Inhibition of morphine metabolism by ketamine

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Non-standard abbreviations:

HPLC, high performance liquid chromatography; IPL, isolated perfused liver; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; UDPGA, uridine 5’-diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase.
Clinical observation of a synergistic effect of ketamine on morphine analgesia remains controversial. Although a pharmacodynamic basis for an interaction has been explored in animal and clinical studies, the possibility of a pharmacokinetic mechanism has not been investigated. Whereas both morphine and M6G are effective analgesics, M3G lacks activity. Thus, changes in the metabolism and disposition of morphine may result in altered response. We firstly investigated the interaction between morphine and ketamine in the isolated perfused rat liver preparation. The clearance of morphine was decreased from 16.8 ± 4.6 mL/min in the control period to 7.7 ± 2.8 mL/min in ketamine treatment period, with the formation clearance of M3G decreasing from 8.0 ± 4.1 mL/min to 2.1 ± 1.1 mL/min. Fractional conversion of morphine to M3G was significantly decreased from 0.46 ± 0.17 in the control period to 0.28 ± 0.14 upon the addition of ketamine. The possible mechanism of the interaction was further investigated in vitro with rat liver microsomes as the enzyme source. The formation of M3G followed single-enzyme Michaelis-Menten kinetics, with a mean apparent $K_m$ of 2.18 ± 0.45 mM and $V_{max}$ of 8.67 ± 0.59 nmol/min/mg. Ketamine inhibited morphine 3-glucuronidation non-competitively, with a mean $K_i$ value of 33.3 ± 7.9 µM. The results demonstrate that ketamine inhibits the glucuronidation of morphine in a rat model.
INTRODUCTION

Ketamine [2-(O-chlorohenyl)-2-(methylamino)cyclohexanone] is a non-competitive N-methyl-D-aspartate receptor antagonist that is used as both an anaesthetic and analgesic agent (Fratta et al., 1980). In recent years, there have been conflicting clinical reports on whether ketamine enhances the analgesic effects of morphine. Several indicated that concurrent use of morphine and ketamine prolonged the duration of morphine-induced analgesia and reduced the consumption and adverse effects of morphine in patients (Bell, 1999; Kannan et al., 2002; Galinski et al., 2007), whereas others reported no additional value when the two were combined for postoperative analgesia (Sakata et al., 2007; Jensen et al., 2008).

The µ opioid receptor antagonist naloxone (10 mg/kg) reversed ketamine-induced analgesia in rodents (Ryder et al., 1978), similar to its effect on morphine analgesia. However, Fratta et al. (1980) indicated that ketamine did not interact with opioid receptors. Animal studies investigating the interaction between morphine and ketamine suggested that a part of the analgesic action of ketamine was achieved through a mechanism similar to that of morphine (Smith et al., 1987). Discussions regarding the mechanism of the morphine-ketamine interaction have focused on either pharmacodynamic effects or clinical observations. However, a potential pharmacokinetic mechanism for the interaction has not been explored.

Morphine undergoes extensive hepatic extraction and metabolism. More than 50% of an oral dose to humans and rodents is excreted in urine as morphine-3-glucuronide (M3G). About 10% is excreted as morphine-6-glucuronide (M6G) in man and several other species, but not in rats (Milne et al., 1996). M6G is a potent µ-receptor agonist while M3G may antagonise the antinociceptive action of morphine and M6G (Milne et al., 1996). Thus, it is possible that altered morphine clearance and formation of morphine glucuronides may influence the pharmacological effect of morphine.
Pharmacokinetic interactions may be examined in vivo and in vitro, the latter using isolated organs and microsomal or cellular preparations. Isolated perfused livers (IPL) allow evaluation of dispositional and metabolic interactions while rat liver microsomal preparations can be used to test for the latter based on interactions at the enzymatic level. The aim of this study was to investigate the impact of ketamine on the metabolism and disposition of morphine using the isolated perfused rat liver and rat hepatic microsomes.
MATERIALS AND METHODS

Chemicals: Morphine (morphine HCl·H₂O; McFarlane Smith, Edinburgh, UK), ketamine (Parnell Laboratories Pty Ltd., NSW, Australia), bovine serum albumin (BSA; ICN Biomedicals Inc., Aurora, Ohio), and uridine 5'-diphosphoglucuronic acid (UDPGA), alamethicin, sodium taurocholate and M3G (all from Sigma Chemical Co., St. Louis, MO) were used as supplied. Other chemicals were of analytical grade and purchased commercially.

Isolated Perfused Liver Preparation: The study was approved by the Institute of Medical and Veterinary Science (IMVS) Animal Ethics Committee (Adelaide, Australia). Five male Sprague-Dawley (SD) rat (310 - 360 g; IMVS, Adelaide, Australia) livers were isolated and perfused in a single-pass mode (Milne et al., 1997). Anaesthesia was induced with 60 mg/kg sodium pentobarbitone. Drug-free Krebs-bicarbonate buffer (pH 7.4) containing glucose (16.5 mM) and sodium taurocholate (8.33 µM) was pumped (30 mL/min) into the liver via the portal vein. After equilibration for 20 min, medium containing morphine (6.7 µM) was perfused (0 - 30 min) and perfusate (1 mL) collected from the cannulated vena cava at 0, 1, 2, 5, 10, 15, 20, 25 and 30 min. The medium was then changed to one containing both morphine (6.7 µM) and ketamine (21 µM) (30 - 70 min). Perfusate was collected at 31, 32 and 35 min and every 5 min thereafter to 70 min. All bile was collected at 5-min intervals. In addition, two rat livers were perfused with medium containing 6.7 µM morphine for 70 min after a 20-min period of equilibration. Liver viability was monitored by assessing its gross appearance (uniformly perfused), bile flow (> 5 µL/min) and the recovery of venous perfusate (> 98% of the volume of inflowing perfusing medium).

The perfusate and bile (diluted 1 in 1000 with water) samples were analysed by HPLC (Shimadzu, Kyoto, Japan) with fluorescence detection (Excitation: 280 nm; Emission: 335...
nm). Samples were directly injected onto a C\textsubscript{18} column (250 × 4.6 mm, Alltech, USA), with a C\textsubscript{18} guard column (7.5 × 3.2 mm, Grace, USA), in an oven set at 45°C. A mobile phase consisting of 2.5% acetonitrile and 97.5% phosphate buffer (20 mM) was used at a flow rate of 1 mL/min. Under these conditions, the retention times for M3G and morphine were 5.9 and 17.3 min, respectively. Calibration curves were linear ($r^2 > 0.999$) for morphine (0.27 – 6.67 µM) and M3G (0.11 – 4.88 µM) in perfusate and bile. Accuracy and precision for measuring quality controls spanning the range for the calibration standards were within 20%.

**Measurement of microsomal M3G formation:** Microsomes were prepared as described by Lear et al. (1991) and stored at -70°C until use. They were preincubated with alamethicin (50 µg/mg protein) on ice for 30 min. Duplicate incubations consisting of microsomes (1 mg protein/mL), morphine, MgCl\textsubscript{2} (5 mM), Tris-HCl (0.1 mM, pH 7.4) and UDPGA (5 mM) in a total volume of 0.2 mL were performed at 37°C for 45 min. The reaction rate was linear with incubation time up to 60 min and protein concentration up to 2 mg/mL. The reaction was terminated by adding 0.2 mL ice-cold 0.1 M HClO\textsubscript{4} and cooling on ice. Samples were centrifuged at 10,000 × g for 10 min. Formed M3G was quantified using the same HPLC system with a UV detector set at 210 nm. Supernatant was injected onto the same column and precolumn as described for the IPL but at ambient temperature. A mobile phase consisting 3% of acetonitrile and 97% of phosphate buffer (20 mM) was used at 1 mL/min. The retention time of M3G was 6.8 min. Calibration curves were linear ($r^2 > 0.996$) for M3G (0.005 – 2.6 µM). The accuracy and precision of quality controls were less than 25%. The intra-day CV at the LLOQ (0.005 µM) was within 8%.

To determine the \( K_m \) and \( V_{max} \) values for M3G formation, duplicate incubations were performed with concentrations of morphine in the range of 0.05 to 10 mM. The effect of
ketamine on M3G formation was assessed at four concentrations of morphine (0.25, 0.5, 1, or 1.5 mM), in the absence (control) or presence of ketamine (20, 50 and 100 µM).

**Calculations and statistical analysis:**

**Experiments with the IPL:** Data from the control period (20 to 30 min) and from the ketamine treatment period (60 to 70 min) were used for calculation of the following pharmacokinetic parameters:

- **Availability of morphine** ($F$)
  \[ F = \frac{C_{\text{out}}}{C_{\text{in}}} \]  
  where $C_{\text{in}}$ and $C_{\text{out}}$ are the concentrations of morphine in inflowing perfusing medium and outflowing perfusate, respectively.

- **Hepatic clearance of morphine** ($CL_{H,M}$)
  \[ CL_{H,M} = \frac{(C_{\text{in}} - C_{\text{out}}) \times Q}{C_{\text{in}}} \]  
  where $Q$ is the flow rate of perfusing medium.

- **Formation clearance of M3G** ($CL_f$)
  \[ CL_f = \frac{R_{\text{perf}} + R_{\text{bil}}}{C_{\text{in}}} \]  
  where $R_{\text{perf}}$ and $R_{\text{bil}}$ are the rates of recovery of M3G in perfusate and bile, respectively.

- **The biliary extraction ratio of hepatically formed M3G** ($E_{b,M3G}$)
  \[ E_{b,M3G} = \frac{R_{\text{bil}}}{R_{\text{bil}} + R_{\text{perf}}} \]  

- **Fractional conversion of morphine to M3G** ($FC_{M\rightarrow M3G}$)
  \[ FC_{M\rightarrow M3G} = \frac{CL_f}{CL_{H,M}} \]  

Pharmacokinetic parameters are presented as mean ± SD. Comparisons between periods were made by paired Student’s t-test, with values of $P < 0.05$ considered significant.
Measurement of M3G formation: In vitro kinetic constants are expressed as mean ± SD. Initial estimates of apparent $K_m$ and $V_{max}$ for M3G formation were obtained from graphical analysis of Eadie-Hofstee plots. The single-enzyme Michaelis-Menten equation was fitted to untransformed data using PRISM 5 (GraphPad Software Inc., San Diego, CA, USA). $K_i$ values were similarly obtained by fitting with the expression for non-competitive inhibition.
RESULTS AND DISCUSSION

The studies presented here provide compelling evidence that ketamine inhibits the conversion of morphine to M3G in rat liver. Morphine and M3G appeared rapidly in the outflowing perfusate and attained steady-state within 10 min of commencing perfusion with morphine alone. Upon addition of ketamine, the concentrations of M3G in the outflowing perfusate decreased markedly whereas those for morphine increased (Figure 1), and an additional 10 to 15 min was required before a new steady-state for M3G and morphine was reached in the outflowing perfusate. In contrast, the concentrations of morphine and M3G in the outflowing perfusate remained constant beyond 20 min during perfusion of the two livers with morphine alone for the entire 70 min (Figure 1). M3G was excreted extensively in bile. The rate of M3G excretion was constant after 15 min, but decreased by approximately 75% following the addition of ketamine. Mean pharmacokinetic parameters during the control and ketamine treatment periods are summarised in Table 1. Ketamine decreased the clearance of morphine by 54% (p<0.05). Hepatic cellular uptake of morphine is partly by an active carrier-mediated system, but with passive diffusion playing an important role after the saturation of transporters beyond 200 µM morphine (Iwamoto et al., 1978). Liver was perfused here with 6.7 µM morphine. Thus, the reduction in hepatic clearance of morphine may result from inhibition by ketamine of the active uptake of morphine or from a reduction in the biotransformation of morphine.

The permeability of M3G through the lipophilic environment of cellular membrane is apparently limited by its polarity. The uptake of M3G from sinusoids to the hepatocytes is negligible compared to the flux of hepatically-formed M3G in the opposite direction. The greater efflux is driven by its accumulation within liver cells after formation from morphine (Evans et al., 1999). Efflux of M3G across the sinusoidal membrane is mediated by multidrug
resistance protein 3 (Mrp3) while efflux across the canalicular membrane is mediated by Mrp2 (van de Wetering et al., 2007). M3G was excreted extensively into bile, but no morphine was detected. The unchanged biliary extraction ratio of M3G suggests that ketamine does not affect M3G efflux, or Mrp2 and Mrp3 are both inhibited to a similar extent.

The fractional conversion of morphine to M3G was significantly impaired in the presence of ketamine (0.46 versus 0.28, P<0.05, Table 1). If ketamine simply blocked hepatic uptake of morphine, the fractional conversion of morphine should remain unchanged. Therefore, it would seem that inhibition of uptake is unlikely to be the only mechanism for the interaction between morphine and ketamine. In addition, the reduced fractional conversion of morphine to M3G suggests that biotransformation of morphine to M3G may be reduced to a greater extent than other metabolic pathways, such as the formation of normorphine (Blanck et al., 1990). To investigate the interaction between morphine and ketamine further, additional experiments examined the impact of ketamine on the formation of M3G by rat liver microsomes.

Glucuronidation, catalysed by UDP-glucuronosyltransferases (UGT), is the most important conjugation reaction in drug biotransformation (Miners and Mackenzie, 1991). Morphine is extensively metabolised in the liver to one or more glucuronides, depending on the species (Milne et al., 1996), but M3G is the only glucuronide formed from natural (-)-morphine by rat microsomal UGTs (Rane et al., 1985). At least two forms of rat liver UGT are involved in the formation of M3G (Ishii et al., 1997). UGT2B7 is the major human UGT catalysing the formation of morphine glucuronides (Stone et al., 2003), but recombinant human UGT1A1 and 1A8 are also known to form M3G (Ohno et al., 2008). However, their activities are low compared to UGT2B7, and UGT1A8 is not expressed in the liver. Figure 2(a) shows a representative Eadie-Hofstee plot for the formation of M3G by activated rat liver microsomes.
M3G formation was well described by the single-enzyme Michaelis-Menten equation, with a mean apparent $K_m$ of $2.18 \pm 0.45$ mM and $V_{max}$ of $8.67 \pm 0.59$ nmol/min/mg.

Figure 2(b) shows that ketamine inhibited the formation of M3G in a non-competitive manner, with a mean apparent $K_i$ of $33.3 \pm 7.9$ µM. This was an unexpected finding given that there was no previous evidence that ketamine is capable of inhibiting drug glucuronidation. Ketamine is metabolised primarily via N-demethylation to norketamine by cytochrome P450 (White and Ryan, 1996). The data suggest that ketamine may occupy a site on the UGT(s) causing a reduction in the formation of M3G. Overall, it can be concluded that the inhibitory effect of ketamine on the formation of M3G by UGT accounted for the interaction observed between these two compounds in the IPL.

Projean et al. (2003) examined the pharmacokinetics of morphine in rats after iv administration of 10 µmol/kg and found that the concentrations of morphine were maintained above 2 µM for up to 1 h. Another study investigated the analgesic effect of morphine in rats, and showed that 3.3 µmol/kg of morphine (sc) provided a delay in the tail-flick reaction time for approximately 2.5 h (Stain et al., 1995). In the IPL, the inflow concentration of morphine in the perfusion medium was 6.7 µM in the control period, which is of the same magnitude as the concentration providing analgesia in rats. A biodisposition study of ketamine in rats showed that the concentration of ketamine was approximately 105 and 16.8 µM in liver and plasma 20 min after an iv dose of 12.6 µmol/kg. With this dosage, the analgesia from ketamine can be maintained for up to 30 min (Marietta et al., 1976). The concentration of ketamine perfusing the liver in our study was similar to the concentration required to achieve analgesia in rats.

Ketamine in perfusate at a concentration of about three-times that of morphine caused a 50% reduction in its hepatic clearance. In contrast, the concentration of ketamine used in the
microsomal study was about 4% of the concentration of morphine. The differences in inhibitory capacity in these two experimental systems might be due to hepatic distributional barriers for ketamine and its extensive metabolism and removal in perfused liver, nonspecific binding of ketamine in microsomes, and/or the inhibitory effects of endogenous inhibitors of glucuronidation present in microsomal incubations (Miners et al., 2010).

In conclusion, this study has shown that ketamine reduces the hepatic clearance of morphine, most likely due to inhibition of the hepatic UGT(s) catalyzing M3G formation. Since M3G and M6G have different pharmacological activities, it is possible that the controversial observation in patients could be partly explained by the change in ratio of M3G and/or M6G to morphine in the body. However, other inhibitory mechanisms of ketamine causing the interaction with morphine cannot be ruled out. Whether ketamine is capable of inhibiting the glucuronidation of other drugs when the two drugs are co-administered \textit{in vivo} warrants investigation.
REFERENCES


LEGENDS FOR FIGURES

**Figure 1.** Concentrations (mean ± SD) of morphine and M3G in outflowing perfusate during perfusion with 6.7 µM morphine (0-30 min) and 6.7 µM morphine plus 21 µM ketamine (30-70 min). Dotted lines show the mean concentrations of morphine and M3G in the two additional rats perfused with morphine throughout 70 min. Solid horizontal line shows the concentration of morphine in the inflow perfusate (6.7 µM).

**Figure 2.** Representative Eadie-Hofstee plot (a) for M3G formation by rat liver microsomes. Representative Dixon plot (b) showing the inhibitory effect of ketamine on the rate of formation of M3G from morphine. The number on the top of each line shows the concentration of morphine (mM).
### Table 1. Pharmacokinetic parameters (mean ± SD) for morphine and M3G in the isolated perfused rat liver during perfusion with morphine alone or morphine plus ketamine.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control period (20 - 30 min)</th>
<th>Ketamine treatment period (60 - 70 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F</strong></td>
<td>0.44 ± 0.14</td>
<td>0.74 ± 0.09*</td>
</tr>
<tr>
<td><strong>CL\textsubscript{H,M} (ml/min)</strong></td>
<td>16.8 ± 4.6</td>
<td>7.7 ± 2.8*</td>
</tr>
<tr>
<td><strong>CL\textsubscript{f} (ml/min)</strong></td>
<td>8.0 ± 4.1</td>
<td>2.1 ± 1.1*</td>
</tr>
<tr>
<td><strong>FC\textsubscript{M→M3G}</strong></td>
<td>0.46 ± 0.17</td>
<td>0.28 ± 0.14*</td>
</tr>
<tr>
<td><strong>E\textsubscript{k,M3G}</strong></td>
<td>0.76 ± 0.16</td>
<td>0.68 ± 0.20</td>
</tr>
</tbody>
</table>

* P < 0.05 compared to the corresponding value in the control period.