

# Effects of mitragynine and 7-hydroxymitragynine (the alkaloids of *Mitragyna speciosa* Korth) on 4-methylumbelliferone glucuronidation in rat and human liver microsomes and recombinant human uridine 5'-diphospho-glucuronosyltransferase isoforms

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## ABSTRACT

**Background:** Glucuronidation catalyzed by uridine 5'-diphospho-glucuronosyltransferase (UGT) is a major phase II drug metabolism reaction which facilitates drug elimination. Inhibition of UGT activity can cause drug-drug interaction. Therefore, it is important to determine the inhibitory potentials of drugs on glucuronidation. **Objective:** The objective was to evaluate the inhibitory potentials of mitragynine, 7-hydroxymitragynine, ketamine and buprenorphine, respectively on 4-methylumbelliferone (4-MU) glucuronidation in rat liver microsomes, human liver microsomes and recombinant human UGT1A1 and UGT2B7 isoforms. **Materials and Methods:** The effects of the above four compounds on the formation of 4-MU glucuronide from 4-MU by rat liver microsomes, human liver microsomes, recombinant human UGT1A1 and UGT2B7 isoforms were determined using high-performance liquid chromatography with ultraviolet detection. **Results:** For rat liver microsomes, ketamine strongly inhibited 4-MU glucuronidation with an  $IC_{50}$  value of  $6.21 \pm 1.51 \mu\text{M}$  followed by buprenorphine with an  $IC_{50}$  value of  $73.22 \pm 1.63 \mu\text{M}$ . For human liver microsomes, buprenorphine strongly inhibited 4-MU glucuronidation with an  $IC_{50}$  value of  $6.32 \pm 1.39 \mu\text{M}$ . For human UGT1A1 isoform, 7-hydroxymitragynine strongly inhibited 4-MU glucuronidation with an  $IC_{50}$  value of  $7.13 \pm 1.16 \mu\text{M}$ . For human UGT2B7 isoform, buprenorphine strongly inhibited 4-MU glucuronidation followed by 7-hydroxymitragynine and ketamine with respective  $IC_{50}$  values of  $5.14 \pm 1.30$ ,  $26.44 \pm 1.31$ , and  $27.28 \pm 1.18 \mu\text{M}$ . **Conclusions:** These data indicate the possibility of drug-drug interaction if 7-hydroxymitragynine, ketamine, and buprenorphine are co-administered with drugs that are UGT2B7 substrates since these three compounds showed significant inhibition on UGT2B7 activity. In addition, if 7-hydroxymitragynine is to be taken with other drugs that are highly metabolized by UGT1A1, there is a possibility of drug-drug interaction to occur.

**Key words:** 4-methylumbelliferone, 7-hydroxymitragynine, buprenorphine, glucuronidation, ketamine, mitragynine

## INTRODUCTION

Ketum or *Mitragyna speciosa* Korth from *Rubiaceae* family is a tropical plant that can be found in the South-East Asia particularly in Thailand and Peninsular of Malaysia.<sup>[1]</sup>

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Traditionally, the ketum leaves have been used as a herbal drug to treat cough, diarrhea, hypertension, and to wean off morphine addicts.<sup>[2,3]</sup> Mitragynine, chemically known as 9-methoxy-corynantheidine, the major alkaloid constituent extracted from ketum leaves and 7-hydroxymitragynine, a minor constituent of ketum had been isolated.<sup>[4]</sup> Mitragynine had been obtained as a major constituent in ketum leaves as 66% of the total alkaloids in the Thai species and as 12% of the total alkaloids in the Malaysian species.<sup>[1]</sup> Meanwhile, 7-hydroxymitragynine is present up

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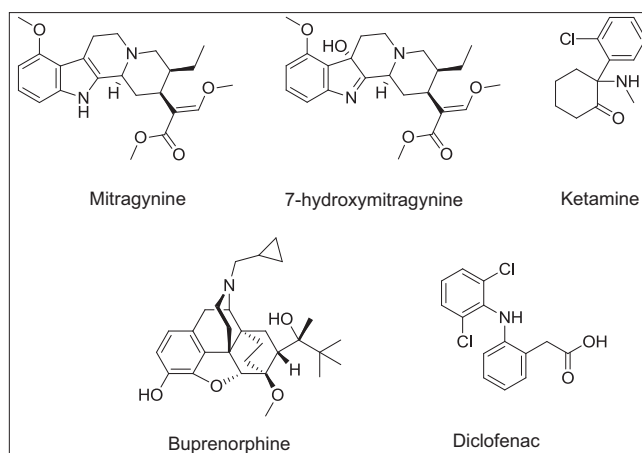


to 2% of total alkaloids content.<sup>[4]</sup> 7-hydroxymitragynine demonstrates 13 times higher analgesic potency than morphine and 46 folds higher potency than mitragynine.<sup>[5]</sup>

Recently, the use of ketum by drug users in Malaysia either as an opium substitute or as a stimulant had escalated.<sup>[6]</sup> It was also reported that 46% of ketum users are concomitantly taking other drugs<sup>[6]</sup> including ketamine which is one of the frequently abused drug.<sup>[7]</sup> In response to the drug abuse problem, buprenorphine, a semi-synthetic opioid was introduced in Malaysia to treat opioid addiction. These situations place individuals being treated with buprenorphine at risk for potentially toxic drug interactions when buprenorphine is ingested concomitantly by ketum users.

Drug-drug interactions may occur through several mechanisms, the main one is through the effects of these drugs on hepatic drug metabolism. Many drug interactions result from the inhibition or induction of enzymes that can have a significant influence on the use and safety of the drugs. Enzyme inhibition refers to the decrease in metabolic enzyme activity because of the presence of an inhibitor. Some drugs may inhibit the enzymes from metabolizing other drugs taken concomitantly causing them to remain active within the body for too long. This can cause high levels of the drug within the body which in turn can lead to adverse reactions and be harmful. Enzyme induction refers to the increase in enzyme activity caused by an inducer. Normally, enzyme induction is related to a reduction in the drug efficacy. However, it may also alter the toxicity of some substances.<sup>[8]</sup> The effects can take place on phase I metabolism (cytochrome P450 [CYP] enzymes) or on phase II metabolism (uridine 5'-diphospho [UDP]-glucuronosyltransferase [UGT] enzymes).

Glucuronidation is a major phase II metabolism reaction that facilitates detoxification and elimination efficiently for many drugs, numerous dietary, and endogenous compounds such as bilirubin and steroids.<sup>[9]</sup> Therefore, it is important to determine the inhibitory potentials of drugs on glucuronidation. The aim of this study is to evaluate the inhibitory potentials of the alkaloids from the ketum plant, which are mitragynine and 7-hydroxymitragynine, and the drug compounds which are ketamine and buprenorphine [Figure 1] on 4-methylumbelliferone (4-MU) glucuronidation in rat liver microsomes, human liver microsomes, and recombinant human UGT isoforms. Despite being a minor constituent of ketum, 7-hydroxymitragynine was chosen since it was found to be the most potent agonistic effect compared to the alkaloids present in ketum which consist of mitragynine, speciogynine, speciociliatine, and paynantheine. This suggests that the opioid effect of ketum is due to 7-hydroxymitragynine.<sup>[10]</sup> In this study, high-performance



**Figure 1:** Chemical structures of potential inhibitory compounds investigated

liquid chromatography (HPLC) will be used to measure the formation rate of 4-MU glucuronide (4-MUG) in the absence and presence of the drugs (potential inhibitors). The concentration of the drugs that reduces UGT enzyme activity by 50% ( $IC_{50}$ ) will be determined. 4-MU was chosen as the substrate because it is glucuronidated by various UGT isoforms and is considered to be a general substrate for UGTs.<sup>[11]</sup>

## MATERIALS AND METHODS

### Chemicals and reagents

4-methylumbelliferone, 4-MU  $\beta$ -D-glucuronide (4-MUG), UDP-glucuronic acid (UDPGA, trisodium salt) and diclofenac (sodium salt) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Ketamine hydrochloride and buprenorphine hydrochloride were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Mitragynine (94.3% purity) was purchased from Chromadex (Irvine, CA, United States). 7-hydroxymitragynine (99% purity) was a kind gift from Assoc. Prof. Dr. Mohd Nizam Mordi (Centre for Drug Research, Universiti Sains Malaysia [USM]). Solvents and other reagents were of analytical reagent grade.

### Enzyme sources

Male Sprague-Dawley rats weighing 150–200 g were obtained from the Animal House Unit of USM. Animals were housed in the animal room under controlled environment (temperature  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and 12 h light and dark cycle) and had free access to food and water *ad libitum*. Animals were acclimatized for 1-week before sacrificed. Animals were maintained and handled following the recommendations of the USM Ethical Committee which approved the design of the animal experiments with reference to the number USM/Animal Ethics Approval/2011/(72)(340).

Rat liver microsomes were prepared by differential centrifugation, as described by Azizi *et al.*<sup>[12]</sup> Briefly, rat livers were removed from the sacrificed animals, washed with ice-cold distilled water followed by ice-cold 67 mM potassium phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl and then homogenized in three volumes of the same solution. The liver homogenates were centrifuged at  $12,500 \times g$  for 20 min at 4°C. The supernatants were further centrifuged at  $100,000 \times g$  for 60 min at 4°C, resulting in the sedimentation of the microsomal pellet, which was resuspended in 300  $\mu$ L of 67 mM potassium phosphate buffer with 1.15% (w/v) KCl and 20% (v/v) glycerol. The rat liver microsomes were kept frozen at -80°C. Protein concentration of the rat liver microsomes was estimated by the method of Lowry *et al.*<sup>[13]</sup> using serum albumin as standard with slight modification according to Pomory.<sup>[14]</sup>

Pooled human liver microsomes were purchased from Sigma-Aldrich (St. Louis, MO, United States) and microsomes from baculovirus-infected insect cells expressing recombinant human UGT1A1 and UGT2B7 were obtained from BD Biosciences (Woburn, MA, United States).

#### 4-Methylumbelliferone glucuronidation assay in rat liver microsomes and human liver microsomes

4-methylumbelliferone glucuronidation was evaluated in microsomal incubation using 4-MU as the probe substrate. Substrate concentration corresponds to the  $K_m$  value.<sup>[15]</sup> Microsomal protein concentration, incubation time and percentage of detergent used (Triton X-100) for rat liver microsomes and human liver microsomes were optimized through range-finding studies (data not shown). The enzyme activity assay mixture (250  $\mu$ L) contained Tris-HCl (100 mM) (pH 7.4),  $MgCl_2$  (5 mM), microsomal protein (0.25 mg/mL for rat liver microsomes or 0.1 mg/mL for human liver microsomes), Triton X-100 (0.01% for rat liver microsomes assay or 0.005% for human liver microsomes assay), UDPGA (3 mM), and 4-MU (substrate) (100  $\mu$ M). After preincubation at 37°C for 5 min in a shaking water bath, reactions were initiated by the addition of UDPGA and were further incubated at 37°C for 30 min. Reactions were then terminated by the addition of 20% (w/v) trichloroacetic acid (50  $\mu$ L). Samples were centrifuged (14,000 rpm for 5 min) and the supernatant fraction (20  $\mu$ L) was injected into the HPLC column for analysis.

#### 4-Methylumbelliferone glucuronidation assays in recombinant human uridine 5'-diphosphoglucuronosyltransferase isoforms (UGT1A1 and UGT2B7)

4-methylumbelliferone glucuronidation was measured using a previously published procedure<sup>[11]</sup> with slight

modifications. The amount of protein, the incubation time and the concentration of 4-MU used in the measurement of 4-MU glucuronidation were described in the method by Uchaipichat *et al.*<sup>[11]</sup> Briefly, incubations (total volume 250  $\mu$ L) contained phosphate buffer (100 mM) (pH 7.4),  $MgCl_2$  (5 mM), cell lysate (83.3  $\mu$ g/mL for UGT1A1 or 62.5  $\mu$ g/mL for UGT2B7), UDPGA (5 mM), and 4-MU (substrate) (100  $\mu$ M for UGT1A1 assay or 350  $\mu$ M for UGT2B7 assay). After preincubation at 37°C for 5 min in a shaking water bath, reactions were initiated by the addition of UDPGA and were further incubated at 37°C for 120 min. Reactions were then terminated by addition of 24% (v/v) perchloric acid (10  $\mu$ L). Samples were centrifuged (6,200 rpm for 10 min) and the supernatant fraction (20  $\mu$ L) was injected into the HPLC column for analysis.

#### 4-Methylumbelliferone glucuronidation inhibition assays

For 4-MU glucuronidation inhibition assays performed in rat liver microsomes, human liver microsomes and recombinant human UGT1A1 and UGT2B7 isoforms, compounds that were investigated were diclofenac (positive inhibitor), mitragynine, 7-hydroxymitragynine, ketamine and buprenorphine. Diclofenac, ketamine and buprenorphine were fully dissolved in water. Mitragynine was prepared in methanol whereas 7-hydroxymitragynine was prepared in dimethyl sulfoxide (DMSO). Inhibitor was added to the incubation assay to reach the final desired concentrations. Final concentrations used for diclofenac, ketamine and buprenorphine in the inhibition screening experiments were 0.01, 0.1, 1, 10, 100, and 1000  $\mu$ M, respectively. However, final concentrations used for mitragynine and 7-hydroxymitragynine were up to only 100  $\mu$ M due to limited solubility of both of the compounds in methanol and DMSO, respectively. The final concentration of both solvents in the incubation mixture was 0.5% (v/v).

#### Apparatus and high-performance liquid chromatography conditions

High-performance liquid chromatography determination of 4-MUG was performed using a method modified from Lewis *et al.*<sup>[16]</sup> The peak areas of 4-MUG were analyzed using reversed-phase HPLC with ultraviolet (UV) detection. The HPLC system used was an Agilent 1200 series instrument consisting of G1322A degasser, G1311A quaternary pump, G1329B autosampler, G1315B diode array detector, and G1316A thermostatted column compartments. Chromatographic separation was achieved using a Gemini NX C18 column (4.6 mm  $\times$  150 mm, 5  $\mu$ m particle sizes). The column was kept at 37°C and the column eluant was monitored by UV absorbance at a wavelength of 316 nm. The mobile phase was delivered at a flow rate of 1 mL/min using gradient elution consisting of solvent A (10% [v/v]

acetonitrile in an aqueous solution of 10 mM triethylamine adjusted to pH 2.5 with 60% [v/v] perchloric acid) and solvent B (acetonitrile) in the (v/v) ratios of 95% A: 5% B from 0 to 7 min, 70% A: 30% B from 7.1 to 12 min, and 95% A: 5% B from 12.1 to 15 min. Concentrations of 4-MUG in incubation samples were quantified by comparison of peak areas to those of standard curves prepared over concentrations ranging from 0.5 to 100  $\mu\text{M}$ .

### Statistical analysis

Results are represented as mean  $\pm$  standard error of mean (SEM) for three independent measurements ( $n = 3$ ).  $\text{IC}_{50}$  values were calculated graphically on the basis of plots created using the GraphPad Prism 6 (Version 6.01, GraphPad Software, Inc., USA) under dose-response inhibition (log [inhibitor] vs. normalized response -- variable slope) model. One-way ANOVA followed by Dunnett test were performed using the same software to determine statistical significance between a range of 0–1000  $\mu\text{M}$  for diclofenac, ketamine and buprenorphine and 0–100  $\mu\text{M}$  for mitragynine and 7-hydroxymitragynine.

## RESULTS

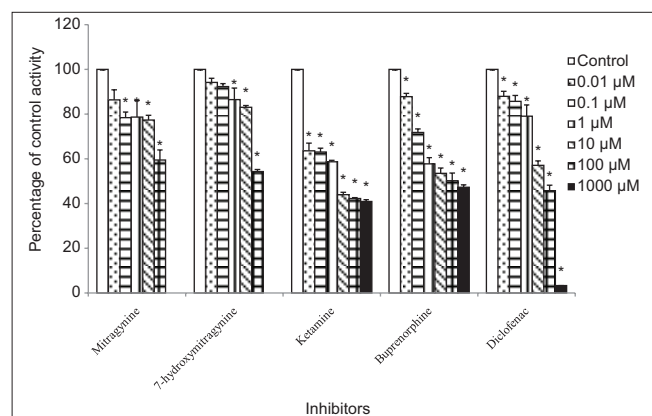
### Inhibition of 4-methylumbelliferone glucuronidation by mitragynine, 7-hydroxymitragynine, ketamine, buprenorphine and diclofenac in rat liver microsomes, human liver microsomes and recombinant human uridine 5'-diphospho-glucuronosyltransferase isoforms (UGT1A1 and UGT2B7)

Figures 2-5 show the effects of five compounds, mitragynine, 7-hydroxymitragynine, ketamine, buprenorphine and diclofenac on the 4-MUG formation by rat liver

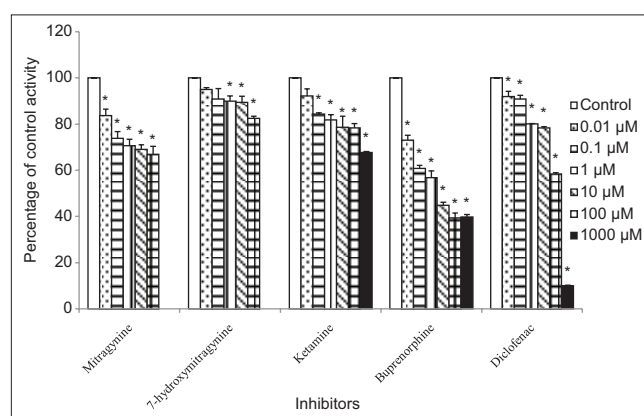
microsomes, human liver microsomes, human UGT1A1 and human UGT2B7 isoforms, respectively.

Using rat liver microsomes, diclofenac which was used as the positive inhibitor gave an  $\text{IC}_{50}$  value of  $21.14 \pm 1.32 \mu\text{M}$  [mean  $\pm$  SEM, Table 1]. Among the other four compounds, ketamine and buprenorphine had the highest inhibition on 4-MU glucuronidation in rat liver microsomes. Buprenorphine showed inhibition with  $\text{IC}_{50}$  value of  $73.22 \pm 1.63 \mu\text{M}$  [mean  $\pm$  SEM, Table 1], meanwhile, ketamine gave an  $\text{IC}_{50}$  value of  $6.21 \pm 1.51 \mu\text{M}$  [mean  $\pm$  SEM, Table 1]. However, the  $\text{IC}_{50}$  values for the mitragynine and 7-hydroxymitragynine could not be determined accurately since their inhibitions on 4-MU glucuronidation were  $<50\%$ . 4-MU glucuronidation was reduced by 7-hydroxymitragynine ranged from  $6\% \pm 2\%$  to  $46\% \pm 1\%$  and mitragynine ranged from  $14\% \pm 4\%$  to  $40\% \pm 5\%$  at concentrations of 0.01–100  $\mu\text{M}$  [Figure 2].

Using human liver microsomes, diclofenac showed inhibition with  $\text{IC}_{50}$  value of  $86.44 \pm 1.28 \mu\text{M}$  [mean  $\pm$  SEM, Table 1]. Buprenorphine showed inhibition with  $\text{IC}_{50}$  value of  $6.32 \pm 1.39 \mu\text{M}$  [mean  $\pm$  SEM, Table 1]. The percentage inhibition of UGT activity is varied from  $16\% \pm 3\%$  to  $33\% \pm 4\%$  for mitragynine,  $8\% \pm 3\%$  to  $32\% \pm 1\%$  for ketamine and  $5\% \pm 1\%$  to  $17\% \pm 1\%$  for 7-hydroxymitragynine at concentrations that ranged from 0.01 to 1000  $\mu\text{M}$  for ketamine whereas 0.01 to 100  $\mu\text{M}$  for mitragynine and 7-hydroxymitragynine [Figure 3]. The  $\text{IC}_{50}$  values for these three compounds toward 4-MU glucuronidation in human liver microsomes were all greater than the highest concentrations used ( $\text{IC}_{50} > 1000 \mu\text{M}$  for ketamine whereas  $\text{IC}_{50} > 100 \mu\text{M}$  for mitragynine and

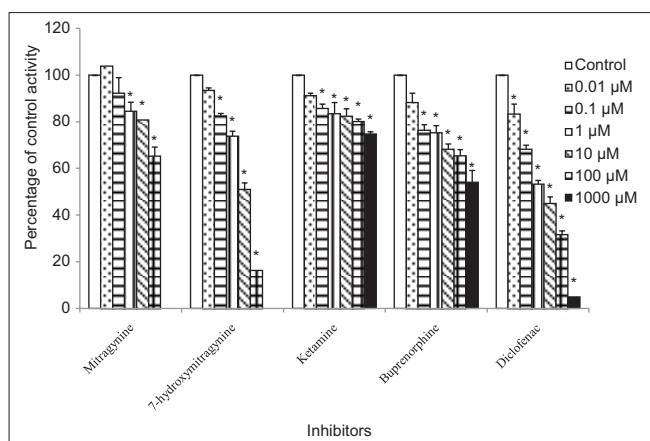


**Figure 2:** Inhibitory effects of mitragynine, 7-hydroxymitragynine, ketamine, buprenorphine, and diclofenac on 4-methylumbelliferone glucuronide formation by rat liver microsomes. Panels to the right show inhibitor concentrations. Each bar represents the mean percentage activity relative to control  $\pm$  standard error of mean for three independent measurements ( $n = 3$ ). Statistical analysis was conducted using one-way ANOVA and Dunnett test.  $*P < 0.05$  versus control (no inhibitor)

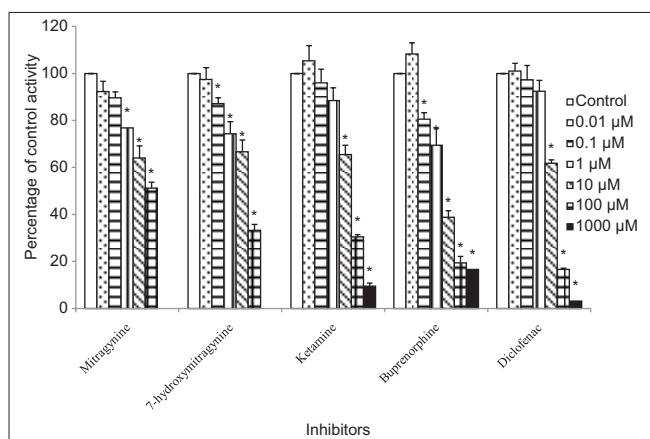


**Figure 3:** Inhibitory effects of mitragynine, 7-hydroxymitragynine, ketamine, buprenorphine, and diclofenac on 4-methylumbelliferone glucuronide formation by human liver microsomes. Panels to the right show inhibitor concentrations. Each bar represents the mean percentage activity relative to control  $\pm$  standard error of mean for three independent measurements ( $n = 3$ ). Statistical analysis was conducted using one-way ANOVA and Dunnett test.  $*P < 0.05$  versus control (no inhibitor)





**Figure 4:** Inhibitory effects of mitragynine, 7-hydroxymitragynine, ketamine, buprenorphine, and diclofenac on 4-methylumbelliferone glucuronide formation by recombinant human UGT1A1 isoform. Panels to the right show inhibitor concentrations. Each bar represents the mean percentage activity relative to control  $\pm$  standard error of mean for three independent measurements ( $n = 3$ ). Statistical analysis was conducted using one-way ANOVA and Dunnett test.  $*P < 0.05$  versus control (no inhibitor)



**Figure 5:** Inhibitory effects of mitragynine, 7-hydroxymitragynine, ketamine, buprenorphine, and diclofenac on 4-methylumbelliferone glucuronide formation by recombinant human UGT2B7 isoform. Panels to the right show inhibitor concentrations. Each bar represents the mean percentage activity relative to control  $\pm$  standard error of mean for three independent measurements ( $n = 3$ ). Statistical analysis was conducted using one-way ANOVA and Dunnett test.  $*P < 0.05$  versus control (no inhibitor)

7-hydroxymitragynine) since inhibition at more than 50% did not occur at the highest concentration [Table 1].

For the human UGT1A1 isoform, diclofenac gave an  $IC_{50}$  value of  $2.34 \pm 1.28 \mu\text{M}$  [mean  $\pm$  SEM, Table 1]. Meanwhile, 7-hydroxymitragynine gave an  $IC_{50}$  value of  $7.13 \pm 1.16 \mu\text{M}$  [mean  $\pm$  SEM, Table 1]. The percentage inhibition of UGT activity varied from  $12\% \pm 4\%$  to  $46\% \pm 5\%$  for buprenorphine and  $9\% \pm 1\%$  to  $25\% \pm 1\%$  for ketamine at concentrations that ranged from 0.01 to  $1000 \mu\text{M}$  and  $8\% \pm 6\%$  to  $35\% \pm 4\%$  for mitragynine at concentrations that ranged from 0.01 to  $100 \mu\text{M}$  [Figure 4]. For these three compounds, accurate  $IC_{50}$  values could not be determined because the maximum inhibition ( $>50\%$ ) did not occur at the highest concentration used. Therefore, the  $IC_{50}$  values for these three compounds toward 4-MU glucuronidation were all greater than the highest concentrations used ( $IC_{50} > 1000 \mu\text{M}$  for buprenorphine and ketamine whereas  $IC_{50} > 100 \mu\text{M}$  for mitragynine) [Table 1].

For the human UGT2B7 isoform, diclofenac gave an  $IC_{50}$  value of  $16.97 \pm 1.11 \mu\text{M}$  [mean  $\pm$  SEM, Table 1]. Among the other four compounds, buprenorphine had the highest inhibition on 4-MUG formation by human UGT2B7 isoform followed by 7-hydroxymitragynine and ketamine. The  $IC_{50}$  values for buprenorphine, 7-hydroxymitragynine, and ketamine were  $5.14 \pm 1.30$ ,  $26.44 \pm 1.31$ , and  $27.28 \pm 1.18 \mu\text{M}$ , respectively [mean  $\pm$  SEM, Table 1]. However, the  $IC_{50}$  value for mitragynine could not be determined accurately since its inhibition on 4-MU glucuronidation was  $<50\%$ . The percentage inhibition by mitragynine against UGT2B7 reducing 4-MU glucuronidation was from  $8\% \pm 4\%$  to  $49\% \pm 3\%$  at concentrations that ranged from 0.01 to  $100 \mu\text{M}$  [Figure 5].

## DISCUSSION

Various drugs have been identified as inhibitors using *in vitro* assays of UGT-mediated glucuronidation reactions

**Table 1: Inhibitory effects of mitragynine, 7-hydroxymitragynine, ketamine, buprenorphine and diclofenac on 4-MUG formation by rat liver microsomes, human liver microsomes and recombinant human UGT1A1 and UGT2B7 isoforms**

Inhibitors	$IC_{50}$ ( $\mu\text{M}$ )			
	Rat liver microsomes	Human liver microsomes	Recombinant human UGT1A1 isoform	Recombinant human UGT2B7 isoform
Mitragynine	$>100$	$>100$	$>100$	$>100$
7-hydroxymitragynine	$>100$	$>100$	$7.13 \pm 1.16$	$26.44 \pm 1.31$
Ketamine	$6.21 \pm 1.51$	$>1000$	$>1000$	$27.28 \pm 1.18$
Buprenorphine	$73.22 \pm 1.63$	$6.32 \pm 1.39$	$>1000$	$5.14 \pm 1.30$
Diclofenac	$21.14 \pm 1.32$	$86.44 \pm 1.28$	$2.34 \pm 1.28$	$16.97 \pm 1.11$

Data represent best-fit  $IC_{50}$  values  $\pm$  SEM for three independent measurements ( $n=3$ ). SEM=Standard error of mean; 4-MUG=4-methylumbelliferone glucuronide

in drug metabolism. In the present study, for all the inhibition assays, diclofenac which is a nonsteroidal anti-inflammatory drug (NSAID) was chosen as the positive inhibitor. Diclofenac, a NSAID is a comparatively potent UGT inhibitor for all UGT isoforms.<sup>[11]</sup> Therefore, the ability of the glucuronidation inhibition assay to detect the effect of test compounds on UGT was demonstrated by performing the assay in the presence of a known UGT inhibitor which is diclofenac.

Under the inhibition assay employed in this study, the result demonstrated that diclofenac, ketamine and buprenorphine had the highest inhibition on 4-MU glucuronidation in rat liver microsomes with the  $IC_{50}$  values of  $21.14 \pm 1.32$ ,  $6.21 \pm 1.51$ , and  $73.22 \pm 1.63 \mu\text{M}$ , respectively. There is a differential degree of inhibition among the compounds. Hence, the  $IC_{50}$  value of diclofenac as a potent UGTs inhibitor was determined in order to compare the  $IC_{50}$ 's of buprenorphine and ketamine with its value. In this study, buprenorphine appears to be about three orders of magnitude less potent than diclofenac. Meanwhile, ketamine appears to be about three orders of magnitude more potent than diclofenac. In contrast, 7-hydroxymitragynine and mitragynine showed <50% inhibition at the highest concentration used. Therefore no  $IC_{50}$  values could be determined. However, 7-hydroxymitragynine and mitragynine gave the percentages inhibition of 46% and 40%, respectively at the highest concentration used. The inhibitory potential shown in rat liver microsomes was in order: Ketamine > buprenorphine > 7-hydroxymitragynine > mitragynine according to the  $IC_{50}$  values and percentage inhibition obtained.

The results obtained using rat liver microsomes showed a good inhibition on 4-MUG formation. In order to compare the inhibitory effects of these five compounds using human models, human liver microsomes were used. The results demonstrated that diclofenac and buprenorphine had the highest inhibition on 4-MU glucuronidation in human liver microsomes with the  $IC_{50}$  values of  $86.44 \pm 1.28$  and  $6.32 \pm 1.39 \mu\text{M}$ , respectively. There is a differential degree of inhibition among these two compounds. Hence, the  $IC_{50}$  value of diclofenac as a potent UGTs inhibitor was determined in order to compare its  $IC_{50}$  to that of buprenorphine. The results illustrate that buprenorphine appears to be about 13 orders of magnitude more potent than diclofenac. However, ketamine, mitragynine, and 7-hydroxymitragynine did not exhibit strong inhibitory effects on 4-MUG formation by human liver microsomes. The inhibitory potential shown in human liver microsomes could be ranked as: Buprenorphine > mitragynine > ketamine > 7-hydroxymitragynine with the respective percentage inhibition of  $60\% \pm 1\%$ ,  $33\% \pm 4\%$ ,  $32\% \pm 1\%$ , and  $17\% \pm 1\%$  at the highest concentration used.

As a comparison, the inhibitory effects of the inhibitors observed for rat liver microsomes and human liver microsomes were different. This is explained by the fact that UGT isoforms are present at different levels in rat liver microsomes and human liver microsomes, thus, contributing to different glucuronidation capacities between the two species.<sup>[17]</sup>

Human liver microsomes used in the current study as the enzymes source contained a mixture of UGT isoforms. In order to investigate which specific UGT isoform was being inhibited by those four compounds in human liver microsomes, two recombinant human UGT isoforms, human UGT1A1, and human UGT2B7 were selected for further study. Both of these UGT isoforms are expressed in human liver. Human UGT1A1 was chosen since this UGT isoform is primarily responsible for the glucuronidation of bilirubin and many important clinical drugs such as irinotecan. In addition, of the top 200 drugs prescribed in the United States in 2002, 15% of the drugs undergo glucuronidation by UGT1A1 as their clearance mechanism, for example, levothyroxine, acetaminophen, and raloxifene.<sup>[18]</sup> Meanwhile, human UGT2B7 was chosen since this UGT isoform glucuronidates endogenous compound such as bile acid and retinoids and several xenobiotics including morphine, zidovudine, and NSAIDs. In fact, UGT2B7 is involved in the metabolism of 35% drugs responsible for glucuronidation of the top 200 prescribed drugs in the United States 2002.<sup>[18]</sup>

Using human UGT1A1 isoform as the source of enzyme, the results showed that 7-hydroxymitragynine had the highest inhibition on 4-MU glucuronidation. However, buprenorphine, ketamine, and mitragynine did not exhibit potent inhibitory effects on human UGT1A1 activity. From a previous report, it has been demonstrated that ketamine showed a weak inhibitory effect on 4-MU glucuronidation in human UGT1A1 isoform.<sup>[19]</sup> It is also reported that buprenorphine had no significant inhibitory effect on bilirubin glucuronidation catalyzed by UGT1A1 except at highly extraordinary concentrations.<sup>[20]</sup> 7-hydroxymitragynine which is the alkaloid that is present in a lesser amount in the ketum extract compared to mitragynine inhibited human UGT1A1 with an  $IC_{50}$  value of  $7.13 \pm 1.16 \mu\text{M}$ . There is no report on the effect of this compound on any UGT isoform activity. However, Azizi *et al.*<sup>[12]</sup> had reported that the ketum plant extracts had no significant inhibition on 4-nitrophenol UGT activity in rat liver microsomes but 4-nitrophenol UGT is mostly catalyzed by UGT1A6. 7-hydroxymitragynine which has a hydroxyl group at the C7 position of mitragynine [Figure 1] is susceptible to metabolism catalyzed by UGT (s). However, this requires further study.

Using human UGT2B7 isoform, the results demonstrated that buprenorphine, ketamine, and 7-hydroxymitragynine exhibited strong inhibition against human UGT2B7 whereas mitragynine exhibited only minor inhibitory effects. Among these three compounds, buprenorphine showed the highest inhibitory effect on 4-MUG formation by human UGT2B7 isoform followed by 7-hydroxymitragynine and ketamine.

In agreement with its effect on human liver microsomes, buprenorphine exhibited a strong inhibitory effect on the human UGT2B7 isoform. Buprenorphine inhibits 4-MUG formation by human UGT2B7 isoform with an  $IC_{50}$  value of  $5.14 \pm 1.30 \mu\text{M}$ . Buprenorphine is a potent opioid analgesic used in the treatment of moderate to severe pain and has also been used for the treatment of drug addiction. The glucuronidation of buprenorphine is primarily carried out by human UGT1A3 followed by human UGT2B7 and human UGT1A1 isoforms.<sup>[21]</sup> Based on the literature, the  $K_m$  value for buprenorphine glucuronidation by human UGT2B7 isoform is  $20.9 \mu\text{M}$ ,<sup>[21]</sup> which is much lower than the  $K_m$  value for 4-MU glucuronidation by human UGT2B7 isoform that is  $335 \mu\text{M}$ .<sup>[11]</sup> Therefore, the affinity of buprenorphine toward human UGT2B7 is much higher than the affinity of 4-MU toward human UGT2B7, which might have resulted in inhibition of 4-MU glucuronidation by human UGT2B7.

7-hydroxymitragynine also had the highest inhibition on 4-MUG formation by human UGT2B7 isoform with an  $IC_{50}$  value of  $26.44 \pm 1.31 \mu\text{M}$ . The inhibition of this compound was also observed in human UGT1A1 isoform in this study but the  $IC_{50}$  value obtained was  $7.13 \pm 1.16 \mu\text{M}$  which was lower than found in human UGT2B7 isoform.

Similar to buprenorphine and 7-hydroxymitragynine, ketamine also inhibit 4-MUG formation by human UGT2B7 isoform in this study. Ketamine had been used clinically as an anesthetic and prescription agent. However, its abuse among drug addicts had recently escalated. In this study, ketamine inhibited the formation of 4-MUG with an  $IC_{50}$  value of  $27.28 \pm 1.18 \mu\text{M}$ . The result observed in this study concurred with a previous study for ketamine in which potent inhibition was observed with human UGT2B7 and human UGT2B15 isoforms with estimated  $IC_{50}$  values of 55 and 95  $\mu\text{M}$ , respectively.<sup>[19]</sup> Previous data had shown that ketamine inhibited the formation of morphine-3-glucuronide in a non-competitive manner. It was interpreted that ketamine might have occupied a site on the UGT (s), which had caused a reduction in the formation of morphine-3-glucuronide and accounted for the interaction observed between these two compounds in the isolated perfused rat liver model.<sup>[22]</sup>

According to Krippendorff *et al.*,<sup>[23]</sup> the potential of CYP450 inhibition of compounds can be classified into low, medium or high potential inhibitors in drug-drug interactions based on their  $IC_{50}$  values. Compounds exhibiting  $IC_{50}$  values  $> 10 \mu\text{M}$  ( $IC_{50} > 10 \mu\text{M}$ ) are low potential inhibitors. Meanwhile, compounds exhibiting  $IC_{50}$  values from 1 to  $10 \mu\text{M}$  ( $1 \mu\text{M} < IC_{50} < 10 \mu\text{M}$ ) are moderate potential inhibitors. However, the compounds exhibiting  $IC_{50}$  values  $< 1 \mu\text{M}$  ( $IC_{50} < 1 \mu\text{M}$ ) are high potential inhibitors. Since the interpretation of UGT drug-drug interactions determined with UGT inhibitors is an ongoing and still unresolved challenge,<sup>[24]</sup> therefore, this classification may also be applicable and sufficient for ranking the compounds as potential inhibitors in the early drug discovery. Further evidence to support this statement can also be obtained from Oechsler and Skopp<sup>[25]</sup> and Venkatakrishnan *et al.*<sup>[26]</sup>

Another way to assess the possible clinical inhibitory potentials against drugs catalyzed mainly by human UGT1A1 and human UGT2B7 is by comparing the  $IC_{50}$  values of buprenorphine, ketamine, and 7-hydroxymitragynine with their therapeutic plasma concentrations on an unbound concentration basis if possible.

It has been reported that the mean plasma concentrations of buprenorphine were 37.52 ng/ml (0.08  $\mu\text{M}$ ) and 6.3 ng/ml (0.012  $\mu\text{M}$ ) after a single intravenous dose (1.2 mg) and a single sublingual dose (16 mg), respectively.<sup>[27,28]</sup> The  $IC_{50}$  value of buprenorphine obtained in this study was 5.14  $\mu\text{M}$  which is much higher than the therapeutic unbound plasma concentration. Therefore, it is highly unlikely that buprenorphine would inhibit human UGT2B7 in clinical use.

Meanwhile, Idvall *et al.*<sup>[29]</sup> reported that a mean plasma concentration of ketamine was 9.3  $\mu\text{M}$  during steady-state anesthesia after induction with 2 mg/kg and a maintenance dose of approximately 40  $\mu\text{g}/\text{kg}/\text{min}$ . After a low-dose (0.125–0.250 mg/kg intravenous) administration, ketamine concentration of  $> 0.42 \mu\text{M}$  (100  $\mu\text{g}/\text{l}$ ) was found.<sup>[30]</sup> The  $IC_{50}$  value of ketamine obtained in this study was 27.28  $\mu\text{M}$  which is higher than the therapeutic unbound plasma concentration in both studies. Therefore, we postulate that ketamine would also not inhibit human UGT2B7 in clinical setting.

Since there are no published reports on the therapeutic plasma concentration of 7-hydroxymitragynine and percent plasma unbound in human, the possible clinical inhibitory potentials of 7-hydroxymitragynine against drugs catalyzed mainly by human UGT1A1 and human UGT2B7 cannot be predicted at present.



## CONCLUSIONS

To summarize, for rat liver microsomes, among the four compounds investigated, the highest inhibition was observed for ketamine followed by buprenorphine. For human liver microsomes, only buprenorphine gave strong inhibitory effect on 4-MU glucuronidation. Using human UGT1A1 isoform, only 7-hydroxymitragynine had the highest inhibitory potential, whereas using human UGT2B7 isoform, the highest inhibition was observed for buprenorphine followed by 7-hydroxymitragynine and ketamine. This *in vitro* study indicates that the interaction of these four compounds with rat liver microsomes, human liver microsomes and recombinant human UGT1A1 and UGT2B7 isoforms is complex and differs among the source of enzymes. However, these data indicate the possibility of drug-drug interaction if 7-hydroxymitragynine, ketamine and buprenorphine are co-administered with drugs that are UGT2B7 substrates since these three compounds showed significant inhibition on human UGT2B7 activity. In addition, there is also possibility of drug interaction if 7-hydroxymitragynine is to be taken with other drugs that are highly metabolized by UGT1A1.

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