The calcium channel blocker diltiazem (DTZ) is used in treatment of hypertension and angina pectoris. It has recently been shown that long-term use of DTZ reduces cardiovascular morbidity and mortality (Hansson et al., 2000). The first-line metabolite desacetyl-DTZ (M1) exhibits approximately 50% of the vasodilating and mortality properties of DTZ (Yabana et al., 1985; Schoemaker et al., 1987; Li et al., 1992). In addition, M1 has been shown to exert an inhibitory effect on thrombocyte aggregation about 3-fold that of DTZ (Kiyomoto et al., 1983). Therefore, the overall clinical effect of DTZ might, in part, be mediated by M1.

Biotransformation of DTZ is substantial and complex and involves deacetylation, N-demethylation, and O-demethylation (Fig. 1). The former process is mediated by esterases, whereas the latter reactions are catalyzed by cytochrome P450 (P450) isoenzymes. More than 10 years ago, Pichard et al. (1990) revealed that the P450 subfamily 3A, which is primarily represented by the isoenzyme CYP3A4 in humans, played an important role in N-demethylation of DTZ. In a recent in vitro study, we showed that the isoenzyme CYP3A4 is involved in O-demethylation of DTZ (Molden et al., 2000). Since the estimated $K_m$ value of DTZ to CYP2D6 ($\approx 200 \mu M$) is considerably higher than that to CYP3A4 (20–50 $\mu M$) (Sutton et al., 1997; Jones et al., 1999; Molden et al., 2000), CYP3A4 probably plays a more prominent role than CYP2D6 in the metabolism of DTZ. However, we have earlier speculated that CYP2D6 might be important in the elimination of the deacetylated DTZ metabolite M1 (Molden et al., 2000; Åsberg et al., 1999).

Both CYP2D6 and CYP3A4 are involved in the further metabolism of M1, and the aim of this in vitro investigation was to characterize transformation of M1 through each of these isoenzymes using immortalized human liver epithelial cells transfected with either CYP2D6 or CYP3A4. The calcium channel blocker diltiazem (DTZ) is used in treatment of hypertension and angina pectoris. It has recently been shown that long-term use of DTZ reduces cardiovascular morbidity and mortality (Hansson et al., 2000). The first-line metabolite desacetyl-DTZ (M1) exhibits approximately 50% of the vasodilating and mortality properties of DTZ (Yabana et al., 1985; Schoemaker et al., 1987; Li et al., 1992). In addition, M1 has been shown to exert an inhibitory effect on thrombocyte aggregation about 3-fold that of DTZ (Kiyomoto et al., 1983). Therefore, the overall clinical effect of DTZ might, in part, be mediated by M1.

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**Experimental Procedures**

**Materials.** Clones of transfected human liver epithelial (THLE) cells were supplied from Dr. Katherine Mace at the Nestlé Research Center (Lausanne, Switzerland). Erythromycin and DTZ were obtained from Sigma (St. Louis, MO), whereas paroxetine was supplied from GlaxoSmithKline (Welwyn Garden City, Hertfordshire, UK). Authentic metabolites of DTZ were gifts from Tanabe Seiyaku (Osaka, Japan). M1 used in the experiments was prepared by hydrolysis of DTZ. Briefly, DTZ was dissolved in concentrated ammonia and incubated for 2 h at 37°C, followed by evaporation of the solvent. The residue was resolved in methanol, and the concentration of M1 in the solution was calibrated against authentic M1. There were no detectable amounts of DTZ, desacetyl-N-de-methyl-DTZ (M2), or desacetyl-O-de-methyl-DTZ (M4) in the prepared stock solution of M1.

**Cell Experiments.** THLE cells, obtained by transfection of the simian virus 40 large tumor antigen gene into primary hepatocytes (Pfeifer et al., 1995), did not express several important P450 isoenzymes, but clones expressing CYP3A4 (T5-3A4) or CYP2D6 (T5-2D6) were produced by subsequent transfection (Mace et al., 1997). Cell-culturing details are presented elsewhere (Molden et al., 2000). M1 was dissolved in the cell culture medium, and its metabolism was studied in the concentration ranges of 2.5 to 100 $\mu M$ (T5-2D6 cells) and 12.5 to 400 $\mu M$ (T5-3A4 cells). CYP2D6 and CYP3A4 activity was expressed as the respective formation of the metabolites M4 and M2 after 90 min (time linear formation), which was related to the protein content of the cells (Bradford, 1976). Samples of the culture medium were analyzed by high-performance liquid chromatography (CE column; UV 238 nm) (Christensen et al., 1999). Formation rates of metabolites in the studied concentration ranges of M1 were modeled by a nonlinear analysis without weighting of the
data (DeltaGraph Pro 3.5; Deltapoint, Inc., Monterey, CA). Calculation of enzyme-kinetic parameters was based on separate estimations of four experiments. In experiments with inhibitors, paroxetine was coincubated in concentrations ranging from 1 to 20 μM, whereas erythromycin was administered in the concentration interval from 12.5 to 400 μM (M1 concentration 100 μM). Approximate IC₅₀ values were visually defined from the graphical presentation of the relative metabolite production in the presence of increasing inhibitor concentrations. In a control experiment, M1 (100 μM) was incubated with non-P450-transfected THLE cells.

**Results**

The metabolite M4 (O-demethylation) was only detected in incubations with T5-2D6 cells, whereas the metabolite M2 (N-demethylation) was exclusively produced by T5-3A4 cells. Formation rates of both M4 and M2 could be described by a nonlinear, single-enzyme Michaelis-Menten model in the studied concentration ranges of M1 (Fig. 2, A and B). The average estimated Kₘ values (±S.D.) from formation rates of M4 (CYP2D6) and M2 (CYP3A4) were 5 ± 2 and 540 ± 188 μM, respectively. Estimated Vₘₐₓ values were almost equal for both metabolites, 0.46 ± 0.09 nmol/min/mg of protein for M4 and 0.56 ± 0.13 nmol/min/mg of protein for M2 (extrapolated). Due to development of cell toxicity when incubating M1 concentrations higher than 500 μM, it was not possible to obtain formation rates of M2 close to Vₘₐₓ.

In agreement with CYP2D6-catalyzing O-demethylation of M1, a dose-dependent reduction in the production of M4 was observed when coincubating M1 and paroxetine (a CYP2D6 inhibitor) in T5-2D6 cells (Fig. 3A). The IC₅₀ value of paroxetine was approximately 3 μM. Erythromycin (a CYP3A4 inhibitor) inhibited the conversion of M1 to M2 in incubations with T5-3A4 cells (Fig. 3B), and the IC₅₀ value was approximately 50 μM. Neither paroxetine nor erythromycin interfered with the detection of any of the analytes.

Based on the cumulative concentration of M1 and its metabolites in the cell culture medium, average (±S.D.) recoveries were 91 ± 7 and 83 ± 8% in experiments with T5-2D6 and T5-3A4 cells, respectively.

**Discussion**

The present in vitro experiments show that the primary deacetylated DTZ metabolite (M1) exhibits approximately 100-fold higher affinity to CYP2D6 compared with CYP3A4. Although the expression of CYP3A4 has been estimated to be approximately 15 times higher than CYP2D6 in human liver (Shimada et al., 1994), it is still likely that the relative contribution of CYP2D6 to the hepatic clearance of M1 is greater than that of CYP3A4. In contrast, since the affinity of DTZ to CYP2D6 is much lower compared with CYP3A4 (Jones et al., 1999; Molden et al., 2000; Sutton et al., 1997), CYP2D6 most probably is of limited importance in the metabolism of DTZ.

In an earlier study with renal transplant recipients treated with DTZ, we reported that a subgroup of the patients showed severalfold higher plasma concentrations of deacetylated DTZ metabolites (i.e., M1 and M2), but not the parent drug, compared with the rest of the study population (Åsberg et al., 1999). Others have also made similar observations (Andren et al., 1988), and in light of the data obtained in the present in vitro study, it could be speculated that patients showing such extensive accumulation of M1 and M2 might be representatives of the population with a deficient CYP2D6 phenotype (approximately

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**Fig. 1.** Chemical structure of diltiazem with the sites of biotransformation and the enzymes catalyzing each reaction indicated by arrows.

**Fig. 2.** Formation rates of the CYP2D6-mediated metabolite M4 in T5-2D6 cells (A) and of the CYP3A4-mediated metabolite M2 in T5-3A4 cells (B) as a function of the initial measured M1 concentration in four separate experiments.

Formation rates were measured as the accumulation of metabolites in the cell culture medium after 90 min of incubation related to the protein (prot) content in the cells. The data were described by a nonlinear, single-enzyme Michaelis-Menten model (regression coefficients, r², are indicated). Average (±S.D.) Kₘ values obtained were 5 ± 2 μM for CYP2D6 and 540 ± 188 μM for CYP3A4.
7% of Caucasians). Coadministration of quinidine, known as a potent inhibitor of CYP2D6, did not significantly increase plasma concentration of DTZ in healthy volunteers (Laganiere et al., 1996). Although the participants in the study with quinidine were not CYP2D6-genotyped, the results support that CYP2D6 plays a secondary role in the biotransformation of DTZ.

In conclusion, the present in vitro results suggest a major involvement of CYP2D6 in the in vivo metabolism of the deacetylated DTZ metabolite M1. Since M1 exhibits pharmacological activity, it might be advisable to consider individual CYP2D6 metabolic capacity when using DTZ, although this needs to be evaluated in controlled clinical studies.

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References


