2019 AACR-NCI-EORTC
Molecular Targets and
Cancer Therapeutics
Abstract C050

Novel, Potent, and Selective Hypoxia-Inducible Factor-2α (HIF-2α) Antagonists

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OVERVIEW

- Preclinical and clinical evidence suggests that HIF-2α is a valid approach to destroy tumor cells, particularly in clear cell renal carcinoma (ccRCC)[1,2].
- Arcus Biosciences is developing novel HIF-2α-specific small-molecule antagonists and investigating the biology of HIF-2α in various cancer and non-cancer cell subsets.
- Here we describe pharmacological properties associated with novel, potent, and selective HIF-2α antagonists and findings related to the understanding of HIF-2α biology in human immune and stromal cells and development of a HIF-2α-specific transcriptional signature.

HIF-2α BIOLOGY & REGULATION

- The solid tumor microenvironment (TME) can be hypoxic and cancer cells require induction of genes associated with metabolism, proliferation, and angiogenesis to survive and metastasize[3].
- The master transcriptional regulators of hypoxia-induced genes are the Hypoxia-Inducible Factor (HIF) proteins[4].
- HIF consists of an oxygen-regulated alpha monomer, of which there are three isoforms (HIF-1α, HIF-2α, and HIF-3α)[4].
- Alpha monomers heterodimerize with a constitutively-expressed beta monomer (HIF-1β/ARNT) using Per-ARNT-SIM (PAS) protein-protein interaction domains[4].
- Disruption of HIF-α/HIF-1β heterodimer formation is an effective means to inhibition of HIF-2α-dependent gene transcription[4].

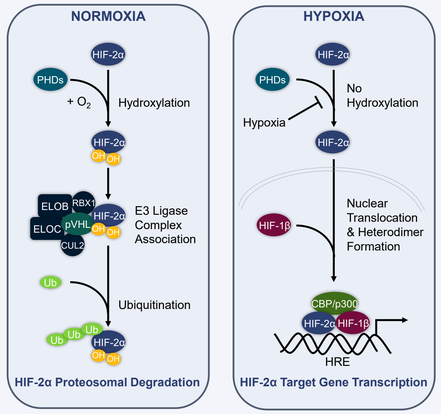
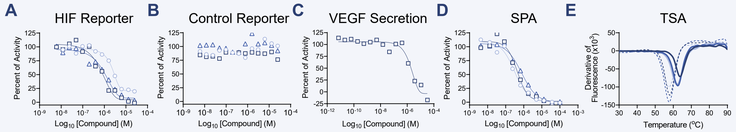


Figure 1. Overview of HIF-2α regulation. In normoxia (left), proline residues present in the oxygen-dependent degradation domain (ODDD) of HIF-2α are hydroxylated by prolyl hydroxylases (PHDs), allowing for recognition by the von Hippel-Lindau (pVHL) E3-ubiquitin ligase complex and subsequent ubiquitination and proteasomal degradation. Upon exposure to low oxygen conditions (hypoxia, right) or in the case of *vhl* mutation or silencing (pseudohypoxia), HIF-2α subunits accumulate and dimerize with HIF-1β/ARNT, resulting in transcription of various gene sets, some of which are pro-tumorigenic, downstream of hypoxia-response element (HRE) DNA binding sites. Adapted from Yu *et al.*[5].

RESULTS

Arcus is Rapidly Optimizing Three Distinct Chemical Series of HIF-2α Antagonists

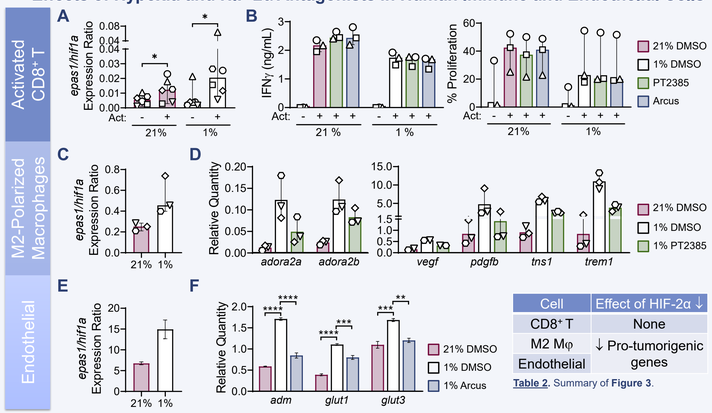


	MST (K_D, μM)	ITC (K_D, μM)
Series 1	1.50	0.44
Series 2	0.66	0.13
Series 3	1.20	0.44

Table 1. Binding Data. MicroScale Thermophoresis (MST). 50 nM human HIF-2α PAS-B domain (PAS-B, AA 240-350) was labeled with Red-Tris-NTA and mixed with varying concentrations of Arcus compounds (3.05 nM – 100 μM). Acquisition was performed at 25 °C with 90% LED and 40% laser power. **Isothermal Titration Calorimetry (ITC).** 100 μM Arcus compounds were titrated into a stirred reaction cell containing 10 μM PAS-B at 25 °C. The reference power was 10 μcal/s and the stirring rate was 750 rpm.

Figure 2. Representative SAR assay examples for Arcus Series 1, Series 2, and Series 3 HIF-2α antagonist compounds. (A & B) HIF and Control Cellular Reporter Assay. 786-O renal adenocarcinoma cells (mutant for VHL and HIF-1α) stably expressing HIF or control CMV luciferase reporter constructs (Qiagen) were treated with Arcus compounds for 20 hours (h) at 37°C 5% CO_2. Data normalization for **A-D** was done using DMSO vehicle controls and IC_{50} quantitation was performed using four parameter curve fitting. **(C) VEGF Protein Secretion Assay.** 786-O cells were treated with Arcus compounds for 24 h at 37 °C 5% CO_2. Media was removed and cells were given fresh media containing Arcus compounds. After an additional 24 h, VEGF in the cell supernatant was quantified by AlphaLISA (Perkin Elmer). **(D) Scintillation Proximity Assay (SPA).** 50 nM PAS-B was incubated at room temperature with Arcus compounds in 2% DMSO for 60 min and 3 μg copper chelate PVT SPA beads for an additional 45 min before to addition of 25 nM ^{3}H-tracer and luciferase measurement. **(E) Thermal Shift Assay (TSA).** Arcus compounds were incubated with PAS-B prior to addition of dye and fluorescence measurement. ΔT_m was calculated by normalizing compound T_m to DMSO T_m. Dotted lines, DMSO only. Symbol legend shown in **Table 1.**

Effects of Hypoxia and HIF-2α Antagonists in Human Immune and Endothelial Cells



Cell	Effect of HIF-2α ↓
CD8+ T	None
M2 Mφ	↓ Pro-tumorigenic genes
Endothelial	

Table 2. Summary of **Figure 3**.

Figure 3. (A & B) Human CD8+ T cells are functional in 1% O_2 and similar to mouse[6], HIF-2α inhibition does not impair function. Primary human CD8+ T cells isolated from the blood of three to six donors (symbols) were left untreated (-) or activated (+) using a αCD2/αCD3/αCD28 bead cocktail in normoxia (21% O_2) or hypoxia (1% O_2) for three days in the presence of 10 μM HIF-2α antagonist. Shown is the **(A)** ratio of HIF-2α/HIF-1α gene expression and **(B)** median ± range of IFNγ secretion as measured by bead array and proliferation capacity as measured by Cell-Trace Violet staining and flow cytometry. Act, activation. *p<0.05. Statistics were calculated using Wilcoxon matched-pairs signed rank test. **(C & D) Exposure of M2-polarized macrophages to hypoxia drives a pro-tumorigenic gene expression profile that is decreased by HIF-2α inhibition.** Primary human CD14+ monocytes isolated from the blood of three donors (symbols) were differentiated in normoxia for six days with M-CSF before polarizing with IL-4 for one day in normoxia or hypoxia in the presence of 10 μM HIF-2α antagonist. Shown is the **(C)** ratio of HIF-2α/HIF-1α gene expression and **(D)** relative quantity of gene transcripts whose products are associated with a suppressive TME (ADORA2A and ADORA2B) and tumor progression (VEGF, PDGFB, TNS1, TREM1). **(E & F) Exposure of HUVECs to hypoxia drives a pro-angiogenic gene expression profile that is decreased by HIF-2α inhibition.** HUVECs were treated with DMSO or 10 μM HIF-2α antagonist for 16 h in normoxia or hypoxia. Shown is the **(E)** ratio of HIF-2α/HIF-1α gene expression and **(F)** relative quantity of gene transcripts associated with angiogenesis. ***p<0.0001, **p<0.001, *p<0.01. Statistics were calculated using one-way ANOVA with Dunnett's multiple comparisons test vs 1% O_2 DMSO control for each gene. Gene expression quantitation done by qPCR ($2^{-\Delta Ct}$). PT2385 was synthesized by Arcus utilizing methodology described in Wehn *et al.*[7].

Selective Downregulation of Pro-tumorigenic HIF-2α-Induced Pathways

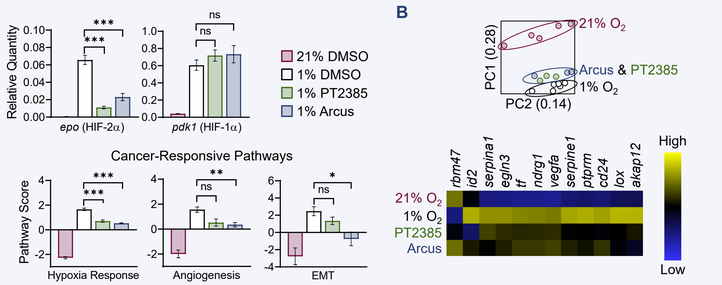


Figure 4. A representative Series 2 compound inhibits HIF-2α-, but not HIF-1α-, mediated transcription of pro-tumorigenic gene sets. Hep3B hepatocellular carcinoma cells (wild-type for VHL and HIF-1α) were treated with 10 μM of PT2385 or Arcus compound and exposed to hypoxia (1% O_2) for 16 h prior to RNA isolation for **(A)** qPCR ($2^{-\Delta Ct}$ method) or **(B & C)** NanoString PanCancer Progression Panel analyses. **(A)** Shown are the mean ± SEM of HIF-2α-specific (*epo*) and HIF-1α-specific (*pdk1*) transcript levels. HIF-2α-specific *serpine1* and HIF-1α-specific *pgk1* genes behave similarly (not shown). Specificity is confirmed genetically in **Figure 6**. **(B)** Shown is a Principal Component (PC) Analysis biplot depicting the top two components of variance and a heat map depicting 12 genes that were significantly changed in hypoxia and reverted by both PT2385 and Arcus compound. **(C)** Shown are the mean ± SEM of Pathway Scores. NanoString analysis was conducted using nSolver Data Analysis Software from NanoString Technologies, Inc. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns=not significant. Statistics were calculated using one-way ANOVA with Dunnett's multiple comparisons test using 1% O_2 control (DMSO or mock transfection) groups. n=3-6 replicates per condition. EMT, epithelial-mesenchymal transition. PT2385 was synthesized by Arcus utilizing methodology described in Wehn *et al.*[7].

Evaluation of the Hep3B HIF-2α Signature ssGSEA Score (HSS) in CCLE and TCGA

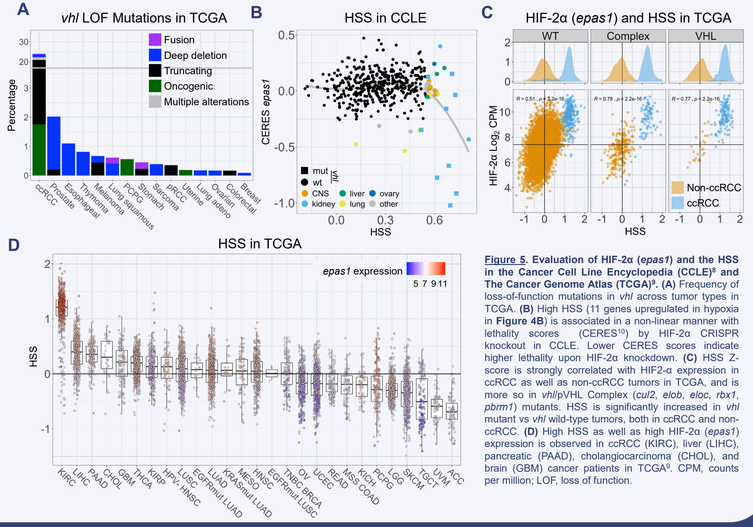


Figure 5. Evaluation of HIF-2α (*epas1*) and the HSS in the Cancer Cell Line Encyclopedia (CCLE)[8] and The Cancer Genome Atlas (TCGA)[9]. **(A)** Frequency of loss-of-function mutations in *vhl* across tumor types in TCGA. **(B)** High HSS (11 genes upregulated in hypoxia in **Figure 4B**) is associated in a non-linear manner with lethality scores ($CERES^{10}$) by HIF-2α CRISPR knockout in CCLE. Lower CERES scores indicate higher lethality upon HIF-2α knockdown. **(C)** HSS Z-score is strongly correlated with HIF-2α expression in ccRCC as well as non-ccRCC tumors in TCGA, and is more so in *vhl*/pVHL Complex (*cul2*, *elob*, *eloc*, *rbx1*, *pbrm1*) mutants. HSS is significantly increased in *vhl* mutant vs *vhl* wild-type tumors, both in ccRCC and non-ccRCC. **(D)** High HSS as well as high HIF-2α (*epas1*) expression is observed in ccRCC (KIRC), liver (LIHC), pancreatic (PAAD), cholangiocarcinoma (CHOL), and brain (GBM) cancer patients in TCGA[9]. CPM, counts per million; LOF, loss of function.

RESULTS

HIF-α Isoform-Specific Signature Development

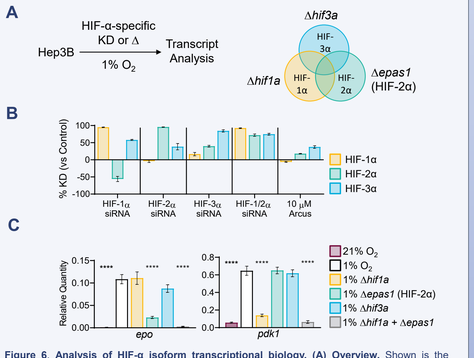


Figure 6. Analysis of HIF-α isoform transcriptional biology. (A) Overview. Shown is the experimental outline for siRNA- (KD) or CRISPR- (Δ) based approaches to derive HIF-α isoform-specific gene signatures and Venn diagram illustrating overlapping gene expression scenarios in each experimental condition. **(B) Cross-regulation between HIF-α isoforms.** Hep3B cells were treated with Arcus compound or transfected with HIF-α isoform-specific siRNA and exposed to hypoxia for 16 h. Shown is the % KD of each HIF-α isoform. **(C) Validation.** Hep3B cells nucleofected with CRISPR protein and control or HIF-α isoform-specific guide RNAs were exposed to hypoxia for 16 h. Shown are the mean ± SEM of *epo* and *pdk1* transcript levels. ****p<0.0001. Statistics were calculated using one-way ANOVA with Dunnett's multiple comparisons test vs 1% O_2 control. Gene expression quantitation done by qPCR ($2^{-\Delta Ct}$). KD, knock-down.

SUMMARY

- Three distinct compound series are undergoing iterative SAR optimization to develop novel HIF-2α antagonists. Representative compounds from each series show both HIF-2α binding and functional activity in cell-based assays **(Figure 2)**.
- In human cells, HIF-2α inhibition does not significantly impact T cell function but does decrease expression of genes that encode proteins implicated in tumorigenicity in macrophages and endothelial cells **(Figure 3)**.
- A representative Series 2 compound selectively inhibited HIF-2α target gene expression in Hep3B cells. NanoString analyses revealed pathway signatures upregulated in hypoxia that were significantly decreased with Arcus antagonist treatment **(Figure 4)**.
- A gene signature derived from pharmacological inhibition of HIF-2α in Hep3B cells was used to evaluate the relationship between expression of HIF-2α or HIF-2α transcriptome genes, VHL/VHL Complex mutational status, and tumor type **(Figure 5)**.
- Deletion of each of the three predominant HIF-α isoforms in Hep3B cells reveals isoform-dependent gene expression profiles **(Figure 6)**.

CITATIONS

1) Wallace *et al.* (2016) Cancer Res 76, 5491-5500.
2) Courtney *et al.* (2018) J Clin Oncol 36, 867-874.
3) Hockel & Vaupel (2001) JNCI 93, 266-276.
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6) Palazon *et al.* (2017) Cancer Cell 32, 669-683.
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9) https://www.cancer.gov/tcga
10) Meyers *et al.* (2017) Nat Genet 49, 1779-784.