

Hepatotoxicity of ketoconazole in Sprague–Dawley rats: glutathione depletion, flavin-containing monooxygenases-mediated bioactivation and hepatic covalent binding

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1. This study has examined ketoconazole (KT)-induced hepatotoxicity *in vivo* and *in vitro*, using male Sprague–Dawley rats with [³H]KT (1.5 µCi mg⁻¹) at 40 and 90 mg KT kg⁻¹ doses. Blood and liver samples were collected from 0 to 24 h for alanine aminotransaminase (ALT), glutathione (GSH) and covalent binding analyses.
2. Covalent binding occurred as early as 0.5 h, peaked at 2 h (0.026 ± 0.01 nmol KT mg⁻¹ protein) and 8 h (0.088 ± 0.04 nmol KT mg⁻¹ protein) for 40 and 90 mg KT kg⁻¹ doses, respectively. ALT levels increased at 0.5 h for the 40 and 90 mg KT kg⁻¹ doses (44.3 and 56.4 U ml⁻¹, respectively) relative to control, 22.7 U ml⁻¹. At 24 h, the 90 mg KT kg⁻¹ dose reduced hepatic GSH levels from 9.92 ± 1.1 to 4.76 ± 0.3 nmol GSH mg⁻¹ protein.
3. The role of the flavin-containing monooxygenases (FMO) utilized Sprague–Dawley microsomes with 1, 10 and 100 µM [³H]KT. Maximum covalent binding occurring at 100 µM KT. Heat inactivation of microsomal FMO significantly decreased covalent binding by 75%, whereas 1 mM GSH significantly reduced covalent binding by 65%.
4. Thus, KT-induced hepatotoxicity is dose- and time-dependent and appears to be FMO mediated, in part, to metabolites that may react with protein and, possibly, GSH.

Introduction

Ketoconazole (KT), an effective broad-spectrum oral anti fungal, has been used in immune-compromised patients since the late 1970s and in recent years has exhibited reliable effectiveness against advanced prostate cancer (Bok and Small 1999). Classified as an azole compound due to the presence of a pyrrole ring within the structure, KT has been shown to inhibit cytochrome P450 (CYP) 3A4 (Back *et al.* 1992), CYP11A (Kurokohchi *et al.* 1992) and is a weak inhibitor of CYP1A and CYP2B (Rodrigues *et al.* 1987a, b). In fungi, KT blocks the conversion of lanosterol to ergosterol by inhibiting the CYP lanosterol 14 alpha-demethylase responsible for the oxidative removal of the [¹⁴C] methyl group from lanosterol resulting in a loss of membrane integrity (Jacobs and Nall 1988, Vanden Bossche *et al.* 1988). KT has also been associated with fatalities (Duarte *et al.* 1984, Janssen 1995) and numerous cases of hepatitis, resulting in increases in serum liver enzymes indicating possible hepatic damage (MacNair *et al.* 1981, Svejgaard and Ranek 1982, Lewis *et al.* 1984, Stricker *et al.* 1986, Benson *et al.* 1988, Knight *et al.*

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1991, Chien *et al.* 1997). Other symptomatic problems include malaise, dark urine, jaundice (Van Parys *et al.* 1987, Cauwenbergh 1989), headache, dizziness, drowsiness and papilloedema (Bok and Small 1999).

The therapeutic concentrations, dosages and duration of treatment for KT vary depending upon the medical diagnosis. The mean peak serum concentrations in humans range from 8 to 19 μM after a 200 mg day⁻¹ dose (Heel *et al.* 1982, Huang *et al.* 1986) and from 13 to 26 μM following a 400 mg day⁻¹ dose (Baxter *et al.* 1986); however, plasma concentrations have been reported to be as high as 94 μM (Brass *et al.* 1982, Sugar *et al.* 1987). In rats, plasma levels of 24 and 62 μM KT resulted from 10 and 20 mg kg⁻¹ doses, respectively (Gascoigne *et al.* 1981). After oral administration in rats, KT and/or its metabolites have shown a higher concentration in the liver than in the plasma (Gascoigne *et al.* 1981, Whitehouse *et al.* 1990).

Specific tissue binding of KT to rat hepatic microsomal fractions was reported to be 89% (Daneshmend and Warnock 1988), indicating possible inhibition of monooxygenase metabolism with the binding of KT to rat liver *in vivo* and *in vitro*. The half-life of KT increases with increasing dose (Gascoigne *et al.* 1981, Daneshmend *et al.* 1983, Huang *et al.* 1986), thus, long-term administration will result in auto-inhibition of metabolism and a potential problem for drug accumulation (Heel *et al.* 1982).

KT-induced hepatitis was first reported in 1981 (MacNair *et al.* 1981) and the first fatality occurred in 1982 due to hepatic coma (Duarte *et al.* 1984). Sudden hepatitis has led to several deaths even though the patients underwent frequent laboratory and clinical evaluations (Duarte *et al.* 1984, Lewis *et al.* 1984, Bercoff *et al.* 1985, Stricker *et al.* 1986). Two other patients that suffered KT-induced hepatitis survived due to liver transplants (Brusko and Marten 1991, Knight *et al.* 1991). An autopsy of the patient who died of KT treatment revealed massive hepatocellular necrosis (Lewis *et al.* 1984). Moreover, a European trial of high dose KT (1200 mg day⁻¹) for prostate cancer resulted in 11 deaths out of 350 participants within 2 weeks of starting the high-dose therapy (Janssen 1995).

Prostate cancer is the second leading cause of cancer death in American men and one in five men will develop prostate cancer during their lifetime (IIT 1999). KT has been found to have inhibitory effects on the precursors of testosterone by inhibiting 17,20-lyase that specifically blocks the synthesis of dehydroepiandrosterone and androstenedione (Bok and Small 1999). High doses of KT of 400 mg/8 h or 600 mg/12 h for 6–9 months result in significant deprivation of androgen, a major factor in prostate cancer (De Coster *et al.* 1996, Bok and Small 1999). The hepatotoxic symptoms that can be mild to severe may develop within 2 days or up to 1 year after the initiation of therapy (Knight *et al.* 1991). The hepatotoxicity appears to be reversible with discontinued use of KT (Janssen and Symoens 1983, Lewis *et al.* 1984, Benson *et al.* 1988, Chien *et al.* 1997).

Challenging problems persist with drug-induced liver injuries such as acute hepatitis. Mechanistic evaluation in the future could aid structural analysis of an entire chemical family such as the azoles. Clinical symptoms, such as increases in serum bilirubin and alanine transaminase (Van Parys *et al.* 1987, Cauwenbergh 1989), indicate cellular damage, but the toxic mechanistic profile of KT is still unclear in humans (Bok and Small 1999) as well as in animals. It is suspected that toxicity responses are not due to an immunoallergic mechanism (Bok and Small 1999) but as the result of a reaction of either KT and/or its metabolites (Lewis *et al.*

1984, Bercoff *et al.* 1985, Stricker *et al.* 1986, Gradon and Sepkowitz 1990, Brusko and Marten 1991). An *in vitro* study using cultured rat hepatocytes has reported that the primary metabolite of KT, *N*-deacetyl-ketoconazole (DAK), is more hepatotoxic than KT (Rodriguez and Acosta 1997a). Continued efforts in our laboratory have established that the primary metabolite, DAK, undergoes flavin-containing monooxygenase (FMO)-mediated metabolism to the *N*-deacetyl-*N*-hydroxyketoconazole (*N*-hydroxy-DAK) metabolite (Rodriguez and Acosta 1997b, Rodriguez *et al.* 1999). The *N*-hydroxy-DAK metabolite is a secondary hydroxylamine, which is susceptible to further FMO-mediated oxidative metabolism, which could produce toxic and reactive metabolites that could be responsible, in part, for the toxicity of KT.

To date, studies investigating the hepatotoxicity associated with KT *in vivo* have not been conducted. The current study evaluates the possible metabolic bioactivation pathways, CYP or FMO, which could be responsible for producing toxic metabolites resulting in hepatic injury. *In vivo* studies have been used to evaluate the comparative changes in serum alanine aminotransaminase (ALT), hepatic glutathione (GSH) and hepatic covalent binding with one another to establish if a relationship exists between metabolism, toxicity and covalent binding. *In vitro* studies will address the involvement of CYP and FMO and GSH on the covalent binding of KT with hepatic microsomes.

Materials and methods

Chemicals and supplies

KT was a generous gift from Janssen Pharmaceuticals (Olen, Belgium), ^3H -KT (5 Ci mmole^{-1} , 98% purity) was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA). Reduced glutathione (GSH), oxidized glutathione (GSSH) reductase, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), NADPH, trichloroacetic acid (TCA), sodium hydroxide, Trizma, ethylenediaminetetraacetic acid (EDTA), sucrose, glycerol, glucose-6-phosphate, glucose 6-phosphate dehydrogenase and NADP⁺ were purchased from Sigma (St Louis, MO, USA). Alanine aminotransaminase (ALT) analysis was conducted with a Sigma Diagnostics Transaminases kit (ALT/GPT), No. 505-P. HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and CytoScint was purchased from ICN (Costa Mesa, CA, USA). Sprague-Dawley (SD) rats were purchased from Simonsen Laboratories, Inc. (Gilroy, CA, USA).

Animal treatment and sample collection

Male SD rats (200–220 g) were treated intraperitoneally (i.p.) with ^3H -KT ($1.5\text{ }\mu\text{Ci mg}^{-1}$) at doses of 40 and 90 mg KT kg^{-1} . Control rats were treated with 0.9% normal saline. Blood and liver samples were collected at 0, 0.5, 1, 2, 4, 8, 12 and 24 h for ALT, GSH and covalent binding analyses. The blood samples and approximately 1.1 g liver were placed on ice for ALT, GSH, covalent binding and protein determination. The remaining liver samples were snap-frozen and placed in a -80°C freezer for metabolite isolation, purification and identification studies. All experiments were performed in triplicate.

In vivo covalent binding studies

After the male SD rats were administered ^3H -KT ($1.5\text{ }\mu\text{Ci mg}^{-1}$) at doses of 40 and 90 mg KT kg^{-1} , approximately 1 g from each of the liver samples was homogenized with Trizma buffer (100 mM Trizma, 1 mM EDTA, 250 mM sucrose, pH 7.5). One ml of the homogenate was mixed with 2 ml ice-cold 0.9 M TCA and centrifuged at $8000g$ at 4°C for 10 min to precipitate the protein. The pellet was then washed three times with ice-cold 0.6 M TCA (2 ml). The protein pellet was then washed six times with ice-cold 80% methanol (2 ml) until the supernatant fraction of the wash achieved background levels of radioactivity. The final protein pellet was dissolved in 1 ml 1 M NaOH at 60°C for 1 h and a 0.3-ml aliquot was placed into scintillation vials containing 2.75 ml scintillation fluid with 1.5% (v/v) glacial acetic acid. Radioactivity was measured in dpms by a Beckman LS6500 scintillation counter (Fullerton,

CA, USA) and normalized to nmol KT mg⁻¹ protein. Protein was determined using the Coomassie PlusTM Assay from Pierce (Rockford, IL, USA). All studies were performed in triplicate.

In vivo glutathione (GSH) studies

Collection of liver samples occurred between 08:30 and 11:30 hours to avoid diurnal pattern variations (Jaeger *et al.* 1973). The method of Roberts and Francetic (1993) was used for GSH analysis. In brief, a 50-mg liver sample was immediately removed from the rat, blotted dry, weighed and placed in 5 ml 5% sulfosalicylic acid (SSA w/v in water). The liver was then homogenized and centrifuged at 3000g for 10 min at 4 °C. The resulting supernatant was diluted 10-fold with sample buffer (100 mM potassium phosphate-buffer, 5 mM EDTA, pH 7.5) and placed on ice. A 300- μ l aliquot of the sample was combined in a vial with 450- μ l sample buffer, 100 μ l GSSG reductase (5 units ml⁻¹ in sample buffer) and 50 μ l DTNB. This sample mixture was then vortexed and 100 μ l was placed into a 96-well plate in duplicate. After a 1-min incubation in the 96-well plate, 100 μ l NADPH (2.4 mM, 2 mg ml⁻¹ in sample buffer) was added to the sample mixture. The 96-well plate was placed in a SpectraMax spectrophotometer, mixed and the absorbance was monitored at 412 nm at time zero and every 30 s for 1.5 min. The change in absorbance per min was determined and converted to μ mol GSH using a calibration curve with known standards. The results are expressed in μ mol GSH g⁻¹ protein.

Alanine aminotransaminase (ALT) assays

For ALT serum determinations, blood samples were collected at 0, 0.5, 1, 2, 4, 8, 12 and 24 h. The blood samples were immediately placed on ice and allowed to coagulate before centrifugation at 4 °C and 8000g. The serum was collected and kept on ice. The ALT was assayed using a modified procedure from Sigma Diagnostic Kit #505-P for small sample volumes. Alanine alpha-ketoglutarate (0.25 ml) was placed into a test tube into a 37 °C water bath. Five microliters of serum and 45 μ l normal saline were added to the test tube containing alanine alpha-ketoglutarate. The sample mixture was gently mixed and placed back into the 37 °C water bath to incubate for 30 min. After the incubation, 0.25 ml of the Sigma color reagent (No. 505-2) were added to the incubation mixture, vortexed (low speed) and left at room temperature (25 °C) for 20 min. A total of 2.5 ml 0.4 N NaOH was then added to the mixture and vortexed. After 5 min, 200 μ l of the sample mixture was placed into a 96-well plate and the absorbance was measured at 505 nm on a SpectraMax 190 spectrophotometer (Sunnyvale, CA, USA). A calibration curve was performed to determine the ALT activity in units ml⁻¹ from the serum samples. ALT catalyses conversion of alpha-ketoglutarate and alanine to glutamate and pyruvate. Comparisons were made with the control rats. All experiments were performed in triplicate.

Microsomal preparations

Liver tissues were removed from male SD rats (200–220 g), immediately placed on ice and homogenized with 0.25 M sucrose, 100 mM Tris and 1 mM EDTA, pH 7.5. Microsomes were prepared by standard differential centrifugation and stored in 10 mM Tris, 1 mM EDTA and 20% glycerol, pH 7.5. The microsomes were frozen in liquid nitrogen and stored at -80 °C. Protein concentration was determined using the Coomassie PlusTM Assay from Pierce (Rockford, IL, USA).

In vitro covalent binding studies

Covalent binding of KT and/or its metabolites to hepatic microsomal protein and protein-dependency analyses were performed to determine linearity. The microsomal incubation mixture consisted of 0.25, 0.5 and 1.0 mg ml⁻¹ microsomal protein, incubation buffer (100 mM glycine-25 mM pyrophosphate buffer, pH 8.8), 1, 10 or 100 μ M KT with ³H-KT (1 μ Ci ml⁻¹), and an NADPH-generating system (10 mM glucose-6-phosphate, 1.0 unit ml⁻¹ glucose-6-phosphate dehydrogenase, 1 mM NADP⁺) at 37 °C in a total volume of 0.5 ml. A negative control without the NADPH-generating system was performed with each experiment. The microsomal incubations were terminated at 0, 0.5, 1, 2, 3, 4 and 8 h with 0.5 ml ice-cold 0.9 M TCA. The protein was precipitated, washed and the radioactivity was determined as described in the *in vivo* covalent binding studies section. All experiments were performed in triplicate.

To evaluate the affect of GSH on the covalent binding of KT and/or its metabolites, co-incubations of 100 μ M ³H-KT (1 μ Ci ml⁻¹) and 1 mM GSH using the microsomal incubations described above were performed in triplicate at 0, 1, 4 and 8 h. To evaluate the effect of FMO on the covalent binding of KT and/or its metabolites, microsomal FMO activity (but not CYP activity) was inhibited by heat inactivation (Ziegler 1980). The microsomes were heated at 50 °C for 90 s before adding them to the incubation mixture containing 100 μ M ³H-KT (1 μ Ci ml⁻¹), 1 mg ml⁻¹ microsomal protein and incubation buffer for a total volume of 0.5 ml. The microsomal incubation was terminated at 0, 1, 4 and 8 h with 0.5 ml 0.9 M TCA. Heat inactivation and incubation with 1 mM GSH was performed with 100 μ M ³H-KT (1 μ Ci ml⁻¹), 1 mg ml⁻¹ microsomal protein and incubation buffer for a total of 0.5 ml

was performed at 0, 1, 4 and 8 h. Comparisons of GSH incubations and heat-inactivated experiments were compared with the normal experimental conditions.

Statistical analysis

Statistical analysis of the ANOVA was performed by SAS statistical program, version 8.01, from SAS Institute, Inc. (Cary, NC, USA) to determine the significance of *in vivo* covalent binding. Tukey's multiple comparison was performed for the *in vivo* ALT study and Dunnett's multiple comparison was performed for the *in vivo* GSH studies. ANOVA analyses were performed for the *in vitro* dose- and time-response studies. Probabilities ≤ 0.05 were considered as statistically significant.

Results

In vivo Studies

Figure 1 shows the 'covalent' or tight binding of [^3H]KT-related material to hepatic proteins from SD rats treated i.p. with KT (40 or 90 mg kg $^{-1}$; 1.5 μCi mg $^{-1}$). The radioactivity was normalized to nmol KT mg $^{-1}$ hepatic protein. The 40 mg kg $^{-1}$ dose represents a low dose that would be expected to produce KT plasma concentrations in the 10–60 μM range, whereas the 90 mg kg $^{-1}$ represents a high-dose treatment for prostate cancer that would produce KT concentrations in the 75–100 μM range. There was a significant difference between KT-derived [^3H] binding to hepatic protein over the 24 h period using ANOVA ($p = 0.014$, $n = 3$). Covalent binding occurred as early as 0.5 h after treatment for both doses. The 40 mg KT kg $^{-1}$ dose peaked at 2 h (0.03 \pm 0.01 nmol KT mg $^{-1}$ protein) with a steady decline thereafter. The 90 mg KT kg $^{-1}$ dose peaked at 8 h (0.09 \pm 0.04 nmol KT mg $^{-1}$ protein), and then there was a steady decline to 24 h (0.04 \pm 0.02 nmol KT mg $^{-1}$ protein).

Figure 2 shows the effect of i.p. administration of KT, 40 or 90 mg kg $^{-1}$, on serum ALT activity in SD rats. Interestingly, there was an immediate increase in ALT levels as early as 0.5 h after treatment for both doses (44.2 and 56.4 U ml $^{-1}$

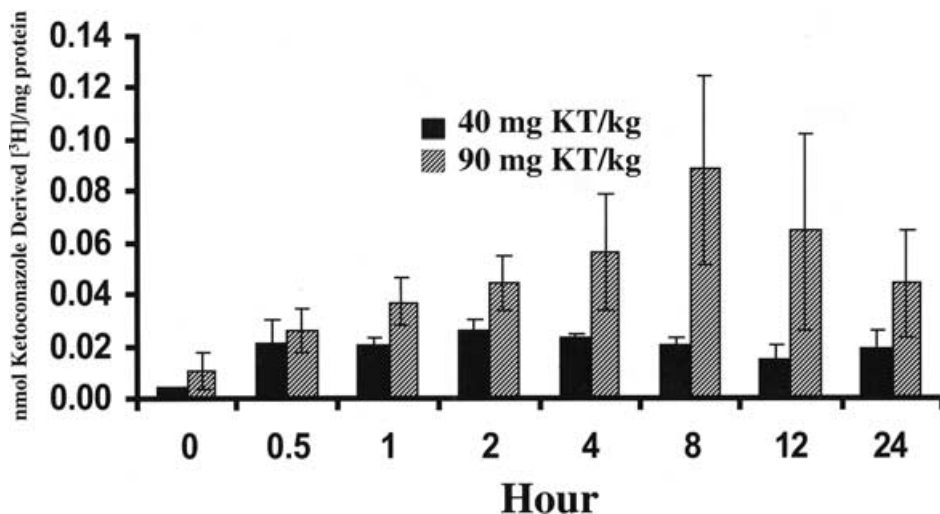


Figure 1. Covalent binding of ketoconazole (KT) to hepatic proteins from Sprague-Dawley rats treated i.p. with ^3H -KT (40 or 90 mg kg $^{-1}$; 1.5 μCi mg $^{-1}$). The radioactivity was normalized to nmol ketoconazole-derived [^3H] mg $^{-1}$ hepatic protein. There was a significant difference between the 40 and 90 mg kg $^{-1}$ treatments over time using ANOVA ($p = 0.014$, $n = 3$).

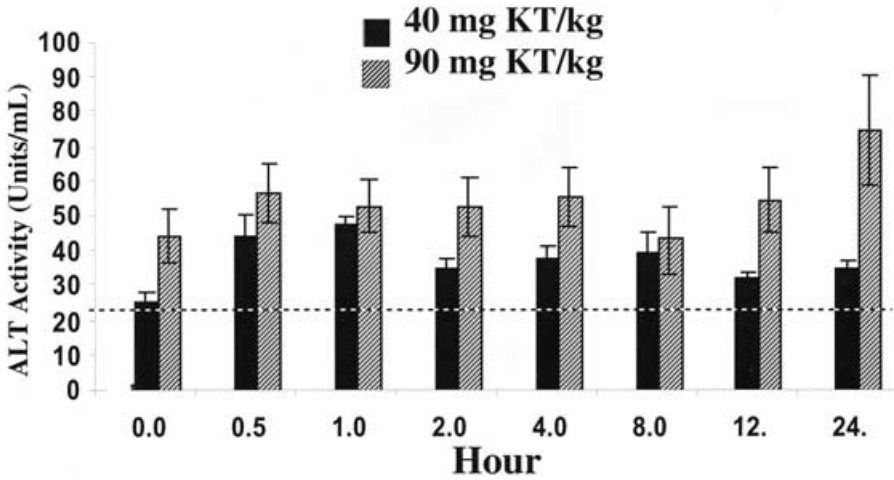


Figure 2. Effect of i.p. administration of ketoconazole (KT) (40 or 90 mg kg⁻¹) on serum alanine aminotransaminase (ALT) activity in Sprague-Dawley rats. The dashed line represents the ALT control value of saline-treated rats, 22.7 ± 9.7 U ml⁻¹. Tukey's multiple comparison indicates significant differences between the 90 mg KT kg⁻¹ dose and control and between the 40 and 90 mg KT kg⁻¹ doses ($p < 0.05$). Data are the mean ± SEM from three determinations.

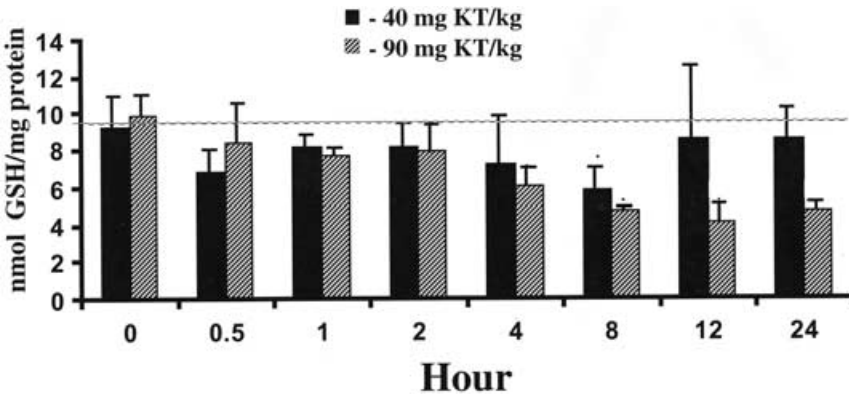


Figure 3. Effect of i.p. administration of ketoconazole (KT) (40 or 90 mg kg⁻¹) on hepatic glutathione (GSH) levels in Sprague-Dawley rats. The dashed line represents the GSH control value of saline-treated rats, 9.66 ± 1.51 nmol GSH mg⁻¹ protein. Dunnett's multiple comparison indicated a significant difference between 40 or 90 mg KT kg⁻¹ dose and control ($p < 0.05$). Data are the mean SEM from six determinations.

for the 40 and 90 mg kg⁻¹ doses, respectively). The control rats treated with normal saline had an ALT of 22.7 ± 9.7 U ml⁻¹. Also, there was an apparent and steady increase in ALT levels from 8 h to 24 h for the 90 mg KT kg⁻¹ dose. There was a significant difference between 90 mg KT kg⁻¹ dose and control using Tukey's multiple comparison ($p < 0.05$). There was also a significant difference between the 40 and 90 mg kg⁻¹ doses at 12 and 24 h after KT administration using Tukey's multiple comparison ($p < 0.05$).

Figure 3 demonstrates the effect of KT on hepatic GSH levels in SD rats. Owing to diurnal variation concerns about GSH levels, all doses were conducted to coordinate extractions between 08:30 and 11:30 hours, when GSH levels would be

at the highest level. The control rats had hepatic GSH levels of 9.66 ± 1.51 nmol GSH mg^{-1} protein. There was a significant difference between the 40 mg KT mg^{-1} and the control rats at 0.5 and 8 h where the GSH levels were 6.89 ± 1.1 nmol GSH mg^{-1} protein and 5.87 ± 1.1 nmol GSH mg^{-1} protein, respectively ($p < 0.05$). Afterwards, the GSH levels increased back to control values. The 90 mg KT kg^{-1} was significantly different from the control rats at 4, 8, 12 and 24 h where the GSH levels were 6.1 ± 0.91 , 4.7 ± 0.19 , 4.17 ± 0.9 and 4.76 ± 0.34 nmol GSH mg^{-1} protein, respectively, using the Dunnett's multiple comparison ($p < 0.05$). At 24 h, there was a significant difference between the 40 and 90 mg kg^{-1} doses, 8.55 ± 1.64 nmol GSH mg^{-1} protein and 4.76 ± 0.34 nmol GSH mg^{-1} protein, respectively ($p < 0.05$).

In vitro studies

Figure 4 shows the covalent binding of ^3H -KT (1, 10 and 100 μM ; 1 $\mu\text{Ci ml}^{-1}$) to hepatic microsomes from SD rats in an *in vitro* NADPH-generating system containing 1 mg ml^{-1} hepatic microsomal protein. Controls without NADPH were used for comparison for each concentration studied. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. All three KT incubation concentrations, 1, 10 and 100 μM , resulted in significant differences with control incubation conditions using an ANOVA ($p \leq 0.01$). As the concentration of KT increased, so did the covalent binding to hepatic protein. In addition, there appeared to be an

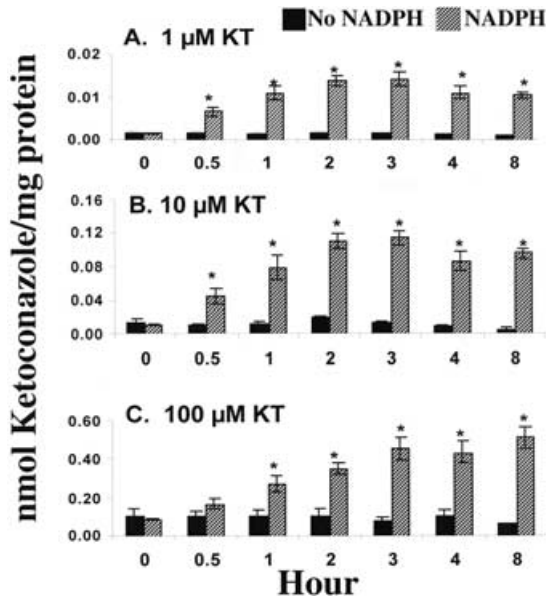


Figure 4. *In vitro* covalent binding of ^3H -ketoconazole (KT) (1 $\mu\text{Ci ml}^{-1}$) to hepatic microsomes from Sprague-Dawley rats in an NADPH-generating system. Controls without NADPH were used for comparison for each concentration. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. In (A), an asterisk indicates a significant difference between the 1 μM KT and control using ANOVA ($p < 0.05$, $n = 6$). In (B), an asterisk indicates a significant difference between the 10 μM KT and control using ANOVA ($p \leq 0.05$, $n = 6$). (C) * Significant difference between 100 μM KT and control using ANOVA ($p \leq 0.05$, $n = 6$).

increase in covalent binding with increasing incubation periods for the 10 and 100 μM KT. The 100 μM KT incubation had the highest covalent binding at 8 h (0.51 ± 0.06 nmol KT mg^{-1} protein). The control levels at 8 h were approximately 10-fold lower (0.06 ± 0.01 nmol KT mg^{-1} protein); thus, indicating an NADPH-dependent mechanism of covalent binding of *KT-derived activity*. The 10 μM KT incubation appears to reach a plateau at 2 h. Thus, there is a dose proportionality between 1 and 10 μM KT and saturation at 100 μM KT that is NADPH dependent. These studies were also performed with protein concentrations of 0.25, 0.5 and 1 mg ml^{-1} and $^3\text{H-KT}$ (10 and 100 μM). There was a linear increase over time (0–8 h) in the covalent binding of KT to the hepatic protein with 0.25 mg ml^{-1} microsomal proteins having a plateau at 2 h (data not shown).

Owing to a decrease in *in vivo* hepatic GSH levels, *in vitro* comparative analyses using microsomal incubations fortified with 1 mM GSH (*in vivo* GSH concentrations) were conducted to determine whether a reduction of covalent binding to hepatic proteins would occur. The *in vitro* covalent binding of 100 μM $^3\text{H-KT}$ ($1 \mu\text{Ci ml}^{-1}$) to hepatic microsomes (1mg ml^{-1}) from SD rats in an NADPH-generating system with 1 mM GSH is shown in figure 5A. Control incubations without GSH were used for comparison for each of the time points evaluated. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. There was a significant reduction of covalent binding to hepatic protein

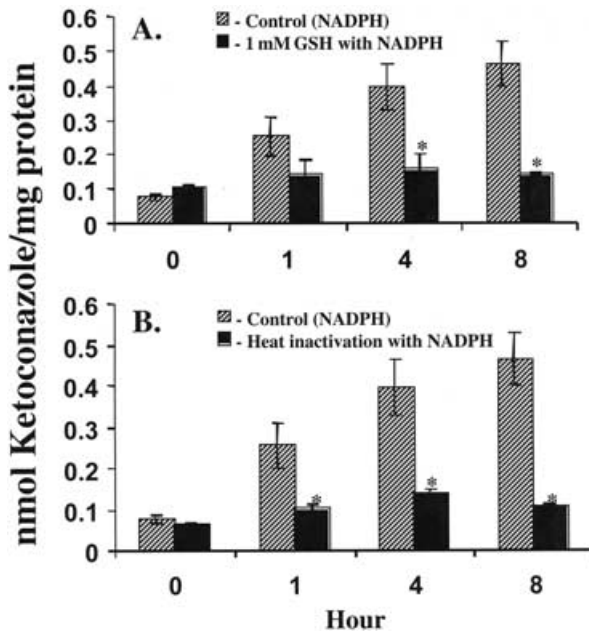


Figure 5. (A) Effect of glutathione (GSH, 1 mM) on *in vitro* covalent binding of ^3H -ketoconazole (KT) (100 μM ; $1 \mu\text{Ci ml}^{-1}$) to hepatic microsomes from Sprague–Dawley rats in an NADPH-generating system. Controls without GSH were used for comparison. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. * Significant difference using ANOVA ($p \leq 0.05$, $n = 6$). (B) Effect of heat inactivation (50 $^{\circ}\text{C}$ for 90 s) on *in vitro* covalent binding of ^3H -ketoconazole (KT) (100 μM ; $1 \mu\text{Ci ml}^{-1}$) to rat hepatic microsomes from Sprague–Dawley rats in an NADPH-generating system. Controls without heat inactivation were used for comparison. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. * Significant difference using ANOVA ($p \leq 0.05$, $n = 6$).

at 4 and 8 h using ANOVA ($p \leq 0.01$); even as early as 1 h after treatment, metabolic activation appears to occur, thus suggesting that KT might also bind to hepatic GSH.

To evaluate the role of microsomal FMO bioactivation of KT to reactive metabolite(s) that may bind to hepatic protein, heat inactivation of microsomal FMO was performed. Microsomal FMO activity was inhibited by heat-inactivation of the microsomes at 50 °C for 90 s, which is an established method to differentiate between FMO and CYP microsomal activities (Ziegler 1980). Figure 5B shows the effect of heat inactivation on *in vitro* covalent binding of 100 μM ^3H -KT (1 $\mu\text{Ci ml}^{-1}$) to SD rat hepatic microsomes in an NADPH-generating system containing 1 mg ml^{-1} microsomal protein. Control incubations without heat inactivation treatment were used for comparison at each time point evaluated. The radioactivity was normalized to nmol KT mg^{-1} microsomal protein. Interestingly, heat inactivation significantly reduced the covalent binding of KT-derived radioactivity to hepatic protein at the 1, 4 and 8 h using ANOVA ($p < 0.05$). The 8 h incubation had a greater than four-fold decrease from $0.46 \pm 0.06 \text{ nmol KT mg}^{-1} \text{ protein}$ to $0.11 \pm 0.01 \text{ nmol KT mg}^{-1} \text{ protein}$. Co-incubation of heat-inactivated microsomal FMO and 1 mM GSH resulted in similar reduction of covalent binding of KT and/or its metabolite(s) to hepatic protein and/or GSH as seen with the heat inactivation studies from 0.46 ± 0.06 to $0.09 \pm 0.01 \text{ nmol KT mg}^{-1} \text{ protein}$ (data not shown).

Discussion

This study is the first to investigate the hepatotoxicity associated with KT *in vivo*. All of the *in vivo* parameters evaluated, ALT levels, hepatic GSH levels and hepatic covalent binding suggest that KT produce hepatic injury as indicated by increases in ALT levels after KT administration (figure 2). This hepatic injury occurred as early as 0.5 h with both the 40 and 90 mg kg^{-1} doses. The 90- mg KT kg^{-1} dose is representative of high-dose KT treatment seen in patients being treated for prostate cancer. The high-dose KT therapy for prostate cancer ranges from 400 mg KT/8 h or 600 mg KT/12 h for a total daily dose of 1200 mg for 6–9 months (De Coster *et al.* 1986, Bok and Small 1999). As shown in figure 2, there is an initial and significant increase in ALT levels for the 40 and 90 mg KT kg^{-1} dose. The 90 mg KT kg^{-1} declines after 4 h and then increases at 12 h and 24 h. In the past, hepatic injury was suspected as being a reaction of KT or its metabolite(s) and not due to an immunoallergic response (Lewis *et al.* 1984, Stricker *et al.* 1986, Bok and Small 1999). Thus, the early release of ALT may be due to the parent compound, KT, whereas the later increase of ALT at 12 and 24 h may be due to reactive metabolite(s).

The present study also investigated the possibility that KT may covalently bind to hepatic macromolecules and/or to hepatic GSH in a dose- and time-dependent response. Figure 1 demonstrates that there was a definite dose-response in KT covalent binding to hepatic SD rat protein. The 90 mg KT kg^{-1} dose produced higher levels of $\text{nmol KT-derived } [^3\text{H}] \text{ mg}^{-1} \text{ protein}$ in comparison with the 40 mg KT kg^{-1} dose over the 24 h time period. Figure 3 indicates that hepatic GSH levels were significantly decreased after a 90 mg KT kg^{-1} dose in a time-dependent manner. The 90 mg KT kg^{-1} dose resulted in lower hepatic GSH levels than the 40 mg KT kg^{-1} dose at 4, 8, 12 and 24 h with the most difference between

the doses being at 24 h. In the 40 mg KT kg⁻¹ dose, the GSH levels appear to decrease from 0 to 8 h, then increase back to control GSH levels at 12 h and 24 h. It is likely the liver was able to recover from low-dose treatment and return to normal hepatic GSH levels. On the other hand, the 90 mg KT kg⁻¹ treatment continued to deplete hepatic GSH at 12 h and 24 h. When comparing the *in vivo* hepatic GSH levels with the covalent binding studies of KT to hepatic protein and the ALT studies, no significant correlations were made between the *in vivo* alterations in GSH and ALT levels (figures 2 and 3, respectively) to the covalent binding (figure 1) that occurred.

It is possible that as KT undergoes oxidative metabolism such that the metabolite(s) of KT may be reacting with GSH because the decrease in GSH occurs hours after exposure to KT rather than earlier. Thus, the parent compound, KT, may be directly binding to hepatic protein whereas the metabolite(s) bind to hepatic protein and hepatic GSH. Earlier studies indicate that the primary metabolite of KT is *N*-deacetylketoconazole (DAK) in mice (Whitehouse *et al.* 1990) and rat (Rodriguez and Acosta 1997b, Rodriguez *et al.* 1999) and DAK is more hepatotoxic in an *in vitro* rat hepatocyte culture system (Rodriguez and Acosta 1997a). Furthermore, DAK is further metabolized by FMO to the *N*-deacetyl-*N*-hydroxyketoconazole, a secondary hydroxylamine (*N*-hydroxy-DAK), metabolite (Rodriguez and Acosta 1997b, Rodriguez *et al.* 1999). *N*-hydroxy-DAK is susceptible to further FMO-mediated oxidative metabolism, where it has been speculated that toxic and reactive metabolites such as the nitrene of KT could be responsible, in part, for the toxicity of KT. Soft nucleophilic sulphur, selenium, nitrogen or phosphorus pharmaceuticals can undergo oxidative metabolism catalysed by FMOs (Ziegler 1988). Hydroxylamines may directly interact with biomacromolecules to impair cellular function (Cashman *et al.* 1999). Therefore, a reduction of GSH would be expected if bioactivation of an electrophilic metabolite such as DAK and *N*-hydroxy-DAK occurred due to the conjugating nature of this cellular scavenger to preserve the integrity of the cell (Hogberg and Kristoferson 1977). Another possibility is DAK undergoes Phase II metabolism by sulfation and/or glucuronide conjugation which would provide good leaving groups resulting in a nitrenium cation that could possibly bind to protein and DNA leading to adduct formation.

The *in vitro* covalent binding studies of KT and/or its metabolite(s) to SD rat hepatic microsomal protein was NADPH-dependent and inhibited by co-incubation with GSH (figures 4 and 5A). Figure 5A demonstrates that the addition of GSH in the microsomal incubation significantly reduced the covalent binding of KT and/or its metabolites to hepatic protein. The 1 mM GSH *in vitro* incubation with KT would prevent the formation of protein- and/or DNA-KT adducts, thereby possibly forming GSH-KT adducts. To differentiate between CYP- or FMO-mediated bioactivation to potential reactive metabolite(s), heat inactivation of microsomal-FMO fractions inactivated FMO activity, but CYP activity remained unaltered (Ziegler 1980). Earlier studies of DAK employed SKF-525A, an inhibitor of CYP activity (Ziegler and Mitchell 1972), and octylamine, a known positive effector for FMO and an inhibitor of CYP (Cashman and Ziegler 1986, Rodriguez and Acosta 1997), resulted in unaltered FMO-mediated metabolism. Figure 5B demonstrates that with NADPH present, a profound and significant reduction in covalent binding of KT and/or its metabolite(s) to hepatic protein by FMO heat inactivation of the rat liver microsomes. Because FMO-mediated

metabolism was inhibited, it is speculated that the metabolite(s) of KT is primarily responsible for the covalent binding to hepatic proteins. Continued efforts are being made to identify whether KT and/or its metabolite(s) bind to hepatic protein and to identify the nature of the protein adduct. Further studies are also being conducted to determine or verify if the binding of KT or its metabolites to GSH is mediated by liver microsomal FMOs or by individual FMO forms. If a GSH adduct is formed, attempts will be made to characterize its structure by mass spectrometry and NMR methods.

In summary, these studies demonstrate that KT-induced hepatotoxicity is dose- and time-dependent and appears to be dependent, in part, upon its bioactivation by FMO to metabolite(s) that may react with protein and, possibly, GSH. Also, GSH appears to be important in the detoxification pathway of KT by the probable conjugation of DAK and the *N*-hydroxy-DAK. Thus, our combined *in vitro* and *in vivo* studies point to potential mechanisms of KT-induced hepatotoxicity that merit further investigation.

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