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

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d) Abbreviations: ß-NADPH, reduced ß-nicotinamide adenine dinucleotide phosphate; CPR, cytochrome P450 NADPH reductase; CrMP, chromium mesoporphyrin IX chloride; MAPK, mitogen-activated protein kinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid monosodium salt; L-NAME, *N*-nitro-L-arginine methyl ester; SnPP, tin protoporphyrin IX; sGC, soluble guanylyl cyclase; ZnPP, zinc protoporphyrin IX.

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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 (CO) formation *in vitro* and *in vivo*, we have shown that azole-containing antifungal drugs are potent HO inhibitors. Terconazole, sulconazole and ketoconazole (KTZ) were the most potent drugs with IC_{50} values of 0.41 ± 0.01 μM, 1.1 ± 0.4 μM and 0.3 ± 0.1 μM for rat spleen microsomal HO activity respectively. Kinetic characterization revealed that KTZ was a non-competitive HO inhibitor. In the presence of KTZ (2.5 μM 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 ($P < 0.005$). 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.

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INTRODUCTION:

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 & 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-tumour effects in prostate cancer (Wilkenson 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 sustaining to about three-quarters 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, 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 tumours such as human hyperplastic and undifferentiated malignant prostate tissue (Maines and Abrahamsson, 1996), and there is mounting evidence that many solid tumours require HO-1(Fang *et al.,* 2004). 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

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number of stimuli including heat shock, heavy metals, heme, ionizing radiation, reactive oxygen species and pro-inflammatory cytokines, while the constitutive HO-2 is mainly expressed in the brain and testes (Maines *et al.,* 1988; Braggins *et al.*, 1986). The HO/CO system has been broadly accepted as an important signalling entity and the products of HO-mediated heme catabolism are involved in the regulation of many physiological processes. CO interacts with multiple targets and may modulate neurotransmission (Hawkins *et al.*, 1994), vascular relaxation (Furchgott *et al.*, 1991), platelet aggregation (Mansouri and Perry 1982), and the mitogen-activated protein kinase (MAPK) signaling pathway leading to anti-apoptotic effects in endothelial cells (Soares *et al.*, 2002), and anti-proliferative effects in smooth muscle cells (Peyton *et al.*, 2002).

In tumour cells *in vitro*, Fang *et al*. (2004a) demonstrated that a well known HO inhibitor, zinc protoporphyrin in its polyethylene glycol-conjugated form (PEG-ZnPP), 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 Zn 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

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effects of other antifungal drugs on HO activity, and investigated the mechanism of KTZ inhibition of HO activity.

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METHODS:

Materials. Antifungal drugs including ketoconazole (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, USA). Fluconazole was obtained from MP Biomedicals, Irvine, CA, USA . Horse heart cytochrome c, L-arginine, L-citrulline, Amberlite IPR-69 column chromatography resin, ethylenediamine tetraacetic acid disodium salt (EDTA), polyethylene glycol-400, hemin chloride, ethanolamine, bovine monoclonoal anti-rat ß-actin antibodies, serum albumin and ß-NADPH were obtained from Sigma Chemical Company (St. Louis, MO, USA). 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

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in physiological saline, snap frozen in liquid nitrogen and then stored at -80 °C prior to 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 x 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). Briefly, reaction mixtures (150 µL) consisting of 100 mM phosphate buffer (pH 7.4), 50 mM methemalbumin and 1 mg/mL protein were pre-incubated with the vehicle (DMSO) in which the drugs were dissolved, or the antifungal drugs at final concentrations ranging from 0.1 µM to 100 µM for 10 minutes at 37°C. Reactions were initiated by adding ß-NADPH at a final concentration of 1 mM and incubations were carried out for an additional 15 minutes 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, USA).

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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 minutes and the baseline VeCO was determined for 85 minutes. A single dose of KTZ (1, 10 or 100 µmol/kg) dissolved in polyethylene glycol-400 was administered intraperitoneally (i.p). Forty minutes following 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 hours. 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 therefore 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,

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1.25 mM CaCl2, 2 mM ß-NADPH and 2 mg/mL cytosolic protein in a total volume of 200 µL. KTZ was tested at final concentrations ranging from 0.001–0.25 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 ^{14}C-L-arginine. Incubations were carried out for 15 minutes 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 nmol ^{14}C-L-citrulline formed/mg protein/hr.

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 minutes 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 following treatment, the animals were anesthetized using halothane and then sacrificed by decapitation. Liver microsomal protein was prepared and separated by SDS-PAGE, and then transferred onto nitrocellulose Immobilon-P

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membranes (Millipore, Bedford, MA, USA) according to previously described methods (Lash *et al.,* 2003). The blots were incubated with a 1:2,000 dilution of the polyclonal anti-human HO-1 (SPA-896) or monoclonoal anti-rat ß-actin antibodies. Peroxidase activity was detected by enhanced chemiluminescence detection kit (Amersham, Toronto, ON, Canada) and the relative HO-1 expression was quantified by optical densitometry using an NIH-imager. Densitometric units were normalized to ß-actin protein expression in all 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) while total protein and ß-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 equation 1 from non-linear regressions in Graph Pad

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Prism (version 3.0), $V = V_{max} \bullet [S]/([S]+K_m)$. Where V = rate of oxidation of methemalbumin (pmol CO/ mg protein/min), [S] = substrate concentration, V_{max} = maximum rate and K_m = substrate concentration at half the maximum 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 non-linear regression of sigmoidal dose-response curves using GraphPad Prism version 3. Data are presented as the mean ± SD from triplicate experiments. Statistical analyses were performed by one-way ANOVA and P values < 0.05 were considered to be statistically significant.

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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 activitities of HO-1 (rat spleen microsomes) and HO-2 (rat brain microsomes) *in vitro* (Table 1). Except for griseofulvin in which an azole or a tetrazole moiety is lacking, all 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 μM, 1.1 ± 0.4 μM 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 hours after treatment (Fig 1). A significant decrease in VeCO was observed for at least 4.5 hours following 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 (AUC) for 3 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, effects of KTZ on *in vitro* catalytic activities of human spleen microsomal HO and CPR, and rat brain NOS were examined. At

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concentrations ranging from 1 μM 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 μM to 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 previously described by Kaminsky *et al.* (1972). A Soret and Q band absorption at 401, 495 and 620 nm was observed (Fig 3, curve 1). Except for 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 while 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 CO/min/mg protein for rat brain and 2.1 ± 0.4 μM and 104 ± 2 pmol

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CO/min/mg protein for rat spleen. In the presence of KTZ (2.5 μM 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 CO/min/mg protein for rat spleen, while in the presence of KTZ (10 μM), the K_m and V_{max} were 2.3 ± 0.7 μM and 20 ± 1 pmol CO/min/mg protein for rat brain and 1.6 ± 0.6 μM and 37 ± 3 pmol 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 ($p < 0.05$), while 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 5). Both KTZ and hemin chloride did not alter the expression of HO-2, the constitutive isozyme (data not shown).

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DISCUSSION:

The major observations of the present study were- 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 non-competitive.

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). While 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. Additionally, 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 as used clinically in humans. Thus, Huang *et al.* (1986) reported that the mean

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maximum 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 middle 400 mg dose, the plasma concentrations stayed above the present IC_{50} values for HO-1 and HO-2 inhibition for 8 hours 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 μM to 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 μM concentrations. Another possibility was that inhibition of HO activity by KTZ was mediated through inhibition of NADPH cytochrome P450 reductase (CPR), which serves as an accessory enzyme during the

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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. Similarly, 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 Xray 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 as to 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

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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 non-competitive and not to be mediated through direct binding to the substrate, interference with the accessory enzyme, CPR, or destruction of HO protein.

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FOOTNOTES:

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LEGENDS FOR FIGURES:

Fig 1: Representative graph of ketoconazole-induced inhibition of CO production by rats *in vivo*. VeCO (μL/kg/hr) 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 heme/kg i.p. at 40 minutes. Data were normalized to the individual baselines for each rat. Inset: Cumulative CO production by AUC calculated during the time represented by the dotted line.

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 in the methods section above. Mean HO, NOS, and CPR activities in control reactions were 45.8 ± 7.7 pmol CO/mg protein/minute, 8.5 ± 3.0 nmol ^{14}C-L-citrulline formed/mg protein/hr and 4.6 ± 0.3 μmol reduced NADPH/min/mg protein, respectively. Data represent the mean ± SD of three experiments. Asterisks (*) 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 2-4, heme/KTZ (mixture of 17 μM heme and 200 μM, 300 μM and 500 μM KTZ respectively); curve 5, KTZ only. The left y-axis represents the Soret region while the inset represents Q bands.

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Fig 4: Kinetics of the inhibition of rat spleen (panel A) and rat brain (panel 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-PAGE 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 ± SD of 3 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; lanes 3, liver microsomes from hemin chloride treated rats. * indicates significant induction of HO-1 by hemin chloride $P < 0.05$.

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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 ± SD (µM), (n = 3)	
		Rat spleen	Rat brain
ketoconazole		0.3 ± 0.1	7 ± 1
terconazole		0.41 ± 0.01	5 ± 3
sulconazole nitrate	• HNO_3	1.1 ± 0.4	49 ± 3
isoconazole		5.6 ± 0.1	32.6 ± 5
miconazole		5.8 ± 0.8	45 ± 16

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econazole nitrate		16 ± 2	49 ± 3
clotrimazole		35 ± 2	> 100
griseofulvin		> 100	> 100
fluconazole		80 ± 6	> 100

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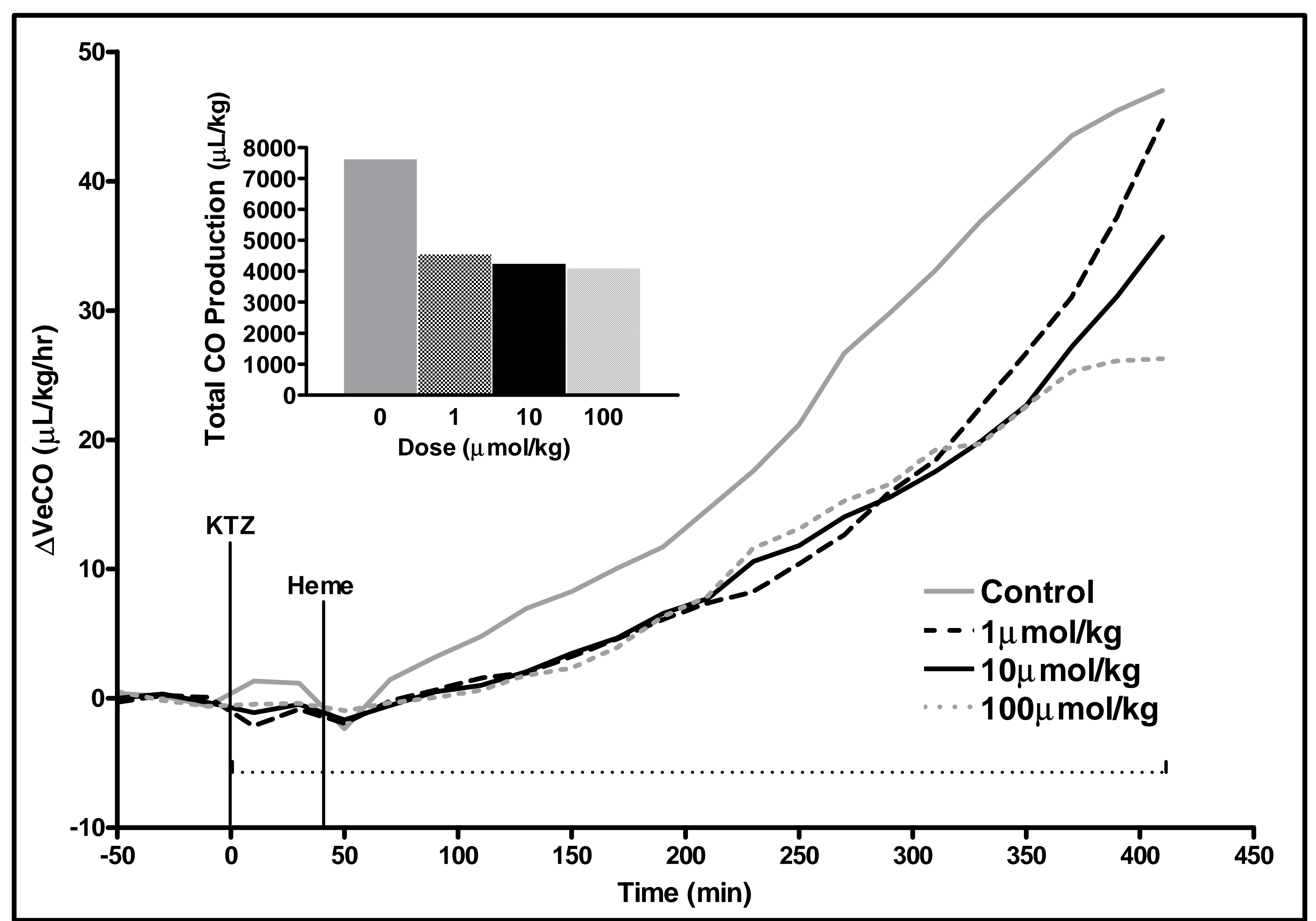

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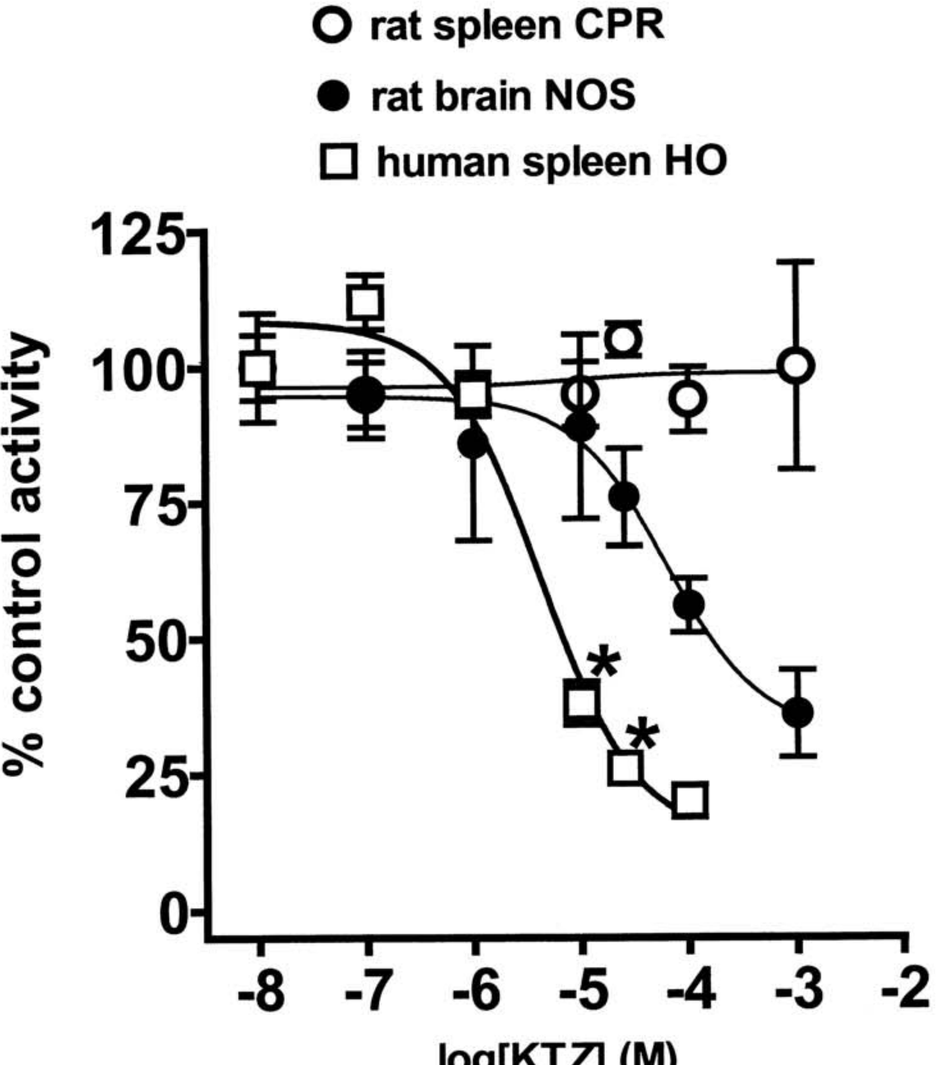

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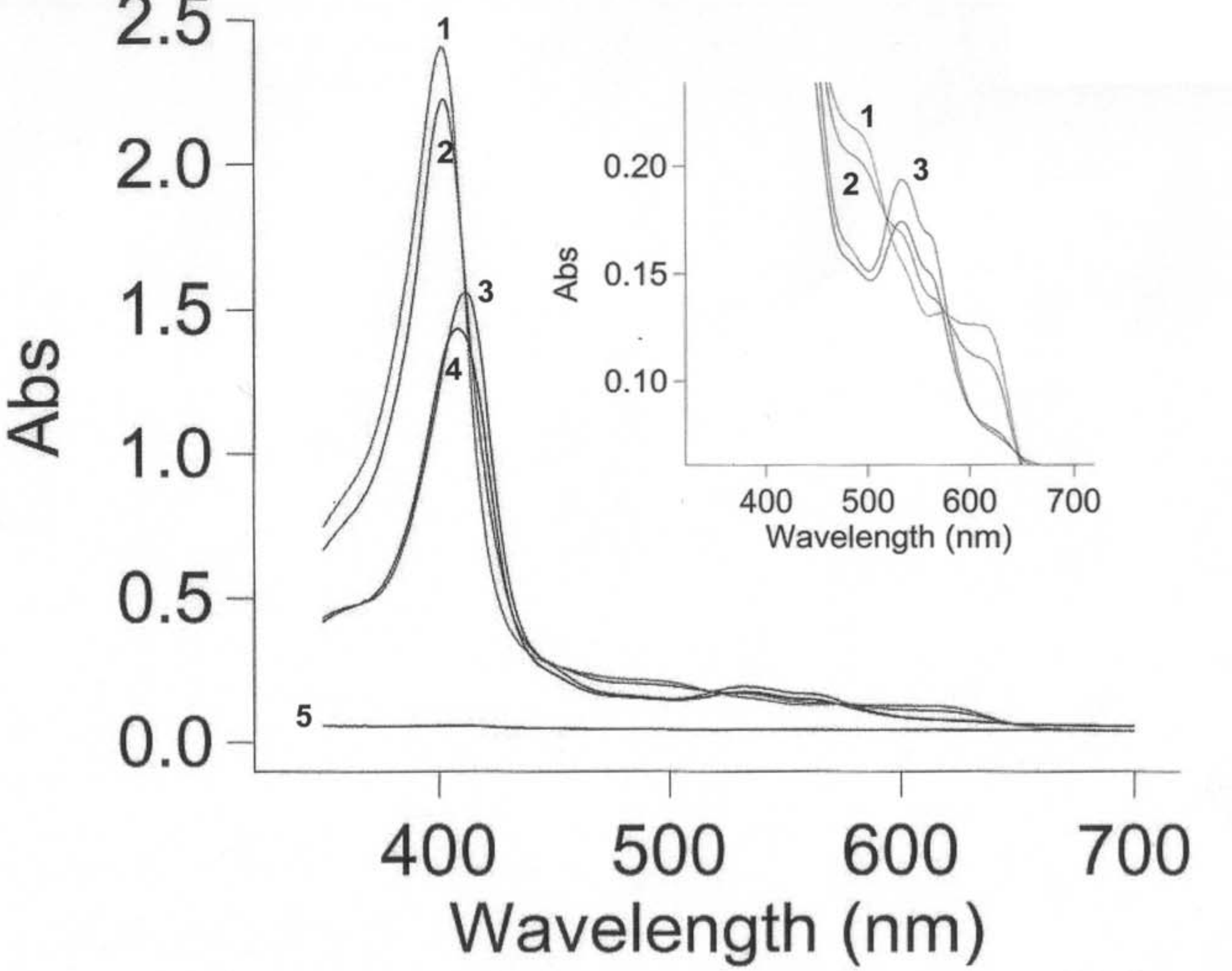

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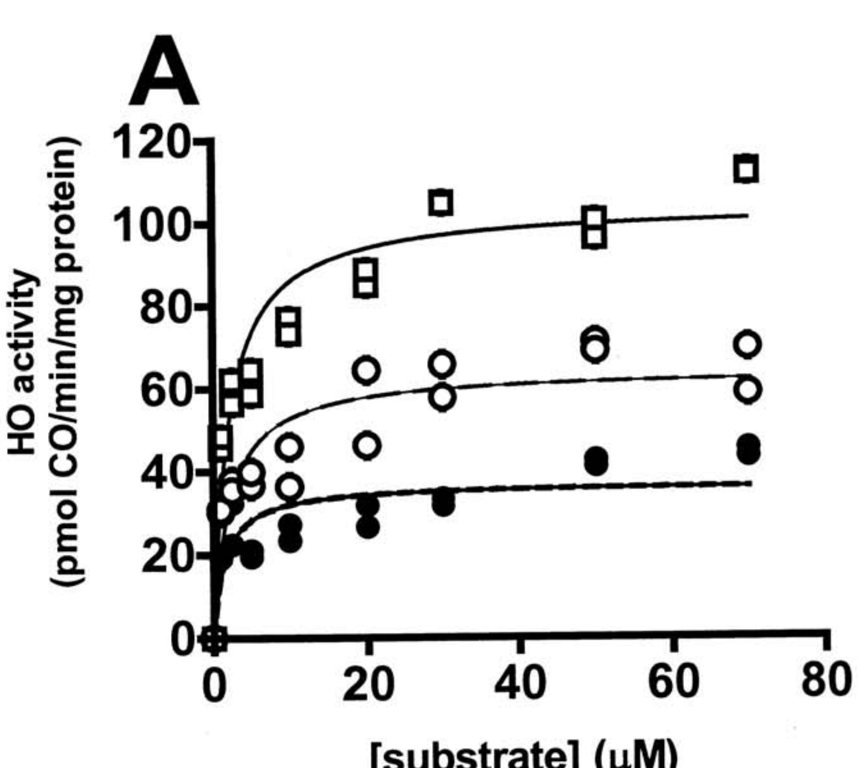

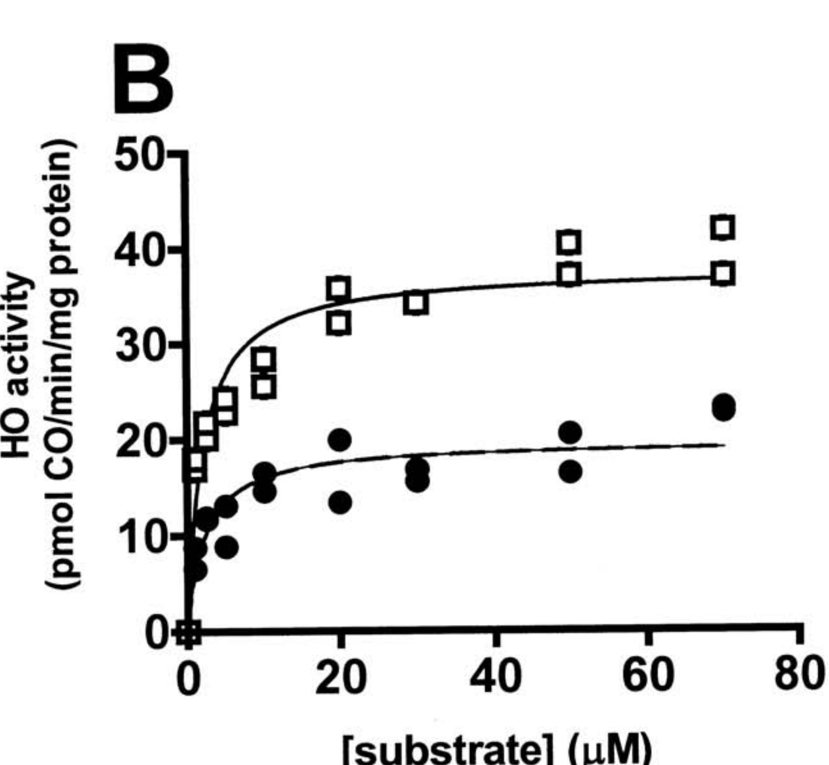

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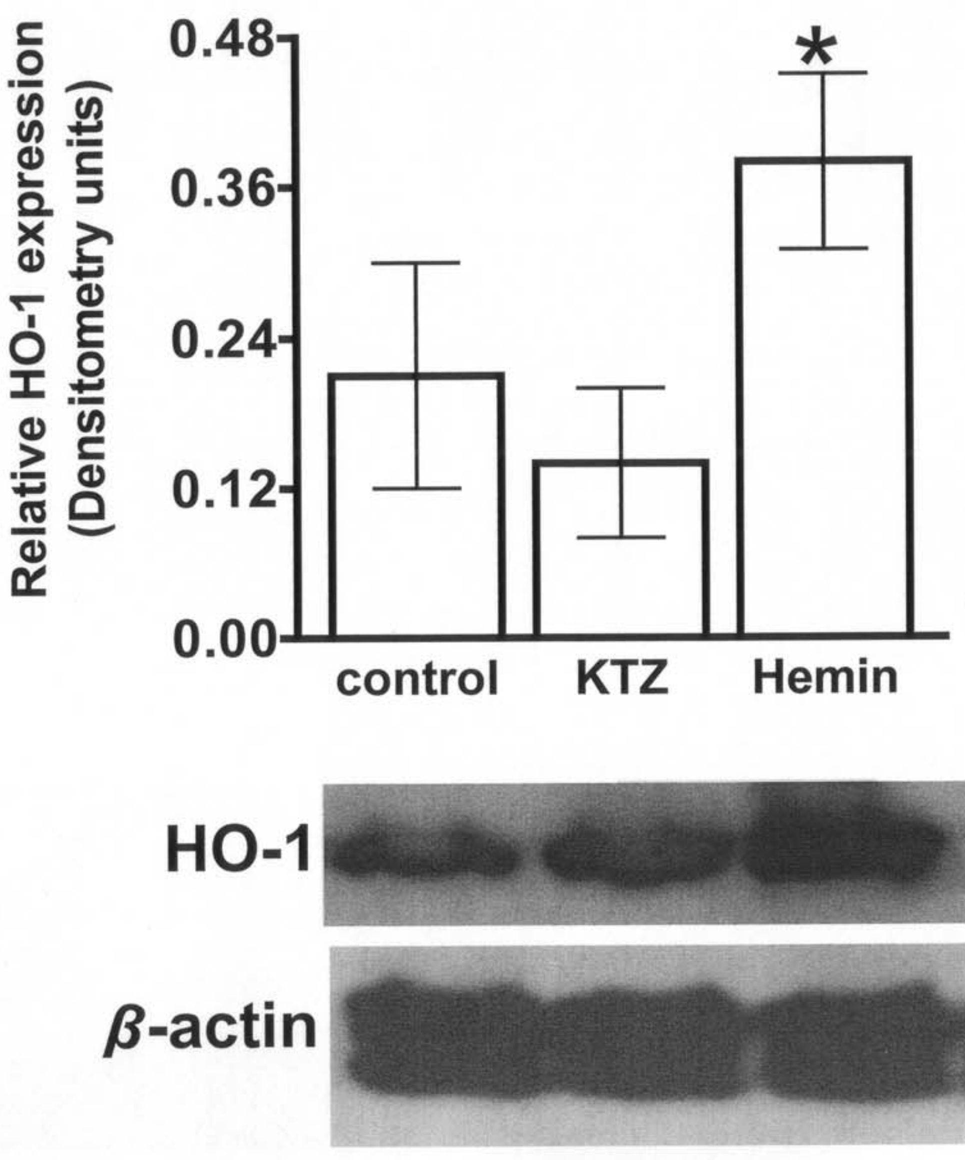

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