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Metabolomics-assisted metabolite profiling of itraconazole in human liver preparations☆

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ABSTRACT

Itraconazole (ITZ) is a first-generation triazole-containing antifungal agent that effectively treats various fungal infections. As ITZ has a better safety profile than that of ketoconazole (KCZ), ITZ has been used worldwide for over 25 years. However, few reports have explored the metabolic profile of ITZ, and the underlying mechanism of ITZ-induced liver injury is not clearly understood. In the present study, we revisited ITZ metabolism in humans, using a non-targeted metabolomics approach, and identified several novel metabolic pathways including *O*-dearylation, piperazine oxidation, and piperazine-*N*,*N*′-deethylation. Furthermore, we explored the formation of reactive ITZ metabolites using trapping agents as surrogates, to assess the possibility of metabolism-mediated toxicity. We found that ITZ and its metabolites did not form any adducts with nucleophiles including glutathione, potassium cyanide, and semicarbazide. The present study expands our knowledge of ITZ metabolism and supports the suggestion that ITZ has a better safety profile than that of KCZ in terms of metabolism-mediated toxicity.

1. Introduction

Itraconazole (ITZ, Fig. 1A) is a first-generation triazole-containing antifungal agent that has been used to treat various fungal infections for over 25 years. ITZ blocks ergosterol synthesis by inhibiting lanosterol-14α-demethylase [1,2]. However, the metabolic characteristics of ITZ have been reported in few literature including hydroxylation, oxidation to ketone, *N*-dealkylation, and dioxolane ring opening [3–5].

Non-targeted metabolomics approaches combined with drug metabolite profiling have been rendered possible by advances in mass spectrometric techniques and have recently been reviewed in detail [6,7]. Such novel approaches are used to evaluate all information acquired from biological samples, in turn enabling unbiased global profiling of drug metabolites (including uncommon metabolites). This overcomes the limitations of conventional metabolite profiling. These approaches have been used successfully to discover reactive metabolites (major causes of idiosyncratic adverse drug reactions [IADR]) using various trapping agents such as glutathione (GSH), potassium cyanide (KCN), and semicarbazide [8–11].

Oral ITZ cause acute liver injury at an incidence of 1 per 10,000 patients [12]. Although ITZ has a better safety profile than those of other azole antifungals such as ketoconazole (KCZ, incidence: 19 per 10,000 patients), the underlying mechanism of its hepatotoxicity remains poorly understood [13]. Moreover, ITZ bioactivation has not been investigated.

In the present work, we carefully explored the metabolic fate of ITZ *in vitro* using metabolomics techniques to develop a systemic view of ITZ metabolism and to derive novel insights into the underlying mechanism of ITZ-induced liver injury from the perspective of bioactivation.

2. Materials and methods

2.1. Materials

ITZ (≥98%), Krebs-Henseleit buffer, reduced nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate, glucose-6-phophate dehydrogenase, $MgCl_2$, GSH, semicarbazide, and KCN were obtained from Sigma-Aldrich (St. Louis, MO, USA). ITZ-d5 (Fig. 1B), keto-ITZ, 3′-hydroxy-ITZ, 2,4-dihydro-4-[4-[4-(4-hydroxyphenyl]-1-

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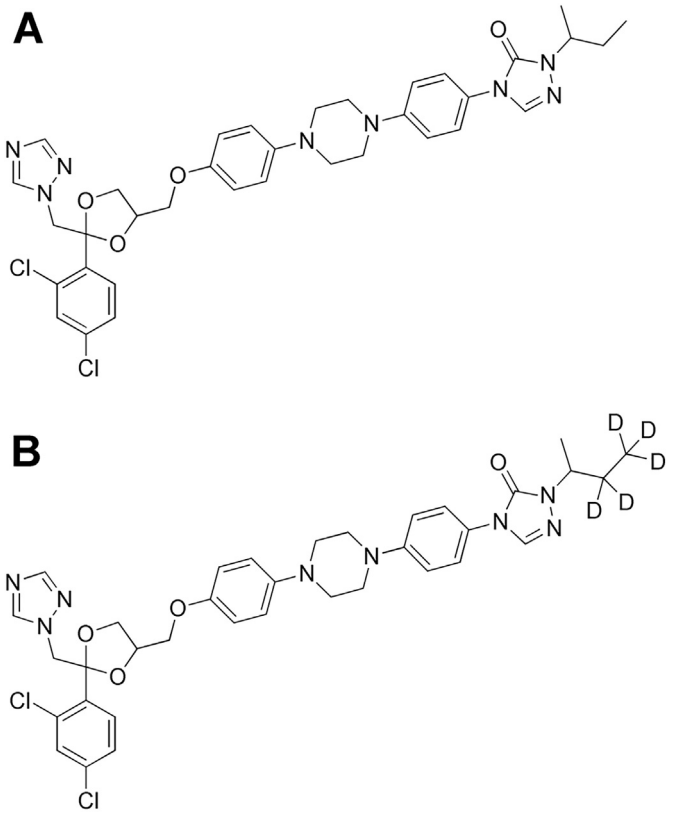

Fig. 1. Chemical structures of (A) itraconazole (ITZ) and (B) ITZ-d5.

piperazinyl]phenyl]-2-(1-methylpropyl)-3H-1,2,4-triazol-3-one, and *N*-desalkyl-ITZ were purchased from Toronto Research Chemicals (Toronto, ON, Canada). LiverPool™ 50-donor pooled cryopreserved human hepatocytes, InVitroGRO™ KHB, and InVitroGRO™ HT Medium were purchased from Bioreclamation IVT (Brussels, Belgium). Acetonitrile, methanol, and water (liquid-chromatography-mass spectrometry grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Other chemicals used were of the highest grade available.

2.2. Incubation with human liver microsomes for metabolite profiling

ITZ (20 μM) was incubated with human liver microsomes (1 mg/mL) in 50 mM potassium phosphate buffer (pH 7.4), with addition of 2 mM NADPH at 37 °C for 60 min. To assist in metabolite identification and structural elucidation, ITZ-d5 (20 μM) was separately incubated with microsomes at 37 °C for 60 min. The reactions were stopped by addition of 200 μL of ice-cold acetonitrile. Control samples were prepared by quenching immediately after addition of NADPH. The incubation mixtures were centrifuged at 15,000 ×*g* for 10 min at 4 °C, and 180 μL aliquots of supernatant were dried in vacuum concentrator. All incubations were performed in sextuplicate. The residues were reconstituted in 100 μL of 10% (v/v) methanol, and 5 μL of the aliquots were injected into a liquid chromatography-high resolution mass spectrometer (LC-HRMS).

2.3. Incubation with human liver microsomes for reactive metabolite profiling

Samples were prepared as described in a previous report [10]. ITZ (30 μM) was incubated with human liver microsomes (1 mg/mL) in 50 mM potassium phosphate buffer (pH 7.4), in the presence or absence of various trapping agents including GSH (2.5 mM), semicarbazide (2.5 mM), and KCN (1.5 mM). Controls were incubated in the absence of NADPH or trapping agents. The reactions were initiated by addition of NADPH and stopped by addition of 200 μL ice-cold acetonitrile at 0 and 60 min, respectively. The incubation mixtures were centrifuged at 15,000 ×*g* for 10 min at 4 °C, and 180 μL aliquots of the supernatant

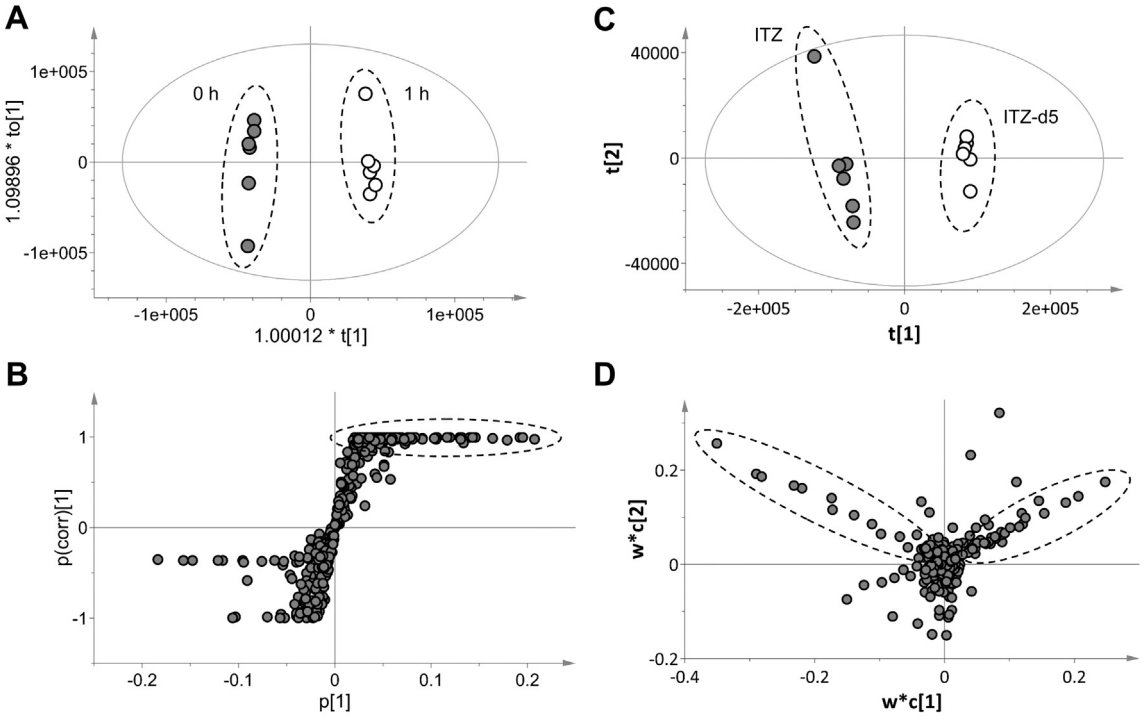

Fig. 2. Multivariate analysis of ITZ metabolites in human liver microsomes. (A) Score scatter plot and (B) loading S-plot obtained *via* orthogonal partial least squares-discriminant analysis of the 0-h and 1-h incubation groups. (C) Score scatter plot and (D) loading scatter plot obtained from partial least squares-discriminant analysis of the ITZ and ITZ-d5 incubation groups. Further structural investigation was performed focusing on the variables highlighted with dotted lines in (B) and (D).