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In vitro ocular metabolism and bioactivation of ketoconazole in rat, rabbit and human

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ABSTRACT

Oral ketoconazole is clinically administered for treatment of severe cases for fungal keratitis. Pharmacodynamics and efficacy of oral and topical (ocular) ketoconazole is explored in rabbit. However, metabolism of ketoconazole in the eye in any species is not well explored in any preclinical species or human. An understanding of ocular drug metabolism in the eye is crucial for ocular therapeutics to facilitate the risk assessment and development of potential drug candidates for the clinic. We aimed to investigate the metabolism of ketoconazole in rat, rabbit and human ocular S9 fractions. Metabolism in liver S9 fractions was also studied for a direct comparison. Eleven putative metabolites were identified in the *in vitro* incubations. Of these metabolites, six were rat ocular whereas eight were present in rabbit and human ocular matrices each. Metabolic pathways in rabbit and human ocular fractions suggested the formation of reactive intermediates in rabbit and human liver and ocular S9 incubations, which was confirmed with trapping studies. Herein, we report eight human ocular metabolites of ketoconazole for the first time. To the best of our knowledge, this is the first report of ocular metabolic pathways and ocular bioactivation of ketoconazole in preclinical species and human.

1. Introduction

The imidazole antifungal, ketoconazole (KT), is a medication that is used to treat a variety of superficial and deep tissue fungal infections since its first approval in the United States in 1981 [1]. Its mode of action is to prevent production of fungal cell membranes [2]. Mycotic keratitis is an ocular condition that is manifested by inflammation, redness, tearing of cornea and can rapidly worsen if not treated. It is believed to be caused by poor upkeep of contact lenses or prolonged exposure to fungi in an outdoor setting [3]. KT has been prescribed orally for the treatment of different types of keratitis [4] alone or in combination with steroids and other antibiotics. KT, 200 mg (once or twice daily) is clinically utilized for treatment of severe cases of *Acanthamoebic* keratitis [5] and *Aspergillus* keratitis [6]. In conjunction with topical antibiotics, KT is also administered orally to treat *Fusarium* keratitis and prevent progression to endophthalmitis [7]. Furthermore, KT has been documented to be effective in preventing recurrent fungal keratitis in patients previously on an intensive topical antifungal regimen [8]. Topical ocular administration of KT for treatment of fungal keratitis has been well tolerated in humans [4] and experimentally, in rabbits [9–12]. Yet, ocular metabolism of KT has never been documented.

Orally administered KT has been linked with hepatotoxicity. Hepatic KT metabolism has been previously studied *in vitro* in rabbit and human [13] and in vivo in mouse [14]. Bioactivation of KT arises due to multiple mechanisms, including but not limited to the formation of the *N*-deacetyl-ketoconazole metabolite and further metabolism by Flavin monooxygenases (FMO) enzymes [15]. Therefore, we decided to examine the ocular metabolism and potential ocular bioactivation of KT with the help of a unique *in vitro* ocular metabolism model recently established in our laboratory [16,17]. Our objective was to document ocular metabolism of KT in S9 fractions obtained from rat, rabbit and human in comparison with liver S9 fractions to contrast our finding from ocular studies

Abbreviations: CID, collision induced dissociation; $[M+H]^+$, protonated molecular ion; FTMS, Fourier transformation mass spectrometry; KT, ketoconazole; RT, retention time; DAK, *N*-deacetylated ketoconazole; FMO, Flavin monooxygenase.

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with those from hepatic metabolism studies documented in the literature. Bioactivation of KT was studied in rabbit and human, specifically because of their pharmacological and clinical relevance.

2. Materials and methods

2.1. Chemicals and reagents

KT was obtained from the compound inventory at Novartis Institutes for Biomedical Research (Basel, Switzerland). Human (mixed gender pool, 11 donors), rabbit (male New Zealand white, 75 donors) and rat (male Sprague–Dawley, 200 donors) ocular S9 (5 mg/mL protein content) were purchased from XenoTech (Lenexa, KS). Rabbit (male New Zealand white, 11 donors) and rat (male Sprague–Dawley, 400 donors) liver S9 (20 mg/mL protein content) were purchased from XenoTech (Lenexa, KS) whereas, human (mixed gender, 50 donors) liver S9 (20 mg/mL protein content) was obtained from BD Bioscience (San Jose, CA). Solvents were HPLC or MS grade and were purchased from Sigma Aldrich (St. Louis, MO, USA) or Mallinckrodt Baker (Phillipsburg, NJ, USA). NADPH and alamethicin from *Trichoderma viride* were purchased from MP Biomedicals (Solon, OH). All remaining chemicals were purchased from Sigma Aldrich.

2.2. In vitro rat, rabbit and human ocular and liver S9 incubations

With the following adjustments, KT ocular and liver S9 incubations were conducted at 1.25 mg/mL protein concentration (1 mL incubation volume) according to commonly accepted protocols, as previously established in our laboratory [16]. All cofactors (SAM, PAPS, Acetyl CoA, etc) including NADPH (1 mM) and UDPGA (5 mM) were added after the two pre-incubations and the reaction was started upon the addition of substrate, KT (10 μM). Aliquots (0.3 mL) were quenched with equal amounts of ice-cold acetonitrile with 0.1% formic acid to stop the reaction at 0 and 1 h. The quenched reactions were vortexed and centrifuged for 5 min at 4630× *g*. The supernatants were transferred to a DW-96 plate, dried down under gentle stream of $N_{2(g)}$ to a pellet, and reconstituted (15× concentration) in the initial mobile phase conditions. The samples were subjected to analysis by LC–MS^n.

2.3. In vitro human and rabbit ocular and liver S9 fractions for iminium ion trapping

Human and rabbit ocular and liver S9 fractions (1 mg/mL protein concentration) were pre-warmed for 3 min in 0.1 M potassium phosphate buffer (pH 7.4) with magnesium chloride, KT (10 μM) and KCN (1 mM KCN:$K^{13}C^{15}N$ 70:30 (v/v)) at 37 °C. NADPH (1 mM) was then added to start the reaction. Reaction mixture was quenched at 0 and 1 h and processed as previously described. The supernatants were transferred to a DW-96 plate and dried down under $N_{2(g)}$ to half the original volume. The samples were then analyzed by LC–MS^n.

2.4. LC–MS^n for metabolism identification

All samples were analyzed on a Thermo Orbitrap-XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with CTC PAL autosampler, a 3× Ti high-performance LC pump (LEAP Technologies, Carrboro, NC)

The analytes were injected and separated on a Waters Symmetry C18 analytical column (5 μm 2.1 × 150 mm; Milford, MA) with a 35 min gradient elution method. Mobile phase A consisted of 10 mM ammonium formate in MS-grade water with 0.1% formic acid. Mobile phase B consisted of acetonitrile with 0.1% formic acid. The sample aliquots were eluted at a flow rate set at 0.25 mL/min. The sample gradient started at 10% B for 2 min and was linearly increased to 90% over 27 min, and held for 3 min. The column was returned to 10% B and held for 3 min before the next injection. LTQ-Orbitrap XL was calibrated in positive electrospray ionization as recommended by the vendor. Detailed MS tune parameters have been described elsewhere [18]. Samples were analyzed with a 35 V capillary voltage, 100 V tube lens, sheath gas of 35 (arbitrary units), auxiliary gas of 5 (arbitrary units), and capillary temperature of 325 °C. Samples were analyzed under data dependent scanning methods detailed in Bushee and Argikar, 2011 [19]. In short, full scans were acquired at 30,000 resolution and CID MS/MS and MS^3 scans were obtained at 7500 resolution. Analysis of the metabolite identification and reactive intermediate trapping samples were manually analyzed with Xcalibur software (version 2.0.7 SP1, Thermo Scientific).

3. Results

3.1. CID product ion spectrum of KT

KT showed a protonated molecular ion $[M+H]^+$ at *m/z* 531. The LC retention time was approximately 17.0 min. The CID product ion spectrum (Fig. 1) showed fragment ions 489, 446, 421, 311 and 255. Fragment ion at *m/z* 489 was formed from the loss of acetyl moiety. Fragment ion 446 was formed by loss of ethyleneamine moiety, whereas, fragment ion 421 was formed by loss of pyrazole moiety from *m/z* 489. Fragment ions 311 and 255 correspond to carbocations containing the pyrazolo and dichlorophenyl structural motifs. The product ion spectrum of KT has been described in depth previously by Whitehouse et al. and Fitch et al. [20,21].

3.2. Structural elucidation of M1 and M6

M1 showed a protonated molecular ion $[M+H]^+$ at *m/z* 545, 14 amu higher than the parent. The LC retention time was approximately 19.5 min and was observed in rabbit and human ocular and liver S9 fractions (Supp. Fig. 1). The retention time for M6 was approximately 15.5 min and was observed across all matrices (Supp. Fig. 6). The addition of 14 amu to parent fragment ion *m/z* 489 indicates oxidation. M1 and M6 were identified as oxidative metabolites of ketoconazole.

3.3. Structural elucidation of M2

M2 showed a protonated molecular ion $[M+H]^+$ at *m/z* 529, 2 amu less than the parent. The LC retention time was approximately 18.6 min and was observed in rat, rabbit and human ocular and liver S9 fractions. The CID MS/MS fragment ion used to elucidate the metabolite was *m/z* 487 and 255 (Supp. Fig. 2). Based on the ion product spectra the site of metabolism was most likely on the piperazine moiety. M2 was identified as hydroxylation followed by dehydration.

3.4. Structural elucidation of M3

M3 showed a protonated molecular ion $[M+H]^+$ at *m/z* 547, 16 amu more than the parent. The LC retention time was approximately 16.7 min and was observed in rat, rabbit and human ocular and liver S9 fractions (Supp. Fig. 3). The addition of 16 amu to parent fragment ion *m/z* 489 to afford *m/z* 505 indicates hydroxylation. M3 was identified as a hydroxylated metabolite of ketoconazole.

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