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

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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 is 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 Kᵢ. 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 Cₘₐₓ 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-D₃ (1α,25-(OH)₂-D₃)-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'-[3H]1'-hydroxymidazolam (1'-[3H]1'-OH MDZ) were provided by Roche Laboratories (Nutley, NJ). N-methyl-N-(2-butyldimethylsilyl) trifluoroacetamide was purchased from Pierce Chemical (Rockford, IL). Acetonitrile and...
ethyl acetate were obtained from Fisher Scientific (Fair Lawn, NJ). Dubelco’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. Untreated polyethylene terephthalate inserts and mouse laminin were obtained from Collaborative Biomedical Products (Bedford, MA). The hormone 1α,25-(OH)2-D3 was obtained from Calbiochem (La Jolla, CA). KTZ [3H(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-D3 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 mm² 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 × 105 cells/cm² 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-D3, 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 cm²). TEER values for all experiments were consistent with previously reported values (14,15).

IC50 Determination

Experiments were performed to determine the IC50 of KTZ in the confluent, 1α,25-(OH)2-D3-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 MDZ (6 µM) 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 + 2 g/dL HSA added to the basolateral side; Condition 3, DM (1.5 mL) on both apical and basolateral sides + 2 g/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-D3. Cells were grown to confluence and were fed medium containing or lacking 1α,25-(OH)2-D3 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 [3H(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,