Structural basis for the allosteric inhibition of hypoxia-inducible factor (HIF)-2 by belzutifan

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**Abbreviations:** HIF, hypoxia-inducible factor; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH-PAS, basic helix-loop-helix-PER-ARNT-SIM; ITC, isothermal titration calorimetry; ccRCC, clear cell renal cell carcinoma; VHL, von Hippel-Lindau; TR-FRET, time-resolved fluorescence energy transfer; FDA, Food and Drug Administration; HRE, hypoxia response element.
Abstract

Hypoxia-inducible factor (HIF)-2α and its obligate heterodimerization partner aryl hydrocarbon receptor nuclear translocator (ARNT), are both members of the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) transcription factor family. Previous studies have identified HIF-2α as a key oncogenic driver in clear cell renal cell carcinoma (ccRCC), rendering it a promising drug target for this type of kidney cancer. Belzutifan is the first HIF-2α inhibitor approved for treating ccRCC and other cancers associated with the von Hippel-Lindau (VHL) disease. However, the detailed inhibitory mechanism of belzutifan at molecular level is still unclear. Here we obtained the crystal structure of HIF-2α-ARNT heterodimer in complex with belzutifan at 2.75 Å resolution. The complex structure shows that belzutifan binds into the PAS-B pocket of HIF-2α, and it destabilizes the dimerization of HIF-2α and ARNT through allosteric effects mainly mediated by the key residue M252 of HIF-2α near the dimer interface. We further explored the inhibitory effects of belzutifan using biochemical and functional assays. The time-resolved fluorescence energy transfer (TR-FRET)-based binding assay showed that belzutifan disrupts the dimerization of HIF-2α and ARNT with a $K_i$ value of 23 nM. The luciferase reporter assay indicated that belzutifan can efficiently inhibit the transcriptional activity of HIF-2α with an IC$_{50}$ value of 17 nM. Besides, the real-time PCR assay illustrated that belzutifan can reduce the expression of HIF-2α downstream genes in 786-O kidney cancer cells in a dose-dependent manner. Our work reveals the molecular mechanism by which belzutifan allosterically inhibits HIF-2α and provides valuable information for the subsequent drug development targeting HIF-2α.

Significance Statement: The bHLH-PAS family of transcription factors are an emerging group of small-molecule drug targets. Belzutifan, originally developed by Peloton Therapeutics, is the first FDA-approved drug directly binding to a bHLH-PAS protein, the
hypoxia-inducible factor (HIF)-2α. Based on the protein-drug complex structure, biochemical binding assays, and functional profiling of downstream gene expression, this study reveals the regulatory mechanism of how belzutifan allosterically destabilizes HIF-2α’s heterodimerization with its obligate partner protein, thus reducing their transcriptional activity that links to tumor progression.
Introduction

Hypoxia-inducible factors (HIFs) are a group of transcription factors that belong to the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) family, each possessing a bHLH domain and two PAS (PAS-A and PAS-B) domains near the protein N-terminus (Wu and Rastinejad, 2017). HIFs can regulate sensing and adaptation of cells to oxygen availability (Wang et al., 1995). Transcriptionally active HIF complexes are heterodimers composed of an oxygen-sensitive α subunit (HIF-α) and a constitutively-expressed β subunit, which is usually called aryl hydrocarbon receptor nuclear translocator (ARNT). In vertebrates, three HIF-α isoforms (HIF-1α, HIF-2α and HIF-3α) encoded by separate genes have been identified (Gu et al., 1998; Tian et al., 1997; Wang et al., 1995). The mechanism of how HIF-α proteins are responsive to cellular oxygen level through the oxygen-dependent hydroxylation and following proteasomal degradation, which is mediated by the von Hippel Lindau (VHL) tumor suppressor via ubiquitination (Huang et al., 1998), has been investigated thoroughly and recognized by the 2019 Nobel Prize in Physiology or Medicine (Zhang et al., 2019a).

HIFs regulate transcription of genes related to cell proliferation (Gordan et al., 2007), angiogenesis (Rankin et al., 2007; Yoshimura et al., 2004), cell migration (Esteban et al., 2006), and resistance to anti-cancer therapies (Zhao et al., 2015), thus they may contribute to tumor survival and serve as potential drug targets. Expression of HIF-2α has been found to correlate with the progression and poor prognosis of several tumors, including head and neck squamous cell carcinoma (Winter et al., 2006), as well as lung (small and non-small cells) (Liu et al., 2006; Luan et al., 2013), colorectal (Yoshimura et al., 2004), liver (Bangoura et al., 2007) and kidney (Xia et al., 2001) cancers. Due to the gene deficiency of VHL, vast majority (about 90%) of patients with clear cell renal cell carcinoma (ccRCC) encounter a stable presence of HIF-α that promotes the survival of cancer cells (Sato et al., 2013). Moreover, it has been found that the HIF-1α isoform is usually missing or mutated in ccRCC, while the
overexpressed HIF-2α functions as a critical driver (Shen and Kaelin, 2013). Therefore, selectively inhibiting the activity of HIF-2α can potentially block the progression of ccRCC.

The identification of HIF-2α inhibitors is a classic structure-based drug discovery process. In 2009, Scheuermann et al. reported the crystal structure of human HIF-2α-ARNT heterodimer containing only the PAS-B domains of both proteins (Scheuermann et al., 2009). This crystal structure revealed a hydrophobic cavity of 290 Å³ within HIF-2α, and enabled a screening campaign for HIF-2α inhibitors targeting the PAS-B cavity (Key et al., 2009). In the following years, they reported more artificial HIF-2α ligands that antagonized the heterodimerization of HIF-2α-ARNT and displayed improved cellular activities (Rogers et al., 2013; Scheuermann et al., 2013; Scheuermann et al., 2015). One of those ligands, N-(3-chloro-5-fluorophenyl)-4-nitro-2,1,3-benzoxadiazol-5-amine (also called 0X3), was used as a starting point for the further design of HIF-2α inhibitors. This work was led by scientists at Peloton Therapeutics who in 2018 disclosed the discovery of PT2385 (Figure 1a), the first HIF-2α small-molecule inhibitor entering clinical trials for the treatment of ccRCC (Wehn et al., 2018). However, in the trial a large proportion of patients were underexposed with little clinical benefit due to the unexpected strong metabolism of PT2385 to its glucuronide metabolite. In 2019, they reported belzutifan (also called PT2977 or MK-6482, Figure 1b), a new HIF-2α inhibitor with improved pharmacokinetic properties by changing the geminal difluoro group of PT2385 to a vicinal difluoro group (Xu et al., 2019). In the same year, Merck Sharp & Dohme (MSD) Corp. acquired Peloton Therapeutics. In August 2021, U.S. Food and Drug Administration (FDA) approved belzutifan for adult patients with VHL disease who require a therapy for associated renal cell carcinoma, central nervous system hemangioblastomas, or pancreatic neuroendocrine tumors, not requiring immediate surgery.

We previously solved the crystal structure of multi-domain HIF-2α-ARNT heterodimer in complex with PT2385 (Wu et al., 2019). However, the structure of HIF-2α-ARNT bound
with belzutifan is still not available, limiting further understanding of its detailed mechanism of action. Here, we report the crystal structure of HIF-2α-ARNT-belzutifan complex. Our results indicate that belzutifan bound into the PAS-B domain pocket of HIF-2α, and disrupted the dimerization of HIF-2α and ARNT through allosteric effects initiated largely by the side-chain conformational change of HIF-2α residue M252. We further determined the inhibitory effects of belzutifan against HIF-2α at both molecular and cellular levels. This work reveals the mechanism of how belzutifan inhibits the transcriptional activity of HIF-2α and provides valuable information for the future design of HIF-2α-targeting drugs.

Materials and methods

Compounds and other materials

Belzutifan and PT2385 were purchased from MedChemExpress (MCE). Tacsimate, PEG3350 and other crystallization reagents were from Sigma-Aldrich. β-D-thiogalactopyranoside and dithiothreitol were from Dingguo Biotechnology. All other reagents and solvents are also commercially available and at analytical grade.

Plasmid construction, protein expression and purification

Recombinant expression plasmids containing the N-terminal bHLH-PAS-A-PAS-B regions of mouse HIF-2α and ARNT proteins, wild-type (WT) pSJ2-HIF-2α (3-361), pSJ2-HIF-2α (3-361) M252A mutant and pMKH-ARNT (82-464), as well as the pSJ2-HIF-2α (241-361) for the single PAS-B domain of HIF-2α used in the binding assay, pMKH-ARNT-GFP (82-464) for GFP-tagged ARNT used in the time-resolved fluorescence energy transfer (TR-FRET) assay, and pCMV-Tag4-HIF-2α full-length plasmids (WT or M252A mutant) used in the real-time PCR assay, were all constructed as described previously (Wu et al., 2019).

Proteins were expressed and purified in a similar manner as previously described (Wu et al., 2019). Briefly, to obtain HIF-2α-ARNT complex proteins, the pSJ2-HIF-2α plasmid and
pMKH-ARNT plasmid were co-transformed into *E. coli* BL21 CodonPlus. Following 0.1 mM β-D-thiogalactopyranoside induction overnight at 16°C, cells were lysed by sonication. Supernatants were purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity resin (GE Healthcare), SP affinity resin (GE Healthcare), followed by size exclusion chromatography using a 16/60 Superdex 200 pg gel-filtration column (GE Healthcare). The heterodimeric proteins of HIF-2α and ARNT-GFP were prepared similarly as described above, except that the pMKH-ARNT-GFP plasmid was used in the place of pMKH-ARNT. Purification of the single PAS-B domain of HIF-2α was conducted similarly, except for the skipped step of cation exchange chromatography.

*Isothermal titration calorimetry (ITC) binding assay*

ITC experiments were performed using a PEAQ-ITC (Malvern) instrument at 25°C. PT2385, belzutifan and the HIF-2α PAS-B proteins were respectively dissolved in a buffer containing 20 mM Tris-HCl (pH 8.0), 400 mM NaCl and 2% DMSO. Then, 40 μL of 100 μM PT2385 or belzutifan was continuously added to titrate the 10 μM HIF-2α protein. The first injection was 0.4 μL, and the twelve subsequent injections were 3.0 μL each. The time interval between two drops was 150 s. The site-binding model was determined by using MicroCal PEAQ-ITC Analysis Software, and binding isotherm data were fitted by the GraphPad Prism software.

*Crystallization, data collection and structure determination*

HIF-2α-ARNT protein complexes were crystallized by mixing equal volume of protein and the reservoir containing Tacsimate (pH 7.0) and PEG3350 using the sitting-drop vapor diffusion method at 16 °C. After a series of trials on soaking conditions, crystals of HIF-2α-ARNT-belzutifan complex were finally obtained by adding compounds (100 μM) to the drops containing *apo* protein crystals and soaking for 8 hours before crystal harvest. Diffraction data were collected at Shanghai Synchrotron Radiation Facility 19U beamline (Zhang et al., 8
2019b), and processed using the XDS program (Kabsch, 2010).

The structure of HIF-2α-ARNT-belzutifan complex was solved by molecular replacement with the program Phaser (McCoy et al., 2007), using the apo HIF-2α-ARNT structure (PDB code 4ZP4) as the search model. Further manual model building was facilitated by using Coot (Emsley et al., 2010), combined with the structure refinement using Phenix (Adams et al., 2010). The final model was validated by MolProbity (Chen et al., 2010). A summary of diffraction data and final statistics can be found in Table 1. Molecular graphics for figures were prepared with PyMOL (The PyMOL Molecular Graphics System, Version 2.3.0, Schrödinger, LLC).

**TR-FRET-based binding assay**

Similar to our previous work, purified protein complexes of GFP-tagged ARNT and HIF-2α were dispensed into 384-well plates, with serial dilutions of belzutifan, in the assay buffer containing 20 mM HEPES (pH 7.5), 400 mM NaCl, 10 mM dithiothreitol and 0.5% Tween-20. After addition of the Terbium-labeled anti-His antibody (Cisbio, 61HI2TLF) into each well, the plate was kept in dark for 3 h. The protein interactions were monitored via the energy transfer signal with a Spark multimode microplate reader (Tecan). The TR-FRET value was determined as a ratio of the signal measured at 520 nm (GFP) to the signal measured at 340 nm (terbium). The apparent $K_i$ value of belzutifan towards HIF-2α-ARNT complex was calculated by plotting the ratios (520 nm/340 nm) against the compound concentrations (Wu et al., 2019).

**Luciferase reporter assay**

786-O cells with a stable-transfected hypoxia response element (HRE)-luc reporter were kindly provided by Prof. Fraydoon Rastinejad at University of Oxford. The cells were seeded in RPMI1640 medium with 10% FBS in 48-well plates, and treated with belzutifan at different concentrations (final DMSO concentration kept at 0.1%) when the cell density was
close to 60%. After 24 h incubation, cells were lysed and analyzed using Steady-Glo Luciferase Assay System (Promega, E2510).

Real-time PCR assay

786-O cells (Dingguo Biotechnology, CS0254) were cultured in RPMI1640 medium with 10% FBS in 12-well plates at 37°C in 5% CO₂. When cells grew to 60% density, different concentrations of belzutifan were added with a final DMSO concentration kept at 0.1%. After 24 h treatment, cells were collected and RNA was extracted using RNAiso Plus (Takara), followed by cDNA synthesis using PrimeScript™ RT reagent Kit with gDNA Erase (Takara).

Hep3B cells (Dingguo Biotechnology, CS0172) were cultured in DMEM medium with 10% FBS in 12-well plates at 37°C in 5% CO₂. When cells grew to 60% density, they were transfected with pCMV-Tag4-HIF-2α plasmids (WT or M252A mutant). Medium was refreshed after 6 h transfection, and cells were treated with 10 μM belzutifan in 0.1% DMSO for 36 h in 5% CO₂ before RNA isolation.

HEK293 cells (Dingguo Biotechnology, CS0001) were cultured in DMEM medium with 10% FBS in 12-well plates at 37°C in 5% CO₂. When cells grew to 60% density, different concentrations of belzutifan were added with the final DMSO concentration kept at 0.1%. Cells were cultured for 24 h in 1% O₂ before RNA isolation.

Real-time PCR was conducted on a step-two system using Hieff qPCR SYBR Green Master Mix (Yeasen). The expression levels of target genes were normalized to the expression of β-actin (ACTB). PCR primers were synthesized by Personalbio Technology as follows: ACTB (F: GCACAGAGCCCTCGCCTT, R: GTTGTCGACGAGCGGCG); CyclinD1 (F: TGGAGCCCGTGAAAAAGAGC, R: TCTCCTTCATCTTAGGCCAC); NDRG1 (F: CTCCGTCAAGAGTTTGATGTCC, R: TCATGCCGATGTCATGGTAGG); EPO (F: GGAGGCCGAGAATATACGAC, R: CCCTGCCAGACTTCTACCG); VEGFA (F:
TACCTCCACCATGCCAAGTG, R: ATGATTCTGCCCTCCTCCTTC); and GLUT1 (F: TCTGGCATCAACGCTGTCTTC, R: CGATACCGGAGCCAATGGT).

Data analysis

Data are expressed as the mean ± standard deviation of the mean (SD). $K_i$ and $IC_{50}$ values were derived from a four-parameter logistic equation.

Results

Binding affinity of belzutifan to HIF-2α protein

As mentioned earlier, belzutifan is the second-generation HIF-2α inhibitor with increased potency and improved pharmacokinetic profile achieved by reduction of Phase 2 metabolism (Xu et al., 2019). To test whether belzutifan maintains a similar binding affinity to its target HIF-2α in comparison with the first-generation inhibitor PT2385, we measured the binding affinities of belzutifan and PT2385 to HIF-2α using an ITC assay with purified proteins of the single PAS-B domain. The calculated $K_D$ value of PT2385 was $10 \pm 4.9$ nM (Figure 1c), while the $K_D$ value of belzutifan was $16 \pm 4.7$ nM (Figure 1d), suggesting no big difference in binding affinities between the two inhibitors. Our results indicate that the structural modification of changing the geminal difluoro group in PT2385 to a vicinal difluoro group in belzutifan had no dramatic influence on their binding affinity to HIF-2α protein. Therefore, the better potency of belzutifan in the clinical trial might largely come from its in vivo stability and resistance to drug metabolism.

Crystal structure of HIF-2α-ARNT-belzutifan complex

To further explore the inhibitory mechanism of belzutifan at the molecular level, we sought to first reveal the structural information about how belzutifan binds into HIF-2α. For crystallization, we co-expressed and purified mouse HIF-2α and ARNT proteins that both contain their N-terminal bHLH and tandem PAS domains (Figure 2a), in a heterodimeric form. It is noteworthy that the originally reported crystal structure of the HIF-2α-ARNT
protein complex only contains two PAS-B domains, which formed a stable dimer with the help from an artificial salt bridge generated by point-mutations (Scheuermann et al., 2009). However, since this artificial salt bridge across the dimer interface introduced additional interactions to further enhance the dimerization, it is hard to interpret how inhibitors binding into the PAS-B domain of HIF-2α exert their dimer-disrupting effects. In contrast, our multi-domain dimeric protein complex was stabilized by native inter-domain interactions, which can likely represent their physiological status in cells and more importantly help delineate the potential allosteric effects of HIF-2α inhibitors (Wu et al., 2015).

Directly through co-crystallization, we were unable to obtain belzutifan-bound HIF-2α-ARNT protein crystals in a good quality, probably due to the strong destabilizing effects of belzutifan (Xu et al., 2019). Therefore, instead we soaked belzutifan into the apo HIF-2α-ARNT protein crystals by a series of trials on the proper drug concentrations as well as duration time, and successfully obtained diffractable crystals. We then solved the structure of HIF-2α-ARNT dimer in complex with belzutifan at 2.75 Å resolution. As shown in Figure 2b, belzutifan binds into the PAS-B domain pocket of HIF-2α, evidenced by the clear electron density maps within the pocket (Figure 2c and 2d). Belzutifan shares a hydrogen bond with the side chain of residue H293, thus stabilizing the binding pose of this drug (Figure 2e). In addition, belzutifan forms a π-π interaction with F254 and numerous hydrophobic interactions with neighboring residues (Figure 2e).

The binding mode of belzutifan is roughly very similar to that of PT2385, which also binds into the PAS-B pocket of HIF-2α and forms a hydrogen bond with H293 (Figure 2f) (Wu et al., 2019). This mode similarity between belzutifan and PT2385 (Figure 2g&h), correlates well with their comparable binding affinities to HIF-2α (Figure 1c&d).

Conformational changes of HIF-2α-ARNT upon belzutifan binding

To reveal the detailed mechanism of how belzutifan disrupts the dimerization of HIF-2α-
ARNT, we first checked the protein conformational changes upon belzutifan binding. When superimposing the belzutifan-bound HIF-2α-ARNT structure onto the unbound “apo” structure (PDB code 4ZP4), we found a clear conformational change near the dimerization interface between HIF-2α and ARNT PAS-B domains (Figure 3a, b). Binding of belzutifan forced the side chain of HIF-2α M252 residue to flip out of the pocket, potentially destroying the stability of the dimerization interface between HIF-2α PAS-B domain and ARNT PAS-B domain. As a result, the loop regions on both sides of the interface also exhibited shifts in their conformation and position (Figure 3b).

Previously we adopted a hydrogen-deuterium exchange mass spectrometry assay, to reveal the overall conformational changes of multi-domain HIF-2α-ARNT protein complex caused by HIF-2α ligands (Wu et al., 2019). In addition to the dimer interface between two PAS-B domains, binding of PT2385 (and another inhibitor named T1001) allosterically induced destabilizing effects at other domain-domain junctions along the interface, as evidenced by the increased H/D exchange rates (Wu et al., 2019). Here since belzutifan binds into HIF-2α in a nearly identical fashion to PT2385 (Figures 2h), it would probably share a similar inhibitory mechanism of reducing the HIF-2 transcriptional activity by allosterically disrupting heterodimerization.

In addition, we also highlighted the key role of HIF-2α M252 residue in mediating the allosteric mechanism in our previous study (Wu et al., 2019). The M252A mutation could abolish inhibitory effects of PT2385 on both HIF-2α-ARNT heterodimerization and the transcriptional activity of downstream genes. Moreover, by comparing various inhibitors we noticed that the movement extent of M252’s side-chain from inside of HIF-2α PAS-B pocket towards the dimer interface, positively correlates with the potency of different inhibitors (Wu et al., 2019). As shown in Figure 3c, in the crystal structure of HIF-2α-ARNT-belzutifan complex, M252 was pushed out of the pocket by belzutifan to a similar position as that by
PT2385, suggesting these two compounds may possess close inhibitory activities in biochemical and cellular systems.

*Determinations of the inhibitory effects on HIF-2α by belzutifan*

To further confirm the allosteric and inhibitory effects of belzutifan on HIF-2α, we conducted both biochemical and functional assays. First, we adopted a TR-FRET-based direct-binding assay, in which interactions between HIF-2α and ARNT could be detected by the energy transfer between donor and acceptor molecules respectively coupled to the two subunits. As shown in Figure 4a and 4b, belzutifan disrupted the dimerization of wild-type HIF-2α and ARNT with a $K_i$ value of approximately 23 nM, while its $K_i$ value for HIF-2α M252A mutant was dramatically increased to about 3.5 μM, supporting the idea that M252 plays a key role in mediating the allosteric regulation of belzutifan on dimerization. It has to be pointed out that the maximum heterodimer disruption ratio was only around 50% in this assay, indicating the disassociation of dimerized HIF-2α-ARNT proteins was not complete even at a very high concentration of belzutifan. These results suggest that in the *in vitro* systems using co-expressed recombinant HIF-2α-ARNT proteins, HIF-2α inhibitors can only partially separate the two subunits, possibly due to the biochemical nature of these dimeric proteins, consistent with our previous hydrogen-deuterium exchange assay showing that inhibitors mainly destabilized HIF-2α-ARNT at the dimer interface regions rather than globally (Wu et al., 2019).

Next, we explored the inhibitory effects of belzutifan on HIF-2α activity in cellular level. HRE (5'-ACGTG-3'), a 5-base pair short motif that often locates in the promoter or enhancer regions of HIF downstream genes, can be recognized by HIF dimers to induce gene expression in response to hypoxia. We used a ccRCC 786-O cell line that was stably transfected with an HRE luciferase reporter to determine the transcriptional activity of HIF-2α. Belzutifan was found to inhibit the HIF-2α activity in a dose-dependent manner, with an
IC$_{50}$ value of 17 nM (Figure 4c), slightly lower than the IC$_{50}$ value of PT2385 (42 nM) measured with an identical assay previously (Wu et al., 2019).

We then determined the inhibitory effects of belzutifan on the mRNA transcription of HIF-2α target genes by a quantitative PCR assay. The overexpression of HIF-2α in the 786-O cell line due to $VHL$ deficiency causes an increased expression of HIF downstream genes, thus improving the survival ability of cancer cells. Belzutifan inhibited the transcription of HIF-2α downstream genes ($CyclinD1$, $NDRG1$, $VEGFA$ and $GLUT1$) in 786-O cells in a dose-dependent manner (Figure 4d). Besides, belzutifan could also inhibit the transcription of HIF-2α downstream genes in the HEK293 cell line, which is a non-cancer cell line (Figure 4e). It is noteworthy that the inhibitory activity of belzutifan in HEK293 cells was weaker than that in 786-O cells, which may be due to the varied responses of different cell lines to HIF-2α signal. To further reveal the key effect of M252 in regulating the interactions between HIF-2α and ARNT, we transfected the full-length HIF-2α WT or M252A mutant plasmids into Hep3B cells and measured the mRNA expression levels of HIF-2α downstream genes. As shown in Figure 4f, the cells transfected with HIF-2α mutant M252A barely showed inhibitory effects by belzutifan, in contrast to the cells transfected with WT HIF-2α.

These above structural, biochemical and functional assays together indicate that belzutifan reduces the transcriptional activity of HIF-2α by allosterically disrupting its dimerization with ARNT, to an extent similar to PT2385 in vitro. Therefore, the efficacy improvement from PT2385 to belzutifan in clinical trials mainly come from the increased resistance to drug metabolism by the chemical modification.

**Discussion**

In this work, we solved the co-crystal structure of HIF-2α-ARNT-belzutifan and revealed the inhibitory mechanism of belzutifan at molecular level. During the crystallization experiments, we found it very challenging to obtain co-crystals of HIF-2α-ARNT-belzutifan.
Routine co-crystallization trials for HIF-2α-ARNT proteins and belzutifan were not successful, and even soaking of belzutifan for a long time would cause the dissolution of preformed protein crystals, which also indicates that the destabilizing effects of belzutifan on the HIF-2α-ARNT complex was fairly strong. Therefore, we had to rely on a proper soaking condition to obtain diffractable crystals, in which likely only limited drug-induced protein conformational changes were tolerated and exhibited. However, we still believe these conformational changes shown in crystal structures (especially those near the dimerization interface) reflect the possible changes in solution, as our previous HDX-MS results correlated with the crystal structures very well (Wu et al., 2019).

Belzutifan is the first HIF-2α small-molecule inhibitor approved by FDA to treat the VHL disease under certain conditions. In addition, several other indications have also been investigated for the application of belzutifan. For instance, the efficacy and safety of belzutifan monotherapy in participants with advanced pheochromocytoma/paraganglioma (PPGL) or pancreatic neuroendocrine tumor (pNET) are undergoing a phase II study (NCT04924075). The safety and efficacy of belzutifan in combination with pembrolizumab and lenvatinib in multiple solid tumors including hepatocellular carcinoma, colorectal cancer, pancreatic ductal adenocarcinoma and biliary tract cancer are currently in a phase II clinical trial (NCT04976634). The New England Journal of Medicine reported a case of treating Pacak-Zhuang Syndrome with belzutifan recently (Kamihara et al., 2021). The genomic testing revealed an EPAS1 (encoding HIF2α) variant, c.1589C→A (p.A530E) in the patients, which led to the decreased HIF-2α protein degradation and enhanced hypoxia-related genes expression. Treatment with belzutifan effectively decreased plasma levels of normetanephrine and chromogranin A, improved the polycythemia, and shrank tumor rapidly. The patients experienced minimal side effects, and no grade 3 or 4 adverse events occurred during 24 months of belzutifan treatment without any interruption or dose modification (Kamihara et al.,
Drug resistance is always a great challenge in the treatment of cancers. A preclinical study in ccRCC tumorgraft mice has showed that prolonged treatment with PT2399, an analog of belzutifan, caused resistance of tumor cells with two leading mutations (G323E of HIF-2α and F446L of ARNT) (Chen et al., 2016). Furthermore, HIF-2α G323E mutation was also identified in ccRCC patients treated with PT2385 (Courtney et al., 2020). Biochemical experiments and in vivo studies revealed that G323E mutation, with a much bulkier side chain, could effectively prevent HIF-2α-ARNT dissociation by blocking the entry of PT2385 into the HIF-2α PAS-B pocket (Courtney et al., 2020; Wu et al., 2019). As a novel pharmacologic agent targeting HIF-2α, belzutifan exhibited a good activity against renal cell carcinoma (RCC) in clinical trials. Although some of the patients treated with belzutifan had progressive disease, the mechanism of possible drug resistance is still unknown (Jonasch et al., 2021). Belzutifan and its analogs are a series of indolols which have great similarities in structure (Xu et al., 2019). Our crystal structure suggests that belzutifan and PT2385 share a similar binding mode and inhibitory mechanism. Therefore, belzutifan and its upcoming analogs may also be affected by HIF-2α G323E and ARNT F446L mutations. Future research may focus on the discovery of HIF-2α inhibitors with novel chemical structures and ideally targeting different binding pockets of HIF-2α.

In addition to tumors, recent studies have revealed that HIF-2α is associated with a series of metabolic diseases. Xie et al reported that intestinal HIF-2α activation contributes to the progression of nonalcoholic fatty liver disease (NAFLD) through a HIF-2α-neuraminidase 3-ceramide axis. Furthermore, PT2385 could effectively improve metabolic disorders in high-fat diet-fed mice, suggesting intestinal HIF-2α may serve as a potential target for the treatment of hepatic steatosis (Xie et al., 2017). In addition, hepatocyte HIF-2α activation also contributes to the NAFLD progression through up-regulation of histidine-rich glycoprotein...
production (Morello et al., 2018). Wu et al reported that intestinal HIF-2α deficiency could reshape the gut microbiome to enhance white adipose tissue thermogenesis through adipose G-protein-coupled bile acid receptor activation, suggesting a new strategy to treat metabolic diseases by HIF-2α inhibition (Wu et al., 2021). These studies imply that HIF-2α inhibitors might play a pivotal role in the treatment of metabolic diseases besides their well-known anti-tumor activity.
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Author contributions

Participated in research design: Wu, Zhuang, Ren

Conducted experiments: Ren, Diao

Performed data analysis: Ren, Diao

Wrote or contributed to writing the paper: Wu, Zhuang
References


Footnotes

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Figure legends

**Figure. 1** Comparison of the HIF-2α-binding affinities between PT2385 and belzutifan. a-b, chemical structures of PT2385 (a) and belzutifan (b). c-d, Binding of PT2385 (c) and belzutifan (d) to the HIF-2α PAS-B domain as measured by ITC. The calculated $K_D$ value of PT2385 was $10 \pm 4.9$ nM, while the $K_D$ value of belzutifan was $16 \pm 4.7$ nM. This experiment was independently repeated twice.

**Figure. 2** Crystal structure of the HIF-2α-ARNT-belzutifan complex. a, Schematic representation showing the domain arrangements of HIF-2α and ARNT. b, Binding position for belzutifan (colored in brown) within the entire HIF-2α–ARNT crystal structure. c, $F_o$–$F_c$ omit map of belzutifan contoured at 3σ. d, $2F_o$–$F_c$ map of belzutifan contoured at 1σ. e-g, Structures of HIF-2α PAS-B domains in complex with belzutifan (e), PT2385 (f) or in apo state (g). h, Comparison of key residue side-chain conformations in the HIF-2α PAS-B domains among apo, belzutifan-bound and PT2385-bound proteins.

**Figure. 3** Allosteric effects of belzutifan revealed by crystal structures. a, The overall arrangement of ARNT (green) and HIF-2α (cyan) PAS-B domains, with the location of dimer interface affected by belzutifan highlighted by an ellipse. b, Conformational differences between belzutifan-bound and apo (PDB ID: 4ZP4, yellow) HIF-2α-ARNT complexes. c, Position comparison of M252 side chain in belzutifan-bound and PT2385-bound (PDB ID: 6E3S, blue) HIF-2α-ARNT complexes.

**Figure 4.** Inhibition of HIF-2α by belzutifan. a-b, Disruption of HIF-2α-ARNT dimers containing WT HIF-2α (a) or M252A mutant (b) by belzutifan measured using TR-FRET. Error bars, mean ± SD; n = 3. The $K_i$ values were derived from a four-parameter logistic equation. $K_i = 23 \pm 1.5$ nM, slope factor $= 1.3 \pm 0.1$ (a); $K_i = 3.5 \pm 0.2$ μM, slope factor $= 1.0 \pm 0.1$ (b). c, Dose-dependent inhibition of HRE luciferase reporter activity by belzutifan. Error bars, mean ± SD; n = 3. The IC$_{50}$ value was derived from a four-parameter logistic equation, IC$_{50} = 17 \pm 1.6$ nM, slope factor $= -1.4 \pm 0.1$. d-e,
Expression of certain HIF-2α target genes in 786-O (d) or HEK293 (e) cells after treatment by belzutifan at various concentrations (0.01, 0.1, 1 or 10 μM). Error bars, mean ± SD; n = 3 (distinct replicates for cell cultures). f, Comparison of the inhibitory effects of 10 μM belzutifan on HIF-2α target genes in Hep3B cells transfected with WT HIF-2α or M252A mutant. Error bars, mean ± SD; n = 3 (distinct replicates for cell cultures).
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Figure 1

(a) PT2385

(b) Belzutifan

(c) Graph showing the change in DP (μcal/s) over time (min).

(d) Graph showing the change in DP (μcal/s) over time (min).

(e) Graph showing the change in ΔH (kcal/mol) with molar ratio.

(f) Graph showing the change in ΔH (kcal/mol) with molar ratio.