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Inhibition of the Enzymatic Activity of Heme Oxygenases by Azole-Based Antifungal Drugs

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ABSTRACT

Ketoconazole (KTZ) and other azole antifungal agents are known to have a variety of actions beyond the inhibition of sterol synthesis in fungi. These drugs share structural features with a series of novel heme oxygenase (HO) inhibitors designed in our laboratory. Accordingly, we hypothesized that therapeutically used azole-based antifungal drugs are effective HO inhibitors. Using gas chromatography to quantify carbon monoxide formation in vitro and in vivo, we have shown that azole-containing antifungal drugs are potent HO inhibitors. Terconazole, sulconazole, and KTZ were the most potent drugs with IC_{50} values of 0.41 ± 0.01, 1.1 ± 0.4, and 0.3 ± 0.1 μM for rat spleen microsomal HO activity, respectively. Kinetic characterization revealed that KTZ was a noncompetitive HO inhibitor. In the presence of KTZ (2.5 and 10 μM), K_m values for both rat spleen and brain microsomal HO were not altered; however, a significant decrease in the catalytic capacity (V_{max}) was observed ($P < 0.005$). KTZ was also found to weakly inhibit nitric-oxide synthase with an IC_{50} of 177 ± 2 μM but had no effect on the enzymatic activity of NADPH cytochrome P450 reductase. Because these drugs were effective within the concentration range observed in humans, it is possible that inhibition of HO may play a role in some of the pharmacological actions of these antimycotic drugs.

The history of pharmacology and therapeutics contains numerous examples of drugs or drug classes that have a known mechanism of action and therapeutic application, which are found subsequently to have other therapeutic applications mediated through different mechanisms of action. Examples of this would be the statins and their effects other than lipid lowering, such as the anti-inflammatory effect (Ray and Cannon, 2005), and the hair growth-stimulating effect of minoxidil, which was originally touted as an antihypertensive agent. Recently, the antifungal agent ketoconazole (KTZ) has been reported to have anti-tumor effects in prostate cancer (Wilkinson and Chodak, 2004). In this application, the rationale for the use of KTZ lies in its ability to interfere with the synthesis of testosterone, which is considered to be associated with approximately 75% of prostate cancers. Interestingly, KTZ has been shown to be an effective adjunctive therapy in patients with androgen-independent prostate cancer where the inhibition of testosterone synthesis would not be anticipated to be a factor (Eichenberger et al., 1989). Of the various potential mechanisms to explain this effect, we considered an involvement of heme oxygenase-1 (HO-1) because an increase in HO-1 protein expression has been observed in a variety of tumors, such as human hyperplastic and undifferentiated malignant prostate tissue (Maines and Abrahamsson, 1996), and there is mounting evidence that many solid tumors require HO-1 (Fang et al., 2004a). Heme oxygenases catalyze the degradation of heme to carbon monoxide (CO), ferrous iron, and biliverdin/bilirubin (Maines, 1997). The inducible stress protein HO-1 is predominantly expressed in the reticuloendothelial cells of the spleen, and its expression is induced by a number of stimuli, including heat shock, heavy metals, heme, ionizing radiation, reactive oxygen species, and proinflammatory cytokines, whereas the constitutive HO-2 is mainly expressed in the brain and testes (Braggins et al., 1986; Maines, 1988). The HO/CO system has been broadly accepted as an important signaling entity, and the products of HO-mediated heme catabolism are involved in the regulation of many physiolog-

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ABBREVIATIONS: KTZ, ketoconazole; HO, heme oxygenase; NOS, nitric-oxide synthase; i.p., intraperitoneally; DMSO, dimethyl sulfoxide; β-NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; CPR, cytochrome P450 NADPH reductase; VeCO, rate of carbon monoxide excretion.

ical processes. CO interacts with multiple targets and may modulate neurotransmission (Hawkins et al., 1994), vascular relaxation (Furchgott and Jothianandan, 1991), platelet aggregation (Mansouri and Perry 1982), and the mitogen-activated protein kinase signaling pathway, leading to antiapoptotic effects in endothelial cells (Soares et al., 2002) and antiproliferative effects in smooth muscle cells (Morita et al., 1997; Peyton et al., 2002).

In tumor cells in vitro, Fang et al. (2004b) demonstrated that a well known HO inhibitor, zinc protoporphyrin, in its polyethylene glycol-conjugated form, exhibited HO inhibitory activity, and this resulted in increased oxidative stress and apoptosis. In the course of designing a series of novel HO inhibitors (Vlahakis et al., 2005; Kinobe et al., 2006), we have synthesized a number of imidazole-dioxolane compounds that share structural features with the azole antifungal agents. This raised the possibility that KTZ might derive its anticancer activity through mimicry of the actions of zinc protoporphyrin, namely HO inhibition.

The hypothesis tested herein is that ketoconazole is an effective inhibitor of HO activity, and this inhibition occurs at normal antifungal therapeutic concentrations. In addition to testing the hypothesis specifically with respect to KTZ, we determined the effects of other antifungal drugs on HO activity and investigated the mechanism of KTZ inhibition of HO activity.

Materials and Methods

Materials. Antifungal drugs including KTZ, terconazole, sulconazole nitrate, isoconazole, miconazole, econazole nitrate, clotrimazole, and griseofulvin were obtained as stock solutions (5 mM) in DMSO from Prestwick Chemical Inc. (Washington, DC). Fluconazole was obtained from MP Biomedicals (Irvine, CA). Horse heart cytochrome *c*, L-arginine, L-citrulline, Amberlite IPR-69 column chromatography resin, EDTA, polyethylene glycol 400, hemin chloride, ethanolamine, bovine monoclonal anti-rat β-actin antibodies, serum albumin, and β-NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). Polyclonal anti-human HO-1 antibodies (SPA-896) were obtained from StressGen (Victoria, BC, Canada). [^{14}C]L-arginine (320 mCi/mmol) and [^{14}C]L-citrulline (58.8 mCi/mmol) were purchased from Mandel/New England Nuclear (Guelph, ON, Canada). All other chemicals were obtained from BDH (Toronto, ON, Canada).

Animals. Male Sprague-Dawley rats (250–300 g) were obtained from Charles River Inc. (Montreal, QC, Canada). The animals were cared for in accordance with principles and guidelines of the Canadian Council on Animal Care and experimental protocols approved by Queen's University Animal Care Committee. Twelve-hour light cycles were maintained, and the animals were allowed unlimited access to water and standard Ralston Purina laboratory chow (Ren's Feed Supplies, Ltd., Oakville, ON, Canada).

Human Spleen Tissue. Sections of human spleen tissue were obtained as surgical dissipate from Kingston General Hospital (Kingston, ON, Canada). Freshly harvested spleens collected for routine histopathological examinations in the course of surgery were washed in physiological saline, snap-frozen in liquid nitrogen, and then stored at −80°C before use.

Preparation of Cytosolic and Microsomal Fractions. Microsomal fractions were prepared from rat brain and spleen and human spleen by differential centrifugation according to procedures described by Appleton et al. (1999). Microsomal fractions (100,000*g* pellet) were washed twice followed by resuspension in buffer containing 100 mM KH_2PO_4, 20% v/v glycerol, and 1 mM EDTA adjusted to pH 7.4 and then stored at −80°C until used. Rat brain cytosol was also prepared for measuring NOS enzymatic activity from whole brains in buffer containing 50 mM HEPES, 1 mM EDTA, and 10 μg/ml leupeptin, pH 7.4. Protein concentration was determined by a modification of the biuret method as described by Marks et al. (1997).

Measurement of HO Enzymatic Activity in Vitro. HO activity in rat spleen and brain and human spleen microsomal fractions was determined by the quantitation of CO formed from the degradation of methemalbumin (heme complexed with albumin) according to the method of Vreman and Stevenson (1988) and Cook et al. (1995). In brief, reaction mixtures (150 μl) consisting of 100 mM phosphate buffer, pH 7.4, 50 mM methemalbumin, and 1 mg/ml protein were preincubated with the vehicle (DMSO), in which the drugs were dissolved, or the antifungal drugs at final concentrations ranging from 0.1 to 100 μM for 10 min at 37°C. Reactions were initiated by adding β-NADPH at a final concentration of 1 mM, and incubations were carried out for an additional 15 min at 37°C. Reactions were stopped by instantly freezing the reaction mixture on pulverized dry ice, and CO formation was monitored by gas chromatography using a TA 3000R Process Gas Analyzer (Trace Analytical, Newark, DE).

Measurement of HO Enzymatic Activity in Vivo. The rate of pulmonary excretion of endogenously produced CO (VeCO) has been used as an index for HO enzymatic activity in vivo (Stevenson et al., 1984; Hamori et al., 1988). In the present study, the effect of KTZ on VeCO in male Sprague-Dawley rats was measured by a flow-through gas chromatography system according to the method described by Hamori et al., (1988) as modified by Dercho et al. (2006). Animals were housed in gas-tight chambers designed for the continuous flow-through of CO-free air (Praxair Canada Inc., Mississauga, ON) at a rate of 130 ml/min. Exhaust gas was directed to the injection valve of a TA 3000R reduction gas analyzer fitted with a 1-ml sample loop. Animals were acclimatized to the chambers for 30 min, and the baseline VeCO was determined for 85 min. A single dose of KTZ (1, 10, or 100 μmol/kg) dissolved in polyethylene glycol 400 was administered intraperitoneally (i.p.). Forty minutes after the administration of KTZ, hemin chloride (30 μmol/kg) dissolved in 0.05% v/v aqueous ethanolamine, pH 7.4, was administered i.p. The animals were then returned to the chambers, and VeCO was measured for an additional 6 h. Control animals were treated with only hemin chloride and polyethylene glycol-400, in which KTZ was dissolved. Treatment with hemin was used to increase CO production and to facilitate detection of an inhibitory effect of KTZ.

Measurement of NOS Enzymatic Activity. The effect of KTZ on rat brain NOS activity in vitro was assayed by monitoring the conversion of [^{14}C]L-arginine into [^{14}C]L-citrulline according to a modification of previously outlined procedures (Brien et al., 1995; Kimura et al., 1996). The reaction mixture consisted of 50 mM HEPES, pH 7.4, 1 mM EDTA, 1.25 mM $CaCl_2$, 2 mM β-NADPH, and 2 mg/ml cytosolic protein in a total volume of 200 μl. KTZ was tested at final concentrations ranging from 0.001 to 0.25 mM, and control reactions contained equivalent amounts of DMSO in which KTZ was dissolved. Total organic solvent concentration was maintained at 1% (v/v) of the final volume in all cases. NOS activity in the reaction mixture was initiated by adding L-arginine/[^{14}C]L-arginine at a final concentration of 30 μM and 35,000 dpm of [^{14}C]L-arginine. Incubations were carried out for 15 min at 37°C, and the reactions were stopped with an equal volume of "quench" buffer (20 mM HEPES and 2 mM EDTA, pH 5.5). Quenched reaction mixtures were loaded on an Amberlite IPR-69 ion-exchange chromatography resin. NOS activity was expressed as nanomoles of [^{14}C]L-citrulline formed/milligram of protein/hour.

Measurement of CPR Enzymatic Activity. Rat spleen microsomal CPR activity was measured by following the NADPH-dependent reduction of horse heart cytochrome *c* in 50 mM phosphate buffer, pH 7.7, containing 0.1 mM EDTA, 1 mM potassium cyanide, 100 μM NADPH, 100 μM cytochrome *c*, and 150 μg/ml microsomal protein according to the method of Yasukochi and Masters (1976). Incubations were done at 25°C for 15 min, and KTZ (1–250 μM) was added to incubation mixtures from concentrated ethanolic stocks.

Reaction rates were determined by reading the absorbance of reduced cytochrome *c* at 550 nm and an extinction coefficient of 0.021 $\mu M^{-1}cm^{-1}$.

HO-1 Protein Expression in Rat Liver Microsomes. To determine the effect of KTZ on HO-1 protein expression in vivo, rats were treated i.p. with KTZ or hemin chloride (100 μmol/kg). Eighteen hours after treatment, the animals were anesthetized using halothane and then sacrificed by decapitation. Liver microsomal protein was prepared, separated by SDS-polyacrylamide gel electrophoresis, and then transferred onto nitrocellulose Immobilon-P membranes (Millipore, Bedford, MA) according to previously described methods (Lash et al., 2003). The blots were incubated with a 1:2000 dilution of the polyclonal anti-human HO-1 (SPA-896) or monoclonal anti-rat β-actin antibodies. Peroxidase activity was detected by enhanced chemiluminescence detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and the relative HO-1 expression was quantified by optical densitometry using an NIH imager. Densitometric units were normalized to β-actin protein expression in all of the samples.

Heme-KTZ Absorption Spectra. The absorption spectra of KTZ-heme complex was recorded on a Varian Cary BIO-100 double beam spectrophotometer according to the method outlined by Huy et al. (2002). For titration of the different concentrations of KTZ (1–500 μM), a solution of 17 μM heme in 40% v/v DMSO and 20 mM HEPES buffer, pH 7.4, at 25°C was used. Under these conditions, heme exists as a monomer with characteristic absorption spectra between 350 and 700 nm (Beaven et al., 1974; Collier et al., 1979).

Kinetic Characterization of the Inhibition of HO. Characterization of the mode of HO inhibition by KTZ was done under conditions for which the rate of HO catalyzed breakdown of methemalbumin was linear with respect to time and protein concentration. In these assays, the rate of CO formation was measured in the presence of varying concentrations of KTZ or substrate (methemalbumin), whereas total protein and β-NADPH concentrations in the reaction mixtures were maintained at 1 mg/ml and 1 mM, respectively. Plots of reaction velocity versus substrate concentration were generated, and Michaelis-Menten parameters were determined in the presence or absence of KTZ using the kinetic model described by eq. 1 from nonlinear regressions in Prism (version 3.0; GraphPad Software, Inc. San Diego, CA),

$$V = V_{max} \times [S]/([S] + K_m) \quad (1)$$

where V = rate of oxidation of methemalbumin (picomoles of CO/milligram of protein/minute), [S] = substrate concentration, V_{max} = maximal rate, and K_m = substrate concentration at half the maximal rate (measure of the affinity of the enzyme for the substrate).

Data Analysis. Inhibition of the catalytic activities of HO, NOS, or CPR was evaluated by the percentage of control activity of each enzyme remaining in the presence of different concentrations of inhibitors with reference to control reactions. IC_{50} values (inhibitor concentration that decreased enzyme activity by 50%) were determined by nonlinear regression of sigmoidal dose-response curves using Prism, version 3.0. Data are presented as the mean ± S.D. from triplicate experiments. Statistical analyses were performed by one-way ANOVA, and P values of <0.05 were considered to be statistically significant.

Results

Effects of Azole-Based Antifungal Agents on in Vitro HO Activity. Commonly used antimycotic drugs, including terconazole, ketoconazole, sulconazole, isoconazole, econazole, miconazole, fluconazole, clotrimazole, and griseofulvin, were screened for the inhibition of the enzymatic activities of HO-1 (rat spleen microsomes) and HO-2 (rat brain microsomes) in vitro (Table 1). With the exception of griseofulvin, in which an azole or a tetrazole moiety is lacking, all of the azole-containing antifungal drugs showed potent inhibition of HO activity, with some selectivity for HO-1 over HO-2. Terconazole, sulconazole nitrate, and ketoconazole were the most potent compounds with IC_{50} values of 0.41 ± 0.01, 1.1 ± 0.4, and 0.3 ± 0.1 μM for HO-1, respectively (Table 1). Sulconazole nitrate was the most selective compound, with a selectivity index (ratio of the IC_{50} value for the inhibition of HO-2 to that of HO-1) of 45.

Effect of KTZ on HO Activity in Vivo. Adult male rats treated with hemin chloride (substrate) and polyethylene glycol (vehicle in which KTZ was dissolved) showed a steady but saturable increase in VeCO. A single KTZ dose (1, 10, or 100 μmol/kg i.p.) led to a concentration- and time-dependent decrease in VeCO for up to 6 h after treatment (Fig. 1). A significant decrease in VeCO was observed for at least 4.5 h after the administration of a single dose of KTZ (100 μmol/kg i.p) ($P < 0.05$) and a maximal decrease in VeCO of approximately 55 ± 2.0% as measured by evaluation of the area under the curve for three different animals.

Effect of KTZ on the Enzymatic Activity of NOS and CPR. To determine the selectivity for the inhibition of HO over CPR and NOS, the effects of KTZ on in vitro catalytic activities of human spleen microsomal HO and CPR and rat brain NOS were examined. At concentrations ranging from 1 to 250 μM, KTZ did not alter CPR activity, whereas both human spleen HO and rat brain NOS activities were inhibited with IC_{50} values of (6.3 ± 1.3 μM, $n = 2$) and (177 ± 3 μM, $n = 3$), respectively (Fig. 2). A limited concentration range (1–25 μM) of KTZ was found to selectively inhibit HO without any significant effect on NOS activity ($P < 0.005$).

Absorption Spectrum of Heme Complexed with KTZ. Heme (17 μM) in 40% (v/v) DMSO exhibited a characteristic absorption spectrum of high-spin ferric complexes assuming a five-coordinate structure with weak axial ligands as described previously by Kaminsky et al. (1972). A Soret and Q band absorption at 401, 495, and 620 nm was observed (Fig. 3, curve 1). With the exception of a slight decrease in the absorption maximum, there were no observable spectral changes when KTZ (1–200 μM, final concentration) was added to heme (17 μM; Fig. 3, curve 2). In the presence of excess KTZ (300–500 μM) in the mixture, however, there was a marked decrease in the absorption maximum and a shift to 412 nm of the Soret band, whereas Q band absorption was seen at 540 nm instead (Fig. 3, curves 3 and 4). In the absence of heme, KTZ in 40% (v/v) DMSO did not exhibit any characteristic spectrum (Fig. 3, curve 5).

Kinetic Characterization of the Inhibition of HO by KTZ. For kinetic analysis and the determination of kinetic constants, conditions were determined for which the rate of the enzymatic oxidation of hemin was linear with respect to time and microsomal protein concentration (data not shown). Nonlinear regression indicated that rat brain and spleen microsomal HO activity conformed to standard Michaelis-Menten kinetics (Fig. 4). In the absence of the drug, values for kinetic constants (K_m and V_{max}) were 2.0 ± 0.4 μM and 38 ± 1 pmol of CO/min/mg protein for rat brain and 2.1 ± 0.4 μM and 104 ± 2 pmol of CO/min/mg protein for rat spleen. In the presence of KTZ (2.5 and 10 μM), K_m values for both rat spleen and brain microsomal HO were not altered, but a significant decrease in the catalytic capacity (V_{max}) was observed ($P < 0.005$). In the presence of KTZ (2.5 μM), the K_m and V_{max} were 2.2 ± 0.6 μM and 64 ± 4 pmol of CO/min/mg

TABLE 1
Inhibitory potency of the azole-based antifungal drugs against rat spleen microsomal and rat brain microsomal HO activity in vitro

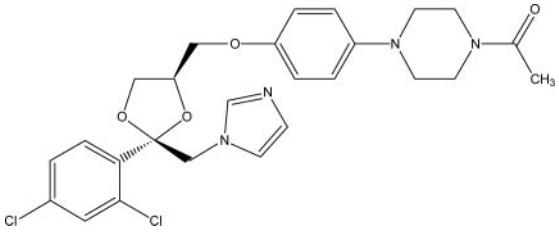
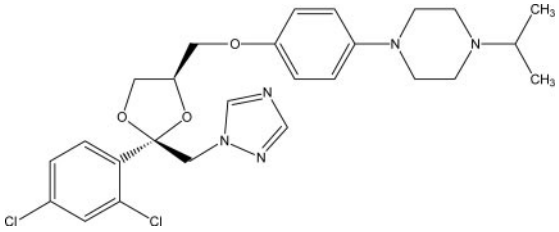
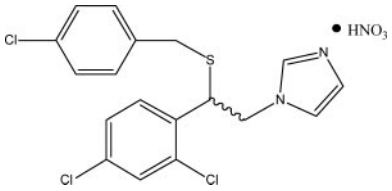
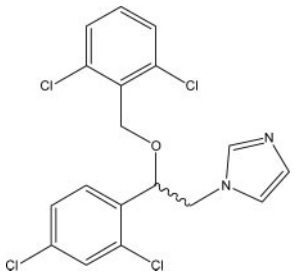
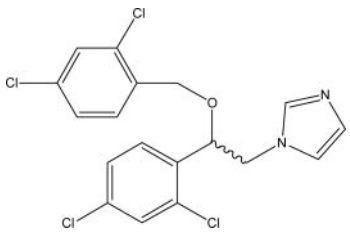
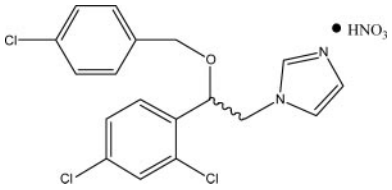
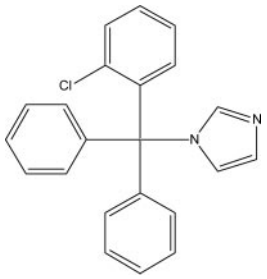
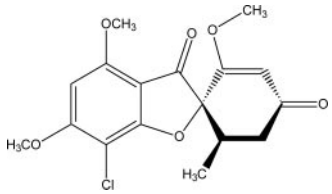
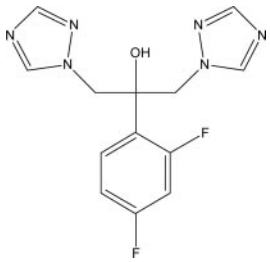
Generic Name	Chemical Structure	IC_{50} Values for the Inhibition of HO Activity in Vitro, Mean ± S.D. (n = 3)	
		Rat Spleen	Rat Brain
		μM	
Ketoconazole		0.3 ± 0.1	7 ± 1
Terconazole		0.41 ± 0.01	5 ± 3
Sulconazole Nitrate		1.1 ± 0.4	49 ± 3
Isoconazole		5.6 ± 0.1	32.6 ± 5
Miconazole		5.8 ± 0.8	45 ± 16
Econazole nitrate		16 ± 2	49 ± 3

TABLE 1—*Continued*

Generic Name	Chemical Structure	IC_{50} Values for the Inhibition of HO Activity in Vitro, Mean ± S.D. (n = 3)	
		Rat Spleen	Rat Brain
		μM	
Clotrimazole		35 ± 2	>100
Griseofulvin		>100	>100
Fluconazole		80 ± 6	>100

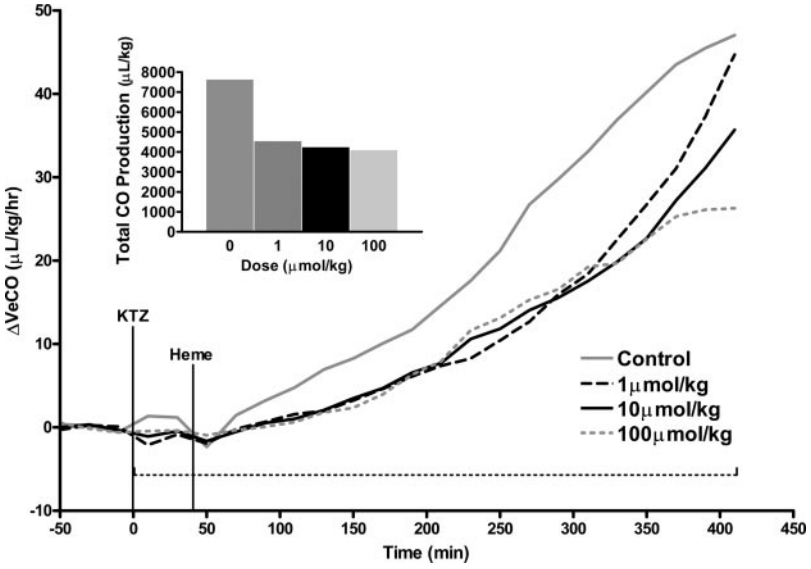


Fig. 1. Representative graph of ketoconazole-induced inhibition of CO production by rats in vivo. VeCO (μl/kg/h) was determined by sampling exhaled air in four adult male Sprague-Dawley rats before and after receiving 1, 10, or 100 μmol/kg ketoconazole or vehicle i.p. at $t = 0$ followed by 30 μmol of heme/kg i.p. at 40 min. Data were normalized to the individual baselines for each rat. Inset, Cumulative CO production by area under the curve calculated during the time represented by the dotted line.

protein for rat spleen, whereas in the presence of KTZ (10 μM), the K_m and V_{max} were 2.3 ± 0.7 μM and 20 ± 1 pmol of CO/min/mg protein for rat brain and 1.6 ± 0.6 μM and 37 ± 3 pmol of CO/min/mg protein for rat spleen (Fig. 4).

Effects of KTZ on HO-1 and HO-2 Protein Expression in Rat Liver Microsomes. To examine whether KTZ had a substantial effect on HO protein expression profile, rat liver microsomal protein, in which both HO-1 and HO-2 are expressed, was used. Under the experimental conditions described above, a single dose of hemin chloride (100 μmol/kg) was found to cause a significant increase in the expression of HO-1 ($P < 0.05$), whereas a similar dose of KTZ (100 μmol/kg) was sufficient to inhibit HO activity in vivo (Fig. 1), without any significant effect on the expression of HO-1 (Fig.

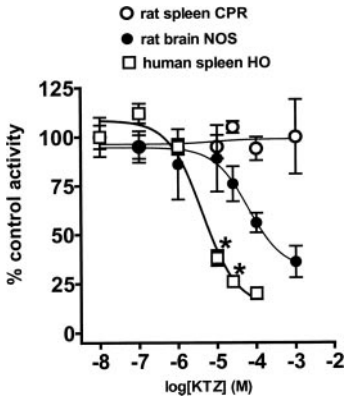


Fig. 2. Effect of KTZ on the catalytic activities of human spleen HO (□), rat brain NOS (●) and rat spleen CPR (○) in vitro. HO, NOS, and CPR enzymatic activity was determined as outlined under *Materials and Methods*. Mean HO, NOS, and CPR activities in control reactions were 45.8 ± 7.7 pmol of CO/mg of protein/min, 8.5 ± 3.0 nmol of [^{14}C]L-citrulline formed/mg of protein/h and 4.6 ± 0.3 μmol of reduced NADPH/min/mg protein, respectively. Data represent the mean ± S.D. of three experiments. * indicate concentrations of KTZ that caused significant inhibition of HO activity without any significant effect on NOS activity; $P < 0.005$.

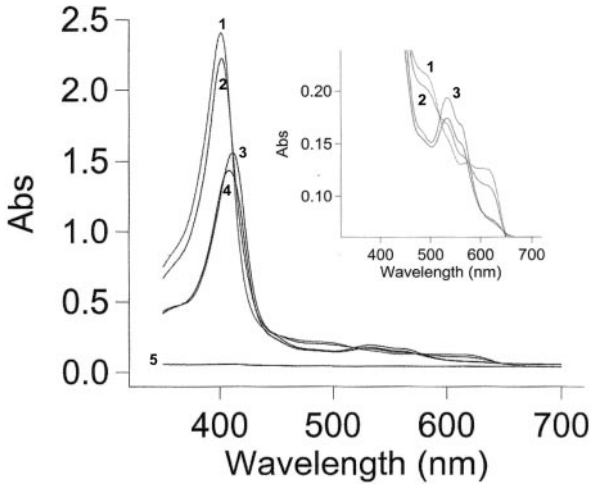


Fig. 3. Absorption spectra of hemin chloride, ketoconazole, and heme in the presence of ketoconazole. Absorption spectral changes to heme (17 μM) in the presence of KTZ were measured in a solution containing 40% v/v DMSO buffered by 20 mM HEPES, pH 7.4. Curve 1, hemin chloride only; curves 2 to 4, heme/KTZ (mixture of 17 μM heme and 200, 300, and 500 μM KTZ, respectively); and curve 5, KTZ only. The left *y*-axis represents the Soret region, whereas the inset represents Q bands.

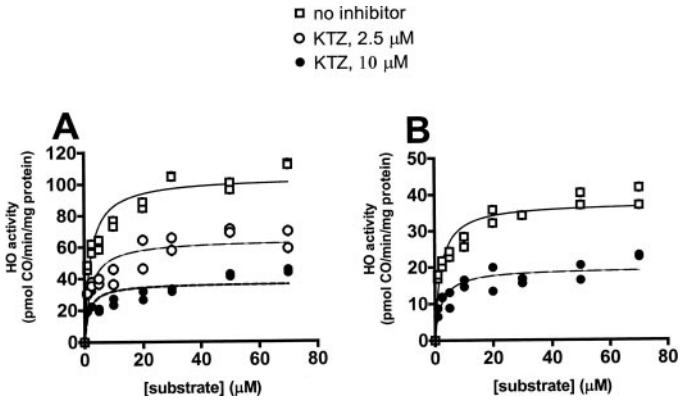


Fig. 4. A and B, kinetics of the inhibition of rat spleen (A) and rat brain (B) microsomal HO enzymatic activity by ketoconazole. HO activity was determined at varying substrate (methemalbumin) concentrations without KTZ (□) and in the presence of KTZ (2.5 μM, ○) or (10 μM, ●). HO-mediated oxidation of heme conformed to standard Michaelis-Menten kinetics.

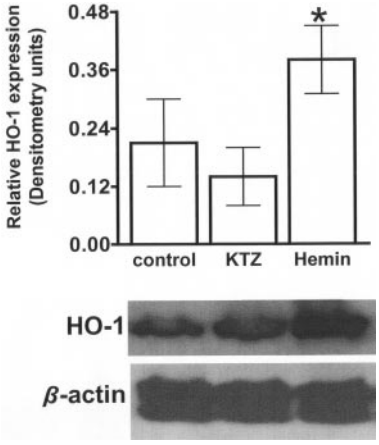


Fig. 5. A representative plot of the effect of ketoconazole on HO-1 protein expression in rat liver. Forty micrograms of microsomal protein was subjected to SDS-polyacrylamide gel electrophoresis and then probed with polyclonal anti-human HO-1 and monoclonal anti-rat β-actin antibodies. Peroxidase activity of the immunoreactive protein bands was detected by enhanced chemiluminescence. Protein loading on gels was normalized to β-actin, and the relative quantity of HO-1 was determined by optical densitometry using an NIH imager. Data represent the mean ± S.D. of three different experiments, and lanes on the blots are represented as follows: lane 1, liver microsomes from control rats; lanes 2, liver microsomes from KTZ-treated rats; and lanes 3, liver microsomes from hemin chloride-treated rats. * indicates significant induction of HO-1 by hemin chloride; $P < 0.05$.

5). Both KTZ and hemin chloride did not alter the expression of HO-2, the constitutive isozyme (data not shown).

Discussion

The major observations of the present study were as follows: ketoconazole, terconazole, and sulconazole inhibited in vitro both HO-1 and HO-2; this inhibition occurred at therapeutically relevant drug concentrations; ketoconazole inhibited HO activity in vivo; and ketoconazole inhibition of both HO-1 and HO-2 was noncompetitive.

The hypothesis tested in the present study was that ketoconazole is an effective inhibitor of heme oxygenase activity, and this inhibition occurs at normal therapeutic concentrations. The observations made herein are consistent with this hypothesis. The results showed that all eight diazole and triazole antifungal agents tested (ketoconazole, terconazole, isoconazole, sulconazole, miconazole, econazole, clotrimazole, and fluconazole) were effective in vitro inhibitors of rat HO activity. Of these eight compounds, five exhibited IC_{50} values of less than 10 μM against rat HO-1 activity, and two, KTZ and terconazole, were similarly potent inhibitors of rat HO-2 (IC_{50} values of less than 10 μM). Although all of the azole-containing drugs tested displayed HO inhibitory capability and some were more potent than KTZ, this drug was selected for further investigation because of its initial dominance in therapeutics and its position as a prototype drug. When human spleen HO activity was tested in the presence of KTZ, inhibition was observed at concentrations below 10 μM. During the in vivo studies with rats, each of the doses of KTZ (1, 10, or 100 μmol/kg) inhibited CO production. In addition, the observed IC_{50} of KTZ was 0.3 μM (0.16 μg/ml) for HO-1 and

7 μM (3.7 μg/ml) for HO-2, which is interesting in light of the plasma concentrations of this drug used clinically in humans. Thus, Huang et al. (1986) reported that the mean maximal plasma concentrations of KTZ were greater than 5, 11, and 20 μg/ml after doses of 200, 400, and 800 mg administered to 12 volunteers. At the 400-mg dose, the plasma concentrations stayed above the present IC_{50} values for HO-1 and HO-2 inhibition for 8 h after dosing. Thus, these data are consistent with the second part of the hypothesis, indicating an inhibitory effect of KTZ on HO activity at usual therapeutic concentrations.

Since one of the mechanisms proposed for other enzyme inhibition by KTZ was binding of the imidazole moiety to heme iron (Vermuyten et al., 1997), we explored the possibility that KTZ-induced inhibition of HO activity might be due to a direct interaction between the KTZ imidazole moiety and heme iron resulting in a complex that is not accessible to the HO catalytic site. If this were the case, one would anticipate that KTZ would influence the characteristic absorption spectrum of heme through formation of a high-spin ferric complex that assumes a five-coordinate structure with weak axial ligands (Kaminsky et al., 1972). The present experiments revealed that the concentration of KTZ that was required to cause changes in the heme spectrum was in the order of 100-fold (300–500 μM) higher than that found to inhibit rat HO activity in vitro. Moreover, kinetic characterization of the inhibition of both HO-1 and HO-2 microsomal isozymes in the current study shows that, in the presence of KTZ, HO activity conformed to standard Michaelis-Menten kinetics with a significant decrease in catalytic capacity but no apparent change in K_m values. These observations are not consistent with KTZ forming a complex with heme at low micromolar concentrations. Another possibility was that inhibition of HO activity by KTZ was mediated through inhibition of NADPH CPR, which serves as an accessory enzyme during the oxidative breakdown of heme and the conversion of NADPH to $NADP^+$ (Yoshida et al., 1980). This idea is not supported by our results, which showed that, even at concentrations as high as 250 μM, KTZ had no substantial effect on the catalytic activity of microsomal CPR. Likewise, KTZ did not alter the quantity of HO protein in an organ that possessed both HO-1 and HO-2 (Fig. 5) as treatment of rats with 100 μmol/kg KTZ resulted in no change in the Western blots. These observations are consistent with KTZ inhibiting HO by binding to the enzyme molecule but not at a site that interferes with the access of the substrate to the active site. Future studies employing X-ray crystallography may shed light on this issue.

The observation that KTZ at therapeutically relevant concentration inhibited HO activity of rat and human tissue broken-cell preparations in vitro and in rats in vivo raises the question whether any of the intended or unintended effects of KTZ in humans is a result of either HO-1 or HO-2 inhibition. In comparison, KTZ was much less potent as an inhibitor of rat brain NOS activity (Fig. 2), which is consistent with previous studies showing azole antifungals to be weak inhibitors of inducible NOS (Vermuyten et al., 1997). Thus, inhibition of NOS by KTZ is less likely to be clinically relevant than inhibition of HO.

Although the mechanism of the antifungal action of the azoles is widely accepted to be mediated via inhibition of fungal sterol 14-α-demethylase, the possibility that inhibition of HO activity is also a contributing factor could be considered. A recent study in *Candida albicans* lends some support to this idea; in this study, it was shown that one source of iron, which is essential for growth, was obtained from heme via HO-catalyzed metabolism (Santos et al., 2003; Pendrak et al., 2004). If KTZ were to interfere with the liberation of iron from heme, it seems possible that this action could contribute to the inhibition of growth of this organism.

In conclusion, the data point to the possibility that KTZ may exert some of its pharmacological activities through inhibition of HO; this might apply also to some therapeutic actions in humans. The mechanism of HO inhibition by KTZ is not clear but seems to be noncompetitive and not mediated through direct binding to the substrate, interference with the accessory enzyme CPR, or destruction of HO protein.

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