

## Using $\beta$ -Lactamase and NanoLuc Luciferase Reporter Gene Assays to Identify Inhibitors of the HIF-1 Signaling Pathway

Thai Khuc, Chia-Wen (Amy) Hsu, Srilatha Sakamuru, and Menghang Xia

### Abstract

The hypoxia-inducible factor 1 (HIF-1) is a transcriptional factor involved in the regulation of oxygen within cellular environments. In hypoxic tissues or those with inadequate oxygen concentrations, activation of the HIF-1 transcription factor allows for subsequent activation of target gene expression implicated in cell survival. As a result, cells proliferate through formation of new blood vessels and expansion of vascular systems, providing necessary nourishment needed of cells. HIF-1 is also involved in the complex pathophysiology associated with cancer cells. Solid tumors are able to thrive in hypoxic environments by overactivating these target genes in order to grow and metastasize. Therefore, it is of high importance to identify modulators of the HIF-1 signaling pathway for possible development of anticancer drugs and to better understand how environmental chemicals cause cancer. Using a quantitative high-throughput screening (qHTS) approach, we are able to screen large chemical libraries to profile potential small molecule modulators of the HIF-1 signaling pathway in a 1536-well format. This chapter describes two orthogonal cell based assays; one utilizing a  $\beta$ -lactamase reporter gene incorporated into human ME-180 cervical cancer cells, and the other using a NanoLuc luciferase reporter system in human HCT116 colon cancer cells. Cell viability assays for each cell line are also conducted respectively. The data from this screening platform can be used as a gateway to study mode of action (MOA) of selected compounds and drug classes.

**Key words** Hypoxia inducible factor 1, Hypoxia response elements, Beta-lactamase, Luciferase, NanoLuc, Reporter gene, Cancer, Genome editing, Drug, Quantitative high-throughput screening, qHTS, Fluorescence resonance energy transfer, FRET

---

### 1 Introduction

Hypoxia-inducible factor (HIF-1), an oxygen-sensitive transcription factor, is the major regulator of cell development and survival in hypoxic, low oxygen tension environments. This transcription factor maintains oxygen homeostasis within cells by activating an array of anti-apoptotic genes implicating angiogenesis, erythropoiesis, glucose metabolism, and vascular expansion [1]. HIF-1 is a heterodimeric basic helix–loop–helix protein [2] consisting of a

hypoxic responsive HIF-1 $\alpha$  subunit and a constitutively expressed HIF-1 $\beta$  subunit, known as the aryl hydrocarbon receptor nuclear translocator (ARNT) [3]. Under normoxic conditions, HIF-1 $\alpha$  is quickly degraded through the ubiquitin–proteasome pathway [4, 5] under direct control of prolyl hydroxylases (PHDs), preventing formation of the transcription complex. However, in decreasing oxygen concentrations, HIF-1 $\alpha$  levels increase exponentially [2] by reduction of prolyl hydroxylase activity [6]. The available HIF-1 $\alpha$  forms dimeric complexes with HIF-1 $\beta$ . The transcriptionally active complex is able to translocate into the nucleus to bind DNA regulatory sequences known as hypoxia response elements (HREs) downstream to promoter regions [1] of target genes that are activated with assistance of transcriptional coactivators such as p300 and the cAMP response element (CREB)-binding protein (CBP) [7].

HIF-1 activity is also involved in the proliferation, metastasis, and pathophysiology associated with cancer. Intratumor hypoxia and overexpression of HIF-1 occurs as a result of increased oxygen consumption in tumor microenvironments. As a result, cancer cells utilize the HIF-1 signaling pathway to activate the same target genes, increasing their aggressiveness and persistence even in hypoxic conditions. Therefore, the hypoxic environment commonly associated with solid tumors can be exploited as a target for potential anticancer drug developments and therapies [8]. Several small molecule inhibitors of HIF-1 are currently in clinical use as anticancer drugs including topotecan (Hycamtin) and vorinostat (Zolinza) [9]. We have utilized a pair of orthogonal cell-based reporter gene assays using a quantitative high-throughput screening approach to rapidly identify small molecule modulators of the HIF-1 signaling pathway [10, 11]. In the first assay, an HRE driven  $\beta$ -lactamase reporter gene (HRE-bla) was stably transfected to human ME-180 cervical cancer cells, and the expressing beta-lactamase can cleave a chemically modified substrate that undergoes fluorescence resonance energy transfer (FRET). The second assay uses a HIF-1 $\alpha$ - NanoLuc luciferase reporter allele stably integrated in human HCT116 colon cancer cells to enable sensitive detection of HIF-1 activity. Both assays have been successfully miniaturized into 1536-well plate formats to profile large chemical libraries including the Tox21 10K chemical collection. Subsequent cell viability assays were conducted for each cell line respectively.

---

## 2 Materials

### 2.1 $\beta$ -Lactamase Reporter Gene Assay

#### 2.1.1 Cell Line and Culture Condition

1. CellSensor<sup>®</sup> HRE-bla ME-180 cell line (Life Technologies, Carlsbad, CA).
2. 0.25% Trypsin–EDTA, phenol red.
3. Dulbecco's Phosphate-Buffered Saline (DPBS).

4. Trypan Blue Solution, 0.4%.
5. Thaw Medium: DMEM medium supplement with 10% dialyzed fetal bovine serum (FBS), 0.1 mM nonessential amino acids (NEAA), and 50U/mL penicillin and 50  $\mu$ g/mL streptomycin.
6. Cell Culture Medium: DMEM medium supplement with 10% dialyzed fetal bovine serum, 0.1 mM nonessential amino acids, 50U/mL penicillin and 50  $\mu$ g/mL streptomycin, and 5  $\mu$ g/mL blasticidin.
7. Assay Medium: Opti-MEM medium supplement with 0.5% dialyzed fetal bovine serum, 0.1 mM nonessential amino acids, and 50 U/mL penicillin and 50  $\mu$ g/mL streptomycin.
8. Cellometer Auto Cell Counter (Nexcelom Bioscience, Lawrence, MA).
9. CO<sub>2</sub> incubator with variable oxygen control.

### 2.1.2 Assay Reagents and Detection

1. Solution A containing LiveBLAzer™ FRET B/G Substrate (CCF4-AM), Solution B, and Solution C (Life Technologies, Carlsbad, CA).
2. Solution D (Life Technologies, Carlsbad, CA).
3. CellTiter-Glo® Luminescent Cell Viability assay (Promega, Madison, WI).
4. 1536-well tissue culture treated black clear bottom microplates.
5. Assay and compound metal lids.
6. PinTool workstation (Wako Automation, San Diego, CA).
7. BioRAPTR flying reagent dispenser (Beckman Coulter, Pasadena, CA).
8. Envision plate reader (PerkinElmer, Waltham, MA).
9. ViewLux plate reader (PerkinElmer, Waltham, MA).
10. Envision bottom mirror: Beta-Lactamase Dual Enh. D425 nm/D490 nm #661, excitation filter: Photometric 405 nm, emission filter: FITC 535 nm and second emission filter: Umbelliferone 460 nm (PerkinElmer, Waltham, MA).

## 2.2 NanoLuc Luciferase Reporter Gene Assay

### 2.2.1 Cell Line and Culture Condition

1. X-Man® HIF1A NanoLuc HCT116 protein reporter cell line (Horizon Discovery, Cambridge, UK).
2. Thaw, Cell Culture, and Assay Medium: RPMI 1640 medium supplement with 10% Hyclone defined fetal bovine serum, 100U/mL penicillin and 100  $\mu$ g/mL streptomycin, and 300  $\mu$ g/mL G418.
3. 0.05% Trypsin-EDTA, phenol red.
4. Dulbecco's Phosphate-Buffered Saline (DPBS).
5. Trypan Blue Solution, 0.4%.

6. Cellometer Auto Cell Counter.
7. CO<sub>2</sub> incubator with variable oxygen control.

### 2.2.2 Assay Reagents and Detection

1. Nano-Glo<sup>®</sup> Luciferase assay (Promega, Madison, WI).
2. CellTiter-Glo<sup>®</sup> Luminescent Cell Viability assay.
3. 1536-well tissue culture treated white wall/solid bottom plates.
4. BioRAPTR flying reagent dispenser.
5. Assay and compound metal lids.
6. PinTool workstation.
7. ViewLux plate reader.

---

## 3 Methods

### 3.1 Thawing Cells

1. Add 9 mL of pre-warmed Cell Culture Medium in a 15 mL conical tube.
2. Thaw a vial of cells in a 37 °C water bath for 1–2 min (*see Note 1*).
3. Transfer thawed cells to the conical tube and gently mix the content.
4. Centrifuge the tube for 4 min at 200×*g* at room temperature.
5. Aspirate supernatant and resuspend cell pellet with 10 mL of Cell Culture Medium.
6. Count total cell number and transfer 2 × 10<sup>6</sup> HIF-1α-NanoLuc HCT116 to a T75 flask and 1 × 10<sup>6</sup> HRE-bla ME-180 cells to a T75 flask with a final medium volume of 10 mL (*see Note 2*).
7. Incubate the flask in a humidified incubator at 37 °C, 5 % CO<sub>2</sub>, and 20 % O<sub>2</sub> until 80–90 % confluence (*see Notes 3 and 4*).

### 3.2 Culturing Cells

1. Aspirate Cell Culture Medium from a T75 flask of cells.
2. Rinse cell layer with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free DPBS (*see Note 5*).
3. Add 2 mL of 0.05 % Trypsin–EDTA to the HIF-1α-NanoLuc HCT116 cell flask and 0.25 % Trypsin–EDTA to the HRE-bla ME-180 cell flask (*see Note 5*).
4. Incubate the flask in a humidified incubator at 37 °C, 5 % CO<sub>2</sub> until all cells are detached as verified by a tissue culture microscope (*see Note 6*).
5. Add 4 mL of Cell Culture Medium to the flask to terminate Trypsin–EDTA action.
6. Transfer the detached cells to a 50 mL conical tube.
7. Centrifuge the tube for 4 min at 200×*g* at room temperature.

8. Aspirate supernatant and resuspend the cell pellet with 10 mL of Cell Culture Medium.
9. Count total cell number and passage cells at 1:10–1:15 ratios twice per week.
10. Incubate the flask in a humidified incubator at 37 °C, 5% CO<sub>2</sub>, and 20% O<sub>2</sub> until 80–90% confluence.

### **3.3 Plating Cells and Compound Treatment**

1. Aspirate Cell Culture Medium from a flask of cells.
2. Rinse cell layer with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free DPBS.
3. Add 2 mL per T75 flask or 6 mL per T225 flask of 0.05% Trypsin–EDTA to the HIF-1 $\alpha$ -NanoLuc HCT116 cell flask and 0.25% Trypsin–EDTA to the HRE-bla ME-180 cell flask.
4. Incubate the flask in a humidified incubator at 37 °C, 5% CO<sub>2</sub> until all cells are detached as verified by a tissue culture scope.
5. Transfer the detached cells to a 50 mL conical tube and measure cell density.
6. Centrifuge the tube for 4 min at 200 $\times g$  at room temperature.
7. Aspirate supernatant and resuspend the cell pellet with 10 mL of pre-warmed assay medium.
8. Pass the cell suspension through a cell strainer and collect the flow through.
9. Count cell number and dilute to 3 $\times 10^5$  HIF-1 $\alpha$ -NanoLuc HCT116 cells per mL and 5 $\times 10^5$  HRE-bla ME-180 cells per mL.
10. Dispense 5  $\mu$ L of 1500 HIF-1 $\alpha$ -NanoLuc HCT116 cells to each well in a 1536-well white wall/solid bottom plate and 5  $\mu$ L of 2500 HRE-bla ME-180 cells to each well in a 1536-well black wall/clear bottom plate using BioRAPTR flying reagent dispenser (*see Note 7*).
11. Place a porous metal lid on top of the assay plate and incubate in a humidified incubator at 37 °C, 5% CO<sub>2</sub>, and 20% O<sub>2</sub> for 6 h.
12. Transfer 23 nL of compound solutions from a control plate and a sample compound plate to the corresponding wells in the assay plate using a Pintool (*see Notes 8 and 9*).
13. Place the porous metal lid back to the assay plate and incubate in a humidified incubator set at 37 °C, 5% CO<sub>2</sub>, and 20% O<sub>2</sub> (normoxia) or 1% O<sub>2</sub> (hypoxia) for 18 h for HIF-1 $\alpha$ -NanoLuc HCT116 cells and 17 h for HRE-bla ME-180 cells.

### **3.4 $\beta$ -lactamase Reporter Gene Assay Multiplexed with Viability Assay**

1. Add 12  $\mu$ L of Solution A to 120  $\mu$ L of Solution B and vortex (*see Note 10*).
2. Add 20  $\mu$ L of Solution D to 1848  $\mu$ L of Solution C and vortex (*see Note 10*).

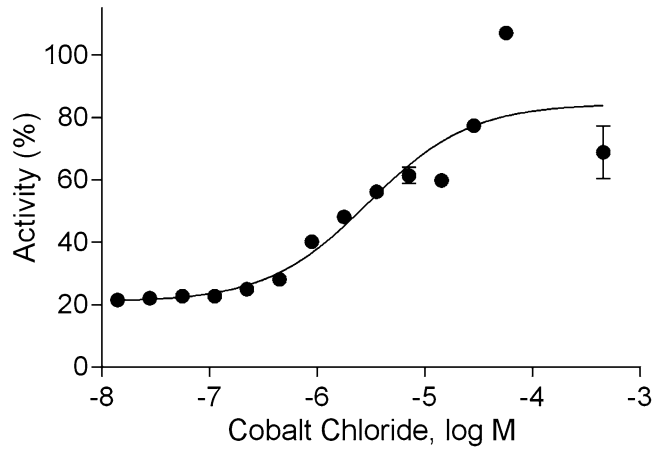
3. Combine solutions from above steps and vortex to create CCF4  $\beta$ -lactamase detection mix (*see Note 10*).
4. Add 1  $\mu$ L of the CCF4  $\beta$ -lactamase detection mix to each 1536 well using BioRAPTR flying reagent dispenser.
5. Place a nonporous lid on the assay plate and incubate in the dark at room temperature for 2.5 h to allow cells to cleave the FRET substrate (*see Note 11*).
6. Read assay plate using an Envision fluorescence plate reader with excitation filters of 405/8 nm and emission filters of 460/25 and 535/20 nm.
7. Calculate the 460 nm to 535 nm fluorescence emission intensity ratio for each well.
8. Add 3  $\mu$ L of CellTiter-Glo Cell viability assay detection reagent to each well using BioRAPTR flying reagent dispenser.
9. Place a nonporous lid on the assay plate and incubate at room temperature in the dark for 30 min.
10. Record luminescence intensity values of CellTiter-Glo viability assay on a ViewLux plate reader (*see Note 12*).

### **3.5 NanoLuc Luciferase Reporter Gene Assay and Viability Assay**

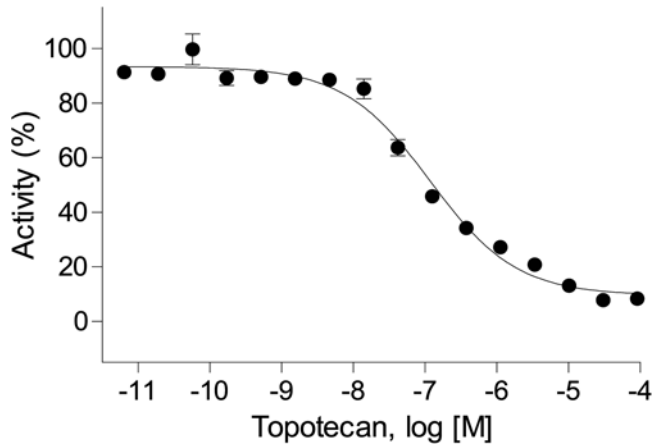
1. Thaw Nano-Glo luciferase assay buffer at 4 °C or room temperature (*see Note 13*).
2. Mix 1 volume of Nano-Glo luciferase assay substrate with 50 volumes of Nano-Glo Luciferase Assay buffer to prepare Nano-Glo luciferase assay detection reagent (*see Note 14*).
3. Add 4  $\mu$ L of Nano-Glo luciferase assay detection reagent or 4  $\mu$ L of CellTiter-Glo Cell viability assay detection reagent to each well in the assay plate using BioRAPTR flying reagent dispenser (*see Note 15*).
4. Place a nonporous lid on the assay plate and incubate at room temperature in the dark for 30 min (*see Note 16*).
5. Record luminescence intensity values of Nano-Glo or CellTiter-Glo viability assay on a ViewLux plate reader.

### **3.6 Data Analysis**

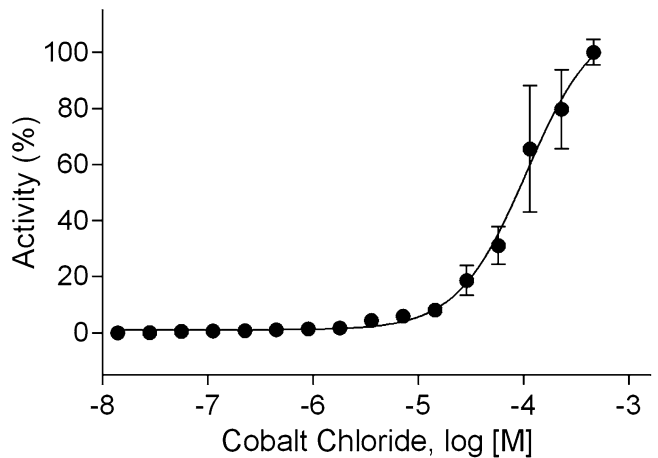
1. Normalize raw plate reads of the  $\beta$ -lactamase reporter gene assay, Nano-Glo luciferase assay, and CellTiter-Glo values to hypoxia mimetic  $\text{CoCl}_2$  control wells (100% activation for normoxia mode), HIF-1 inhibitor topotecan control wells (100% inhibition for hypoxia mode), viability control tetraoctylammonium bromide (TOAB) wells (0% viability), and DMSO-only wells (0% activation/inhibition and 100% viability).
2. Fit concentration–response curves of normalized data points to determine  $\text{IC}_{50}$  and efficacy values using a four-parameter Hill equation in GraphPad Prism software (Figs. 1, 2, 3, and 4).



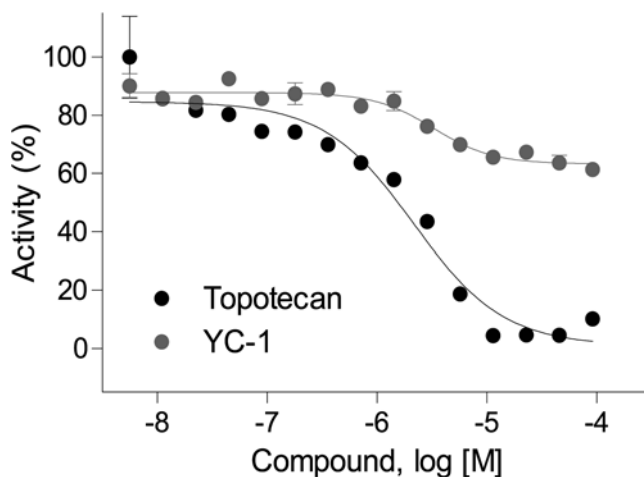
**Fig. 1** Concentration–response curves of cobalt chloride in HRE-bla normoxia mode assay. Each data point is presented as mean  $\pm$  SD from duplicates



**Fig. 2** Concentration–response curves of topotecan in HRE-bla hypoxia mode assay. Each data point is presented as mean  $\pm$  SD from duplicates



**Fig. 3** Concentration–response curves of cobalt chloride in HIF-1 $\alpha$ -NanoLuc normoxia mode assay. Each data point is presented as mean  $\pm$  SD from duplicates



**Fig. 4** Concentration–response curves of topotecan and YC-1 in HIF-1 $\alpha$ -NanoLuc hypoxia mode assay. Each data point is presented as mean  $\pm$  SD from duplicates

---

## 4 Notes

1. When thawing cells in water bath do not overthaw cells. Leaving a small ice pellet will ensure not overthawing and increase cell viability.
2. To ensure accurate cell counts for plating, it is a good idea to count three times and take the average cell density.
3. Gently shake cell flask and place on a flat surface inside the incubator to ensure even cell growth.
4. Do not allow cells to reach 100% confluence which might affect assay performance.
5. When washing cells with DPBS or adding Trypsin–EDTA, make sure to cover all areas of the flask.
6. To ensure increased cell viability, do not leave cells in trypsin for more than 4 min. It is also helpful to gently tap the flask to help cells detach.
7. Be sure to wash the BioRaptr dispensing tips with 70% ethanol and distilled water before use to avoid contamination.
8. Be sure to wash the PinTool workstation tips using and dimethyl sulfoxide (DMSO) and methanol before use to avoid contamination.
9. Allow assay plates cool down to room temperature prior to addition of compound solutions or detection reagents; therefore, there is less evaporation which might affect assay performance.
10. Gently mix to avoid bubbling.



11. Be sure the nonporous metal lid is on the assay plate properly to prevent evaporation and light interference.
12. Adjust exposure time and make sure luminescence intensity values are within the linear dynamic range of the plate reader used.
13. Use fresh Nano-Glo Assay Reagent in each experiment for best assay performance. At room temperature, the reagent will be 10% to 50% less active in approximately 8 h. The reagent will slightly lose activity (<10%) over 2 days of storage at 4 °C.
14. To recover most volume of Nano-Glo luciferase assay substrate, spin down the vial of Nano-Glo luciferase assay substrate before opening it.
15. It is feasible to use CellTiter-Blue viability assay reagent for multiplexing with the NanoLuc assay.
16. Wait for at least 3 min to collect NanoLuc luminescence readings. The half-life of Nano-Glo luminescence is approximately 120 h. We found the 1536-well HIF-1 $\alpha$ -NanoLuc assay reached the maximum luminescence intensity between 30 and 45 min after adding detection reagent.

## References

1. Xia M, Bi K, Huang R, Cho MH, Sakamuru S, Miller SC, Li H, Sun Y, Printen J, Austin CP, Inglese J (2009) Identification of small molecule compounds that inhibit the HIF-1 signaling pathway. *Mol Cancer* 8:117. doi:10.1186/1476-4598-8-117
2. Jiang BH, Semenza GL, Bauer C, Marti HH (1996) Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O<sub>2</sub> tension. *Am J Phys* 271(4 Pt 1):C1172–C1180
3. Wang GL, Semenza GL (1995) Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* 270(3):1230–1237
4. Huang LE, Gu J, Schau M, Bunn HF (1998) Regulation of hypoxia-inducible factor 1 $\alpha$  is mediated by an O<sub>2</sub>-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* 95(14):7987–7992
5. Salceda S, Caro J (1997) Hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 272(36):22642–22647
6. van Uden P, Kenneth NS, Rocha S (2008) Regulation of hypoxia-inducible factor-1 $\alpha$  by NF- $\kappa$ B. *Biochem J* 412(3):477–484. doi:10.1042/bj20080476
7. Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML (2002) Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 295(5556):858–861. doi:10.1126/science.1068592
8. Huang W, Huang R, Attene-Ramos MS, Sakamuru S, Englund EE, Inglese J, Austin CP, Xia M (2011) Synthesis and evaluation of quinazolin-4-ones as hypoxia-inducible factor-1 $\alpha$  inhibitors. *Bioorg Med Chem Lett* 21(18):5239–5243. doi:10.1016/j.bmcl.2011.07.043
9. Hu Y, Liu J, Huang H (2013) Recent agents targeting HIF-1 $\alpha$  for cancer therapy. *J Cell Biochem* 114(3):498–509. doi:10.1002/jcb.24390
10. Xia M, Huang R, Sun Y, Semenza GL, Aldred SF, Witt KL, Inglese J, Tice RR, Austin CP (2009) Identification of chemical compounds that induce HIF-1 $\alpha$  activity. *Toxicol Sci* 112(1):153–163. doi:10.1093/toxsci/kfp123
11. Hsu CW, Huang R, Khuc T, Shou D, Bullock J, Grooby S, Griffin S, Zou C, Little A, Astley H, Xia M (2015) Identification of approved and investigational drugs that inhibit hypoxia-inducible factor-1 signaling. *Oncotarget* 7(7):8172–8183