Cysteinyl leukotrienes (cysLTs) are 5-lipoxygenase pathway products of arachidonate that induce bronchoconstriction, vascular hyperpermeability, mucosal edema accumulation, and mucus secretion. Pranlukast is a selective cysLTs receptor antagonist, and a 225 mg twice-daily dose has been used to treat bronchial asthma and allergic rhinitis in Japan.

The absorption fraction of pranlukast in human is approximately 20%, based on the excretion ratio of the unchanged form in the feces following oral administration (the absolute bioavailability has not been reported). The terminal elimination half-life of pranlukast in plasma is approximately 2 h. Pranlukast is minimally excreted in the urine. The major metabolic pathway of pranlukast is shown in Fig. 1. The plasma-binding of pranlukast is more than 99%, and the major binding protein is albumin.

To date, it has been very difficult to predict drug–drug interactions with pranlukast in the clinical setting due to a lack of information. Therefore, we conducted in vitro experiments using human liver microsomes to allow us to evaluate the potential for drug–drug interactions, based on the metabolism and the inhibitory effects of pranlukast.

MATERIALS AND METHODS

**Materials** Pranlukast and 6-methyl-4-oxo-8-[4-(4-phenylbutoxy)benzoylamino]-2-(tetrazol-5-yl)-4H-1-benzopyran (ONO-RS-425, an internal standard for pranlukast), were synthesized at Ono Pharmaceutical Co., Ltd. (Osaka, Japan). α-Naphthoflavone, quinidine, 4-hydroxybenzoic acid n-butyl ester and 4-hydroxybenzoic acid n-amyl ester were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Tranilcypromine, terfenadine, metoprolol, tolbutamide, erythromycin and roxithromycin were pur-
chased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Midazolam and clarithromycin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-Cyano-7-ethoxycoumarin (CEC), 7-methoxy-4-trifluoromethylcoumarin, 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC), sulpha-phenazole, human B-lymphoblastoid cells expressing human cytochrome P450 (CYP), and anti-CYP and flavin-containing monooxygenase (FMO) antibodies were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Human liver microsomes were purchased from Xenotech LLC (Lenexa, KS, U.S.A.) and KAC Co., Ltd. (Kyoto, Japan).

**Metabolism of Pranlukast with Human B-Lymphoblastoid Cells Expressing Human CYPs**

Reaction mixtures consisting of 1 μmol/l pranlukast, 2 mg/ml of human microsomes from B-lymphoblastoid cells expressing human CYPs and 2 mmol/l reduced nicotinamide adenine dinucleotide phosphate (NADPH) in 100 mmol/l potassium phosphate buffer (pH 7.4) containing 5 mmol/l MgCl₂ were incubated for 120 min at 37 °C. The enzyme reactions were terminated with the addition of ethanol containing an internal standard (ONO-RS-425). The data are presented as the means of duplicate samples. Pranlukast levels in the incubation mixtures were quantified based on the peak areas.

**Metabolism of Pranlukast with Human Microsomes Containing Anti-CYP or FMO Antibodies**

Anti-CYP or FMO antibodies (1 μg protein of antibody/1 μg protein of microsome) were mixed with human liver microsomes before the enzyme reactions. Reaction mixtures consisting of 1 μmol/l pranlukast, 0.2 mg/ml human liver microsomes and 2 mmol/l NADPH in 100 mmol/l potassium phosphate buffer (pH 7.4) containing 5 mmol/l MgCl₂ were incubated for 30 min at 37 °C. The enzyme reactions were terminated with the addition of ethanol containing an internal standard (ONO-RS-425). The data are presented as the means of duplicate samples. Pranlukast levels in the incubation mixtures were determined by an HPLC-UV detection method described above.

**Effect of Ketoconazole on Pranlukast Metabolism**

Reaction mixtures consisting of 0.2 mg/ml human liver microsomes and 2 mmol/l NADPH in 100 mmol/l potassium phosphate buffer (pH 7.4) containing 5 mmol/l MgCl₂ were preincubated for 5 min at 37 °C. Ketoconazole (final concentrations: 1, 3, 10 and 30 μmol/l) and pranlukast (final concentration: 1 μmol/l) was used at a flow rate of 1.0 ml/min. Pranlukast was quantified based on the peak areas.

**Effect of Macrolides on Pranlukast or Midazolam Metabolism in Human Liver Microsomes**

Reaction mixtures consisting of 0.2 mg/ml human liver microsomes and 2 mmol/l NADPH in 100 mmol/l potassium phosphate buffer (pH 7.4) containing 5 mmol/l MgCl₂ were preincubated for 5 min at 37 °C. Macrolide solutions were added to the mixtures and incubated for an additional 30 min at 37 °C, followed by the addition of pranlukast or midazolam and another 37 °C incubation for 30 or 6 min. The enzyme reactions were terminated with the addition of ethanol containing an internal standard (ONO-RS-425 for pranlukast, 4-hydroxybenzoic acid n-butyl ester for midazolam). The final pranlukast and midazolam concentrations were 1 and 5 μmol/l, respectively, whereas clarithromycin, erythromycin and roxithromycin concentrations ranged from 1 to 100 μmol/l. The data are presented as the means of duplicate samples. Pranlukast and midazolam levels in the incubation mixture were determined by an HPLC-UV detection method. For pranlukast measurements, the method was as described above. For midazolam measurements, the HPLC system consisted of an Agilent model 1100 series set at 220 nm and a TSK-GEL ODS-120T reverse-phase column (150×4.6 mm inner diameter, TOSOH CORPORATION, Tokyo, Japan). A gradient with mobile phase A (18/1/1 mixture of 10 mmol/l sodium acetate/acetonitrile/methanol) and mobile phase B (2/1 mixture of methanol/acetonitrile) was used at a flow rate of 1.0 ml/min. The percent of B changed as follows: 48% (0 min), 48% (9 min), 58% (10 min), 58% (18 min), 100% (20 min) and 100% (25 min). Midazolam was quantified based on the peak areas.

**Effect of Pranlukast on Tolbutamide Metabolism**

Reaction mixtures consisting of 0.2 mg/ml human liver microsomes and 2 mmol/l NADPH in 100 mmol/l potassium phosphate buffer (pH 7.4) containing 5 mmol/l MgCl₂ were preincubated for 5 min at 37 °C. Tolbutamide (final concentrations: 200, 400 and 800 μmol/l) and pranlukast (final concentrations: 1, 3, 10 and 30 μmol/l) solutions were added to the mixture and incubated for an additional 90 min at 37 °C. The enzyme reactions were terminated with acetic ether containing hydrochloric acid and an internal standard (4-hydroxybenzoic acid n-butyl ester). 4-Hydroxytolbutamide levels in the incubation mixture were determined by an HPLC-UV detection method. The HPLC system consisted of an Agilent model 1100 series set at 230 nm and a LiChroCART 250-4 (MERCK). A gradient with mobile phase A (690/310/1 mixture of water/acetonitrile/perchloric acid) and mobile phase B (600/400/1 mixture of acetonitrile/water/perchloric acid) was used at a flow rate of 1.0 ml/min. The percent of B changed as follows: 0% (0 min), 0% (5 min), 75% (20 min), 0% (20.1 min) and 0% (25 min). 4-Hydroxytolbutamide was quantified based on the peak areas.

**Effect of Pranlukast on Terfenadine Metabolism**

Reaction mixtures consisting of 4.5 μmol/ml human CYP3A4-expressing microsomes and 2 mmol/l NADPH in 100 mmol/l potassium phosphate buffer (pH 7.4) containing 5 mmol/l MgCl₂ was preincubated for 5 min at 37 °C. Terfenadine was added into reaction mixtures at a concentration of 1 or 10 μmol/l. The data are presented as the means of duplicate samples.
(final concentrations: 1, 2, 5 and 10 μmol/l) and pranlukast (final concentrations: 0, 1 and 5 μmol/l) solutions were added to the mixture and incubated for additional 0, 10, 20 and 30 min at 37 °C. The enzyme reactions were terminated with the addition of methanol containing an internal standard (metoprolol). Terfenadine concentrations in the incubation mixture were determined by an HPLC-UV detection method. The HPLC system consisted of a model LC module 1 (Waters) and fluorescence detector (Hitachi) set at EX 230 nm and Em 280 nm, and a YMC-Pack A-302 (YMC). The mobile phase (89/118 mixture of acetonitrile/50 mmol/l ammonium acetate (pH 5.1)) was used at a flow rate of 1.2 ml/min. Terfenadine was quantified based on the peak areas.

RESULTS

Metabolism of Pranlukast in Expressed Recombinant CYP Isoforms The metabolism of pranlukast was investigated in microsomes derived from human B-lymphoblastoid cells expressing human CYP isoforms (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) based on the elimination rate of the unchanged form after incubations of pranlukast (1 μmol/l) at 37 °C (Fig. 2). Pranlukast was primarily metabolized by CYP3A4, and some metabolism by CYP2C8 was also confirmed.

Inhibition of Pranlukast Metabolism by Anti-CYP Antibodies, Anti-FMO Antibody and Ketoconazole CYP isoforms involved in the metabolism of pranlukast were examined by the elimination rate of the unchanged form after incubation of pranlukast (1 μmol/l) with human liver microsomes in the presence or absence of anti-CYP or anti-FMO antibodies at 37 °C (Fig. 3). The percent inhibition by anti-CYP3A4 and anti-CYP2C8 antibodies was approximately 44% and 19%, respectively. Figure 4 shows the effect of the specific CYP3A4 inhibitor, ketoconazole, on pranlukast metabolism. One to 3 μmol/l of ketoconazole inhibited pranlukast metabolism in human liver microsomes by approximately 45 to 56%.

Effect of Macrolides on Pranlukast and Midazolam Metabolism Figure 5 shows the effect of macrolides (clarithromycin, erythromycin and roxithromycin), irreversible inhibitors of CYP3A4, on pranlukast and midazolam metabolism. The apparent dissociation constant (Kapp) and the maximum inactivation rate constant (kinact) of macrolides on the metabolism of midazolam, a typical CYP3A4 substrate, were calculated and compared with the inhibitory activity of macrolides against pranlukast. Pranlukast metabolism was slightly inhibited by preincubation of a microsomal reaction mixtures with macrolides for 30 min. However, the macrolides had little effect on pranlukast metabolism, and therefore the Kapp and kinact could not be calculated. In contrast, midazolam metabolism was inhibited by macrolides in a dose- and preincubation-time-dependent manner. The Kapp and kinact were 24.1 μmol/l and 0.0472 min⁻¹, respectively, for clarithromycin, and 12.1 μmol/l and 0.0390 min⁻¹, respectively, for erythromycin.

Effect of Pranlukast on CYP Metabolism Table 1 shows the effect of pranlukast on the metabolism of fluorescent substrates by human B-lymphoblastoid cells expressing human CYP isoforms (CYP1A2, CYP2C9, CYP2C19 and CYP2D6). Pranlukast inhibited the metabolism by CYP2C9, with an IC50 value <1 μmol/l. Figure 6 shows the effect of pranlukast on tolbutamide metabolism by human liver micro-
Tolbutamide hydroxylation by CYP2C8 and/or 2C9 was competitively inhibited by co-incubation with pranlukast, with a $K_i$ value of 3.9 µmol/l. Pranlukast was primarily metabolized by CYP3A4, and thus, pranlukast might inhibit metabolism of other compounds mediated by CYP3A4. Figure 7 shows the effect of pranlukast on terfenadine metabolism by human B-lymphoblastoid cells expressing the human CYP3A4 isoform. Terfenadine metabolism by CYP3A4 was competitively inhibited by co-incubation with pranlukast, with a $K_i$ value of 4.1 µmol/l.

### Table 1. Effect of Pranlukast on Metabolism by Human B-Lymphoblastoid Cells Expressing Human CYPs

<table>
<thead>
<tr>
<th>CYP isoforms</th>
<th>Fluorescent substrate</th>
<th>Concentration of substrate</th>
<th>Concentration of pranlukast</th>
<th>Positive control</th>
<th>Concentration of positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>CEC</td>
<td>10 µmol/l</td>
<td>0.0</td>
<td>98.3</td>
<td>α-NF</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>7-MFC</td>
<td>75 µmol/l</td>
<td>56.5</td>
<td>93.6</td>
<td>SUL</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>CEC</td>
<td>25 µmol/l</td>
<td>5.0</td>
<td>49.1</td>
<td>TCP</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>AMMC</td>
<td>1.5 µmol/l</td>
<td>5.0</td>
<td>60.0</td>
<td>QN</td>
</tr>
</tbody>
</table>

CEC, 3-cyano-7-ethoxycoumarin; 7-MFC, 7-methoxy-4-trifluoromethylcoumarin; AMMC, 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin; α-NF, α-naphthoflavone; SUL, sulphaphenazole; TCP, tranylcypromine; QN, quinidine.

**Comparison between $K_i$ Value and the Concentration of Pranlukast in Plasma** The concentration of inhibitor at
Pranlukast concentrations were 0 (closed circle), 200 (closed diamond), 400 (closed triangle) and 800 (closed square) μmol/l, respectively. Human liver microsomes were incubated with terfenadine and pranlukast in the presence of 20 μmol/l NADPH for 5 min. Each data point presents the mean of duplicate determinations.

**DISCUSSION**

Pranlukast was primarily metabolized by CYP3A4, and slightly metabolized by CYP2C8 and other enzymes, as demonstrated using expressed recombinant enzymes, anti-CYP and anti-FMO antibodies. The contribution of CYP3A4 to the metabolism of pranlukast in vitro was deemed to be less than 50%, based on results with human liver microsomes in the presence of anti-CYP3A4 antibodies or ketoconazole. It was suggested that pranlukast was partly metabolized by other enzymes included in the microsomes. In order to estimate the increase in the area under the plasma concentration–time curve (AUC) of pranlukast by irreversible inhibition (mechanism-based inhibition), the effect of pre-incubation on pranlukast metabolism was investigated using the CYP3A4 mechanism-based inhibitors, clarithromycin, erythromycin and roxithromycin (macrolides). When macrolides were added to the reaction mixtures at 100 μmol/l, approximately 40% of pranlukast metabolism was inhibited after preincubation for 30 min. This result was reasonable, considering the relative contribution of CYP3A4 to the metabolism of pranlukast. However, because the slopes were small for each inhibitor when metabolic activities were plotted relative to pre-incubation times, the $K_{i}^*$ and $K_{max}$ values could not be calculated. Midazolam metabolism was inhibited by macrolides in a concentration- and preincubation time-dependent manner. The inhibitory activities of clarithromycin and erythromycin on midazolam metabolism were greater than that of roxithromycin in this study, suggesting that the in vitro metabolism results would reflect in vivo interactions. Since the inhibitory activities of all tested macrolides on pranlukast metabolism were very weak, it is considered that the in vivo effects of macrolides on pranlukast metabolism would be small.

Pranlukast metabolism was minimally affected by CYP3A4 inhibitors, because the contribution of CYP3A4 was only 50% of the total metabolism. It was estimated that the increase of the AUC for pranlukast would be double at most, even when pranlukast was coadministered with strong CYP3A4 inhibitors. The safety of pranlukast with a dose of 450 mg (twice the clinical dose) has been confirmed. Therefore, based on the above, it was considered that co-administration with a CYP3A4 inhibitor would not lead to adverse effects.

Additionally, the effects of pranlukast on metabolism by CYP2C9 and CYP3A4 were investigated. Liu et al. reported that pranlukast inhibited CYP2C9-mediated metabolism, but did not inhibit CYP3A4-mediated metabolism. With respect to CYP3A4-mediated metabolism, there are multiple active sites on the enzyme, and the effect of inhibitors varies from substrate to substrate. Midazolam and testosterone are typical CYP3A4 substrates with different active sites. We investigated the inhibitory effects of pranlukast on CYP3A4 metabolism by both substrates. The metabolism of midazolam and testosterone was inhibited by 30 to 100 μmol/l and 3 to 30 μmol/l of pranlukast, respectively (data not shown). We calculated $K_i$ values of pranlukast with terfenadine, the metabolism of which is related to both active sites involved in midazolam and testosterone metabolism. Liu et al. suggested that pranlukast did not inhibit CYP3A4 metabolism because midazolam was used as the substrate. Additionally Liu et al. and we used tolbutamide for CYP2C9-mediated metabolism. Hydroxylation of tolbutamide is mediated through CYP2C8, CYP2C9 and CYP2C19, but mainly CYP2C8 and 2C9. Therefore inhibition of tolbutamide hydroxylation by pranlukast is mainly based on CYP2C8 and/or 2C9 metabolism.

It is thought that the concentration of pranlukast in the liver is higher than that in the plasma after oral administration. To avoid a false-negative estimation due to underestimation of the concentration of inhibitor in the prediction of drug–drug interactions, we estimated $[I]_{in, max,u}$. If the $[I]_{in, max,u}/K_i$ ratio is less than 0.2, in vivo drug–drug inter-
actions were not reported, or there was no interaction predicted. The $[I]_{\text{in, max}}/K_i$ ratio for pranlukast was sufficiently small, and the possibility of interaction in vivo was considered to be extremely low when CYP2C8 and/or 2C9 or CYP3A4 substrates are co-administered.

REFERENCES