Table 1. Accumulation of gentamicin in the inner ear

Incubation time (days)	Gentamicin concentration Medium* (μg/ml)	Explant	Ratio†
1	0.12	1.10	9.2
1	1.6	3.8	2.4
1	17.0	11.6	0.7
3	0.19	2.11	11.1
5	0.09	1.50	16.7
5	0.19	2.57	13.5
5	2.1	8.5	4.0
5	13.7	38.6	2.8

Otic explants were cultured in two sets of independent experiments and analyzed as described in Materials and Methods. Both sets of experiments were incorporated into this table. In the first set, three explants were incubated per condition; in the second set, six explants each were incubated for 3 and 5 days at 0.19 μg gentamicin/ml medium. Otic explants were analyzed individually for their gentamicin and protein content (see Fig. 1 for gentamicin concentrations/mg protein and standard deviations of the first set). For the weight determination, the three otic explants that were cultured together per dish were weighed together at the end of the incubation. To obtain gentamicin concentrations/mg explant, total gentamicin for these explants was calculated and divided by their combined weight.

* Concentration at the end of the culture period. There were no significant differences in drug concentrations between the beginning and the end of the incubation. Numbers are means of at least duplicate determinations (each within 10% of the mean).

† Ratio of gentamicin concentrations in otic explants to those in the culture medium.

micin concentrations were up to 16-fold higher in the otic explants (Table 1).

The results clearly indicate that the uptake of gentamicin into the inner ear proceeds against a concentration gradient, suggesting an active (i.e. energy-dependent) transport. Although its characteristics have yet to be elucidated, the gentamicin uptake appears to obey Michaelis–Menten kinetics. The 5-day time points (Lineweaver–Burk analysis, correlation coefficient, $r^2 = 0.98$) yielded a K_m of 1.9 μM. In remarkably good agreement, K_m calculated from the 1-day time points ($r^2 = 0.98$) was 1.5 μM. The presence of a high-affinity uptake system for gentamicin in inner ear tissues has important implications for an understanding of the ototoxic actions of aminoglycoside antibiotics.

In summary, the developing inner ear in culture took up gentamicin from the surrounding medium against a concentration gradient with an apparent K_m of approximately 1.7 μM. Inner ears of CBA/C57 hybrid mouse embryos were explanted at gestational day 16 and maintained in organ culture for 1, 3 or 5 days in media containing from 0 to 17 μg gentamicin/ml. Gentamicin concentrations in the otocysts increased with incubation time and exceeded the drug concentration in the medium up to 16-fold. This is the first suggestion that tissues of the inner ear may actively transport aminoglycoside antibiotics by a high-affinity uptake system.

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Ketoconazole hepatotoxicity: an *in vitro* model

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Ketoconazole is the newest in a series of imidazole-derivative antifungal agents. It has a broad spectrum of activity against both superficial and systemic mycoses [1–3]. Ketoconazole is distinct from other antifungal imidazoles in clinical use. It is the only one that is absorbed in sufficient quantity to achieve therapeutic blood levels. Thus, it is effective when given orally [2, 4]. It does not induce its own metabolism *in vivo*, nor that of other drugs *in vitro* [5]. Like other imidazoles, however, it inhibits the metabolism of some drugs in clinical situations [6, 7]; recent reports have suggested that it does so by inhibiting the microsomal P-450 enzyme system [8].

As clinical use of ketoconazole increased, reports of adverse hepatic reactions appeared [9–11]. There are two general categories of reactions. Asymptomatic elevations of hepatocellular enzymes occur in 5–10% of patients at

some time during the course of ketoconazole therapy and have not been associated with any long-term sequelae [10]. Clinically symptomatic hepatitis occurs in an estimated 1 of 12,000 to 15,000 patients treated with ketoconazole [10, 11]. These patients present with jaundice, anorexia, nausea and vomiting, as well as a marked elevation of hepatic enzyme levels. Recovery usually follows withdrawal of ketoconazole. Because the reported cases of ketoconazole hepatotoxicity do not exhibit manifestations of hypersensitivity, it is felt that these clinical reactions are most likely due to a metabolic idiosyncrasy [11].

A model that would enable us to define aspects of ketoconazole metabolism which result in hepatotoxic effects would increase our understanding of the problem, but more importantly it would permit more detailed investigations of the possible metabolic idiosyncrasies that could be responsible. Intact animal models of ketoconazole hepatotoxicity have not been helpful, in that liver toxicity in animals appears only as a part of a generalized reaction that leads to death [12]. This study was designed to investigate the usefulness of rat hepatocyte monolayer cultures in studying ketoconazole hepatotoxicity.

Materials and methods

Hepatocytes were isolated from adult male Sprague–Dawley rats, using a slight modification of the methods of Berry and Friend [13] and Bissel *et al.* [14]. Prior to cell harvest, rats were pretreated with sodium phenobarbital (80 mg/kg/day) intraperitoneally for 4 days, with piperonyl butoxide (2000 mg/kg) intraperitoneally 60 min before hepatocyte isolation, or with sterile saline (0.5 ml) intraperitoneally 60 min before hepatocyte isolation. Three rats were used in each group. The livers were perfused *in situ* with sodium bicarbonate buffered balanced salt solution containing 0.05% collagenase (Cooper Biochemical Corp., Malvern, PA). Parenchymal cells were washed and separated from nonparenchymal cells and debris, and then suspended in Waymouth MB 752 culture medium (Sigma Chemical Co., St. Louis, MO) containing gentamicin (50 μg/ml) (Schering Corp., Kenilworth, NJ), insulin (1.0 μM), corticosterone (1.0 μM), and testosterone (1.0 μM) (all from the Sigma Chemical Co.). Cell viability of greater than 90% was confirmed by trypan blue exclusion [15]. The cells were then diluted to 10^6 cells/ml in the Waymouth medium. Suspensions of 2.5 ml were placed in collagen-coated 60 × 15 mm plastic petri dishes and allowed to form a monolayer in a humidified incubator at 37° in an atmosphere of 5% CO_2 and 95% air. Twenty-four hours later, the plated cells were washed with fresh Waymouth medium and then allowed to incubate for 6 hr in 2.5 ml of the medium, which contained varied concentrations of ketoconazole.

Ketoconazole (Janssen Pharmaceutica Inc., Piscataway, NJ) was dissolved as a 5 mg/ml solution in 0.05 M hydrochloric acid (reagent grade). Then immediately prior to treatment of the hepatocyte monolayer cultures, this stock solution was diluted directly into the Waymouth culture medium to final concentrations of 20–100 μg/ml.

After the 6-hr treatment period, the medium was aspirated off the monolayer cultures and passed through a 3-μm Millipore filter. The cells were lysed with 2.5 ml deionized water, scraped from the plate, sonicated for 30 sec, then centrifuged for 10 min at 9000 *g*. Lactate dehydrogenase (LDH) in the filtered medium and in the cell supernatant fraction was measured by the method of Wroblewski and LaDue [16]. An LDH index (the ratio of LDH in the medium filtrate to total LDH in the medium plus the cell supernatant fraction) was used to evaluate the severity of cell injury due to the drug toxicity. LDH release into the culture medium has been shown to correlate well with cell membrane disruption or damage under the conditions described [15]. Preliminary experiments with ketoconazole in the concentrations used in this study demonstrated that it does not interfere with the LDH assay.

Cytochrome P-450 levels were determined using the method of Omura and Sato [17].

All control LDH index values were adjusted to 5%, with the experimental values corrected accordingly, to allow comparison of values from multiple rats. Data were analyzed using one-way analysis of variance and the Newman–Keuls multiple range test.

Results and discussion

Ketoconazole exhibited hepatotoxicity that was manifested by an increase in the LDH index, as shown in Fig. 1. The toxicity was dose dependent, increasing steadily across the range of 30–70 μg/ml. No toxicity was seen at concentrations of less than 30 μg/ml. When measured over time, toxicity began to appear after 1 hr of exposure to ketoconazole and reached a maximum at 6 hr, as shown in Fig. 2. The hydrochloric acid vehicle alone, in the concentrations used, did not increase the LDH index above control values (data not shown).

Pretreatment with sodium phenobarbital prior to cell harvest significantly ($P < 0.001$) decreased the toxicity of ketoconazole across the entire range of concentrations, as shown in Fig. 1. Piperonyl butoxide pretreatment, on the other hand, had no effect on the toxicity of ketoconazole. Measurements of cytochrome P-450 showed a 3- to 4-

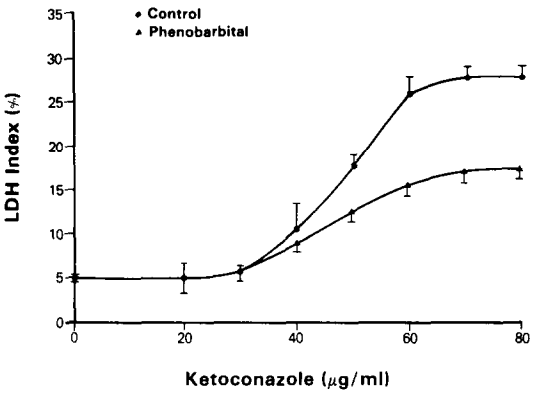

Fig. 1. Ketoconazole hepatotoxicity as measured by the LDH index. Key: (●) toxicity of ketoconazole alone in hepatocyte cultures, and (▲) toxicity of the same doses of ketoconazole after pretreatment of the hepatocytes with phenobarbital. Each point represents the mean ± S.D. of fifteen culture plates, five from each of three rats.

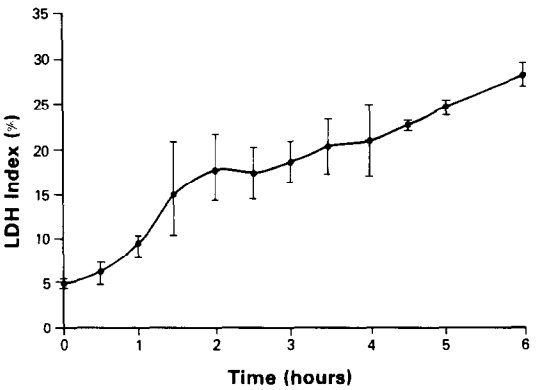

Fig. 2. Time response of ketoconazole toxicity in the hepatocyte cultures as measured by the LDH index. The concentration of ketoconazole was 80 μg/ml. Each point represents the mean ± S.D. of fifteen culture plates, five from each of three rats.

fold increase of the levels in the sodium phenobarbital pretreated cells compared to saline pretreatment and a 40–50% reduction with piperonyl butoxide pretreatment (data not shown).

These data show that ketoconazole was hepatotoxic in an *in vitro* model of rat hepatocyte monolayer cultures. The toxicity was dose dependent across a concentration range that approached the serum drug levels seen in clinical practice [4, 18]. Phenobarbital pretreatment, which induces the hepatic microsomal cytochrome P-450 enzyme system, significantly decreased the toxicity of ketoconazole. The microsomal enzyme system is responsible in large part for the extensive oxidative metabolism of ketoconazole *in vivo* [4, 19]. Because the induction of this system, which presumably enhances the metabolism of ketoconazole, decreased toxicity, it appears that the parent compound was responsible for the observed hepatotoxicity. The mechanism(s) by which this toxicity occurs remains unknown; however, several possibilities exist.

It is possible that ketoconazole caused the toxicity by directly interfering with membrane sterol synthesis, resulting in weakened or damaged cell membranes as has been shown in candida species [20, 21]. In fact, ketoconazole has been shown to inhibit the synthesis of one steroid, testosterone, in mammalian cells at concentrations similar to those showing toxicity in the hepatocyte system [22, 23]; those studies used subcellular organelle fractions, or, when intact cells were used, the time that was required to show measurable inhibition of steroid synthesis ranged from 4 to 24 hr [21]. In the current experiment, however, the rapid onset of measurable toxicity in hepatocytes made it unlikely that inhibition of steroid synthesis was responsible for the toxicity.

A second possibility is that ketoconazole caused toxicity by inhibiting the hydrogen peroxide degrading enzymes, such as catalase or cytochrome *c* peroxidase, thus leading to an accumulation of hydrogen peroxide and oxidant injury to the cell. There is evidence that this occurs in mycotic organisms [20]. It could also account for the sporadic incidence of clinical hepatic reactions. Presumably in most people, other peroxidative enzymes or antioxidant systems would compensate for the suppressed catalase or cytochrome *c* peroxidase activity; however, those with compromised antioxidant systems, possibly from either genetic or environmental causes, would be more susceptible to injury.

These possibilities related to the mechanism(s) of ketoconazole hepatotoxicity remain to be tested and verified. The model of toxicity in the rat hepatocyte monolayer culture system offers a means to investigate these and other possibilities. Ultimately, such studies should lead to rational preventive measures.

In summary, an *in vitro* model of ketoconazole hepatotoxicity was developed, utilizing cultured rat hepatocytes. In this system ketoconazole hepatotoxicity was dose dependent and reproducible. Stimulation of the cytochrome P-450 enzyme system protected against the toxicity, suggesting that the parent drug is the toxin. This model offers the opportunity to study further the mechanism(s) of ketoconazole hepatotoxicity.

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