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# Discovery of novel anti-angiogenesis agents. Part 11: Development of PROTACs based on active molecules with potency of promoting vascular normalization

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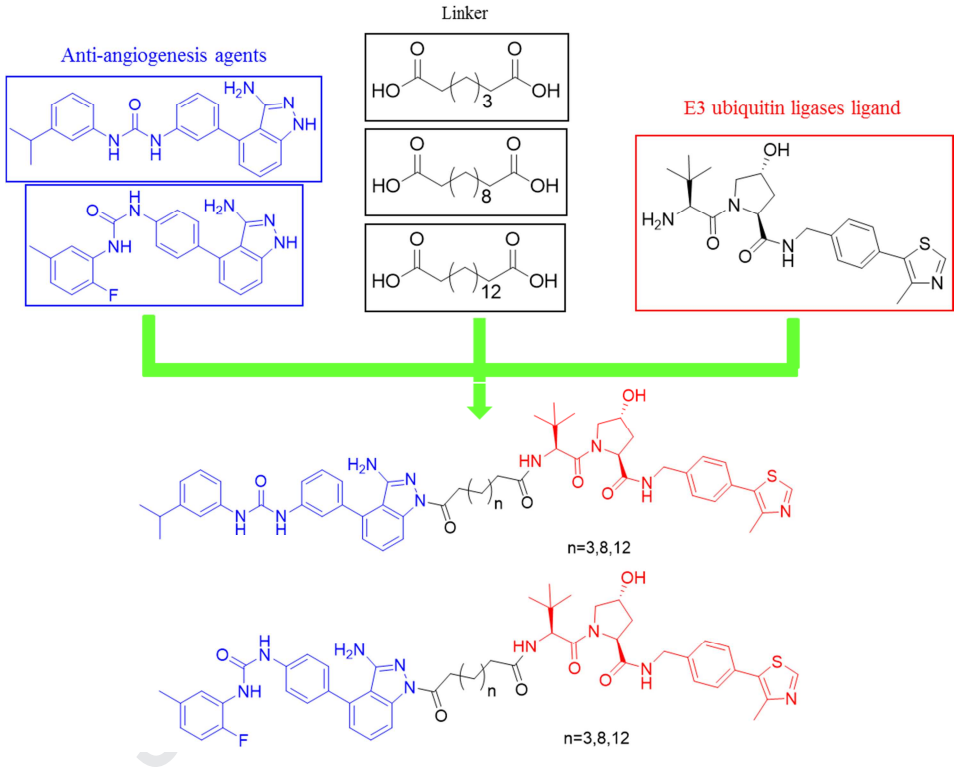


As a continuation to our previous research, a series of novel PROTACs were developed based on the potent anti-angiogenesis agents.

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# Discovery of novel anti-angiogenesis agents. Part 11: Development of PROTACs based on active molecules with potency of promoting vascular normalization

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ABSTRACT

Vascular normalization induced by anti-angiogenic agent appears to be a promising strategy. Our recent investigation is focused on the discovery of anti-angiogenesis agents with potency of promoting vascular normalization. Herein, we reported the design, synthesis and preliminary evaluation of proteolysis-targeting chimera (PROTACs) based on the previously developed anti-angiogenesis agents. Two PROTACs exhibited potent VEGFR-2 inhibition and anti-proliferative activity against HUVECs. Moreover, they were capable of reducing protein levels of VEGFR-2 in EA.hy926 cells without significant cytotoxicity. These novel PROTACs could be used to normalize the abnormal vessels, resulting in efficient delivery of drugs.



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## 1. Introduction



Pathological angiogenesis plays a critical role in numerous diseases and has been recognized as a defining characteristic of cancers. Vasculature abnormal is inadequate for delivery of drugs or oxygen and is becoming an urgent problem in clinic [1]. Vascular normalization induced by anti-angiogenesis agents appears to be a promising strategy and has been proposed as an emerging paradigm in the intervention for pathological angiogenesis [2].

Our recent study is focused on the discovery of novel anti-angiogenesis agents. We have identified biphenyl-aryl ureas incorporated with salicyladoxime as angiogenesis inhibitors. Subsequently, we developed diaryl ureas incorporated with aromatic-heterocyclics as next generation angiogenesis inhibitors *via* structure-guided core-refining approach. Biological evaluations identified a novel anti-angiogenesis agent, **S7** (**Figure 1**) [3-7]. It displayed selective inhibitory activities against angiogenesis-related receptor tyrosine kinase (RTK) and proliferation of human umbilical vein endothelial cells (HUVECs). Moreover, **S7** exhibited vascular normalization potency in transgenic zebrafish model. In summary, we developed a novel anti-angiogenesis agent with potency of promoting vascular normalization.

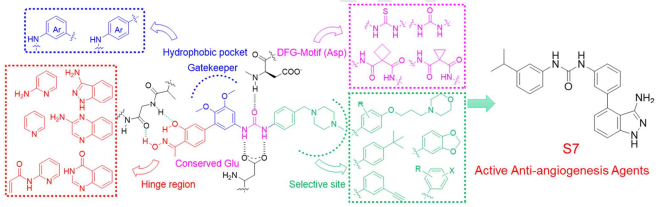



***Fig. 1.*** *Discovery of active anti-angiogenesis agent with vascular normalization potential.*

As well known, traditional occupancy-based RTK inhibitors are designed to specifically activate or inactivate protein functions. They have inherent limitations owing to high concentration needed to ensure sufficient inhibition and potential off-target side effects [8]. Moreover, resistance to RTK inhibitors is responsible for the limited benefit in clinic. As an alternative, induced protein degradation might overcome these limitations and offers a novel catalytic mechanism to irreversibly inhibit protein function by targeting protein destruction [9]. The targeted degradation of proteins via the ubiquitin-proteasome system (UPS) is an emerging modality in small molecule drug discovery. Recent reports of proteolysis targeting chimeras (PROTACs) have demonstrated that this technology can effectively decrease the cellular levels of target protein [10]. PROTACs are bifunctional molecules composed of target protein ligand and E3 ubiquitin ligase ligand *via* various linkers (**Figure 2**). These hetero-bifunctional molecules could recruit an E3 ubiquitin ligase to a given substrate protein resulting in ubiquitination and degradation [11].

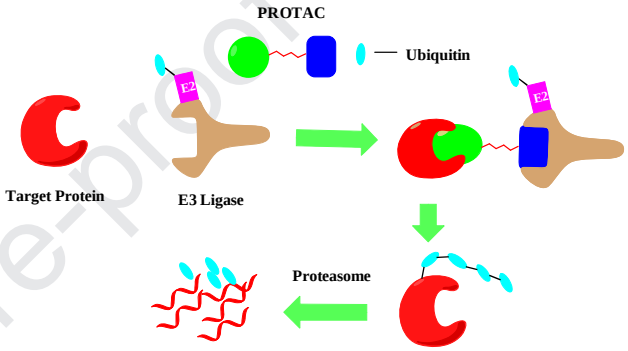



***Fig. 2.*** *The design strategy of novel PROTAC based on active anti-angiogenesis agent.*

Our findings suggested that anti-angiogenesis agents were potential candidates for treatment of vasculature abnormal. Emerging PROTACs technique converts target inhibitors into advantageous target degraders. With the aims to develop novel PROTACs for vasculature abnormal [12]. We designed and synthesized six PROTACs based on our previously developed anti-angiogenesis agents as well as the potent VEGFR-2 inhibitor, ABT-869. The pharmacophores and scaffolds of two inhibitors are similar with each other. The novel PROTACs are composed of anti-angiogenesis agents and E3 ubiquitin ligases ligand (VH 032) with different linkers. As the linker in a PROTAC plays a key role for its degradation potency and efficacy, we have performed extensive optimization of the linker (**Figure 3**).

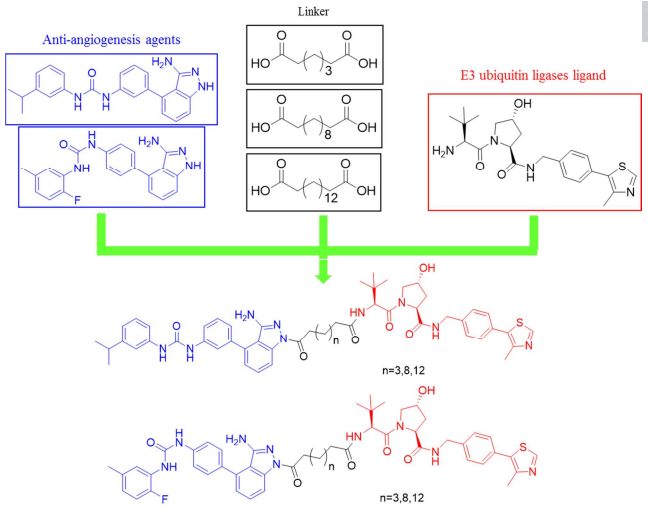


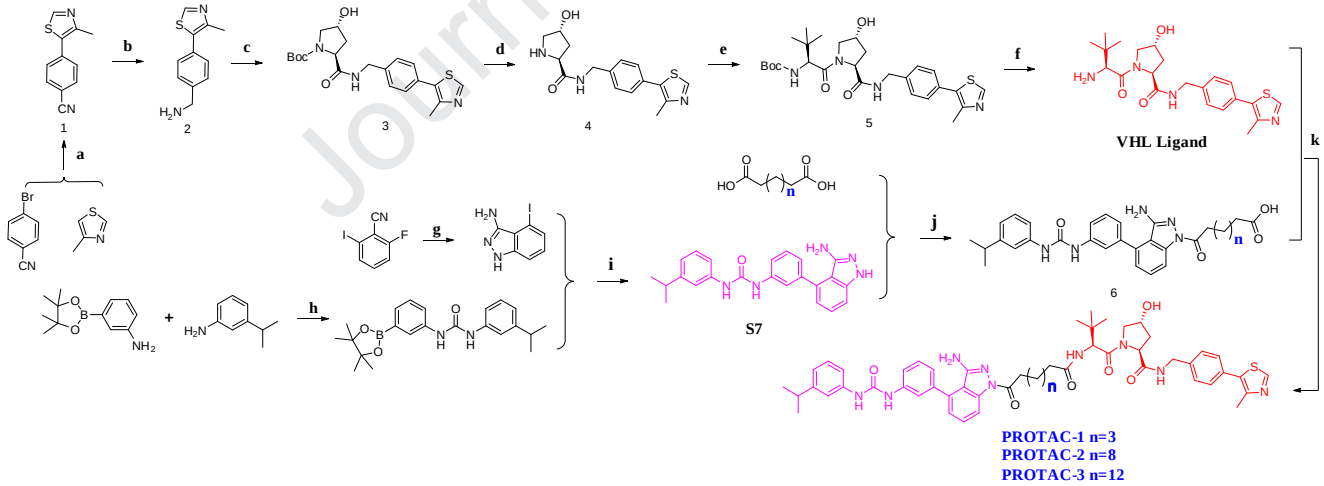

***Fig. 3.*** *The structures of novel PROTAC based on potent anti-angiogenesis inhibitors.*

Herein, we performed the design, synthesis, and biological evaluation of novel PROTACs based on new angiogenesis inhibitors. Among them, PROTAC-2 and PROTAC-5 displayed potent inhibition against VEGFR-2 and anti-proliferative activities against human umbilical vein endothelial cells (EA.hy926). They were capable of specifically reducing protein levels of VEGFR-2. Moreover, the new PROTACs exhibited no significant cytotoxicity. These PROTACs could be used to normalize the abnormal vessels, resulting in efficient delivery of drugs and oxygen.

## 2. Results and discussion

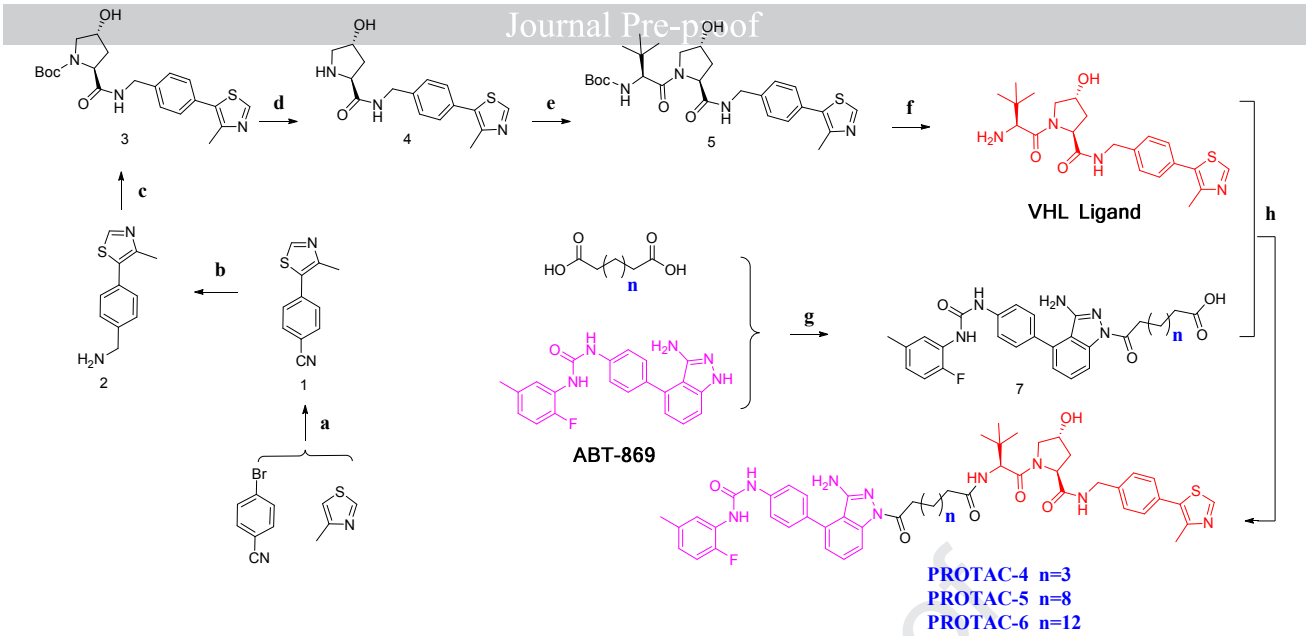
### *2.1 Chemistry*

The length and composition of linker is one of the critical parameters affecting degrader potency [13]. Therefore, we focused on extensive optimization of the linker. The general synthetic procedures for the title PROTACs **1-6** were outlined in **Scheme 1** and **Scheme 2** [14]. VH032 is commonly functionalized as von Hippel-Lindau (VHL) ligand for PROTACs. According to the synthetic schemes, E3 ubiquitin ligases ligand (VH032) was prepared as previously described [15]. Target protein ligand **S7** and ABT-869 were prepared according to the synthetic scheme previously reported [16]. Condensation of target protein ligand with various di-acid in the presence of PyBop afforded the key intermediates (**6**) or (**7**). Finally, the title compounds (PROTAC-1~PROTAC-6) were yielded by connecting the key intermediates with VHL ligand [17].




**Scheme 1.** Synthetic route of the title PROTACs **PROTAC-1~PROTAC-3**.

***Reagents and conditions***: a. $Pd(OAc)_2$, KOAc, DMAc, 150℃; b. $NaBH_4$, $CoCl_2$, MeOH, 0℃; c. Boc-Hyp-OH, DIPEA, HATU, THF; d. TFA, $CH_2Cl_2$; e. Boc-*L*-tert-leucine, DIPEA, HATU, THF; f. TFA, $CH_2Cl_2$; g. EtOH, $NaHCO_3$, $NH_2NH_2 \cdot H_2O$; h. BTC, $Et_3N$, DCM; i. $Pd(PPh_3)_4$, $Na_2CO_3$, $H_2O$, dioxane; j. PyBop, TEA, DMF; k. HATU, DIPEA, DMF.




**Scheme 2.** Syntheitc route of the title PROTACs **PROTAC-4~PROTAC-6**.

***Reagents and conditions*:** a. $Pd(OAc)_2$, KOAc, DMAc, 150℃; b.$NaBH_4$, $CoCl_2$, MeOH, 0℃; c.Boc-Hyp-OH, DIPEA, HATU, THF, rt; d.TFA, $CH_2Cl_2$, rt; e. Boc-*L*-tert-leucine, DIPEA, HATU, THF, rt; f. TFA, $CH_2Cl_2$, rt; g. PyBop, TEA, DMF, rt; h. HATU, DIPEA, DMF, rt.

All the title compounds are characterized by HPLC, LC-MS, $^{1}$H-NMR, $^{13}$C-NMR, and melting point analysis (**Supplementary Material**).

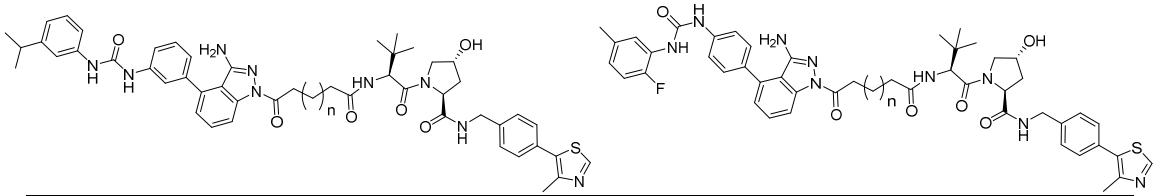
*2.2 Biology assays*

In order to demonstrate that these PROTACs retained the biological activities of anti-angiogenesis agents, we firstly tested the enzymatic inhibitory activity of PROTACs against VEGFR-2. Subsequently, their inhibition against viability of HUVECs (EA.hy926) was evaluated to prove the anti-angiogenic potency. In parallel, ABT-869 was used as positive control. Moreover, molecular docking, western blot assay, cell cycle assay, and apoptosis detection assay were also performed to investigate the action mechanism of these novel PROTAVs [18].

*2.2.1. Angiogenic receptor tyrosine kinases inhibition assay*

The inhibitory activities of PROTACs on VEGFR-2 were evaluated using ADP-Glo Kinase Assay Kit (Promega, Wisconsin, USA) [19]. The kinase inhibition evaluation results were depicted in **Table 1**. Kinase assays indicated that most of PROTACs exhibited considerable enzymetic inhibitory potency. In special, PROTAC-2, PROTAC-5, PROTAC-6 displayed potent inhibitory activity against VEGFR-2. The results indicated that the new PROTACs could still exhibit VEGFR-2 inhibition, especially for PROTAC-6. However, three of them exhibited the largest magnitude of reduction in enzymatic inhibition.

**Table 1.** Structures and kinase inhibitory activities of title PROTACs (n=3).



| Compounds | n | $IC_{50}$ (nM) | Compounds | n | $IC_{50}$ (nM) |
|---|---|---|---|---|---|
| PROTAC-1 | 3 | >1 μM | PROTAC-4 | 3 | >1 μM |
| PROTAC-2 | 8 | 208.151±49.62 | PROTAC-5 | 8 | 208.657±15.421 |
| PROTAC-3 | 12 | >1 μM | PROTAC-6 | 12 | 80.418±1.678 |
| S7 | | 6.86±0.916 | ABT-869 | | 8.404±0.759 |

*2.2.2. Effects of PROTACs on EA.hy926 and toxicity assay* With the aim to determining the anti-angiogenic effect of

these PROTACs, we evaluate the anti-proliferative activity against HUVECs (EA.hy926, VEGFR-2 positive) [20]. Highly consistent with RTK inhibitory activity, three of PROTACs displayed dose-dependent inhibition against EA.hy926 viability with $IC_{50}$ values ranging from 32 μM to 133 μM (**Table 2**). Both PROTAC-2 and PROTAC-5 concentration-dependently inhibited proliferation of HUVECs with similar potencies ($IC_{50}$=32.793±4.971 μM and 38.650±3.876 μM, respectively). On the other hand, PROTAC-1 and PROTAC-3 were much less active at anti-proliferative potency, which was consistent to their weaker activities in the VEGFR-2 inhibition. The results demonstrated that these PROTACs might display anti-angiogenic potency through decreasing viability of HUVECs (EA.hy926).

**Table 2.** Anti-proliferative activity of title PROTACs against EA.hy926 cells (n=3).

| Compounds | $IC_{50}$ (μM) | Compounds | $IC_{50}$ (μM) |
|---|---|---|---|
| PROTAC-1 | >100 | PROTAC-4 | 133.248±15.463 |
| PROTAC-2 | 32.793±4.971 | PROTAC-5 | 38.650±3.876 |
| PROTAC-3 | >100 | PROTAC-6 | ND* |
| S7 | 51.554±1.511 | ABT-869 | 2.480±0.480 |

**ND = Not Determined.*

Given the potent activity of these PROTACs in decreasing viability of HUVECs (EA.hy926) bearing VEGFR-2, we also assessed the anti-proliferative activity against HEK 293 cells (VEGFR-2 negative) to ensure against general toxicity. According to the results shown in **Figure 4**, these PROTACs displayed negligible toxicity toward HEK 293 cells. The results suggested that all PROTACs exhibited no significant cytotoxicity against VEGFR-2 negative cells, which indicated that their potency was VEGFR-2 dependent.

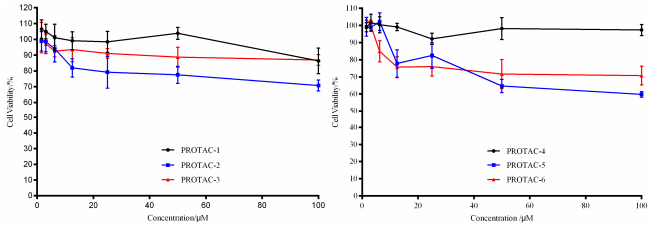



***Fig. 4.*** *Cell proliferation assays and toxicity of PROTACs against HEK 293 cells (n=3).*

*2.2.3. Molecular docking study*

To identify the binding mode of PROTACs with E3 ubiquitin ligases and VEGFR-2, molecular docking studies were performed using Sybyl-X (Version 2.0, Tripos Inc. St. Louis, MO) [21]. PROTAC-2 and PROTAC-5 were constructed and optimized using Powell's method with a Tripos force field. Molecular modeling was performed using Surflex-dock module. E3 ubiquitin ligases (PDB ID: 1LM8) and VEGFR-2 (PDB ID: 4ASD) were identified as the active site. The binding mode of PTOTAC-2 and PROTAC-5 with E3 ubiquitin ligases and VEGFR-2 were described respectively in **Figure 5**. As expected, VHL ligand and small molecule inhibitors in PROTACs bound tightly with E3 ubiquitin ligases and VEGFR-2, respectively. Therefore, the length and flexibility of linker are suitable for the binding of inhibitors with corresponding protein.

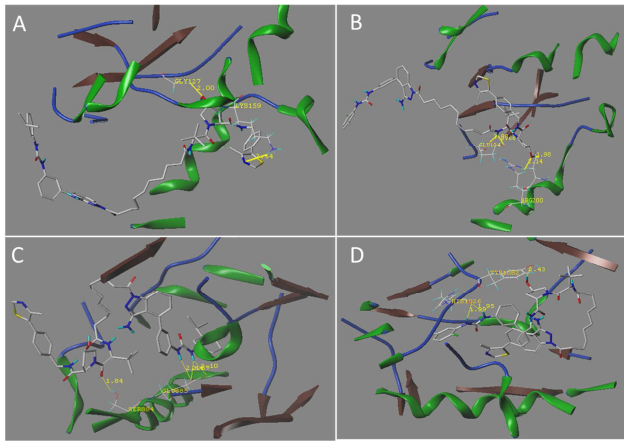



***Fig. 5.*** *The binding mode of PROTACs with E3 ubiquitin ligases (A and B) and VEGFR-2 (C and D).*

*2.2.4. Western blot assay*

In order to demonstrate the protein degradation activity of PROTACs, we investigated the abundance of VEGFR-2 in EA.hy926 cells [22]. Based on the results of kinase assay and anti-proliferation assay, potent PROTACs were selected for protein degradation assay. We performed dose-response and time-course investigation with PROTACs and evaluated their degradation kinetics in EA.hy926 cells in a time course. As shown in **Figure 6**, these PROTACs could reduce protein levels of VEGFR-2. PROTAC-2 was capable of reducing protein levels by 70% at 80 μmol/L, efficient degradation of VEGFR-2 was observed at 72 h after treatment; PROTAC-5 was capable of reducing

protein levels by 60% at 40 μmol/L, the efficient degradation of VEGFR-5 was observed as early as 24 h after treatment and was nearly complete afer 48 h. PROTAC-5 exhibited concentration-dependent VEGFR-2 degradation potency.

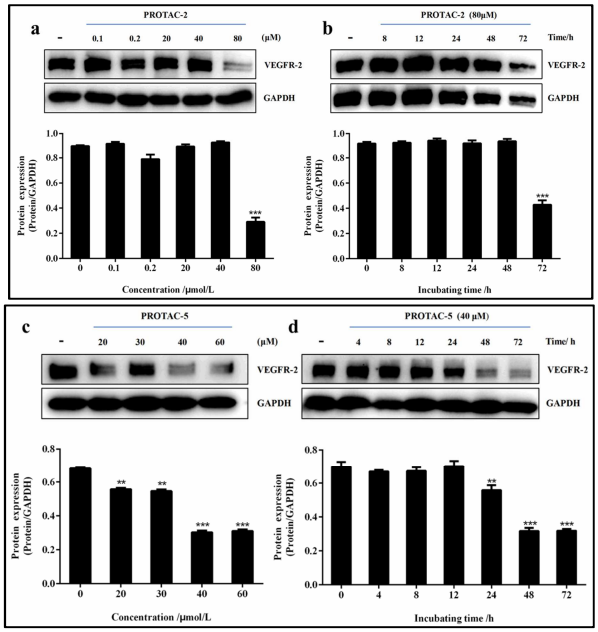



***Fig. 6.*** *Dose- and Time-dependent degradation of VEGFR-2 for PROTAC-2 and PROTAC-5 (**P<0.01, ***P<0.001).*

*2.2.5. Cell cycle assay*

To further explore the effects of PROTAC-5 on the cell cycle of HUVECs, cell-cycle profiles of EA.hy926 were assessed with flow cytometry analysis [23]. As shown in **Figure 7**, the results indicated that PROTAC-5 could obviously prolong the S phase at 20 μM. PROTAC-5 treatment could induce a significant increase of cells in the S-phase and corresponding decreased proportion of cells in G1-phase. Therefore, the anti-proliferative activities of PROTAC-5 might due to cell cycle arrest.

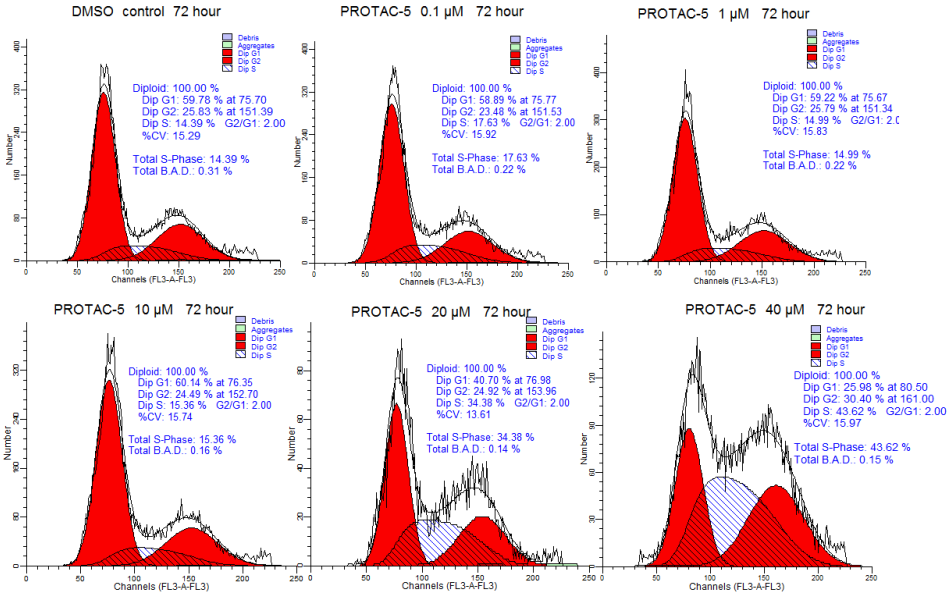



***Fig. 7.*** *Effects of PROTAC-5 on HUVECs (EA.hy926) cell cycle progress.*

*2.2.4. Apoptosis detection assay*

To further investigate the effect of PROTAC-5 on the apoptosis of HUVECs (EA.hy926), we performed flow cytometry analysis and apoptosis detection assays [24]. A substantial and dose-dependent proapoptotic activity in HUVECs for 72 h was observed. PROTAC-5 exhibited pro-apoptosis potency on HUVECs. The late apoptotic HUVECs (5.34%) of negative control group were depicted in **Figure 8**. After treatment with PROTAC-5 for 72 h, the percentage of late apoptotic cells was 6.94%, 8.32%, 15.6%, 48.7%, and 64.2%, respectively. The results suggested that PROTAC-5 could induce late apoptosis of HUVECs in a dose dependent manner with concentration of 10 μM-40 μM.

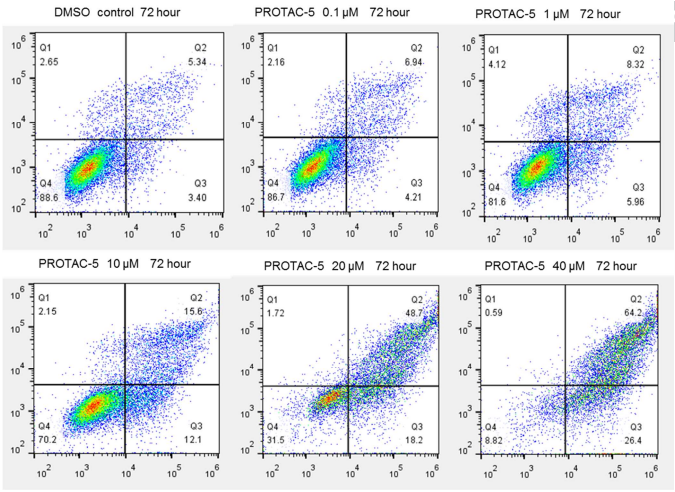



***Fig. 8.*** *Effects of PROTAC-5 on HUVECs (EA.hy926) cell apoptosis by flow cytometry measurement.*

## 3. Conclusion

PROTACs is a powerful approach for inducing protein degradation and is an emerging modality in small molecule drug discovery. In this investigation, we have applied the PROTACs concept to explore the potential of anti-angiogenesis agents in vascular normalization. Six novel PROTACs were developed based on previously reported anti-angiogenesis agents. Two of them, PROTAC-2 and PROTAC-5, displayed potent inhibitory activity against VEGFR-2 and considerable anti-proliferative activities against human umbilical vein endothelial cells (EA.hy926). In addition, they were capable of specifically reducing protein levels of VEGFR-2. Meanwhile, all the PROTACs exhibited no significant cytotoxicity against HEK293 cells. PTOTAC-5 could induce the increase of cells in the S-phase. It exhibited pro-apoptosis effects on VEGFR-2 positive HUVECs with negligible cytotoxicity against VEGFR-2 negative HEK 293 cells. Taken together, these results are in keeping with our previous findings and provide strong evidences to support the potential therapeutic usage of PROTAC-2 and PROTAC-5 in vascular normalization.

## 4. Experimental section

*4.1. Chemistry: General procedure*

All commercial chemicals and solvents were purchased and used without further purification unless otherwise indicated. All anhydrous solvents were dried according to the standard methods and partly of them were freshly distilled prior to use. Melting points were measured on an X-4 micromelting apparatus. $^{1}$H NMR and $^{13}$C NMR spectra were measured on a Bruker Advance 400 at 400 and 100 HZ, respectively. Chemical shifts (δ) in DMSO-$d_6$ were referenced to tetra-methyl silane (TMS 0.0 ppm). The solvent peak and $H_2O$ peak were set to 2.50 ppm and 3.33 ppm. Mass spectra were partly measured using liquid chromatography mass spectrometry (LC-MS) with electrospray ionization (ESI), while others were measured using gas chromatography mass spectrometry (GC-MS) with electron impact ionization (EI). All reactions progress was monitored by TLC ($GF_{254}$), and visualized with UV (254 nm) except Boc-protection of amino acids using ninhydrin reaction. All compounds were purified by chromatography with silica gel (300-400 mesh).

*4.1.1 General procedure for the synthesis of title compounds PROTAC-1~PROTAC-6.*

*4.1.2 4-(4-methylthiazol-5-yl)benzonitrile (**1**)*

To a solution of 4-bromobenzonitrile (3.0 g, 16.48 mmol, 1 eq) and $Pd(OAc)_2$ (4 mg, 0.16mmol, 0.1mol%) in DMAc (16mL) were added KOAc (3.24g, 33 mmol, 2 eq) and 4-methylthiazole (3.26 g, 2.98 mL, 33 mmol, 2 eq). The resulting mixture was heated to 150 ℃ and stirred 10 hours. The mixture was diluted with water and extracted with DCM (3×). The combined organic phases were washed with saturated sodium chloride solution and dried overnight with $NaSO_4$, and evaporated under reduced pressure to give the corresponding cyano derivate as oil substance, and the white product (**1**) was obtained by column chromatography (2.84 g, 86.16%). LC-MS (EI, m/z): 200.04.

*4.1.3 (4-(4-methylthiazol-5-yl)phenyl)methanamine (**2**)*

The compound (**1**) (0.54 g, 2.7mmol, 1eq) in methanol (15mL) was cooled to 0 ℃. $CoCl_2$ (0.52 g, 4.05 mmol, 1.5 eq) was added, followed by portionwise addition of $NaBH_4$ (0.51g, 13.5 mmol, 5 eq). The resulting mixture was stirred for 90 min, the reaction was quenched with water and ammonium hydroxide, and the mixture was extracted with DCM (6×). The combined organic phases were dried over $NaSO_4$ and evaporated under reduced pressure to give a

dark-brown oil which was purified by column chromatography to yield the product (2) as a yellow oil (0.18 g, 32.63%). LC-MS (EI, m/z): 204.07.

*4.1.4 (2S,4R)-tert-Butyl-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidine-1-carboxylate (**3**)*

Boc-L-Hyp-OH (0.68 g, 2.94 mmol, 1 eq), PyBOP (1.68 g, 3.23 mmol, 1.1 eq) was added in DMF (10 mL), and the solution was stirred at 0 ℃. TEA (0.814mL, 5.87 mmol, 2 eq) was added dropwise, and the mixture was stirred for 5 min at 0 ℃, then the compound (**2**) (0.6 g, 2.94 mmol, 1 eq) was added, and the mixture was stirred overnight at room temperature. Water was added, and the mixture was extracted with ethyl acetate (3×). The combined organic phases were washed with brine (2×), dried over $NaSO_4$, and evaporated under reduced pressure to give the corresponding crude, which was purified by column chromatography purification to yield the key intermediate (**3**) (1.22 g, 99.49%). LC-MS (EI, m/z): 417.17.

*4.1.5 (2S,4R)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl) pyrrolidine-2-carboxamide (**4**)*

The compound (**3**) (0.86 g, 2.06 mmol)) was dissolved and stirred in DCM (30 mL) at 0 ℃, and trifluoroacetic acid (3 mL) was added to the solution. After stirring the mixture for 1 hour at room temperature, the reaction was completed, and the pH was adjusted to 7 with saturated $NaHCO_3$ solution. Then the mixture was extracted with DCM (3×), dried over $NaSO_4$, and the solvents were evaporated under reduced pressure to give the corresponding crude compound (**4**) (0.65 g). LC-MS (EI, m/z): .17.12.

*4.1.6 tert-Butyl((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazole-5-yl)-benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)-carbamate (**5**)*

Boc-*L*-tert-leucine (0.47 g, 2.04 mmol, 1 eq), HATU (0.85 g, 2.24 mmol 1.1 eq) was added in DCM (30 mL), and the solution was stirred at 0 ℃. DIPEA (1.425mL, 8.16 mmol, 4 eq) was added dropwise, and the mixture was stirred for 10 min at 0 ℃, then the compound 10 (0.65 g, 2.04 mmol, 1 eq) was added, and the mixture was stirred overnight at room temperature. Water was added, and the mixture was extracted with DCM (3×). The combined organic phases were washed with brine (2×), dried over $NaSO_4$, and evaporated under reduced pressure to give the corresponding crude, which was purified by column chromatography purification to yield the key intermediate (**5**) (0.82 g, 75.45%). LC-MS (ESI) $[M+H]^+$: 531.30.

*4.1.7 (2S,4R)-1-((S)-2-Acetamido-3,3-dimethyl butanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl) benzyl)pyrrolidine-2-carboxamide (**VHL ligand**)*

The compound (**5**) (0.82 g, 1.55 mmol)) was dissolved and stirred in DCM (30 mL) at 0 ℃, and triethylamine (3 mL) was added to the solution. After stirring the mixture for 2 hour at room temperature, the reaction was completed, and the pH was adjusted to 7 with saturated $NaHCO_3$ solution. Then the mixture was extracted with DCM (3×), dried over $NaSO_4$, and the solvents were evaporated under reduced pressure to give the corresponding crude which was purified by column chromatography purification to yield the VHL ligand (0.53 g, 79.73%). LC-MS (ESI) $[M+H]^+$: 431.21. $^1$H NMR (400MHz, DMSO-$d_6$) δ 9.00 (s, 1H), 8.74-8.77 (t, 1H), 7.40 (s, 4H), 5.31 (s, 1H), 4.52-4.57 (t, 1H), 4.39-4.47 (m, 2H), 4.21-4.27 (q, 1H), 4.00-4.06 (q, 1H), 3.90 (s, 1H), 2.45 (s, 1H), 2.09-2.14 (m, 1H), 1.16-1.19 (t, 2H), 1.02 (s, 9H).

*4.1.8 5-(3-amino-4-(3-(3-(3-isopropylphenyl)ureido)phenyl) -1H-indazol-1-yl)-5-oxopentanoic acid (**6**)*

Compound (**S7**) and ABT-869 were prepared according to the synthetic scheme previously reported. Subsequently, heptanedioic acid (0.12 g, 0.778 mmol, 2eq), PyBOP (0.33 g, 0.648 mmol, 2 eq) was added in DCM (30 mL), and the solution was stirred at 0 ℃. TEA (4 eq) was added dropwise, and the mixture was stirred for 5 min at 0 ℃, then S7 (0.1 g, 0.259 mmol) or ABT-869 (1 eq) was added, and the mixture was stirred overnight at room temperature. The volatile organic solvent is spun out under vacuum conditions. Then water was added, and the mixture was extracted with ethyl acetate (3×). The combined organic phases were washed with brine (2×), dried over $NaSO_4$, and evaporated under reduced pressure to give the

corresponding crude, which was purified by column chromatography purification to yield the key intermediate (6) (0.11 g, 80.36%). LC-MS (ESI) $[M+H]^+$: 528.30.

*4.1.9 (2S,4R)-1-((S)-2-(7-(3-amino-4-(3-(3-(3-isopropylphenyl)ureido)phenyl)-1H-indazol-1-yl)-7-oxoheptanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide* ***(PROTAC-1)***

The compound (6) (0.15 g, 0.284 mmol), HATU (0.16 g, 0.426 mmol) was added in DCM (30 mL), and the solution was stirred at 0 ℃. DIPEA (193 μL, 1.137 mmol) was added dropwise, and the mixture was stirred for 10 min at 0 ℃, then VHL ligand (0.13 g, 0.284 mmol) was added, and the mixture was stirred overnight at room temperature. The volatile organic solvent is spun out under vacuum conditions. Then water was added, and the mixture was extracted with DCM (3×). The combined organic phases were washed with brine (2×), dried over $NaSO_4$, and evaporated under reduced pressure to give the corresponding crude, which was purified by column chromatography purification to yield the final PROTAC-1 (0.12 g, 44.9%). mp: 115-117 ℃, LC-MS (ESI) $[M+H]^+$: 940.55. $^1$H NMR (400MHz, DMSO-$d_6$) δ 8.98 (s, 1H), 8.86 (s, 1H), 8.71 (s, 1H), 8.55-8.58 (t, 1H), 8.34-8.36 (d, 1H), 7.87-7.89 (d, 1H), 7.68 (s, 1H), 7.60-7.64 (t, 1H), 7.35-7.47 (m, 6H), 7.17-7.26 (m, 3H), 7.07-7.09(d,1H), 6.85-6.88 (d, 2H), 5.29 (s, 2H), 5.13-5.14 (d, 1H), 4.53-4.59 (t, 1H),4.41-4.45 (t, 2H), 4.35 (s, 1H), 4.19-4.25 (q, 1H), 4.01-4.06 (q, 1H), 2.96-3.00 (t, 2H), 2.79-2.88 (m, 1H), 2.45 (s, 3H), 2.12-2.19 (t, 3H), 1.36 (s, 8H), 1.18-1.20 (d, 6H), 0.93 (s, 9H). $^{13}$C NMR (101 MHz, DMSO) δ 172.54, 172.43, 172.22, 170.19, 153.13, 152.08, 151.86, 149.52, 148.19, 140.46, 140.32, 139.97, 139.49, 138.78, 136.81, 132.99, 131.64, 130.38, 130.21, 130.11, 129.80, 129.11, 128.82, 127.89, 126.53, 125.38, 125.25, 122.68, 120.51, 119.00, 118.57, 116.78, 116.58, 116.43, 114.73, 69.34, 59.16, 56.81, 42.12, 38.41, 35.66, 34.85, 34.78, 33.96, 30.89, 28.83, 26.85, 25.72, 24.43, 24.34, 16.41.

The title compounds PROTAC-2~PROTAC-6 were prepared by using the general procedure described above.

*4.1.10 (2S,4R)-1-((S)-2-(12-(3-amino-4-(3-(3-(3-isopropylphenyl)ureido)phenyl)-1H-indazol-1-yl)-12-oxododecanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide* ***(PROTAC-2)***

Yield: 48.9%, mp: 107-109 ℃, LC-MS (ESI) $[M+H]^+$: 1010.45. $^1$H NMR (400MHz, DMSO-$d_6$): δ 8.98 (s, 1H), 8.86 (s, 1H), 8.71 (s, 1H), 8.55-8.58 (t, 1H), 8.34-8.36 (d, 1H), 7.83-7.86 (d, 1H), 7.68 (s, 1H), 7.60-7.64 (t, 1H), 7.36-7.47 (m, 7H), 7.19-7.25 (m, 3H), 7.07- 7.09 (d, 1H), 6.86-6.88 (d, 1H), 5.28 (s, 2H), 5.13-5.13 (d, 1H), 4.54-4.56 (d, 1H), 4.41-4.47 (q, 2H), 4.36 (s, 1H), 4.20-4.25 (q, 1H), 4.01-4.06 (q, 1H), 2.97-3.00 (t, 2H), 2.81-2.88 (m, 1H), 2.45 (s, 3H), 2.00-2.14 (m, 3H), 1.68-1.72 (m, 2H), 1.19-1.50 (m, 22H), 0.94 (s, 9H). $^{13}$C NMR (101 MHz, DMSO) δ 172.58, 172.43, 172.27, 170.18, 153.13, 152.05, 151.91, 149.52, 148.18, 140.45, 140.31, 139.97, 139.95, 138.78, 136.80, 131.63, 130.21, 130.10, 129.80, 129.14, 129.10, 127.88, 125.25, 122.68, 120.51, 118.99, 118.56, 116.77, 116.55, 116.42, 114.72, 69.33, 59.15, 56.81, 56.74, 42.11, 38.41, 35.67, 35.33, 34.87, 33.96, 29.42, 29.38, 29.33, 29.22, 29.20, 29.12, 26.83, 25.91, 24.70, 24.34, 16.41.

*4.1.11 (2S,4R)-1-((S)-2-(16-(3-amino-4-(3-(3-(3-isopropylphenyl)ureido)phenyl)-1H-indazol-1-yl)-16-oxohexadecanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide* ***(PROTAC-3)***

Yield: 54.1%, mp: 100-102 ℃, LC-MS (ESI) $[M+H]^+$: 1066.60. $^1$H NMR (400MHz, DMSO-$d_6$): δ 8.98 (s, 1H), 8.86 (s, 1H), 8.70 (s, 1H), 8.55-8.58 (t, 1H), 8.34-8.36 (d, 1H), 7.83-7.86 (d, 1H), 7.68 (s, 1H), 7.60-7.64 (t, 1H), 7.35-7.47 (m, 7H), 7.19-7.26 (m, 3H), 7.08-7.09 (d, 1H), 6.85-6.87 (d, 1H), 5.27 (s, 2H), 5.13-5.14 (d, 1H), 4.53-4.56 (d, 1H), 4.41- 4.47 (q, 2H), 4.35 (s, 1H), 4.18-4.24 (m, 1H), 4.01-4.06 (q, 1H), 2.96-3.00 (t, 2H), 2.79-2.87 (m, 1H), 2.45 (s, 3H), 1.99-2.13 (m, 3H), 1.68-1.71 (m, 2H), 1.23-1.49 (m, 24H), 1.18-1.20 (d, 6H), 0.94 (s, 9H). $^{13}$C NMR (101 MHz, DMSO) δ 172.59, 172.42, 172.28, 170.19, 153.12, 152.05, 151.91, 149.52, 148.18, 140.45,

140.32, 139.97, 139.95, 138.78, 136.80, 131.63, 130.20, 129.80, 129.14, 129.10, 127.89, 125.24, 122.68, 120.52, 119.00, 118.58, 116.78, 116.56, 116.43, 114.72, 69.33, 59.15, 56.80, 56.74, 42.13, 38.41, 35.68, 35.33, 34.85, 33.96, 29.48, 29.33, 29.21, 29.16, 29.12, 26.84, 25.90, 24.70, 24.33, 21.84, 16.40, 12.25.

*4.1.12 (2S,4R)-1-((S)-2-(7-(3-amino-4-(4-(3-(2-fluoro-5-methylphenyl)ureido)phenyl)-1H-indazol-1-yl)-7-oxoheptanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide* ***(PROTAC-4)***

Yield: 61.2%, mp: 124-126 ℃, LC-MS (ESI) $[M+H]^+$: 930.45. $^1$H NMR (400MHz, DMSO-$d_6$): δ 9.28 (s, 1H), 8.98 (s, 1H), 8.57 (s, 2H), 8.31- 8.33 (d, 1H), 8.00-8.02 (d, 1H), 7.87-7.89 (d, 1H), 7.58-7.64 (m, 3H), 7.37-7.43 (m, 6H), 7.10-7.19 (m, 2H), 6.81-6.84 (m, 1H), 5.21 (s, 2H), 5.14 (d, 1H), 4.53-4.56 (d, 1H), 4.41- 4.47 (t, 2H), 4.35 (s, 1H), 4.19-4.25 (q, 1H), 3.63-3.70 (t, 2H), 2.45 (s, 3H), 2.29 (s, 3H), 2.12-2.19 (m, 1H), 2.02-2.06 (m, 1H), 1.87-1.94 (m, 1H), 1.67-1.75 (m, 3H), 1.50-1.61 (m, 2H), 1.34-1.39 (m, 2H), 1.24 (m, 2H), 0.91 (s, 9H). $^{13}$C NMR (101 MHz, DMSO) δ 172.57, 172.44, 172.20, 170.19, 152.62, 152.18, 151.90, 149.61, 148.18, 140.34, 140.15, 139.96, 136.61, 134.00, 131.64, 131.57, 130.19, 130.10, 129.97, 129.10, 127.89, 127.47, 127.36, 125.43, 123.33, 123.26, 121.46, 118.55, 116.66, 114.92, 114.38, 69.35, 59.17, 56.81, 42.13, 38.40, 35.66, 35.24, 34.76, 28.83, 26.84, 25.72, 24.44, 21.24, 16.40.

*4.1.13 (2S,4R)-1-((S)-2-(12-(3-amino-4-(4-(3-(2-fluoro-5-methylphenyl)ureido)phenyl)-1H-indazol-1-yl)-12-oxododecanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide* ***(PROTAC-5)***

Yield: 58.1%, mp: 116-118 ℃, LC-MS (ESI) $[M+H]^+$: 1000.55. $^1$H NMR (400MHz, DMSO-$d_6$): δ 9.28 (s, 1H), 8.99 (s, 1H), 8.58 (s, 2H), 8.31-8.33 (d, 1H), 8.01-8.03 (d, 1H), 7.85-7.87 (d, 1H), 7.58-7.64 (m, 3H), 7.40-7.43 (m, 6H), 7.10-7.19 (m, 2H), 6.83 (m, 1H), 5.21 (s, 2H), 5.14 (s, 1H), 4.54-4.56 (d,1H), 4.43-4.45 (t, 2H), 4.36 (s, 1H), 4.21-4.25 (q, 1H), 3.63-3.70 (t, 2H), 2.45 (s, 3H), 2.29 (s, 3H), 2.00-2.12 (m, 3H), 1.91-1.92 (m, 1H), 1.66-1.71 (m, 2H), 1.43-1.55 (m, 2H), 1.17-1.36 (m, 14H), 0.93 (s, 9H). $^{13}$C NMR (151 MHz, DMSO) δ 172.57, 172.42, 172.24, 170.19, 152.62, 152.16, 151.91, 151.57, 149.99, 148.19, 140.34, 140.16, 139.98, 136.62, 134.02, 131.63, 131.57, 130.20, 130.11, 129.98, 129.11, 127.89, 127.47, 127.40, 125.43, 121.44, 118.53, 116.65, 115.09, 114.96, 114.38, 69.33, 59.16, 56.81, 56.74, 42.12, 38.42, 35.67, 35.33, 34.86, 29.42, 29.37, 29.33, 29.21, 29.20, 29.12, 26.84, 25.91, 24.72, 21.25, 16.41.

*4.1.14 (2S,4R)-1-((S)-2-(16-(3-amino-4-(4-(3-(2-fluoro-5-methylphenyl)ureido)phenyl)-1H-indazol-1-yl)-16-oxohexadecanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide* ***(PROTAC-6)***

Yield: 55.6%, mp: 105-107 ℃, LC-MS (ESI) $[M+H]^+$: 1056.60. $^1$H NMR (400MHz, DMSO-$d_6$): δ 9.27 (s, 1H), 8.98 (s, 1H), 8.57 (s, 2H), 8.30-8.32 (d, 1H), 8.00-8.02 (d, 1H), 7.83-7.86 (d, 1H), 7.57-7.64 (m, 3H), 7.37-7.43 (m, 6H), 7.10-7.19 (m, 2H), 6.82 (m, 1H), 5.19 (s, 2H), 5.13-5.14 (d, 1H), 4.53-4.56 (d, 1H), 4.41-4.47 (t, 2H), 4.35 (s, 1H), 4.19-4.24 (q, 1H), 3.63-3.70 (t, 2H), 2.45 (s, 3H), 2.29 (s, 3H), 1.99-2.12 (m, 2H), 1.87-1.94 (m, 1H), 1.67-1.71 (m, 2H), 1.23-1.49 (m, 25H), 0.94 (s, 9H). $^{13}$C NMR (101 MHz, DMSO) δ 172.60, 172.42, 172.24, 170.19, 152.61, 152.15, 151.97, 151.90, 148.18, 140.34, 140.15, 139.97, 136.61, 133.99, 131.63, 131.57, 130.19, 130.10, 129.97, 129.10, 127.89, 127.48, 127.37, 125.42, 123.24, 121.44, 118.53, 116.64, 115.11, 114.92, 114.36, 69.33, 59.16, 56.80, 56.74, 42.11, 38.41, 35.68, 35.33, 34.84, 29.48, 29.33, 29.21, 29.17, 29.12, 26.84, 25.90, 24.71, 21.24, 16.40.

*4.2. In vitro VEGFR-2 inhibition assays [24]*

The *in vitro* enzymatic inhibition assays against VEGFR-2 was carried out by ADP-Glo™ kinase assay (Promega, Madison, WI) according to the manufacturer's instructions, with ABT-869 as positive control. General procedures were as follows: VEGFR-2 kinases (0.6 ng/μL) was incubated with substrates (0.2 μg/μL), tested title compounds ($1.2 \times 10^{-4}$-12 μM) and ATP (50 μM) in a final

buffer of Tris 40 mM, $MgCl_2$ 10 mM, BSA 0.1 mg/mL, and DTT 1 mM in a 384-well plate with a total volume of 5 μL. The assay plate was incubated at 30°C for 1 h. Once the kinase reaction was complete, 5 μL of ADP-Glo reagent was added into each well to terminate the reaction and deplete the remaining ATP. The mixture was incubated at room temperature for another 40 min. At the end, 10 μL of kinase detection reagent was added into each well and incubated for 30 min, to convert ADP to ATP and allow the newly synthesized ATP to be measured using luciferase/luciferin reaction. The luminescence generated was read by a VICTORX multiple plate reader. The signal was correlated with the amount of ATP present in the reaction and was inversely correlated with the kinase activity.

*4.3. Anti-proliferative activity against human vascular endotheial cell (EA.hy926) [25].*

The viability of HUVEC (EA.hy926) was assessed using standard MTT method with ABT-869 as positive control. Briefly, EA.hy926 cells were harvested and plated in a 96-well plate at the density of $1\times10^5$ cells for each well, and cultured in DMEM medium with 10% FBS in humidified 5% $CO_2$. For MTT assay, the operation detail was as follows: The plate was incubated in 5% $CO_2$ at 37℃ for 48 h. The test compounds at serial diluted concentrations were added into the cells solution and incubated for 24 h. Subsequently, MTT was added into each well at a final concentration of 0.5 mg/mL and incubated for 2 h at 37℃. Supernatant was discarded, and 150 μL DMSO was added to each well. After shaking 15 min, absorbance values at 450 nm were determined by a microplate reader (Bio-Rad, Hercules, USA). The viability rate was calculated as experimental OD value/control OD value. The $IC_{50}$ values were calculated according to inhibition ratios.

*4.4. Western blotting assay [26].*

Protein content was determined with a bicinchoninic acid (BCA) assay and equal protein amounts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at a constant voltage. Protein was transferred onto an Immobilon-P transfer membrane (Millipore). For western blot analyses, the following antibodies were used: GAPDH (1:10000, Proteintech), VEGFR-2 (1:500, CST). Quantification was performed using the software distribution ImageJ. EA.hy926 cells were treated with vehicle (DMSO) or tested title compounds at desired concentration for 48 h. The cells were lysed in SDS sample buffer, collected, and normalized using BCA protein assay kit before being diluted in SDS loading buffer. Then the samples containing equal amounts of protein were separated by SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes and blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20. Membranes were incubated with antibodies Bcr-Abl and p-Bcr-Abl at 4℃ overnight. The membranes were washed with PBS three times, and incubated with the appropriate anti-HRP secondary antibodies for 2 h at room temperature. Finally, immunoreactive proteins were visualized using the enhanced chemiluminescence system from Pierce Chemical.

*4.5. Molecular docking modeling [27]*

In order to investigate the interaction between the title compounds and VEGFR-2 kinases, molecular docking was performed using Surflex-Dock Module of Sybyl-X (version 2.0, Tripos Inc, St.Louis, MO). Crystal structures of VEGFR-2 (PDB ID: 4ASD) and E3 ubiquitin ligases (PDB ID: 1LM8) were imported, and protein structure preparation was carried out as follows: removing waters and other small molecules, repairing sidechain, and adding hydrogen atoms, and minimized with Pullman charges under Tripos Force Field. Then the corresponding ligand in the prepared protein structure was extracted and used to define the binding cavity for molecules. The residues in a radius 5.0 Å around the ligand were selected as active site and generated as docking promotal. The title molecules were drawn with Sketch and minimized by Powell's method for 1000 iterations under Tripos Force Field with Gasteiger-Huckel charges. In the end, the optimized molecules were docking into the active sites of protein promotal using ligand-based mode with other docking parameters as default.

*4.6. Cell Cycle Assay.*

HUVEC cells ($5\times10^5$ cells/mL) were seeded in six-well plates and treated with compounds at different concentrations for 24 h. The cells were then harvested by trypsinization and washed twice with cold PBS. After centrifugation and removal of the supernatants, cells were resuspended in 400 μL of 1×PBS buffer. After adding 10 μL of PI the cells were incubated at room temperature for 15 min in the dark. The stained cells were analyzed by a flow cytometer (BD Accuri C6).

*4.7. Apoptosis Detection Assay.*

HUVEC cells ($5\times10^5$ cells/mL) were seeded in six-well plates and treated with compounds at different concentrations for 48 h. The cells were then harvested by trypsinization and washed twice with cold PBS. After centrifugation and removal of the supernatants, cells were resuspended in 400 μL of 1×binding buffer which was then added to 5 μL of annexin V-FITC and incubated at room temperature for 15 min. After adding 10μL of PI the cells were incubated at room temperature for another 15 min in the dark. The stained cells were analyzed by a flow cytometer (BD Accuri C6).

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# Highlights

- Six PROTACs have been developed based on anti-angiogenesis agents.
- PROTACs exhibited potent VEGFR-2 inhibition and anti-proliferative activity.
- PROTACs exhibited no significant cytotoxicity against HEK293 cells.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: