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Utilization of an In Vivo Reporter for High Throughput Identification of Branched Small Molecule Regulators of Hypoxic Adaptation

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SUMMARY

Small molecules inhibiting hypoxia inducible factor (HIF) prolyl hydroxylases (PHDs) are the focus of drug development efforts directed toward the treatment of ischemia and metabolic imbalance. A cell-based reporter produced by fusing HIF-1α oxygen degradable domain (ODD) to luciferase was shown to work as a capture assay monitoring stability of the overexpressed luciferase-labeled HIF PHD substrate under conditions more physiological than in vitro test tubes. High throughput screening identified novel catechol and oxyquinoline pharmacophores with a "branching motif" immediately adjacent to a Fe-binding motif that fits selectively into the HIF PHD active site in in silico models. In accord with their structure-activity relationship in the primary screen, the best "hits" stabilize HIF1α, upregulate known HIF target genes in a human neuronal line, and exert neuroprotective effects in established model of oxidative stress in cortical neurons.

INTRODUCTION

Hypoxia is a common etiology of cell injury in human disease, including stroke, myocardial infarction, and solid tumors. Over the past two decades, cell adaptation to hypoxia has emerged as a well-defined active process. Each cell of a multicellular organism can respond to hypoxia by building up hypoxia inducible factor (HIF), a ubiquitous transcription factor capable of activating a battery of genes including genes involved in glucose uptake and metabolism, extracellular pH control, angiogenesis, erythropoiesis, mitogenesis, and apoptosis. The discovery of HIF opened new horizons for the treatment of ischemia and cancer: upregulation of HIF levels has been shown to be beneficial for ischemic diseases, stem cell proliferation (Zhang et al., 2006), and transplantation (Liu et al., 2009), whereas downregulation of elevated HIF, a marker of most aggressive cancers, represents a new approach for cancer treatment.

HIF consists of two subunits, HIF-1α and HIF-1β; HIF-1α is rapidly degraded under normoxic conditions, whereas HIF-1β is stable (Wang et al., 1995; Wang and Semenza, 1995). HIF levels are regulated primarily by posttranslational modification of conserved proline residues. Hydroxylation of Pro564 and/or 402 residues in HIF-1α is a prerequisite for its interaction with the von Hippel-Lindau (VHL) protein yielding a complex that provides HIF ubiquitinylation and subsequent proteasomal degradation (Kaelin, 2005). Hydroxylation of Pro564 occurs prior to Pro402 (Chan et al., 2005), though some experiments contradict this finding (Villar et al., 2007). Hydroxylation of HIF-1α Asn803 blocks its interaction with transcriptional proactivator p300 (Lando et al., 2002). In both cases HIF hydroxylation is executed by α-KG dependent non-heme iron dioxygenases, HIF prolyl-4-hydroxylase (PHD1-3 isozymes) and asparaginyl hydroxylase (or the so-called FIH, factor inhibiting HIF) (Hirota and Semenza, 2005).

HIF1 also upregulates a number of prodeath proteins, and thus HIF1 upregulation can be either prodeath or prosurvival. However, recent evidence (Siddiq et al., 2005; Knowles et al., 2004; Baranova et al., 2007) strongly suggests that PHDs and FIH are important targets for medical intervention for a number of conditions, including chronic anemia and stroke. PHD inhibitors abrogate the ability of HIF1-mediated transactivation of BNIP3 and PUMA to potentiate oxidative death in normoxia (Aminova et al., 2008). Although new targets for intervention in the HIF pathway are constantly emerging, the latter observation justifies the search for PHD inhibitors rather than for other types of HIF activators. New substrates have been recently identified for PHD1 (e.g., Rpb1, large subunit of RNA polymerase II [Mikhaylova et al., 2008]) responsible for the fundamental enzymatic activity of the complex, synthesizing all cellular mRNAs) and PHD3 (e.g., β_2-adrenergetic receptor [Xie et al., 2009], whose sustained downregulation is associated with heart failure and asthma) placing HIF PHDs into the focus of drug development efforts. Despite characterization of HIF PHDs as a potential target for anti-ischemic therapy, few high throughput screening (HTS) results for PHD inhibitors are publically available.

In this work, we developed a novel application for an approach elegantly validated by Kaelin and Livingston's group for visualization of HIF stabilization in transgenic mice (Safran et al., 2006) and used it for the purposes of HTS. The reporter system

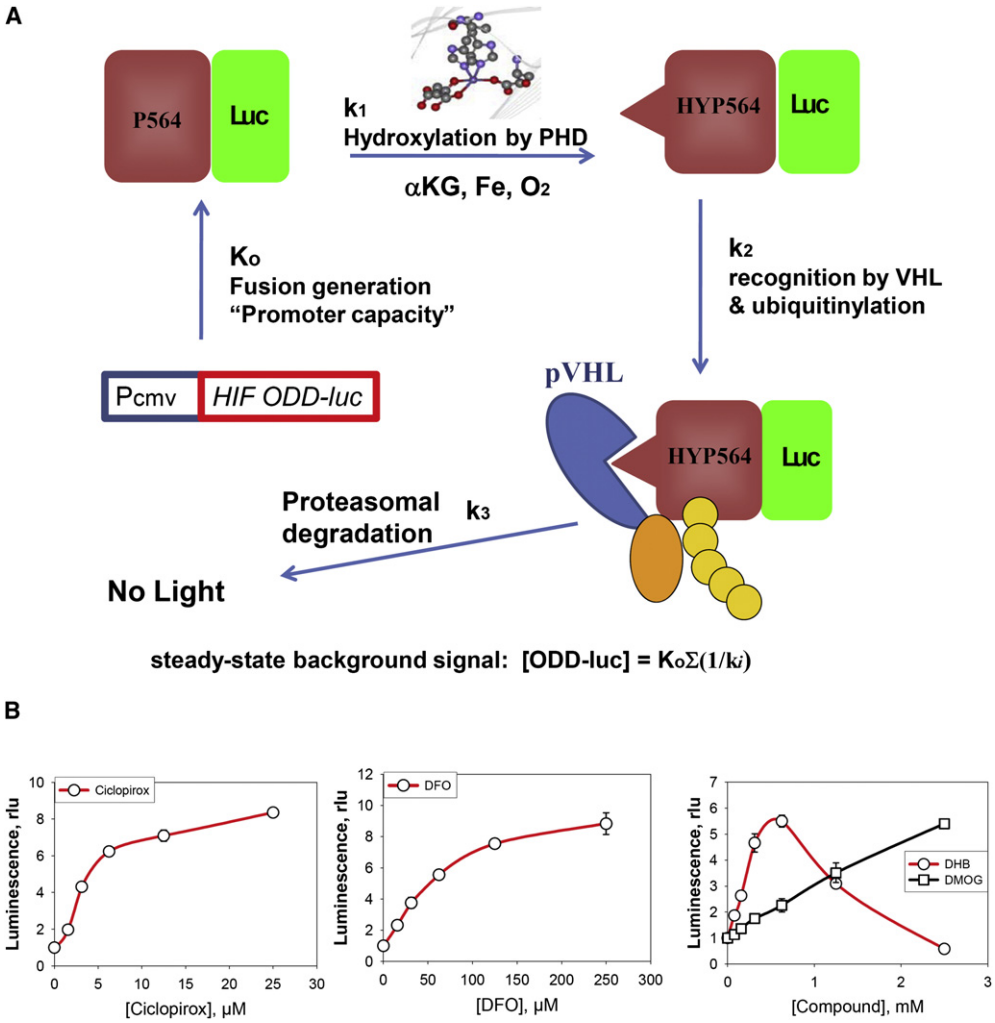

Figure 1. Mechanism of Reporter Activation and Response to Canonical HIF PHD Inhibitors

(A) Schematic presentation of reporter performance showing key steps/potential sites of inhibition.

(B) Reporter response to canonical HIF PHD inhibitors: ciclopirox, DFO, DMOG, and DHB.

All values are presented as mean ± SEM. Calculation of activation parameters from the titration curve in shown in Figure S1.

consists of the HIF-1α gene fragment encoding the oxygen degradable domain (ODD) containing the key proline residue followed by luciferase gene (*luc*). The regulation of luciferase protein stability in this reporter system is the same as the physiological activation of HIF: hydroxylation of oxygen-degradable domain (ODD, which contains 530-653 amino acids [aa] of HIF1-α) results in recognition of the ODD-luc fusion protein by VHL followed by its ubiquitinylation and proteasomal degradation (Figure 1A), and as we present below, the approach proved to be productive for HTS purposes.

We performed a cell-based HTS of 85,000 compounds for HIF protein stabilizers to identify those working as specific HIF PHD inhibitors. The most intriguing finding from the primary screen of 85,000 compounds was the group of branched 8-hydroxyquinoline derivatives whose binding mode into the active site of PHD2 resembles that of the HIF peptide. The biological effects of the newly identified hits—i.e., HIF1 protein stabilization, induction of HIF1-regulated genes such as vascular endothelial growth factor (VEGF), lactate dehydrogenase (*LDHA*), and phosphoglycerate kinase 1 (*PGK1*), neuroprotection in homocysteic acid (HCA) cellular model of oxidative stress—are in good agreement with their activation effects in the reporter assay.

RESULTS

Development and Optimization of the ODD-luc Reporter System

The reporter cell lines constitutively expressing ODD-luc (human neuroblastoma, SH-SY5Y) were stable for more than 1 year without significant change in their response to canonical PHD inhibitors such as deferoxamine (DFO), dihydroxybenzoate (DHB), dimethyloxalylglycine (DMOG), and ciclopirox (Figure 1B). The dependence of the ODD-luc reporter signal on inhibitor concentration has a sigmoid shape (Figure 1B and Figure S1 available online), which is characterized by maximum activation, IC_{50} and a "concentration lag," which likely reflects the presence