Note

Effect of Hydrocotarnine on Cytochrome P450 and P-glycoprotein

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Summary: The injectable form of oxycodone contains hydrocotarnine that is supposed to potentiate the analgesic effect of oxycodone with unknown mechanism(s). In this study, the effects of hydrocotarnine on the cytochrome P450 (CYP) and P-glycoprotein (P-gp) were investigated. Hydrocotarnine did not induce a significant change in the metabolic activities of CYP2C9, 2C19, and 2E1 in an in vitro study using human CYP recombinants. Although weak inhibitory effects were observed on CYP3A4 and 2D6, these interactions did not seem to be clinically relevant. Hydrocotarnine also did not cause a significant change in the ATPase activity of human P-gp membranes, suggesting that it is not an inhibitor of P-gp. Furthermore, mice were intraperitoneally injected with hydrocotarnine for 14 days and the mRNA levels of major CYP isozymes and P-gp in the liver and small intestine were determined by real-time RT-PCR. As a result, none of the mRNAs investigated showed a significant change in their levels by hydrocotarnine treatment. In conclusion, it is unlikely that the potentiation of oxycodone effect by hydrocotarnine involves its effect on CYP and P-gp. The findings also demonstrate that hydrocotarnine is unlikely to cause drug interactions via CYP or P-gp.

Keywords: hydrocotarnine; oxycodone; cytochrome P450; P-glycoprotein; drug interaction

Introduction

Oxycodone, an opioid analgesic, has been shown to be metabolized mainly by cytochrome P450 (CYP) 3A4 and CYP2D6.1) Some of the metabolites of oxycodone exhibit a higher analgesic potency than the parent drug.2) It is also reported that oxycodone is a substrate of P-glycoprotein (P-gp), one of the major drug transporters.3) The injectable form of oxycodone contains hydrocotarnine which supposedly potentiates the analgesic effect of oxycodone. However, the mechanism of its potentiating effect as well as the possibility of hydrocotarnine causing drug interactions are unknown. In the present study, the effects of hydrocotarnine on the major CYP isozymes and P-gp were investigated both in vitro and in vivo.

P-gp, a member of the ATP-binding cassette (ABC) transporter family, is located in the cell membrane and transports drugs using the energy of ATP hydrolysis. P-gp is involved in modulating the absorption, distribution and excretion of xenobiotics in various tissues. The inhibition of CYP or P-gp by another drug could increase the plasma concentration of their substrates, leading to the increased pharmacological effects and unwanted side effects or toxicities. Furthermore, in case of drugs with active metabolites, such as oxycodone, the magnitude of pharmacological effects could also be affected by the change in plasma levels of the active metabolites due to the enzyme inhibition/induction.

The inhibitory effects on CYP activity can be evaluated using recombinant enzymes and their fluorometric probe substrates.4) In the present study, microsomes from insect cells expressing human CYP3A4, 2C9, 2C19, 2D6, and 2E1 were used to investigate the effect of hydrocotarnine on the activity of these CYP isozymes. The effect on P-gp activity was evaluated in an ATPase assay using human P-gp membranes.5) The possibility of hydrocotarnine being inhibitors and substrates of P-gp was evaluated in the presence and absence of verapamil (a P-gp substrate), respectively. Finally, the effect of repeated administration of hydrocotarnine on the expression levels of major CYP isozymes and P-gp were investigated in a mouse in vivo study.

Methods

Materials High throughput inhibitor screening kits for CYP3A4, 2C9, 2C19, 2D6, and 2E1 and human P-gp membranes were purchased from BD Gentest (Woburn, MA, USA). Hydrocotarnine hydrochloride was provided.
Effect of Hydrocotarnine on CYP and P-gp

**CYP inhibition assay** The inhibitory effects of hydrocotarnine on CYP activities were determined using black 96-well microtiter plates (Nalge Nunc International, Roskilde, Denmark) and high throughput inhibitor screening kits for CYP3A4, 2C9, 2C19, 2D6, and 2E1 as described on the BD Gentest website (http://www.gentest.com). The experimental conditions for each CYP isozyme are summarized in Table 1. Briefly, after 50 μL of hydrocotarnine solutions (0.00155–155 μM) or positive control inhibitors (in <8% acetonitrile) were added to 50 μL of the cofactor solution (NADP<sup>+</sup>, MgCl<sub>2</sub>, glucose-6-phosphate, glucose–6-phosphate dehydrogenase, control protein in potassium phosphate buffer, pH 7.4) and preincubated at 37°C for 10 min, the reaction was initiated by the addition of 100 μL prewarmed enzyme/substrate mixture. Reactions were terminated by addition of 75 μL of a stop solution (0.5 M Tris base:acetonitrile = 1:4) at the various times given in Table 1. Fluorescence in each well was measured using a microplate reader (GENios FL, Tecan Japan Co., Ltd, Tokyo, Japan) with the excitation and emission wavelengths shown in Table 1. The IC<sub>50</sub> values were calculated by linear interpolation.

**ATPase assay** The ATPase activity of human P-gp membranes was determined by measuring inorganic phosphate liberation according to the procedure reported by Sarkadi et al. A sample of human P-gp membranes was suspended in 10 μL incubation medium containing 50 mM Tris-Mes (pH 6.8), 2 mM DTT, 50 mM KCl, 2 mM EGTA, 2 mM ouabain, and 5 mM sodium azide. This medium was mixed by Takeda Pharmaceutical Co., Ltd. (Osaka, Japan). Adenosine-5′-triphosphate magnesium salt (MgATP), DL-dithiothreitol (DTT), ethylene glycol-bis-(beta-aminoethyl)ether)-N,N′,N′-tetraacetic acid (EGTA), malachite green, 2-[N-Morpholino]-ethane-sulfonic acid (Mes) hydrate, ouabain, polyvinyl alcohol, sodium molybdate dehydrate, sodium orthovanadate, trichloroacetic acid, and (±)-verapamil hydrochloride were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Acetonitrile, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), dimethylsulfoxide (DMSO), hydrochloric acid (HCl), potassium chloride (KCl), quinidine sulphate dihydrate, sodium azide, sodium dihydrogenphosphate dehydrate, and sulfuric acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were of analytical grade.

**Table 1. Experimental conditions for the CYP inhibition assay**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CYP3A4</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2D6</th>
<th>CYP2E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate Concentration (μM)</td>
<td>50</td>
<td>75</td>
<td>25</td>
<td>1.5</td>
<td>70</td>
</tr>
<tr>
<td>Incubation Time (min)</td>
<td>30</td>
<td>45</td>
<td>30</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Positive Control</td>
<td>verapamil</td>
<td>SFZ</td>
<td>TCP</td>
<td>quinidine</td>
<td>DDTC</td>
</tr>
<tr>
<td>Excitation/Emission Wavelength (nm)</td>
<td>409/530</td>
<td>409/530</td>
<td>410/460</td>
<td>390/460</td>
<td>390/460</td>
</tr>
</tbody>
</table>

BFC: 7-benzyloxy-trifluoromethylcoumarin, MFC: 7-methoxy-4-trifluoromethylcoumarin, CEC: 3-cyano-7-ethoxycoumarin, AMMC: 3-(2-(N,N-diethyl-N-methylamino)ethyl)-7-methoxy-4-methylcoumarin, SFZ: sulfaphenazole, TCP: tranylcypromine, DDTC: diethyldithiocarbamic acid

The liberated inorganic phosphate was measured by a method described by Carter & Karl. Briefly, 42 μL of solution A (2 M HCl: 0.1 M sodium molybdate = 4:3), 18 μL of solution B (0.042% (w/v) malachite green solution in 1% (w/v) polyvinyl alcohol), and 120 μL of solution C (7.8% (v/v) sulfuric acid) were added and the mixture was allowed to stand at room temperature for 1 h, after which the absorbance was measured at a wavelength of 630 nm using a microplate reader (Model 550, Bio-Rad, Hercules, CA). The amount of inorganic phosphate liberated in 30 min was estimated from the difference in phosphate levels between 0-min (reaction stopped immediately) and 30-min incubation periods and the ATPase activity (nmol/mg protein/min) was calculated.

**Mouse in vivo study** Male ddY mice (6 weeks old) were intraperitoneally injected with hydrocotarnine (0.4 mg/kg) or saline once daily for 14 days. The mRNA levels of major CYP isozymes (CyP2c29, 2c37, 2d9, 2d22, 2e1, 3a11 and 3a13) and P-gp (Abcb1a and Abcb1b) in the liver and small intestine were determined by real-time RT-PCR using 18S rRNA as a housekeeping gene.

**RNA preparation from the mouse tissues:** Mice were sacrificed and the liver and the upper part of small intestine were harvested for preparation of RNA. Each of the samples (about 50 mg tissue) was homogenized in 1 mL of TRI reagent (Sigma-Aldrich Japan, Tokyo, Japan) with POLYTRON tissue homogenizer (Microtec Co Ltd, Chiba, Japan). RNA extraction from the tissue homogenate was performed according to the protocol of TRI reagent attachment. To check the quality and quantity of RNA, ultraviolet (UV) absorbance at 260 nm and 280 nm was determined.

**Real-time RT-PCR:** RNA (1 μg) was reverse transcribed into cDNA, using High capacity cDNA synthesis kit (Applied Biosystems, Tokyo, Japan) according to the manufacture’s instructions.

For real-time PCR reaction, a mastermix of the following reaction components was prepared to the indicated end-concentration: 15 μL water, 25 μL iQ SYBR Green dition of 20 μL of 5% trichloroacetic acid.

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Table 2. Primer sequences for the real-time RT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Accession Number</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
<th>Amplicon Size (bp)</th>
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<tr>
<td>Cyp2c29</td>
<td>NM_007815</td>
<td>ATCTGGCTCTTTGACG</td>
<td>AGTAGGCTTTGAGCCAAATAC</td>
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<tr>
<td>Cyp2c37</td>
<td>NM_010001</td>
<td>GCACTAATGGAATGGGCTTGG</td>
<td>GCAAGCGTCTTCTCTGAAAGC</td>
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<tr>
<td>Cyp2d9</td>
<td>NM_010006</td>
<td>AGTCCTCCCTTTACTCCCTGAG</td>
<td>CGCAAGATATCCGGAGTGC</td>
<td>51</td>
</tr>
<tr>
<td>Cyp2d22</td>
<td>NM_019823</td>
<td>GACACCTCTTACAGCCCTAACA</td>
<td>GAACCTGGCTATCTGACT</td>
<td>101</td>
</tr>
<tr>
<td>Cyp2e1</td>
<td>NM_021282</td>
<td>TCCCTAATGCTCGCCCTGAG</td>
<td>GAATCAGAAGGCTTTGGA</td>
<td>189</td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>NM_007818</td>
<td>CGGCCCTCTGACGCACTAG</td>
<td>CTTTGCCCTGCGCTCAAGT</td>
<td>260</td>
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<tr>
<td>Cyp3a13</td>
<td>NM_007819</td>
<td>CCGCCTGCTCGGAGGCAGT</td>
<td>CCGGAGGGTGTGAGAGTTAGT</td>
<td>192</td>
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<tr>
<td>Abcb1a</td>
<td>NM_011076</td>
<td>CCCCGAGATTGCAGCTAC</td>
<td>ACTTCAACAGAGCAGCTAC</td>
<td>76</td>
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<tr>
<td>Abcb1b</td>
<td>NM_011075</td>
<td>GACGTGACTATCGAGGAGT</td>
<td>GCCCTCCTATCGAGGAGTTA</td>
<td>114</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>X00686</td>
<td>GTCTGTGATGCGCTTAGT</td>
<td>AGCTTAGCCGCCATTAC</td>
<td>177</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of hydrocotarnine on the CYP activities in vitro

The inhibitory effects of hydrocotarnine (○) or positive control inhibitors (●: verapamil for CYP3A4, sulfaphenazole for CYP2C9, transylcypromine for CYP2C19, quinidine for CYP2D6, diethyldithiocarbamic acid for CYP2E1) were investigated using recombinant CYP isozymes. Data are expressed as means±SE (n=3).

Supermix (Bio-Rad Laboratories, Tokyo, Japan), 3 µL forward primer (300 nM) (Invitrogen, Tokyo, Japan) and 3 µL reverse primer (300 nM) (Invitrogen); The sequence of each primer is shown in Table 2. PCR mastermix (46 µL) was filled in each well of the Multiplate® PCR Plates 96-well clear (Bio-Rad Laboratories) and 4 µL cDNA (0.5 ng reverse transcribed RNA for 18S rRNA, 10 ng for others) was added as PCR template. Well plate was placed into the My iQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories). The following real-time PCR experimental run protocol was used: denaturation program (95°C for 3 min), amplification and quantification program repeated 40 times (95°C for 15 s, 56°C for 30 s, 72°C for 30 s with a single fluorescence measurement), melting curve program (60–95°C with a heating rate of 0.5°C per 10 seconds and a continuous fluorescence measurement) and finally a cooling step to 4°C. The threshold cycles (Ct) were used to quantify the mRNA expression levels of samples with 18S rRNA normalization.

Statistical analyses The results are shown as means±SE. Student t-test was used to evaluate the statistical significance.

Results

Effect of hydrocotarnine on the CYP activity in vitro The effects of hydrocotarnine on the activities of CYP3A4, 2C9, 2C19, 2D6, and 2E1 are shown in Figure...
and the IC_{50} values for the CYP inhibition by hydrocotarnine and positive controls are summarized in Table 3. For each CYP isozyme, the inhibitory effect of hydrocotarnine was smaller than the respective positive control inhibitor. Hydrocotarnine (0.000388–38.8 μM) did not induce a significant change in the metabolic activities of CYP2C9, 2C19, and 2E1. Although weak inhibitory effects were observed on CYP3A4 and 2D6, the inhibition rate was less than 50% at the concentrations below 3.88 μM.

**Effect of hydrocotarnine on the ATPase activity in vitro**  

The effects of hydrocotarnine on the ATPase activity of human P-gp membranes are shown in Figure 2. The ATPase activity was inhibited by orthovanadate both in the absence and presence of verapamil. On the other hand, hydrocotarnine did not affect the ATPase activity at the concentrations of 0.000388–38.8 μM in the absence of verapamil, suggesting that hydrocotarnine is not a substrate of P-gp. The results in the presence of 50 μM verapamil showed a similar pattern as that in its absence, which suggests that hydrocotarnine does not in-

### Table 3. The IC_{50} values for the CYP inhibition by hydrocotarnine and positive controls

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC_{50} (μM)</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydrocotarnine</td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>18.4</td>
<td>0.594 (verapamil)</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>—</td>
<td>0.230 (SFZ)</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>—</td>
<td>6.51 (TCP)</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>6.63</td>
<td>0.00779 (quinidine)</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>—</td>
<td>0.111 (DDTC)</td>
</tr>
</tbody>
</table>

The IC_{50} values were calculated by linear extrapolation using the data indicated in Fig. 1.

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**Fig. 2. Effect of hydrocotarnine on the ATPase activity in vitro**  
The effects of hydrocotarnine (△) or orthovanadate (◆) on the ATPase activity was investigated using human P-gp membranes in the presence (△) or absence (◆) of verapamil. Data are expressed as means ± SE (n = 3).

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**Fig. 3. Effect of hydrocotarnine treatment on the CYP and P-gp mRNA levels in the mouse liver**  

mRNA levels were measured by the real-time RT-PCR after treatment of mice with saline (open column) or hydrocotarnine 0.4 mg/kg (closed column) once daily for 14 days. Data are expressed as means ± SD (n = 5). N.D.: not detected.
Fig. 4. Effect of hydrocotamine treatment on the CYP and P-gp mRNA levels in the mouse small intestine
mRNA levels were measured by the real-time RT-PCR after treatment of mice with saline (open column) or hydrocotamine 0.4 mg/kg (closed column) once daily for 14 days. Data are expressed as means ± SD (n = 5).

Effect of hydrocotamine on the CYP and P-gp expression in vivo  Figure 3 shows the mRNA expression levels of CYP isozymes and P-gp in the liver of mice treated with hydrocotamine (0.4 mg/kg) or saline once daily for 14 days. Abcb1a was not detected in the liver samples. None of the CYP isozymes or P-gp investigated showed a significant difference in their mRNA expression levels between hydrocotamine-treated and control mice. The mRNA levels of CYP isozymes or P-gp were also unchanged in the small intestine of the corresponding mice (Fig. 4).

Discussion

The effects of hydrocotamine on the activities of major CYP isozymes and P-gp have been investigated in this study to elucidate the mechanism of its potentiating effect on oxycodone analgesia and to evaluate the possibility that hydrocotamine affects the pharmacokinetics of other drugs when administered concomitantly.

The major metabolic pathway of oxycodone is reported to be CYP3A4-mediated N-demethylation to noroxycodone which has a lower affinity for human μ-opioid receptor than oxycodone.1,2) Oxycodone also undergoes CYP2D6-mediated O-demethylation to oxymorphone which is a potent ligand for human μ-opioid receptor with 40-times higher affinity compared with oxycodone.1,2) Although the plasma concentration of oxymorphone is reported to be much lower than that of oxycodone after oral administration of oxycodone in healthy human volunteers,2) changes in the CYP activities induced by factors such as concomitant drugs or disease states could lead to the alteration of pharmacokinetics, thus altering the pharmacological effects of oxycodone.

In the in vitro studies using recombinant CYP isozymes, hydrocotamine (0.000388–38.8 μM) did not induce a significant change in the metabolic activities of CYP2C9, 2C19, and 2E1 (Fig. 1). Although weak inhibitory effects were observed on CYP3A4 and 2D6 with IC₅₀ values of around 18.4 μM and 6.63 μM, respectively (Table 3), these interactions did not seem to be clinically relevant considering that the steady-state plasma concentration of hydrocotamine in clinical practice is usually less than 1 ng/mL (4.5 nM).2,9,10)

The effect of hydrocotamine on P-gp was investigated in this study by an ATPase assay using human P-gp membranes. The ATPase assay has been reported to allow more efficient screening for substrates and inhibitors of P-gp compared with other in vitro assays such as transcellular transport assay.11) Hydrocotamine (0.000388–38.8 μM) did not cause a significant change in the ATPase activity both in the presence and absence of verapamil (Fig.
suggesting that hydrocotarnine is neither a substrate nor an inhibitor of P-gp.

In the mouse in vivo study, the mRNA levels of Cyp2c29, 2c37, 2d9, 2d22, 2e1, 3a11, 3a13 and Abcb1b in the liver and small intestine and Abcb1a in the small intestine were unchanged by hydrocotarnine treatment for 14 days (Figs. 3 and 4). Although no information is available on the elimination pathways of hydrocotarnine either in mice or in humans, these findings suggest that hydrocotarnine or its metabolite(s) does not have the potential to affect the expression levels of major CYP iso-zymes and P-gp. Further studies would be necessary to confirm the lack of in vivo effects of hydrocotarnine on the protein expression and activities of CYP and P-gp. In conclusion, it is unlikely that the potentiation of the oxycodone effect by hydrocotarnine involves its effect on CYP and P-gp. The findings of this study also demonstrate that hydrocotarnine is unlikely to cause drug interactions via CYP or P-gp. Although changing the administration route of oxycodone needs caution with only the injection product containing hydrocotarnine, the findings of the present study provide some useful information for avoiding unwanted drug interactions.

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References


