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Discovery and biological evaluation of proteolysis targeting chimeras (PROTACs) as an EGFR degraders based on osimertinib and lenalidomide

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ABSTRACT

Epidermal growth factor receptor (EGFR) is one of the important and valuable drug targets. Overexpression of EGFR is associated with the development of many types of cancer. In this study, three PROTACs small molecules (**16a-16c**) were designed, synthesized and evaluated for their cytotoxicity against the growth in different NSCLC cell line and the degradation effect. The bioassay results indicated that **16c** has a good inhibition in PC9 cells and H1975 cells, and the corresponding IC_{50} value was 0.413 μM and 0.657 μM, respectively. Western blotting results demonstrated that compound **16c** could serve as an effective $EGFR^{del19}$-targeting degrader in PC9 cells.

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Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK), belongs to the ErbB family, which plays a fundamentally important role in cell proliferation, opposing apoptosis, survival and migration[1]. Overexpression of EGFR is associated with the development of many types of cancer, such as pancreatic cancer, breast cancer, glioblastoma multiforme, head and neck cancer, and non-small cell lung cancer (NSCLC)[2]. Overexpression and mutation of EGFR are closely related to the development of NSCLC, and is also one of the important and valuable drug targets in NSCLC. The activating mutations in the EGFR tyrosine kinase domain (L858R mutation and exon-19 deletion) have been identified as oncogenic drivers for NSCLC[3], and the first-generation EGFR tyrosine kinase inhibitors (EGFR-TKIs) gefitinib[4] and erlotinib[5] have approved for NSCLC patients with EGFR activation mutations. However, most of NSCLC patients have developed acquired resistance after 9 to14 months with gefitinib or erlotinib clinical therapy treatment[6, 7], a secondary threonine[790] to methionine[790] mutation (T790M), is also called "gatekeeper" T790M mutation[8,9]. Therefore, the second-generation EGFR-TKIs afatinib[10] and the third-generation EGFR-TKIs osimertinib (AZD9291)[11] were developed to overcome the resistance. In particular, osimertinib have achieved significant clinical response rate in NSCLC patients with EGFR T790M[12, 13]. However, there are several studies reported a tertiary Cys797 to Ser797 (C797S) point mutation with osimertinib clinical therapy up to now[14].

Proteolysis Targeting Chimeras (PROTACs) are heterobifunctional small molecules, first proposed by Crews groups[15]. It contains one ligand for the protein of interest (POI), another for E3 ubiquitin ligase (cereblon (CRBN), von Hippel-Lindau (VHL) and mouse double minute 2 (MDM2) et al) and connected by a linker[16]. Different with other small molecule inhibitors, PROTACs can target and degrade the proteins rather than inhibits them[17]. At present, a series of small molecular compounds were synthesized and successfully targeted and induced the degradation of kinases based on PROTACs technology, such as AR[18, 19], BTK[20, 21], ER[22, 23] and BET[24, 25], etc. Specifically, the drug of ARV-110 targeted to the androgen receptors (AR) has been approved for clinical phase I and shows a good tolerance[26].

Epidermal growth factor-RTK (EGF-RTK), as a transmembrane protein, is hard to be degraded. Crews groups firstly demonstrated that RTKs, such as HER2, c-Met and EGFR, were able to be degraded induced by PROTACs [27]. In this study, lapatinib, gefitinib and afatinib were used as a ligand for the POI and von Hippel-Lindau (VHL) was selected as an E3 ligase recruiting ubiquitin. Recently, the Zhang group reported a series of PROTAC compounds to induce the degradation of EGFR on the basis of a novel fourth-generation EGFR-TKI and VHL[28]. The Jin and Xiong groups described the discovery of a novel E3 ligase VHL-recruiting EGFR degrader (MS39) and E3 ligase CRBN-recruiting EGFR degrader (MS154) base on gefitinib[29]. (Answer 4 to reviewer 1)

We employed the binding mode data that a published T790M structure (PDB code: 3IKA) with osimertinib (**Fig. 1**). (Answer 4 to editor) The X-ray crystal structure of $EGFR^{T790M}$ bound to osimertinib showed that the acrylamide group of osimertinib forms a covalent bond to Cys797 of EGFRT790M, the aminopyrimidine core forms two hydrogen bonds with Met793, the methylated indole is adjacent to the gatekeeper Met790 and the dimethylamine moiety is exposed to the solvent channel [11, 30]. (Answer 1 to reviewer 1) Thus, the dimethylamine side chain in AZD9291 was considered as the binding site to lenalidomide via a linker for the design of potential

EGF tion of EGFR degradation induced by PROTACs are relatively limited. Herein, we wish to develop a novel class of small-molecule EGFR degraders employing lenalidomide PROTAC with EGFR inhibitor osimertinib as POI ligand through the different linkers. As well, their corresponding biological activity was evaluated, such as anti-proliferative activity, degradation of EGFR and cell health and cycle.

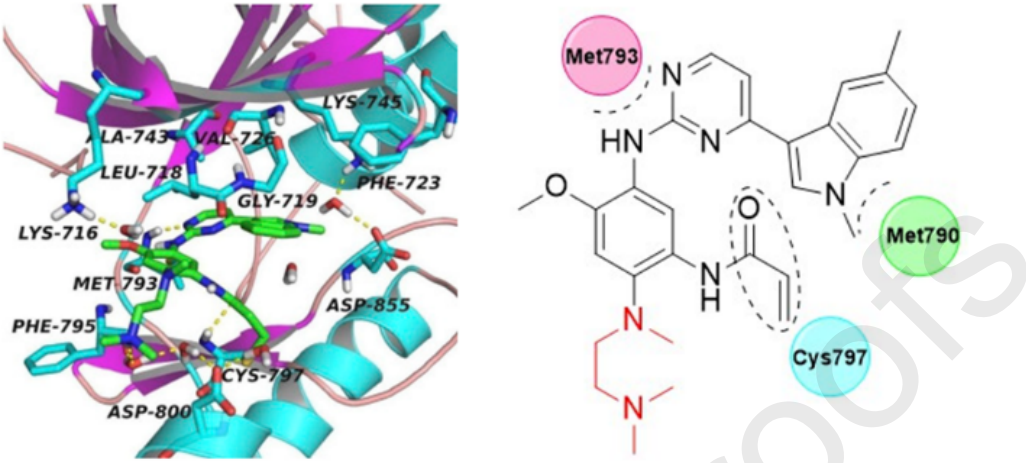
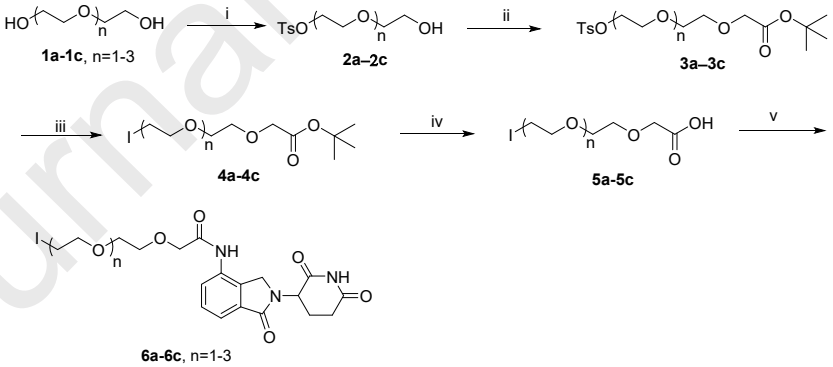


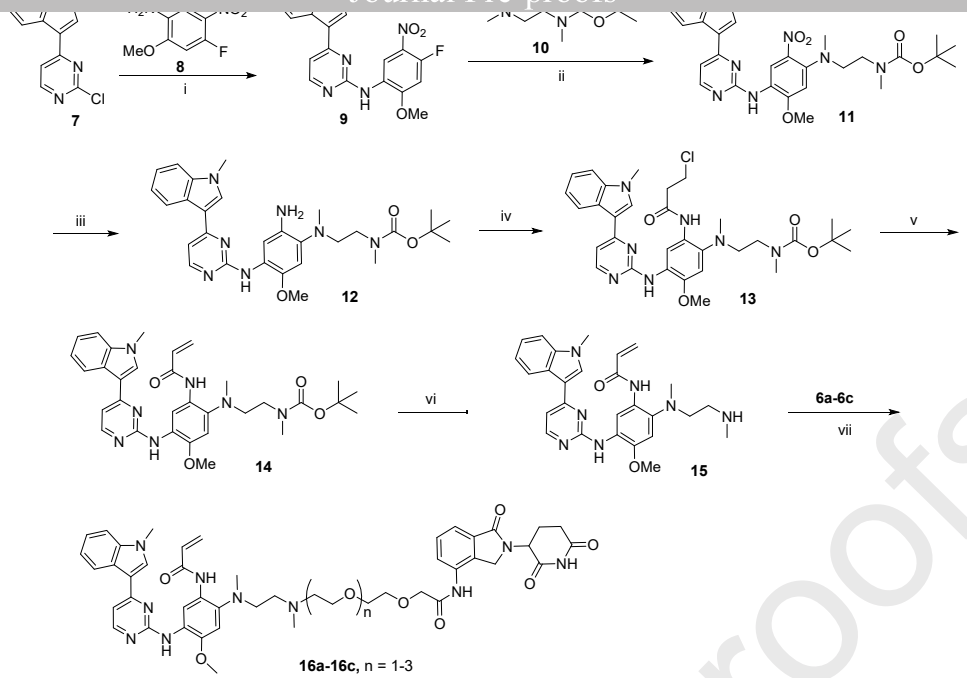
Fig. 1. The binding interactions between osimertinib and EGFR^{T790M}

The synthetic route of compounds **6a-6c** was showed in Scheme 1. Compounds **1a-1c** reacted with tosyl chloride (TsCl) provided **2a-2c**, followed by the Williamson alkylation with *tert*-butyl bromoacetate to give **3a-3c** in the presence of KOH and TBAB. The treatment of **3a-3c** with NaI in acetone produced the iodide compounds **4a-4c**, which was subjected to remove the Boc- protecting group by TFA. Then, the compounds **5a-5c** reacted with oxaloyl chloride and *in situ* amidated with lenalidomide in one pot to give compounds **6a-6c**.

The synthetic route of intermediate **15** was described in Scheme 2. Nucleophilic substitution of compound **7** and **8** produced **9** under acidic condition in an excellent yield. Subsequently, compound **9** reacted with *tert*-butyl methyl(2-(methylamino)ethyl) carbamate **10** to give **11**, followed by the reduction of nitro group with the treatment of iron powder and NH_4Cl to afford compound **12**. Furthermore, the amidation of **12** with 3-chloropropionyl chloride and then elimination expelled HCl to produce acrylamide derivative **14** in the presence of triethylamine under reflux. Finally, after removal of Boc- protecting group with treatment of TFA, alkylation of **15** with iodide compounds **6a-6c** resulted in the desired PROTACs **16a-16c**, respectively (Scheme 2).



Scheme 1. General synthesis of compounds **6a-6c**. Reagents and conditions: (i) TsCl, Et_3N, CH_2Cl_2, 0 ℃ to r.t, 35%-48% yield; (ii) *tert*-butyl bromoacetate, TBAB, KOH, toluene, r.t, 60%-73% yield; (iii) NaI, acetone, reflux, 92%-97% yield; (iv) TFA, CH_2Cl_2, r.t, 73%-80% yield; (v) a) $(COCl)_2$, DMF(cat.), 0 ℃; b) lenalidomide, DMF, r.t, 40%-50%. (Answer 3 to reviewer 1)



Scheme 2. Synthesis of PROTACs **16a~16c**. Reagents and conditions: (i) *p*-TsOH, 1,4-dioxane, reflux, 95% yield; (ii) DIPEA, 90 ℃, 82% yield; (iii) Fe, NH_4Cl, EtOH, H_2O, 57% yield; (iv) 3-chloropropionyl chloride, Et_3N, DMF, 80% yield; (v) Et_3N, MeCN, 80 ℃, 70% yield; (vi) TFA, DCM, 94% yield; (vii) **6a-6c**, DIPEA, DMF, 90 ℃, 53%-62% yield. (Answer 3 to reviewer 1)

Next, we evaluated the biological activity of these compounds in vitro. The antiproliferative activities in vitro of the PROTACs **16a-16c** against human cancer cells PC9 cells ($EGFR^{Del19}$), HCC827 cells ($EGFR^{Del\ E746\text{-}A750}$, also known as $EGFR^{Del19}$) and H1975 cells ($EGFR^{L858R/T790M}$) were evaluated by MTT assay. The results were summarized in Table 1. As shown in Table 1, the length of the linker had pronounced effects on anti-proliferative activity. All the tested compounds exhibited good biological activities against human cancer cells PC9 cells, HCC827 cells and H1975 cells. Especially, compound **16c** has simultaneously better inhibitory effect on PC9 cells and H1975 cells and its corresponding IC_{50} value is 0.413 μM and 0.657 μM, respectively. Furthermore, compounds **16a-16c** were allowed to determine EGFR degradation activity against PC9 cells and H1975 cells.

The western blotting analysis revealed that all the tested compounds **16a-16c** displayed moderate to potent degradation activity against PC9 cells and H1975 cells at the concentration of 10 μM (Fig. 2). According to the anti-proliferative activity of the compounds **16a-16c**, compound **16c** was preferred to be selected to further determine the degradation of EGFR in PC9 cells. We set six concentration gradients ranged from 0.1 μM to 10 μM to determine the degradation activity and calculated the DC_{50} values (Fig. 2B). The results revealed that compound **16c** can induce degradation of EGFR in a concentration dependent manner in PC9 cells. From the concentration-dependent of compound **16c**, we observed a distinct "hook effect'' on EGFR degradation and reached the maximum degradation rate (Dmax = 68%) with treatment for 6 h at the concentration of 0.3 μM. The corresponding DC_{50} value of compound **16c** is 0.161 μM. (Answer 1 to reviewer 2)

Moreover, the time-dependent degradation activities of compound **16c** at the concentration of 0.3 μM were also investigated (Fig. 2C). The amount EGFR protein was decreased as the administration time was prolonged. Wherein compound **16c** reached the maximum degradation rate (Dmax = 56%) at 6 h. These data suggests that the degradation of transmembrane protein EGFR is a very time-consuming process. Unexpectedly, compound **16c** stimulated the PC9 cells to cause a stress effect and increased the expression of EGFR for a certain period of time (Fig. 2C).

In order to further investigate the mechanism of EGFR protein degradation, the proteasome inhibitor MG-132 and lenalidomide were pretreated with the cells before the treatment of the compound **16c**, respectively. As shown in Fig. 2D, the protein degradation property of compound **16c** was disrupted and almost disappeared due to the presence of MG-132 and lenalidomide. The results suggested that the EGFR protein was likely to be degraded through ubiquitination. In addition, the immunofluorescence microscopy analysis illustrated that compound **16c** could also effectively induce the degradation of EGFR protein (Fig. 3).

Table 1.
Antiproliferative activities of compounds against different cells (n = 3, X ± SD).

compound	IC_{50}(μM)		
	PC9 Cells	HCC827 Cells	H1975 Cells
16a	1.425 ± 0.052	1.874 ± 0.098	1.388 ± 0.556
16b	1.117 ± 0.105	1.611 ± 0.139	1.698 ± 0.614
16c	0.413 ± 0.087	1.344 ± 0.112	0.657 ± 0.008

T icro Confocal/Widefield after the treatment with 0.5 μM of compound **16c** for 24 h. As shown in Fig. 4, the results demonstrated that the degrader **16c** could arrest the PC9 cells in G0/G1 phase and caused 38.6% of apoptosis in PC9 cell.

In conclusion, we have developed a new class of PROTACs EGFR degraders which were based on osimertinib and lenalidomide. All of the compounds were evaluated for antiproliferative effects in vitro, and showed a significant inhibitory effect in PC9 cells, HCC827 cells and H1975 cells. The western blotting assays analyzed the effect of EGFR degradation and the results showed that compound **16c** could effectively degrade EGFR protein through ubiquitination and reached the maximum degradation rate (D_{max} = 68%) in PC9 cells. Meanwhile, compound **16c** could significantly induce the apoptosis of PC9 cells and arrest the cells in G0/G1 phase. These findings demonstrated that $EGFR^{Del19}$ in PC9 cells can be effectively targeted for degradation by our designed PROTACs. On the basis of the potent degradation activity on $EGFR^{Del19}$ in PC9 cells demonstrated here, further evaluations of degradation activity on $EGFR^{C797S}$ are ongoing in our lab and the data will be reported in due courses.

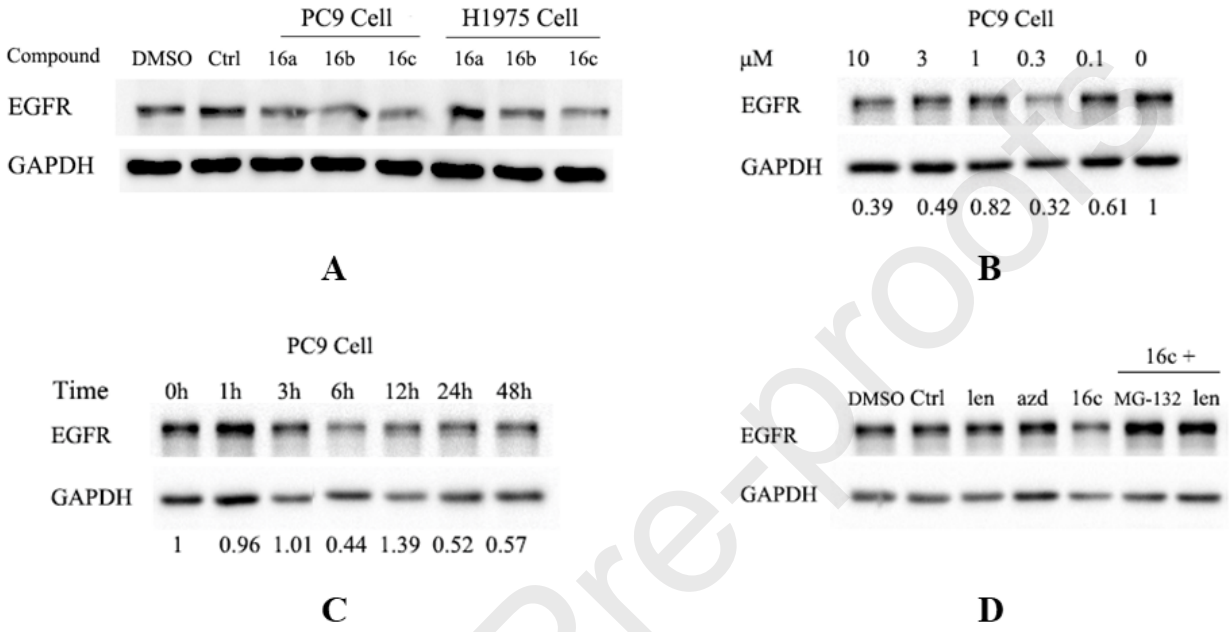


Fig. 2. The western blotting analysis of EGFR protein degradation. (A) PC9 Cells and H1975 cells were treated for 24 h with the concentration 10 μM. (B) The concentration-dependent EGFR degradation by PROTAC **16c** in PC9 cells. Cells were treated for 6h with the concentration from 0.1 μM to 10 μM. (C) The time-dependent degradation by PROTAC **16c** in PC9 cells. Cells were treated with 0.3 μM for indicated time points. (D) The mechanistic of EGFR degradation with PROTAC **16c**, PC9 Cells were pretreated with lenalidomide (len, 10 μM), and MG-132 (10 μM) for 2 h, followed by 6 h treatment with 0.3 μM of PROTAC **16c**.

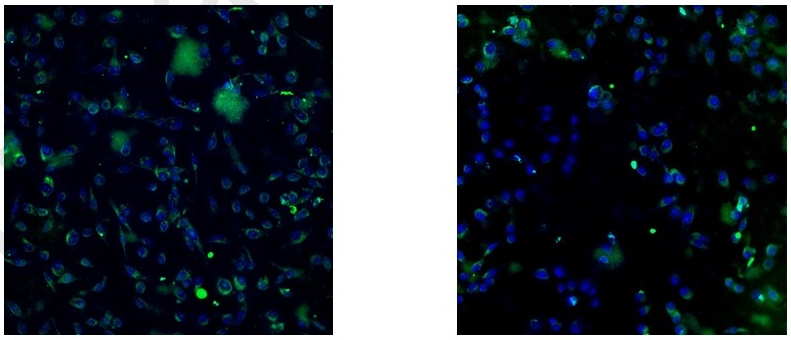


Fig. 3. Representative immunofluorescence microscopy images of EGFR (green) internalization in response to compound **16c** (0.3 μM) at 24 h and DAPI nuclear stain in blue.

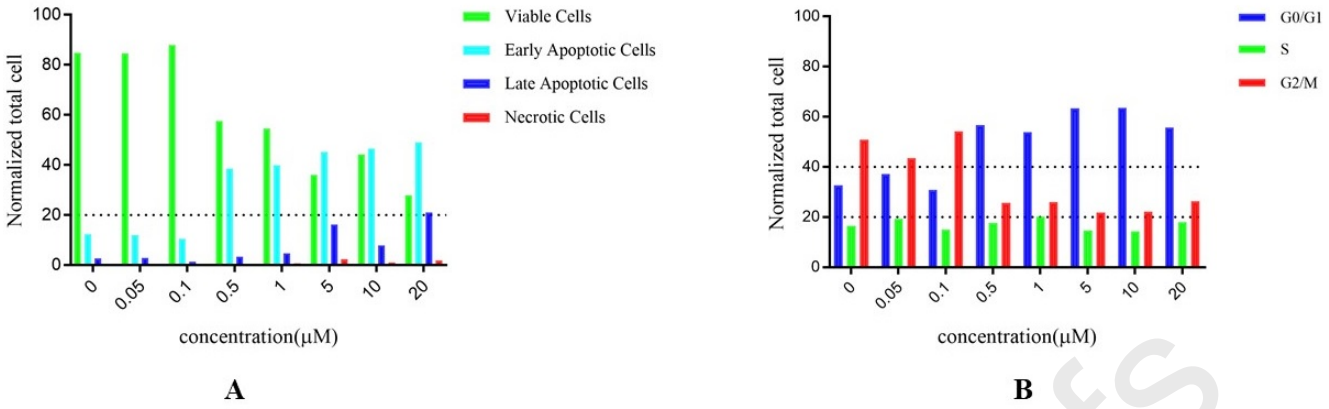


Fig. 4. The cell health and cycle of compound **16c** in PC9 cells

Declaration of Competing Interest

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.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at (Answer 5 to editor)

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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:

Graphical Abstract

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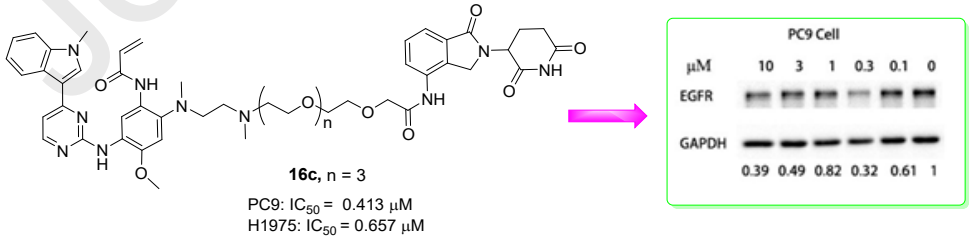

Highlights

- We designed and synthesized novel PROTACs by tethering the third generation EGFR-TKI osimertinib with CRBN ligands.
- Compounds **16c** (D_{max} = 68%) potently degraded $EGFR^{Del19}$ in PC9 cells.
- Compounds **16c** could significantly induce the apoptosis of PC9 cells and arrest the cells in G0/G1 phase.