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Short Communication

Deacetylated ketoconazole: a major ketoconazole metabolite isolated from mouse liver

L.W. WHITEHOUSE,* A. MENZIES, B. DAWSON, J. ZAMECNIK and W.-W. SY

Biotechnology Section, Drug Identification Division, Bureau of Drug Research, Health Protection Branch, Banting Research Center, Tunney's Pasture, Ottawa, Canada K1A 0L2

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Introduction

Ketoconazole, an imidazole-piperazine compound effective against a wide range of fungal pathogens, was first marketed in the United Kingdom in 1981 as an orally active antimycotic agent by Janssens Pharmaceutica Inc. It is becoming increasingly apparent, however, that ketoconazole (Nizoral) is also therapeutically useful in the treatment of other conditions such as cancer of the prostate [1], precocious puberty [2], hirsutism [3], and Cushing's Syndrome [4] giving it the potential for more widespread use.

Ketoconazole has been reported to be extensively metabolized into a number (>22) of metabolites with hepatic microsomal enzymes playing the major rôle in these biotransformation reactions [5, 6]. Oxidation, cleavage and degradation of the imidazole and piperazine rings, *O*-dealkylation, and aromatic hydroxylation were the suggested metabolic pathways involved in its biotransformation. Surprisingly, there is little published information to support these metabolic pathways and the failure to observe oxidative *O*-dealkylation in a recent study by Remmel *et al.* [7] only serves to raise questions regarding ketoconazole's metabolic disposition.

Recent studies from this laboratory have indicated that ketoconazole caused pathological changes (osmiophilic lysosomal inclusion forming a finger print pattern) in the livers of male Swiss Webster mice characteristic of phospholipidosis [8]. Energy dispersive X-ray microanalysis of these inclusions for the detection of chlorine indicated that they contained higher concentrations of chlorine than other cellular organelles or fluids (unpublished observation). These data suggested that ketoconazole or a metabolite with the chlorinated ring was concentrated in the liver. This paper describes the isolation and characterization of a novel metabolite of ketoconazole from hepatic tissue following a treatment regimen associated with ketoconazole induced phospholipidosis.

Materials and Methods

Animals

Male Swiss Webster mice weighing 25 ± 3 g purchased from Charles River Laboratories, Inc. (Montreal JOL 1XO, Canada), were acclimatized to the animal facility for at least 1 week on inert bedding (Beta Chips) with free access to food and water. Mice were treated with a 0.25% (w/v) Gum Tragacanth suspension (10 ml kg^{-1}) of ketoconazole (350 mg kg^{-1} po × 7 days). Twenty-four hours after the last dose of ketoconazole the animals were killed by decapitation and their livers collected and held on ice.

Extraction of liver

Each liver was homogenized in 5 ml of distilled water using a Tissumizer (Tekmar Co., Cincinnati, OH, USA). Homogenates

* Author to whom correspondence should be addressed.

were extracted with 20 ml of chloroform–methanol (2:1), followed by a wash with 2.0 ml of chloroform. Pooled organic extracts were stored at −20°C until processed.

Isolation and purification of the ketoconazole metabolite

Extracts were dried under nitrogen at 37°C and the resulting residue partitioned between hexane (15 ml) and methanol (7 ml) to remove neutral lipids. The residue resulting from the methanol fraction was dissolved in mobile phase (chloroform–methanol–water, 120:25:1), and injected (Waters U6K injector) onto a LiChroprep Si 60 column (Merck) equipped with a Waters M 6000 pump having a flow rate of 2 ml min^{-1} and a 440 detector set at 254 nm. Fractions were collected using an LKB fraction collector. Aliquots from each fraction were spotted on silica gel G plates, developed in chloroform–methanol–water (75:25:1) and the unknown ketoconazole metabolite visualized using an acetic acid–sulphuric acid–anisaldehyde (98:2:1) spray followed by heating. Fractions containing the ketoconazole metabolite were pooled, streaked on preparative silica gel G TLC plates and re-run in chloroform–methanol–ammonium hydroxide (75:25:3). The band of interest, located using iodine, was scraped from the plates, eluted with mobile phase, dried under nitrogen and redissolved in chloroform. The final purification step was by HPLC on a Waters resolve radial pak cartridge (5 μm, 8 mm × 10 cm) using chloroform–methanol–ammonium hydroxide (200:25:1.5) at a flow rate of 1 ml min^{-1}. The fractions containing the ketoconazole metabolite were pooled, dried, dissolved in chloroform and stored at −70°C until analysed.

Quantitation of de-N-acetyl ketoconazole

A Beckman System Gold HPLC equipped with an Altex injector connected to a 10 cm Whatman Partisil 5 ODS-3 column, and a 164 Beckman detector set at 254 nm was used to quantitatively assay liver extracts for deacetylated ketoconazole. Liver tissue (300 mg) homogenized in 2.7 ml of 0.1 M phosphate buffer (pH 9.5) was extracted with 10 ml of chloroform–methanol (2:1). The extract was dried under nitrogen and then redissolved in mobile phase. The mobile phase was methanol–ammonium hydrogen phosphate (50 mm) (80:20), pH 3.2 (before TEA addition) with both solutions containing 0.1% triethylamine (TEA) to reduce the retention of de-*N*-acetyl ketoconazole on the column. Authentic de-*N*-acetyl ketoconazole added to duplicate samples was used to assess extraction recoveries.

Chemical synthesis of de-N-acetyl ketoconazole

Ketoconazole (2.05 g) was added to a solution of sodium methoxide in methanol (1.65 g sodium in 125 ml methanol) and the solution was heated under reflux for 2 days. The solution was then cooled to 25°C, treated with water (13 ml) and the mixture evaporated to dryness on a rotary evaporator. The residue was passed through a silica gel column, eluted with methanol–chloroform using a step gradient starting with 10% methanol and increasing to 50%. The deacetylated ketoconazole was obtained as a white powder (0.78 g, m.p. 168–170°C) by recrystallization from ethanol. Its identity was confirmed by MS and various 1D- and 2D-NMR techniques.

Spectrometric analysis

Infrared spectra were obtained on a Nicolet model 60SX FT–IR. Sample and standards were run as 0.03% KBr disks. All NMR spectra were recorded in $CDCl_3$ at 300 K on a Bruker AM400 spectrometer equipped with an Aspect 3000 computer and process controller, using DISNMR version 870101. Standard microprograms from the Bruker software Library were employed. Chemical shift assignments were made by comparison with those for ketoconazole [9].

Mass spectra were obtained on a Finnigan 4610B quadrupole GC–MS, using a direct exposure probe (DEP). The mass spectrometer was scanned from 34 to 750 amu at 1 s per cycle in the electron impact mode using 45 eV electron energy and emission current of 0.32 mA.

Results and Discussion

Chromatographic studies

A metabolite with the chromatographic characteristics tabulated in Table 1 was isolated from mouse hepatic tissue. Its chromatographic characteristics were identical to those of authentic de-*N*-acetyl ketoconazole prepared chemically from ketoconazole. This metabolite was found to be the major metabolite in the liver at 24 h following the last dose

Table 1
Chromatographic characteristics of ketoconazole (KC) and its predominant metabolite (de-*N*-acetyl ketoconazole, DAKC) isolated from the livers of male Swiss Webster mice

Method	Solid phase	Mobile phase	Retention DAKC	Retention KC
TLC	Silica gel G	$CHCl_3$–CH_3OH–H_2O (75:25:1)	0.16	0.73
TLC	Silica G	$CHCl_3$–CH_3OH–NH_4OH (75:25:3)	0.68	0.85
HPLC	Silica 5 μ Radial Pak Cartridge	$CHCl_3$–CH_3OH–NH_4OH (200:25:1.5) 1 ml min^{-1}	8.03	3.20
HPLC	Partisil 5 ODS-3	CH_3OH + TEA (1%)–phosphate buffer (0.1 M, pH 3.2) + TEA (1%) (80:20)	6.52	2.85

Retention on TLC was expressed as R_f and on HPLC in min.

of a 7-day ketoconazole dosing regimen (350 mg kg^{-1} day po). Quantitative analysis of liver samples by LC indicated that the tentatively identified de-*N*-acetyl ketoconazole was present at a concentration of 1.79 ± 0.34 mg g^{-1} of tissue (n = 5). Material having an LC retention time similar to ketoconazole was also isolated from the liver, however, its structure was confirmed by NMR not to be ketoconazole. These results indicated that at 24 h following treatment with ketoconazole, accumulation of the parent drug in mouse liver was minimal, whereas the hepatic levels of de-*N*-acetyl ketoconazole were significant, demonstrating the potential of this metabolite for accumulation in the liver.

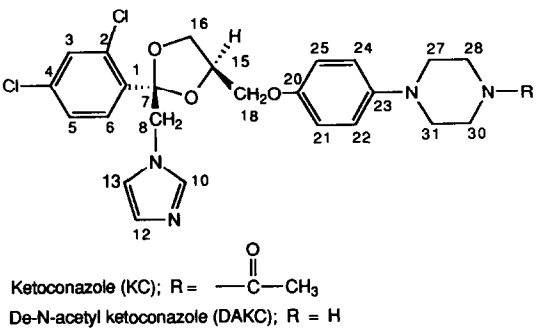

Figure 1
Structure and numbering scheme for ketoconazole and its hepatic *N*-deacetylated metabolite de-*N*-acetyl ketoconazole.

Spectroscopic identification

Infra-red spectroscopy. The structure of the tentatively identified *N*-deacetylated ketoconazole was established by spectrometric analysis. The structure and numbering scheme used are given in Fig. 1. A comparison of the IR bands of the unknown metabolite with ketoconazole indicated that the band in ketoconazole at 1647 cm^{-1} indicative of the acetylcarbonyl group on the piperazine ring, was absent in the metabolite isolated from the liver. This suggested that the unknown was a deacetylated metabolite of ketoconazole. Comparison of the IR for the metabolite with authentic de-*N*-acetyl ketoconazole obtained by synthesis supported the assigned structure.

NMR spectroscopy. A 400 MHz ^{1}H-NMR spectrum of the metabolite indicated that below δ3.8 the spectrum was very similar to that of ketoconazole. The only signals at higher field for ketoconazole, which were not in the metabolite spectrum, were due to the piperazine ring and acetyl protons. In the metabolite there was an eight-proton singlet at δ3.1 and a one proton, broad singlet at δ1.67 in this region. These are consistent with protons on a rapidly inverting piperazine ring with one secondary nitrogen. The assignments for the proton chemical shifts are given in Table 2. The 100 MHz ^{13}C-NMR spectrum of the metabolite was also consistent with it being de-*N*-acetyl ketoconazole — its carbon-13 signals corresponded closely to those for ketoconazole [9], except for the absence of the acetyl signals and the presence of only two signals for the piperazine carbons rather than the four signals observed with ketoconazole [9]. The ^{13}C-NMR chemical shift assignments are presented in Table 2. Both proton and carbon-13 NMR spectra for the metabolite were identical to those for the synthesized de-*N*-acetyl ketoconazole, except for the position of the secondary amine proton which appeared at δ1.67 and δ2.20, respectively.

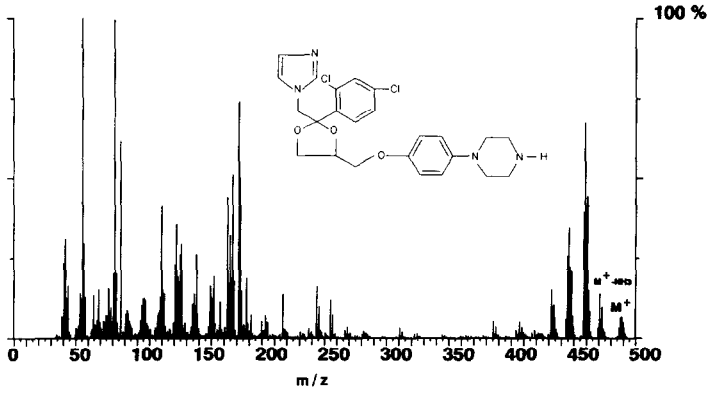

Figure 2
Mass spectrum of the hepatic *N*-deacetylated metabolite of ketoconazole.

Table 2
^{1}H and ^{13}C chemical shifts of de-*N*-acetyl ketoconazole (δH and δC from TMS ± 0.01)*

Site	δH	δC
1		134.64
2		133.00
3	7.46 (d)	131.36
4		135.86
5	7.24 (dd)	127.21
6	7.57 (d)	129.52
7		108.02
8	4.45 (a)	51.33
10	7.50 (dd)	138.80
12	6.99 (dd)	128.61
13	6.96 (dd)	121.11
15	4.33 (m)	74.83
16	3.87 (dd), 3.73† (m)	67.63
18	3.73† (m), 3.32 (dd)	67.79
20		152.31
21, 25	6.76 (m)	115.23
22, 24	6.88 (m)	118.10
23		146.78
27, 31	3.07 (s)	51.79
28, 30	3.07 (s)	46.27
29	1.67 (s)	

*Solvent: $CDCl_3$.
†Values approximate due to peak overlap.
s, d, q, m, dd — singlet, doublet, quartet, multiplet, doublet of doublets.

Mass spectrometry. Mass spectrometry of the hepatic metabolite of ketoconazole gave a molecular ion at 488 *m/z* with the characteristic two chlorine molecular ion cluster (Fig. 2). Further fragmentation patterns, such as the neutral loss of NH_3 to give 471 *m/z*, and further fragmentation of the remaining ethyl groups of the cleaved piperazine ring to yield ions at 419, 432, 446 and 459 *m/z*, respectively, supported the de-*N*-acetyl ketoconazole structure assignment, and was identical to the chemically synthesized product.

Possible biological implications. Brasseur *et al.* [10] have studied the orientation of ketoconazole and de-*N*-acetyl ketoconazole inserted in a model lipid monolayer, and demonstrated that deacetylation of ketoconazole caused a drastic membrane conformational change. It was postulated that deacetylation of ketoconazole could enhance the destabilizing membrane influence of the parent compound. The enhanced destabilizing influence of de-*N*-acetyl ketoconazole observed by Brasseur *et al.* [10], taken in conjunction with the finding of this study that de-*N*-acetyl ketoconazole is a major hepatic metabolite in the mouse, offers a possible explanation for the pathogenesis of ketoconazole induced phospholipidosis observed in mice [8].

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References

[1] J. Trachtenberg and A. Pont, *Lancet* **2**, 433–435 (1984).
[2] F.J. Holland, L. Fishman, J.D. Bailey and A.T.A. Fazekas, *N. Engl. J. Med.* **312**, 1023–1028 (1985).
[3] D. Carvalho, D. Pignatell and C. Resende, *Lancet* **2**, 560 (1985).
[4] M.E.B. Paola-Loli and M. Tagliaferri, *J. Clin. Endocr. Metab.* **63**, 1365–1371 (1986).
[5] E.W. Gascoigne, G.J. Barton, M. Michaels, W. Meuldermans and J. Heykants, *Clin. Res. Rev.* **1**, 177–187 (1981).
[6] A.L. Hume and T.M. Kerkering, *Drug Intel. Clin. Pharm.* **17**, 169–174 (1983).
[7] R.P. Remmel, K. Amoh and M.M. Abdel-Monem, *Drug Metab. Dispos.* **15**, 735–739 (1987).
[8] L.W. Whitehouse, R. Mueller and A. Pakuts, *The Toxicologist* **8**, 225 (1988).
[9] B.A. Dawson, *Can. J. Spectrosc.* In press.
[10] R. Brasseur, C. Vandenbosch, H. Van den Bossche and J.M. Ruysschaert, *Biochem. Pharmacol.* **32**, 2175–2180 (1983).

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