Substrate Specificity of Opioid Compounds to UDP-Glucuronosyltransferase (UGT), hUGT2B7 and Bovine Microsomal UGT

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(Received February 2, 2005; Accepted March 10, 2005)

We studied the substrate specificity of some opioid derivatives of 5,9-dimethyl-2′-hydroxybenzomorphan [1], composed of A, B, and D ring-systems of morphine, for human UDP-glucuronosyltransferase 2B7 (hUGT2B7), a typical glucuronidation enzyme to morphine and for bovine microsomal UGT. The group of nitrogen atom on the D ring (pyperidine ring) in [1] was modified with alkyl, alkenyl, alkynyl and aralkyl hydrocarbon substituents. hUGT2B7 did not react with the compounds with methyl and isopropyl groups on the nitrogen atom, but reacted with those having longer alkyl substituents of more than 3 carbon chains. Substances with alkenyl and isobutyl substituents are the best substrates (the Km value, 15 and 25 μM, respectively). Opioids with alkynyl and aralkyl hydrocarbon substituents are of low affinity (the Km value, 119 and 542 μM, respectively). Meanwhile, bovine enzyme did not react with opioid substances having methyl and isopropyl groups, like hUGT2B7. Bovine enzyme reacted well with opioid substances with alkenyl and alkynyl substituents on the same level as alkyl substituents. Thus, a clear difference between human UGT2B7 and bovine microsomal UGT was found in the reactivity of alkynyl group and this comes from species specificity. For development of effective opioid drugs, these results suggest that opioid compounds with short carbon substituents are better to maintain the effective level in the blood for a longer time, with low glucuronidation activity, as well as maintaining the analgesic potency of each drug.

Key words —— opioid, morphine, UDP-glucoronosyltransferase, pentazocine

INTRODUCTION

Most xenobiotics, such as drugs, non-nutrient chemicals in foods, and pollutants, are absorbed and then metabolized by the phase I enzymes, then the phase II enzymes, and finally excreted through transporters. Many drugs are ordinarily lipophilic and stay longer in lipophilic membranes composed of lipid matrix. During the course of metabolism of these lipophilic drugs, the phase I metabolizing enzymes functionalize lipophilic drugs by addition of active residues, such as hydroxyl group (-OH), to drugs and then the phase II metabolizing enzymes conjugate with water-soluble substances, such as UDP-glucurononic acid (UDPGA) for UDP-glucuronosyltransferase (UGT), sulfuric acid for sulfotransferase (ST), GSH for glutathione S-transferase (GST), to functionalize drugs.[1)] These soluble conjugates are excreted in bile and urine by transporters. The UGT is the most functional enzyme among those phase II enzymes. UGT transfers glucuronic acid in UDPGA to ligands to make water-soluble glucuronides.[2)] The C-terminal portion of UGT plays some roles, such as binding with UDPGA and with membrane. Meanwhile, the N-terminal half of UGT plays roles in ligand recognition, providing substrate specificity, and oligomerization.[3)]

UGT is a membrane-bound enzyme, differing from the other phase II enzymes, such as ST and GST. UGT localizes within the smooth endoplasmic reticulum (sER) and the majority of the protein is not on the cytosolic, but the lumenal, side of ER membrane, unlike cytochrome P-450.[4)] From this orientation of UGT in sER, UGT in microsomal preparations is activated by addition of some detergents, such as Triton X-100, suggesting UGT is in-

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side of microsomes.[5,6] UGT has also species specificity and organ specificity. The level of human bilirubin metabolizing UGT is 4.5 times more than that of bovine UGT.[7] UGT is in the most in liver,[8] and also found in kidney, gastric duct, lung, and brain.[9] There are many UGT species and they are classified into the UGT1 and UGT2 families, with a molecular mass of 50–57 kDa.[10] hUGT1A family gene is found on chromosome 2, and 9 kinds of UGT1 are made by alternative splicing between several exon 1 and a constant exon 2–5. Also, the hUGT 2 subfamily gene is found on chromosome 4.[11,12]

Endogenous ligands for UGT are bilirubin, bile acid and steroid hormones. There is much medicinal interest in the metabolism of bilirubin by UGT1A1 (jaundice) and morphine (an anodyne) by UGT2B7.[5,11] UGT monomers interact with each other to produce homo- or hetero-oligomer (dimer and/or tetramer) on sER.[13–17] There are some reports suggesting that UGT is present and acts as monomer.[10,18] It has been suggested that Gilbert's syndrome comes from a dominant negative phenomenon by oligomerization composed of inactive monomer mutants.[19] In guinea pigs, the dimer of UGT is more active for morphine metabolism.[17] Opioid drugs are metabolized by UGT in liver, and morphine is metabolized by hUGT2B7 to the major product, morphine-3-glucoronide (M3G), and the minor product, morphine-6-glucuronide (M6G).[20,21] The anodyne activity is disappeared in M3G, but the activity of M6G was 800-times stronger than the activity of original morphine.[22] The ratio of M6G to M3G differs among species, and high in humans and guinea pigs, and low in rats, mice and dogs.[2]

Codeine is demethylated by CYP2D6 to become morphine, and then conjugated with UDPGA to glucuronide. Pentazocine, a synthetic opioid, is well absorbed and metabolized, and the main metabolites are cis alcohol, carboxylic acid, and the glucuronide, which have no analgesic activity and are excreted through the kidney.[23] The activity of opioid metabolism differs among people, resulting in deviation of the anodyne activity. Buprenorphine is a derivative of oripavine and acts as a ligand to the μ receptor. The analgesic activity of buprenorphine continues for longer time than that of morphine at a low dose administered by injection or suppository.[24] The main metabolite of buprenorphine is glucoronide by UGT2B7.[25] hUGT2B7 also reacts with carcinogenic substances, as well as morphine.[26,27] Recently, the active site on UGT2B7 to opioid compounds was investigated for the amino-terminal 119 residues and the binding mechanism was studied by NMR.[28] Sanchez *et al*., reported that the *N*-alkyl on aglycon played an important role.[20] King *et al*., reported that the interaction sites of morphine with UGT2B7 were both of the *N*-alkyl group on the D ring and 3-OH group on the A ring.[25]

In this report, we studied substrate specificity of UGT2B7 with synthetic opioid compounds. The structure of these compounds is shown in Fig. 1 and Table 1. TL3 has a similar structure to buprenorphine, and TL9 to pentazocine. We also compare the glucuronidation activity between human and bovine UGT.

MATERIALS AND METHODS

UDP-Glucuronosyltransferase —— Human UGT2B7 in supersomes is a product of Gentest. Bovine liver microsomes were prepared as previously reported.[29] Briefly, fresh bovine liver was minced and mixed in 4-fold with 0.25 M sucrose-10 mM Tris–HCl (pH 7.4). The extract was centrifuged at 8000 $\times$ *g* and the supernatant was centrifuged at 100000 $\times$ *g* for 1 hr. The precipitate at 100000 $\times$ *g* (microsomes) was suspended in 0.1 M Tris–HCl-10% glycerol and centrifuged once more at 100000 $\times$ *g*. The precipitate was resuspended in 0.1 M Tris–HCl (pH 7.5)-10% glycerol and stored at –80°C.

Microassay of UGT Activity —— The microassay method of UGT activity in this study was carried out according to the previous report[29]; as follows. A reaction mixture of 50 μl contained 2 mM [^{14}C]UDPGA (0.02 nCi/nmol) and opioid analogs in ethanol at various concentrations in 0.1 M Tris-maleate (pH 7.5) and 5 mM $MgCl_2$. The reactions were started by the addition of microsomes (protein 30 μg) and incubated at 37°C for 30 min. The reaction was stopped by the addition of 100 μl ethanol. The mixture was centrifuged at 8000 $\times$ *g* for 5 min and the supernatant was concentrated by evaporation under vacuum. The residual material was dissolved in 70% ethanol and spotted onto silica plates (Merck silica gel 60, Merck KGaA, Darmstadt, Germany) for thin layer chromatography. The TLC plate was developed in *n*-butanol : H_2O : acetone : glacial acetic acid : 30% ammonium = 70 : 60 : 50 : 18 : 1.5. The radioactivity of the [^{14}C] products on the thin layer was quantitatively analyzed using the radio-image analyzer Fuji BAS2500 (Fuji Film, Tokyo, Japan). The [^{14}C] conjugates were found in upper positions on

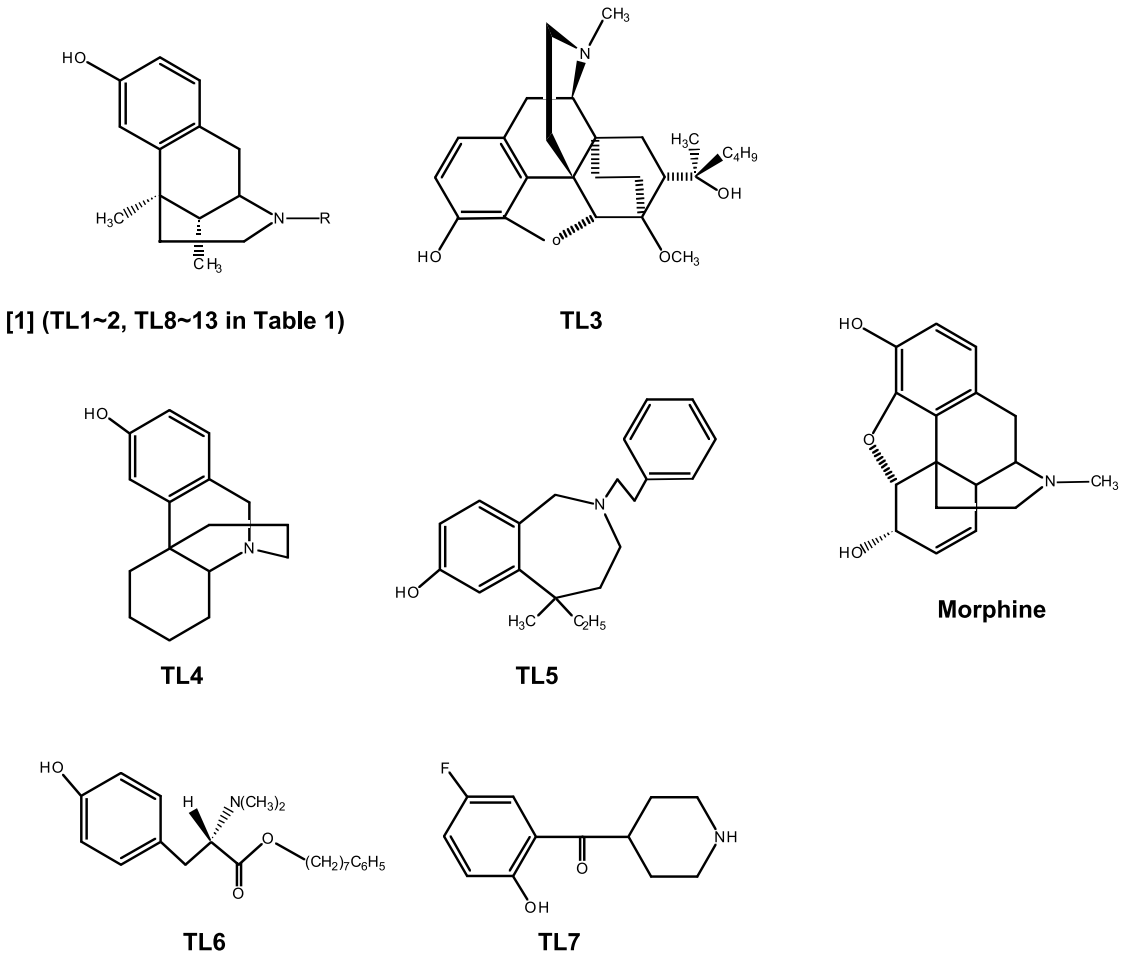

Fig. 1. Chemical Structure of Opioid Substances Used in this Study

Table 1. Structure of R Residues on the Nitrogen Atom in [1]

Compounds	R residues
TL1	$-CH(CH_3)_2$
TL2	$-CH_2CH(CH_3)_2$
TL8	$-CH_3$
TL9	$-CH_2CH=C(CH_3)_2$
TL10	$-CH_2C\equiv CH$
TL11	$-CH_2C(CH_3)_3$
TL12	$-CH_2C_6H_5$
TL13	-H

the thin layer and UDPGA was found in lower positions on the thin layer.[29] Kinetic parameters were calculated from the amount of radioactivity of the conjugates. Androsterone was used as positive ligand for UGT reaction.

Opioid Substances —— Opioid substances TL1, 2, 8–13 are derivatives of 5,9-dimethyl-2′-hydroxybenzomorphan [1], Fig. 1, and contain some substituents on the nitrogen atom of [1] as shown in Table 1. These are synthesized from TL13[30] by addition of those substituents and the method of synthesis was published according to a previous report.[31–33] TL3–7 were different from the structure of [1] but related to the basic structure of morphine. TL3,[34] TL4,[35] TL5,[36] and TL6[37] were prepared by the described methods. The method of synthesis of TL7 will be published elsewhere in details.

RESULTS

Figure 1 shows the structures of opioid substances used in this study. Table 1 shows the structure of some derivatives (TL1, 2, 8–13) from benzomorphan [1] and contains the structure of R substituents. In Fig. 1, there are the structure of some other substances (TL3–7) having an anodyne effect, opioid agonists to the μ receptor, and affinity to serotonin receptor (TL7). Figure 2 shows the result of

autoradiography of the products with TL9 and Fig. 3 shows the Lineweaver-Burk plot of TL9 from the results in Fig. 2.

Table 2 shows the summary of kinetic parameters of TL1, 2, 8–13 for human UGT2B7, as well as the parameters of androsterone and morphine reported.[3] TL9 (pentazocine) with dimethylpropenyl group as the R substituent on the nitrogen atom of [1] has the strongest affinity among those derivatives, and hUGT2B7 has a similar affinity to androsterone of a typical ligand. Surprisingly, TL1 and TL8, having methyl or isopropyl group as the R substituent, did not show substrate activity for UGT2B7. Interestingly, TL13 without an alkyl group also showed weak substrate activity for hUGT2B7. Meanwhile, compounds having long alkyl groups (longer than 3 carbons) as the R substituents, such as TL2, TL9 and TL11, show higher affinity to hUGT2B7. The R substituents on TL2 is one methylene longer than that of TL1 and the affinity of TL2 to hUGT2B7 is stronger than that of TL1. These results showed that the R substituent of longer alkyl residues on TL2 and 9 is better for binding to UGT2B7 than that of short alkyl residues, such as on TL1, TL8, and TL13. These results suggest that the hydrophobic atmosphere surrounding the nitrogen atom is an important factor of substrates that recognized by hUGT. The position of nitrogen atom in TL1 may be covered by two methyl residues in isopropyl residue but the position N2 in TL2 may be opened from the shield of this methyl group to increase interaction of amine with the enzyme. Longer alkyl residues may have the same effect. Derivatives having benzyl group (TL12) have low affinity, because the aromatic ring has a higher unsaturated π-electronic density than the density of TL11. TL10, having the alkynyl group, has a moderate affinity. Finally, the kinetic parameters of TL2, TL9 and androsterone show high Vmax/Km values but the parameters of morphine[3] are low.

Table 3 shows the summary of kinetic parameters of opioid compounds for bovine microsomal UGT. Overall results with bovine UGT are similar with those obtained with hUGT2B7. TL2 and TL9 have higher affinities for bovine UGT. TL1 and TL8 showed no affinity to either bovine and human

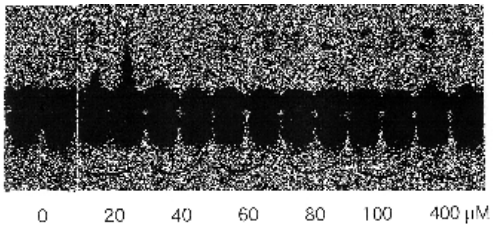

Fig. 2. Pattern of Autoradiography of Glucuronides in Duplicates of TL9 with Bovine Microsomes

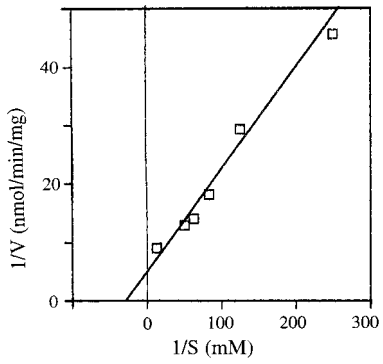

Fig. 3. Lineweaver-Burk Plots of Glucuronidation of TL9 with Bovine Microsomes

Table 2. Summary of Kinetic Parameters of Opioid Substances with hUGT2B7

Compounds	Km (μM)	Vmax (nmol/min/mg protein)	Vmax/Km
TL1	—	ND[a]	—
TL2	25	1.4	56
TL8	—	ND[a]	—
TL9	15	1.2	80
TL10	119	0.30	2.5
TL11	50	0.33	6.6
TL12	542	0.78	1.4
TL13	269	0.11	0.4
Androsterone	15	1.3	86
Morphine	351, 633[b]	—	—
Buprenorphine	22[b]	—	—

a) ND is "not detected." *b*) Data from Reference 3.

Table 3. Summary of Kinetic Parameters of Opioid Substances with Bovine UGT

Compounds	Km (μM)	Vmax (nmol/min/mg protein)	Vmax/Km
TL1	—	ND[a)]	—
TL2	108	1.4	13
TL8	—	ND[a)]	—
TL9	31	0.16	5.2
TL10	42	0.21	5.0
TL11	244	0.64	2.6
TL12	276	0.95	3.4
TL13	42	0.23	5.5
Androsterone	150[b)]	0.28	1.9

a) ND is "not detected." *b*) Data from Reference 3.

Table 4. Comparison between Kinetic Parameters of TL3–7 with hUGT2B7 and Bovine UGT

Compounds	hUGT2B7		Bovine UGT	
	Km (μM)	Vmax (nmol/min/mg protein)	Km (μM)	Vmax (nmol/min/mg protein)
TL3	38	1.1	230	0.3
TL4	—	ND[a)]	—	ND[a)]
TL5	68	0.8	62	2.5
TL6	55	1.1	77	2.4
TL7	—	ND[a)]	—	ND[a)]

a) ND is "not detected."

UGTs, and TL12 has low affinity for bovine enzymes. Meanwhile, some differences between human and bovine UGTs were found with TL11 and TL13. The substrate activity of TL11 was lower for bovine UGT and the activity of TL13 was higher for bovine UGT. The activity for androsterone was also significantly different between human and bovine UGTs (high for human UGT and low for bovine UGT). Human enzyme has a high affinity to alkyl moiety (TL11) and bovine enzyme has a high affinity to alkynyl moiety (TL10). Thus, some species specificity to those opioid analogs in glucuronidation reaction was detected.

Table 4 is the summary of kinetic parameters of glucuronidation with opioid analogs (TL3–7) for hUGT2B7 and bovine UGT. Comparison of these results in Table 4 shows a similar pattern between human and bovine UGTs. The affinities of TL5 and TL6 for human enzymes are similar to that of bovine UGT. UGT interacts with *p*-OH residue on TL6. TL5 and TL6 are good agonists for μ receptor. Meanwhile, the affinity (Km, 230 μM) of TL3 for bovine UGT was lower than that (Km, 38 μM) for human UGT, and similar results were found with TL2 and androsterone, as shown in Tables 2 and 3. This suggests that human UGT has a stronger affinity to hydrophobic residues on substrates. hUGT2B7 was a preparation expressed in baculovirus-infected insect cells and the Vmax values in Table 4 were slightly lower than the levels obtained with bovine microsomal UGT. It is well known that there is deviation of Vmax values of hUGT2B7 by the product lots from Gentest, therefore we did not compare those Vmax values in this study. Neither TL4 nor TL7 were substrates for hUGT2B7 and bovine microsomal UGT, since the caged nitrogen atom in TL4 was blocked by the carbon framework and the enzymes could not recognize the nitrogen. The enzymes could not recognize the OH group of TL7, because the OH group should make the same intramolecular interaction with the ortho-substituted carbonyl group by hydrogen bonding, as the interaction of COOH and OH groups in salicylate.

DISCUSSION

Morphine is a well known aglycone for UGT2B7 and has methyl group on the nitrogen atom. On Table 2, TL8 having methyl group on the nitrogen atom was not a substrate for hUGT2B7. As shown in Table 2, the affinity of morphine to hUGT2B7 is

very low (Km value, 350–630 μM),[3] compared with those of opioid analogs a having high affinity (Km values of TL2 and TL9 are 10–20 μM). We believe that the low affinity of morphine to UGT comes from the electron density surrounding the rings C and E, and the methyl group on the nitrogen atom. However, this low affinity of morphine to hUGT2B7 is a useful characteristic, because it takes a longer time to conjugate morphine with glucuronide, thus a high drug level is maintained in blood. Therefore, in Tables 2–4, it is possible that low reactive analogs, such as TL12 and TL13 are better than high reactive analogs, such as TL2 and TL9, at maintaining a high dose in the blood for long time. However, It is probable that high reactive and rapid excretion is better in some diseases. The $T_{1/2}$ value of TL9 (pentazocine) by oral administration is 98–192 min, showing rapid excretion. The $T_{1/2}$ value of buprenorphine, near TL3, is also 232–383 min by intravenous administration.[24,25] These results suggest that TL3 and TL9 are good substrates of the phase II drug metabolizing enzyme. In Tables 2–4, we showed species-specificity for substrates of *O*-glucuronidation. For cattle disease, this difference must be considered for cure and development of animal drugs.

Morphine interacted with μ receptors at a concentration (Ki value) of 1.7 nM,[38] and the Km value for hUGT2B7 was 350–630 μM.[3] The key structures on morphine interacting with the μ receptors are an aromatic ring, an amine residue in the D ring, and a T-type structure between the A + B rings and D ring, as well as the OH group on the A ring. The μ receptors interact with methyl-, allyl-, cyclopropylmethyl-, and cyclobutylmethyl-groups, binding with the nitrogen atom in the D ring.[30–34] Meanwhile, human and bovine UGTs did not interact with methyl- (TL8) and isopropyl groups (TL1). Thus, substrate specificity for UGT as shown in this study is clearly different from the substrate specificity and affinities for μ receptors as anodynes.[39] Thus, we considered the structure of opioids from the activity of the anodyne and metabolism to maintain an appropriate drug concentration in blood. We propose an explanation for no-activity of TL8 and high activity of TL2 and TL11. There are some hydrophobic pockets on UGT departing (approximately 6 angstrom) from the interaction site with the nitrogen on drugs.

Acknowledgements A part of this study was supported by the San-Ei Gen Foundation for Food Chemical Research.

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