A modular PROTAC design for target destruction using a degradation signal based on a single amino acid

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Proteolysis targeting chimeras (PROTACs) are bivalent molecules that bring a cellular protein to a ubiquitin ligase E3 for ubiquitination and subsequent degradation. Although PROTAC has emerged as a promising therapeutic means for cancers as it rewire the ubiquitin pathway to destroy key cancer regulators, the degradation signals/pathways for PROTACs remain underdeveloped. Here we append single amino acids, the simplest degradation signal, to a ligand specific for estrogen-related receptor α (ERRα) and demonstrate their utility in ERRα knockdown via the N-end rule pathway and also their efficiency in the growth inhibition of breast cancer cells. The modular design described offers unique advantages including smaller molecular size with shortest degradation sequences and degradation speed modulation with different amino acids. Our study expands the repertoire of limited ubiquitin pathways currently available for PROTACs and could be easily adapted for broad use in targeted protein degradation.

Results and discussion

We reason that a short binding motif to a potent ubiquitin ligase may greatly improve the utility of the PROTAC approach. The shortest degradation motif is a single amino acid, a destabilizing residue (e.g. Arg, His, Lys, Leu, and Ile) at the N terminus of a protein that targets the substrate to a ubiquitin ligase termed Ubr1 for rapid degradation via the N-end rule pathway, the first ubiquitin-dependent degradation pathway identified (12–14). We synthesized adaptor molecules starting with Arg or His and fused with a flexible linker (15) to a ligand

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for estrogen-related receptor α (ERRα) (3) (Fig. 1A), a nuclear receptor that is a major regulator of several critical metabolic pathways and also is a prognostic marker of breast cancer (16). ERRα inhibition has been shown to reduce the proliferation of breast tumor cells in vitro and in vivo. We found that both compounds Arg-TERRa and His-TERRa reduced the endogenous ERRα level in the MCF-7 metastatic breast cancer cell line (Fig. 1B). To assess whether the ERRα decrease was due to protein turnover, we treated the cells with cycloheximide to block protein synthesis and collected the samples at various time points for Western blot analysis. Both Arg-TERRα and His-TERRα triggered ERRα degradation (Fig. 1C and Fig. S1).

We then evaluated whether PROTAC-induced ERRα degradation is mediated by the proteasome. We found that Arg-TERRα- or His-TERRα–triggered ERRα turnover was compromised upon the treatment of the proteasome inhibitor MG132 (Fig. 2, A and B, and Fig. S2), suggesting the involvement of the proteasome in ERRα turnover. To ascertain that ERRα is degraded by the N-end rule pathway, we took advantage that dipeptides starting with a destabilizing residue (e.g., Arg, His) can block the degradation of N-end rule substrates through their competition for the ubiquitin ligase Ubr1 (13, 14). We found that the PROTAC-exposed ERRα level was increased upon the addition of the dipeptide Arg-Ala or His-Ala, but not the control peptide Ala-Ala bearing a stabilizing residue (Fig. 2, C and D, and Fig. S3).

We next examined the biological effects of the PROTAC molecules in MCF-7 cells. Both compounds led to decreased proliferation of MCF-7 cells (Fig. 3A). As cell migration is key to tumorigenesis, we then evaluated whether the compounds affect cell motility by the scratch wound healing method and the ThinCert cell migration assay. We found both Arg-TERRα and His-TERRα reduced MCF-7 cancer cell migration and wound repair (Fig. 3, B and C, and Fig. S4). Furthermore, we monitored the expression of several markers of epithelial-mesenchymal transition (EMT) that is crucial for cancer cell migration and invasion. PROTACs led to increased expression of E-cadherin and reduced expression of N-cadherin, SNAIL, and fibronectin (Fig. 3D), indicating EMT is repressed upon ERRα depletion. Combined, these results suggest that Arg-TERRα and His-TERRα could effectively modulate ERRα and be explored for anticancer purpose.

Our study demonstrates that the N-end rule-based PROTACs efficiently trigger target destruction and inhibit the proliferation of breast cancer cells. The advantage of our approach lies in the use of the simplest degradation signal—single amino acid, which can be any one of the 13 destabilizing residues in the N-end rule pathway (13, 14) that in turn may lead to different degradation speeds. The PROTAC method presented here overcomes limitations of existing approaches with significantly smaller molecular size of E3 targeting moiety, likely leading to higher permeability and better efficacy, and a modulatable potent degradation activity, allowing better control and broader application. Furthermore, the N-end rule degradation pathway is universally present and constitutively active, making it ideally suited for the PROTAC approach. This modular design expands the repertoire of limited ubiquitin pathways.
ACCELERATED COMMUNICATION: N-end rule-based PROTACs

**Experimental procedures**

**Cell cultures**

The MCF-7 cell line (ATCC, Manassas, VA) was maintained at 37 °C with 5% CO₂ in Dulbecco's modified Eagle's medium with 2 mM L-glutamine that was modified to contain 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1.5 g/liter of sodium bicarbonate. It was also supplemented with 10% fetal bovine serum, 10 μg/ml of bovine insulin, and 100 units/ml of penicillin/streptomycin. All drug treatment studies were supplemented with 2% FBS. Functional assays, including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), migration assays were done in 10% FBS in Dulbecco's modified Eagle's medium.

**Peptides and compounds**

Details of Arg-TERRα and His-TERRα syntheses are described under supporting information. Dipeptides Arg-Ala and His-Ala currently available for PROTACs and could be easily adapted for broad use in targeted protein degradation.
were purchased from Bachem Americas (Torrance, CA). MG132 is a proteasome inhibitor obtained from Calbiochem (Gibbstown, NJ). Cycloheximide and dipeptide Ala-Ala were purchased from Sigma.

**Cell proliferation assay**

The MTT method was used to measure living cells through mitochondrial dehydrogenase activity (Sigma). Cells were plated in a 96-well plate, 5000 cells/100 μl/well. After 24 h, the cells were treated with PROTACs in fresh medium. At the indicated time point, the medium was removed and DMSO was added as MTT solubilization solution, followed by 100 μl of stop solution. Absorbance was measured at 550 nm.

**Western blot analysis**

Cells were lysed in RIPA buffer (Sigma) with the addition of protease inhibitors tablet and phosphatase inhibitors mixture (Sigma). Lysates were resolved by SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and then probed with antibodies as indicated. Antibodies against ERRs, E-Cadherin, N-Cadherin, Snail, and GAPDH were obtained from Cell Signaling (Danvers, MA). Fibronectin antibody was obtained from Sigma.

**Wound-healing assay**

Cells were allowed to grow to near confluence in 6-well dishes. A uniform scratch was then made down the center of the plate using a 200-μl micropipette tip, followed by washing twice with PBS. The same marked field of the scratch wound was photographed using an Olympus light microscope (×4 objective) at the indicated time points. The width of the scratch wound was measured at three different areas with ImageJ software.

**Migration assay**

MCF-7 cells (1 × 10^4) were seeded on an 8 μm pore size Thin-cert for 24-well plates (Greiner Bio-One) in serum-free media. PROTAC compounds were added to the bottom chamber as chemoattractant. After 48 h, cells on the top of the membrane were removed with a cotton swab. The migrated cells at the bottom side were washed with PBS, fixed with 70% ethanol, and stained using 0.1% crystal violet to visualize the migrated cells. Migrated cells attached to the lower side of the membrane were enumerated using a light microscope at ×10 magnification.

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