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(2R)-[(4-Biphenylsulfonyl)amino]-N-hydroxy-3-phenylpropionamide (BiPS), a Matrix Metalloprotease Inhibitor, Is a Novel and Potent Activator of Hypoxia-Inducible Factors

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Abstract

Hypoxia-inducible factors (HIFs) are unstable heterodimeric transcription factors and decisive elements for the transcriptional regulation of genes important in the adaptation to low-oxygen conditions. Hypoxia is the ubiquitous inducer of HIFs, stabilizing the α -subunit and permitting the formation of a functional HIF complex. Here, we identify (2R)-[(4-biphenylsulfonyl)amino]-N-hydroxy-3-phenylpropionamide (BiPS), a commercially available metalloprotease-2 and -9 inhibitor, as a rapid and potent inducer of HIFs. We show that in different cell lines, BiPS induces the HIF- α subunit by inhibiting its degradation through stabilization of its labile oxygen-dependent degradation domain. This is achieved through the inhibition of HIF-1 α hydroxylation. The HIF-1 complex, formed after BiPS treatment, is capable of DNA binding and activation of HIF target genes, including the expression of vascular endothelial growth factor. Because novel HIF activators have generated considerable interest in the possible treatment of different ischemic diseases, we believe that BiPS and derivative molecules could have strong therapeutic potential.

Molecular oxygen is crucial for aerobic energy metabolism and cell survival. Hypoxia-inducible transcription factors (HIFs) are decisive elements in the adaptation to low-oxygen conditions by permitting the expression of numerous genes involved in angiogenesis, anaerobic glycolysis, and cell survival (Semenza, 2003). HIFs are heterodimers composed of an oxygen-sensitive subunit, HIF-1 α , HIF-2 α , or HIF-3 α and a constitutive HIF- β subunit. HIF-1 α is expressed ubiquitously and has been highly characterized. The regulation of HIF-2 α , also named HLF, EPAS1, HRF, or MOP2, is similar to HIF-1 α , but its expression is more restricted (Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Tian et al., 1997). HIF-3 α has been less characterized and could act as a repressor of HIF-1 and HIF-2 activities (Gu et al., 1998; Makino et al., 2001, 2002).

Under normal oxygen conditions, the α subunit is hydroxylated through the action of HIF prolyl hydroxylases (PHDs). This hydroxylation occurs on two specific proline residues (Pro402 and Pro564 on human HIF-1 α) contained in its oxygen-dependent degradation domain (ODDD). Hydroxylation of HIF- α allows the binding of pVHL, the product of the von Hippel-Lindau tumor suppressor gene. As the recognition component of an E3 ubiquitin ligase complex, pVHL allows for HIF- α polyubiquitination and subsequent proteosomal degradation (Schofield and Ratcliffe, 2004). Furthermore, HIF- α is hydroxylated on a

specific asparagine residue (Asn803 on human HIF-1 α) contained in its C-terminal transactivation domain by another dioxygenase, factor-inhibiting HIF. Asn803 hydroxylation inhibits HIF transcriptional activity by preventing binding of the coactivator p300/cAMP response element-binding protein-binding protein (Mahon et al., 2001). Inactivated by hypoxia, HIF hydroxylases are dependent on oxygen and 2-oxoglutarate (2-OG) as substrates and ascorbate and iron as cofactors. Therefore, in most if not all cell types, the lack of oxygen permits the stabilization of HIF- α and the formation of a transcriptionally active HIF complex. Given the importance of HIF in response to ischemic conditions, the development of novel HIF inducers/activators has gained strong interest because of their therapeutic potential in the treatment of different ischemic disorders (Khan et al., 2003; Simons and Ware, 2003; Hewitson and Schofield, 2004).

In this study, we present (2*R*)-[(4-biphenylsulfonylethyl)-amino]-*N*-hydroxy-3-phenylpropionamide (BiPS) as a potent activator of both HIF-1 and HIF-2. This compound, derived from *N*-sulfonylamine acid, was originally designed as an inhibitor of matrix metalloproteinases (MMP) -2 and -9 (Tamura et al., 1998). A number of studies using this compound have demonstrated potent effects in different *in vivo* and *in vitro* situations. These include reducing lung colonization by Lewis lung carcinoma cells in mice (Tamura et al., 1998), blocking lymphocyte migration across endothelial cells (Deem and Cook-Mills, 2004), inhibiting transforming growth factor- β -induced cataract formation in rat lens (Dwivedi et al., 2006), blocking the invasion of mouse brain microvessel endothelial cells in Matrigel (Fears et al., 2005), and impeding the migration of smooth muscle cells (Lin et al., 2007).

In addition to its well-characterized effects on MMP activity, here we show that BiPS can also induce HIFs. In the present study, we demonstrate that BiPS stabilizes HIF- α protein by blocking HIF- α hydroxylation. This increase in HIF- α protein levels leads to the formation of active HIF complexes and the expression of HIF-target genes. Therefore, our study characterizes BiPS as a novel and potent activator of HIF complexes, which could be of particular interest in the chemical pharmacology of the HIF signaling cascade.

Materials and Methods

Materials

BiPS, also known as MMP-2/MMP-9 Inhibitor II (Tamura et al., 1998), was from Calbiochem/EMD Chemicals (Gibbstown, NJ). Cobalt chloride (CoCl₂), dimethyl-2-OG, and MG132 were from Sigma (St. Louis, MO). pGL3 (R2.2) 3HRE-tk-LUC luciferase reporter vector was generated in our laboratory (Lauzier et al., 2007). GST-HIF-1 α 344–582, pVHL-HA, and CMV-Luc-ODD constructs were kindly provided by Drs Jacques Pouyssegur (Institute of Signaling, Developmental Biology and Cancer Research, Université de Nice, Nice, France), Peter Ratcliffe (University of Oxford, Oxford, England), and Richard K. Bruick (University of Texas, Austin, TX), respectively.

Cell Culture

VSMCs were isolated from thoracic aorta of 6-week-old male Wistar rats (Owens et al., 1986) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (50 U/ml penicillin, 50 U/ml streptomycin). Bovine aortic endothelial cells (BAECs) were isolated from calf aortas and cultured in DMEM containing 10% FBS and antibiotics. HeLa cells were cultured in DMEM containing 10% heat-inactivated FBS and antibiotics. All cells were cultured in a humid atmosphere (5% CO₂, 95% air) and serially passaged upon reaching confluence. Hypoxic conditions were obtained by placing cells in a sealed hypoxic workstation (Ruskin, Bridgend, UK). The

oxygen level in this workstation was maintained at 1% with the residual gas mixture containing 94% nitrogen and 5% carbon dioxide. All media for cell culture were from Invitrogen (Carlsbad, CA) unless otherwise indicated.

Western Blot Analysis

Cells were lysed in 2× Laemmli sample buffer. Protein concentration was determined by Lowry assay. Cell extracts (25 µg) were loaded on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (Immobilon-P; Millipore Corporation, Billerica, MA) or nitrocellulose membranes (Hybond C Extra; GE Healthcare Life Sciences, Piscataway, NJ). Anti-HIF-1 α and anti-HIF-2 α antisera were raised in rabbits immunized against the last 20 amino acids of the C termini of each human protein (Richard et al., 1999). Anti-hydroxylated HIF-1 α raised against hydroxylated Pro402 and hydroxylated Pro564 of the human HIF-1 α sequence were obtained as described previously (Chan et al., 2005). Monoclonal anti-HIF-1 α and anti-GST antibodies and polyclonal anti-HIF-1 β antibody were from Novus Biologicals (Littleton, CO). Monoclonal HA.11 antibody was from Convince (Emeryville, CA). Total polyclonal p42/p44 MAPK antibody was from Millipore Biotechnology (Lake Placid, NY) and used as a loading control. Horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies were from Pro-mega (Madison, WI). Proteins were visualized with an enhanced chemiluminescence system (GE Healthcare Life Sciences) or with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Western blots were quantified using Odyssey quantification software or ImageJ (available at <http://rsb.info.nih.gov/ij>).

RNA Interference

To down-regulate HIF- α protein expression in HeLa cells, small interfering RNA (siRNA) duplexes targeting human HIF-1 α (accession number NM_001530; sense, 5'-AGGA-CAAGUCACAACAGGAUU-3') and human HIF-2 α (accession number NM_001430; sense, 5'-GGGUCAGGUAGUAAGUGGCUU-3') were obtained from Ambion (Austin, TX). As a control, *Silencer Negative Control #1 siRNA* was used (Ambion). HeLa cells were transfected with siRNA duplexes (20 nM) by calcium phosphate precipitation.

Luciferase Assay

HeLa cells, seeded in six-well plates, were transiently transfected by calcium phosphate precipitation with pGL3 (R2.2) 3HRE-tk-LUC luciferase reporter vector (1 µg/well) or CMV-Luc-ODD (0.5 µg/well). *Renilla reniformis* luciferase expression vector (250 ng/well) was also used as a control for transfection efficiency. At 30 h after transfection, cells were stimulated, and luciferase assays were performed using the Dual Luciferase Reporter Assay System (Pro-mega). Results were quantified with a Luminoskan Ascent microplate reader with integrated injectors (Thermo Electron, Milford, CA). Results are expressed as a ratio of firefly luciferase activity over *R. reniformis* luciferase activity. Experiments are an average $\pm$ S.E.M. of triplicate data and representative of three independent experiments performed on different cell cultures.

Transcription Factor Enzyme-Linked Immunoassay

Experiments were performed as described previously (Blouin et al., 2004). In brief, high-bind NeutrAvidin-coated 96-well strip plates (Pierce Biotechnology, Rockford, IL) were incubated with a 5'-biotinylated 26 base pair double-stranded DNA oligonucleotide for 1 h at room temperature. This sequence contains the wild-type or mutant (underlined) HIF-1 binding motif described previously (Semenza and Wang, 1992). Sequences used here were 5'-GATCGCCCTACGTGCTGTCTCAGATC-3' for W26 wild-type sequence and 5'-GATCGCCCTAAAAAGCTGTCTCAGATC-3' for M26 mutant sequence. Nuclear extracts

from HeLa cells were incubated with oligonucleotides, and HIF-1 complexes bound to DNA were detected using anti-HIF-1 α or anti-HIF-1 β antibodies (Novus Biologicals), horseradish peroxidase-conjugated secondary antibodies, and TMB-ONE (Pro-mega). Experiments are an average $\pm$ S.E.M. of triplicate data representative of three independent experiments performed on different cell cultures.

pVHL Capture Assay

HeLa cells were grown to confluence and stimulated as indicated. Cells were washed once in phosphate-buffered saline and twice in buffer containing 20 mM Tris, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, and 1 mM dithiothreitol. Cells were then suspended and lysed using a Dounce homogenizer. Cytoplasmic extracts were cleared by centrifugation (20,000g). Extracts (250 μ g) were incubated with Sepharose-bound GST-HIF-1 α 344–582 (30 μ g) for 1 h at room temperature, washed with NETN buffer (150 mM NaCl, 0.5 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Igepal, and 100 μ M deferoxamine), and incubated overnight with in vitro translated pVHL-HA in NETN at 4°C. Samples were washed with NETN, denatured in 2 $\times$ Laemmli sample buffer, resolved in SDS-polyacrylamide gels (12%), and revealed by Western blotting with anti-HA and anti-GST antibodies.

Iron Chelation Assays

Iron chelation potential was determined using a FerroZine-based iron measurement assay (Stokey, 1970; Fish, 1988). Ferrous chloride (FeCl₂, 20 μ M) was incubated with BiPS (75 μ M) or deferoxamine (50 μ M) for 30 min. FerroZine (20 mg/ml) and ammonium acetate (1 mg/ml) was then added to the reaction mixture and incubated at 37°C for 30 min. Iron was measured by determining sample absorbance at 562 nm by spectrophotometry. The amount of free iron in the solution was determined by comparison with a FeCl₂/FerroZine standard curve. Experiments are an average $\pm$ S.E.M. of triplicate data representative of three independent experiments.

Results

The effect of BiPS on HIF-1 α and HIF-2 α protein induction was evaluated in different cell lines including the established human HeLa cell line and primary cultures of BAECs and rat aortic VSMCs. As seen in Fig. 1, BiPS induced HIF- α protein levels in all cell lines tested. In HeLa cells, a treatment with 10 μ M BiPS for 2 h caused a detectable increase of both HIF-1 α and HIF-2 α proteins, whereas maximal induction was observed between 75 and 100 μ M BiPS (Fig. 1, top). BiPS was more potent in BAEC because 5 μ M led to a detectable increase of both HIF- α proteins, whereas maximal induction was observed at 25 μ M (Fig. 1, middle). In VSMCs, 10 μ M BiPS led to a detectable increase of HIF-1 α protein, whereas maximal induction was observed at 50 μ M BiPS (Fig. 1, bottom). However, we did not detect HIF-2 α protein levels in VSMCs treated with BiPS or under hypoxic conditions. HIF-1 α and HIF-2 α protein induction by BiPS was also observed in the macrophage-like cell line NR8383, human embryonic kidney 293 cells, the murine endothelial cell line 1G11 and mouse embryonic fibroblasts (results not shown). It is interesting to note that the maximal induction of HIF- α after BiPS treatment was comparable with treatments under hypoxic conditions or in the presence of CoCl₂, two main inducers of HIF- α . Finally, other MMP-2/9 inhibitors, such as GM6001 (galarin) and MMP-2/9 inhibitor I, did not induce HIF- α subunits (results not shown). These results indicate that the effect of BiPS on HIF- α induction was independent of MMP-2/9 inhibition and identify BiPS as a novel inducer of HIF- α subunits in normoxic cells.

The kinetics of HIF- α protein induction under BiPS treatment were rapid and comparable with hypoxic HIF- α induction. In HeLa cells, a detectable increase of HIF-1 α was observed

by 30 min, and maximal induction was attained after 2 h of treatment with 75 μ M BiPS (Fig. 2). It is interesting that although HIF-1 α levels were maintained in hypoxia for more than 6 h, treatment of cells with BiPS maintained HIF-1 α levels for up to 4 h and then subsequently decreased to basal levels. HIF-2 α protein induction by BiPS followed a similar kinetic pattern to HIF-1 α . These results suggest that like hypoxia, BiPS stabilizes HIF- α instead of exploiting transcriptional or translational mechanisms for protein induction as seen for other normoxic inducers.

To directly evaluate the effect of BiPS on HIF- α subunit stabilization, HeLa cells were transfected with a construct encoding a luciferase protein chimera destabilized by the HIF-1 α ODDD (Salnikow et al., 2004). In these conditions, changes in luciferase activity by treatment with BiPS would indicate the specific stabilization of HIF-1 α 's ODDD. As expected, the treatment of cells with CoCl₂ (Fig. 3A) or the proteasome inhibitor MG132 (results not shown) strongly increased luciferase activity. It is noteworthy that treatment with BiPS also increased luciferase activity to levels similar to those observed during MG132 and CoCl₂ treatments. This result indicates that BiPS stabilizes the HIF-1 α ODDD.

Because decreased pVHL binding leads to increased HIF- α stabilization, we examined pVHL binding to HIF-1 α after BiPS treatment. HeLa cells were treated with BiPS or CoCl₂ followed by the preparation of cytosolic extracts and in vitro hydroxylation of GST-HIF-1 α (344–582). After incubation with in vitro translated pVHL and GST pull-down, we observed that the treatment of cells with BiPS strongly prevented pVHL binding to HIF-1 α (Fig. 3B). It is interesting that the inhibition of pVHL binding with BiPS was more potent than with CoCl₂ treatment. This result suggests that BiPS inhibits PHD-mediated HIF- α hydroxylation. To confirm this, we evaluated the level of HIF-1 α hydroxylation using two specific antibodies against hydroxylated proline residues, Pro402 and Pro564 (Chan et al., 2005). For this experiment, cells were pretreated with MG132 and maintained in the presence of CoCl₂ or BiPS. As expected, the treatment of cells with CoCl₂ led to a near complete inhibition of HIF-1 α protein hydroxylation of both proline residues (Fig. 3C). The treatment of cells with BiPS also strongly decreased both HIF-1 α Pro402 and Pro564 hydroxylation. Taken together, these results indicate that BiPS is a potent inhibitor of PHD activity.

We then wanted to investigate the mechanism by which BiPS inhibits PHD activity. Because PHD requires Fe(II) to hydroxylate HIF- α subunits, we wanted to evaluate possibility that BiPS could inhibit PHD activity by acting as an iron chelating agent. In an iron chelation assay, deferoxamine, a well known iron chelator and HIF- α stabilizing agent, was able to chelate more than 80% of the iron present in our experimental conditions (Fig. 4A). However, BiPS did not act as an iron chelating agent because total free iron was completely accessible for reaction with FerroZine. This result suggests that BiPS does not inhibit PHD activity through iron chelation. PHDs belong to a large group of enzymes that use 2-oxoglutarate as a cosubstrate (Schofield and Ratcliffe, 2004). We wanted to determine whether BiPS could interfere with the binding of 2-oxoglutarate to PHD. We therefore performed a pVHL capture assay in which 2-OG was added to the cellular extracts. In these conditions, the addition of 2-OG increased pVHL binding to HIF-1 α (Fig. 4B). It is interesting that the treatment of cellular extracts with BiPS blocked 2-OG-induced pVHL binding to HIF-1 α . These results indicate that BiPS decreases PHD activity by interfering with 2-OG binding.

To determine the effect of BiPS treatment on HIF activity, we first evaluated its effect on the formation of an active HIF-1 transcription complex. To perform these studies, we used a HIF-1 transcription factor enzyme-linked immunoassay (Blouin et al., 2004). Nuclear extracts from HeLa cells maintained in hypoxic conditions or in the presence of BiPS both

demonstrated increased DNA-binding activity for HIF-1 α and HIF-1 β (Fig. 5, W26). To control for specificity, we substituted the W26 double-stranded DNA oligonucleotide sequence with a sequence mutated on two essential residues of the HIF-1-binding sequence (Fig. 5, M26). In this case, very little HIF-1 binding could be observed. This result indicates that BiPS stabilizes HIF-1 α and permits the formation of the HIF-1 complex and binding to target hypoxia response element (HRE) sequences.

We next attempted to determine whether BiPS treatment led to HIF-mediated transcriptional activity using a HRE-controlled reporter assay. HeLa cells were transiently transfected with a 3HRE-tk-LUC reporter vector followed by treatment of cells with BiPS or in hypoxic conditions for 6 h. Under these conditions, hypoxia led to an 11-fold induction in luciferase activity (Fig. 6). It is interesting that BiPS was more potent than hypoxia for activating HIF and increased reporter activity by 15.4-fold. We then studied the effect of BiPS treatment on the expression of a well known HIF-1-activated target gene, vascular endothelial growth factor (Ebert et al., 1995; Liu et al., 1995; Forsythe et al., 1996; Mazure et al., 1996). As seen in Fig. 7, the treatment of HeLa cells with BiPS strikingly increased VEGF expression. As in HRE reporter studies, BiPS was more potent than hypoxia for activating the expression of VEGF mRNA. To demonstrate the implication of HIF complexes in the increased expression of VEGF by BiPS, we used siRNA technology to deplete cells of HIF-1 α and HIF-2 α . The silencing of HIF-1 α protein strongly reduced the expression of VEGF, whereas the silencing of HIF-2 α caused a modest inhibition of the expression of this gene. Finally, the silencing of both HIF- α isoforms led to an additive inhibitory effect on VEGF mRNA expression. Effective silencing of HIF-1 α and HIF-2 α protein levels in HeLa cells is shown in the bottom of Fig. 7. Taken together, our results identify BiPS as a potent activator of HIF complexes.

Discussion

HIFs are decisive elements in the transcriptional regulation of genes implicated in cellular and physiological adaptation to hypoxic stress. The products of these genes are involved in several adaptive responses, including angiogenesis and erythropoiesis. A complex process, angiogenesis is regulated by several endogenous proteins and can be deregulated in tumor cell progression and during ocular and inflammatory pathologies. Angiogenesis may also be ineffective during ischemic stress, leading to tissue destruction. In this regard, the first therapeutic attempts to promote angiogenesis used the delivery of VEGF alone. This led to the production of leaky blood vessels and was proven to be ineffective in clinical trials (Ferrara, 2004). As a powerful regulator of many endogenous proangiogenic molecules, HIFs are considered appealing pharmacological targets for therapeutic angiogenesis. Different groups have shown the strong potential of HIF complexes in promoting vascularization in ischemic tissues (Li et al., 2000; Vincent et al., 2000; Elson et al., 2001; Shyu et al., 2002; Willam et al., 2002). In addition, HIFs are strong regulators of erythropoiesis by inducing erythropoietin (Epo) production. A recombinant form of this specific hormone is commonly used for its ability to treat anemia. However, the high cost of this therapy, combined with the need for parenteral administration and the development of anti-Epo antibodies, has triggered the development of different approaches to stimulate erythropoiesis. Therefore, the discovery of novel HIF-inducing compounds should lead to new strategies to promote angiogenesis and treat anemia. It is interesting that specific PHD inhibitors have been shown to successfully increase the hemoglobin levels in rhesus macaques (Hsieh et al., 2007) and Epo levels in mice (Kasiganesan et al., 2007). Here, we have identified BiPS, an MMP-2/9 inhibitor, as a novel and potent activator of HIFs. BiPS stabilizes HIF- α protein by blocking HIF α hydroxylation. This increase in HIF- α protein levels leads to the formation of active HIF complexes and the expression of HIF target

genes. Therefore, BiPS or derivative molecules could have interesting therapeutic options for HIF-regulated responses.

To our knowledge, the only compounds currently used to stabilize and activate HIFs are inhibitors of PHD activity. The hydroxylase activity of these enzymes is modulated by their cofactors, namely O₂, 2-OG, and Fe(II). The identification of PHD cofactors has led to the identification/design of compounds able to increase HIFs, including compounds that chelate Fe(II) (deferrioxamine) or compete with 2-OG binding to PHD (Hewitson and Schofield, 2004). Because BiPS can efficiently inhibit HIF-1 α hydroxylation and pVHL binding without chelating iron, we believe that BiPS functions by interfering with the binding of 2-OG to PHD. This is supported by the demonstration that BiPS blocks the potentiation of pVHL binding by 2-OG. In addition, using a docking simulation software (AutoDock, available at <http://autodock.scripps.edu/>), we performed a binding simulation of 2-OG and BiPS to PHD2, which corresponds to the current understanding of the 2-OG/PHD2 binding model (McDonough et al., 2006). In this simulation, the most probable position for BiPS binding to PHD2 was the 2-OG binding site in the cavity containing the Fe(II) atom. The biphenyl group of BiPS was positioned inside the cavity, whereas the two oxygen atoms of the sulfur group interacted with the Fe(II) atom. Furthermore, this model predicted that the PHD2 binding energy of BiPS is 1.65 times higher than 2-OG (mean of -7.009 ± 0.3 kcal/mol for BiPS and -4.245 ± 0.04 kcal/mol for 2-OG). Taken together, experimental and in silico evidence strongly suggests that BiPS induces and activates HIF by interfering with the binding of the PHD cofactor, 2-OG, leading to PHD inhibition.

We have successfully used BiPS for HIF induction in a variety of cell types. These characteristics reveal BiPS as an interesting compound for researchers interested in studying the HIF system. As a potent inhibitor of MMP-2/9, BiPS was used by many different groups that were unaware of its effect on HIF activity. BiPS was used to study migration and invasion properties of cells (Deem and Cook-Mills, 2004; Fears et al., 2005; Lin et al., 2007), to study the colonization of cancer cells in mice (Tamura et al., 1998), and to evaluate the remodeling and inflammation of airways during asthma (Lee et al., 2004). Depending on tissues targeted by BiPS the concentrations used, HIF activation could play an important role in the responses obtained using BiPS as an MMP-2/9 inhibitor. We believe that researchers considering the use of BiPS in their studies should strongly consider this possibility.

In this study, we present evidence that BiPS is a novel and potent PHD inhibitor in addition to its known role as an MMP-2/9 inhibitor. A PHD inhibitor, BiPS prevents pVHL binding to HIF- α and its subsequent degradation. In addition, BiPS permits the transcriptional activity of HIFs and the expression of target genes. Because PHD inhibitors are now recognized as potential therapeutic drugs in the treatment of anemia and ischemic diseases, we strongly believe that BiPS and derivative molecules could have strong therapeutic potential.

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ABBREVIATIONS

HIF	hypoxia-inducible factor
PHD	hypoxia-inducible factor prolyl hydroxylase
ODDD	oxygen-dependent degradation domain
pVHL	von Hippel Lindau protein
MMP	matrix metalloprotease
BAEC	bovine aortic endothelial cell
VSMC	vascular smooth muscle cell
2-OG	2-oxoglutarate
BiPS	(2 <i>R</i>)-[(4-biphenylsulfonyl)amino]- <i>N</i> -hydroxy-3-phenylpropionamide
MG132	<i>N</i> -benzoyloxycarbonyl (<i>Z</i>)-Leu-Leu-leucinal
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
siRNA	small interfering RNA
NETN buffer	NaCl, EDTA, Tris, Igepal, and deferoxamine
HA	hemagglutinin
HRE	hypoxia response element
PAGE	polyacrylamide gel electrophoresis
MAPK	mitogen-activated protein kinase
DMSO	dimethyl sulfoxide
GM6001	galardin

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Fig. 1. HIF- α induction by BiPS. Quiescent VSMC, quiescent BAEC, or HeLa cells were maintained under control conditions, in hypoxic conditions (1% O₂), in the presence of CoCl₂ (200 μ M), or various concentrations of BiPS or vehicle (DMSO 0.08%) for 2 h. Total cell extracts (25 μ g) were resolved by SDS-PAGE (8%) and immunoblotted using anti-HIF-1 α , anti-HIF-2 α , or anti-p42/p44 MAPK antibodies.

Fig. 2.

Time course for HIF- α induction by BiPS. HeLa cells were maintained under control conditions, in hypoxic conditions (1% O₂), or in the presence of 75 μ M BiPS or vehicle (DMSO 0.08%) for different periods of time. Total cell extracts (25 μ g) were resolved by SDS-PAGE (8%) and immunoblotted using anti-HIF-1 α , anti-HIF-2 α , or anti-p42/p44 MAPK antibodies.

Fig. 3.

Stabilization of HIF-1 α by BiPS. A, HeLa cells were transfected with 0.5 μ g of CMV-luc-HIF-1 α -ODDD and 250 ng of an expression vector coding for *R. reniformis* luciferase. At 40 h after transfection, cells were maintained under control conditions, hypoxic conditions (1% O₂), or in the presence of BiPS (75 μ M) or MG132 (5 μ M) for 6 h. Cells were lysed, and luciferase activity was measured. Results are expressed as a ratio of beetle luciferase activity to *R. reniformis* luciferase activity and are an average $\pm$ S.E.M. of at least three independent experiments performed in triplicate. B, cytoplasmic extracts from HeLa cells treated as indicated were incubated with HIF-1 α (344–582) GST protein-coupled Sepharose beads. Samples were then incubated overnight in the presence of in vitro translated pVHL and resolved by SDS-PAGE (12%). Immunoblotting was performed using anti-HA (pVHL) and anti-GST antibodies. C, HeLa cells were treated with MG132 (25 μ M) for 2 h and followed by the addition of CoCl₂ (200 μ M) or BiPS (75 μ M) for an additional 2 h. Total cell extracts (25 μ g) were resolved by SDS-PAGE (8%) and immunoblotted using anti-hydroxylated Pro402 (P402-OH), anti-hydroxylated Pro564 (P564-OH), anti-HIF-1 α , or anti-p42/p44 MAPK antibodies.

Fig. 4.

Modulation of PHD activity by BiPS. A, FeCl_2 ($20 \mu\text{M}$) was incubated with BiPS ($75 \mu\text{M}$) or deferoxamine ($50 \mu\text{M}$) for 30 min. FerroZine (20 mg/ml) and ammonium acetate (1 mg/ml) were then added to the reaction mixture and incubated at 37°C for 30 min. Results are expressed as total free iron as determined by comparison with a FeCl_2 /FerroZine standard curve and are an average $\pm$ S.E.M. of three independent experiments. B, cytoplasmic extracts from HeLa cells were supplemented with dimethyl-2-oxoglutarate ($33 \mu\text{M}$) and/or treated with BiPS ($100 \mu\text{M}$) for 20 min before incubation with HIF-1 α (344–582) GST protein-coupled Sepharose beads. Samples were then incubated overnight in the presence of in vitro translated pVHL and resolved by SDS-PAGE (12%). Immunoblotting was performed using anti-HA (pVHL) and anti-GST antibodies.

Fig. 5.

Increased HIF-1 nuclear complex formation by BiPS. HeLa cells were maintained under control conditions, in hypoxic conditions (1% O₂), or in the presence of BiPS (75 μM) for 2 h. Nuclear protein extract (10 μg) was incubated in a 96-well plate coated with an oligonucleotide containing the wild-type (W26) or mutant (M26) HIF-1-binding site. The presence of HIF-1 transcription complex was evaluated using anti-HIF-1α and anti-HIF-1β antibodies. Results are expressed as the fold increase of absorbance at 450 nm over control conditions and are an average ± S.E.M. of at least three independent experiments performed in triplicate.

Fig. 6. HIF-dependent reporter gene activation by BiPS. HeLa cells (six-well plate) were transfected with 1 μ g of a pGL3 (R2.2) 3HRE-TK reporter construct and 250 ng of an expression vector coding for *R. reniformis* luciferase to normalize transfection efficiency. At 40 h after transfection, cells were maintained under control conditions, in hypoxic conditions (1% O₂), or in the presence of BiPS (75 μ M) or vehicle (DMSO 0.08%) for 6 h. Cells were lysed, and luciferase activity was measured. Results are expressed as a ratio of beetle luciferase activity to *R. reniformis* luciferase activity and are an average $\pm$ S.E.M. of at least three independent experiments performed in triplicate.

Fig. 7.

HIF-dependent VEGF expression by BiPS. BAECs were transfected with 20 nM siRNA oligonucleotides targeting HIF-1 α , HIF-2 α , or with a control siRNA. At 40 h after transfection, cells were maintained under control conditions, in hypoxic conditions (1% O₂), or in the presence of BiPS (75 μ M) or vehicle (DMSO 0.08%) for 2 h. Top, total RNA was extracted and resolved on formaldehyde/agarose gels. Northern blot was performed using a specific radiola-beled VEGF probe. An 18S RNA probe was used as a control for gel loading. Bottom, total cell extracts (25 μ g) were resolved on SDS-PAGE (8%) and immunoblotted using anti-HIF-1 α and anti-HIF-2 α antibodies.