Effect of γ-Oryzanol on Cytochrome P450 Activities in Human Liver Microsomes

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The effects of γ-oryzanol, a drug mainly used for the treatment of hyperlipidaemia, on several cytochrome P450 (CYP) specific reactions in human liver microsomes were investigated to predict drug interactions with γ-oryzanol in vivo from in vitro data. The following eight CYP catalytic reactions were used in this study: CYP1A1/2-mediated 7-ethoxyresorufin O-deethylation, CYP2A6-mediated coumarin 7-hydroxylation, CYP2B6-mediated 7-benzoxazlyresorufin O-debenzylation, CYP2C8/9-mediated tolbutamide methylhydroxylation, CYP2C19-mediated S-mephenytoin 4'-hydroxylation, CYP2D6-mediated bufuralol 1'-hydroxylation, CYP2E1-mediated chlorozoxazone 6-hydroxylation, and CYP3A4-mediated testosterone 6β-hydroxylation. γ-Oryzanol had little inhibitory effects on CYP activities, indicating that this compound would not be expected to cause clinically significant interactions with other CYP-metabolized drugs at expected therapeutic concentrations.

Key words γ-oryzanol; in vitro drug–drug interaction; human liver microsome

g-oryzanol is a substance extracted from rice bran oil, and has been widely used clinically due to its effectiveness in treating autonomic imbalance and menopausal disorders. In addition, it has been reported that γ-oryzanol also has a cholesterol-lowering effect.1–3) γ-Orzanol is mainly metabolized to ferulic acid in animals and humans (Fig. 1).4–7) Following a single oral administration of γ-oryzanol at 300 mg to humans, the peak plasma concentrations of γ-oryzanol and ferulic acid were 37.6 ng/ml and 36.6 ng/ml, respectively.4) Ferulic acid was excreted in urine at 2.4–2.8% of the dose/d but γ-oryzanol was not detected.

Most oxidative metabolism of drugs is catalyzed by cytochrome P450 (CYP) enzymes, which comprise a large family of hemoproteins.8,9) More than 15 isozymes have been identified in human liver, and several forms play important roles in the metabolism of drugs.10) It is important to identify the enzymes responsible for the metabolism of drugs and to investigate further whether a drug has an inhibitory effect on the catalytic activity of each CYP enzyme to consider possible drug interactions. A previous in vitro study showed that γ-oryzanol was hydrolyzed to ferulic acid in rat liver, intestine, pancreas and kidney preparations, with possible involvement of carboxylesterase.11) However, it is unclear whether γ-oryzanol has an inhibitory effect on CYP catalytic activities. In this study, we examined the characteristic properties of γ-oryzanol on the catalytic activities of CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 using specific reaction probes for the enzymes.

MATERIALS AND METHODS

Chemicals γ-Oryzanol was supplied by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Coumarin, tolbutamide, chlorozoxazone, glucose-6-phosphate disodium salt; hydride, and β-NADP sodium salt were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were obtained from the following sources: 7-ethoxyresorufin, 4-hydroxytolbutamide, S-(+)-mephenytoin, (±)-4'-hydroxy-mephenytoin, 1'-hydroxybufuralol maleate salt, (±)-bufuralol hydrochloride salt, 6-hydroxychlorzoxazone, and 6β-hydroxytestosterone from Salford Ultrafine Chemical & Research Ltd. (Guildhall Close, Manchester, U.K.); 7-benzoxylresorufin from Molecular Probes (Eugene, OR, U.S.A.); 7-hydroxycoecmarin from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); resorufin, testosterone, and magnesium chloride hexahydrate from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); and glucose-6-phosphate dehydrogenase (approximately 350 U/mg, 1 mg/ml) from Boehringer Mannheim GmbH (Mannheim, Germany). All other reagents and solvents were of high analytical grade.

Liver Microsome Preparation Pooled human liver microsomes from 10 donors were prepared at the Biomedical Research Institute, Human and Animal Bridge Discussion Group (HAB, Chiba, Japan). Human liver samples were legally procured from the National Disease Research Interchange (NDRI, PA, U.S.A.) through the international partnership between the NDRI and HAB. The study was conducted in accordance with the Declaration of Helsinki. Human livers were homogenized with 0.25 M sucrose containing 3 mM Tris and 0.1 mM EDTA (pH 7.4), and microsomes were isolated by differential centrifugation using the common method.12) Washed microsomes were resuspended in 100 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol at protein concentrations of 20 mg/ml.

Determination of Human CYP Activities 7-Ethoxyresorufin O-deethylation activity by CYP1A1/2, coumarin 7-hydroxylase activity by CYP2A6, 7-benzoxylresorufin O-debenzylation activity by CYP2B6, tolbutamide methylhydroxylation activity by CYP2C8/9, S-mephenytoin 4'-hydroxylation activity by CYP2C19, bufuralol 1'-hydroxylation activity by CYP2D6, chlorozoxazone 6-hydroxylation activity by CYP2E1, and testosterone 6β-hydroxylation activity by CYP3A4 were determined in the presence of CYP2C19, CYP2D6, CYP2E1, and CYP3A4 using specific reaction probes for the enzymes.

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determined as previously described.\textsuperscript{13}

Standard incubation mixtures of 0.5 ml contained microsomal protein (0.1—0.5 mg), 0.1 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, NADPH-generating system (2.5 mM β-NADP, 25 mM glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, and 10 mM magnesium chloride), and substrates with or without γ-oryzanol. γ-Oryzanol was dissolved in dimethyl sulfoxide and added to incubations at a volume of 5 μl. The precipitate of test compound was not visually observed in the reaction system. Substrates were dissolved in the following solvents: 7-ethoxyresorufin and 7-benzoxylresorufin in dimethyl sulfoxide; coumarin and bufuralol in ethanol; tolbutamide, S-mephenytoin, and testosterone in methanol; and chlorzoxazone in 1% (w/v) aqueous sodium carbonate. They were added to incubations at a volume of 5 μl.

Product formation was determined using HPLC and a fluorescence spectrophotometer. Enzyme incubations and metabolite analysis were carried out in duplicate.

Assay methods were validated in this study. Calibration curves for resorufin, 7-hydroxycoumarin, 4-hydroxybufuralol, 6-hydrochlorzoxazone, and 6β-hydroxysterosterone were established with respective calibration ranges of 0.2—200 μM (γ = 0.9996), 0.025—5 μM (γ = 1.0000), 0.05—10 μM (γ = 0.9998), 0.025—5 μM (γ = 0.9996), 0.025—5 μM (γ = 0.9995), 0.25—100 μM (γ = 0.9994), and 0.03—30 μM (γ = 0.9999).

The substrate concentrations used to estimate the kinetic parameters for each assay were 7-ethoxyresorufin 0.01—10 μM, coumarin 0.1—100 μM, 7-benzoxylresorufin 0.03—10 μM, tolbutamide 25—1000 μM, S-mephenytoin 5—500 μM, bufuralol 5—200 μM, chlorzoxazone 10—400 μM, and testosterone 10—250 μM. For the determination of residual activity in the presence of γ-oryzanol (1—30 μg/ml), the concentrations of substrates were 7-ethoxyresorufin 0.5 μM, coumarin 2 μM, 7-benzoxylresorufin 1 μM, tolbutamide 100 μM, S-mephenytoin 100 μM, bufuralol 20 μM, chlorzoxazone 100 μM, and testosterone 100 μM. Selective CYP inhibitors were used in this study to validate that the assays were working properly. 7,8-Benzoflavone,\textsuperscript{14} furafylline,\textsuperscript{15} ketoconazole,\textsuperscript{22} which are inhibitors of CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, respectively, inhibited the respective enzyme activities (data not shown). Diethylthiocarbamate is also known to be a specific inhibitor of CYP2A6,\textsuperscript{43} and the current study confirmed the potent inhibitory capability of this compound on CYP2A6-mediated metabolism (data not shown).

**Data Analysis** Apparent \( K_m \) and \( V_{max} \) values for the formation of metabolites were calculated using nonlinear regression analysis on the computer program WinNonLin Standard (Version 2.1, Scientific Consulting, Inc., Apex, NC, U.S.A.).

**RESULTS AND DISCUSSION**

Multiple-drug therapy is a common therapeutic practice, particularly for patients with various diseases. Whenever two or more drugs are administered concurrently, there is the possibility of drug interactions. Many drug interactions are clinically caused by inhibition of drug-metabolizing enzymes, CYPs, leading to decreased metabolic clearance and increased exposure to the inhibited drug. The inhibition of CYP enzymes should thus be examined to assess the potential for drug interactions.

The current study examines the *in vitro* ability of γ-oryzanol to inhibit the metabolism of substrates for CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. γ-Oryzanol had almost no inhibitory effects on CYP-mediated pathways (Table 1).

After an oral dose of 600 mg in healthy volunteers, the peak plasma concentration range of γ-oryzanol was 21.0 to 106.8 ng/ml.\textsuperscript{4} Moreover, the steady-state peak plasma concentration of γ-oryzanol was 111.7 ng/ml after repetitive oral administration of γ-oryzanol at 100 mg three times a day for 10 d.\textsuperscript{5} To extrapolate *in vitro* inhibition data to *in vivo* situations, free concentrations of drug and inhibitor at the site of metabolism are important.\textsuperscript{23,24} However, it is difficult to evaluate the free concentration of γ-oryzanol at an enzyme site due to the lack of information about the plasma protein binding of γ-oryzanol. The lower plasma level of γ-oryzanol compared with the highest tested concentration (30 μg/ml) suggested that γ-oryzanol would cause little or no inhibitory effect on the metabolism of drugs metabolized by CYPs even if intracellular binding or accumulation of γ-oryzanol in hepatocytes occurs. In addition, there are no clinical reports indicating that γ-oryzanol causes drug–drug interactions with other CYP-metabolized drugs.

In conclusion, the results of the *in vitro* experiments performed with human liver microsomes indicate that γ-oryzanol has little inhibitory effect on CYP-mediated catalytic activities and that this compound, at anticipated thera-

<table>
<thead>
<tr>
<th>CYP</th>
<th>Reaction</th>
<th>( K_m ) (μM)</th>
<th>( V_{max} ) (pmol/min/mg protein)</th>
<th>% Control activity 1 μg/ml</th>
<th>3 μg/ml</th>
<th>10 μg/ml</th>
<th>30 μg/ml</th>
</tr>
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<tbody>
<tr>
<td>CYP1A1/2</td>
<td>7-Ethoxyresorufin O-deethylation</td>
<td>0.519</td>
<td>15.0</td>
<td>85</td>
<td>86</td>
<td>86</td>
<td>84</td>
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<tr>
<td>CYP2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>1.73</td>
<td>163</td>
<td>86</td>
<td>86</td>
<td>85</td>
<td>81</td>
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<tr>
<td>CYP2B6</td>
<td>7-Benzoxylresorufin O-debenzylation</td>
<td>1.59</td>
<td>4.83</td>
<td>93</td>
<td>98</td>
<td>85</td>
<td>71</td>
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<tr>
<td>CYP2C8/9</td>
<td>Tolbutamide methylhydroxylation</td>
<td>442</td>
<td>84.9</td>
<td>96</td>
<td>100</td>
<td>98</td>
<td>98</td>
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<tr>
<td>CYP2C19</td>
<td>S-Mephenytoin 4’-hydroxylation</td>
<td>86.0</td>
<td>8.54</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>96</td>
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<tr>
<td>CYP2D6</td>
<td>Bufuralol 1’-hydroxylation</td>
<td>21.7</td>
<td>61.0</td>
<td>109</td>
<td>105</td>
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<td>CYP2E1</td>
<td>Chlorzoxazone 6-hydroxylation</td>
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<td>2600</td>
<td>96</td>
<td>102</td>
<td>104</td>
<td>96</td>
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<td>CYP3A4</td>
<td>Testosterone 6β-hydroxylation</td>
<td>136</td>
<td>5540</td>
<td>99</td>
<td>98</td>
<td>94</td>
<td>91</td>
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</table>

The control activities were 7.25, 74.8, 1.44, 8.52, 2.39, 10.9, 109, and 1490 pmol/min/mg for CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4-catalyzed reactions, respectively. The values of the percent of control activity are the average of duplicate determinations (control = 100%).

Table 1. Effect of γ-Oryzanol on Cytochrome P450-Catalyzed Oxidase Activities in Human Liver Microsomes
peutic concentrations, would not be expected to cause clinically significant interactions with other CYP-metabolized drugs.

REFERENCES

1) Kuzuya F., Yoshimine N., Kato S., Fujita K., Ushigome H., Geriatric Medicine, 18, 519—524 (1980).