FLAVIN-CONTAINING MONOOXYGENASE-MEDIATED METABOLISM OF N-DEACETYL KETOCONAZOLE BY RAT HEPATIC MICROSONES

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Although ketoconazole is extensively metabolized by hepatic microsomal enzymes, the route of formation and toxicity of suspected metabolites are largely unknown. Reports indicate that N-deacetyl ketoconazole (DAK) is a major initial metabolite in mice. DAK may be susceptible to successive oxidative attacks on the N-1 position by flavin-containing monooxygenases (FMO) producing potentially toxic metabolites. Previous laboratory findings have demonstrated that postnatal rat hepatic microsomes metabolize DAK by NADPH-dependent monooxygenases to two metabolites as determined by HPLC. Our current investigation evaluated DAK’s metabolism in adult male and female rats and identified metabolites that may be responsible for ketoconazole’s hepatotoxicity. DAK was extensively metabolized by rat liver microsomal monooxygenases at pH 8.8 in pyrophosphate buffer containing the glucose 6-phosphate NADPH-generating system to three metabolites as determined by HPLC. The initial metabolite of DAK was a secondary hydroxylamine, N-deacetyl-N-hydroxyketoconazole, which was confirmed by liquid chromatography/mass spectrometry and NMR spectroscopy. Extensive metabolism of DAK occurred at pH 8.8 in pyrophosphate buffer (female 29% and male 53% at 0.25 h; female 55% and male 57% at 0.5 h; and female 62% and male 66% at 1.0 h). Significantly less metabolism of DAK occurred at pH 7.4 in phosphate buffer (female 11%, male 17% at 0.25 h; female 20%, male 31% at 0.5 h; and female 27%, male 37% at 1 h). Heat inactivation of microsomal-FMO abolished the formation of these metabolites from DAK. SKF-525A did not inhibit this reaction. These results suggest that DAK appears to be extensively metabolized by adult FMO-mediated monooxygenation.

Ketoconazole (KT)1 and other azoles (fluconazole and itraconazole) are prominent broad-spectrum oral antifungal agents used in the treatment of systemic mycoses, especially in patients who are susceptible to opportunistic systemic fungal infections (Ringel, 1990). KT and related azoles exert their antifungal actions by inhibiting lanosterol 14α-demethylase which is responsible for the conversion of lanosterol to ergosterol in fungi (Van Den Bossche et al., 1980). KT has been shown to have similar inhibitory effects on mammalian cytochrome P-450 (CYP) enzymes responsible for the conversion of lanosterol to cholesterol, a major component of membranes in mammals (Strandberg et al., 1987). In addition to its antymycotic activity, an inhibitory effect on the synthesis of testosterone in both testicular and adrenal cells makes KT a suitable candidate for treatment of androgen-dependent diseases such as advanced prostate cancer (Pont et al., 1984; Jubelirer and Hogan, 1989; Vogelzang and Kennealey, 1992). Also, KT has been shown to be a strong selective in vitro inhibitor of CYP3A (Maurice et al., 1992).

KT has been reported to be extensively metabolized to a large number of metabolites, with hepatic-microsomal enzymes playing the major role in the biotransformation reactions (Gascoigne et al., 1981; Daneshmend and Warnock, 1988). The metabolic pathways suggested include CYP-mediated oxidation, cleavage, degradation and scission of the imidazole and piperazine rings, oxidative O-dealkylation, and aromatic hydroxylation (Heel et al., 1982; Daneshmend and Warnock, 1988). The major metabolite in mice, deacetyl ketoconazole (DAK), Fig. 1, accumulates to significant levels in hepatic tissues, whereas accumulation of KT in the mouse liver is minimal (Whitehouse et al., 1994a,b). Moreover, two N-oxide metabolites of KT have been isolated from mouse liver (Whitehouse et al., 1994a). The formation of the aforementioned metabolites could be CYP- or flavin-containing monooxygenase (FMO)-mediated.

Due to KT’s extensive therapeutic usage, there have been numerous documented cases of KT-induced hepatotoxicity (Bercoff et al., 1985; Benson et al., 1988; Brusko and Marten, 1991). Rechallenge of KT in patients resulted in enhanced toxicity (Van Parys et al., 1987). The biochemical features related to KT hepatotoxicity generally tend to be hepatocellular injury in 57% of the patients and cholestatic injury in 43% of the patients (Stricker et al., 1986). Many reports indicate that the type of hepatic injury is zone 3 necrosis (Stricker et al., 1986; Benson et al., 1988). The overall incidence of clinical hepatitis appears to be on the order of 0.01 to 0.1% of the patients and many cases appear to be subclinical (Stricker et al., 1986). The actual incidence is probably greater due to underreporting (Stricker et al., 1986; Knight et
The hepatotoxicity was usually reversible when the drug was discontinued but recovery usually took as long as 3 to 6 months (Janssen and Symoens, 1983; Benson et al., 1988). Apoptosis through the p53-dependent pathway may be a mechanism by which KT induces toxicity in cultured rat liver cells (Ho et al., 1998).

Because the clinical literature has clearly demonstrated that KT can be toxic to the liver resulting in serious adverse reactions, new azole derivatives have been developed for antimycotic activity and for the treatment of prostate cancer. The newer azole derivatives, fluconazole derivatives have been developed for antimycotic activity and for the treatment of prostate cancer. The newer azole derivatives, fluconazole derivatives have been developed for antimycotic activity and for the treatment of prostate cancer. The newer azole derivatives, fluconazole derivatives have been developed for antimycotic activity and for the treatment of prostate cancer. The newer azole derivatives, fluconazole derivatives have been developed for antimycotic activity and for the treatment of prostate cancer. The newer azole derivatives, fluconazole derivat...
at 1.94. The major metabolite sample (~100 μg) was dissolved in 40 μl of solvent, then transferred to a Shigemi, Inc. microtube (Allison Park, PA) for accumulation of data. DAK and the model compound samples were prepared in standard 5-mm NMR tubes in ~0.4 ml of solvent.

**Liquid Chromatography/Mass Spectrometry (LC/MS).** Three microliters of a concentrated 1-h incubation sample was injected onto a chromatography system consisting of a Perkin Elmer ABI 140 B syringe pump, a Rheodyne 8125 injector with a 5-μl sample loop, and a 3-mm × 150-mm, 5-μm particle Waters C18 symmetry column. The solvents were 0.005 M ammonium formate (pH 4.5) and acetonitrile, which were delivered at 400 μl/min and programmed from 20% acetonitrile to 60% acetonitrile in 30 min. The individual HPLC peaks were directly analyzed with a Perkin Elmer Sciex triple quadruple ion spray mass spectrometer (Ontario, Canada) using an atmospheric pressure chemical ionization source. Assignments are characterized by LC/MS and 1H NMR spectrometry. The results of the LC/MS indicated the molecular weights of DAK (mw 489), M1 (mw 503), M2 (mw 505), and M3 (mw 517); data not shown. Table 1 shows the 1H NMR spectrum of DAK and M2.

The results of DAK’s metabolism from the various male hepatic-microsomal incubations are presented in Fig. 4. There was no metabolism of DAK from the microsomal incubation that did not contain the NADPH-generating system. Heat inactivation of FMO, which will abolish FMO activity while preserving CYP activity (Ziegler, 1980), completely inhibited the metabolism of DAK. The male rat hepatic microsomes displayed the same metabolic profile (data not shown). No significant differences were seen between the heat-inactivated FMO incubation and the incubation without the NADPH-generating system for both the male and female hepatic microsomes ($p > .05$).

Extensive metabolism occurred by 1 h (>60%) with the male and female hepatic microsomes with the FMO pyrophosphate buffer system containing the NADPH-generating system with SKF-525A, an inhibitor of CYP (male 39%, female 27% at 0.25 h; male 66%, female 56% at 0.5 h; and male 66%, female 65% at 1 h). There were no significant differences ($p > .05$) between the FMO pyrophosphate buffer system with and without SKF-525A for the male and female microsomes at all time points except for the 0.25-h male hepatic microsomes ($p < .05$). Thus, the metabolism of DAK was not significantly altered with the addition of SKF-525A. In contrast, significantly less metabolism of DAK occurred in the CYP phosphate buffer, pH 7.4 (male 17%, female 11% at 0.25h; male 31%, female 20% at 0.5 h; and male 37%, female 27% at 1 h). There was statistical significance ($p < .05$) with the male and female hepatic microsomes at all time points for the CYP phosphate buffer system compared with the FMO pyrophosphate buffer system.

Figures 5 and 6 show the percentages of metabolites formed after incubation with DAK from male hepatic microsomes. Results shown in Fig. 5 demonstrate that metabolism of DAK was greater in the pyrophosphate buffer, pH 8.8 (optimum conditions for FMO metabolism) than in the CYP phosphate buffer system, pH 7.4. The female hepatic microsomes displayed similar results. In addition, M2 appeared to be the initial metabolite formed from DAK in the pyrophosphate buffer as early as 0.25 h (30%), continued to increase at 0.5 h (43%), and then began to decrease by 1 h (39%). Unlike the male hepatic microsomes, M2 formation from the female hepatic microsomes in the pyrophosphate buffer increased over time to 22% at 0.25 h, 47% at 0.5 h, and 48% at 1 h. As M2 decreased over time from the male hepatic microsomes, there was an increase in metabolite formation of M1 and M3. M1 began to appear as early as 0.25 h (5%) and was present at 0.5 h (14%) and at 1.0 h (12%). M3 also began to appear at 0.25 h (3%) and continued to increase over time to 8% at 0.5 h and 14% at 1 h. As for the female hepatic microsomes, M1 remained at 5% over the time period evaluated. In contrast, M3 began to increase over time to 2% at 0.25 h (2%), 6% at 0.5 h, and 9% at 1h.

The metabolism of DAK in the CYP phosphate buffer was not as extensive as the FMO pyrophosphate buffer. M2 appeared to be the initial metabolite formed from DAK in phosphate buffer as early as 0.25 h (10%) and increased at 0.5 h (23%) and 1.0 h (24%). M2 formation from the female hepatic microsomes also increased over time in the phosphate buffer to 5% at 0.25 h, 16% at 0.5 h, and 20% at 1 h. M1 began to appear as early as 0.25 h (5%) and was present...
at 0.5 h (8%) and 1 h (7%) in the male hepatic-microsomal incubations. M3 also began to appear at 0.25 h (2%), and increased at 0.5 h (7%), and was still present at 1.0 h (6%). As for the female hepatic microsomes in the phosphate buffer, M1 began to appear as early as 0.25 h (9%) and was present at 0.5 h (3%) and 1.0 h (3%). M3 began to increase over time to 2% at 0.25 h, 2% at 0.5 h, and 4% at 1 h. Thus, metabolite formation was greatly reduced in the phosphate buffer compared with the pyrophosphate buffer.

In Fig. 6, the metabolism of DAK or the formation of its metabolites were not significantly altered (p > .05) with the addition of SKF-525A, a CYP inhibitor, in the male liver microsomes at 0.5 h and 1.0 h; however, there was a significant decrease in metabolism at 0.25 h (p < .01). No significance in DAK metabolism was seen for the female microsomes at all time points evaluated (p > .05). In addition, metabolite formation was not significantly altered (p > .05) in the presence of SKF-525A. Similarly, the use of anti-rat NADPH IgG reductase to inhibit microsomal-CYP in the incubation mixture did not alter the metabolism of DAK or its metabolite formation (data not shown). Thus, the CYP inhibitors did not display any significant inhibitory effect in hepatic microsomal metabolism of DAK. These results suggest that DAK is primarily converted to three metabolites, one of which is the piperazine secondary hydroxylamine, by FMO and not by CYP in the rat liver microsomes.

Discussion

KT’s primary metabolite, DAK, appears to be the major metabolite formed and accumulated in liver (Whitehouse et al., 1990). Our earlier studies demonstrated that DAK may be responsible, in part, for the hepatotoxicity associated with KT (Rodriguez and Acosta, 1997b). DAK was more cytotoxic than KT in a time- and dose-response relationship using postnatal (8- to 10-day-old) rat hepatocytes, sug-
The NADPH-generating system at optimum conditions for FMO pyrophosphate buffer, pH 8.8 (solid line), and CYP phosphate buffer, pH 7.4 (dashed line). DAK, □: M1, ●; ○: M2, ▲, △ (piperazine 2'-hydroxylamine); and M3, ◆, ○. Error bars represent S.E.M. of three experiments performed in triplicate. The data support the secondary hydroxylamine (A) as the major metabolite. Furthermore, hydroxylamine formation from 1-phenylpiperazine has been reported; however, we have now identified a DAK metabolite that is apparently generated by FMO using rat hepatic microsomes.

The major metabolite of DAK (mw 489) was determined by LC/MS analysis to have a mw of 505, which indicates an increase in mass of 16 mass units, consistent with addition of a single oxygen atom to the structure. The 1H NMR spectrum of this metabolite in acetonitrile-d6 showed only minor variations in chemical shifts for protons in the imidazole, dichlorophenyl, glycerol, and disubstituted benzene portions of the structure compared with DAK, but significant chemical shift changes were observed in the region corresponding to the piperazine ring (Table 1). The piperazine ring is therefore the site of oxidation. Three possible structures can be envisioned, the secondary hydroxylamine (A), the tertiary N-oxide (B), or a ring-hydroxylated structure (C), Fig. 7. The third structure, which would likely be an equilibrium mixture of cyclic and open chain forms if stable enough to be isolated, would require seven different carbon-bound protons, with the methine proton at the site of oxidation significantly deshielded relative to the remaining protons. The 1H NMR spectrum, eight protons are observed as four two-proton signals, eliminating structure C as a possibility. The N-oxide B would be expected to show significant effects on not only the piperazine ring protons, but also the disubstituted benzene ring protons (Whitehouse et al., 1994a). The shifts for the benzene ring protons in M2 are unaltered from those of DAK (Table 1). The data support the secondary hydroxylamine (A) as the major metabolite. Further evidence for structure A was obtained by synthesizing a secondary hydroxylamine model compound (Bilski and Ganem, 1983), 1-hydroxy-4-phenylpiperazine, from 1-phenylpiperazine and recording its NMR spectra. The 1H chemical shift changes and overall patterns observed on conversion of 1-phenylpiperazine to 1-hydroxy-4-phenylpiperazine were very similar to those seen when converting DAK to its major metabolite (Table 2). Furthermore, hydroxylamine formation from 1-phenylpiperazine has previously been reported in rat liver homogenates (Achari and Beckert, 1983). We therefore conclude that M2, N-deacetyl-N-hydroxyketonazole (cis-4-[4-[(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl)methyl]-1,3-dioxolan-4-yl]methoxy]phenyl]-1-hydroxypiperazine; N-hydroxy-DAK), has the secondary hydroxylamine structure A.

LC/MS experiments also indicated that M1 had a mw of 503, which is consistent with formation of a nitrene from M2 (loss of 2 mass units). Earlier studies have shown that hepatic microsomal-FMO catalyzed the formation of nitrones from hydroxylamines (Kadubur et al., 1973). Formation of M1 increased in a time-dependent manner after formation of N-hydroxy-DAK (Fig. 3). As the incubation time period increased, N-hydroxy-DAK decreased and M1 and M3 began to be detected by HPLC. This supports further FMO-mediated me-
abolished system. Lastly, heat inactivation of microsomal FMO completely because extensive metabolism occurred by 0.25 h, it is possible that containing the NADPH-generating system with and without SKF-525A.

Because extensive metabolism occurred by 0.25 h, it is possible that the end product, N-hydroxy-DAK, may have inhibited the reaction as early as 0.25 h, resulting in nonlinear kinetics. Metabolism of DAK in CYP phosphate buffer was significantly less compared with the FMO pyrophosphate buffer system. Moreover, both male and female displayed similar metabolic profiles of DAK in CYP phosphate buffer system. Lastly, heat inactivation of microsomal FMO completely abolished N-hydroxy-DAK, M1, and M3 formation. Therefore, male and female rat hepatic microsomes appear to metabolize DAK by FMO.

N-hydroxy-DAK was the predominant metabolite of DAK formed from both male and female hepatic microsomes. N-hydroxy-DAK appeared to undergo subsequent metabolism to M1 and M3 as indicated by the decrease of N-hydroxy-DAK and an increase in M1 and M3 formation. Although M1 and M3 were formed from N-hydroxy-DAK from female hepatic microsomes, metabolism formation was less than the male. We speculate that gender differences between male and female rats may play a role with DAK’s metabolite formation. Cher- rington et al. (1998) has reported that hepatic FMO3 and FMO5 are gender-independent in rat, whereas FMO1 appears to be selective to the male rat. Thus, DAK may be a substrate for hepatic FMO1, FMO3, and FMO5, whereas N-hydroxy-DAK may be a substrate for FMO3 and FMO5. Therefore, M1 and M3 could still be generated from female hepatic microsomes but not to the same extent seen with male hepatic microsomes.

Also, metabolism of DAK was greater in the FMO pyrophosphate buffer than in the CYP phosphate buffer system for both male and female hepatic microsomes. There was no significant differences in M1 and M3 formation between the male and female in the CYP phosphate buffer system. Furthermore, DAK’s metabolism or formation of its metabolites were not significantly altered in the presence of SKF-525A, a CYP inhibitor. Similarly, use of anti-rat NADPH IgG reductase to inhibit microsomal CYP in the incubation mixture did not alter metabolism of DAK or its metabolites. Thus, these data support that metabolism of DAK is not CYP mediated.

Lastly, our earlier study with postnatal (8- to 10-day-old) rat hepatic microsomes demonstrated that DAK was a substrate for FMO (Rodriguez and Acosta, 1997a). Metabolism of DAK occurred as early as 0.25 h (29%) up to 43% at 1 h in the FMO buffer. N-hydroxy-DAK was formed as early as 0.25 h (19%) and increased to 30% by 1 h. Also, M1 and M3 began to increase over the incubation period, (M1, 9% and M3, 6% at 0.25 h; M1, 9% and M3, 7% at 0.5 h; and M1, 11% and M3, 7% at 1.0 h). As seen with the adults, minimal metabolism of DAK and formation of its metabolites occurred in the CYP phosphate buffer. DAK was metabolized by 25% at 1.0 h with metabolite formation: N-hydroxy-DAK, 10%; M1, 11%, and M3, 3% formed by 1.0 h in the CYP phosphate buffer. Postnatal rats may display similar characteristics as mice in which FMO1 and FMO5 are present in hepatic tissue, whereas FMO3 was detected 2 weeks postpartum (Cherrington et al., 1998). Thus, postnatal rat hepatic microsomes possess hepatic FMO enzymes necessary for FMO-mediated metabolism as seen with adult microsomes.

In conclusion, we propose that bioactivation of DAK through oxidation attack by FMO on the piperazine ring generates a secondary hydroxylamine, N-hydroxy-DAK, which is susceptible to further FMO-mediated oxidative attacks. These products of further FMO metabolism may lead to toxic consequences. Continued efforts are being made to identify the remaining metabolites (M1 and M3) apparently generated by FMO. Moreover, current studies with recombinant human FMO3 confirm FMO-mediated metabolism of DAK to N-hydroxy-DAK (manuscript in preparation). Other metabolites may have been formed from DAK that were not detected by the HPLC method used in the present study. Lastly, if both oxidation and reduction reactions occur rapidly, tissue NADPH concentrations may be perturbed (Ziegler, 1988). NADPH could be depleted during metabolism of DAK to N-hydroxy-DAK and M1 and M3. Loss of cellular NADPH would affect a number of cellular processes and may be responsible, in part, for toxicity. Thus, it is possible that DAK’s metabolism to N-hydroxy-DAK by FMO may play a role in the hepatotoxicity associated with KT.

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References


