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14. ABSTRACT

The hypoxia-inducible factors (HIFs) are proteins that have remained largely unexplored as possible targets for cancer therapeutics; yet there is widespread recognition these proteins directly contribute to the progression of many types of human solid tumors. Having previously discovered that the HIF proteins contain small-molecule binding pockets within their architectures, we sought to identify small-molecules that bind and act directly through the HIF proteins to block their functions. Over the first year of funding, we produced large quantities of highly pure HIF proteins (both HIF-1alpha/ARNT and HIF-2alpha/ARNT) and used these proteins for conducting high-throughput screens with 32,000 different small-molecules. The screen was efficient and a mass-spectrometry based assay, and the "hits" obtained were further confirmed by a counter-screen using another protein unrelated to HIFs. To understand the relative binding affinities of the hits, we carried out a biochemical assay to measure the equilibrium dissociation constants of the small molecules in binding to the HIF complexes. Over the coming year, we plan to examine these small-molecules in a number of cell-based studies to identify which ones are potent inhibitors that may be useful as possible use as anti-cancer therapies.

15. SUBJECT TERMS

Hypoxia-inducible factors, mass-spectrometry, drug discovery, kidney cancer

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1. INTRODUCTION:

The subjects of this proposal are the hypoxia-inducible factors (HIFs) proteins, HIF-1alpha and HIF-2alpha, both of which function as transcription factors controlling genetic programs required for driving solid tumor growth in cancers of kidney, pancreas, stomach, colon and skin. The scope of the proposal involves generating large quantities of purified HIF-1alpha and HIF-2alpha proteins and examining chemical libraries consisting of small molecules, to find directly binding ligands for each protein. The scope of the proposal further involves selecting among the putative ligands those that can inhibit HIF-1 alpha /ARNT and/or HIF-2 alpha/ARNT functions in cellular assays, and to characterize activities, specificities, and mechanisms of

2. KEYWORDS:

Hypoxia-inducible factors, mass-spectrometry, high-throughput screen, Cancer.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

There were two major Specific Aims for the original project, and no major changes were made in pursuing these two goals.

1) Identify new classes of drug-like molecules that directly target HIF-1alpha/ARNT and HIF-2alpha/ARNT heterodimers with high affinity and specificity using biochemical approaches.

2) Validate and establish the subset of HIF-alpha/ARNT ligands that robustly inhibit HIF functions in cell culture assays.

What was accomplished under these goals?

We previously detailed within our 2017 annual report that the main objectives of Aim 1 were successfully met. Here, we again briefly summarize those studies, and then further relate the findings to our more recent accomplishments, under Aim 2.

For Aim 1, we conducted studies to identify new classes of small-molecules that could directly target and bind to HIF-1alpha/ARNT and HIF-2alpha/ARNT heterodimers with high affinity and specificity using biochemical approaches. This work included producing 15 mg of highly purified proteins, as well as additional proteins in their superfamily, using large-scale *E. coli* expression systems and extensive purification steps. We then subjected both HIF-1alpha/ARNT and HIF-2alpha/ARNT heterodimers to several high-throughput screens to identify binding ligands. The main screen utilized affinity-selection mass spectrometry (AS-MS), and a chemical library of 32,000 small-molecules. This work allowed us to identify putative ligands successfully, which we then further studied over the past twelve months in the context of Aim 2, to complete the major goals of the proposal.

Shown in our previous (2017) annual report were examples of SDS-PAGE gels displaying the level of purity obtained for each heterodimer, showing their suitability for screening. We also showed a representative cross-section of some of the AS-MS data that we obtained from our screens, when employing each HIF-alpha/ARNT heterodimer. For counter-screening, we employed the same set of 32,000 compounds, but with the related heterodimer of Aryl hydrocarbon receptor (AHR) with ARNT. This counter-screen allowed us to remove “non-specific hits” for HIF-1alpha/ARNT and HIF-2alpha/ARNT. Finally, we successfully established a biochemical binding assay (SPR) to measure the direct binding of the small- molecule to HIF-alpha/ARNT heterodimers. We also developed a more sensitive binding assay, based on microscale thermophoresis (MST) to make these measurements. Therefore, in Aim 1, we met our stated goals of identifying ligands capable of direct and specific binding to each HIF heterodimers with characterized biochemical equilibrium binding constant.

For Aim 2, we conducted follow-up studies over the past year, by specifically utilizing the small-molecule ligands ("hits") that were generated from the Aim 1 studies. These Aim 2 studies were largely based on additional biochemical and cell-culture based methods that could inform us about the selectivity and cellular effects of the compounds, and whether they were antagonists (inhibitors) or agonists (activators).

As originally proposed under our specific aims, we relied heavily on dose-response cell-based reporter transcription assays to assess which compounds (among the "hits" identified in Aim 1) were inhibitors *versus* activators. However, this work required us to first establish clear benchmarks for defining such activities. For a benchmark for inhibitor activity, we relied on the compound PT2385, a selective inhibitor of HIF-2alpha. Accordingly, we began by applying our complementary biochemical and cell-based assays to this compound (**Fig. 1**).

Using the recombinant PAS-B domain of HIF-2alpha, we found that PT2385 (**Fig. 1a**) binds to this protein with an equilibrium binding constant (K_d) of 167 nM, as measured by the MST (microscale thermophoresis) method (**Fig. 1b**). Using a fluorescence polarization assay, we further examined if PT2385 might change the binding affinity of HIF-2alpha/ARNT heterodimer for its hypoxia response element (HRE) (**Fig. 1c**). In the presence of 0 μM, 0.1 μM, 1 μM and 10 μM PT2385, the DNA binding affinities observed for the heterodimer were 65 nM, 96 nM, 118 nM and 339 nM, respectively, showing a dose-dependent inhibition caused by PT2385. However, even at high and saturating concentrations of PT2385, the DNA binding affinity was not completely abolished.

Then we used cell-culture methods on this compound in a dose-dependent manner, using the 786-O cell line stably transfected with the HRE luciferase reporter. We measured an IC_{50} value of 42 nM for PT2385's inhibition on the Hypoxia-response element (HRE)-driven transcription (**Fig. 1d**). A qPCR assay monitoring the expression of endogenous target genes of HIF-2 (*VEGF*, *CyclinD1*, *GLUT1* and *NDRG1*) further revealed the dose-dependent inhibitory activity of this compound in 786-O cells (**Fig. 1e**). The expression of a HIF-1alpha specific target gene (*PGK1*) was not affected by PT2385, in support of its selectivity for HIF-2alpha. We had previously showed that three residues differed between the corresponding PAS-B pockets of HIF-2α and HIF-1α, that would allow chemical ligands to be highly selective for HIF-2α *versus* HIF-1a.

Using co-immunoprecipitation (co-IP) studies, we confirmed that PT2385 worked as an inhibitor by destabilizing the heterodimeric association between overexpressed HIF-2alpha and ARNT subunits in HEK293T cells (**Fig. 1f**). This protein-protein disruption required a higher concentration of ligand (1-10 μM) in our case given that we had overexpressed its target protein, compared to the concentrations seen to disrupt the dimerization between endogenous HIF-2alpha and ARNT proteins (0.1-1 μM) in 786-O cells previously. Nevertheless, given that DNA-binding property of HIF-2alpha/ARNT was not completely abrogated biochemically even in high concentrations of PT2385, we concluded that a complete dissociation of two subunits into monomers is not likely to occur *in vitro*. However, the positioning and/or stabilities of the subunits within the context of their heterodimer are sufficiently altered and destabilized by PT2385 to reduce transcriptional function *in vivo*.

While our data clearly revealed the mechanism of action used by PT2385 was based on partial destabilization of the heterodimer, it remained unclear to us whether other distinct classes of antagonists would rely on this same mechanism of action. Therefore, we shifted away from this benchmark compound to novel HIF-2alpha antagonists that derived from our screens, to understand and compare their mechanism of action to PT2385. An interesting hit had emerged from the use of the LOPAC chemical library in a screen, was S(-)-*p*-bromotetramisole (designated as compound T1001 (shown in **Fig. 2a**). We found T1001 could increase the protein melting temperature of the complex (T_m) by approximately 1.5 °C, compared to PT2385's T_m upshift of 6.7 °C/ We further discovered that T1001 could directly bind to the PAS-B domain of HIF-2alpha with a K_d of about 246 nM, as measured by the MST method (**Fig. 2b**). By examining its cellular effects on the expression of known HIF-2alpha target genes in 786-O cells (**Fig. 2c**), we found that compared to PT2385, T1001 was a weaker antagonist.

To carefully map the precise binding and inhibitory mechanism of T1001, we also obtained the co-crystal structure of HIF-2alpha/ARNT in complex with T1001. The "omit" maps in **Fig. 2d** show that T1001 binds into the same PAS-B domain of HIF-2alpha as PT2385, making use of a similar constellation of interacting residues within the protein pocket. We conducted cell-culture based dose-dependent activity studies of T1001 by examining known target gene expressions HIF-2alpha and HIF-1alpha regulated genes, and these studies confirmed its activity as a selective HIF-2alpha inhibitor (**Fig. 2e**).

In addition to studies with these two HIF-binding antagonists (PT2385 and T1001), we further characterized the binding mode of a previously know antagonist (0X3). We made further comparison of HIF-2alpha/ARNT crystal structures bound to each of PT2385, T1001 and 0X3. All of these ligands rely on a similar constellation of amino-acid residues for their binding, but their abilities to physically displace M252 from the pocket are notably different (**Fig. 2f**). PT2385 causes the most significant displacement for M252 side-chain, forcing it completely out of the PAS-B pocket. 0X3 has a moderate impact, and T1001 produces the least displacement. The extents of movement by M252 from inside the pocket and toward the dimer interface can be correlate to the relative potencies of these antagonists (**Fig. 2g**).

Among the other hits we identified from AS-MS and other screening efforts was an activator (agonist). The compound, which we designated as compound M1001, increased the T_m of HIF-2alpha/ARNT complex by approximately 0.8 °C in the thermal shift assay (**Figure 3**). The structure of M1001 is shown in **Fig 4a**, and this compound was subsequently found to physically interact with the HIF-2alpha PAS-B domain by MST (**Fig. 4b**). 786-O cells treated with M1001 showed modestly increased expression of known HIF-2alpha target genes, producing the opposite response previously observed with PT2385 (**Fig. 4c**). These data together indicated to us that M1001 directly binds to HIF-2alpha and has the properties of a weak agonist.

In summary, the research we conducted over past two years allowed us to identify new compounds and carefully investigate their activities, binding sites and allosteric mechanisms as HIF binding modulators. We found these compound to differentially influence the stability of HIF-alpha/ARNT complexes by altering the protein-protein interactions between HIF proteins and their heterodimeric partners ARNT. To delineate this allosteric mechanisms of actions, we relied on a number of complementary biochemical and cell-based approaches. By comparing the crystal structures of HIF-2alpha/ARNT in complex with various antagonists (including PT2385, T1001 and 0X3), we found through crystallographic studies that ligand-induced movement of HIF-2alpha pocket residue M252's side-chain allosterically destabilized the heterodimeric complex formed between HIF-2alpha and ARNT. The relative potency of the antagonists we employed correlated with the degree to which they could physically displace an amino-acid (M252) from inside of pocket and point it towards the dimer interface, providing important new guidance for future improvement of HIF-alpha directed antagonists through medicinal chemistry. The PT2385 class is a potent inhibitor already being developed by others as an anti-cancer drug, but the resistance mutations that are known arise due to its exposure could limit its long-term effectiveness, and necessitate additional classes of antagonists to be used for prolonged therapy. Therefore, the discovery of multiple new classes of chemical antagonists with *in vivo* and clinical promise remains highly desirable going forward.

What opportunities for training and professional development has the project provided?

Two postdoctoral scientists were trained over the past two years. A) Dalei Wu contributed to the project's goals over the past year in all aspects, and subsequently took up a new position as Professor in China. B) Jingping Lu contributed to the project, and has received training in mass-spectrometry based high-throughput screening and biochemical binding studies involving proteins and small-molecules.

How were the results disseminated to communities of interest?

The PI presented lectures with data obtained over the past year, a) Conference on "Adaptations to Hypoxia in Physiology and Disease" in Whistler, British Columbia, Canada., b) ASBMB annual meeting in Chicago, USA, c) Dana-Farber Cancer Institute (Boston, USA), and Oxford University (Oxford, UK).

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The findings show for the first time, that it is possible to inhibit the HIF proteins using small-molecules. The findings also show for the first time, that the mechanism of action for inhibiting these proteins is through disruption of their dimer interfaces. Both of these findings are viewed as proof-of-principle that should more widely (other laboratories and pharmaceutical industry) encourage drug-discovery against the HIF-1alpha/ARNT and HIF-2alpha/ARNT complexes, for a variety of unmet human diseases and particularly for cancers.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: Publications, conference papers, and presentations

Journal publications.

1. Wu D, **Rastinejad F**. Structural characterization of mammalian bHLH-PAS transcription factors. *Curr Opin Struct Biol*. 2017 Apr;43:1-9. doi: 10.1016/j.sbi.2016.09.011. Epub 2016 Oct 6. PMID:27721191. Federal support acknowledged.

2. Wu D, Su X, Potluri N, Kim Y, **Rastinejad F**. NPAS1-ARNT and NPAS3-ARNT crystal structures implicate the bHLH-PAS family as multi-ligand binding transcription factors. *Elife*. 2016 Oct 26;5. pii: e18790. doi: 10.7554/eLife.18790. PMID: 27782878 Federal support acknowledged.

(A third manuscript is currently under preparation and expected to be published within next few months: “Bidirectional Modulation of HIF-2 Activity through Chemical Ligands”, by Dalei Wu, Xiaoyu Su, Jingping Lu, Sheng Li, Becky L. Hood, Stefan Vasile, Nalini Potluri, Xiaotong Diao, Youngchang Kim, Sepideh Khorasanizadeh, **Fraydoon Rastinejad).**

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Fraydoon Rastinejad
Project Role: PI
Researcher Identifier: 0000-0002-0784-9352
Nearest person month worked: 2.20
Overall project management (together with co-I), including oversight, interpretation and quality control of all data, generated through all tasks and subtasks, and ensuring alignment of the team's daily activities with specific goals and timelines of the grant application. Contribution to Project: In SA1, Subtask 2: conduct separate AS-MS screens using 32,000 small-molecule library for each of HIF-1α /ARNT and HIF-2α /ARNT purified heterodimers. Subtask 3: Counter-Screen all hits from Subtask 2 against AHR/ARNT heterodimer and eliminate non-specific binding molecules (i.e. small molecules that also show binding to AHR/ARNT). In SA2, Subtask 4: RT-qPCR to evaluate target gene expression in response to inhibitors in cancer cell lines Subtask 4: Evaluate target engagement of compounds in cells using si-RNA.

Name: Sepideh Khorasanizadeh
Project Role: Co-Investigator
Researcher Identifier: 0000-0003-03742-0524
Nearest person month worked: 2.20
Co-management of team's daily activities. Training of scientists and review of data generated related to the biochemical, biophysical, and cell-based studies. Contribution to Project: In SA1, Measure the Kd values for top hits with HIF-1α/ARNT and HIF-2α /ARNT using biochemical binding assays. In SA2, Subtask 2: Cytotoxicity assay. Subtask 3: Co-IP studies to see if molecules disrupt heterodimer stability in cells. Subtask 4: RT-qPCR to evaluate target gene expression in response to inhibitors in cancer cell lines. Subtask 4: Evaluate target engagement of compounds in cells using si-RNA.

Name: Chandra Vikas
Project Role: Staff Scientist
Researcher Identifier: 0000-0002-0768-0673
Nearest person month worked: 0.60
Contribution to Project: Participated in SA1 subtasks 1 and 2, by helping to develop the necessary purification strategy and quality control steps required to generate high-quality proteins for AS-MS screens. He additionally contributed to the SPR assay implementation in measuring the Kd values of ligands for HIF proteins (SA1).

Name: Jingping Lu
Project Role: Postdoctoral Associate
Researcher Identifier: 0000-0001-5620-0819
Nearest person month worked: 1.80
Contribution to Project: Assisted with SA1 subtasks 2 and 3, which involved producing the assay development and implementation related to mass-spectrometry based high-throughput screening and counter-screening.

Name: Nalini Potluri
Project Role: Research Assistant II
Researcher Identifier: 0000-0002-4921-4742
Nearest person month worked: 3.00
Contribution to Project: In SA1, Subtask 1: Produce 15 milligrams of highly purified (>99%) HIF-1α/ARNT and HIF-2/ARNT heterodimers, as well as AHR/ARNT (for counter-screening) to be employed in Affinity-Selection Mass-Spectrometry (AS-MS) Screens and biochemical binding studies. Measure the Kd values for top hits with HIF-1α/ARNT and HIF-2α /ARNT using biochemical binding assays. In SA2, Subtask 1: Dose-response cell-based reporter transcription assays. Subtask 4: RT-qPCR to evaluate target gene expression in response to inhibitors in cancer cell lines. Subtask 4: Evaluate target engagement of compounds in cells using si-RNA.

Name: Xiaoyu Su
Project Role: Research Assistant II
Researcher Identifier: 0000-0002-0509-1007
Nearest person month worked: 0.60
Contribution to Project: Participated in preparing for meeting the goals of SA2 subtasks 1 and 3, by developing the necessary cell-based assay tools, reagents and techniques to initiate and carry out a) dose-reponse cell based reporter transcription assays, b) co-immunoprecipitation studies.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

A new NIH R01 grant was funded on June 1, 2018 to the PI and co-I. This grant, 1 R01 DK118297, is entitled "Identification of hypoxia-inducible factor-2alpha activators for chronic kidney disease anemia. The goal is to identify small-molecules that can specifically enhance *EPO* gene expression through transcriptional activation of HIF-2alpha. There is no direct overlap.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Nothing to report.

QUAD CHARTS: Nothing to report.

9. APPENDICES: Appendix 1 contains Figures 1-4.

APPENDIX (Figures 1-4)

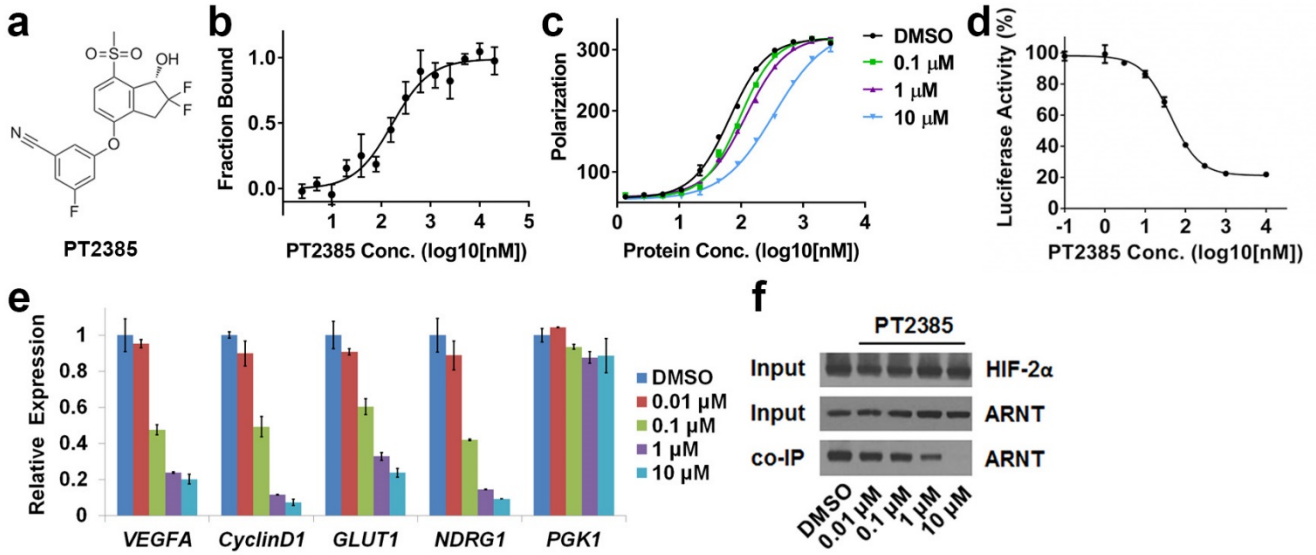

Figure 1 Inhibition of HIF-2alpha by compound PT2385. a, Chemical structure of PT2385. **b**, Binding of PT2385 to the HIF-2alpha PAS-B domain (K_d about 167 nM) as measured by MST. **c,** Reduced HRE DNA binding of the HIF-2alpha/ARNT protein complex by PT2385 as tested by fluorescence polarization. **d**, Dose-dependent inhibition of HRE luciferase reporter activity by PT2385. **e**, Expression of HIF-2 target genes in 786-O cells after treatment with PT2385 at various concentrations. **f**, Co-IP results showing the dose-dependent disrupting effect of PT2385 on the dimerization between overexpressed full-length HIF-2alpha and ARNT proteins.

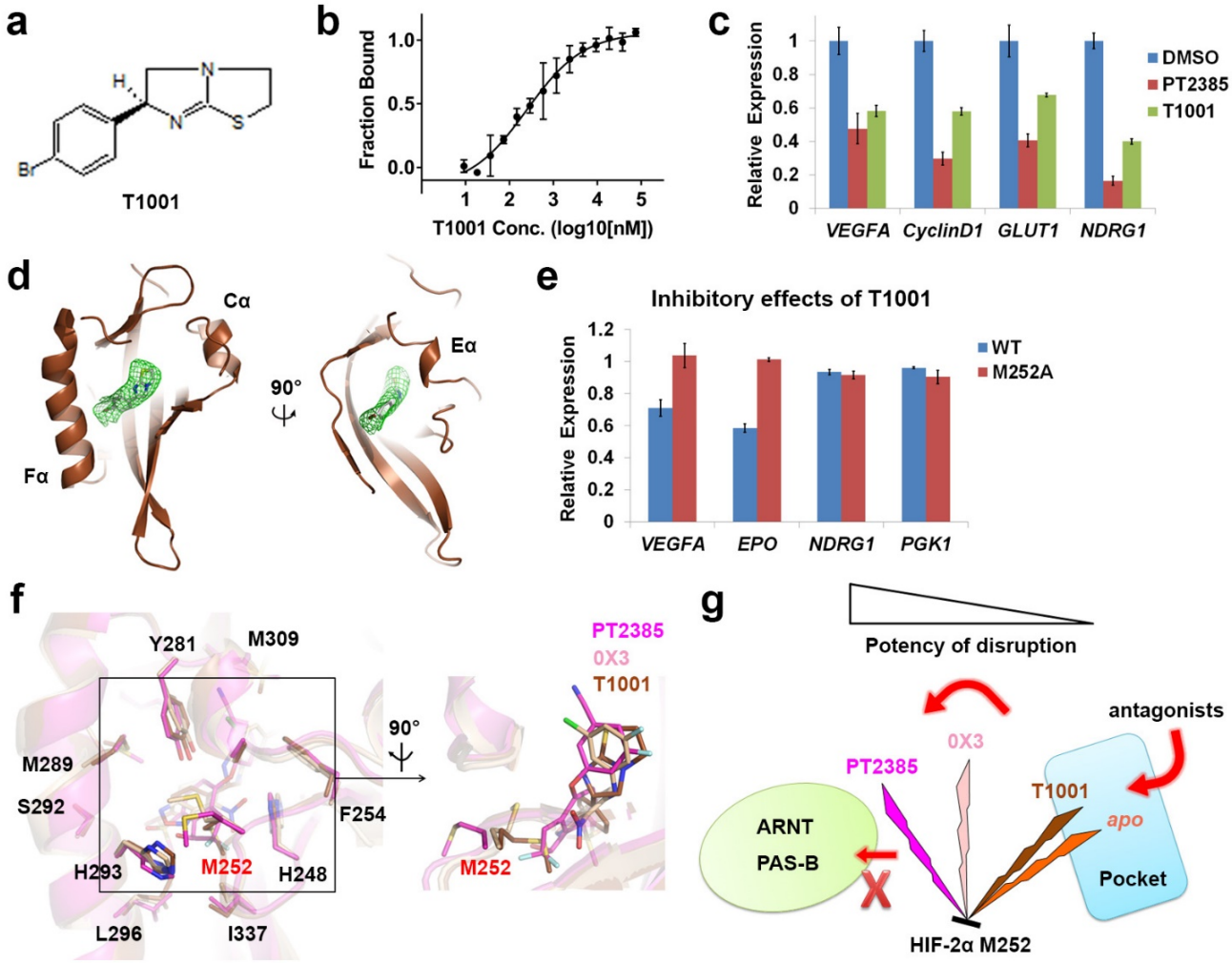

Figure 2 Newly identified antagonist T1001 points to the same mechanism of action that was observed for compound PT2385. a, Chemical structure of T1001. **b**, Binding of T1001 to the HIF-2alpha PAS-B domain (K_d about 246 nM) measured by MST. **c**, Inhibition on the expression of HIF-2alpha target genes in 786-O cells by PT2385 and T1001. **d**, Binding site of T1001 within the HIF-2alpha PAS-B pocket, with green meshes showing the $F_O - F_C$ omit map contoured at 3.0σ. **e**, Comparison of T1001's inhibitory effects on expression of HIF-2alpha target genes in HEK293T cells transfected with wild-type HIF-2alpha or M252A mutant. **f**, Comparison of HIF-2alpha residues surrounding antagonists in the PAS-B structures. The ligands and residues are in magenta, pink and brown, for PT2385, 0X3 and T1001 respectively. **g**, A mechanistic diagram showing how antagonist binding leads to the movement of M252 to disrupt HIF-2alpha/ARNT heterodimers. The proximate positions of M252 side-chain in *apo* (orange) and antagonist-bound structures are compared and related to the disruption potency.

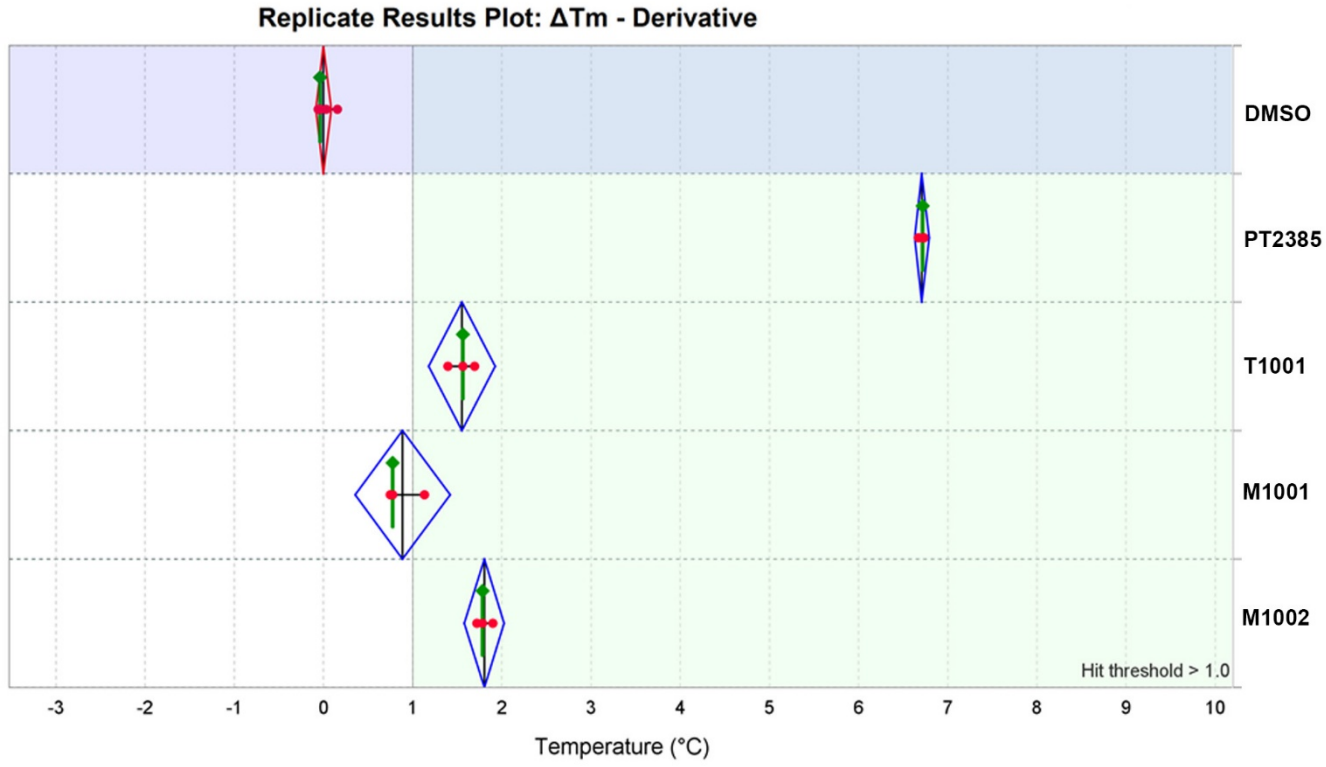

Figure 3. Protein thermal shift assays measuring the changes in T_m values of HIF-2alpha/ARNT complex proteins in the presence of various ligands compared with DMSO. The concentration of protein complex in this assay was about 1.5 μM, and all the antagonists or agonists were tested at 10 μM.

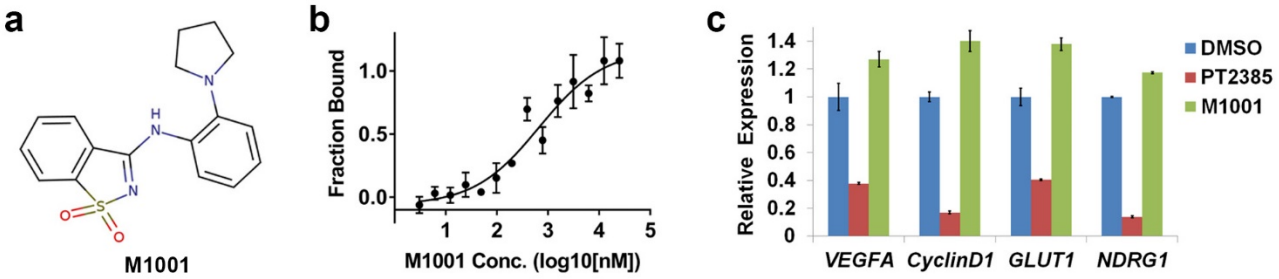

Figure 4 The amechanism of action for compound M1001. a, Chemical structure of M1001. **b**, Binding of M1001 to the HIF-2alpha PAS-B domain (K_d about 667 nM) measured by MST. **c**, Opposite effects on the expression of HIF-2alpha target genes in 786-O cells by PT2385 and M1001.