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Selectivity of imidazole–dioxolane compounds for *in vitro* inhibition of microsomal haem oxygenase isoforms

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1 Haem oxygenases (HO) are involved in the catalytic breakdown of haem to generate carbon monoxide (CO), iron and biliverdin. It is widely accepted that products of haem catabolism are involved in biological signaling in many physiological processes. Conclusions to most studies in this field have gained support from the judicious use of synthetic metalloporphyrins such as chromium mesoporphyrin (CrMP) to selectively inhibit HO. However, metalloporphyrins have also been found to inhibit other haem-dependent enzymes, such as nitric oxide synthase (NOS), cytochromes P-450 (*CYPs*) and soluble guanylyl cyclase (sGC), induce the expression of HO-1 or exhibit varied toxic effects.

2 To obviate some of these problems, we have been examining non-porphyrin HO inhibitors and the present study describes imidazole–dioxolane compounds with high selectivity for inhibition of HO-1 (rat spleen microsomes) compared to HO-2 (rat brain microsomes) *in vitro*. (2*R*,4*R*)-2-[2-(4-chlorophenyl)ethyl]-2-[(1*H*-imidazol-1-yl)methyl]-4-methyl-1,3-dioxolane hydrochloride) was identified as the most selective inhibitor with a concentration of 0.6 μM inhibiting HO-1(inducible) by 50% compared with 394 μM for HO-2 (constitutive).

3 These compounds were found to have no effects on the catalytic activities of rat brain NOS and lung sGC, but were potent inhibitors of microsomal *CYP*2E1 and *CYP*3A1/3A2 activities.

4 In conclusion, we have identified imidazole–dioxolanes that are able to inhibit microsomal HO *in vitro* with high selectivity for HO-1 compared to HO-2, and little or no effect on the activities of neuronal NOS and sGC. These molecules could be used to facilitate studies on the elucidation of physiological roles of HO/CO in biological systems.
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Abbreviations: CO, carbon monoxide; CrMP, chromium mesoporphyrin IX chloride; *CYP*, cytochromes P450; GTP, guanosine triphosphate; HEPES, 1-piperazineethane sulphonic acid, 4-(2-hydroxyethyl)-monosodium salt; HO, haem oxygenase; L-NAME, *N*-nitro-L-arginine methyl ester; NMR, nuclear magnetic resonance; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PMSF, phenylmethylsulphonyl fluoride; sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; SnPP, tin protoporphyrin IX; ZnPP, zinc protoporphyrin IX

Introduction

In mammals, carbon monoxide (CO) is formed mainly *via* the action of haem oxygenase (HO) on its substrate, haem (Maines, 1997; Abraham & Kappas, 2005). Under normal physiological conditions, some 85% of the CO produced by humans derives from haem, while the remainder arises through processes that do not involve haem, such as lipid peroxidation (Vreman *et al*., 1998). While the majority of haem oxidation takes place in the liver and spleen during the housekeeping degradation of haem, the formation of CO from haem occurs in significant quantities in other structures such as blood vessels, kidneys and the brain. In fact, the brain, testes and spleen are organs with some of the highest specific activities of HO in the body (Maines, 1988). HO activity is attributable to two isozymes, HO-1 (molecular weight $\sim$32 kDa, a stress protein induced by a number of stimuli including heat shock, heavy metals, haem, and reactive oxygen species) and HO-2 (molecular weight $\sim$36.5 kDa and constitutive). Tissues such as spleen, which are rich in reticuloendothelial cells, are thought to contain maximally upregulated levels of HO-1 under physiological conditions, while the hippocampus and vascular endothelial cells possess its counterpart HO-2.

A substantial portion of our present comprehension of the role of CO and HO is a result of the judicious use of drugs, especially the HO inhibitors. To date, virtually all such studies have exploited competitive HO inhibitors of the metalloporphyrin class, such as tin protoporphyrin (SnPP) (Vreman *et al*., 1993; Johnson *et al*., 1997; Tulis *et al*., 2001). The close structural similarity between the HO substrate, haem, and the metalloporphyrin HO inhibitors may contribute to a problem of lack of selectivity for HO. Haem plays an important role in many biologically relevant molecules, where it functions in the active site of enzymes or as a regulatory prosthetic group.

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Examples of the former would be cytochromes P450 (*CYPs*) and nitric oxide synthase (NOS), and an example of the latter would be soluble guanylyl cyclase (sGC). The use of metalloporphyrins has been criticised because of their ability to inhibit enzymes other than HO, leading some investigators to doubt the validity of the interpretation of the results obtained using these drugs (Luo & Vincent, 1994; Meffert *et al*., 1994). Moreover, Trakshel *et al*. (1992) have reported that *in vivo* treatment of rats with SnPP inhibited a number of adrenal *CYP* activities; similarly, Hajdena-Dawson *et al*. (2003) found that zinc protoporphyrin (ZnPP) strongly induced transcription of HO-1 in cell cultures. At the same time, other investigators have argued that these molecules are useful if they are used cautiously with great attention to the concentration or doses used (Zakhary *et al*., 1997).

Our laboratories have contributed to this debate by comparing the potency of various metalloporphyrins on HO, sGC and NOS. Thus, Appleton *et al*. (1999) found that chromium mesoporphyrin (CrMP) was one of the more useful drugs in broken cell preparations where a 10 μM concentration inhibited HO by 90%, with little or no effect on sGC or NOS; at a concentration of 100 μM, CrMP inhibited NOS by 60%. It was concluded that metalloporphyrin inhibitors of HO can be selective, and useful, when used at the appropriate concentrations. Nevertheless, the range of concentrations in which selectivity is observed is limited; this proscribes the utility of the metalloporphyrins and raises uncertainty in the interpretation of data. Furthermore, some metalloporphyrins have been reported also to be photosensitizers (Keino *et al*., 1990), capable of generating CO (Vreman *et al*., 1990) and potent inhibitors of lipid peroxidation (Day *et al*., 1999). If these drugs are employed in experiments involving experimental models at higher levels of integration such as cultured cells or intact animals, uncertainty is introduced because the concentration of the drug at the location of a given enzyme cannot be known with certainty as in the case of broken cell preparations. Even with these caveats, the metalloporphyrins have been depended upon for the elucidation of the function of HO. For example, it is quite widely accepted that the HO/CO system plays a regulatory role in the perfusion of the human placenta. This acceptance is based substantially on the observations that SnPP, an HO inhibitor, increased perfusion pressure in a concentration-dependent manner between 35 and 350 μM (Lyall *et al*., 2000). In recognition of the other pharmacological activities of the metalloporphyrins, NOS and cyclooxygenase activities were blocked with L-NAME (*N*-nitro-L-arginine methyl ester) and meclofenamate, respectively. Nevertheless, there are still significant concerns about the use of the metalloporphyrin HO inhibitors and the interpretations based on their use (Cary & Marletta, 2001).

To increase the validity of the observations and concepts in this field, it is important to have available more selective HO inhibitors that can be exploited to generate data whose interpretation is not complicated by the confounding effects of the metalloporphyrins. This has led us to focus on molecules that are structurally dissimilar to haem, and our working hypothesis is that non-porphyrin inhibitors of HO, which have minimal effects on NOS and sGC, can be identified. DeNagel *et al*. (1998) first reported that azalanstat, an imidazole–dioxolane compound designed for the inhibition of mammalian lanosterol 14α-demethylase (Walker *et al*., 1993), inhibited the activity of HO-1. Subsequently, Vreman *et al*. (2002) reported on azalanstat as a potent inhibitor of HO-1 and HO-2 in tissues of newborn and adult mice. Using azalanstat as a lead compound, we have reported on the synthesis and initial screening of the HO-1 and HO-2 isozyme inhibitory activity of several analogues with chemical structures which are quite different from those of the metalloporphyrins (Vlahakis *et al*., 2005). The present communication describes studies on the selectivity of these imidazole–dioxolane compounds as inhibitors of the enzymatic activity of HO-1 and HO-2 compared with NOS, sGC and selected *CYP*s.

Methods

Animals

Brain, liver, lung and spleen tissue were obtained from adult male Sprague–Dawley rats (250–300 g) purchased from Charles River Inc. (Montreal, QC, Canada). Rats were maintained on 12 h light cycles and *ad libitum* access to water and standard Ralston Purina laboratory chow 5001 (Ren's Feed Supplies, Ltd, Oakville, ON, Canada). All 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.

Human spleen tissue

Human spleen tissue was obtained as surgical waste from Kingston General Hospital (Kingston, ON, Canada). Segments of 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 prior to use.

Preparation of brain, spleen and liver microsomal fractions

Brain, liver and spleen microsomal fractions were prepared for HO and *CYP* activity assays according to previously described procedures (Appleton *et al*., 1999). Briefly, tissue homogenate (15% w v^{-1}) was prepared in ice-cold buffer (20 mM KH_2PO_4, 135 mM KCl and 0.1 mM EDTA, pH 7.4) using a 60S Sonic Dismembrator (Fisher Scientific Ltd, Ottawa, ON, Canada). Microsomal fractions were obtained by differential centrifugation of the homogenate at $10{,}000 \times g$ for 20 min at 4°C, followed by centrifugation of the supernatant at $100{,}000 \times g$ for 60 min at 4°C. Microsomes ($100{,}000 \times g$ pellet) were resuspended in buffer (100 mM KH_2PO_4, 20% v v^{-1} glycerol and 1 mM EDTA adjusted to pH 7.4) and then stored at –80°C until used. Spleen microsomes were used as a source of HO-1 (Braggins *et al*., 1986; Maines, 1988), while brain microsomes were used as a source of HO-2 (Trakshel *et al*., 1988).

Preparation of brain and lung cytosolic fractions

Cytosolic fractions for rat brain and lung tissue were prepared for measuring NOS and soluble guanylyl cyclase (sGC) activities, respectively. Whole brains from five rats were pooled and homogenised in ice-cold buffer (50 mM HEPES (1-piperazineethane sulphonic acid, 4-(2-hydroxyethyl)-monosodium salt (HEPES)), 1 mM EDTA and 10 μg ml^{-1} leupeptin,

pH 7.4) according to the procedure outlined by Appleton *et al*. (1999). For sGC activity assays, rats were pretreated with heparin (3 mg kg^{-1} for 60 min), anaesthetised with halothane and then killed by decapitation. Lungs were perfused with phosphate-buffered saline, excised and then homogenised in buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM benzamidine HCl and 1 mM PMSF. Cytosolic fractions were prepared by differential centrifugation of the homogenate as described for microsomal preparation above. Protein concentration of all the cytosolic and microsomal fractions was determined by a modification of the biuret method as described by Marks *et al*. (1997).

Measurement of HO-1 and HO-2 protein expression

In total, 40 μg of rat spleen and brain tissue homogenate protein (10,000 $\times$ *g* supernatant fraction) were subjected to sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, and then the protein was transferred onto nitrocellulose Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) according to the method of Laemmli (1970). To block nonspecific binding sites, membranes were incubated in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 10% (w v^{-1}) skimmed milk powder at 4°C for 16–18 h. The blots were then incubated with a 1 : 2000 dilution of the polyclonal anti-human HO-1 (SPA-896, StressGen, Victoria, BC, Canada) or anti-human HO-2 (SPA-897, StressGen) antibodies. The specificity of anti-HO antibodies under these conditions was confirmed previously (Lash *et al*., 2003). Membranes were subsequently incubated with a peroxidase-labeled goat anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, U.S.A.). Peroxidase activity was detected by enhanced chemiluminescence detection kit according to the manufacturer's instructions (Amersham, Toronto, ON, Canada). All gels were calibrated with prestained, broad-range molecular weight markers (Bio-Rad, Hercules, CA, U.S.A.). Rat recombinant human HO-1 (SPP 730) or HO-2 (NSP-550, StressGen) were also used as standard markers. Relative HO-1 and HO-2 expression was quantified by optical densitometry using an NIH imager. To ensure uniform protein loading on all the gels, membranes that were used for HO quantification were stripped in buffer (200 mM glycine, pH 2.6), blocked as described above and then probed with a mouse antibody against β-actin. Densitometric units for HO-1 and HO-2 expression were normalised to β-actin protein expression in all the samples.

Measurement of HO enzymatic activity

HO activity in rat spleen and brain microsomal fractions was determined by the quantitation of CO formed from the degradation of methemalbumin, that is, haem complexed with albumin according to Vreman & Stevenson (1999) and Cook *et al*. (1995). Incubations for HO activity analysis were carried out under conditions for which the rate of CO formation (pmol CO mg protein^{-1} min^{-1}) was linear with respect to time and microsomal protein concentration. Briefly, reaction mixtures (150 μl) consisting of 100 mM phosphate buffer (pH 7.4), 50 μM methemalbumin and 1 mg ml^{-1} protein were preincubated with the vehicle (ethanol or water in which the inhibitors were dissolved), or inhibitors at final concentrations ranging from 0.1 to 1000 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 pulverised dry ice and CO formation was measured by gas chromatography using a TA 3000R Process Gas Analyzer (Trace Analytical/Ametek, Newark, DE, U.S.A.).

Measurement of NOS enzymatic activity in rat brain cytosolic fraction

A radiometric assay monitoring the conversion of ^{14}C-L-arginine into ^{14}C-L-citrulline was used to assess the effect of novel HO inhibitors on the catalytic activity of NOS in rat brain cytosolic fractions 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^{-1} cytosolic protein in a total volume of 200 μl. Stock solutions of the different HO inhibitors and L-NAME, a selective NOS inhibitor, were prepared in water, ethanol or 1% aqueous ethanolamine and were used at final concentrations in the range of 1–1000 μM. Control reactions for each inhibitor contained equivalent amounts of the vehicle in which the inhibitor was dissolved and the total organic solvent concentration was maintained at $\leqslant$1% (v v^{-1}) of the final volume in all cases. Samples were preincubated for 10 min at 37°C and 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 d.p.m. ^{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 nmol ^{14}C-L-citrulline formed mg protein^{-1} h^{-1}.

Measurement of sGC catalytic activity in rat lung cytosolic fraction

sGC activity in rat lung cytosolic fraction was measured following the formation of cGMP in reaction mixtures containing 50 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 2 mM 3-isobutyl-1-methylxanthine, 1 mg ml^{-1} BSA, 1 mM L-cysteine, 2 mg ml^{-1} cytosolic protein, 100 μM SNP and 200 μM GTP in a total volume of 1 ml. Novel HO inhibitors or ODQ, a selective sGC inhibitor, were added to the reaction mixture at final concentrations in the range of 1–1000 μM. Control reactions contained an equivalent amount of vehicle in which the inhibitor was dissolved. Incubations were carried out for 30 min at 37°C in a shaking water bath. Under these conditions, cGMP formation was found to be linear with respect to time and cytosolic protein. Reactions were stopped with an equal volume of 125 mM zinc acetate/125 mM sodium carbonate solution, samples were centrifuged at 12,000 $\times$ *g* for 10 min at 4°C. cGMP in the supernatant was quantified by competitive enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A.) according to the manufacturer's instructions. sGC activity was expressed as pmol of cGMP formed mg protein^{-1} min^{-1}.

Measurement of CYP*2E1 and* CYP*3A1/3A2 enzymatic activity*

To determine the *in vitro CYP*2E1 and *CYP*3A1/3A2 catalytic activities, untreated and dexamethasone-treated Sprague–Dawley rats were used, respectively. Dexamethasone was dissolved in DMSO and animals were treated i.p. ($5\,mg\,kg\,day^{-1}$ for 4 days). The animals were killed, the livers were perfused and microsomes were prepared according to procedures described above. *CYP*3A1/3A2-catalysed erythromycin *N*-demethylase activity was determined by the spectrophotometric measurement of formaldehyde according to previously published methods (Nash, 1953; Kim *et al*., 2001), whereas the hydroxylation of *p*-nitrophenol by *CYP*2E1 was determined by the spectrophotometric measurement of 4-nitrocatechol (Koop, 1986). The effects of HO inhibitors on *CYP*2E1 and *CYP*3A1/3A2 activities were tested at concentrations in the range of 0.1–100 μM.

Data analysis

Inhibition of the catalytic activities of HO, NOS, sGC, *CYP*2E1 and *CYP*3A1/3A2A by azalanstat and its structural analogues (compounds II and III) 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 GraphPad Prism version 3. Data are presented as the mean $\pm$ s.d. from triplicate experiments. Statistical analyses to compare IC_{50} values were performed by one-way ANOVA. *P*-values $\leqslant 0.05$ were considered to be statistically significant. To express the relatively greater effect on HO-1 compared with HO-2, we calculated a selectivity index (IC_{50} rat brain/IC_{50} rat spleen).

Materials

Azalanstat (compound I) (2*S*,4*S*)-2-[2-(4-chlorophenyl)ethyl]-2-[(1*H*-imidazol-1-yl)methyl]-4-[{(4-aminophenyl)thio}methyl]-1,3-dioxolane dihydrochloride) and its analogues (compounds II, (2*R*,4*R*)-2-[2-(4-chlorophenyl)ethyl]-2-[(1*H*-imidazol-1-yl)-methyl]-4-methyl-1,3-dioxolane hydrochloride and III, 2-[2-(4-chlorophenyl)ethyl]-2-[(1*H*-imidazol1-yl)methyl]-1,3-dioxolane hydrochloride) were synthesised and then characterised by elemental analysis, mass spectrometry and nuclear magnetic resonance spectroscopy according to Vlahakis *et al*. (2005 and unpublished observations). Dexamethasone, 1-piperazineethane sulphonic acid, HEPES, 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), *p*-nitrophenol, sodium nitroprusside, *N*-nitro-L-arginine methyl ester (L-NAME), cyclic guanosine monophosphate (cGMP), guanosine triphosphate (GTP), L-arginine, L-citrulline, Amberlite IPR-69 column chromatography resin, ethylenediamine tetra-acetic acid disodium (EDTA), phenylmethylsulphonyl fluoride (PMSF), benzamidine HCl, 3-isobutyl-1-methylxanthine, heparin, leupeptin, hemin, ethanolamine, bovine serum albumin and reduced β-nicotinamide adenine dinucleotide were obtained from Sigma Chemical Company (St Louis, MO, U.S.A.). ^{14}C-L-arginine ($320\,mCi\,mmol^{-1}$) and ^{14}C-L-citrulline ($58.8\,mCi\,mmol^{-1}$) were purchased from Mandel/New England Nuclear (Guelph, ON, Canada). All other chemicals were at least reagent grade and were obtained from BDH (Toronto, ON, Canada).

Results

Relative expression of HO-1 and HO-2 in rat spleen and brain microsomes

HO protein in brain tissue was predominantly attributable to HO-2 although the expression of HO-1 was detectable (Figure 1). The predominant isozyme in the spleen was HO-1 with very minimal expression of HO-2, and β-actin protein expression showed no variation in the rat spleen and brain tissue (Figure 1). These data are consistent with previous findings, which indicate that spleen and brain tissue from untreated adult rats may serve as a native source of mammalian HO-1 (Braggins *et al*., 1986; Maines, 1988) and HO-2 (Trakshel *et al*., 1988), respectively.

Inhibition of in vitro *HO activity by imidazole–dioxolane compounds*

Using human and rat spleen tissue as a source of HO-1 and rat brain as a source of HO-2, imidazole–dioxolanes compounds I, II and III, and CrMP were studied for the *in vitro* inhibition of

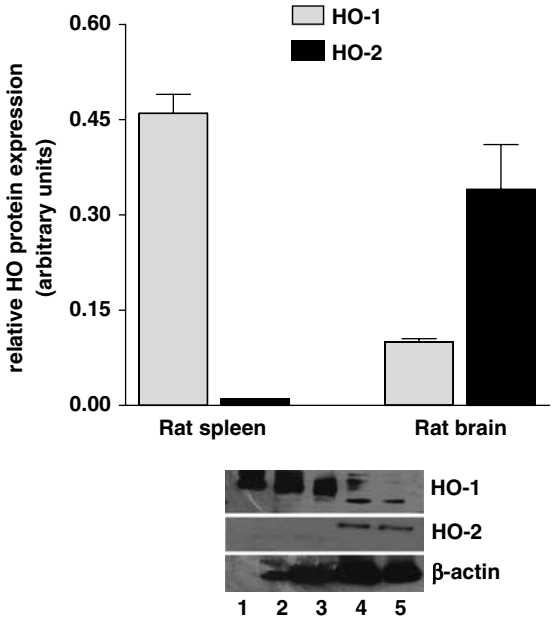
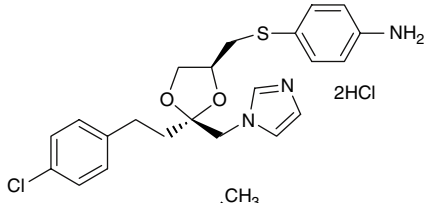
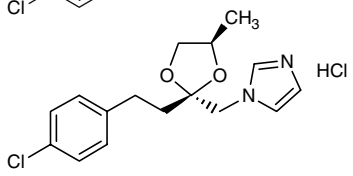
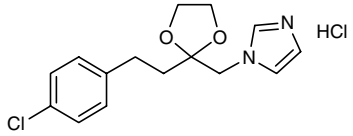
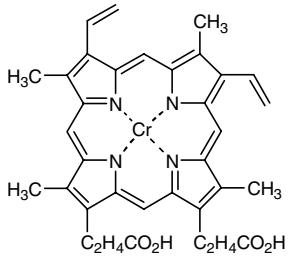


Figure 1 Relative HO-1 and HO-2 protein expression in rat spleen and brain tissue. In total, 40 μg of $10{,}000 \times g$ supernatant protein were subjected to SDS–PAGE and then probed with polyclonal anti-human HO-1 and anti-human HO-2 antibodies. Membranes were subsequently incubated with a peroxidase-labelled goat anti-rabbit IgG secondary antibody. Peroxidase activity was detected by enhanced chemiluminescence. Protein loading on gels was normalised to the expression of β-actin and the relative HO-1 and HO-2 expression was quantified by optical densitometry using an NIH imager. Data represent the mean of two different experiments and lanes on the blots are represented as follows: lane 1, recombinant human HO-1; lanes 2–3, rat spleen homogenate; lanes 4–5, rat brain homogenate.

Table 1 Inhibitory potency of compounds I, II and III, and CrMP against HO-1 (spleen mirosomes) and HO-2 (brain microsomes) activity *in vitro*

Code	*Chemical structure*	*Rat spleen*	*Rat brain*	*Selectivity index (IC_{50} rat brain/IC_{50} rat spleen)*
I		5.8±1.0	28.0±8.2	5
II		0.6±0.2	394.2±5.0	657
III		2.8±1.3	>100	
CrMP		1.4±0.3	0.7±0.4	0.5

HO-1 and HO-2 activities, respectively (Table 1). CrMP inhibited HO activity in all the tissue microsomes examined with similar inhibition potency for HO-1 (rat and human spleen) and HO-2 (rat brain). The imidazole–dioxolane compounds, however, showed selectivity for the inhibition of HO-1 over HO-2. Compound II was the most selective inhibitor towards HO-1 with a selectivity index of 657, whereas the parent compound I had a selectivity index of 5. Similarly, compound III showed at least a 30-fold greater potency for the inhibition of HO-1 in comparison to HO-2. Total microsomal HO activity was generally higher in the spleen than the brain, and average activities in control reactions in the human spleen, rat spleen and rat brain microsomes were 63.2±8.5, 91.8±8.3 and 41.3±7.5 pmol CO mg protein^{-1} min^{-1}, respectively.

Effect of imidazole–dioxolane-based HO inhibitors on NOS, sGC, CYP*3A1/3A2 and* CYP*2E1 activities*

Since metalloporphyrin HO inhibitors have been reported to inhibit hemoprotein enzymes, the effects of the imidazole–dioxolane compounds on *in vitro* catalytic activities of other haem-dependent enzymes such as NOS, sGC and selected *CYP*s were examined. It was found that for inhibitor concentrations ranging from 1 to 1000 μM, compound I did not alter NOS activity, whereas both HO-1 and HO-2 activities were nearly completely inhibited by a concentration of 100 μM (Figure 2). For compounds II and III at concentrations ranging from 1 to 100 μM, substantial inhibition of HO-1 was observed without any significant effect on HO-2 and NOS activities ($P>0.01$) (Figure 2b and Figure 2c). The IC_{50} values for inhibition of NOS by CrMP, compound II and compound III were 7.8±0.1, 439±3, and 584±34 μM, respectively. Consistent with the work of Appleton *et al.* (1999), we have also shown that CrMP is a potent HO-1 and HO-2 inhibitor with a limited concentration range (1–10 μM) which does not affect NOS activity *in vitro* (Figure 2d). A similar trend was observed in the effects of these imidazole–dioxolane-based HO inhibitors and CrMP on sGC activity *in vitro* (Figure 3). sGC activity was not affected by compounds I, II and III at concentration ranging from 1 to 1000 μM. At 100 μM, however, CrMP inhibited more than 50% of the *in vitro* sGC activity, whereas concentrations as high as 1000 μM led to a total loss of sGC activity.

Contrary to the observed effects on NOS and sGC activities *in vitro*, the imidazole–dioxolanes studied herein were found to be potent *CYP*3A1/3A2 (Figure 4) and *CYP*2E1 (Figure 5) inhibitors. The IC_{50} values for the inhibition of *CYP*3A1/3A2 by compounds II (3.1±0.5 μM, $n=3$) and III (2.1±0.3 μM, $n=3$) were significantly lower than that of compound I (48±0.2 μM, $n=3$) ($P<0.05$). Generally, the catalytic activity of *CYP*2E1 was inhibited to a greater extent than that of *CYP*3A1/3A2 by compound II. The IC_{50} values for the inhibition of *CYP*2E1 by compounds II and III were (1.8±0.2 μM, $n=3$) and (4.2±0.01 μM, $n=3$), respectively. Compound I had no effect on *CYP*2E1 activity at concentration ranging from 1 to 1000 μM.

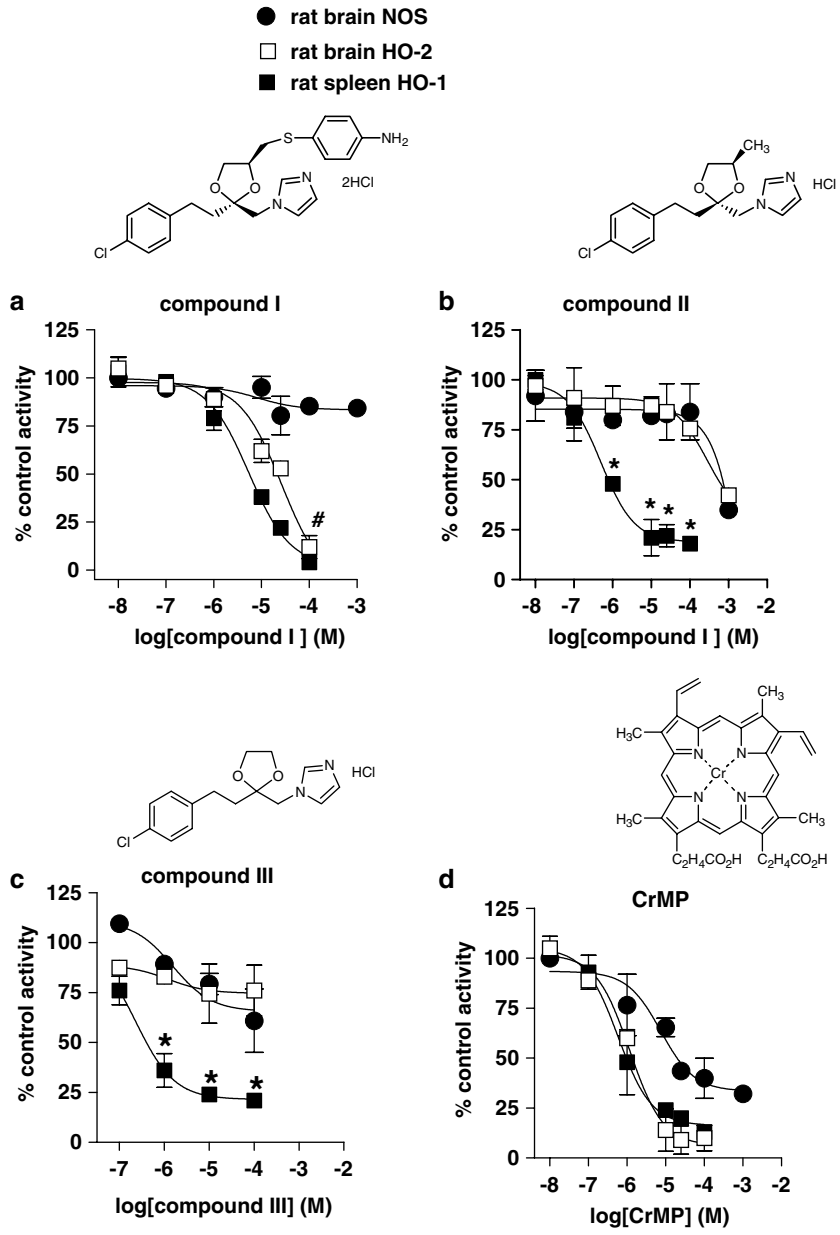


Figure 2 Effect of azalanstat (compound I) (a), compound II (b), compound III (c) and CrMP (d) on the *in vitro* NOS, HO-1 and HO-2 activities. HO and NOS catalytic activities were determined as outlined in the Methods section above. Average NOS, HO-1 and HO-2 activities in control reactions were 12.1 ± 0.6 nmol ^{14}C-L-citrulline formed mg protein^{-1} h^{-1}, 91.8 ± 8.3 and 41 ± 7.5 pmol CO mg protein^{-1}min^{-1}, respectively. The IC_{50} values for inhibition of NOS by CrMP, compound II and compound III were 7.8 ± 0.1, 439 ± 3, and $584 \pm 34\,\mu$M, respectively. Data represent the mean $\pm$ s.d. of three experiments. Asterisks (*) indicate concentrations of compound II and compound III that caused significant inhibition of HO-1 activity without any significant effect on HO-2 and NOS activities $P < 0.001$, while (#)denotes concentrations of azalanstat (compound I) that caused significant inhibition of both HO-1 and HO-2 with no significant effect on NOS $P < 0.001$.

Discussion

As mentioned above, we articulated the working hypothesis for this research 'non-porphyrin inhibitors of HO that have minimal effects on NOS and sGC can be identified'. To this end, the major findings are: the verification of non-porphyrin compounds that inhibit HO activity; the inability of these compounds to inhibit rat brain NOS and rat lung sGC; their ability to inhibit some cytochromes P450 and the ability of some compounds to distinguish between HO-1 and HO-2.

In order to test the hypothesis, we evaluated a number of imidazole–dioxolanes for their ability to inhibit HO activity in rat spleen (HO-1) and brain (HO-2) microsomes. As we looked ahead to *in vivo* studies, we chose these sources of HO instead of purified, soluble preparations because they should behave more like the enzymes *in vivo*. For the present study, three imidazole–dioxolanes were examined in comparison with a representative metalloporphyrin to illustrate their potential as tools in the investigation of CO/HO function. As mentioned above, the use of metalloporphyrins to elucidate the functional

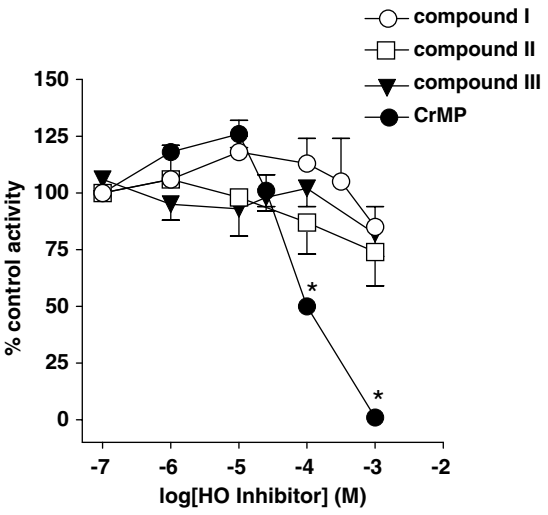


Figure 3 Effect of compounds I, II and III, and CrMP on sGC activity *in vitro*. sGC activity was determined as outlined in the Methods section above. Average sGC activity in control reactions was 1.05 ± 0.14 pmol cGMP mg protein^{-1} min^{-1}. The IC_{50} values for inhibition of sGC by compounds I, II and III were higher than 1000 μM, but 100 μM for CrMP. Data represent the mean $\pm$ s.d. of three experiments. Asterisks (*) indicate concentrations of CrMP that caused significant inhibition of sGC activity, $P < 0.05$.

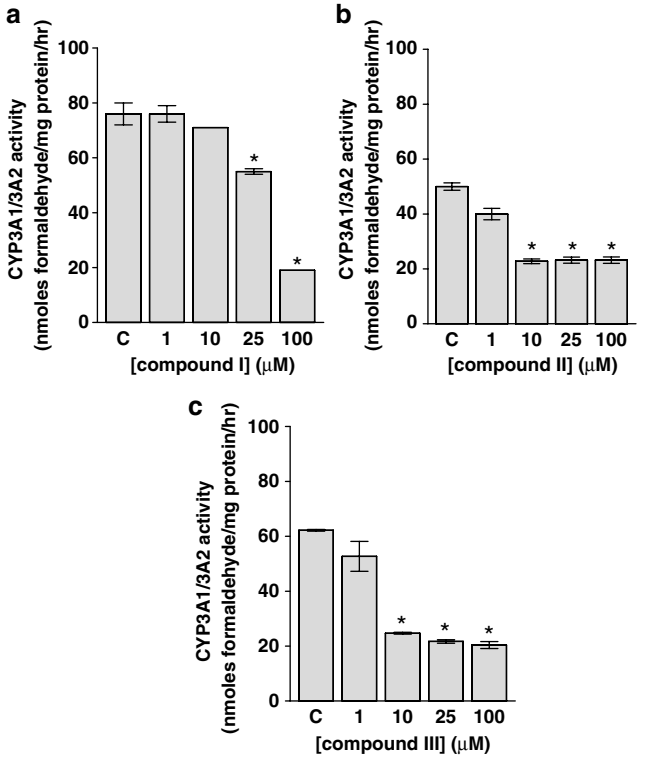


Figure 4 Effect of compounds I (a), II (b) and III (c) on the *in vitro* *CYP*3A1/3A2 activity. *CYP*3A1/3A2 activity was determined from erythromycin *N*-demethylase as outlined in the Methods section above. Average activity in control reactions was 62.5 ± 9 nmol formaldehyde mg protein^{-1} h^{-1}, $n = 5$. The IC_{50} values for inhibition of *CYP*3A1/3A2 by compounds I, II and III were 48.1 ± 0.2, 3.1 ± 0.5 and 2.1 ± 0.3 μM, respectively. Data represent the mean $\pm$ s.d. of three experiments. Asterisks (*) indicate concentrations of compounds I, II and III that caused significant inhibition of *CYP*3A1/3A2 activity, $P < 0.05$.

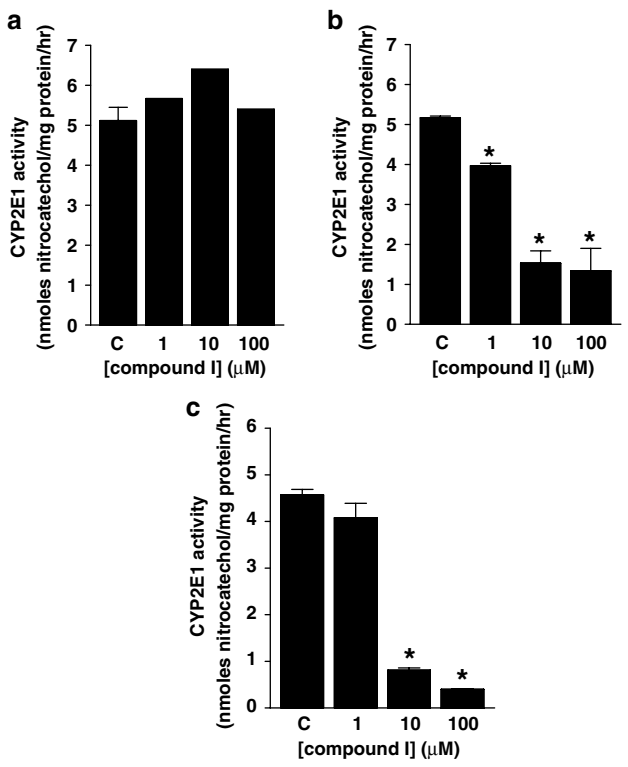


Figure 5 Effect of compounds I (a), II (b) and III (c) on the *in vitro* *CYP*2E1 catalytic activity. *CYP*2E1 activity was determined from the hydroxylation of *p*-nitrophenol by rat liver microsomes as outlined in the Methods section above. Average activity in control reactions was 6.8 ± 2.0 nmol 4-nitrocatechol mg protein^{-1} h, $n = 4$. The IC_{50} values for inhibition of *CYP*2E1 by compounds II and III were 1.8 ± 0.2 and 4.2 ± 0.01 μM, respectively. Compound I had no effect on *CYP*2E1 activity at all concentrations tested. Data represent the mean $\pm$ s.d. of three experiments. Asterisks (*) indicate concentrations of compounds II and III that caused significant inhibition of *CYP*3A1/3A2 activity, $P < 0.05$.

roles of the CO/HO system is compromised by their effects on NOS and sGC activity. The present results indicate that this problem is obviated when the imidazole–dioxolanes were used because they did not inhibit rat brain NOS or rat lung sGC activity. These studies to address the question of their effect against NOS were conducted using rat brain (NOS). Although the effects of these imidazole–dioxolanes on other forms of NOS (endothelial and inducible) was not examined, the data presented here allows us to make direct comparisons to our previous study on the selectivity of the metalloporphyrins (Appleton *et al*., 1999). In that study, we found that CrMP was selective for HO in a limited range of concentrations *in vitro*. In comparison, the present study revealed that imidazole–dioxolanes were much more selective. For example, the lead compound I (azalanstat) had IC_{50} values of 6 and 28 μM against rat HO-1 and HO-2, respectively; at concentrations as high as 1 mM, we were unable to detect any inhibition of NOS activity. Compound II was similarly effective against HO-1 with an IC_{50} of 0.6 μM, and no observable inhibition of NOS at 100 μM. To this point, the data support the hypothesis that these non-porphyrin HO inhibitors exhibited no propensity to inhibit NOS activity. Studies to investigate the mechanisms of inhibition of HO by the imidazole–dioxolanes are being undertaken but most of the preliminary data are not consistent with a simple

competitive inhibition and favour an irreversible, noncompetitive mode of inhibition, as has been observed by DeNagel *et al*. (1998).

In a parallel set of experiments, it was observed that the present imidazole–dioxolane HO inhibitors also lacked the ability to inhibit rat lung sGC activity. Thus, compound I had no effect on sGC at concentrations as high as 1000 μM. Similarly, compound II was observed not to inhibit sGC. Accordingly, these observations lend further support to the hypothesis as these drugs did not inhibit sGC activity. Of the properties exhibited by the present compounds, their lack of effect on NOS and sGC activities may be the most useful in terms of investigating the CO/HO system as a signaling pathway. This is because this system shares a number of characteristics with the NO/NOS signaling pathway. For example, CO and NO generated from the enzymatic activity of HO and NOS, respectively, are known activators of sGC. Consequently, the production and release of either CO or NO may result in the elevation of intracellular cGMP concentration. As employed in experiments on vascular function, such as the cGMP-mediated dilation of blood vessels (Furchgott & Jothianandan, 1991), the limited selectivity of metalloporphyrins compromises our ability to differentiate among effects mediated through the CO/HO system, the NO/NOS system or sGC.

In contrast to the absence of significant inhibition of sGC and NOS by the imidazole–dioxolanes (compounds I, II and III), we found that *in vitro* *CYP*3A1/3A2 and *CYP*2E1 activities were inhibited by these drugs (Figures 4 and 5). Thus, the enzyme inhibitory activity of imidazole–dioxolanes was not specific for HO. As these compounds share some structural features with ketoconazole, which is a known inhibitor of *CYP*3A, the inhibition of *CYP*3A by the present compounds was not unexpected. Nevertheless, it is interesting that the catalytic activity of *CYP*2E1 was inhibited to a greater extent than that of *CYP*3A1/3A2. *CYP*3A1/3A2 were considered for this study because of their structural similarities with human *CYP*3A gene subfamily, which is responsible for the biotransformation of many drugs. The *CYP*2E gene subfamily, on the other hand, is equally important for metabolic clearance of xenobiotics, with comparable catalytic activity across a wide range of animal species. Thus, these *CYP* isoforms were selected as representative of some of the drug-metabolising *CYP* enzymes. Compounds II and III were more potent *CYP*3A1/3A2 inhibitors than was compound I. This indicates that the 4-aminophenylthio functional group of compound I has only a minimal role in *CYP*3A1/3A2 enzyme–inhibitor interaction. Interestingly, ketoconazole has a large functional group at the location of the 4-aminophenylthio moiety and is known to inhibit *CYP* and lanosterol demethylase; the latter is a *CYP* and has been reported to be inhibited by compound I (Walker *et al*., 1993). We are undertaking further structure–activity studies of the imidazole–dioxolane series of HO inhibitors, including many novel compounds, to identify molecules with improved selectivity.

In this context, the low potencies of compounds II and III towards inhibition of HO-2 activity compared with that of compound I are interesting. These observations are interpreted to mean that a specific pharmacophore in these imidazole–dioxolane compounds may account for the observed differences in HO protein–inhibitor interaction. Modification of compound I by removal (compound III) or replacement, with a methyl group (compound II), of the 4-aminophenylthio moiety led to more than a magnitude-fold increase in the selectivity for HO-1 inhibition. Therefore, it appears that the 4-aminophenylthio moiety in these compounds is important for the interaction with HO-2, but not HO-1. Collectively, these results emphasize the existence of structural differences in HO-1 and HO-2 isoforms, and how these differences may be exploited by using chemical effectors to study the different HO isozymes. Since HO-1 is inducible and its regulation is influenced by a multitude of physiological as well as pathological conditions, there is enormous research interest in this isozyme.

The ability of a number of the present imidazole–dioxolanes to inhibit HO-1 much more effectively than HO-2 will be of interest to investigators whose research focuses on HO-1. Thus, compound II was found to be approximately 600-fold more potent in inhibiting the inducible form of HO than the constitutive form. In the metalloporphyrin series of HO inhibitors, we did not observe such a degree of selectivity for inhibition of HO-1; the eight-fold selectivity that the metalloporphyrins exhibited was towards inhibition of HO-2 compared with HO-1 (Vreman *et al*., 1993; 1996; Wong *et al*., 2000).

One of the questions that interested us was the possibility of differential responses when these imidazole–dioxolanes were tested against HOs of different species. In spite of the interspecies differences in HO protein sequences and/or structures (Schacter *et al*., 1990; Rotenberg and Maines, 1991; Trakshel *et al*., 1991), the imidazole–dioxolane compounds studied herein showed similar effects on HO-1 obtained from rat (Table 1) and human spleen (data not shown). As a result, we are optimistic that these new drugs will find utility in studies of HO in other mammalian species so that the physiological function of the CO/HO system can be further elucidated.

In conclusion, we have identified imidazole–dioxolanes that are able to inhibit rat and human HO, as well as *CYP*3A1/3A2 and *CYP*2E1 activities, with little or no effect on rat brain NOS and rat lung sGC. Furthermore, a subset of these agents revealed high selectivity for inhibition of HO-1 compared to HO-2. We propose that these molecules may be used as important pharmacological tools to elucidate the physiology and pharmacology of HO-1 in mammals.

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