Differential *in vitro* inhibition of M3G and M6G formation from morphine by (R)- and (S)-methadone and structurally related opioids

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Aims

To determine the *in vitro* kinetics of morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) formation and the inhibition potential by methadone enantiomers and structurally related opioids.

Methods

M3G and M6G formation kinetics from morphine were determined using microsomes from five human livers. Inhibition of glucuronide formation was investigated with eight inhibitors (100 μM) and the mechanism of inhibition determined for (R)- and (S)-methadone (70–500 μM) using three microsomal samples.

Results

Glucuronide formation displayed single enzyme kinetics. The M3G V_{max} (mean $\pm$ SD) was 4.8-fold greater than M6G V_{max} (555 ± 110 vs. 115 ± 19 nmol mg^{-1} protein h^{-1} ; $P = 0.006$, mean of difference 439; 95% confidence interval 313, 565 nmol mg^{-1} protein h^{-1}). K_m values for M3G and M6G formation were not significantly different (1.12 ± 0.37 vs. 1.11 ± 0.31 mM; $P = 0.89$, 0.02; -0.29 , 0.32 mM). M3G and M6G formation was inhibited ($P < 0.01$) with a significant increase in the M3G/M6G ratio ($P < 0.01$) for all compounds tested. Detailed analysis with (R)- and (S)-methadone revealed noncompetitive inhibition with (R)-methadone K_i of 320 ± 42 μM and 192 ± 12 μM for M3G and M6G, respectively, and (S)-methadone K_i of 226 ± 30 μM and 152 ± 20 μM for M3G and M6G, respectively. K_i values for M3G inhibition were significantly greater than for M6G for (R)-methadone ($P = 0.017$, 128; 55, 202 μM) and (S)-methadone ($P = 0.026$, 75; 22, 128 μM).

Conclusions

Both methadone enantiomers noncompetitively inhibited the formation of morphine's primary metabolites, with greater inhibition of M6G formation compared with M3G. These findings indicate a mechanism for reduced morphine clearance in methadone-maintained patients and reduced relative formation of the opioid active M6G compared with M3G.

Introduction

Morphine is the gold standard opioid for the treatment of moderate to severe acute and chronic pain. Morphine is primarily cleared via glucuronidation to morphine-

3-glucuronide (M3G) and morphine-6-glucuronide (M6G). M6G is a potent μ -opioid agonist [1] with clinical studies suggesting it contributes to analgesia after the administration of morphine [2, 3]. M3G has no anal-

gesic activity, but has been shown to cause excitation and attenuation of opioid effects in rats [4–6]. Sjögren and coworkers [7] proposed that reduced morphine efficacy in chronically treated cancer patients may be due to increased plasma M3G concentrations, or an increase in the M3G/M6G plasma concentration ratio. Only limited human studies investigating intravenous (i.v.) administration of M3G alone have been performed. Preliminary assessment of the toxicity and pharmacokinetics of i.v. M3G reported no excitatory effects [8]; however, investigation of M3G combined with morphine or M6G by this group reported M3G may have been associated with mild symptoms that reflected neuroexcitation in several of the subjects, a finding not observed when morphine or M6G were administered alone [9].

Although the *in vitro* glucuronidation of morphine has been widely studied [10–14], few studies have investigated the simultaneous formation of both metabolites in human liver microsomes. Comparison between these studies is further complicated due to variation in incubation conditions and the method of microsome activation. The mean single enzyme K_m and V_{max} values reported for M3G formation range from 0.9 to 5.2 mM and 56 to 402 nmol mg⁻¹ protein h⁻¹, respectively, and for M6G from 0.51 to 1.23 mM and 7.8 to 88.8 nmol mg⁻¹ protein h⁻¹, respectively [11, 12, 14]. Investigations with expressed recombinant human UDP-glucuronosyltransferase (UGT) enzymes have shown UGT1A1, 1A3, 1A6, 1A8, 1A9, 1A10 and 2B7 all metabolize morphine to M3G [15–17]. However, only UGT2B7 has been shown to form M6G [17, 18]. The relative contribution of these isoforms to the overall glucuronidation of morphine is still unknown. Only one investigation using human liver microsomes has shown the possible involvement of more than one UGT isoform in M3G formation, with the suggested involvement of a high-affinity, low-capacity enzyme (with a mean K_m and V_{max} of 5.3 μ M and 18 nmol mg⁻¹ protein h⁻¹) and low-affinity, high-capacity enzyme (with mean K_m and V_{max} of 1203 μ M and 653 nmol mg⁻¹ protein h⁻¹) [19].

Methadone, a μ -opioid receptor agonist, is the most widely used substitution treatment for opioid dependence [20] and is becoming increasingly accepted as a treatment for cancer pain [21]. Methadone exists as two stereo-isomeric forms, the levorotatory R-(–)-methadone and the dextrorotatory S-(+)-methadone. Methadone is commonly administered as a 50 : 50 racemic mixture of the two enantiomers; however, only the (R)-enantiomer has μ -opioid agonist activity [22, 23]. Doverty and coworkers [24] reported that morphine was ineffective at reducing pain detection and threshold in

methadone maintenance patients subjected to the cold-pressor test. In this investigation, it was also observed that methadone-maintained subjects had higher plasma morphine concentrations than non-opioid-dependent controls when given the same i.v. dose, suggesting decreased morphine clearance in this population.

Methadone is primarily metabolized by the cytochrome P450 enzyme system, specifically CYP3A4, to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) [25–27]. Although not a substrate for glucuronidation itself, methadone inhibits the glucuronidation of 3'-azido-3'-deoxythymidine (AZT) *in vitro* [28]. This agrees with data from clinical studies showing that individuals taking methadone, in addition to AZT, have a reduced clearance of AZT [29, 30]. The possibility of similar inhibition of morphine glucuronidation by methadone has not yet been investigated either *in vitro* or *in vivo*. In clinical use are several other phenylheptylamine opioids similar in chemical structure to methadone, including dextropropoxyphene and levo- α -acetylmethadol (LAAM), which is metabolized to the pharmacologically active nor-LAAM and dinor-LAAM. Consequently, the aim of this study was to investigate the inhibition of morphine glucuronidation to M3G and M6G by methadone and structurally related opioids *in vitro* using human liver microsomes.

Materials and methods

Chemicals

Alamethicin (from *Trichoderma viride*), uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA) and M3G were purchased from Sigma Chemical Co. (St Louis, MO, USA). Morphine sulphate B.P. was a kind gift from GlaxoSmithKline (Port Fairy, Australia). Racemic (*rac*)-methadone as the hydrochloride salt and (R)- and (S)-methadone as the free bases were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). *Rac*-EDDP was purchased from Alltech-Applied Science Laboratories (State College, PA, USA). LAAM, nor-LAAM, dinor-LAAM, all as hydrochloride salts, were obtained from Ultrafine Chemicals (Manchester, UK). Dextropropoxyphene was obtained from Fisons Pharmaceuticals (Thornleigh, Australia). M6G was synthesized by the method of Yoshimura *et al.* (1968), with identity and purity as previously reported [31]. Acetonitrile [HiPerSolv™ for high-performance liquid chromatography (HPLC)] was obtained from BDH (Poole, UK). Milli-Q water (Millipore, Bedford, MA, USA) was used in all buffer and mobile phase preparations and control incubations. All other reagents and chemicals were obtained from commercial sources and were of analytical grade.

Human liver microsomes

Ethics approval for the study was obtained from the Committee on the Ethics of Human Experimentation of the University of Adelaide and the Human Ethics Committee of the Royal Adelaide Hospital. Human liver samples (HLS) were obtained during partial hepatectomy from patients (internal code: HLS nos 18, 21, 34, 36, 46) who had given their written informed consent for their tissue to be used. Donors ranged in age from 31 to 70 years, four were female and all had clinical chemistry measurements within normal ranges prior to surgery and all tissue samples appeared normal based on gross histology. Microsomes were prepared using differential centrifugation based on the method of Nelson *et al.* [32] using sucrose–Tris buffer (0.25 M sucrose and 0.05 M Tris–HCl, pH 7.4). Protein and total cytochrome P450 content were determined using previously established methods [33, 34]. Liver samples and microsomes were stored at -80°C until required.

Microsomal incubations

The *in vitro* formation of M3G and M6G was investigated in human liver microsomes ($n = 5$; HLS nos 18, 21, 34, 36, 46). Optimal incubation conditions were determined prior to the commencement of the kinetic and inhibition studies (see below). All incubations were performed in duplicate. In the first step, 200 μg of microsomal protein, 6 μg alamethicin, 0.1 M Tris–HCl buffer and 5 mM MgCl_2 were preincubated on ice for 20 min. Morphine (0.1–10 mM; $n = 9$ concentrations) was then added and the mixture preincubated at 37°C for 20 min. The reaction was then initiated with the addition of 10 mM UDPGA (giving a final incubation volume of 200 μl) and incubated at 37°C for 20 min. The reaction was stopped by the addition of 250 μl of ice-cold acetonitrile and placed on ice for 10 min, then centrifuged at 16 000 g for 5 min to separate the precipitated protein and supernatant.

Optimization of incubation conditions

Optimal microsome activation by alamethicin (2, 4, 6, 8, 12, 16 and 20 μg) was assessed with 1 and 10 mM morphine. The influence of incubation time and microsomal protein content on M3G and M6G formation was assessed with 0.2 and 2 mM morphine.

M3G and M6G quantification

The supernatant (50 μl) and 10 mM NaH_2PO_4 (100 μl) were mixed and 30 μl injected for HPLC analysis. Chromatography conditions for the quantification of M3G and M6G were adapted from a previous method by Milne *et al.* [31], which described the quantification of

M3G and M6G in plasma. Analysis was performed with a Shimadzu (Kyoto, Japan) HPLC system equipped with LC-10AS pump, a SCL-10A VP system controller, SPD-10A VP UV-Vis detector, SIL-10A auto injector, and a Nova-Pak 4 μC_{18} 5×100 mm column with a Waters radial compression module (Waters Assoc., Lane Cove, Australia). The mobile phase comprised 23% acetonitrile, 0.01 M NaH_2PO_4 , and 230 mg l^{-1} lauryl sulphate (sodium salt), adjusted to pH 2.6 with 85% orthophosphoric acid. Interassay performance was assessed using quality control (QC) samples prepared in duplicate containing low (LQC, 11 and 1.6 μM), medium (MQC, 40 and 8 μM) and high (HQC, 110 and 16 μM) concentrations of M3G and M6G, respectively. The lower limits of quantification (LLOQ) for M3G and M6G were 5.0 and 1.0 μM , respectively. Interassay accuracy and precision (mean accuracy $\pm$ % coefficient of variation) for M3G ($n = 5$) was $102 \pm 9\%$, $100 \pm 7\%$, $101 \pm 4\%$ and $102 \pm 4\%$, whilst for M6G ($n = 5$) it was $95 \pm 6\%$, $101 \pm 7\%$, $101 \pm 4\%$ and $105 \pm 10\%$ for LQC, MQC, HQC and LLOQ, respectively. Intraassay accuracy and precision for M3G ($n = 8$) was $106 \pm 6\%$, $99 \pm 10\%$, $99 \pm 6\%$ and $104 \pm 4\%$, whilst for M6G ($n = 8$) it was $100 \pm 6\%$, $99 \pm 10\%$, $98 \pm 6\%$ and $102 \pm 6\%$ for LQC, MQC, HQC and LLOQ, respectively.

Inhibition studies – structurally related opioids

Liver microsomes ($n = 3$; HLS nos 34, 36, 46) were coincubated with a range of opioids structurally related to methadone, in order to investigate their effect on M3G and M6G formation from morphine (1 mM, based on the average K_m observed during formation). Inhibitors used were (R)-, (S)- and *rac*-methadone, *rac*-EDDP, LAAM, nor and dinor-LAAM and dextropropoxyphene, all at a final incubation concentration of 100 μM . All inhibitors were prepared in MilliQ water, which was also used in control incubations. Incubation conditions did not change from the morphine kinetic studies except that the inhibitor was incubated with the reaction mixture for 20 min at 37°C prior to the preincubation with morphine.

Methadone inhibition characterization

Determination of the inhibition constant (K_i) and the mechanism of inhibition of M3G and M6G formation from morphine (0.3–2.5 mM; $n = 4$ concentrations) was further characterized for (R)- and (S)-methadone at 0, 70, 100, 200 and 500 μM in human liver microsomes ($n = 3$, HLS nos 18, 36, 46). Incubation conditions were the same as those for the previous inhibition studies.

Data analysis

The rates of M3G and M6G formation (V) from the kinetic studies were expressed as $\text{nmol mg}^{-1} \text{ protein h}^{-1}$. To obtain estimates of $V_{\max}$ and K_m , Eadie–Hofstee plots ($V/[S]$ vs. V) were constructed and a single-enzyme and two-enzyme Michaelis–Menten equations were fitted to the unweighted data using nonlinear regression (Prism®, Version 4.0; GraphPad Software Inc., San Diego, CA, USA). Visual inspection of the Eadie–Hofstee plots, goodness of fit of the predicted line to the observed data and an F -test comparing the sums of squared deviations were used to determine whether a single- or two-enzyme model provided the best fit to the data. $V_{\max}$ and K_m values for M3G and M6G formation were compared using a paired T -test with results expressed as P -value; mean difference and 95% confidence interval (CI). Apparent intrinsic clearance (Cl_{int}) was calculated as $V_{\max}/K_m$. Inhibition data were expressed as a percent inhibition compared with control formation.

Statistical significance of the observed inhibition of M3G and M6G formation and comparison of the M3G/M6G ratio was assessed using a repeated measures one-way ANOVA with a Dunnett's multiple comparisons posthoc test. The mechanism of (R)- and (S)-methadone inhibition and estimates for K_i were determined by fitting the data to Michaelis–Menten competitive, noncompetitive and uncompetitive inhibition equations [35]. The best model was determined by visual inspection of the goodness of fit of the predicted line to the observed data and the comparison of the Akaike's Information Criteria. Comparisons of K_i values between (R)- and (S)-methadone inhibition and M3G and M6G formation were assessed using a paired T -test and results expressed as P -value; mean difference and 95% CI for the difference between means. Statistical significance was set at $P < 0.05$.

Results

Maximal activation, as determined by maximal formation of M3G and M6G, was observed with 6 μg of alamethicin for both metabolites at both morphine concentrations, and remained maximal up to 20 μg alamethicin (Figure 1). Formation of both metabolites was observed to be linear for times up to 60 min and for amounts of microsomal protein from 40 to 400 μg .

The formation of M3G and M6G at the morphine concentrations investigated produced linear Eadie–Hofstee plots (Figure 2A,C), and subsequently a single enzyme Michaelis–Menten equation provided the best fit to the data ($P < 0.05$; Figure 2B,D). Kinetic parameters for the formation of M3G and M6G are shown in Table 1. The M3G $V_{\max}$ was a mean of 4.8-fold greater than M6G $V_{\max}$ ($P = 0.006$, mean of difference 439; 95% CI for the difference between means 313, 565 $\text{nmol mg}^{-1} \text{ protein h}^{-1}$). In contrast, K_m values for M3G and M6G formation were not significantly different ($P = 0.89$, 0.02; -0.29 , 0.32 mM).

M3G and M6G formations were significantly inhibited ($P < 0.01$) by 100 μM (R)-, (S)- and *rac*-methadone, *rac*-EDDP, LAAM, nor- and dinor-LAAM and dextro-propoxephene (Figure 3). All inhibitors caused a significantly ($P < 0.03$) greater reduction in M6G formation compared with the inhibition in M3G formation except *rac*-EDDP ($P = 0.12$). As a consequence, all inhibitors caused a significant increase ($P < 0.01$) in the M3G/M6G formation ratio compared with the control formation ratio (Table 2).

Further investigation of methadone inhibition of morphine metabolism showed M3G and M6G formation was significantly inhibited ($P < 0.05$) by all concentrations of (R)- and (S)-methadone tested (70, 100, 200 and 500 μM). Data for both methadone enantiomers were best described by the noncompetitive model of inhibition for both M3G and M6G formation (Figure 4A,B).

Figure 1

The effect of various concentrations of alamethicin on the formation of M3G (■) and M6G (▲) from 10 mM (A) and 1 mM (B) morphine in human liver microsomes (0, 4, 8 μg alamethicin, $n = 4$ replicates; 2, 6, 12, 16, 20 μg alamethicin, $n = 2$ replicates). Data are expressed as mean $\pm$ SD

Figure 2

Eadie-Hofstee plot (A) and Michaelis-Menten plot (V vs. $[S]$ mM) (B) of M3G (■) formation from morphine. Eadie-Hofstee plot (C) and Michaelis-Menten plot (V vs. $[S]$ mM) (D) of M6G (▲) formation from morphine. All plots using HLM no. 18. Solid line represents the single enzyme Michaelis-Menten equation fitted to the data

Table 1

Kinetic parameters for M3G and M6G formation from morphine by human liver microsomes (HLM from five livers)

Kinetic parameters	no. 18	no. 21	HLM no. 34	no. 36	no. 46	Mean $\pm$ s.d.
<i>M3G formation</i>						
K_m (mM)	1.1	1.8	0.9	1.1	0.8	1.1 ± 0.4
V_{max} (nmol mg^{-1} protein h^{-1})	538	691	379	613	553	555 ± 115
Cl_{int} (μl h^{-1} mg^{-1})	489	384	421	557	691	509 ± 122
<i>M6G formation</i>						
K_m (mM)	1.2	1.5	1.2	1.1	0.6	1.1 ± 0.3
V_{max} (nmol mg^{-1} protein h^{-1})	126	123	89	135	104	115 ± 19
Cl_{int} (μl h^{-1} mg^{-1})	105	82	74	123	173	111 ± 40

V_{max} is the maximum rate of formation, K_m is the concentration of substrate at which half the V_{max} is obtained and Cl_{int} is the apparent intrinsic clearance calculated as V_{max}/K_m .

Results from these experiments resulted in a (mean $\pm$ SD) (R)-methadone K_i of 320 ± 42 μM and 192 ± 12 μM for M3G and M6G, respectively, and for (S)-methadone K_i of 226 ± 30 μM and 152 ± 20 μM for M3G and M6G, respectively. (S)-methadone had a significantly lower K_i than (R)-methadone for M6G formation ($P = 0.03$, 40;

9, 71 μM) and also had a decreased K_i for M3G formation ($P = 0.07$, 94; -22, 210 μM). In addition, K_i values for M3G inhibition were significantly greater than for M6G for both (R)-methadone ($P = 0.017$, 128; 55, 202 μM) and (S)-methadone ($P = 0.026$, 75; 22, 128 μM).

Discussion

This study aimed to investigate the *in vitro* glucuronidation of morphine and to characterize the inhibition of this glucuronidation by *rac*-methadone, its enantiomers and related phenylheptylamine opioids. The formation kinetics (K_m and V_{max}) for both M3G (1.1 mM and 555 nmol mg⁻¹ protein h⁻¹, respectively) and M6G (1.1 mM and 115 nmol mg⁻¹ protein h⁻¹, respectively) were comparable to previously reported values of 0.9–5.2 mM and 56–402 nmol mg⁻¹ protein h⁻¹ for M3G and 0.5–1.2 mM and 8–89 nmol mg⁻¹ protein h⁻¹ for M6G [11, 12, 14].

The number of UGT isoforms involved and their relative contribution to M3G and M6G formation, both *in*

vitro and *in vivo*, are still unclear. Although multiple UGT isoforms (i.e. UGT1A1, 1A3, 1A6, 1A8, 1A9, 1A10 and 2B7) have the ability to catalyse M3G formation in isolated enzyme systems, only UGT2B7 forms M6G [15–18]. The present study using morphine concentrations of 0.1–10 mM produced linear Eadie–Hofstee plots, consistent with the involvement of a single enzyme or multiple enzymes with similar affinity constants, and is in agreement with the majority of previous studies using human liver microsomes. In contrast, the only group to date to investigate morphine glucuronidation at morphine concentrations below 0.1 mM (2.5–5000 μ M) observed nonlinear Eadie–Hofstee plots for the formation of M3G in human liver microsomes and concluded that two enzymes were involved [19]. The present study was limited to concentrations above 0.1 mM as morphine concentrations below this would

Figure 3

Percentage inhibition of M3G (□) and M6G (■) formation (compared with control formation) by the individual methadone enantiomers and structurally related opioids ($n = 3$ human liver microsomes). Data are expressed as mean $\pm$ SD. * $P < 0.01$ compared with formation when no inhibitor was present (control). # $P < 0.03$ compared with inhibition of M3G formation

Table 2

Effect of methadone and structurally related opioids on the ratio of M3G formation to M6G formation from 1 mM morphine ($n = 3$ human liver microsomes)

Inhibitor (100 μ M)	M3G/M6G formation ratio (mean $\pm$ SD)
Control (MilliQ water)	4.6 $\pm$ 0.2
(S)-methadone	5.6 $\pm$ 0.2*
(R)-methadone	5.5 $\pm$ 0.2*
Racemic methadone	5.6 $\pm$ 0.2*
Racemic EDDP	5.1 $\pm$ 0.3*
LAAM	5.4 $\pm$ 0.1*
Nor-LAAM	5.2 $\pm$ 0.1*
Dinor-LAAM	5.2 $\pm$ 0.1*
Dextropropoxyphene	5.5 $\pm$ 0.3*

* $P < 0.01$ compared with no inhibitor (control).

Figure 4

Michaelis–Menten noncompetitive inhibition plot of M3G (A) and M6G (B) formation from morphine in the presence of 0 (■), 70 (▲), 100 (▼), 200 (◆), and 500 (●) μ M of inhibitor ((R)-methadone). All plots using HLM no. 36. Solid lines represent the noncompetitive inhibition Michaelis–Menten equation fitted to the data

have resulted in greater than 20% substrate consumption, thus producing inaccurate Eadie–Hofstee and Michaelis–Menten plots. Determination of enzyme involvement is further complicated by the lack of knowledge of the binding site(s) on UGT2B7, with evidence for both single-site [36] and two-site binding [17]. Multiple binding sites within the one enzyme may result in atypical kinetics normally observed with multiple enzyme activity.

Isolated enzyme studies using expressed UGT2B7 systems [17, 18] produce M3G and M6G at a similar ratio to that seen in the current study using human liver microsomes and thus it is likely that UGT2B7 is largely responsible for morphine metabolism *in vitro*. The formation ratio of the glucuronide metabolites in our study is also highly similar to that seen after i.v. morphine administration to subjects receiving dialysis for kidney failure, who therefore have minimal glucuronide removal, thus eliminating changes in ratio due to differences in M3G and M6G renal clearance [37]. We therefore postulate that UGT2B7 is the predominant enzyme involved in both *in vitro* and *in vivo* morphine glucuronidation and conclude that *in vitro* human liver microsome studies afford a good representation of *in vivo* morphine metabolism in humans.

Racemic methadone, its individual enantiomers and several structurally related compounds significantly inhibited the formation of both M3G and M6G. In addition, further investigation showed that both (R)- and (S)-methadone noncompetitively inhibited the formation of both M3G and M6G. Noncompetitive inhibition of morphine metabolism by nortriptyline has previously been demonstrated [38]. However, this is the first time methadone has been shown to inhibit glucuronidation noncompetitively. (S)-methadone inhibition was shown to be statistically significantly greater than that of the (R)-enantiomer; however, the magnitude of the difference was minor (<30% difference in K_i).

Methadone, although not a substrate for glucuronidation itself, inhibits the *in vitro* glucuronidation of AZT [28], which is also a substrate for UGT2B7 [39]. These *in vitro* data are supported by increased plasma AZT concentrations in methadone-maintained subjects [29, 30]. These studies provide further evidence that methadone inhibited UGT2B7 activity in the current study, resulting in decreased M3G and M6G formation, and may explain the observation by Doverty and coworkers [24] of increased morphine plasma concentrations in methadone-maintained subjects, compared with control subjects, when administered the same i.v. dose. Preliminary pharmacokinetic analysis of these *in vivo* data by our laboratory has confirmed decreased morphine clearance in these subjects (unpublished).

It is of interest that cytochrome P450 2D6, although not a major enzyme involved in methadone metabolism, is also inhibited by methadone both *in vitro* [40] and *in vivo* [41]. Together, these findings are of note, as such metabolic inhibition interactions are not immediately apparent from current knowledge of methadone metabolism pathways.

In the current investigation, we also observed a significantly lower K_i value for the formation of M6G compared with M3G, resulting in greater inhibition of M6G formation and a significantly increased M3G/M6G formation rate ratio. These data suggest that M3G and M6G are formed from morphine by either a single enzyme with multiple binding sites or multiple enzyme isoforms with similar K_m values. Furthermore, these binding sites/isoforms are inhibited by methadone to different extents. Alteration of the M3G/M6G formation ratio has previously been observed after the inhibition of morphine glucuronidation by various drugs and metal ions in human liver microsome systems [38, 42]. We have also observed an increase in the M3G/M6G ratio in methadone-maintained subjects administered morphine compared with non-opioid-dependent controls [43], suggesting that methadone differentially inhibits morphine glucuronidation to M3G and M6G *in vivo* in a stereoselective manner.

The extrapolation of these *in vitro* data to a numerical estimate of effect *in vivo* is difficult. *In vitro*–*in vivo* correlations for drugs that undergo glucuronidation have not yet been performed with much success, with data from *in vitro* morphine glucuronidation studies consistently underpredicting *in vivo* clearance [11, 44, 45]. Drugs that competitively inhibit CYP3A enzymes *in vivo* generally have K_i values equal to or less than their clinical plasma concentrations [46]. Methadone therapeutic plasma concentrations are commonly between 50 and 600 ng ml⁻¹ (0.16 and 1.92 μ M) [47], with apparent methadone K_i values reported in the present study being considerably larger. However, the present study has demonstrated noncompetitive inhibition by methadone. Therefore, *in vivo* inhibition will be dependent on the affinity with which methadone binds to the enzyme and also on the extent of accumulation of methadone at this site after chronic administration. Thus, plasma methadone concentration is less important in determining inhibition of morphine clearance compared with competitive inhibition. The apparent K_i values reported here may also be an overestimation of actual K_i values due to possible nonspecific binding of methadone to the microsomes, as is common for other basic amine drugs [48]. Also, *in vivo* methadone concentrations in the liver are likely to be larger than plasma concentrations, as

methadone is highly accumulated in this tissue. This is supported by post mortem investigation of methadone maintenance subjects by Drummer and coworkers [49], who reported liver to blood *rac*-methadone ratios ranging between 3.2 and 61 : 1.

Morphine displays high hepatic extraction and thus hepatic clearance is mainly limited by hepatic blood flow. Mazoit *et al.* [50] have reported a morphine extraction ratio of 0.65 and total morphine clearance values that exceed hepatic blood flow, indicating extrahepatic morphine metabolism (e.g. kidney and brain). Although not explored in this study, it is possible that methadone will also inhibit glucuronide formation at extrahepatic sites.

The clinical significance of the inhibition of morphine glucuronidation in the treatment of pain remains complex. Inhibition of morphine clearance would result in increased plasma morphine concentrations, which would be expected to increase analgesia. However, M6G is a more potent μ opioid agonist [1] and a number of studies have shown its contribution to analgesia, particularly after a high dose or during chronic morphine therapy [2, 3, 51, 52]. In contrast, several animal studies have shown that M3G attenuates the opioid effects of morphine [5, 6, 53, 54], although this has yet to be well demonstrated in humans. Thus, decreased M6G formation combined with an increase in the M3G/M6G ratio may result in decreased analgesia and an increase in excitatory effects. In addition, it should not be overlooked that methadone, and structurally related compounds, may also reduce the clearance of other drugs that undergo glucuronidation by UGT2B7, leading to toxicity or reduced clinical efficacy of these drugs if the conjugates are active.

In conclusion, this study has shown that both enantiomers of methadone noncompetitively inhibit the formation of M3G and M6G from morphine *in vitro*. We provide data that indicate other drugs structurally related to methadone also inhibit morphine glucuronidation to a similar extent. These findings may explain the previously observed increases in plasma morphine concentrations in methadone-maintained subjects [24]. As UGT2B7 is the only UDP-glucuronosyltransferase to catalyse the formation of both metabolites, these results suggest that methadone may inhibit this UGT isoform, in agreement with observations of methadone inhibition of AZT metabolism *in vitro* and *in vivo*. Further investigations showed that inhibition of morphine glucuronidation mediated by methadone resulted in greater inhibition of M6G formation compared with M3G and an increase in the M3G/M6G formation ratio. Future research should investigate the inhibition of glucuronide

formation in recombinant expressed UGT isoforms and in individuals receiving methadone and structurally related compounds to determine the effect of this inhibition on the pharmacokinetics and therapeutic response of drugs that undergo glucuronidation.

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