N-Deacetyl ketoconazole-induced hepatotoxicity in a primary culture system of rat hepatocytes

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Received 4 June 1996; revised 27 September 1996; accepted 4 October 1996

Abstract

Ketoconazole (KT) is an azole antifungal agent that has been associated with hepatotoxicity. The mechanism of its hepatotoxicity has not yet been resolved. It has been suggested that a reactive metabolite may be the cause of toxicity because the hepatic injury does not appear to be mediated through an immunological mechanism. Several metabolites of KT have been reported in the literature of which the deacetylated metabolite, N-deacetyl ketoconazole (DAK), is the major metabolite which undergoes further metabolism by the flavin-containing monooxygenases (FMO) to form a potentially toxic dialdehyde. The objective of this study was to evaluate DAK's cytotoxicity and the role of FMO in a primary culture system of rat hepatocytes. Cytotoxicity was evaluated by measuring the leakage of the cytosolic enzyme, lactate dehydrogenase (LDH), into the medium and by assessing mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The cultures were exposed to various concentrations of DAK (20-160 μM) for 0.5-4 h. There was a significant increase (P < 0.05) in LDH leakage and an immediate decrease in MTT reduction (P < 0.05) as early as 0.5 h. The MTT reduction assay appeared to be more sensitive than the LDH assay in that lower concentrations were needed to observe a 50% reduction of MTT (107, 90, 75, 58 μM DAK at 0.5, 1.0, 2.0 and 4.0 h, respectively). The concentrations to observe 50% LDH leakage from the hepatocytes were 155, 133, 100, 70 μM DAK at 0.5, 1.0, 2.0 and 4.0 h, respectively. Moreover, cotreatment with methimazole, a competitive substrate for FMO, produced a significant decrease (P < 0.05) in % LDH leakage as early as 0.5 h, when compared to cells treated solely with DAK. Also, the toxicity was significantly (P < 0.05) enhanced as early as 0.5 h by n-octylamine, a known positive effector for FMO. These results demonstrate that DAK is a more potent cytotoxicant than its parent compound, KT, as reported previously by our laboratory (Rodriguez and Acosta, Toxicology, 96: 83-92, 1995) and its toxicity was expressed in a dose- and time-dependent manner. Furthermore, DAK's cytotoxicity was enhanced with n-octylamine and suppressed with methimazole, suggesting a role for FMO in the toxicity of the metabolite. © 1997 Elsevier Science Ireland Ltd. All rights reserved

Keywords: Azole derivatives; Flavin-containing monooxygenases; Hepatocytes; Hepatotoxicity; Ketoconazole; Metabolite; Methimazole; N-Deacetyl ketoconazole; n-Octylamine; Primary cultures

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1. Introduction

Ketoconazole (KT), Fig. 1, was the first oral antifungal agent in a series of azole-derivatives with a broad spectrum of activity against both superficial and systemic mycoses. KT exerts its antifungal actions by blocking the conversion of lanosterol or 24-methylenedihydrolanosterol to ergosterol in fungi (Vanden Bossche et al., 1988). The specific point of chemical intervention appears to involve inhibition of the cytochrome P-450 enzyme responsible for the oxidative removal of the C-14 methyl group of lanosterol by lanosterol 14α-demethylase (Vanden Bossche et al., 1988). Moreover, the action of KT is not only limited to the cytochromes of fungi because inhibition is also seen with mammalian cytochrome P-450. For example, KT has been shown to have similar inhibitory effects on the cytochrome P-450 enzymes responsible for the conversion of lanosterol to cholesterol (Strandberg et al., 1987).

There have been numerous documented cases of KT-induced hepatitis (Bercoff et al., 1985; Stricker et al., 1986; Lake-Bakaar et al., 1987; Benson et al., 1988; Cauwenbergh, 1989; Brusko and Marten, 1991). Many reports indicate that the type of hepatic injury is zone 3 necrosis (Bercoff et al., 1985; Stricker et al., 1986; Lake-Bakaar et al., 1987; Benson et al., 1988). The overall incidence of clinical hepatitis appears to be in the order of 0.01–0.1% of the patients, and many cases appear to be subclinical, detected only by routine liver function studies (Svejgaard and Ranek, 1982; Stricker et al., 1986). Thus, the actual incidence is probably greater than that reported in the literature (Stricker et al., 1986; Knight et al., 1991). Similarly, there have been documented reports of hepatotoxicity related to fluconazole (Grant and Clissold, 1990; Bodey, 1992; Wells and Lever, 1992) and itraconazole treatment (Lavrjisen et al., 1992; Hann et al., 1993). Moreover, it has been reported that KT induces hepatic phospholipidosis in mice (Whitehouse et al., 1990). At present, the mechanism of azole-induced hepatic necrosis is unknown (Bercoff et al., 1985; Lake-Bakaar et al., 1987; Brusko and Marten, 1991). The absence of hypersensitivity and eosinophil-rich infiltration in liver biopsies suggests that the KT-induced liver lesion might be related to a direct action of the parent compound or to a reactive metabolite (Bercoff et al., 1985; Stricker et al., 1986; Lake-Bakaar et al., 1987; Brusko and Marten, 1991).

Despite the world-wide usage of KT, the role of KT and its metabolite(s) in KT-induced hepatotoxicity has not yet been explored. There have been few published studies on the metabolism of KT or its metabolites in animals or humans. 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; Daneshmand and Warnock, 1988). The metabolic pathways suggested in KT's biotransformation include oxidation, cleavage, degradation, and scission of the imidazole and piperazine rings, oxidative O-dealkylation, and aromatic hydroxylation (Gascoigne et al., 1981; Heel et al., 1982; Daneshmand and Warnock, 1988); however, the actual identification of the metabolites undergoing the suggested metabolic pathways have not been reported in open literature. More recently, KT's metabolites have been isolated and identified from mouse liver (Whitehouse et al., 1990, 1994a). Of KT’s metabolites, the N-deacetyl ketoconazole (DAK) (Fig. 1) appears to be the major metabolite formed in mice (Whitehouse et al., 1990, 1994a). Furthermore, our laboratory has demonstrated that DAK is further metabolized by purified hog liver flavin-containing monooxygenases (FMO) and by hepatic guinea pig and postnatal rat microsomal-FMO to form a potentially toxic dialdehyde (Rodriguez et al., 1995). The bioactivation of DAK by FMO may ultimately be responsible for KT’s hepatotoxicity. Because of the potential bioactivation of DAK by FMO, investigations of DAK’s cytotoxicity using positive effectors and competitive substrates of FMO in an established model system were initiated.

In order to evaluate the cellular mechanism(s) of KT-induced hepatotoxicity, an in vitro model for KT-induced hepatotoxicity has been established in our laboratory: a primary culture system of rat hepatocytes (Rodriguez and Acosta, 1995). We evaluated the possible role of FMO on DAK’s toxicity in our culture system using methi-
mazole, a competitive substrate for FMO, and 
n-octylamine, a known positive effector for FMO, 
as well as an inhibitor of cytochrome P-450 
(Cashman and Ziegler, 1986). Positive effectors 
and competitive substrates of FMO have been 
used in primary cultures of mouse astrocytes to 
evaluate the role of FMO (Di Monte et al., 1991). 
For this present study, the indices used to evalu-
ate the direct cytotoxicity of DAK were plasma 
membrane integrity, as assessed by the leakage of 
lactate dehydrogenase (LDH) into the culture 
medium, and mitochondrial reduction of 3-(4,5- 
dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bro-
mide (MTT).

2. Materials and methods

2.1. Materials

Newborn bovine serum was obtained from 
Summit Biologicals (Fort Collins, CO). Williams’ 
medium E, hydrocortisone-water soluble, insulin, 
and albumin fraction V were tissue culture grade 
from Sigma Chemical Co. (St. Louis, MO). Colla-
genase Type II was obtained from Worthington 
Biochemical Corp. (Freehold, NJ). Fungizone® 
(amphotericin B) was from E.R. Squibb and Sons, 
Inc. (Princeton, NJ). Streptomycin and penicillin 
were from Roering-Pfizer (New York, NY). N-
Deacetyl ketoconazole was a gift from Janssen 
Pharmaceutica (Beerse, Belgium). n-Octylamine 
was purchased from Eastman Organic Chemicals. 
All other reagents were obtained from Sigma 
Chemical Co. (St. Louis, MO). Plastic culture 
dishes were obtained from Falcon Becton-Dickin-
son (Rutherford, NJ).

2.2. Animals

Sprague-Dawley rats, were bred and main-
tained in the Animal Resources Center at the 
University of Texas at Austin. They were housed 
in temperature (25°C)- and light cycle (12 h)-con-
trolled rooms. Water and feed were provided ad 
libitum.

2.3. Rat hepatocyte culture procedure

The isolation and culturing of the rat hepa-
tocytes were done according to a method developed 
in our laboratory (Acosta et al., 1980) and later 
modified (Rodriguez and Acosta, 1995). Briefly, 
the hepatocytes were obtained from postnatal 
male and female Sprague–Dawley rats (i.e. 8–11 
days of age). The rats were decapitated and the 
heart removed to prevent back-flow of blood dur-
ing the perfusion. The liver was perfused by retro-
grade perfusion of the portal vein with a needle 
connected to a hypodermic syringe containing 
warm Hank’s balanced salt solution (HBSS) with 
albumin fraction V (3 mg/ml) and collagenase 
Type II (80 Units/ml). After several mechanical 
dissociations and centrifugations of the tissue ho-
mogenates, the resulting pellets were resuspended 
in 4 ml of Williams’ medium E which contained 
0.001% (w/v) insulin, 0.005% (w/v) hydrocorti-
sone, 10% (v/v) newborn bovine serum, (1 mg/ml) 
albumin, (200 Units/ml) potassium penicillin G, 
(200 µg/ml) streptomycin sulfate, and (4 µg/ml) 
amphotericin B. The cell suspension was strained 
through a 100 mesh stainless-steel screen into a 
beaker; then, cell viability was determined with 
the trypan blue dye exclusion test. The total num-
ber of cells were counted with a hematocytometer 
and the cell suspension was diluted to a volume of 
0.75 × 10^6 cells/well in Falcon 24-well multiplates. 
Cultures were grown in a CO₂ incubator with 95% 
air and 5% CO₂ at 37°C and maintained at a pH 
of 7.2–7.4. After 24 h from the initial plating, the 
culture plate was gently shaken in a circular mo-
sion such that debris was removed during the 
replacement of fresh incubation medium. The in-
cubation medium was replenished every 24 h. 
Hepatocytes were grown in culture to confluency 
for 2 days prior to drug treatment.

2.4. Chemical exposure

We initiated a study in which experiments were 
conducted on the second day after the initial 
plating of the hepatocytes. A stock solution of 20 
mM DAK was prepared by dissolving DAK in 
0.01 N HCl and diluting with Williams’ medium 
E. Then, the stock solution of DAK was diluted
with serum-free and antibiotics-free medium to yield final concentrations ranging from 20 to 160 μM DAK, pH 7.4. Ten millimolar stock solutions were prepared for n-octylamine and methimazole by dissolving in Williams’ medium E. n-Octylamine and methimazole final concentrations were 250 μM and 100 μM, respectively.

2.5. Lactate dehydrogenase assay

The LDH enzymatic activities were assayed using a procedure previously developed by our laboratory for cell cultures (Mitchell et al., 1980). Briefly, the reaction mixture contained 2.5 ml of warm 0.1 M phosphate buffer (pH 7.4), 0.2 ml 0.25% (w/v) reduced NADH solution, 0.2 ml 1.0% (w/v) sodium pyruvate solution, and a 0.1 ml test sample. The test samples were obtained from the media after the 0.5-, 1-, 2- and 4-h treatments with DAK. The reaction mixture was mixed and immediately placed in a Beckman DU-40 spectrophotometer with the kinetic Soft-Pac software at 30°C. The absorbance at 340 nm was recorded at intervals of 30 s for 3 min. LDH catalyzes the reductive conversion of pyruvate to lactate with simultaneous oxidation of NADH to NAD⁺. The rate of disappearance of NADH in the presence of sodium pyruvate is directly proportional to LDH activity in the sample. Enzyme leakage into the medium was expressed as a percentage of total cellular LDH.

2.6. MTT assay

The MTT (3-(4,5-dimethythiazole-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay was done according to Mosmann (1983). The MTT assay is based on the reduction of the soluble yellow MTT tetrazolium salt to a blue MTT formazan product by mitochondrial dehydrogenases. It is a general measurement of mitochondrial dehydrogenase activity and cell viability. This assay was conducted immediately after the 0.5-, 1-, 2- and 4-h drug treatments. Briefly, MTT was dissolved in serum- and antibiotic-free culture medium at a concentration of 0.5 mg/ml and filtered to remove small amounts of insoluble residue. MTT-containing medium was added to each well in a volume of 0.5 ml and incubated for 2 h. Thereafter, the supernatant was removed and 1.25 ml of 0.4 N-HCl-isopropanol (1:24, v/v) were added to dissolve and solubilize the intracellular MTT formazan product for a period of 30 min at room temperature in the dark. Afterwards, the absorbance of the formazan solution was read at a wavelength of 570 nm on the Beckman DU-40 spectrophotometer. Results were expressed as a percentage of control cultures.

2.7. Statistical analysis

All data represent a minimum of three separate experiments with triplicate determinations and were expressed as the mean ± standard deviation unless otherwise indicated. The statistical significance of the difference of mean values of treated and control groups was evaluated by a two-way completely randomized factor analysis of variance (ANOVA) with significance set at P < 0.05 with respect to LDH leakage and MTT formazan formation.

3. Results

3.1. LDH assay

Treatment of the cultures with DAK resulted in a concentration- and time-dependent increase in LDH leakage from the cells into the culture medium (Fig. 2). There were no significant differences among 20–80 μM DAK-treated cells when compared to the control cultures at 0.5–1.0 h. However, 60 μM DAK at 4 h (28%) and 80 μM DAK at 2–4 h (24% and 75%, respectively) resulted in a significant increase of LDH leakage (P < 0.05) when compared to control. The higher concentrations of 120 μM and 160 μM DAK resulted in a significant dose-dependent increase from control at all time points monitored. Also, there was a significant time-dependent increase (P < 0.05) in LDH leakage from 0.5 h seen at concentrations ≥ 120 μM DAK; 1 h at concentrations ≥ 120 μM DAK; 2 h at concentrations ≥ 80 μM DAK; and 4 h at concentrations ≥ 40 μM DAK.
Co-treatment of cell cultures with DAK and n-octylamine for 0.5 h resulted in a significant ($P < 0.05$) increase of LDH leakage when compared to cultures treated with DAK alone (Fig. 3). This observation continued at 1 h of exposure where there was significant leakage of LDH in co-treated cells, DAK plus n-octylamine (Fig. 4). Moreover, the use of a competitive substrate of FMO, methimazole, resulted in a significant ($P < 0.05$) decrease of LDH leakage as early as 0.5 h (Fig. 3). This observation continued at the 1-h exposure period where there was a significant decreased LDH leakage in co-treated cells, DAK plus methimazole (Fig. 4). These observations were also seen at 2 and 4 h; however, there were no significance differences observed (data not shown). DAK, methimazole and n-octylamine did not interfere with the LDH assay (data not shown).

### 3.2. MTT assay

Treatment of the cell cultures with DAK resulted in a concentration- and time-dependent decrease in mitochondrial reduction of MTT (Fig. 5). There were no significant differences among 20 μM and 40 μM DAK-treated cells when compared to the control cultures at 0.5–2.0 h. However, 40 μM DAK produced a significant decrease
Fig. 4. Percent LDH leakage at 1.0 h from the hepatocytes after treatment with N-deacetyl ketoconazole and co-treatment with either 100 μM methimazole or 250 μM n-octylamine. The LDH leakage values are expressed as a percentage of total cellular LDH in order to standardize values between different cultures. The error bars are the standard deviation of three cultures (n = 9). A completely randomized factor ANOVA resulted in significant treatment- and dose-interaction (*P < 0.05) from N-deacetyl ketoconazole-treated cells (*).

(P < 0.05) at 4 h when compared to control. Moreover, 60 μM DAK produced a significant decrease in MTT reduction from control (P < 0.05) at all time points monitored except at 1 h. At 0.5 h, 120 and 160 μM DAK produced a significant decrease in mitochondrial reduction of MTT from control (34–18%, respectively). Furthermore, there was a significant time-dependent decrease (P < 0.05) in MTT reduction from 0.5 h seen at concentrations ≥ 60 μM DAK; 1 h at concentrations ≥ 80 μM DAK; 2 h at concentrations ≥ 60 μM DAK; and 4 h at concentrations ≥ 40 μM DAK. Less than 10% reduction of MTT occurred after 2 h exposure to 120 μM DAK and 4 h exposure to 80 μM DAK. DAK did not interfere with the MTT assay (data not shown).

4. Discussion

The in vitro model established for the evaluation of KT-induced hepatotoxicity in a primary culture system of rat hepatocytes (Rodriguez and Acosta, 1995) was used to evaluate DAK's cytotoxicity. The model used a variety of cytotoxic assays which assess plasma membrane integrity and mitochondrial function. Furthermore, the primary culture system developed in our laboratory is closer in its biochemical and physiologic responses to cells of the same origin in the intact animal because the culture system retains differentiated functions and responses characteristic of intact tissue in vivo (Acosta et al., 1985). Also, the use of the rat for the in vitro cell culture system may provide a more sensitive model for the evaluation of KT-associated hepatotoxicity because the rat, among the mouse, guinea pig and dog, was the most susceptible to death when KT was administered orally (Heel et al., 1982).

Treatment of the hepatocytes with DAK resulted in a concentration- and time-dependent increase of LDH leakage from the cells into the culture medium (Fig. 2). The 160 μM DAK exposure to hepatocytes produced greater than 50% LDH leakage as early as 0.5 h (P < 0.05). The concentration required of KT to reach greater than 50% LDH leakage was 175 μM at 1.0 h; 138 μM at 2.0 h; and 100 μM at 4 h (Rodriguez and Acosta, 1995). The concentration required of DAK to reach greater than 50% LDH leakage was 155 μM at 0.5 h; 133 μM at 1.0 h; 100 μM
at 2.0 h; and 70 μM at 4 h. Co-treatment of cell cultures with DAK and n-octylamine resulted in a significant (P < 0.05) increase of LDH leakage as early as 0.5 h (Fig. 3) and continued at the 1-h exposure period (Fig. 4). Moreover, co-treatment with DAK and methimazole resulted in a significant (P < 0.05) decrease of LDH leakage as early as 0.5 h (Fig. 3) and continued at the 1-h exposure period (Fig. 4). The observations at later time points, 2 and 4 h, did not result in any significant differences, possibly due the potent cytotoxic effect of DAK on the liver cells. This study demonstrated that DAK was directly toxic to liver cells in a dose- and time-dependent manner and that its toxicity was enhanced with n-octylamine and suppressed with methimazole. The concentrations of KT’s metabolite, DAK, have not yet been reported in either animals or humans; however, the therapeutic concentrations of KT vary on the dosage and duration of treatment that is dependent on the medical diagnosis with peak serum concentrations ranging from 40 μM to 94 μM in normal human (Brass et al., 1982; Huang et al., 1986; Sugar et al., 1987). Furthermore, autoinhibition of KT in vivo may result in higher plasma/tissue concentrations (Heel et al., 1982; Huang et al., 1986; Daneshmend and Warnock, 1988). In addition to the LDH assay, the treatment of the cell cultures with DAK also resulted in a significant (P < 0.05) concentration- and time-dependent decrease in mitochondrial reduction of MTT (Fig. 5). The 120 μM and 160 μM DAK concentrations produced less than 50% reduction of MTT by the liver cells as early as 0.5 h. Moreover, the concentration of DAK to reach less than 50% reduction of MTT was 107 μM at 0.5 h; 90 μM at 1.0 h; 75 μM at 2.0 h; and 58 μM at 4.0 h. In comparison, the concentration of KT to reach less than 50% reduction of MTT was 160 μM at 1.0 h; 113 μM at 2.0 h; and 90 μM at 4.0 h (Rodriguez and Acosta, 1995).

As previously mentioned, the mechanism(s) of the hepatotoxicity associated with KT has not been explored; thus, the mechanism of its toxicity is unknown. The early onset of cytotoxicity seen with KT in our earlier studies (Rodriguez and Acosta, 1995) suggested that the parent compound may be responsible for the observed in vitro toxicity rather than a metabolite. However, this present study revealed that the deacetylated metabolite of KT was the more potent cytotoxic, thus supporting the possibility of a reactive metabolite being responsible for the hepatotoxicity. Moreover, DAK, like lipophilic 4-alkylpiperazines, is susceptible to successive oxidative attacks by the flavin-containing monoxygenases (FMO) on the N-1 position, producing potentially toxic ring-opened dialdehydes (Rodriguez et al., 1995). Thus, the increased susceptibility of the liver cells to the deacetylated compound may result from DAK’s metabolism by FMO. DAK’s toxicity was enhanced with n-octylamine and decreased with methimazole. Thus, these data support a role for FMO in the metabolism of DAK in a primary culture system of postnatal rat hepatocytes. Although the literature has demonstrated that male rats have higher FMO activity than female (Skett et al., 1980; Dannan et al., 1986; Lemoine et al., 1991), this phenomenon occurs after puberty. Thus, 8- to 11-day-old rats have similar enzyme activity. Also, we have established immunochemically the presence of FMO and its enzyme activity in our primary culture system (Rodriguez et al., 1996). In a previous study (Rodriguez et al., 1996), we showed that FMO enzyme activity was present from microsomes obtained from cultured postnatal hepatocytes following methimazole-dependent oxygen uptake. The results were 250 ± 34 and 433 ± 33 nM O2/min/mg protein for days one and two, respectively (Rodriguez et al., 1996). Thus, it is possible that KT’s deacetylated metabolite, after metabolism by FMO, may ultimately be responsible for KT’s hepatotoxicity.

Secondly, the results of this experiment demonstrated that the MTT reduction assay, which measures mitochondrial dehydrogenase activity, was more sensitive than the LDH assay for the evaluation of DAK’s cytotoxicity. This may indicate that the mitochondria may be a cellular target of DAK’s toxicity. In our previous paper, the cytotoxicity of KT affected the LDH and MTT assays equally (Rodriguez and Acosta, 1995). Furthermore, the MTT results show an increase of MTT reduction at the low concentrations of DAK (20 μM) for all time points monitored; however, none
were significant from control. Thus, low DAK concentrations may stimulate mitochondrial dehydrogenase activity while higher concentrations may inhibit the enzyme activity.

Also, the possibility of the parent compound’s inhibition of sterol synthesis in mammals cannot be excluded. It is known that KT inhibits the conversion of lanosterol or 24-methylated hydroxysterol to ergosterol and results in the loss of membrane integrity, which becomes increasingly permeable and progressively deteriorates (Heel et al., 1982). Thus, KT’s inhibitory effect on sterol synthesis may alter plasma membrane integrity resulting in cell death. Lastly, the hepatotoxicity of KT may be the result of autoinhibition. As reviewed in our earlier paper (Rodriguez and Acosta, 1995) KT has been demonstrated to inhibit a number of cytochrome P-450 enzymes involved in steroidogenesis and drug metabolism (Loose et al., 1983; Santen et al., 1983; Sheets and Mason, 1984; Meredith et al., 1985; Rodrigues et al., 1987; Maurice et al., 1992). Also, KT’s half-life is dose-dependent and increases during long-term treatment in normal human volunteers (Heel et al., 1982; Daneshmend and Warnock, 1988). Moreover, it has been reported that KT’s deacetylated metabolite, DAK, was the primary metabolite that accumulates in the livers of mice after a 7-day dosing regimen (Whitehouse et al., 1990). The accumulation in mice may be due to KT’s inhibitory effect on cytochrome P-450, as well as on FMO (Rodriguez et al., 1995), thus preventing further metabolism of the metabolite. This autoinhibition of KT after long-term administration may play a role in the hepatotoxicity documented in the clinical literature.

In summary, our in vitro model of KT hepatotoxicity with a primary culture system of postnatal rat hepatocytes demonstrated that DAK is directly toxic to liver cells in a distinct time- and dose-response relationship. In addition, the results showed that DAK is a potent toxicant when compared to KT on an equimolar basis. Lastly, DAK’s toxicity was enhanced with octylamine and suppressed with methimazole, thus suggesting that FMO may play a role in KT-induced hepatotoxicity.

Acknowledgements

We acknowledge Dr Daniel M. Ziegler for his helpful comments and suggestions. Rosita J. Rodriguez was a recipient of a NIGMS Pre-Doctoral Fellowship (1994–1996) sponsored by NIDDK grant #1 F31 DK09063-01. Daniel Acosta, Jr. was a Burroughs Wellcome Scholar in Toxicology.

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