Comparison of ketoconazole- and fluconazole-induced hepatotoxicity in a primary culture system of rat hepatocytes

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Abstract

Ketoconazole (KT) and fluconazole (FLU) areazole antifungal agents with a broad spectrum of activity against both superficial and systemic mycoses. KT is also an anticancer agent in the treatment of advanced prostate cancer. In many clinical and retrospective studies, KT has been reported to cause liver damage, i.e. chemical hepatitis. Histologic analysis of KT induced hepatotoxicity shows massive centrilobular necrosis in which the hepatotoxicity was not thought to be mediated through an immunological mechanism. According to the medical literature, the pattern of hepatic injury appears to be primarily of the hepatocellular type. Because of the documented reports of KT and FLU hepatotoxicity, a cytotoxicity comparison of KT and FLU was implemented. The objective of this comparison was to evaluate the cytotoxicity of these azoles such that future mechanistic investigations of hepatotoxicity could be performed. The relative hepatotoxicity of KT and FLU was evaluated using primary cultures of postnatal rat hepatocytes. Cytotoxicity was evaluated by measuring the leakage of the cytosolic enzyme, lactate dehydrogenase (LDH), into the medium; by assessing mitochondrial reduction of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); by assessing lysosomal uptake of neutral red (NR); and by gross morphology (phase contrast microscopy). The cultures were exposed to various concentrations of KT (56-188 μM) for 0.5-4 h and to various concentrations of FLU (50 μM to 1.0 mM) for 0.5-6 h. There was a significant increase (P < 0.05) in LDH leakage and a large decrease in MTT reduction and lysosomal uptake of NR at 4 h for KT. One millimolar FLU had minimal effects on the LDH leakage and MTT reduction. These results demonstrate that KT is a more potent cytotoxicant than FLU; and its toxicity was expressed in a dose- and time-dependent manner.

Keywords: Azole derivatives; Fluconazole; Ketoconazole; Hepatocytes; Hepatotoxicity; Primary cultures

1. Introduction

Ketoconazole (KT) or (±)-cis-1-acetyl-4-[4-[1][2-(2,4-dichlorophenyl)-2-(1-H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl][methoxy]- phenyl]piperazine was the first oral antifungal agent in a series ofazole-derivatives with a broad spectrum of activity against both superficial and systemic mycoses. Since the development of KT in the late 1970s, KT and other azoles (fluconazole and itraconazole) have become prominent broad spectrum oral antifungal agents in the treatment of systemic mycoses, especially in patients with the acquired immune deficiency syndrome who are susceptible to opportunistic systemic fungal infec-
tions. KT and the related azoles exert their antifungal actions by blocking the conversion of lanosterol or 24-methylenedihydrolanosterol to ergosterol in fungi (Van 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 (Van Bossche et al., 1988). Furthermore, the action of KT is not only limited to the cytochromes of fungi because inhibition is also seen with a number of mammalian cytochromes. 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, a major component of membranes in mammals (Strandberg et al., 1987). In addition to its antifungal activity, an inhibitory effect on the synthesis of testosterone in both testicular and adrenal cells makes KT a suitable candidate for the treatment of androgen-dependent diseases such as advanced prostate cancer (Santen et al., 1983; Pont et al., 1984).

With worldwide usage of KT, 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). Rechallenge of KT in patients resulted in enhanced hepatotoxicity (Van Parys et al., 1987; Cauwenbergh, 1989). 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). However, this estimation is debatable because the exact number of treated patients and the durations of treatment are not known (Janssen and Symoens, 1983). In addition, under reporting of symptomatic cases is probable; therefore, the actual incidence is probably greater (Stricker et al., 1986; Knight et al., 1991).

Similarly, there have been documented reports of fatal hepatic necrosis related to fluconazole (FLU) treatment (Grant and Clissold, 1990; Bodey, 1992) and of a dose-dependent/rechallenge of FLU induced-hepatotoxicity (Wells and Lever, 1992). Furthermore, the newly released azole, itraconazole, has been reported to cause drug-induced hepatitis (Lavrijsen et al., 1992; Hann et al., 1993). Also, a study on adverse events associated with chronic itraconazole therapy in 189 patients reported 22 deaths after implementing itraconazole treatment (Tucker et al., 1990). The authors felt that the deaths were not related to itraconazole. At present, the mechanism of azole-induced hepatic necrosis is unknown (Bercoff et al., 1985; Buchi et al., 1986; Lake-Bakaar et al., 1987; Brusko and Marten, 1991). The absence of clinical manifestations 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). Because of the many documented cases of azole-induced hepatotoxicity and because of their important use in the treatment of systemic mycotic infections and prostate cancer, investigations on the mechanism of hepatotoxicity of KT and FLU may provide essential information in the research development of other azole derivatives.

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. For this present study, the indices used to evaluate the direct cytotoxicity of KT and FLU were: (i) plasma membrane integrity as assessed by the leakage of lactate dehydrogenase (LDH) into the culture medium; (ii) mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); (iii) subcellular perturbations of lysosomes with the neutral red (NR) assay; and (iv) gross morphology using phase contrast microscopy.

2. Materials and methods

2.1. Materials

Newborn bovine serum was obtained from JRH Biosciences (Lenexa, KS). Williams’ medium E,
hydrocortisone-21-hemisuccinate, insulin, and albumin fraction V were tissue culture grade from Sigma Chemical Co. (St. Louis, MO). Collagenase 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 Roerig-Pfizer (New York, NY). Ketoconazole was from Research Diagnostics, Inc. (Flanders, NJ). Fluconazole was a generous gift from Pfizer Inc. (Groton, CT). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Plastic culture dishes were obtained from Falcon Becton-Dickinson (Rutherford, NJ).

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

2.3. Rat hepatocyte culture procedure
The isolation and culturing of the rat hepatocytes were done according to a method developed in our laboratory (Acosta et al., 1980) and later modified (Davila et al., 1990). Briefly, the hepatocytes were obtained from postnatal Sprague-Dawley rats (i.e., 8-11 days of age). The rats were decapitated and the heart removed to prevent back-flow of blood during the perfusion. The liver was perfused by retrograde perfusion of the portal vein with a hypodermic syringe containing approximately 2 ml of 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 homogenates, the resulting pellets were resuspended in 4 ml of Williams' medium E which contained 0.001% (w/v) insulin, 0.005% (w/v) hydrocortisone, 10% (v/v) newborn bovine serum, (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 number of cells were counted with a hematocytometer, and the cell suspension was diluted to a volume of 0.5 × 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 motion such that debris was removed during the replacement of fresh incubation medium. The incubation medium was replenished every 24 h. Hepatocytes were grown in culture to near confluency for 3 days prior to drug treatment.

2.4. Chemical exposure
We initiated a study in which experiments were conducted on the third day after the initial plating of the hepatocytes. For the toxicity studies, stock solutions of 18.8 mM KT and 19.6 mM FLU were prepared by dissolving the compounds in N,N-dimethyl formamide (DMF) and Williams' medium E. Then, the stock solutions of KT and FLU were diluted with serum-free and antibiotics-free medium to yield final concentrations ranging from 56 to 188 μM KT and 50 μM to 1 mM FLU. The final treatment concentration of DMF was 0.25% (v/v) DMF/culture medium.

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 KT and 0.5-, 1-, 2-, 4, and 6-h treatments with FLU. The reaction mixture was mixed and immediately placed into 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-, 4-, and 6-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. Neutral red assay

The neutral red (NR) assay was conducted according to the procedure of Borenfreund et al. (1985) to assess lysosomal homeostasis and cell viability. The test was conducted immediately after the 0.5-, 1-, 2-, and 4-h treatment with KT. Briefly, the cells were incubated with serum-free NR containing medium (50 μg/ml) for an additional 3 h at 37°C after the designated treatment. After the cells were washed with 1% formaldehyde–1% calcium chloride to remove unincorporated NR and to enhance the attachment of cells to the culture plates, 1.25 ml 1% acetic acid–50% ethanol were added to each well for 30 min to extract the dye from the cells. The optical density of NR extracts was measured at a wavelength of 540 nm on the Beckman DU-40 spectrophotometer. Results were expressed as a percentage of control cultures.

2.8. Morphological evaluation

Gross cellular morphological alterations were observed at 0.5, 1, 2, and 4 h for the KT treatment by a method previously described by our laboratory (Welder-Butler et al., 1985). Two random areas were observed for each well and 3–4 wells were examined in each culture. The hepatocyte cultures were monitored with an inverted phase contrast microscope (NIKON TMS-F) equipped with a Nikon FX-35A camera and a UFX-II photographic control system.

2.9. Statistical analysis

All data represent a minimum of three separate experiments with quadruplicate determinations and were expressed as the mean ± standard error (S.E.M.) 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, NR uptake, and MTT formazan formation. The data from three to four different cultures were used in the determination of statistical significance.

3. Results

3.1. LDH assay

Treatment of the cultures with KT resulted in a concentration- and time-dependent increase in LDH leakage from the cells into the culture medium (Fig. 1). There were no significant differences among the DMF, 56-PM and 75-FM KT treated cells when compared to the control cultures for all of the time points monitored. At a concentration of 95 μM, KT caused a slight increase of LDH leakage after 2 h compared to controls, but the 4-h treatment resulted in a significant increase (24% LDH leakage). At 0.5 h, 188 μM KT produced significant LDH leakage (26%) and greater than 50% LDH leakage at 1, 2 and 4 h. Cell death occurred after 4 h of exposure to 188 μM KT (93% LDH leakage). For the hepatocytes exposed to 50 μM to 1.0 mM FLU for 0.5–6 h, there was a significant increase of LDH leakage only at 2 h when compared to controls (Fig. 2). The increase was not of biological significance because the leakage was minimal (<5%). The statistical significance was probably due to the tightness of the data. KT and FLU did not interfere with the LDH assay (data
Fig. 1. Percent LDH leakage from the hepatocytes after treatment with KT. 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 S.E.M. of 3 cultures (n = 9). A two-way completely randomized factor ANOVA resulted in significant time and dose interaction (P < 0.005).

Fig. 2. Percent LDH leakage from the hepatocytes after treatment with FLU. 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 S.E.M. of 3 cultures (n = 9). A two-way completely randomized factor ANOVA resulted in a significant time interaction (P < 0.005) and a significant time and dose interaction (P < 0.05).
not shown). Lastly, control experiments with DMF showed that the incubation concentration of 0.25% (v/v) DMF did not result in any significant increase of LDH leakage when compared to cells exposed to culture medium only.

### 3.2. MTT and NR assay

Treatment of the cell cultures with KT resulted in a concentration- and time-dependent decrease in mitochondrial reduction of MTT and NR uptake into lysosomes (Figs. 3 and 4, respectively). At a concentration of 95 μM, KT caused a significant decrease in both mitochondrial reduction of MTT (49%) and NR uptake (30%) after 4 h. At 0.5 h, 188 μM KT produced a significant decrease in mitochondrial reduction of MTT (63%) and NR uptake (43%). Cell death occurred after a 4-h exposure to 188 μM KT. For the hepatocytes exposed to 50 μM to 1.0 mM FLU for 0.5–6 h, there was no significant decrease in the reduction of MTT at all of the concentrations and time points monitored (Fig. 5). Because the LDH and MTT assays did not reveal any cytotoxicity to the cultures by FLU, the NR assay was not performed. KT and FLU did not interfere with the MTT or NR assays (data not shown). Lastly, control experiments with DMF showed that the incubation concentration of 0.25% (v/v) DMF did not result in a decrease in mitochondrial reduction of MTT or lysosomal uptake of NR when compared to control cells.

### 3.3. Morphological observations

Morphological observations of KT (56 μM for all times) revealed the presence of vacuoles and granules when compared to the control cells. At higher concentrations (75–95 μM KT for 2 h), rounding of cells and an increase in vacuoles were noticed; and at 115 μM KT for 2 h, there was cell detachment from the culture plates. The detachment of cells from the culture plates was also characteristic of cells treated with 95 μM KT for 4 h. There was almost complete rounding of cells and formation of clusters, thus leaving few cells at-

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**Fig. 3.** Percent mitochondrial reduction of MTT after treatment with KT. The MTT reduction values are expressed as a percentage of control hepatocytes in order to standardize values between different cultures. The error bars are the S.E.M. of 3 cultures (n = 10). A two-way completely randomized factor ANOVA resulted in significant time and dose interaction (P < 0.005).
Fig. 4. Percent lysosomal uptake of neutral red (NR) of the hepatocytes after treatment with KT. The NR uptake values are expressed as a percentage of control hepatocytes in order to standardize values between different cultures. The error bars are the S.E.M. of 3 cultures (n = 12). A two-way completely randomized factor ANOVA resulted in significant time and dose interaction (P < 0.005).

Fig. 5. Percent mitochondrial reduction of MTT after treatment with FLU. The MTT reduction values are expressed as a percentage of control hepatocytes in order to standardize values between different cultures. The error bars are the S.E.M. of 3 cultures (n = 9). A two-way completely randomized factor ANOVA resulted in no significance.

Because of the concerns of KT-induced hepatotoxicity and poor penetration into the cerebral spinal fluid, new azole derivatives are currently being developed. The new azole derivatives recently released on market appear to be less hepatotoxic than KT; nonetheless, there have been reports of drug-induced hepatitis with FLU (Grant and Clissold, 1990; Bodey, 1992; Trujillo et al., 1994) anditraconazole (Lavrijsen et al., 1992; Hann et al., 1993). In addition, FLU has been reported to result in a dose-dependent/rechallenge of FLU-
induced hepatotoxicity (Wells and Lever, 1992). The objective of this study was to compare the relative hepatotoxicity of the newly released azole derivative, FLU, to the well-documented hepatotoxic KT.

The establishment of an in vitro model for KT-induced hepatotoxicity was performed in a primary culture system of rat hepatocytes. The model was established through a series of cytotoxicity assays which evaluated several indices such as plasma membrane integrity and mitochondrial and lysosomal stability. The choice of the rat for our in vitro model system involves a couple of factors. First, the acute in vivo toxicity studies of KT in animals using LD₅₀ values revealed that the rat, among the mouse, guinea pig and dog, was the most susceptible to death when KT was administered orally (Heel et al., 1982). Also, there has been a dose and time-dependent cytotoxicity report utilizing adult rat hepatocytes in vitro (Buchi et al., 1986). 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). Therefore, in vitro cell culture studies utilizing rats may provide a more sensitive model for the evaluation of KT-induced hepatotoxicity.

Treatment of the hepatocytes with KT resulted in a concentration- and time-dependent increase of LDH leakage from the cells into the culture medium (Fig. 1). The 188-μM KT exposure to hepatocytes produced significant LDH leakage as early as 0.5 h with greater than 50% total LDH leakage after a 1-h exposure. The onset of cell death occurred 2 h earlier with the postnatal hepatocyte culture than with the adult hepatocytes in vitro model (Buchi et al., 1986). The early onset of toxicity to the postnatal hepatocyte culture correlates with the clinical literature which has shown that children maybe more susceptible to the hepatotoxic effects of KT (MacNair et al., 1981; Tkach and Rinaldi, 1982). This study demonstrated that KT was directly toxic to liver cells in a dose- and time-dependent manner. As for the hepatocytes exposed to 50 μM to 1.0 mM FLU for 0.5–6 h, the results indicate that there does not appear to be any biologically significant damage to the hepatocytes despite the high concentrations tested and time points monitored (Fig. 2). The evaluation of the FLU concentrations, 50 μM to 1.0 mM, was primarily to contrast the two azole derivatives on an equimolar basis and are not representative of in vivo concentrations for FLU. The therapeutic concentrations for KT vary on the dosage and duration of treatment that is dependent on the medical diagnosis (i.e., fungal infection or prostate cancer). KT peak serum concentrations have been reported to range from 40 μM to 94 μM in normal human volunteers (Brass et al., 1982; Huang et al., 1986). Higher concentrations of KT maybe obtained in vivo due to the autoinhibition and increased half-life with increasing doses and duration of treatment with KT (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 KT also resulted in a significant (P < 0.05) concentration- and time-dependent decrease in mitochondrial reduction of MTT and lysosomal uptake of NR (Figs. 3 and 4, respectively). Once again, the hepatocytes exposed to 50 μM to 1.0 mM FLU for 0.5–6 h did not result in any significant decrease in the reduction of MTT for each of the concentrations and time points monitored (Fig. 5).

In addition to the cytotoxic assays implemented, the morphological observations of KT- and FLU-treated hepatocytes correlated with the results seen with cytotoxicity assays. The low concentrations, 56 μM KT, produced vacuoles and granules when compared to the control cells. At higher concentrations (75–95 μM KT for 2 h), there was rounding of cells and an increase in vacuoles; and cell detachment from the culture plates at the higher concentrations at 95 μM KT for 4 h, 115 μM KT for 2 h, and at 188 μM KT for 1, 2, and 4 h. As for the FLU-treated cells, there appeared to be no morphological alterations of the hepatocytes when compared to control cells for each of the concentrations and time points monitored. Thus, these morphological observations correlate with the cytotoxicity results of the LDH, MTT reduction, and NR assays. Lastly, control experiments with DMF showed that the incubation concentration of
0.25% (v/v) DMF did not result in any significant increase of LDH leakage or a decrease in mitochondrial reduction of MTT or lysosomal uptake of NR when compared to cells exposed to culture medium only.

As previously mentioned, the mechanism(s) of the hepatotoxicity associated with KT remains unknown, however, several possibilities exist. First, the early onset of toxicity of KT may suggest that the parent compound may be responsible for the toxicity rather than a metabolite. However, the possibility of a reactive metabolite cannot be excluded. Because KT is primarily metabolized by the liver by oxidation of the imidazole ring, degradation of the oxidized imidazole, oxidative O-dealkylation, oxidative degradation of the piperazone ring and aromatic hydroxylation to a large number of metabolites (Heel et al., 1982), further investigations using inducers and inhibitors of the cytochrome P-450 needs to be implemented to establish whether the parent compound, KT, or a metabolite is the cause of toxicity. Secondly, KT may directly interfere with membrane sterol synthesis in mammals. In fungi, the inhibition of the conversion of lanosterol or 24-methylenedihydrolanosterol to ergosterol results in the loss of membrane integrity, which becomes increasingly permeable and progressively deteriorates (Heel et al., 1982). Furthermore, KT has been reported to have an inhibitory effect on the conversion of lanosterol to cholesterol, a major component of membranes in mammals (Strandberg et al., 1987). Thus, KT may directly cause alteration in the sterol synthesis in the mammalian membrane resulting in the loss of membrane integrity. Thirdly, the hepatotoxicity of KT may be the result of autoinhibition. KT has been demonstrated to inhibit a number of other 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). In addition, the hepatic injury reported in the clinical literature occurs after 2 weeks of treatment and appears to be located in acinar zone 3 where the cytochrome P-450 is predominately located. Because KT's half-life is dose-dependent and increases during long-term treatment in normal human volunteers, autoinhibition of metabolism of KT has been suggested (Heel et al., 1982; Daneshmend and Warnock, 1988). This autoinhibition of KT after long-term administration may play a role in the hepatotoxicity documented in the clinical literature.

Our in vitro model of KT hepatotoxicity using a primary culture system of postnatal rat hepatocytes demonstrated that KT is directly toxic to liver cells in a distinct time- and dose-response relationship. Thus, the results clearly demonstrate that KT is a potent toxicant when compared to FLU on an equimolar basis. The in vitro model developed by our laboratory will allow for a detailed investigation of the hepatotoxic mechanism(s) associated with KT.

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