NOVEL AZOLE-BASED HEME OXYGENASE INHIBITORS: IN VIVO CHARACTERIZATION AND THERAPEUTIC APPLICATION IN CANCER

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A thesis submitted to the Department of Pharmacology and Toxicology in conformity with the requirements for the degree of Doctor of Philosophy

Queen’s University
Kingston, Ontario, Canada
April 2010

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ABSTRACT

Heme degradation is catalyzed through the action of heme oxygenases (HO), present as inducible (HO-1) and constitutive (HO-2) isoforms, resulting in the endogenous production of carbon monoxide (CO), biliverdin and iron. Numerous studies attempting to elucidate the physiological roles of these products have relied on metalloporphyrin inhibitors of HO; however, they have limited utility, as they are known to inhibit other hemoproteins. Previous work in our laboratory has led to the identification of a number of azole-based compounds that inhibit HO in vitro, including several that have demonstrated selectivity for HO-1. The principal goal of this project was to characterize lead compounds in vivo and investigate their therapeutic potential as anti-cancer agents. The first objective was to develop a method to screen novel azole-based HO inhibitors (abHOi) in vivo. To this end, we describe a non-invasive method of measuring CO exhalation (VeCO) as a measure of in vivo HO activity. Using the VeCO method, we found that the inhibition of HO by abHOi was dose- and time-dependent, and was comparable to the effectiveness of the classical metalloporphyrin HO inhibitor, zinc protoporphyrin. Moreover, abHOi inhibited HO by a reversible, non-competitive mechanism and had no effect on HO-1 or HO-2 protein expression. Interestingly, structurally similar azole antifungal drugs were found to inhibit HO activity at therapeutically relevant doses, which raises the possibility that some of the anti-mycolic and anti-tumourigenic effects of drugs like ketoconazole may be mediated, in part, by the inhibition of HO. Our last objective was to investigate the effectiveness of abHOi in in vitro and in vivo models of tumour angiogenesis, growth and metastasis. We found that the HO-1-selective abHOi, QC-15, decreased cancer cell viability and inhibited
endothelial cell sprouting from aortic rings and capillary-like endothelial cell tube formation in vitro. In an orthotopic breast cancer model, treatment of tumour-bearing mice with QC-15 reduced primary tumour volume and lung metastasis, but not tumour angiogenesis. These results demonstrate that abHOi are valuable pharmacological tools in the elucidation of the physiological roles of HO and its products and, barring serious toxicity, promising therapeutic entities for the treatment of metastatic breast cancer.
CO-AUTHORSHIP

Chapter 1 and 4

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Robert shared in the writing of the manuscripts and was responsible for the in vitro screening of azole-based antifungal drugs for inhibition of HO-1, HO-2, NOS and CPR.

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Chapter 5

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Maaike and Steve were important co-investigators for the in vivo tumour study. Maaike helped with the administration of drugs and caliper measurement of tumours (figure 5.8). She also participated in the necropsy and prepared tissue homogenates and performed the Western blot for figure 5.17. Steve remained blind to the treatment groups and objectively identified tumour volume from ultrasound images and metastases from biophotonic images (figures 5.11, 5.12, 5.15).

Dr Bruce E. Elliott, Department of Pathology, Queen’s University, Kingston, Ontario, Canada.

Dr Elliott was an important mentor in the planning of the in vivo tumour study. He also remained blind to the treatment groups and objectively identified the lung metastases in the histological sections for figure 5.12.
ACKNOWLEDGEMENTS

I can say with great certainty, that I would not be writing this today without the support of my family. For my Mom and Dad, whose encouragement over the years has allowed me to pursue all of my ambitions, I am truly grateful. To my Dad, I owe the gift of an inquisitive mind. His garage and basement full of junk were always a treasure chest of parts for the latest science project. I will never forget the model of the futuristic, sustainable house we built when I was in elementary school. To this day, I am still not sure who had more fun working on it… And to my Mom, whose persistence and organization provided the necessary balance to the chaos of my Dad. I would have never been able to complete this thesis without the focus that I learned from her. And of course, how could I forget my little sisters, Alicia and Lindsay, who, no doubt, were the test subjects of my first ‘scientific experiments’. To my Oma and Opa, who always believed I could achieve anything I set my sights on. And lastly, to my Nonna, I can finally answer her question “So, when are you gonna be doctor?”

To my friends, you’ve made grad school more fun than I could have ever imagined. Whether it was part of Phat Pharm, Buddy Jesus, Yukon Cornelius, Teamo Supremo, T-Pain or Pharm Animals you are all my Super Best Friends. Some of my favourite times have been spent with you including barbecues on the balcony, Casino Night, the Annual Wolfe Island Flip Cup Championships and of course, the Elixer patio. I’m already looking forward to our reunion next year!

A special thanks to the members of the Nakatsu lab, past and present. Robert, I hope you know how important of a mentor you were for me early in my career. I always
looked up to you and only hope that I can encompass the integrity you brought to all your research. To Maaike and Steve, for the first 3 years of my thesis all I wanted was to work on a big project as part of a team; thanks for making that happen, I couldn’t ask for better partners. To Brian McLaughlin, it has been a pleasure. I very much appreciate your willingness to help no matter what the situation. You were definitely my ‘go to’ guy when it came to any questions I had.

Finally, none of this would have been possible without my mentor and supervisor, Dr. Kanji Nakatsu. Your impeccable ability to employ just the right amount of guidance, while allowing me the freedom to pursue my ideas, has made me the scientist I am today. I am also grateful for your patience. I cannot count the amount of abstracts, manuscripts and applications that I dropped off on your desk at 4:30 p.m. that needed to be edited and submitted by 5:00 p.m. (including this thesis). Outside of the lab, you have also been an important role model and friend. I will miss Saturday morning hockey with the guys (not the waking-up part) and summer barbecues at Desert Lake. I can only hope that I still get an invite after I graduate!
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LIST OF ABBREVIATIONS

abHOi: azole-based heme oxygenase inhibitor
AUC: area under the curve
BSA: bovine serum albumin
BW: body weight
cGMP: cyclic guanosine monophosphate
CO: carbon monoxide
COHb: carboxyhemoglobin
CoPP: cobalt protoporphyrin
CORM: carbon monoxide-releasing molecule
CPR: cytochrome P450 reductase
CYP450: cytochromes P450
CXCR4: CXC chemokine receptor 4
DMEM: Dulbecco’s Modified Eagle medium
DMSO: dimethyl sulfoxide
DPM: disintegrations per minute
EDTA: ethylenediaminetetraacetic acid
EBM-2: endothelial basal medium-2
EGFP: enhanced green fluorescence protein
EGM-2: endothelial growth medium-2
F12K: Kaighn's Modification of Ham's F-12 medium
FBS: Fetal bovine serum
GFR: growth factor-reduced
GT: guanosine-thymidine
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO: heme oxygenase
HO-1: heme oxygenase-1
HO-2: heme oxygenase-2
hEPC: human endothelial progenitor cell
HMEC-1: human microvascular endothelial cells
hMSC: human mesenchymal stem cell
HUVEC: human umbilical vein endothelial cell
IP: intraperitoneal
IV: intravenous
K$_{Ca}^+$: calcium-dependent potassium channels
KTZ: ketoconazole
L-NAME: N-nitro-L-arginine methyl ester
mRNA: messenger ribonucleic acid
MSC: mesenchymal stem cell
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW: molecular weight
β-NADPH: Nicotinamide-Adenine Dinucleotide Phosphate: Reduced
NF-κB: nuclear factor-κB
NO: nitric oxide
Nrf2: nuclear factor–erythroid 2-related factor 2
PBS: phosphate-buffered saline
PDGF: platelet-derived growth factor
PIGF: placental growth factor
ppm: parts per million
RGA: reduction gas analyzer
RNA: ribonucleic acid
RNAi: ribonucleic acid interference
ROS: reactive oxygen species
RPMI-1640: Roswell Park Memorial Institute medium
SD: standard deviation
SDF-1α: stromal cell-derived factor-1
SDS-PAGE: sodium dodecylsulphate–polyacrylamide gel electrophoresis
SEM: standard error of the mean
sEng: soluble endoglin
SF: serum-free
sFlt-1: soluble fms-like tyrosine kinase-1
sGC: soluble guanylyl cyclase
siRNA: short interfering ribonucleic acid
SnPP: tin protoporphyrin
sVEGFR-1: soluble vascular endothelial growth factor receptor-1
TGF-β1: transforming growth factor-beta-1
VeCO: rate of pulmonary excretion of endogenously produced carbon monoxide

VEGF: vascular endothelial growth factor
ZnPP: zinc protoporphyrin
CHAPTER 1

GENERAL INTRODUCTION

Adapted from


*Canadian Journal of Physiology and Pharmacology* (86) 577-599
1.1 HEME OXYGENASES

Heme oxygenases catalyze the degradation of heme to form carbon monoxide (CO), biliverdin and ferrous iron (Figure 1.1), and are present in most tissues as a combination of inducible (HO-1) and constitutive (HO-2) isoforms (Tenhunen et al., 1968). Originally considered to be waste products of heme catabolism, CO and biliverdin (which is rapidly reduced to bilirubin) have demonstrated important roles in many physiological and pathological processes (Furchgott & Jothianandan, 1991; Otterbein, 2002).

![Figure 1.1 Heme oxygenase reaction](image)

1.1.1 Regulation of Heme Oxygenases

While most heme oxidation takes place in the liver and spleen, significant HO activity is detected in all organs and cell types including neurons, germ cells and leukocytes. In mammals, two active HO isoforms (HO-1 and HO-2) have been identified (Maines, 1988; Ewing & Maines, 1992). Under basal conditions, HO-1 (a 32 kDa heat-shock protein) is expressed predominantly in tissues that are rich in reticuloendothelial cells such as the spleen (Braggins et al., 1986; Maines, 1988). The expression of HO-1 is known to be upregulated by numerous stimuli including its substrate (heme), ultraviolet irradiation and various oxidants, heavy metals, toxins, cytokines and hormones, as well as therapeutic agents such as non-steroidal anti-inflammatory drugs, anti-diabetic
thiazolidinediones and statins. Pathways that have been shown to be involved include serine-threonine kinases (extracellular signal regulated kinases 1/2, c-Jun-N-terminal kinase, and p38-MAPK) (Elbirt et al., 1998; Shan et al., 1999; Alam et al., 2000; Chen & Maines, 2000), protein kinase C, calcium-calmodulin-dependent protein kinase, phosphoinosit (Terry et al., 1999; Tacchini et al., 2001) and protein kinase A (Durante et al., 1997; Immenschuh et al., 1998). The best-characterized pathways are those that involve heat-shock proteins, nuclear factor-κB (NF-κB), nuclear factor–erythroid 2-related factor 2 (Nrf2), and activator protein–1 (Alam & Den, 1992; Lavrovsky et al., 1994; Camhi et al., 1995; Alam et al., 1999). In contrast, HO-2 is constitutively expressed but it is considered to be a member of the glucocorticoid-regulated family of proteins that is specifically upregulated by adrenal glucocorticoids (Weber et al., 1994; Raju et al., 1997; Liu et al., 2000), opioids (Li & Clark, 2000) and estrogens (Tschugguel et al., 2001).

1.1.2 Heme Oxygenase and Cell Signalling

Over the past several years, evidence has accumulated revealing a major role of the HO/CO system in cytoprotection. Induction of HO-1 protein synthesis and activity has been characterized as a strong response to oxidative stress. The overall effect of HO action is to decrease the cellular concentration of cell toxicants and increase the concentration of cell protectants. Thus, HO activity decreases the cellular levels of heme, which is a pro-oxidant that catalyzes the decomposition of organic peroxides leading to the generation of alkyl peroxyl radicals that initiate membrane lipid peroxidation (Ryter & Tyrrell, 2000; Akaike et al., 1992; Sawa et al., 1999). The complementary effects of
elevating cellular concentrations of CO, biliverdin/bilirubin and iron are described below in terms of their cellular effects.

*Carbon Monoxide:* CO has been observed to activate, directly or indirectly, a number of intracellular signalling pathways that result in benefits for cells via its anti-inflammatory, anti-proliferative, anti-apoptotic and anti-thrombotic effects. Mechanisms that underlie most of these effects of CO include one or a combination of the following: increase in the synthesis of cyclic guanosine monophosphate (cGMP) through a direct activation of soluble guanylyl cyclase (sGC) (Furchgott & Jothianandan, 1991; Stone & Marletta, 1994), stimulation of calcium-dependent potassium channels (K\(_{\text{Ca}}^+\)) (Wang et al., 1997b), modulation of the activation of various mitogen-activated protein kinases that initiate a cascade of transcription regulatory mechanisms aimed at protection against oxidative stress (Otterbein et al., 2000; Amersi et al., 2002; Ryter & Choi, 2005; Wu & Wang, 2005). Other cellular targets of CO that may be unrelated to cytoprotection but are mediated via an interaction with a heme moiety resulting in inhibition include: hemoglobin, myoglobin, prostaglandin endoperoxide synthase, nitric oxide synthase (NOS), catalase, peroxidases, respiratory burst oxidase, pyrrolases, cytochrome c oxidase, cytochromes P450 (CYP450) and tryptophan dioxygenase (Wu & Wang, 2005). An intriguing feature of the interaction of CO with NOS is the cross talk that exists between the NOS and HO systems through the common interaction of NO and CO with sGC. The effectiveness of CO as an activator of sGC is only a fraction of that of NO; CO may be a partial agonist (and hence a partial antagonist) in this role (Furchgott & Jothianandan, 1991; Stone & Marletta, 1994). As a result, CO is most likely to activate sGC in cells or tissues with low NO production, and has the potential to decrease NO
activation of sGC (Kajimura et al., 2002; Teran et al., 2005). Conversely, studies in our laboratory (Kinobe et al., 2004) have shown that peroxynitrite, a product of upregulation in NO synthesis, directly inhibits HO activity thus decreasing the release of CO.

**Biliverdin and Bilirubin:** Biliverdin and bilirubin are products of HO activity with strong anti-oxidant properties because of their ability to scavenge free radicals including superoxide, peroxides, hydroxides, hypochlorous acid, singlet oxygen, nitroxides and peroxynitrite (Stocker et al., 1987; Stocker & Peterhans, 1989; Wagner et al., 1993; Kaur et al., 2003). One mechanism by which this could occur involves an oxidant taking C-10 methylene electrons from bilirubin, converting it to the low energy, resonance-stabilized compound, biliverdin; the latter is reduced rapidly back to bilirubin by biliverdin reductase (Baranano et al., 2002; Sedlak & Snyder, 2004). This allows for the reutilization of extremely low tissue bilirubin concentrations to quench much higher concentrations of oxidants in a manner that is similar to the cellular glutathione reductase system. On the other hand, there is evidence that biliverdin is not the major product of the reaction between bilirubin and oxyradicals, dioxygen and singlet oxygen species (Stocker, 2004). Thus, other plausible chemical mechanisms for regenerating bilirubin that do not require biliverdin reductase have been proposed (McDonagh & Assisi, 1972). While the mechanisms underlying the anti-oxidant properties of biliverdin and bilirubin may require further elucidation, the importance of these anti-oxidants has garnered substantial attention.

The direct or indirect effects of bilirubin on cellular oxidants have far reaching implications in the context of oxidative stress-mediated diseases. Sedlak and Snyder (2004) have addressed the association of low serum bilirubin concentrations with several
pathological conditions, including peripheral vascular disease (Kangas et al., 1999; Papadakis et al., 1999; Cerne et al., 2000; Ishizaka et al., 2001; Krijgsman et al., 2002), ischemic heart disease (Morita et al., 2001; Shimomura et al., 2002; Vitek et al., 2002), congestive heart failure (Hokamaki et al., 2004), respiratory distress, circulatory failure, sepsis, aspiration, and asphyxia (Benaron & Bowen, 1991).

Iron: In comparison to CO and biliverdin/bilirubin, the cytoprotective effects of free iron are less obvious because it can promote the production of intracellular reactive oxygen species (ROS) via lipid peroxidation chain reactions, nitric oxide-dependent nitrosylation of thiols, and the Haber-Weiss reaction (Halliwell & Gutteridge, 1992). The increase in ROS may cause oxidative preconditioning that triggers the activation of redox-sensitive signalling pathways with important consequences for inflammation, mitochondrial biogenesis, apoptosis and cell survival (Balla et al., 1993; Vile et al., 1994; Bilban et al., 2008). Most prominent is the ability of iron to bind to iron regulatory proteins (IRP1 and IRP2) thus influencing the stability of mRNAs corresponding to proteins such as ferritin H and L, transferrin receptor-1 and ferroportin-1 that are critical for the processing and trafficking of iron (Hentze & Kuhn, 1996; Domachowske, 1997). This mechanism alone accounts for most of the increase in the synthesis of ferritin that sequesters free intracellular iron following the induction of HO-1 activity (Vile & Tyrrell, 1993; Vogt et al., 1995).
1.2 TOOLS FOR THE ELUCIDATION OF THE PHYSIOLOGICAL ROLES OF HEME OXYGENASES

A primary approach to studying the role of any component of a biological system has been to determine the effects of perturbations to the component in question. Two that have stood the test of time are spontaneous genetic alterations in the form of genetic disease and the application of carefully selected drugs. A more recent and paradigm-altering complementary tool has been laboratory generated genetically altered animals. Genetic animal models in the form of HO-1 or HO-2 gene knockout as well as transgenic animals overexpressing HO have enhanced our current understanding of the HO/CO system.

1.2.1 Effects of Genetic Deficiency in HO-1 Activity

The most compelling evidence for the distinctive physiological role of HO-1 as well as HO-2 has emerged from studies with targeted deletion or disruption of HO-1 or HO-2 protein expression. In mouse models, total ablation of HO-1 activity was associated with anemia and abnormally low serum iron levels, macromolecular oxidative tissue damage, chronic inflammation and increased susceptibility to cardiac ischemia reperfusion injury, chronic renovascular hypertension, acute renal failure, atherosclerotic lesion formation, and a markedly reduced reproductive potential (Poss & Tonegawa, 1997; Shiraishi et al., 2000; Nath et al., 2001; Yoshida et al., 2001; Wiesel et al., 2001; Yet et al., 2003). Similarly, in the well-documented case of human HO-1 deficiency, a Japanese boy was diagnosed with anemia, leukocytosis, thrombocytosis, coagulopathies, tissue amyloid deposits and enhanced endothelial cell damage (Yachie et al., 1999; Kawashima et al., 2002). As a result, a thorough analysis of the human HO-1 gene
identified two potentially functional polymorphisms in the promoter region characterized by guanosine-thymidine (GT)$_n$ repeats and a single nucleotide (thymidine to adenosine) change in position 413 which modulates the level of HO-1 activity in response to a given stimulus (Yamada et al., 2000; Hirai et al., 2003; Ono et al., 2003). Individuals with the homozygous expression of the long (GT)$_n$ alleles (greater than 32 base pairs), show an inherent inability to transcriptionally-activate HO-1. These functional human HO-1 polymorphisms are associated with several haematological, cardiovascular, renal, pulmonary, neurological and obstetrical diseases (Exner et al., 2004).

Support for the physiological properties of HO-1 has also been generated by use of transgenic mouse models that overexpress HO-1 in a tissue specific manner. Expressing the human or rat HO-1 gene under the cardiac-specific mouse alpha-myosin heavy chain promoter was found to be protective against cardiac ischemia-reperfusion injury and apoptosis (Yet et al., 2001; Vulapalli et al., 2002), and allograft heart transplant rejection (Araujo et al., 2003). As most of the above mentioned studies documented the consequences of HO-1 disruption or activation with minimal or no interference with HO-2, it is possible to conclude that HO-1 serves important physiological functions independently of HO-2 activities.

1.2.2 Effects of HO-2 Gene Deletion

A limited number of studies have investigated the specific role of constitutively expressed HO-2 in physiological functions. Selective genetic deletion of the mouse HO-2 gene is associated with a host of pathophysiological conditions including: oxidative stress-induced neuronal cell death (Dore et al., 1999b; Chang et al., 2003; Chen &
Regan, 2004; Regan et al., 2004; Basuroy et al., 2006; Qu et al., 2007), cerebral ischemic injury (Dore et al., 2000; Goto et al., 2003; Namiranian et al., 2005), decreased tolerance and mechanical allodynia following chronic opioid administration (Liang et al., 2003), reduced nonadrenergic, noncholinergic neurotransmission in the enteric nervous system (Zakhary et al., 1997; Xue et al., 2000; Watkins et al., 2004), and ejaculatory abnormalities (Burnett et al., 1998). Noteworthy here is the fact that the tissue distribution of abnormalities linked to HO-2 ablation are limited to the nervous and male reproductive systems where HO-2 is expressed predominantly with little or no expression of HO-1 under basal conditions. This indicates that under certain circumstances such as ischemic brain injury (Dore et al., 1999a), HO-2 may serve specific roles that may not be compensated for by HO-1 activity. Systematically designed studies to compare effects of genetic deletions or the disruption of HO-1 as well as HO-2 activities in the same disease models are lacking but would be useful to confirm these contentions.

1.2.3 Catalytically-Independent Physiological Effects of HO-1 and HO-2

Another important consideration is that the physiological effects of HO isozymes may not be entirely attributed to their enzymatic activities, and genetic manipulations offer an added advantage in studying these aspects of the HO/CO system. A catalytically inactive HO-2 mutant (histidine-45-alanine) with an inability to bind heme in the active site was found to be protective against hydrogen peroxide-induced cytotoxicity in human embryonic kidney cells (Kim & Dore, 2005), and catalytically inactive HO-1 cDNA upregulated its own expression following exposure to oxidative stress in cultured NIH 3T3 cells (Lin et al., 2008). Additionally, a number of posttranslational protein modifications as well as protein-protein interactions may be involved in the overall
regulation of HO activity. This is illustrated by a reciprocal modulation of activities between HO-2 and calcium/calmodulin kinase and protein kinase C (Dore et al., 1999a; Boehning et al., 2003; Boehning et al., 2004). More recently it has been shown that hypoxia and other inducers of HO-1 result in protease-mediated C-terminal truncation of HO-1, its nuclear translocation and reduction of activity followed by the activation of important transcription factors for oxidative stress (Lin et al., 2007). Likewise, a potential HO-1–HO-2 binding interaction that results in decreased overall HO activity has been reported (Weng et al., 2003). Although the in vivo existence of HO-1/HO-2 protein interactions is yet to be validated, this process may represent a useful negative feedback mechanism for limiting HO activity when there is excessive activation of HO protein. On the whole, these studies indicate that there are some scantily understood, physiologically relevant, but catalytically-independent effects of HO-1 or HO-2 that warrant further investigation.

1.2.4 Challenges to the Use of Genetically Modified Animals

Knockout animal models have given us a unique window into the functions of HO-1 and HO-2. They provide a view of animals with chronic protein neutralization (lack of HO-1 or HO-2) unclouded by limitations associated with drug delivery (such as dosing, duration of action and efficacy); nevertheless they are imperfect, bringing an array of limiting and challenging anomalies. Most notable for HO-1 knockout animals is a high incidence of embryonic lethality coupled with poor survival rates because of their inability to handle stress (Poss & Tonegawa, 1997). The most commonly used technique for generation of HO-1 and HO-2 gene knockout models involves transfection of transformed gene constructs into early embryonic stem cells (Poss et al., 1995). This
precludes the assessment of developmental or tissue specific effects as the knockout animals carry the null allele globally and throughout life. A further practical limitation is the substantial cost associated with generating and maintaining knockout animal colonies. Thus, the complementary application of both pharmacological and genetic approaches provides a view that is truly greater than the sum of its parts.

1.2.5 HO-1 and HO-2 Gene Knockdown - RNA Interference

The discovery and development of RNA interference (RNAi) has become an attractive alternative to knockout animals when incomplete inhibition of gene expression (knockdown) is desired. Briefly, RNAi gene knockdown involves the creation of double-stranded short (20-25 base pairs) interfering RNA (siRNA) segments complimentary to the mRNA of interest. When transfected into cells the siRNA stimulates host cell machinery to destroy endogenous complementary mRNA and consequently decreases gene expression (Hamilton & Baulcombe, 1999; Elbashir et al., 2001). Using vector delivery systems with tissue- or substrate-specific promoters, tissue-specific and conditional gene knockdown is possible. This technique has been used in the HO/CO field to circumvent some of the difficulties associated with using HO-1 null mice. Segments of siRNA have been designed to specifically target either HO-1 or HO-2 isozyme and used to effectively reduce mRNA and protein expression in human embryonic kidney (HEK293A) (Williams et al., 2004; Miralem et al., 2005), THP-1 monocytes (Hsu et al., 2008), Hela and HepG2 cells (Ding et al., 2006) in vitro. The use of siRNA knockdown is also possible in vivo. Zhang et al. (2004) demonstrated that intranasal administration of HO-1 siRNA duplexes 16 hours before ischemia-reperfusion injury successfully reduced HO-1 protein expression and increased apoptosis in lung
Intravenous injection of HO-1 siRNA was successful in decreasing cobalt protoporphyrin (CoPP)-induced HO-1 activity in joint, lung and liver but not spleen tissue in mice (Benallaoua et al., 2007). With the use of lentiviral vectors it is also possible to transduce cells in vivo achieving sustained HO-1 knockdown, lasting up to 5 days after a single dose (Siner et al., 2007). However, RNAi is subject to off-targeting, where genes with incomplete complementarity are unintentionally downregulated. Using computational biology, it has been estimated that 10% of siRNA sequences are subject to off-target effects that can lead to misinterpretation of data and potential toxicity (Qiu et al., 2005). The development of new siRNA design algorithms should reduce these incidences. Although it seems that genetic approaches to altering gene expression and enzyme activity show much promise as scientific tools, pharmacological agents will likely remain the gold standard of treatment for the time being.
1.3 PHARMACOLOGICAL APPROACHES FOR MODULATION OF HEME OXYGENASE ACTIVITY

Much of our current knowledge of the physiological importance of the HO/CO system has been facilitated by the use of pharmacological approaches including HO mimetics (CO, biliverdin or bilirubin and inducers of HO expression) and chemical inhibitors of HO enzymatic activity. The use of drugs obviates some of the difficulties of using genetic approaches mentioned above and opens the door to therapeutic applications because of the historical acceptance of drug therapy. The HO/CO field is approaching a stage of maturity for which translation into practical treatments for human diseases can be contemplated.

1.3.1 Heme Oxygenase Activators and Mimetics

The first class of compounds used in HO research acted at the transcriptional level to increase enzyme activity or expression. The most frequently used HO inducers are CoPP and the HO substrate, heme. Heme and heme-analogues, such as CoPP, are likely to bind heme-responsive elements in the HO promoter region thereby increasing transcription. Interestingly, CoPP is a stronger inducer of HO expression than heme, producing almost twice as much mRNA over 4 hours (Smith et al., 1993). Conversely, during the initial 6 hours following administration, heme produces a greater increase in activity because it acts as a substrate as well as an inducer (Dercho et al., 2006). Caution must be taken when exploiting these porphyrins, as there are a number of well-known side effects associated with their use. As stated earlier, free heme is a pro-oxidant which has been shown to activate intracellular, vascular and endothelial leukocyte adhesion molecules leading to leukocyte recruitment, one of the hallmarks of inflammation.
Liver toxicities were also apparent in rats 24 hours after administration of 5 mg/kg CoPP as evidenced by increased serum levels of glutamic-pyruvate transaminase and lactate dehydrogenase, markers of hepatocellular injury (Schmidt, 2007).

An alternative to increasing HO expression and activity is to directly administer the products of HO degradation or agents that can mimic their action. This approach has led many researchers to explore the therapeutic potential of biliverdin/bilirubin and CO in various models of disease. For example, the presence of 10 nM bilirubin prevented H$_2$O$_2$-mediated oxidative stress-induced cell death in rat primary hippocampal neurons (Dore et al., 1999b). Perfusion with low levels (0.1-10 µM) of bilirubin has also been used to prevent ischemia-reperfusion injury in rat heart, liver and kidney in vitro (Clark et al., 2000; Fondevila et al., 2004; Adin et al., 2005). The beneficial effects of exogenous bilirubin may be organ or model specific however, as the protective effects were not duplicated with in vivo models of ischemia-reperfusion injury in the heart and kidney. It is also possible that the cytoprotective actions of HO are mediated through the synergistic effects of bilirubin and CO as suggested by Kirby et al. (2007) and demonstrated by Nakao et al. (2005), such that both are necessary to confer protection. The additive effects of biliverdin/bilirubin and CO have also been noted in models of organ transplantation. Bach and colleagues showed that treating the donor before, and the recipient after, organ transplant with biliverdin, inhaled CO (20 ppm) or both resulted in marked increases in survival over 7 (hepatic, cardiac), 14 (small bowel) and 100 (renal) days (Fondevila et al., 2004; Nakao et al., 2004; Nakao et al., 2005).

There is genuine interest in using CO as a therapeutic agent in cases such as lung
disease, sepsis, and organ transplantation as highlighted by various laboratories (Ryter & Choi, 2006; Hoetzel et al., 2007). Given the potential toxicity associated with CO inhalation, there is also constant debate as to whether or not a true safe, effective concentration exists (Choi & Dolinay, 2005; Thom et al., 2005). Carboxyhemoglobin (COHb) levels in healthy non-smoking adults lie between 0.3 and 3%, corresponding to a concentration of 0-6 ppm exhaled in the breath (Smith et al., 1986). Suggested doses of CO for therapeutic purposes range from 20 to 500 ppm (Song et al., 2003; Sarady et al., 2004) depending on exposure time and desired outcome, and result in COHb levels from 5 to 44%. At the low end of the range, 5% COHb may not result in any noticeable symptoms in healthy subjects. COHb levels of 44% are associated with severe headache, nausea, vomiting and syncope, while concentrations greater than 50% may induce coma and even death (Stewart, 1976). Clinical trials are currently underway investigating the pharmacokinetics and safety of CO inhalation in patients with kidney transplants, chronic obstructive pulmonary disease and lung inflammation. With regard to mode of delivery, CO gas can be administered via a ventilator circuit, facemask, or nasal cannula in much the same way as the therapeutic delivery of NO. A novel method of targeted tissue delivery of CO may be through the use of CO-releasing molecules (CORM). The most recent of these, CORM-3 [Ru(CO)₃Cl(glycinate)] and CORM A-1 [Na₂(H₃BCO₂)], are water-soluble and spontaneously release CO at physiological pH (Clark et al., 2003; Motterlini et al., 2005). These compounds have been used widely in HO/CO research and are good candidates for therapeutic development with phase I clinical trials proposed for 2010 (Motterlini et al., 2005).
1.3.2 Porphyrin-Based Heme Oxygenase Inhibitors

By and large, metalloporphyrins (such as tin protoporphyrin, SnPP; zinc protoporphyrin, ZnPP; and chromium mesoporphyrin) have been the major pharmacological tools used for decreasing HO activity, and a substantial amount of information on the role of HO in physiology has been elucidated by the use of these compounds (Vreman et al., 1993; Johnson et al., 1997; Lyall et al., 2000; Tulis et al., 2001). Nonetheless, increasing evidence shows that metalloporphyrins are associated with a wide range of undesired effects, mainly related to their close structural similarity to the substrate of HO, heme. In its association with a variety of proteins, heme has a breadth of functions ranging from participating in the principal activity (for example, binding oxygen in hemoglobin; catalyzing the transfer of atomic oxygen in CYP450) to regulating enzyme activity (sGC) to acting as the substrate (HO). A corollary to this is that metalloporphyrins, as close structural analogues of heme, have a number of activities in addition to their ability to inhibit HO. Most metalloporphyrins, including ZnPP and SnPP, have the ability to inhibit activities of some isoforms of NOS and sGC (Ignarro et al., 1984; Ny et al., 1995; Chakder et al., 1996), and G protein-coupled receptor-mediated smooth muscle relaxation by disrupting coupling of the receptor with adenylate cyclase or particulate guanylyl cyclase (Ny et al., 1995; Undem et al., 1996). Also, metalloporphyrins have been suggested to induce a variety of other effects including: the inhibition of voltage-dependent calcium channels via the production of free radicals (Linden et al., 1993), stimulation of the immune system by inducing mitogenicity in immune T cells (Novogrodsky et al., 1989), exertion of anti-inflammatory properties via inhibition of interleukin-1 activity (Nagai et al., 1992) and induction of HO-1 (Sardana &
Kappas, 1987; Morioka *et al.*, 2006; Abate *et al.*, 2007). All these effects directly interfere with crucial signalling pathways that may involve CO as well as other metabolic products of HO activity. For instance, CO directly activates both sGC and voltage-dependent calcium channels while bilirubin exerts anti-inflammatory properties. The lack of selectivity therefore, has led some laboratories (Luo & Vincent, 1994; Grundemar & Ny, 1997; Cary & Marletta, 2001) to conclude that metalloporphyrins should not be used to establish a role for the HO/CO system in physiological processes. Moreover, several of the non-specific effects of metalloporphyrins occur within the concentration range used in many studies on the physiological role of the HO/CO system. A previous student in the laboratory investigated the selectivity of several metalloporphyrins and found that a number of them were useful tools *in vitro*, provided that they were used in a defined concentration range (Appleton *et al.*, 1999). Thus, the challenges presented by the lack of selectivity of the metalloporphyrins have presented obstacles towards progress in this field but they cannot be said to invalidate the work even if they limit interpretations of the data.

1.3.3 Non-Porphyrin-Based Heme Oxygenase Inhibitors

To enhance progress in the HO/CO field, it is important to have available more selective HO inhibitors that can be exploited to generate data whose interpretations are not complicated by the confounding effects of the metalloporphyrins. Some alternatives to the metalloporphyrins have been suggested. In this vein, Cuturi *et al.*, (1999) identified a novel peptide (RDP1258) with HO inhibitory activity. Unfortunately, these peptides were found also to potently induce the expression of HO-1, an action that compromises their utility as HO inhibitors. In other studies, the inhibition of HO activity
by azalanstat, an imidazole-dioxolane, non-porphyrin compound, was observed (DeNagel et al., 1998; Morisawa et al., 2008). Using azalanstat as a lead compound, we initiated a program to design novel HO inhibitors that were not based on the porphyrin nucleus with the objective of creating more selective HO inhibitors. To date, our laboratory has synthesized and partially evaluated more than 300 imidazole-based compounds for their effects on HO activity (Vlahakis et al., 2005; Kinobe et al., 2006b; Vlahakis et al., 2006; Roman et al., 2007; Vlahakis et al., 2009; Roman et al., 2010). For the in vitro evaluation of HO inhibitory activity, rat spleen and rat brain were used as the source of native HO-1 and HO-2, respectively. The most pertinent properties of these compounds compared with the metallloporphyrins are that they do not inhibit NOS or sGC activities except at higher (millimolar) concentrations; they do not induce the expression of NOS, sGC, HO-1 or HO-2; and some of these compounds are very selective for HO-1 with selectivity indices greater than 300 relative to HO-2 in vitro (The basis for QC-15 and QC-56 being referred to as HO-1-selective inhibitors; see Chapter 3: Discussion for further discourse). Thus, these compounds should be invaluable for investigations of the involvement of HO in physiological processes. On the other hand, a substantial amount of work remains to be done to fully characterize these newer imidazole-based HO inhibitors. One of the main objectives of this thesis is to determine the pharmacokinetic and pharmacodynamic properties of a lead azole-based heme oxygenase inhibitor in vivo.
1.4 EXCESSIVE ACTIVATION OF THE HEME OXYGENASE-CARBON MONOXIDE SYSTEM

A growing body of literature cautions that over-activation of the HO/CO system may have detrimental effects to normal homeostasis and that HO-1, in particular, may not be exclusively cytoprotective but may contribute to tissue injury under certain unfavourable circumstances (Platt & Nath, 1998). Direct clinical toxicity of high doses of inhaled CO has been known for decades and the underlying mechanisms are primarily attributed to the high affinity of CO for hemoglobin and the resulting competition with oxygen, which is essential for tissue respiration (Yoon et al., 1998; Von Burg, 1999; Gorman et al., 2003). On the other hand, a host of clinical conditions with complex and enigmatic etiologies are associated with excessive upregulation of HO and products (iron, CO and bilirubin) of its metabolic activity.

1.4.1 Adverse Effects of Released Ferrous Iron

Free ferrous iron, as released by the enzymatic activity of HO, is a pro-oxidant that initiates oxidative stress and tissue injury (Dugue & Meunier, 1985; Wardman & Candeias, 1996). Accordingly, it has been proposed that there is a threshold to the induction and overexpression of HO-1 related to the accumulation of reactive iron released in the degradation of heme (Suttner & Dennery, 1999). Lending support to this idea, disruption of HO-1 expression was found to protect mice against hyperoxic lung injury; it was suggested that disruption of HO-1 protected against hyperoxia by diminishing iron-mediated generation of toxic reactive intermediates (Dennery et al., 2003). These findings have been corroborated by other studies in which the administration of an HO inhibitor, Zn-deuteroporphyrin-IX-2,4-bisethylene glycol,
resulted in the amelioration of pulmonary fibrosis in the bleomycin model of acute lung injury and fibrotic lung disorder (Atzori et al., 2004). Furthermore, in comparative studies of wild type and HO-1 knockout mice, induction of HO-1 resulted in the release of free iron that exacerbated inflammation, edema and brain injury following intracerebral hemorrhage (Wagner et al., 2000; Huang et al., 2002; Koeppen et al., 2004; Wang & Dore, 2007). The deleterious effects of an upregulation in the expression of HO-1 are also implicated in experimental allergic encephalomyelitis where extravasated blood is present in the brain, and iron released by HO activity from this heme source may not be adequately sequestered by ferritin allowing for iron-mediated tissue damage (Chakrabarty et al., 2003). Besides the acute hemorrhagic conditions mentioned above, an intriguing connection of HO-mediated release of free iron to neurodegenerative conditions such as Alzheimer’s and Parkinson’s disease has been made. Schipper and colleagues (2000) suggested that chronic overexpression of HO-1, and attendant liberation of intracellular free iron and CO, may contribute to the aberrant patterns of brain iron deposition and mitochondrial insufficiency documented in aging-related neurodegenerative disorders. All these studies support the notion that limiting heme or hemoglobin breakdown by inhibiting HO activity coupled with the use of iron chelators may offer a novel therapeutic strategy for the management of several brain injuries. In the same manner, pharmacological or genetic strategies that target HO-2 instead of HO-1 showed beneficial cytoprotective effects from heme-mediated oxidative injury in hemorrhagic nervous system disorders (Rogers et al., 2003; Regan et al., 2004; Qu et al., 2005). This indicates that the adverse effects of excessive HO activation are not solely attributed to the inducible HO-1 isozyme. In tissues that express predominantly HO-2,
such as the brain and the spinal cord, HO-2 as opposed to HO-1 may serve as a therapeutic target. Thus, in terms of therapeutic interventions for these brain disorders, a real potential exists for HO inhibitors with enhanced selectivity for the HO-2 isozyme.

1.4.2 Adverse Effects of Bilirubin

The injurious effects of undue activation of HO are not limited to the release of free ferrous iron as both CO and bilirubin may exhibit or aggravate disease-like conditions depending on body tissue and circumstances. In neonates and individuals with impaired hepatic disposition of bilirubin, such as seen in Crigler–Najjar type I syndrome for example, overexpression of HO-1 can lead to hyperbilirubinemia and the accompanying jaundice, kernicterus and encephalopathy. This neuronal-specific, cytotoxic effect of unconjugated bilirubin occurs through a mitochondrial-dependent pathway linking the opening of a permeability transition pore and release of cytochrome c, which initiates apoptotic cell death (Kappas et al., 1993; Rodrigues et al., 2000). Indeed, over a decade ago, the use of metalloporphyrins as therapeutic agents against hyperbilirubinemia was recommended (Drummond & Kappas, 1981; Galbraith et al., 1992; Martinez et al., 2001). Despite this potential clinical application of the metalloporphyrins, phototherapy and exchange blood transfusion have been the mainstay of managing hyperbilirubinemia (Tiribelli & Ostrow, 1996; Dennery, 2005). This may be due to the lack of selectivity associated with metalloporphyrins as therapeutic drug candidates. The most prominent undesired side effect of metalloporphyrins has been the induction of cutaneous photosensitivity arising from singlet oxygen damage (Land et al., 1988). In this context, a class of HO inhibitors that does not have a porphyrin nucleus may offer a useful pharmacological adjunct to presently available therapeutic strategies for controlling
episodes of severe jaundice.

1.4.3 Adverse Effects of Carbon Monoxide

Involvement of CO in the detrimental effects of excessive HO activation has been investigated in several studies. Exposure to low concentrations of CO was found to enhance apoptosis in porcine granulosa cells (Harada et al., 2004), and smooth muscle cells both in vitro and in vivo (Tulis et al., 2001; Liu et al., 2002). The detailed mechanisms that underlie these effects are not fully understood but some of the molecular interactions that may lead to deleterious effects of CO are being studied. For instance, it has been shown that in Jurkat T cells, Fas/CD95-induced cell death is augmented by exposure to CO and this is mediated by inhibition of extracellular regulated kinase and mitogen-activated protein kinase signalling pathways (Song et al., 2004). CO is also known to activate sGC and this may be detrimental in neuropathic pain where enhanced HO-2 activity in the brain and the spinal cord with an attendant CO-mediated elevation of cGMP may participate in thermal hyperalgesia and mechanical allodynia (Li & Clark, 2000; Li & Clark, 2003). Moreover, under these circumstances, the pain and mechanical allodynia were attenuated by SnPP (Li & Clark, 2001). Other situations where HO/CO can be considered to be detrimental to the organism would be those in which a pathology is dependent or is enhanced by the action of HO/CO. One such situation that has attracted considerable research attention is the development and progression of malignancies.
1.5 POTENTIAL THERAPEUTIC APPLICATIONS OF HEME OXYGENASE INHIBITORS

1.5.1 Heme Oxygenase and Tumour Growth

Cancer cells are unique in the sense that they proliferate uncontrollably. This, combined with the fact that they often lack a sufficient blood supply can result in cells from solid tumours becoming hypoxic and nutrient deficient. A number of laboratories have revealed the upregulation of HO-1 in certain solid tumours, such as prostate tumours (Maines & Abrahamsson, 1996), cerebral glioblastomas and astrocytomas (Hara et al., 1996), hepatocellular carcinomas (Abdel Aziz et al., 2008; Sass et al., 2008), renal cell carcinomas (Goodman et al., 1997), oral squamous cell carcinomas (Tsuji et al., 1999; Chang et al., 2004; Lee et al., 2008) and nasopharyngeal carcinomas (Shi & Fang, 2008), suggesting that increased HO-1 activity may be an important adaptation to these stresses in malignant tissue.

Upregulation of HO-1 in human cancer cells using viral transduction has been used to highlight the role of HO-1 in the growth, metastasis and survival of solid tumours in experimental animals. Subcutaneous implantation of a pancreatic cancer cell line (Panc-1) overexpressing human HO-1 in immune-deficient mice demonstrated accelerated tumour growth and increased occurrence of metastasis, effects that were completely abolished with HO-inhibition (Sunamura et al., 2003). Similar effects have been observed with B16 and Sk-mel188 melanoma cells (Was et al., 2006) and are usually associated with decreased survival of mice.

More recently, downregulation of HO-1 mRNA expression using siRNA was found to cause pronounced growth inhibition as well as increased sensitivity to radiotherapy and
chemotherapy of pancreatic cancer cells (Berberat et al., 2005) and SW 480 colon cancer cells (Fang et al., 2003; Fang et al., 2004). These results were further validated in vivo where HO-1 inhibition by siRNA resulted in increased cellular damage and apoptosis in tumour cells while reducing their proliferation and growth in a mouse orthotopic model of human hepatocellular carcinoma (Sass et al., 2008).

The metalloporphyrin class of HO inhibitors has been used in several in vivo models of tumour growth and mirrored the anti-tumour effects seen with HO-1 knockdown by siRNA. Targeted inhibition of HO by polyethylene glycol-conjugated ZnPP reduced the growth of Sarcoma-180 tumours in mice (Fang et al., 2003). Nowis et al. (2008) observed a statistically significant retardation of C-26 adenocarcinoma growth 6 days after the commencement of ZnPP treatment, culminating in a 70% reduction in tumour volume at the end of treatment (Nowis et al., 2008). These results further demonstrate that HO is an important target for anti-tumour therapy.

1.5.2 Tumour Vascularization

Angiogenic tumour vasculature represents one of the phenotypic hallmarks of most solid tumours (Folkman, 2007). The development of a vascular supply is essential for the supply of oxygen and nutrients necessary to drive tumour growth. Moreover, the tumour vasculature also plays a central role in the development of a metastatic phenotype. The tumour-associated vasculature represents an important target for novel cancer therapies and can be used to complement more traditional approaches, which have focused primarily on neoplastic cells.

As solid tumours grow they eventually reach a size (1–2 mm$^3$) whereby oxygen and
nutrient delivery by diffusion alone is insufficient. Regions of hypoxia develop to which
tumour cells respond by secreting diffusible pro-angiogenic factors in an effort to direct
blood vessel formation (Folkman et al., 1989). This represents a critical point in tumour
development, *i.e.*, the ‘angiogenic switch’. Under normal physiological circumstances,
more than 99% of endothelial cells are quiescent (Augustin et al., 1994). During
angiogenesis, endothelial cells are switched from a resting state to one of rapid growth in
a tightly controlled process involving a number of pro- and anti-angiogenic factors.
Examples of the former include vascular endothelial growth factor (VEGF), transforming
growth factor-beta-1 (TGF-β1), angiopoietin-1, basic fibroblast growth factor, platelet-
derived growth factor, hepatocyte growth factor and insulin-like growth factor-1 while
the latter include soluble VEGF receptor-1 (sVEGFR-1), soluble endoglin (sEng),
angiopoietin-2, angiostatin and vasostatin (Carmeliet & Jain, 2000). Each of these
factors represents a potential target for anti-angiogenic therapy.

1.5.2.1 Cellular Mechanisms of Tumour Vascularization: Role of Endothelial
Progenitor Cells

For over 30 years, the most widely accepted view whereby tumours acquire a blood
supply was the result of directed endothelial sprouting from pre-existing blood vessels,
termed angiogenesis (Ausprunk & Folkman, 1977). However, it is now apparent that
tumour vascularization is the result of several mechanisms including vasculogenesis,
vessel co-option, intussusception, vasculogenic mimicry and lymphangiogenesis (Dome
et al., 2007). Vasculogenesis, which is defined as formation of a *de novo* vascular system
from human endothelial progenitor cells (hEPC), was originally thought to play a critical
role in blood vessel formation only during embryogenesis. There has been controversy
surrounding the potential importance of hEPCs in the developing tumour vasculature, particularly because of the scarcity of these cells. Nevertheless, recent evidence has demonstrated that despite their low numbers, these bone marrow-derived circulating hEPCs are pivotal to the progression of avascular tumours becoming lethal and metastatic (Gao et al., 2008). A contributing factor to the difference in opinion is likely due to the difficulty associated with distinguishing hEPCs from mature endothelial cells. Phenotypic definition by specific molecules (Id1, VE-cadherin, VEGFR-2, CD31, CD13, and c-Kit, but absence of CD11b and CD45) has been used previously (Nolan et al., 2007), however, some of these markers are expressed by subsets of hematopoietic lineages as well (Bertolini et al., 2006). One characteristic feature of hEPCs is that unlike mature endothelial cells, hEPCs have significant potential for clonogenic expansion (Yoder et al., 2007). Since the initial discovery of circulating hEPCs (Asahara et al., 1999), numerous studies have observed that patients with various malignancies such as lung (Dome et al., 2006), hepatocellular (Ho et al., 2006), breast (Kollet et al., 2001) and colorectal (Willett et al., 2004) cancers, and myeloma multiplex (Zhang et al., 2005), myelofibrosis (Massa et al., 2005), non-Hodgkin’s lymphoma (Igreja et al., 2007), acute myeloid leukemia (Wierzbowska et al., 2005), and malignant gliomas (Zheng et al., 2007) have significantly higher levels of circulating hEPCs compared to normal individuals. For these reasons hEPCs are considered to represent another important target in tumour angiogenesis and metastasis.
1.5.2.2 Cellular Mechanisms of Tumour Vascularization: Role of Mesenchymal Stem Cells

There is a growing body of evidence to demonstrate that progressive tumours are associated with an infiltration of bone marrow-derived stem cells. Hypoxia along with mutations in cancer cells results in the secretion of chemotactic signals and pro-angiogenic factors that play a prominent role in the recruitment, migration and engraftment of putative vascular cells. It is believed that these cells may provide the groundwork for the development of the tumour vasculature. However, the identity of these factors and their source is unknown. Nonetheless, the contribution that bone marrow-derived stem cells make to tumour vascularization deserves our attention and represents an important target in anti-vascular therapies.

The tumour stromal microenvironment plays a critical role in facilitating the growth and spread of epithelial tumours (Bissell & Radisky, 2001). The stroma (mesenchyme) consists of several cell types ranging from fibroblasts, myofibroblasts, pericytes, mast cells, mesenchymal cells and macrophages. Recent evidence demonstrates that bone marrow-derived human mesenchymal stem cells (hMSC) are attracted to, and migrate towards the stroma of developing tumours (Dvorak, 1986; Hall et al., 2007). In a pre-clinical study, stromal fibroblasts present in human breast carcinomas were shown to promote tumour growth and angiogenesis through stromal cell-derived factor 1 (SDF-1α) secretion (Orimo et al., 2005). It is believed that SDF-1α secretion acts directly on CXC chemokine receptor 4 (CXCR4) located on mammary cancer cells resulting in increased proliferation. Moreover, CXCR4, the cognate receptor for the SDF-1α ligand is also found on circulating EPCs, which may be recruited to the tumour stroma contributing to
vascularization. Overexpression of SDF-1α results in the recruitment of blood circulating hEPCs and enhancement of vascularization (Yamaguchi et al., 2003) acting via HO-1-dependent mechanisms (Deshane et al., 2007). Karnoub and colleagues demonstrated that hMSCs within the tumour stroma were responsible for initiating the metastatic spread of breast cancer cells in a mouse model (Karnoub et al., 2007). The mechanism whereby hMSCs within the tumour microenvironment induce the metastatic spread of breast cancer cells is poorly understood. It is possible that hMSCs secrete pro-angiogenic growth factors that act to recruit circulating hEPCs to the tumour stroma resulting in tumour blood vessel formation. hMSCs represent another potential target for HO-inhibition-based anti-tumour therapy.

1.5.2.3 Heme Oxygenase and Vascular Endothelial Growth Factor

VEGF is touted as a key pro-angiogenic factor in tumour angiogenesis. Many human tumour biopsies exhibit enhanced expression of VEGF mRNAs by malignant cells and VEGF receptor mRNAs in adjacent endothelial cells. The first clinically useful therapy to inhibit VEGF-signalling and consequently the first anti-angiogenic drug was bevacizumab (Avastin), a humanized VEGF monoclonal antibody for combination therapy of colorectal, non-small cell lung and metastatic breast cancer. Since the introduction of bevacizumab, the FDA has approved other anti-angiogenic drugs such as sorafenib and sunitinib. They represent a different class of anti-angiogenic drugs that target multiple receptor tyrosine kinases, including VEGF receptors and platelet-derived growth factor (PDGF) receptors (Faivre et al., 2007). At least 20 angiogenesis inhibitors are currently being tested in human clinical trials. Most are in early phase I or II clinical studies with a few in phase III testing. With the exception of VEGF-Trap, a decoy VEGF
receptor, most of these are small-molecule-receptor-tyrosine kinase inhibitors (Verheul & Pinedo, 2007).

Recently, HO-1 has been demonstrated to be crucial in orchestrating VEGF-signalling in endothelial cells as well as supporting cells of the vasculature. Of the three HO metabolites (CO, biliverdin, iron), CO had the greatest influence on VEGF resulting in a 20-fold increase in VEGF protein (Dulak et al., 2002). In vascular smooth muscle cells, inhibition of HO activity by SnPP completely prevented cytokine- and hypoxia-induced VEGF generation while stimulation of HO-1 activity by hemin, enhanced VEGF production. Zinc deuteroporphyrin, another potent inhibitor of HO, decreased basal VEGF synthesis by approximately 80% (Dulak et al., 2002). Similar results were seen in human microvascular endothelial cells (HMEC-1) where hemin-induced VEGF production was completely prevented by incubation with SnPP; an effect not seen with copper protoporphyrin, which has no effect on HO activity. Additionally, overexpression of HO-1 in HMEC-1 using a plasmid elevated the secretion of VEGF protein into the culture media (Jozkowicz et al., 2002). These effects are associated with activation of the VEGF promoter.

1.5.2.4 Heme Oxygenase and Soluble fms-like Tyrosine Kinase-1

As described earlier, angiogenesis is a complex process and does not rely solely on the activity of one factor. Resistance to anti-angiogenic therapy can involve the upregulation of alternative pro-angiogenic factors such as basic fibroblast growth factor and SDF-1α or the downregulation of endogenous anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1; also known as soluble VEGFR-1) and sEng.
Approaches to overcome resistance might include combination therapy with drugs that inhibit other relevant targets, or single drugs that simultaneously inhibit multiple crucial targets. As a target, HO is especially attractive because it is involved in the regulation of more than one angiogenic factor. In addition to VEGF protein, HO can regulate the production of the soluble VEGF receptor, sFlt-1. Soluble Flt-1 binds and inhibits VEGF and placental growth factor (PlGF) and therefore acts as an endogenous anti-angiogenic factor. Tumours from human osteosarcoma G-292 cells transfected with retroviral vectors encoding soluble Flt-1 showed marked growth inhibition in an *in vivo* murine model (Yin *et al.*, 2008). Additionally, adenovirus-mediated delivery of sFlt-1 caused decreases in tumour growth and angiogenesis in a mouse xenograft model of multiple myeloma (Liu *et al.*, 2007). Cudmore *et al.* (2007) have shown that human umbilical vein endothelial cells (HUVEC) overexpressing HO-1 release significantly less sFlt-1. Conversely, inhibition of HO-1 activity using HO-1-siRNA or the HO-1 inhibitor, SnPP, resulted in increased VEGF-E-stimulated sFlt-1 production. Baseline levels of sFlt-1 are also higher in HO-1 knockout mice (Cudmore *et al.*, 2007). Results from these studies suggest that targeted inhibition of HO-1 activity may inhibit angiogenesis by increasing levels of the anti-angiogenic factor sFlt-1.
1.5.3 Statement of the Problem

From the knowledge base summarized above, it is evident that HO and its products, CO, bilirubin and iron, are important signalling molecules and participate in many physiological and pathological conditions. What is also clear is that much of that data was generated with the use of non-selective metalloporphyrin HO inhibitors, which limit the interpretation and the conclusions drawn from these experiments. There is also a growing interest in the specific role of HO-1 and as such, the need for an isozyme-selective HO inhibitor has never been greater. Of particular interest is the role of HO-1 in cancer biology. Although the etiology and mechanisms involved in malignancy are diverse, it is clear that some solid tumours demonstrate a dependence on HO-1 for growth, angiogenesis and metastasis and would therefore be likely to respond to anti-HO-1 therapy. For the first time we have access to compounds that can selectively inhibit HO-1 and will therefore address some of these important questions in the subsequent chapters.
1.6 HYPOTHESES AND OBJECTIVES

The general hypothesis for this thesis is that the azole-based heme-oxygenase inhibitors, QC-10 and QC-15, have pharmacological properties that make them useful research tools and potential therapeutic entities. Specifically, we hypothesize that the pharmacodynamic and pharmacokinetic properties of QC-10 and QC-15 make them useful alternatives to zinc protoporphyrin as the leading pharmacological tools used to elucidate the physiological and pathological roles of carbon monoxide and heme oxygenase. As a therapeutic entity, we hypothesize that treatment with QC-15 will inhibit cancer cell proliferation and angiogenic- and metastatic- like properties in vitro and tumour growth, angiogenesis and metastasis in vivo.

Objective 1: To develop and validate a non-invasive method to determine total heme oxygenase activity in rodents in vivo.

Objective 2: To characterize lead azole-based heme oxygenase inhibitors in terms of their in vivo activity, time course of action, selectivity, effect on HO-1 and HO-2 protein expression, mechanism of binding and reversibility.

Objective 3: To determine whether azole-based anti-fungal drugs inhibit heme oxygenase at therapeutically relevant concentrations.

Objective 4: To determine the effects of lead azole-based heme oxygenase inhibitors, QC-10 and QC-15, on in vitro models of cancer cell viability and angiogenesis.

Objective 5: To determine the effects of lead azole-based heme oxygenase inhibitor, QC-15, on an in vivo model of breast cancer growth, angiogenesis and metastasis.
1.7 STATEMENT CONCERNING QC-15 AND QC-56

Although several different azole-based heme oxygenase inhibitors were evaluated during the course of my thesis work, all of the experiments were designed to assess the properties of the lead HO-selective and HO-1-selective compounds, QC-10 and QC-15, respectively. There are a few experiments where QC-56 was used in place of QC-15. Originally, QC-56 was considered the lead HO-1-selective inhibitor; however, due to concerns regarding intellectual property, QC-15 was selected as a substitution for future experiments. QC-15 and QC-56 are identical in chemical structure except for a chlorine-bromine substitution (Figure 1.2). Previously published data, as well as data presented in this thesis where both QC-15 and QC-56 were used in the same experiments, conclude that both compounds exhibit equivalent pharmacokinetic and pharmacodynamic properties. Therefore, in the few experiments where only QC-56 was evaluated, it is a reasonable assumption that QC-15 would demonstrate similar results.

Figure 1.2 Chemical structure of QC-15 and QC-56 azole-based heme oxygenase inhibitors
CHAPTER 2

DETERMINATION OF *IN VIVO* CARBON MONOXIDE PRODUCTION IN LABORATORY ANIMALS VIA EXHALED AIR

INTRODUCTION

Once thought of as simply a waste product of heme catabolism, carbon monoxide (CO) continues to garner attention as its role is elucidated in physiological processes such as neurotransmission (Verma et al., 1993), olfactory function (Ingi & Ronnett, 1995), prevention of apoptosis (Liu et al., 2003) and ischemia-reperfusion injury (Katori et al., 2002; Vulapalli et al., 2002), as well as regulation of inflammation (Otterbein, 2002) and vascular tone (Furchgott & Jothianandan, 1991; Wang et al., 1997a).

Under normal physiological conditions, CO production occurs primarily through the oxidation of heme (Berk et al., 1974); a process which is controlled by the activity of heme oxygenase (HO) (Tenhunen et al., 1968) and the availability of heme substrate (Vreman et al., 2000). This process also generates equimolar amounts of iron and biliverdin, with the latter being quickly reduced to bilirubin via biliverdin reductase (Vreman et al., 1988). HO exists as inducible (HO-1) and constitutive (HO-2) isoforms and has been found in every nucleated cell in the body, which attests to HO's physiological importance (Vreman et al., 2002). Aside from its various physiological roles, HO is involved in a number of pathological processes as well. For example, it has been proposed that HO is necessary for the growth of certain solid tumours (Fang et al., 2003), progression of *Plasmodium falciparum* infection (Srivastava et al., 1998), and the development of hyperbilirubinemia (Galbraith et al., 1992; Yao & Stevenson, 1995). Integral to the further study of the physiological and pathological roles of HO (and its products), as well as the development of subsequent therapeutic strategies, is the ability to accurately assess the many aspects of the heme degradation pathway.
Much of the research concerning the functional contribution of HO to life has been focused on the determination of HO enzyme activity in vitro. This is accomplished through measurements of bilirubin and/or CO (Vreman et al., 2002). Bilirubin formation can be quantified photometrically using the difference in light absorption at 454 nm and 530 nm (Tenhunen et al., 1968; McNally et al., 2004) or by liquid scintillation counting following $^{14}$C-heme degradation and separation of $^{14}$C-bilirubin by thin layer chromatography (Sierra & Nutter, 1992). $^{14}$CO bound to hemoglobin can also be quantitated by liquid scintillation counting following administration of the heme precursor $^{14}$C-glycine to tissue (Motterlini et al., 1998; Sammut et al., 1998).

*In vitro* measurements of CO generation have also been a very versatile tool for assessing the potential of tissue preparations to degrade heme and the effects of HO inhibitors and inducers (Vreman et al., 2002). Both of these well-established methods involve incubating heme and NADPH with tissue homogenates or sonicates or broken or intact (Murphy et al., 1993) cell preparations in sealed reaction vials and quantitating the CO generated in the headspace by gas chromatography (GC) (Vreman & Stevenson, 1988; Vreman et al., 2000). This *in vitro* approach is invaluable in providing indices of a tissue's or cell’s capacity for degrading heme via the HO reaction. However, estimates of *in vivo* functioning of HO as indexed by CO production require studies with intact animals.

Being a relatively small, uncharged molecule, CO produced in cells freely crosses membranes, and equilibrates in the blood where it binds to hemoglobin to form carboxyhemoglobin (COHb). COHb is subsequently transported to the lungs where the CO is exchanged for $O_2$ and excreted in the breath. Under conditions of physiological
equilibrium, there exists a strong linear relationship between COHb and the rate at which CO is excreted (Fallstrom, 1968; Ostrander et al., 1976). Therefore, VeCO measurements represent an estimate of the rate of endogenous CO production or HO activity. The method was first reported by Sjöstrand in 1949 when he determined the endogenous formation rate of CO in man (Sjöstrand, 1949). Subsequently, it has been refined and used primarily as a research tool to elucidate the mechanism of the heme degradation pathway in the formation of bilirubin and development of jaundice or hyperbilirubinemia in newborns (Yao & Stevenson, 1995). The method has been used with human newborns (Landaw & Winchell, 1966; Bartoletti et al., 1979), monkeys (Vreman et al., 1990), mice (Stevenson et al., 1987), and rats (Vreman et al., 1993) for the study of jaundice, inhibitors of HO (metalloporphyrins, peptides), and xenobiotic degradation (Avertin®, methylene chloride) (Vreman et al., 2002). Nevertheless, this method has never been fully described in detail for general practice in the fields of pharmacology and toxicology.

Thus, in this communication, we describe the method for measuring VeCO as an index for the rates of heme degradation and CO production, and the effects that several established HO-manipulating drugs have on the VeCO of small laboratory animals. We also provide practical details for the assembly and use of the VeCO system.
MATERIALS AND METHODS

Small Animal Chambers

The mouse chamber (Figure 2.1A) consists of a modified 50 mL conical polypropylene tissue culture tube (10 x 2.5 cm i.d.; BD Biosciences, Franklin Lakes, NJ) with a screw cap. The pointed end and screw cap were fitted with a 10-32 to 1/16" brass NPT hose fitting (Bay Pneumatic Inc., San Carlos, CA) for attachment of 1/8 x 1/16" i.d. silicone tubing. Teflon® tape (Dupont, Wilmington, DE) was wrapped around the fitting threads to ensure a gas-tight seal. A small wad of cotton or other absorbent material was placed behind the animal before capping the tube in order to absorb urine and trap feces produced during experimental time period.

The rat chamber (Figure 2.1B) consists of a transparent, 770 mL acrylic plastic cylinder [20 x 7 cm i.d. x 3 mm (wall), Hendrik J. Vreman, Scientific Instruments & Services, Los Altos, CA] sealed at both ends with square acrylic endplates. The front endplate is permanently glued to the cylinder, while the back plate is removable to allow for animal access and cleaning. The chamber endplates are held together by four 22.5 cm x 1/4-20 NPT threaded galvanized steel rods and matching nuts. The removable endplate contains a recessed silicone gasket to ensure a gas-tight seal. The center of each endplate was fitted with a 10-32 to 1/16" brass NPT hose fitting for the attachment of 1/8 x 1/16" i.d. silicone inlet and outlet gas tubing. To maintain a clean surface for the animal, a removable stainless steel 4 x 4 mesh-welded cloth platform (19.5 x 4.5 cm) was affixed above the bottom of the cylinder, allowing for the passage of urine and feces to be absorbed into a strip of cotton or other absorbent material, placed under the platform.
Figure 2.1 Small Animal Chambers. The mouse chamber (A) consists of a 50 mL polypropylene centrifuge tube with screw cap which is fitted with hose fittings to accommodate connection of inlet and outlet tubing for air delivery and exhaust, respectively. The rat chamber (B) consists of a Plexiglas® cylinder with acrylic end plates held together by threaded galvanized steel rods and nuts. The removable entry plate was fitted with a recessed seal of silicone rubber and both plates have 1/16" brass hose connectors for connection of 1/16 i.d. x 1/4" silicone rubber inlet and outlet tubing.
Inlet Gas

The animal chambers are designed for the continuous flow-through of gas in order to allow real-time monitoring of CO production (Figure 2.2). Both types of chambers are supplied with a pressure- and flow-regulated supply of CO-free compressed medical grade air (Praxair, Danbury, CT and Praxair Canada Inc., Mississauga, ON) at a rate of 2.0 ± 0.05 and 0.4 ± 0.05 mL/min/g body weight (BW) for mouse and rat, respectively, through a flow restrictor proximal to the inlet port facing the animal. The inlet air may contain low (< 1 ppm) levels of CO as long as its concentration is known and the measured VeCO is corrected for it. Alternatively, the inlet air may be scrubbed of CO by passing it through a heated (120 °C) catalytic combustion filter (Trace Analytical, Inc., Menlo Park, CA) containing Hopcalite (a mixture of MnO and CuO, Scientific Instruments & Services), which oxidizes CO to CO₂.

Sampling Exhaust Gas

Exhaust gas from the animal chamber is first passed through a pressure control device, which consists of a 1/16" i.d. T-tubing connector, of which the downward pointing leg, fitted with a 10 cm length of 1/16" silicone tubing, is placed in an approximately 3 cm column of water in an upright test tube. The slight water pressure will cause the chamber exhaust gas to flow through the horizontal legs of the T when the analyzer valve with the sample loop is being loaded and there is no restriction in the flow path. However, during injection phase, when the sample loop and the valve's exhaust are closed to the chamber exhaust gas, this gas will then vent through the vertical T-leg and the short column of water, thus keeping the animal chamber at nearly ambient pressure.
Figure 2.2 Diagram of the VeCO determination apparatus. (a) compressed air tank; (b) two-stage pressure regulator; (c) flow controller; (d) hopcalite catalytic filter; (e) gas distribution manifold; (f) animal chamber; (g) exhaust outlet pressure control device; (h) chemical moisture trap; (i) CO analyzer (gas chromatograph, reduction gas detector, and chart recorder/integrator).
(3 cm H₂O). Distal to the exhaust outlet pressure control device is a moisture trap which consists of a 1 mL (for the mouse) or a 3 mL (for the rat) syringe filled to near capacity with desiccant [Anhydron®, magnesium perchlorate, (Fisher Scientific, Santa Clara, CA) laced with Coomassie Blue indicator (Eastman Kodak CO, Rochester, NY)] and contained between two plugs of glass fibre, to remove moisture from the chamber exhaust gas before it reaches the analyzer.

**Gas Analysis**

The exhaust gas line is connected to the reduction gas analyzer (RGA) injection valve fitted with a 2 mL sample loop. The analyzer is programmed through a timer (Scientific Services & Products) to load the sample loop with dehumidified chamber exhaust gas (during the 90 seconds it takes to analyze the previous sample), inject it onto the GC separating column by rerouting the GC carrier gas through the loop for 15 seconds, followed by analysis lasting 90 seconds. The analyzer (Model RGA2) consists of a gas chromatograph equipped with a 13X molecular sieve column, operated at 150 °C at a flow rate of 60 mL/min, and a reduction gas detector (Robinson and Robbins, 1968; Trace Analytical, Inc., formerly of Menlo Park, CA). Similar equipment and service is now available from Ametek, Inc. Newark, DE and Peak Laboratories, LLC, Mountain View, CA). The column separates CO from other possible exhaled gases such as H₂ (produced by bacteria in the gut of the animals) on the basis of molecular size (retention times of 0.7 and 0.25 minutes, respectively). The carrier gas with CO flows over a heated (260 °C) bed of HgO in the detector and reduces the HgO to volatile elemental Hg. The Hg-containing carrier stream is then passed through a 10 cm optical cuvette with associated UV light source and photodiode for measuring the absorbance of the Hg at
254 nm. The very high molar absorptivity of Hg gives the RGA its great sensitivity (parts per billion or nL CO/L). The detector CO (and H₂) peak signal is processed by an integrating recorder (Shimadzu Instruments, Columbia, MD) and displayed as peak profiles, height, and area (mV x sec). One VeCO measurement requires less than 2 minutes. The analyzer is calibrated each experimental day using 0 to 500 µL aliquots of certified CO standard (10.82 ppm, Scott Specialty Gases, Inc., Troy, MI). Standard curves (mV x sec vs. ppm) are linear from 0 to 1.08 ppm (representing 0 to 200 µL standard gas).

**Sampling from Multiple Animal Chambers**

To conduct experiments on several animals simultaneously, a number of animal chambers can be connected in parallel. Up to 12 animals in chambers can be studied simultaneously by connecting the gas source to a 12-position, polyvinyl chloride gas manifold with 10-32 to 1/16" NPT brass hose fittings (31 x 3 cm i.d., Scientific Services & Products). By capping unused outlet ports, this device can be used with up to 12 animals.

**Determination of the Validity of the Method**

We tested this method under normal (control), HO-1-induced, and HO-inhibited conditions, and demonstrated its sensitivity through the ability to distinguish a range of doses of compounds known to affect HO activity. This section outlines the protocol for one application of this method, *i.e.*, determining the effect of modulators of HO activity on the VeCO.
Animals

Prior to the commencement of this investigation, ethics approval was obtained through the Animal Care Committees of Stanford University and Queen’s University in accordance with the Institutional guidelines of Stanford University and the Canadian Council on Animal Care. Seven adult male BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). They had a mean weight of 20±0.5 g. Four adult male Sprague-Dawley rats and 3 adult male C57BL/6 mice purchased from Charles River Laboratories (Montreal, QC) had mean weights of 350 ± 10.0 and 20 ± 0.5 g, respectively. During their one-week acclimation period, the mice and rats were fed standard laboratory chow (ProLab RMH 3000, Agway Ltd., Syracuse, NY and LabDiet® 5001, Ren's Feed and Supply Ltd., Oakville, ON, Canada, respectively) and water ad libitum under a 12-hour light/dark cycle in a temperature-controlled room (25 °C).

Reagents

Because some of the metalloporphyrins are known to be light sensitive, solutions were prepared under subdued light on the day of experimentation (Vreman et al., 1993). Bovine hemin chloride (Sigma-Aldrich, St. Louis, MO), zinc protoporphyrin (ZnPP), and cobalt protoporphyrin (CoPP) (both from Frontier Scientific Inc., Logan, UT) were each dissolved in 500 µL of 10% w/w (1.6 M) ethanolamine (Sigma-Aldrich) and then diluted to 8 mL by the addition of 0.9% saline. It was then carefully titrated under stirring with 1 M HCl to a pH of 7.4, followed by addition of saline to 10.0 mL to yield 10.0 mM solutions for administration to the experimental animals.
Experimental Procedures

At 5:00 pm on the day prior to experimentation, food was removed from the animal’s cage. This food restriction was implemented in order to minimize the non-enzymatic, oxidative production of CO that can arise from feces and urine (Levitt et al., 1989). Restricting the food intake also decreases H\textsubscript{2} gas production which, when produced at high levels, can affect the separation of the CO peak and decrease the baseline resolution on the chromatogram. On the day of experimentation, mice (n = 7) or rats (n = 4) were placed in chambers and allowed to acclimate for 30 minutes before sampling began. Ten to twelve sequential chamber outlet air samples were analyzed during the next 150 minutes to determine baseline VeCO levels for each animal. Animals (n = 1 for each treatment) were then administered 5, 10, or 20 µmol/kg BW of either ZnPP or CoPP, intravenously (IV) via the tail vein. The seventh mouse served as a heme control and received an equal volume of metalloporphyrin vehicle. Subsequently, the mice were dosed with an IV injection of 30 µmol heme/kg BW. Following treatments, animals were returned to their chambers and sampling was continued (t = 0); a time course of CO production after treatment was recorded during the next 9 hours. Each animal's chamber was sampled and analyzed once every 10 to 15 minutes for the duration of the experiment.

Data for Figure 2.5 were obtained under the same experimental conditions described above with the exception that rats and mice were administered heme intraperitoneally (IP) and were not subject to metalloporphyrin administration.
Considerations

It is important to ensure constant and accurately known gas flow through the chambers. Not only does it provide animals with sufficient oxygen and remove CO$_2$ and CO, it also determines the extent of dilution of the breath CO sample stream. Fluctuations in flow rate can significantly affect the measured VeCO. A flow controller (Valco Instruments Co. Inc., Houston, TX) was used to provide a well-regulated flow rate with less than 1% fluctuation.

An opaque cover is placed over the chambers for the duration of the experiment to minimize stress on the animals, avoid possible photosensitizing effects of the administered drugs, and prevent photo-oxidative production of CO from the feces and urine (Levitt et al., 1989).

Calculations

VeCO [μL CO excreted per hour per kg body weight (μL/hr/kg BW)] is calculated as follows:

\[
\text{VeCO} = \left( \frac{\text{ppm (μL/L) CO in exhaust gas}}{} \times \frac{\text{Chamber Flow Rate (L/hr)}}{} \right) \times \frac{\text{Animal Weight (kg)}}{}
\]

The area under the curve (AUC) of the VeCO/time profiles in Figures 2.3 and 2.4 represent the accumulated CO produced above calculated baseline levels for the time period specified and was calculated using the trapezoidal rule (Notari et al., 1987).
RESULTS

The dose response and time courses of CO excretion for mice given ZnPP or CoPP, followed by 30 µM heme/kg BW appear in Figures 2.3 and 2.4, respectively. There was a pronounced elevation in VeCO immediately after heme administration, which reached a maximum of 197 µL/hr/kg BW after 75 minutes in the control (heme only) mouse.

Figure 2.3 demonstrates the ability of the method to detect variations in the rates of heme degradation due to HO inhibition as represented by the VeCO profiles. VeCO decreased with increasing doses of ZnPP, eventually reaching 32% inhibition with the 20 µmol/kg BW dose as determined by the AUC.

We also assessed the method’s sensitivity with respect to detection of small changes in CO production due to enzyme induction and increased substrate availability. Figure 2.4 depicts the time course of VeCO with increasing doses of the HO-1 inducer, CoPP. A 20 µmol CoPP/kg BW dose resulted in an 18% increase in VeCO as illustrated by the change in AUC.

Figure 2.5 illustrates the effects of heme administration to 4 Sprague-Dawley rats and 3 C57BL/6 mice. An IP injection of 30 µmol heme/kg BW increased VeCO to 429% and 563% respectively above baseline after 6 hours. The intra-day variability was determined by using the baseline VeCO data; for C57BL/6 Mice (n = 15) the values were 38.39 ± 1.97 uL/hr/kg BW, and for Sprague-Dawley Rats (n = 4) they were 17.15 ± 1.28 uL/hr/kg BW. The baseline values for assessing inter-day variability in C57BL/6 Mice
(n = 7) were 34.19 ± 2.70 uL/hr/kg BW, and in Sprague-Dawley Rats (n = 4) were 16.91 ± 1.37 uL/hr/kg BW.
Figure 2.3 VeCO (µL/hr/kg BW) time course for 4 adult male BALB/c mice before and after receiving 5, 10, or 20 µmol ZnPP/kg BW or vehicle IV at t = 0, immediately followed by 30 µmol heme/kg BW IV at 150 minutes. The average baseline (37 µL CO/hr/kg BW) was determined from the average VeCO from 0–150 minutes for each animal. Inset: Change in CO production (µL/kg BW) as compared to control animals determined by the AUC during the time period represented by the bordered line (t = 150–470 minutes).
Figure 2.4 VeCO (µL/hr/kg BW) time course for 4 adult male BALB/c mice before and after receiving 5, 10, or 20 µmol CoPP/kg BW or vehicle IV at $t = 0$, immediately followed by 30 µmol heme/kg BW IV at 150 minutes. The average baseline (38 µL CO/hr/kg BW) was determined from the average VeCO from 0–150 minutes for each animal. Inset: Change in CO production (µL/kg BW) as compared to control animals determined by the AUC during the time period represented by the bordered line ($t = 150–470$ minutes).
Figure 2.5 ΔVeCO (µL/hr/kg BW) time course of four adult male Sprague-Dawley rats (●) and three C57BL/6 mice (▼) after receiving 30 µmol heme/kg BW IP at 105 minutes. Data were normalized by subtracting the individual baseline values (determined from the average VeCO from 0–100 minutes) from the VeCO of each animal.
DISCUSSION

The measurement of a component in breath provides a distinct advantage over monitoring levels of other substances in the body tissues or fluids, because exhaled gas can be collected continuously with no disturbance to the subject. If the endogenous production of the gas is tied closely to a specific metabolic pathway, one can measure and use the rate of excretion of the gas as an estimate of the pathway activity. CO is an example of such a gas and is produced primarily through the heme degradation pathway catalyzed by HO. Over 85% of CO production in the body is mediated by HO, thus, quantitation of CO is an accurate means of estimating heme degradation, and CO and bilirubin formation (Bartoletti et al., 1979). The fact that CO is derived mainly through the actions of HO allows the determination of perturbations in this pathway when other non-heme CO-generating processes (i.e., photoreactivity, lipid peroxidation) have been controlled for and/or heme-derived processes are at steady state.

Except perhaps for the spleen, endogenous HO capacity in tissues appears to be in excess of substrate availability, which is the limiting factor in the generation of CO under normal conditions (Vreman et al., 2002). Since in vitro studies using tissue homogenates or sonicates involve the addition of excess substrate (heme), such results should be interpreted as measures of tissue HO capacity rather than an indication of endogenous rates of HO activity.

The method of determining the VeCO of mice and rats provides an example of a non-invasive technique of estimating the cumulative effects of organ HO activity towards the pool of available substrate (heme), which is demonstrated by the excretion of CO in
Variations in endogenous CO production in response to a heme dose following administration of known xenobiotics were reflected in changes in the animals’ VeCO (Figures 2.3, 2.4, 2.5). Administration of increasing concentrations of ZnPP, an established competitive inhibitor of HO activity (Vreman et al., 1993), resulted in decreased CO excretion (Figure 2.3). On the other hand, administration of CoPP resulted in dose-dependent increases in measured VeCO (Figure 2.4). CoPP is known to cause induction of HO-1 mRNA (Smith et al., 1993) while interfering with the formation of cytochromes P450 (Guzelian & Bissell, 1976) which could lead to increased HO-1 protein and heme availability, respectively, resulting in increased CO production. The results in Figure 2.5, which confirm earlier published results (Stevenson et al., 1979), demonstrate that the method is valid for the study of heme-mediated increases in VeCO in rat and mouse.

This method offers another approach to measure CO production in vivo in biological systems. Where other techniques for quantitation of HO catalyzed heme degradation products (bilirubin, COHb) in biological samples can be subject to interference (McNally et al., 2004), VeCO samples are relatively clean and do not require extensive sample preparation prior to quantitation. VeCO measurements can also be useful for comprehensive time course analyses, such as the examination of drugs that are thought to affect HO activity. In situations such as these where experiments require repetitive sampling, other methodologies are limited by the number of blood samples that can be drawn from an animal, such as for the determination of COHb (Rodgers et al., 1996). Measuring VeCO is non-invasive in that it does not require the collection of blood or tissue for analyte quantitation.
Another application of VeCO measurements is for the assessment of the progression of certain pathological states. Diseases such as sickle-cell anaemia (Sears et al., 2001), thalassemias (Solanki et al., 1988), glucose-6-phosphate dehydrogenase deficiency (Seidman et al., 1995) and hyperbilirubinemia (Yao & Stevenson, 1995; Vreman et al., 2002) can result in increasing levels of CO production, which correlate with the severity of the disease. Increased levels of CO production have been demonstrated to occur in these patient populations and have been studied with a variation of the VeCO method, the measurement of end tidal CO (Vreman et al., 1996a).

We used a GC with an RGA as a means to quantitate the VeCO in our system. It has high sensitivity (up to 1 ppb or nL/L) without sacrificing accuracy and is more efficient with respect to time and cost when compared to mass spectrometry (Marks et al., 2002; Vreman et al., 2002). This methodology, originally developed for use with human neonates, has also been used with monkeys, and with rodents because of their low cost, availability, size, and the ability to manipulate physiological conditions with various xenobiotics. Their small size and rapid breathing rates also allow more immediate detection of variations of CO production. The flexibility of this method allows it to theoretically accommodate almost any type of subject, simply by constructing an adequately sized chamber and adjusting gas flow and sampling rates. For example, slight modifications have allowed this method to determine the CO production from single-celled microorganisms (Engel et al., 1972). This method has been used clinically to estimate bilirubin production as a means to identify newborns susceptible to develop neonatal jaundice (Vreman et al., 2002). It is important to note that there are numerous variables that can alter HO activity including but not limited to the age, diet, and size of
the subject. The VeCO values reported were determined under the strict conditions outlined in this manuscript and are intended only as an example. For other applications, it will be necessary to establish new baselines.

As is demonstrated herein, VeCO can be used to accurately assess the efficacy and potency of inducers and/or inhibitors of HO activity \textit{in vivo}. Quantitating VeCO with the modified flow-through method is yet another tool available to elucidate the physiological roles of CO/HO \textit{in vivo} as well as their involvement in specific pathological states (Landaw, 1976).
ACKNOWLEDGEMENTS

This work was supported by the Canadian Institutes of Health Research and the Christopher Hess Research Fund and the H. M. Lui Research Fund (DKS, HJV, RJW).

RAD is a recipient of a Canadian Institutes of Health Research Studentship through the Gasotransmitter Research and Training Program.
CHAPTER 3

IN VIVO SELECTIVITY AND CHARACTERIZATION OF NOVEL AZOLE-BASED HEME OXYGENASE INHIBITORS

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To be submitted to the British Journal of Pharmacology
INTRODUCTION

Carbon monoxide (CO) is classically thought of as an environmental toxicant, however, over the last 20 years, research investigating physiological CO-signalling has changed this view dramatically. CO is produced endogenously from the catabolism of heme by the enzyme heme oxygenase (HO), which also produces biliverdin and iron (Tenhunen et al., 1968). Present as HO-1 (inducible) and HO-2 (constitutive) isoforms, HO and its products have been shown to be involved in many biological processes such as apoptosis (Liu et al., 2003), inflammation (Otterbein, 2002), angiogenesis (Sunamura et al., 2003; Deshane et al., 2007) and vascular tone (Sammut et al., 1998), to name a few.

CO acts similarly to another gaseous molecule, nitric oxide (NO). Both have a strong affinity for binding to heme iron and, therefore, interact with heme-containing proteins such as soluble guanylyl cyclase (sGC) (Stone & Marletta, 1994), nitric oxide synthase (NOS) and the cytochromes P450 (CYP450). CO is known to be a partial agonist for sGC (Schmidt, 1992), which is, in fact, the primary downstream effector for the vasodilatory effects of NO in the vasculature (Ignarro, 1990). Moreover, crosstalk between CO and NO is important to their function and they may mutually regulate their respective synthetic enzymes, HO and NOS (Lee & Yen, 2009). As a result of their similarity, distinguishing the biological roles of CO and NO is not trivial.

The research that has contributed much of our current understanding of the functions of HO and CO has liberally employed the metalloporphyrin-class of HO inhibitors, namely zinc-protoporphyrin (ZnPP) and tin-protoporphyrin. While these
inhibitors have proved to be an invaluable tool in HO/CO research, they must be used with prudence when investigating the involvement of HO in biological systems (Appleton et al., 1999). Metalloporphyrins have a close structural similarity to heme, the substrate for HO, and therefore bind reversibly in the active site, acting as competitive inhibitors (Vreman et al., 1989). As a result of this structural similarity, the metalloporphyrins have a tendency to bind and inhibit other targets of heme and heme-containing proteins such as NOS (Meffert et al., 1994), sGC (Ignarro et al., 1984) and CYP450 (Maines & Trakshel, 1992; Stout & Becker, 1988), which has led to some controversy concerning the interpretation of experimental results (Grundemar & Ny, 1997; Danziger, 2009). Furthermore, metalloporphyrin-based HO inhibitors do not demonstrate isozyme selectivity with regards to inhibition of HO-1 or HO-2.

As research in the area of CO-signalling continues to grow, so does the complexity of the questions asked. It has become apparent that HO-1 and HO-2 are differentially-regulated and have distinct roles in vivo; therefore tools that can isolate and examine these individual effects are becoming increasingly important. Genetic approaches such as gene-deletion and use of siRNA have helped in this regard but still have limited use with respect to temporal inhibition in vivo. We believe there is a clear need for more selective pharmacological inhibitors of HO and have thus attempted to address this in our research program. To date, our laboratory has identified over 300 novel azole-based compounds with a wide range of potency, efficacy and selectivity with respect to HO inhibition (Vlahakis et al., 2005; Kinobe et al., 2006b; Vlahakis et al., 2006; Roman et al., 2007; Vlahakis et al., 2009; Roman et al., 2010). These compounds, dubbed QC-xx, have demonstrated significant and selective inhibition of HO in purified HO-1 and HO-2
protein (Vukomanovic et al., 2010), tissue microsomes (Kinobe et al., 2006b) and cell culture systems (Kinobe et al., 2007).

This manuscript characterizes some of the effects of these novel azole-based HO inhibitors (abHOi) in vivo. We have chosen to investigate the analogues: QC-10 (imidazole-alcohol), QC-15 (chlorophenyl-imidazole-dioxolane), QC-56 (bromophenyl-imidazole-dioxolane), QC-86 (triazole-ketone) and QC-99 (methylated-imidazole) (Figure 3.1) based on their structural characteristics and in vitro efficacy and selectivity and have compared their in vivo efficacy to ZnPP, the most widely used metalloporphyrin. We show that abHOi demonstrate dose and time-dependent inhibition of HO for up to 12 hours in vivo. These compounds bind in a reversible manner, but contrary to metalloporphyrins, they do not induce HO-1 or HO-2 protein expression. At the highest dose tested in vivo (100 µMol/kg), QC-15 and QC-86 demonstrated HO selectivity; in that they did not inhibit NOS. However, the compounds tested to date do not demonstrate significant in vivo HO-1 isozyme selectivity. This study provides the necessary information to facilitate the use of azole-based selective HO inhibitors for in vivo research.
Figure 3.1 Chemical structures of azole-based heme oxygenase inhibitors. QC-10 (imidazole-alcohol), QC-15 (chlorophenyl-imidazole-dioxolane), QC-56 (bromophenyl-imidazole-dioxolane), QC-86 (triazole-ketone) and QC-99 (methylated-imidazole)
MATERIALS AND METHODS

Animals

Male C57BL/6 mice (18-22 g) and Sprague-Dawley rats (250-300 g) were purchased from Charles River Inc. (Montreal, QC, Canada). Male and female HO-1 knockout and wildtype (129sv x Balb/c) mice were a generous gift from the laboratory of Dr. Luis Melo, Queen’s University. Mice and rats were maintained on 12-hour light cycles and had ad libitum access to water and standard Ralston Purina mouse (5010) and laboratory (5001) chow, respectively (Ren’s Feed Supplies Ltd., Oakville, ON, Canada). All animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care and experimental protocols were approved by the Queen’s University Animal Care Committee.

Materials and Reagents

The abHOi: QC-10 (4-(4-chlorophenyl)-1-(1H-imidazol-1-yl)butan-2-ol hydrochloride), QC-15 (2-[2-(4-chlorophenyl)ethyl]-2-[(1H-imidazol-1-yl)methyl]-1,3-dioxolane hydrochloride), QC-56 (2-[2-(4-bromophenyl)ethyl]-2-[(1H-imidazol-1-yl)methyl]-1,3-dioxolane hydrochloride), QC-86 (4-phenyl-1-[1,2,4]triazol-1-yl-butan-2-one hydrochloride) and QC-99 (1-(2-methy-imidazol-1-yl)-4-phenyl-butan-2-one hydrochloride) were synthesized and then characterized by elemental analysis, mass spectrometry and nuclear magnetic resonance spectroscopy in the laboratory of a co-investigator, Dr. W. A. Szarek (Vlahakis et al., 2006; Roman et al., 2007; Roman et al., 2010). ZnPP (8,13-bis(vinyl)-3,7,12,17-tetramethyl-21H,23H-porphine-2,18-dipropionic acid zinc(II)) was purchased from Frontier Scientific, Inc. (Logan, UT, U.S.A.). Anti-
HO-1 (SPA-895) and anti-HO-2 (SPA-897) polyclonal antibodies and recombinant rat HO-1 (SPP 730) and human HO-2 (NSP-550) protein were obtained from Stressgen Biotechnologies Corp. (Victoria, BC, Canada) now Assay Designs, Inc. (Ann Arbor, MI, U.S.A.). N-nitro-L-arginine methyl ester (L-NAME), ethylenediamine tetra-acetic acid disodium (EDTA), hemin chloride, ethanolamine, bovine serum albumin (BSA) and reduced β-nicotinamide adenine dinucleotide (β-NADPH) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Glycerol, potassium hydroxide (KOH), potassium phosphate monobasic (KH$_2$PO$_4$) and sodium chloride (NaCl) were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada). Stock solutions of all chemicals were prepared fresh on the day of the experiment.

**Determination of In Vivo Carbon Monoxide Production in Mice via Exhaled Air**

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 HO inhibitors on VeCO and total CO production in mice 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). Mice 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 50 mL/min. Exhaust gas was directed to the injection valve of a TA 3000R reduction gas analyzer (Trace Analytical/Ametek, Newark, DE, U.S.A.) fitted with a 750 µl sample loop. Mice were acclimatized to the chambers for 30 minutes and the baseline VeCO was determined for 90 minutes. After drugs were administered, animals were returned to the chambers and VeCO was measured for a minimum of three additional hours. In all cases, treatment
with 30 µmol/kg heme (in the form of methemalbumin; 1.5 mM hemin chloride, 0.15 mM BSA dissolved in 0.05% v/v aqueous ethanolamine, pH 7.4) was administered intravenously (IV) via the tail vein to increase CO production and facilitate detection of an inhibitory effect of the HO inhibitors.

**Preparation of Tissue Microsomes and Quantification of Protein Expression**

Spleen and liver microsomal fractions were prepared according to previously described procedures (Appleton et al., 1999). Briefly, tissue homogenate (15% w/v) was prepared in ice-cold 20 mM KH$_2$PO$_4$ buffer (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 x g for 20 minutes at 4 °C, followed by centrifugation of the supernatant at 100,000 x g for 60 minutes at 4 °C. Microsomes (100,000 x g pellet) were resuspended in buffer (100 mM KH$_2$PO$_4$, 20% v/v glycerol and 1 mM EDTA, adjusted to pH 7.4) and then stored at -80 °C until used. In total, 20 µg of mouse liver was subjected to sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. The protein was transferred onto polyvinylidene difluoride Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) according to the method of Laemmli (1970) and nonspecific binding sites were blocked by incubating the membranes in PBS (pH 7.4) containing 10% (w/v) skimmed milk powder at 4 °C for 16–18 hours. The blots were then incubated with a 1:2000 dilution of the anti-HO-1 or anti-HO-2 antibodies. 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, low-range molecular weight markers (Bio-Rad, Hercules, CA, U.S.A.). Recombinant HO-1 and HO-2 protein 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 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 (Sigma).

**In Vitro Heme Oxygenase Activity Assay**

*In vitro* HO activity was determined by the quantification of CO formed from the degradation of methemalbumin according to the method of Vreman and Stevenson (1988). Briefly, HO reaction mixtures (150 µL, consisting of 0.5 mg protein/mL rat spleen microsomal fraction, 50 mM methemalbumin and 100 mM phosphate buffer (pH 7.4)) were dispensed into 3.5 mL amber glass vials (Chromatographic Specialties Inc., Brockville, ON, Canada), which were sealed with rubber septa and screw caps. Atmospheric CO was purged from the vials using CO-free air and the HO-mediated heme breakdown was initiated by the addition of β-NADPH at a final concentration of 1 mM and incubation at 37 °C. After 15 minutes, the reaction was stopped by instantly freezing the reaction mixture on pulverized dry ice and CO formation was measured by gas chromatography using a TA 3000R Process Gas Analyzer (Trace Analytical/Ametek).

**Data Analysis**

The dose- and time-dependent inhibition of HO by QC-10, the comparison of HO inhibitor efficacy, the effect of modulators of HO activity on HO-1 protein expression
and the effect of abHOi on NOS activity were all analyzed by one-way analysis of variance followed by a Dunnett’s post-hoc test. Treated vs. control conditions from the reversibility assay were analyzed by paired Student’s t-test. The effects of treatment and mouse phenotype were compared using two-way analysis of variance. All statistics were performed using Prism, version 4.0. $p$-values of $< 0.05$ were considered to be statistically significant.
RESULTS

Dose-Dependent Inhibition of In Vivo Heme Oxygenase Activity by an Azole-Based Heme Oxygenase Inhibitor

A single dose of QC-10 (1, 10, or 100 µmol/kg) or vehicle control (saline) was administered intraperitoneally (IP), after baseline VeCO was determined, and mice were returned to their chambers for 90 minutes. Heme (30 µmol/kg) was then administered IV and VeCO was measured for 5 hours. The administration of QC-10 resulted in a dose-dependent decrease in HO activity, as measured by VeCO (Figure 3.2). The addition of heme was followed by an immediate and marked increase in CO excretion that peaked at 90 minutes with a maximal VeCO of 187 µL/min/kg body weight. The area under the curve (AUC) above baseline was calculated for the first 90 minutes following heme administration to compare the total CO production for each dose (Figure 3.2 inset). Administration of 1, 10 and 100 µmol/kg QC-10 decreased HO activity by 8%, 24% and 85%, respectively, compared to control. Doses of 10 and 100 µmol/kg resulted in a statistically significant inhibition of HO activity in vivo ($p < 0.01; n = 4$).

Comparison of Relative Efficacies of Metalloporphyrin- and Azole-based Heme Oxygenase Inhibitors In Vivo

A selection of azole-based HO inhibitors and the representative metalloporphyrin-based HO inhibitor, ZnPP, were chosen (based on chemical structure and in vitro potency and selectivity) to test their ability to inhibit HO activity in vivo at a dose of 100 µmol/kg. Figure 3.3 compares the in vivo HO activity measured by the total heme-induced CO excretion over 90 minutes from mice after they received the indicated HO inhibitor.
Figure 3.2 *In vivo* inhibition of heme oxygenase activity by QC-10. HO activity was measured by CO excretion (VeCO; µL/hr/kg) of adult male C57BL/6 mice before and after receiving 1, 10, or 100 µmol/kg QC-10 or saline (control) IP 90 minutes prior to an injection of 30 µmol heme/kg IV at T = 0. Inset: Total CO production (µL/kg BW) as determined by the AUC from 0–90 minutes. Data were normalized to individual baseline values for each mouse. *p < 0.01; n = 4.
Figure 3.3 Efficacy of heme oxygenase inhibitors \textit{in vivo}. Mice were administered 100 µmol/kg HO inhibitor or saline (control) IP 90 minutes prior to an injection of 30 µmol heme/kg IV at \( t = 0 \). Heme oxygenase activity was estimated by measuring the total CO production (µL/kg BW) as determined by the AUC for 90 minutes after heme administration. Data were normalized to individual baseline values for each mouse (*\( p < 0.01; n = 3–12 \)). Inset: Correlation between HO inhibition \textit{in vivo} and HO-1 and HO-2 inhibition \textit{in vitro} (*\( p < 0.05 \)). Data are presented as the percent inhibition of HO-1 (closed) and HO-2 (open) for QC-10 (▼, △), QC-56 (●, ○), QC-15 (■, □), QC-86 (▲, △) and QC-99 (◆, ◇).
All compounds that were active in vitro also significantly inhibited HO activity in vivo (p < 0.01; n = 3–12). QC-10 was found to be the most efficacious compound at 100 µmol/kg, inhibiting total HO activity by 85%. Moreover, QC-99, which was inactive in vitro, did not produce a significant change in HO activity in vivo. The inset in Figure 3.3 illustrates the statistically significant correlation between in vivo HO and in vitro HO-1 (r² = 0.844) and HO-2 (r² = 0.937) efficacy (p < 0.05).

**Time Course of In Vivo Inhibitory Activity of Azole-based HO inhibitors**

To assess the time course of abHOi activity in vivo, mice were given an IP injection of 100 µmol/kg QC-10 after baseline VeCO were established. Mice then received 30 µmol/kg heme IV immediately (t = 0) or after 0.5, 1, 2, 4, 6, 8, or 12 hours and the resulting VeCO was measured for 90 minutes. HO activity assessed immediately after QC-10 administration was decreased by 80% while maximal HO inhibition (85%) occurred between 1 and 1.5 hours after drug administration. HO activity gradually returned to baseline levels over the 12-hour test period but remained significantly suppressed for at least 8 hours (Figure 3.4; p < 0.01; n = 3–12).

**Effect of Heme Oxygenase Inhibitors on HO-1 and HO-2 Protein Expression In Vivo**

To assess the effects of HO inhibitors on HO-1 and HO-2 protein expression in vivo 15 C57BL/6 mice were randomized to 1 of 5 treatment groups destined to receive either 100 µmol/kg ZnPP, QC-10, QC-15; 30 µmol/kg heme; or vehicle (saline) on days 1, 3, and 5. On day 6, mice were sacrificed by cervical dislocation and perfused with saline; organs were harvested, snap-frozen in liquid nitrogen and stored at -80 °C. Liver HO-1 protein expression from microsomal fractions was assessed by SDS–PAGE and
Figure 3.4 Time course of heme oxygenase inhibition by QC-10. C57BL/6 mice were administered 30 µmol heme/kg IV 0, 0.5, 1, 1.5, 2, 4, 6, 8 or 12 hours after receiving 100 µmol/kg QC-10 HO inhibitor or vehicle control (saline) IP. Heme oxygenase activity was estimated by measuring the total CO (µL/kg BW) excretion as determined by the AUC for 90 minutes after heme administration. Data were normalized to individual baseline values for each mouse. * indicates a statistically significant difference from vehicle control, p < 0.01; n = 3–12.
Western Blot analysis. Heme, a known inducer of HO-1, caused an over 800-fold increase in HO-1 protein (Figure 3.5A). The metalloporphyrin, ZnPP, also induced a statistically significant increase in HO-1 protein of over 300-fold ($p < 0.01$). Neither of the abHOi, QC-10 or QC-15 had any effect on HO-1 protein levels. Figure 3.5B illustrates a representative western blot. None of the compounds tested had any effect on HO-2 protein expression (data not shown).

**Reversible Binding of Azole-Based Heme Oxygenase Inhibitors**

To test the binding reversibility of a given abHOi, HO protein samples were incubated with QC-56 and subjected to dilution and dialysis assays. In the dialysis assay, parallel HO reaction mixtures were preincubated with 10 µM QC-56 for 10 minutes at 37 °C. Experimental samples were loaded into Slide-a-lyzer® dialysis cassettes (10 kDa molecular weight cutoff; Thermo Scientific, Rockford, IL, U.S.A.) and dialyzed against 250 volumes of dialysis buffer (50 mM methemalbumin in 100 mM phosphate buffer) designed to promote the dialysis of unbound QC-56. Control samples were treated identically except 10 µM QC-56 was added to the dialysis buffer to prevent dialysis of QC-56 from the reaction mixture. Samples were dialyzed for 24 hours at 4 °C with two changes of buffer. HO reaction mixtures subject to QC-56 dialysis showed significantly higher HO activity compared to controls (Figure 3.6A; $p < 0.01$; n = 3–4). In the dilution assay, a control sample (HO reaction mixture with 10 µM QC-56) and a pre-concentrated (10x concentrated reaction mixture with 100 µM QC-56) were incubated for 10 minutes at 37 °C in gas-tight vials to allow inhibitor–enzyme binding. The pre-concentrated sample was then diluted to the same concentration and volume as the control sample and both were purged of atmospheric CO. The HO enzymatic reaction was initiated and CO
Figure 3.5 A) HO-1 protein induction by heme and heme oxygenase inhibitors. Adult male C57BL/6 mice (n = 3) were injected IP with 30 µmol/kg heme, 100 µmol/kg ZnPP, 100 µmol/kg QC-10, 100 µmol/kg QC-15 or saline (control) on day 1, 3, 5. Mice were euthanized on day 6; livers were excised and microsomal fractions were subjected to SDS–PAGE and Western blotting for HO-1. * indicates a statistically significant difference compared to control (p < 0.01). Columns represent the average of 3 mice, bars represent the standard error of the mean. B) Representative Western blot.
Figure 3.6 Reversible binding of the heme oxygenase inhibitor QC-56. A) Rat spleen microsomes were mixed with 50 µM methemalbumin and 10 µM QC-56 and loaded into 10 kDa dialysis cassettes incubated in 250 volumes of 100 mM KH₂PO₄ buffer containing 50 µM methemalbumin with (control) or without (dialysis) 10 µM QC-56. Samples were incubated at 4 °C for 24 hours and dialysis buffer was changed twice. After dialysis, HO activity was measured by the average CO (pmol) produced from the microsomes incubated at 37 °C for 15 minutes after the addition 1 mM NADPH. B) Rat spleen microsomes were incubated with methemalbumin (50 µM, 500 µM) and QC-56 (10 µM, 100 µM) for control and pre-concentrated, respectively. After 10 minutes the pre-concentrated sample was diluted to the same concentration of methemalbumin and QC-56 as the control and both samples were administered 1 mM NADPH, further incubated for 10 minutes and CO production was measured as an estimate of HO activity. * indicates a statistically significant difference compared to control (p < 0.01). Columns represent the average of 3–4 experiments; bars represent the standard error of the mean.
formation was measured as described above. HO reaction mixtures pre-concentrated with 100 µM QC-56 for 10 minutes followed by dilution to 10 µM did not result in a statistically significant different level of HO activity compared to reaction vials with a constant inhibitor concentration of 10 µM (Figure 3.6B). Experiments were carried out a minimum of 3 times in triplicate.

**Effect of Azole-Based Heme Oxygenase Inhibitors on Nitric Oxide Synthase Activity *In Vivo***

Urine nitrate levels were measured as a means to quantify *in vivo* NOS activity in mice after treatment with abHOi. Mice were restricted access to food for 12 hours before the experiment to eliminate food-derived nitrates that can cause high background nitrate in the urine. Twelve male C57BL/6 mice were randomized to receive either 100 µmol/kg QC-15, QC-86 or vehicle (saline) IP. A parallel group of mice were administered the nitric oxide synthase inhibitor, L-NAME (40 mg/kg) as a positive control. Three hours after drug administration, urine was collected on plastic wrap according to the method of Kurien and Scofield for one hour (Kurien & Scofield, 1999). Urine was immediately snap-frozen in liquid nitrogen and stored at -80 °C. Urine nitrate measurement was performed using a commercially available colorimetric kit (Cat. No. 780001, Cayman Chemical Co., Ann Arbor, MI, U.S.A.). L-NAME administration resulted in a statistically significant 62% reduction in urine nitrate (data not shown). However, there was no difference in urine nitrate after administration of 100 µmol/kg QC-15 or QC-86 (Figure 3.7).
Figure 3.7  Azole-based heme oxygenase inhibitors do not inhibit nitric oxide synthase in vivo. Male C57BL/6 mice were injected IP with 100 µmol/kg QC-15, QC-86 or vehicle (saline) and urine was collected for one hour, 3 hours after drug administration. Columns represent the average of 4 mice; bars represent the standard error of the mean. There was no statistically significant difference between the groups.
In Vivo Heme Oxygenase Isozyme Selectivity of an Azole-Based Inhibitor

To assess the HO-1 isozyme selectivity of QC-56 in vivo, the degree of total HO inhibition after QC-56 administration in wild type and HO-1 knockout mice was compared. CO production from wild type mice represents the sum of HO-1 and HO-2 activity whereas CO production from HO-1 knockout mice is largely the result of HO-2 activity. Therefore, an HO-1-selective inhibitor would be expected to have much less efficacy in the HO-1 knockout animal. In vivo HO activity was quantified using the VeCO method described above. Total CO excretion for 90 minutes after heme administration was determined by calculating the AUC above baseline VeCO for each mouse. Total CO excretion was inhibited 32% and 64% by 10 µmol/kg and 100 µmol/kg QC-56, respectively, in wild-type mice. Mice lacking expression of functional HO-1 protein demonstrate a statistically significant 31% reduction in total HO activity as measured by CO production (Figure 3.8; p < 0.01; n = 3). Treatment of HO-1 knockout mice with 10 µmol/kg and 100 µmol/kg QC-56 resulted in dose-dependent inhibition of total heme oxygenase activity (24% and 61% inhibition, respectively; p < 0.05; n = 3). There was no significant difference in the degree to which 10 µmol/kg or 100 µmol/kg QC-56 inhibited the HO-1 knockout compared to the wild type mice.
Figure 3.8  Heme oxygenase-1 isozyme selectivity of QC-56 in vivo. Wild type (■) and HO-1 knock out (□) 129sv x Balb/c mice were administered 10 or 100 µmol/kg QC-56 HO inhibitor or vehicle control (saline) IP 60 minutes prior to an injection of 30 µmol heme/kg IV at $t = 0$. HO activity was estimated by measuring total CO production ($\mu$L/kg BW), as determined by the AUC, for 90 minutes after heme administration. Data were normalized to individual baseline values for each mouse. * and $\psi$ indicate a statistically significant difference compared to wild type ($p < 0.01$) and knockout ($p < 0.05$) control, respectively; $n = 3–4$. 
DISCUSSION

A representative selection of abHOi analogues was chosen from our library of compounds to characterize their inhibitory effects on HO in vivo. We hypothesized that these compounds would inhibit in vivo HO activity in a dose- and time-dependent manner and that the degree of inhibition would be comparable to that of the gold-standard metalloporphyrin-based HO inhibitor, ZnPP. We sought to further explore the mechanism of inhibition and the effect that repeated in vivo administration would have on HO-1 and HO-2 protein expression. Finally, we examined the NOS and HO isozyme selectivity of abHOi in vivo.

QC-10 was chosen to test in vivo because of numerous properties that increased its likelihood of being an effective in vivo inhibitor. With respect to bioavailability, QC-10 adheres to Lipinski’s rule of 5 (cLogP = 2.01 using OSIRIS property explorer; MW = 287.18 g/mol; H-bond donors = 1; H-bond acceptors = 3) (Lipinski et al., 2001) and is soluble in water and ethanol. It was also one of the most potent inhibitors of both HO-1 (IC$_{50}$ of 0.2 ± 0.02 µM) and HO-2 (IC$_{50}$ of 4.8 ± 0.6 µM) and did not significantly inhibit NOS or sGC activity in vitro (Vukomanovic et al., 2010). When administered IP to mice, QC-10 dose-dependently decreased total HO activity (Figure 3.2); up to 85% with a 100 µmol/kg dose. Moreover, this dose was well tolerated and has been administered to mice on alternating days for up to 6 weeks without any overt signs of toxicity (Dercho, unpublished observations). The time course of HO inhibition in Figure 3.4 shows that the inhibitory effects of QC-10 occur immediately after a single IP dose and reaches maximal inhibition after 1 hour. This suggests that QC-10 is rapidly
absorbed and distributed to tissues containing active HO. CO production and HO activity continue to be significantly inhibited (58%) when measured from 8 to 9.5 hours after administration and, after 12 to 13.5 hours, HO activity returned to 85% of control, although this was not statistically significant. These data highlight the functional time course of inhibition of these novel azole-based compounds in the whole animal.

In addition to QC-10, a number of other abHOi were selected to evaluate their in vivo efficacy. QC-15 and QC-56 are structurally similar analogues, differing only by a chlorine-bromine substitution; they were the most selective HO-1 inhibitors according to their relative IC\textsubscript{50} values for HO-1 (rat spleen microsomes) and HO-2 (rat brain microsomes) inhibition in vitro (Kinobe et al., 2006b). Figure 3.3 shows that QC-15 and QC-56 also demonstrated potent inhibition in vivo with reductions in total HO activity at 100 µmol/kg of 62% and 65%, respectively. In contrast to the aforementioned imidazoles (QC-10, QC-15, QC-56), QC-86 is part of a newer series of triazole compounds, which, unlike their imidazole counterparts, do not inhibit CYP3A4 or CYP2E1 (Hum, unpublished data), which is a common trait among azole-based compounds (Ahmed, 2000). QC-86 was found to be a less a potent HO inhibitor in vitro compared to the imidazoles and this trend was also true in vivo. QC-99 was selected as a negative control compound because it has close structural similarity to the other azoles tested but lacks any inhibitory activity in the in vitro HO assays. In QC-99, the 2-nitrogen of the imidazole is methylated. This increased steric hindrance is thought to prevent the coordination of the compound to the heme molecule and thus prevent binding. Similar to the in vitro data, QC-99 did not demonstrate any inhibitory activity in vivo. In fact, as the inset scatter plot in Figure 3.3 shows, efficacy of azole-based HO
inhibitors *in vitro* is a reliable predictor of efficacy *in vivo*. Lastly, the most commonly used metalloporphyrin, ZnPP, was also evaluated at a dose of 100 µmol/kg to compare the *in vivo* efficacy to the azole-based HO inhibitors. It was found to be equally efficacious (64% inhibition) to QC-15 and QC-56, but significantly less than QC-10. It is important to note that the experimental conditions of the *in vivo* HO assay may underestimate the inhibitory capacity of competitive inhibitors like ZnPP. ZnPP binds in the active site of HO thereby competing for binding with substrate. Increasing the heme concentration would shift the binding equilibrium of the two molecules in the direction of heme and therefore decrease the efficacy of a given concentration of ZnPP. Azole-based inhibitors bind in a non-competitive fashion and therefore binding affinity is independent of substrate concentration (Vukomanovic *et al.*, 2010). In the above experiment, it was necessary to administer heme to the mice prior to inhibitor testing to increase enzymatic activity and facilitate compound comparison. In an experiment performed at endogenous heme concentrations, however, one would predict that ZnPP would appear relatively more efficacious compared to abHOi.

In a recent publication, Roman *et al.*, (2010) discuss the co-crystallization of human HO-1 bound to the azole-based HO inhibitor, QC-86 (Roman *et al.*, 2010). This afforded a complex in which the inhibitor binds to the distal side of the heme and displaces a critical water ligand deemed essential for catalysis. It does so without competing with heme for binding to HO, corroborating the enzyme kinetic data from Vukomanovic *et al.*, (2010), which describes the abHOi as having a non-competitive binding mechanism. The experiments in Figure 3.6 were designed to determine the binding reversibility of these compounds and extend our understanding of the mechanisms of abHOi binding. The
dilution assay (Figure 3.6B) shows that when an HO reaction mixture was preincubated with 100 µM abHOi under conditions suitable for drug binding, and subsequently diluted to 10 µM the resulting HO activity was not significantly different from the control sample that contained a constant 10 µM inhibitor concentration. If the azole-based compounds bound to HO irreversibly, the HO activity in the pre-concentrated sample would have been significantly reduced. To further explore this hypothesis we conducted a complimentary experiment where HO reaction mixtures were incubated with 10 µM abHOi, loaded into dialysis cassettes and placed in a buffer designed to allow the dialysis of the unbound inhibitor. When compared to controls (where buffer did not allow inhibitor dialysis) the dialyzed HO reaction mixtures had significantly increased HO activity, comparable to samples incubated without abHOi. The results from these experiments suggest that the abHOi bind in a reversible manner. This is what would be predicted based on the work of Rahman et al., (2010) which concluded that abHOi bind through hydrogen bonding and van der Waals forces alone (Rahman et al., 2010).

With respect to their utility as pharmacological tools, the most relevant criticism of metalloporphyrin-based HO inhibitors is their lack of selectivity for HO. They are known to inhibit NOS (Meffert et al., 1994), which is involved in many of the same proposed biological signalling pathways as HO (Stone & Marletta, 1994). We have previously identified a number of abHOi that do not inhibit NOS at concentrations necessary for HO inhibition in vitro (Kinobe et al., 2006b). For abHOi to be considered more useful tools for the study of HO and CO biology, they should demonstrate selectivity for HO in vivo as well. Figure 3.7 shows that even the highest dose of abHOi used to inhibit HO activity did not inhibit nitrate production and, therefore, NOS activity
in vivo. A second criticism of metalloporphyrin HO inhibitors is that they are known to strongly induce HO-1 protein expression. This was confirmed in Figure 3.5 where mice administered repeated doses of ZnPP and expression of HO-1 in the liver greatly increased. Under the same experimental conditions, neither QC-10 nor QC-15 had any affect on in vivo HO-1 or HO-2 protein expression. These data will be important to consider when choosing between metalloporphyrins and abHOi for in vivo administration. As our knowledge base grows with regards to the function of HO and CO, there is increasing evidence for the distinct roles of the individual isozymes, HO-1 and HO-2. Along with genetic approaches, selective pharmacological inhibitors are an effective way to study these differences. Our most selective in vitro HO-1 inhibitor was chosen to determine if isozyme selectivity could also be achieved with in vivo administration. The experiment highlighted in Figure 3.8 was designed such that an HO-1-selective compound would be significantly less efficacious in an HO-1 knockout compared to a wildtype mouse. Our results show that the relative efficacy of 10 or 100 µmol/kg QC-56 was not significantly different between the two types of mice and therefore did not confer HO-1 selectivity at these doses. The physicochemical properties of QC-56 predict a high bioavailability in vivo (cLogP = 2.33 using OSIRIS property explorer; MW = 373.67 g/mol; H-bond donors = 0; H-bond acceptors = 4) (Lipinski et al., 2001). Thus, the intracellular concentration of QC-56 at the site of action may exceed the concentration range identified as being selective for HO-1 inhibition in vitro. It is also possible that the apparent loss of selectivity in vivo was due to the metabolic removal of a crucial functional group, rendering a functional HO-inhibiting metabolite without isozyme selectivity. Although we are actively investigating the structure-activity
relationship between the new abHOi analogues and HO, we are unable to say with certainty which functional groups are responsible for the documented *in vitro* selectivity of QC-56.

**Conclusion**

As we continue the pursuit of more potent, efficacious and selective HO inhibitors, this manuscript characterizes the pharmacodynamic properties of the lead abHOi thus far. It also highlights some key advantages such as greater HO selectivity and negligible effect on HO-1 protein expression. The identification of these novel compounds now provides a more selective alternative to metalloporphyrin HO inhibitors and will prove to be an invaluable pharmacological tool in HO/CO research. Future investigation is directed towards identifying more potent and selective abHOi and testing them in disease states in which selective inhibition of HO may prove to be a useful therapeutic strategy.
ACKNOWLEDGEMENTS

The authors would like to thank Miriam Katzman and Kristin McCabe for technical assistance. This work was supported by the Canadian Institutes of Health Research. RAD is a recipient of a Canadian Institutes of Health Research Studentship through the Gasotransmitter Research and Training Program.
CHAPTER 4

INHIBITION OF THE ENZYMATIC ACTIVITY OF HEME OXYGENASES BY AZOLE-BASED ANTIFUNGAL DRUGS

Adapted from:


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 (Rogaine®), which was originally touted as an antihypertensive agent. Recently, the antifungal agent ketoconazole (KTZ) has been reported to have anti-tumor effects in prostate cancer (Wilkinson & Chodak, 2004). In this application, the rationale for the use of KTZ lies in its ability to interfere with the synthesis of testosterone, which is considered to be associated with approximately 75% of prostate cancers. Interestingly, KTZ has been shown to be an effective adjunctive therapy in patients with androgen-independent prostate cancer where the inhibition of testosterone synthesis would not be anticipated to be a factor (Eichenberger & Trachtenberg, 1989; Eichenberger et al., 1989a).

Of the various potential mechanisms to explain this effect, we considered an involvement of heme oxygenase-1 (HO-1) because an increase in HO-1 protein expression has been observed in a variety of tumors, such as human hyperplastic and undifferentiated malignant prostate tissue (Maines & Abrahamsson, 1996), and there is mounting evidence that many solid tumors 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 number of stimuli, including heat shock, heavy metals, heme, ionizing radiation, reactive oxygen species, and pro-inflammatory cytokines, whereas the constitutive HO-2 is mainly expressed in the brain and testes (Braggins et al., 1986; Maines, 1988). The HO/CO system has been broadly accepted as an important 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 & Jothianandan, 1991), platelet aggregation (Mansouri & Perry, 1982), and the mitogen-activated protein kinase signalling pathway, leading to anti-apoptotic effects in endothelial cells (Soares et al., 2002) and anti-proliferative effects in smooth muscle cells (Morita et al., 1997; Peyton et al., 2002).

In tumor cells in vitro, Fang et al. (2004) demonstrated that a well-known HO inhibitor, zinc protoporphyrin (ZnPP), in its polyethylene glycol-conjugated form, exhibited HO inhibitory activity, and this resulted in increased oxidative stress and apoptosis (Fang et al., 2004). In the course of designing a series of novel HO inhibitors (Vlahakis et al., 2005; Vlahakis 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 ZnPP, namely HO inhibition.

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

Materials

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

Animals

Male Sprague-Dawley rats (250–300 g) and male C57BL/6 mice (18–22 g) were obtained from Charles River Inc. (Montreal, QC, Canada). The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care and experimental protocols were approved by the Queen’s University Animal Care Committee. Mice and rats were maintained on 12-hour light cycles and had \textit{ad libitum} access to water and standard Ralston Purina mouse (5010) and laboratory (5001) chow, respectively (Ren’s Feed Supplies Ltd., Oakville, ON, Canada).
Human Spleen Tissue

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

Preparation of Cytosolic and Microsomal Fractions

Microsomal fractions were prepared from rat brain and spleen and human spleen by differential centrifugation according to procedures described by Appleton et al., 1999. Microsomal fractions (100,000 x g pellet) were washed twice followed by resuspension in buffer containing 100 mM KH$_2$PO$_4$, 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 brain 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 Heme Oxygenase 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). In brief, reaction mixtures (150 µL) consisting of 100 mM phosphate buffer, pH 7.4, 50 mM methemalbumin, and 1 mg/mL protein were preincubated with the vehicle (DMSO), in which the drugs were dissolved, or the antifungal drugs at final concentrations ranging from 0.1 to 100 µM for 10 minutes 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 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/Ametek).

**Measurement of Heme Oxygenase 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., 1989). 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., (1989) as modified by Dercho et al. (2006). Mice and rats 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 50 or 130 mL/min, respectively. Exhaust gas was directed to the injection valve of a TA 3000R reduction gas analyzer (Trace Analytical/Ametek) 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 (IP). Forty minutes after the administration of KTZ, 30 µmol/kg heme (hemin chloride dissolved in 0.05% v/v aqueous ethanolamine, pH 7.4) was administered IP. The animals were then returned to the chambers, and VeCO was measured for an additional 6 hours. Control animals were treated with only heme and polyethylene glycol-400, in which KTZ was dissolved. Treatment with heme was used to increase CO production and to facilitate detection of an inhibitory effect of KTZ.
Measurement of Nitric Oxide Synthase Enzymatic Activity

The effect of KTZ on rat brain NOS activity in vitro was assayed by monitoring the conversion of $[^{14}\text{C}]$-L-arginine into $[^{14}\text{C}]$-L-citrulline according to a modification of previously outlined procedures (Brien et al., 1995; Kimura et al., 1996). The reaction mixture consisted of 50 mM HEPES, pH 7.4, 1 mM EDTA, 1.25 mM CaCl$_2$, 2 mM $\beta$-NADPH, and 2 mg/mL cytosolic protein in a total volume of 200 $\mu$L. KTZ was tested at final concentrations ranging from 0.001 to 0.25 mM, and control reactions contained equivalent amounts of DMSO in which KTZ was dissolved. Total organic solvent concentration was maintained at 1% (v/v) of the final volume in all cases. NOS activity in the reaction mixture was initiated by adding L-arginine/$[^{14}\text{C}]$-L-arginine at a final concentration of 30 $\mu$M and 35,000 dpm of $[^{14}\text{C}]$-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 nanomoles of $[^{14}\text{C}]$-L-citrulline formed/milligram of protein/hour.

Measurement of Cytochrome P450 Reductase Enzymatic Activity

Rat spleen microsomal cytochrome P450 reductase (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 $\mu$M NADPH, 100 $\mu$M cytochrome c, and 150 $\mu$g/mL microsomal protein according to the method of Yasukochi and Masters (Yasukochi & Masters, 1976). Incubations were performed at 25 °C for 15 minutes, and KTZ (1–250 $\mu$M) 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 \( \mu \text{M}^{-1}\text{cm}^{-1} \).

**Data Analysis**

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

Effects of Azole-Based Antifungal Agents on In Vitro Heme Oxygenase Activity

Commonly used antimycotic drugs, including terconazole, ketoconazole, sulconazole, isoconazole, econazole, miconazole, fluconazole, clotrimazole, and griseofulvin, were screened for the inhibition of the enzymatic activities of HO-1 (rat spleen microsomes) and HO-2 (rat brain microsomes) in vitro (table 4.1). With the exception of griseofulvin, in which an azole moiety is lacking, all of the antifungal drugs tested showed potent inhibition of HO activity, with some selectivity for HO-1 over HO-2. Terconazole, sulconazole nitrate, and ketoconazole were the most potent compounds with IC$_{50}$ values of 0.41 ± 0.01, 1.1 ± 0.4, and 0.3 ± 0.1 µM for HO-1, respectively (table 4.1). 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 Ketoconazole on Heme Oxygenase Activity In Vivo

Adult male mice and rats treated with heme (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 IP) led to a concentration- and time-dependent decrease in VeCO for up to 6 hours after treatment (Figure 4.1). A significant decrease in VeCO was observed for at least 4.5 hours after the administration of a single dose of KTZ (100 µmol/kg IP) ($p < 0.05$) and a maximal decrease in VeCO of approximately 50 ± 2.2% and 55 ± 2.0% for mice and rats, respectively, as measured by evaluation of the average area under the curve for three different animals.
IC₅₀ values for the inhibition of HO activity *in vitro*, mean ± SD (µM), (n = 3)

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Chemical structure</th>
<th>Rat spleen</th>
<th>Rat brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>ketoconazole</td>
<td><img src="image1.png" alt="Chemical structure" /></td>
<td>0.3 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>terconazole</td>
<td><img src="image2.png" alt="Chemical structure" /></td>
<td>0.41 ± 0.01</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>sulconazole nitrate</td>
<td><img src="image3.png" alt="Chemical structure" /></td>
<td>1.1 ± 0.4</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>isoconazole</td>
<td><img src="image4.png" alt="Chemical structure" /></td>
<td>5.6 ± 0.1</td>
<td>32.6 ± 5</td>
</tr>
<tr>
<td>miconazole</td>
<td><img src="image5.png" alt="Chemical structure" /></td>
<td>5.8 ± 0.8</td>
<td>45 ± 16</td>
</tr>
</tbody>
</table>
### Table 1. Inhibitory potency of the azole-based antifungal drugs and griseofulvin against rat spleen microsomal and rat brain microsomal HO activity in vitro.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibitory Potency (IC50, μM)</th>
<th>Rat Spleen</th>
<th>Rat Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>econazole nitrate</td>
<td>16 ± 2</td>
<td>49 ± 3</td>
<td></td>
</tr>
<tr>
<td>clotrimazole</td>
<td>35 ± 2</td>
<td>&gt; 100</td>
<td></td>
</tr>
<tr>
<td>fluconazole</td>
<td>80 ± 6</td>
<td>&gt; 100</td>
<td></td>
</tr>
<tr>
<td>griseofulvin</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1  Ketoconazole-induced inhibition of CO production by mice (A) and rats (B) *in vivo*. VeCO (µL/hr/kg) was determined by sampling exhaled air in four adult male C57Bl/6 mice (A) or Sprague-Dawley rats (B) before and after receiving 1, 10, or 100 µmol/kg ketoconazole or vehicle IP at \( t = 0 \) followed by 30 µmol of heme/kg IP at 40 minutes. Data were normalized to the individual baseline values for each animal. Inset, Cumulative CO production by area under the curve calculated during the time represented by the dotted line.
Effect of Ketoconazole on the Enzymatic Activity of Nitric Oxide Synthase and Cytochrome P450 Reductase

To determine the selectivity for the inhibition of HO over CPR and NOS, the effects of KTZ on in vitro catalytic activities of human spleen microsomal HO and CPR and rat brain NOS were examined. At concentrations ranging from 1 to 250 µM, KTZ did not alter CPR activity, whereas both human spleen HO and rat brain NOS activities were inhibited with IC$_{50}$ values of (6.3 ± 1.3 µM, $n = 2$) and (177 ± 3 µM, $n = 3$), respectively (Figure 4.2). A limited concentration range (1–25 µM) of KTZ was found to selectively inhibit HO without any significant effect on NOS activity ($p < 0.005$).
Figure 4.2 Effect of KTZ on the catalytic activities of human spleen HO (□), rat brain NOS (●) and rat spleen CPR (○) in vitro. HO, NOS, and CPR enzymatic activity was determined as outlined under Materials and Methods. Mean HO, NOS, and CPR activities in control reactions were 45.8 ± 7.7 pmol of CO/mg of protein/min, 8.5 ± 3.0 nmol of \[^{14}\text{C}]-\text{L}-\text{citrulline}\) formed/mg of protein/hr and 4.6 ± 0.3 µmol of reduced NADPH/min/mg protein, respectively. Data represent the mean ± SD of three experiments. * indicates concentrations of KTZ that caused significant inhibition of HO activity without any significant effect on NOS activity; \(p < 0.005\).
DISCUSSION

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

The hypothesis tested in the present study was that ketoconazole is an effective inhibitor of HO activity, and this inhibition occurs at normal therapeutic concentrations. The observations made herein are consistent with this hypothesis. The results showed that all eight imidazole 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 \(\mu\)M 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 \(\mu\)M). Although all of the azole-containing drugs tested displayed HO inhibitory capability and some were more potent than KTZ, this drug was selected for further investigation because of its initial dominance in therapeutics and its position as a prototype drug. When human spleen HO activity was tested in the presence of KTZ, inhibition was observed at concentrations below 10 \(\mu\)M. During the in vivo studies with rats and mice, each of the doses of KTZ (1, 10, or 100 \(\mu\)mol/kg) inhibited CO production. In addition, the observed IC\(_{50}\) of KTZ was 0.3 \(\mu\)M (0.16 \(\mu\)g/mL) for HO-1 and 7 \(\mu\)M (3.7 \(\mu\)g/mL) for HO-2, which is interesting in light of the plasma concentrations of this drug used clinically in humans. Thus, Huang et al. (1986) reported that the mean maximal plasma concentrations of KTZ
were greater than 5, 11, and 20 µg/mL after doses of 200, 400, and 800 mg administered to 12 volunteers (Huang et al., 1986). At the 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.

Another possibility was that inhibition of HO activity by KTZ was mediated through inhibition of NADPH CPR, which serves as an accessory enzyme during the oxidative breakdown of heme and the conversion of NADPH to NADP (Yoshida et al., 1980). This idea is not supported by our results, which showed that, even at concentrations as high as 250 µM, KTZ had no substantial effect on the catalytic activity of microsomal CPR.

The observation that KTZ, at therapeutically relevant concentrations, inhibited HO activity of rat and human tissue broken-cell preparations in vitro and in rats and mice in vivo raises the question whether any of the intended or unintended effects of KTZ in humans is a result of either HO-1 or HO-2 inhibition. In comparison, KTZ was much less potent as an inhibitor of rat brain NOS activity (Figure 4.2), which is consistent with previous studies showing azole-based antifungal drugs to be weak inhibitors of inducible NOS (Vermuyten et al., 1997). Thus, inhibition of NOS by KTZ is less likely to be clinically relevant than inhibition of HO.

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

In addition to fungal cytochromes P450 (CYP450), azole-based drugs such as KTZ are effective inhibitors of mammalian CYP450 as well. This class of enzymes is involved in a variety of metabolic processes including the bioactivation of drugs, the metabolism of xenobiotics and the synthesis of steroids. Pharmacological manipulation of these processes has been utilized as a treatment in many types of cancer. Specifically, prostate and breast carcinomas, two of the most prevalent forms of cancer, happen to be steroid-dependent malignancies. In three-quarters of cases of prostate cancer, high levels of the androgen, 5α-dihydrotestosterone sustained the growth of cancerous tissue (Nesbit & Baum, 1950). Androgen lowering therapy is well-practiced and there have been numerous studies identifying the effectiveness of KTZ in the treatment of androgen-dependent cancers. What these studies have failed to consider is what effects the inhibition of HO by KTZ has with respect to tumour growth. According to Maines and Abrahamsson, HO-1 levels in prostate tissue fluctuate with the different states of proliferation and differentiation of the cell. There was an increase in HO-1 expression in hyperplastic and undifferentiated malignant prostate tissue when compared with normal tissue; the most notable increase in epithelial cells (Maines & Abrahamsson, 1996). It was also shown that HO-1 activity could protect tumours cells against oxidative stress and prevent apoptosis in AH136B experimental solid tumour tissues (Tanaka *et al.*, 2004).
If HO-1 activity is necessary for the progression of prostate tumours perhaps the inhibition of HO-1 activity is partially responsible for the anti-tumour effects seen with KTZ treatment. The concept of HO inhibition as an anti-tumour strategy has been studied in experimental solid tumour models. Fang and colleagues demonstrated that polyethylene glycol-conjugated zinc protoporphyrin (PEG-ZnPP) exhibited HO inhibitory activity resulting in increased oxidative stress and apoptosis of tumour cells in vitro. In vivo, PEG-ZnPP accumulated preferentially in solid tumours and suppressed the growth of Sarcoma 180 tumours in mice (Fang et al., 2003). The most compelling evidence for an alternative anti-tumour mechanism of KTZ comes from studies that used KTZ in patients with androgen-independent prostate cancer. Eichenberger and Trachtenberg discovered that in 57% of patients who had not responded to conventional hormonal manipulation, high-dose ketoconazole (400 mg 3x/day) was able to stabilize their progressive cancer (Eichenberger & Trachtenberg, 1989). Wilkenson and Chodak found that even intermediate doses of KTZ (300 mg 3x/day) resulted in at least a 50% lowering in prostate-specific antigen levels (marker of prostate size) in over half of patients with androgen-independent prostate cancer (Wilkinson & Chodak, 2004). When administered directly to hormone-independent prostate cancer cell lines PC-3 and DU-145, KTZ caused a greater than 90% reduction in tumour cell growth (Eichenberger et al., 1989b). These studies suggest that KTZ is exhibiting anti-tumour activity, which is independent of CYP450-inhibited androgen reduction.

This knowledge should be used when planning future studies and interpreting results involving these compounds. When prescribing azole-based drugs as therapeutics, special consideration should be given to the additional effects that HO inhibition may
have on the progression of the disease. The possible role that inhibition of HO-1 and HO-2 has in the therapeutic outcomes of diseases utilizing these treatments remains to be investigated.
ACKNOWLEDGEMENTS

We thank Tracy Gifford for technical assistance. This work was supported by the Canadian Institutes of Health Research. RAD is a recipient of a Canadian Institutes of Health Research Studentship through the Gasotransmitter Research and Training Program.
CHAPTER 5

AZOLE-BASED HEME OXYGENASE INHIBITORS
AS ANTI-TUMOURIGENIC AND ANTI-METASTATIC COMPOUNDS IN VIVO IN AC2M2 BREAST CANCER


To be submitted to The British Journal of Cancer
INTRODUCTION

Heme oxygenase (HO) exists as either the inducible (HO-1) or the constitutive (HO-2) form of the enzyme (Braggins et al., 1986). Together these rate-limiting enzymes are involved in the conversion of heme into biliverdin, carbon monoxide (CO), and free iron (Fe$^{2+}$) (Tenhunen et al., 1968). Originally, these by-products were stigmatized as waste, whereas now they can be appreciated as important physiological effectors. Generally, the attributes of HO-1 and its products are considered cytoprotective (antioxidant, anti-inflammatory, anti-apoptotic) (Wu & Wang, 2005; Ryter et al., 2006; Abraham & Kappas, 2008; Dulak et al., 2008; Loboda et al., 2008). While these cytoprotective effects are generally viewed as beneficial to healthy cells, they may be detrimental to a patient with cancer by promoting or facilitating tumour survival.

Major tools in the elucidation of HO biology have been the metalloporphyrin HO inhibitors such as zinc protoporphyrin (ZnPP) (Vreman et al., 1993). While these drugs have allowed much progress to be made in understanding the role of the HO/CO system, they have limited utility because of their ability to inhibit other heme-dependent enzymes such as nitric oxide synthase (NOS) and soluble guanylyl cyclase (sGC). They are also potent inducers of the HO enzyme itself (Chapter 3, Figure 3.5). As potential therapeutic compounds, the metalloporphyrins have other limitations including poor solubility and risk of phototoxicity (Hintz et al., 1990). Our laboratory has been successful in the design, synthesis and development of selective, non-porphyrin-based HO inhibitors. To date we have synthesized and tested over 300 azole-based HO inhibitors (abHOi) and identified a number of HO-selective and HO-1-selective analogues (Figure 5.1)
Figure 5.1 Chemical structures of heme oxygenase inhibitors and heme. QC-15 (HO-1 Selective), QC-56 (HO-1 Selective), QC-10 (HO Selective), QC-99 (Non-functional Analogue).
(Vlahakis et al., 2005; Vlahakis et al., 2006; Kinobe et al., 2007; Roman et al., 2007; Kinobe et al., 2008). At concentrations capable of HO inhibition, these compounds did not affect other heme-proteins such as NOS and sGC (Kinobe et al., 2006b). In vivo, these water-soluble compounds demonstrated dose- (Chapter 3, Figure 3.2) and time-dependent (Chapter 3, Figure 3.4) inhibition of HO activity after intravenous (IV) and intraperitoneal (IP) administration. They are also well tolerated after repeated dosing and, unlike the metalloporphyrins, do not induce HO protein expression (Chapter 3, Figure 3.5, Chapter 5, Figure 5.17). The favourable properties of abHOi make them promising compounds for investigation in disease models where the inhibition of HO is predicted to be beneficial, such as cancer.

Various laboratories have reported an upregulation of HO-1 expression in a number of cancers including: prostate tumours (Maines & Abrahamsson, 1996), cerebral glioblastomas and astrocytomas (Hara et al., 1996), oligodendrogliomas (Deininger et al., 2000), lymphosarcomas (Schacter & Kurz, 1982), malignant vertical growth melanomas (Torisu-Itakura et al., 2000), oral squamous cell carcinomas (Tsuji et al., 1999; Chang et al., 2004), chronic myeloid leukemia (Mayerhofer et al., 2004), and renal cell carcinomas (Goodman et al., 1997). To validate some of these observations as well as provide theoretical support for the involvement of HO activity in the progression of tumours, some recent studies have shown that downregulation of HO-1 mRNA expression using short interfering ribonucleic acid caused pronounced growth inhibition as well as increased sensitivity to radiotherapy and chemotherapy of pancreatic cancer cells (Berberat et al., 2005) and SW 480 colon cancer cells (Fang et al., 2003; Fang et al., 2004). Targeted inhibition of HO by polyethylene glycol-linked ZnPP resulted in an
inhibition of the growth of solid Sarcoma-180 tumours in mice (Fang et al., 2003). Precise mechanisms as to how the HO/CO system promotes tumour growth are not fully understood but widespread processes including the induction of resistance to stress and apoptosis (Doi et al., 1999; Chen et al., 2004; Liu et al., 2004; Was et al., 2006) altered expression of cell cycle and differentiation genes (Clark et al., 1997; Koiso et al., 2000; Chauveau et al., 2005; Zwerina et al., 2005), and the promotion of angiogenesis (Cherrington et al., 2000; Dulak et al., 2002; Hirai et al., 2003; Sunamura et al., 2003; Cisowski et al., 2005; Deshane et al., 2007) have been suggested. Significant knowledge gains have been made recently in uncovering the mechanisms that regulate and support tumour vasculogenesis and HO-1 has been established as an important player (Dulak et al., 2008). Loss of function studies have shown that the absence of HO-1 severely impaired human endothelial progenitor cell (hEPC) tube formation and migration, in part due to a deviant response to stromal cell-derived factor-1 (Loboda et al., 2008). Several reports have also identified interplay in the expression-regulation relationship between HO-1 and vascular endothelial growth factor (VEGF) (Dulak et al., 2002; Jozkowicz et al., 2002; Suzuki et al., 2003; Bussolati et al., 2004; Bussolati & Mason, 2006; Jazwa et al., 2006).

There is an abundance of evidence validating HO-1 as a novel therapeutic target in angiogenesis and cancer. The experiments described herein were designed to test the hypothesis that the HO-1-selective abHOi, QC-15, has anti-angiogenic and anti-tumourigenic properties in vitro and will, thus, inhibit mammary tumour angiogenesis, growth, and metastasis in vivo.
MATERIALS AND METHODS

Animals

Female Swiss Nude (NTac:NIHS--Foxn1nu) mice (6 weeks of age) were purchased from Taconic Farms Inc. (Germantown, NY, U.S.A.) and were acclimatized for one week prior to use. Mice were maintained on a 12-hour light cycle and had ad libitum access to water and autoclaved Ralston Purina mouse (5010) chow (Ren’s Feed Supplies Ltd., Oakville, ON, Canada). All animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care and experimental protocols were approved by the Queen’s University Animal Care Committee.

Materials and Reagents

The abHOi: QC-10 (4-(4-chlorophenyl)-1-(1H-imidazol-1-yl)butan-2-ol hydrochloride), QC-15 (2-[2-(4-chlorophenyl)ethyl]-2-[(1H-imidazol-1-yl)methyl]-1,3-dioxolane hydrochloride), QC-56 (2-[2-(4-bromophenyl)ethyl]-2-[(1H-imidazol-1-yl)methyl]-1,3-dioxolane hydrochloride) and QC-99 (1-(2-methy-imidazol-1-yl)-4-phenyl-butan-2-one hydrochloride) were synthesized and then characterized by elemental analysis, mass spectrometry and nuclear magnetic resonance spectroscopy in the laboratory of a co-investigator, Dr. W. A. Szarek (Vlahakis et al., 2005; Vlahakis et al., 2006). ZnPP (8,13-bis(vinyl)-3,7,12,17-tetramethyl-21H,23H-porphine-2,18-dipropionic acid zinc(II)) was purchased from Frontier Scientific, Inc. (Logan, UT, U.S.A.). Anti-HO-1 (SPA-895) polyclonal antibody and recombinant rat HO-1 (SPP 730) protein were obtained from Stressgen Biotechnologies Corp. (Victoria, BC, Canada) now Assay Designs, Inc. (Ann Arbor, MI, U.S.A.). Ethylenediamine tetra-acetic acid disodium
(EDTA), hemin chloride, ethanolamine, bovine serum albumin (BSA) phosphate-buffered saline (PBS), bovine monoclonal anti-rat β-actin antibody and reduced β-nicotinamide adenine dinucleotide (β-NADPH) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Glycerol, potassium hydroxide (KOH), potassium phosphate monobasic (KH₂PO₄) and sodium chloride (NaCl) were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada). Stock solutions of all chemicals were prepared fresh on the day of the experiment.

**Preparation of Porphyrins and Azole-Based Heme Oxygenase Inhibitors**

All drug solutions were prepared fresh on treatment days. Stock solutions of the porphyrins, heme and ZnPP, were prepared under subdued light conditions. The porphyrins were each dissolved in ethanolamine (1.6 M; Sigma Aldrich) such that the final ethanolamine concentration would be 0.05% (v/v). The volume was increased to 1500 µL by the addition of 0.9% saline. The solution was then carefully titrated under stirring with 1 M HCl to a pH of 7.4 followed by the addition of saline to yield a 75 mM solution for administration to the experimental animals. Stock solutions of the abHOi were prepared by dissolving in 0.9% saline to yield a 100 mM solution for administration to the experimental animals.

**In Vitro Cell Culture**

PC3, Du145, Hela, HepG2, MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.); human mesenchymal stem cells (hMSC) and human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (Basel, Switzerland) and hEPCs were isolated from the blood of an adult male
according to the method of Brunt et al. (2007). Hela and HepG2 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma) with 10% fetal bovine serum (FBS, Sigma); Du145 and MCF-7 cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640, Sigma) with 5% FBS; MDA-MB-231 cells were maintained in RPMI-1640 with 10% FBS; PC3 cells were maintained in Kaighn's Modification of Ham's F-12 Medium (F12K, Sigma) with 10% FBS; hMSCs were maintained in Minimum Essential Medium-Alpha with GlutaMAX™ (Invitrogen, Burlington, ON, Canada) with 10% FBS; and hEPCs and HUVECs were maintained in endothelial cell growth medium (EGM-2, Lonza) with full supplements (EGM-2 bullet kit: 2% FBS, 0.4% hFGF-2, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% hEGF, 0.04% hydrocortisone, 0.1% ascorbic acid, 0.1% heparin, and 0.1%-GA-100). In all conditions cells were grown in media free of antibiotics in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

**Cell Viability Assays**

The effects of abHOi alone and in combination with chemotherapeutics on cell viability were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay as previously described (Hall & Heckel, 1990). In brief, cells were seeded at a density between 1.0×10⁴ and 1.0×10⁵ cells/well (such that untreated control cells would reach confluence at the end of the experiment) in a 24-well plate and allowed to attach overnight. The medium was then aspirated and cells were incubated with fresh serum-free (SF) medium containing the various agents (cytotoxic compounds or HO inhibitors) for 48 hours. Cells were washed in sterile PBS and then incubated with SF-medium containing 0.5 mg/mL MTT for 2 hours. Formazan formed
from the reduction of MTT by mitochondrial dehydrogenases was dissolved in anhydrous isopropanol and the absorbance was measured spectrophotometrically at 570 nm.

**Aortic Endothelial Cell Sprouting Assay**

The aortic ring endothelial cell sprouting preparation is an *ex vivo* assay designed to assess the effects of pro- or anti-angiogenic factors originally developed by Nicosia and Ottinetti (1990). Briefly, the thoracic and abdominal aorta is harvested from a rat, cleaned of fibro-adipose tissue, cut into 0.5 mm ring segments and embedded in growth factor-reduced (GFR) Matrigel® (BD Biosciences, Mississauga, ON, Canada) in a 24-well cell culture plate. Embedded rings were covered in SF-F12K media containing varying concentrations (1–100 µM) of the QC-15, or vehicle (water) control. Photographs were taken every 24 hours using a phase contrast microscope, and microvessel density was quantified using ImagePro® 6.0 software. Results were expressed as a percentage of the microvessel density of untreated rings.

**Capillary-like Endothelial Tube Formation Assay**

Delta-T glass-bottom culture dishes (Bioptechs Inc, Butler, PA, U.S.A.) were coated with a thin layer of GFR-Matrigel® and incubated at 37 °C for 20 minutes to allow the GFR-Matrigel® to solidify before bathing in SF-endothelial basal medium-2 (EBM-2, without supplemental growth factors). Du145 or PC3 tumour cells (6×10⁵) were then evenly spread over GFR-Matrigel® and incubated for 2 hours to allow for cell attachment. hEPCs (3×10⁵) were labeled with a red-fluorescent membrane dye (PKH26, Sigma) for identification and spread evenly over the tumour cell culture. The co-culture was placed back in the cell incubator for two hours to allow the hEPCs to attach and form
the capillary-like endothelial tube network. Finally, hMSCs ($3 \times 10^5$) were added to the co-culture along with additional SF-EBM-2 media containing either 100 µM QC-15 or vehicle (water) control. The co-cultures were photographed 12 hours later using phase contrast as well as fluorescent microscopy. The resulting images were used to perform qualitative analyses.

**Cell Migration Wound-Healing Assay**

To assess hEPC and tumour cell migration, the wound-healing assay of Todaro *et al.*, (1965), as modified by Liang *et al.*, (2007) was used. Briefly, cells were labeled with Hoechst nuclear stain and plated at $1 \times 10^5$ cells/well in 48-well tissue culture plates in complete media. After 24 hours, cultures were wounded (cells removed) by streaking cell monolayers with a 200 µL pipette tip down the middle of each well. The wells were washed with SF-media, followed by incubation for 24 hours with SF-media containing either abHOi, ZnPP or vehicle control (1% DMSO). Phase contrast and fluorescent photographs were taken immediately after the wound area was made (0 hours) and after 24 hours; cell migration was quantified using ImagePro® Software 6.0. For AC2M2 cells, migration was quantified by counting the number of cells that migrated into the wound area. The migration of hEPCs was quantified by taking the sum of the distance of each cell from the edge of the wound area.

**In Vivo Tumour Model**

A highly metastatic mouse breast carcinoma cell line (AC2M2) was selected by three times serial passage of a lung metastatic nodule following intra-mammary injection of a mouse breast carcinoma cell line (SP1) into syngeneic mice (Elliott *et al.*, 1992;
Elliott et al., 2005). Using a lentiviral vector, these cells were transduced with a gene encoding enhanced green fluorescence protein (EGFP), and maintained in DMEM containing 10% FBS. On the day of tumour inoculation, $7.5 \times 10^5$ EGFP-AC2M2 cells were harvested in sterile PBS. Female Swiss Nude mice were anaesthetized with inhaled isoflurane and a 1 cm incision was made between the linea alba and fat pad #4. The underlying breast tissue was exposed and 10 µL of EGFP-AC2M2 cells ($7.5 \times 10^3$) was injected just above the draining lymph node with a Hamilton® syringe. The wound was closed with surgical metal clips and mice were allowed to recover for 7 days before staples were removed. After 7 days, mice were subject to high frequency ultrasound using a Vevo 770® high-resolution imaging system (VisualSonics, Toronto, ON, Canada) to assess tumour take and initial tumour volume. Only mice that had developed a single solid tumour were included in the study. Mice were ranked and grouped into tiers of 5 mice each based on initial tumour size. One mouse was randomly selected from each tier for each of 5 treatment groups such that the mean initial tumour volume of each treatment group was the same. Mice received either 100 µmol/kg QC-15, 100 µmol/kg QC-10, 75 µmol/kg ZnPP, 75 µmol/kg heme or vehicle (saline) control by IP injection on days 1, 3 and 5 of a 7-day cycle for the duration of the study. Tumour volume was assessed by either high frequency ultrasound (days 1–4) or by measuring the greatest longitudinal diameter (length) and the greatest transverse diameter (width) by external caliper (days 5–14) and applying the modified ellipsoidal formula (Euhus et al., 1986)

$$\text{Tumour volume} = \frac{1}{2} (\text{length} \times \text{width}^2)$$

Tumour vascularization was assessed after two weeks of treatment by imaging the tumours with high frequency ultrasound before and after injecting mice IV with a
microbubble contrast agent (Vevo MicroMarker™, VisualSonics). Areas with increased contrast represented microvessels and were quantified using the provided software. Primary tumours were resected on day 15 to allow time for outgrowth of metastases to the lungs as described previously (Demicheli et al., 2008). Mice continued to receive treatments following the same dosing schedule for an additional 10 days at which point mice were euthanized by halothane inhalation followed by cervical dislocation. Lungs were immediately harvested and imaged in a Lightools Research (Encinitas, CA, U.S.A.) photo cabinet equipped with a ORCAII ER cooled camera (Hamamatsu Co, Bridgewater, NJ, U.S.A.) to measure photon flux from EGFP-AC2M2 lung metastasis. Sections of brain, spleen, liver, kidneys, heart, mesenteric, axillary and draining lymph nodes were fixed in 10% buffered formalin and embedded in paraffin blocks for histology. The balance of the harvested organs were snap-frozen in liquid nitrogen and stored at -80 °C for biochemical analysis.

**Determination of In Vivo Carbon Monoxide Production in Mice via Exhaled Air**

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., 1989). In the present study, the effect of HO inhibitors on VeCO and total CO production in mice 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). Mice 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 50 mL/min. Exhaust gas was directed to the injection valve of a TA 3000R reduction gas analyzer (Trace Analytical/Ametek) fitted with a 750 µl sample loop. Mice were acclimatized to
the chambers for 30 minutes, and the baseline VeCO was determined for 15 minutes.

**Preparation of Tissue Homogenate and Quantification of Protein Expression**

Primary tumours and mouse livers were thawed and tissue homogenate (30% w/v) was prepared in ice-cold RIPA buffer (0.150 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.05% Tris(hydroxymethyl) aminomethane base pH 8.0, and 0.1% protease inhibitor cocktail added fresh) using a 60S Sonic Dismembrator (Fisher Scientific Ltd., Ottawa, ON, Canada). The samples were centrifuged at 10,000 x g at 4 °C for 20 minutes and the supernatant was stored at -20 °C until used. Protein concentrations were determined using the Biuret method, as modified by Marks *et al.* (1997). In total, 25 µg of AC2M2 tumour homogenate and 30 µg of mouse liver tissue homogenate were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. Proteins were transferred onto polyvinylidene fluoride Immobilon-P membranes (Millipore, Bedford, MA, USA) according to the method of Laemmli (1970). Nonspecific binding sites were blocked by incubating the membranes in Tris-buffered saline Tween-20 (TBS-T; 0.02M Tris base, 0.14M NaCl, 0.075% Tween-20, pH 7.6) containing 5% (w/v) skimmed milk powder at room temperature for 1 hour. The blots were then incubated with a 1:5000 dilution of the polyclonal anti-rat HO-1 (SPA-895; Assay designs, Ann Arbor, MI, USA) antibody overnight at 4 °C. Subsequently, the membranes were incubated with either a peroxidase-labeled goat anti-rabbit IgG secondary antibody (BioRad Laboratories Ltd., Mississauga, ON, Canada). Peroxidase activity was detected by enhanced chemiluminescence detection kit according to the manufacturer’s instructions (LumiGlo; Mandel Scientific Company Inc., Guelph, ON, Canada). All gels were calibrated with
prestained low-range molecular weight markers (BioRad). Relative HO-1 expression was quantified by optical densitometry using an NIH imager. To ensure uniform protein loading on all gels, membranes used for HO quantification were stripped in buffer (25 mM glycine, 1% SDS, pH 2.0), blocked as described above, and probed with a 1:10,000 dilution of the monoclonal anti-β-Actin (mouse IgG1 isotype; Sigma Aldrich, St. Louis, MO, USA).

Data Analysis

The EC$_{50}$ values of QC-56 on cancer cell viability (concentration that decreased cell viability by 50%) were determined by nonlinear regression of sigmoidal concentration-response curves using Prism, version 4.0. The effect of QC-15 on the density of endothelial cell sprouting from aortic rings was assessed by one-way analysis of variance and Dunnett’s post-hoc test. The effects of abHOi on hEPC and AC2M2 were presented as a percent of the vehicle-treated control and assessed by one-way analysis of variance and Dunnett’s post hoc test. The effect of QC-15 on final AC2M2 tumour volume was assessed by analysis of co-variance and unpaired Student’s t-test. The effects of QC-15 on tumour vascularization and metastasis were assessed by unpaired Student’s t-test. The effects of treatment on tumour HO-1 protein expression and VeCO were assessed by one-way analysis of variance and Dunnett’s post-hoc test. 

$p$-values of $< 0.05$ were considered to be statistically significant.
RESULTS

Concentration-Dependent Cytotoxicity of Cancer Cells by an Azole-Based Heme Oxygenase Inhibitor

Cancer cells are known to experience high levels of oxidative stress and it has been suggested that certain solid tumours upregulate HO-1 expression and activity as an adaptive response. We sought to investigate whether the HO-1-selective abHOi, QC-56 had any cytotoxic effects on cancer cell lines known to express HO-1. Figure 5.2 illustrates the concentration-dependent decrease in cell viability of human breast (MCF-7, MDA-MB-231), prostate (PC3, Du145), liver (HepG2) and cervical (Hela) cancer cells after incubation with various concentrations (0.1 to 500 µM) of QC-56 for 48 hours. HepG2 cells were the most susceptible to QC-56 cytotoxicity with an EC\textsubscript{50} of 97 µM while MCF-7 were the least susceptible (EC\textsubscript{50} = 217 µM). With the exception of PC3, a concentration of 500 µM QC-56 resulted in a greater than 90% reduction in cancer cell viability. Cell viability was assessed by the MTT method and expressed as a percentage of the optical density in untreated cells from at least three experiments carried out in duplicate.

No Synergistic Activity Between an Azole-Based Heme Oxygenase Inhibitor and Cytotoxic Chemotherapeutics

In addition to their primary mechanism of action, some cytotoxic cancer drugs are known to increase oxidative stress in cancer cells. Here we assessed whether inhibition of an important anti-oxidant pathway (HO-1) by administration of a non-cytotoxic concentration of QC-56 would increase the cytotoxicity of camptothecin, etoposide,
Figure 5.2. Concentration-response curves of the cell viability of various human tumour cell lines after increasing concentrations of a selective HO-1 inhibitor (0.1–500 µM QC-56). Cells were plated in 24-well plates and given 24 hours to attach before a 48-hour incubation with the inhibitor. Cell viability was assessed by the MTT method and presented as a percent of the control (n = 4). Error bars represent the standard error of the mean. Inset: Cell viability EC$_{50}$ of QC-56 for each tumour cell line.
doxorubicin or cisplatin. Based on the results from the concentration-response cell viability curves in Figure 5.2, a concentration of 10 µM QC-56 (~90–100% viability in all cancer cell lines) was determined to be non-cytotoxic. Figure 5.3 shows the concentration-response curves for each chemotherapeutic in Hela, HepG2, Du145, PC3, MCF-7 and MDA-MB-231 human cancer cell lines in the presence or absence of 10 µM QC-56. In all cases, there was no significant difference in cell viability with the addition of 10 µM QC-56 and therefore no synergistic cytotoxicity. Cell viability was expressed as a percentage of the optical density in untreated cells from three experiments carried out in duplicate.

Azole-Based Heme Oxygenase Inhibitors Reduce In Vitro Angiogenesis and Vasculogenesis

It has been suggested that VEGF, sFlt-1, sEng and SDF-1α may contribute to the regulation of angiogenesis through HO-1-dependent mechanisms; therefore we investigated whether QC-15, a selective HO-1-inhibitor, decreased capillary-like endothelial tube formation in two in vitro models of angiogenesis. First, we used the aortic ring preparation because it represents many of the individual elements of angiogenesis (migration, invasion, endogenous angiogenic signalling) into one assay. Capillary-like endothelial cell outgrowth was evident after 48 hours in all treatment groups (Figure 5.4A), however, analysis using ImagePro 6.0 revealed that treatment with 100 µM QC-15 resulted in a significant reduction (62%) in the density of vessel outgrowth. The effect of 100 µM QC-15 persisted through to the 72- and 96-hour time points as well, resulting in 67% and 69% reduction in vessel densities, respectively. There was also a concentration-response effect of QC-15 evident at all three time points,
Figure 5.3 Concentration-Response curves showing the viability of Hela (A), HepG2 (B), Du145 (C), PC3 (D), MCF-7 (E) and MDA-MB-231 (F) human cancer cells after incubation with 0.01 µM–100 µM of various chemotherapeutics (camptothecin, etoposide, doxorubicin and cisplatin). Cells were incubated for 48 hours in the presence (---) or absence (—) of a non-cytotoxic concentration of a selective HO-1 inhibitor (10 µM QC-56). Cell viability was assessed by the MTT method and presented as a percent of the control (n = 4). Error bars represent the standard error of the mean.
Figure 5.4 QC-15 inhibits endothelial cell sprouting in aortic rings. Rat aortic rings were cultured in Matrigel® bathed in media containing increasing concentrations of a selective HO-1 inhibitor (1 µM-100 µM QC-15). Aortic rings were photographed after 48 (A), 72 (B) and 96 (C) hours. Endothelial cell sprouting was quantified using ImagePro® software. Each column represents the mean of five experiments. Error bars represent the standard error of the mean. * indicates a statistically significant difference of \( p < 0.05 \) from control (\( n = 4–5 \)). Right: Representative aortic rings from control conditions.
which resulted in a significant reduction in vessel density at the lowest concentration of QC-15 (1 µM) after 96 hours (Figure 5.4C).

Tumour cells and the surrounding microenvironment are known to be important contributors to tumour angiogenesis and vasculogenesis in vivo through the production of growth factors. To better recapitulate this complex microenvironment in vitro we modified the well-established Matrigel® capillary-like endothelial tube formation assay to include the co-culture of cancer cells as well as hMSCs (which are thought to act as perivascular cells involved in stabilizing the fragile network of endothelial cells). In the control condition, Du145 or PC3 human prostate cancer cells were plated on GFR-Matrigel® followed 2 hours later by hEPCs. The hEPCs began to organize into a network of capillary-like tubes over the following two hours and there was evidence of tumour cell migration towards this growing network of cells. hMSCs were then added and after a 12-hour incubation the hEPC network was complete. In the Du145-hEPC-hMSC co-culture, Du145 cells migrated towards, and organized evenly, along the entire length of the hEPC network (Figure 5.5A) whereas in the PC3-hEPC-hMSC co-culture the PC3 cells tend to aggregate at the nodes where the hEPC tubes connect (Figure 5.5C). When 100 µM QC-15 was added to the co-culture media in either case, it completely prevented the development of the hEPC network and resulted in the formation of cell clumps (Figure 5.5B, 5.5D).

To further investigate one of the possible mechanisms that may contribute to the anti-angiogenic/anti-vasculogenic effects seen in the previous two assays we examined the effects of some of the lead abHOi on hEPC migration. The wound-healing assay measures the ability of confluent quiescent endothelial cells to break cell-cell contacts
Figure 5.5 QC-15 inhibits endothelial cell capillary-like tube formation. Adult human endothelial progenitor cells (red) were co-cultured with human mesenchymal stem cells and (A, B) Du145 or (C, D) PC3 human prostate cancer cells and incubated with vehicle (dH₂O; A, C) or 100 µM QC-15 (B, D) for 12 hours.
and migrate to fill the open space created by the wound, mimicking the *in vivo* situation during blood vessel damage. Although treatment with QC-10 (50 μM) or QC-15 (50 μM) caused a modest reduction of wound closure (13% and 11%, respectively; Figure 5.6) these results were not statistically significant. From these data it is unlikely that the anti-angiogenic effects of QC-15 were due to the inhibition of hEPC migration alone.

**The Anti-Tumourigenic and Anti-Metastatic Effects of an HO-1-Selective Azole-Based Heme Oxygenase Inhibitor *In Vivo***

Based on the results of the *in vitro* assays we wanted to investigate whether the combination of the cytotoxic and anti-angiogenic effects of QC-15, seen *in vitro*, would translate into a reduction in tumour growth and angiogenesis *in vivo*. The AC2M2 mouse breast cancer model was chosen as this cancer cell line has high expression of HO-1 protein. In addition, an *in vitro* cell proliferation assay revealed a concentration-dependent reduction in AC2M2 cell proliferation by the HO-selective, QC-10, and the HO-1-selective, QC-15, abHOi (Figure 5.7). Moreover, QC-99, a close structural analogue of QC-10 and QC-15, devoid of HO inhibitory activity, had no effect on cell proliferation suggesting that the effects seen with QC-10 and QC-15 were mediated through their inhibition of HO.

Implantation of EGFP-AC2M2 breast cancer cells into the mammary fat pad of female Swiss Nude mice resulted in the rapid development of solid tumours with the mean volume of tumours from vehicle-treated mice reaching 810 mm³, 21 days after implantation. Treatment of mice with 100 μmol/kg QC-15 (HO-1-selective) beginning 7 days after inoculation (considered day 1) resulted in a statistically significant reduction in
Figure 5.6  Effect of heme oxygenase inhibitors on spontaneous human endothelial progenitor cell migration. Cells were plated in 48-well plates and given 24 hours to reach confluence. A lesion was made in the centre of the well creating a wound and cells were rinsed and replaced with media containing an HO inhibitor. The sum of the distance of each cell from the edge of the wound area after 24 hours was quantified using ImagePro® software and presented as a percent of the control of three independent experiments performed in at least triplicate. Error bars represent the standard error of the mean.
Figure 5.7 Concentration-response effect on the proliferation of AC2M2 breast cancer cells incubated with various concentrations (1–100 µM) of the selective HO-1 inhibitor QC-15 (▼), selective HO inhibitor QC-10 (▲), and the non-functional azole-analogue QC-99 (■). Cells were plated in 24-well plates and given 24 hours to attach before a 48-hour incubation with the inhibitor. Live cell counts were assessed by the trypan blue exclusion assay and presented as a percent of the control. There was no difference in the number of dead cells between the groups. Error bars represent the standard error of the mean.
final tumour volume after two weeks (day 15) of treatment compared to vehicle-treated controls (Figure 5.8 inset, Figure 5.9). Interestingly, mice treated with 100 µmol/kg, QC-10 (HO-1 and HO-2 selective), showed no difference in final tumour volume. Treatment with the classical metalloporphyrin-based HO inhibitor ZnPP (75 µmol/kg) showed a modest reduction in tumour volume, although not statistically significant, while tumours from heme-treated mice (75 µmol/kg) were not different from vehicle treated controls (Figure 5.8). Mice were monitored closely throughout the study for signs of distress or deteriorating health caused by any of the treatments, however no changes in behaviour were noted and there were no significant differences in mouse body weights between the treatment groups (Figure 5.10). The reduction in tumour volume in the QC-15-treated group was independent of a reduction in tumour angiogenesis as data from Figure 5.11 show no difference in tumour vascularization between QC-15 and vehicle-treated mice. Remarkably, the most striking effects of QC-15 were with respect to lung metastases. Table 5.1 highlights the metastatic incidence rates (mice with the presence of at least one lung metastasis). QC-15 and heme-treated mice had the lowest incidence rates at 67% whereas 100% of QC-10 and vehicle-treated mice had lung metastases. The number of lung metastases was quantified using biophotonic detection of EGFP-labeled AC2M2 cells in the lung as well as histological examination of hematoxylin and eosin-stained lung sections. Both methods resulted in the same trends highlighted in Figure 5.12. There was a statistically significant reduction (88%) in the number of lung metastases in mice treated with QC-15 compared to controls (Figures 5.12, 5.13, 5.14). There was also a marked decrease in the number of lung metastases due to treatment with ZnPP. As a measure of the total burden of lung metastases, we quantified the photon flux
Figure 5.8 Time course of treatment effect on primary AC2M2 tumour volume. Tumour volume was measured using high frequency ultrasound (day 1-4) and external calipers (day 5-14). On day 15 primary tumours were removed and volume was determined by water displacement. Inset: Final tumour volume. Treatment with QC-15 resulted in a statistically significant reduction in tumour volume ($p < 0.05$; $n = 5$).
Figure 5.9. Primary AC2M2 tumours from mice receiving (A) 100 µM QC-15 or (B) saline control on day 1, 3 and 5 of a 7 day cycle. Primary tumours were surgically removed on day 15 (after 2 cycles) and prepared for biochemical and histological analyses.
Figure 5.10  Effect of treatment on mouse body weight. Mice were weighed immediately before treatment. There were no significant differences in body weight among the treatment groups.
Figure 5.11 Tumour vascularization. Percent vascularization of tumours was assessed by high frequency ultrasound imaging enhanced by administration of a microbubble contrast agent. There was no difference in tumour vascularization in mice treated with QC-15 (n = 3)
Table 1. Lung Metastatic Incidence Rates of AC2M2 Tumour Cells

<table>
<thead>
<tr>
<th>Incidence of Metastases</th>
<th>Control</th>
<th>QC-15</th>
<th>QC-10</th>
<th>ZnPP</th>
<th>Heme</th>
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<tbody>
<tr>
<td></td>
<td>100% (5/5)</td>
<td>67% (4/6)</td>
<td>100% (5/5)</td>
<td>83% (5/6)</td>
<td>67% (4/6)</td>
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Figure 5.12  QC-15 reduces the number of distinct metastatic events to the lung. Individual metastases were quantified in the whole lung using bioluminescent imaging (crossed) as well as histology on prepared formalin fixed- H&E stained-lung sections. Quantification was performed by at least two individuals who were blind to the treatment groups. Treatment with QC-15 resulted in a statistically significant reduction in the number of metastatic events ($p < 0.05$; n = 5–6).
Figure 5.13 Representative histological lung tissue sections from (A, C) control and (B, D) QC-15-treated mice showing AC2M2 metastases (arrows). Ten days after primary tumour removal, mice were euthanized and lungs were removed, fixed in 10% buffered formalin and paraffin-embedded. Sections were stained with hematoxylin and eosin and examined for the presence of metastases.
Figure 5.14  Representative biophotonic images showing EGFP-labeled AC2M2 metastases (arrows) in the lungs of (A, C) control and (B, D) QC-15-treated mice. Ten days after primary tumour removal, mice were euthanized and lungs were removed and photon flux was measured using a Hamamatsu ORCAII ER cooled camera.
from the EGFP-AC2M2 cells in the lungs densitometrically and found that once again, there was a statistically significant reduction in lung metastases in mice treated with QC-15, similar to that of mice treated with ZnPP (Figure 5.14, 5.15) when compared to vehicle. QC-10-treatment, however, had no effect on the metastatic burden in the lungs. Tumour cell detachment and migration are necessary components to the development of distant metastasis in vivo. To further investigate these potential mechanisms by which QC-15 and ZnPP exerted their anti-metastatic effects in vivo we tested their ability to inhibit AC2M2 cell migration in the wound-healing assay. Figure 5.16 shows a significant decrease in cell migration of AC2M2 cells after treatment with QC-15 (26%) and ZnPP (18%) but lack of effect of QC-10. These results suggest that inhibition of tumour cell detachment and migration may partially explain the decrease in lung metastases seen in the in vivo model. Treatment with either QC-10 or QC-15 did not effect HO-1 protein expression in the tumours, however both ZnPP and heme resulted in statistically significant increases of 109% and 201%, respectively (Figure 5.17). Total body HO activity measured by VeCO one hour after treatment was significantly higher in mice treated with 75 µmol/kg heme (Figure 5.18A). There appeared to be a modest reduction in VeCO by QC-10, however this difference was not statistically significant. After 24 hours, the increase in VeCO caused by heme administration was diminished (Figure 5.18B) to levels no different than those of the controls.
Figure 5.15 QC-15 reduces the burden of metastases in the lung. The extent of metastases in the lung was quantified by measuring the bioluminescence from EGFP-containing AC2M2 lung metastases. Treatment with QC-15 resulted in a statistically significant reduction in the size of metastases in the lung ($p < 0.05; n = 5–6$).
Figure 5.16  Inhibition of AC2M2 breast cancer cell migration by heme oxygenase inhibitors. Cells were plated in 48-well plates and given 24 hours to reach confluence. A lesion was made in the centre of the well creating a wound and cells were rinsed and replaced with media containing HO inhibitors. The migration distance of cells into the wound was quantified after 24 hours by ImagePro® software and presented as a percent of the control. Treatment with QC-15 or ZnPP resulted in a statistically significant reduction in cell migration (*p < 0.05; n = 5). Error bars represent the standard error of the mean.
Figure 5.17  HO-1 protein expression in primary tumours. Primary tumours were surgically removed 24 hours after drug administration on day 23. Homogenates were prepared and subjected to SDS–PAGE and Western blotting for HO-1. Data are presented as percent HO-1 protein expression of control mice. HO-1 protein expression in tumours from ZnPP- and heme-treated mice was statistically significantly higher than that of control ($p < 0.01$). Columns represent the average of 6 mice; bars represent the standard error of the mean.
Figure 5.18 Whole animal HO activity 1 and 24 hours after drug treatment. HO activity was measured by the average CO excretion (VeCO; µL/hr/kg) over 15 minutes. Mice in the heme treated group had a statistically significant increase in HO activity 1 hour after drug administration ($p < 0.05$; $n = 6$). There were no significant differences in HO activity 24 hours after treatment.
DISCUSSION

The data presented herein suggest that novel HO-1-selective abHOi possess several properties \textit{in vitro} and \textit{in vivo} that make them attractive therapeutics for the treatment of metastatic cancer. We show that HO-1-selective abHOi decrease the viability and proliferation of several different tumour cell lines \textit{in vitro}. This effect translated into a significant inhibition of AC2M2 mammary tumour growth \textit{in vivo} after just two weeks of treatment. In this aggressive lung-metastatic model, mice treated with the HO-1-selective abHOi, QC-15, showed a considerable reduction in the incidence, number and total burden of lung metastases, an effect that may be partially mediated by the inhibition of tumour cell detachment and migration. In several \textit{in vitro} models of angiogenesis, QC-15 inhibited the formation and growth of capillary-like endothelial tube networks independent of any effect on endothelial cell migration. However, these anti-angiogenic effects did not result in a reduction in tumour vascularization in the AC2M2 tumour model. When compared to currently available chemotherapeutic drugs, the HO-1-selective abHOi do not seem to offer any advantages in terms of potency or additional effectiveness. When administered in addition to cytotoxic concentrations of etoposide, camptothecin, doxorubicin or cisplatin, QC-56 did not increase the effectiveness or potency and therefore there does not appear to be any synergistic effects between their mechanisms of action. Nevertheless, the marked anti-metastatic effects of QC-15 represent an important milestone in its development as a cancer drug and warrants further investigation into the mechanism of action responsible for these effects.
These results are consistent with those generated by Alaoui-Jamali et al., (2009) who showed that treatment of tumour-bearing mice with a structurally similar HO-1-selective abHOi (QC-56, also known as OB-24) inhibited primary tumour growth and metastases in an orthotopic model of prostate cancer (Alaoui-Jamali et al., 2009). The most remarkable result from their work was the apparent synergistic effect between QC-56 and the anti-microtubule agent, paclitaxel (Taxol®) on PC3 prostate tumour growth. In our examination of the synergistic effects of QC-56 and chemotherapeutics in vitro (Figure 5.3) we did not observe any significant synergistic effects in any of the cell lines tested, including PC3 prostate cancer cells. A possible explanation for this discrepancy is that the synergistic effect observed by Alaoui-Jamali et al., (2009) was due to a pharmacokinetic effect that may not be manifest in the in vitro synergistic studies represented in Figure 5.3. The primary route of metabolism of taxanes such as paclitaxel is via CYP450 3A4 biotransformation and recent work in our laboratory has shown that QC-15 and QC-56 inhibit CYP450 3A4 in the therapeutic range used for HO inhibition. Inhibition of paclitaxel metabolism by QC-56 would result in increased circulating levels of this taxane and could explain the increased therapeutic response. In our own pilot study investigating the therapeutic potential of an HO-1-selective abHOi in prostate cancer, mice receiving a combination of QC-15 and the taxane, docetaxel, had to be removed from the study due the development of paralytic ileus, a common side effect seen with the overdose of taxanes. Since, neither the QC-15 alone nor the docetaxel alone group showed any of the same symptoms it appeared as though the abHOi was augmenting the effects of the taxane. This interaction may also explain synergistic effects of QC-56 and paclitaxel on tumour growth reported by Alaoui-Jamali et al.,
(2009) where the administration of QC-56 increased the bioavailability of paclitaxel resulting in increased efficacy of the treatment.

Although most cancers are described in terms of their primary site of origin it is the metastatic outgrowth that proves to be the most difficult to treat and in many cases is responsible for the associated mortality. Here we observed that treatment of AC2M2 tumour-bearing mice with QC-15 significantly reduced the incidence, frequency and burden of lung metastases. These data are supported by the findings of Alaoui-Jamali et al., (2009) who showed that QC-56 treatment prevented lung metastases in an orthotopic prostate cancer model and by Sunamura et al., (2003) who showed that HO-1 overexpression increased, and HO inhibition by stannous mesoporphyrin decreased the occurrence of metastasis in a lung colonization model in SCID mice (Sunamura et al., 2003). The metastatic process comprises many steps including tumour cell migration and invasion into local tissue, dissemination through the lymphatic and venous systems, implantation, extravasation into the distant tissue and growth of the established tumour emboli. Each one of these processes represents a potential target for anti-metastatic therapy. Data from Figure 5.16 suggest that the anti-metastatic effect observed of QC-15 and ZnPP may be, in part, due to the inhibition of tumour cell detachment and migration. Recently, HO-1 has been shown to induce matrix metalloprotease-1 expression in human breast cancer cells (Kim et al., 2009); this protease activity is responsible for degradation of the extracellular matrix necessary for cell migration. Therefore, it is possible that reduction in HO-1-mediated matrix metalloprotease-1 expression by QC-15 and ZnPP may be partially responsible for their anti-metastatic effects and should be explored further.
The effect of reduction in viability of tumour cells was consistent across all tumour cell lines tested in the cell viability assays highlighted in Figure 5.2. Previous work in the field has revealed that inhibition of HO by ZnPP also reduces the viability of tumour cells to comparable levels. Interestingly, these effects occur at 100-fold lower concentrations despite the fact that the potency of ZnPP with respect to HO inhibition is similar to that of QC-56 (Chapter 3, Figure 3.3). It is possible that the increased cytotoxicity of ZnPP is independent of HO inhibition. Metalloporphyrins, and in particular ZnPP, are known to be phototoxic and have been shown to oxidize organic molecules such as lipids, proteins, and nucleic acids in the presence of light (Hintz et al., 1990; Scott et al., 1990). Although efforts are made to minimize exposure of ZnPP solutions and cell cultures to light, it is likely that some photo-oxidation of ZnPP occurred and this may partially explain the increased potency seen with ZnPP. In the in vivo AC2M2 orthotopic mammary tumour model, where phototoxicity is less likely occur, treatment with QC-15 and ZnPP showed similar inhibition of primary tumour volume. What is more interesting from these data is the lack of effect of QC-10 treatment on primary tumour growth. Previous work in our lab revealed that QC-10 was a more potent and efficacious inhibitor of both HO-1 and HO-2 in vitro and total HO in vivo when compared to QC-15 and ZnPP (Chapter 3, Figure 3.3). Based on this, one would have predicted that treatment with QC-10 would have resulted in a greater reduction in tumour volume. This evokes the possibility that the effects seen with QC-15 and ZnPP may be independent of HO inhibition. Many laboratories have published work showing that low levels of endogenous NO can be anti-apoptotic and cytoprotective. In addition to HO, ZnPP is also a known inhibitor of NOS and its downstream mediator, sGC, therefore the decrease
in NO signalling may render the tumour cells more susceptible to cell death and result in decreased tumour volume. To explain the difference in the effects of QC-15 and QC-10 we must consider their respective HO selectivity. The major structural difference between QC-15 and QC-10 is the substitution of the hydroxyl group on QC-10 with a 1,3-dioxolane on QC-15 at the carbon-2 position which is thought to interfere with inhibition of HO-2 and therefore confer selectivity for HO-1. The different effect of QC-10 and QC-15 may suggest different roles for HO-1 and HO-2 in tumour cells such that the inhibition of HO-2 may negate the anti-tumour effects of HO-1 inhibition. It is also possible that the dioxolane substitution on QC-15 results in an undetermined off-target effect. We plan to further explore this possibility by synthesizing the non-functional analogue of QC-15 via methylation of the nitrogen in the imidazole that is crucial to HO binding. If this structurally similar analogue, devoid of HO inhibitory activity, retains the ability to inhibit tumour growth it would suggest the involvement of an off-target effect and perhaps the presence of a novel therapeutic target for cancer treatment.

This study represents an important step in demonstrating the utility of abHOi, specifically QC-15, in the treatment of metastatic cancer. Whether the inhibition of tumour growth or the marked anti-metastatic effects are entirely due to HO-1 inhibition or whether other unidentified targets are involved remains to be seen. Although QC-15 treatment did not inhibit tumour vascularization in this model, the effects on angiogenesis in vitro should not be discounted. Currently indicated anti-angiogenic therapies, such as bevacizumab, are indicated in specific types of cancer that respond to their particular mode of action. It is possible therefore that if tested in an alternative tumour model
where angiogenesis is driven by a different subset of angiogenic factors, treatment with QC-15 may show greater efficacy. Overall, these data support previous work revealing a role for HO-1 inhibition in the treatment of cancer. Future studies should be directed towards elucidating the exact mechanisms responsible for these anti-tumourigenic and anti-metastatic effects.
ACKNOWLEDGEMENTS

The authors would like to thank Jalna Meens and Daniel Werry for technical assistance and the laboratory of Dr. Peter Greer for carrying out the lentiviral transduction of the EGFP gene into the AC2M2 cells. This work was supported by the Canadian Institutes of Health Research and the Ontario Institute of Cancer Research. RAD is a recipient of a Canadian Institutes of Health Research Studentship through the Gasotransmitter Research and Training Program.
CHAPTER 6

GENERAL DISCUSSION
In comparison with what is known about the first established gasotransmitter, NO, and its synthetic enzyme, NOS, the field of CO and HO is still in its infancy. That being said, we are at an exciting point in our progression of knowledge that has seen a dramatic increase in interest and important discoveries in recent years. This was all made possible by the advancement of genetic tools and most importantly, the identification and characterization of a group of enzyme inhibitors dubbed the metalloporphyrins (Vreman et al., 1993; Vreman et al., 1996b). These compounds are used ubiquitously throughout the field to the point that it is difficult to find a paper investigating HO that has not employed their use. Unfortunately, there are a number of limitations of metalloporphyrin-use as HO inhibitors. Metalloporphyrins are structurally similar to heme, the substrate for HO, and predictably act as competitive inhibitors by replacing heme in the binding pocket of HO. Not surprisingly, the metalloporphyrins also demonstrate the ability to inhibit other heme-dependent enzymes such as NOS and sGC (Ignarro et al., 1984; Ny et al., 1995; Chakder et al., 1996). The fact that NOS and sGC were found to be involved in many of the same biochemical pathways as HO/CO led many to state that the metalloporphyrins should not be used to establish a messenger role for CO (Luo & Vincent, 1994; Grundemar & Ny, 1997; Cary & Marletta, 2001). Previous work in this laboratory investigated the selectivity of several metalloporphyrins and found that a number of them were useful tools in vitro, provided that they were used in a defined concentration range (Appleton et al., 1999). Thus, the challenges presented by the lack of selectivity of the metalloporphyrins have hindered progress in this field and have left investigators with less confidence in interpreting their observations.
As the field has matured, so has the depth and complexity of the questions we are asking. It has become evident that the two isozymes of HO, HO-1 and HO-2, are not redundant and have distinct roles in the body (Maines, 1988; Ewing & Maines, 1992). They are found in different ratios depending on tissue type, are encoded by different genes and respond to a diverse set of stimuli in different conditions. Our laboratory has identified a novel set of azole-based compounds that have demonstrated the ability to inhibit HO \textit{in vitro} (Vlahakis \textit{et al.}, 2005; Kinobe \textit{et al.}, 2006b; Vlahakis \textit{et al.}, 2006; Roman \textit{et al.}, 2007; Vlahakis \textit{et al.}, 2009; Roman \textit{et al.}, 2010). Moreover, specific modifications to functional groups have led to analogues with increased selectivity for the HO-1 isozyme. These compounds have the potential to make a profound impact on the field of HO/CO research by, for the first time, allowing the investigation of the individual roles of HO-1 without the confounding effects of HO-2, NOS and sGC inhibition.

Adaptation in science is a slow process and the acceptance of a new technique or tool can be met with reluctance and even resistance. For these reasons, one of the main goals of this thesis was to provide the necessary characterization and comparative studies to aid in the acceptance and dissemination of these novel HO inhibitors into the field of HO/CO research. To evaluate the effectiveness of these compounds \textit{in vivo}, we first established a method of quantifying HO activity \textit{in vivo} through the non-invasive measurement of CO production (Dercho \textit{et al.}, 2006; Chapter 2). This method was validated by manipulating HO activity using known inducers and inhibitors of HO activity in both rat and mouse animal models and demonstrating the resulting change in CO production. Compounds with substantive potency or selectivity \textit{in vitro} were screened \textit{in vivo} using the VeCO method and resulted in the selection of QC-10
(HO-selective inhibitor) and QC-15/QC-56 (HO-1-selective inhibitors) as lead compounds destined for further characterization (Chapter 3). The work described in Chapter 3 demonstrated that abHOi were functional HO inhibitors in vivo and that the relative efficacies in vitro were excellent predictors of the relative efficacies in vivo. They demonstrated dose- and time-dependent inhibition of HO with a capacity to inhibit HO comparable to the classical metalloporphyrin HO inhibitor, ZnPP. Enzyme kinetic studies showed that inhibition of HO-1 by QC-56 occurred by way of a reversible, non-competitive mechanism. These observations have since been confirmed with QC-10, QC-15 and QC-86, leading us to conclude that this binding mechanism is universal to this class of compounds (Vukomanovic et al., 2010). Unlike the metalloporphyrin HO inhibitors, repeated administration of QC-10 or QC-15 to mice in vivo did not result in a change of HO-1 or HO-2 protein expression. With respect to enzyme selectivity, abHOi did not inhibit NOS in vivo, confirming earlier in vitro work done in our laboratory (Kinobe et al., 2006b). The only discrepancy between the in vitro and in vivo characterization was with respect to isozyme selectivity. In vitro, QC-56 had a HO-1 selectivity index of over 50, meaning that the IC$_{50}$ value for inhibition of HO-1 was at least 50 times lower than the IC$_{50}$ value for inhibition of HO-2. When tested in vivo, however, QC-56 did not demonstrate selective inhibition of HO-1. Chapter 3 discusses several possible explanations for this discrepancy; still, further experiments are needed before this issue can be resolved.

In the ongoing search for more effective and selective abHOi, it was noted that many azole-based antifungal drugs, such as ketoconazole, share a common pharmacophore with the abHOi. Although their primary mode of action is accepted to be
the inhibition of fungal sterol 14-α-demethylase, several of the azole-based anti-fungal drugs tested demonstrated effective inhibition of HO in vitro and at therapeutically relevant concentrations in vivo (Kinobe et al., 2006a; Chapter 4). This raises the possibility that inhibition of HO activity is also a contributing factor in the pharmacological actions of these antimycotic drugs. One such case is the use of ketoconazole as an inhibitor of androgen synthesis in hormone-dependent-prostate cancer (Wilkinson & Chodak, 2004). Moreover, clinicians have reported that ketoconazole treatment remains effective in hormone-refractory prostate cancer (Eichenberger & Trachtenberg, 1989; Eichenberger et al., 1989a). In Chapter 4, we suggest that this effect may be, in part, due to the inhibition of HO. This led us to the studies in Chapter 5 examining the effects of the HO-1-selective abHOi, QC-15 in in vitro and in vivo models of tumour growth, angiogenesis and metastasis. Treatment of tumour-bearing mice with QC-15 in an orthotopic model of metastatic breast cancer resulted in significant reductions in primary tumour volume and striking inhibition of the number and growth of lung metastases.

Although the evaluation of tumour angiogenesis in Chapter 5 revealed no difference in tumour vascularization between QC-15-treated and vehicle-treated mice, the marked anti-angiogenic effects seen in the in vitro models of angiogenesis cannot be ignored. As described in the Introduction, angiogenesis is a complex and tightly regulated process involving many redundant signalling molecules. The relative importance of specific angiogenic and anti-angiogenic mediators can be quite different in tumour angiogenesis when compared to angiogenesis resulting from wound healing, inflammation, or growth and development. Therefore, abHOi have therapeutic potential and warrant investigation
in other *in vivo* models of angiogenesis where the signalling molecules mediated by HO are perhaps of greater importance. There are several disease states other than cancer for which anti-angiogenic therapy is currently approved. In fact, excessive angiogenesis underlies some of the most common causes of blindness, including neovascular glaucoma, age-related macular degeneration, diabetic macular edema, proliferative diabetic retinopathy, corneal neovascularization, and pterygium (Adamis *et al.*, 1999). With respect to ophthalmic disease, ranibizumab (Lucentis®) and pegaptanib (Macugen®) are currently the only approved anti-angiogenic therapies and are specifically indicated for the treatment of age-related macular degeneration. Nevertheless, bevacizumab (Avastin®) is used extensively ‘off-label’ due to its lower cost. All three therapies work by binding circulating VEGF, thus preventing it from interacting with VEGF receptors. Aside from the cost associated with using these biologics, they must be administered by intravitreal injection, which can be quite painful. A small molecule angiogenic inhibitor, such as QC-15, would overcome both of these limitations. There is clearly room for improvement with respect to treating these ophthalmic conditions and, therefore, the testing of QC-15 in a model of age-related macular degeneration would be a logical next step.

In addition to cancer and the ophthalmic diseases described above, HO activity may also play an important role in the pathogenesis of infectious conditions such as malaria. Most pathogenic *Plasmodium* species have evolved strategies to detoxify free hematin (Ginsburg *et al.*, 1998; Platel *et al.*, 1999), such as the polymerization of hemin into hemozoin, although some of the parasites in this group also possess functional HO (Srivastava *et al.*, 1992; Srivastava & Pandey, 1995). Therefore, the treatment of malaria
represents another potential therapeutic application of abHOi and should be studied further.

The data generated and presented in this thesis highlight the beginning of a new chapter in HO/CO research. With the validation of these novel pharmacological tools comes the potential for exciting and groundbreaking research elucidating the distinct physiological and pathological roles of HO-1 and HO-2. As we continue to discover the involvement of HO in disease, so too will we identify novel therapeutic applications for azole-based heme oxygenase inhibitors. Surely, this is only the beginning…
REFERENCES


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