Canadian Journal of Physiology and Pharmacology
Revue canadienne de physiologie et pharmacologie

Differential inhibition of rat and mouse microsome heme oxygenase by derivatives of imidazole and benzimidazole

Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2017-0236.R1
Manuscript Type:	Article
Date Submitted by the Author:	05-May-2017
Complete List of Authors:	Hum, Maaïke; Queens University, Biomedical & Molecular Sciences McLaughlin, Brian; Queens University, Biomedical & Molecular Sciences Kong, Xianqi; Queens University, Chemistry Vlahakis, Jason; Queens University, Chemistry Vukomanovic, Dragic; Queens University, Biomedical & Molecular Sciences Szarek, Walter; Queens University, Chemistry Nakatsu, Kanji; Queens University, Biomedical & Molecular Sciences
Is the invited manuscript for consideration in a Special Issue?:	N/A
Keyword:	heme oxygenase, second-generation inhibitors, microsomes, rat, mouse

SCHOLARONE™
Manuscripts

**Differential inhibition of rat and mouse microsome heme oxygenase by
derivatives of imidazole and benzimidazole**

Maaïke Hum¹, Brian E. McLaughlin¹, Xianqi Kong², Jason Z. Vlahakis²,
Dragic Vukomanovic¹, Walter A. Szarek², and Kanji Nakatsu¹

Departments of Biomedical and Molecular Sciences¹ and Chemistry²,
Queen's University, Kingston, Ontario, Canada

Corresponding Author: Brian McLaughlin, Department of Biomedical and Molecular
Sciences, 18 Stuart Street, Queen's University, Kingston, ON, K7L 3N6, Canada;
Phone: 613-533-6107; Fax: 613-533-2022; E-mail: bm3@queensu.ca

Abstract

Metalloporphyrin, heme oxygenase (HO) inhibitors have made an important contribution to elucidating the role of HO in physiological processes. Nevertheless, their off-target effects have drawn substantial criticism, which prompted us to develop non-porphyrin, azole-based inhibitors of HO. These second-generation HO inhibitors were evaluated using spleen and brain microsomes from rats as native sources of HO-1 and HO-2, respectively. Recently, the use of azole-based inhibitors of HO has been extended to other mammalian species and, as a consequence, it will be important to characterize the inhibitors in these species. The goal of this study was to compare the inhibitory profile of imidazole- and benzimidazole-based inhibitors of HO in a breast cancer-implanted mouse to that of an untreated rat. For spleen and brain microsomes from both species, HO protein expression was determined by western blotting and concentration-response curves for imidazole- and benzimidazole-derivative inhibition of HO activity were determined using a headspace gas-chromatographic assay. It was found that the effects on HO activity by imidazole and benzimidazole derivatives were different between the two species and were not explained by differences in HO expression. Thus, the HO inhibitory profile should be determined for azole derivatives before they are used in mammalian species other than rats.

Key Words: Heme oxygenase; second-generation inhibitors; microsomes; rat; mouse.

Introduction

Heme oxygenase (HO; EC 1.14.14.18) activity is attributed to two genetically distinct isozymes, namely, HO-1 and HO-2 (Maines 1988). Both isozymes catabolize heme through a reaction that yields equimolar amounts of carbon monoxide (CO), ferrous iron and biliverdin, which is subsequently converted to bilirubin (Ryter et al. 2006). HO degradation of heme is the major endogenous source of CO (Maines 1988), which is recognized as a cellular signaling molecule with anti-inflammatory, anti-proliferative, anti-apoptotic and anti-coagulant properties (Wu and Wang 2005; Ryter et al. 2006).

Metalloporphyrin inhibitors of HO have made an important contribution to our current knowledge of the role of HO in physiological processes (Kinobe et al. 2008; Schulz et al. 2012). Nevertheless, various off-target effects of metalloporphyrins, including inhibition of nitric oxide synthase and soluble guanylyl cyclase, and induction of HO-1 (Grundemar and Ny 1997; Kinobe et al. 2008), prompted our research group to design non-porphyrin HO inhibitors based on the lead compound, azalanstat (DeNagel et al. 1998; Vreman et al. 2002). Accordingly, a series of imidazole derivatives were synthesized (Vlahakis et al. 2005; Vlahakis et al. 2006; Roman et al. 2007), followed by the synthesis of additional azole-based inhibitors of HO (Vlahakis et al. 2009; Roman et al. 2010a; Roman et al. 2010b; Vlahakis et al. 2012; Vlahakis et al. 2013). Selected imidazole derivatives had little or no effect on nitric oxide synthase or soluble guanylyl cyclase activity (Kinobe et al. 2006), did not induce the expression of HO-1 protein (Kinobe et al. 2008; Csongradi et al. 2010), and had a range of inhibitory effects on P450 activity (Hum et

al. 2010). Another research group has subsequently synthesized additional azole derivatives as HO inhibitors (Sorrenti et al. 2012; Pittalà et al. 2013; Salerno et al. 2013; Salerno et al. 2015).

These second-generation azole-based HO inhibitors were designed and characterized using spleen and brain microsomes from rats, as native sources of HO-1 and HO-2, respectively (Kinobe et al. 2006). Subsequently, selected imidazole derivatives have been used as research tools to study the physiological roles of HO in other mammalian species (Kinobe et al. 2007; Di Francesco et al. 2009; Csongradi et al. 2010; Csongradi et al. 2012) and tested as therapeutics in mouse and human cancer cell lines (Alaoui-Jamali et al. 2009; Salerno et al. 2013; Salerno et al. 2015). Interpretation of these data were made on the assumption that the azole-based HO inhibitors affected the HO isozymes of other mammals similarly to that of the rat.

The present study addresses this assumption by comparing the effects of selected imidazole and benzimidazole derivatives on the inhibition of HO activity in spleen and brain microsomes from a breast cancer-implanted mouse to those of an untreated rat. Chromium mesoporphyrin (CrMP) was included for comparison to a first-generation metalloporphyrin HO inhibitor. Based on the similarity of the amino acid sequence and the highly conserved catalytic core of HO proteins from rat and mouse (Rotenburg and Maines 1991; Matsumoto et al. 1996; Maines 1997), it was hypothesized that enzymatic activity of HO in spleen and brain microsomes from these two species would be similarly affected by azole-based inhibitors of HO.

Materials and Methods

Drugs and Solutions. The following compounds, QC-220, (1-(adamantan-1-yl)-2-(1*H*-imidazol-1-yl)ethane hydrochloride) (see description of synthesis below); QC-282, (1-[4-(benzyl)phenyl]-2-(1*H*-imidazol-1-yl)ethanone hydrochloride) (Roman et al. 2010b, compound 38); QC-291, (1-{[2-(naphthalen-2-yl)-1,3-dioxolan-2-yl]methyl}-1*H*-imidazole hydrochloride) (Roman et al., 2010b compound 80); QC-2350, (1-(2-phenylethyl)-2-(pyrrolidin-1-ylmethyl)-1*H*-benzimidazole dihydrochloride) (Vlahakis et al. 2013, compound 30); and QC-2356, (1-(3-nitrobenzyl)-2-(pyrrolidin-1-ylmethyl)-1*H*-benzimidazole dihydrochloride) (Vlahakis et al. 2013, compound 17) were synthesized and characterized by elemental analysis, mass spectrometry and nuclear magnetic resonance spectroscopy. See Fig. 1 for chemical structures of the non-selective inhibitor of HO isozymes, QC-282, the HO-1 selective inhibitors QC-220 and QC-291, and the HO-2 selective inhibitors QC-2350 and QC-2356. Ethylenediaminetetraacetic acid disodium salt (EDTA), hemin, bovine serum albumin (BSA), β -nicotinamide adenine dinucleotide 2'-phosphate tetrasodium salt (NADPH), Triton-X-100, sodium deoxycholate, Tris base, protease inhibitor cocktail and monoclonal anti- β -actin were obtained from Sigma-Aldrich Canada Co. (Toronto, Canada). CrMP IX was purchased from Frontier Scientific Inc. (Logan, USA) and isoflurane was acquired from Pharmaceutical Partners of Canada, Inc. (Richmond Hill, Canada). Anti-HO-1 (ADI-SPA-895) and anti-HO-2 (ADI-SPA-897) polyclonal antibodies were obtained from Enzo Life Sciences (Farmingdale, USA). All other chemicals were at least reagent grade and were purchased from Fisher Scientific (Ottawa, Canada). Stock solutions of methemalbumin

(1.5 mM hemin and 0.15 mM BSA) and of 1.5 mM CrMP were prepared as described previously (Appleton et al. 1999).

Synthesis of 1-(Adamantan-1-yl)-2-(1*H*-imidazol-1-yl)ethane hydrochloride

(QC-220). Under a nitrogen atmosphere, a sample of 1-(adamantan-1-yl)-2-(1*H*-imidazol-1-yl)ethanone (Rahman et al. 2008, compound 3) (451 mg, 1.85 mmol, 1 equiv) was combined with potassium hydroxide (1.24 g, 22.02 mmol, 12 equiv), ethylene glycol (3.7 mL, 4.12 g, 66.38 mmol, 36 equiv) and 98% hydrazine hydrate (1.1 mL, 1.12 g, 34.25 mmol, 19 equiv). The mixture was heated at 100°C with stirring for 24 h. To this mixture was added more 98% hydrazine hydrate (0.5 mL) and the mixture was heated at 195°C for 7 h. After cooling to room temperature, a saturated aqueous solution of sodium carbonate was added, and the mixture was extracted three times with ethyl acetate. The combined organic extracts were washed twice with a saturated aqueous solution of sodium carbonate, then once with brine. The organic extract was dried (anhydrous Na₂SO₄) and concentrated to a colorless oil. The oil (*R*_f = 0.24, EtOAc) was purified by flash column chromatography on silica gel (EtOAc) to give the free base form of the product as a white solid (190 mg). To a solution of this solid in EtOH (2 mL) was added a solution of 37% aqueous HCl (100 mg, 1.02 mmol, 1.24 equiv) in EtOH (2 mL). The mixture was concentrated; to the residual material was added diethyl ether and the mixture was concentrated again. The residual material was dried under high-vacuum, giving the title compound (213 mg, 43%) as a white solid: mp 153–154 °C; ¹H NMR (400 MHz, CD₃OD): δ 1.58–1.83 (m, 14H), 1.99 (s, 3H), 4.25–4.33 (m, 2H), 7.56 (s, 1H), 7.69 (s, 1H), 9.01 (s, 1H); ¹³C NMR (100 MHz, CD₃OD): δ 30.0, 33.1, 38.0, 43.1, 45.4, 46.2, 121.0, 123.4, 136.2; HRMS (ESI) [M-Cl]⁺ Calculated for C₁₅H₂₃N₂: 231.1861. Found: 231.1852.

Animals. Male Sprague-Dawley (CrI:CD(SD)) rats (250-300 g) and Female Swiss Nude (CrI:NU-Foxn1^{nu} - NU/NU) mice (4-6 weeks of age) were purchased from Charles River Inc. (Montreal, Canada). This strain of male rats was the same as that used during the development of our second-generation HO inhibitors. Female nude mice were selected because they are widely used in research on cancer, which was one of the earliest targets suggested for the application of HO inhibitors. The mouse spleen and brain tissues used in this study were harvested from saline-treated control mice that were part of a previously conducted animal study, in which the mice had mouse breast carcinoma (AC2M2) cells (7.5×10^3) surgically implanted into a mammary fat pad as described previously (Elliott et al. 1992). At the end of the experiment, mice were euthanized by isoflurane inhalation followed by cervical dislocation, after which the spleen and brain were harvested, flash frozen in liquid nitrogen and stored at -80°C. Rats and mice were maintained on a 12 h light:12 h dark cycle with lights on at 7:00 am and were given *ad libitum* access to water and LabDiet Laboratory Rodent Diet #5001 or LabDiet Autoclavable Mouse Breeder Diet #5021, respectively (Ren's Feed and Supply, Oakville, Canada). All animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care and all experimental protocols were approved by the Queen's University Animal Care Committee.

Preparation of Tissue Microsomes. Microsomal fractions of spleen and brain tissue harvested from rat and mouse were prepared using differential centrifugation of tissue homogenate, as previously described (Appleton et al. 1999; Kinobe et al. 2006). Briefly, on the day of microsomal preparation, rats were euthanized by isoflurane inhalation followed by

decapitation, after which the spleen and brain were harvested. Mouse spleen and brain tissues were harvested as described above and stored at -80°C until they were used. Tissues were homogenized (15% w/v) in ice-cold homogenizing buffer (20 mM KH_2PO_4 , 135 mM KCl and 0.1 mM EDTA; adjusted to pH 7.4 at 4°C with 1 M KOH) using a 60S Sonic Dismembrator (Fisher Scientific, Ottawa, Canada). The homogenate was centrifuged at $10,000 \times g$ for 20 min at 4°C , after which the supernatant was collected and centrifuged at $100,000 \times g$ for 60 min at 4°C . The resulting microsomal pellet was resuspended in phosphate-glycerol buffer (100 mM KH_2PO_4 adjusted to pH 7.4 with 1 M KOH, 20% glycerol and 10 mM EDTA) using a Potter-Elvehjem homogenizer with a Teflon pestle. Microsomes were aliquoted and stored at -80°C until used. Protein concentration was determined using a modification of the Biuret method, as previously described (Marks et al. 1997).

HO Activity Assay. NADPH-dependent CO formation as an index of HO activity in spleen and brain microsomal fractions was determined by quantifying CO production from the degradation of methemalbumin using gas chromatography, as described previously (Vreman and Stevenson 1988; Cook et al. 1995; Kinobe et al. 2006; Vlahakis et al. 2006). Briefly, reaction mixtures (150 μL) consisting of 100 mM phosphate buffer (pH 7.4), 50 μM methemalbumin and spleen microsomes (0.5 mg/mL protein) or brain microsomes (1.0 mg/mL protein) were preincubated in sealed 1.5-mL amber vials, with a QC compound or CrMP at final concentrations ranging from 0.01 to 100 μM , or with vehicle (water for QC compounds or 0.5% (v/v) ethanolamine for CrMP), for 10 min at 37°C . During this time, headspace of the sealed vials was purged with CO-free air. Reactions were initiated by adding 1 mM NADPH and incubations were carried out for 15 min (rat tissue microsomes) or 20 min (mouse tissue

microsomes) at 37°C. Reactions were terminated by instantly freezing the samples on powdered dry ice and CO formation was measured by gas chromatography using a ta3000R Process Gas Analyzer (Trace Analytical/Ametek, Newark, USA). NADPH-dependent formation of CO in each sample was calculated by subtracting the value for CO produced in samples not containing NADPH and then interpolating the corrected peak area value on the linear CO standard curve (10 – 164 pmol of CO). The activity of HO following incubation with each concentration of QC compound or CrMP was expressed as a percentage of total HO activity that was determined from the analysis of vehicle samples.

Quantification of Protein Expression. Brain and spleen microsomes from rat and mouse were diluted using radioimmunoprecipitation assay buffer (0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 0.05% Tris base, adjusted to pH 8.0; with addition of 0.1% protease inhibitor cocktail on day of experiment). Expression of HO-1 and HO-2 protein in rat and mouse tissues was determined using a modification of the method previously described by Kinobe et al. (2006). Specifically, microsome samples (10 µg protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel (Laemmli 1970) and the proteins were transferred to polyvinylidene fluoride Immobilon-P membranes (Millipore, Billerica, USA). Nonspecific binding sites were blocked by incubating the membranes in Tris-buffered saline /Tween-20 (0.02 M Tris base, 0.14 M NaCl and 0.075% Tween-20, adjusted to pH 7.6) containing 5% (w/v) skimmed milk powder at room temperature for 1 h. The western blots were then incubated with a 1:5,000 dilution of the polyclonal anti-HO-1 antibody overnight at 4°C. Subsequently, the membranes were incubated with a peroxidase-labelled goat anti-rabbit IgG secondary antibody (Bio-Rad Laboratories Ltd.,

Mississauga, Canada). Peroxidase activity was detected using an enhanced chemiluminescence detection kit according to the manufacturer's instructions (LumiGlo; Mandel Scientific Company Inc., Guelph, Canada). All gels were calibrated with pre-stained low-range molecular weight markers (Bio-Rad Laboratories Ltd., Mississauga, Canada). Relative HO-1 expression was quantified by optical densitometry using the ImageJ program (National Institutes of Health, Bethesda, USA). The anti-HO-1 antibody was removed from membranes using stripping buffer (25 mM glycine and 1% SDS, adjusted to pH 2.0), blocked as described above, and probed with a 1:5,000 dilution of the polyclonal anti-HO-2 antibody, as described for anti-HO-1. Relative HO-2 expression was quantified by optical densitometry as described above for HO-1. To ensure uniform protein loading on all gels, membranes were reprobed with monoclonal anti- β -actin antibody and densitometry units for HO-1 and HO-2 expression were normalized to β -actin protein expression.

Data Analysis. All statistical analyses were performed using Prism® Version 7 (GraphPad Software Inc., San Diego, USA). IC₅₀ (inhibitor concentration that decreased enzyme activity by 50%) values were calculated as part of the nonlinear regression of concentration-response curves analysis. The maximum effect values for each inhibitor were determined by recording the minimum residual enzyme activity (EA_{res}) achieved for each concentration-response curve. Accordingly, the most effective inhibition of enzyme activity is indicated by the smallest residual enzyme activity or EA_{res}. The data are presented as group means $\pm$ SD for three or four experiments, as indicated in the figure captions. Parametric statistical analysis was conducted by unpaired Student's *t* test, one-way analysis of variance (ANOVA) or two-way ANOVA, depending on which statistical test was appropriate. For a significant *F* statistic

($p < 0.05$), the ANOVAs were followed by a post test to determine which experimental groups were statistically different ($p < 0.05$). Specifically, the one-way ANOVA was followed by a Tukey's post test and the two-way ANOVA was followed by a Tukey's post test when there was a significant F statistic for both variables and a Sidak's post test when there was a significant F statistic for one variable, as recommended by the Prism software.

Draft

Results

Expression of HO-1 and HO-2 Protein in Spleen and Brain Microsomes from Rat and Mouse. The predominant HO isozyme in spleen microsomes from both rat and mouse was HO-1 (Fig. 2), with a 13-fold and 5-fold greater amount of HO-1 compared to HO-2, respectively. In brain microsomes from rat, the amount of HO-2 protein expressed was 3-fold greater than HO-1 protein, while there was no apparent difference in the protein expression of HO-1 and HO-2 in mouse brain (Fig. 2).

Inhibitory Profile for Non-Selective Inhibitors of HO Isozymes Was Comparable Between Rat and Mouse Microsomes for CrMP, but Not for QC-282. The metalloporphyrin CrMP, a non-selective inhibitor of HO isozymes, inhibited HO activity in spleen and brain microsomes from rat and mouse similarly over a 0.01 to 100 μ M concentration range. The one exception was that minimum residual enzyme activity (EA_{res}) for mouse brain microsomes was statistically ($p < 0.05$) greater than that for rat brain microsomes (Fig. 3A). In contrast, the characterization of the imidazole derivative QC-282 as a non-selective inhibitor of HO isozymes, based on a similar IC_{50} between rat spleen and brain microsomes, was confirmed in the present study for rat spleen and brain microsomes (Fig. 3B). However, for mouse, QC-282 was less potent (IC_{50}) and significantly ($p < 0.05$) less effective (EA_{res}) as an inhibitor of HO activity in both spleen and brain microsomes of mouse as compared to rat (Fig. 3B).

HO-1 Selective Inhibitors QC-220 and QC-291 Were Less Effective in Mouse Microsomes. The selective inhibition of HO-1 activity by two imidazole derivatives, QC-220

and QC-291, was confirmed in the present study, whereby for both compounds IC_{50} and EA_{res} were significantly ($p < 0.05$) smaller for rat spleen than for rat brain microsomes (Fig. 4). These results were indicative of an increase of inhibitory potency and efficacy of QC-220 and QC-291 on HO activity in rat spleen microsomes. For the mouse, there did not appear to be selective inhibition of HO activity in spleen microsomes compared with brain microsomes by QC-220 or QC-291, as for the most part, both compounds were similarly effective between mouse spleen and brain microsomes. Comparing between the two species, it was found that based on IC_{50} and EA_{res} , the HO-1 selective inhibitors QC-220 and QC-291 were less potent and less effective inhibitors of HO activity in mouse spleen compared to rat spleen microsomes (Fig. 4).

HO-2 Selective Inhibitors Were Similarly Effective in Rat and Mouse Microsomes.

QC-2350 and QC-2356 are benzimidazole derivatives that were previously characterized as HO-2 selective inhibitors using rat tissue microsomes. This was confirmed in the present study, whereby for both compounds the IC_{50} and EA_{res} for rat brain microsomes were smaller compared to rat spleen microsomes (Fig. 5), which was indicative of a greater inhibitory potency and efficacy of QC-2350 and QC-2356 on HO activity in rat brain compared with rat spleen microsomes. Similar observations were made in the case of mouse microsomes, whereby QC-2350 and QC-2356 inhibitory potency and efficacy were greater in mouse brain microsomes, as determined by the decrease in IC_{50} and significant ($p < 0.05$) decrease in EA_{res} values for mouse brain compared to mouse spleen microsomes. Comparing between the two species revealed that IC_{50} and EA_{res} values for the HO-2 selective inhibitors QC-2350 and QC-2356 were similar for rat brain compared to mouse brain microsomes. An exception was that EA_{res} for

QC-2356 in mouse spleen and brain microsomes was significantly ($p < 0.05$) different than that in rat spleen and brain microsomes.

Draft

Discussion

The major observations of the present study were that (a) NADPH-dependent CO formation, an index of HO activity, was less effectively inhibited by QC-282 (non-selective HO inhibitor) in mouse spleen and brain microsomes as compared with rat spleen and brain microsomes; (b) HO activity was less effectively inhibited by QC-220 and QC-291 (HO-1 selective inhibitors) in mouse spleen microsomes than in rat spleen microsomes; (c) HO activity was inhibited in a similar manner by QC-2350 and QC-2356 (HO-2 selective inhibitors) in rat and mouse brain microsomes; and (d) mouse brain microsomes contained approximately equal amounts of HO-1 and HO-2 protein.

The hypothesis addressed in the present study was that selected imidazole and benzimidazole inhibitors of HO would inhibit enzymatic activity of HO in spleen and brain microsomes obtained from rats and mice in a similar manner. This was based on the amino acid sequence homology between rat and mouse being greater than 80% for HO-1 (Rotenburg and Maines 1991; Maines 1997) and 95% for HO-2 (Matsumoto et al. 1996), and that the catalytic core of both HO isozymes from rat and mouse contain a highly conserved 24-amino acid segment (Rotenburg and Maines 1991, Matsumoto et al. 1996). Furthermore, inhibition of HO activity by the metalloporphyrin CrMP was similar between spleen and brain preparations from both rats and mice, as previously observed (Kinobe et al. 2006; Wong et al. 2011). With regards to the azole-based inhibitors of HO, observations made with QC-2350 and QC-2356 supported the hypothesis in that these drugs selectively inhibited brain microsomal HO activity, while having little effect on that of spleen microsomes of both rat and mouse. This was consistent with the classification of these drugs as HO-2 selective inhibitors.

Conversely, the observations made with QC-220, QC-291 and QC-282 were not as predicted by the hypothesis. In the case of QC-220 and QC-291, originally classified as HO-1 selective inhibitors, the inhibition of HO activity by these compounds was less potent and less effective in mouse spleen microsomes compared to rat spleen microsomes; for QC-282, originally classified as a non-selective inhibitor of HO, the inhibition of HO activity by this compound in both spleen and brain microsomes of mouse was less potent and less effective than that for comparable rat microsomes. These observations clearly did not support the hypothesis and the reason for this difference between species is difficult to explain considering the high degree of homology of the HO enzymes, particularly at the conserved catalytic core (Rotenberg and Maines 1991; Matsumoto et al. 1996; Maines 1997). Furthermore, the non-competitive mechanism for inhibition of rat HO activity by our azole-based compounds, and the manner in which these compounds bind to the heme-HO complex (Rahman et al. 2008), would be more predictive of 100% inhibition rather than significantly less as shown in Fig. 3B and Fig. 4. It was expected that the maximum extent of inhibition of HO activity would be similar in rat and mouse spleen and brain microsomes, although not necessarily at the same drug concentrations.

These observations do not appear to be explained by the relative quantities of HO-1 and HO-2 protein determined by western blotting. The lesser effectiveness of the HO-1 selective inhibitors QC-220 and QC-291 in inhibiting mouse spleen compared to rat spleen HO activity was not consistent with the greater relative quantity of HO-1 protein in both mouse and rat spleen microsomes. Also inconsistent was the selective inhibition of HO activity in mouse brain compared to mouse spleen microsomes by the HO-2 selective inhibitors QC-2350 and QC-2356 even though the relative quantity of HO-1 and HO-2 protein in mouse brain microsomes was similar.

The lesser effectiveness of QC-282 against mouse microsomes raises a practical concern when considering applications in which extensive inhibition of both HO isozymes is desired in mice or other mammalian species. A similar concern was raised in the case of QC-220 and QC-291, wherein the extent of inhibition of HO activity in mouse spleen microsomes was substantially lower than that of the rat; which in turn resulted in no selective inhibition of mouse spleen microsomal HO activity compared to mouse brain microsomes.

After considering the problems identified through the use of microsomal preparations of HO, one might ask the question, “Why not dispense with microsomes completely and conduct all these experiments using recombinant enzymes, which are pure and not subject to the complexity of microsomes?” A major reason for retaining microsomes as a biological model for studying heme oxygenases is that microsomal enzymes are largely bound to cell membranes, usually by virtue of a hydrophobic tail embedded in a lipophilic membrane. Thus, microsomes may retain these enzymes in a milieu that is more representative of that found in living cells. One of the drugs synthesized by our team, QC-15, was originally identified as a HO-1 selective inhibitor using rat spleen and brain microsomes, (Kinobe et al. 2006, compound III; Vlahakis et al. 2006, compound 5). When this drug was tested against recombinant HO-1 and HO-2 protein from rat (Sugishima et al. 2007) and human (Vukomanovic et al. 2010), it was found not to be selective for HO-1; nevertheless it should be noted that these recombinant forms of HO were truncated, which favours the crystal formation necessary for x-ray analysis. Furthermore, QC-15 inhibition of HO activity was less effective in human recombinant HO-1 compared to rat spleen microsomal HO activity; accordingly 10 μ M QC-15 inhibited HO activity $34 \pm 1.7\%$ and $74 \pm 1.4\%$ of control, respectively (Vukomanovic et al. 2010). We interpret such differences as indicating that the lipid milieu of microsomal membranes affects the interaction of the embedded

HO with drugs such as inhibitors. In this case, the presence of lipid membranes appears to enhance inhibition of HO-1 in rat spleen microsomes. Huber et al. (2009) have reported a similar critical importance of membrane lipids; in their case they found that the maximum enzyme activity of recombinant full-length HO-1 was increased by 3-fold when the enzyme preparation was pre-incubated with phospholipids.

In conclusion, the inhibitory profile for the azole-based inhibitors of HO studied herein was not consistent between spleen and brain microsomes from a breast cancer-implanted mouse as compared to an untreated rat. This serves to remind us of the caveat to consider interspecies differences in drug response when designing an experiment. Therefore, it is important to test the effects of azole-based inhibitors on CO formation using homogenates or microsomes of the proposed experimental model before proceeding with a full study. Further, as first-generation metalloporphyrin and second-generation azole-based inhibitors of HO have different off-target effects, these two groups of drugs may be productively used as complementary pharmacological tools in studying the role of HO in physiological processes.

Conflict of Interest

The authors declare that there is no conflict of interest associated with this work.

Acknowledgements

This work was supported by the Ontario Institute of Cancer Research grant No. 08NOV-142. M.H. received a doctoral fellowship from the Canadian Breast Cancer Foundation, Ontario Region.

Draft

References

Alaoui-Jamali, M.A., Bismar, T.A., Gupta, A., Szarek, W.A., Su, J., Song, W., Xu, Y., Xu, B., Liu, G., Vlahakis, J.Z., Roman, G., Jiao, J., and Schipper, H.M. 2009. A novel experimental heme oxygenase-1-targeted therapy for hormone-refractory prostate cancer. *Cancer Res.* **69**(20): 8017-8024.

Appleton, S.D., Chretien, M.L., McLaughlin, B.E., Vreman, H.J., Stevenson, D.K., Brien, J.F., Nakatsu, K., Maurice, D.H., and Marks, G.S. 1999. Selective inhibition of heme oxygenase, without inhibition of nitric oxide synthase or soluble guanylyl cyclase, by metalloporphyrins at low concentrations. *Drug Metab. Dispos.* **27**(10): 1214-1219.

Cook, M.N., Nakatsu, K., Marks, G.S., McLaughlin, B.E., Vreman, H.J., Stevenson, D.K., and Brien, J.F. 1995. Heme oxygenase activity in the adult rat aorta and liver as measured by carbon monoxide formation. *Can. J. Physiol. Pharmacol.* **73**(4): 515-518.

Csongradi, E., Vera, T., Rimoldi, J.M., Gadepalli, R.S.V., and Stec, D.E. 2010. *In vivo* inhibition of renal heme oxygenase with an imidazole-dioxolane inhibitor. *Pharmacol. Res.* **61**(6): 525-530.

Csongradi, E., Storm, M.V., and Stec, D.E. 2012. Renal inhibition of heme oxygenase-1 increases blood pressure in angiotensin II-dependent hypertension. *Int. J. Hypertens.* **2012**: 497213.

DeNagel, D.C., Verity, A.N., Madden, F.E., O'Yang, C., Sangameswaran, L., and Johnson, R.M. 1998. Identification of non-porphyrin inhibitors of heme oxygenase-1. *Soc. Neurosci.* **24**: 2058.

Di Francesco, L., Totani, L., Dovizio, M., Piccoli, A., Di Francesco, A., Salvatore, T., Pandolfi, A., Evangelista, V., Dercho, R.A., Seta, F., and Patrignani, P. 2009. Induction of prostacyclin by steady laminar shear stress suppresses tumor necrosis factor- α biosynthesis via heme oxygenase-1 in human endothelial cells. *Circ. Res.* **104**(4): 506-513.

Elliott, B.E., Tam, S.-P., Dexter, D., and Chen, Z.Q. 1992. Capacity of adipose tissue to promote growth and metastasis of a murine mammary carcinoma: effect of estrogen and progesterone. *Int. J. Cancer* **51**(3): 416-424.

Grundemar, L., and Ny, L. 1997. Pitfalls using metalloporphyrins in carbon monoxide research. *Trends Pharmacol. Sci.* **18**(6): 193-195.

Huber, W.J. III, Scruggs, B.A., and Backes, W.L. 2009. C-Terminal membrane spanning region of human heme oxygenase-1 mediates a time-dependent complex formation with cytochrome P450 reductase. *Biochemistry* **48**(1): 190-197.

Hum, M., McLaughlin, B.E., Roman, G., Vlahakis, J.Z., Szarek, W.A., and Nakatsu, K. 2010. The effects ofazole-based heme oxygenase inhibitors on rat cytochromes P450 2E1 and 3A1/2 and human cytochromes P450 3A4 and 2D6. *J. Pharmacol. Exp. Ther.* **334**(3): 981-987.

Kinobe, R.T., Vlahakis, J.Z., Vreman, H.J., Stevenson, D.K., Brien, J.F., Szarek, W.A., and Nakatsu, K. 2006. Selectivity of imidazole-dioxolane compounds for *in vitro* inhibition of microsomal haem oxygenase isoforms. Br. J. Pharmacol. **147**(3): 307-315.

Kinobe, R.T., Ji, Y., Vlahakis, J.Z., Motterlini, R., Brien, J.F., Szarek, W.A., and Nakatsu, K. 2007. Effectiveness of novel imidazole-dioxolane heme oxygenase inhibitors in renal proximal tubule epithelial cells. J. Pharmacol. Exp. Ther. **323**(3): 763-770.

Kinobe, R.T., Dercho, R.A., and Nakatsu, K. 2008. Inhibitors of the heme oxygenase-carbon monoxide system: on the doorstep of the clinic? Can. J. Physiol. Pharmacol. **86**(9): 577-599.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**(5259): 680-685.

Maines, M.D. 1988. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J. **2**(10): 2557-2568.

Maines, M.D. 1997. The heme oxygenase system: A regulator of second messenger gases. Annu. Rev. Pharmacol. Toxicol. **37**:517-554.

Marks, G.S., McLaughlin, B.E., Vreman, H.J., Stevenson, D.K., Nakatsu, K., Brien, J.F., and Pang, S.C. 1997. Heme oxygenase activity and immunohistochemical localization in bovine pulmonary artery and vein. J. Cardiovasc. Pharmacol. **30**(1): 1-6.

Matsumoto, M., Saito-Ohara, F., Hoshi, M., and Kurata, S. 1996. Cloning and sequencing of mouse complementary DNA for heme oxygenase-2. *J. Biochem.* **120**(6): 1079-1081.

Pittalà, V., Salerno, L., Romeo, G., Modica, M.N., and Siracusa, M.A. 2013. A focus on heme oxygenase-1 (HO-1) inhibitors. *Curr. Med. Chem.* **20**(30): 3711-3732.

Rahman, M.N., Vlahakis, J.Z., Szarek, W.A., Nakatsu, K., and Jia, Z. 2008. X-ray crystal structure of human heme oxygenase-1 in complex with 1-(adamantan-1-yl)-2-(1*H*-imidazol-1-yl)ethanone: a common binding mode for imidazole-based heme oxygenase-1 inhibitors. *J. Med. Chem.* **51**(19): 5943-5952.

Roman, G., Riley, J.G., Vlahakis, J.Z., Kinobe, R.T., Brien, J.F., Nakatsu, K., and Szarek, W.A. 2007. Heme oxygenase inhibition by 2-oxy-substituted 1-(1*H*-imidazol-1-yl)-4-phenylbutanes: effect of halogen substitution in the phenyl ring. *Bioorg. Med. Chem.* **15**(9): 3225-3234.

Roman, G., Rahman, M.N., Vukomanovic, D., Jia, Z., Nakatsu, K., and Szarek, W.A. 2010a. Heme oxygenase inhibition by 2-oxy-substituted 1-azolyl-4-phenylbutanes: effect of variation of the azole moiety. X-ray crystal structure of human heme oxygenase-1 in complex with 4-phenyl-1-(1*H*-1, 2, 4-triazol-1-yl)-2-butanone. *Chem. Biol. Drug. Des.* **75**(1): 68-90.

Roman, G., Vlahakis, J.Z., Vukomanovic, D., Nakatsu, K., and Szarek, W.A. 2010b. Heme oxygenase inhibition by 1-aryl-2-(1*H*-imidazol-1-yl/1*H*-1,2,4-triazol-1-yl) ethanones and their derivatives. *ChemMedChem* **5**(9): 1541-1555.

Rotenberg, M.O., and Maines, M.D. 1991. Characterization of a cDNA-encoding rabbit brain heme oxygenase-2 and identification of a conserved domain among mammalian heme oxygenase isozymes: possible heme-binding site? *Arch. Biochem. Biophys.* **290**(2): 336-344.

Ryter, S.W., Alam, J., and Choi, A.M.K. 2006. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol. Rev.* **86**(2): 583-650.

Salerno, L., Pittalà, V., Romeo, G., Modica, M.N., Siracusa, M.A., Di Giacomo, C., Acquaviva, R., Barbagallo, I., Tibullo, D., and Sorrenti, V. 2013. Evaluation of novel aryloxyalkyl derivatives of imidazole and 1,2,4-triazole as heme oxygenase-1 (HO-1) inhibitors and their antitumor properties. *Bioorg. Med. Chem.* **21**(17): 5145-5153.

Salerno, L., Pittalà, V., Romeo, G., Modica, M.N., Marrazzo, A., Siracusa, M.A., Sorrenti, V., Di Giacomo, C., Vanella, L., Parayath, N.N., and Greish, K. 2015. Novel imidazole derivatives as heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2) inhibitors and their cytotoxic activity in human-derived cancer cell lines. *Eur. J. Med. Chem.* **96**:162-172.

Schulz, S., Wong, R.J., Vreman, H.J., and Stevenson, D.K. 2012. Metalloporphyrins – an update. *Front. Pharmacol.* **3**:68.

Sorrenti, V., Guccione, S., Di Giacomo, C., Modica, M.N., Pittalà, V., Acquaviva, R., Basile, L., Pappalardo, M., and Salerno, L. 2012. Evaluation of imidazole-based compounds as heme oxygenase-1 inhibitors. *Chem. Biol. Drug. Des.* **80**(6): 876-886.

Sugishima, M., Higashimoto, Y., Oishi, T., Takahashi, H., Sakamoto, H., Noguchi, M., and Fukuyama, K. 2007. X-ray crystallographic and biochemical characterization of the inhibitory action of an imidazole-dioxolane compound on heme oxygenase. *Biochemistry* **46**(7): 1860-1867.

Vlahakis, J.Z., Kinobe, R.T., Bowers, R.J., Brien, J.F., Nakatsu, K., and Szarek, W.A. 2005. Synthesis and evaluation of azalanstat analogues as heme oxygenase inhibitors. *Bioorg. Med. Chem. Lett.* **15**(5): 1457-1461.

Vlahakis, J.Z., Kinobe, R.T., Bowers, R.J., Brien, J.F., Nakatsu, K., and Szarek, W.A. 2006. Imidazole-dioxolane compounds as isozyme-selective heme oxygenase inhibitors. *J. Med. Chem.* **49**(14): 4437-4441.

Vlahakis, J.Z., Hum, M., Rahman, M.N., Jia, Z., Nakatsu, K., and Szarek, W.A. 2009. Synthesis and evaluation of imidazole-dioxolane compounds as selective heme oxygenase inhibitors: effect of substituents at the 4-position of the dioxolane ring. *Bioorg. Med. Chem.* **17**(6): 2461-2475.

Vlahakis, J.Z., Lazar, C., Roman, G., Vukomanovic, D., Nakatsu, K., and Szarek, W.A. 2012. Heme oxygenase inhibition by α -(1*H*-imidazol-1-yl)- ω -phenylalkanes: effect of introduction of heteroatoms in the alkyl linker. *ChemMedChem* **7**(5): 897-902.

Vlahakis, J.Z., Vukomanovic, D., Nakatsu, K., and Szarek, W.A. 2013. Selective inhibition of heme oxygenase-2 activity by analogs of 1-(4-chlorobenzyl)-2-(pyrrolidin-1-ylmethyl)-1*H*-benzimidazole (clemizole): exploration of the effects of substituents at the *N*-1 position. *Bioorg. Med. Chem.* **21**(21): 6788-6795.

Vreman, H.J., and Stevenson, D.K. 1988. Heme oxygenase activity as measured by carbon monoxide production. *Anal. Biochem.* **168**(1): 31-38.

Vreman, H.J., Wong, R.J., Stevenson, D.K., and Clark, J.D. 2002. Azalanstat (RS-1607): evidence for a novel class of potential heme oxygenase inhibitors. *Pediatr. Acad. Soc. PAS2002*:1980.

Vukomanovic, D., McLaughlin, B., Rahman, M.N., Vlahakis, J.Z., Roman, G., Dercho, R.A., Kinobe, R.T., Hum, M., Brien, J.F., Jia, Z., Szarek, W.A., and Nakatsu, K. 2010. Recombinant truncated and microsomal heme oxygenase-1 and -2: differential sensitivity to inhibitors. *Can. J. Physiol. Pharmacol.* **88**(4): 480-486.

Wong, R.J., Vreman, H.J., Schulz, S., Kalish, F.S., Pierce, N.W., and Stevenson, D.K. 2011. *In vitro* inhibition of heme oxygenase isoenzymes by metalloporphyrins. *J. Perinatol.* **31**(Suppl 1): S35-S41.

Wu, L., and Wang, R. 2005. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol. Rev.* **57**(4): 585-630.

Figure Captions

Fig. 1. Chemical structures of azole-based inhibitors of HO used in this study, namely, the non-selective inhibitor of HO isozymes, QC-282; HO-1 selective inhibitors QC-220 and QC-291; and HO-2 selective inhibitors QC-2350 and QC-2356.

Fig. 2. Relative HO-1 and HO-2 protein expression in spleen and brain microsomes from A) rat and B) mouse were normalized to the expression of β -actin. C. Representative western blots showing HO-1, HO-2 and actin bands for rat brain and spleen, and mouse brain and spleen. Data were presented as group means $\pm$ S.D. ($n = 3$). * $p < 0.05$ compared with corresponding HO-1 protein.

Fig. 3. Inhibition of HO activity by HO non-selective inhibitors CrMP, a metalloporphyrin, and QC-282, an imidazole derivative, in spleen and brain microsomes from rat and mouse. Concentration-response curves show the inhibitory effect of A) CrMP and B) QC-282 on HO activity in rat spleen ($n = 4$) compared to mouse spleen ($n = 3$) microsomes and in rat brain ($n = 4$) compared to mouse brain ($n = 3$) microsomes. The IC_{50} and EA_{res} values for each concentration-response curve are summarized in the associated tables. The data are presented as group means $\pm$ S.D. Not Detectable, concentration curve did cross 50% of control activity by a concentration of 100 μ M compound. * $p < 0.05$ compared with rat spleen and † $p < 0.05$ compared with rat brain.

Fig. 4. Inhibition of HO activity by HO-1 selective inhibitors QC-220 and QC-291, both imidazole derivatives, in spleen and brain microsomes from rat and mouse. Concentration-response curves show the inhibitory effect of A) QC-220 and B) QC-291 on HO activity in rat spleen ($n = 4$) compared to mouse spleen ($n = 3$) microsomes and in rat brain ($n = 4$) compared to mouse brain ($n = 3$) microsomes. The IC_{50} and EA_{res} values for each concentration-response curve are summarized in the associated tables. The data are presented as group means $\pm$ S.D. Not Detectable, concentration curve did cross 50% of control activity by a concentration of 100 μ M compound. * $p < 0.05$ compared with rat spleen, † $p < 0.05$ compared with rat brain and ‡ $p < 0.05$ compared with mouse spleen.

Fig. 5. Inhibition of HO activity by HO-2 selective inhibitors QC-2350 and QC-2356, both benzimidazole derivatives, in spleen and brain microsomes from rat and mouse. Concentration-response curves show the inhibitory effect of A) QC-2350 and B) QC-2356 on HO activity in rat spleen ($n = 4$) compared to mouse spleen ($n = 3$) microsomes and in rat brain ($n = 4$) compared to mouse brain ($n = 3$) microsomes. The IC_{50} and EA_{res} values for each concentration-response curve are summarized in the associated tables. The data are presented as group means $\pm$ S.D. Not Detectable, concentration curve did cross 50% of control activity by a concentration of 100 μ M compound. * $p < 0.05$ compared with rat spleen, † $p < 0.05$ compared with rat brain and ‡ $p < 0.05$ compared with mouse spleen.

A. Non-selective Inhibitor of HO Isozymes **QC-282****B. HO-1 Selective Inhibitors** **QC-220** **QC-291****C. HO-2 Selective Inhibitors** **QC-2350** **QC-2356**

Fig. 1. Chemical structures of HO inhibitors.

167x170mm (300 x 300 DPI)

A. Rat **B. Mouse** **C. Western Blot**

Fig. 2. HO protein expression

124x222mm (300 x 300 DPI)

Fig. 3. Inhibition of HO activity by non-selective inhibitors CrMP and QC-282

197x240mm (300 x 300 DPI)

Fig. 4. Inhibition of HO activity by HO-1 selective inhibitors QC-220 and QC-291

197x240mm (300 x 300 DPI)

Fig. 5. Inhibition of HO activity by HO-2 selective inhibitors QC-2350 and QC-2356

197x241mm (300 x 300 DPI)