STIMULATING RETINAL BLOOD VESSEL PROTECTION WITH HYPOXIA-INDUCIBLE FACTOR STABILIZATION: IDENTIFICATION OF NOVEL SMALL-MOLECULE HYDRAZONES TO INHIBIT HYPOXIA-INDUCIBLE FACTOR PROLYL HYDROXYLASE (AN AMERICAN OPHTHALMOLOGICAL SOCIETY THESIS)

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ABSTRACT

Purpose: To discover novel small molecules that inhibit hypoxia-inducible factor (HIF) prolyl hydroxylase (PHD), a key enzyme that regulates the posttranslational stability and hence activity of HIF.

Methods: NIH3T3 cell line stably transfected with firefly luciferase under a HIF-1-inducible promoter was used to screen a Chembridge library of 34,000 small molecules of molecular weight 250 to 550 Da. Positive hits were considered at 4.5-fold higher luminescence than control. Selected compounds were validated in vitro. The most effective dose was then used to treat mice expressing firefly luciferase fused to the oxygen-dependent degradation domain (lucODD) in order to determine the location of the receptor for systemic treatment with small-molecule HIF PHD inhibitors.

Results: Twenty-three novel small molecules were discovered, the majority of which were hydrazones and hydrazines. Of the 23 compounds, each had different selectivity for expression of erythropoietin or vascular endothelial growth factor, two angiogenic, HIF-regulated gene products. In addition, each showed different selectivity for hepatocytes or kidney, or both or neither, when injected intraperitoneally in an in vivo reporter gene assay.

Conclusion: The discovery of multiple small molecules that inhibit HIF PHD identifies new reagents to develop strategies to prevent the degradation of HIF by its selective PHD. These molecules are novel hypoxia mimetics that may provide new strategies to protect retinovasculature from hyperoxia.

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INTRODUCTION

Oxygen is paradoxical because its central role in biological systems as the final electron acceptor during respiration is tempered by its toxicity in high concentrations. Precise molecular systems have evolved to cope with this dual nature through redox protective systems and through transcription factors called hypoxia-inducible factors (HIFs), which are regulated by oxygen-responsive prolyl hydroxylase (PHD) enzymes. Hypoxia-inducible factors are a family of transcription factors that control the expression of multiple genes that compensate for decreased partial pressures of oxygen in tissues. These disparate gene products increase glycolysis and hematopoiesis and induce angiogenesis and vasculogenesis. All of these factors are particularly important to the growth and development of the fetus, which is relatively hypoxic with respect to the mother and, in fact, has lower partial pressures of oxygen in peripheral tissues in comparison to an adult (for a review, see Park and associates[1]). This physiologic hypoxia drives growth. The response of HIF PHD to oxygen tension creates a critical enzymatic control for blood vessel development, repair, and maintenance because it regulates the stability of the alpha subunit of the mature heterodimeric HIF protein. Prolyl hydroxylases are therefore central to the basic biology of ischemic retinovascular disease and, in particular, disease phenotypes caused by hyperoxia, such as retinopathy of prematurity (ROP).

The gene family of HIF includes three homologous alpha subunits, named HIF-1, -2, and -3. Hypoxia-inducible factor-1 and -2 are homologous heterodimers composed of inducible alpha and constitutive beta subunits.[2-5] The stability of the alpha subunit is regulated by PHDs, which induce hydroxylation on two proline residues (Pro-402 and Pro-564) within the oxygen-dependent degradation domain (ODD) of the alpha subunit of HIF.[6-9] Hydroxylation makes the alpha subunit a substrate of von Hippel–Lindau protein if the key proline residue is hydroxylated within the ODD. The von Hippel–Lindau protein poly-ubiquitinates HIF-α to create a degradation signal that targets the alpha subunit to the 26S proteasome (Figure 1).[10,11] Because PHD uses the cofactor oxoglutarate, its inhibition can be induced by inhibitory oxoglutarate analogues, such as dimethyloxaloylglycine (DMOG), which competitively inhibits the hydroxylation of HIF-1α and -2α by displacing the endogenous oxoglutarate cofactor.[12-14] Inhibition of HIF PHD can also be induced by iron chelation, because the hydroxylase has an Fe metal center. Inhibition of the HIF PHD therefore blocks hydroxylation of HIF alpha subunit in the oxygen degradation domain, resulting in increased stability of the HIF protein[8,9] (Figure 1).[15,16] Hypoxia-inducible factor is also modified in an oxygen-dependent mechanism by hydroxylation of asparagines in the C-terminal transactivation domain catalyzed by factor-inhibiting HIF (for a review, see Dioum and associates[17]); this produces a hyperoxic response by blocking a necessary interaction between HIF-α and p300, a nuclear protein cofactor of transcription. Both HIF PHD and factor-inhibiting HIF (HIF asparaginyl hydroxylase) are members of the superfamily of Fe(II) and 2-oxoglutarate-dependent oxygenases. In addition to hydroxylation, lysine acetylation induces HIF catabolism to a lesser extent, making it a substrate of SIRT-1, which promotes deacetylation and stabilization of HIF.[18] Hypoxia-inducible factors have also been implicated in overriding hypoxia-induced decrease in translation in general by forming a complex with the RNA-binding protein RBM4 and the cap-binding protein eIF4E2, an eIF4E homologue at the ribosomal hypoxia response element. Although this does not regulate the catabolism of HIF, it demonstrates that the activity of HIF-regulated transcripts may require the alpha subunit for translation as well as transcription.[19]

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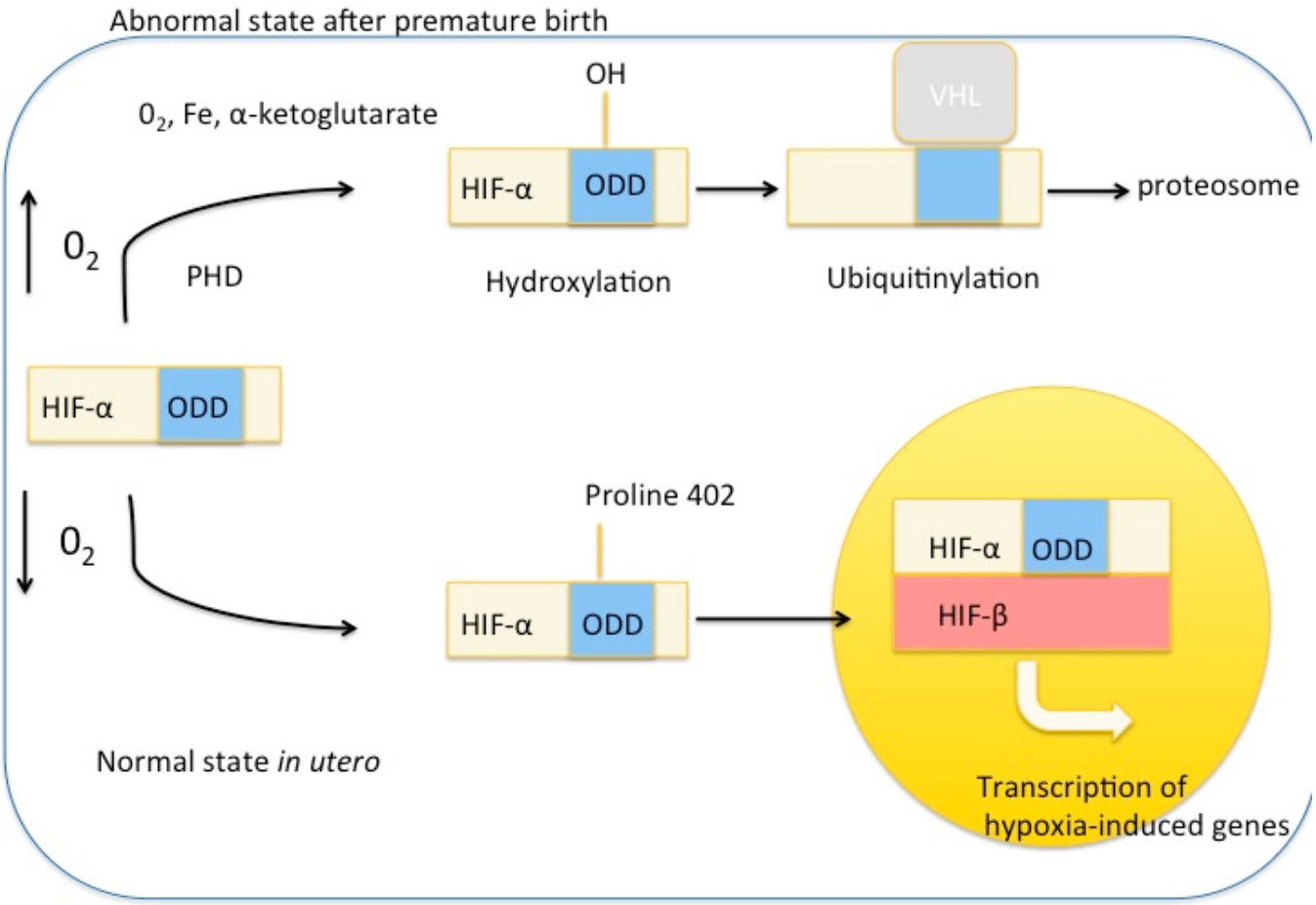

FIGURE 1

Angiogenesis is controlled by oxygen through strict regulation of hypoxia-inducible factor 1 (HIF-1). Normoxia or hyperoxia activates a prolyl hydroxylase (PHD), which hydroxylates the alpha subunit of HIF within the oxygen-dependent degradation domain (ODD). This makes the alpha subunit of HIF a substrate of the von Hippel–Lindau (VHL) protein, which targets the alpha subunit of HIF to the proteasome by ubiquitination. Hypoxia inhibits the PHD, increasing the stability of HIF-α by preventing hydroxylation of the ODD, enabling it to bind the HIF beta subunit (also known as ARNT) to form the complete and active dimer, which translocates to the nucleus and binds the hypoxia response element to direct the transcription of hypoxia-induced genes. Inhibition of the PHD by small molecule inhibitors serves as a hypoxia mimetic.

Studies using HIF-1 knockouts have been hard to complete because nonconditional knockouts do not allow viable fetuses. However, conditional knockouts and knockdown mice have suggested the critical role of HIFs in retinovascular development.[20] In addition, Western blot analysis and immunohistochemistry in retina have demonstrated higher concentrations of protein in hypoxic regions of the developing retina and cell-specific stabilization of isoforms in inner and outer retina and in Müller cells.[21] Although it is becoming clear that these proteins affect retinovascular development, the role that PHDs play is not known. Their ability to rapidly regulate the posttranslational stability of HIF suggests that their action is an exciting area of research to understand the basic mechanisms of development and perturbation of development by premature birth and oxygen supplementation.

Prolyl hydroxylases are an evolutionary conserved subfamily of dioxygenases that use Fe(II) and ascorbate as cofactors and oxoglutarate and oxygen as cosubstrates (for a review, see Fong and Takeda[22]). Prolyl hydroxylases have been identified in plants, prokaryotes, and mammalian cells. In the latter group, 4 gene isoforms have been identified: PHD1/EGLN2/HPH3, PHD2/EGLN1/HPH2, PHD3/EGLN3/ HPH1, and a recently characterized sequence named as P4HTM. Knockout studies of PHD1, 2, and 3 have revealed the central role that PHDs play in development and erythropoiesis.[23,24] Prolyl hydroxylase-2 is hypothesized to be the major significant isoform in most tissues because PHD-1 and -3 knockout mice survive, whereas PHD-2 knockout mice die at mid gestation. Major defects in knockouts include underdeveloped myocardium and poor labyrinth branching in the placenta. Conditional

Cre/lox knockouts have demonstrated severe polycythemia with increases in hematopoietic stem cells in both liver and spleen.

Oxygen has long been recognized as causative to ROP. The earliest cause and effect between oxygen and ROP was developed by Campbell[25] and also Ryan,[26] who saw a direct link between oxygen exposure and ROP. Campbell observed that those children whose parents were wealthy enough to purchase oxygen developed ROP at a greater rate than children from destitute parents who could not purchase it. Crosse and Evans further reported the dose response between oxygen and severity of ROP, which was confirmed by Patz,[27,28] who randomly assigned infants to high or low oxygen, and then Kinsey,[29] who conducted a multicenter trial in conjunction with Patz to prove the causal effect of high oxygen.

These studies led to the hypothesis by Ashton,[30,31] given a mechanistic basis by Alon and associates[32] and Pierce and associates,[33] that ROP has two phases that are dependent on the abnormal down-regulation and subsequent up-regulation of the oxygen-regulated expression of cytokines, later identified as vascular endothelial growth factor (VEGF) and erythropoietin (Epo). Phase I begins at birth when the infant is placed into hyperoxia, which results in a reduction in the secretion of VEGF that is associated with oxygen-induced vascular obliteration. Phase II is a hypoxic state created by weaning of oxygen supplementation and increased retinal metabolic demand exacerbated by vessel loss from phase I. Phase II is characterized by an overexpression of growth factors, such as VEGF, from the ischemic retina, resulting in pathological neovascularization.[34]

Two studies in the mid 1990s proved that administering VEGF during phase I hyperoxia was protective and could reduce the severity of oxygen-induced retinopathy in mice.[32,33] In addition to VEGF and Epo, IGF-1 and IGFBP3 have been implicated as soluble factors that protect blood vessels (for a review, see Hartnett and Penn[35]). Insulin-like growth factor-1 itself may have both a direct effect on blood vessel growth and an effect on HIF activity. Slomiany and Rosenzweig[36] have reported that IGF-1 increases the nuclear translocation and activity of HIF. It is therefore a sensible hypothesis that HIF stabilization during hyperoxia treatment in early gestational age could induce the normal and sequential development of retinovasculature, even during hyperoxia, to deprive ROP of its ischemic substrate. The strategy of hypoxia mimetics could therefore stimulate the HIF transcription factor to effect global gene expression and might be a more powerful technique than using gene expression to express only one gene. Stimulating a transcription factor could induce the sequential and orderly development of blood vessels, maintaining vascular repair and maintenance because a set of genes is expressed in a coordinated physiologic pattern, in contrast to ectopic unregulated expression of a single gene.

In fact, when an activator of HIF (inhibitor of HIF PHD) was administered systemically in phase I in preclinical studies, a robust protection of retinal vessels was observed. This validated the concept that preservation of HIF activity may be critical for retinovascular protection. An interesting finding in this study was that PHD inhibition, when administered systemically, activated the liver to function as an endocrine organ, which preserved retinal capillary beds.[37]

The concept of a two-step process in ischemia-induced vasoproliferation now offers the chance to intervene before ischemia occurs in ROP and perhaps in diverse diseases such as diabetic retinopathy. With respect to premature infants, it may well be that efforts to promote retinovascular development will have benefit to other organ systems, such as the lung and gastrointestinal tract. The challenge is to perfect the timing of new drugs that pharmaceutically precondition the eye, to determine whether these drugs are best applied locally or systemically, and to discover how to develop agents specific enough either to target particular vascular beds or to restrict their activity to the normal development, maintenance, and repair of blood vessels.

In order to further investigate the hypothesis that ischemia could be prevented by stabilizing HIF PHD, a screen of potential inhibitors was undertaken to develop new tools to refine the hypothesis that HIF stabilization could act to preserve retinal capillaries during the causative phase of ischemia. Selected compounds were validated primarily by Western blot and then secondarily by measuring the concentration of HIF-regulated gene products that they induced in cell culture. These small molecules were then analyzed for PHD inhibition by following proline hydroxylation within the oxygen-dependent degradation domain of the alpha subunit of HIF. Because we have previously shown that the liver is a target of systemic PHD inhibition and that this inhibition protects the retina, these small molecules were screened using the luciferase-oxygen-dependent degradation domain (luc-ODD) mouse to see which of the 23 small molecules targeted the liver and which did not, in order to identify reagents that could further prove whether or not the hepatic HIF target is necessary and sufficient to induce protection of retinal blood vessels against hyperoxia.

METHODS

All animal experiments were performed under strict observance of Institutional Animal Committee for Use and Care protocols and conformed to the guidelines established by Association for Research in Vision and Ophthalmology.

SMALL-MOLECULE LIBRARY SCREENING

Reporter System

Screening for compounds that induce HIF-1 activity was performed using NIH3T3 cell line stably expressing the reporter gene of firefly luciferase under a HIF-1-inducible promoter (NIH3T3/HIF-luc, Panomics, Santa Clara, California). The NIH3T3/HIF-luc cell line was obtained by cotransfection of pHIF-luc (Panomics P/N LR0128) and pHyg into murine fibroblast NIH3T3 cells, followed by hygromycin selection. This promoter contains hypoxia response element, to which HIF-1 binds and transactivates gene expression.

HIF Reporter Cells

Mouse fibroblast cell line NIH-3T3 with integrated reporter (NIH3T3/HIF-luc) was maintained in Dulbeccos' Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 2 mM glutamine, 100 units/mL penicillin G, 100 µg/mL streptomycin sulfate

(all from Invitrogen, Carlsbad, California), and 100 μg/mL hygromycin B (Roche Diagnostics Corp, Indianapolis, Indiana). Cells used for screening were between 5th and 15th passage.

Chemical Library

A chemical DiverSet library of 34,000 chemical compounds was purchased from Chembridge (San Diego, California). The library consists of handcrafted drug-like organic molecules with molecular weight in a range of 250 to 550 Da, which were dissolved in dimethyl sulfoxide (DMSO) at concentration 5 mg/mL or 10 to 20 mM, depending on molecular weight.

Screening Procedure

Cells were plated in 96-well plates (opaque white with transparent bottom; Costar-Corning, Lowell, Massachusetts) with 5×10^4 cells in 100 μL per well and incubated overnight in tissue culture incubator (37ºC, 5% CO_2). The library is formatted in 96-well format with 80 compounds per plate. After overnight incubation, 0.2 μL of compounds in DMSO were added per well to final concentrations of 5 to 15 μM. Equal amounts of DMSO or desferrioxamine mesylate (0.1 to 0.5 mM) were used as negative and positive controls, respectively. After 24 hours, a cocktail of lysis and reporter buffer with luciferin (Bright-Glo; Promega, Madison, Wisconsin) was added, and luciferase activity was measured as chemiluminescence using Wallac 1420 VICTOR2 multilabel reader (PerkinElmer, Waltham, Massachusetts). Compounds that induced the reporter stronger than 4.5-fold comparing to negative control were considered to be primary hits.

VALIDATION OF PRIMARY HITS

The validation consists of determining whether drug-like molecules selected from the Chembridge library can induce increase of endogenous HIF protein in cell cultures.

Treatment of Hep3B and MIO-M1 Cells

Hepatoma cell line (Hep3B)[38] was purchased from ATCC (Manassas, Virginia). Müller cells (MIO-M1) were kindly provided by Dr G. A. Limb (Moorfields Institute of Ophthalmology, London, United Kingdom).[39] Cells were plated at a density of 2×10^4 cells per well in 6-well cell culture clusters in DMEM supplemented with 400 mM glutamine and 10% fetal calf serum. Prior to stimulation, cells were placed in serum-free media for 24 hours. A dose response of Chembridge compounds was constructed from DMSO stock solutions (10 mg/mL). Cells were treated with media alone, cobalt chloride and desferoxamine as positive controls, and various doses of Chembridge compounds. Cells were harvested in each particular experiment by scraping them into 2 mL chilled PBS and pelleting them in a 15-mL conical tube at 500 rpm for 5 minutes. Cell lysates were made by directly resuspending cells in 2× sample buffer with 1 mM dithiothrytol.

HIF Western Blot Analysis

Cell lysates were subjected to 4% to 20% SDS-PAGE and electro-transferred to PVDF membrane for immunoblotting. Membranes were blocked with 5% nonfat dried milk in TBS and hybridized with anti-HIF-1α (BD Transduction Laboratories, San Diego, California) or anti-HIF-2α antibody (Novus Biologicals, Littleton, Colorado) and after washing and secondary antibody hybridization, exposed by chemiluminescence (Western Lightning; PerkinElmer).

VEGF and Epo ELISA

Fifty microliters of culture media obtained from MIO-M1 or Hep3B cells treated with Chembridge compounds was added 1:1 to sample diluent provided by the manufacturer of mouse VEGF or Epo colorimetric ELISA (R&D Systems, Minneapolis, Minnesota). VEGF or Epo measurements were performed according to the manufacturer's instructions.

Detection of HIF-1α Hydroxylation

Müller cells were plated at a density of 10^5 cells per dish in 100-mm Petri dishes and cultured as described above. Confluent cell cultures were then treated with selected Chembridge compounds at a dose of 3 μg/mL or with 200 μg/mL DMOG as a positive control. During the last 2 hours of the 6-hour incubation with the tested compounds, cell cultures were given 10 μM MG-132, a proteasome inhibitor, in order to prevent degradation of hydroxylated HIF. Cellular protein extracts were obtained, resolved on SDS-PAGE, and blotted as described above. Accumulation of hydroxylated and total HIF-1α was detected using anti-hydroxy-HIF-1α (Pro564; Cell Signaling Technology, Danvers, Massachusetts) and anti-HIF-1α (BD Transduction Laboratories) antibodies, respectively.

Luciferase Oxygen-Dependent Degradation Domain Mouse (Luc-ODD)

The Luc-ODD mouse is a transgenic mouse that constitutively expresses a fusion protein composed of luciferase fused to the ODD, the substrate of HIF PHD. If the prolyl hydroxylase enzyme hydroxylates the ODD, the luciferase protein is catabolized. This construct therefore serves an in vivo reporter gene that creates luminescence in tissues where HIF PHD is inhibited and HIF is stabilized. Increased luminescence demonstrates inhibition of PHD, whereas decreased luminescence demonstrates lack of inhibition of PHD. Adult mice were intraperitoneally injected with small molecules at a concentration of 3 μg/gm followed by injection of 50 μg/gm luciferin. Live whole-animal imaging was performed using a Xenogen IVS 200 (Xenogen, Berkley, California) after administration of an intraperitoneal dose of ketamine HCl, 50 mg/kg, and xylazine HCl, 5 mg/kg (Bioniche Pharma, Lake Forest, Illinois).

RESULTS

Twenty-three compounds were isolated from 34,000 total small molecules, which were screened with a high-throughput system dependent on a gene reporter activity assay composed of the luciferase gene fused to the hypoxia response element (Figure 2 and Appendix). Seventeen of these compounds were hydrazines and hydrazones, the most potent of which were hydrazones. These molecules contained a central C=N-C structure flanked by benzyl rings and are known to be potent activators of HIF. Because the PHD enzymes require a Fe metallocenter, hydrazones are efficient at inhibiting hydroxylation by forming a 2:16-coordinated Fe complex in solution that very likely creates Fe deprivation.

Hypoxia-inducible factor expression was analyzed primarily by immunoanalysis, an example of which is provided in Figure 3. A very potent induction was discovered at extremely low doses. For example, 0.1 μg/mL induced HIF stabilization, which is 100 to 1000 times more potent than DMOG, which is active at 200 μg/mL in vitro. A dose response between 0.1 and 10 μg/mL demonstrated increasing concentrations of HIF-1α showing high activity with little toxicity to Müller cells in culture. Even though these compounds are extremely hydrophobic, they were initially dissolved as a concentrated mixture in DMSO, 10 mg/mL, and then diluted 1000 times culture media for 24 hours. Bright field microscopy of live cell cultures revealed no cell death.

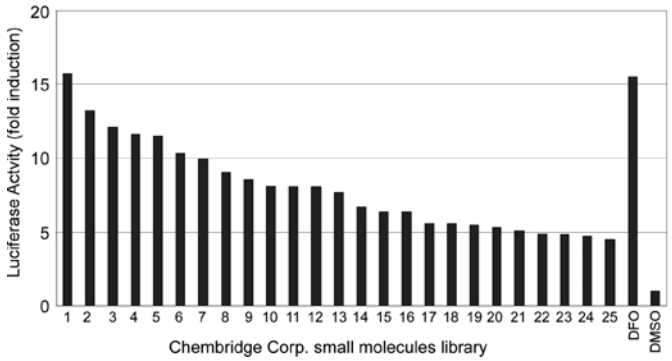

FIGURE 2

High-throughput reporter assay at 10 μg/mL used to identify small molecules that inhibit hypoxia-inducible factor prolyl hydroxylase (HIF PHD). Twenty-three new small molecules were discovered by the high-throughput assay using NIH3T3 cell line stably expressing the reporter gene of firefly luciferase under a HIF-1-inducible promoter (NIH3T3/HIF-luc; Panomics, Santa Clara, California). See Appendix for a list of the chemical names of these 23 compounds in the same order as in this Figure.

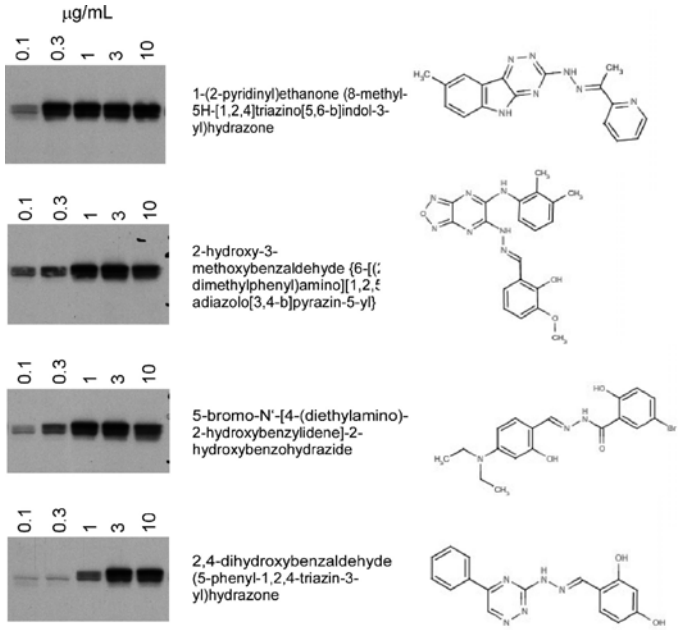

FIGURE 3

Dose response for hypoxia-inducibe factor prolyl hydroxylase (HIF PHD) inhibition in MIO-M1 by select compounds. The above compounds are a family of small molecules called hydrazones that have a characteristic $R_1R_2C{=}NNH_2$ structure. These compounds are extremely effective at inhibiting HIF PHD (stabilizing HIF-1α). Note that at 0.1 μg, these compounds are potent inhibitors of HIF PHD (stabilizing HIF-1α).

All of the 23 compounds were next tested to determine their effect on the expression of erythropoietin, a gene product regulated by HIF, in liver-derived Hep3B cells (Figure 4). At a concentration of 1.0 μg/mL, 22/23 compounds induced Epo expression but to varying degrees that did not follow the same hierarchy as their ability to stimulate luciferase activity in the reporter assay. This finding could be explained by either disparate or selective binding of the small molecules to a particular isoform of PHD or, less likely, toxicity of the compounds to cells in culture. In the case of the first four small molecules, no toxicity was observed in a previous dose-response experiment and yet compound d4 was much more potent at stimulating Epo secretion than d2 and d3, which were more potent at inducing HIF in the cell-based reporter assay (Figure 4).

Figure 5 shows up-regulation of HIF-1α and -2α protein stability in Müller cells. In order to see if there was selectivity of these

compounds with respect to either isoform or expression of gene products, various small molecules of the original 23 compounds were tested for their ability to stabilize HIF and drive VEGF and Epo expression in a dose-response fashion in hepatocytes (Hep3b cells), as seen in Figure 6 (VEGF) and Figure 7 (Epo). Compound d5 was able to drive the highest level of VEGF and Epo expression in hepatocytes (Figures 6 and 7), but was less effective than d1 in inducing VEGF expression in Müller cells (Figure 8). This finding may represent selectivity of this compound for a particular PHD isoform or may imply that there is selective expression of these isoforms that is tissue-dependent. Note that Müller cells do not secrete Epo, and therefore no data is available with regard to the induction of Epo from Müller cells. The remainder of the compounds tested in this dose-response experiment followed the same hierarchical order established by the high-throughput cell-based system.

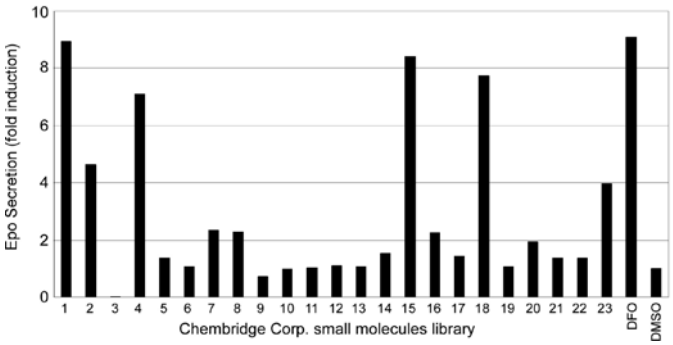

FIGURE 4

Erythropoietin secretion induced by each compound at concentration of 1.0 µg/mL. The differential effect of novel hypoxia-inducible factor prolyl hydroxylase (HIF PHD) inhibitor small molecules on erythropoietin production was analyzed by ELISA. Human hepatoma cell line Hep3B cultures were treated with 1.0 µg/mL of each compound shown in Figure 2 for 20 hours, followed by harvesting of cells and collection of conditioned media. Culture media were analyzed separately for levels of erythropoietin using ELISA (R&D Systems, Minneapolis, Minnesota).

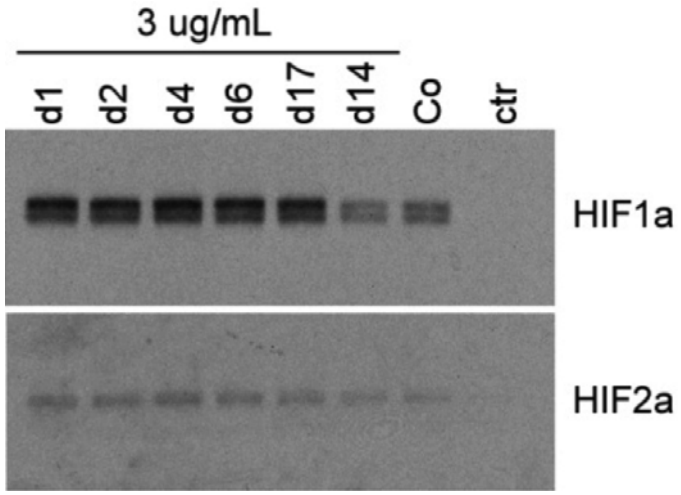

FIGURE 5

Induction of hypoxia-inducible factor (HIF)-1α and -2α in cultured human Müller cells. Select novel small molecules inhibit HIF propyl hydroxylase (stabilizer of HIF-1α and -2α) in cultured human Müller cells. The effect of the small molecules is greater at inducing HIF-1α stabilization than -2α stabilization.

The effect that these compounds had on HIF stability was next tested to determine whether they acted through the canonical pathway of inhibition of PHD within the ODD and subsequent catabolism in the proteasome. By using an inhibitor of the proteosome, it is possible to track the hydroxylation pattern of HIF by using an antibody specific to proline hydroxylation (hyp-HIF1α). Figure 9 shows that compounds d1, d2, d4, d6, d17, d14, and a positive control compound, DMOG, were able to inhibit proline hydroxylation, which correlated nicely to the amount of HIF found by immunoanalysis. Figure 9 demonstrates the lack of HIF-1α hydroxylation after various small molecules were used to treat cells in culture, proving that these compounds are stabilizing HIF by preventing the hydroxylation of proline residues within the alpha subunit, thereby proving that these compounds work by inhibiting the hydroxylase.

One of the truly astonishing findings of previous investigations of systemic PHD inhibition is that the maximal enzyme inhibition is localized to the liver and that the liver directs protection of retinovasculature. This implies that the liver is induced to protect retinal vasculature. Using the luc-ODD mouse, compounds 1, 15, and 21 were found to be the most potent at inducing hepatic luminescence. Figure 10 demonstrates the wide selectivity of these compounds. For example, DMOG is selective for the liver, whereas hydralazine, an antihypertensive medication, is selective for the kidney. In comparison, compound d4 targets both the kidney and the liver, whereas d15 targets the liver. Inasmuch as we have reported that DMOG targets the liver and prevents oxygen-induced retinopathy, liver specificity in conjunction with high Epo induction makes d1 and d15 excellent candidate molecules.

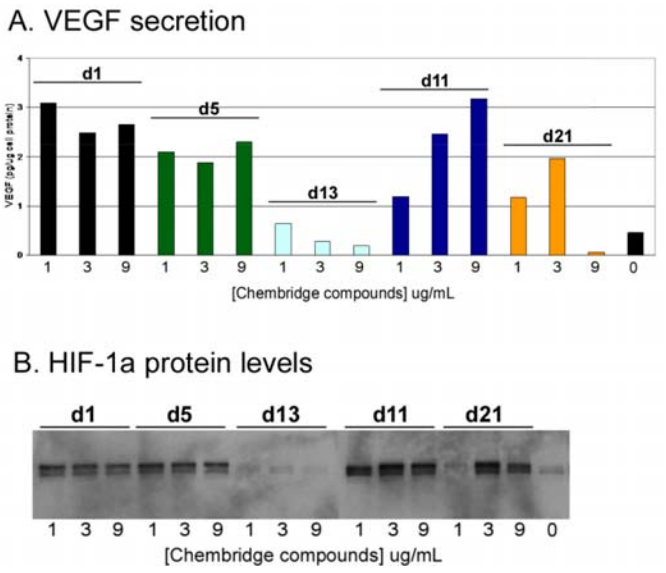

FIGURE 6

Relationship between vascular endothelial growth factor (VEGF) secretion and hypoxia-inducible factor (HIF)-1α levels in MIO-M1 cells. Expression of gene products that HIF regulates, such as VEGF, correlates to the concentration of HIF protein. Human cultured Müller cells were obtained from G. A. Limb, Moorfields Institute. The concentration of small molecule compounds is provided on the x-axis. Each ELISA was run in quadruplicate.

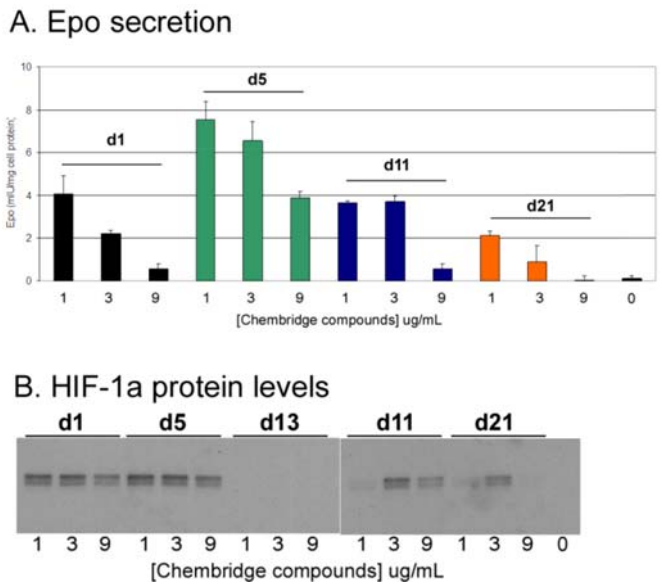

FIGURE 7

Relationship between erythropoietin protein expression and hypoxia-inducible factor prolyl hydroxylase (HIF PPD) inhibition in Hep3B cells. Hep3b cells respond to select small molecules by secreting both vascular endothelial growth factor and erythropoietin. Note that compound d5 is a stronger inducer in hepatocytes than in Müller cells. The concentration of small molecule inhibitors is provided on the x-axis. The level of HIF-1α stability qualitatively corresponds to these gene products. Error bars are given in standard error. ELISA was run in quadruplicate.

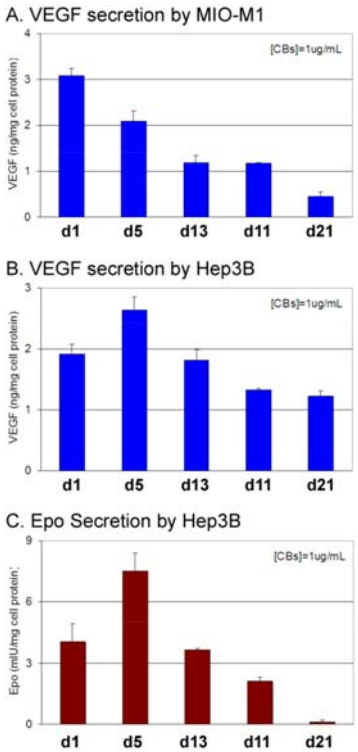

FIGURE 8

Relative activities of select small molecule compounds at 1 μg/mL in MIO-M1 and Hep3B cells. Compounds that induce erythropoietin expression from hepatocytes (Hep3b cells) may be candidate molecules for preventing oxygen-induced retinopathy. At the lowest concentration, there is differential expression of VEGF and Epo based on cell type for compound d5. ELISA was run in quadruplicate. Error bars denote standard error.

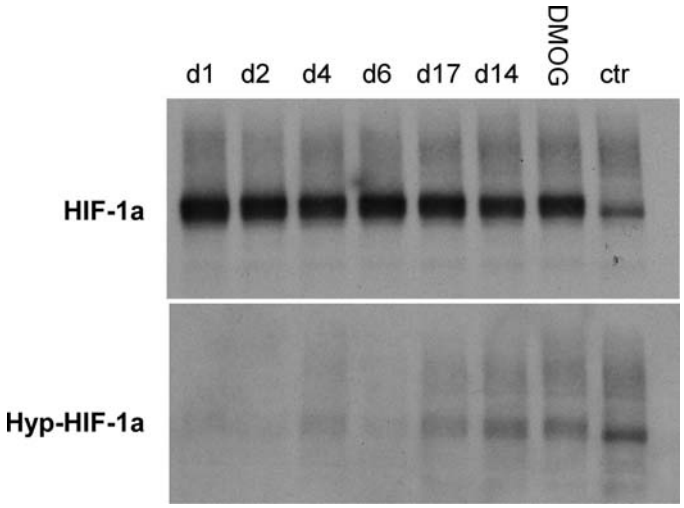

FIGURE 9

Small molecules act through the canonical pathway of propyl hydroxylase inhibition and blockade of oxygen-dependent degradation domain (ODD) hydroxylation. The proteosome inhibitor MG-132 was used to inhibit hypoxia-inducible factor (HIF) degradation to reveal the amount of ODD hydroxylation, recognized by an antibody specific to hydroxylated HIF-1α (hyp-HIF1α). Compounds d1 and d6 are more potent than even dimethyloxaloylglycine (DMOG) at inhibiting ODD hydroxylation.

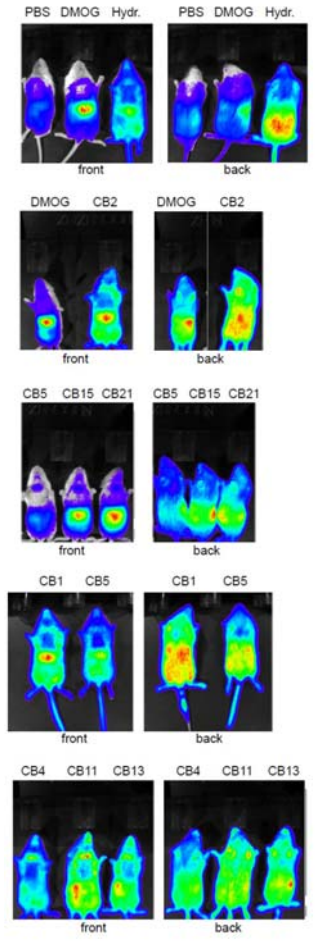

FIGURE 10

Examination of hepatic specificity of selected small molecules using whole-animal imaging of the luciferase oxygen-dependent degradation domain (luc-ODD) transgenic mouse. The use of systemic hypoxia-inducible factor prolyl hydroxylase (HIF PHD) inhibitors prevents retinovascular obliteration but targets the liver, at least in the case of dimethyloxaloylglycine (DMOG). Figure demonstrates differential selectivity of small-molecule inhibitors of PHD. DMOG targets the liver, whereas hydralazine, a potent hydrazone antihypertensive agent, targets the kidney. Compound 4 targets both kidney and liver, whereas compound 15 targets the liver. The luc-ODD mouse developed by Kaelin enables live whole-animal imaging. Selected Chembridge compounds were injected intraperitoneally at a dose of 3 μg/gm followed by injection of luciferin intraperitoneally at a concentration of 50 μg/gm body weight. These images were taken at 6 hours postinjection of Chembridge compounds and 30 minutes after injection of luciferin. High luminescence denotes maximal HIF PHD inhibition. The outline of the liver can be seen in the ventral view and on the right side of the dorsal view. Analogously, the outline of the kidney can be seen strongest in the retroperitoneal space for hydralazine. DMOG was used as a positive control and was administered at a concentration of 200 μg/gm body weight and shows strong uptake in the liver.

DISCUSSION

The long-term goal of this research is to develop translational strategies for the prevention of ischemia. This thesis identifies unique hydrazones, hydrazines, and a quinolone and carboxylate that are useful reagents to study the use of hypoxia mimetics in preventing blood vessel destruction.

One important question that arose after our initial investigation of PHD inhibition in the oxygen-induced retinopathy model is how best to develop drug delivery using this strategy. For example, how does systemic therapy compare to local therapy? With respect to ROP, ocular pathology is an organ-specific manifestation of systemic pathology that has a similar hyperoxic pathogenesis. Asikanen and associates[40] have shown that bronchopulmonary dysplasia, a disease of the alveoli, can be reduced by pharmaceutical hypoxic preconditioning, suggesting that lung disease of the premature infant has a stepwise progression much like ROP. Hyperoxia-induced inhibition of HIF and subsequent cessation of alveolar development in the lung resembles the effect that oxygen has on the retina, and the destructive fibrosis that causes a chronic restrictive lung disease in premature infants resembles the proliferative and cicatricial phase of ROP stage 3 and higher.[41] Therefore, one could consider that systemic PHD inhibition might offer the hope that both the lung and eye will benefit from a single intervention.

Our original exploration of systemic therapy revealed a curious result: the target of systemic in vivo treatment was hepatic PHD. This suggests that the liver might act as an endocrine organ, secreting HIF-regulated gene products such as VEGF and Epo, which protect retinovasculature during hyperoxia. We have used this knowledge to rapidly screen the novel small molecules described here to identify those agents that have a liver trophism. The new hydrazone compounds described are hence invaluable for continuing our research into the best route of drug delivery and whether specificity for a particular PHD isoform or tissue tropism is important, because many target the liver and yet have a molecular structure that suggests they would function well as a topical preparation or eye drop. Their hydrophobicity and polarity suggest that they may penetrate cornea as eye drop preparations and serve as a control to test the advantages of systemic vs local drug delivery. We are currently testing these molecules and have thus far noted that their hydrophobicity creates a sustained action in comparison to water-soluble DMOG.

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APPENDIX. CHEMICAL NAMES OF THE HIGHEST INDUCERS OF LUMINESCENCE IN THE CHEMBRIDGE LIBRARY SCREEN (AS SEEN IN FIGURES 2 AND 4)

CHEMBRIDGE ID/d NUMBER	CHEMICAL NAME
5617521/d1	1-(2-pyridinyl)ethanone (8-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)hydrazone
5612881/d2	2-hydroxy-3-methoxybenzaldehyde (6-[(2,3-dimethylphenyl)amino][1,2,5]oxadiazolo[3,4-b]pyrazin-5-yl)hydrazone
5658176/d3	5-[3-nitro-4-(1-piperidinyl)benzylidene]-2-thioxo-1,3-thiazolidin-4-one
5653105/4	2,4-dihydroxybenzaldehyde (5-phenyl-1,2,4-triazin-3-yl)hydrazone
5636024/5	5-bromo-N'-[4-(diethylamino)-2-hydroxybenzylidene]-2-hydroxybenzohydrazide
5489566/d6	1-(2-pyridinyl)ethanone 5H-[1,2,4]triazino[5,6-b]indol-3-ylhydrazone
5785187/d7	3-(1-adamantyl)-N'-(3-allyl-2-hydroxybenzylidene)-1H-pyrazole-5-carbohydrazide
5624806/d8	5-hydroxy-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde 2-pyridinylhydrazone
5692217/d9	isopropyl 5-[(3-chlorobenzoyl)amino]-4-cyano-3-methyl-2-thiophenecarboxylate
5561661/d10	3-(9-acridinylamino)-2-(5-chloro-1,3-benzoxazol-2-yl)acrylaldehyde
5791245/d11	2-(1H-benzimidazol-2-ylthio)-N'-[4-(diethylamino)-2-hydroxybenzylidene]acetohydrazide
5788741/d12	N'-(3-allyl-2-hydroxybenzylidene)-3-tert-butyl-1H-pyrazole-5-carbohydrazide
5702018/d13	isopropyl 4-cyano-5-[(3-fluorobenzoyl)amino]-3-methyl-2-thiophenecarboxylate
6045523/d14	N'-[1-(1-hydroxy-2-naphthyl)ethylidene]-3-methyl-1H-pyrazole-5-carbohydrazide
5952082/d15	N'-(2,4-dihydroxybenzylidene)-5-nitro-3-phenyl-1H-indole-2-carbohydrazide
6048106/d16	6-phenyl-2,3-dihydropyrazolo[1,5-a]thieno[3,2-d]pyrimidin-9-ol
5788475/d17	N'-(3-allyl-2-hydroxybenzylidene)-3-(2-naphthyl)-1H-pyrazole-5-carbohydrazide

APPENDIX. CONTINUED

CHEMBRIDGE ID/d NUMBER	CHEMICAL NAME
6049305/d18	N-(3,4-dimethylphenyl)-2,3-dihydro-1,4-benzodioxine-2-carboxamide
6050495/d19	10-acetyl-2-nitro-10H-indolo[3,2-b]quinolone
6048007/d20	2-(1,3-benzothiazol-2-yl)-4-(([2-(3,4-dimethoxyphenyl)ethyl]amino)methylene)-5-(trifluoromethyl)-2,4-dihydro-3H-pyrazol-3-one
5949310/d21	N'-(2,4-dihydroxybenzylidene)-5-methyl-3-phenyl-1H-indole-2-carbohydrazide
6049746/d22	2-(1,3-benzothiazol-2-yl)-4-[(dimethylamino)methylene]-5-(trifluoromethyl)-2,4-dihydro-3H-pyrazol-3-one
6050628/d23	methyl 4-([(3-oxo-2-thiomorpholinyl)acetyl]amino)benzoate

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