Journal of Pharmaceutical Sciences 105 (2016) 1318–1326

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmacokinetics, Pharmacodynamics and Drug Transport and Metabolism

Ketoconazole Stereoisomers Differentially Induce Cytochrome P450 1A1 Between Human Hepatoma HepG2 and Mouse Hepatoma Hepa1c1c7 Cells

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ARTICLE INFO

Article history:
Received 15 October 2015
Revised 10 December 2015
Accepted 11 December 2015
Available online 6 February 2016

Keywords:
cytochrome P450
dose-response
drug metabolizing enzymes
enzyme kinetics
enzymes
hepatic clearance
hepatic metabolism
kinetics

ABSTRACT

Ketoconazole (KTZ) has 2 chiral centers with the therapeutically active form being a racemic mixture of 2 *cis*-enantiomers, namely, (2R,4S)-(+)-KTZ and (2S,4R)-(−)-KTZ. The aims of the present study were to examine the effects of (+)-KTZ, (−)-KTZ, and (±)-KTZ on aryl hydrocarbon receptor activation and subsequently CYP1A1 induction in both human HepG2 and murine Hepa1c1c7 hepatoma cells, and to further test their inhibitory effect using recombinant human and mouse CYP1A1 enzymes. Our results demonstrated that (+)-KTZ induced human CYP1A1 more than (−)-KTZ, whereas on the other hand (−)-KTZ induced murine Cyp1a1 more than (+)-KTZ at the mRNA, and activity levels. Human CYP1A1 showed higher affinity to 7ER compared with murine Cyp1a1 (K_m values 13.29 nM for human vs. 168.1 nM for murine). The intrinsic clearance values for human and murine CYP1A1 were 194.1 and 87.6 μL/pmol P450/min, respectively, whereas, V_{max} values were 2.58 and 14.73 pmol/pmol P450/min, respectively. (+)-KTZ and (−)-KTZ directly inhibited CYP1A1 activity by noncompetitive mechanism. The affinity of (−)-KTZ to interact with human CYP1A1 and murine Cyp1a1 was significantly different from (+)-KTZ, as the K_i values for human CYP1A1 and murine Cyp1a1 were 199.4 and 413.7 nM, respectively, for (+)-KTZ, and 269.3 and 230.8 nM, respectively, for (−)-KTZ.

Introduction

Ketoconazole (KTZ) is an imidazole antifungal agent that acts via inhibiting lanosterol 14 α-demethylase (CYP51). Inhibition of CYP51 by KTZ is a critical step to prevent the conversion of lanosterol to ergosterol, which leads to the accumulation of 14-α-methylsterols with successive inhibition of fungal growth. The favorable effect of KTZ is not only limited to CYP51 as it also inhibits multiple enzymes involved in adrenal cortisol synthesis and has been efficiently used in the treatment of hypercortisolemia (Cushing's syndrome).[1] Furthermore, KTZ inhibits the synthesis of testosterone in both testicular and adrenal cells, making it an attractive tool for the treatment of prostate cancer.[2] Unfortunately, since its introduction onto the market in the late 1980s and early 1990s of the past century, increasing evidence of KTZ-associated hepatotoxicity and hepatic tumors have been also reported.[3,4] Furthermore, KTZ has been historically used as an index inhibitor of CYP3A isozymes in drug-drug interaction studies.[5] In October 2013, the Food and Drug Administration had advised against the use of KTZ in clinical drug-drug interaction studies due to the serious potential liver injuries and adrenal gland problems associated with its administration.[6]

Looking at its chemical structure, it is apparent that KTZ has 2 chiral centers with the therapeutically active form being a racemic mixture of 2 *cis*-enantiomers, namely, (2R,4S)-(+)-KTZ and (2S,4R)-(−)-KTZ. The 2 other *trans*-enantiomers have also been previously evaluated for their selectivity in inhibiting a number of cytochrome P450 enzymes (CYPs) including CYP51.[7] In this regard, it was shown that the *cis*-enantiomers are far more potent inhibitors of CYP51 than their *trans*-enantiomer counterparts. KTZ also inhibits several CYPs, including CYP2C9, CYP2C19, and CYP3A4, resulting in potential drug-drug interactions with other medications.[8-10] We were the first to report the significant difference in the pharmacokinetic

Conflict of interest: there is no conflict of interest.

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http://dx.doi.org/10.1016/j.xphs.2015.12.009
0022-3549/

profile between (+)- and (−)-KTZ *in vivo*, highlighting the enantioselective interaction of KTZ with metabolizing enzymes.[11] Of interest, the enantioselective inhibition of CYP3A4 and CYP3A5 by KTZ has been previously reported.[8] KTZ has also been shown to have an antagonistic effect on human glucocorticoid receptor[12] while having an agonistic effect toward both pregnane X receptor and aryl hydrocarbon receptor (AhR), henceforth its effects on xenobiotic-metabolizing enzymes pathways are multifaceted.[13,14]

With respect to its effect on CYP1A1, we have previously reported that KTZ and other antifungal agents are inducers of *CYP1A* genes in human and murine cancer cell lines through an AhR-dependent mechanism.[14] Furthermore, a recent study has demonstrated that (+)-KTZ is a more potent agonist for human AhR than (−)-KTZ with a subsequent increase in CYP1A1 mRNA, protein, and catalytic activity levels.[13] To this end, it has been fundamentally generalized that human and other species AhR and CYP1A1 behave similarly but not identically in response to different agonists or antagonists and/or inducers/inhibitors, respectively. Despite these generalizations, there is in fact species-specific differences in AhR-ligand binding and function. For example, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) binding to the mouse AhR has been shown to be antagonized by several di-ortho polychlorinated biphenyls; however, these compounds only partially antagonized the rat AhR and did not exhibit any antagonistic effect on the human or guinea pig AhRs.[15] More importantly, benzimidazole drugs such as omeprazole, thiabendazole, and lansoprazole can significantly activate AhR in human HepG2 cells, while they were unable to activate AhR in mouse Hepa1c1c7 cells further highlighting the species-specific differences between the 2 species.[16] The species-specific differences are not only limited to AhR-ligand binding and subsequent activation but are also extended to CYP1A1 catalytic activity. In this regard, it has been previously demonstrated that mouse Cyp1a1 (mCyp1a1) shares 80% homology with human CYP1A1 (hCYP1A1),[17] therefore giving rise for the species-specific substrate preference and responses to direct acting CYP1A1 inhibitors across the 2 species. For example, it has been previously reported that Hepa1c1c7 cells show ~4.8 times more TCDD-induced B[a]P hydroxylase activity (per unit of mCyp1a1 mRNA) than HepG2 cells exhibit for induced B[a]P hydroxylase activity (per unit of hCYP1A1 mRNA).[18]

Therefore, the objectives of the present study were to examine the effects of (+)-KTZ, (−)-KTZ, and (±)-KTZ on AhR activation and subsequently CYP1A1 induction in both human hepatoma HepG2 cells and murine hepatoma Hepa1c1c7 cells. In addition, we examined the direct inhibitory effect of (+)-KTZ, (−)-KTZ, and (±)-KTZ on recombinant human CYP1A1 and murine Cyp1a1 using the specific substrate 7-ethoxyresorufin (7ER).

Materials and Methods

Materials

7ER, fluorescamine, racemic ketoconazole [(±)-KTZ], and Dulbecco's Modified Eagle Medium were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent, high-capacity cDNA reverse transcription (RT) kit, and SYBR Green PCR Master Mix were purchased from Life Technologies (Carlsbad, CA). pRL-CMV plasmid and dual luciferase assay reagents were obtained from Promega (Madison, WI). Human xenobiotic responsive element (XRE)-driven luciferase reporter plasmid (pGudLuc 6.1) and mouse XRE-driven luciferase reporter plasmid (pGudLuc1.1) were generously provided by Dr. M.S. Denison (University of California, Davies, CA). Human CYP1A1- and mouse Cyp1a1-containing cell microsomes supplemented with nicotinamide adenine dinucleotide phosphate (NADPH)—P450 reductase (Supersomes) were obtained from Gentest (Woburn, MA); cytochrome b5 and NADPH-P450 reductase were obtained from Oxford Biomedical Research (Oxford, MI). All other chemicals were purchased from Fisher Scientific (Toronto, ON).

Collection of KTZ Enantiomers From (±)-KTZ

The 2 *cis*-enantiomers, (2R,4S)-(+)-KTZ and (2S,4R)-(−)-KTZ, were isolated from the commercially available (±)-KTZ. A previously published normal-phase high-performance liquid chromatography method was used, where the 2 enantiomers were isocratically separated on ChiralPak AD column (Diacel Chemical Industries Ltd, Fort Lee, NJ) using 0.1% diethylamine in hexane, absolute ethanol, and 2-propanol (70:20:10).[19] The eluates corresponding to each KTZ enantiomer were collected, dried, and quantified using published reverse-phase high-performance liquid chromatography method.[20]

Cell Culture

Murine hepatoma Hepa1c1c7 and human hepatoma HepG2 cell lines, ATCC number CRL-2026 and HB-8065 (Manassas, VA), were maintained in Dulbecco's Modified Eagle's Medium, without phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20-μM L-glutamine, 50 μg/mL amikacin, 100 IU/mL penicillin, 10 μg/mL streptomycin, 25 ng/mL amphotericin B, 0.1-mM nonessential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 cell-culture flasks at 37°C in a 5% CO_2 humidified incubator.

Chemical Treatments

Cells were treated in serum-free medium with various concentrations of (−)-KTZ, (+)-KTZ, or (±)-KTZ (0.2-5 μM) or TCDD (1 nM) as described in figure legends. KTZ was dissolved in ethanol while TCDD was dissolved in dimethylsulfoxide, and both were maintained at −20°C until use. In all treatments, the ethanol and dimethylsulfoxide concentrations did not exceed 0.05% (vol/vol).

RNA Extraction and cDNA Synthesis

Six h after incubation with the test compounds, cells were collected and total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. Thereafter, first-strand cDNA synthesis was performed using the high-capacity cDNA RT kit (Life Technologies) according to the manufacturer's instructions. Briefly, 1.5 μg of total RNA from each sample was added to a mix of 2.0 μL 10 × RT buffer, 0.8 μL 25 × deoxyneucleotide mix (100 mM), 2.0 μL 10 × RT random primers, 1.0-μL MultiScribe reverse transcriptase, and 3.2-μL nuclease-free water. The final reaction mix was kept at 25°C for 10 min, heated to 37°C for 120 min, heated to 85°C for 5 min, and finally cooled to 4°C.

Quantification by Real-time PCR

Quantitative analysis of specific mRNA expression was performed by real-time PCR, by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 system (Applied Biosystems). Twenty-five microliter reactions contained 0.1 μL of 10 μM forward primer and 0.1 μL of 10 μM reverse primer (40-nM final concentration of each primer), 12.5 μL of SYBR Green Universal Mastermix, 11.05 μL of nuclease-free water, and 1.25 μL of cDNA sample. Primers and probes for mouse Cyp1a1