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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Design, synthesis, and biological evaluation of small molecule PROTACs for potential anticancer effects

Yang Da[1] · Shaodong Liu[1] · Pei Lin[1] · Feng Wang[2] · Renjie Yan[2] · Yongzhi Shu[2] · Jun Lin[1]

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Abstract

Androgen receptor (AR) reactivation was closely related with the recurrence of human prostate cancer. Currently, several limitations of AR inhibitors impede their application, such as the acquired drug resistance and the potential off-target binding. Recently, proteolysis targeting chimera (PROTAC) has been used to treat various diseases by degrading target proteins, which has shown higher selectivity than the corresponding inhibitor. In this study, PAP508 was developed as a novel PROTAC degrader of AR proteins. The results suggested the effect of PAP508 on AR protein depended on the action of proteasome, furthermore, the degradation effect was concentration- and time-dependent manner in LNCaP and VCaP cells. PAP508 can inhibit the proliferation, migration, and invasion of prostate cancer cells. These data suggested that PAP508 is a potent and efficacious compound for the development of PROTAC targeted AR.

Keywords Prostate cancer · PROTAC · Androgen receptor · Targeted protein degradation

Introduction

Prostate cancer (PCa) refers to the epithelial malignant tumor that occurs in the prostate, which has become one of the most common tumors in elderly men. It is the leading cause of cancer death in Europe and the United States (Jemal et al. 2008). Androgen deprivation therapy (ADT) is usually used as the main treatment and shows effects in its early stage. However, disease progression was inevitable despite the significant decline in serum testosterone levels (Ross et al. 2008). Furthermore, most patients progressed to the castration-resistant prostate cancer (CRPC). The androgen receptor (AR), ligand-induced transcription factor, was expressed in primary PCa and cells metastases. CRPC is thought to be mediated by a variety of mechanisms, such as AR signaling reactivation (Marcelli et al. 2000). AR expression is usually increased in CRPC, and the preferred treatment of CRPC was enzalutamide (androgen blockers) and abiraterone (androgen synthesis inhibitors). But, long-term use of these inhibitors or conventional ADT result in missense mutations in the ligand-binding domain of the AR, which eventually evolve into resistance to these molecules in patients (Joseph et al. 2013; Bohl et al. 2005). Moreover, inhibitors even lead to protein accumulation in some special cases (Lu et al. 2015).

Recently, many studies have shown that degrading a protein is better than inhibiting it for the anticancer activities (Sakamoto 2005; Neklesa et al. 2017). Proteolysis targeting chimera (PROTAC) as a promising technology recruited human inherent enzymatic systems to mediate the ubiquitination and subsequent degradation of proteins. Simply, ubiquitin tags are delivered to target proteins via initial conjugation to an E1 ubiquitin-activating enzyme followed by transfer to an E2 ubiquitin-conjugating enzyme which then rely on ubiquitin E3 ligases in order to deliver their ubiquitin cargo to the desired protein. Then ubiquitin-tagged proteins are recognized and cleaved by the proteasome. Some macromolecular polypeptide PROTACs were explored in the early stage (Sakamoto et al. 2001; Schneekloth et al. 2004). In addition, this irreversible effect is to eliminate all functional activities of the protein, rather than only inhibit a single. This approach has been used to

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✉ Jun Lin
junlin@jiangnan.edu.cn

[1] School of Pharmaceutical Science, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China

[2] Shanghai Meizer Pharmaceuticals Co., LTD, 797 Puxing Road, Shanghai 201114, China

degrade multiple protein targets using those E3 ligases proven amenable for the development of PROTACs (Buckley et al. 2015; Lai et al. 2016; Bondeson et al. 2017). But the polypeptide PROTAC had a high molecular weight and unstable peptide bonds, leading to many problems in synthesis, purification, and stability. In comparison, small molecules can access a wide range of organs and sites of action and be produced with relatively low cost of goods via a well understood development path (Churcher 2018). Given this, the design of small molecule PROTAC as our focus included two parts for binding the target protein and E3 ubiquitin ligase. And the small molecule PROTAC targeting AR was developed and named PAP508 in our study, which may help PCa treatment.

Material and methods

Chemical synthesis

All reagents and materials were obtained from commercial sources and were used without further purification. Thin-layer chromatography performed on silica gel 60 F_{254} plates was used to monitor the reaction. Column chromatography was performed using silica gel of size 200–300 mesh. All ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance III 400 with tetramethylsilane as the internal standard, and dimethylsulfoxide (DMSO-d6) as the solvent. The mass spectra were carried out on Thermo Scientific Quantum Access max LC-MS spectrometer. All reactions were carried out using predried glassware under nitrogen atmosphere. The data for all tested compounds are in Supporting Information.

General synthetic procedure of target compounds

To a solution of 2-(2,6-dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione (99 mg, 0.360 mmol, 1.2 eq), RU-59063 (116 mg, 0.300 mmol, 1.0 eq), and triphenylphosphine (102 mg, 0.390 mmol, 1.3 eq) in anhydrous tetrahydrofuran (5 mL) under nitrogen atmosphere, DIAD (91 mg, 0.450 mmol, 1.5 eq) was added dropwise in ice bath. The reaction mixture was stirred at room temperature for 0.5 h. The reaction solution was concentrated under reduced pressure and the residue was purified by column chromatography on silica gel to afford the target compound PAP508.

4-(3-(4-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)butyl)-4,4-dimethyl-5-oxo-2-thioxoimidazolidin-1-yl)-2-(trifluoromethyl)benzonitrile (PAP508)

^{1}H NMR (DMSO-d6, 400 MHz): δ = 11.11 (s, 1H, NH-12′), 8.33 (d, J = 8.3 Hz, 1H, Ar-H), 8.24 (d, J = 1.7 Hz, 1H, Ar-H), 8.01 (m, 1H, Ar-H), 7.81 (dd, J = 8.5, 7.3 Hz, 1H, Ar-H), 7.52 (d, J = 8.5 Hz, 1H, Ar-H), 7.45 (d, J = 7.2 Hz, 1H, Ar-H), 5.08 (m, 1H, H-10′), 4.28 (t, J = 5.9 Hz, 2H, CH_2), 4.06–3.70 (m, 2H, CH_2), 2.88 (td, J = 16.5, 13.7, 5.1 Hz, 1H, CH_2), 2.70–2.48 (m, 2H, CH_2), 2.06–2.01 (m, 1H, CH_2), 2.01–1.92 (m, 2H, CH_2), 1.91–1.81 (m, 2H, CH_2), 1.54 (s, 6H, CH_3-14, CH_3-15).

^{13}C NMR (DMSO-d6, 101 MHz): δ = 178.6 (C=O), 175.8 (C=O), 173.2 (C=S, C-13), 170.4 (C=O), 167.3 (C=O), 165.8 (C=O), 156.3 (Ar-C, C-1′), 138.6 (Ar-C, C-5), 137.5 (Ar-C), 136.4 (Ar-C), 134.5 (Ar-C), 133.7 (Ar-C), 131.4 (q, J_{CF} = 32.5 Hz, C-3), 128.5 (q, J_{CF} = 4.7 Hz, C-2), 122.7 (q, J_{CF} = 273.8 Hz, C-8), 118.6 (Ar-C), 116.7 (Ar-C), 115.7 (CN, C-7), 115.5 (Ar-C), 108.8 (q, J_{CF} = 1.9 Hz, C-4), 69.1 (Al-C), 65.7 (Al-C), 49.2 (Al-C), 49.0 (Al-C), 43.7 (Al-C), 31.4 (Al-C), 26.4 (Al-C), 24.9 (Al-C), 22.6 (CH_3), 22.4 (CH_3).

ESI, m/z: 642.1$[M+H]^+$.

Cell cultures

LNCaP and VCaP cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. LNCaP cells were cultured in RPMI1640 medium supplemented with 10% FBS-fetal bovine serum (Gibco Life Technologies, USA) and 1% penicillin and streptomycin. VCaP cells were cultured in Dulbecco's modified eagle medium supplemented with 10% FBS and 1% penicillin and streptomycin. The cells were cultured in a 37 °C incubator with 5% CO_2.

Cell proliferation assay

Cell viability of LNCaP and VCaP was evaluated by cell counting kit-8 (CCK-8) assay. LNCaP cells (5000 cells/well) and VCaP cells (10,000 cells/well) were inoculated in 96-well culture plates with the medium containing 10 nM R1881 (Nekoonam et al. 2018). Different doses of the compounds were added, and the cells were incubated for 168 h. Following the addition of CCK-8, 96-well plates were incubated for 4 h, and the absorbance was determined in an ELX800 microplate reader (BioTek, Winooski, VT, USA) at 450 nm.

Western blot analysis

The harvested cells were lysed in RIPA Buffer (pierce™, REF: 89900) and separated on 8% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. And then, incubated with the primary antibodies (Cell Signaling, Androgen receptor D6F11) diluted according to the manufacturer's instructions at 4 °C overnight. After washed with TBST for three times and incubated with the second antibody (Cell Signaling, Anti-rabbit IgG, HRP-linked

Antibody) at room temperature for 1 h, the expression of protein was detected by electrochemiluminescence assay.

Cell invasion assay

LNCaP cells were added into the top chambers of 24-well transwell plates (Corning Incorporated, 3422, USA) with serum-free medium while the bottom chambers were added 20% FBS medium incubated for 48 h, cells that migrated to the bottom surface of the top chamber membranes were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 10 min. Then images were generated with a microscope (Motic BA210).

Wound-healing assay

LNCaP cells (3 × 105) were added into the 24-well transwell plates with ibidi-Culture-Insert (Eubio 80241) and incubated for 48 h. After removing the Culture-Insert, test compounds or DMSO were added into 24 wells. Following incubation at 37 °C, 5% CO_2 atmosphere, the cells were allowed to migrate. Images were generated with a microscope (Motic BA210).

Results and discussion

Chemistry

RU-59063 was chosen as the AR binding moiety because of its high potency and selectivity for AR. RU-59063 is an N-substituted arylthiohydantoin and was confirmed to be an androgen activator with a dose-dependent manner (Teutsch et al. 1994; Cadilla and Turnbull 2006; Ran et al. 2015; Tran et al. 2009). Although its efficacy of androgen activation is lower than dihydrotestosterone, RU-59063 has high affinity for the human AR (Ki = 2.2 nM; Ka = 5.4 nM) and 1000-fold selectivity for the AR over other nuclear steroid hormone receptors (Cadilla and Turnbull 2006; Liu et al. 2010). For the E3 ligase binding moiety, we used thalidomide. Thalidomide as immunomodulatory drug (Celgene) can bind to the cereblon (CRBN) protein, and then CRBN activates the activity of the CRBN E3 ubiquitinated ligase complex (Fischer et al. 2014). The phthalimide was included in thalidomide part of the small molecule. And this structure as an E3 ligase recruitment ligand hijacked CRBN to degrade the target protein.

Synthesis route of the novel compound was shown in Fig. 1. 2-(2,6-dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione (4-hydroxy thalidomide) reacted with RU-59063 by the Mitsunobu reaction to get PAP508 as a novel PROTAC.

Antiproliferative activity in LNCaP and VCaP cells

Using enzalutamide as positive control, the antiproliferative activity of PAP508 was determined in LNCaP and VCaP cells. The result suggested that PAP508 showed better antiproliferative activity than enzalutamide, especially in LNCaP cells with higher drug concentration (Fig. 2a), and its IC_{50} (50% of cell growth inhibition) value was 3.073 μM. For VCaP cells, PAP508 and enzalutamide showed the similar inhibition effects, and their IC_{50} values were 0.127 and 0.224 μM, respectively (Fig. 2b). These results suggest that cells showed different sensitivity to the novel compound PAP508. The phenomenon that the effect of PROTAC on inhibition was superior to the corresponding inhibitor existed in studies of PROTAC technology for degrading multiple protein targets (Burslem et al. 2018; Sun et al. 2018). Previous studies have suggested that the obvious advantage of protein degradation agent is that the reduction in AR protein prevents reactivation by alternate

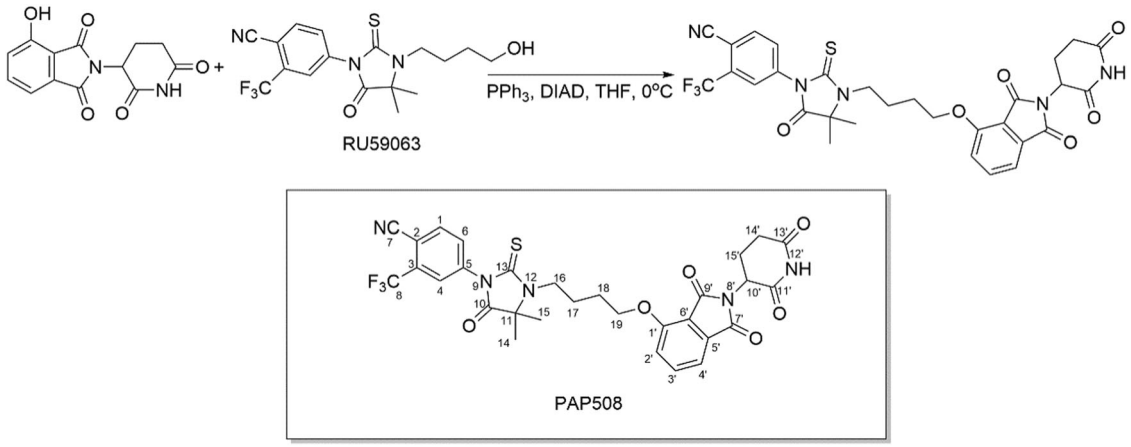

Fig. 1 Synthetic route of PAP508

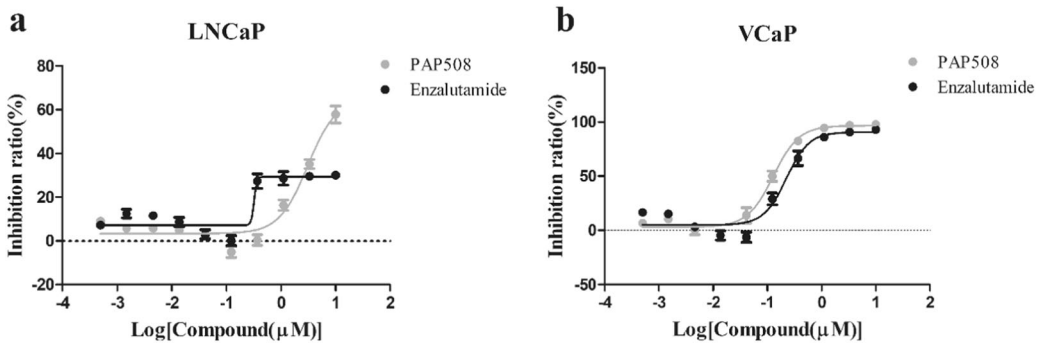

Fig. 2 The inhibition of cell proliferation was detected by CCK-8 assay. **a** LNCaP cells treated with increasing concentrations of PAP508 for 168 h. **b** VCaP cells treated with increasing concentrations of PAP508 for 168 h

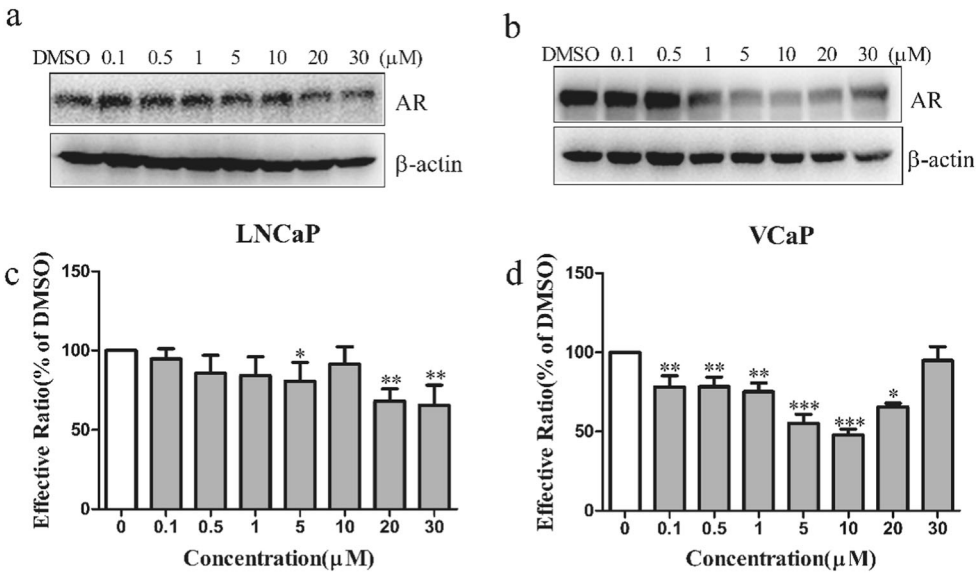

Fig. 3 PAP508-concentration-dependent AR degradation was detected by western blot analysis. **a** LNCaP cells treated with exponentially increasing concentrations of PAP508 for 24 h were detected by Western blot analysis. **b** VCaP cells treated with exponentially increasing concentrations of PAP508 for 24 h were detected by western blot analysis. **c** Grayscale analysis of AR protein levels in LNCaP cells. **d** Grayscale analysis of AR protein levels in VCaP cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control (DMSO). Value was expressed as mean ± SD

mechanisms, thereby achieving better antiproliferative activity (Ponnusamy et al. 2017). These results indicated that the degradation of AR could be crucial for the enhanced antiproliferative effect.

Effects of PAP508 on AR protein degradation

To determine the degradation of PAP508 and RU-59063 on AR protein, the total proteins of LNCaP and VCaP cells were extracted for western blot analysis. Firstly, the effect of compound concentration on the AR protein degradation was investigated in LNCaP and VCaP. Compared with the control (DMSO), PAP508 could significantly reduce the expression level of AR in both cells (Fig. 3a, b). PAP508 exerted prominent action at the concentration of 5 μM and higher. Interestingly, the optimal concentration of the PAP508 in AR degradation is different on two cell lines. Western blot analysis showed attenuated protein when the concentration of the PAP508 was increased on LNCaP cells, and the amount of protein was reduced by 65% in the best (Fig. 3a, c). While the optimal concentration of the PAP508 was 10 μM in VCaP cells (Fig. 3b, d). In VCaP cells, the AR protein degradation reached 47% in the dose of 10 μM, but degradation effect began to ease up with the growing concentration of the PAP508 due to high-dose hook effect (Roy et al. 2017). Compared with PAP508, 30 μM RU-59063 showed no degradation action on AR proteins of LNCaP cells (Fig. S1). Then, time-dependent manner was explored to assess the temporal degradation of AR fusing proteins by PAP508 in LNCaP (Fig. 4a, c) and VCaP cells (Fig. 4b, d). For VCaP cells, the AR was significantly degraded after treated by 10 μM PAP508 for 12 h. The maximum of AR degradation was at 16 h. Similarly, the degradation of PAP508 on AR protein firstly increased (from 2 to 16 h) and then decreased (from 16 to 24 h) in both two cells (Fig. 4c, d). Based on these results, the optimal degradation time is around 16 h.

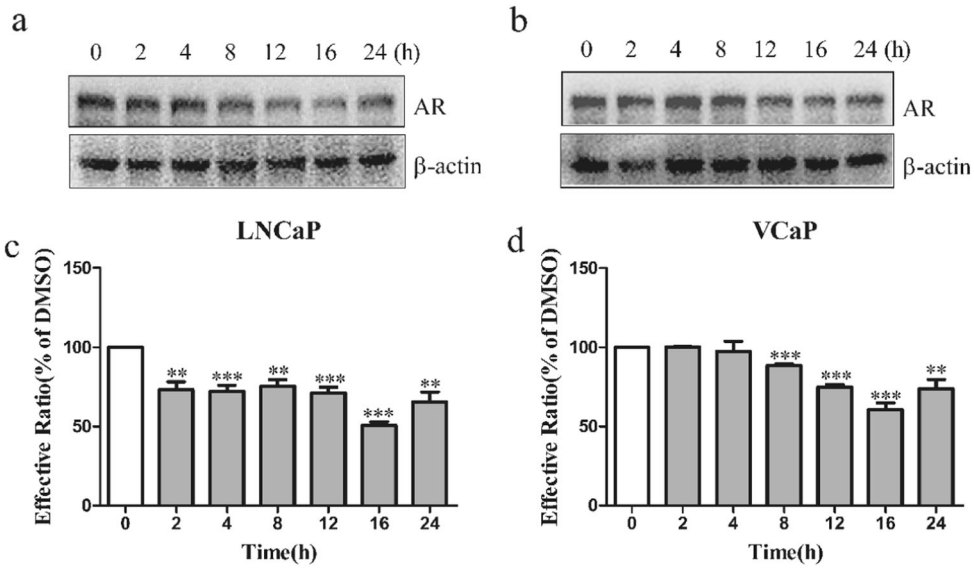

Fig. 4 PAP508-time-dependent AR degradation was detected by western blot analysis. **a** LNCaP cells were treated with 30 μM PAP508 for 0–24 h. **b** VCaP cells were treated with 10 μM PAP508 for 0–24 h. **c** Grayscale analysis of AR protein levels in LNCaP cells. **d** Grayscale analysis of AR protein levels in VCaP cells. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control (DMSO). Value was expressed as mean ± SD

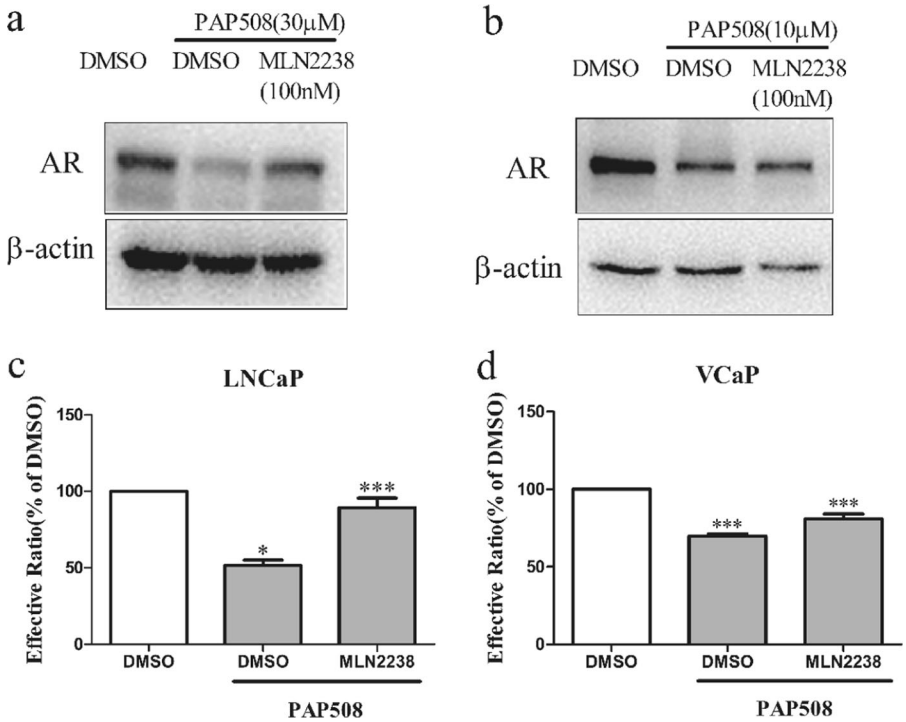

Fig. 5 PAP508-induced androgen receptor (AR) degradation was inhibited by proteasome inhibitors. **a** LNCaP cells were pretreated with proteasome inhibitor for 2 h, followed by treatment with 30 μM PAP508 for 24 h. AR expression was detected by western blot analysis. **b** VCaP cells were pretreated with proteasome inhibitor for 2 h, followed by treatment with 10 μM PAP508 for 24 h. AR expression was detected by western blot analysis. **c** Grayscale analysis of AR protein levels in LNCaP cells. **d** Grayscale analysis of AR protein levels in VCaP cells. $^{*}P < 0.05$, $^{***}P < 0.001$

The result suggested that PAP508 had excellent degradation effect against the both two cells, but its cell specificity needs to be investigated further. CRPC was lethal, but existing treatment options were limited. And CRPC progression was still dependent on reactivation of the AR signaling pathway (Schalken and Fitzpatrick 2016; Scher et al. 2004). The AR transcriptional activity can be reactivated in CRPC through the intracellular conversion of adrenal androgens to testosterone (Stanbrough et al. 2006). The strong degradation effect of PAP508 on AR may significantly slow down the recurrence of cancer. It also provided a new idea to break through the bottleneck of current inhibitor design.

Protease inhibitors weaken the degradation effects of PAP508 on AR

To explore the mechanism of AR degradation induced by PAP508, we performed a rescue experiment in LNCaP cells by using Ixazomib (MLN2238). Firstly, LNCaP cells

were pretreated by this proteasome inhibitor (100 nM) for 2 h. Subsequently, the cells were incubated with PAP508 (30 μM) for 24 h. Finally, AR protein was determined by western blot analysis. The results showed comparable AR protein degradation in the proteasome inhibitor (MLN2238) group and the DMSO control group. In contrast, AR protein in the positive control (PAP508) were significantly reduced (Fig. 5c). Obviously, MLN2238, a proteasome inhibitor, can significantly block AR degradation (Fig. 5a). And VCaP cells showed the similar inhibition phenomenon (Fig. 5b, d). MLN2238 is a selective, potent, and reversible proteasome inhibitor, which inhibits the chymotrypsin-like proteolytic (β5) site of the 20S proteasome (Kupperman et al. 2010). The ligand that binds to E3 ligase in PROTAC molecule has competitive inhibitory effect on proteasome inhibitors, but its potential mechanism needs to be investigated further.

PAP508 effectively inhibit cell migration and invasion

Compared with the control, cell invasion in transwell assays was observably inhibited for both LNCaP and VCaP cells (Fig. 6). PAP508 reduced cell invasion could be due to the increased apoptotic potential of the cells. Subsequently, healing assay showed that PAP508 significantly slowed down the healing rates of LNCaP cells (Fig. 6c). After 168 h, mobility was reduced by nearly 30% when compared with the control group (Fig. 6d). Migration and invasion are important processes in cancer progression. Some evidence has demonstrated that AR involved in regulation of various cellular events, migration, invasion, proliferation, and apoptosis, and played important roles in primary PCa and in metastases (Culig and Santer 2014; Tang et al. 2017). And in PCa, AR overexpression partly reverses the tumor-suppressive effects of miR-381, which inhibits migration and invasion of PCa cells (Wang et al. 2017). So the degradation of the AR protein by PAP508 induction could directly reduce the rate of cell migration and invasion.

Conclusion

In this study, PAP508 was developed as a novel PROTAC degrader of AR proteins, and then its effect on the degradation of AR protein was investigated in PCa cells. PAP508 was competent of effectively inducing degradation of AR protein, and excellent inhibition of cell growth, migration, and invasion in the LNCaP and VCaP cells. The result of rescue experiment suggested that AR protein degradation induced by PAP508 is proteasome dependent. The results showed that PAP508 is promising for potential future applications, yet further studies are required to gain more insight into the mechanism of binding and degradation of target protein.

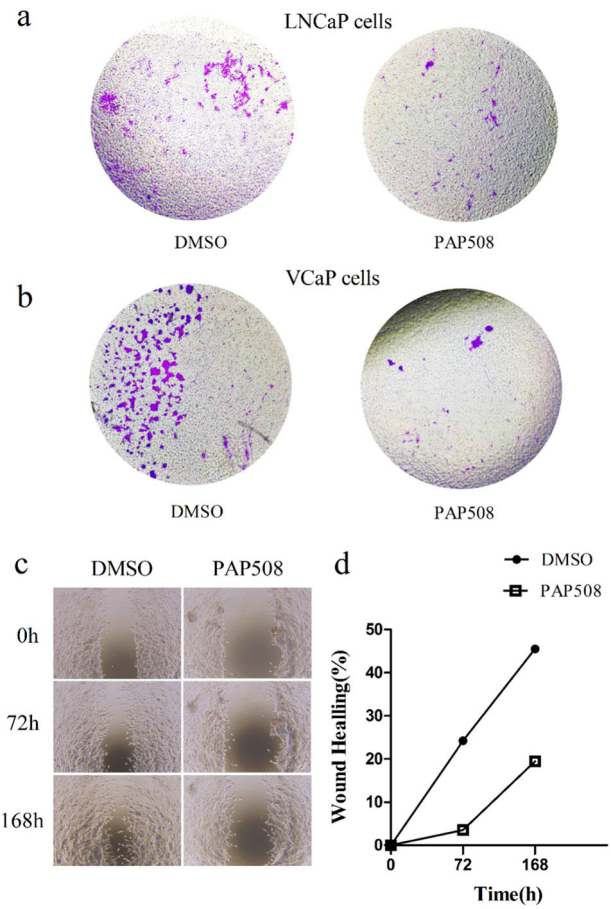

Fig. 6 PAP508 inhibited cell migration and invasion. **a** Transwell invasion assay were performed in LNCaP cells treated with 30 μM PAP508 for 48 h. **b** Transwell invasion assay were performed in VCaP cells treated with 10 μM PAP508 for 48 h. **c** Wound-healing assay were performed in LNCaP cells treated with 30 μM PAP508. **d** Migration rate was measured by the area of migration cells in LNCaP cells

Statistical analysis

The data are presented as mean ± standard deviation (SD) of three independent experiments. Student's *t* tests were performed to compare individual treatment to the control, and comparisons between different treatments were analyzed by one-way analysis of variance with a significance level of $P < 0.05$.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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