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Induction of apoptosis in MDA-MB-231 breast cancer cells by a PARP1-targeting PROTAC small molecule†

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Poly (ADP-ribose) polymerase-1 (PARP1) is a major member of the PARP superfamily that is involved in DNA damage signalling and other important cellular processes. Here we report the development of a small molecule targeting PARP1 based on the PROTAC strategy. In the MDA-MB-231 cell line, the representative compound 3 can induce significant PARP1 cleavage and programmed cell death.

Poly (ADP-ribose) polymerases (PARPs) are DNA-dependent nuclear enzymes that transfer negatively charged ADP-ribose moieties from cellular nicotinamide-adenine-dinucleotide (NAD+) to a variety of protein substrates.[1–3] Poly (ADP-ribose) polymerase-1 (PARP1) is a fundamental member of the enzyme superfamily.[4] Following DNA damage, PARP1 can rapidly sense and bind to single-strand breaks (SSBs). Then, SSBs are repaired by the base excision repair (BER) pathway. Inactivation of SSB repair by PARP1 inhibition can cause the formation of DNA double-strand breaks after stalling and collapse of progressing DNA replication forks.[5–7]

Due to its pivotal role in DNA damage response, PARP1 is considered as a suitable therapeutic target for the potential treatment of cancers.[8] The rationale for PARP-targeting therapeutics in cancer treatment is the selective induction of synthetic lethality in some BRCA1/2 mutant cancers (especially, ovarian cancer and breast cancer).[9–11] Currently, a number of PARP1 inhibitors, such as olaparib,[12–14] niraparib[15,16] and iniparib[17,18], are under development in different stages of clinical trials.[19,20] Among these inhibitors, olaparib has been approved for the treatment of germline BRCA mutated (gBRCAm) advanced ovarian cancer and gBRCAm metastatic breast cancer. Although the synthetic lethal strategy is very promising, BRCA1 or BRCA2 mutant cancers only account for 3–5% of all breast cancers and a greater proportion of ovarian cancers.[21] For the remaining part of breast cancers especially triple negative breast cancer (TNBC), in consideration of its poor prognosis, high resistance to standard treatment and complicated heterogeneity, other therapeutics with novel mechanisms are still highly needed.[22]

Proteolysis-targeting chimeras (PROTACs) have emerged as a novel and valuable tool for the chemical knockdown of a protein of interest. The concept can be dated back to 2001 when Crews reported the first example of seeking to artificially target a protein to an E3 complex for ubiquitination and degradation.[23] PROTAC molecules (PROTACs) are heterobifunctional small molecules consisting of a target protein binding ligand, an E3 recruiting ligand and an interval linker (Fig. 1A). These molecules are capable of bringing the target protein close to an E3 ligase of interest, causing consequent degradation of the target protein.[24] In 2015, the Bradner[25] and Crews[26] groups independently demonstrated the efficient degradation of BRD4 by

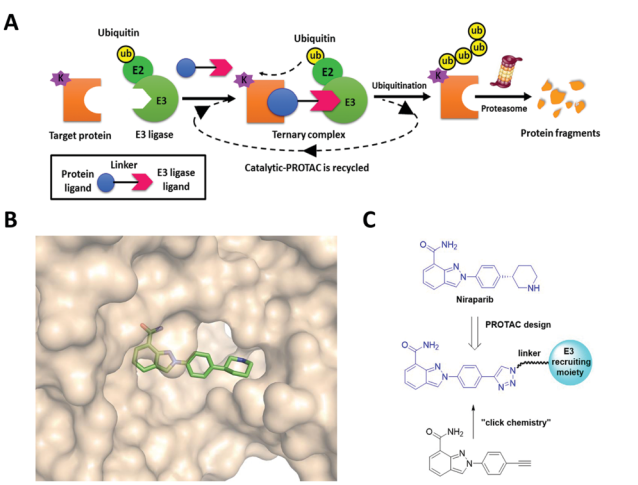

Fig. 1 Design of PROTACs for PARP1. (A) Schematic representation of protein degradation mediated by proteolysis targeting chimeras (PROTACs). (B) Crystal structure of niraparib bound to the PARP1 catalytic domain (PDB code: 4R6E). (C) A "click chemistry" strategy was utilized to construct the PROTAC candidates targeting PARP1.

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PROTACs employing pomalidomide as the CRBN E3 ligase ligand, which has encouraged more to develop PROTACs targeting other proteins, such as ALK,[27,28] BCR-ABL,[29,30] CDK9,[31,32] and TRIM24.[33] In our previous study, we developed efficient PROTACs targeting BTK, which are also effective to degrade mutant C481S BTK and may be used as a novel therapy for ibrutinib-resistant B-cell malignancies induced by mutation.[34] Considering the promising therapeutic utility of degrading PARP1, the successful examples of PROTACs reported so far have encouraged us to develop potential PARP1 degraders.

To develop PARP1 targeting PROTACs, a PARP1 ligand should be tethered to an E3 ubiquitination ligase ligand through a flexible linker. By simultaneously binding to PARP1 and E3 ligase, the PROTACs should bridge the unnatural interaction between PARP1 and the E3 ligase to enable the ubiquitination and subsequent proteasome degradation of PARP1. To initiate the study, niraparib was selected as the PARP1 binding moiety. The analysis of the crystal structure of niraparib in the complex with PARP1 (Fig. 1B) suggests that the piperidine ring on niraparib experiences the opening of the ligand binding pocket and thus may represent a suitable site for modification without losing too much binding affinity. As a consequence, intermediate **6**, an alkyne analogue of niraparib was designed as a synthetic precursor to PARP1 PROTACs. The synthesis of **10** is depicted in Fig. 2. The radical benzylic bromination of **6** followed by the oxidation of benzyl bromide yielded **7**. Then, substitution of the nitro group with the azido group gave **8**. Heating 1c with commercially available 4-ethynylaniline at 100 °C in DMF under acidic conditions generated intermediate **9**. Subsequent ammonolysis of **9** produced intermediate **10**. The advantage of employing intermediate **10** as a precursor is that the desired PROTACs can be readily obtained from intermediate **10** through a versatile copper assisted click reaction and the yielded 1,2,3-triazol group may to some extent mimic the binding mode of the piperidine ring of niraparib to PARP1 (Fig. 1C). As for the E3 ligase binding part, **11**, a racemic mixture of nutlin-3 (enantiomer a IC_{50} = 13.6 μM, enantiomer b IC_{50} = 0.09 μM), was synthesized using the MDM2 recruiting moiety based on a literature reported method.[35] Afterwards, the condensation of **11** with amine **12** yielded the azido intermediate **13**. Finally, through a ''click chemistry'' method, an enantiomeric mixture of compound **3** was prepared as a potential PARP1 degrader. In addition, an alkyne analogue of olaparib was synthesized as another PARP1 binding ligand. Other E3 ligase binding ligands targeting CRBN or VHL were also employed in this study. Subsequently, through the ''click chemistry'' strategy, PARP1 targeting PROTAC candidates **1**, **2**, **4** and **5** were synthesized as well (for details, please see the ESI†).

All PROTACs were then tested for PARP1 degradation in TNBC cell lines. Among the different tested TNBC cell lines (Fig. S1, ESI†), it was found that compound **3** could selectively induce significant PARP1 cleavage in the MDA-MB-231 cell line (Fig. 3A). Control assays clearly demonstrated that niraparib, nutlin-3 or their combination at equimolar or higher concentrations could not induce the cleavage of PARP1 in MDA-MB-231 cells (Fig. 3A).

To explore whether the cleavage of PARP1 is dependent on binding to both PARP1 and MDM2, we pre-treated MDA-MB-231 cells with niraparib at 50 μM or nutlin-3 at 20 μM. It was found that niraparib abolished compound **3** induced PARP1 cleavage successfully while nutlin-3 only partially blocked PARP1 cleavage (Fig. S2A and B, ESI†). Higher concentrations of nutlin-3 might be more effective in blocking the PARP1 cleavage, however it is toxic to the cells. As MDM2 is a RING-type E3 ubiquitin ligase,[36] we used MLN4924, an inhibitor of the NEDD8-activating enzyme

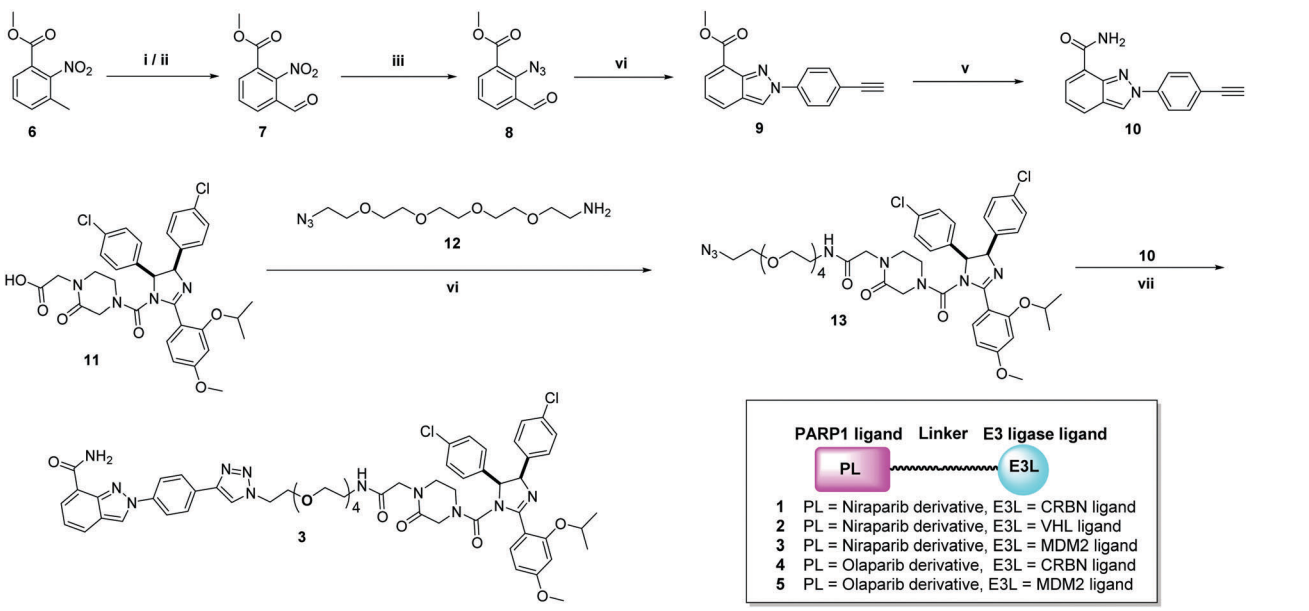

Fig. 2 The synthesis of compound **3** and schematic representation of compounds (i) NBS, AIBN, MeCN, reflux, and 3 h. (ii) 4-Methylmorpholine *N*-oxide, MeCN, and 3 h. (iii) NaN_3, DMF, 80 °C, and 1 h. (iv) 4-Ethynylaniline, HOAc, DMF, 100 °C, and 12 h. (v) Ammonium hydroxide, EtOH, 70 °C, and 11 h. (vi) (a) HATU, Et_3N, DMF/DCM, and 5 min; (b) 14-azido-3,6,9,12-tetraoxatetradecan-1-amine, and 5 h. (vii) $CuSO_4$, sodium ascorbate, DMF/H_2O, and 6 h.

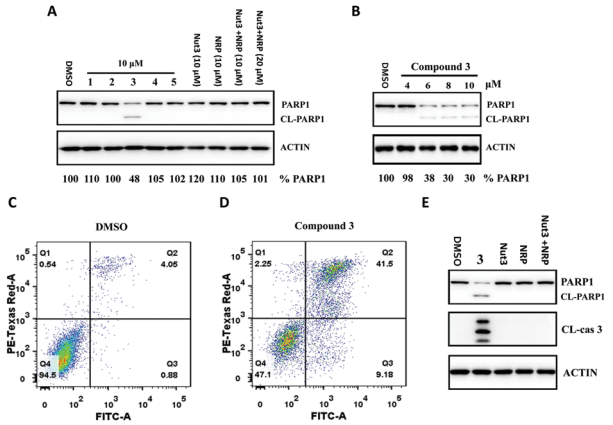

Fig. 3 Characterization of compound **3** mediated PARP1 degradation and apoptosis in MDA-MB-231 cells. (A) Immunoblot of PARP1 and actin following 24 h incubation with DMSO or indicated small molecules. (B) Immunoblot of PARP1 and actin following 24 h incubation with DMSO or the indicated concentrations of compound **3** in MDA-MB-231 cells. (C and D) MDA-MB-231 cells were treated with DMSO and compound **3** (10 μM) for 24 h, and apoptosis was assayed by flow cytometry after annexin-V/PI co-staining. The annexin V/PI intensity dot plots show a significantly increased dot intensity in the Q2 region after treatment with compound **3** (10 μM) for 24 h. (E) Immunoblot for PARP1, cleaved caspase 3 and actin after treatment with DMSO, compound **3** (10 μM), nutlin-3 (10 μM), niraparib (10 μM) or a combination of nutlin-3 (10 μM) and niraparib (10 μM) for 24 h.

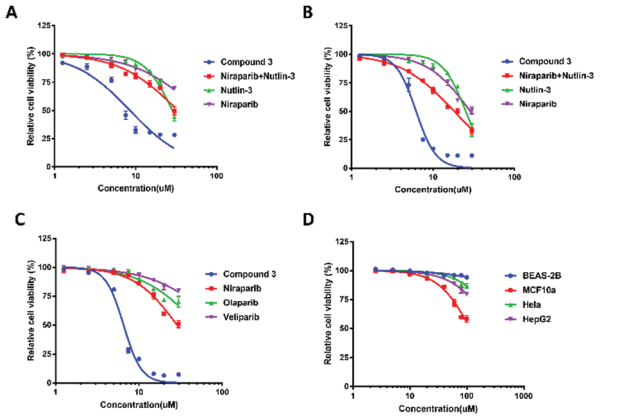

Fig. 4 Effect of compound **3** and PARP1 inhibitors on the cell viability. (A and B) CCK-8 assay was performed after incubation of MDA-MB-231 cells (5×10^3 cells per well) with serially diluted compound **3**, niraparib, nutlin-3 or in combination for 24 h and 48 h in 96-well plates in triplicate. (C) CCK-8 assay was performed after incubation of MDA-MB-231 cells (5×10^3 cells per well) with serially diluted compound **3**, niraparib, olaparib and veliparib for 48 h in 96-well plates in triplicate. (D) Viability of BEAS-2B, MCF10A, HeLa and HepG2 cells that were cultured with serially diluted compound **3**, niraparib, nutlin-3 or in combination for 48 h in 96-well plates was measured by a CCK-8 assay. Data are mean ± SD of 3 independent experiments.

(NAE), to pre-treat MDA-MB-231 cells at 1 μM for 4 h, which effectively blocked compound **3**-induced PARP1 cleavage (Fig. S2C, ESI†). These results verified that PARP1 cleavage induced by compound **3** was dependent on both specific ligand-binding and proteasome.

Given that PARP1 cleavage is a well-known marker for cell apoptosis,[37–39] we conjectured that PARP1 cleavage induced by compound **3** in MDA-MB-231 cells is a characterization of cell apoptosis. To verify this hypothesis, we performed flow cytometric analysis to evaluate the occurrence of apoptosis. After incubation of 10 μM compound **3** for 24 h and then staining with annexin V-FITC/PI, we could significantly detect enhanced PS externalization in MDA-MB-231 cells (Fig. 3C and D). Furthermore, the appearance of cleaved caspase-3 also indicated that compound **3** could induce an enhanced apoptotic response in MDA-MB-231 cells (Fig. 3E). Under the same conditions, niraparib and nutlin-3 alone or in combination failed to externalize PS (Fig. S3, ESI†) and induce any detectable caspase-3 cleavage (Fig. 3E).

Next, we examined the effect of compound **3** on the cell viability in the MDA-MB-231 cell line. Treatment with compound **3** at 10 μM inhibited the cell growth by about 70% at 24 h, whereas, only marginal or no inhibitory effects were observed upon treatment with niraparib, nutlin-3 alone or in combination (IC_{50} = 8.45 ± 0.54 μM, 33.22 ± 1.7 μM, 28.47 ± 0.75 μM and 60.91 ± 4.5 μM, respectively) (Fig. 4A). Upon prolonging the treatment time to 48 h, treatment with compound **3** at 10 μM produced 80–90% inhibition, whereas, niraparib, nutlin-3 or their combination produced about 30%, 30% and 40% inhibition, respectively (IC_{50} = 6.12 ± 0.204 μM, 18.2 ± 0.35 μM, 25.19 ± 0.62 μM and 28.64 ± 0.89 μM, respectively) (Fig. 4B). In addition, we also compared compound **3** with other PARP1 inhibitors, including olaparib and veliparib, both of which exhibited only 10% inhibition at 10 μM after 48 h treatment (IC_{50} = 6.55 ± 0.16 μM, 28.6 ± 0.89 μM, 50.1 ± 4.8 μM and 90.88 ± 10.29 μM, respectively) (Fig. 4C). Furthermore, we used MCF10A, a non-tumorigenic breast epithelial cell line, BEAS-2B, a normal bronchial epithelium cell line, HeLa cells and HepG2 cells to evaluate the cytotoxicity of compound **3**. Surprisingly, compound **3** showed 40% inhibition or no toxic effect on the cell viability even at 100 μM after incubation for 48 h. Meanwhile, there was no significant change of the PAPR1 protein in theses cell lines detected by immunoblot (Fig. S4, ESI†), which indicated that compound **3** exhibited very high selectivity (Fig. 4D).

In conclusion, we have reported the development of a PROTAC-type small molecule based on a niraparib derivative and nutlin-3 derivative. The small molecule specifically induces PARP1 cleavage and cell apoptosis in the MDA-MB-231 cell line, the mechanism of which should be due to the PARP1 degradation or some other complicated reasons. We propose that some genes may be mutated and defective in MDA-MB-231 cells, which leads to synthetic lethal effects to make cells undergo suicide in the presence of compound **3**. According to our observations, compound **3** is 5-fold more potent than niraparib, olaparib and veliparib in MDA-MB-231 cells. Besides, compound **3** exhibited no cytotoxicity to the normal breast epithelial cells. Considering the complicated heterogeneity of triple negative breast cancer (TNBC), these PARP 1-targeting PROTAC-type compounds are of great potential application value for the therapy of the MDA-MB-231 cells-like subtype of TNBCs. The detailed mechanism study is underway in our laboratory and will be reported in due course.

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Conflicts of interest

There are no conflicts to declare.

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