Inhibition of Morphine Glucuronidation in the Liver Microsomes of Rats and Humans by Monoterpenoid Alcohols

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Morphine is an important drug used to alleviate moderate to severe pain. This opiate is mainly metabolized by glucuronidation to a non-analgesic metabolite, morphine-3-glucuronide (M-3-G) and an active metabolite morphine-6-glucuronide (M-6-G). To understand the modulation of morphine glucuronidation activity by environmental factors, the effect of endogenous and food-derived compounds on morphine uridine 5′-diphosphate (UDP)-glucuronosyltransferase (UGT) in rat and human microsomes was evaluated examining the 50% inhibitory concentration (IC_{50}). The liver microsomes from Sprague-Dawley rats (RLM) and humans (HLM, 150 donors, pooled microsomes) were used as enzyme sources. Of 27 compounds tested, monoterpenoid alcohols, such as borneol and iso-borneol, exhibited a strong inhibitory effect on morphine glucuronidation in rat liver microsomes (RLM), whereas we failed to detect any inhibitory effect of endogenous substances including amino acids and sugars. The substances which have the ability to inhibit the activity in RLM are also inhibitory toward morphine glucuronidation in HLM and UGT2B7 baculosomes. However, the difference was that while the strongest inhibitory effect was observed for iso-menthol in HLM, borneol was the strongest inhibitor of the activity mediated by RLM. Although zidovudine is a typical substrate of UGT2B7, the inhibition of morphine glucuronidation by zidovudine was far weaker than that of monoterpenoid alcohols. These results demonstrate that dietary and supplementary monoterpenoid alcohols modify the pharmacokinetics and pharmacodynamics of morphine through inhibition of UGT2B7.

Key words uridine 5′-diphosphate-glucuronosyltransferase; glucuronidation; morphine; borneol; iso-menthol; monoterpenoid alcohol

Morphine is an important analgesic for relieving moderate to severe pain (WHO). The major metabolic pathway for morphine is glucuronidation leading to the formation of morphine-3-glucuronide (M-3-G) and morphine-6-glcuronide (M-6-G).[1] While M-3-G is a non-analgesic metabolite, M-6-G is a far more potent analgesic than the parent morphine.[2] M-3-G is the major metabolite of morphine in many species. However, the ability to form M-6-G varies from species to species,[3] and relatively higher activity was detected in humans[4,5] and guinea pigs.[3] Glucuronidation is catalyzed by uridine 5′-diphosphate (UDP)-glucuronosyltrasferase (UGT) which is expressed mainly in the liver and gastro-intestinal tract.[6] UGT is a family of enzymes which transfer the glucuronic acid moiety of the co-substrate, UDP-glucuronic acid (UDPGA), to an acceptor substrate.[7] Previous studies have suggested that, among UGT isoforms, UGT2B1 and UGT2B7 contribute to morphine glucuronidation in rats and humans, respectively.[8,9]

The accelerated action of narcotics is recommended for curing cancer pain because withdrawal symptoms rarely happen even following the opiate treatment of patients with pain.[10] However, a problem associated with the clinical use of narcotics is that the mortality of patients due to overdosing is increasing.[11] As has been established, the pharmacological effect of morphine varies widely among individuals.[12] Therefore, it is important to construct more effective and safer regimens of morphine use.

There are genetic polymorphisms of the μ-opioid receptor,[13–15] UGT2B7[15–19] and ATP-binding cassette transporter B1 (ABCB1)[14] all of which are possible candidates for explaining the inter-individual differences in the effect of morphine. However, it seems unlikely that the inter-individual differences in the efficacy of morphine can be fully explained by the above mechanisms. We have suggested that a protein-protein interaction between cytochrome P450 3A4 (CYP3A4) and UGT2B7 alters the regio-selectivity of UGT2B7-catalyzed morphine glucuronidation.[20] Furthermore, we found an enhancement of morphine glucuronidation by acyl-CoAs.[21] In this study, we further extended the likely candidates and examined endogenous and food-derived compounds in an attempt to identify inhibitory substances. Since ethanol is known to be a substrate for UGT2B7,[22] ethanolamine derivatives related to phospholipids were considered as having a potential effect on the glucuronidation. Plant polyphenols, quercetin and curcumin, are expected to reverse morphine tolerance and dependence.[23,24] In addition, quercetin, resveratrol and curcumin are known substrate of UGTs.[25–27] Furthermore, borneol is a substrate of UGT2B21 which is involved in morphine glucuronidation in guinea pig liver.[28] Monoterpenoid alcohols like borneol are major constituents of plant oil that are often ingested in the diet.[29,30] Taking the above information into consideration, we selected 27 compounds including monoterpenoid alcohols and polyphenols (Fig. 1) and examined their inhibitory effect on morphine glucuronidation in rats and humans.

MATERIALS AND METHODS

Materials Morphine hydrochloride was purchased from Takeda Pharmaceutical Industry, Co., Ltd. (Osaka, Japan).

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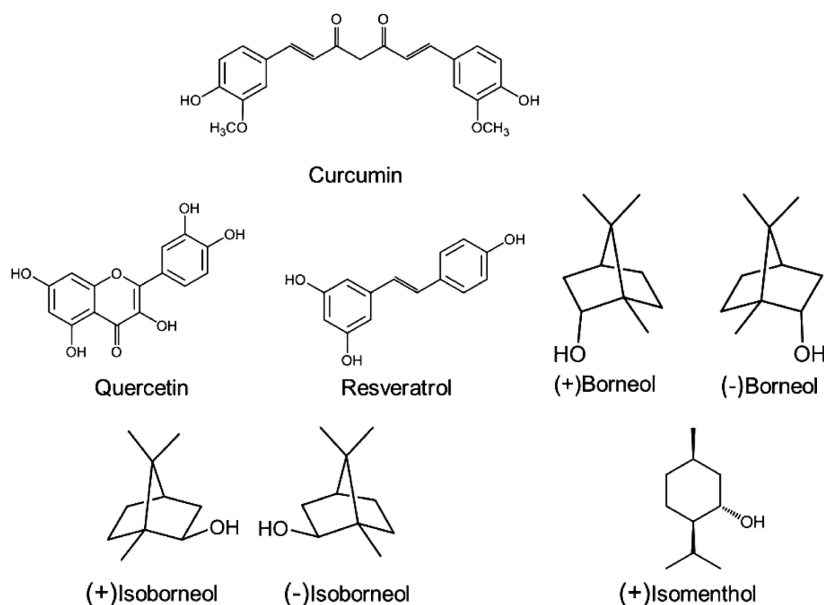
Fig. 1. Structures of the Representative Food-Derived Compounds Examined in This Study

L-α-Phosphatidylcholine (egg yolk, type XI-E), (−)borneol, (±)borneol, (±)iso-borneol, (−)isomenthol, azidothymidine (zidovudine), hyodeoxycholic acid, quercetin, resveratrol, DL-carnitine, L-serine and curcumin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). UDPGA trisodium salt, 4-methylumbelliferone-β-D-glucuronide (4-MU), 4-hydroxybiphenyl (4-OHBP), L-carnitine, betaine, choline, CTP, taurine, propionic acid, pyridoxal-5′-phosphate and polyoxyethylene cetyl alcohol ether (Brij-58) were purchased from Nakalai Tesque (Kyoto, Japan). M-3-G and M-6-G were synthesized by the method described previously.[31] All other reagents were of the highest grade commercially available.

Enzyme Sources Animal experiments in this study were conducted following the approval of the Ethics Committee for Animal Experiments of Kyushu University. Male Sprague-Dawley rats (5 weeks-old) were purchased from Kyudo (Kumamoto, Japan) and they were maintained for one week with free access to water and a suitable diet under a 7 a.m. to 7 p.m. light/dark cycle. The liver was removed and perfused with ice-cold saline, and then the microsomes were prepared by differential centrifugation as described previously.[32] The resulting microsomes were re-suspended in 0.25 M sucrose. Pooled human liver microsomes (HLM) from 150 donors were obtained from BD Gentest (Woburn, MA, U.S.A.). UGT2B7 baculosomes were prepared as follows. UGT2B7 cDNA[33] was subcloned into pFastBac1 and then the recombinant baculovirus was prepared by bac-to-bac methods (Invitrogen). Microsomes were prepared by differential centrifugation from Sf-9 cells infected with recombinant UGT2B7 virus according to the previously reported methods.[34]

Determination of UGT Activity The activity of 3- and 6-glucuronidation of morphine was determined by the published methods.[21,32] Frozen microsomes were thawed and pretreated with a detergent, Brij-58 (0.25 mg/mg protein), on ice for 30 min prior to use. In the assays, the enzyme sources at the protein amounts indicated were used: RLM (15 µg protein), HLM (50 µg protein) and UGT2B7 baculosomes (50 µg protein). Determination limits for M-3-G and M-6-G were 2
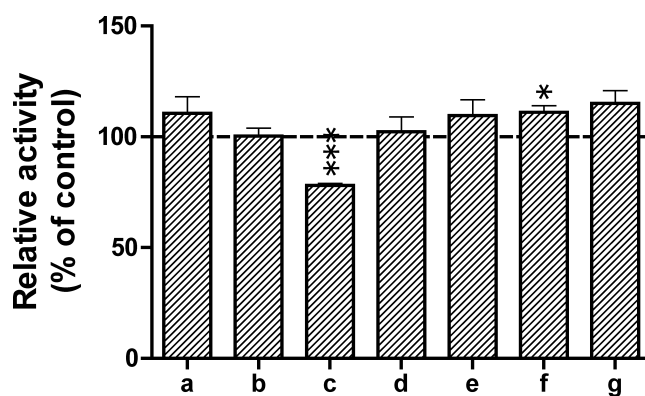

Fig. 2. Effects of the Solvents Used in This Study on M-3-G-Forming Activity

The concentration of morphine and UDPGA was fixed at 4 and 2 mM, respectively. Brij-58-treated RLM were used as the enzyme source. The activity relative to the control (0.083 mM sucrose) (10.1 nmol/min/mg protein) is shown. Each column represents the mean±S.D. of triplicate assay. Significantly different from control; * $p<0.05$; *** $p<0.001$. Solvents used to dissolve test compounds were as follows: (a) 0.079 mM sucrose–3.34 mM NaOH, (b) 0.079 mM sucrose–0.66% ethanol, (c) 0.079 mM sucrose–1.33% ethanol, (d) 0.079 mM sucrose–0.66% ethanol–0.83 mM NaOH; (e) 0.079 mM sucrose–0.66% acetone–0.83 mM NaOH; (f) 0.079 mM sucrose–0.66% acetone–1.66 mM NaOH, (g) 0.075 mM sucrose–1.83% DMSO–1.67 mM NaOH. Each value is a final concentration.

and 5 nmol/injection, respectively.

Other Methods The protein concentration was determined by the method of Lowry et al.[35] using bovine serum albumin as a standard. Calculation of statistical significance, kinetic parameters and IC$_{50}$ was carried out using the computer software, GraphPad Prism 5.04 (GraphPad, La Jolla, CA, U.S.A.).

RESULTS

Effect of Solvents on Morphine UGT Activity in RLM Prior to examining the effect of various substances on morphine UGT activity, the effect of solvent cocktails (Table 1) for dissolving test compounds was investigated. The solvents

Table 1.   The Endogenous and Food-Derived Compounds Used in This Study and Their Solvents

Compounds	Solvent	Compounds	Solvent
CTP	0.083 mM sucrose	Pyridoxal-5′-phosphate	0.079 mM sucrose, 3.34 mM NaOH
L-Serine			
Ethanol		Quercetin	0.075 mM sucrose, 1.83% DMSO,
Ethanolamine		Resveratrol	1.67 mM NaOH
Betaine		Curcumin	
Choline		(−)Borneol	0.079 mM sucrose, 0.66% ethanol
L-Carnitine		(±)Borneol	
DL-Carnitine		(±)Isoborneol	
β-Alanine		(+)Isomenthol	0.079 mM sucrose, 1.33% ethanol
Taurine		Zidovudine	
Propionic acid		Hyodeoxycholic acid	0.079 mM sucrose, 0.66% ethanol,
N-Acetyl-L-tyrosine		4-Methylumbelliferone	0.83 mM NaOH
Glucose		4-Hydroxybiphenyl	
Fructose			
4-Nitrophenol			

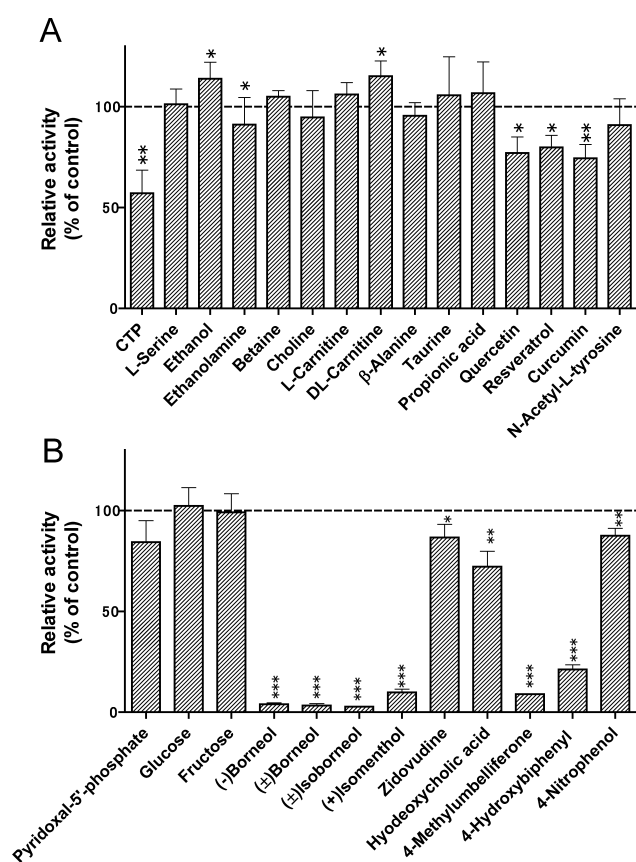The concentration of solvent is a final concentration.

Fig. 3.   Effect of Endogenous and Food-Derived Compounds on M-3-G-Forming Activity in RLM

Control activity with vehicle was determined (see Fig. 1, Table 1), and then the activity relative to the control is shown. The concentration of morphine and UDPGA was fixed at 4 and 2 mM, respectively. Each column represents the mean±S.D. of triplicate assays. Significantly different from control; *$p<0.05$, **$p<0.01$, ***$p<0.001$. The concentrations of inhibitors were as follows: 1000 μM: CTP, L-serine, ethanol and choline; 500 μM: ethanolamine, betaine, L-carnitine, DL-carnitine, β-alanine, taurine, propionic acid, N-acetyl-L-tyrosine, pyridoxal-5′-phosphate, (±)borneol, (−)borneol, (±)isoborneol, (+)isomenthol, zidovudine, hyodeoxycholic acid, 4-MU, 4-OHBP and 4-nitrophenol; 100 μM: quercetin, resveratrol and curcumin; 8.3 mM: glucose and fructose.

Table 2.   The IC$_{50}$ Values of Endogenous and Food-Derived Compounds for M-3-G Activity Catalyzed by RLM

Compounds	IC$_{50}$ (μM)	Compounds	IC$_{50}$ (μM)
Pyridoxal-5′-phosphate	>500	CTP	>1000
N-Acetyl-L-tyrosine	>500	L-Serine	>1000
Glucose	>1000	Ethanol	>1000
Fructose	>1000	Ethanolamine	>1000
(−)Borneol	20.34±0.39	Betaine	>1000
(±)Borneol	23.24±0.29	Choline	>1000
(±)Isoborneol	14.01±0.30	L-Carnitine	>1000
(+)Isomenthol	22.72±0.63	DL-Carnitine	>1000
Zidovudine	>1000	β-Alanine	>1000
Hyodeoxycholic acid	>500	Taurine	>1000
4-Methylumbelliferone	92.38±1.4	Propionic acid	>1000
4-Hydroxybiphenyl	202.7±5.2	Quercetin	>100
4-Nitrophenol	>500	Resveratrol	>100
		Curcumin	>1000

UGT activity was assayed in the presence of Brij 58 (0.25 mg/mg protein). Morphine and UDPGA concentrations were fixed at 4 and 2 mM, respectively. Value represents the estimated value±S.D.

used did not inhibit M-3-G formation in RLM except for 0.079 mM sucrose–1.33% ethanol (Fig. 2).

Effect of Endogenous and Food-Derived Compounds on Morphine UGT Activity in RLM   The K_m for the M-3-G-forming activity of Brij-58-treated RLM was calculated to be 3.80±0.14 mM. Based on this, the present study used a substrate concentration of 4.0 mM which is close to the K_m. Figure 3 shows the effect of 27 compounds examined in this study. Although their concentration could not be exactly the same, because of different solubility, three plant polyphenols, i.e., quercetin, resveratrol and curcumin, exhibited significant inhibition. CTP also had a suppressant effect. These are substrates of UGT or their inhibitory effects have already been reported in publications.[25–27,36] All the stereoisomeric borneols and isoborneols, classified as monoterpenoid alcohols, strongly
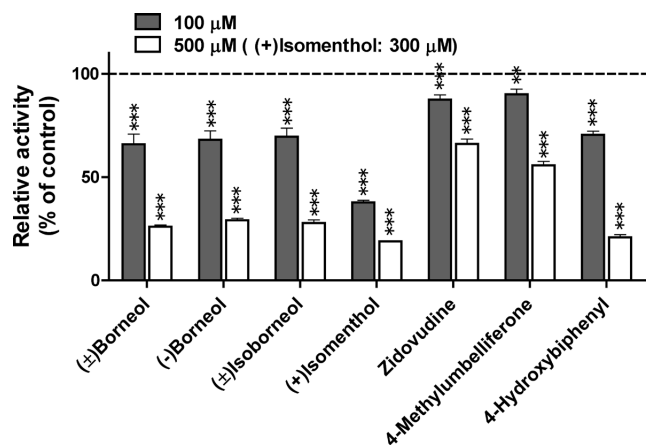
Fig. 4. Effects of Monoterpenoid Alcohols on M-3-G-Forming Activity in HLM

The activity relative to the control is shown. The concentration of morphine and UDPGA was fixed at 2 mM. Significantly different from control; **$p<0.01$, ***$p<0.001$. The inhibitors used and their final concentration (in parenthesis) were as follows: (±)borneol, (−)borneol, (±)isoborneol, zidovudine, 4-MU, 4-OHBP (100, 500 μM); (+)isomenthol (100, 300 μM). Each bar represents the mean±S.D. of triplicate assay.
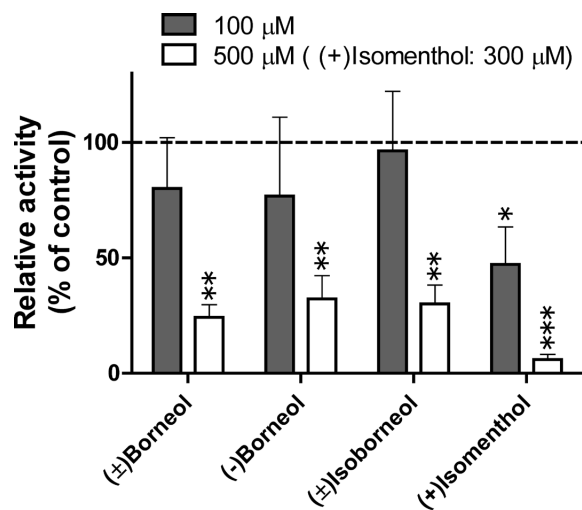

Fig. 5. Effects of Monoterpenoid Alcohols on M-3-G-Forming Activity by Recombinant UGT2B7

The activity relative to the control is shown. The concentration of morphine and UDPGA was fixed at 2 mM. Significantly different from control; *$p<0.05$, **$p<0.01$, ***$p<0.001$. The inhibitors used and their final concentration (in parenthesis) were as follows: (±)borneol, (−)borneol, (±)isoborneol (100, 500 μM); (+) isomenthol (100, 300 μM). Each bar represents the mean±S.D. of triplicate assay.

Table 3. The IC$_{50}$ Values of (±)Borneol, (±)Isoborneol and (+)Isomenthol for Morphine Glucuronidation Activity in HLM

Compounds	IC$_{50}$ (μM)	
	M-3-G	M-6-G
(±)Isoborneol	207.4±3.8	153.1±6.6
(±)Borneol	184.9±6.0	100±12
(+)Isomenthol	52.66±2.19	N.D.[a]

HLM (50 μg protein) pretreated with Brij-58 (0.25 mg/mg protein) were used for the assay. The concentration of morphine and UDPGA was fixed at 2 mM. Value represents the estimated value±S.D. a) N.D.: not determined.

inhibited M-3-G formation by RLM (Fig. 3B). However, endogenous substances, including amines, amino acids, sugars and acids, exhibited only a weak or no inhibitory effect on morphine UGT activity as far as the tested concentration used (Figs. 3A, B). Regarding the compounds showing an inhibitory potential, their 50% inhibitory concentration (IC$_{50}$) was determined (Table 2). The IC$_{50}$ values for (±)borneol, (−)borneol, and (+)isomenthol were about 20 μM, and the lowest IC$_{50}$ (14 μM) was observed for (±)isoborneol. As zidovudine, hyodeoxycholic acid, 4-MU, 4-OHBP and 4-nitrophenol are substrates of UGT, they exhibited a significant inhibitory effect. Among them, the strongest inhibitory effect was observed with 4-MU followed by 4-OHBP. Although zidovudine and morphine glucuronidation are thought to be catalyzed by the same UGT isoform, zidovudine, exhibited only a minor inhibitory effect comparable with hyodeoxycholic acid and 4-nitrophenol. The inhibitory effect of monoterpenoid alcohols was far stronger than that of zidovudine (IC$_{50}$ > 1000 μM).

Effect of Monoterpenoid Alcohols on Morphine UGT Activity in HLM and UGT2B7 The K_m of M-3-G formation mediated by Brij-58-treated HLM was calculated to be 2.04±0.06 mM. Thus, the substrate concentration used to examine the inhibitory effect on HLM was set at 2.0 mM. Figure 4 shows the effect of four monoterpenoid alcohols on the M-3-G formation by HLM. These all exhibited inhibitory effects on M-3-G formation, and these effects were more marked compared with zidovudine and 4-MU. The IC$_{50}$ values of (±)borneol and (±)isoborneol for M-3-G formation in HLM were 8- and 15-times higher than those for RLM (Tables 2, 3). (±)Borneol and (±)isoborneol also inhibited the M-6-G-forming activity of HLM, and this conjugating activity seems to be somewhat more sensitive to these inhibitors than that for M-3-G formation (Table 3). In terms of the inhibition of HLM-catalyzed M-3-G formation, (+)isomenthol had the lowest IC$_{50}$ of the three inhibitors. Finally, we examined whether monoterpenoid alcohols inhibit morphine glucuronidation by recombinant UGT2B7 (Fig. 5). In agreement with their effects on HLM, (+)isomenthol exhibited a strong inhibitory

effect. Although the IC$_{50}$ was not determined, it appears to be about 100 μM or less. The observation that the M-3-G formation activity was almost abolished by 300 μM (+)isomenthol would support the above view. As our preliminary experiment showed that UGT2B7 catalyzes the glucuronidation of (+)isomenthol,[37] this substance seems to compete with another substrate such as morphine in the inhibition of UGT2B7.

DISCUSSION

This study demonstrated that monoterpenoid alcohols exhibit an inhibitory effect on morphine UGT both in RLM and HLM. The inhibitory effects of monoterpenoid alcohols were more marked on RLM than on HLM. In HLM, although the inhibitory effect was observed for both M-3-G and M-6-G formation, the latter reaction seems to be a little more sensitive to the inhibitors compared with the former one. Since UGT2B7 is considered as the major UGT involved in M-6-G formation in humans,[9] it is highly likely that monoterpenoid alcohols are UGT2B7 inhibitors. Although UGT2B7 is also a major isoform involved in M-3-G formation, other isoforms make a minor contribution to this reaction.[9] Whether monoterpenoid alcohols non-specifically inhibit other isoforms of human UGT remains unknown. Concerning the selectivity in inhibition of rat UGTs, UGT2B1 plays a major role in

morphine glucuronidation.[38] Thus, monoterpenoid alcohols are undoubtedly UGT2B1 inhibitors, but their effects on other rat isoforms are again unclear. While UGT2B12 catalyzes the glucuronidation of monoterpenoid alcohols, this UGT lacks morphine glucuronidaiton activity.[39] Thus, it is likely that some UGT isoforms, such as UGT2B12, are resistant to inhibition by monoterpenoid alcohols. However, because borneol at a high concentration completely inhibited morphine UGT in RLM, this compound appears to inhibit all UGT isoforms involved in morphine glucuronidation in RLM.

Monoterpenoid alcohols which we found to have strong inhibitory effects on morphine UGT are rich in plant essential oils. When a Chinese cough medicine, Qingyan Diwan, is given orally to patients, the blood concentration of borneol reaches as much as 130μM.[40] Plant essential oils are also often used for aromatherapy, and the content of borneol in the oils from ginger and rosemary is reported to be 5.6%[29] and 2.7%,[30] respectively. Similarly, the borneol content in the essential oil from turmeric is approximately 0.5%, which is rather higher than the curcumin content.[41] The inhibitory concentration of monoterpenoid alcohols needed for the inhibition of liver UGT seems to be higher than the expected value after ingestion of vegetable oils. However, the concentration in the gasterointestinal tract after ingestion of plant oils may be sufficient for the inhibition of intestinal UGTs. In addition to this, zidovudine, a probe substrate of UGT2B7, exhibited an IC_{50} greater than 1000μM for morphine glucuronidation in HLM (Table 2). The K_m for zidovudine glucuronidation in HLM was reported as 2.4 mM.[42] Similarly, the K_m of morphine glucuronidation in HLM is also in the range of only a few mM.[20] Despite such larger K_m values, glucuronidation is the major metabolic pathway of morphine and zidovudine. A Michaelis–Menten parameter obtained from *in vitro* glucuronidation often underestimates drug clearance. As the IC_{50} values of monoterpenoid alcohols on morphine glucuronidaiton are far lower than that of zidovudine, it would be likely that monoterpenoid alcohols significantly interfere with morphine glucuronidation *in vivo*.

As described before, the monoterpenoid alcohols used in this study may have a stronger inhibitory effect on the formation of M-6-G than M-3-G. Therefore, it is possible that monoterpenoid alcohols reduce respiratory suppression and delirium which are the main adverse effects due to M-6-G. Although we used liver microsomes as enzyme sources in this study, it is of interest to know whether monoterpenoid alcohols affect morphine glucuronidation in the brain. The central nervous system has neurotransmitters which are metabolized by UGT, and this is another issue of interest for the pharmacological effects of monoterpenoid alcohols. For example, monoterpeoid alcohols may cause the activation of neurons through inhibiting the biotransformation of neurotransmitters.

In this study, we examined the inhibitory effects focusing on a limited number of monoterpenoid alcohols. However, there are various kinds of monoterpenoid alcohols, such as menthol, geraniol and linalool, which are ingested through the intake of plant oils. Therefore, it would be necessary to clarify the inhibition of UGT using an increased number of monoterpenoid alcohols and related substances. Menthol, a UGT2B7 substrate,[43] is being widely used as an additive or constituent of foods, cigarettes and cool compresses. Although we did not examine menthol in this study, its inhibitory effect

on morphine glucuronidation is conceivable. Furthermore, our preliminary study showed that isomenthol, a substance analogous to menthol, is a substrate of UGT2B7.[37] Isomenthol had the strongest inhibitory effect on HLM and UGT2B7 (Table 3, Fig. 5). (+)Isomemthol and menthol are UGT2B7 substrates, and they seem to have rough similarities to morphine in stereo-structure as far as the comparison by Jmol software is considered. On this basis, isomenthol and menthol may compete with morphine at the substrate binding site of UGT2B7. Taking these data into consideration, menthol probably inhibits UGT2B7-catalyzed morphine glucuronidation. Non-steroidal anti-inflammatory drugs (NSAIDs), like diclofenac and indomethacin, are also UGT2B7 substrates.[33,44] Thus, inclusion of menthol in cool compresses may enhance the pharmacological activity of NSAIDs. NSAIDs can be metabolized to their acyl glucuronides and CoA-derivatives.[45–47] These CoA-derivatives are suspected to contribute to the adverse effects through their binding to macromolecules. If monoterpenoid alcohols suppress the formation of acyl glucuronides, this would lead to the compensatory production of acyl-CoA derivatives. In agreement with this, borneol inhibits the detoxification of NSAIDs catalyzed by UGTs in human hepatocytes.[48] As mentioned above, UGT2B7 is one of the major UGT isoforms involved in the glucuronidation of NSAIDs.[33,44] Thus, it is plausible that borneol and related monoterpenopid alcohols inhibits the UGT2B7-catalyzed glucuronidation of NSAIDs and other substrates. Therefore, it is necessary to examine monoterpenoid alcohol–drug interactions from a toxicological viewpoint.

It has been reported that smoking cigarettes containing menthol interferes with nicotine glucuronidation[49] the catalysts of which are assumed to be UGT2B10 and UGT1A4.[50,51] Monoterpenoid alcohols interfere with propofol glucuronidation catalyzed by UGT1A9.[52] In this case, (±)borneol exhibits the strongest inhibitory effect. Therefore, it is suggested that although monoterpenoid alcohols inhibit UGTs in a loosely-specific fashion, some isoforms including UGT2B7 and 1A9 are favorable targets. Further comprehensive studies are necessary to clarify the selectivity in the inhibitory effect of monoterpenoid alcohols on UGT and its implication for the safe clinical use of medicines.

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