Short Communication

Effects of ketamine on human UDP-glucuronosyltransferases in vitro predict potential drug-drug interactions arising from ketamine inhibition of codeine and morphine glucuronidation

Verawan Uchaipichat, Pritsana Raungrut, Nuy Chau, Benjamas Janchawee, Allan M. Evans and John O Miners

Department of Clinical Pharmacology, Flinders University School of Medicine, Adelaide, Australia (VU, PR, NC, JOM)
Faculty of Pharmaceutical Science, Khon Kaen University, Khon Kaen, Thailand (VU)
Department of Biomedical Sciences, Prince of Songkla University, Hat Yai, Thailand (PR, BJ)
School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, Australia (AME)
Running title: Ketamine inhibition of codeine and morphine glucuronidation

Address for correspondence:

Professor John O. Miners

Department of Clinical Pharmacology
Flinders University School of Medicine
Flinders Medical Centre
Bedford Park, SA 5042
Australia

Telephone: 61-8-82044131
Fax: 61-8-82045114
Email: john.miners@flinders.edu.au

Number of text pages: 20
Number of Figures: 2
Number of Tables: 1
Number of References: 24

Word count: Abstract, 216; Introduction, 396; Results and Discussion, 1012

Abbreviations: BSA, bovine serum albumin; C6G, codeine-6-β-D-glucuronide; COD, codeine; HLM, human liver microsomes; IV-IVE, in vitro – in vivo extrapolation; KTM, ketamine; LTG, lamotrigine; 4MU, 4-methylumbelliferone; MOR, morphine; M3G, morphine 3-β-D-glucuronide; M6G, morphine 6-β-D-glucuronide; UGT, UDP-glucuronosyltransferase
Abstract

The selectivity of UGT enzyme inhibition by KTM and the kinetics of KTM inhibition of human liver microsomal morphine (MOR) and codeine (COD) glucuronidation were characterized here to explore further a pharmacokinetic basis for the ketamine (KTM) – opioid interaction. With the exception of UGT1A4, KTM inhibited the activities of recombinant human UGT enzymes in a concentration-dependant manner. However, IC\textsubscript{50} values were < 100 µM only for UGT2B4, UGT2B7 and UGT2B15. UGT2B7 catalyzes MOR 3- and 6- glucuronidation and the 6-glucuronidation of COD, with an additional substantial contribution of UGT2B4 to the latter reaction. Consistent with the effects of KTM on the activities of recombinant UGT2B enzyme activities, KTM competitively inhibited human liver microsomal MOR and COD glucuronidation. K\textsubscript{i} values for KTM inhibition of MOR 3- and 6- glucuronidation and COD 6- glucuronidation by human liver microsomes supplemented with 2% BSA were 5.8 ± 0.1 µM, 4.6 ± 0.2 µM, and 3.5 ± 0.1 µM, respectively. Based on the derived inhibitor constants, in vitro – in vivo extrapolation was employed to predict the effects of anesthetic and analgesic doses of KTM on MOR and COD clearances. Potentially clinically significant interactions (> 50% increases in the in vivo AUC ratios) with MOR and COD were predicted for anesthetic doses of KTM, and for a sub-anesthetic dose of KTM on COD glucuronidation.
The N-methyl-D-aspartate (NMDA) receptor antagonist ketamine (KTM) has been employed clinically as a dissociative anesthetic for more than four decades. However, KTM additionally exerts analgesic effects. In particular, there is evidence supporting the use of KTM as an adjuvant analgesic in several chronic pain states. Notably, KTM has been reported to provide improved pain relief in cancer patients with sub-optimal analgesic response to high dose morphine (MOR), with a concomitant reduction in opioid dose requirement and adverse effects (Bell 1999; Fitzgibbon and Viola, 2005). Although the improvement in MOR response due to KTM is generally believed to arise from attenuation of opioid tolerance and opioid–induced pain sensitivity following NMDA receptor blockade, it has recently been demonstrated that KTM inhibits the clearance of MOR via 3-glucuronidation in the isolated perfused rat liver preparation (Qi et al., 2010). Furthermore, KTM inhibited MOR 3-glucuronidation by rat liver microsomes.

As in the rat, MOR undergoes extensive hepatic glucuronidation in humans. Elimination by 3- and 6-glucuronidation comprise 57% and 10% of MOR systemic clearance, respectively (Hasselstrom and Sawe, 1993). UGT2B7 is the enzyme primarily responsible for hepatic MOR 3- and 6-glucuronidation (Stone et al., 2003). Like MOR, codeine (COD) is extensively glucuronidated in humans, with approximately 80% of the dose excreted in urine as COD 6-glucuronide (C6G) (Yue et al., 1991). UGT2B7 also glucuronidates COD, although there is an additional substantial contribution of UGT2B4 to C6G formation (Raungrut et al., 2010).

In order to explore further a pharmacokinetic basis for the potentiation of opioid analgesia by KTM, this study characterized the selectivity of UGT enzyme inhibition by KTM and the kinetics of KTM inhibition of human liver microsomal MOR and COD glucuronidation.
Inhibition studies with human liver microsomes (HLM) as the enzyme source were conducted in the presence and absence of BSA (2%). BSA sequesters long-chain unsaturated fatty acids released from the microsomal membrane during the course of an incubation, which act as potent inhibitors of UGT2B4 and UGT2B7 (Rowland et al., 2007 and 2008; Raungrut et al., 2010). Thus, Ki values generated in the presence of BSA provide more accurate prediction of drug-drug interaction potential in vivo (Rowland et al., 2006; Uchaipichat et al., 2006; Raungrut et al., 2010). Based on the derived inhibitor constants, in vitro – in vivo extrapolation (IV-IVE) was employed to predict the likelihood of clinically significant interactions between KTM and MOR and COD.

Materials and Methods

Materials

Alamethicin (from *Trichoderma viride*), codeine (COD), bovine serum albumin (BSA), ketamine (KTM), 4-methylumbelliferone (4MU), 4-methylumbelliferone β-D-glucuronide, morphine 3-β-D-glucuronide (M3G), and UDP-glucuronic acid (UDPGA; trisodium salt) were purchased from Sigma Aldrich (Sydney, Australia); morphine hydrochloride (MOR) from GlaxoSmithKline (Melbourne, Australia); codeine-6-β-D-glucuronide (C6G) from Toronto Research Chemicals (North York, ON, Canada); morphine 6-β-D-glucuronide (M6G) from Salford Ultrafine Chemicals (Manchester, UK); and Supersomes® expressing UGT 2B4, 2B7, 2B15 and 2B17 from BD Gentest (Woburn, MA, USA). Lamotrigine (LTG) and lamotrigine N2-β-D-glucuronide were a gift from the Wellcome Research laboratories (Beckenham, UK). Solvents and other reagents were of analytical reagent grade.

**Human liver microsomes (HLM) and recombinant UGT proteins.** Human livers were obtained from the human ‘liver’ bank of the Department of Clinical Pharmacology, Flinders
Medical Centre. Approval for the use of human liver tissue in xenobiotic metabolism studies was obtained from the Flinders Clinical Research Ethics Committee. HLM were prepared by ultracentrifugation according to Bowalgaha et al. (2005) and pooled by mixing equal protein amounts of microsomes from five livers (HL7, HL10, HL12, HL13, and HL40). HLM were activated by preincubation with alamethicin (50 μg/mg protein) on ice for 30 min before use in incubations (Boase and Miners, 2002). UGT1A cDNAs (viz. 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, and 1A10) were stably expressed in a human embryonic kidney cell line (HEK293), as described previously (Uchaipichat et al., 2004). Due to the relatively low activity of UGT 2B4, 2B7, 2B15 and 2B17 expressed in HEK293 cells, UGT2B enzymes expressed in insect cells (Supersomes) were employed in inhibition studies.

**Inhibition of recombinant UGT activities by ketamine.** KTM inhibition of recombinant UGT enzyme activity was assessed using 4MU (UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, 2B15 and 2B17), LTG (UGT1A4), and COD (UGT2B4) as the ‘probe’ substrates. Incubation and analytical conditions were as described previously (Uchaipichat et al., 2004; Rowland et al., 2006; Raungrut et al., 2010). Substrate concentrations corresponded to the K_m or S_50 values for each enzyme/substrate combination. Concentrations of KTM used in the inhibition screening experiments were 0, 10, 100, 500, and 1000 μM. Data were compared with activities determined in the absence of KTM, and the inhibitory effects are reported as percentage of control activity for duplicate measurements.

**Codeine 6-glucuronidation and morphine 3- and 6-glucuronidation by pooled HLM.** C6G formation by pooled HLM, in the absence and presence of 2% (w/v) BSA, was measured according to Raungrut et al. (2010). For studies of MOR glucuronidation, the
incubation mixture (in a total volume of 200µl) contained phosphate buffer (0.1 M, pH 7.4), MgCl₂ (4 mM), pooled activated HLM (0.5 mg/ml), and MOR, in the absence and presence of 2% (w/v) BSA. After a 5 min preincubation, reactions were initiated by the addition of UDPGA (5 mM) and continued at 37 °C in a shaking water bath for 30 min. Reactions were terminated by the addition of 2 and 8 µl of 70% (v/v) HClO₄ for incubations performed in the absence and presence of 2% BSA, respectively. Samples were cooled on ice for 20 min and then centrifuged at 5000 x g for 10 min at 10°C. A 10µl aliquot of the supernatant fraction was injected into the HPLC column. HPLC was performed using an Agilent 1100 series instrument (Agilent Technologies, Sydney, Australia) fitted with a NovaPak C18 column (3.9 x 150 mm, 4 µm particle size; Waters, Milford, MA). Column eluant was monitored by fluorescence detection at excitation and emission wavelengths of 235nm and 345nm, respectively. The mobile phase, delivered at a flow rate of 1 ml/min, consisted of 100% acetonitrile in distilled water (A) and 1-octanesulfonic acid (4 mM), acetonitrile (5%) and glacial acetic acid (1%) in distilled water (B). Initial conditions were 4% phase A - 96% phase B followed by a linear gradient over 10 min to 9% phase A - 91% phase B, which was held constant for 1 min. Mobile phase A was then increased to 25%, which was held for 0.8 min, before returning to the starting conditions. Retention times for M3G, M6G and morphine were 7.0, 10.3 and 13.6 min, respectively. Concentrations of M3G and M6G in incubation samples were quantified by comparison of peak areas to those of standard curves prepared over the concentration ranges 0.5 - 20 and 0.5- 5 µM, respectively.

**Ketamine inhibition of human liver microsomal morphine and codeine glucuronidation.**

Inhibition of human liver microsomal (pooled) C6G, M3G and M6G formation was determined at four KTM concentrations (see Figure 2 for concentrations) at each of three COD or MOR concentrations (see Figure 2) in the presence and absence of BSA (2% w/v) to
determine mechanism of inhibition and $K_i$ values. COD and MOR concentrations spanned the $K_m$ values for each substrate (Raungrut et al., 2010; Chau and Miners, unpublished results).

**Non-specific binding of ketamine to human liver microsomes.** The binding of KTM to HLM and to HLM plus 2% BSA was characterized by equilibrium dialysis according to the general procedure of McLure et al. (2000). One side of dialysis cell contained KTM in phosphate buffer (0.1M, pH 7.4), while the other side contained a suspension of either pooled HLM (1 mg/ml) or a combination of 2% BSA and HLM (1 mg/ml). KTM binding was investigated over the concentration ranges 10-250 μM and 2-50 μM for samples containing HLM and HLM plus 2% BSA, respectively. Following dialysis at 37°C for 4 hr, a 200 μl aliquot was collected from each cell and treated with 500 μl of ice-cold methanol containing 4% glacial acid. Samples were chilled on ice for 20 min and subsequently centrifuged at 13,000 x g for 5 min at 4 °C. An aliquot of the supernatant fraction was analyzed by HPLC. HPLC was performed using an Agilent 1100 series instrument (Agilent Technologies) fitted with a NovaPak C18 column (3.9 × 150 mm, 5 μm particle size; Waters). Mobile phase, delivered at flow rate 1 ml/min, comprised of a 1:1 mixture of 30 mM phosphate buffer containing triethylamine (pH 7.2) and acetonitrile. Column eluant was monitored by UV absorbance at 215 nm. The retention time of KTM was 2.5 min. The KTM concentrations of dialysis samples were quantified by comparison of peak areas to those of a standard curve prepared over the concentration range 2-250 μM. The fraction unbound of KTM in incubations ($f_{\text{unb}}$) was calculated as the drug concentration in the buffer compartment divided by the drug concentration in the protein compartment.

**Data analysis.** All data points represent the mean of duplicate estimates (< 10% variance). $K_i$ values for KTM inhibition of MOR and COD glucuronidation by pooled HLM were
calculated using Enzfitter (Biosoft, Cambridge, UK). Expressions for competitive, uncompetitive, noncompetitive and mixed inhibition were fit to experimental data. Goodness of fit was assessed from comparison of the F statistic, $r^2$ values, standard error of the parameter fit, and 95% confidence intervals. $K_i$ values are reported as the parameter ± SE of the parameter estimate.

**IV-IVE.** The predicted magnitude of the inhibition of MOR and COD hepatic clearance by KTM was calculated as the predicted ratios of the areas under the plasma concentration - time curves with ($AUC_i$) and without ($AUC$) KTM co-administration,

$$\frac{AUC_i}{AUC} = \frac{1}{\frac{fm}{1+[I]/K_i}+(1-fm)}$$

(equation 1)

where $[I]$ is the inhibitor concentration; $fm$ is the fraction of victim drug (COD or MOR) cleared along each pathway, and $K_i$ is the inhibition constant generated in vitro. COD fractional clearance via 6-glucuronidation was taken as 80% (Yue et al., 1991), and the fractions of MOR 3- and 6-glucuronidation as 0.57 and 0.10, respectively (Hasselstrom and Sawe, 1993). The inhibitor concentration ($[I]$) in vivo was taken as either the total or unbound concentrations of KTM in plasma following anesthetic and analgesic doses (see Results and Discussion). The mean unbound fraction of KTM in the plasma of healthy subjects has been reported as 0.73 (Dayton et al., 1983).

**Results and Discussion**

The binding of KTM to HLM alone was negligible across the concentration range investigated ($fu_{inc} = 0.98 \pm 0.02$). However, binding of KTM to HLM plus 2% BSA was 21% ($fu_{inc} = 0.79 \pm 0.02$), which was independent of KTM concentration. The concentration of KTM added to incubations containing BSA was corrected for $fu_{inc}$ when calculating $K_i$ values from
inhibition studies. Previous results from this laboratory have demonstrated that COD and MOR do not bind significantly to HLM in the absence and presence of 2% BSA (Raungrut et al. 2010; Chau and Miners, unpublished data).

KTM inhibition of recombinant human UGT activities was assessed using 4-MU (UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, 2B15 and 2B17), LTG (UGT1A4) and COD (UGT2B4) as the ‘probe’ substrates. With the exception of UGT1A4, KTM inhibited all UGT enzymes in a concentration dependant manner (Figure 1). However, greatest inhibition was observed with UGT 2B4, 2B7 and 2B15 as the enzyme sources; respective estimated IC$_{50}$ values were 69, 55, and 95 µM, while the IC$_{50}$ values obtained for the other enzymes were an order of magnitude higher. As indicated in Methods, inhibition studies with the UGT2B enzymes were conducted with Supersomes as the enzyme source whereas UGT1A enzymes were expressed in HEK293 cells. To exclude expression system dependent effects of KTM, inhibition of UGT2B enzymes expressed in HEK293 cells (Uchaipichat et al., 2004) was also tested. Similar inhibition of UGT2B7 and UGT2B15 was observed (data not shown), although the low activity of UGT2B4 in HEK293 cell lysate precluded meaningful interpretation of inhibition data.

As noted in the Introduction, UGT2B7 catalyzes COD and MOR glucuronidation while UGT2B4 additionally contributes to C6G formation. On the basis of the data shown in Figure 1 and the previous report of KTM inhibition of MOR 3-glucuronidation in the rat (Qi et al., 2010), KTM inhibition of human liver microsomal MOR 3- and 6- glucuronidation and COD 6-glucuronidation was characterized kinetically. Effects of KTM on each of the 3 glucuronidation pathways were modeled well using the equation for competitive inhibition (Figure 2). K$_{i}$ values determined for MOR 3- and 6- glucuronidation and COD
glucuronidation in the absence of BSA were 40 ± 0.7 µM, 35 ± 0.7 µM, and 52 ± 0.8 µM, respectively. Addition of BSA (2%) to incubations resulted in 85% to 93% reductions in $K_i$ values; 5.8 ± 0.1 µM, 4.6 ± 0.2 µM, and 3.5 ± 0.1 µM for KTM inhibition of MOR 3- and 6-glucuronidation and COD 6- glucuronidation, respectively. As noted previously, KTM binding to HLM plus 2% BSA was accounted for in the calculation of $K_i$ values.

IV-IVE approaches have been applied successfully to predict in vivo clearance and inhibitory drug-drug interaction potential for compounds eliminated by glucuronidation (Miners et al., 2004, 2006 and 2010). In particular, IV-IVE predicted the magnitude of the fluconazole – zidovudine and valproic acid – lamotrigine interactions (Rowland et al., 2006; Uchaipichat et al., 2006) and identified a number of potential interactions resulting from inhibition of COD 6-glucuronidation (Raungrut et al., 2010). Since these interactions arise from inhibition of UGT2B7 and UGT2B4, successful prediction of the change in the AUC of the victim drug requires the use of $K_i$ values generated in the presence of BSA because sequestration of inhibitory long chain unsaturated fatty acids is necessary to accurately measure an inhibitor constant. Interestingly, the decrease in the $K_i$ for KTM inhibition of C6G glucuronidation observed in the presence of BSA (52 µM to 3.5 µM) was larger than the approximate 7-fold reduction in $K_is$ for MOR 3- and 6- glucuronidation. The larger decrease in the $K_i$ for COD glucuronidation presumably reflects differential effects of long chain unsaturated fatty acids (and hence BSA) on UGT2B4 and UGT2B7; both enzymes contribute to C6G formation (Raungrut et al., 2010), whereas UGT2B7 is the dominant enzyme responsible for MOR 3- and 6- glucuronidation (Stone et al., 2003).

Predicted effects of KTM on MOR and COD clearances via glucuronidation were determined for anesthetic and analgesic doses. A mean KTM plasma concentration of 9.3 µM was
reported during steady-state anesthesia following induction with 2 mg/kg and a maintenance
dose of approximately 40 µg/kg/min (Idvall et al., 1979). When used as an adjuvant
analgesic, sub-anesthetic doses of KTM are typically administered by subcutaneous infusion,
but plasma concentrations appear not to have been reported when KTM is administered in
this manner. However, a peak KTM plasma concentration of 2.7 µM has been observed in
patients administered 0.5 mg/kg epidurally (Xie et al., 2003). Following low dose (0.125 –
0.250 mg/kg) iv administration, Clements and Nimmo (1981) found that a KTM
concentration > 0.42 µM (100 µg/L) was required for analgesia (defined as pain relief for > 5
min).

Substitution of the above values (for [I]) in equation 1 predict approximate 60% and 140%
increases in the AUCs for MOR and COD, respectively, following an anesthetic dose of
KTM (Table 1). Potentially clinically significant inhibition of COD clearance was also
predicted for a sub-anesthetic dose given epidurally (54% increase in AUC ratio), but a lesser
effect was predicted for the MOR AUC. No interaction was predicted for low dose KTM
given intravenously. As expected, smaller increases in AUCs were predicted when unbound
KTM concentrations were used for IV-IVE. Previous studies of drug-drug interaction
potential have generally reported optimal prediction of the AUC ratio using total drug
concentration (e.g. Ito et al., 2004; Rowland et al., 2006).

In summary, data presented here demonstrates that KTM inhibits human UGT2B4, UGT2B7
and UGT2B15. Consistent with the known involvement of UGT2B4 and UGT2B7 in MOR
and COD metabolism, KTM inhibited the glucuronidation of these compounds by HLM. K_i
values generated in the presence of BSA predicted potential inhibition of opioid clearance
following anesthetic and possibly sub-anesthetic doses of KTM, supporting the hypothesis
that a pharmacokinetic mechanism may contribute to KTM – opioid interactions.

Furthermore, KTM may potentially precipitate interactions with other compounds since UGT2B7 contributes to the metabolism of numerous other drugs, including anticancer agents and NSAIDs, and endogenous compounds such hydroxy-steroids (Jin et al., 1997; Kiang et al., 2005; Miners et al., 2010).
Authorship Contributions

Participated in research design: Miners, Evans, Uchaipichat, Raungrut, Janchawee

Conducted experiments: Uchaipichat, Raungrut, Chau

Performed data analysis: Uchaipichat, Miners

Wrote or contributed to the writing of the paper: Miners, Uchaipichat, Evans
References


DMD #39727


Footnote

This study was supported by a grant from the National Health and Medical Research Council of Australia. VU was the recipient of an Australian Education International Endeavour Fellowship and PR was supported in part by a Prince of Songkla University Graduate Studies grant.
Legends for Figures

Figure 1. Effects of ketamine (0, 10, 100, 500 and 1,000 µM) on the activities of recombinant human UGT enzymes. 4MU was used as the probe substrate, except for UGT1A4 (LTG) and UGT2B4 (COD). The 4MU, LTG and COD concentrations correspond to the known $K_m$ or $S_{50}$ values for each substrate/enzyme combination. Each bar represents the mean of duplicate measurements.

Figure 2. Dixon plots for ketamine inhibition of COD 6-glucuronidation and MOR 3- and 6-glucuronidation by pooled HLM in the absence (panels A, C, E) and presence (panels B, D, F) of BSA (2%). Each point represents the mean of duplicate estimates while lines are from model fitting.
Table 1. Predicted fold increase in the AUCs of codeine and morphine based on plasma ketamine concentrations reported following anesthetic and analgesic doses

<table>
<thead>
<tr>
<th>Reference</th>
<th>KTM dose</th>
<th>Total plasma [KTM] (µM)a</th>
<th>Predicted fold increases in codeine and morphine AUC ratios based on reported total and unbound in vivo ketamine concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Codeine ([KTM]_total)</td>
</tr>
<tr>
<td>Idvall et al. (1979)</td>
<td>2mg plus 41µg/min/kg iv</td>
<td>9.3</td>
<td>2.39</td>
</tr>
<tr>
<td>Xie et al. (2003)</td>
<td>0.5mg/kg epidurally</td>
<td>2.7</td>
<td>1.54</td>
</tr>
<tr>
<td>Clements and Nimmo (1981)</td>
<td>0.125-0.25 mg/kg iv</td>
<td>0.42c</td>
<td>1.09</td>
</tr>
</tbody>
</table>

a. Unbound ketamine concentration calculated as fu x total plasma concentration

b. Morphine AUC ratios represent the sum of the separate ratios for the 3- and 6- glucuronidation pathways

c. Pain threshold increased > 5 min when total plasma ketamine concentration > 100µg/L
Figure 1.
Figure 2

A) Morphine-3-glucuronide (-BSA)

B) Morphine-3-glucuronide (+BSA)

C) Morphine-6-glucuronide (-BSA)

D) Morphine-6-glucuronide (+BSA)

E) Codeine 6-glucuronidation (-BSA)

F) Codeine 6-glucuronidation (+BSA)