Comparison of CYP3A Activities in a Subclone of Caco-2 Cells (TC7) and Human Intestine

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Purpose. To compare the activity of the CYP3A enzyme expressed by TC7, a cell culture model of the intestinal epithelial cell, to the activity of human intestinal CYP3A4, using terfenadine as a substrate.
Methods. The metabolism of terfenadine was investigated in intact cells and microsomal preparations from TC7, human intestine, and liver. The effect of two CYP3A inhibitors, ketoconazole and troleandomycin (TAO), on the metabolism of terfenadine was also examined.
Results. Only hydroxy-terfenadine was detected in TC7 microsomal incubations. In contrast, azacyclonol and hydroxy-terfenadine were detected in human intestinal and hepatic microsomal incubations. The K_m values for hydroxy-terfenadine formation in TC7 cells, intestine and liver microsomes were 1.91, 2.5, and 1.8, μM respectively. The corresponding V_{max} values were 2.11, 61.0, and 370 pmol/min/mg protein. K_m values for azacyclonol in intestinal and hepatic samples were 1.44 and 0.82 μM and the corresponding V_{max} values were 14 and 60 pmol/min/mg protein. The formation of hydroxy-terfenadine was inhibited by ketoconazole and TAO in human intestine and TC7 cell microsomes. The K_m and V_{max} values for terfenadine metabolism in intact TC7 cells were similar to those from TC7 cell microsomes.
Conclusions. Our results indicate that TC7 cells are a potentially useful alternative model for studies of CYP3A mediated drug metabolism. The CYP3A expressed by TC7 cells is not CYP3A4, but probably CYP3A5, making this cell line suitable for studies of colonic drug transport and metabolism.

KEY WORDS: TC7; Caco-2; CYP3A; terfenadine; intestinal metabolism.

INTRODUCTION

The small intestine plays an important role in man as an extrahepatic site of drug metabolism (1–3). Most intestinal drug metabolizing enzymes belong to the P450 enzymes superfamily (3). CYP3A4 accounts for the majority of the total intestinal P450 enzymes (4,5) and constitutes a biochemical barrier to the oral absorption of numerous compounds (1,2). CYP3A5 represents the predominant P450 in human colon (6). Differences in the activities of these enzymes could enhance intersubject variability in oral bioavailability. Thus, a greater understanding of the gastrointestinal distribution and regulation of CYP3A enzymes should help predict potential bioavailability problems and intestinal drug-drug interactions. Unfortunately, studies on intestinal P450 enzyme regulation are difficult to perform *in vivo,* partly because of the rapid maturation of enterocytes (3). Therefore, an *in vitro* model to study intestinal drug metabolism would be useful.

Following their characterization as a model for intestinal permeability (7), Caco-2 cells have been widely used for studying intestinal drug transport (8,9). Increasing recognition of the prevalence of small intestinal drug metabolism has led to the characterization of drug metabolizing enzymes in Caco-2 cells. Caco-2 cells express phenol sulfotransferase (10), UDP-glucuronyl transferase (11) and P450 enzymes (11,12).

A more recent study described a Caco-2 subclone (TC7) which expresses a CYP3A enzyme (13). Although Northern and Western blot analysis from that study indicated that this enzyme is different from small intestinal CYP3A4, functional data on this enzyme is not available. The objective of this work was to compare the CYP3A activity in TC7 cell microsomes with the CYP3A4 activity in human intestine. Terfenadine was used in this study as the model compound to probe CYP3A activity in intact TC7 cells and TC7 microsomes (14,15). It has been reported that the metabolism of terfenadine azacyclonol and hydroxy-terfenadine is mediated by CYP3A4 (14–17).

We investigated terfenadine metabolism in TC7 cells and human intestinal and hepatic microsomes. Michaelis-Menten parameters associated with the formation of hydroxy-terfenadine in TC7 microsomes were compared with those from human jejunum and liver. The activity of CYP3A enzyme was examined using appropriate enzyme inhibitors and antibodies. We conclude that TC7 cells express an enzyme that different from CYP3A4, probably CYP3A5 (6).

MATERIALS AND METHODS

Materials

Terfenadine, troleandomycin (TAO) glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and $NADP^+$ were purchased from Sigma Chemical Co. (St. Louis, MO). Hydroxy-terfenadine and azacyclonol were from Phoenix International (Montreal, Quebec, Canada). Ketoconazole was from Research Diagnostics (Flanders, NJ). Polyclonal antibody to rat CYP3A2 and control (pre-immune) IgG and M107r microsomes were purchased from Genetest Corporation (Woburn, MA). Human intestine and liver samples were obtained through the Organ Procurement Agencies (Philadelphia, PA). Caco-2-TC7 cells were a generous gift from Dr. Alain Zweibaum (INSERM U178, Cedex, France). Rat tail collagen (Type I) was purchased from Collaborative Research Inc. (Bedford, MA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Grand Island, NJ). Fetal bovine serum (FBS), penicillin (10000 units/ml)-streptomycin (10000 μg/ml) solution, trypsin (0.25%)-EDTA (1 mM), non-essential amino acids (NEAA, 100X), L-glutamine (200 mM), Dulbecco's phosphate buffered saline (DPBS), and Hank's balanced salts solution (HBSS) were from JRH Bioscience (Lenexa, KS). Transwell™ clusters, 24.5 mm

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ABBREVIATIONS: CYP3A4, cytochrome P450 3A4; CYP3A, cytochrome P450 3A; CYP3A5, cytochrome P450 3A5; TAO, troleandomycin; DMEM, Dulbecco's modified Eagle's medium; NEAA, non essential amino acids; HBSS, Hank's balanced salts solution; FBS, fetal bovine serum.

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in diameter and with 0.4 μm pore size, were from Costar Corp. (Bedford, MA).

Preparation of TC7, Human Intestine and Liver Microsomes

TC7 cells were plated in T150 flasks between passages 25 and 50. The cells were cultured for 15–20 days in DMEM supplemented with 20% FBS, 1% NEAA, 1% L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cultures were maintained in an atmosphere of 5% CO_2 and 90% relative humidity. On the days of the experiments, the cells were washed twice with HBSS (pre-equilibrated at 37°C), scraped and sonicated with twenty 5-sec bursts using an ultrasonic cell disrupter. The cell homogenate was centrifuged at 9,000 g for 20 min, and then at 100,000 g for 60 min. The microsomal pellet was stored at −70°C until used.

The small intestine was opened and cleaned by washing with ice cold HBSS. The tissue was cut into segments. Mucosal cells of the small intestine were scraped with a glass slide and homogenized. The liver was also homogenized. Homogenates from the intestine and the liver were centrifuged as described above and the microsomal pellets were stored separately at −70°C until used.

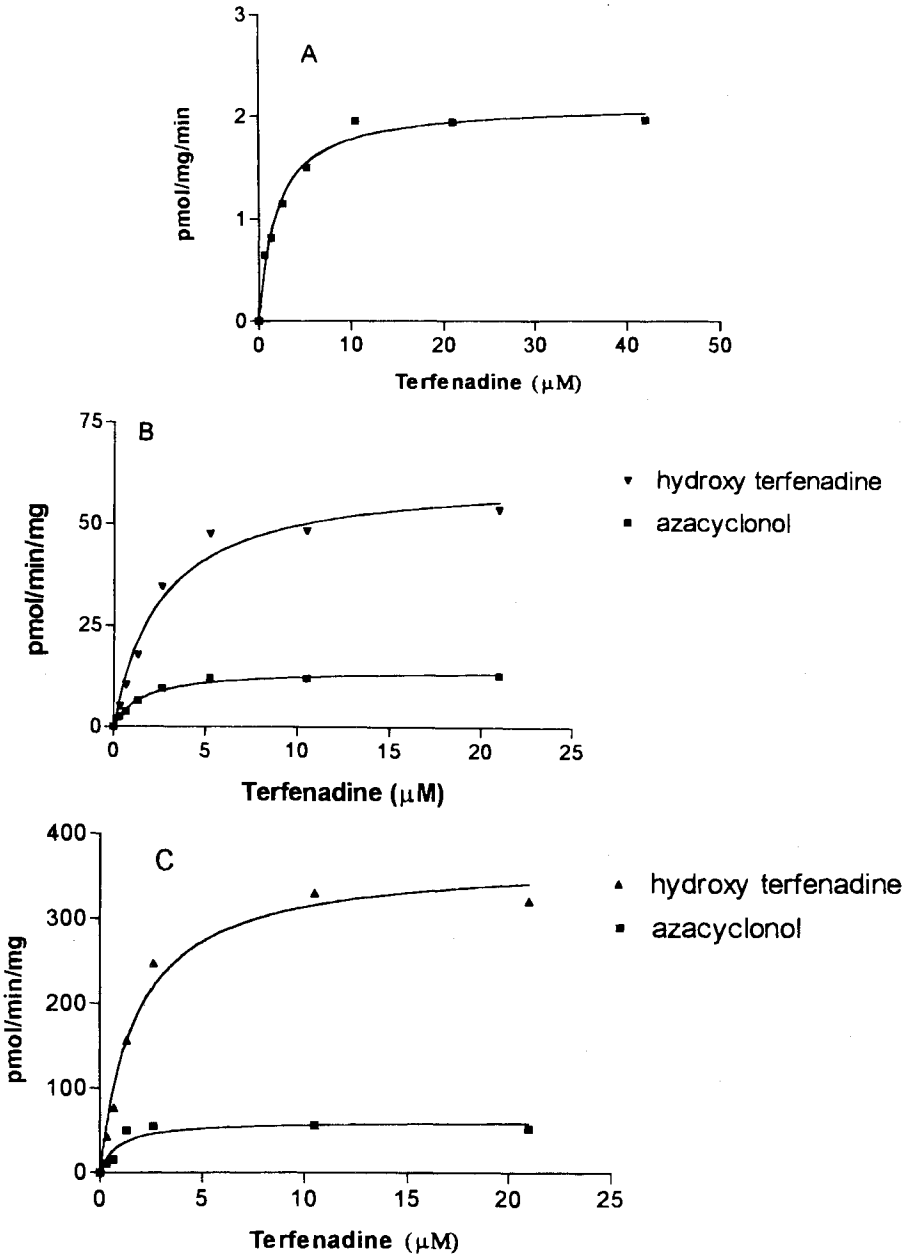

Fig. 1. Terfenadine hydroxylation in different systems. TC7 cells (A), human jejunum (B), and human liver (C) microsomes were incubated at 37°C for 45, 30 and 5 min, respectively, with terfenadine concentrations ranging from 1 to 21 μM. Each point represents the average of duplicate determinations.

Incubation Conditions

Microsomal protein was incubated in a final volume of 500 μl that contained 100 mM potassium phosphate buffer, 5 mM $MgCl_2$, and 20 μM terfenadine. The amounts of protein used for TC7 or human intestine or liver were; 1.0, 0.25 and 0.125 mg, respectively. The mixture was pre-incubated for 3 min and the reaction was started with a NADPH regenerating system (25 unit/ml glucose-6-phosphate dehydrogenase; 10 mM $NADP^+$; 100 mM glucose 6-phosphate). After 45, 30 and 5 min, for TC7 cells, human intestine, and liver, respectively, the reaction was terminated with 3 ml of methylene chloride. Then 100 μl of buffer containing 1 M Na_2CO_3, 10 mM EDTA and 2 M NaCl was added and the mixture was vortexed for 5 min and centrifuged at 2000 g for 10 min. The organic phase was transferred to new tubes and then evaporated to dryness under a stream of nitrogen. The sample was reconstituted in mobile phase and injected into an HPLC.

Inhibition studies were performed with TC7 and human intestine microsomes. The kinetic parameters were also determined with human liver. Chemical inhibitors were dissolved in methanol. Various concentrations of ketoconazole were added at the same time as the substrate. TAO was preincubated for 30 min with the NADPH regenerating system before the substrate was added to start the reaction. The final concentration of methanol in the incubation mixture was 1% (v/v). Control experiments showed that methanol 1% (v/v) did not inhibit drug metabolism. The polyclonal antibody was preincubated with the microsomal protein at room temperature for 20 min. The substrate and NADPH regenerating system were then added and the incubation was conducted as described above. The experiments were conducted in duplicate because of the limited amount of TC7 cell and human intestinal microsomes.

Metabolism in Intact TC7 Cells

TC7 cells were cultured as decribed in the *Preparation of TC7, human intestine and liver microsomes* section and used between passages 25 and 50. Prior to experiments, the monolayers were washed with HBSS. Both sides of the cell monolayers were bathed in HBSS containing terfenadine (20 μM) alone or with an inhibitor. The incubation was at 37°C for 180 min. After incubation, the apical and basolateral solutions were pooled and an aliquot was analyzed by HPLC, the monolayers were washed twice with ice-cold HBSS, and the filters containing the cells were cut and frozen until analysis. The amounts of terfenadine that remained cell-associated or in the incubation buffer were measured by HPLC. For terfenadine measurements, the filters containing the cells were immersed in 5.0 ml of methanol and vortexed for 3.0 min. The mixture was centrifuged at 3000 g for 10 min and one ml of the supernatant was transferred to another tube and evaporated to dryness. The samples were reconstituted in mobile phase and injected into the HPLC.

High Performance Liquid Chromatographic Analyses of Terfenadine and its Metabolites

The HPLC system consisted of a Waters Powerline 600 controller, a Waters 710 WISP autoinjector, a Supelco cyano column (250 × 4.6 mm internal diameter, 5μm particle size, Supelco, Inc. Bellefont, PA) and a Hitachi L-7480 fluorescence detector. Excitation and emission wavelengths were set at 230 nm and 280 nm, respectively. Data were collected using Waters Millennium data acquisition software (version 2.1). The mobile phase consisted of acetonitrile/methanol/12 mM ammonium acetate buffer pH 4.3 (30/30/40, v/v/v) and with a flow rate of 1.3 ml/min. Retention times of terfenadine (21 min), azacyclonol (9.4 min) and hydroxy-terfenadine (12.8 min) were confirmed by comparison with retention times of reference standards. Quantification was done using a standard curve of peak area vs compound concentration.

Table 1. Kinetics of Terfenadine Oxidation in Microsomes from TC7 Cells, Human Jejunum, and Human Liver

	Metabolite			
	Hydroxy-terfenadine		Azacyclonol	
Origin of microsomes	K_m (μM)	V_{max} (pmol/min/mg)	K_m (μM)	V_{max} (pmol/min/mg)
TC7 Cells	1.91 (0.26)[a]	2.11 (0.07)	—	—
Human Jejunum	2.50 (0.50)	61.0 (3.89)	1.44 (0.19)	14 (0.51)
Human Liver	1.80 (0.33)	370 (20.72)	0.82 (0.41)	60 (7.80)

Note: The K_m and V_{max} values were obtained by fitting the concentration dependent activity data of duplicate determinations to the one site binding equation of Graph Pad™

[a] Numbers in parenthesis are standard error of the estimates.

Kinetic Analyses

The K_m and V_{max} values were obtained by fitting the concentration dependent activity data to the one site binding equation of Graph Pad™ (Graph Pad software Inc., San Diego, CA).

RESULTS

Following the incubation of TC7 cell microsomes with terfenadine (20 μM) the only metabolite detected was hydroxy-terfenadine. In contrast, two metabolites (azacyclonol and hydroxy-terfenadine) were detected in incubations with human intestinal or hepatic microsomes.

The formation of hydroxy-terfenadine in TC7 cell microsomes was linear with increasing protein concentration between 0.25 and 3.0 mg/ml and with incubation time up to 60 min (data not shown). The formation of both hydroxy-terfenadine and azacyclonol in human intestinal microsomes was also linear with respect to time up to 60 min. The rates of formation of hydroxy-terfenadine and azacyclonol increased linearly with microsomal protein concentration up to 1.0 mg/ml and 2.0 mg/ml, respectively (data not shown).

The relative affinity of CYP3A for terfenadine (K_m) and the maximal reaction rate (V_{max}) for terfenadine oxidation in TC7 microsomes were 1.91 μM and 2.11 pmol/min/mg protein, respectively (Figure 1A, Table 1). The K_m and V_{max} values for hydroxy-terfenadine and azacyclonol formation in intestinal microsomes were 2.5 μM and 61 pmol/min/mg protein and 1.44 μM and 14 pmol/min/mg protein, respectively (Figure 1B, Table 1).

Human liver microsomes catalyzed the formation of both azacyclonol and hydroxy-terfenadine (Figure 1C and Table 1).

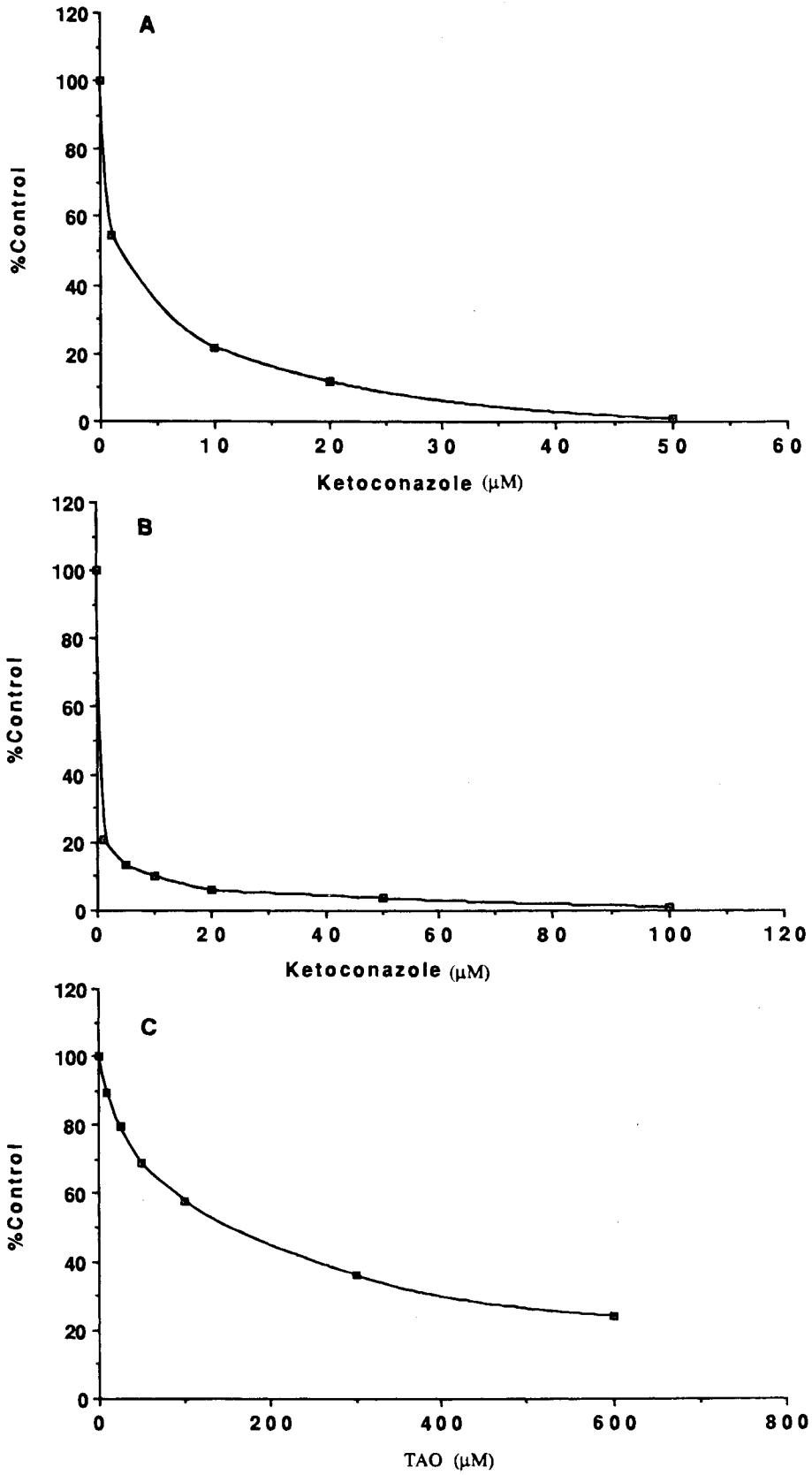

Fig. 2. Inhibition of hydroxy-terfenadine formation by ketoconazole and TAO. TC7 cells (A) or human jejunal microsomes (B) were incubated with ketoconazole (1–100 μM) at 37°C for 45 and 30 min, respectively. TC7 cells (C) or human jejunum (D) microsomes were incubated with TAO (10–600 μM) at 37°C for 45 and 30 minutes, respectively. Each point represents the average of duplicate determinations.

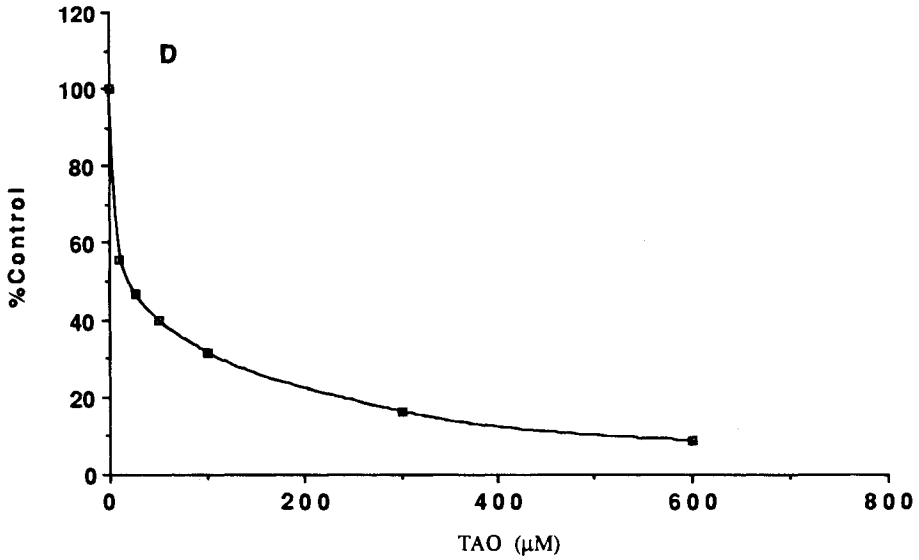

Fig. 2 Continued.

The apparent K_m and V_{max} values of hydroxy-terfenadine and azacyclonol were 1.8 μM and 370 pmol/min/mg protein and 0.82 μM and 60.0 pmol/min/mg protein, respectively.

Inhibition of Terfenadine Hydroxylation by Ketoconazole and TAO

Ketoconazole inhibited the oxidation of terfenadine in TC7 (Figure 2A) and human intestine (Figure 2B) in a concentration dependent manner. The IC_{90} for hydroxy-terfenadine in human intestine and TC7 microsomes were 10 μM and ~ 20 μM, respectively. TAO (300 μM) inhibited (65%) terfenadine hydroxylation in TC7 cell microsomes (Figure 2C). In human intestinal microsomes TAO (300 μM) inhibited (80%) terfenadine hydroxylation (Figure 2D). The strong inhibition of terfenadine oxidation by ketoconazole and TAO suggests the involvement of CYP3A4-related enzyme in these reactions.

Effect of Rat Anti-CYP3A2 on Terfenadine Oxidation

To determine the role of CYP3A in terfenadine hydroxylation, microsomal samples from TC7 cells, human intestine and M107r microsomes (obtained from cells transfected with human CYP3A4 cDNA) were pre-incubated with a polyclonal antibody against rat liver CYP3A2. This antibody was shown previously to recognize CYP3A4 in human liver microsomes (Product Information, Gentest Corporation). The effect of the antibody on terfenadine oxidation in M107r microsomes was used as a positive control for antibody reactivity. The formation of hydroxy-terfenadine was inhibited by 90% at 2.5 mg of IgG/mg protein in M107r samples and by 60% at 5 mg IgG/mg protein in human intestine. However, the anti-CYP3A (5 mg IgG/mg protein) did not inhibit the TC7 terfenadine oxidation. Preimmune IgG had no effect on the formation of hydroxy-terfenadine by any of the microsomal samples.

Terfenadine Metabolism in Intact TC7 Cells

The formation of terfenadine increased linearly with time up to 210 min. After a 180 min incubation, 50% of the terfenadine applied to the cells was associated with the cell monolayer, 40% was in the incubation buffer, and 5% was converted to hydroxy-terfenadine.

To determine the concentration dependence of terfenadine hydroxylation, TC7 cell monolayers were incubated with different concentrations of terfenadine (1 to 42 μM) for 180 minutes. The apparent K_m was 1.65 μM and the apparent V_{max} was 1.5 pmol/min/mg cellular protein (Figure 3A). Ketoconazole, at concentrations higher than 10 μM, abolished the formation of hydroxy-terfenadine (Figure 3B). In contrast, 600 μM TAO inhibited hydroxy-terfenadine formation by only 30% (Figure 3C). A lesser inhibitory effect by TAO in the intact TC7 cells than TC7 cell microsomes, may be due to limited permeation in intact cells.

DISCUSSION

The comparisons of metabolite formation, kinetic parameters, and inhibition in TC7 cell microsomes and human intestine suggest that the CYP3A in TC7 cells is not CYP3A4 enzyme, but probably CYP3A5 (6).

The first step was to determine whether TC7 cells could metabolize terfenadine. Human intestine and liver were used for comparison. The formation of hydroxy-terfenadine and azacyclonol by human hepatic microsomes agrees with two recent studies (14,15). During incubation of terfenadine with human intestinal microsomes, the same two terfenadine metabolites; hydroxy-terfenadine and azacyclonol, were formed. The metabolism of terfenadine to hydroxy-terfenadine in TC7 cells constitutes evidence of the expression of a CYP3A enzyme in these cells. The lack of formation of azacyclonol was more puzzling. One possibility is that azacyclonol was formed, but not in sufficient amounts to be detected. Another explanation could be that the CYP3A enzyme expressed by TC7 cells is different from intestinal CYP3A4.

Hydroxy-terfenadine formation in TC7 cell microsomes exhibited Michaelis-Menten kinetics with K_m and V_{max} values of 1.91 μM and 2.11 pmol/min/mg protein. The K_m value for TC7 microsomes is similar to those for human intestinal (2.5

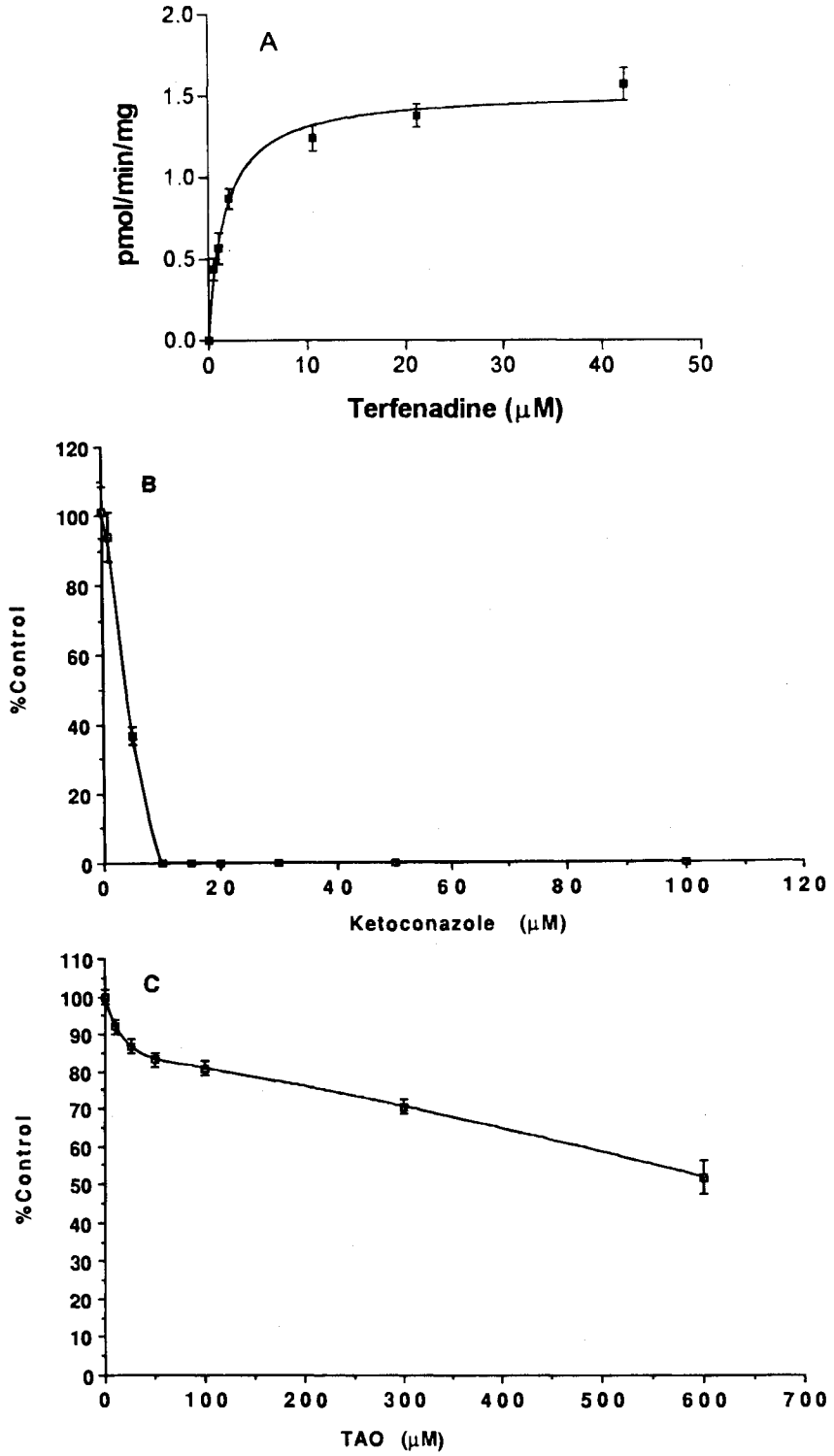

Fig. 3. Metabolism of terfenadine to hydroxy-terfenadine in intact TC7 cells. (A) Terfenadine (1 to 42 μM) was incubated with TC7 cells. (B) Terfenadine (20 μM) was incubated with ketoconazole (1–100 μM). (C) Terfenadine (20 μM) was incubated with TAO (10–600 μM). Incubations were done at 37°C for 180 min. Data represent mean±SD (n = 3).

μM) and hepatic (1.8 μM) microsomes. This suggests that the affinity for terfenadine of TC7 CYP3A is similar to that of human intestinal and hepatic CYP3A4. However, the V_{max} value for hydroxy-terfenadine formation in TC7 cells was 30 and 175-fold lower than that for human jejunum and liver, respectively. This difference in V_{max} values indicates either lower expression or lower efficiency of the CYP3A enzyme in TC7 cells.

The IC_{90} associated with the inhibition of hydroxy-terfenadine in TC7 cell microsomes by ketoconazole (20 μM) was comparable to that in human jejunal microsomes (10 μM). Ketoconazole is a relatively general inhibitor of CYP3A isozymes at low concentration (17). Thus, the complete inhibition of hydroxy-terfenadine formation by ketoconazole (a concentrations higher than 50 μM) further suggests the involvement of CYP3A in the hydroxy-terfenadine formation in TC7 cell microsomes.

TAO, a more specific inhibitor of CYP3A4 (14) inhibited the formation of hydroxy-terfenadine in both TC7 cell (~ 60% at 300 μM) and human jejunal microsomes (~ 80% at 300 μM). The lower effect of TAO in TC7 cell microsomes could be due to the lower activity of CYP3A in TC7 cells, which could result in lower activity of the formation of the TAO intermediate that causes the inhibition.

Terfenadine metabolism was also investigated in intact TC7 cells, because this cell line could be used as in vitro model of intestinal drug transport and drug metabolism simultaneously. The apparent K_m (1.65 μM) and V_{max} (1.5 pmol/min/mg protein) values in intact TC7 cells were comparable to K_m (1.91 μM) and V_{max} (2.11 pmol/min/mg protein) values from TC7 microsomes.

During the course of this work it was shown, based on Western and Northern blot analyses, that CYP3A5 is the major cytochrome P450 3A expressed in TC7 cells (6). In the same study, it was shown that CYP3A5 is the dominating CYP3A isoform in human colon (6). The amino acid sequences of CYP3A4 and CYP3A5 have 84% identity (18) and the substrate specificities of CYP3A4 and CYP3A5 overlap somewhat. For instance CYP3A5 formed 6β-hydroxylation of testosterone although at a much lower rate than CYP3A4 (19). CYP3A5 is also able to metabolize nifedipine at rate comparable to that of CYP3A4 (18). Contrary to CYP3A4, which metabolizes erythromycin and quinidine, CYP3A5 metabolizes these substrates poorly (20). In addition, CYP3A5 has shown different regioselectivity for midazolam oxidation compared to CYP3A4 (21). Although CYP3A5 and CYP3A4 show some similarity in substrate specificity, they differ in some of their catalytic properties. This is consistent with the formation of hydroxy-terfenadine and the lack of formation of azacyclonol by TC7 cells. Furthermore, a CYP3A4 blocking antibody did not inhibit CYP3A activity in TC7 microsomes, but inhibited terfenadine hydroxylation in human intestine supporting the notion that the major CYP3A enzyme in TC7 cells is CYP3A5 (6).

In summary, the CYP3A activity in TC7 cells was compared with CYP3A4 activity in human intestine. TC7 CYP3A catalyzed the formation of hydroxy-terfenadine, but no azacyclonol was detected, indicating that CYP3A in TC7 cells is different from that in human intestine. The differences in metabolite profiles and lack of inhibition by a CYP3A-blocking antibody suggest that TC7 cells express an enzyme that is not CYP3A4, but probably CYP3A5, the CYP3A isoenzyme of human colon (6). The expression of CYP3A5 in TC7 cells is comparable to that in the human colon (6). This, together with the well differentiated characteristics of TC7 monolayers grown on Transwell filters, should make these cells an excellent model for studies of drug transport and CYP3A5 metabolism. Studies on the relative influence of CYP3A5 and P-glycoprotein as barrier to drug transport should be of particular interest.

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