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Short Communication

INHIBITION OF UDP-GLUCURONOSYLTRANSFERASE 2B7-CATALYZED MORPHINE GLUCURONIDATION BY KETOCONAZOLE: DUAL MECHANISMS INVOLVING A NOVEL NON-COMPETITIVE MODE

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Running title: Inhibition of UGT2B7 by Ketoconazole

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ABBREVIATIONS: P450, cytochrome P450; UGT, UDP-glucuronosyltransferase;
UDPGA, UDP-glucuronic acid; M-3-G, morphine-3-glucuronide; M-6-G,
morphine-6-glucuronide; ER, endoplasmic reticulum; DMSO, dimethyl sulfoxide.

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ABSTRACT

Glucuronidation of morphine in humans is predominantly catalyzed by UDP-glucuronosyltransferase 2B7 (UGT2B7). Since our recent research suggested that cytochrome P450s (P450s) interact with UGT2B7 to affect its function [Takeda S. *et al.* (2005) *Mol Pharmacol* **67**:665-672], P450 inhibitors are expected to modulate UGT2B7-catalyzed activity. To address this issue, we investigated the effects of P450 inhibitors (cimetidine, sulfaphenazole, erythromycin, nifedipine, and ketoconazole) on the UGT2B7-catalyzed formation of morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G). Among the inhibitors tested, ketoconazole was the most potent inhibitor of both M-3-G and M-6-G formation by human liver microsomes. The others were less effective except that nifedipine exhibited an inhibitory effect on M-6-G formation comparable to ketoconazole. Neither addition of NADPH nor solubilization of liver microsomes affected the ability of ketoconazole to inhibit morphine glucuronidation. In addition, ketoconazole had an ability to inhibit morphine UGT activity of recombinant UGT2B7 freed from P450. Kinetic analysis suggested that the ketoconazole-produced inhibition of morphine glucuronidation involves a mixed-mechanism. Codeine potentiated inhibition of morphine glucuronidation by ketoconazole. On the contrary, addition of another substrate, testosterone, showed no or a minor effect on ketoconazole-produced inhibition of morphine UGT. These results suggest that 1) metabolism of ketoconazole by P450 is not required for inhibition of UGT2B7-catalyzed morphine glucuronidation; and 2) this drug exerts its inhibitory effect on morphine UGT by novel mechanisms involving competitive and non-competitive inhibition.

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Introduction

A number of endogenous and exogenous compounds are biotransformed by Phase I and Phase II drug-metabolizing enzymes (Oguri et al., 1994; Radomska-Pandya et al., 1999; Ritter, 2000). The cytochrome P450 (P450) and UDP-glucuronosyltransferase (UGT) families are two major enzyme groups responsible for Phase I and II reactions. UGT-catalyzed glucuronidation is assumed to play a role in up to 35% of phase II reactions. UGT is anchored to the endoplasmic reticulum (ER) membrane by the transmembrane domain, and the main body of the enzyme, including the catalytic site, is believed to be present on the luminal side of the ER. This model of type-I topology causes “latency”, a characteristic feature of UGT, whereby UGT activity is activated by disruption of the microsomal membranes with detergent. Among the UGTs identified in humans, UGT2B7 is of particular interest because it is a major isoform involved in morphine glucuronidation (Coffman et al., 1997). To date, UGT2B7 is the sole enzyme reported capable of forming morphine-6-glucuronide (M-6-G) (Coffman et al., 1997), a metabolite having more potent analgesic activity than morphine. Although increasing evidence suggests that there are a number of factors that affect morphine UGT activity (Narayan et al., 1991; Ishii et al., 2001; Antonilli et al., 2003; Ishii et al., 2004; Ishii et al., 2005; Takeda et al., 2005a; Takeda et al., 2005b), little is known about inhibitors of UGT2B7.

Experimental evidence in humans indicates that the glucuronidation of acetaminophen, codeine, zidovudine, carbamazepine, lorazepam, and propafenone is influenced by concomitantly administered drugs (Kiang et al., 2005). However, the glucuronidation of morphine is not affected by any of the drugs examined so far (Kiang

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et al., 2005). While diclofenac is a potent inhibitor of codeine glucuronidation *in vitro* ($K_i = 7.9 \mu\text{M}$, Ammon et al., 2000), it has only a low ability to inhibit the glucuronodation of morphine by human liver microsomes ($IC_{50} = \text{about } 500 \mu\text{M}$, King et al., 2001). On the other hand, using human liver microsomes, it has been reported that ketoconazole, a P450 inhibitor (Wrighton and Ring; 1994), competitively inhibits the glucuronidation of the UGT2B7 substrate zidovudine with a K_i of $80 \mu\text{M}$ (Sampol et al., 1995). A recent study by Yong et al., also using human liver microsomes, showed that ketoconazole inhibited UGT1A-mediated glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38) and cDNA-expressed UGT1A isoforms (Yong et al., 2005). Since glucuronidation of zidovudine is catalyzed by human liver microsomal UGT (Sampol et al., 1995), it is conceivable that UGT2B7 is inhibited by ketoconazole. However, there are no data which demonstrates the direct inhibition of UGT2B7 by ketoconazole.

Since P450s are suggested to interact with UGT including UGT2B7 to modulate its function (Taura et al., 2000; Fremont et al., 2005; Ishii et al., 2005; Takeda et al., 2005a; Takeda et al., 2005b), it is conceivable that P450 inhibitors modulate UGT2B7-catalyzed reactions. To examine this possibility, we carried out a comparative study to clarify whether P450 inhibitors are able to inhibit morphine UGT activity catalyzed by hepatic microsomes and recombinant UGT2B7. The results indicated that among the inhibitors tested ketoconazole is the strongest inhibitor for UGT2B7-mediated morphine glucuronidation.

Materials and Methods

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Chemicals and enzyme sources. UDP-glucuronic acid (UDPGA, trisodium salt) was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Morphine hydrochloride and codeine phosphate were obtained from Takeda Chemical Ind. Co., Ltd. (Osaka, Japan). NADPH, ketoconazole, sulfaphenazole, egg yolk L- α -phosphatidylcholine, saccharolactone, and bovine serum albumin (BSA)(fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G) were synthesized in our laboratory (Yoshimura et al., 1968). Cimetidine, erythromycin, and nifedipine were purchased from Nacalai Tesque Co. (Kyoto, Japan). Pooled human liver microsomal samples were obtained together with agreement for their use from donors from Gentest (BD Science, Woburn, MA, USA), and stored at -8°C until use. Recombinant UGT2B7 microsomes (SupersomesTM) were obtained from Gentest, BD Science, Woburn, MA, USA. Protein assay reagents were provided by Bio-Rad (Hercules, CA, USA). All other reagents were of analytical grade and used without further purification.

Glucuronidation assay. Morphine glucuronidation activity was determined by the method described elsewhere (Takeda et al., 2005a). Briefly, the assay mixture consisted of 50 mM Tris-HCl buffer (pH 7.8), 10 mM MgCl_2 , 0.45 mg protein/ml human liver microsomes or recombinant UGT2B7 microsomes, 2 mM UDPGA, 5 mM saccharolactone and morphine in a final volume of 300 μl . Morphine was dissolved in H_2O and added to the incubation mixture at the desired concentrations. Sonicated L- α -phosphatidylcholine, at a concentration of 0.5 mg/ml, was included in all assays. Solubilization of the microsomes with sodium cholate was performed by the methods

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described previously (Taura et al., 2000; Takeda et al., 2005a). In a separate experiment, 1 mM NADPH was added to above reaction mixture. The reaction at 37°C was started by adding ice-cold microsomes, stopped by the addition of 100 μ l ice-cold 1 M trichloroacetic acid, and then the mixture was chilled on ice and centrifuged. An aliquot of the supernatant was analyzed by high-performance liquid chromatography (Ishii et al., 2001). Formation of M-3-G and M-6-G in relation to time and protein concentration was linear under the conditions used. No production of M-3-G and M-6-G was observed in blank experiments which were performed in the absence of UDPGA.

Inhibition studies. Prior to the inhibition studies, the apparent Michaelis-Menten values (K_m) of M-3-G and M-6-G formation were determined using human liver microsomes and cDNA-expressed UGT2B7 microsomes as the enzyme source. The activity was measured at eight morphine concentrations ranging from 0.1 to 5 mM. The apparent K_m of morphine glucuronidation was determined by the Michaelis-Menten equation by the nonlinear regression method using SigmaPlot software (SPSS Inc., Chicago, IL). Inhibition of human liver microsomal UGT-catalyzed morphine glucuronidation by inhibitors was examined at a morphine concentration of 3.8 mM. The inhibitory effects on morphine glucuronidation of cimetidine, sulphaphenazole, erythromycin, nifedipine, and ketoconazole were investigated at concentrations of 6.25, 12.5, 25, 50, 100, and 250 μ M. Cimetidine and codeine was dissolved in H₂O. The others drugs were dissolved in dimethyl sulfoxide (DMSO) the concentration of which did not exceed 0.5% (v/v) in the incubation mixture. The reference incubation contained the same volume of solvent. It was confirmed that

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the 0.5% DMSO had little effect on morphine glucuronidation (data not shown).

Control incubations were carried out by omitting substrate.

Data analysis. The concentration of the inhibitor that is required to produce 50% inhibition of the enzymatic activity (IC_{50}) was determined from the curves plotting enzymatic activity versus inhibitor concentrations. The inhibition mechanism was estimated from the figure plotting $1/V$ versus $1/S$ (Segel, 1993). The apparent inhibitory constant (K_i) was determined from the x-intercept of a replot of the mean slopes of the double reciprocal plot versus inhibitor concentration. The K_i and the mode of inhibition were determined by fitting the kinetic data to a mixed inhibition model using nonlinear regression analysis using the SigmaPlot enzyme kinetics module. The mode of inhibition that best described the data was determined by comparing r^2 values for the fit of the various models. The K_i/K_m ratio was used to compare the relative inhibition potential by ketoconazole for UGT1A1/9 or UGT2B7-mediated reactions (Murray and Butler, 1996). Differences were considered significant when p value was calculated to be less than 0.05. All statistical analyses were performed by Scheffe's F test which is a type of posthoc test for analyzing results of ANOVA testing. These calculations were done using Satview5.0J software (SAS Institute Inc., USA).

Results

Effects of P450 inhibitors on morphine glucuronidation. The inhibitory effect of five P450 inhibitors, cimetidine, sulfaphenazole, erythromycin, nifedipine and

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ketoconazole, on the activity of morphine UGT was examined using human liver microsomes as the enzyme source. From kinetic analyses in the absence of inhibitor, the K_m values for M-3-G and M-6-G formation were determined to be 2.66 ± 0.32 and 2.62 ± 0.46 mM, respectively. The apparent K_m values for morphine glucuronidation were similar between the human liver microsomes and cDNA-expressed UGT2B7 microsomes (about 3 mM). Although M-3-G formation can be mediated by UGT isoforms other than UGT2B7 at high morphine concentrations ($5 \text{ mM} <$)(Court et al., 2003), the pooled human liver microsomes used in this study exhibited apparent monophasic Michaelis-Menten kinetics. The monophasic nature of M-3-G formation is reasonable because morphine is a highly specific substrate of UGT2B7 and is poorly glucuronidated by other UGTs (Coffman et al., 1997; Court et al., 2003; Stone et al., 2003). These results were consistent with the reports by other workers (Fisher et al., 2000; Court et al., 2003). The effects of the five drugs on the activity of morphine glucuronidation by human liver microsomes were determined at a morphine concentration around the K_m . Ketoconazole inhibited both M-3-G and M-6-G formation, with apparent IC_{50} s of 196 and 168 μM , respectively (Fig. 1). However, cimetidine, sulfaphenazole, and erythromycin did not exhibit any inhibitory effect on morphine glucuronidation (data not shown). Although nifedipine produced a reduction in M-6-G formation (IC_{50} : 186 μM), the IC_{50} value for M-3-G formation could not be determined because of the weak inhibition (Fig. 1). It should be noted that there have been no reports describing the glucuronidation of ketoconazole and nifedipine by UGTs including UGT2B7. Fig. 2A illustrates the Dixon plots for the inhibition of M-3-G formation by ketoconazole. The Dixon plot was characterized by straight lines at

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different fixed substrate concentrations intersecting in the second quadrant (Fig. 2A). The Dixon plot was not able to distinguish between the inhibition-types. A replot of the slopes produced a straight line not going through the origin, suggesting a “linear mixed-type” mode of inhibition (Fig. 2C)(Segel, 1993). Double reciprocal plots of the substrate concentration data ($1/V$ vs $1/S$) in the presence of fixed concentrations of ketoconazole showed that both the slopes and the y-intercepts increased along with increasing inhibitor concentrations, consistent with a “mixed-type” mechanism and this was also found for the M-6-G inhibition (data not shown)(Segel, 1993). The K_i of ketoconazole for M-3-G formation was calculated to be $118 \pm 27 \mu\text{M}$. In the case of the M-6-G formation catalyzed by human liver microsomal UGT, ketoconazole also exhibited a “linear mixed-type” inhibition (Figs. 2B and 2D) with an apparent K_i of $108 \pm 35 \mu\text{M}$.

Effect of NADPH on the Inhibition of morphine glucuronidation by

ketoconazole. It has been suggested that the inhibition of CYP2D6 by cimetidine is likely due to a metabolite(s) rather than the parent compound (Madeira et al., 2004). Similarly, anti-fungal compounds are suggested to exert their inhibitory effects on P450s through oxidative metabolism by P450 (Isoherranen et al., 2004). To examine the hypothesis that the potency of morphine UGT inhibitors is also modified by oxidative metabolism, we examined the inhibitory effects of cimetidine and ketoconazole in the presence of NADPH, an obligate co-factor of P450. The results showed that cimetidine does not exhibit any inhibitory effect on the activity of hepatic microsomal morphine UGT, even in the presence of NADPH (Fig. 3A). Similarly, NADPH did not influence the inhibitory effect of ketoconazole (Fig. 3A). This result

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does not support the possibility that ketoconazole requires oxidative metabolism to inhibit morphine glucuronidation by human liver microsomes.

Inhibition by ketoconazole of morphine glucuronidation catalyzed by solubilized microsomes and recombinant UGT. To examine the possibility that the condition of the microsomal membrane may affect the inhibition of morphine glucuronidation, an inhibition study was performed for cimetidine and ketoconazole using solubilized microsomes. The results (Fig. 3B) showed that ketoconazole inhibits the M-3-G and M-6-G formation catalyzed by solubilized microsomes in a concentration-dependent manner with IC_{50} values comparable to those obtained after incubation with intact microsomes (Fig. 1). Cimetidine had hardly any effect on the activity obtained with both microsomal preparations (data not shown). When recombinant UGT2B7 expressed in insect cells was used as the enzyme, ketoconazole (100 μ M) significantly reduced morphine glucuronidation at both the 3- and 6-positions (Fig. 3C). This observation, together with the data showing that NADPH failed to affect the inhibitory effect of ketoconazole (Fig. 3), suggested that ketoconazole directly inhibits UGT2B7-catalyzed morphine glucuronidation without requiring pre-activation by P450.

Effect of codeine and testosterone on the inhibition by ketoconazole of morphine glucuronidation. As shown in Fig. 2, the inhibition mode of ketoconazole for M-3-G and M-6-G formation was suggested to be a “mixed-type” consisting of competitive and noncompetitive inhibition. Since codeine is a substrate for UGT2B7 (Coffman et al., 1997), it is reasonable to expect that the competitive phase in ketoconazole-produced inhibition of morphine glucuronidation should be additively

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affected by codeine. To assess this possibility, codeine was added to the incubation mixture containing pooled human liver microsomes, ketoconazole, UDPGA, and morphine. When codeine was added to the incubation mixture not containing ketoconazole, higher concentrations over 2.5 mM of this opioid were needed for the inhibition both of M-3-G and M-6-G formation (Fig. 4A and 4B). Codeine at lower concentrations below 1.0 mM did not exert any inhibitory effect (Fig. 4A and 4B). However, in the presence of ketoconazole, codeine reduced the activities of M3-G and M-6-G formation more markedly than did the opioid alone. Such effect of codeine was observed even at the least concentration (0.25 mM). Similarly, the degree of the inhibition with ketoconazole plus Codeine was significantly greater than that of ketoconazole alone (Fig. 4A and 4B). Combination of ketoconazole and codeine seemed to produce a inhibitory effect which was synergic rather than additive. On the contrary, testosterone, another UGT2B7 substrate, exhibited a quite different effect from codeine on ketoconazole inhibition. Although testosterone itself inhibited morphine glucuronidation, this compound hardly exhibited any additive and/or synergic effect on ketoconazole-produced inhibition (Fig. 4C and 4D). As far as M-6-G formation was concerned, testosterone significantly weakened the inhibitory effect of ketoconazole (Fig. 4A and 4B). Taken together, it is suggested that ketoconazole affects not only morphine binding site in UGT2B7 but also other possible effector site(s).

Discussion

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Several drugs have been reported thus far as inhibitors of UGT-mediated glucuronidation (Kiang et al., 2005). However, to our best knowledge, UGT-selective inhibitors have not been known. For example, diclofenac is a potent UGT inhibitor, but the effect is not selective to this enzyme (Kiang et al., 2005). Since CYP3A4 and other forms of P450 interact with UGT2B7 to modulate the function (Taura et al., 2000; Fremont et al., 2005; Ishii et al., 2005; Takeda et al., 2005a; Takeda et al., 2005b), it seemed worthwhile to investigate if the inhibition of P450 function gives rise to a reduction in morphine glucuronidation. This was examined in this study, using P450 inhibitors including ketoconazole capable of inhibiting CYP3A4 and other forms of P450 (Ong et al., 2000). However, the above hypothesis was not supported by observations that ketoconazole inhibits morphine glucuronidation catalyzed by a UGT2B7 preparation not containing P450 (Fig. 3C) and NADPH does not cause any modification toward the ketoconazole inhibition of morphine glucuronidation by hepatic microsomes (Fig. 3A). However, NADPH itself produced a minor, but significant, increase in M-3-G formation by human liver microsomes. The mechanism whereby NADPH stimulates UGT function remains to be determined.

Biphasic kinetics of M-3-G formation by human liver microsomes and/or recombinant UGT2B7 has been reported (Miners et al., 1988; Stone et al., 2003), although neither our experiments nor those of other workers observed the same form of kinetics (Fisher et al., 2000; Court et al., 2003; Takeda et al., 2005a). The K_m values of the low- and high-affinity phases in the kinetics of M-3-G formation have been reported to be 2.5 μM and 798 μM , respectively (Miners et al., 1988). If the kinetics of UGT2B7-mediated glucuronidation is biphasic, the inhibition by ketoconazole of

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UGT2B7 function observed in our study may only affect the low affinity-phase reaction.

Inhibitory effects of ketoconazole on UGT function that have been reported so far are summarized in Table 1. On the basis of data shown in this article, the mode of inhibition of M-3-G and M-6-G formation by ketoconazole is suggested to be a mixed-type (Fig. 2 and computer fitting program, Table 1). Since ketoconazole is a CYP3A4 inhibitor and morphine is a substrate both of CYP3A4 (Projean et al., 2003) and UGT2B7, it is conceivable that ketoconazole shares structural similarities with morphine and functions as a competitive inhibitor for morphine glucuronidation. If ketoconazole only affects substrate binding to the catalytic site, addition of codeine which is a UGT2B7 substrate would result in additive inhibitory effects. However, although codeine itself had no effect on morphine UGT activity at 0.25-1.0 mM concentrations, the same concentrations of codeine potentiated ketoconazole-produced inhibition. Thus, codeine is suggested to modify enzyme structure to facilitate the inhibitory effect by ketoconazole. Similarly, binding of testosterone may alter UGT structure so that the inhibitory effect of ketoconazole is decreased somehow. It has been suggested that UGT2B7 has an opioid binding site in its N-terminal domain (Coffman et al., 2001). In addition, a possible steroid binding site has been proposed in UGT2B7 (Coffman et al., 2001). Taken together, it is suggested that ketoconazole binds to a site(s) distinct from opioid-and testosterone-binding sites in the UGT2B7. If this is true, the following may be the reason why codeine synergistically enhances the inhibitory effect of ketoconazole: that is, ketoconazole may bind to its specific site to modulate efficacy and/or specificity in the interaction of opioid and its association site. Ketoconazole is suggested to be a competitive inhibitor

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of zidovudine UGT in human liver microsomes (Sampol et al., 1995, Table 1). Since zidovudine glucuronidation is catalyzed by UGT2B7 (Court et al., 2003), it is reasonable to consider that ketoconazole also directly inhibits UGT2B7-catalyzed zidovudine glucuronidation. Although ketoconazole is not a UGT substrate (Yong et al., 2005), this drug appears to bind to the substrate binding site of UGT2B7. This assumption is supported by our present data together with the above information showing that ketoconazole inhibits UGT2B7 in a competitive manner. Further studies are necessary to clarify the clinical impact and significance of the ketoconazole-produced inhibition of UGT.

The K_i values of ketoconazole for M-3-G and M-6-G formation were far greater than the maximal serum concentrations (7.9–11.7 μM , 1-2 h) of this anti-fungal agent given to patients at a dose of 200 mg /man/day (Huang et al., 1986). However, several lines of investigation described below seem to support the *in vivo* inhibitory effect of ketoconazole. Firstly, the K_i values of ketoconazole for the inhibition of M-3-G and M-6-G formation were close to the K_i (80 μM) seen for the inhibition of zidovudine glucuronidation (Sampol et al., 1995). In addition, fluconazole, another azole drug, is known to affect the bioavailability of zidovudine *in vivo* (Kiang et al., 2005). Secondly, azole anti-fungal agents including ketoconazole are effectively taken up from plasma into liver cells, and this leads to an increase in the hepatic concentration of the inhibitor to a level which is much higher than the plasma concentrations (von Moltke et al., 1998). This observation supports the view that ketoconazole inhibits UGT activity *in vivo* although its plasma concentration is lower than the K_i . In support of this, it has been reported that ketoconazole significantly increase the *in vivo* level of active

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metabolite (SN-38) of irinotecan, an anti-cancer drug (Yong et al., 2005). When K_i/K_m ratio is used as index (Murray and Butler, 1996), ketoconazole seems to be a more potent inhibitor for morphine glucuronidation than SN-38-glucuronidation (Table 1). Further studies are necessary to clarify the clinical impact (and significance) by ketoconazole.

Human hepatic UGT1A1 and 1A3 also catalyze morphine-3-glucuronidation, although their activities are lower than UGT2B7 (Stone et al., 2003). Therefore, it is reasonable to assume that the estimated K_i of ketoconazole with regard to morphine UGT activity by human liver microsomes is a combination of K_i values toward different UGTs. Therefore, the combined K_i should vary as a function of the molar ratio of the UGT1A1, 1A3 and 2B7 expressed in the liver. However, due to the lower expression of UGT1A3 mRNA in the liver (5–20-fold less than other UGT1As; Mojarrabi et al., 1996), this UGT would not substantially contribute to the glucuronidation of morphine. The contribution of UGT1A1 to M-3-G formation is also less expected (Stone et al., 2003). It has been reported that while neither UGT1A1 nor UGT1A3 catalyze M-6-G formation (Stone et al., 2003), UGT2B7 catalyzes this reaction (Coffman et al., 1997). Thus, it is suggested that the K_i of ketoconazole for M-6-G formation reflects the effect on the UGT2B7-catalyzed reaction.

It has been reported that estradiol-17-glucuronidation by human liver UGT is modulated by polymethoxyflavonoides, nobiletin and tangeretin (Williams et al., 2002). In this report, enzyme activities were used as a marker for UGT2B7. However, nobiletin and tangeretin (100 μ M) had no effect on the activity of human microsomal morphine-UGT (data not shown). Thus, it seems that the inhibitory effect of

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ketoconazole to UGT2B7 is relatively specific for morphine glucuronidation. In this study, although we focused on the inhibitory effect of P450 inhibitor toward morphine-UGT (UGT2B7), the activity of human liver microsomal morphine-UGT was a little but significantly stimulated by erythromycin and sulfaphenazole (data not shown). It has been suggested that estradiol-3-glucuronidation activity by human liver UGT1A1 is stimulated by the substrate of this UGT (Williams et al., 2002). Thus, it is likely that the interaction of P450 substrates and UGTs including UGT2B7 is complex.

Taking all these findings into consideration, we have shown that ketoconazole is a potent inhibitor of UGT2B7-catalyzed morphine glucuronidation *in vitro* as well as of P450-catalyzed oxidation. Our findings also suggest that further studies are needed to identify potential drug–drug interactions based on the inhibition and stimulation of UGT.

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REFERENCES

- Ammon S, von Richter O, Hofmann U, Thon K-P, Eichelbaum M, and Mikus G (2000)
In vitro interaction of codeine and diclofenac. *Drug Metab Dispos* **28**: 1149–1152.
- Antonilli L, Suriano C, Paolone G, Badiani A, and Nencini P (2003) Repeated
exposures to heroin and/or cadmium alter the rate of formation of morphine
glucuronides in the rat. *J Pharmacol Exp Ther* **307**:651–660.
- Coffman BL, Rios GR, King CD, and Tephly TR (1997) Human UGT2B7 catalyzes
morphine glucuronidation. *Drug Metab Dispos* **25**:1–4.
- Coffman BL, Kearney WR, Green MD, Lowery RG, and Tephly TR (2001) Analysis of
opioid binding to UDP-glucuronosyltransferase 2B7 fusion proteins using nuclear
magnetic resonance spectroscopy. *Mol Pharmacol* **59**:1464–1469.
- Court MH, Krishnaswamy S, Hao Q, Duan SX, Patten CJ, Von Moltke LL, and
Greenblatt DJ (2003) Evaluation of 3'-azido-3'-deoxythymidine, morphine, and
codeine as probe substrates for UDP-glucuronosyltransferase 2B7 (UGT2B7) in
human liver microsomes: specificity and influence of the UGT2B7*2 polymorphism.
Drug Metab Dispos **31**:1125–1133.
- Fisher MB, Campanale K, Ackermann BL, VandenBranden M, and Wrighton SA
(2000) In vitro glucuronidation using human liver microsomes and the
pore-forming peptide alamethicin. *Drug Metab Dispos* **28**:560–566.
- Fremont JJ, Wang RW, and King CD (2005) Co-immunoprecipitation of

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UDP-glucuronosyltransferase (UGT) isoforms and cytochrome P450 3A4. *Mol Pharmacol* **67**:260–262.

Huang YC, Colaizzi JL, Bierman RH, Woestenborghs R, and Heykants J (1986) Pharmacokinetics and dose proportionality of ketoconazole in normal volunteers. *Antimicrob Agents Chemother* **30**:206–210.

Ishii Y, Miyoshi A, Watanabe R, Tsuruda K, Tsuda M, Yamaguchi-Nagamatsu Y, Yoshisue K, Tanaka M, Maji D, Ohgiya S, and Oguri K (2001) Simultaneous expression of guinea pig UDP-glucuronosyltransferase 2B21 and 2B22 in COS-7 cells enhances UDP-glucuronosyltransferase 2B21-catalyzed morphine-6-glucuronide formation. *Mol Pharmacol* **60**:1040–1048.

Ishii Y, Miyoshi A, Maji D, Yamada H, and Oguri K (2004) Simultaneous expression of Guinea pig UDP-glucuronosyltransferase 2B21 (UGT2B21) and 2B22 in COS-7 cells enhances UGT2B21-catalyzed chloramphenicol glucuronidation. *Drug Metab Dispos* **32**:1057–1060.

Ishii Y, Takeda S, Yamada H, and Oguri K (2005) Functional protein-protein interaction of drug metabolizing enzymes. *Front Biosci* **10**:887–895.

Isoherranen N, Kunze KL, Allen KE, Nelson WL, and Thummel KE (2004) Role of itraconazole metabolites in CYP3A4 inhibition. *Drug Metab Dispos* **32**:1121–1131.

Kiang TK, Ensom MH, and Chang TK (2005) UDP-glucuronosyltransferases and clinical drug-drug interactions. *Pharmacol Ther* **106**:97–132.

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- King C, Tang W, Ngui J, Tephly T, and Braun M (2001) Characterization of rat and human UDP-glucuronosyltransferases responsible for the in vitro glucuronidation of diclofenac. *Toxicol Sci* **61**:49–53.
- Madeira, M., Levine, M., Chang, T. K. H., Mirfazaelian, A., and Bellward, G. D. (2004) The effect of cimetidine on dextromethorphan O-demethylase activity of human liver microsomes and recombinant CYP2D6. *Drug Metab Dispos* **32**:460–467.
- Miners JO, Lillywhite KJ, and Birkett DJ (1988) In vitro evidence for the involvement of at least two forms of human liver UDP-glucuronosyltransferase in morphine-3-glucuronidation. *Biochem Pharmacol* **37**:2839–2845.
- Mojarrabi B, Butler R, and Mackenzie PI (1996) cDNA cloning and characterization of the human UDP glucuronosyltransferase, UGT1A3. *Biochem Biophys Res Commun* **225**:785–790.
- Murray M and Butler AM (1996) Enhanced inhibition of microsomal cytochrome P450 3A2 in rat liver during diltiazem biotransformation. *J Pharmacol Exp Ther* **279**:1447–1452.
- Narayan SS, Hayton WL, and Yost GS (1991) Chronic ethanol consumption causes increased glucuronidation of morphine in rabbits. *Xenobiotica* **21**: 515–524.
- Oguri K, Yamada H, and Yoshimura H (1994) Regiochemistry of cytochrome P450 isozymes. *Annu Rev Pharmacol Toxicol* **34**:251–79.
- Ong, C. E., Coulter, S., Birkett, D. J., Bhasker, C. R., and Miners, J. O. (2000) The

DMD#9738

xenobiotic inhibitor profile of cytochrome P4502C8. *Br J Clin Pharmacol*

50:573–580.

Projean, D., Morin, P. E., Tu, T. M., and Ducharme, J. (2003) Identification of CYP3A4 and CYP2C8 as the major cytochrome P450s responsible for morphine N-demethylation in human liver microsomes. *Xenobiotica* **33**: 841–854.

Radomska-Pandya A, Czernik PJ, Little JM, Battaglia E, and Mackenzie PI (1999) Structural and functional studies of UDP-glucuronosyltransferases. *Drug Metab Rev* **31**:817–899.

Ritter JK (2000) Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. *Chem-Biol Interact* **129**:171–193.

Sampol E, Lacarelle B, Rajaonarison JF, Catalin J, and Durand A (1995) Comparative effects of antifungal agents on zidovudine glucuronidation by human liver microsomes. *Br J Clin Pharmacol* **40**:83–86.

Segel IH (1993) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. John Wiley and Sons Inc., New York.

Stone AN, Mackenzie PI, Galetin A, Houston JB, Miners JO (2003) Isoform selectivity and kinetics of morphine 3- and 6-glucuronidation by human UDP-glucuronosyltransferases: evidence for atypical glucuronidation kinetics by UGT2B7. *Drug Metab Dispos* **31**:1086–1089. Erratum in: *Drug Metab Dispos* (2003) **31**:1541.

DMD#9738

Takeda S, Ishii Y, Iwanaga M, Mackenzie PI, Nagata K, Yamazoe Y, Oguri K, and

Yamada H (2005a) Modulation of UDP-glucuronosyltransferase function by cytochrome P450: evidence for the alteration of UGT2B7-catalyzed glucuronidation of morphine by CYP3A4. *Mol Pharmacol* **67**:665–672.

Takeda S, Ishii Y, Mackenzie PI, Nagata K, Yamazoe Y, Oguri K, and Yamada H

(2005b) Modulation of UDP-glucuronosyltransferase 2B7 function by cytochrome P450s In vitro: differential effects of CYP1A2, CYP2C9 and CYP3A4. *Biol Pharm Bull* **28**:2026–2027.

Taura K, Yamada H, Hagino Y, Ishii Y, Mori M, and Oguri K (2000) Interaction

between cytochrome P450 and other drug-metabolizing enzymes: evidence for an association of CYP1A1 with microsomal epoxide hydrolase and UDP-glucuronosyltransferase. *Biochem Biophys Res Commun* **273**:1048–1052.

von Moltke, L. L., Greenblatt, D. J., Schmider, J., Wright, C. E., Harmatz, J. S., and

Shader, R. I. (1998) In vitro approaches to predicting drug interactions in vivo. *Biochem Pharmacol* **55**:113–122.

Williams JA, Ring BJ, Cantrell VE, Campanale K, Jones DR, Hall SD, and Wrighton

SA (2002) Differential modulation of UDP-glucuronosyltransferase 1A1 (UGT1A1)-catalyzed estradiol-3-glucuronidation by the addition of UGT1A1 substrates and other compounds to human liver microsomes. *Drug Metab Dispos* **30**:1266–1273.

Wrighton SA and Ring BJ (1994) Inhibition of human CYP3A catalyzed 1'-hydroxy

DMD#9738

midazolam formation by ketoconazole, nifedipine, erythromycin, cimetidine, and nizatidine. *Pharm Res* **11**:921–924.

Yong WP, Ramirez J, Innocenti F, and Ratain MJ (2005) Effects of ketoconazole on glucuronidation by UDP-glucuronosyltransferase enzymes. *Clin Cancer Res* **11**:6699–6704.

Yoshimura H, Oguri K, and Tsukamoto H (1967) The synthesis of codeine and morphine glucuronides. *Tetrahedron Lett* **4**:483–486.

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Footnote

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Legends to figures

FIG. 1. Morphine 3- and 6-glucuronide formation activity of human liver microsomes in the presence of nifedipine and ketoconazole.

The activities of M-3-G and M-6-G formation in the absence of inhibitors were 312.3 ± 12.0 and 49.0 ± 5.9 pmol/min/mg protein, respectively. See the *Materials and Methods* section for details of the assay method. Each plot represents the mean \pm S.D. of triplicate determinations.

FIG. 2. Dixon plots of the inhibition by ketoconazole of morphine glucuronidation catalyzed by human liver microsomes.

Effect of ketoconazole (25 μ M to 500 μ M) on the M-3-G (A) and M-6-G (B) formation was estimated using four concentrations of morphine (closed circle, 0.5 mM; open square, 1.0 mM; closed triangle, 2.0 mM; and open triangle, 5.0 mM). Replots of the slopes of Dixon plot (slopes vs. $1/[S]$) are shown in panels (C)(M-3-G) and (D)(M-6-G). Details of the assay conditions are described in the *Materials and Methods* section. Each plot represents the mean of duplicate measurements. In experiment (B), M-6-G-formation in the presence of 0.5 mM morphine and 0.5 mM ketoconazole was not determined because of the low activity.

FIG. 3. Inhibitory effect of ketoconazole on morphine glucuronidation by solubilized (A) and NADPH-supplemented human liver microsomes (B), and on the activity catalyzed by UGT2B7 expressed in insect cells (C)

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In (A), effect of NADPH on the ketoconazole-induced inhibition of morphine glucuronidation by human liver microsomes was assessed. The values are expressed as a percentage of the control which was determined in the absence of NADPH or ketoconazole (UDPGA alone). Each bar represents the mean \pm S.D. of triplicate determinations. * Significantly different from UDPGA alone ($p < 0.01$). Control (UDPGA alone) activities of M-3-G and M-6-G formation were 352.0 ± 9.4 and 48.6 ± 3.5 pmol/min/mg protein, respectively. In (B), morphine 3- and 6-glucuronidations were determined using solubilized human liver microsomes in the presence of ketoconazole. The values are expressed as percentages relative to the control which was determined in the absence of ketoconazole. Each plot represents the mean \pm S.D. of triplicate determinations. In (C), Incubation was performed with insect cell microsomes expressing recombinant UGT2B7, 3.8 mM morphine and 2 mM UDPGA for 60 min. The values are expressed as percentages relative to the control which was determined in the absence of ketoconazole. Details of the assay conditions are described in the *Materials and Methods* section. Each bar represents the mean \pm S.D. of triplicate determinations. * Significantly different compared with the control group ($p < 0.01$). Control activities of M-3-G and M-6-G formation were 351.4 ± 7.0 and 53.2 ± 6.2 pmol/min/mg protein, respectively.

FIG. 4. Effect of codeine and testosterone on the inhibition of human liver microsomal morphine glucuronidation by ketoconazole.

In (A) and (B), morphine glucuronidation (A: M-3-G, B: M-6-G) by pooled human liver microsomes was examined in the presence of either ketoconazole (KTC), codeine or

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their combination. Control activities of M-3-G and M-6-G formation were 293.2 ± 16.3 and 54.4 ± 12.4 pmol/min/mg protein, respectively. In (C) and (D), morphine glucuronidation (C: M-3-G, D: M-6-G) by pooled human liver microsomes was examined in the presence of either ketoconazole (KTC), testosterone (TEST) or their combination. Inhibitor concentrations are shown in the figure. Details of the assay conditions are described in the *Materials and Methods* section. Each bar represents the mean \pm S.D. (triplicate determinations) of the relative activity to the control. * Significantly different ($p < 0.05$) from control which was determined in the absence of inhibitor. #Significantly different ($p < 0.05$) from the activity in the presence of KTC alone, codeine alone, or TEST alone. In panels A and B, the comparison between codeine alone vs. codeine plus KTC was made for pairs with same concentration of codeine. In panels C and D, the comparison between TEST alone vs. TEST plus KTC was made for pairs with same concentration of TEST. N.S., not significant.

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TABLE 1

Kinetic parameters for the *in vitro* inhibition of UGT-mediated glucuronidation by ketoconazole

Glucuronide formed (enzyme source)	K_m	K_i (μM)	K_i/K_m	Inhibition-type	Reference
M-3-G (HLM)	2.66 ± 0.32 (mM)	118 ± 27	0.04	Mixed	This work
M-6-G (HLM)	2.62 ± 0.46 (mM)	108 ± 35	0.04	Mixed	This work
SN-38-G (rUGT1A1) ^a	33.5 ± 2.8 (μM)	3.3 ± 1.1	0.1	Competitive	Yong et al., 2005
SN-38-G (rUGT1A9) ^a	39.6 ± 1.3 (μM)	31.9 ± 4.7	0.81	Competitive	Yong et al., 2005
Zidovudine-G (HLM)	3.7 (mM)	80	0.02	Competitive	Sampol et al., 1995

^arUGT1As indicate cDNA-expressed UGT1A isoforms. HLM indicates human liver microsomes. The apparent K_m and K_i values of morphine UGT-catalyzing M-3-G/M-6-G formation were determined using microsomal preparations from pooled human liver, as described in *Materials and Methods*.

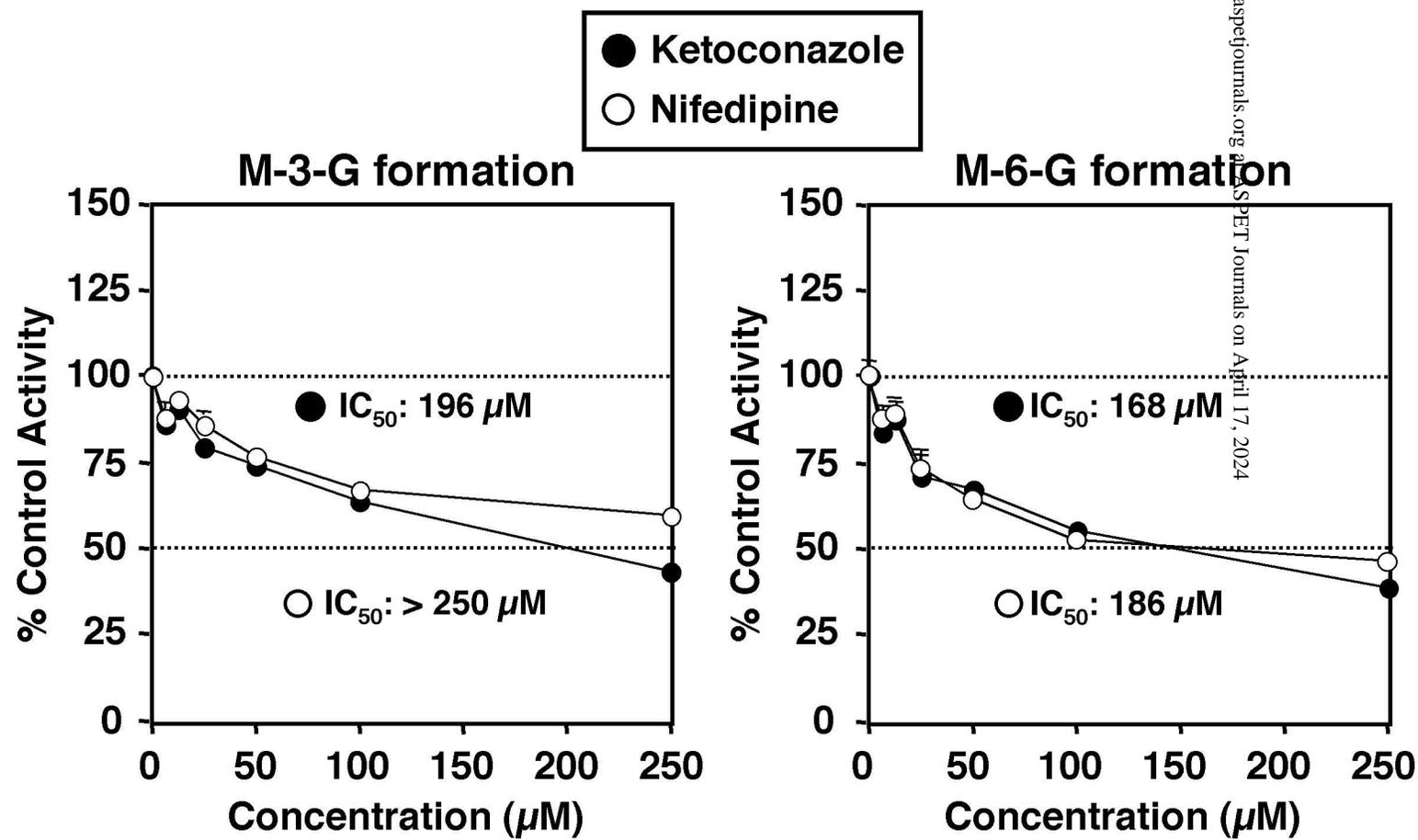


Fig. 1

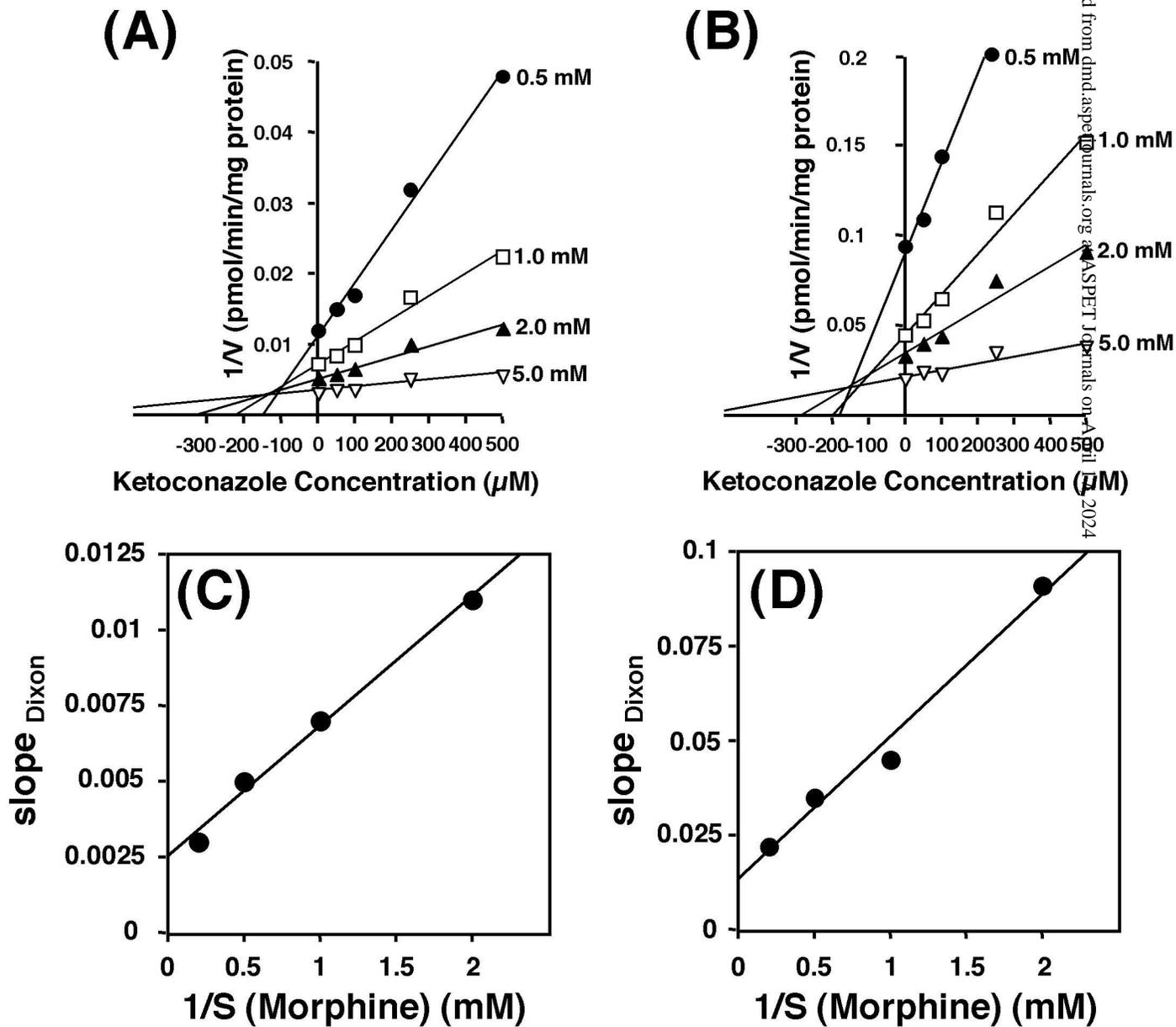


Fig. 2

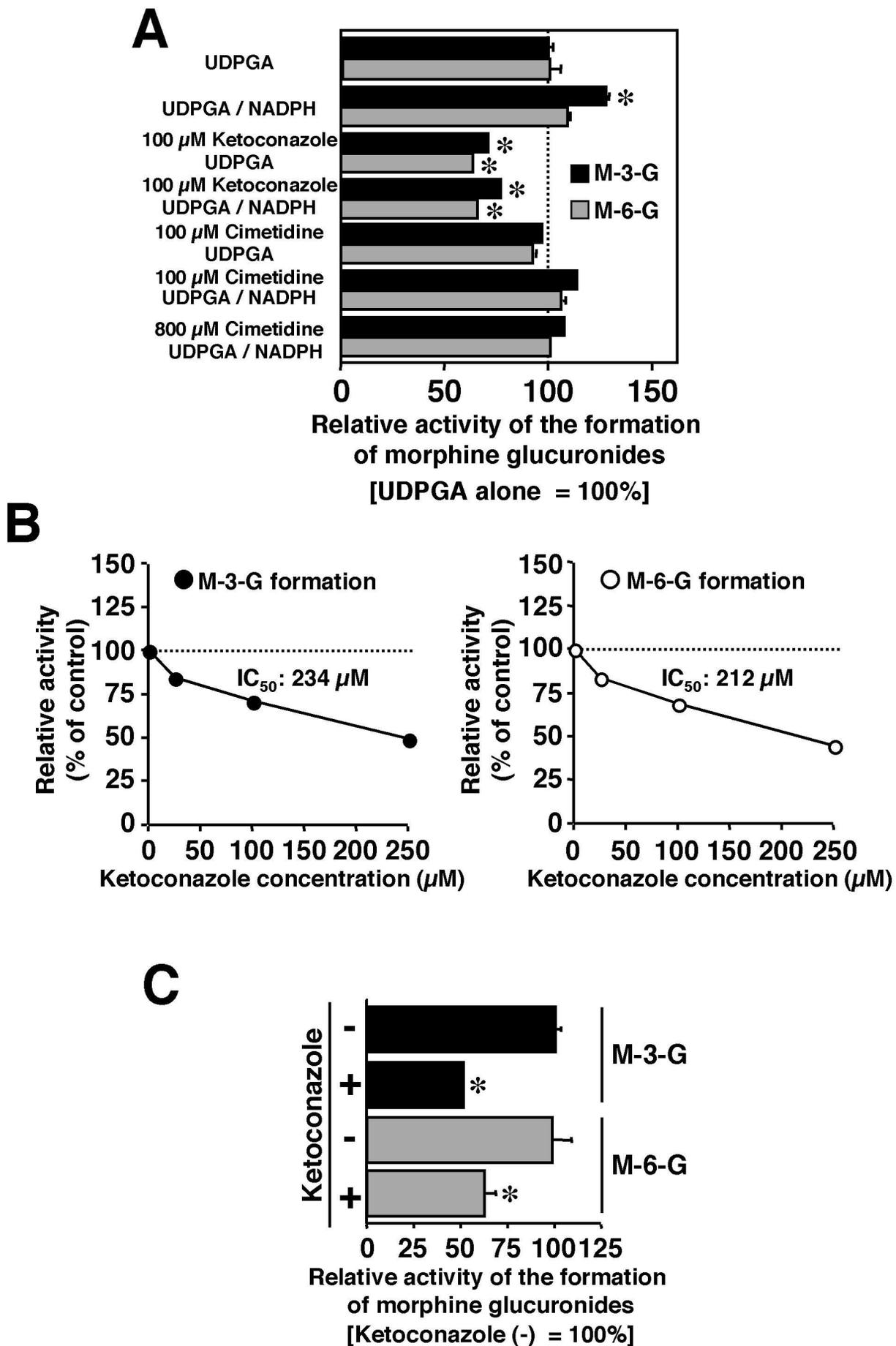


Fig. 3

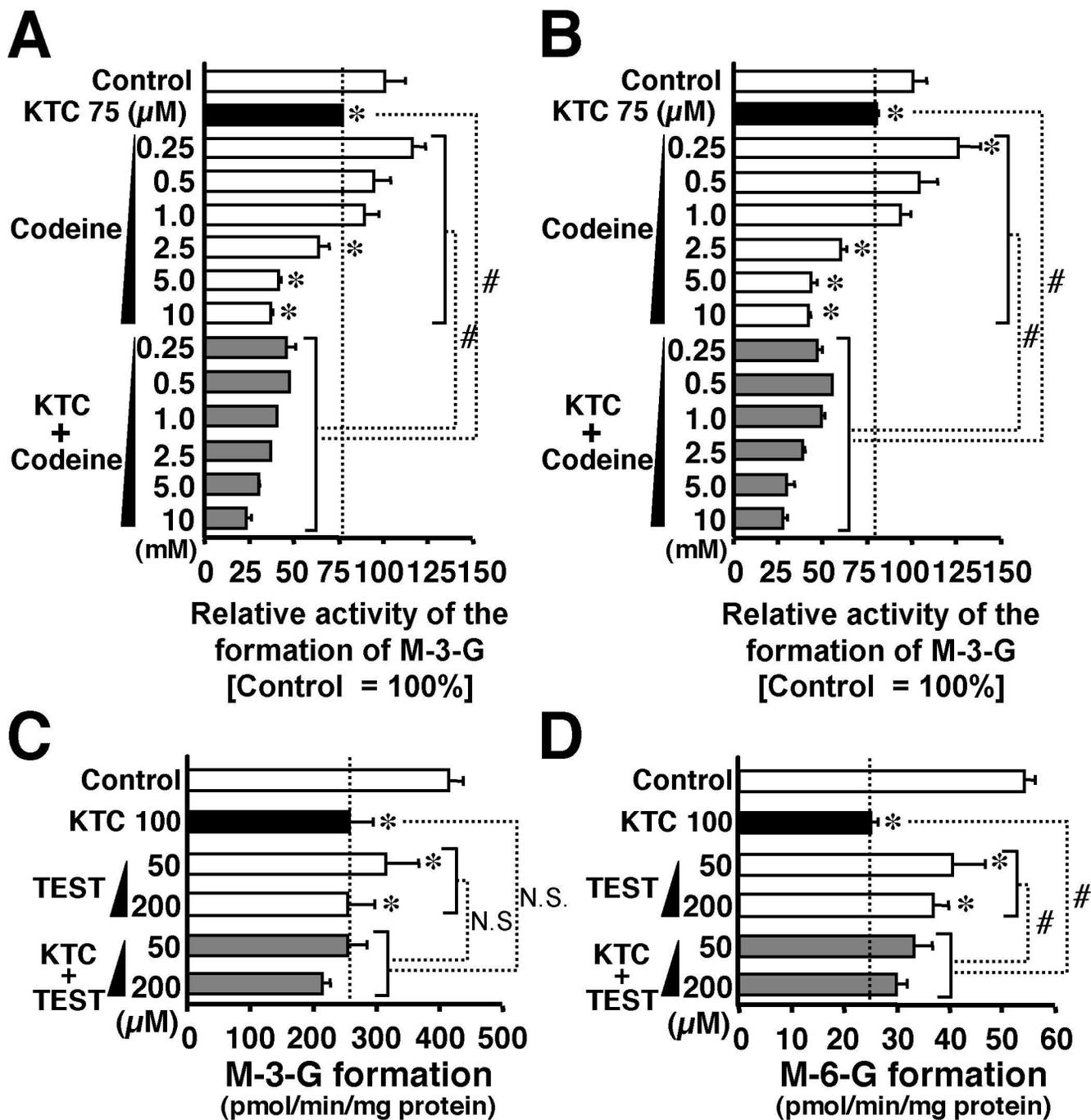


Fig. 4