Persistent Inhibition of CYP3A4 by Ketoconazole in Modified Caco-2 Cells

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Purpose. The intestinal metabolism of some CYP3A substrates can be altered profoundly by co-administration of the potent inhibitor, ketoconazole. The present research was conducted to test the hypothesis that, unlike the inhibition kinetics observed with isolated microsomes, inhibition of CYP3A4 by ketoconazole in an intestinal cell monolayer is time-dependent and slowly reversible.

Methods. Confluent, 1α,25-dihydroxy Vitamin D_3-treated Caco-2 cells were exposed to 1 μM ketoconazole for two hours (Phase I) and then washed three times with culture medium containing no inhibitor. This was followed by a second incubation period (Phase II) that varied in the composition of the apical and basolateral culture medium: Condition 1, apical/basolateral differentiation medium (DM); Condition 2, apical/basolateral DM + basolateral 2g/dL Human Serum Albumin (HSA); Condition 3, apical/basolateral DM + apical/basolateral 2 g/dL HSA. After various lengths of time for the second phase (0 to 4 hours), both apical and basolateral medium were exchanged with fresh DM. Midazolam (6 μM) was included in the apical medium for determination of CYP3A4 activity (Phase III).

Results. Two-way ANOVA of the data revealed persistent inhibition of CYP3A4 under Conditions 1 and 2 ($p < 0.001$). In contrast, cells treated under Condition 3 exhibited rapid reversal of CYP3A4 inhibition. The level of CYP3A4 activity observed was inversely correlated with the amount of ketoconazole remaining in the cell monolayer at the end of Phase II.

Conclusions. These studies provide mechanistic evidence that ketoconazole can be sequestered into the intestinal mucosa after oral administration, producing a persistent inhibition of first-pass CYP3A4 activity.

KEY WORDS: CYP3A4; Caco-2; ketoconazole; midazolam; drug metabolism.

INTRODUCTION

Cytochrome P450 3A4 (CYP3A4) metabolizes a wide range of chemically diverse compounds. The enzyme is expressed in mature enterocytes of the small intestine and in hepatocytes of the liver (1,2). *In vivo* studies with MDZ (3,4) and cyclosporine (5,6) revealed that both hepatic and intestinal CYP3A4 can contribute to a first-pass metabolic extraction after oral administration of the drug. If the extent of intestinal and hepatic first-pass metabolism are significant, as demonstrated with MDZ (3), inhibition of these processes during drug polytherapy may be subject to differing dose and time-dependencies.

It is generally assumed that the magnitude of inhibition of hepatic and intestinal drug metabolism will depend on unbound concentrations of inhibitor in the hepatocyte or enterocyte, relative to the inhibitor K_i. Although inhibition of hepatic first-pass metabolism should also reflect the unbound inhibitor concentration in plasma, a similar relationship may not apply to first-pass metabolism in the enterocyte, particularly when the inhibitor is dosed orally and close in time to an oral dose of the substrate. Although the time of administration of substrate, relative to inhibitor, may also affect the magnitude of interaction observed in the liver, the impact is likely to be more pronounced at the level of the intestine.

Differences in the degree and time-course of CYP3A inhibition observed at the level of the intestine *versus* the liver have been reported for various substrate-inhibitor pairs. Studies that examined the effect of grapefruit juice indicated that only intestinal CYP3A was inhibited, whereas hepatic CYP3A activity remained unchanged (7–9). Further, the reduction in duodenal mucosal CYP3A4 levels that occurred after grapefruit juice ingestion may have been a consequence of greater gut luminal exposure relative to systemic exposure (9). Similarly, a pharmacokinetic analysis of an interaction between the CYP3A4 and P-glycoprotein substrate, cyclosporine, and KTZ indicated that intestinal bioavailability of cyclosporine may have been preferentially altered (compared to hepatic bioavailability) by KTZ even though the inhibitor dose was administered ten hours before the substrate (10). The C_{max} for KTZ in healthy individuals is generally achieved 1.5 to 2 hours after an oral dose (11, 12), and small intestine transit time is approximately 3.5 hours (13). Thus, the magnitude and timing of the interaction suggest that the inhibitory effect on intestinal CYP3A/P-gp extended well beyond the residence time of the KTZ dose in the small intestine, and that it is not directly dependent on circulating KTZ blood levels.

To examine the possibility of time-dependent inhibition of intestinal CYP3A, we utilized 1α,25-dihydroxy-vitamin-D_3 (1α,25-$(OH)_2$-D_3)-modified Caco-2 monolayers (14) as a surrogate model. Experiments were conducted to determine whether inhibition of CYP3A4 following pulsed exposure to KTZ would persist after removal of the inhibitor from the monolayer perfusing medium, and whether this persistence was related to a sequestration of KTZ within the intracellular matrix.

MATERIALS AND METHODS

Materials

Dimethylsulfoxide (DMSO) and purified Human Albumin (Fraction V) (HSA) were purchased from Sigma Chemicals (St. Louis, MO). KTZ was acquired through Research Diagnostics (Flanders, NJ). Midazolam (MDZ), 1′-hydroxymidazolam (1′-OH MDZ), and 1′[$^2H_2$]-hydroxymidazolam (1′-[$^2H_2$]1′-OH MDZ) were provided by Roche Laboratories (Nutley, NJ). *N*-methyl-*N*-(*t*-butyl-dimethylsilyl) trifluoroacetamide was purchased from Pierce Chemical (Rockford, IL). Acetonitrile and

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ABBREVIATIONS: HSA, human serum albumin; FBS, fetal bovine serum; DMEM, Dubelcco's Modified Eagle Medium; CYP3A, Cytochrome P450 3A; NEAA, non-essential amino acids; HBSS, Hanks Balanced Salt Solution; TEER, transepithelial electrical resistance; DMSO, dimethylsulfoxide; MDZ, midazolam; 1′-OH MDZ, 1′-hydroxymidazolam; 1α,25-$(OH)_2$-D_3, 1α,25-di-hydroxy vitamin-D_3.

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ethyl acetate were obtained from Fisher Scientific (Fair Lawn, NJ). Dubelcco's Modified Eagle Medium (DMEM), non-essential amino acids (NEAA), penicillin, streptomycin, and Hanks Balanced Salt Solution (HBSS) were obtained from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Upon receipt, it was warmed to 37°C, heat inactivated for 30 minutes at 57°C, placed on ice for 10 minutes and subsequently stored for further use at −20°C. Uncoated polyethylene terephthalate inserts and mouse laminin were obtained from Collaborative Biomedical Products (Bedford, MA). The hormone 1α,25-$(OH)_2$-D_3 was obtained from Calbiochem (La Jolla, CA). KTZ [^{3}H(G)] dissolved in ethanol was purchased from American Radiolabeled Chemicals (St. Louis, MO); its specific activity was 5 Ci/mmol, with 99% radiochemical purity. EcoLite™ was obtained from ICN Pharmaceuticals (Costa Mesa, CA). Stock solutions of MDZ and KTZ were prepared in DMSO. A concentrated 1α,25-$(OH)_2$-D_3 stock solution (250 μM) was prepared in ethanol.

Caco-2 Cell Culture Conditions

The Caco-2 subclone, P27.7 (14), was obtained at passage 12 and grown to confluence on 100 mm^2 culture dishes as described previously (15). All experiments were performed with cells at passage numbers 19 to 22. For each experiment, cells were seeded onto laminin coated inserts at a density of 5.2×10^5 cells/cm^2 and grown to confluence in complete growth medium. Once confluence was achieved, they were cultured with fresh differentiation medium (DM) replaced every 48 hours for a period of two weeks. DM consisted of the following: DMEM, 0.1 mM NEAA, 100 units/mL sodium penicillin, 100 μg/mL streptomycin, 0.1 μM sodium selenite, 3 μM zinc sulfate, 45 nM DL-α-tocopherol, 0.25 μM 1α,25-$(OH)_2$-D_3, and 5% heat-inactivated FBS.

Monolayer Integrity

Prior to the initiation of each experiment, cells were allowed to reach room temperature (~ 22 °C). Resistance (ohm) was measured for each insert using a Millicell electrical resistance system (Millipore, Bedford, MA). Unseeded laminin-coated inserts were used for determination of background resistance. Transepithelial electrical resistance (TEER) was defined as the product of the background-corrected resistance and the surface area of the insert (4.2 cm^2). TEER values for all experiments were consistent with previously reported values (14,15).

IC_{50} Determination

Experiments were performed to determine the IC_{50} of KTZ in the confluent, 1α,25-$(OH)_2$-D_3-treated Caco-2 monolayer. The volume of the apical and basolateral compartments were 1.5 mL each and the incubation temperature was maintained at 37 °C. For the first experiment, KTZ and MDZ were dissolved in DMSO and added simultaneously to the apical compartment medium (final solvent concentration in apical DM of 1%, v/v). Control inserts were treated with 1% DMSO (v/v) and no inhibitor. KTZ (0, 0.01, 0.1, 1, and 10 μM) and 6 μM MDZ were co-incubated for 20 minutes.

A second experiment was conducted to determine whether the inhibitory effect of KTZ would persist after a brief exposure of the Caco-2 monolayer to the drug. KTZ (0, 0.01, 0.1, 1, and 10 μM) was added to the apical compartment medium (DM) and the monolayer was incubated for 2 hours. Control inserts were treated with 1% DMSO (v/v) and incubated for the same length of time. Subsequently, cells were rinsed 3 times with DMEM and followed with the addition of fresh DM to apical and basolateral compartments. MDZ (6 μM) was added into the apical medium for assessment of CYP3A activity. At the end of a 20-minute incubation period, apical and basolateral medium were collected. Cells were gently scraped away from the solid support into 1 mL of DMEM after a single rinse of the monolayer (apical and basolateral sides) with 1 mL of DMEM. All samples were stored at −20°C pending analysis of 1′-OH-MDZ by GC-MS, as described previously (14).

Evaluation of Persistent CYP3A4 Inhibition

This set of cell culture experiments consisted of three phases: Inhibition (Phase I), Washout (Phase II) and Activity Assessment (Phase III), as depicted in Fig. 1. All incubations were conducted at 37°C. The basic culture medium was DM, which contains 5% FBS, and the basic wash medium was DMEM, which does not contain FBS. During Phase I, cells were incubated for 2 hours with 1 μM KTZ dissolved in DMSO (1% in DM, v/v) or with the dose vehicle alone (1% DMSO in DM, v/v); both were applied apically. At the end of two hours, apical and basolateral medium were removed and both sides of the monolayer were rinsed three times with 1 mL DMEM. Incubation conditions for Phase II varied in the composition of the apical and basolateral culture medium: *Condition 1*, DM (1.5 mL) on both apical and basolateral sides; *Condition 2*, DM (1.5 mL) on both apical and basolateral sides + 2g/dL HSA added to the basolateral side; *Condition 3*, DM (1.5 mL) on both apical and basolateral sides + 2g/dL HSA and to both apical and basolateral sides. At various times during Phase II (0+, 0.5, 1, 1.5, 2, or 4 hours), the apical and basolateral medium were removed. For time 0+ sampling, cells were exposed briefly (about 1 minute) to apical and basolateral medium under Conditions 1, 2, or 3. At the indicated time, both sides of the monolayer were rinsed once with 1 mL DMEM and then 1.5 mL of fresh DM was added to each compartment. MDZ (6 μM) was included in the apical medium for the initiation of the activity phase of the experiment (Phase III). After incubation with MDZ for 20 minutes, apical and basolateral medium as well as cell scrapings were collected and stored at −20°C, pending analysis of MDZ and 1′-OH-MDZ. Each experimental condition was performed in triplicate.

Intracellular Ketoconazole Content

Two sets of experiments were conducted to evaluate intracellular KTZ levels in Caco-2 cells treated or not treated with 1α,25-$(OH)_2$-D_3. Cells were grown to confluence and were fed medium containing or lacking 1α,25-$(OH)_2$-D_3 for a period of two weeks, as described above. On the given experiment day, cells were treated according to the conditions depicted in Fig. 1. The medium volume in the apical and basolateral compartments was 1.5 mL each. Phase I consisted of exposure to an apical dose of radiolabeled KTZ [^{3}H(G)] (~ 0.8 μM) in DM, which contained 5% FBS, for a period of 2 hours. DM without the inhibitor was placed basolaterally. At the end of two hours, aliquots of the apical and basolateral medium were collected,

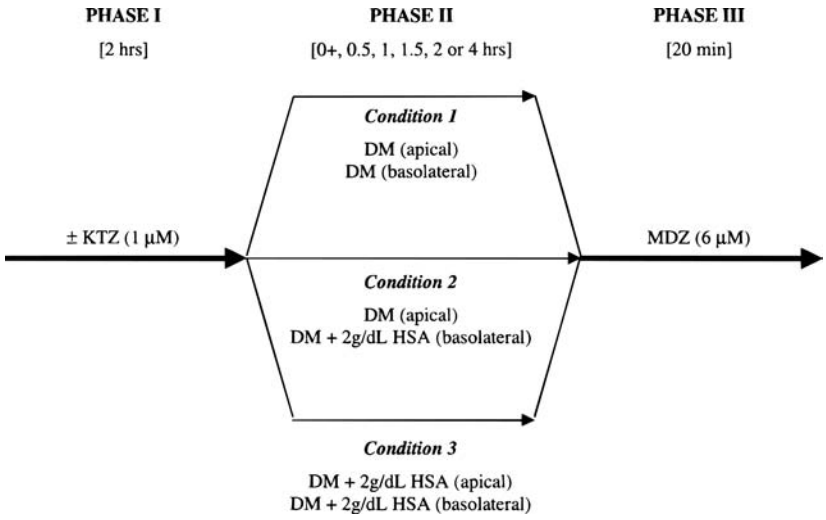

Fig. 1. Experimental design for evaluation of persistent inhibition with KTZ. Phase I: cell monolayers were incubated with 1 μM KTZ in 1% DMSO or with 1% DMSO (control), for 2 hr. Phase II: KTZ- and solvent-treated cells were incubated for variable lengths of time (0–4 hrs), with one of three different culture conditions; Condition 1, DM in the apical and basolateral compartments; Condition 2, DM in the apical compartment and DM + 2 g/dL HSA in the basolateral compartment; Condition 3, DM + 2 g/dL HSA both in apical and basolateral compartments. Phase III: all monolayers were incubated for 20 min after addition of 6 μM MDZ to the apical compartment to assess CYP3A4 catalytic activity.

placed in a scintillation vial, and EcoLite™ scintillation fluid (5 mL) was added to samples. Both sides of the cell monolayer were washed three times with 1 mL of DMEM. Subsequently, the apical and basolateral medium was replaced with DM or DM supplemented with 2 g/dL HSA, according to Conditions 1, 2, or 3. At the end of 4 hours, apical and basolateral medium were collected. Cells were washed once with DMEM, followed by the collection of cell scrapings in 1 mL of DMEM. EcoLite™ scintillation fluid (5 mL) was added to apical and basolateral samples as well as cell scrapings and radioactivity was measured using a Packard 2200CA Liquid Scintillation Analyzer (Downers Grove, IL).

Determination of KTZ Free Fraction

The free fraction of KTZ was determined in DM, DM + 2g/dL HSA, and DM + 4g/dL HSA, using an adaptation of a previously described equilibrium dialysis method (3). Briefly, [^{3}H(G)] KTZ was added to the culture medium at a nominal concentration of 1 μM (pH 7.4, 300 μL total volume) and placed on one side of a 12,000–14,000 molecular weight cut off dialysis membrane (Spectra/Por-2, VWR). Sodium phosphate buffer (67 mM, pH 7.4, 300 μL total volume) was placed on the opposite side. Samples were equilibrated for 4 hours in a 37°C water bath. At the end of 4 hours, buffer and protein chambers were sampled. Scintillation fluid (5 mL) was added to each sample and radioactivity was measured using a Packard 2200CA Liquid Scintillation Analyzer (Downers Grove, IL). The free fraction was calculated as a ratio of ^{3}H-KTZ in the buffer side over ^{3}H-KTZ in the protein side.

Statistics

To examine the persistence of CYP3A4 inhibition, two-way analysis of variance (ANOVA) was performed to assess the effects of Phase I KTZ treatment and Phase II KTZ washout time on MDZ 1′-hydroxylation, using Systat® for Windows, Version 5 (Evanston, IL). A value of $p < 0.05$ was considered to be significant. The amount of KTZ (based on specific activity) found in apical, basolateral or cellular compartments under Conditions 1, 2, or 3 after four hours of Phase II was compared using one-way ANOVA (SPSS, Version 8.0, Chicago, IL). For each experiment, post hoc comparisons were made using the Bonferroni correction.

RESULTS

IC_{50} Determination

The effect of KTZ on MDZ 1′-hydroxylation in 1α,25-$(OH)_2$-D_3-modified Caco-2 cells is shown in Fig. 2. Apically-applied KTZ was a potent inhibitor of MDZ hydroxylation, with an approximate IC_{50} value of 0.3 or 0.4 μM after pre-incubation and removal of KTZ from both compartments or co-incubation of KTZ and MDZ, respectively. Results for the coincubation of MDZ with KTZ were similar to those reported previously for Caco-2 cell culture monolayers (14). For comparison, the non-competitive K_i of KTZ for CYP3A4-dependent MDZ 1′-hydroxylation in liver and intestinal microsomes was found to be 0.025 μM (16). The higher apparent K_i observed in the monolayer may be due to differences in free concentrations of KTZ in the cell culture medium (and presumably intracellular milieu) which contained 5% FBS, compared to a microsomal incubation containing no soluble proteins and a low amount of protein and membrane (i.e., minimal nonspecific binding).

Apical and basolateral MDZ concentrations were also measured in the presence and absence of KTZ to rule out an effect

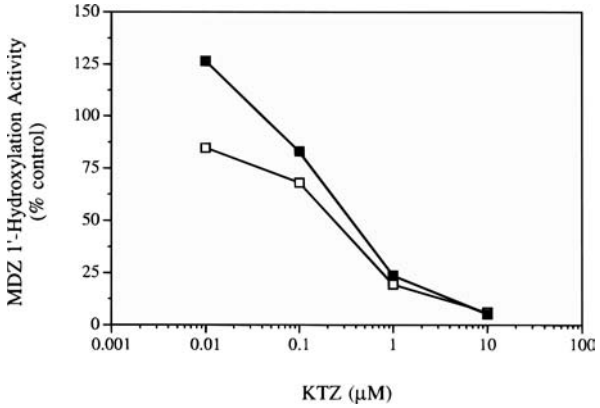

Fig. 2. Concentration-dependent inhibition of CYP3A4 activity in Caco-2 cells by KTZ. Closed squares represent co-incubation of cells with an apical dose of KTZ and 6 μM MDZ. Open squares represent pre-incubation of KTZ for 2 hours, followed by an exchange of extracellular culture medium prior to the addition of 6 μM MDZ. Both KTZ and MDZ were added to the apical compartment. MDZ 1′-hydroxylation activity was assessed from a 20 minute incubation. Data shown are mean of duplicate determinations.

of KTZ on MDZ flux. The apical to basolateral MDZ concentration ratio at the end of a 20-minute incubation with 6 μM MDZ was the same with and without KTZ administration (data not shown).

Evaluation of Persistent CYP3A4 Inhibition

Based on results from the IC_{50} determination, a 1 μM apical KTZ dose concentration was chosen for subsequent experiments that investigated the time-dependent nature of CYP3A4 inhibition. Once again, simple removal of KTZ from the bulk extracellular medium after a 2-hour exposure period and washing of the monolayer with DMEM did not readily reverse the inhibition of CYP3A4 (Fig. 3). Furthermore, an extension of the washout period with DM (contained 5% FBS) to 4 hours (Phase II, Condition 1) had only a limited effect. Following 0+, 0.5, 1, 1.5, 2, and 4 hours of incubation with DM, production of 1′-OH-MDZ, normalized to the activity of a baseline insert (Phase II, time 0+, control group), was 43, 60, 61, 50, 56, and 50% for the KTZ treatment group, compared to 100, 111, 127, 135, 98, and 102% for the parallel control groups. When analyzed by two-way ANOVA for the effect of time (Phase II) and treatment (Phase I) on CYP3A4 activity, both prior KTZ treatment and the length of the KTZ washout time were found to be significant ($p < 0.001$ and $p = 0.012$, respectively).

KTZ is highly bound to plasma proteins; $f_{unbound} \sim 0.01$ (17). For some experiments, HSA was added to the extracellular medium to provide a drug binding sink analogous to that which exists *in vivo*. A concentration of 2 g/dL, rather than the normal serum concentration of 4 g/dL, was employed to avoid excessive variability in MDZ activity associated with the presence of residual albumin in the apical compartment during the activity assessment period. The free fraction of KTZ in DM (contains 5% FBS), DM (5% FBS) + 2 g/dL HSA and DM (5% FBS) + 4 g/dL HSA was found to be 41.5, 5.0 and 3.8%, respectively.

Cells pre-treated with KTZ and subjected to an incubation under Phase II, Condition 2 (DM on the apical side and DM + 2 g/dL HSA on the basolateral side) exhibited a MDZ 1′-hydroxylation activity that was 37, 68, 60, 80, 60, and 79% of baseline activity after 0+, 0.5, 1, 1.5, 2, and 4 hours, respectively (Fig. 4). For comparison, control cells that were never exposed to KTZ exhibited a MDZ 1′-hydroxylation activity that was 100, 121, 100, 114, 92, and 95% of the baseline activity at parallel times of Phase II. Analysis of the data obtained under Condition 2 by two-way ANOVA revealed that both prior KTZ treatment and Phase II incubation time produced a significant effect on CYP3A activity ($p < 0.001$ and $p = 0.002$, respectively).

When 2 g/dL HSA was added to DM on both apical and basolateral sides during Phase II (Condition 3), cells pre-treated

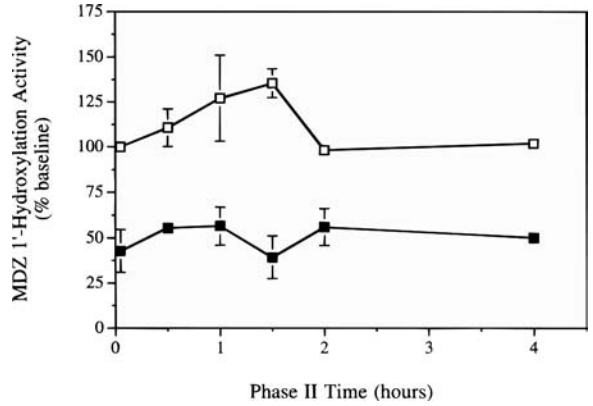

Fig. 3. Time-dependent inhibition of CYP3A4 by KTZ under Phase II, Condition 1. During Phase II, DM was added to both apical and basolateral compartments and incubated for the indicated time. MDZ 1′-hydroxylation activities measured at the end of Phase II were normalized to the activity of a baseline control insert for that day (time 0 = 100%). Open squares represent data from cells treated with 1% DMSO during Phase I, closed squares represent cells treated with 1 μM KTZ in DMSO during Phase I. Data shown are mean ± SD from experiments performed in triplicate.

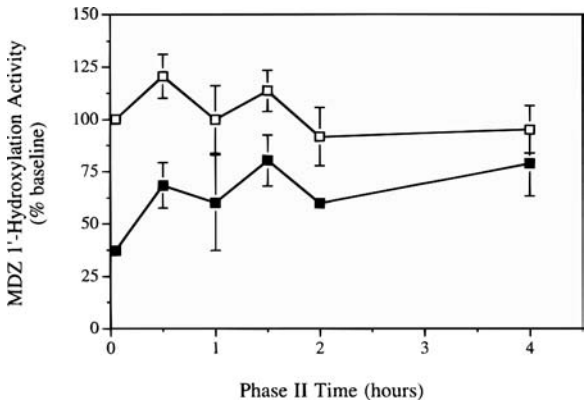

Fig. 4. Time-dependent inhibition of CYP3A4 by KTZ under Phase II, Condition 2. During Phase II, DM and DM + 2 g/dL HSA were added to the apical and basolateral compartments, respectively, and incubated for the indicated time. MDZ 1′-hydroxylation activities measured at the end of Phase II were normalized to the activity of a baseline control insert for that day (time 0 = 100%). Open squares represent data from cells treated with 1% DMSO during Phase I, closed squares represent cells treated with 1 μM KTZ in DMSO during Phase I. Data shown are mean ± SD from experiments performed in triplicate.

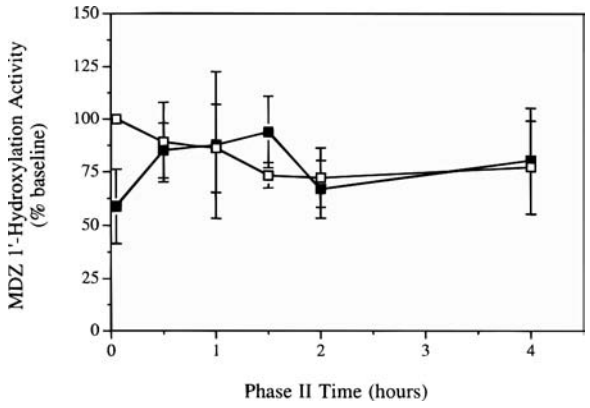

Fig. 5. Time-dependent inhibition of CYP3A4 by KTZ under Phase II, Condition 3. During Phase II, DM + 2 g/dL HSA was added to both the apical and basolateral compartments and incubated for the indicated time. MDZ 1′-hydroxylation activities measured at the end of Phase II were normalized to the activity of a baseline control insert for that day (time 0 = 100%). Open squares represent data from cells treated with 1% DMSO during Phase I, closed squares represent cells treated with 1 μM KTZ in DMSO during Phase I. Data shown are mean ± SD from experiments performed in triplicate.

with KTZ exhibited a CYP3A4 activity that was 59, 85, 87, 73, 73, or 77% of baseline activity after 0+, 0.5, 1, 1.5, 2, and 4 hours of incubation (Fig. 5). In comparison, control inserts exhibited 100, 89, 87, 94, 67, and 80% of baseline activity at parallel times. Analysis of data from cells treated under Condition 3 showed that neither KTZ pre-treatment nor Phase II washout time were significant factors affecting CYP3A4 activity ($p = 0.509$ and $p = 0.604$, respectively). Thus, KTZ inhibition appeared to be rapidly reversed by the addition of HSA to the apical medium during Phase II.

The comparative effect of KTZ for all three Phase II conditions, illustrated as a percent of the respective parallel control activity (minus KTZ in Phase I), is depicted in Fig. 6. For cells treated under Condition 1, CYP3A4 was still inhibited after 4 hours of Phase II incubation. Treatment under Condition 2 resulted in CYP3A4 inhibition that only slowly reversed, with a return to 81% of control activity after 4 hours. For Condition 3, after time 0+, when the cells were briefly exposed to apical medium containing HSA, MDZ-1′-OH activity had already rebounded to 59% of control. This was in contrast to Conditions 1 and 2, where activity at time 0+ was 43 and 37% of control, respectively. Further, after 30 minutes of Phase II incubation, CYP3A activity for Condition 3 cells had returned to 97% of control. It was noted that the activity of some of the control (minus KTZ) cells treated under Condition 3 was reduced slightly, in comparison to baseline. This may have been the result of MDZ binding to residual HSA in the apical compartment.

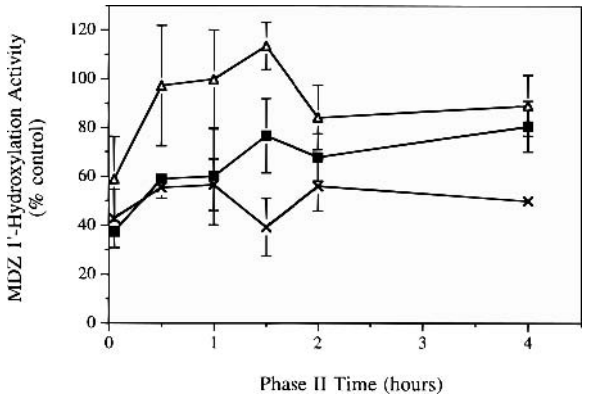

Fig. 6. Comparative effects of extracellular HSA on the persistence of KTZ inhibition in Caco-2 cell cultures. Each data point represents the mean ratio (±SD) of midazolam 1′-hydroxylation activity for a KTZ-treated insert divided by the activity of a parallel control insert, multiplied by 100. Data from cells incubated under Conditions 1, 2, and 3 are denoted by the symbol x, closed square and open triangle, respectively.

Intracellular Ketoconazole Concentrations

To test whether the persistent inhibition of CYP3A4 activity after KTZ pre-treatment was due to sequestration of KTZ in the cell matrix, 1α,25-$(OH)_2$-D_3-treated cells were dosed with radiolabeled KTZ under conditions identical to those described for Phases I and II. The amount of KTZ equivalents (based on the initial specific radioactivity) in apical and basolateral compartments, measured at the end of the 2-hour Phase I incubation period, are shown in Table 1. As expected, KTZ equivalents in the apical and basolateral compartments, 2 hours after the administration of 1 μM KTZ to the apical compartment, were very similar for the three Phase II treatment groups. Approximately 57, 53 and 59%, and 14.5, 13.7 and 13.4% of the administered KTZ dose, for Conditions 1, 2 and 3, was found in the apical and basolateral compartments, respectively.

KTZ equivalents found in the apical, basolateral and cellular compartments after incubation of 1α,25-$(OH)_2$-D_3-cultured cells for 4 hours under Phase II conditions are also shown in Table 1. The sum of these values, 15.5, 15.1 and 16.6% of the administered KTZ dose for Conditions 1, 2 and 3, respectively, represents the amount remaining in the system after removal of the Phase I apical and basolateral medium and the three washes with DMEM. The total measured recovery of the administered KTZ dose was 87, 82 and 89% for Conditions 1, 2 and 3, respectively. The remainder of the dose was presumed to have been discarded with wash buffer between Phase I and II. During Phase II, most of the drug (> 80%) was removed from the cellular matrix by the end of the 4-hour incubation interval. However, cellular amounts of KTZ equivalents were highest (29.4 ± 2.0 pmol) for Condition 1 (minus HSA). In contrast, cellular amounts of KTZ equivalents were 11.6 ± 0.6 pmol when HSA was added to the basolateral medium (Condition 2), and were even lower, 4.4 ± 0.3 pmol, when HSA was placed both apically and basolaterally (Condition 3). One-way ANOVA showed that apical and basolateral KTZ equivalents after 4 hours of Phase II were statistically different for the three conditions ($p < 0.001$).

Measured intracellular KTZ equivalents under the three different Phase II incubation conditions corresponded well with the degree of inhibition of MDZ 1′-hydroxylation observed in activity experiments (Figure 6). That is, the least inhibition was found with the lowest intracellular KTZ equivalents (Condition 3), whereas the greatest inhibition was found with the highest intracellular KTZ equivalents under Condition 1. Placement of HSA in the basolateral compartment only (Condition 2), which

Table 1. Distribution of Ketoconazole After Pulsed Administration to Caco-2 Cells Cultured with 1α,25-$(OH)_2$-D_3

	Ketoconazole Equivalents[a] (pmol)		
	Condition 1 DM (A/B)	Condition 2 DM (A/B), HSA (B)	Condition 3 DM & HSA (A/B)
Phase I, t = 2 hours			
Apical	648 (36.4)	637 (33.7)	697 (70.2)
Basolateral	164 (10.7)	165 (14.4)	157 (19.2)
Phase II, t = 4 hours			
Apical	96.4 (2.1)	51.7 (2.6)[b]	149 (1.1)[b,c]
Basolateral	50.2 (5.3)	119 (4.6)[b]	41.5 (3.1)[b,c]
Cellular	29.4 (2.0)	11.6 (0.6)[b]	4.4 (0.3)[b,c]

[a] Ketoconazole equivalents are based on specific radioactivity of the ketoconazole dose. Data are presented as the mean (standard deviation) of triplicate determinations, and were subjected to one-way ANOVA with pairwise comparisons using SPSS, Version 8.0, (Chicago, IL).
[b] Statistically different from Condition 1, Phase II, $p < 0.001$.
[c] Statistically different from Condition 2, Phase II, $p < 0.001$.

is more representative of the *in vivo* state of intestinal mucosa, had an intermediate effect on the redistribution of KTZ into the apical and basolateral compartments during Phase II, and an intermediate effect on CYP3A4 activity.

Given its high affinity for KTZ, inclusion of HSA into the culture medium during Phase II had a predictable effect on the redistribution of KTZ from the cellular matrix. Under Condition 1, the apical to basolateral KTZ concentration ratio at 4 hours was 1.9. When 2 g/dL HSA was placed basolaterally (Condition 2), the apical to basolateral ratio decreased to 0.43. Placement of HSA on both the apical and basolateral sides (Condition 3) yielded an apical to basolateral ratio of 3.6.

To confirm that KTZ bound to CYP3A4 represented only a minor fraction of the total cellular inhibitor content, Caco-2 monolayers that had not been cultured with 1α,25-$(OH)_2$-D_3 were dosed with radiolabeled KTZ and treated in a manner identical to that described above for 1α,25-$(OH)_2$-D_3-cultured cells. Results indicate that the absence of CYP3A4 in the Caco-2 monolayer (14) had no appreciable effect on the uptake and distribution of KTZ under Phase I and Phase II incubation conditions (Table 2). Measured recovery of the administered KTZ dose was 84, 85 and 87% for Conditions 1, 2, and 3, respectively.

DISCUSSION

Ketoconazole is a potent inhibitor of CYP3A4-catalyzed drug metabolism, both *in vitro* and *in vivo*. Studies with cyclosporine, tacrolimus and tirilazad (10,18,19) suggest that KTZ will inhibit intestinal CYP3A4 enzyme and that this effect may persist beyond the period of inhibitor absorption from the intestinal lumen. The present results obtained with Caco-2 cell cultures suggest that the inhibitory effect of KTZ in an intact cell monolayer system may not be rapidly reversible. Pulsed treatment of the Caco-2 extracellular culture medium with KTZ produced an inhibition of intracellular CYP3A4 activity that was only slowly reversed when a drug binding sink (HSA) was added to the basolateral compartment, mimicking the *in vivo* state. Inhibition of CYP3A4 by KTZ was not irreversible, as indicated by the return to control activity when HSA was added to the apical medium (a non-physiological state). We also showed that the persistent inhibition of CYP3A activity coincided with sequestration of KTZ equivalents within the Caco-2 cell monolayer (Table 1). Based on previous estimates of CYP3A4 content in 1α,25-$(OH)_2$-D_3-treated Caco-2 cells (15), the molar KTZ/CYP3A4 ratio at the end of Phase II was approximately 9.8, 3.8 and 1.5 under Conditions 1, 2, and 3, respec-

Table 2. Distribution of Ketoconazole After Pulsed Administration to Caco-2 Cells Cultured Without 1α,25-$(OH)_2$-D_3

	Ketoconazole equivalents[a] (pmol)		
	Condition 1 DM (A/B)	Condition 2 DM (A/B), HSA (B)	Condition 3 DM & HSA (A/B)
Phase I, t = 2 hours			
Apical	602 (11.8)	603 (4.9)	582 (15.0)
Basolateral	185 (17.0)	183 (13.9)	184 (10.9)
Phase II, t = 4 hours			
Apical	118 (2.1)	59.8 (0.6)[b]	186 (0.5)[b,c]
Basolateral	40.6 (4.5)	133 (5.2)[b]	52.8 (6.6)[b,c]
Cellular	38.2 (2.9)	19.7 (1.5)[b]	8.9 (0.4)[b,c]

[a] Ketoconazole equivalents are based on specific radioactivity of the ketoconazole dose. Data are presented as the mean (standard deviation) of triplicate determinations, and were subjected to one-way ANOVA with pairwise comparisons using SPSS, Version 8.0, (Chicago, IL).
[b] Statistically different from Condition 1, Phase II, $p < 0.001$.
[c] Statistically different from Condition 2, Phase II, $p < 0.001$.

tively. The corresponding inhibition of CYP3A4 activity was 50, 19 and 11%. Similar amounts of KTZ were found in the cell monolayer at the end of Phase II in cells that were not treated with 1α,25-$(OH)_2$-D_3, and presumably expressed negligible amounts of CYP3A4 (Table 2). Together, these data suggest that most of the inhibitor that resides within the active cell monolayer is not associated with CYP3A4, although occupancy/inhibition of CYP3A4 appears related to total KTZ levels. However, since total radioactivity was measured, one can not rule out the possibility that a KTZ metabolite, and not unchanged parent drug, could be inhibiting CYP3A4 in a persistent manner. KTZ behaves as a noncompetitive type inhibitor of CYP3A4 with a high affinity K_i (~25 nM) in human liver and intestinal microsomes (16). In modified Caco-2 cells, KTZ may form a tight complex with ferrous P450 3A4. Indeed, the binding of other antifungal agents (clotrimazole and miconazole) to reduced rat microsomal P450 will only slowly reverse after the addition of carbon monoxide (20). However, for inhibition of CYP3A4 in the Caco-2 cell to be sustained for several hours, the off-rate from the enzyme binding site would have to be exceedingly slow. It is conceivable that KTZ is sequestered nonspecifically into cellular lipids, even after its removal from the extracellular medium, and that some may redistribute to the enzyme after the addition of MDZ, leading to the formation of a stable complex. A major driving force for inhibition under this mechanism, and that of direct partitioning from a lipid domain to the active site of CYP3A4, would be the initial (first-pass) exposure to high concentrations of KTZ and sequestration of the inhibitor into cellular lipids, with relatively slow redistribution to the basolateral (i.e., vascular) compartment.

In summary, we have shown that KTZ causes a persistent inhibition of CYP3A4 in a modified Caco-2 cell culture system. Our findings with the cell system mimic the persistent inhibition of intestinal first-pass by KTZ that is observed *in vivo*. Attempts to predict the magnitude of an *in vivo* drug-drug interaction from experimental data generated with *in vitro* microsomal systems are employed widely in drug development. If this finding applies to other potent CYP3A inhibitors, it may further complicate our ability to make quantitative *in vitro* to *in vivo* predictions of a drug-drug interaction from subcellular tissue fractions alone.

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