect GUS activity. Mouse, rat, and human feces were tested. Fecal S9 fractions were prepared with sonication and without sonication (suspension). Different reaction conditions including, buffer pH, Mg^{2+} concentration, and feces collection time were tested. The relative reaction activity of pNPG, reaction rates, and reaction kinetics for wogonoside were calculated. The results showed that sonication increased total protein yield during enzyme preparation. Fresh feces showed the highest hydrolysis activities when compared to feces collected after 24hrs and after 7 days. The pH of the reaction system increased the activity in 0.69-1.32, 2.9-12.9, and 0.28-1.56 folds for mouse, rat, and human at three different concentrations of wogonoside, respectively. The Vmax for wogonoside hydrolysis was 2.37±0.06, 4.48±0.11, and 5.17±0.16 μmol/min/mg and Km was 6.51±0.71, 3.04±0.34, and 0.34±0.047 μM for mouse, rat, and human, respectively. The inter-individual difference was significant (4-6 folds) using inbred rats as the model animal. Therefore, for an optimized hydrolysis reaction, sonication should be

included in preparation of the enzyme, fresh feces should be used to avoid activity loss and the buffer pH should be appropriate according to the specie of animal being used.

To determine if SN-38G hydrolysis by bacterial GUS activity can be altered, we prepared fecal enzymes using feces collected from rats at different condition including F344 rats at different ages (4 and 10 weeks old), different breed of rats (Pirc and F344), rats before and after irinotecan administration. The results showed that GUS activity is increased after the administration of irinotecan. Younger showed higher GUS activity when compared to the older ones and the increased in GUS activity noticed in the young rats increased by two folds after administration of Irinotecan. We therefore suspect that age may have a synergistic effect on diarrhea induced by CPT-11. Pirc rats, a type of rat that is known for inflammation of their colon mucosa showed higher GUS activity when compared with the wildtype (F344). This might be the reason why Pirc rat have high incidence of diarrhea when CPT-11 is administered to them.

Having determined that hydrolysis of SN-38G by bacterial GUS can be altered, we decided to see if this manipulation can result in reduced incidence of irinotecan-induced diarrhea. We established an irinotecan-induced diarrhea model using F344 rats. We used an herbal formula Xiao-Chai-Hu-Tang (XCHT) to treat the rats 3 days prior to CPT-11 injection and their fecal samples were collected for 9 days afterwards. The results showed that with XCHT treatment (1.8g/kg p.o.), bacterial GUS against SN-38G hydrolysis were significantly decreased (Vmax 0.4umol/min/mg) when compared to that of the rats without XCHT treatment (Vmax 1.3umol/min/mg). In vitro study also showed that XCHT can also inhibit GUS activity. Toxicity results showed that with XCHT treatment, irinotecan-induced diarrhea was attenuated. Rats given XCHT treatment showed only grade 1 diarrhea

for up to 9 days after CPT-11 injection and rats without XCHT treatment showed severe diarrhea (grade 3 and 4) by day 5 after CPT-11 injection. Therefore, it can be said that XCHT alleviates diarrhea by reducing the amount GI microflora available to deconjugate SN-38G to SN-38.

In conclusion, we were able to clearly show that bacterial GUS enzyme is the major culprit in the development of irinotecan-induced diarrhea and its manipulation can result in the alleviation of irinotecan-induced diarrhea This knowledge can be of help in the management of chemotherapy induced diarrhea by alleviating this dose limiting toxicity.

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LIST OF EQUATIONS

LIST OF ABBREVIATIONS

%	Percentage
<	Less Than
µG	Microgram
µL	Microlitre
µM	Micromolar
µm	Micron
µmol	Micromole
ALK	Anaplastic Lymphoma Kinase
AMP	Adenosine Monophosphate
BCRP	Breast Cancer Resistance Protein
BRAT	Bananas, Rice, Applesauce, And Toast
□	Temperature Degree in Celsius
CE	Carboxylesterase
CID	Chemotherapy Induced Diarrhea
CLint	Intrinsic Clearance
CPT-11	Camptothecin-11
CT	Computed Tomography
DPD	Dihydropyrimidine Dehydrogenase

DTO	Deodorized Tincture of Opium
EGFR	Epidermal Growth Factor Receptor
FDA	US Food and Drug Administration
FIG.	Figure
FU	5-Fluorouracil
G	Gram
GI	Gastrointestinal
GUS	Glucuronidase
IACUC	Institutional Animal Care and Use Committee
IBS	Irritable bowel syndrome
IFL	5-Fluorouracil and Leucovorin
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Monopotassium phosphate
KM	Michaelis Constant
KPI	Potassium Phosphate
MG	Milligram
Mg^{2+}	Magnesium ion
MIN	Minute

mL	Milliliter
Mm	Millimolar
NCI	National Cancer Institute
NCI CTCAE	National Cancer Institute Common Terminology Criteria for Adverse Events
OD	Optical Density
PGP	P-Glycoprotein 1
pNPG	4-Nitrophenyl-B-D- Glucopyranoside
RCF/G	Relative Centrifugal Force or G force
RPM	Revolutions per Minute
RT	Retention Time
S	Seconds
S9	Supernatant fraction obtained from an organ homogenate by centrifuging at 9000g
SN-38	7-Ethyl-10-Hydroxy Camptothecin
SN-38G	SN-38 Glucuronide
TKI(s)	Tyrosine Kinase Inhibitor(s)
UGT	UDP-Glucuronosyltransferase
UGT1A1	Uridine Diphosphoglucuronate-Glucuronosyltransferase 1A1

UPLC	Ultra Performance Liquid Chromatography
US	United States
UV	Ultraviolet
VIP	Vasoactive Intestinal Polypeptide
VMAX	Maximum Velocity
XCHT	Xiao-Chai-Hu-Tang
β-GUS	β-Glucuronidase

VITA

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CHAPTER 1

INTRODUCTION

Diarrhea is a very damaging condition in which patients experience watery stools or a continuous requirement of a bowel movement. This medical issue remains for some days and may disappear without using any medicine. There are two types of diarrhea: Acute & Chronic. Its acute form happens when the diarrhea remains for 1-2 days while the chronic form lasts for at least four weeks with three or more loose stools per day. A person can experience such condition because of irritable bowel syndrome (IBS), inflammatory bowel disease (Crohn disease and ulcerative colitis), malabsorption syndromes in which food cannot be digested and absorbed, and chronic infections by viruses or bacteria.

Chemotherapy-induced diarrhea is a common side effect but is an understudied area in cancer management (McQuade et. al., 2016; Richardson & Dobish, 2017; Tarricone et. al., 2016). This problem is especially significant with irinotecan hydrochloride (CPT-11), a prodrug of SN-38 (7-ethyl-10-hydroxy camptothecin) used in treating metastatic colon cancer as well as other types of cancers (e.g., lung, pancreatic). Irinotecan causes acute diarrhea (instantly after the use of drug) or in some cases diarrhea that is delayed. Its Immediate-onset acute form has cholinergic properties and has various symptoms or signs of cholinergic excess, involving, abdominal cramping, Rhinitis, Lacrimation & Salivation. (Arbuckle 2000). It is reported that more than 80% of patients treated with irinotecan experienced diarrhea, with up to 40% of severe (grade 3 and 4) diarrhea (Rougier & Mitry, 2001). Grade 1 & 2 Chemotherapy-induced diarrhea (CID) experienced during anticancer treatments can cause draw back in chemotherapy as this condition can lead to delayed

treatment (30 to 70 % of patients), dose reduction (21to 40 % of patients), or entire treatment discontinuation (4 to 20 % of patients) (Wu et. al., 2019).

In the liver, CPT-11 is largely activated to SN-38 by carboxylesterase (CEs) and then detoxified to SN-38 glucuronide (SN-38G) (fig 1) by UDP-glucuronosyltransferase (UGT). Then through biliary excretion, irinotecan and its metabolites are transported to the small intestine. In the intestinal tract, SN-38G is hydrolyzed back to SN-38 through the action of β-glucuronidase (β-GUS) produced by the intestinal bacteria (Yamamoto et. al., 2008). Then, accumulation of SN-38 in the intestinal tract produces intestinal mucosal injury, resulting in delayed-onset diarrhea. Although there are multiple sources that can contribute to intestinal SN-38 such as the direct conversion of CPT-11 to SN-38 by intestinal CEs (Khanna et. al., 2000). The excretion of SN-38G from the liver (via bile) to the intestine where it is hydrolyzed to SN-38 by β-GUS in the intestinal content (Kurita et. al., 2011) is one of the major ones and as such a major factor contributing to the incidence of diarrhea.

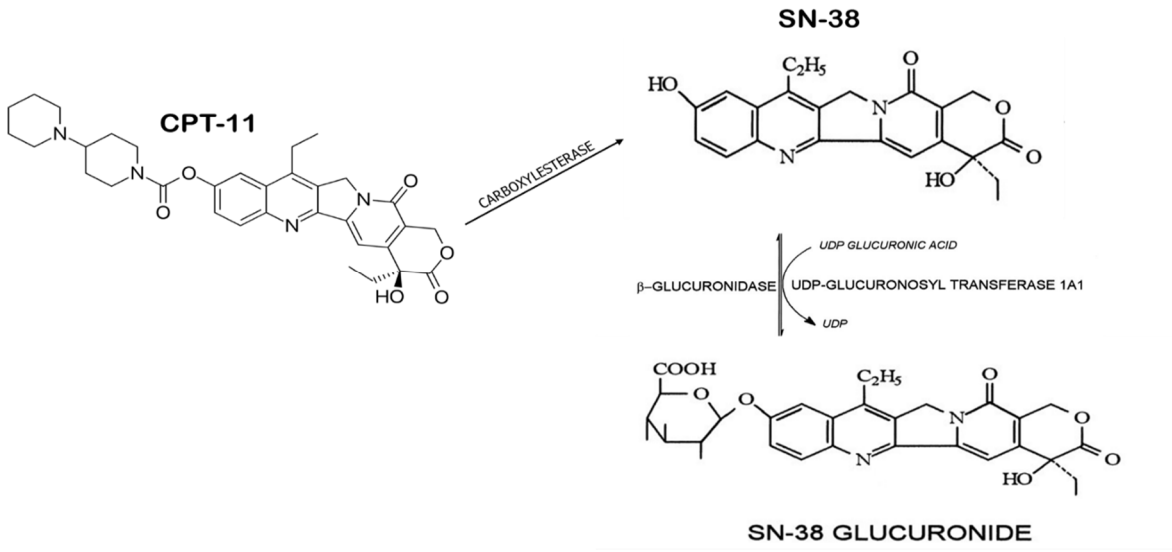


Figure 1: *Metabolism and Chemical Structures of CPT-11, SN-38 and SN-38 Glucuronide*

Different anti-diarrhea medications (e.g., loperamide, octreotide, tincture of opium) have been recommended, but diarrhea is still a major concern in a large number of the patients treated with irinotecan as they do not respond well to these treatments (Desai et. al., 2005; Jiang et. al., 2012)

There are a few researches on reducing SN-38 concentration by manipulating β-GUS activities. For example, the use of antibiotics (Alimonti et. al., 2003; Flieger et. al., 2007), use of probiotics (Mego et. al., 2015), use of bacterial β-GUS specific inhibitor (Fittkau et. al., 2004) and activated charcoal (Michael et. al., 2004). However, these approaches have several drawbacks in the sense that their results so far have been inconsistent, and most of them have single mode of action which might not be helpful since the pathogenesis of irinotecan-induced diarrhea is not yet thoroughly understood (Deng et. al., 2017). Also, Intestinal biota play essential roles in carbohydrate metabolism, vitamin production, and the processing of bile acids, sterols, and xenobiotics (Cummings & Macfarlane, 1997; Guarner & Malagelada, 2003).

The role of gut microflora in health has gained increasing attention in the past decades (Frick & Autenrieth, 2013; Tuohy et. al., 2005). One of the major benefits from intestinal microflora is that the commensal bacteria could generate beneficial metabolites, such as cancer preventive compound equol for daidzein and compound k from ginsenosides, to the host from dietary components through different biotransformation pathways (Yang et. al., 2015). These metabolites either accumulate in the gastrointestinal system or reach distant organs, which are associated with certain physiological or pathological effects (e.g., gastrointestinal inflammation and carcinogenesis (Zhang et. al., 2018). Glucuronide hydrolysis is one of the major metabolism pathways catalyzed by

microflora. Many nutritional dietary components exist as glycoside or glucuronide forms which must be hydrolyzed before absorption. For example, it is well-known that dietary components existing as glucuronide or glycoside forms, such as soy isoflavone genistin, daidzin, are usually hydrolyzed into aglycones by intestinal microflora to facilitate absorption (Coldham et. al., 2002). In addition, some important endogenous compounds (e.g., bilirubin) also undergo glucuronide hydrolysis pathways to form entero-hepatic recycling (Yang et. al., 2015; Rigato et. al., 2005), an important physiological phenomenon. Glucuronide hydrolysis has shown to be promising as pharmaceutical scientists explore ways to design glucuronide pro-drugs for colon drug delivery and also improve its water solubility as glucuronide hydrolysis by intestinal microflora is the key step towards the release of pro-drugs (Prijovich et. al., 2002).

It is well known that glucuronide hydrolysis is catalyzed by β-glucuronidases (GUS) expressed in intestinal microbiota and there are at least 279 GUS isoforms expressed in the gut microflora and the structures of human microbiome GUS was reviewed and the atlas was published recently (Pollet et. al., 2017). However, at functional level, glucuronide hydrolysis by microbial GUS is an under-studied area. GUS activity studies using in vivo model is complex and difficult because the hydrolysis product (i.e., aglycone) can be conjugated back into glucuronides by highly expressed UGTs in the GI tract. Enzyme-mediated reaction is a well-accepted in vitro model to evaluate enzyme activity. However, it is difficult to isolate or purify GUS from microflora. Recombinant microbial GUS might be available, but a single GUS isoform could not reflect the reaction occurring in the gut as there are so many isoforms. Theoretically, bacteria culture seems to be the best approach to study the total activity of GUS in microflora and it is doable to obtain gut bacteria from

feces. However, most gut bacteria can only be cultured under anaerobic condition and some bacteria are not culturable even under anaerobic condition (Galeano-Castañeda et. al., 2019), which limit the application. Thus, total proteins prepared from feces (e.g., fecal S9 fractions, fecal suspensions) are usually used as the enzyme sources in in-vitro studies in literature to determine glucuronide hydrolysis in the gut (Choi et. al., 2011; Phruksawan et. al., 2018). However, conditions used in these studies are not standardized and results from different research groups may not be comparable as different reaction conditions are used.

We hypothesize that bacteria beta-glucuronidase plays an important role in the irinotecan induced diarrhea and manipulation of intestinal bacterial GUS will alleviate the irinotecan-induced diarrhea. We conducted three specific aims to test our hypothesis;

- Establish an optimized method to evaluate microflora hydrolysis activity.
- Determine the conditions in which SN-38G hydrolysis by bacterial GUS can be altered.
- Determine diarrhea attenuation by manipulating intestinal bacterial GUS using an herbal formula Xiao-Chai-Hu-Tang (XCHT).

CHAPTER 2

LITERATUE REVIEW

2.1 Chemotherapy Induced Diarrhea

2.1.1 Overview

With roughly 14.1 million new cancer cases and 8.2 million cancer deaths in 2012 alone (Torre et. al., 2015), cancer is a leading cause of death worldwide (Torre et. al., 2015; Jemal et. al., 2011). Underlying factors such as population aging and growth coupled with the adoption of high-risk lifestyle choices such as smoking, physical inactivity, and westernization of diets have been identified to contribute to the increasing incidence of cancer worldwide (Jemal et. al., 2011). It is now projected that by 2025 more than 20 million people will be affected by cancer (Torre et. al., 2015).

Throughout the course of treatment, majority of cancer patients receive curative or palliative chemotherapeutic intervention (Benson et. al., 2004; Arafa et. al., 2010). Even though chemotherapy has greatly improved overall survival in many types of cancer, the clinical application of this otherwise beneficial therapy is greatly hindered by cytotoxic side-effects (Wang et. al., 2013).

Major complications of Gastrointestinal (GI) side-effects such as nausea, vomiting, ulceration, abdominal pain, bloating, constipation and, especially, diarrhea are causing delays, adjustments, and discontinuation of treatment while greatly affecting quality of life in several cancer patients (Benson et. al., 2004; Denlinger & Barsevick, 2009). Specific chemotherapeutic agents have been associated with heightened incidence of GI side-effects (Table 1), and these incidences have been reported to be as high as 40% in patients receiving standard dose chemotherapy and 100% in patients receiving high dose chemotherapy (McQuade et. al., 2014).

Table 1. Gastrointestinal Side Effects of Chemotherapy

Mechanisms	**Chemotherapeutic Agents**	**Cancer type**	**GI Side effects**
Alkylating Agents	Cisplatin	Lung, Breast, Stomach, Liver, Colorectal	Nausea, Vomiting, Diarrhea, Constipation
	Cyclophosphamide	Breast	Nausea, Vomiting, Diarrhea, Abdominal pain
	Oxaliplatin	Colorectal, Breast Stomach	Nausea, Vomiting, Diarrhea, Constipation
Antimetabolites	5-flurouracil	Breast, Stomach, Liver, Colorectal	Nausea, Vomiting, Diarrhea, Abdominal pain
	Capecitabine	Colorectal, Breast Stomach	Nausea, Vomiting, Diarrhea
	Gemcitabine	Lung, Breast	Nausea, Vomiting, Diarrhea, Constipation, Abdominal pain
	Methotrexate	Breast	Nausea, Vomiting, Diarrhea, Abdominal pain

Anthracycline	Doxorubicin	Breast, Lung, Liver	Nausea, Vomiting, Diarrhea, Abdominal pain, GI Ulceration
Immunomodulating Agent	Thalidomide	Kidney, Myeloma	Nausea, Vomiting, Diarrhea, Constipation
Mitotic Inhibitors	Cabazitaxel	Prostate	Nausea, Vomiting, Diarrhea, Abdominal pain
	Docetaxel	Prostate, Breast, Lung, Stomach	Nausea, Vomiting, Diarrhea
	Paclitaxel	Prostate, Breast, Lung, Stomach	Nausea, Vomiting, Diarrhea
	Vincristine	Breast, Lung	Abdominal pain, Constipation
Topoisomerase Inhibitor	Irinotecan	Colorectal, Breast, Lung, Stomach	Nausea, Vomiting, Acute and Delayed Diarrhea

McQuade et. al. 2016

CID can be experienced in patients with carcinoid tumors, carcinoid syndrome, gastrointestinal tumors, and hormone-producing tumors. Patients more susceptible to diarrhea are those undergoing high-dose chemotherapy and those receiving radiation therapy to abdominal and pelvic areas. Certain chemotherapy, biotherapy, and targeted therapies and regimens are associated with greater risk of diarrhea. In patients receiving

chemotherapy, the incidence of diarrhea can range from 50%–90%, and this condition can be a dose-limiting toxicity for certain chemotherapeutic agents.

Additionally, the prevalence of chronic post-treatment diarrhea amongst cancer survivors has been estimated to be as high as 49% with episodes continuing up to 10 years after treatment has ended (Denlinger & Barsevick, 2009). The pathophysiology of CID is likely to be complex, involving several overlapping inflammatory, secretory and neural mechanisms, therefore the underlying mechanism is unclear.

2.1.2 Clinical Manifestation

CID typically begins with an increasing frequency of bowel movements and/or a loosening of stool consistency. It is usually accompanied by excessive gas and/or intestinal cramping. As the CID progresses, it can become severe, with frequent watery stools. CRD can be debilitating and, in some cases, life-threatening. Findings in such patients include volume depletion, acute kidney injury, and electrolyte disorders such as hypokalemia, metabolic acidosis, and depending on water intake, hyponatremia (increased water intake that cannot be excreted because of the hypovolemic stimulus to the release of antidiuretic hormone) or hypernatremia (insufficient water intake to replace losses). In the setting of chemotherapy-induced immunosuppression, infection is worsened. For instance, life-threatening sepsis, can result due to breach of the intestinal mucosa. Given the risk for dehydration and infection, severe CID frequently requires hospital admission for adequate supportive care (Arbuckle et. al., 2000; Dranitsaris et. al., 2005; Carlotto et. al., 2013; Elting & Shih, 2004). Other emanations of CID include increased cost of care, reduced quality of life, treatment delays, and diminished compliance with treatment regimens,

which may compromise long-term results if the chemotherapy is being administered with curative intent (Arbuckle et. al., 2000).

2.1.3 Clinical Assessment

The severity of CID is often described using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) grades; the latest version (v5.0) is outlined in the table below (table 2). Severity is determined by the number of stools per day or an increase in ostomy output compared with baseline, the need for hospitalization, and the effect on activities of self-care. It is extremely critical to determine the patient's baseline bowel pattern when grading the severity of CID. Although adequate for study purposes, these criteria have been criticized because they do not consider the volume or the duration of diarrhea (Benson et. al., 2004). Other validated CID measurement scales have been developed but are not in general use (Mertz et. al., 1995).

Table 2: Common Toxicity Criteria for Diarrhea Grading

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Diarrhea	Increase of less than 4 stools per day over baseline. Mild increase in ostomy output	Increase of 4-6 stools per day over baseline. Moderate increase in ostomy output compared with baseline. Limiting	Increase of greater than 7 stools per day over baseline. Incontinence. Hospitalization. Severe increase in ostomy output compared to baseline. Limiting	Life threatening consequences. Urgent intervention indicated.	Death

	compared with baseline.	instrumental ADL – shopping, using phone, preparing meals	self-care ADL – bathing, dressing, using toilet, taking medication and not bedridden.		

Common Terminology Criteria for Adverse Events (CTCAE), Version 5.0, November 2017, National Institutes of Health, National Cancer Institute (NCI)

Evaluation of the patient with CID begins with a history to determine the severity according to the NCI CTCAE grades (table 2). The history should include questions concerning foods or drugs that might play a contributory role (which are usually avoided in patients with CID) (table 3) and the volume and duration of diarrhea should also be determined (32).

Because mucosal injury caused by chemotherapy may lead to a temporary lactase deficiency, the ingestion of milk-containing foods may be an important trigger for diarrhea and should be specifically queried.

Table 3: Food and Medications to Avoid in Chemotherapy Induced Diarrhea

Food Products	**Medications**
Milk and Dairy products	Bulk Laxatives
Spicy Foods	Promotility Drugs
Alcohol	Stool Softeners
Caffeine	Siberian ginseng and Plantago seeds
High Fiber and High Fat foods	High doses of vitamin C

Some fruit juices (e.g., prune and orange juices)	Green Tea

Wadler et. al 1998

Laboratory testing such as a complete blood count and a standard chemistry screening, should be used to address both the complications and causes of CID. Cultures of blood and stool, as well as a diagnostic testing for toxin-producing strains of *Clostridium difficile* are suggested for patients with severe (grade 3 or 4) diarrhea, persistent mild to moderate (grade 1 or 2) diarrhea, or diarrhea accompanied by neutropenia, fever, or blood in the stools (Uptodate).

Radiographic imaging is not typically needed in most patients with acute CID. However, for patients who have fever, bloody diarrhea or peritoneal signs, abdominal imaging (usually computed tomography [CT]) can be important to identify potential complications, such as bowel perforation, abscess, or neutropenic enterocolitis, as well as to dismiss causes of diarrhea not associated with chemotherapy (e.g., bowel ischemia). Surgical consultation may also be needed in these cases too. Abdominal radiographs, which can document the extent of stool, are useful in cases of suspected overflow diarrhea. Endoscopy is not indicated in most cases but should be considered for refractory cases and for patients who develop chronic diarrhea or bloody diarrhea (Uptodate).

2.2 Current Approaches for Chemotherapy Induced-Diarrhea Management

CID is a common major and dose-limiting toxicity in cancer patients and is most often linked with fluoropyrimidines and irinotecan. The frequency of severe (grade 3 or 4) diarrhea with these agents ranges from 5 to 44 percent, and the rates differ according to the dose, the specific agents administered, and the schedule of administration.

Treatment for CID includes nonpharmacologic and pharmacologic mediations to slow the diarrhea, as well as careful serial evaluation to assess response to therapy and to rule out other risk factors that would require targeted intervention or hospitalization.

2.2.1 Uncomplicated Chemotherapy Induced Diarrhea

Patients with mild to moderate (grade 1 or 2) diarrhea are usually classified as uncomplicated because they have no moderate to severe abdominal cramping, worse nausea/vomiting, decreased performance status, fever, frank bleeding, or suspected dehydration. This kind of patients can initially be managed conservatively at home with oral hydration, dietary modification, and antidiarrheal therapy (usually loperamide 4 mg to start, and then 2 mg after each loose stool). If symptoms persist after 24 hours but are not worsened, patients should be evaluated and assessed for hydration status, and neutropenia. The stool is sent for culture and diagnostic testing for toxin-producing strains of *Clostridium difficile*. In the absence of an added risk factor for complicated diarrhea, chest pain, or prior admission for CID, outpatient octreotide is used as the next step of treatment. Persistent mild to moderate diarrhea despite the addition of octreotide, any progression to severe (grade 3 or 4) diarrhea, or the development of an added risk factor for complicated diarrhea should prompt admission to the hospital for further evaluation and treatment (www.uptodate.com).

2.2.2 Complicated Chemotherapy Induced Diarrhea

Patients with complicated diarrhea are those that have grade 3 or 4 diarrhea, and those with grade 1 or 2 diarrhea associated with moderate to severe abdominal cramping, nausea/vomiting, deteriorating performance status, fever, sepsis, neutropenia, frank bleeding, or dehydration (Andreyev et. al., 2014). Most of these patients warrant admission

for intravenous fluids, octreotide, monitoring of cardiovascular status, serial assessment of electrolytes, and antibiotics, if needed. However, selected patients who have grade 3 diarrhea that has not yet been treated adequately with loperamide, who are well hydrated, and who have no worrisome signs or symptoms may be managed at home initially (Andreyev et. al., 2014).

If it has not been done, cultures of stool and diagnostic testing for toxin-producing strains of *Clostridium difficile* should be performed. For patients with bloody diarrhea, at least two potential pathogens, enterohemorrhagic *Escherichia coli* and Entamoeba histolytica, warrant additional testing. In addition to culture, bloody stools should be checked for Shiga toxin, and fecal leukocytes or lactoferrin, if available; if the fecal leukocyte/lactoferrin test is negative, then test for intestinal amebiasis should be done. Patients with fever, peritoneal signs, or bloody diarrhea should have an urgent computed tomography (CT) scan of the abdomen and pelvis, and a surgical consultation (Andreyev et. al., 2014). If the patient is neutropenic, avoidance of surgery is prudent, if possible. Blood cultures are recommended for neutropenic patients with CID or for those with fever or bloody diarrhea.

2.2.3 Reducing and Withholding Chemotherapy.

For most chemotherapeutic agents such as Irinotecan, treatment is usually withheld for grade 2 or worse diarrhea and is only resumed after toxicity resolves. Guidelines about CID published in response to septic deaths that occurred with weekly irinotecan chemotherapy concluded that patients must be free from diarrhea for at least 48 hours and not require antidiarrheals above baseline before retreatment with irinotecan (Benson et. al., 2004). This is a critical principle that is usually applied to treatment of patients with CID due to any

cytotoxic chemotherapy. There is need for dose reduction for any patient who develops grade 2 or worse diarrhea (particularly complicated diarrhea) during a prior cycle of therapy. The United States (US) prescribing information generally includes recommendations for dose modification for individual drugs. For instance:

- Patients taking Anti-epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs; afatinib, erlotinib, gefitinib) with grade 2 diarrhea, can continue the drug. But, if the patient does not respond to loperamide by 48 hours, the drug should be temporarily discontinued until diarrhea returns to grade 1, after which the drug can be resumed with a dose reduction. However, for grade 3 or 4 diarrhea, TKI is withheld until diarrhea reaches grade 1, then it is resumed usually with a dose reduction. If the diarrhea does not get back to grade 1 within 2weeks, the drug will be permanently discontinued.
- The US prescribing information for Irinotecan recommends that therapy for patients with grade 2 or worse diarrhea should be delayed for 1-2weeks to allow for recovery to baseline and if the patient has not recovered from this therapy-related toxicity after a 2-week delay, therapy should be discontinued.
- For lapatinib, the US prescribing information recommends that the drug be interrupted in patients with grade 3 diarrhea, or grade 2 complicated diarrhea, and resumed with a dose reduction after recovery to grade 1. In the case of grade 4 diarrhea, lapatinib is permanently discontinued.
- For Ceritinib - the anaplastic lymphoma kinase (ALK) inhibitor, the US prescribing information recommends that the drug be withheld for severe or intolerable

diarrhea, despite optimal antidiarrheal therapy, and resuming with a dose reduction once resolved.

- The US prescribing information for Capecitabine recommends that the drug be temporarily interrupted for grade 2 or worse diarrhea until resolved to grade 1.

2.2.4 Nonpharmacologic Measures

Initial nonpharmacologic measures include avoidance of foods that might aggravate the diarrhea (table 3) and aggressive oral rehydration with fluids that contain water, salt, and sugar (since glucose promotes intestinal sodium absorption) (Ilnyckyj, 2001). Due to possible mucosal damage and loss of absorptive surfaces, patients should ingest easy-to-digest food until the CID resolves. Patients should be advised to follow a "BRAT" diet (ie, bananas, rice, applesauce, and toast) (O'Brien et. al., 2005). Fresh fruits and vegetables should be avoided, as they will worsen diarrhea, except for bananas, which can be binding. High-osmolar dietary supplements should be avoided (Benson et. al., 2004). Patients with CID should follow a lactose-free diet until CID resolves as they may be temporarily lactose intolerant (Parnes et. al., 1994). Particularly if diarrhea is severe, a clear liquid diet can provide bowel rest and may decrease the volume of diarrhea. Alcohol and caffeine should be avoided, as they are dehydrating and stimulates gastrointestinal (GI) tract motility respectively. Patients should be instructed to stop other medications and supplements that could cause diarrhea, such as stool softeners, laxatives, aloe, saw palmetto, Siberian ginseng, plantago seeds, high doses of vitamin C, and green tea (O'Brien et. al., 2005).

2.2.5 Pharmacologic Measures

The mainstays of pharmacologic therapy for CID are opioids. Loperamide (Imodium) and diphenoxylate-atropine (Lomotil) are the most used, and both are US Food and Drug Administration (FDA)-approved for this complication. Both loperamide and diphenoxylate-atropine can be used for effective control of acute and chronic diarrhea of various causes but there are no randomized trials directly comparing loperamide with diphenoxylate in patients with CID. However, efficacy data from double-blind crossover studies comparing these agents in a variety of settings of non-chemotherapy-related acute and chronic diarrhea suggest that loperamide is the more effective agent, providing more rapid control of diarrhea and prolonging the time to first recurrence of diarrhea (Pelemans & Vantrappen, 1976; Palmer et. al., 1980; Jaffé, 1977); furthermore, Loperamide has a more favorable side effect profile, especially lacking central narcotic activity (Shee & Pounder, 1980). Loperamide is the preferred approach in several published guidelines for treatment of CID (Benson et. al., 2004; Andreyev et. al., 2014; Vehreschild et. al., 2013; Maroun, 2007).

The various pharmacologic agents that can be used for CID are Loperamide, Diphenoxylate-atropine, Octreotide, Deodorized tincture of opium, Anticholinergic drugs, Racecadotril, Budesonide, Oral antibiotics, and Uridine triacetate.

- **Loperamide.** It is usually recommended for initial therapy of CID. As a synthetic opioid it acts by binding to mu opiate receptors in the intestinal wall and inhibiting peristalsis thereby inhibiting the release of acetylcholine through activation of the mu opioid receptors. This slows GI transit time, allowing more time for absorption of water in the intestine (Awouters et. al., 1993). Inhibition of acetylcholine release

also leads to antisecretory activity because muscarinic acetylcholine receptors exist on secretory epithelial cells in the intestinal wall. Therefore, loperamide reduces fluid and electrolyte loss, decreases fecal volume, and increases stool consistency. Loperamide is rapidly absorbed from the GI tract and extensively metabolized by the liver, thus the amount of loperamide entering the systemic circulation is minimal (systemic bioavailability is only 0.3%), and the blood brain barrier prevents any absorbed loperamide from entering the central nervous system (Andreyev et. al., 2014). As such, it has a much wider margin between central and peripheral opioid action than do other centrally acting opioids, such as fentanyl, codeine, and even diphenoxylate (Awouters et. al., 1993). The main adverse effect of Loperamide is constipation. The typical dose of loperamide for CID is an initial 4 mg, followed by 2 mg every four hours or after every loose stool. Two randomized placebo-controlled trials established the efficacy of this dose of loperamide in a variety of settings of both acute and chronic diarrhea (Mainguet & Fiasse, 1977; Galambos et. al., 1976). This dose has been used empirically for patients with CID and has been shown to be effective. However, it is only fairly effective for severe (grade 3 or 4) CID (Cascinu et. al., 2000). In a study among patients with grade 1 to 4 diarrhea induced by 5-fluorouracil (FU) chemotherapy, loperamide was given at 4mg for the first dose, followed by 4mg every eight hours (i.e., 16 mg per 24 hours) for up to 48 hours. This led to the resolution of grade 1 or 2 diarrhea in 84% of patients but was effective in only 52% of patients with grade 3 or 4 diarrhea (Cascinu et. al., 2000). More aggressive regimens may be recommended for severe or complicated diarrhea (e.g., 4 mg initially, then 2 mg

every two hours or 4 mg every four hours until diarrhea free for 12 hours). Although the package directions for Loperamide use states that the dose should not exceed 16 mg (eight tablets) in any 24-hour period, patients should be note that loperamide is not absorbed but is excreted in stool, and thus the risk of overdose in the setting of CID is unlikely (Andreyev et. al., 2014).

- **Diphenoxylate-atropine.** This is a synthetic opioid that is chemically related to meperidine. It acts by inhibiting excessive GI motility and GI propulsion. Commercial preparations of Diphenoxylate contain a subtherapeutic amount of atropine to discourage abuse. Excessive diphenoxylate-atropine can result in symptoms of excess cholinergic and central narcotic effects, including drowsiness, flushing, dry mouth, tachycardia, dilated pupils, rash, and nausea (Penfold & Volans, 1977). It is also commonly used to treat acute and chronic diarrhea and it has a rapid onset of action (Palmer et. al., 1980). There are no published data on the use of diphenoxylate-atropine in the setting of diarrhea refractory to loperamide, but as diphenoxylate-atropine is another opioid, it is unlikely that it will be effective for patients with severe diarrhea despite aggressive loperamide use.
- **Octreotide.** It is usually recommended for any patient with CID that is refractory to loperamide because it is unlikely that higher doses of loperamide will be beneficial (Benson et. al., 2004). Octreotide is approved by the FDA for the treatment of diarrhea associated with VIP-secreting tumors and symptoms caused by carcinoid syndrome. For initial octreotide dose, most guidelines suggest 100 to 150µg subcutaneously three times daily, with escalation to 500µg three times daily and, rarely, higher (up to 2000µg three times daily), as needed for refractory cases

(Benson et. al., 2004; Andreyev et. al., 2014; Vehreschild et. al., 2013; Maroun, 2007; Harris et. al., 1995). Octreotide, an artificial somatostatin analog, is effective for the control of diarrhea related to several conditions, like carcinoid syndrome, short bowel syndrome, vasoactive intestinal polypeptide (VIP)- and gastrin-secreting tumors. Octreotide reduces diarrhea via two mechanisms:

A. By acting directly on epithelial cells to reduce the secretion of pancreatic and GI hormones, such as VIP, serotonin, gastrin, secretin, and pancreatic polypeptide (Lamberts et. al., 1996).
B. It can prolong intestinal transit time, promote intestinal absorption, and decrease secretion of fluids and electrolytes (Edwards et. al., 1986; Dueno et. al., 1987; Maton et. al., 1985).

Absorption after subcutaneous injection is rapid and complete, but the duration of effect is 6 to 12 hours, necessitating multiple daily doses (Petrelli et. al., 1993). However, there is a long-acting depot suspension of Octreotide (Sandostatin long-acting release) that is released slowly and is available for intramuscular use. This slow-release-long-acting octreotide is designed to be injected every four weeks. Peak serum levels occur after one hour of intramuscular injection, these slowly decline over 3-5 days, then slowly increase again and reach a plateau over 2-3 weeks.

Patients with CID from fluoropyrimidines, irinotecan, and 5-Fluorouracil-based chemoradiotherapy who are failing to respond to loperamide, or diphenoxylate-atropine has benefitted from Octreotide (Petrelli et. al., 1993;

Wadler et. al., 1995; Casinu et. al., 1992; Topkan & Karaoglu, 2006; Barbounis et. al., 2001; Zidan et. al., 2001). In one study of 32 patients with grade 2 or 3 CID (from different chemotherapeutic agents, including 5-Fluorouracil, irinotecan, cyclophosphamide, methotrexate, and cisplatin) who were refractory to loperamide and treated with octreotide (100µg three times daily for three days, then 50µg three times daily for three days), complete resolution of diarrhea was obtained in 94% of the patients. This resolution occurred within 24 hours in 5, within 48 hours in 14, and within 72 hours in 11 patients. 19 patients were successfully treated as outpatients. No adverse effects related to octreotide were observed in this study, although others report local pain at the injection site, flatulence, nausea, fatigue, weakness, and constipation (Zidan et. al., 2001).

The optimal dose has not been established, but most guidelines suggest initiating therapy with 100 or 150µg three times daily. There are some data that suggest a dose-response relationship with escalating doses provides better control of diarrhea (Petrelli et. al., 1993; Wadler et. al., 1995; Barbounis et. al., 2001; Goumas et. al., 1998). For instance, the safety of octreotide at subcutaneous doses of 50 to 2500µg three times daily for five days was evaluated in a dose-escalation study conducted in patients with grade 2 or worse diarrhea associated with FU therapy (Wadler et. al., 1995). The maximum tolerated dose was 2000µg three times daily. According to the study, there was a great correlation between dose and total resolution of diarrhea, and a greater chance of completing therapy as the dose increased, thereby supporting the upward titration of octreotide dose up to 2000µg three times daily. The side effects of octreotide are generally mild, e.g., bloating,

cramping, flatulence, and fat malabsorption. Hypersensitivity-like reactions and hypoglycemia can occur at higher doses (2500μg three times daily) (Wadler et. al., 1995). Octreotide should be discontinued when diarrhea has resolved to avoid the development of ileus (Barbounis et. al., 2001). Based on the high cost of octreotide and the overall effectiveness of loperamide when administered at therapeutic doses, octreotide is typically reserved as second-line therapy for patients who do not respond to high-dose loperamide.

- **Deodorized tincture of opium (DTO).** This contains the equivalent of 10 mg/mL morphine and is a popularly used antidiarrheal agent, despite the absence of literature reports supporting efficacy for treatment of CID. The recommended dose is 10 to 15 drops in water every 3-4 hours (Cascinu et. al., 1993). An alternative is paregoric (camphorated tincture of opium) a less concentrated preparation that contains the equivalent of 0.4 mg/mL morphine. The recommended dose is 5 mL (one teaspoonful) in water every 3-4 hours.
- **Anticholinergic drugs.** They are not normally used because of their side effects. However, they can be beneficial when diarrhea is associated with significant cramping.
- **Racecadotril.** It inhibits enkephalinase by blocking epithelial cyclic adenosine monophosphate (AMP)-mediated secretion. It has modest activity in patients with irinotecan-induced diarrhea (Saliba et. al., 1998).
- **Budesonide**. This is an orally administered synthetic steroid that is topically active. It has a low systemic activity due to extensive first-pass metabolism in the liver. It has proven to be beneficial to patients suffering from diarrhea caused by

inflammatory bowel disease and collagenous colitis. At least one report suggests possible efficacy for oral budesonide in patients with diarrhea caused by irinotecan or FU (Lenfers et. al., 1999). In a phase I study involving 14 patients with loperamide-refractory grade 3 or 4 diarrhea, severity was reduced by at least two grades in 86% of the irinotecan-treated patients and 57% of those receiving FU. One setting in which budesonide may be effective is in patients receiving aflibercept, which has been associated with microscopic (lymphocytic) colitis (Ghiringhelli et. al., 2015).

- **Oral antibiotics**. Currently, there is no agreement on the suitable role for oral antibiotics in patients with CID. According to the 2004 guidelines from Benson and colleagues, it is recommended to start oral antibiotics for uncomplicated mild to moderate diarrhea that persisted after 12 to 24 hours of initial loperamide therapy. However, these guidelines were issued around the time that deaths were reported in patients with CID from irinotecan in conjunction with bolus FU and leucovorin (the IFL regimen), many of which occurred in the setting of neutropenia. Unfortunately, this regimen is no longer desirable (Andreyev et. al., 2014). The current recommendation is to use intravenous antibiotics for patients with fever, neutropenia, peritoneal signs, bloody diarrhea or hypotension.
- **Uridine triacetate.** Also known as vistonuridine, is an orally administered prodrug of uridine, used to treat for overdose of fluoropyrimidines including 5-fluorouracil (FU) and capecitabine. It is also recommended for individuals with ongoing severe fluoropyrimidine-related toxicity (diarrhea, myelosuppression, neurotoxicity, cardiotoxicity, or mucositis) who are identified within or later than 96 hours after

the last chemotherapy dose. Uridine triacetate was studied in 173 adult and pediatric patients who were treated in two separate trials and had either received an overdose of FU or capecitabine (n = 147) or had life-threatening toxicities within 96 hours after receiving FU (Ma et. al., 2016). Overall, 96% of patients (five were lost to follow-up) treated with uridine triacetate survived to 30 days, and 38% were able to resume chemotherapy within 30 days. All 5 deaths were in patients who initiated uridine triacetate treatment beyond 96 hours after the last dose of the fluoropyrimidine. Uridine triacetate has been shown to prevent fatalities in mice that are treated with FU after receiving an inhibitor of dihydropyrimidine dehydrogenase (DPD) (Von Borstel et. al., 2010). Therefore, treatment with uridine triacetate should be beneficial to DPD enzyme-deficient patients who develop early severe toxicity after receiving the first dose of a fluoropyrimidine if the deficiency is identified soon enough after the drug is administered. Uridine triacetate has received orphan drug designation for treatment of capecitabine or FU overexposure from the European Medicines Agency (EMA). In the United States, uridine triacetate is approved for emergency use within 96hrs following a FU or capecitabine overdose (U.S. Food and Drug Administration). However, there are articles of patients with severe toxicity from FU or capecitabine responding to uridine triacetate administered 7 and 21 days after the last fluoropyrimidine dose (Baldeo et. al., 2018; Zurayk et. al., 2017). It is therefore suggested that uridine triacetate should be given to any patient with severe toxicity from fluoropyrimidine chemotherapy and suspected DPD or thymidylate synthetase enzyme deficiency regardless of the duration of time since the last dose of chemotherapy. The

recommended dose and schedule for uridine triacetate in adults is 10g orally every six hours for 20 doses. The recommended dose and schedule for pediatric patients is 6.2 g/m^2 of body surface area, orally, every six hours for 20 doses. Finally, for nonemergency toxicities, uridine triacetate should not be administered as it may interfere with the efficacy of fluoropyrimidine treatment.

2.2.6 Prophylactic Measures

Prophylactic treatment for CID is not a standard approach and it is not usually recommended. Several studies have investigated the potential benefit of prophylactic antidiarrheal therapy in patients treated with a variety of regimens, mostly bolus FU plus leucovorin and irinotecan but the results have not been consistent.

- **Activated Charcoal**. This may be effective in prevention of irinotecan-induced diarrhea (Michael et. al., 2004). The incidence of grade 3 or 4 diarrhea increased from 7 to 25 percent between cycles 1 and 2 in a study where 28 patients received activated charcoal during the first treatment cycle but not the second, and more patients required 10 or more tablets of loperamide without prophylaxis.
- **Oral alkalization.** In a Japanese case-control study of patients undergoing irinotecan and cisplatin chemotherapy, a complicated regimen of oral alkalization of the intestinal lumen in conjunction with control of defecation may have been beneficial (Takeda et. al., 2001).
- **Probiotics**. An exciting but investigational strategy is oral administration of "probiotics," microorganisms such as the *Lactobacillus* species. Due to the success of this approach in children plagued by diarrheal illnesses, as well as for antibiotic-induced diarrhea, a trial was conducted in 150 patients receiving two different FU-

based chemotherapy regimens, in which the random assignment was to receive or not receive *Lactobacillus rhamnosus* GG and/or fiber supplementation (Österlund et. al., 2007). Patients who received Lactobacillus supplements had significantly less grade 3 and 4 diarrhea and required fewer hospitalizations and dose reductions due to bowel toxicity, but this group also had a trend toward a higher number of neutropenic complications as fiber intake did not alter chemotherapy toxicity. Interpretation of this trial is limited by the lack of stratification for method of chemotherapy administration. A randomized, placebo-controlled trial of probiotics for patients treated with irinotecan was closed early for insufficient accrual. Among the 46 patients enrolled, those on probiotics had less diarrhea and less severe diarrhea, but the results are not statistically significant (Mego et. al., 2015). Further studies are needed to assess the overall benefit of such interventions.

- **Lafutidine.** Lafutidine is a histamine antagonist that has been shown to reduce FU-induced mucosal injury (Tashima et. al., 1998). Twenty-two patients with resected stage II or III gastric cancer were randomly assigned to adjuvant S1 chemotherapy or S1 plus lafutidine. The overall incidence of diarrhea significantly reduced from 83 to 10 percent due to the addition of lafutidine to chemotherapy. Larger studies are needed to confirm these findings.
- **Calcium Aluminosilicate Clay.** Calcium aluminosilicate clay, a cation exchange absorbent, did not prevent severe irinotecan-induced diarrhea in a randomized trial of 100 patients (Kee et. al., 2014).
- **Palifermin.** Palifermin, a keratinocyte growth factor, administered intravenously for three days before and after chemotherapy was found to reduce severe diarrhea

from 26 to 8 percent in 155 patients with acute myelogenous leukemia undergoing induction chemotherapy (Bradstock et. al., 2014).

- **Glutamine.** Whether glutamine is beneficial to prevent CID is unclear; studies have had mixed results (Rotovnik Kozjek et. al., 2011; Daniele, 2001; Miraghajani et. al., 2015; Decker-Baumann et. al., 1999). In three studies, glutamine (oral in two, parenteral in one) did not reduce the incidence of severe diarrhea in patients undergoing chemoradiation for rectal cancer (Rotovnik Kozjek et. al., 2011), in patients receiving leucovorin-modulated FU chemotherapy for colorectal cancer (Decker-Baumann et. al., 1999), or in breast cancer patients treated with doxifluridine. However, modest benefit was suggested in a small (n = 70), randomized, placebo-controlled trial in which oral glutamine (18g/day) administered prophylactically to patients treated with leucovorin-modulated FU decreased the duration of diarrhea but had no significant impact on severity; treated patients also took fewer loperamide tablets after one cycle of chemotherapy (Daniele, 2001).

2.2.7 Pharmacogenetic and Pharmacokinetic Measure

Strategies that have been used to reduce CID include initial dosing of irinotecan based on uridine diphosphoglucuronate-glucuronosyltransferase 1A1 (UGT1A1) genotype, pharmacogenetic testing for mutations and polymorphisms in the fluoropyrimidine-metabolizing enzymes DPD and thymidylate synthetase, and pharmacokinetically guided dosing of FU (Iyer et. al., 1999). However, none of these approaches has been widely adopted.

2.3 Irinotecan Disposition and Diarrhea Mechanism

Irinotecan, a camptothecin derivative is a prodrug for SN-38 that suppresses topoisomerase-I, an enzyme that is functional in DNA replication. It is active against a broad spectrum of malignancies, including colon, stomach, and lung cancers. Unfortunately, its widespread use has been limited by frequent and often severe gastrointestinal toxicities, such as diarrhea.

Preclinical models, pharmacokinetic studies, and clinical observations have yielded some critical insights into the pathophysiology of these side effects. Unfortunately, gastrointestinal toxicities remain a major problem with the clinical use of irinotecan, despite new pathophysiologic insights and advances in treatment. The gastrointestinal side effects of irinotecan administration can be divided into early and late diarrhea.

Early diarrhea occurs in 80% of patients within the first 24 hours of irinotecan administration and is cholinergically mediated (i.e., related to increased motility). It is usually accompanied emesis, diaphoresis, abdominal cramping, and, less commonly, hyperlacrimation and rhinorrhea (Bleiberg & Cvitkovic, 1996). In a study of patients treated with 250 mg/m2 of irinotecan bimonthly, it was reported that diarrhea occurred within the first 2 hours, and median duration was approximately 30 minutes (Rothenberg et. al., 2001).

Late diarrhea is that which occurs after 24 hours of irinotecan administration. It is not cholinergically mediated but, instead, appears to be multifactorial, with contributions from dysmotility and secretory factors, as well as a direct toxic effect on the intestinal mucosa. Late diarrhea is a common dose-limiting side effect and can be particularly dangerous in elderly or debilitated patients who experience other toxicities, such as

neutropenia (Rothenberg et. al., 2001). Late diarrhea occurs in 60-80% of patients in most US phase I and II trials and this is usually dose dependent (Bleiberg & Cvitkovic, 1996; Rothenberg et. al., 2001, 1996; Rougier & Mitry, 2001). The incidence of severe (grade 3 or 4) diarrhea in these studies varies from 20% to 40%.

In general, most cases of CID occur through three different pathophysiologic mechanisms:

- Secretory Diarrhea - increased secretion of electrolytes, caused by luminal secretagogues or reduced absorptive capacity due to surgery or epithelial damage caused by chemotherapy drugs. For instance, both FU and irinotecan can cause damage to the intestinal mucosa, leading to loss of epithelium and secretory diarrhea (Ikuno et. al., 1995).
- Osmotic Diarrhea - increased intraluminal osmotic substances. Approximately 10% of patients being treated with FU have decreased expression of the enzyme lactase in their intestinal brush border, leading to lactose intolerance and causing osmotic diarrhea (Parnes, 1994).
- Altered gastrointestinal motility as seen in early diarrhea.

Etiology of irinotecan-induced diarrhea may be found in the complex pharmacology and metabolism of the drug (Fig 2). Irinotecan is a prodrug that is converted to an active form, SN-38, by carboxylesterases, which in humans are found predominantly in the liver (Haaz et. al., 1997). SN-38 is further metabolized to its inactive form - SN-38 glucuronide (SN-38G), by an isoform of hepatic uridine diphosphate glucuronosyltransferase (UGT1A1) in a process called glucuronidation (Ando et. al., 1998). This enzyme also catalyzes the glucuronidation of bilirubin and is deficient in Gilbert's syndrome (Bosma

et. al., 1995). SN-38G can also be deconjugated back to SN-38 in the gut by bacterial glucuronidases (GUS), which may result in increased exposure of the intestinal epithelium to toxic SN-38 thereby causing intestinal mucosal injury, which results in delayed-onset diarrhea. A correlation between intestinal bacterial beta-glucuronidase activity and the site of epithelial damage in rats exposed to irinotecan has been reported (Takasuna et. al., 1996).

Severe irinotecan toxicity in two patients with Gilbert's syndrome, in which glucuronidation is deficient has been reported (Wasserman et. al., 1997). This finding indicates the importance of glucuronidation in the detoxification of irinotecan and its metabolites. Therefore, patients with Gilbert's syndrome, may be at high risk for irinotecan-induced diarrhea.

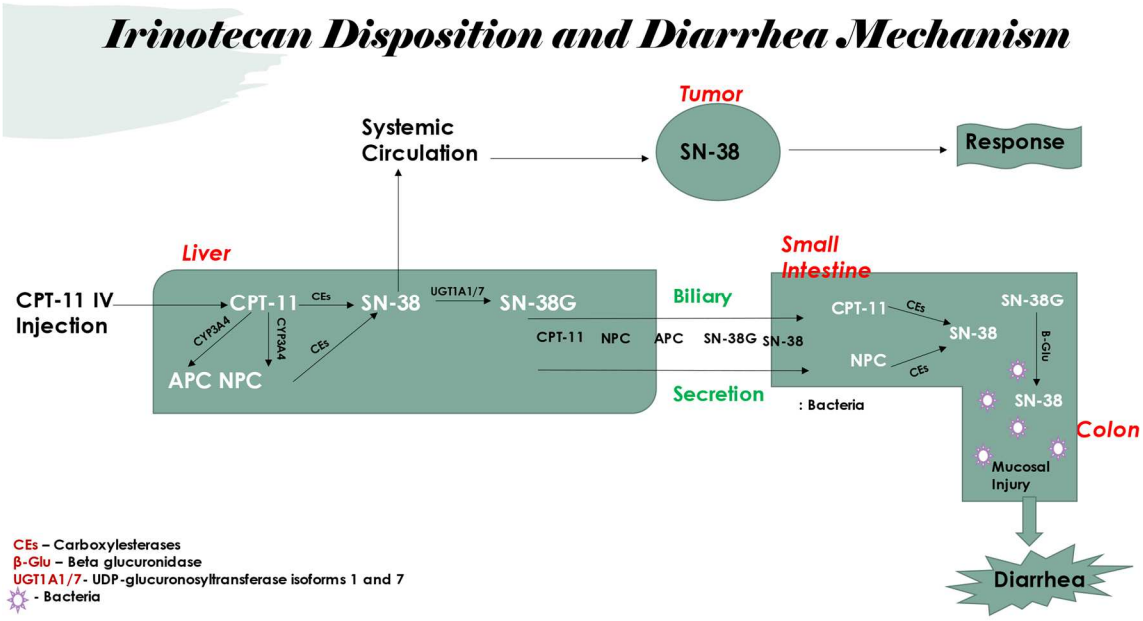


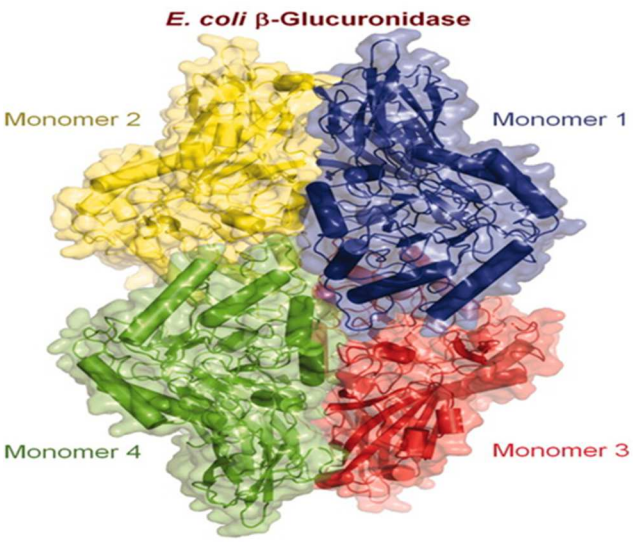
Figure 2. *Irinotecan Disposition and Diarrhea Mechanism*

Irinotecan and its metabolites may have additional effects on the intestinal mucosa that might result to diarrhea. In a normal intestine, secretion of fluid is driven by active

secretion of chloride which is actively transported into the cell across the basolateral membrane by the Na^+:K^+:2Cl- cotransporter and then exits the cell via chloride channels along the electrochemical gradient (Epstein et. al., 1989). It has been demonstrated that colonic chloride secretion in rats, as measured with using chambers as a short-circuit current, is stimulated by irinotecan (Sakai et. al., 1995).

2.4 Effect of Bacterial β-GUS on Concentration of SN38 in the Gut

The human gut microbiota consists of complex and dynamic microbial community. The use of various carbon sources provides the gut bacteria with competitive advantages that increase their chances for survival and/or abundance (Quercia et. al., 20142). Many gut microbes obtain glucuronic acid by processing glucuronides—the end products of glucuronidation, a major detoxification pathway in mammalian liver (Ritter, 2000).



Wallace et. al., 2010

Figure 3: *Crystal Structure of Bacterial β-glucuronidase Tetramer*

A xenobiotic is detoxified in the liver by addition of glucuronic acid to increase its solubility for excretion out of the body, once the glucuronide enters the intestine, a gut

bacterial GUS removes the glucuronide group as a carbon source, producing reactivated SN-38 in situ (Ritter, 2000). This then results in toxicities such as diarrhea or epithelial injury and increases the lifetime of the compound in circulation. Therefore, the activities of bacterial GUS enzyme affect the physiological activities and toxicities of various drugs such as irinotecan and this enzyme is believed to be the main culprit that causes abundance of SN38 in the intestinal lumen (Wallace et. al., 2010).

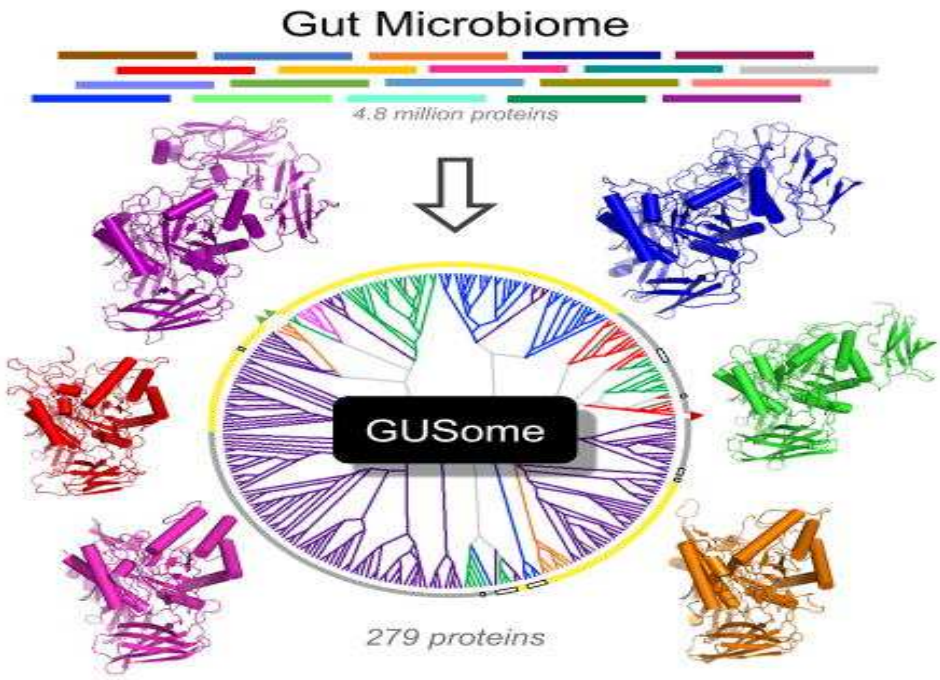
There have been several studies aimed to alleviate irinotecan induced diarrhea by inhibiting the activities of bacterial GUS in the intestinal lumen. However, these approaches have several drawbacks in the sense that their results so far have been inconsistent, and most of them have single mode of action which might not be helpful since the pathogenesis of irinotecan-induced diarrhea is not yet thoroughly understood (Deng et. al., 2017).

- Use of Antibiotics - the feasibility of using antibiotics to reduce GI bacteria levels prior to CPT-11 treatment has been examined (Flieger et. al., 2007), but this approach is not recommended. This is because intestinal biota plays essential roles in carbohydrate metabolism, vitamin production, and the processing of bile acids, sterols, and xenobiotics (Cummings, J. H., & Macfarlane, G. T. 1997). Thus, the removal of GI bacteria is not recommended for patients already challenged by neoplastic growths and chemotherapy. In addition, elimination of symbiotic GI flora increases the chances of infections by pathogenic bacteria, including enterohemorrhagic Escherichia coli and Clostridium difficile (Nord et. al., 1984).

- Use of Probiotics – The use of probiotics to reduce incidence of diarrhea in patients undergoing irinotecan chemotherapy has been reported (Mego et. al., 2015). However, the study was prematurely terminated due to slow accrual.
- Use of Bacterial β-GUS specific inhibitor – There are reports of the use of bacterial GUS inhibitors to reduce irinotecan-induced toxicity in animal models (Fittkau et. al., 2004; Kurita et. al., 2011) but this approach is yet to be translated in human studies and as such its efficiency is still unsure.

2.5 Measurement of Bacteria β-GUS Activity

It has been reported that the activity of bacteria GUS is associated with higher drug toxicities in the GI tract, development of cancer, and increased incidence of Crohn's disease and colitis because glucuronide hydrolysis is catalyzed by β-glucuronidases (GUS) expressed in intestinal microbiota (Pellock et. al., 2017). The structures of human microbiome GUS were reviewed, and the atlas was published recently, and it was reported that there are at least 279 GUS isoforms expressed in the gut microflora (Pollet et. al., 2017).



Pollet et. al 2017

Figure 4: *Human Microbe Glucuronidase*

This makes the composition of gut microflora complex, and difficult to study the activity of GUS using fresh microflora. Therefore, bacteria culture seems to be the best approach to study the activity of GUS, although some of the bacteria may not culturable. As a result of this, total proteins prepared from feces (e.g., fecal S9 fractions) or fecal suspensions are usually used in invitro studies of GUS activity (Huang et. al., 2013; Niu et. al., 2012; Hasan et. al., 2017; Taylor et. al., 2019; Cao et. al., 2011; Cui et. al., 2016; Tao et. al., 2016; Lee et. al., 2002). The only downside to this approach is that the conditions used in these studies are not standardized and results from different research groups may or may not be comparable. Concerns about experimental conditions include:

- Optimum feces collection time – Different studies collected their fecal samples at different times. For example, some collected the feces fresh almost immediately after defecation (Huang et. al., 2013; Taylor et. al., 2019; Cao et. al., 2011; Tao et.

al., 2016), some collected feces couple of hours or 1 day after defecation (Lee et. al., 2002; 116), others collected feces up to a duration of 7days (Hasan et. al., 2017), and while some did not even mention the duration after defecation the feces were collected (Niu et. al., 2012; Cao et. al., 2011).

- Enzyme preparation method – Different methods were used for the preparation of enzyme from the fecal samples. Some studies reported sonication of the fecal solution before finally centrifuging (Niu et. al., 2012; Taylor et. al., 2019; Lee et. al., 2002; 116,), others prepared their fecal enzymes without sonicating (Hasan et. al., 2017; Cao et. al., 2011; Cui et. al., 2016; Tao et. al., 2016), while other studies reported extraction of the fecal enzymes by method of liquid-liquid extraction (Hasan et. al., 2017).
- Optimum buffers condition – The most used extracting solvent for GUS analysis through fecal samples is phosphate buffer (Niu et. al., 2012; Cui et. al., 2016; Tao et. al., 2016), however, some studies use methanol to extract fecal enzymes (Huang et. al., 2013; Cao et. al., 2011). It is important to consider the pH of the buffer as pH alteration may lead to the degradation of some metabolites (e.g., through hydrolysis) and at the same time may affect extraction efficiency. The most common buffer pH value used is 7 (Lee et. al., 2002; S9 Fraction Wikipedia), but we cannot tell if that pH is the best for the efficient extraction of these enzymes as there's been no comparison of other buffer pH values.
- Centrifugation Conditions – Another important factor in the preparation of enzyme from fecal samples is the removal of particulates by centrifugation and in most cases, two centrifugation cycles are normally used. The first centrifugation is

performed after extraction with buffer and the 2nd is done just before analysis of the extracts. The centrifugation duration varies from 10min to 30min, and the centrifugation speeds were different with no clear reason why in some cases (Cui et. al., 2016) the centrifuge speed of 9000g was not used since this is the speed used for the extraction of enzymes involved in phase II metabolism (S9 Fraction Wikipedia).

Another way to remove particulate is filtration and some studies adopt this (Huang et. al., 2013). However, filtration was not found to significantly affect extraction yield, but leads to cleaner, particulate free extracts (Deda et. al., 2015).

- Anaerobic condition necessary – Since the natural habitat of these enzymes is anaerobic, some studies carry out the fecal preparation and analysis in anaerobic conditions (Cui et. al., 2016; Tao et. al., 2016) and some do not think it is necessary to do so (Huang et. al., 2013; Cao et. al., 2011),

2.6 Xiao-Chai-Hu-Tang (XCHT)

Xiao-Chai-Hu-Tang (XCHT) is an old Chinese herbal formula utilized in China and Japan for many years. It contains seven medicinal herbs: Bupleurum root, Scutellaria root, Ginseng root, Glycyrrhiza root, Pinellia tuber, Jujube fruit, and Ginger rhizome. This herbal combination makes it very effective towards multiple pathways and targets.

In traditional Chinese medicine, XCHT has been used to treat a variety of diseases such as the disorders of the liver and gallbladder. Common side-effects experienced with XCHT treatment include alternating chills and fever, but the medication is well-tolerated by most patients. The relatively low toxicity of XCHT is an important characteristic for

potential use of this extract in combinatorial therapies. Ancient scholars believed the mechanism of action of XCHT involved eliminating pathogens through enhanced liver function and improved digestion (Zhao et. al., 2017).

XCHT is safe, has multiple mode of action, and has the potential to affect microflora. According to studies, Scutellaria (Wu et. al., 2019), Ginseng (Guo et. al., 2015) and Glycyrrhizia (Zhang et. al., 2018) have shown to affect the gut microflora.

CHAPTER 3

DESIGN OF STUDY

3.1 General Hypothesis

We hypothesize that bacteria beta-glucuronidase plays an important role in the irinotecan induced diarrhea and manipulation of intestinal bacterial GUS will alleviate the irinotecan-induced diarrhea. We conducted three specific aims to test our hypothesis.

3.2 Specific Aims

3.2.1 Aim 1

Establish an optimized method to evaluate microflora hydrolysis activity.

Rationale. A few investigations have been published regarding drug or nutritional glucuronides hydrolysis by microflora using enzymes (e.g., fecal S9 fraction) prepared from feces. However, the conditions and methods used in these studies were different and it is unknown whether the results are comparable.

We will determine the experimental conditions that affect GUS activity evaluation and will establish an optimized method to evaluate SN-38G hydrolysis.

At the end of this aim, we expect to have established an optimized in vitro method to study glucuronide hydrolysis by intestinal microflora and will use the established condition to evaluate SN-38G hydrolysis.

Experimental Design. We will prepare fecal enzymes using feces collected from rats, mice, and humans at different conduction using different methods. Then we will incubate wogonoside and pNPG with fecal S9 fractions to determine evaluate GUS activity and optimize the condition used for SN-38G hydrolysis.

3.2.2 Aim 2

Determine if SN-38G hydrolysis by bacterial GUS can be altered.

Rationale. SN-38G hydrolysis by GUS will release SN-38 in the colon, which will increase local SN-38 concentrations and enhance epithelium damage. Although SN-38G can be hydrolyzed by bacterial GUS, whether GUS against SN-38G hydrolysis can be manipulated is understudied. In this aim, we propose to incubate SN-38G with fecal S9 fractions prepared with feces from different animals or animals with different treatment. If GUS activity could be altered, we will be able to manipulate it using agents for diarrhea attenuation.

Experimental Design. We will prepare fecal enzymes using feces collected from Pirc rats, which have inflammation in the intestine, F344 rats (wild type), F344 rats at different ages, and F344 rats treated with irinotecan and incubate SN-38 with these fecal S9 fractions to determine the variation of SN-38G hydrolysis.

3.2.3 Aim 3

Determine diarrhea attenuation by manipulating intestinal bacterial GUS using an herbal formula Xiao-Chai-Hu-Tang (XCHT).

Rationale. Many factors (e.g., UGTs, Pgp, BCRP) are involved in SN-38 disposition. Whether manipulating intestinal bacterial GUS activity can affect irinotecan-induced diarrhea is not entirely understood. We propose to use XCHT to manipulate intestinal bacteria for diarrhea attenuation. The reason we select to use XCHT is because some of the ingredient in this herbal formula was reported to be effective in altering the composition of microflora.

Experimental Design. We will treat F344 rats with XCHT and determine the fecal S9 faction GUS activity against SN-38G hydrolysis using the diarrhea model. Diarrhea severity with and without XCHT treatment will be evaluated to demonstrate the impact of manipulate intestinal bacteria on irinotecan-induced diarrhea.

3.3 Materials

3.3.1 Chemicals and Drugs

- 4-Nitrophenyl β-D-glucopyranoside (pNPG) (purity >98) was purchased from Sigma Aldrich (St. Louis, MO).
- Deionized water was produced in the lab with a Barnstead TM Smart2Pure TM water purification system (Thermo Fisher Scientific, Waltham, MA).
- Formic acid was purchased from Sigma Aldrich (St. Louis, MO).
- UPLC grade water and UPLC grade Acetonitrile were purchased from Sigma Aldrich (St. Louis, MO).
- Monopotassium phosphate (KH_2PO_4), Dipotassium phosphate (K_2HPO_4) and KPI buffer was made using KH_2PO_4 and K_2HPO_4 and the pH was adjusted using sodium hydroxide and hydrochloride. All of which were purchased from VWR (Radnor, PA).
- Saline was purchased from was purchased from Sigma Aldrich (St. Louis, MO).
- SN-38 Glucuronide and SN-38 were purchased from Sigma Aldrich (St. Louis, MO).
- Wogonoside and Wogonin were purchased from Sigma Aldrich (St. Louis, MO).
- XCHT was made in Dr Hu's lab in the pharmaceutical science department of University of Houston.

3.3.2 Supplies

- Autosampler injection vials (clear, 2 mL) purchased from Agilent (Santa Clara, CA) were used to hold samples for injection in the UPLC.

- Face masks obtained from AlphaProTech, Inc (Salt Lake City, UT) were worn when performing experiments especially the ones involving animals.
- Kim wipes from Kimtech were used to wipe and clean messes made during experiment.
- Lab coat and safety googles from were used as protective gears during experiments
- Manual pipettes (5 mL, 10 mL, and 25 mL) purchased from VWR (Radnor, PA) were used for volume transfers.
- Microcentrifuge tubes (amber and clear, 1.5 mL) purchased from VWR (Radnor, PA) were used to prepare working standard solution, sample preparations for UPLC analysis.
- Pipette tips (10 µL, 20 µL, 250 µL, 1000 µL) purchased from Ranin Instrument (Oakland, CA) were used along with the appropriate pipettor for measurements and transfers.
- Powder-free latex examination gloves purchased from VWR (Radnor, PA) were worn as personal protective equipment during experiments.
- Storage containers 250 mL, 600 mL and 1000 mL purchased from VWR (Radnor, PA) were used to store mobile phases used for UPLC analyses.

3.3.3 Equipment, Apparatus and Software

- Empower 3® software was used to control and acquire data from the UPLC system.
- Eppendorf Centrifuge 5427 R was used to centrifuge the fecal samples and working solutions to enable collection of supernatants.
- Graph Pad Prism v8.0.2 (Graph Pad Software Inc, La Jolla, CA) was used to generate plots and for statistical analysis of data.

- Incubating Microplate shaker from VWR was used to incubate plates during hydrolysis.
- Magnetic stirrer (PC-351) purchased from Corning Co. (Coring, NY) was used to stir solutions as required.
- Multimode Detector DTX 880 from Beckman Coulter was used to read the absorbance of working solutions on plate.
- Negative 80□ Refrigerant from Thermo fisher Scientific was used to store the fecal samples after preparation.
- Ohaus PA64 - Pioneer Analytical Balance (65 g x 0.1 mg) purchased from Mettler Toledo (Columbus, OH) were used to weigh all chemicals and drugs.
- Pipettors purchased from Ranin Instrument (Oakland, CA) and Eppendorf (Hamburg, Germany) were used along with the appropriate pipette tip for measurements and transfers.
- Shel Lab® SWBR17 Shaking Water Bath, 17 Liter Capacity, 115V was used to incubate tubes containing working solutions during hydrolysis.
- Ultrasonicator from Thermo Fisher Scientific was used to sonicate fecal samples during preparation.
- Vortex machines (Vortex-Genie 2) purchased from Scientific Industries (Bohemia, NY, USA) were used for mixing of liquid samples and preparations.

3.3.4 Animals

The animal protocol used in this work was reviewed and approved by the Texas Southern University Institutional Animal Care and Use Committee (IACUC). All animal experiments were carried out with strict adherence to the National Institute of Health

"Guide for the Care and Use of Laboratory Animals, 8th Edition". C57BL6 mice (male, 8 weeks) were obtained from Jackson lab (Bar Harbor, ME), Pirc rats (male, 4 weeks old) and F344 rats (male, 4 weeks and 10 weeks old) were bought from Charles River (Wilmington, MA) and the human fecal sample came from a healthy volunteer. The rats were housed under normal conditions: free access to food and water, room temperature and a 12 h: 12 h light and dark cycle (light from 6 am to 6 pm daily). The rats were allowed to acclimatize to the vivarium for 7 days before animal experiments.

3.4 Methods

3.4.1 Feces collection

Mice and rats' feces were collected for enzyme preparation. Briefly, animals were fed with regular AIN-93 diet for at least one week after receipt from the vendors. To collect feces, the cages of the animals were cleaned out and feces were collected within 24hrs unless in cases where we needed the feces that are older than 24hours. Human feces were collected from a female adult healthy volunteer. The collected feces were prepared immediately and in cases where it couldn't be prepared immediately the feces were stored at -80□ until preparation.

Different time points to determine the impact of collection time on GUS activity. Briefly, to collect the fresh feces, animals were sacrificed and the fresh feces in the colon were collected immediately for enzyme preparation. To collect Day 1 feces, animals were housed in the cages with new bedding and feces were collected 24 hours after changing the bedding for enzyme preparation. To collect Day 7 feces, animals were housed in the cages

with new beddings for 24 hours, then animals were moved out and the feces were collected on Day 7 for enzyme preparation.

3.3.3 Fecal enzyme Preparation

The fecal enzyme was prepared using two different procedures according to those reported previously. Briefly, for sonication method (Niu et. al., 2013), the pooled feces (1g) were mixed with 10 ml ice-cold 50mM KPI buffer solution (pH 7.4) and swirled vigorously for 60 seconds followed by centrifugation at 1,000 rpm and 4°C for 10 min. The pellets were further washed using 5 ml of 50mM KPI buffer solution, then the supernatant was sonicated in ice water bath for 30 min and centrifuged at 9,000 × g at 4°C for 30 min. The final supernatant, which is called S9 fraction, was aliquoted and stored at −80°C. Protein concentrations were determined by the BCA protein assay kit using bovine serum albumin as the standard.

For suspension method (Cui et. al., 2016), 1g of feces was mixed with 10 ml of cold 50mM KPI buffer solution (pH 7.4). The mixture was vigorously swirled for 60 s and centrifuged at 9,000 × g at 4°C for 30min. The supernatant, which is also called S9 fraction, was aliquoted and stored at −80°C. Protein concentrations were determined by the BCA protein assay kit using bovine serum albumin as the standard.

3.3.4 pNPG Hydrolysis Assay

The pNPG hydrolysis was evaluated following the procedure reported previously with slightly modification (Biernat et. al., 2019; Kaur et. al., 2007; Wei et. al., 2018). Briefly, a reaction mixture (200 µL) in KPI buffer containing 50µuL of enzymes (1 mg/ml, final concentration 0.25mg/ml) and 20 µL of pNPG (10 mM, final concentration 1 mM) in a 96-well plate was incubated in an incubator shaker at 37 °C for 30 min. Then, the reaction

was terminated by adding 20 μL of 6% formic acid in acetonitrile. The plate was read using a microplate reader at 405 nm. The relative hydrolysis rate was evaluated using the OD values. Each experiment was conducted in triplicate.

3.3.4 Hydrolysis Reaction

The incubation procedures for measuring hydrolysis enzyme's activities were followed, a similar procedure used in the previous publications (Niu et. al., 2013). Briefly, certain volume of the thawed fecal enzymes was transferred into 50 mM KPI buffer to achieve a final concentration of 10μg/ml. Wogonoside or SN-38G was added to the system to achieve certain concentrations. The system (100μl) was then incubated at 37□ for 30mins in a water bath with orbital shaker. The reaction was stopped by adding 25μl of 6% formic acid in acetonitrile, followed by centrifugation (15,000 rpm, 15 min, 4 °C). The supernatant (10μl) was injected into UPLC for analysis.

3.3.5 Quantification of Wogonoside and Wogonin Using UPLC

Wogonin and wogonoside were analyzed by a common UPLC chromatographic method: system, Waters Acquity UPLC with photodiode array detector and Empower software; column, BEH C18, 1.7μm, 2.1 × 50 mm; mobile phase B, 100% acetonitrile, mobile phase A, 0.1% formic acid in water (pH 2.5); flow rate 0.5 ml/min; gradient, 0 to 1.0 min, 10–50% B, 1 to 1.5min, 50-55% B, 1.5 to 2.5 min, 55–70% B, 2.5 to 2.8min, 70-10%, 2.8 to 3.0 min, 10–10% B; detection wavelength, 273 nm; and injection volume, 10μl. Standard curve samples containing wogonoside and the metabolite wogonin in the same matrix were prepared and injected into UPLC for quantification. Rutin (0.2μM in acetonitrile) was used as the internal standard.

3.3.6 Quantification of SN-38 and SN38 Glucuronide using UPLC

SN-38 and SN38 Glucuronide were analyzed by a common UPLC chromatographic method: system, Waters Acquity UPLC with photodiode array detector and Empower software; column, BEH C18, 1.7μm, 2.1 × 50 mm; mobile phase B, 100% acetonitrile, mobile phase A, 0.1% formic acid in water (pH 2.5); flow rate 0.5 ml/min; gradient, 0 to 1.0 min, 10–15% B, 1 to 2.5min, 15-45% B, 2.5 to 4.5 min, 45–10% B, 4.5 to 5.0min, 10-10%, detection wavelength, 375 nm; and injection volume, 10μl. Standard curve samples containing SN-38Glu and the metabolite SN-38 in the same matrix were prepared and injected into UPLC for quantification. Rutin (0.2μM in acetonitrile) was used as the internal standard.

3.3.7 Optimization of Incubation Buffer and $MgCl_2$ Concentration

To determine the impact of Mg^{2+} on GUS activity, pNPG was incubated with fecal enzymes in KPI buffer containing 0, 1, 2, 5, 8, 10 mM of magnesium chloride following the protocol described above. The results were read at 405 nm after reaction and the relative reaction rates were calculated using the OD values. Similarly, to determine the impact of pH on GUS activity, pNPG was incubated with fecal enzymes in KPI buffer without Mg^{2+} at different pH to determine the relative reaction rates.

To determine wogonoside hydrolysis using buffers with different concentrations of Mg^{2+} or at different pH, wogonoside at three different concentrations were incubated with fecal enzymes for 30 min at 37□. Samples were then prepared according to the procedure described above and injected into UPLC to quantitate the metabolite concentrations.

3.3.8 Kinetics of wogonoside and SN38 Glucuronide hydrolysis

To determine the kinetics of wogonoside and SN-38Glu hydrolysis by fecal enzymes, the optimized conditions were used to obtain the reaction rates, which were expressed as amounts of metabolite (i.e., wogonin or SN-38) formed per min per mg protein (μmol/min/mg) as shown below.

$$Hydrolysis\ Rate = \frac{Amount\ of\ Metabolite}{Time\ x\ Enzyme\ Amt}$$

Kinetic parameters were then obtained according to the profile of Eadie-Hofstee plots using the standard Michaelis-Menten equation:

$$V = \frac{V_{max} \times C}{K_m + C}$$

where Km is the Michaelis-Menten constant, and Vmax is the maximum rate of forming wogonin. GraphPad Prism software (version 7.3 for Windows; GraphPad Software, La Jolla, CA) was used. Visual inspection of fitted functions was used to select the best-fit enzyme kinetic model. The results of triplicate incubations are presented as the mean ± S.D.

3.3.9 Statistical Analysis

Two-tailed t tests or one-way ANOVA were used to evaluate statistical differences. Differences were considered significant when p values were less than 0.05. Statistical comparisons were performed using GraphPad Prism software (version 7.3 for Windows).

CHAPTER 4

RESULTS AND DISCUSSION

Aim 1. Establish an approach to evaluate microflora hydrolysis activity

4.1 Confirmation of Metabolites

4.1.1 Confirmation of Wogonoside Metabolite using UPLC

The metabolite of wogonoside was confirmed by UPLC. The results showed that after incubation, an additional peak was observed at retention time of 5.08 min in UPLC analysis (Fig 4A). The UV spectra of this additional peak was as similar as that of wogonoside (Fig 4B and 4C), suggesting that the skeleton of the metabolite is as similar as that of wogonoside. Additionally, the retention time of the metabolite peak is identical with that of standard compound wogonin. When wogonin was spiked into the sample, the peak at RT=5.08 min increased according. Therefore, this metabolite is identified as wogonin.

Quantification was conducted in UPLC. The test linear response range for wogonoside and wogonin was 0.39 - 100μM (total 9 concentrations). Analytical methods for each compound were validated for inter-day and intra-day variation using six samples at three concentrations (40, 15 and 0.5μM). Precision and accuracy for both compounds were in the acceptable range of 0.11%–3.97%, and at 88.19%–104.62%, respectively.

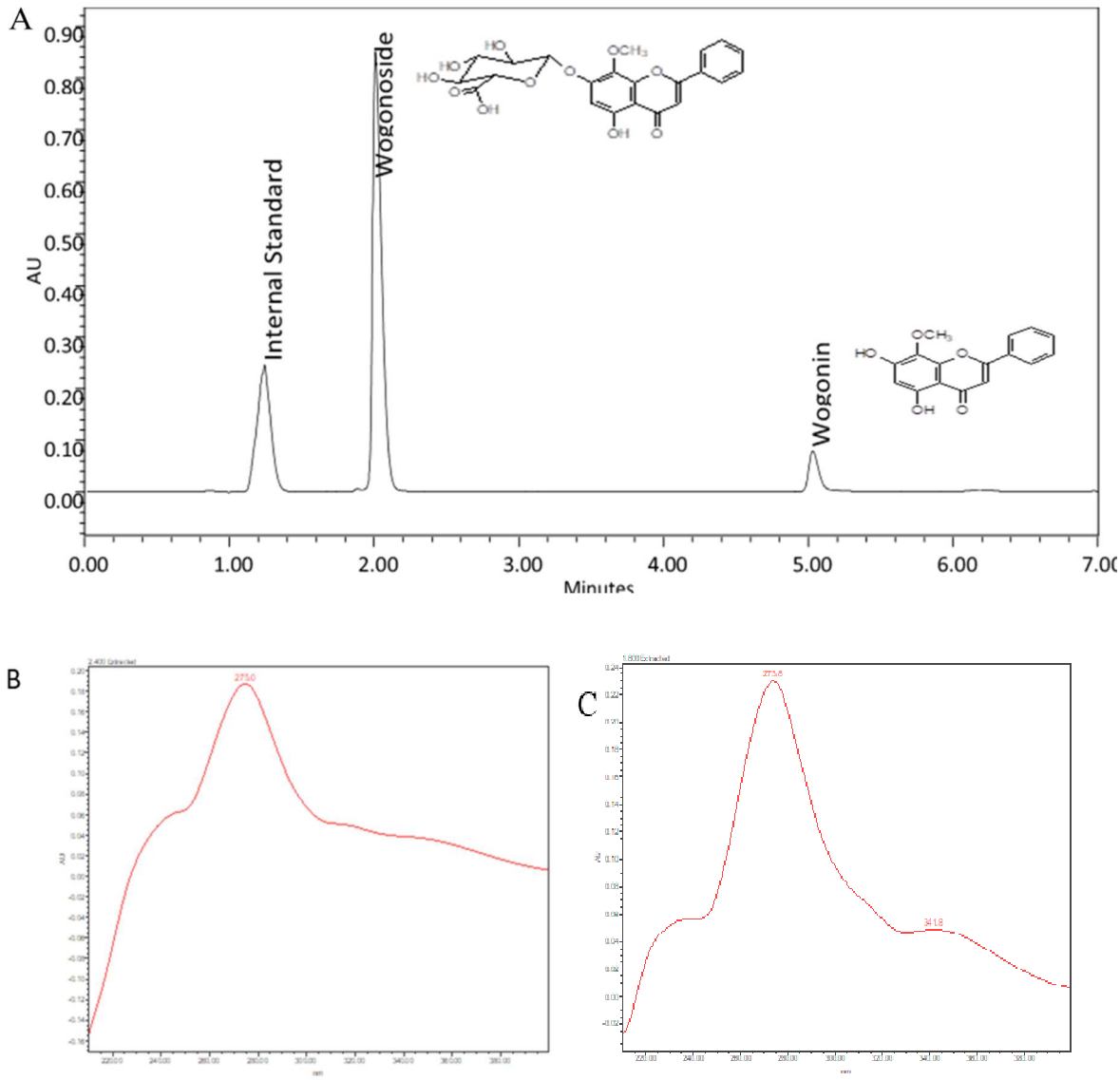


Figure 5: *Chemical Structures, UV and, a Representative UPLC Chromatogram of Wogonoside and Wogonin.*

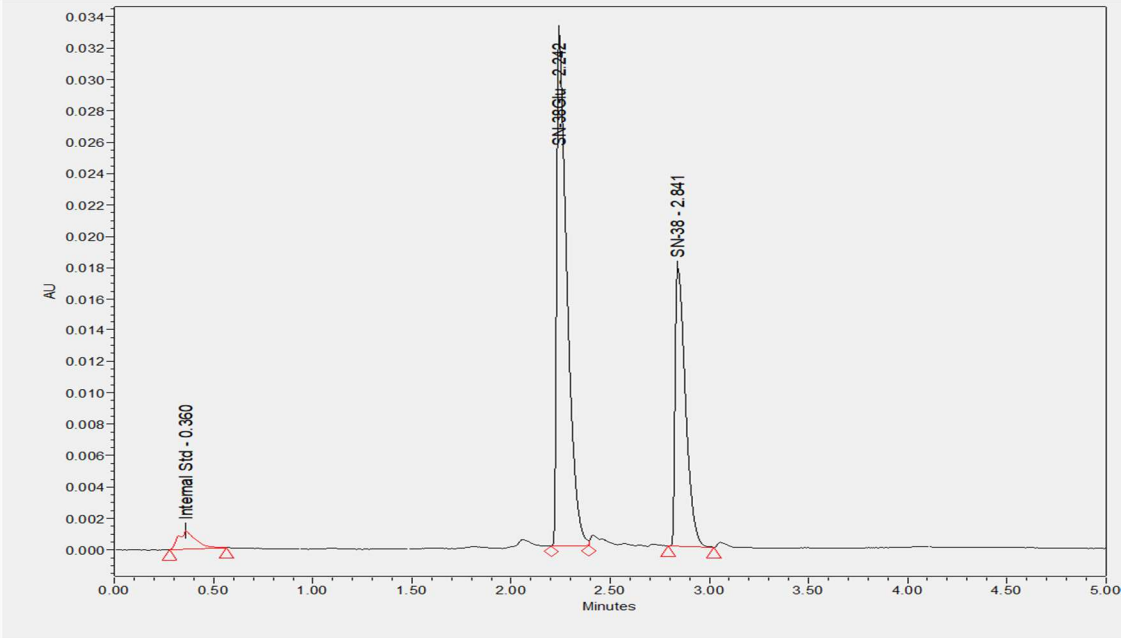
4.1.2 Confirmation of SN38-Glucuronide Metabolite using UPLC

The metabolite of SN38-Glucuronide was confirmed by UPLC. The results showed that after incubation, an additional peak was observed at retention time of 2.84 min in UPLC analysis (Fig 5A). The UV spectra of this additional peak was as similar as that of

SN38-Glucuronide (Fig 5B), suggesting that the skeleton of the metabolite is as similar as that of SN38-Glucuronide. Additionally, the retention time of the metabolite peak is identical with that of standard compound SN38. When SN38 was spiked into the sample, the peak at RT=2.84 min increased according. Therefore, this metabolite is identified as SN38.

Quantification was conducted in UPLC. The test linear response range for SN38-Glucuronide and SN38 was 0.39 - 100μM (total 9 concentrations). Analytical methods for each compound were validated for inter-day and intra-day variation using six samples at three concentrations (40, 15 and 0.5μM). Precision and accuracy for both compounds were in the acceptable range of 0.11%–3.97%, and at 88.19%–104.62%, respectively.

A



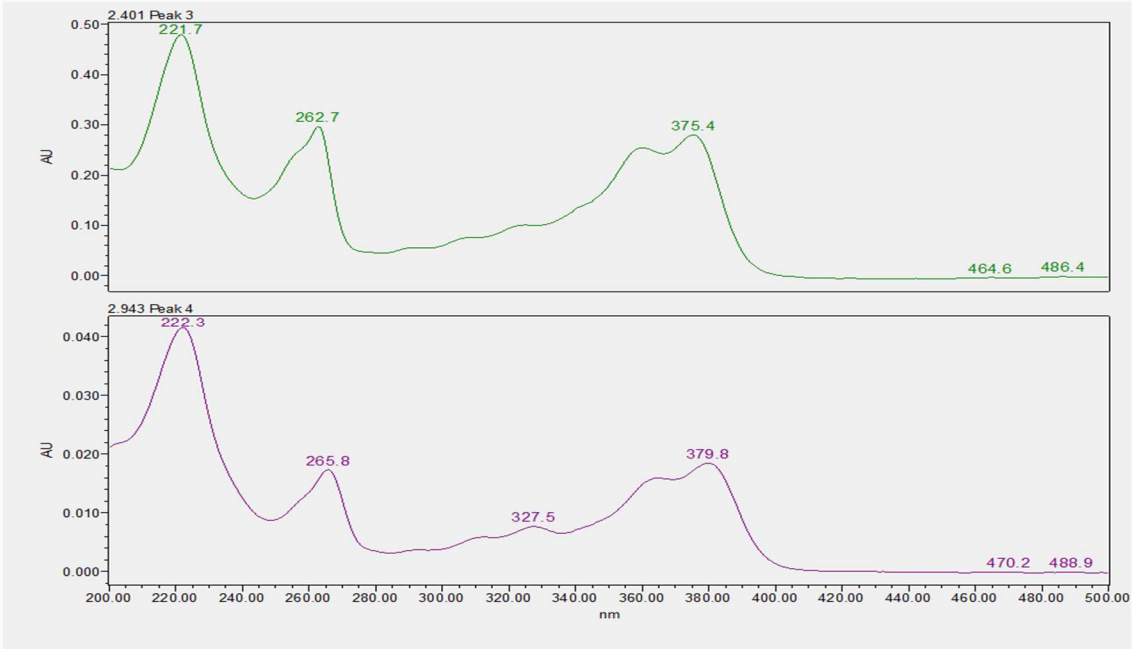


Figure 6: *UV and, a Representative UPLC chromatogram of SN38-Glucuronide and SN38.*

4.2 Total Protein Concentrations were Different Between Sonication and Suspension Preparations

The protein concentration was quantified using the BCA kit following the protocols provided by the manufacture. The results showed that the total protein concentration in the S9 fractions are slightly higher than those suspensions for mice, rats, and humans (Fig. 7). Since same amounts of feces and same volume of buffers were used in these two procedures, it can be concluded that the protein extraction yield is higher when sonication was applied.

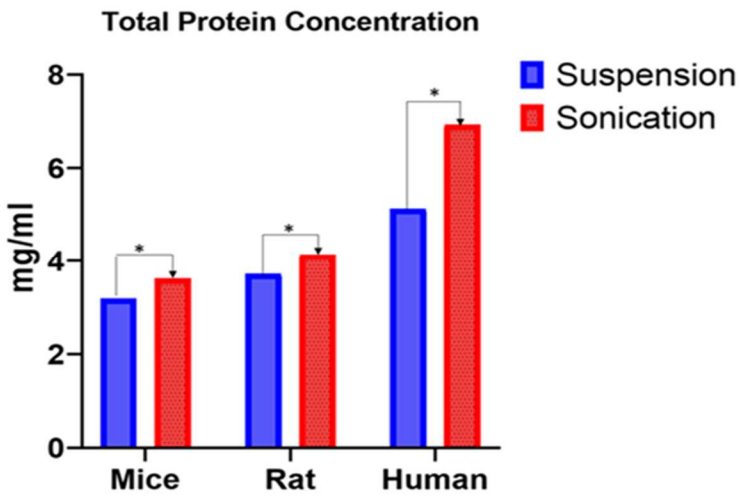


Figure 7: *Total Protein Concentrations Prepared from Feces with Sonication or without Sonication (Suspension)*

4.3 Preparation Method Affected GUS Activity

To determine the impact of fecal enzyme preparation on GUS activity, p-NPG and wogonoside were incubated with enzyme prepared from different species with or without sonication. The results showed that the relative reaction rates of pNPG were significantly higher using enzymes prepared with sonication in all three species (fig 8a). Similarly, the wogonoside hydrolysis rates were also higher using enzymes prepared with sonication in all three species at all three concentrations (fig. 8b, c, d).

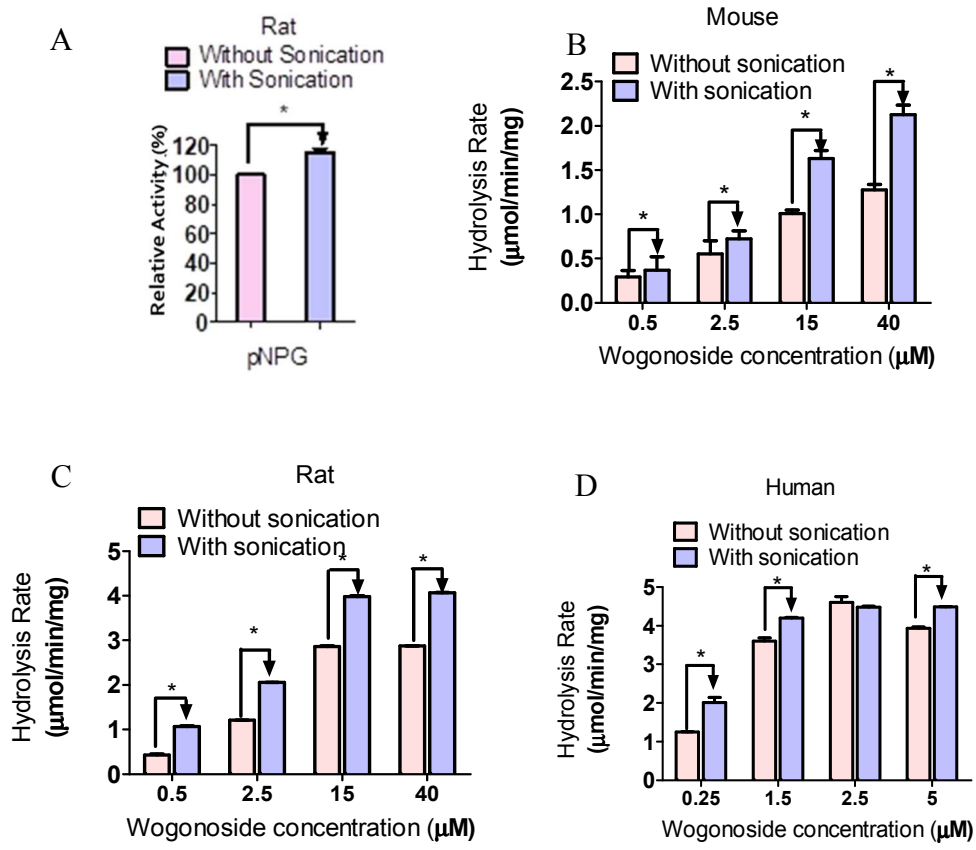


Figure 8: *Impact of Enzyme Preparation Method on pNPG (A) and Wogonoside Hydrolysis (B mouse, C rat, D human)*

4.4 Feces Collection Time Affected Enzyme Activity

To determine the impact of feces collection time on GUS activity, pNPG and wogonoside were incubated with enzymes prepared from rat feces collected at different time. The results showed that pNPG relative hydrolysis rates were highest when fresh feces were used. Activity was decreased gradually on Day 1 and 7 (Fig. 9a). For wogonoside, the results also showed that the hydrolysis rates at all three concentrations were significantly higher when using enzyme prepared from fresh feces than those of Day 1 and

7. Additionally, enzyme prepared using Day 1 feces was significantly higher than that of Day 7 (Fig. 9b). These findings suggested that activity may decrease once the feces is out of the colon and fresh feces should be collected to avoid activity loss probably due to death of bacteria during storage in the cages.

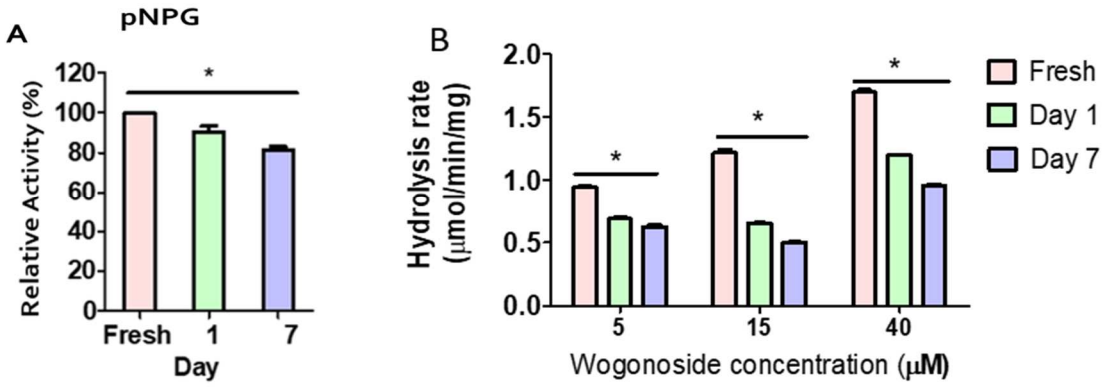


Figure 9: *Impact of Feces Collection Time on pNPG (A) and Wogonoside (B) Hydrolysis using Rat Fecal Enzyme*

4.5 Magnesium Ions Affected Enzyme Activity

To determine the impact of magnesium on the GUS activity, hydrolysis buffers containing different concentrations of magnesium ions were tested. The results showed that the relative hydrolysis rates for pNPG were slightly different using buffers with different concentrations of Mg^{2+} (<50%, Fig. 10). We then determined the impact of magnesium on wogonoside hydrolysis and the results showed that the hydrolysis rates were also slightly different at different concentrations (<30%, Fig. 10). These findings suggest that the impact of magnesium ions on GUS activity was minor, which is different with its reverse

glucuronidation reaction mediated by UGTs with 5-fold difference at different concentrations of Mg^{2+} (116).

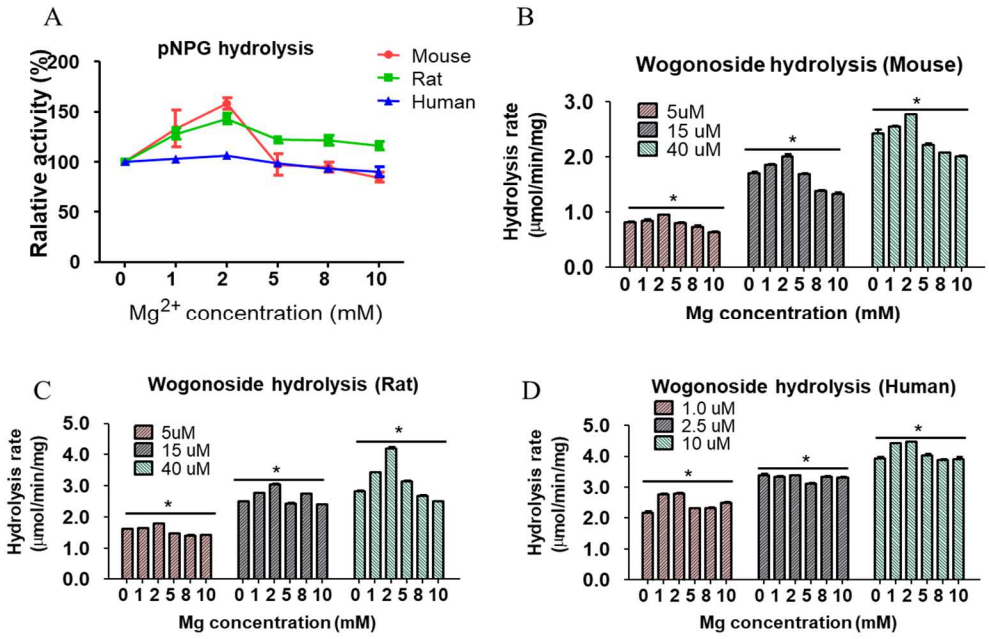


Figure 10: *Impact of Mg2+ on pNPG and Wogonoside Hydrolysis*

4.6 Buffer pH Value Affected Enzyme Activity

To determine the impact of buffer pH on GUS activity, pNPG and wogonoside were incubated with different enzymes in KPI buffers at different pH. The results showed that for rat and mouse, the relative hydrolysis rates of pNPG were highest at pH 6.5 and for human the highest rate is at pH 7.4 (Fig. 11). For wogonoside, the results are as similar as those of pNPG: the hydrolysis rates were highest at pH 6.5, then 5.5 at three different concentrations for both mouse and rat, while pH 7.4 is the best pH to facilitate wogonoside hydrolysis for human (Fig. 11).

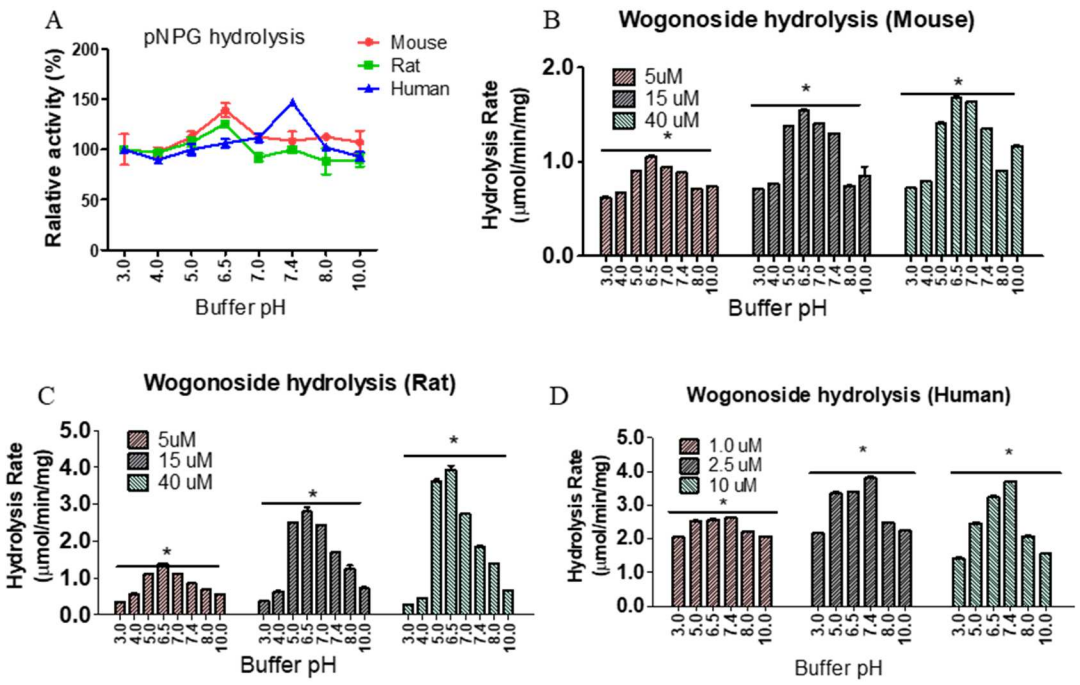


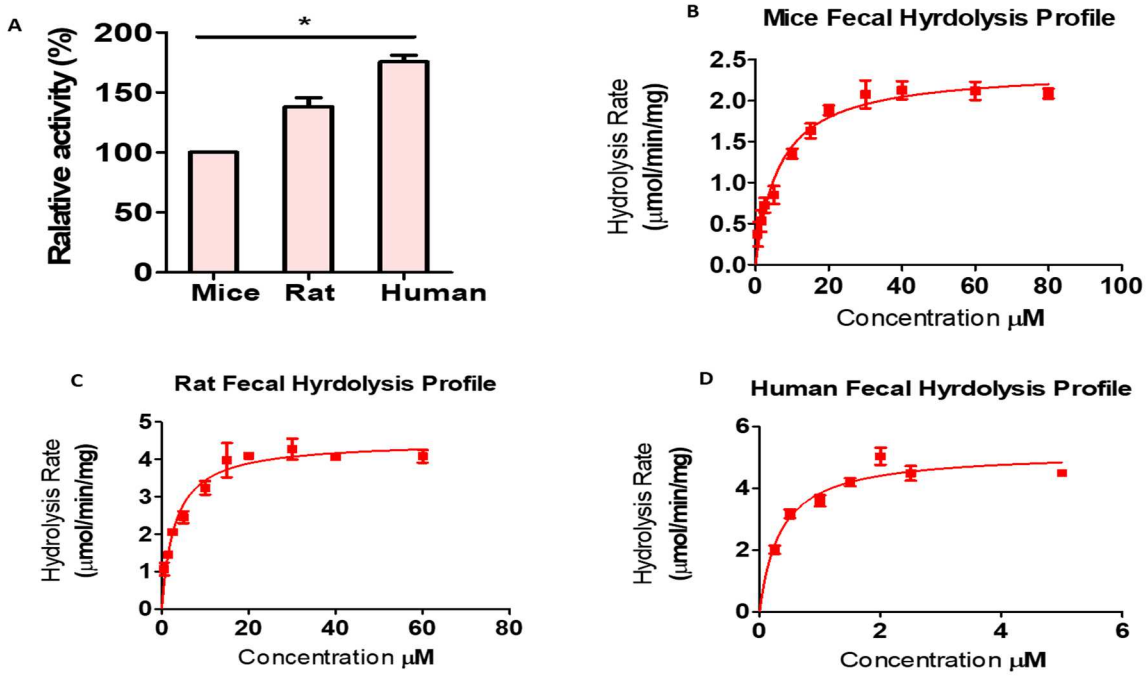
Figure 11*: Impact of pH on pNPG (A) and Wogonoside (B, Mouse, C, Rat, D, Human) Hydrolysis*

4.7 Hydrolysis Rates are Different Across Species

To compare the GUS activity across species, pNPG was incubated with enzymes prepared from mouse, rat, and human feces. The results showed that the relative hydrolysis rates were different across these three species. Rat and human enzymes are 138% and 175% respectively of that of mouse.

To quantitatively described GUS activity further, wogonoside at different concentrations were incubated with three types of the enzymes. The metabolic rates and the kinetic parameters were calculated (Fig. 12). The results showed that for mice, rats, and human feces, the hydrolysis followed classic Michaelis-Menten kinetics. However, the

hydrolysis rates are highly different across these three species. The Vmax for mouse, rat, and human were 2.37±0.06, 4.42±0.12, and 5.17±0.16μmol/min/mg and the Km were 6.51±0.71, 6.51±0.71, and 0.34±0.047μM, respectively.



Mouse			Rat			Human		
Vmax (μmol/min/mg)	Km (μM)	CLint (L/min/mg)	Vmax (μmol/min/mg)	Km (μM)	CLint (L/min/mg)	Vmax (μmol/min/mg)	Km (μM)	CLint (L/min/mg)
2.37±0.06	6.51±0.71	0.36	4.48±0.11	3.04±0.34	1.47	5.17±0.16	0.34±0.047	15.21

Figure 12: *pNPG (A) and Wogonoside (B, Mouse, C, Rat, D, Human) Hydrolysis using Enzymes from Different Species*

4.8 Hydrolysis Rates are Highly Different Across Individuals

To determine individual variation, we prepared enzymes using feces collected from different rats and incubated the fecal enzymes with wogonoside at three different concentrations. The results showed that the hydrolysis rates are highly different across individuals. The reaction rates in rat # 6 is the lowest and in rat #9 is the highest. The maximal difference between these two rats were 6.15, 5.38, and 4.51-fold at low (5μM), medium (15μM), and high (40μM) concentrations, respectively (Fig. 13).

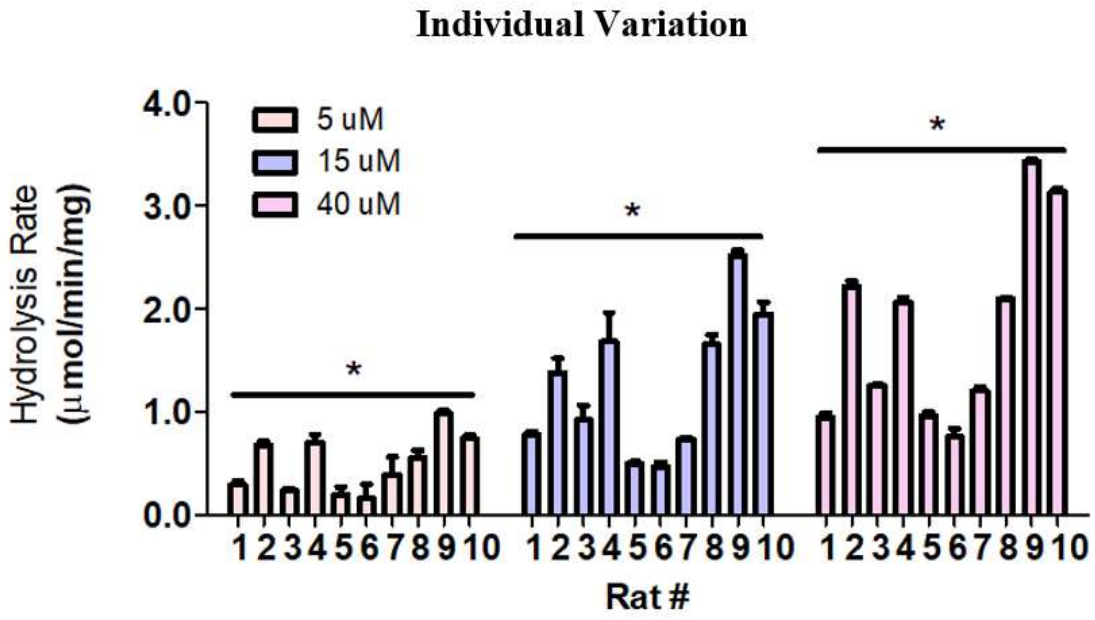


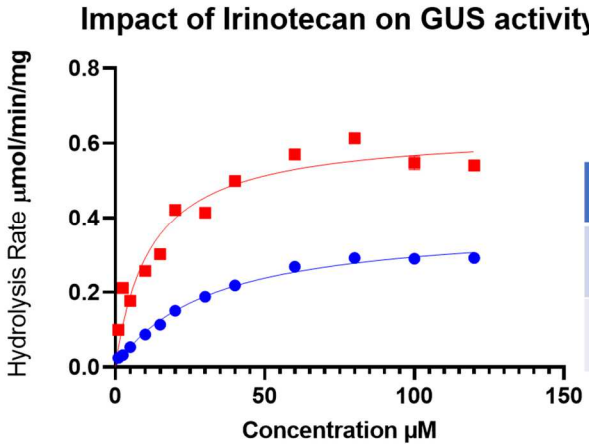
Figure 13: *Individual Rat Variation for Wogonoside Hydrolysis*

<u>Aim 2</u>. Determine SN-38G hydrolysis by bacterial GUS using the optimized conditions.

This aim is to determine if SN-38G hydrolysis using fecal S9 from different conditions and sources are different.

4.9 GUS Activity Increases After Irinotecan Administration

To determine if irinotecan has any impact on GUS activity, feces of F344 rats were collected before irinotecan injection and after the injection which was given in 3 doses, one dose daily. The analysis showed that there was a significant increase in GUS activity in the feces collected after 3days than those collected before the irinotecan injection (fig 14). This increase was also reflective in the intrinsic clearance.



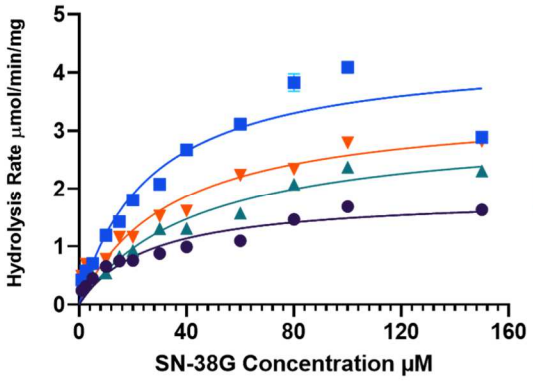
	Vmax (µmol/min/mg)	Km (µM)	CLint (ml/min/mg)
Before CPT-11	0.39±0.02	31.61±3.61	12.34
After CPT-11	0.63±0.05	11.78±3.70	53.48

Figure 14: *Effect of Irinotecan on GUS Activity*

4.10 Juvenile Rats may be More Susceptible to Irinotecan-induced Diarrhea than Adult Rats

In the experiment to see if age has any effect on GUS activity, we discovered that young rats (4 weeks old) displayed higher GUS activity after CPT-11 administration when compared to older rats (10 weeks old) (fig 15). Also, the increase in GUS activity after the administration of irinotecan in the young rats was more than 2 folds unlike that in older rats which was a slight increase (fig 15). This goes to show that age might have a synergistic

effect on irinotecan's ability to induce diarrhea as it drastically increases GUS enzymes in the gut.

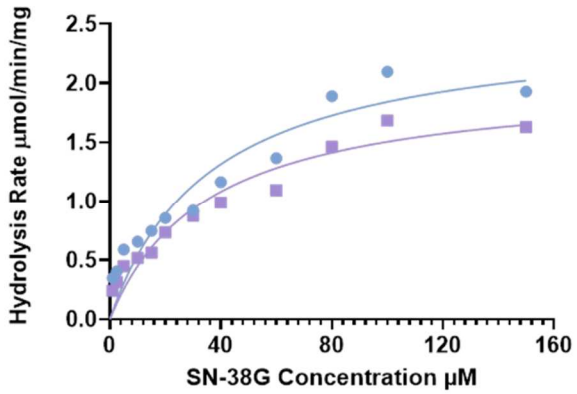


	Vmax (µmol/min/mg)	Km (µM)	CLint (ml/min/mg)
Young Before/After CPT-11	1.9/4.4	25.8/26.0	73.6/169.2
Adult Before/After CPT-11	3.1/3.5	43.6/34.1	71.1/102.6

Figure 15: *Effect of Age on GUS Activity*

4.11 Pirc Rats Showed Higher GUS Activity than F344 Rats

Two different rat breeds were compared to see if breed has any effect on GUS activity. The rat breeds were F344 and Pirc rats. Pirc is a type of rat that have higher incidence of diarrhea due to intestinal inflammation. It was discovered the Pirc rats displayed higher GUS activity when compared to F344 rats (fig 16) although the difference between the GUS activities in the rats were not significant.



	Vmax (µmol/min/mg)	Km (µM)	Clint (ml/min/mg)
Pirc	2.5	36.4	68.7
F344	2.0	35.3	56.7

Figure 16: *Effect of Rat Breed on GUS Activity*

Aim 3. Determine diarrhea attenuation by manipulating intestinal bacterial GUS using an herbal formula Xiao-Chai-Hu-Tang.

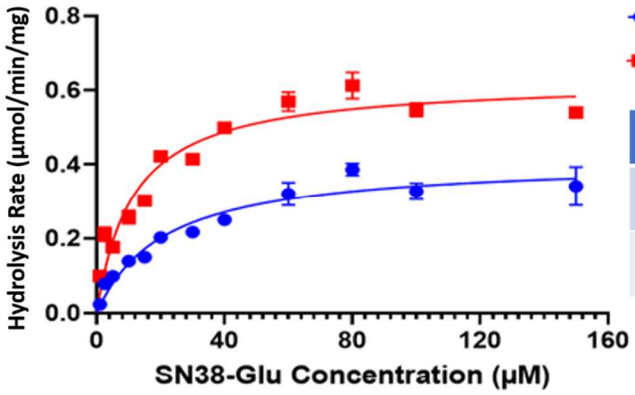
This aim is to determine the impact of XCHT on GUS activity and diarrhea.

4.12 XCHT Reduced GUS Enzyme Activity In Vitro

To determine the effect of XCHT on GUS activity in vitro, different concentrations of SN-38G was incubated with fecal S9 fraction in the presence and absence of XCHT at 37□ for 30mins. The result showed that in the presence of XCHT, the intrinsic clearance (CLint) of SN-38G was significantly decreased from 52.6±0.17 to 19.0±0.12 mL/min/mg, suggesting that XCHT components can inhibit GUS.

$$CLint = \frac{Vmax}{km}$$

It could also be seen that the activity of GUS reduced in the presence of XCHT (fig 17). This was also evident in the reduction of Vmax when XCHT is present.



	Vmax (µmol/min/mg)	Km (µM)	Clint (ml/min/mg)
XCHT	0.4	21.1	19.0
Saline	0.6	11.4	52.6

Figure 17: *XCHT Inhibition Effect on GUS Activity In Vitro*

4.13 XCHT also Reduced GUS Activity In Vivo After Administration of CPT-11

To see the effect of XCHT on GUS activity on different days after CPT-11 injection, the fecal samples collected at different days from the rat models treated with XCHT and Irinotecan injection were prepared and incubated with SN38-Glu at 37□ for 30mins. The results gotten were analyzed and the hydrolysis profile of GUS at different days were determined. In figure 18, it could be seen that XCHT reduced GUS activity immediately after CPT-11 injection until the end of the experiment (8 days after CPT-11 injection). From 2 days after CPT-11 injection, there was no more significant difference between the hydrolysis profiles of the rest of the days. This could be due to the system reaching equilibrium.

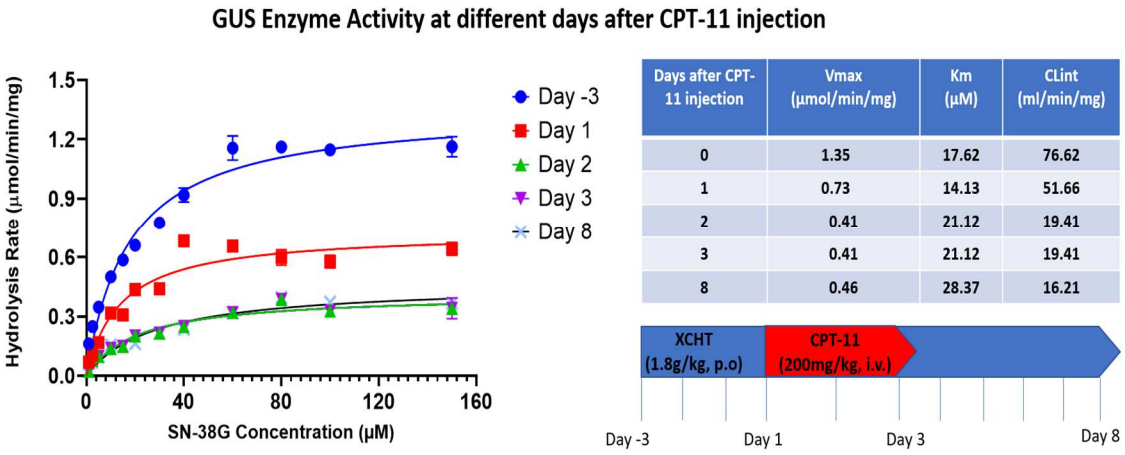


Figure 18: *XCHT Inhibition Effect on GUS Activity In Vivo After Administration of CPT-11*

4.14 XCHT is Shown to Alleviate Irinotecan-induced Diarrhea in Rats

F344 rats were used as models to evaluate the diarrhea preventive efficacy of Xiao-Chai-Hu-Tang (XCHT). The rats were divided into two groups and one group was given XCHT treatment (1.8g/kg p.o.) while the other was given Saline treatment. After 3days all the rats were given CPT-11 (Irinotecan – 200mg/kg) i.v. injection and their feces were collected daily for 9 days and the diarrhea severity was evaluated using the fecal conditions. The rat group treated with saline developed severe diarrhea (grade 3 and 4) by day 5 while the group treated with XCHT didn't develop up to grade 2 diarrhea throughout the days of collection (fig 19).

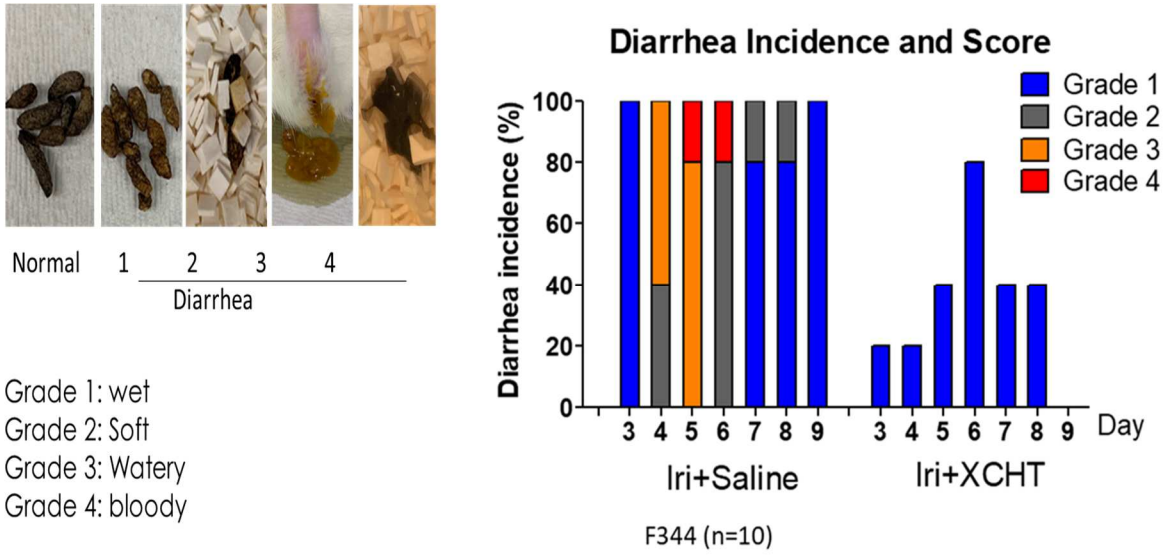


Figure 19: *Impact of XCHT on Irinotecan-induced Diarrhea in Rats (This experiment was conducted in Dr. Ming Hu's lab)*

CHAPTER 5

SUMMARY AND CONCLUSION

One of the dose-limiting toxicity of irinotecan is Diarrhea. This is caused majorly by the conversion of SN-38Glu back to its active form SN-38 in the intestine by beta glucuronidase enzyme. Thus, metabolism, such as glucuronides hydrolysis, by intestinal microflora plays an important role in a host's health. This is evident in the increasing number of papers that have been published to study drug and nutritional components metabolism by intestinal microflora. We therefore believe that studying the role of GUS enzyme and reduction of its activity can help with the alleviation of irinotecan-induced diarrhea.

To effectively study the role and activity of GUS enzyme it is important that we determine an effective and optimized method to do this. Enzyme-mediated reaction is a major and widely used model to study in vitro metabolic capability and specificity and lots of useful information have been reported using this model. However, different protocols have been used in enzyme-mediated reaction when studying microflora hydrolysis and lack of standardization of incubation conditions is one of the main factors affecting interstudy variability. Our first aim of this study was to develop and optimize the conditions of glucuronide hydrolysis by microflora using enzyme-mediated reactions. We achieved this by identifying and implementing methodological and technical improvements to reduce the discrepancies in determination of microflora hydrolysis activity across studies. The standard GUS activity probe pNPG and a common natural occurring dietary glucuronide wogonoside were used as our model compounds. We evaluated and optimized the conditions that may impact enzyme activity. The analytical method was developed and

validated (Figure 5). We found that sonication during enzyme preparation increased the yield of enzyme extraction from feces when compared to the suspension method which does not involve sonication (Figure 7). The reason we investigated sonication is because it is usually used to disrupt cellular or bacterial membranes and release the cells/ bacterial contents (Moore et. al., 2016; Taktak-BenAmar et. al., 2017; Sharma & Bisht, 2016; Prauchner et. al., 2013). Then an intriguing question is whether sonication will affect the GUS activity since proteins' structures may be influenced by sonication (Stathopulos et. al., 2004). To answer this question, we incubated the enzymes prepared with or without sonication with p-NPG and wogonoside and the results showed that for both p-NPG and wogonoside the hydrolysis rates are significantly higher using enzymes prepared with sonication (Fig. 8). These findings suggested that sonication didn't affect GUS activity and should be applied for better active enzyme extraction. We also found that GUS activity was decreased gradually when feces was exposed to air at room temperature (Fig. 9), suggesting bacteria could die in feces and it is better to collect the feces as early as possible to ensure adequate GUS activity. For in vitro GUS activity evaluation, not like phase 1 and phase 2 studies, the favorite reaction buffer condition remains unclear. It is well-known that magnesium is by far the most frequently found metal ion cofactor in enzymatic systems and is associated with enzyme activity in mammalian cells (Badee et. al., 2019), probably because magnesium ion can form stable complexes with phosphate-containing species, including ATP (Cowan, 2002). We then determined the GUS activity using KPI buffers containing magnesium ions at 0-10 mM, which was used in glucuronidation activity evaluation in literature (Badee et. al., 2019; Walsky et. al., 2012). Interestingly, the results showed magnesium ion concentration affected GUS activity for both pNPG and

wogonoside hydrolysis (Fig. 10) but the variations with different concentration of Mg2+ was only slightly different (<33%). Therefore, based on these data, we concluded that magnesium is not necessary for glucuronides hydrolysis by GUS. Other than magnesium, buffer pH is also an important factor affecting enzyme activity (Chang et. al., 2009). We then determined the impact of buffer pH on pNPG and wogonoside hydrolysis. The results showed that GUS activities for both pNPG and wogonoside were highly different at different pH (Fig. 11), which is similar as UGT activity as shown in literature (Chang et. al., 2009). In addition, different species have different favorite pH values. The rat and mouse fecal GUS were most active at pH 6.5, while human fecal GUS was most active at pH 7.4, probably because the pH value in the gut of the host is different where it was reported that human colon pH is around 6.0-7.4 and mouse and rat were 5.0-6.5 (McConnell et. al., 2008; Fallingborg, 1999). For drug metabolism, difference between species are frequently reported and translational potential is always challenged in preclinical models (Toutain et. al., 2010; Caldwell, 1982; Dybing & Huitfeldt, 1992). However, microflora GUS activity across species has never been reported previously. We determined the relative hydrolysis activity using pNPG and the results showed that human fecal enzyme was more active than that of rat and then than that of mouse (Fig 12). To further elucidate the difference, we determined wogonoside hydrolysis kinetics using three enzymes under the optimized conditions. The results also showed that human fecal GUS activity was significantly higher than that of rat and mouse (Fig. 12). Interestingly, the order of fecal GUS activity in these three species is as similar as fecal reduction activity reported previously using a bacteria culture system (Manning et. al., 1988), probably because the microbial composition in human feces is different with that in rodent (Nagpal

et. al., 2018). The fecal GUS activity is different across human, mouse, and rat, however, it is debatable whether mouse and rat can be used as preclinical models to study GUS activity and predict what will happen in human. For wogonoside, the difference in Vmax is only 2-3 folds, which is less than individual variations. However, the difference in Km is significantly different (20-fold). For the GUS activity probe pNPG, the difference is only 50%. More compounds should be used in future to full elucidate the variation across species. Individual variation is another concern in preclinical studies. Therefore, we determined the variation across individual using rat feces collected from 10 inbred rats, which were housed under the same condition and eating the same food. The results showed that fecal GUS difference across individuals is significant (4~6 folds) (fig. 13), suggesting that experimental condition should be well controlled, especially diet, which will have noteworthy impact on gut microflora (Flint, 2012; Conlon & Bird, 2014; Zhang et. al., 2018). On the other hand, this individual variation did not exceed the individual variations of phase I and II enzymes (e.g., CYPs and UGTs) activities (Fang et. al., 2018; den Braver-Sewradj et. al., 2018), revealing that fecal enzymes still can be used as an in vitro model to study drug metabolism when pooled feces are used.

In general, drug metabolism by microflora can be investigated by incubating the drug with colon contents (i.e. feces) in vitro (van de Steeg et. al., 2018; Imai et. al., 2019; Koistinen et. al., 2019), even though there is no assurance that the results obtained in in vitro studies could fully reflect the metabolism in vivo. It is difficult to collect contents from the colon without sacrificing animals, therefore, feces are usually used as the source of gut microflora. Since feces only contain around 50% bacteria (Stephen & Cummings, 1980), enzymes prepared from feces are commonly used instead of feces to study

microflora metabolic activity (Ervin et. al., 2019; Bhatt et. al., 2020; Sun et. al., 2019). Having developed and optimized the method to determine and evaluate GUS activity, the next step was to determine if any difference exist when fecal S9 gotten from different animals or animals with different treatment will affect GUS activity. The knowledge of this will help us know if GUS against SN-38G hydrolysis can be manipulated as this is an understudied area.

The first factor we checked was to see the effect of the drug - irinotecan on GUS activity. We discovered that administration of irinotecan alone displayed an increase in GUS activity after 3 days when compared to animals that did not receive the drug (fig 14). This is agreement with the discovery made by Bhatt et. al. (Bhatt et. al., 2020) that a single dose of irinotecan increased GUS activity.

We also were able to discover that factors such as age may impact the incidence of irinotecan-induced diarrhea as it has major effect on GUS activity. Younger rats (4 weeks old) showed higher GUS activity more than their older counterparts (10 weeks old) before administration of irinotecan (fig 15). 3 days after the administration of irinotecan, the younger rats showed more than 2 folds increase in GUS activity and the older rats also showed increase in GUS activity, but the change wasn't as drastic as that noticed in younger rats (fig 15). This suggests that age might have a synergistic effect on GUS activity in young animals. To check the impact of breed, we used Pirc rat (F344/NTac-Apc am1137), a type of rat that have higher incidence of diarrhea due to mucosal damage in its colon (Tortora et. al., 2020), as suspected displayed higher GUS activity when compared to F344 rats (fig 16), although the difference was not statistically significant. This suggests that GUS plays an important role in the occurrence of diarrhea in these rats.

Although the above results indicate that GUS activity can be manipulated by altering some factors and conditions, what is still unknown is whether the manipulation of GUS enzyme can affect irinotecan-induced diarrhea. We therefore decided to determine the attenuation of diarrhea by manipulating intestinal bacterial GUS using an herbal formula Xiao-Chai-Hu-Tang (XCHT). We chose XCHT because it's been reported to significantly decrease the exposure of SN38 in the gut (Sun et. al., 2019) as well as some of its herbal content have been individually shown to affect gut microflora (Guo et. al., 2015; Zhang et. al., 2018). and we propose that this herbal supplement will alter GUS enzymes in the intestine. F344 rats were divided into two groups and one group was administered XCHT while the other was given saline. The feces of the rats were prepared and furthered analyzed to determine the presence of GUS in them and it was determined that the fecal samples from rats given XCHT displayed reduced GUS activity when compared to those treated with saline. This was also reflective in the invitro study carried out in the lab were fecal samples were incubated with CPT-11 and XCHT or CPT-11 and saline. The samples with CPT-11 and XCHT showed reduced GUS enzyme activity (fig 17). When the fecal samples collected on different days after the rats administered with XCHT were analyzed and compared, we determined that there was reduced GUS activity as the days progressed until the system was saturated and there was no significant decrease anymore (fig 18). Since XCHT has proven to reduce GUS activity, we compared the feces collected from the rat models given XCHT treatment with the ones that had no XCHT treatment. We found out that irinotecan-induced diarrhea was alleviated in rats given XCHT, but this was not the case in rats that were not given XCHT treatment (fig 19). The rats that were given saline (in place of XCHT) developed severe diarrhea (grade 3 and 4)

by day 5 while the rats given XCHT did not develop even grade 2 diarrhea till the last day of the experiment. This result is in concurrent with the study by Sun et. al. 2019 that stated that XCHT has the potential of alleviating irinotecan induced diarrhea. These findings clearly show that the inhibition of GUS resulted in reduced incidence of irinotecan-induced diarrhea.

In conclusion, we were able to clearly show that the GUS enzyme is the major culprit in the development of irinotecan-induced diarrhea and its manipulation can result in the alleviation of irinotecan-induced diarrhea This knowledge can be applied in the management of chemotherapy induced diarrhea by alleviating this dose limiting toxicity.

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