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LETTER TO THE EDITOR

DiPTAC: A degradation platform *via* directly targeting proteasome



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To the Editor:

Targeted protein degradation (TPD) has emerged as a powerful therapeutic strategy for the treatment of various diseases, including cancer, that are associated with aberrant high levels of pathogenic proteins¹. Recently, several strategies have been investigated to address TPD, including targeting E2, E3 for POI degradation², or employing DUBs to stabilize POI³. Among them, proteolysis targeting chimeras (PROTACs) have emerged as the predominant approach in both preclinical and clinical investigations by connecting an E3 recruiting ligand to a POI ligand⁴.

Proteasome, the primary protease responsible for proteolysis in eukaryotes, is a more central and widely present component in the UPS, consisting mainly of the 19S and 20S subcomplexes⁵. Recently, heterobifunctional molecules targeting the 19S subunit RPN13 or PSMD2 for POI degradation *via* 26S proteasome were reported^{6–8}. Compared to targeting the 19S regulatory particle, targeting the 20S core particle enables simultaneous delivery of the POI to both the 20S and 26S proteasomes. In this study, several small molecular proteasome activators that allosterically bind to the α -subunits of the 20S proteasome were selected as ligands for proteasome recruitment⁹. The innovative degradation strategy that anchors the proteasome is referred to as Direct-Proteasome Targeting Chimera (DiPTAC), utilizing the POI endogenous degradation system, circumventing the uncertainties associated with E2s or E3s.

1. Screening of the appropriate 20S proteasome ligand for DiPTAC

Heterobifunctional molecules were designed by conjugating different proteasome agonists such as ursolic acid (UA) and oleanolic acid (OA) to the ligand of POIs with a linker (Fig. 1A). Firstly, we detected the proteasome agonistic activities of the proteasome ligands in the molecular assay (Supporting Information Table S1). Then, the exogenous Halotag and endogenous CDK9 system were conducted to screen for the appropriate ligands targeting the 20S proteasome.

A series of heterobifunctional molecules targeting Halotag were evaluated in 293T cells expressing HA-EGFP-HaloTag2 fusion protein, using the Incucyte system, where mCherry served as an internal control (Supporting Information Fig. S1A and S1B). Immunoblotting (Fig. S1C) also revealed that these DiPTAC molecules exhibited diverse degrees of efficacy in degrading the fusion protein, whereas treatment with proteasome agonists alone did not demonstrate this capability (Fig. S1D), indicating the feasibility of DiPTAC for TPD. Subsequently, a series of potential CDK9 DiPTACs based on the CDK9 inhibitor SNS-032 were evaluated (Fig. S1E). Among them, 10 μ mol/L of SNS-UA, SNS-OA, and SNS-oxo-OA exhibited a certain level of degradation capacity towards CDK9 (Fig. S1F). Based on the degradation outcomes of both Halotag and CDK9, OA was selected as the proteasome ligand for the subsequent experiments (Fig. 1B, Supporting Information Table S2).

To provide additional evidence supporting OA as a proteasome ligand for the construction of DiPTAC, we designed and synthesized an AMC-modified OA derivative AMC-OA. Immunofluorescence analysis demonstrated the co-localization of AMC-OA with PSMA6, a subunit of the proteasome, in the cells (Fig. 1C). Next, cycloheximide (CHX) chase assay revealed that HLT-OA could significantly reduce HALOTAG protein stability, while SNS-OA could significantly reduce CDK9 protein stability (Fig. S1G and S1H). And, proteasome inhibitor could effectively reverse the HLT-OA and SNS-OA induced protein degradation

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Figure 1 DiPTAC: a degradation platform *via* directly targeting proteasome. (A) Schematic of the DiPTAC design concept. (B) Degradation levels of CDK9 and Halotag by DiPTAC molecules utilizing various proteasome agonists as proteasome ligands. $n = 3$, biologically independent experiments, data are presented as a mean of the degradation level (mean $\pm$ SD are shown in Table S2). (C) Immunofluorescence colocalization analysis of AMC-OA (Green) with PSMA6 (Red) in 293T cells, scale bar = 50 μ m. (D) Chemical structures of YQ-25. (E) Immunoblot following indicated times of incubation with DMSO or 10 μ mol/L YQ-25 in NCI-H1299 cells. (F) Immunoblot following 12 h of incubation with DMSO or indicated concentrations of YQ-25 in NCI-H1299 cells. (G) Quantified CDK9 levels in Fig. 1F. DC_{50} was calculated by nonlinear regression. (H) Quantified mean fluorescence intensity (MFI) of CDK9 in NCI-H1299 cells by Opera Phenix after treatment with indicated concentrations for 8 h, $n = 6$, six technical replicates from two independent experiments were performed for each condition. (I) Global quantitative proteomic analysis of NCI-H1299 cells after treatment with 10 μ mol/L YQ-25 for 8 h. A volcano plot showing protein content ($\log_2$) as a function of significance level ($\log_{10}$), the differentially expressed proteins were identified as $\log_2FC > 1.5$ or < -1.5 and FDR-adjusted P value < 0.01 . Blue markers represent significantly downregulated proteins, red markers represent significantly upregulated proteins. (J, K) 293T cells were pretreated with DMSO, YQ-25, or YQ-30 for 2 h and lysed. CETSA was performed with gradient temperature, and samples were analyzed by Western blot. (L) Quantified CDK9 levels of immunoblot in 293T cells treated with AMC-OA or YQ-25. (M) Quantified CDK9 levels of immunoblot in 293T cells treated with SNS-032 or YQ-25. (N) Immunoblot in 293T cells treated with YQ-25 after pre-treatment with PYR-41 or MLN4924 or PS341. (O) Quantified CDK9 levels in Fig. 1N. (P) Quantified CDK9 levels of immunoblot in NCI-H1299 cells treated with YQ-25 or CDK9-PROTAC following RNA interference of PSMD6 or PSMD2. (Q) Immunoblot in 293T cells treated with YQ-25 or CDK9 PROTAC following over-expression of CDK9 WT or CDK9 mut proteins. (R) Quantified CDK9 levels in Fig. 1Q. (S) Immunoblot in NCI-H1299 cells treated with CDK9 PROTAC or YQ-25. (T-U): NCI-H1299 cells treated with CDK9 PROTAC or YQ-25 were incubated with Incucyte[®] Annexin V Red Dye, and fluorescence emission was measured in the IncuCyte imaging platform every 2 h for 22 h at 37 $^{\circ}$ C. (T) Real-time apoptosis measurements are shown. (U) Real-time cell proliferation measurements are shown as cell confluence. All qualified data are biologically independent experiments and presented as mean $\pm$ SD ($n = 3$). P values were calculated using a t -test. ns, not significant. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

(Fig. S1I and S1J), demonstrating that DiPTAC degrades POI in a proteasome-dependent manner. These observations demonstrated that the OA-based DiPTAC strategy can be employed for the degradation of both exogenous (HALOTAG) and endogenous (CDK9) proteins.

2. The optimization of CDK9 DiPTAC

OA and SNS-032 were selected and retained as direct ligands for targeting the proteasome and CDK9, respectively, and various types of linkers were screened for degrader optimization (Supporting Information Figs. S2 and S3). Notably, among the tested compounds, YQ-25 exhibited the most remarkable activity (Fig. 1D), which degraded CDK9 in a time- and dose-dependent manner (Fig. 1E and F, Supporting Information Fig. S4A and S4B) with a half-degrading concentration value (DC_{50}) of $\sim 2.96 \mu\text{mol/L}$ in NCI-H1299 cells (Fig. 1G). Visualization and quantification of CDK9 by immunofluorescence assay following YQ-25 treatment showed significantly diminished CDK9 signals (Fig. 1H, Fig. S4C and S4D). As expected, YQ-25 treatment could significantly reduce CDK9 protein stability (Fig. S4E and S4F).

To unbiasedly explore the degradation targets of YQ-25, we performed a global quantitative proteomic on NCI-H1299 cells treated with $10 \mu\text{mol/L}$ of YQ-25 for 8 h. A total of 6910 proteins were detected in the proteome, of which CDK9 protein levels were significantly reduced (Fig. 1I, Supporting Information Table S3). Moreover, YQ-25 demonstrated effective degradation of CDK9 in diverse tumor cell lines (Fig. S4G). Overall, these findings provide compelling evidence for the efficacy and selectivity of YQ-25 in degrading CDK9.

Then, CETSA experiments showed that YQ-25 induced thermal stabilization of both CDK9 and PSMA6 in cell lysates (Fig. 1J and K), indicating the direct engagement of YQ-25 with CDK9 and proteasome in a physiological environment. To assess the interaction between YQ-25 and PSMA6 more comprehensively, we conducted microscale thermophoresis (MST) experiments. The finding revealed that the equilibrium dissociation constant (K_D) for the binding of YQ-25 to PSMA6 was determined to be $1.69 \mu\text{mol/L}$ (Supporting Information Fig. S5A).

To assess the ligand dependency of CDK9 degradation, the ligand occupancy experiments were performed and the results revealed that YQ-25-induced degradation was significantly attenuated after 2 h of occupancy with either SNS-032 or AMC-OA (Fig. 1L and M, Fig. S5B and S5C), demonstrating that both CDK9 and proteasome binding are necessary for degradation. To further establish the dependence of YQ-25-induced CDK9 degradation on proteasome binding, we utilized 18β -glycyrrhetic acid, an OA analogue that lacks agonist effect on the 20S proteasome, to develop a negative compound, YQ-30 (Fig. S5D). As anticipated, YQ-30 did not induce CDK9 degradation (Fig. S5E, Supporting Information Table S4). Consistently, YQ-30 exhibited interaction with CDK9 but failed to bind to PSMA6 in cell lysates (Fig. 1J and K) and was unable to bind to PSMA6 in MST assay (Fig. S5F). Such evidence indicated that YQ-25 induced CDK9 degradation by bringing CDK9 into proximity with PSMA6.

3. CDK9 DiPTAC induced CDK9 degradation in proteasome-dependent and ubiquitin-modification-independent manner

Pre-treatment with either the ubiquitin-activating enzyme (E1) inhibitor PYR-41 or the proteasome inhibitor PS341 was able to

rescue CDK9 degradation by YQ-25 in both 293T and NCI-H1299 cells (Fig. 1N and O, Supporting Information Fig. S6A and S6B). While, the NEDD8-activating enzyme inhibitor MLN4924 could significantly reverse the degradation effect of CDK9 PROTAC (Fig. S6C), as previously reported¹⁰, but could only slightly rescue the degradation of CDK9 induced by YQ-25 (Fig. 1N and O, Fig. S6A and S6B). MLN4924 inhibits Cullin-RING ligase (CRL) E3s indirectly by blocking Cullin neddylation, which is required for the activity of the holoenzyme ubiquitin ligase. The interesting results indicated that not all the endogenous E3 ubiquitin ligases of CDK9 are CRL E3s.

PSMD2 is a subunit of the 19S proteasome and the siPSMD2 cells harbor an abnormally high ratio of 20S to 30S/26S proteasomes¹¹. Consistently, accumulation of polyubiquitinated proteins was observed in PSMD2 knockdown cells, whereas not in PSMA6 knockdown cells (Fig. S6D, Supporting Information Table S5). PSMA6 knockdown could partially reverse YQ-25-induced CDK9 degradation, suggesting that YQ-25 requires binding to PSMA6 in order to facilitate the proximity of CDK9 to the proteasome. PSMD2 knockdown could partially reverse the PROTAC-induced CDK9 down-regulation, aligning with that PROTAC induced POI tagged with Ub, then are destined for the 26S proteasome for degradation. Intriguingly, PSMD2 Knockdown does not reverse YQ-25-induced degradation, as DiPTAC directly delivers the target proteins to the proteasome, either the 20S or 26S, without necessitating recognition by the ubiquitin receptor on the 19S subunit (Fig. 1P, Fig. S6E).

To minimize the potential impact on overall intracellular ubiquitination caused by PYR-41, we constructed a specific mutation in the potential ubiquitination sites of CDK9. Five potential ubiquitination sites (K3, K56, K151, K164, and K178) were predicted using GPS-Uber¹² and were then mutated to arginine. Subsequently, the mutant CDK9 and wild-type CDK9 were overexpressed in both 293T and NCI-H1299 cells. CDK9-PROTAC-induced degradation was significantly abolished in CDK9-mutant cells, compared to wild-type CDK9 cells, as expected. Intriguingly, the mutation in the ubiquitination modification sites of CDK9 showed little effect on YQ-25-induced CDK9 degradation (Fig. 1Q and R, Fig. S6F and S6G), indicating that the protein degradation induced by DiPTAC was not dependent on the ubiquitination modification.

4. The combination effect of CDK9 DiPTAC and CDK9 PROTAC

Since YQ-25 was able to increase the proximity of CDK9 to the proteasome, we hypothesized that DiPTAC could improve the degradation of artificially ubiquitinated CDK9 induced by the PROTAC molecule. To investigate this hypothesis, co-treatment with YQ-25 was performed with 100 nmol/L CDK9 PROTAC, at which concentration minimal degradation of CDK9 was observed. The result showed that the maximum degradation of CDK9 protein was achieved when both compounds were used (Fig. 1S). The transcription-associated kinases phosphorylate serine residues located within the hepta-repeat sequence Y1S2P3T4S5P6S7 of RPB1, the largest subunit of RNA polymerase II (Pol II), and the CDK9/CYCLIN T complex phosphorylates serine 2 to recruit other factors that are necessary for productive elongation. Consistent with the degradation, the combination also induced a significant reduction in serine 2 phosphorylation, while serine 5 and serine 7 were not noticeably unaffected (Fig. S6H). Afterward, we examined the impact of co-treatment on tumor cell

viability and observed that following 22 h of incubation, co-administration of YQ-25 and CDK9 PROTAC significantly promoted apoptosis in NCI-H1299 cells (Fig. 1T), as well as inhibited cell proliferation (Fig. 1U).

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Author contributions

Yutong Tu, Qian Yu, Yubo Zhou, Jiankang Zhang, and Jia Li designed the research. Yutong Tu carried out the experiments and performed data analysis. Mengna Li, Lixin Gao, Jialuo Mao, Jingkun Ma, Xiaowu Dong, Jinxin Che, Chong Zhang, Linghui Zeng, Huajian Zhu, Jiaan Shao, Jingli Hou, and Liming Hu participated part of the experiments. Qian Yu provided experimental drugs and quality control. Yutong Tu, Qian Yu, Yubo Zhou, and Jiankang Zhang wrote the manuscript. Yutong Tu, Qian Yu, Yubo Zhou, and Jiankang Zhang revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2024.09.003>.

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