# Inhibitory Effects of Sulfonylureas and Non-Steroidal Anti-Inflammatory Drugs on In-Vitro Metabolism of Canagliflozin in Human Liver Microsomes

A thesis submitted by

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In partial fulfillment of the requirements for the degree of

Master of Science

in

Pharmacology and Drug Development

Tufts University

Sackler School of Graduate Biomedical Sciences

May, 2017

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## Abstract

Canagliflozin, a sodium-glucose transport protein 2 (SGLT2) inhibitor, is a novel drug used to treat type 2 diabetes mellitus (T2DM). A Phase II glucuronide conjugation reaction is the primary elimination pathway of canagliflozin. Uridine diphosphate glucuronosyltransferases (UGTs) are the responsible enzymes for the glucuronidation reactions that occur in Phase II metabolism. The glucuronidation of canagliflozin results in formation of two major inactive metabolites, M7 and M5, that are catalyzed by UGT1A9 and UGT2B4, respectively. Canagliflozin is commonly co-administered with other antidiabetic agents such as sulfonylureas. Until now, there has been no documented in vitro drug-drug interactions (DDIs) study conducted to assess the inhibitory effect of other drugs on the metabolism of canagliflozin. Moreover, the inhibitory effects of sulfonylureas against UGTs isoenzymes are not well established. In this study, the inhibitory effect on the metabolism of the two metabolites of canagliflozin, M7 and M5, has been investigated using three sulfonylurea drugs as inhibitors, including chlorpropamide, glimepiride, and gliclazide. Additionally, two non-steroidal anti-inflammatory drugs were included as positive controls which are known for their inhibitory effect against UGT1A9 (niflumic acid) and UGT2B4 (diclofenac). The rate of formation for M7 and M5 metabolites was monitored by HPLC after incubation of canagliflozin as a substrate with and without inhibitors at different concentrations. The IC50 values were calculated for all inhibitors by using five different individual human liver microsomes (HLMs) including pooled HLMs. Ki values were calculated for niflumic acid by using additional three individual HLMs and a pooled sample.

Among sulfonylureas, glimepiride showed the most potent inhibitory effect against M7 metabolite formation with an IC50 value of 88 ± 4 µM (mean ±SE), compared to chlorpropamide and gliclazide with IC50 values of more than 500µM. Diclofenac inhibited M5 metabolite formation, which is catalyzed by UGT2B4, more than M7, with IC50 values of 32 ± 13µM for M5 and 80 ± 13 µM for M7. Niflumic acid showed no inhibition activity against M5 formation, but showed relatively selective inhibitory potency against M7 formation with an IC50 value of 1.9 ± 0.03µM and a Ki value of 0.8±0.25 µM. The results of this study suggest that there is unlikely to be a metabolic interaction between canagliflozin and sulfonylureas. This study also demonstrates a possible clinical interaction between niflumic acid and canagliflozin. The low Ki value of niflumic acid, when compared to its maximum plasma therapeutic concentration, suggest the possibility of a clinical drug interaction which could be of therapeutic impotence.

## Acknowledgements

First and foremost, I want to thank and express my sincere gratitude to my advisor Dr. David J. Greenblatt for giving me this opportunity to work in his lab. I appreciate all his contributions of time, expert guidance, and funding to make my master's research project. The door of Greenblatt's office was always open whenever I needed advice or had a question on my research. I am especially grateful for the confidence he put in me and the freedom he gave me to choose my thesis topic. I am proud to be one of his students, and it was my honor to complete my research work under his supervision.

My sincere thanks go to Amin Hussain, who graduated from the same program last year, he introduced me to the lab techniques, and he was always there to listen and to give advice. Thanks also go to my colleagues in Greenblatt's lab, Novera Alam, Vaughn Youngblood, and Yunmi Park. Their corporation, support, and friendship will never be forgotten.

I would also like to acknowledge Dr. John Castellot as the second reader of this thesis, and I am gratefully indebted to his valuable comments on this thesis.

Finally, I must express my very profound gratitude to my parents, Ghazi Algeelani and Ramziah Khojah and to my sister and brother, Nessrin and Wail and to my lovely roommates, Aseel Jambi, Abeer Aleid, Shatha Bajowaiber, Bayan Alhefzi, and Ebtesam Alharbi. They provided me with unfailing support and continuous encouragement. This accomplishment would not have been possible without them. Thank you.

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# List of Abbreviations

| | |
|---|---|
| AUC | The area under the curve |
| CYP | Cytochromes P450 |
| DDIs | Drug-drug interactions |
| HLMs | Human liver microsomes |
| HPLC | High Performance Liquid Chromatography |
| NSAIDs | Nonsteroidal anti-inflammatory drugs |
| PD | Pharmacodynamics |
| PK | Pharmacokinetics |
| SGLT2 | Sodium-glucose co-transporter 2 |
| T2DM | Type 2 diabetes mellitus |
| UDPGA | Uridine-diphosphate-glucuronic acid |
| UGT | Uridine 5'-diphospho-glucuronosyltransferase |
| NFA | Niflumic acid |
| CANA | Canagliflozin |

## Chapter 1: Introduction

According to the American Diabetes Association, diabetes mellitus was the 7th leading cause of death in the United States in 2010. It is estimated that 9.3% of the global population (29.1 million people) have diabetes based on the national diabetes statistics report. Type 2 diabetes mellitus (T2DM) is the most prevalent type of diabetes. It accounts for about 90% of all adult people with diabetes [1]. T2DM is a chronic condition of high blood glucose level, which occurs as a result of varying degrees of insulin deficiency and resistance. Untreated diabetes mellitus can lead to serious health complications include damage to the heart, kidneys, eyes, extremities, and nervous system.

Since the discovery of insulin in the 1920s and until the last 20 years, most of the medications for type 2 diabetes mellitus had a focus on reducing hepatic glucose production, increasing insulin secretion from the pancreas, or increasing receptor sensitivity in peripheral tissues. Currently, we have about 11 different categories of pharmacological therapies designed to control hyperglycemia. However, there are still difficulties in achieving the target blood glucose level in many patients. Therefore, there is a need for new agents with different mechanisms of action that can be synergistically more effective in controlling and reducing hyperglycemia for patients with chronic T2DM.

Canagliflozin is a novel hypoglycemic drug, approved by the FDA in March 2013 to treat adults with type 2 diabetes mellitus (T2DM)[2]. Unlike other hypoglycemic agents, canagliflozin has a unique mechanism of action as it does not work on the secretion/ absorption of insulin or glucose. Canagliflozin inhibits renal glucose reabsorption by

inhibiting the subtype 2 sodium-glucose cotransporter (SGLT2) in the proximal kidney tubule. It increases the excretion of glucose urine, and consequently, reduces the glucose level in blood[3]. The recommended starting dose is 100 mg, and it can be increased to 300 mg once daily. Canagliflozin can be given as a monotherapy or in combination with other antidiabetic medications[4]. However, it is commonly prescribed in combination with sulfonylureas.

Possible drug-drug interactions (DDIs) are of concern in combination drug therapy. DDIs are classified into pharmacokinetic (PK) and pharmacodynamic (PD) interactions. PK interactions occur when one drug interacts with the PK properties of the other co-administered drug, and that includes absorption, distribution, metabolism, or excretion. In PD interactions, the drug only interacts with the target organ of the combined drug without altering its PK. PK interactions, especially metabolism-mediated drug-drug interactions, are well documented and have contributed to many instances of drug recall and market withdrawals[5].

Hypoglycemic agents, including canagliflozin and sulfonylureas, undergo hepatic metabolism, a biotransformation process that occurs mainly in the liver. There are two phases of hepatic metabolism; Phase I includes oxidation, reduction, and hydroxylation reactions, and Phase II includes conjugation reactions. These metabolic reactions produce more hydrophilic, more polar, and less therapeutically active metabolic products of drugs so that they can be excreted in the urine or the bile more easily. Some drugs undergo either Phase I or Phase II metabolism. However, the majority undergo sequential Phase I followed by Phase II.

Phase l metabolism involves mainly cytochrome P450 (CYP) oxidation mediated reactions, and is the responsible metabolic reaction for most clinically significant drug-drug interactions. However, Phase II metabolism also plays an important role in the biotransformation of many drugs and endogenous compounds. In Phase II metabolism, drugs or metabolites from Phase I are attached to large polar endogenous molecules forming more hydrophilic conjugated compounds. After conjugation, the polarity and water solubility of the compound is increased, and thus enhances the extent of its excretion out of systemic circulation into urine or bile. Glucuronidation, sulfation, methylation, acetylation, glutathione, and amino acid conjugation are the primary forms of conjugation reactions. Glucuronidation is the most common and the most important form which accounts for about 35% of the Phase 2 metabolic reactions [6].

Glucuronidation reactions occur by the transfer of glucuronic acid from UDP-glucuronic acid to a functional group on the substrate, and forming O, C, N, or S-glucuronide [7]. This reaction is catalyzed by specific enzymes called uridine 5'-diphospho-glucuronosyltransferases (UGTs). Many UGT isoforms exist: UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, and 2B15 have been shown to play the most important roles in drug metabolism. Most Phase II metabolic interactions happen as a result of inhibition or induction of these enzymes. However, the induction of Phase II enzymes, including UGTs, is less important compared to Phase I CYP450s.

Some substrates are metabolized by specific UGT isoforms. For example, the glucuronidation of endogenous bilirubin is relatively selective for UGT1A1, which is the principal enzyme responsible for the removal of bilirubin from the body. Mutations in the UGT1A1 gene may cause accumulation of unconjugated bilirubin in the blood, which in

one specific case is known as Crigler-Najjar syndrome [8]. Nonetheless, the substrate specificity for UGT isoforms is not as well established as for CYP450 isoforms. One reason is the lack of data on UGT isoform specific inhibitors that can be used as positive controls for in-vitro metabolism studies. The only drug that has been established by FDA as a UGT1A1 inhibitor is atazanavir; however, it also inhibits CYP3A [9]

Studying inhibitory drug interactions in Phase II metabolism is challenging but necessary. DDIs studies begin with an in-vitro system that includes human liver tissue. The results of these in-vitro studies can indicate the need to assess the potential risk of interactions in in-vivo systems [10]. Understanding the mechanism of enzyme inhibition is critical to understand the clinical DDIs. The mechanism of enzyme inhibition can be either irreversible or reversible (competitive, noncompetitive, uncompetitive, or mixed). Irreversible inhibition is also called mechanism-based or time-dependent inactivation, wherein the inhibitor reacts with the enzyme and forms a covalent bond. Reversible inhibition is more common, and it can be further classified into four types. With competitive inhibition, substrate and inhibitor both compete to bind at the same enzyme site. In contrast to competitive inhibition, noncompetitive inhibition involves inhibitor binding to to a different site of the free enzyme. The inhibition can also be a mixture of competitive and noncompetitive. Uncompetitive inhibition is the last type, in which the inhibitor can bind only to the substrate-bound enzyme complex [11].

The PK and the metabolic profile of canagliflozin are essential to understand the potential for metabolic DDIs. In a mass-balance study of canagliflozin in animals and humans [12], the drug was mainly metabolized by UGT enzymes. The major metabolic pathway of canagliflozin in humans was O-glucuronidation, accounting for about 30% of

the administered dose. Glucuronidation of canagliflozin produces two major plasma metabolites, M5 and M7; glucuronic acid is attached at the 2′ and 3′ positions of the 2-hydroxymethyl-tetrahydropyrantriol moiety of M5 and M7, respectively (Figure 1). UGT1A9 is the specific isoenzyme that responsible for M7 metabolite, and UGT 2B4 is responsible for M5 metabolite formation.

A review article published in 2014 analyzed the potential DDI of SGLT2 inhibitors, including canagliflozin, with other antidiabetic, cardiovascular and other medications of interest. This review included all clinical studies that performed from 2008 to October 2013, and it showed no clinically relevant pharmacokinetic DDIs. However, most of the studies in this review were conducted on healthy volunteers, and mostly after a single acute administration of the SGLT2 inhibitor [13].

In 2015 another clinical study was conducted to assess the DDI based on the metabolic profile of canagliflozin. As canagliflozin is mainly metabolized by UGTs, they analyzed the canagliflozin exposures with the co-administration of drugs that induce or inhibit UGTs such as rifampin and probenecid, respectively. There was no important clinical interaction detected for probenecid, but rifampin produced a moderately reduction in the canagliflozin plasma concentration [14]. Moreover, probenecid might be considered as a general UGT inhibitor, and it is not selective for UGT1A9 which forms the primary metabolite of canagliflozin, M7. No DDI was detected in another study conducted in the same year to assess the potential effect of canagliflozin on the PK of metformin, glyburide, and simvastatin, which are the common medications that are used with canagliflozin. Beyond that, the effects of these drugs on canagliflozin plasma levels have not been assessed [15]. Canagliflozin inhibited UGT1A isoenzymes, especially

UGT1A1, UGT1A9, and UGT1A10 (IC50 values ≤ 10 μM), in an in-vitro study that evaluated the inhibitory effect of SGLT2 inhibitors on UGTs enzymes using human liver microsomes [16].

No in vitro study has evaluated the inhibitory effect of other drugs on the metabolism of canagliflozin. Since canagliflozin is commonly used with other antidiabetics and especially sulfonylureas, in-vitro Phase II DDI studies involving canagliflozin and sulfonylureas are needed. One study has shown that 100μM glimepiride (a second-generation sulfonylurea) inhibits UGT1A9 by about 70% [17]. UGT1A9 is the enzyme responsible for formation of the principal metabolite of canagliflozin, M7.

The aim of this project was to study the possible inhibitory effect of other anti-diabetic drugs on the metabolism of canagliflozin. For the first time, canagliflozin was used as a substrate to test the inhibitory effect of other antidiabetics sulfonylureas and positive controls using the in-vitro system based on human liver microsomes. Three types of sulfonylureas were used as inhibitors: chlorpropamide from the first generation, and glimepiride and gliclazide from the second and the third generations. Two positive controls have been used to validate the model system. These have been established previously for their potent inhibitory activity toward UGT2B4 and UGT1A9 enzymes, which mediate the formation of M5 and M7, respectively. Niflumic acid has been used as a potent and selective inhibitor of UGT1A9. For UGT2B4, there is no established selective inhibitor, so diclofenac has been used as a general inhibitor of the UGT2B subfamily.

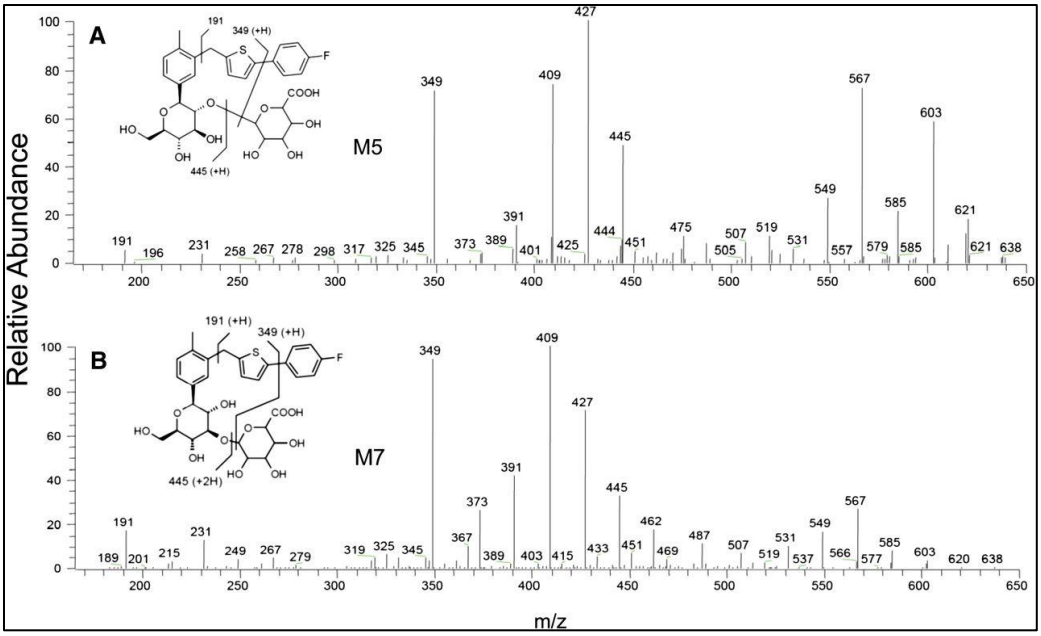



Figure 1.1: Chemical structure and product mass spectra of the M5 and M7 metabolites of canagliflozin [12].

# Chapter 2: Materials and Methods

## 2.1. Chemicals and reagents

Canagliflozin were purchased from Advanced ChemBlocks Inc (Burlingame, CA, USA) Methanol, acetonitrile, potassium phosphate, and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Telmisartan, glimepiride, glipizide, chlorpropamide, alamethicin, UDP-glucuronic acid (UDPGA, trisodium salt) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

## 2.2. Human liver microsomes

Human liver samples were obtained from the National Disease Research Interchange (Philadelphia, PA, USA). The samples were mechanically homogenized and centrifuged at 10 000× g for 20 min. The microsomal pellets from the human liver were then suspended in 0.1 M potassium phosphate buffer containing 20% glycerol and kept at −80°C until used according to the method of von Moltke et al. [18]. Four different HLMs

from adult Caucasian males, and one pooled HLM from 30 donors with mixed gender and ethnicity, were used in the concentration range of 0.5-1mg/ml (Table 2.1).

Table 2.1: Demographic characteristics of HLMs.

| Liver ID | Age | Sex | Ethnicity | Activity of 100µM Propofol glucuronidation (Catalyzed by UGT1A9) nmoles/mg/min |
|---|---|---|---|---|
| **FGH** | 33 | M | C | 0.75 |
| **774** | 32 | M | C | 2.32 |
| **792** | 34 | M | C | 1.51 |
| **839** | 35 | M | C | 2.16 |
| **Pooled (30-donors)** | Mixed (2-75) | Mixed (M, F) | Mixed (A, C, H,) | Max: 5.61 Min: 0.43 |

HLMs, human liver microsomes; M, male; F, female; A, African; C, Caucasian; H, Hispanic. Michael H. Court data, from Greenblatt's lab,2004.

### 2.3. In vitro glucuronidation of canagliflozin

For canagliflozin glucuronidation, the dried substrate (varying concentrations of canagliflozin) was incubated. The incubation mixture contained: 5 mM $MgCl_2$, 50 mM phosphate buffer (PH 7.4), 50µg alamethicin for each 1mg protein, 0.5mg/ml HLM, and 5mM UDPGA. The total volume of the incubation mixture in each sample was 140µl. First, the detergent, alamethicin, was added to the HLM and was placed on ice for 5 min to allow pore formation. Then 100 µl of the pre-incubation mixture was added to the dried substrate and incubated in a shaking water bath at 37°C for 10 min. To start the reaction, 40µl of UDPGA was added to the samples and incubated for another 60 min. The metabolic reaction was terminated by placing the samples on ice for 2 min and adding 20 µl of stop solution (telmisartan + acetonitrile 1:10). Telmisartan was added as an internal standard (IS). After adding the stop solution, the mixture was vortexed and

centrifuged at 13000 rpm for 10 min. Finally, the supernatant was injected into the HPLC for analysis.

Linearity of canagliflozin metabolite (M5, M7) formation with respect to incubation time was evaluated to determine the best incubation time (Figure 2.1). The substrate concentration had been chosen based on the reaction velocity versus concentration relationship conforming to Michaelis–Menten kinetics (Figure 2.1). Canagliflozin 200 µM was the best concentration that produces sufficient metabolite formation for quantifying enzyme activity even when the enzyme is 95% inhibited. Thus, 200µM canagliflozin was the constant substrate concentration that was used in the in-vitro inhibition assay to determine the IC50 of all inhibitors tested.

**2.4. Chromatographic conditions**

HPLC analysis of canagliflozin glucuronidated metabolites was performed using an Agilent Technologies Series 1100 system. Compounds were separated using a reverse phase 300 × 3.9-mm µBondapak C18 column (Waters Associates, Milford, MA). The mobile phase was isocratic, consisting of acetonitrile and 20 mM potassium phosphate buffer (55: 45, v/v). pH was adjusted to 3.2 by addition of 50% formic acid, and the mixture filtered through a Millipore membrane filter before use. A flow rate of 1mL/min was used throughout the analysis. The volume of the injection was 20 µl, and the eluents were detected by a fluorescence detector at 280nm excitation and 325nm emission wavelengths according to the method of Iqbal et al. [19].

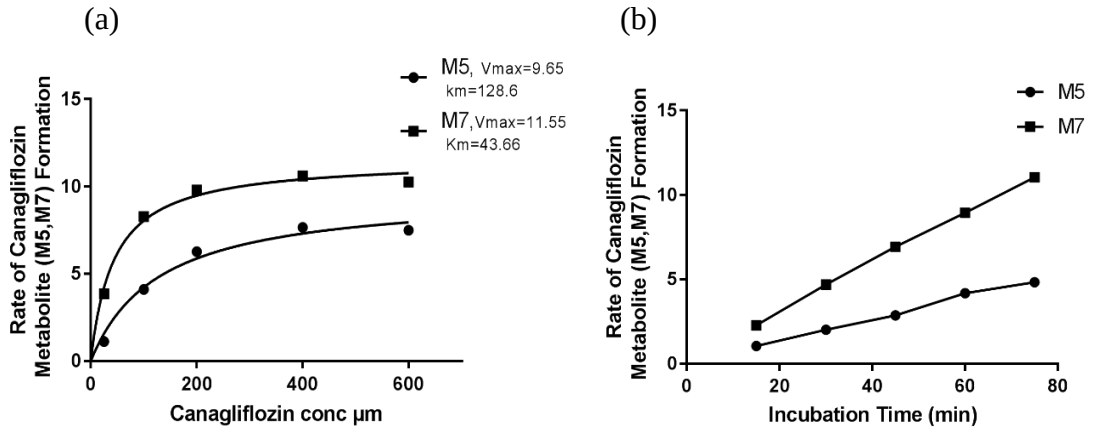



Figure 2.1: Rate of formations of canagliflozin metabolites M5 and M7. Pooled human liver microsomes were used. (a) Curves represent nonlinear regression model using Michaelis-Menten equation (described in Eq.1.) to determine the reaction velocity for the two metabolites in relation to substrate concentration. (b) The linear relationship between incubation time and formation of canagliflozin metabolites.

## 2.5. IC50 determination assay

In vitro inhibition of canagliflozin glucuronidation was done using the same method as described above, except different concentrations of inhibitors were added to the incubation mixture before drying the solvent. The inhibitory effects of known UGT 1A9 and UGT 2B4 inhibitors were evaluated as positive controls. Niflumic acid (0.1-30 µM) was used as a specific UGT1A9 inhibitor to test the inhibition of the M7 metabolite of canagliflozin. There is no specific inhibitor for UGT2B4, so diclofenac (10-200µM) was used to evaluate the inhibition of the other metabolite, M5. Varying concentrations of sulfonylureas, glimepiride (10-1000µM), Gliclazide (50-2000µM), and chlorpropamide (20-1500µM) were used as inhibitors in three separate sets of incubation tubes. Control incubations without inhibitor were also done to confirm baseline reaction velocities. One concentration (at the IC50 concentration) of the positive control was included with each study run to validate the system. The three sets of inhibitors were evaluated in parallel and underwent the same in vitro incubation conditions that described above. All incubations were carried out in duplicate for each concentration.

### 2.6. Time-dependent inhibition assay

For the purpose of testing whether the inhibition is time-dependent or not, pre/no pre-incubations were conducted for the inhibitors with an IC50 of less than 100µM. Based on these criteria, three inhibitors were studied in this assay. Two concentrations were used for each inhibitor: glimepiride (10,100 µM), diclofenac (10,100 µM), and niflumic acid (0.5,10 µM). In this assay, one set of the inhibitor with the substrate (canagliflozin) underwent the same previous no pre-incubation process. For the other set, the pre-incubation technique was used, in which the inhibitor is exposed to the HLM and the cofactor and pre-incubated for 15 min before the addition of substrate. The two sets of pre/no pre-incubation were conducted in parallel at the same time and under the same incubation conditions. The percentage of inhibition of each concentration was compared between the two conditions. The inhibition is considered time-dependent if the pre-incubation enhances the extent of inhibition. In the case of the time-dependent inhibition model, there will be an irreversible interaction between the inhibitor and the enzyme, leading to inactivation of the enzyme.

### 2.7. Ki determination assay for niflumic acid

Since niflumic acid produced significant reversible inhibition of M7 metabolite formation, an additional Ki assay was done to determine the inhibition constant. The Ki value defines the inhibitory affinity of niflumic acid for UGT1A9, and is independent of the substrate concentration. This assay was done as described above, using 6 additional concentrations of substrate (canagliflozin 10, 25, 50, 100, 200, and 400µM) incubated with 6 different concentrations of inhibitor (niflumic acid 0.1, 0.5, 1, 5,10, 30 µM) in duplicate samples. Two individual HLMs plus pooled HLM were included in this assay.

The inhibition type was determined by using a mixed competitive- non-competitive model as described below.

**2.8. Data analysis**

The Michaelis-Menten equation (Eq.1) was fitted to metabolite formation rates using Graph Pad Prism 7.02 software.

$$V = \frac{Vmax\ [S]}{Km+[S]} \qquad \text{(Eq.1)}$$

where $V$ is the relative metabolite formation velocity. Iterated variables were*:* $Vmax$, the maximum reaction velocity; and $Km$, the Michaels–Menten constant. $S$ is the substrate concentration.

The IC50 value is typically defined as the concentration of the inhibitor at which the velocity of metabolite formation is half the velocity without inhibitor [20]. For reversible inhibition, this is equivalent to the inhibitor concentration that decreases the metabolite formation rate by 50%. The inhibitory potential of the inhibitors was determined by measuring the decrease in metabolite formation of canagliflozin by the enzymes UGT1A9 and UGT2B4 compared to the vehicle control. Non-linear regression analysis was performed to fit the 3-parameter equation (Eq.2) to the experimental data using Graph Pad Prism 7.02 software.

$$Rv = 100(1 - \frac{Emax.[I]^b}{[I]^b+IC^b}) \qquad \text{(Eq.2)}$$

where $Rv$ is the percentage ratio of metabolite formation velocity relative to the velocity of the control without inhibitor; $[I]$ is the concentration of the inhibitor, Emax is the maximum degree of inhibition, and b is an exponent. The actual IC50 was calculated in the following equation (Eq.3) where IC is the estimated IC50 from the program.

$$IC50 = \frac{IC}{(2\ Emax-1)^{\frac{1}{b}}} \qquad \text{(Eq.3)}$$

To determine Ki, the mixed competitive–noncompetitive inhibition equation (Eq.4) was fitted to the data points by nonlinear regression using Graph Pad Prism 7.02 software.

$$V = \frac{Vmax.[s]}{[s]\left(1+\frac{[I]}{\alpha.Ki}\right)+Km\ (1+\frac{[I]}{Ki})} \qquad \text{(Eq.4)}$$

Iterated variables were: Vmax, the maximum reaction velocity; Km, the Michaels–Menten constant; Ki, the inhibition constant; and α is a value always greater than zero, indicating the 'mix' of competitive and noncompetitive mechanisms.

To describe the variability of data, the mean and standard error of mean (SEM) were used after averaging the duplicates for each sample.

# Chapter 3: Results

## 3.1 In-vitro metabolism of canagliflozin

Following the in vitro incubation of canagliflozin, the two main glucuronide metabolites M5 and M7 were detected in addition to the peak corresponding to remaining canagliflozin. The retention times for canagliflozin, M5, M7, and IS were 4.6, 3.26, 3.76, and 5.88 min respectively (Figure 3.1). Since the peak height of the two metabolites represent the glucuronide metabolite formation rate, they were not detected in the absence of UDPGA, and increased with increasing canagliflozin concentration and incubation time with UDPGA added (Figure 2.1). The M7 metabolite distinguished from M5 by using a selective UGT1A9 inhibitor (niflumic acid) which selectively inhibited the M7 peak at 3.76 min, and had no effect on the M5 metabolite formed by UGT2B4. Diclofenac is not a selective inhibitor of UGT2B4, but it generally inhibits the UGT2B subfamily. It produced inhibition of the M5 peak at 3.26 min more than M7 (Figure 3.2).

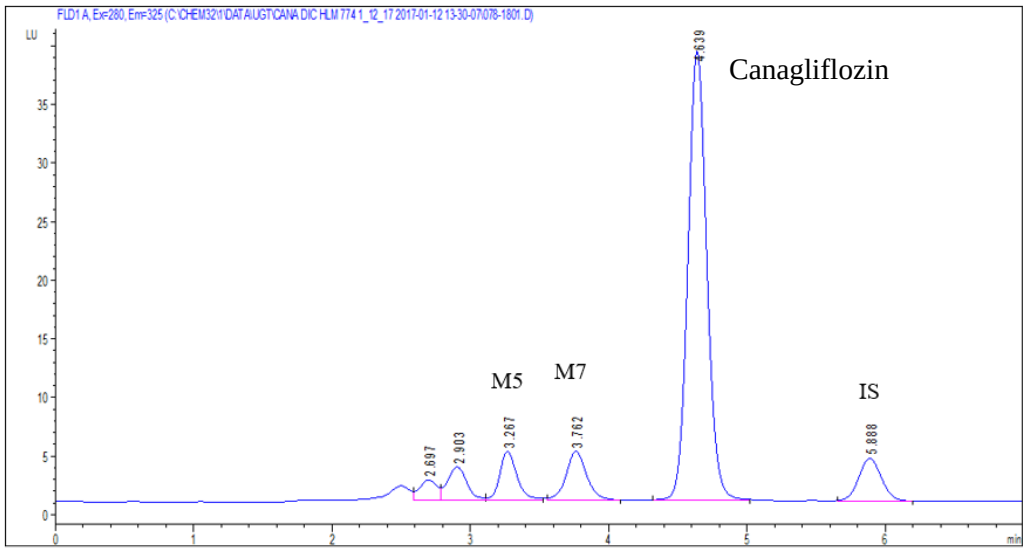



Figure 3.1: Chromatogram from a human microsome incubation with canagliflozin 200µM and UDPGA, showing peaks corresponding to canagliflozin and its metabolites, M5, M7, and the internal standard (IS) telmisartan.

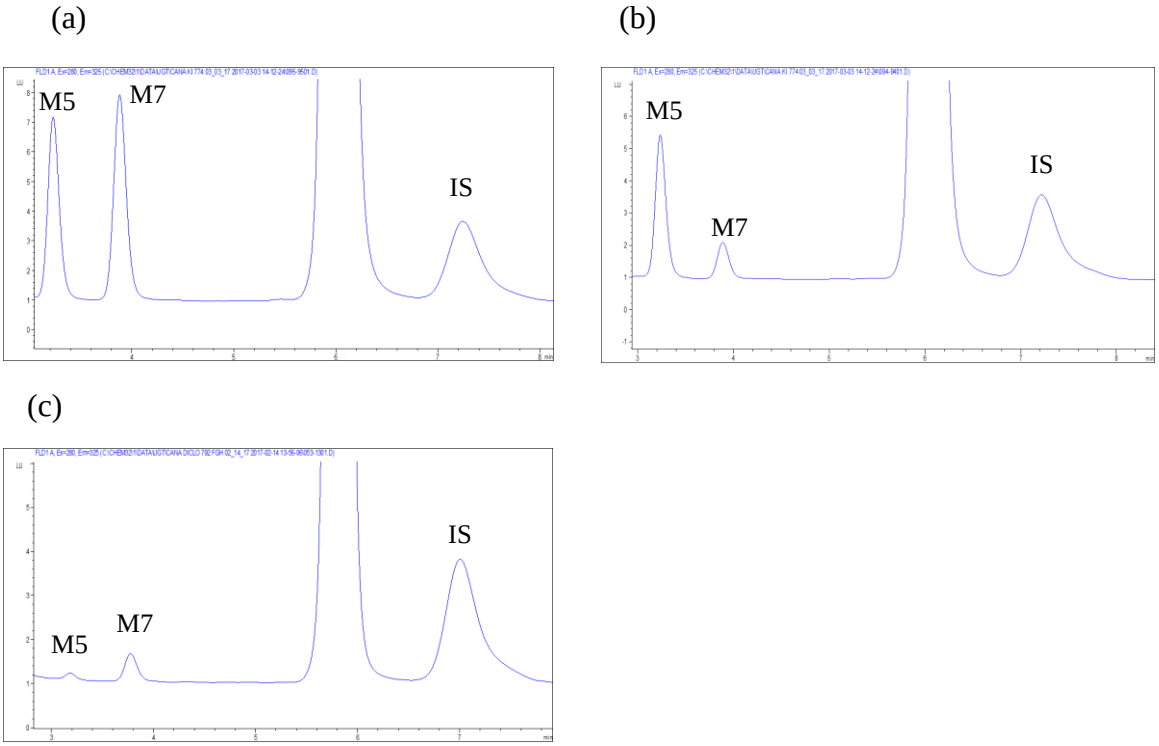



Figure 3.2: Chromatogram from a human microsome incubation with canagliflozin 200µM and positive control inhibitors. (a)The zero-inhibitor control (b) 30µM niflumic acid was added as an inhibitor that selectively inhibited the peak of M7 and had no effect on the peak of M5 (b) 200µM diclofenac was added as an inhibitor that inhibited the peak of M5 more than M7.

### 3.2. The inhibition of canagliflozin glucuronidation by positive controls

Niflumic acid and diclofenac were used as positive controls. Niflumic acid was anticipated to inhibit M7, as it is considered to be a selective inhibitor for UGT1A9. Diclofenac was expected to inhibit M5 because of its known inhibitory activity toward the UGT2B subfamily. Results indicated that niflumic acid produced potent inhibition of the activity of UGT1A9, with an IC50 value of 1.86 ± 0.03µM. Niflumic acid produced negligible inhibition of M5 metabolite formation (Figure 3.3). Diclofenac inhibited M5 more than M7, with IC50 values of 31.7 ±12.5 and 80 ±13.4µM respectively (Figure 3.3).

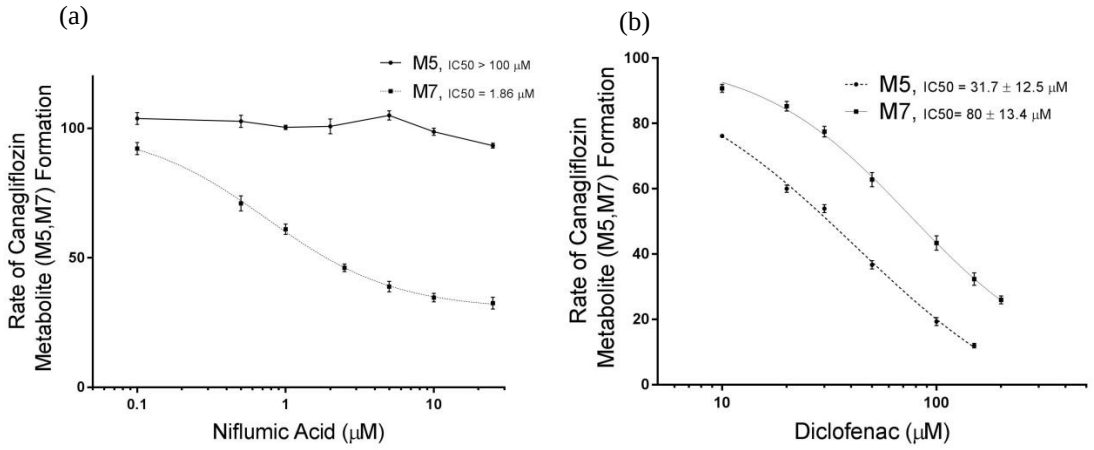
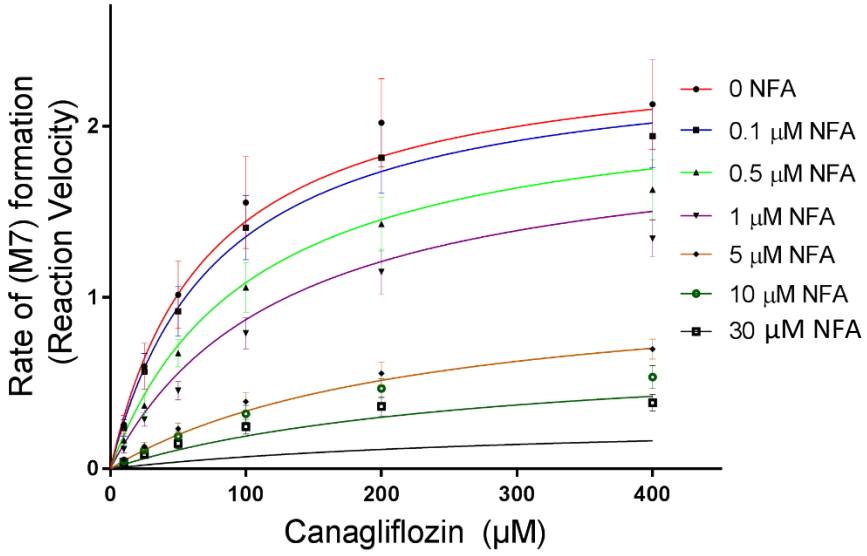



Figure 3.3: IC50 inhibition curves for positive controls ((a) niflumic acid, (b) diclofenac) vs. formation of canagliflozin glucuronides M5 and M7. The canagliflozin concentration was fixed at 200µM. The y-axis indicates the rate of metabolite formation, expressed as a percentage ratio relative to the control containing no inhibitor. Points represent mean ± SE of averaging the duplicates of each sample then averaging these values across 5 different HLMs including one pooled.

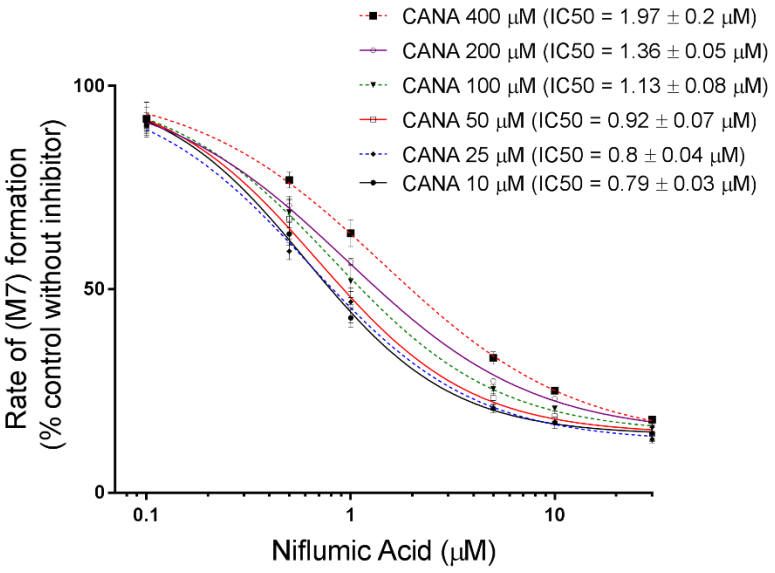
### 3.3. Ki Determination of niflumic acid against M7 metabolite

To further characterize the inhibition of niflumic acid toward M7 metabolite of canagliflozin, additional enzyme kinetic experiments were performed with varying concentrations of canagliflozin and niflumic acid. Based on nonlinear regression analysis using Graph Pad Prism 7.02 software, niflumic acid showed mixed competitive and noncompetitive inhibition against the formation of M7 metabolite with a mean Ki of 0.81

±0.25 µM, km of 71.1 ±10.4 µM, α of 5.0 ±3.5, and Vmax of 2.47± 0.11(Figure 3.4 a). Figure 3.4 b shows that IC50 values become larger with increasing concentrations of substrate, indicating that the inhibition has at least some contribution of a competitive mechanism.




(a)




(b)

Figure 3.4: The rate of M7 metabolite formation with various concentrations of canagliflozin and niflumic acid. Lines represent the functions determined by nonlinear regression based on (a) mixed model inhibition equation (Eq 4) (b) IC50 is increasing with increasing canagliflozin concentrations (Eq 2,3). Points represent mean ±SE of duplicate samples of 3 different HLMs including pooled.

### 3.4. The inhibition of canagliflozin glucuronidation by sulfonylureas

Three different sulfonylureas were used in this study to compare their possible inhibitory effect on the formation of canagliflozin metabolites. Chlorpropamide and gliclazide showed no significant inhibition against either M5 or M7 formation, with IC50 values of more than 500 µM. Glimepiride showed more inhibitory activity than chlorpropamide and gliclazide against canagliflozin metabolites, and especially M7 with an IC50 of 88 ± 4 µM (Figure 3.5)

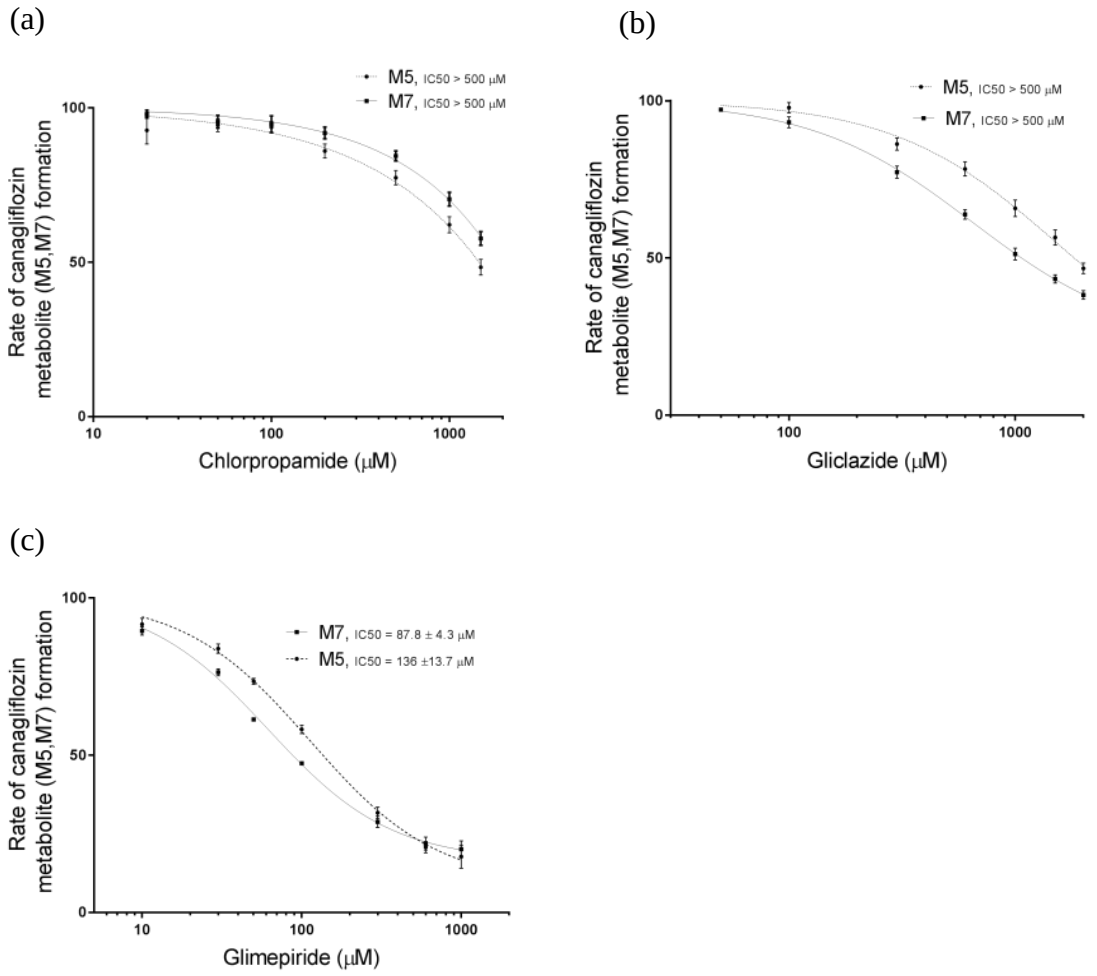
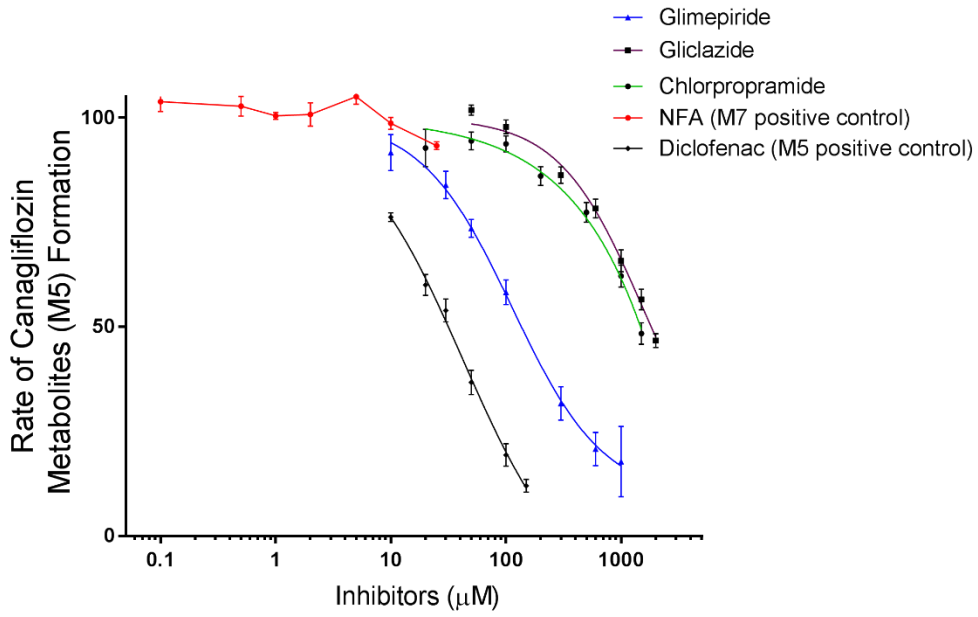



Figure 3.5: IC50 Inhibition curves for sulfonylureas (a) chlorpropamide, (b) gliclazide, (c) glimepiride, vs formation of canagliflozin glucuronides M5 and M7.

(a)




(b)

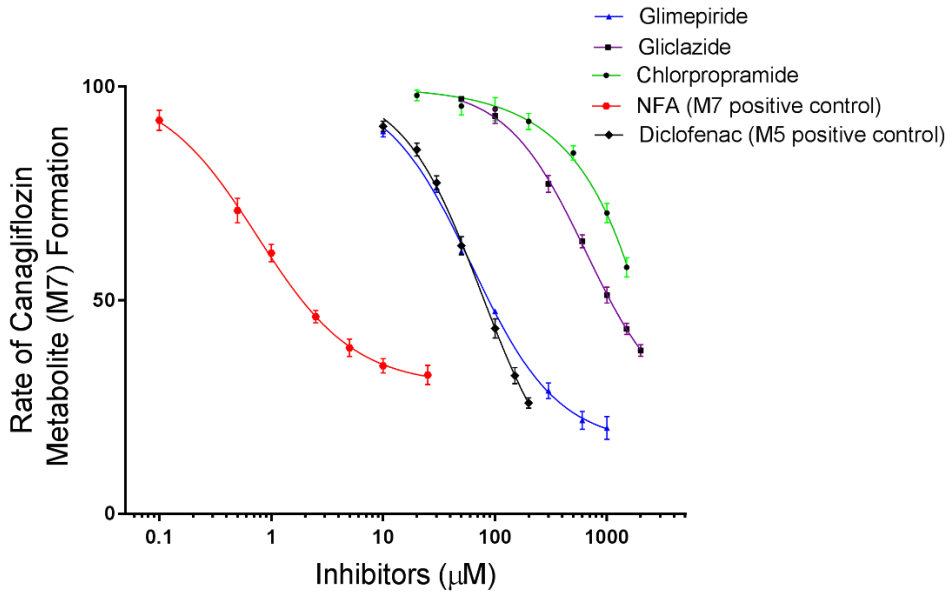



Figure 3.6: IC50 inhibition curves for all sulfonylureas (chlorpropamide, gliclazide, and glimepiride) and positive controls (diclofenac and niflumic acid) vs. (a) Rate of M5 formation (b) Rate of M7 formation.

### 3.5. Inhibition by niflumic acid, diclofenac, and glimepiride is reversible

No significant difference was observed in the extent of inhibition between pre-and no pre-incubation conditions for the three inhibitors. These results indicate that the inhibition of M7 by niflumic acid and glimepiride and M5 by diclofenac is reversible and not time-dependent (Figure 3.7)

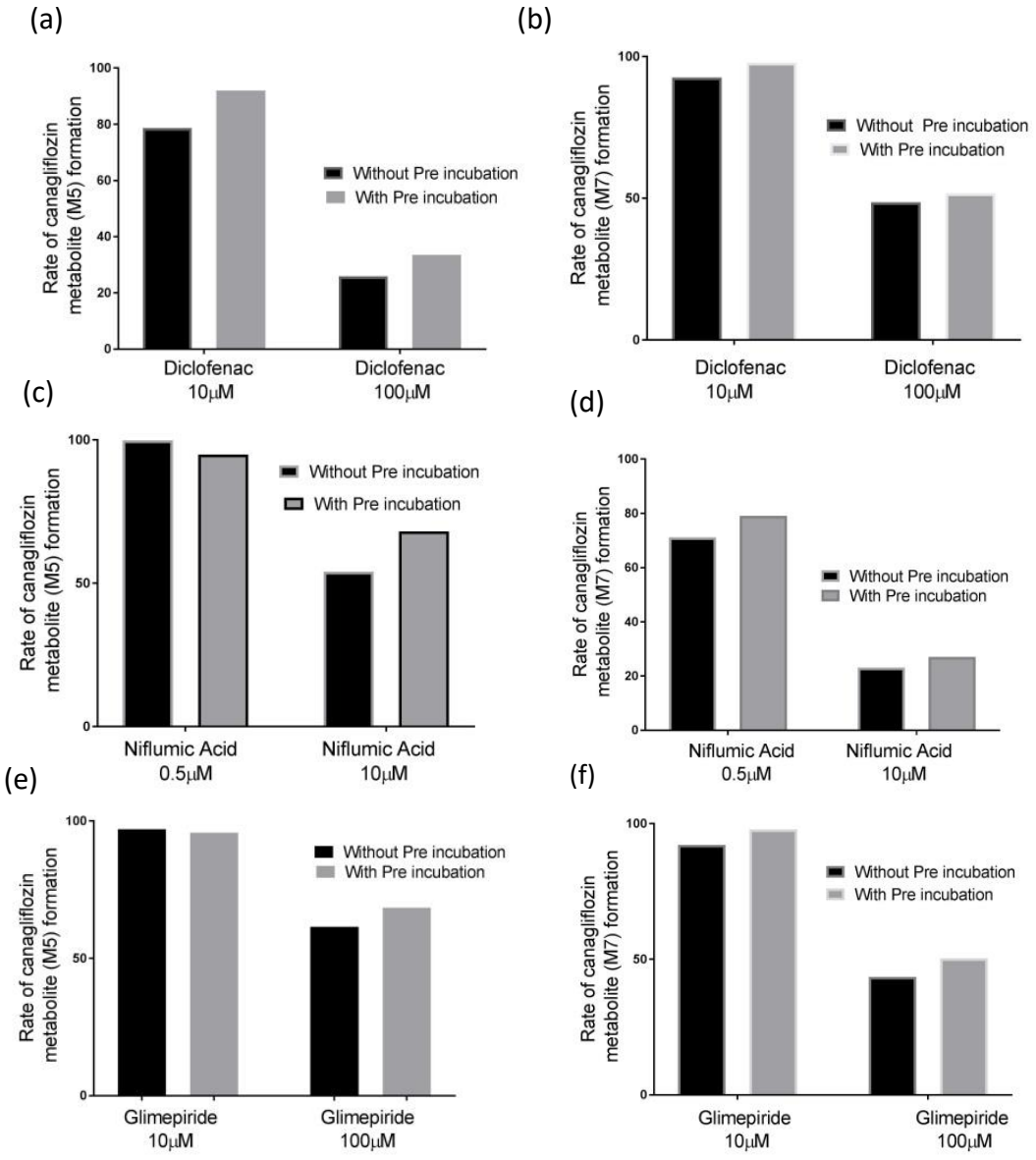



Figure 3.7: The effect of the pre vs. no pre-incubation conditions on the formation rate of M5 and M7 in the presence of two concentrations of diclofenac (a,b) niflumic acid (c,d) or glimepiride (e,f) from duplicate samples of pooled HLMs.

Table 3.1: IC 50 values for sulfonylureas and NSAIDs against canagliflozin metabolites.

| | **IC50 (Mean ± SE) µM** | |
|---|---|---|
| **Inhibitors** | **M5** | **M7** |
| Diclofenac | 32 ± 12.5 | 80 ±13 |
| Niflumic acid | > 100 | 1.86 ± 0.03 |
| Chlorpropamide | >500 | >500 |
| Glimepiride | 135 ± 14 | 88 ± 4 |
| Gliclazide | >500 | >500 |

The substrate concentration of Canagliflozin was 200µM. Data represent mean ± SE of averaging the duplicates of each sample then averaging these values across 5 different HLMs including one pooled.

# Chapter 4: Discussion

This is the first in vitro study conducted to test the effect of other drugs on canagliflozin metabolism by UGTs. This study showed that there is no significant inhibition of the two primary metabolites of canagliflozin, M5, and M7 by sulfonylureas. Glimepiride showed more inhibition potency than chlorpropamide and gliclazide, but the IC50, the concentration required to produce 50% inhibition, of glimepiride was much higher than the usual in-vivo therapeutic plasma concentrations. It has been reported that the therapeutic plasma concentration of glimepiride after a single dose of 8mg is 551±152 ng/ml (1.12 ±0.3 µM), compared to the IC50 values of 87µM for M7and 136µM for M5 [21]. This indicates that the potential drug-drug interaction risk between canagliflozin and sulfonylureas is low.

In one clinical study, probenecid was tested as an inhibitor of canagliflozin clearance. There was a 13% increase in the mean Cmax, and a 21% increase in systemic

exposure. The authors did not consider this difference to be clinically important, and no dose adjustment was recommended. They also suggested that the potential glucuronidation inhibitory effect of other drugs on the PK profile of canagliflozin is unlikely to be clinically significant because canagliflozin is metabolized by two parallel UGT isoenzyme pathways, and glucuronidation is generally high capacity- low affinity [14]. However, they did not test a selective, potent UGT1A9 inhibitor. In the present in vitro study, niflumic acid, a potent UGT1A9 inhibitor, was used as a positive control and produced substantial inhibition of M7 metabolism formation at concentrations below therapeutic plasma levels. The in vitro IC50 for niflumic acid was 1.86 ±0.03 µM, compared to typical plasma concentrations in the range of 2-35 µg/ml (7-124 µM)[22]. Based on these findings, we proceeded further and determined the Ki value for niflumic acid against the M7 metabolite of canagliflozin, and characterized the type of inhibition.

Determination of Ki is also required to predict the potential risk of DDI in vivo by using the [I]/Ki ratio, where [I] is the maximum in vivo plasma level of the inhibitor and Ki is the inhibitor's in vitro inhibition constant [23]. The FDA has suggested [I]/Ki of more than 0.1 as a boundary for judgment for whether a clinical trial should be initiated [24].

Our findings indicated that the Ki value for niflumic acid was 0.8±0.25 µM. Considering 124µM as the maximum in vivo plasma concentration, the [I]/Ki ratio was calculated to be 155 which far exceeds 0.1. This result indicates a potential risk of drug-drug interaction between canagliflozin and niflumic acid, and in vivo clinical drug-drug interaction study might be required to assess the importance of this interaction.

The α value reflects the mix of competitive versus noncompetitive inhibition mechanisms. This value is always greater than zero. If α is equal to one, noncompetitive

inhibition would be suggested, in which the inhibitor does not bind to the active site of the enzyme. The value of α is very large in competitive inhibition, in which inhibitor and substrate compete for the same binding site on the enzyme. In uncompetitive inhibition, α is very small, and it occurs when the inhibitor does not bind to the free enzyme, but only binds to the substrate-enzyme complex. In this study, α for niflumic acid was 5± 3.5 which indicates mixed inhibition where niflumic acid can bind to both the free enzyme, UGT1A9, and to the UGT1A9-canagliflozin complex.

Niflumic acid is a non-steroidal anti-inflammatory drug used to treat musculoskeletal and joint disorders[25]. This drug is currently not available in the US, but is available in various other countries around the world. Until now, there have been no studies indicating an interaction between niflumic acid and canagliflozin. However, it has been established that niflumic acid is a potent in vitro UGT1A9 inhibitor. The inhibitory effect of seven non-steroidal anti-inflammatory drugs (NSAIDs) was tested on UGT1A9 activity using recombinant human UGT1A9 and 4-methylumbelliferone as a substrate. Among the seven NSAIDs, niflumic acid showed the most potent inhibitory effect [26]. Niflumic acid also inhibits the activity of UGT1A9 when HLM was used as a source of the enzyme [27]. Another study demonstrated that niflumic acid selectively inhibits UGT1A9 at low concentration (2.5 μM) and at higher concentrations (50-100μM), it might inhibit other UGT isoenzymes such as UGT1A1 and UGT 2B15 [28].

These findings indicate that a drug-drug interaction between niflumic acid and canagliflozin is likely, and an in vivo study is needed to verify the significance of this DDI. The magnitude and the clinical importance are difficult to predict, since

canagliflozin is metabolized in parallel to form the M5 metabolite in addition to M7. The drug is also excreted unchanged to a significant extent [12].

In vitro studies remain the most cost-effective and extensively used method to screen for potential DDIs. However in vitro models as a predictive approach for DDIs has some limitations, including the accurate estimation of the actual concentrations of the inhibitor or the substrate that is exposed to the enzyme in vivo. Also, it is difficult to predict the risk of metabolic DDIs from in vitro studies in the case of the following: the drug's clearance is highly dependent on hepatic blood flow, the presence of concurrent induction, or the contribution of extrahepatic metabolism. Additionally, the predictability of DDIs from in vitro to in vivo in the case of mechanism-based (time dependent) inhibition is usually not straightforward [29]. Thus, under- or over-prediction of the DDI magnitude from in-vitro studies is always a possibility.

UGT1A9, which is the responsible isoenzyme for M7 formation from canagliflozin, is expressed in human kidneys more than human liver [30]. The function of the kidneys is not limited to excretion of drugs, but can also include metabolic reactions such as glucuronidation. Therefore, studies using human kidney microsomes as a source of UGT1A9 are needed. Future research that investigates the effect of UGT1A9 and UGT2B4 polymorphisms on the metabolism of canagliflozin is also recommended.

In summary, this study demonstrates no apparent potential interaction present between two major classes of antidiabetic drugs. Niflumic acid was demonstrated to inhibit UGT1A9 with reduced formation of the M7 metabolite of canagliflozin. Thus, clinical monitoring should be done when niflumic acid is co-administered with drugs that are mainly metabolized by UGT1A9 including canagliflozin.

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