A Novel and Potent Inhibitor of Dimethylarginine Dimethylaminohydrolase: A modulator of cardiovascular nitric oxide

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List of nonstandard abbreviations:

ADMA = asymmetric dimethylarginine
BH₄ = tetrahydrobiopterin
DDAH = dimethylarginine dimethylaminohydrolase
ECs = endothelial cells
ESI = electrospray ionization
FDA = food and drug administration
HMVECs = human microvascular endothelial cells
HTBC = high throughput bioscience center
LOPAC = library of pharmacologically active compounds
LPS = lipopolysaccharide
MABP = mean arterial blood pressure
MS = mass spectrometry
NIH = National Institutes of Health
NO = nitric oxide
NOS = nitric oxide synthase
SMTC = S-methyl-thiocitrulline
VEGF = vascular endothelial growth factor
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ABSTRACT

PD 404182, a heterocyclic iminobenzothiazine derivative, is a member of the Library of Pharmacologically Active Compounds (LOPAC) that is reported to possess antimicrobial and antiinflammatory properties. In this study, we used biochemical assays to screen LOPAC against human dimethylarginine dimethylaminohydrolase isoform 1 (DDAH1), an enzyme that physiologically metabolizes asymmetric dimethylarginine (ADMA), an endogenous and competitive inhibitor of nitric oxide (NO) synthase (NOS). We discovered that PD 404182 directly and dose-dependently inhibits DDAH. Moreover, PD 404182 significantly increased intracellular levels of ADMA in cultured primary human vascular endothelial cells (ECs) and reduced lipopolysaccharide (LPS)-induced NO production in these cells suggesting its therapeutic potential in septic shock-induced vascular collapse. In addition, PD 404182 abrogated the formation of tube-like structures by ECs in an in vitro angiogenesis assay, indicating its anti-angiogenic potential in diseases characterized by pathologically excessive angiogenesis. Furthermore, we investigated the potential mechanism of inhibition of DDAH by this small molecule and found that PD 404182, which has striking structural similarity to ADMA, could be competed by a DDAH substrate, suggesting that it is a competitive inhibitor. Finally, our enzyme kinetics assay showed time-dependent inhibition and our inhibitor dilution assay showed that the enzymatic activity of DDAH did not recover significantly after dilution, suggesting that PD 404182 might be a tightly bound, covalent, or an irreversible inhibitor of human DDAH1. This proposal is supported by mass spectrometry studies with PD 404182 and glutathione.
INTRODUCTION

We and others have applied high throughput screening (HTS) to discover small molecules that regulate the nitric oxide (NO) synthase (NOS)/dimethylarginine dimethylaminohydrolase (DDAH) pathway (Ghebremariam et al., 2012; Ghebremariam et al., 2013; Hartzoulakis et al., 2007; Kotthaus et al., 2008; Linsky and Fast, 2011; Linsky et al., 2011; Linsky and Fast, 2012; Wang et al., 2009). This scientific interest stems from the essential biological role that the NOS/DDAH pathway plays in metabolic, cardiovascular, pulmonary, immune and nervous systems (Leiper and Nandi, 2011; Palm et al., 2007). The substrate L-arginine is converted to NO by the enzymatic activity of each of the three NOS isoforms which are expressed in multiple cell types including vascular endothelial cells (ECs), fibroblasts, macrophages and lung epithelial cells. The first isoform, neuronal NOS (nNOS; NOS I), is constitutively expressed in the cytosol of neuronal cells and is physiologically involved in neurotransmission (Bryan et al., 2009). Overproduction of NO in the central nervous system has been implicated in tension-type and cluster headaches as well as migraine attacks (Lassen et al., 1998; Olesen, 2010). Type II NOS (iNOS; NOS II) is an inducible isoform predominantly expressed in alveolar macrophages and other immune cells and plays a critical role in physiological immune defense (Pechkovsky et al., 2002). The third isoform of NOS (eNOS; NOS III) is expressed in endothelial cells and is a major determinant of vascular tone, vascular growth, and interaction of the vessel wall with circulating blood elements (Dinerman et al., 1993).

The eNOS isoform produces small amounts of NO in a highly regulated manner. For example, the tractive force of fluid flow activates eNOS, leading to flow-mediated vasodilation which reduces endothelial shear stress (Cooke et al., 1991). In addition, physiological concentrations of NO inhibit platelet and immune cell adherence to vessel walls, and maintain quiescence of the underlying vascular smooth muscle (Cooke and Tsao, 1993). By contrast, iNOS is induced by cytokines, and is not regulated by hemodynamic stimuli. Notably, the catalytic activity of
iNOS is 1000-fold greater than eNOS. As a result, iNOS generally outstrips the local L-arginine supply. As a result, in addition to oxidizing L-arginine to NO, iNOS donates electrons to oxygen, generating superoxide anion, O$_2^-$; Because NO and O$_2^-$ are highly reactive, they combine rapidly to form the peroxynitrite anion (OONO$^-$). This reactive molecule is useful in destroying pathogens. However, inappropriate activation of iNOS may be involved in peroxidation of lipids in cell membranes and nitration of tyrosines in signaling proteins, leading to impaired cell signaling, tissue injury and further inflammation. In addition, peroxynitrite anion activates matrix metalloproteinases (MMPs), which degrade collagen (Galis and Khatri, 2002). This activation of MMPs contributes to loss of normal tissue architecture leading to pathological conditions. Parenthetically, the nitrosative stress produced by iNOS activation may also induce endothelial dysfunction with impaired regulation of vascular tone as in septic shock (Julou-Schaeffer et al., 1990; Landry and Oliver, 2001; Lorente et al., 1993; Nandi et al., 2012). This could be in part due to “uncoupling” of eNOS (Munzel et al., 2005). The oxidative stress reduces levels of tetrahydrobiopterin (BH$_4$), a cofactor that is necessary for proper eNOS dimerization and oxidation of L-arginine to NO. As a result, the “uncoupled” eNOS transfers electrons to oxygen, generating more superoxide anion, which promotes further inflammation and tissue injury.

The activity of each of the NOS isoforms is decreased by an endogenous inhibitor, asymmetric dimethylarginine (ADMA). ADMA is derived from proteolysis of nuclear proteins containing methylated arginine residues; a chemically related endogenous inhibitor is monomethylarginine or MMA, but this is a less common. Free ADMA (and MMA) molecules are largely degraded (~80%) within the cell by DDAH (Palm et al., 2007) into citrulline and dimethylamine (in the case of MMA, monomethylamine). DDAH is widely expressed in mammalian cells in one of two isoforms (DDAH1 and DDAH2) (Palm et al., 2007). The activity of DDAH is reduced by oxidative stress that occurs in various cardiovascular disorders (Palm et al., 2007; Stuhlinger et al., 2001). The reduced expression of DDAH causes an increase in circulating ADMA levels and
consequent suppression of NOS activity. By contrast, DDAH is overly active in some diseases including idiopathic pulmonary fibrosis (IPF) (Janssen et al., 2013; Pullamsetti et al., 2011). Notably, DDAH expression has been reported to be induced by inflammatory cytokines (Ueda et al., 2003), which may explain its elevation in pulmonary fibrosis. Therefore, inhibition of DDAH activity using small molecules may increase local ADMA concentration and place a brake on the activity of iNOS.

In this study, our HTS for DDAH inhibitors revealed that the small molecule PD 404182 is a potent inhibitor of human DDAH1 activity and elevates cellular ADMA levels. In addition, PD 404182 reduced lipopolysaccharide (LPS)-induced elevation of NO. Moreover, PD 404182 abrogated angiogenic response of vascular ECs. We speculate that PD 404182 has therapeutic potential in diseases characterized by overproduction of NO.
MATERIALS AND METHODS

High throughput screening of DDAH modulators

The Stanford High Throughput Bioscience Center (HTBC) maintains a diverse collection of compounds gathered from various sources including the Food and Drug Administration (FDA) and the National Institutes of Health (NIH). The Library of Pharmacologically Active Compounds (LOPAC) is a subset of this collection which consists of about 1,200 bioactive compounds obtained from Sigma-Aldrich (product # LO1280). We screened this library as part of a larger screen described recently (Ghebremariam et al., 2012). In brief, recombinant human DDAH1 was produced in a bacterial system, purified using affinity chromatography, and was confirmed by Western blot and mass spectrometry. A biochemical assay was developed to monitor DDAH enzymatic activity by studying conversion of ADMA into L-citrulline and to identify small molecules that alter its activity (Ghebremariam et al., 2012). Initially, a single concentration screen was performed with the LOPAC and compounds that decreased DDAH activity by 30% or more compared to controls were confirmed using a 7-point dose response as described (Ghebremariam et al., 2012). Hits were further validated using an orthogonal assay and freshly prepared compounds as described below.

Validation of DDAH inhibition by PD 404182

The small molecules that consistently showed modulation of DDAH enzymatic activity in the colorimetric assay described above were cross-validated using a fluorimetric assay that uses a synthetic DDAH substrate, S-methyl-thiocitrulline (SMTC) (Ghebremariam et al., 2012; Linsky and Fast, 2011). The catabolism of SMTC produces methanethiol (CH₃-SH); a product that can be quantified fluorimetrically upon reaction with a maleimido-coumarin containing reagent (Ghebremariam et al., 2012). Comparison of fluorescence intensity with controls allowed the identification of small molecules that regulate DDAH enzymatic activity. Of the several inhibitors
of DDAH activity, PD 404182 was identified as a novel and potent antagonist that inhibited DDAH activity in a dose-dependent fashion as described below.

**Cellular ADMA and NO measurements**
To further confirm our in vitro finding and to validate the efficacy of PD 404182 as a viable DDAH antagonist, we performed cell culture studies of vascular endothelial cells to measure intracellular ADMA and NO levels, indicators of cellular DDAH activity. Briefly, human dermal microvascular endothelial cells (HMVECs; female donor, Lonza, Walkersville, MD; cat # CC-2543) were cultured using standard techniques in cell culture flasks until ~60% confluency. Next, the cells were rinsed with PBS and fresh media was mixed with vehicle, PD 404182 or L-257 [a known and selective DDAH1 inhibitor; (Leiper et al., 2007; Nandi et al., 2012); kindly provided for this study by Dr. James Leiper (Imperial College London)] at 20 µM final compound concentration. The cells were cultured for 24 hours (with or without 100 ng/mL of bacterial LPS) and the effect of the compounds on cellular ADMA and NO was studied in the cell lysate. The concentration of ADMA and NO was quantified using ELISA-based biochemical assays following the recommendation of the suppliers (DLD Diagnostika for ADMA and Assay Designs for NO).

**In vitro angiogenesis assay**
One of the characteristic features of endothelial cells (ECs) is their ability to form capillary-like tubes and cord-like structures, an in vitro model of angiogenesis, when seeded in Matrigel (Kiuchi et al., 2008). This ability of ECs to form tubes can be enhanced or inhibited by pharmacological agents that increase or decrease NO production respectively. Accordingly, 6-well plates were coated with Matrigel (BD Biosciences) at 37°C for 45 min prior to seeding HMVECs. The cells were treated with PD 404182 (50 – 100 µM); L-257 (50 – 100 µM) or vehicle and incubated at 37°C (5% CO₂) for 18 hours to allow tube formation. The next day, the
effect of the different pharmacological agents on tubulogenesis was assessed microscopically by scanning the wells. Representative images of each condition were captured from non-overlapping fields. Meanwhile, a cell viability study was performed using the lactate dehydrogenase (LDH)-release assay (Sigma cat # TOX7) by incubating PD 404182 or L-257 with ECs at increasing compound concentration (10 to 300 µM each).

**Enzymatic activity competition assay**

In order to examine whether PD 404182 is an active-site competitive DDAH1 inhibitor, we assessed the effect of increased substrate concentration on the enzymatic reaction. First, we incubated DDAH (30 nM final concentration) with PD 404182 (10 µM final concentration) or vehicle (diluted DMSO). Next, various concentrations of SMTC (50 µM to 1 mM) were added to the wells (triplicate of each condition) to initiate the reaction. Fluorescence intensity, proportional to DDAH enzymatic activity, was monitored and DDAH activity in the PD 404182 containing wells was calculated relative to the vehicle control wells.

**Reversibility study**

To study whether DDAH1 inhibition by PD 404182 can be reversed, we performed a jump dilution assay as described (Copeland et al., 2011). In brief, PD 404182 (at 10X or 100X its IC_{50} value) was pre-incubated with high concentration of DDAH (3 µM) for 1 hour at room temperature prior to a jump dilution by 100-fold with a solution containing the substrate SMTC. Subsequently, the recovery of DDAH enzymatic activity was evaluated by comparing data points for statistical significance.

**Kinetic characterization**

One of the classic properties of irreversible enzymatic inhibitors is the ability to progressively inhibit enzymatic activity over a period of time. To address if PD 404182 shows this
characteristic, we performed kinetic analyses by following inhibition of the conversion of SMTC into the product methanethiol in the presence of the compound (10 µM final concentration), vehicle or ebselen (a known irreversible DDAH1 inhibitor (Linsky et al., 2011)) over a period of several hours. The percent inhibition over time for PD 404182 and ebselen was calculated relative to the vehicle treated wells and was expressed as Mean ± SEM.

**Electrospray ionization mass spectrometry (ESI/MS) study**

The interaction of PD 404182 with glutathione was examined by ESI/MS. In brief, PD 404182 was dissolved in DMSO to make a 50 mM stock concentration. Next, the compound was reacted with glutathione in 50 mM pH 7.7 sodium borate buffer by incubating for 2 hours at room temperature; both compounds were at 5 mM. Subsequently, the samples were subjected to HPLC-MS on an Agilent 1260 HPLC equipped with an Agilent 6140 spectrometer. The column was a Synergi 4 µm Hydro-RP 80 Å 30 x 2.0 mm with initial conditions of 95% A (0.1% formic acid in water)/5% B (0.1% formic acid in acetonitrile) held 0.3 minutes then ramped to 100% B in 1.2 minutes at a flow rate of 1.5 mL/min. Ionization was in both positive and negative mode, with detection from 90-800 m/z. The column temperature was 40 °C and the injection volume was 1 µL. The UV wavelength was set to 254 nm. Experiments were also performed at pH 8.5 and 9.5, with similar results.
RESULTS

PD 404182 inhibits human DDAH1 enzymatic activity

The enzymatic activity of human DDAH1 was significantly and dose-dependently inhibited by PD 404182 (Figure 1) with an IC$_{50}$ = 9 µM. Interestingly, PD 404182 possesses significantly higher in vitro potency than the substrate-like DDAH1 inhibitor L-257 (IC$_{50}$ = 20 µM (Leiper et al., 2007)) in modulating DDAH enzymatic activity (Figure 2) suggesting its feasibility as a probe to develop novel classes of inhibitors of human DDAH1.

PD 404182 increases cellular ADMA concentration

The inhibition of DDAH enzymatic activity by PD 404182 in biochemical assays in vitro was corroborated by cell based assays that quantify components of the DDAH/NOS pathway. In this study, PD 404182 caused significant elevation of the endogenous substrate ADMA in endothelial cells (ECs) incubated with the small molecule (Figure 3). Treatment of ECs with PD 404182 resulted in a roughly 70% increase in cellular levels of ADMA compared to the 60% increase seen in the cells treated with L-257.

PD 404182 reduces LPS-induced NO production

One of the consequences of septic shock resulting from bacterial infection is uncontrolled increase in iNOS-derived vascular NO production resulting in pathological decline in blood pressure. Since iNOS can be induced by bacterial lipopolysaccharide (LPS) (Lowenstein et al., 1993), we studied the effect of PD 404182 on LPS induced NO synthesis. In this study, LPS markedly increased NO production by the vascular ECs. Pre-incubation with PD 404182 significantly attenuated the increment in NO and nearly reversed NO concentrations to normal levels (Figure 4).
PD 404182 inhibits *in vitro* angiogenesis

NO plays a significant role in physiological (as in wound healing) and pathological (as in cancer metastasis) angiogenesis. Inhibitors of DDAH would limit NO production by elevating ADMA levels. Here, we found that PD 404182 is a potent inhibitor of EC angiogenesis (Figure 5). The compound concentration used in this study (50 to 100 µM) did not perturb cell membrane integrity (as demonstrated by lack of LDH leakage into the conditioned media (Supplemental Figure 1) or induce cytotoxicity as demonstrated by standard Trypan Blue staining; a finding that is validated by a previous report on several human cell lines (Chamoun et al., 2011). Interestingly, PD 404182 has been reported to inhibit EC sprouting in response to the potent pro-angiogenic protein vascular endothelial growth factor (VEGF) (Kalen et al., 2009) suggesting its therapeutic potential in pathological angiogenesis. The anti-angiogenic potency of PD 404182 is relatively modest compared to NOS inhibitors (Iwasaki et al., 1997; Pfeiffer et al., 1996; Takaoka et al., 2013) and other anti-angiogenic agents including inhibitors of tyrosine kinase, fibroblasts growth factor (FGF) and its receptors, proteinases, prostaglandins, integrins and adhesion molecules that have been tested in cancer malignancy and metastatic studies (Davis, 2008).

**PD 404182 is a competitive DDAH inhibitor**

Our study of competition between DDAH substrate and PD 404182 for occupancy of the active site in DDAH revealed that PD 404182’s inhibitory action can be significantly competed away by increasing the substrate concentration (Figure 6) suggesting that PD 404182 inhibited DDAH activity, at least in part, by residing in the active site of the enzyme. This finding indicates that PD 404182 is a competitive inhibitor of human DDAH1. However, our attempt to reverse inhibition of DDAH activity by diluting away the inhibitor did not show significant recovery of enzymatic activity (Figure 7) suggesting that PD 404182 might be an irreversible or slowly-dissociating inhibitor. These findings are consistent with the time-dependent inhibition of DDAH
by PD 404182 (Supplemental Figure 2). In addition, our electrospray ionization mass spectrometry (ESI/MS) study of PD 404182 with glutathione, a cysteine-containing compound, revealed the formation of new products. The observed masses of these products correspond to cyanylated glutathione and a disulfide-bonded adduct (Supplemental Figure 3 and discussion below) (Degani and Patchornik, 1974). These results suggest that PD 404182 is inherently reactive to thiol groups such as the cysteine in the active site of DDAH1.
DISCUSSION

Preclinical and clinical studies indicate an important role of the NOS/DDAH pathway in a number of acute and chronic cardiovascular, immune, neurological and pulmonary diseases. It is therefore logical to pharmacologically exploit this pathway for the management of such disorders. Inhibition of DDAH activity would be expected to increase endogenous ADMA which would in turn inhibit iNOS and reduce nitrosative stress.

Recently, dimethylarginine dimethylaminohydrolase (DDAH) has been implicated in IPF. DDAH is upregulated in lung tissue from patients with IPF (Janssen et al., 2013; Pullamsetti et al., 2011), and would be expected to increase iNOS activity by reducing ADMA levels. The increased expression of DDAH would unleash iNOS, so as to generate toxic nitrosative radicals and increased lung injury (Genovese et al., 2005). Notably, bleomycin induces greater fibrosis and impairment of lung function in mice that overexpress DDAH (Pullamsetti et al., 2011). By contrast, inhibition of DDAH or iNOS ameliorates the lung injury induced by bleomycin (Pullamsetti et al., 2011). Other inflammatory diseases where iNOS overactivity contributes to lung pathobiology include asthma and COPD (Batra et al., 2007; Seimetz et al., 2011).

In the present study, we identified a novel bioactive small molecule, PD 404182, that regulates ADMA and NO levels through inhibition of DDAH enzymatic activity. It appears that PD 404182 inhibits human DDAH1 activity competitively with substrate. Inhibitor dilution and time-dependent kinetic experiments show that PD 404182 is likely a competitive but slowly-dissociating or irreversible inhibitor. Interestingly, the compound bears structural similarity with the endogenous substrate ADMA (Figure 8) suggesting that it may interact covalently with the catalytic site of DDAH. The intriguing mechanism of PD 404182 interaction with sulfhydryl-containing compounds such as glutathione suggests that PD 404182 might transfer a cyano group to the sulfhydryl moiety on the protein. Protein cyanylation and cyanocysteine-mediated
cyclization have been previously reported (Takahashi et al., 2007; Takenawa et al., 1998), although this was not known to be a characteristic of PD 404182. This mechanism may explain the low recovery of DDAH activity upon inhibitor dilution (Figure 7). Crystallographic and protein mass-spectrometry studies will ultimately provide greater insight into this question.

In addition to its inhibition of DDAH, PD 404182 has been reported to have antibacterial, antiviral, antiinflammatory, and anti-angiogenic properties (Birck et al., 2000; Chamoun et al., 2011; Kalen et al., 2009; Sansom, 2001) as well as the ability to regulate mammalian circadian rhythms (Isojima et al., 2009). First discovered as an inhibitor of 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase (KDO 8-P synthase; in vitro inhibition constant, $K_i$, of less than 1 µM), an enzyme important for the synthesis of LPS and gram negative bacterial cell walls (Birck et al., 2000), PD 404182 has been considered as a candidate antibiotic against gram-negative bacteria (Sansom, 2001). Recently, it was reported to have antiviral effects against human immunodeficiency virus (HIV) and hepatitis C virus (HCV) through an unknown mechanism (Chamoun et al., 2011). As a result of this promising polypharmacological potential, a number of facile synthetic methods for lead optimization have been proposed (Mizuhrara et al., 2010; Mizuhara et al., 2012a; b; 2013).

In this study, we extend the pharmacological potential of PD 404182 by demonstrating its ability to directly regulate human DDAH1 enzymatic activity in biochemical and cell biological studies. It is intriguing that PD 404182 significantly reduced LPS-induced NO overproduction, as it also has antibacterial activity against LPS-containing gram negative bacteria (such as *E. coli*). Such an agent could simultaneously oppose bacterial infection while reducing the adverse effect of LPS on blood pressure (Dellinger et al., 2008). In the US alone, about 200,000 people die annually from sepsis-related complications, and sepsis costs over 16 billion dollars in direct and indirect medical expenses (Angus et al., 2001; Jones, 2006; Weycker et al., 2003).
Recently, Nandi et al. (Nandi et al., 2012) demonstrated the therapeutic potential of inhibiting DDAH1 to regulate blood pressure in a mouse model of endotoxic shock induced by LPS (Nandi et al., 2012). In their study, the selective DDAH1 inhibitor L-257 significantly prevented the drop in mean arterial blood pressure (MABP) in response to LPS treatment. In our study of vascular ECs treated with LPS, PD 404182 was as effective as L-257 in suppressing the LPS-induced spike in NO (Figure 4). Although our in vitro enzymatic assay study shows that PD 404182 (IC$_{50}$ = 9 µM) is about 2-fold more potent against human DDAH1 than L-257 (IC$_{50}$ = 20 µM (Leiper et al., 2007)), they possess similar potency in cell culture studies of ADMA and NO levels, perhaps due to the dynamic regulation of these endogenous molecules by a cascade of biochemical pathways, and/or due to cellular availability of these molecules (Meijer et al., 1990; Palm et al., 2007). However, because PD 404182 also possesses potent antibacterial activity (Birck et al., 2000; Sansom, 2001), in addition to reducing NO synthesis in response to LPS, it may be a better candidate for some forms of septic shock.

Our finding that PD 404182 has anti-angiogenic effects is consistent with a previous report demonstrating inhibition of VEGF-induced sprouting of endothelial cells (Kalen et al., 2009). This property of PD 404182 might be useful in the development of drugs or as adjuvant therapy to reduce DDAH and/or NO-dependent cancer metastasis (Wink et al., 1998). NO is known to be involved in tumor progression and carcinogenesis resulting in increased vascularity (angiogenesis) and spread of cancer (tumorigenesis) (Fukumura et al., 2006). In addition, glioma tumor cells that overexpress DDAH have been reported to show increased VEGF and NO production and present a more aggressive phenotype showing increased proliferation, vascularity and tumor blood volume when implanted into animals (Kostourou et al., 2002; Kostourou et al., 2003). Furthermore, the DDAH/NOS machinery is upregulated in human prostate cancer (Vanella et al., 2011). However, any application of a DDAH antagonist must also take into account the physiological importance of eNOS in the systemic vasculature. Thus
for most indications, it is likely that antagonists of DDAH would be administered with limitations in space and time (eg. intermittent aerosolized administration to the lung). Nevertheless, given the lack of optimal therapies for several oncologic, cardiovascular and pulmonary diseases a novel therapeutic avenue is welcome, and antagonists of DDAH activity deserve further study.
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Authorship Contributions

Participated in research design: Ghebremariam, Erlanson and Cooke.

Conducted experiments: Ghebremariam and Erlanson

Performed data analysis: Ghebremariam, Erlanson and Cooke.

Wrote or contributed to the writing of the manuscript: Ghebremariam, Erlanson and Cooke.
References


Footnotes

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Conflict of Interest: YTG and JPC are inventors on patents, owned by Stanford University, that protect the use of agents that modulate the NOS/DDAH pathway for therapeutic application.

Reprint Requests:

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Figure Legend:

Figure 1: Dose-dependent inhibition of DDAH activity by a novel small molecule: PD 404182. The biochemical conversion of the artificial substrate SMTC into CPM in the presence of PD 404182 is shown. The mean % inhibition at 4 hours, in reference to vehicle control, from duplicate experiments is shown. The X-axis shows final compound concentration.

Figure 2: Validation of DDAH inhibition by PD 404182. In vitro production of L-citrulline from the endogenous substrate ADMA in the presence of vehicle (DMSO) control, PD 404182 or L-257 is shown. Compounds were used at 20 µM final concentration each. Data is Mean ± SEM from duplicate experiments (*p<0.05).

Figure 3: Intracellular inhibition of DDAH activity by PD 404182. Cellular ADMA concentration in vascular endothelial cells (ECs) following treatment with vehicle or the indicated small molecules at 20 µM each. Data is Mean ± SEM from duplicate experiments (*p<0.05).

Figure 4: The effect of PD 404182 on nitric oxide (NO) production. Human vascular endothelial cells were treated with vehicle, PD 404182 or L-257 in the presence of LPS (100 ng/mL; Sigma) for 24 hours. Total nitrite (NOx) was measured using Griess reaction. Data is Mean ± SEM from duplicate experiments. *p<0.05 compared to non-LPS control. *+p<0.05 compared to vehicle + LPS.

Figure 5: PD 404182 attenuated endothelial tube formation. Human microvascular endothelial cells were seeded on Matrigel and treated with vehicle, PD 404182 or L-257 for 18 hours and formation of tube-like structure was visualized microscopically. Representative images are from duplicate experiments.
**Figure 6:** Competition Assay. DDAH was incubated with PD 404182 (10 µM) or vehicle for 30 minutes and various substrate (SMTC) conc. was added to initiate the reaction. Fluorescence intensity was measured and DDAH activity was calculated relative to vehicle controls. Data is Mean ± SEM from triplicate experiments. *p<0.05 Vs 50 µM; **p<0.05 between 100 µM and 1000 µM substrate concentration. The Michaelis constant (Km) for SMTC is about 3 µM (Wang et al., 2009).

**Figure 7:** Inhibitor dilution assay demonstrating low recovery of DDAH activity. PD 404182 (IC₅₀ ~ 9 µM) was pre-incubated with DDAH at high concentration and then serially diluted as described above. The recovery of DDAH activity upon dilution of the inhibitor was evaluated. Data is Mean ± SEM from triplicate experiments. ns = not significant.

**Figure 8:** Schematic of the structural resemblance of PD 404182 with the endogenous NOS inhibitor and DDAH substrate ADMA. The chemical similarities between the two are highlighted in bold. A putative reaction of PD 404182 with the active site cysteine (Cys 273) of human DDAH-1 is also proposed. The active site cysteine first forms a covalent intermediate with PD 404182. This can fragment to release “thiol 192” (a species with a molecular weight of 192), leaving the cysteine cyanylated. This could then form a mixed disulfide with thiol 192 or other thiols.
Fig-6

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1hr at RT pre-incubation prior to 100-fold dilution with substrate containing buffer

100X DDAH
10X or 100X IC₅₀ Inhibitor

1X DDAH
0.1X or 1 X IC₅₀ Inhibitor

% DDAH Activity

ns

9 µM

0.9 µM

[PD 404182]

DDAH (0.3 µM)