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Development of Cell-based Assays for the Identification and Characterization of Inhibitors of Mutant G323E Hypoxia Inducible Factor-2α (HIF-2α).

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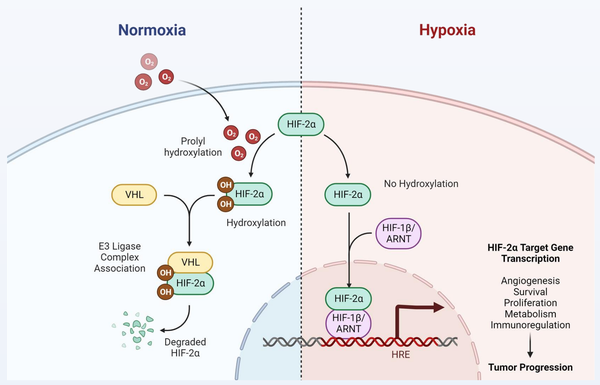
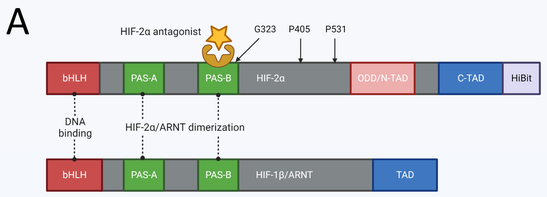
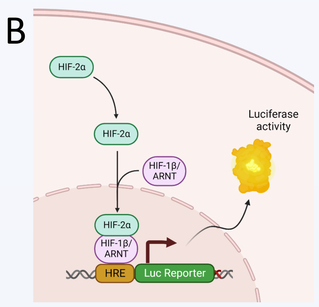
INTRODUCTION

The role of Hypoxia Inducible Factor-2α (HIF-2α) in the hypoxia response pathway has garnered attention as a target for the treatment of von Hippel-Lindau (VHL) mutated clear cell renal cell carcinoma (ccRCC). Belzutifan is a HIF-2α small molecule inhibitor, approved by the FDA in 2021, for the treatment of VHL disease. Reported objective responses to HIF-2α inhibitors tend to be long and potential mechanisms of adaptive resistance have not been reported; however, during the development of first-generation HIF-2α inhibitor, PT2385, drug resistance was observed in a patient that led to the identification of the G323E HIF-2α gatekeeper mutant.[1] We developed cell-based assays to determine the feasibility of generating compounds that inhibit HIF-2α with the G323E mutation. Using previously reported normoxia stable HIF-2α mutations, HEK-293 cells were engineered to express stable wild type or G323E mutant HIF-2α. These cells were transfected with a hypoxia response element (HRE) luciferase reporter to measure the ability of compounds to inhibit the HIF-2α luciferase response. Identified inhibitors were further characterized by VEGF secretion in wild type or CRISPR generated 786-O G323E mutant renal cells and in a protein thermal shift assay (TSA). The assays developed provide a platform for screening against and identifying small molecule inhibitors of the G323E HIF-2α mutant.



MATERIALS AND METHODS





HEK-293 HIF-2α Luciferase Reporter Assay

Figure 1a) The P405A/P531A mutations have been reported to stabilize HIF-2α in normoxia.[2]

- Constructs for stable or G323E HIF-2α were generated with a C-terminal HiBit tag to allow monitoring of protein expression and clone selection.
- HEK-293 cells were transfected with either stable or G323E HIF-2α to achieve protein stability under normoxia.
- Clones expressing stabilized HIF-2α with or without G323E mutation were identified utilizing the Nano-Glo® HiBiT Lytic Detection System (Promega).

Figure 1b) Stabilized positive expressing clones were transfected with HRE luciferase reporter to measure HIF-2α activity.

- Endogenous HIF-2α from HEK-293 is quickly degraded in normoxia condition. Minimal HRE-reporter activity is detected in parental cells.
- Cells were incubated with compound for 24 hours.
- After 24 hours, One-Glo (Promega) was added, and luminescence read.

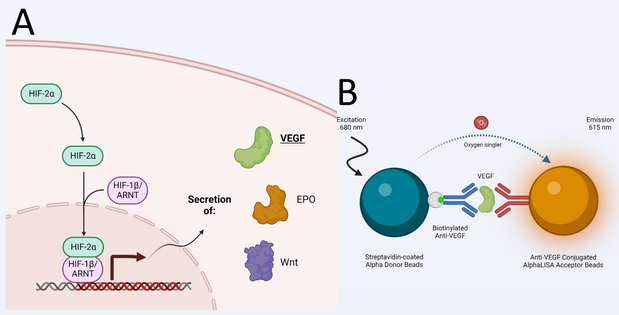
786-O VEGF Secretion Assay

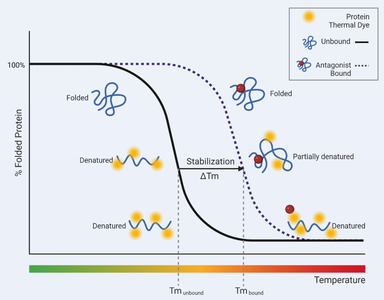
Figure 2a) 786-O renal cells lack a functional VHL gene allowing for constitutive HIF-2α activity. This endogenous HIF-2α activity made for an ideal system to measure HIF-2α inhibition via downstream VEGF secretion.

- Utilizing CRISPR, a 786-0 cell line was generated with the G323E HIF-2α mutation.

Figure 2b) VEGF secretion measured by AlphaLISA (Perkin Elmer).

- Wild type and CRISPR generated G323E 786-O cells were treated with HIF-2α inhibitors over 48 hours. After compound treatment, the supernatant was collected, and VEGF secretion was measured utilizing the VEGF AlphaLISA.



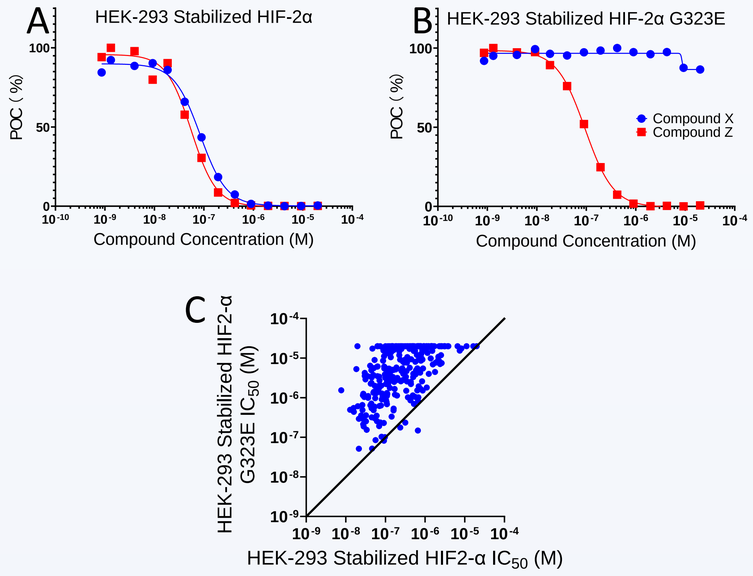


HIF-2α Thermal Shift Assay

Figure 3) Using purified PAS-B domain for wild type or G323E HIF-2α inhibitor binding was measured using a thermal shift assay.

- In the presence of Protein Thermal Shift (Thermo) dye, protein is exposed to an increasing temperature gradient. As protein denatures, the dye binds to exposed hydrophobic regions increasing fluorescence generating a melting curve.
- The presence of inhibitor binding stabilizes the protein at higher temperatures, shifting the melting point (Tm) for the protein. This shift in Tm is proportional to the binding affinity.

RESULTS



HEK-293 Luciferase Reporter

Figure 4a) and 4b) Select compound dose responses in reporter assays.

- Compound Z demonstrates inhibition against the stabilized HIF-2α and the stabilized G323E HIF-2α.
- Compound X is selective for wild type HIF-2α and not G323E.

Figure 4c) Potency correlation of select inhibitors against the stable wild type and stable G323E HIF-2α.

- Compounds demonstrate selectivity towards the wild type HIF-2α.
- Wide range of potencies is observed against the G323E mutant.

786-O VEGF Secretion

Figure 5a) and 5b) Select compound dose response in VEGF secretion assay.

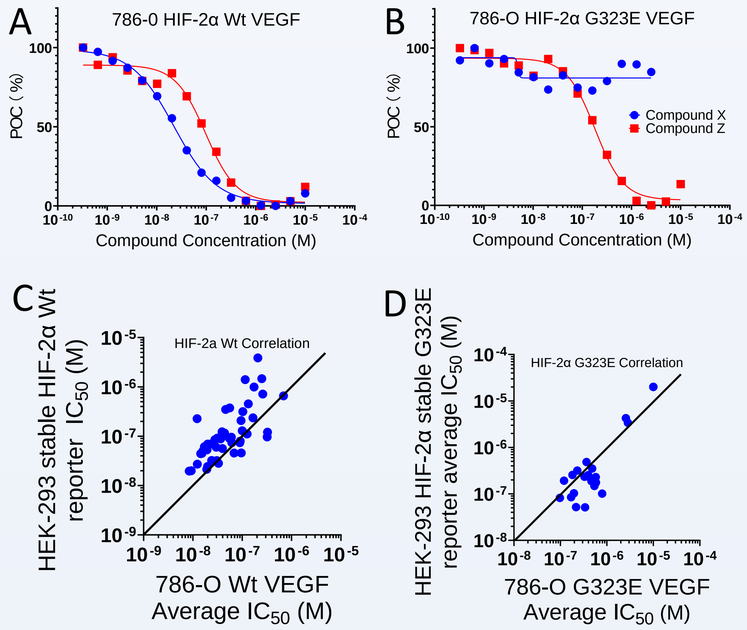
- The same compounds dosed in the reporter assay recapitulate their results in the 786-O VEGF assay.

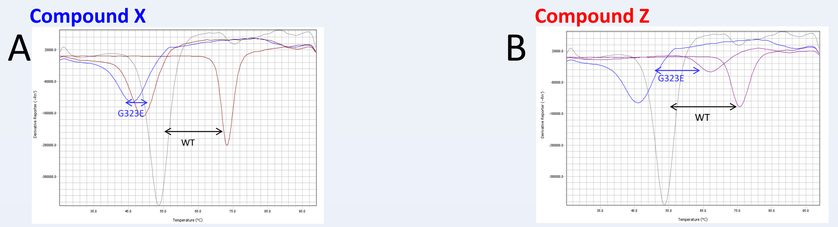
Figure 5c) Compound correlation between wildtype assays.

- Compounds appear slightly more potent in the VEGF assay.
- Good correlation is observed between select inhibitors comparing the reporter assay to the VEGF assay.

Figure 5d) Compound correlation between G323E assays

- Good correlation is observed for select compounds when comparing the luciferase reporter. assay to the 786-O VEGF.





Thermal Shift Assay

Figure 6a) and 6b) Thermal shift assay melting curve of PAS-B wildtype or G323E domain in the presences of select inhibitors.

- Compound X shows minimal shift in ΔTm against the G323E mutant compared to wild type HIF-2α.
- Compound Z generates a significant ΔTm against both the wild type and the G323E mutant confirming binding of inhibitor to the PAS-B domain of both wild type and G323E HIF-2α.

CONCLUSION

- Expression of normoxia stable HIF-2α in HEK-293 cells and subsequent development of luciferase reporter assay allowed for testing and identification of inhibitors against both wild type and G323E HIF-2α.
- In addition, inhibition of endogenous HIF-2α activity was measured in CRISPR generated 786-O using VEGF AlphaLISA.
- The data recapitulates the results of both the wildtype and G323E across cell-based assays.
- Binding of inhibitors to G323E mutants was confirmed by thermal shift assay and subsequently compound binding mode was identified via X-ray crystallography.[3]

References:

1) Courtney K. et al. Clin Cancer Res 15 February 2020; 26 (4): 793–803.
2) Fujita, N. et al. J Bone Miner Res. 2012 Feb; 27(2): 401–412.
3) Shia, S et al. Partially open conformation of the G323E mutated HIF-2α PASB domain captured by X-ray crystallography. In: Proceedings of the AACR-NCI-EORTC Virtual International Conference on Molecular Targets and Cancer Therapeutics; 2023 Oct 11-15; Boston, MA. Philadelphia (PA): AACR; Mol Cancer Ther 2023;22.
4) Illustrations created with BioRender.com (2024).