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LETTER TO THE EDITOR

Optimizing TPD-based oncology drug development: the critical role of PDR distribution in POI-positive cancer cells

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Tumor organoid

To the editor:

Targeted protein degradation (TPD) drugs are designed to link the protein of interest (POI) and protein degradation regulator (PDR), thus aiding in the degradation of the POI through cellular degradation mechanisms, including the ubiquitin–proteasomal and lysosomal pathways. The lysosomal pathway can be further divided into the endosomal–lysosomal degradation pathway and the autophagic–lysosomal degradation pathway. Proteolysis-targeting chimeras (PROTAC), molecular glue, and chaperone-mediated protein degradation (CHAMP) are designed for targeting the ubiquitin–proteasomal degradation pathway. Technologies utilizing the endosomal–lysosomal pathway for protein degradation include AbTAC and PROTAB, while those leveraging the autophagic–lysosomal pathway include autophagosome-tethering compound (ATTEC), autophagy-targeting chimera (AUTOTAC), and chaperone-mediated autophagy-based degraders (CMA). Recently, *Acta Pharmaceutica Sinica B* reported a new molecular glue that uses damage specific DNA binding protein 1 (DDB1) as an E3 ligase to degrade phosphoglycerate dehydrogenase (PHGDH)[1]. We have noticed an increasing trend in using novel E3 ligases, rather than traditional E3 ligases like cereblon (CRBN) and von hippel-lindau (VHL), for designing molecular glue and PROTAC drugs. The main reason for selecting new E3 ligases is to find tissue-specific E3 ligases to enhance the tissue selectivity of protein degraders and reduce on-target toxicity. In addition to finding tumor-specific E3 ligases, we believe that the co-expression of E3 ligases and target proteins in the same tumor cells is crucial for selecting appropriate E3 ligases as protein degraders for oncology drug development. Unlike traditional small molecule drugs that only require the presence of the POI in cells, protein degraders need both the POI and the corresponding E3 ligase to be present in cells to degrade target proteins and achieve drug efficacy.

However, most protein degradation drugs currently target tumors, which are heterogeneous. This results in E3 ligases not being expressed in all tumor cells. For example, CRBN and VHL are common E3 ligases used in PROTAC drug design, but our previous study found that in prostate cancer (PC), breast cancer (BC), and acute myeloid leukemia (AML) tumor cells, the percentage of $CRBN^{+}POI^{+}$or $VHL^{+}POI^{+}$double-positive cells does not reach 50%[2]. This means that at least fifty percent of tumor cells will not respond to these PROTAC drugs. Therefore, when developing anti-cancer protein degraders, E3 ligases expressed in the majority of POI-positive tumor cells should be selected for designing E3 ligase warheads. In this study, we used single-cell RNA sequencing data from PC, BC, and AML to analyze the percentage of double-positive ($E3\ ligase^{+}POI^{+}$) cancer cells in POI-positive (POI^{+}) cancer cells in PROTAC and molecular glue strategies, aiming to identify which E3 ligases are most highly expressed in POI-positive tumor cells.

1. Eight newly identified E3 ligases are expressed in more than 50% of POI^{+} PC cells, but none surpassed 50% in POI^{+} BC and AML cells

Currently, numerous PROTAC and molecular glue drugs are undergoing clinical trials, including PC, BC and AML. Most of these

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drugs use androgen receptor (AR), estrogen receptor 1 (ESR1) and G1 to S phase transition 1 (GSPT1) as POI in PC, BC and AML, respectively. As the percentage of $E3^+POI^+$ cancer cells in POI^+ cancer cells was less than 50% for the listed PROTAC or molecular glue drugs in clinical trials, evidenced by our bioinformatics analysis (Supporting Information Table S1), we endeavored to identify an alternative E3 that is expressed in the majority of POI^+ cancer cells. First, we assessed the E3 sourced from clinical drugs, PROTAC experimental data or the most promising E3s identified by Liu et al.[3] and Kannt et al.[4]. Our findings revealed that the percentage of $SH3RF1^+AR^+$ cancer cells in AR^+ cancer cells was 74.56%, much higher than the known $E3^+AR^+$ used in clinical trials, such as $CRBN^+AR^+$ and VHL^+AR^+ (Fig. 1A and Supporting Information Fig. S1). SH3 domain-containing ring finger 1 (SH3RF1) is a protein that is part of the SH3RF family, known for containing SH3 domains and a RING finger domain. The RING finger domain in SH3RF1 suggests it can function as an E3 ubiquitin ligase. The RING domain typically mediates the interaction with E2 enzymes and facilitates the transfer of ubiquitin to target proteins. SH3RF1 functions as a negative post-translational regulator of atypical calmodulin 1 (FAT1), which is indispensable for the regulation of cell proliferation[5]. SH3RF1 exhibits the ligandability of $E3^3$ and facilitates K63-linked ubiquitination of SHVV P at the K264 site[6], suggesting SH3RF1 functions as an E3 ubiquitin ligase. In our analysis, we found in addition to SH3RF1, X-linked inhibitor of apoptosis (XIAP), CCR4−NOT transcription complex subunit 4 (CNOT4), DDB1, baculoviral IAP repeat containing 2 (BIRC2), tripartite motif containing 24 (TRIM24), ring finger protein 114 (RNF114), and helicase like transcription factor (HLTF) are highly expressed in AR^+PC cells, with double-positive rates exceeding 50%. Notably, drugs using XIAP, DDB1, BIRC2, and RNF114 as E3 ligases are already under development. For example, PROTAC drugs that recruit XIAP E3 ligase have been demonstrated to effectively degrade B-cell lymphoma-extra (BCL-X) in the malignant T-cell lymphoma cell line MyLa 1929[7]. (*R*)-CR8 is a cell cycle protein-dependent kinase (CDK) inhibitor that acts as a molecular glue-degrading compound. The CDK-bound form of CR8 has a solvent-exposed pyridyl portion that induces the formation of a complex between CDK12−cyclin K and the cullin4 (CUL4) adaptor protein, DDB1. This complex bypasses the need for a substrate receptor and presents cell cycle protein K for ubiquitylation and degradation. The PROTAC was also developed with the objective of targeting bromodomain containing 4 (BRD4) and by targeting DDB1. The AR PROTAC was shown to degrade the AR protein in prostate cancer cells[8]. The synthesized covalent ligand EN219, which targets RNF114 and mimics the mode of action of the natural product nimbolide, has been observed to degrade novel substrate proteins in an RNF114-dependent manner, to degrade therapeutically relevant targets, and to demonstrate the most potent BRD4 degradation in 231MFP breast cancer cells, and a strong BCR-ABL degradation in K562 leukemia cells[9]. The objective of the design and synthesis of an inducer of CRABPs degradation was to utilize the ubiquitin E3 ligase activity of BIRC2. The compound has been demonstrated to inhibit the migration of neuroblastoma IMR-32 cells in cellular systems[10].

Unfortunately, no E3 ligase is expressed in more than 50% of ESR1-positive breast cancer cells. Among these cells, RNF114 is the most expressed, with $RNF114^+ESR1^+$ cells making up 38.14%. Similarly, no E3 ligase is expressed in more than 50% of GSPT1-positive AML cells. The aryl hydrocarbon receptor (AHR) is the most expressed in these cells, with AHR^+GSPT1^+ cells accounting for 35.37% of $GSPT1^+$ AML cells (Fig. 1A). PROTAC and molecular glue use E3 ligase to degrade POI. However, our sequencing analysis revealed that no E3 ligase is expressed in more than 50% of POI-positive tumor cells in BC and AML. Therefore, PROTAC or molecular glue may not be suitable for developing oncology drugs for BC and AML. Alternative protein degradation strategies for these cancers need to be explored (Fig. 1A).

2. HSP90AB1 is expressed in over 80% of POI-positive tumor cells, highlighting the promise of CHAMP

In addition to the ubiquitin−proteasomal system, lysosomes offer crucial protein degradation pathway. Emerging technologies capitalize on lysosomal degradation, including ATTEC AUTOTAC, and CMA (Supporting Information Fig. S2A and S2B). In addition, LYTAC targets extracellular and membrane proteins, and it connects to the target protein at one end and the cation-independent mannose-6-phosphate receptor (CI-M6PR) at the other end to target POI for lysosomal degradation. ATTEC features one end targeting POI and the other end targeting microtubule-associated protein 1A/1B light chain 3 (MAP1LC3B), a specific protein on the surface of autophagosomes. This facilitates the wrapping of POI into autophagosomes, ultimately translocating it to the lysosome for degradation. AUTOTAC comprises a sequestosome 1 (SQSTM1) ligand at one end and a POI ligand at the other, connected by a linker. Simultaneous binding of AUTOTAC to both SQSTM1 and POI forms a ternary complex, inducing a conformational change in SQSTM1. This triggers SQSTM1 oligomerization and exposes the MAP1LC3B binding domain, mediating POI degradation *via* the lysosomal pathway. CMA, a novel TPD technology, utilizes heat shock cognate protein 70 (HSC70, also known as HSPA8). CMA is a peptide drug comprising three functional domains: a cell membrane-penetrating sequence, a POI-binding sequence and a CMA targeting motif. The CMA targeting motif contains KFERQ sequence specifically recognizes and binds to HSC70. The HSC70−CMA−POI complex then binds to lysosome-associated membrane protein 2A (LAMP2A), promoting LAMP2A multimerization and subsequent POI translocation to the lysosome for degradation.

To compare their theoretical degradation efficiencies in degrading intracellular POIs, the clinical POIs, AR, ESR1 and GSPT1 were used in subsequent analysis of PC, BC and AML, respectively. In PC, the percentage of PDR^+AR^+ cancer cells in AR^+ cancer cells for ATTEC, CMA, AUTOTAC, and PROTAC/molecular glue were 61.62%, 55.16%, 55.04% and 41.90%, respectively (Fig. 1B). In BC, the percentage of PDR^+ESR1^+ cancer cells in $ESR1^+$ cancer cells for ATTEC, CMA, AUTOTAC and PROTAC/molecular glue was 42.47%, 39.57%, 34.64% and 21.82%, respectively (Fig. 1B). These results suggest that ATTEC might be a more effective TPD technology for the treatment of PC and BC. In AML, the percentage of PDR^+GSPT1^+ cancer cells in $GSPT1^+$ cancer cells for CMA was 44.14%, which was higher than that of PROTAC/molecular glue, ATTEC and AUTOTAC (Fig. 1B). These findings indicate that in PC, ATTEC drugs elicited better responses in AR^+ tumor cells compared to PROTAC drugs. However, in BC and AML, no PDR was expressed in more than 50% of POI-positive tumor cells. This suggests that theoretically, the therapeutic efficacy of ATTEC, CMA, and AUTOTAC in BC and AML may be constrained.

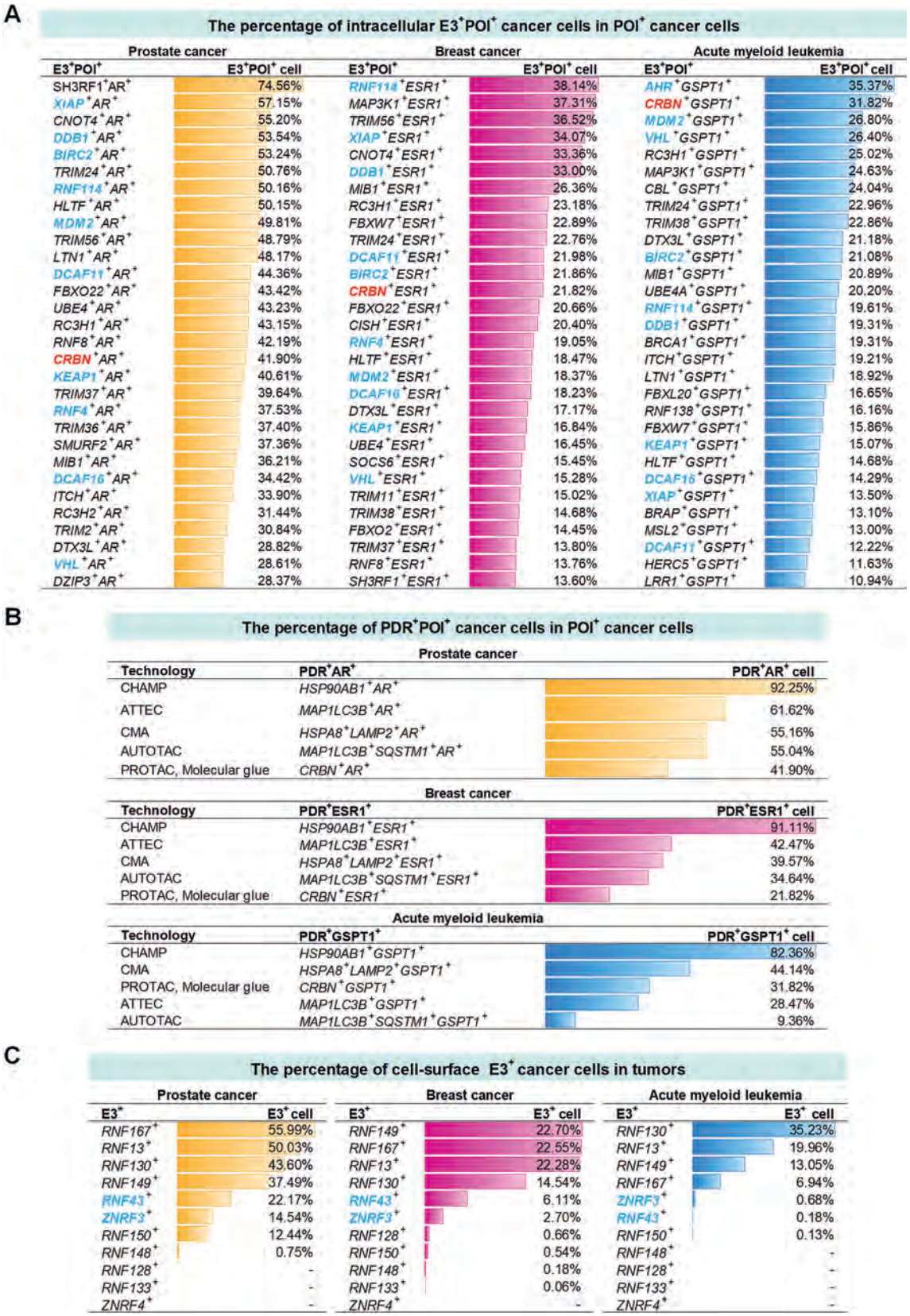

Figure 1 Optimization of degradation efficiency of protein degradation regulation. (A) The percentage of $E3^{+}POI^{+}$ cancer cells in POI^{+} cancer cells in PC, BC and AML. E3s in red present E3s of PROTAC or molecular glue used in clinical trials. E3s in blue present E3s explored in PROTAC or molecular glue experiments. Full list was shown in Fig. S1. (B, C) Comparison of degradation efficiencies of TPD technologies for degradable POIs. (B) The percentage of $PDR^{+}POI^{+}$ cancer cells in POI^{+} cancer cells in PC, BC and AML. (C) The percentage of cell-surface $E3^{+}$ cancer cells in tumors. These E3s belong to The PA-TM RING family as *RNF43*. E3s in blue present the first E3s explored in PROTAB or AbTAC experiments.

Unlike CMA, CHAMP utilizes molecular chaperones for protein degradation and ultimately degrades the target protein through the ubiquitination−proteasome system. This system consists of a conjugate of the target protein, a linker, and a conjugate of the molecular chaperone heat shock protein 90 (HSP90). HSP90 binds to multiple E3 scaffolding proteins and these scaffolding proteins bind to a variety of E3 substrate-binding proteins[2]. The unique feature of CHAMP technology is that multiple E3s can be recruited for protein degradation (Fig. S2B). In the cytoplasm, there exist two isoforms of HSP90 protein: heat shock protein 90 alpha family class A member 1 (HSP90 AA1) and heat shock protein 90 alpha family class B member 1 (HSP90 AB1). These isoforms share 86% amino acid sequence identity. In PC, BC and AML, the proportions of *HSP90AA1*$^{+}$POI $^{+}$ cancer cells were 62.78%, 85.04%, and 84.14%, respectively (Supporting Information Fig. S3). Meanwhile, *HSP90AB1*$^{+}$POI $^{+}$ cancer cells accounted for 92.25%, 91.11%, and 82.36% of the above cancers, respectively (Fig. 1B). HSP90 are highly expressed in majority of cancer cells, and HSP90 could form complex with multiple E3s, providing unique advantages for CHAMP technology in cancer therapy.

3. Optimizing PDR selection for enhancing degradation efficiency

TPD drugs leverage natural intracellular degradation systems to degrade proteins of interest. This approach enables the targeting of previously inaccessible or undruggable proteins, expanding the therapeutic landscape. However, current PROTAC and molecular glue technologies primarily utilize the E3, CRBN. The proportion of cancer cells expressing CRBN, as well as the corresponding target proteins, is relatively low in PC, BC and AML of cancer cells[2]. This issue is prevalent across many TPD technologies, including ATTEC, CMA, and AUTOTAC. Additionally, ring finger protein 43 (RNF43) and zinc and ring finger 3 (ZNRF3) are the main transmembrane E3s used in antibody-based PROTAC (AbTAC) and proteolysis-targeting antibodies (PROTAB) (Fig. S2B). One arm of AbTAC/PROTAB connects the transmembrane E3 (mainly RNF43 and ZNRF3), while the other end connects the POI to be degraded, then RNF43/ZNRF3 can induce endocytosis of POIs into cells by ubiquitination of POIs. The POIs can then be degraded by lysosomes or proteasomes. Study shows that proteins of the PA-TM-RING family can also be used for designing PROTAB. Our findings indicate that the percentage of ring finger protein 167-positive (*RNF167*$^{+}$), ring finger protein 13-positive (*RNF13*$^{+}$), ring finger protein 130-positive (*RNF130*$^{+}$) or ring finger protein 149-positive (*RNF149*$^{+}$) cancer cells in tumors is significantly higher—ranging from 2-fold to 50-fold—than that of *RNF43*$^{+}$ or *ZNRF3*$^{+}$ cancer cells in tumors (Fig. 1C). This suggests that these transmembrane E3 ligases may offer better degradation efficiency than RNF43 and ZNRF3, which are currently used in AbTAC and PROTAB drug design. Therefore, we propose that the selection of PDR should depend on the distribution of the corresponding POI within the tumor. Bioinformatics analysis should be employed to select the appropriate PDR before developing TPD (Supporting Information Fig. S4).

Once TPD-based oncology drug synthesized, their anti-cancer activity is typically characterized by cancer cell lines. Notably, the expression levels of POI and PDR in these cell lines tend to remain stable, thereby ensuring relatively consistent anti-cancer efficacy of TPD drugs. However, tumor cells exhibit significant heterogeneity in the clinic, with inconsistent expression patterns of POI and PDR across different cells. In instances where PDR is lacking in tumor cells, POI degradation cannot be achieved, allowing tumor cells to persist and survive. Tumor organoids, derived from patient tumor cells, replicate the complex cellular architecture and genetic diversity of the original tumors. This offers a more accurate and versatile model for mimicking tumor heterogeneity. Considering their advantages, tumor organoids should be utilized in future TPD drug screening efforts to enhance the prediction of TPD drug efficacy (Fig. S4).

Protein degradation mechanisms can vary significantly among different cancer cells. Even within the same type of cancer, the expression of specific E3 ligases can differ. Studies showed that the expression landscape of E3 ligases varies in tumors[3], suggesting that distinct cancer cells may require different E3 ligases for effective protein degradation. Researchers could also pay more attentions to CHAMP for its unique advantages over other technologies. We found the expression ratio of HSP90 in cancer cells is high, and it can bind to various E3 scaffolding proteins. These scaffolding proteins bind to multiple substrate-binding proteins, resulting in a high theoretical ratio of CHAMP-targeted degradation. In the future, studies are required for an in-depth examination of the interaction between the HSP90 protein and E3, as well as the contribution of molecular chaperones to the degradation of targeted proteins. Nevertheless, HSP90 has the capacity to bind to proteins beyond E3. If the CHAMP−HSP90 complex interacts with other proteins, it fails to degrade POI. Further investigation is necessary to ascertain the specific binding of the CHAMP−HSP90 complex with E3 for the degradation of the POI.

Although the ratio of POI and PDR double-positive cancer cells to POI-positive cancer cells should not be the sole criterion for evaluating the efficacy of targeted protein degradation technologies, it can be very useful as one of the screening conditions in pre-drug design research. Researchers should consider PDRs or POIs with higher expression rates in cancer cells as a foundation for design. We propose that, when multiple PDRs are available, bioinformatics analysis can help narrow down the selection of PDRs. The intracellular interactions of proteins are inherently complex, and the expression rate of double-positive cells cannot does not provide a comprehensive indicator of the actual efficiency of degradation proteins in different cancer cells. The precise processes occurring within cancer cells, where interactions between distinct proteins regulate degradation, may deviate from our initial analytical expectations. The actual frequency of degradation is not fully represented by the probabilities derived from single-cell data. Despite these complexities, our proposal in this paper may pave the way for new advancements in targeted protein degradation therapies.

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Author contributions

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Conflicts of interest

Authors declare that they have no competing interest.

Appendix A. Supplementary information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2025.02.016.

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