Department of Urology[1], Nanfang Hospital Southern Medical University; Guangdong Provincial Key Laboratory of New Drug Screening[2], School of Pharmaceutical Science, Southern Medical University, Guangzhou, PR China

Enhanced antitumor efficacy by combining afatinib with MDV3100 in castration-resistant prostate cancer

Jianhua Li[1,#], Huanxian Wu[2,#], Shidong Lv[1], Dongling Quan[2], Danni Yang[2], Jiahuan Xu[2], Boyu Chen[2], Baofang Ou[2], Shaoyu Wu[2,*], Qiang Wei[1,*]

Received October 6, 2021, accepted November 11, 2021

**Corresponding authors: Dr. Qiang Wei, Department of Urology, Nanfang Hospital Southern Medical University,1838 North Guangzhou Avenue, Guangzhou, Guangdong, 510515, PR China*
qwei@smu.edu.cn
Dr. Shaoyu Wu, Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Science, Southern Medical University, Guangzhou, Guangdong, 510515, PR China
wushaoyu@smu.edu.cn
[#]These authors contributed equally to this work.

Pharmazie 77: 59-66 (2022) *doi: 10.1691/ph.2022.1948*

Background: Patients with prostate cancer often develop resistance to androgen deprivation therapy, a condition called castration-resistant prostate cancer (CRPC). Enzalutamide (MDV3100) can prolong the survival of patients with CRPC after chemotherapy, but ~50% of patients eventually relapse and develop resistance to MDV3100. Thus, it is necessary to explore new treatment methods to improve the therapeutic effect of MDV3100. Tyrosine kinases play an essential role in the pathogenesis of CRPC. *Methods:* MTT assay was used to detect the inhibitory effects of MDV3100 and tyrosine kinase inhibitor on prostate cancer cells. CompuSyn version 1.0 was used to calculate the combination index (CI) values using the Chou-Talalay method. Clone formation and EdU assay were used to detect the effect of afatinib combined with MDV3100 on the proliferation of 22Rv1 cells. RT-qPCR and Western blot were used to explore the mechanism of drug combination. The aim of the present study was to determine the effects of several tyrosine kinase inhibitors (TKIs) when used in combination with MDV3100 *in vitro*. *Results:* The results demonstrated that TKIs combined with MDV3100 exerted a synergistic effect on a variety of PCa cells. Afatinib combined with MDV3100 could suppress the proliferation and migration of 22RV1 cells, as well as cause their cell cycle arrest and apoptosis. Mechanistically, afatinib effectively reduced the protein expression levels of HER2 and HER3 and inhibited EGFR phosphorylation, thereby enhancing the effect of MDV3100 and suppressing CRPC. *Conclusions:* These findings suggested that afatinib treatment improved the effect of MDV3100 on 22RV1 cells, highlighting this drug as a potential therapeutic strategy for patients with CRPC.

1. Introduction

Prostate cancer (PCa) is the second most common cancer among male patients with a high mortality rate worldwide (Sung et al. 2021). Castration (surgical or medical) is an important therapeutic approach to PCa (Nevedomskaya et al. 2018; Evans 2018; Wade and Kyprianou 2018). Activation of the androgen receptor (AR) pathway has a crucial effect on the occurrence and development of PCa (Feng and He 2019; Jamroze et al. 2021). Therefore, androgen deprivation therapy (ADT), which is aimed at reducing serum androgen levels by antagonizing AR receptors, is widely used to treat PCa and to prevent its metastasis (Perlmutter and Lepor 2007; Knudsen and Scher 2009). Moreover, ADT is an effective treatment for patients with PCa whose condition cannot be managed using surgery alone. Although the application of ADT has achieved great success in PCa treatment, >80% of patients develop CRPC after 12-18 months of ADT treatment, and the survival of patients does not exceed two years if they progress to castration-resistant PCa (CRPC)(Pienta and Bradley 2006; Ojo et al. 2015).

The mechanisms underlying PCa resistance to castration are unclear but are considered to be diverse (Harris et al. 2009; Feldman and Feldman 2001; Sharifi 2013; Green et al. 2012). In addition to amplification (Coutinho et al. 2016), mutation (Tilley et al. 1996; Taplin et al. 1995) and overexpression of co-regulators of AR (Yeh et al. 1999), crosstalk between AR and other signaling pathways plays an important role in the development of CRPC (Craft et al. 1999). Tyrosine kinases (TKs) play an important role in this process. EGFR can activate the MAPK signaling pathway, as well as interfere with AR function and regulate the androgen response (Zhu and Luo 2020). A clinical study involving 2,497 patients with PCa demonstrated that 18% of these patients exhibited high EGFR expression levels. And high expression of EGFR was associated with high tumor grade and advanced stages, as well as shorter progression-free survival (Craft and Shostak 1999). In addition, clinical data suggested that EGFR signaling could activate AR and enhance AR-mediated tumor growth, particularly in patients receiving ADT.

HER2 is a member of the receptor tyrosine kinase superfamily. Compared with tumors with normal hormone expression, HER2 is often overexpressed in CRPC samples. HER2 stabilizes AR and promotes the binding of AR to DNA by activating the MAPK and AKT pathways (Schlomm et al. 2007; Chuang et al. 2010; Agus et al. 2002; Zhang et al. 2001). A clinical study involving 2,525 PCa samples indicated that the HER2 gene was hardly amplified in PCa tissue samples, although its protein expression levels were marginally upregulated. HER2 expression positively correlates with that of Ki67 and is significantly associated with poor patient prognosis (Minner et al. 2010). Moreover, the HER2 protein is overexpressed in PCa, and increased HER2 levels in metastatic

Table 1: TK inhibitors used in clinical and classification (Data from www.clinicaltrials.gov)

Drug	Targets	Conditions	NCT Trial
Gefitinib	ERBB1	Prostate Cancer; Non-Metastatic Prostate Cancer; Locally Advanced Prostate Cancer; Hormone Refractory Prostate Cancer	NCT00483561 NCT00085566 NCT00635856 NCT00265070 NCT00241475 NCT00025116 NCT00242918 NCT00239291 NCT00319787 NCT00418080 NCT02208583
Erlotinib	ERBB1	Prostate Cancer	NCT00148772 NCT00272038 NCT00996502 NCT00203424 NCT00087035
Dacomitinib	ERBB1 ERBB2 ERBB4	Castration-Resistant Prostate Carcinoma	NCT03878524
Afatinib	ERBB1 ERBB2 ERBB4	Hormone-refractory Prostate Cancer; Prostatic Neoplasms; Prostate Carcinoma; Recurrent Prostate Carcinoma; Castration-Resistant Prostate Carcinoma	NCT01320280 NCT00706628 NCT02465060 NCT03878524
Sorafenib	EGFR PDGFRB KIT VEGFR2	Prostate Cancer; Metastatic Prostate Cancer; Adenocarcinoma of the Prostate; Stage II/ III Prostate Cancer	NCT00924807 NCT00090545 NCT00694291 NCT00589420 NCT00619996 NCT00405210 NCT00452387 NCT00466752 NCT00093457 NCT00424385 NCT00414388 NCT00430235 NCT02642913 NCT00326898 NCT00703638

patients with CRPC are associated with increased risk of death. VEGFR can interact with and activate AR in low or even undetectable androgen levels (Wu et al. 2017).

TK inhibitors (TKIs) have been extensively studied in patients with PCa (Table 1). Erlotinib could prolong the prostate-specific antigen (PSA) doubling time in patients with metastatic CRPC (mCRPC) in a phase-I clinical trial, and the effective rate was 31% (Nabhan et al. 2009). In a phase-II clinical trial of erlotinib combined with docetaxel, the PSA index of 23% of patients was reduced by >50% (Gross et al. 2007). Additionally, in a phase-II clinical trial, lapatinib reduced the PSA levels in 50 % of patients with mCRPC (Whang et al. 2013). The results of phase-II clinical trials demonstrated that, compared with the placebo, carbozantinib could significantly improve the progression-free survival of

Table 2: The effect of TK inhibitors and MDV3100 on proliferation of PCa cells

Drug	IC_{50} (μM)				
	LNCap	C4-2	PC3	DU145	22RV1
Androgen-dependent	**+**	**-**	**-**	**-**	**-**
AR axis	**+**	**+**	**-**	**-**	**+**
Gefitinib	41.16 ± 1.16	>100	21.62 ± 2.82	>100	21.05 ± 4.09
Erlotinib	62.90 ± 20.08	>100	18.12 ± 3.77	>100	4.78 ± 0.36
Sorafenib	0.31 ± 0.03	1.22 ± 0.26	3.45 ± 0.03	4.87 ± 1.19	13.33 ± 9.82
Afatinib	0.39 ± 0.38	2.46 ± 0.92	5.60 ± 0.01	1.34 ± 0.65	0.82 ± 0.02
Dacomitinib	0.97 ± 0.40	27.04 ± 10.36	5.42 ± 0.003	2.83 ± 0.85	2.10 ± 0.42
MDV3100	1.74 ± 0.46	49.17 ±9.91	>100	64.15 ± 6.59	39.78 ± 16.36

patients with CRPC (Corn et al. 2020). Sorafenib also exhibited antitumor activity in a phase-II clinical trial for patients with CRPC (Dahut et al. 2008).

Enzalutamide (MDV3100) is a second-generation AR antagonist that competitively binds AR (Rice et al. 2019). The affinity of MDV3100 to AR is 5-8 times stronger compared with that of bicalutamide, a first-generation AR antagonist. Preclinical *in vitro* studies suggested that MDV3100 could reduce androgen binding to AR, inhibit AR transport to the nucleus and prevent the binding of AR to androgen response elements. This reduces the recruitment of AR and coactivators and the expression of AR target genes (Tran et al. 2009). Phase-I and -II clinical trials indicated that MDV3100 could inhibit the AR signaling pathway in patients with CRPC. Moreover, the results of phase-III clinical trials indicated that MDV3100 prolonged the survival time of patients with CRPC after chemotherapy and delayed the PSA doubling time (Scher et al. 2012). However, although ~50% of patients with CRPC are sensitive to treatment with enzalutamide, the rest eventually relapse, and some patients experiencing disease recurrence develop drug resistance independent of AR activity (McNamara et al. 2019). Therefore, it is necessary to develop an effective treatment that can improve the therapeutic effect of MDV3100.

As aforementioned, TKs are essential for CRPC pathogenesis. Therefore, TKIs combined with MDV3100 may have therapeutic effects in CRPC. In the present study, the combination of MDV3100 and TKIs significantly inhibited the viability of PCa cells. Moreover, the molecular mechanism underlying the effects of MDV3100 in combination with TKIs was examined.

2. Investigations and results

2.1. TKIs and MDV3100 effectively inhibit the proliferation of PCa cell lines

The ability of various compounds to inhibit proliferation was evaluated in the LNCap, C4-2, PC3, DU145 and 22RV1 cell lines using an MTT assay. As shown in Table 2, MDV3100 moderately inhibited the proliferation of PC3 and DU145 cells, both of which are androgen-independent PCa cell lines. Moreover, it also inhibited proliferation in the androgen-dependent LNCap, 22RV1 and C4-2 cell lines, with IC_{50} values of 1.74, 39.79 and 49.17 μM, respectively. Interestingly, TKIs significantly inhibited cancer cell proliferation. Compared with first-generation TKIs (gefitinib and erlotinib), second-generation TKIs (afatinib and dacomitinib) and the multi-target kinase inhibitor sorafenib, resulted in increased inhibition of proliferation in PCa cells, with IC_{50} <10 μM.

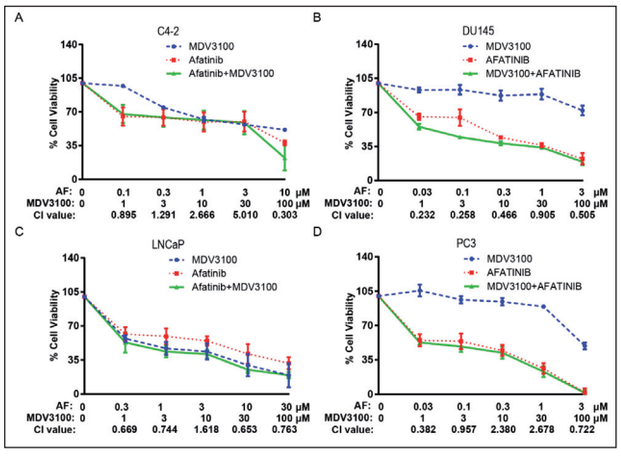

Fig. S1: Comparison of afatinib (AF) and MDV3100 in (A) C4-2, (B) DU145, (C) LNCap and (D) PC3 cell lines. Cell viability was measured using MTT. The combi-nation index (CI) values for the drug combinations at various combinations in various cells. CI values were calculated with CompuSyn software using the Chou-Talalay method.

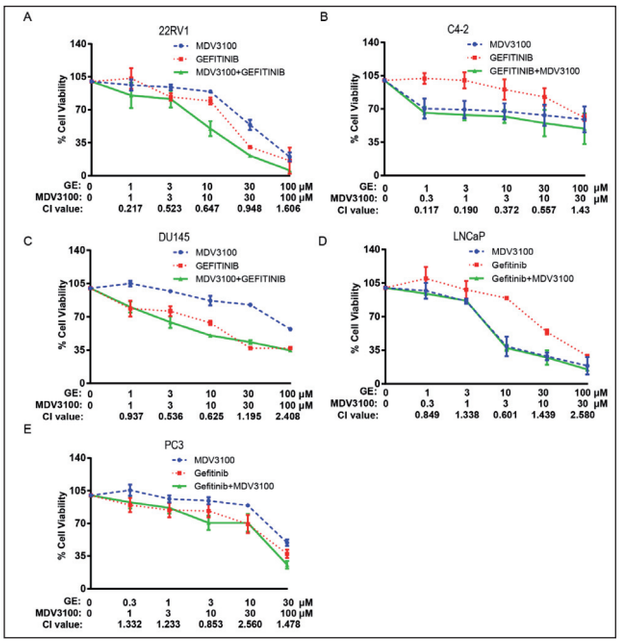

Fig. S2: Comparison of gefitinib (Ge) and MDV3100 in (A) 22RV1, (B) C4-2, (C) DU145, (D) LNCap and (E) PC3 cell lines. Cell viability was measured using MTT. The combination index (CI) values for the drug combinations at various combinations in various cells. CI values were calculated with CompuSyn software using the Chou-Talalay method.

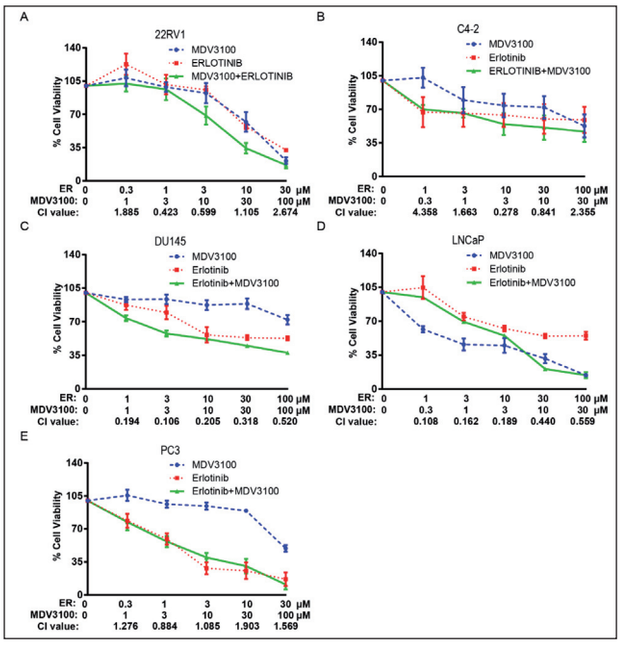

Fig. S3: Comparison of eriotinib (ER) and MDV3100 in (A) 22RV1, (B) C4-2, (C) DU145, (D) LNCap and (E) PC3 cell lines. Cell viability was measured using MTT. The combination index (CI) values for the drug combinations at various combinations in various cells. CI values were calculated with CompuSyn software using the Chou-Talalay method.

2.2. MDV3100 and afatinib synergistically inhibit the viability of PCa cells

TKIs combined with MDV3100 showed synergistic effects in various PCa cells (Fig. S1-S6). In the DU145 cell line, MDV3100 combined with afatinib, erlotinib or sorafenib achieved satisfactory effects. What's more, gefitinib, erlotinib and afatinib showed a synergistic inhibitory effect on cell proliferation in 22RV1 cells. The combination of afatinib with MDV3100 significantly inhibited

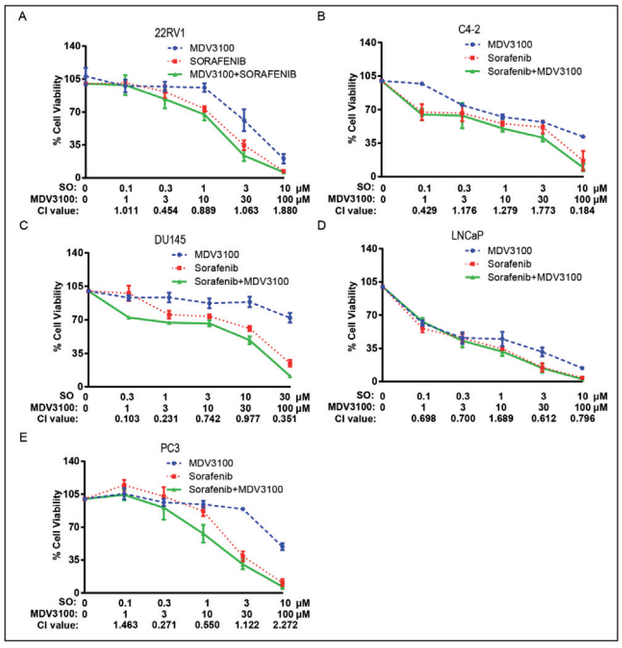

Fig. S4 Comparison of sorafenib (SO) and MDV3100 in (A) 22RV1, (B) C4-2, (C) DU145, (D) LNCap and (E) PC3 cell lines. Cell viability was measured using MTT. The combination index (CI) values for the drug combinations at various combinations in various cells. CI values were calculated with CompuSyn software using the Chou-Talalay method.

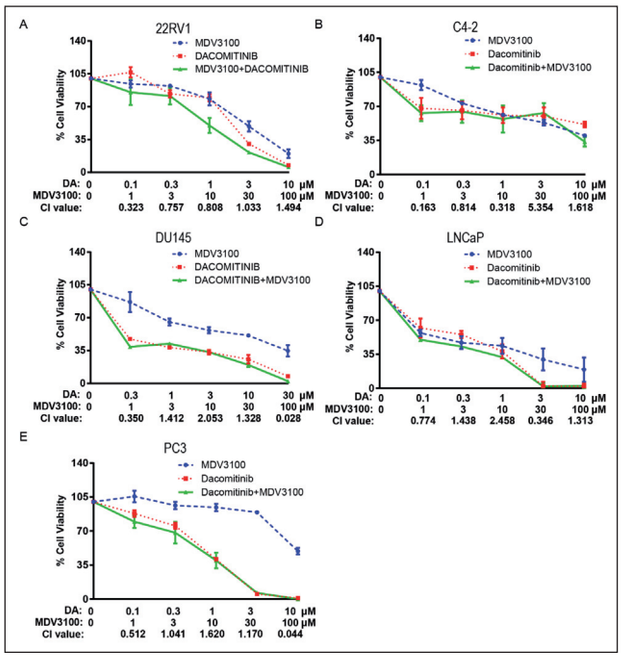

Fig. S5: Comparison of dacomitinib (DA) and MDV3100 in (A) 22RV1, (B) C4-2, (C) DU145, (D) LNCap and (E) PC3 cell lines. Cell viability was measured using MTT. The combination index (CI) values for the drug combinations at various combinations in various cells. CI values were calculated with CompuSyn software using the Chou-Talalay method.

viability in 22RV1cells (Fig. 1A). In addition, CompuSyn software was used to determine whether there was synergy between the two drugs. CompuSyn calculates CI values using the Chou-Talalay algorithm, with CI values <1 considered to indicate synergy. The calculated CI values are shown in Fig. 1B. Afatinib and MDV3100 exerted a strong synergistic effect on 22RV1 cells; thus, this drug combination was used in subsequent experiments. Following treatment with 10 μM MDV3100 and 0.3 μM afatinib, the viability of 22RV1 cells was <50%, and the combined CI value was <0.3,

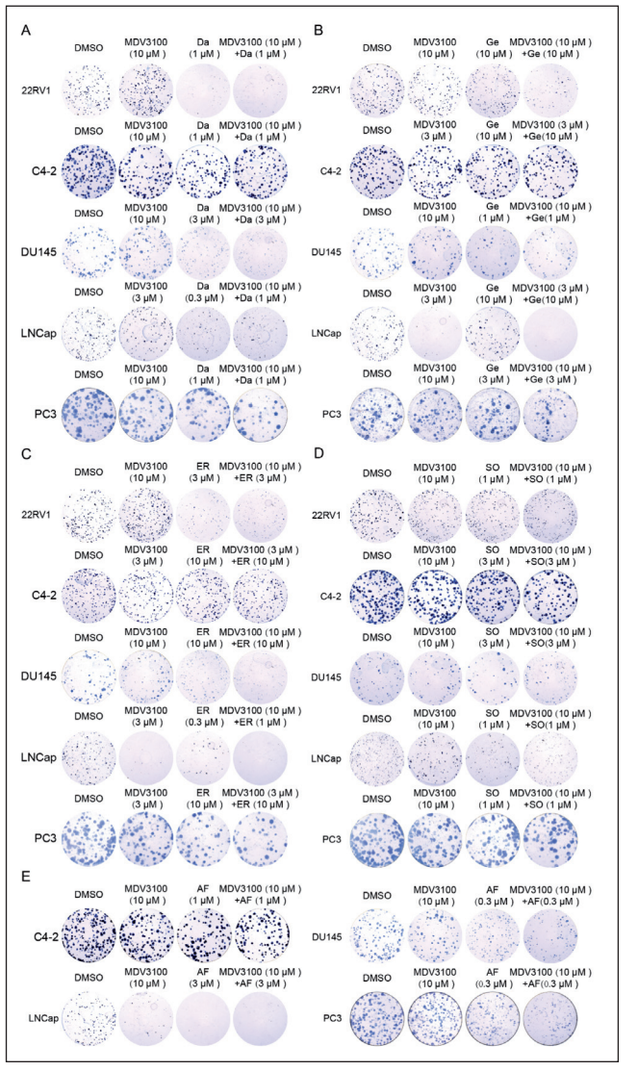

Fig. S6: Cloning formation experiment was used to verify the inhibitory effect of drug combination on prostate cancer cells. (A) Dacomitinib (Da); (B) Gefitinib (Ge); (C) Eriotinib (ER); (D) Sorafenib (SO); (E) Afatinib (AF).

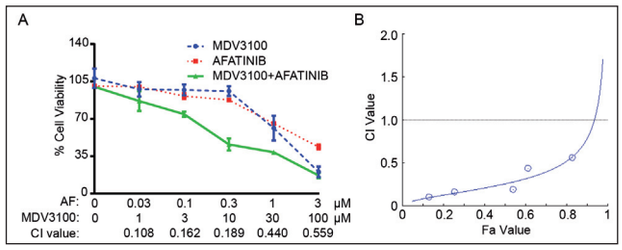

Fig. 1: Afatinib combined with MDV3100 synergistically inhibited 22RV1 cells. (A) 22RV1 cells were seeded in 96-well plates and treated with afatinib (0.03, 0.1, 0.3, 1 and 3 μM), MDV3100 (1, 3, 10, 30 and 100 μM) and the combination (0.03 μM afatinib+1 μM MDV3100; 0.1 μM afatinib+3 μM MDV3100; 0.3 μM afatinib+10 μM MDV3100; 1 μM afatinib+30 μM MDV3100; 3 μM afatinib+100 μM MDV3100) for 72 h. Cell viability was measured using MTT. (B) Combination index (CI) of different drug combinations in 22RV1 cells. CI values were calculated by the Chou-Talalay algorithm with CompuSyn software.

demonstrating strong synergy. In colony formation assays, the number of 22RV1 cell clones was significantly reduced following treatment with 0.3 μM afatinib and 10 μM MDV3100 (Fig. 2A and B). In addition, the combination of afatinib and MDV3100 reduced the frequency of EdU^+ cells compared with either drug alone (Fig. 2C and D). This finding indicates that afatinib combined with MDV3100 exert synergistic effects on the viability of PCa cells.

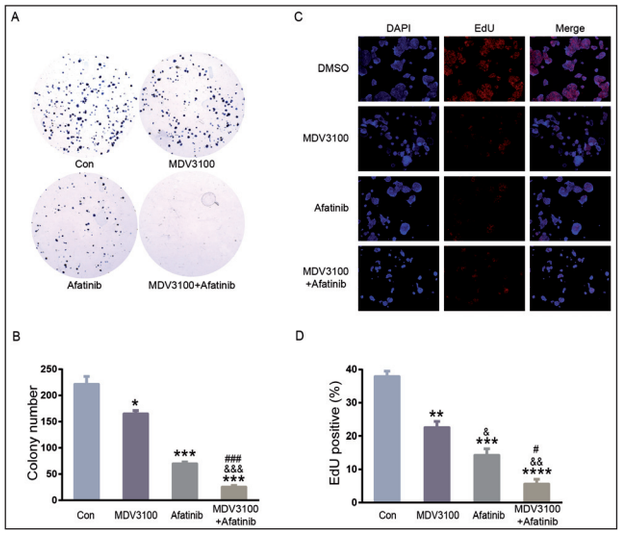

Fig. 2: MDV3100 and afatinib synergistically inhibit proliferation of 22RV1 cells. (A) 22RV1 cells were seeded in the 6-well plate. After 24 h, 0.3 μM afatinib and/or10 μM MDV3100 were added to the media. Colonies were stained by the 0.25% trypan blue and (B) colony number was counted. (C) The proliferative capacity of 22RV1 cells was detected by EdU assays after treatment with 0.3 μM afatinib and/or10 μM MDV3100. (D) The proliferation activity was evaluated by the ratio of EdU-stained cells (red fluorescence) to DAPI-stained cells (blue fluorescence). Data represent the average of three independent experiments. *(**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.001$* vs control (Con); *(&&) $p < 0.01$* vs MDV3100 group; *(##) $p < 0.01$, (#) $p < 0.05$* vs afatinib group.

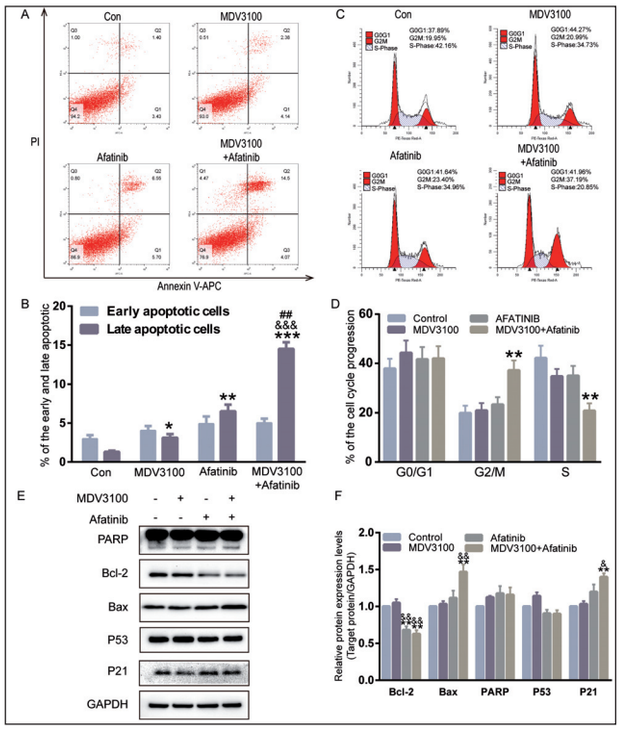

Fig. 3: MDV3100 and afatinib synergistically induce 22RV1 cell apoptosis and cycle arrest. (A) Cells were incubated with 0.3 μM afatinib and/or10 μM MDV3100 for 48 h and detected by flow cytometry after stained by APC-Annexin V/PI. (B) Cells were incubated with 0.3 μM afatinib and/or 10 μM MDV3100 and detected by flow cytometry after stained by PI. Percentage values of cells (C and D) represent the mean ± S.D. of the three independent experiments; (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ vs control (DMSO). (&&&) $p < 0.001$ vs MDV3100 group; (##) $p < 0.01$ vs afatinib group. (E, F) Western blot analysis of cell apoptotic and cycle-associated protein levels in 22RV1 cells upon treatment with 0.3 μM afatinib and/or10 μM MDV3100 for 48 h.

2.3. *MDV3100 and afatinib synergistically induce 22RV1 cell apoptosis and cell cycle arrest*

The aforementioned experiments suggested that afatinib combined with MDV3100 synergistically inhibited the viability of 22RV1 cells. To determine whether the combination of these two drugs could promote apoptosis, 22RV1 cells were incubated with afatinib and/or MDV3100 for 48 h, and apoptosis was analyzed using flow cytometry. The results indicated that the percentage of apoptotic cells following combined treatment with afatinib and MDV3100 was significantly increased compared with treatment with either compound alone (Fig. 3A and B). To examine the mechanism underlying the increased apoptosis observed following combined treatment, the expression levels of apoptosis-associated proteins was detected using western blotting. The results demonstrated that Bcl-2 protein expression levels significantly decreased in the combination treatment group compared with the single-agent treatment groups. Moreover, the combination treatment increased the levels of cleaved poly(ADP-ribose) polymerase 1 (PARP) under the same conditions (Fig. 3E and F). To determine whether the combined treatment group induces cell cycle arrest, cell cycle distribution was analyzed using flow cytometry. Cell cycle progression in the combined treatment group was blocked in the G_2/M phase. (Fig. 3C and D). Moreover, the expression levels of cell cycle-related proteins were analyzed using western blotting. The protein expression levels of p21 increased in the combined treatment group. However, the combination of the two drugs did not influence the protein expression levels of p53 (Fig. 3E and F).

2.4. *MDV3100 and afatinib synergistically inhibit 22RV1 cell migration*

To examine whether a combination treatment could reduce cell migration, Transwell migration assays were carried out. The results suggested that the number of migrating cells following combined treatment with afatinib and MDV3100 was significantly reduced compared with that observed with either treatment alone (Fig. 4A). In addition, although afatinib did not influence the protein expression of N-cadherin, it upregulated that of E-cadherin (Fig. 4C).

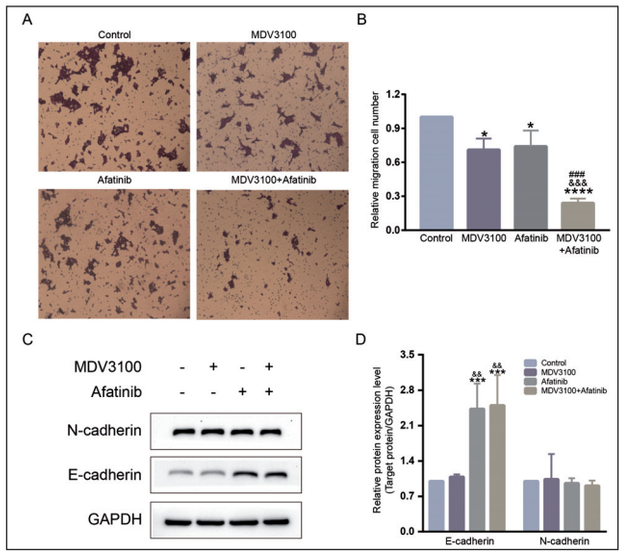

Fig. 4: MDV3100 and afatinib synergistically reduce 22RV1 cell migration. (A) The effect of afatinib combined with MDV3100 on the migration of 22RV1 cells was detected by Transwell assay. (B) The relative migration cell number was calculated by comparing with the control. Percentage values of migration cell number represents the mean±S.D. from three independent experiments; (*) $p < 0.05$, (****) $p < 0.0001$ vs control (DMSO). (&&&) $p < 0.001$ vs MDV3100 group; (###) $p < 0.001$ vs afatinib group. (C) Western blot analysis of N-cadherin and E-cadherin protein expression levels in 22RV1 cells upon treatment with 0.3 μM afatinib and/or10 μM MDV3100 for 48 h. (D) Percentage values of relative protein expression level represent the mean ± S.D. from three independent experiments; (***) $p < 0.001$ vs control (DMSO). (&&) $p < 0.01$ vs MDV3100 group.

2.5. MDV3100 and afatinib synergistically inhibit the AR and RTK signaling pathways

The potential molecular mechanism underlying the synergy between afatinib and MDV3100 was examined. Western blot analysis indicated that the protein expression levels of HER2 and HER3 were upregulated following treatment with MDV3100, consistent with the results of Masaki Shiota et al. However, the use of afatinib effectively reduced the protein expression of HER2 and HER3 and inhibited EGFR phosphorylation (Fig. 5B). Moreover, RT-qPCR demonstrated results similar to those of the western blot analysis (Fig. 5A). In addition, combined treatment with afatinib and MDV3100 significantly reduced the protein expression levels of AR and PSA compared with treatment with either compound alone (Fig. 5D and E).

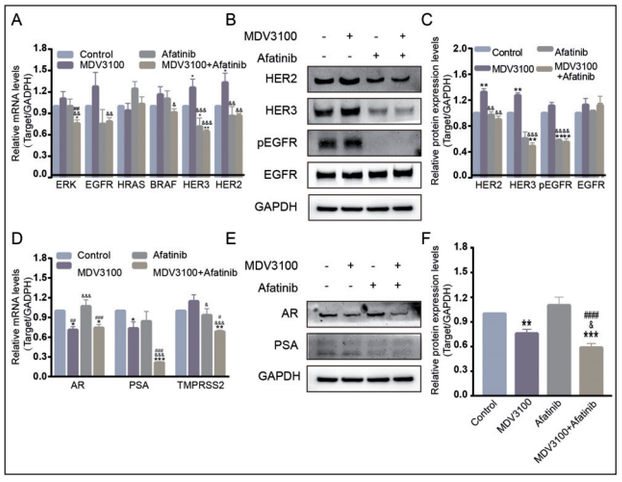

Fig. 5: MDV3100 and afatinib synergistically inhibit prostate cancer progression by inhibiting AR and receptor tyrosine kinase signaling pathways. The mRNA (A, D) and protein (B, E) expression level of receptor tyrosine kinase and downstream signaling pathways of AR of the 22RV1 that treated with 0.3 μM afatinib and/or10 μM MDV3100 for 48 h. (C, F) Percentage values of relative protein expression level represents the mean±S.D. from three independent experiments; (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$vs control (DMSO); (&) $p < 0.05$, (&&) $p < 0.01$, (&&&) $p < 0.001$, (&&&&) $p < 0.0001$vs MDV3100 group; (#) $p < 0.05$, (##) $p < 0.01$, (###) $p < 0.001$, (####) $p < 0.0001$ vs afatinib group.

3. Discussion

Despite recent diagnostic and therapeutic advances, the incidence of PCa has reached 7.3%, second only to breast, lung and colorectal cancer according to global cancer statistics (Sung and Ferlay 2021). ADT and surgical castration constitute an important treatment option for patient with PCa. However, numerous patients develop CRPC (Buttigliero et al. 2015). MDV3100 is a competitive oral AR antagonist that blocks its nuclear translocation and DNA binding to androgen response elements. This compound can greatly improve the metastasis-free survival of patients with high-risk, non-metastatic CRPC (Scher and Fizazi 2012). However, 22% of patients with mCRPC do not respond to MDV3100 treatment. The remaining 78% respond initially, although increased PSA levels can be observed after a median time of 11.2 months (Beer et al. 2014). Therefore, it is urgent to develop new drug treatment methods.

Previous studies have shown that TKs increase AR signal transduction, thereby promoting the progression of CRPC in a ligand-independent manner (Kung 2011). TKIs alone or in combination with other drugs inhibit the development of PCa *in vitro* and *in vivo* (Yuan et al. 2020; Jathal et al. 2019; Arai et al. 2018), which suggests that TKs may be a potential target for drug therapy of PCa. Tingting Chen et al. reported that afatinib could weaken AKT activation and increase the expression level of FOXO3A in prostate cancer. In addition, the combination of afatinib and GW3965 simultaneously increased and activated LXR-α, which led to an increase of tumor suppressors, and eventually inhibited tumor progression (Chen et al. 2020). The present study suggested that TKIs inhibited the proliferation of PCa cells and that some drug combinations improved the sensitivity of PCa cells to MDV3100. Indeed, afatinib effectively reduced the protein expression of HER2 and HER3 following MDV3100 treatment and inhibited EGFR phosphorylation. Moreover, the combination of these two drugs significantly reduced AR and PSA protein expression levels. These results indicated that MDV3100 and afatinib synergistically inhibited PCa progression by inhibiting AR, TKs and their downstream signaling pathways. In addition, the combination of these drugs exerted synergistic effects against the proliferation and migration of 22RV1 cells. This may have been achieved by increasing the protein expression of E-cadherin, arresting the cell cycle at the G_2/M phase and promoting apoptosis by downregulating Bcl-2 expression and increasing levels of cleaved PARP. What's more, Phase I clinical trial results showed that afatinib has a favourable and time-independent pharmacokinetic profile that is consistent across a range of patient populations, and has a low potential for drug–drug interactions via cytochrome P450. Intrinsic factors such as age, ethnicity, and hepatic function do not affect the pharmacokinetics of afatinib, and effects of sex, weight and renal function status are within the variability range of afatinib exposure (Wind et al. 2017).

Clinical trials involving single or combined TKIs for the treatment of PCa are ongoing. The available findings have shown some encouraging results. For example, erlotinib (Nabhan and Lestingi 2009; Gross and Higano 2007), carbozantinib (Corn and Zhang 2020) and sorafenib (Dahut und Scripture 2008) have shown prospect of clinical application in a number of clinical trials on PCa. However, the therapeutic effects achieved in some clinical trials were not optimal. For example, in a phase-II clinical trial of docetaxel combined with gefitinib for the treatment of patients with hormone-refractory metastatic PCa, the response rate and duration of response were consistent with those seen with docetaxel monotherapy, which may be due to the heterogeneity of the disease (Salzberg et al. 2007). Despite afatinib was already tested in CRCP in phase II clinical trial in CRPC patients without clinical signs of efficacy. However, studies have shown that CRPC patients are highly heterogeneous. The effect of afatinib depends on the amplification expression or mutation of EGFR and HER2 (Qian et al. 2020; Tamura et al. 2018; Watanuki et al. 2014). It was reported that PXR could be a biomarker of response to kinase inhibitors in castration-resistant prostate cancer. The stable expression of PXR in 22RV1 cells used in this study led to higher sensitivity to afatinib (Molife et al. 2014). This requires the development of improved and more individualized treatments and medication regimen.

In conclusion, the present study identified a potential approach to improving the therapeutic efficacy of MDV3100 in PCa. The findings demonstrated that the combination of MDV3100 with afatinib may hold promise as a strategy for the treatment of CRPC.

4. Experimental

4.1. Cell culture

The human prostatic cancer cells PC-3, DU145, 22RV1, LNCap, and C4-2 were purchased from Guangzhou Cellcook Biotech Co., Ltd. All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplied with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and placed in a 37 °C in 5% CO_2 incubator for culture.

4.2. Drugs

Tyrosine kinase (TK) inhibitors gefitinib (cat. no. 125560), erlotinib (cat. no. 341249), dacomitinib (cat. no. 1525219), afatinib (cat. no.1232626), sorafenib (cat. no. 297281) and the androgen receptor antagonist enzalutamide (MDV3100, cat. no. 1239111) were purchased from J&K Scientific Ltd. All drugs were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 100 mM and then diluted to desired concentrations with DMSO and stored at -80 °C. RPMI1640 medium was used to dilute the stock solution to the required concentration for *in vitro* studies.

4.3. MTT assay

PCa cells in the logarithmic growth phase were seeded into 96-well plates ($2x10^3$ cells /well in a 100-µl volume) and incubated at 37 °C in a 5% CO_2 incubator overnight, in order to allow cells to adhere to the culture plate. After 24 h, different concentration gradients of various compounds were added to each group. All wells were set up in duplicate. The control group was treated with 0.1% DMSO. The cells were continuously treated for 72 h, then supplemented with 10 µl MTT solution for 4 h. After the reaction was terminated, the culture medium was discarded. The formazan was dissolved in DMSO. The absorbance was read in each well at a wavelength of 570 nm using an Infinite M1000 Pro plate reader (Tecan Group, Ltd.). The inhibition rate was calculated as follows: Inhibition (%) = [(control cells – treated cells) / (control cells – blank)] x100%. The IC_{50} values were determined using the Prism 7 statistical software (GraphPad Software, Inc. USA). The synergistic effect between TKIs and MDV3100 was evaluated based on cell viability. CompuSyn version 1.0 (ComboSyn, Inc. Paramus, NJ, USA), was used to calculate the combination index (CI) values using the Chou-Talalay method. Drug combinations with CI values <1 were considered to act synergistically (Chou 2010).

4.4. Colony formation assays

For colony formation assays, the cells were plated in 6-well cell culture cluster at a density of $5x10^2$ cells/well. After 24 h, PCa cells were treated with 0.3 µM afatinib and/or 10 µM MDV3100 for 48 h. The control group received 0.1% DMSO. Following treatment, the medium was replaced with fresh complete medium. Subsequently, the medium was replaced regularly. When visible cell colonies were observed, the samples were fixed and stained using Trypan blue solution (75% methanol; 25% acetic acid; 0.25% Trypan blue) for 15 minutes at room temperature, washed with PBS and completely dried at room temperature before determining the number of colonies.

4.5. EdU cell proliferation assays

The cells were plated into 96-well plates at a density of $2x10^3$ cells/well. After 24 h, cells were treated with 0.3 µM afatinib and/or 10 µM MDV3100. The control group was treated with 0.1% DMSO. After 48 h of treatment, the cells were treated with EdU (Guangzhou RiboBio Co., Ltd.) for 5 h and stained using the Apollo reaction cocktail and DAPI according to the manufacturer's instructions (Guangzhou RiboBio Co., Ltd.). Immediately after staining, the cells were examined under a inverted microscope Axio Observer A1 (Carl Zeiss, Germany).

4.6. Flow cytometry

Apoptosis and cell cycle progression were evaluated in 22RV1 cells using flow cytometry. 22RV1 cells were plated in 6-well plates at a density of $2x10^5$ cells/well for 24 h. The cells were then treated with 0.3 µM afatinib and/or 10 µM MDV3100 for 48 h. To analyze apoptosis, the cells were collected and stained using Annexin V-APC and propidium iodide (PI) (Nanjing KeyGen Biotech Co., Ltd.; cat. no. KGA1030) according to the manufacturer's instructions. For the cell cycle analysis, cells were fixed with 70% ethanol at -20 °C overnight. After fixation, the cells were collected and stained with the PI solution. Apoptosis and cell cycle progression were analyzed using a FACSCanto II flow cytometer (BD Biosciences).

4.7. Transwell assays

The migration of 22RV1 cells was determined using Transwell assays. 22RV1 cells ($1.2x10^5$ cells/well) were seeded into the upper chamber of a Transwell in serum-free culture medium containing 0.3 µM afatinib and/or 10 µM MDV3100 and cultured for 48 h. A volume of 700 µl RPMI-1640 medium containing 10% FBS was added to the lower chamber. Subsequently, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. After staining, the cells were examined under an optical microscope.

4.8. Western blot analysis

22RV1 cells (1.5×10^5 cells) were seeded in 6-well plates. After 24 h, cells were incubated with 0.3 µM afatinib and/or 10 µM MDV3100 for 48 h, and whole cell lysates were extracted for Western blot. Immunoblot analysis was conducted as described previously (Wu et al. 2013). The antibodies were used, including PARP (CST, 9532, USA), p53 (CST, 9282, USA), p21 (CST, 2947, USA), N-cadherin (CST, 4061, USA), E-cadherin (CST, 3195, USA), HER2 (CST, 2165, USA), HER3 (CST, 12708, USA), p-EGFR (CST, 2234, USA), EGFR (CST, 4267, USA), AR (CST, 5153, USA), PSA (CST, 5365, USA), GAPDH (CST, 2118S, USA), Bax (CST, 5023S, USA), Bcl-2 (CST, 4223S, USA), horseradish peroxidase-conjugated secondary antibodies (CST, 7074, USA). Each experiment was repeated at least three times.

4.9. Reverse transcription-quantitative PCR (RT-qPCR)

22RV1 cells were treated with 0.3 µM afatinib and/or 10 µM MDV3100, and RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed to cDNA using 5X PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd.). Subsequently, RT-qPCR was performed using SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.). The thermocycling conditions were as follows: Initial denaturation at 95 °C, followed by 35 cycles at 94 °C for 45 s, 60 °C for 34 s, 72 °C for 60 s and a final extension step at 72 °C for 10 min. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are provided in Table SI.

Table S1: PCR primer sequences

Gene	Sequence	
GAPDH	Forward	5'- GCACCGTCAAGGCTGAGAAC-3'
	Reverse	5'- TGGTGAAGACGCCAGTGGA-3'
ERK	Forward	5'- ACACCAACCTCTCGTACATCGG-3'
	Reverse	5'- TGGCAGTAGGTCTGGTGCTCAA-3'
EGFR	Forward	5'- AACACCCTGGTCTGGAAGTACG-3'
	Reverse	5'- TCGTTGGACAGCCTTCAAGACC-3'
HRAS	Forward	5'- ACGCACTGTGGAATCTCGGCAG-3'
	Reverse	5'- TCACGCACCAACGTGTAGAAGG-3'
BRAF	Forward	5'- AACGAGACCGATCCTCATCAGC-3'
	Reverse	5'- GGTAGCAGACAAACCTGTGGTTG-3'
HER2	Forward	5'- GGAAGTACACGATGCGGAGACT-3'
	Reverse	5'- ACCTTCCTCAGCTCCGTCTCTT-3'
HER3	Forward	5'- CTATGAGGCGATACTTGGAACGG-3'
	Reverse	5'- GCACAGTTCCAAAGACACCCGA-3'
AR	Forward	5'- ATGGTGAGCAGAGTGCCCTATC-3'
	Reverse	5'- ATGGTCCCTGGCAGTCTCCAAA-3'
PSA	Forward	5'- CGCAAGTTCACCCTCAGAAGGT -3'
	Reverse	5'- GACGTGATACCTTGAAGCACACC -3'
TMPRSS2	Forward	5'- CCTCTAACTGGTGTGATGGCGT-3'
	Reverse	5'- TGCCAGGACTTCCTCTGAGATG-3'

4.10. Statistical analysis

Standard software (SPSS 13.0 for Windows) was used for statistical analysis. Measurement data were expressed as mean±standard deviation (SD). Gaussian distribution test was conducted. One-way analysis of variance (ANOVA) was used to test the differences among the groups. $P<0.05$ was considered statistically significant.

Funding: This work was supported by the Natural Science Foundation of Guangdong Province, China (No. 2018A030313405, NO. 2021A1515011023), the Science and Technology Planning Project of Guangdong Province, China (No. 2017A050501020) and National Natural Science Foundation of China (No. 81872092).

Authors' contributions: Shaoyu Wu, Qiang Wei and Shidong Lv conceived and designed the experiments; Huanxian Wu, Jianhua Li, Dongling Quan, Danni Yang performed the experiments; Jiahaun Xu and Boyu Chen analyzed the data; Huanxian Wu and Shaoyu Wu wrote the paper. All authors have read and approved the manuscript.

Competing interests: The authors declare that they have no competing interests.

References

Agus DB, Akita RW, Fox WD, Lewis GD, Higgins B, Pisacane PI, Lofgren JA, Tindell C, Evans DP, Maiese K, Scher HI, Sliwkowski MX (2002) Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell 2: 127–137.

Arai S, Jonas O, Whitman MA, Corey E, Balk SP, Chen S (2018) Tyrosine kinase inhibitors increase MCL1 degradation and in combination with BCLXL/BCL2 inhibitors drive prostate cancer apoptosis. Clin Cancer Res 24: 5458–5470.

Beer TM, Armstrong AJ, Rathkopf DE, Loriot Y, Sternberg CN, Higano CS, Iversen P, Bhattacharya S, Carles J, Chowdhury S, Davis ID, de Bono JS, Evans CP, Fizazi K, Joshua AM, Kim CS, Kimura G, Mainwaring P, Mansbach H, Miller K, Noonberg SB, Perabo F, Phung D, Saad F, Scher HI, Taplin ME, Venner PM, Tombal B, Investigators P (2014) Enzalutamide in metastatic prostate cancer before chemotherapy. New Engl J Med 371: 424–433.

Buttigliero C, Tucci M, Bertaglia V, Vignani F, Bironzo P, Di Maio M, Scagliotti GV (2015) Understanding and overcoming the mechanisms of primary and acquired resistance to abiraterone and enzalutamide in castration resistant prostate cancer. Cancer Treat Rev 41: 884–892.

Chen T, Xu J, Fu W (2020) EGFR/FOXO3A/LXR-alpha axis promotes prostate cancer proliferation and metastasis and dual-targeting LXR-alpha/EGFR shows synthetic lethality. Front Oncol 10: 1688.

Chou TC (2010) Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 70: 440–446.
Chuang TD, Chen SJ, Lin FF, Veeramani S, Kumar S, Batra SK, Tu YP, Lin MF (2010) Human prostatic acid phosphatase, an authentic tyrosine phosphatase, dephosphorylates ErbB-2 and regulates prostate cancer cell growth. J Biol Chem 285: 23598–23606.
Corn PG, Zhang M, Nogueras-Gonzalez GM, Xiao L, Zurita AJ, Subudhi SK, Tu SM, Aparicio AM, Coarfa C, Rajapakshe K, Huang S, Navone NM, Lin SH, Wang G, Ramachandran S, Titus MA, Panaretakis T, Gallick GE, Efstathiou E, Troncoso P, Logothetis C (2020) A phase II study of cabozantinib and androgen ablation in patients with hormone-naive metastatic prostate cancer. Clin Cancer Res 26: 990–999.
Coutinho I, Day TK, Tilley WD, Selth LA (2016) Androgen receptor signaling in castration-resistant prostate cancer: a lesson in persistence. Endocr Relat Cancer 23: T179–T197.
Craft N, Shostak Y, Carey M, Sawyers CL (1999) A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat Med 5: 280–285.
Dahut WL, Scripture C, Posadas E, Jain L, Gulley JL, Arlen PM, Wright JJ, Yu Y, Cao L, Steinberg SM, Aragon-Ching JB, Venitz J, Jones E, Chen CC, Figg WD (2008) A phase II clinical trial of sorafenib in androgen-independent prostate cancer. Clin Cancer Res 14: 209–214.
Evans AJ (2018) Treatment effects in prostate cancer. Mod Pathol 31: S110–S121.
Feldman BJ, Feldman D (2001) The development of androgen-independent prostate cancer. Nat Rev Cancer 1: 34–45.
Feng Q, He B (2019) Androgen receptor signaling in the development of castration-resistant prostate cancer. Front Oncol 9: 858.
Green SM, Mostaghel EA, Nelson PS (2012) Androgen action and metabolism in prostate cancer. Mol Cell Endocrinol 360: 3–13.
Gross M, Higano C, Pantuck A, Castellanos O, Green E, Nguyen K, Agus DB (2007) A phase II trial of docetaxel and erlotinib as first-line therapy for elderly patients with androgen-independent prostate cancer. BMC cancer 7: 142.
Harris WP, Mostaghel EA, Nelson PS, Montgomery B (2009) Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. Nat Clin Pract Urol 6: 76–85.
Jamroze A, Chatta G, Tang DG (2021) Androgen receptor (AR) heterogeneity in prostate cancer and therapy resistance. Cancer Lett 518: 1–9.
Jathal MK, Steele TM, Siddiqui S, Mooso BA, D'Abronzo LS, Drake CM, Whang YE, Ghosh PM (2019) Dacomitinib, but not lapatinib, suppressed progression in castration-resistant prostate cancer models by preventing HER2 increase. Br J Cancer 121: 237–248.
Knudsen KE, Scher HI (2009) Starving the addiction: new opportunities for durable suppression of AR signaling in prostate cancer. Clin Cancer Res 15: 4792–4798.
Kung HJ (2011) Targeting tyrosine kinases and autophagy in prostate cancer. Horm Cancer 2: 38–46.
McNamara MA, George DJ, Ramaswamy K, Lechpammer S, Mardekian J, Schultz NM, Wang L, Baser O, Huang A, Freedland SJ (2019) Overall survival by race in chemotherapy-naïve metastatic castration-resistant prostate cancer (mCRPC) patients treated with abiraterone acetate or enzalutamide. J Clin Oncol 37: 212–212.
Minner S, Jessen B, Stiedenroth L, Burandt E, Kollermann J, Mirlacher M, Erbersdobler A, Eichelberg C, Fisch M, Brummendorf TH, Bokemeyer C, Simon R, Steuber T, Graefen M, Huland H, Sauter G, Schlomm T (2010) Low level Her2 overexpression is associated with rapid tumor cell proliferation and poor prognosis in prostate cancer. Clin Cancer Res 16: 1553–1560.
Molife LR, Omlin A, Jones RJ, Karavasilis V, Bloomfield D, Lumsden G, Fong PC, Olmos D, O'Sullivan JM, Pedley I, Hickish T, Jenkins P, Thompson E, Oommen N, Wheatley D, Heath C, Temple G, Pelling K, de Bono JS (2014) Randomized Phase II trial of nintedanib, afatinib and sequential combination in castration-resistant prostate cancer. Future Oncol 10: 219–231.
Nabhan C, Lestingi TM, Galvez A, Tolzien K, Kelby SK, Tsarwhas D, Newman S, Bitran JD (2009) Erlotinib has moderate single-agent activity in chemotherapy-naive castration-resistant prostate cancer: final results of a phase II trial. Urology 74: 665–671.
Nevedomskaya E, Baumgart SJ, Haendler B (2018) Recent advances in prostate cancer Treatment and Drug Discovery. Int J Mol Sci 19: 1359.
Ojo D, Lin X, Wong N, Gu Y, Tang D (2015) Prostate cancer stem-like cells contribute to the development of castration-resistant prostate cancer. Cancers 7: 2290–2308.
Perlmutter MA, Lepor H (2007) Androgen deprivation therapy in the treatment of advanced prostate cancer. Rev Urol 9 Suppl 1: S3–S8.
Pienta KJ, Bradley D (2006) Mechanisms underlying the development of androgen-independent prostate cancer. Clin Cancer Res 12: 1665–1671.
Qian K, Wang G, Ju L, Liu J, Luo Y, Wang Y, Peng T, Chen F, Zhang Y, Xiao Y, Wang X (2020) A novel germline EGFR variant p.R831H causes predisposition to familial CDK12-mutant prostate cancer with tandem duplicator phenotype. Oncogene 39: 6871–6878.
Rice MA, Malhotra SV, Stoyanova T (2019) Second-generation antiandrogens: from discovery to standard of care in castration resistant prostate cancer. Frontiers Oncol 9: 801.
Salzberg M, Rochlitz C, Morant R, Thalmann G, Pedrazzini A, Roggero E, Schonenberger A, Knuth A, Borner M (2007) An open-label, noncomparative phase II trial to evaluate the efficacy and safety of docetaxel in combination with gefitinib in patients with hormone-refractory metastatic prostate cancer. Onkologie 30: 355–360.
Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, de Wit R, Mulders P, Chi KN, Shore ND, Armstrong AJ, Flaig TW, Flechon A, Mainwaring P, Fleming M, Hainsworth JD, Hirmand M, Selby B, Seely L, de Bono JS, Investigators A (2012) Increased survival with enzalutamide in prostate cancer after chemotherapy. N Engl J Med 367: 1187–1197.
Schlomm T, Kirstein P, Iwers L, Daniel B, Steuber T, Walz J, Chun FH, Haese A, Kollermann J, Graefen M, Huland H, Sauter G, Simon R, Erbersdobler A (2007) Clinical significance of epidermal growth factor receptor protein overexpression and gene copy number gains in prostate cancer. Clin Cancer Res 13: 6579–6584.
Sharifi N (2013) Mechanisms of androgen receptor activation in castration-resistant prostate cancer. Endocrinology 154: 4010–4017.
Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Ca-Cancer J Clin 71: 209–249.
Tamura S, Wang Y, Veeneman B, Hovelson D, Bankhead A, 3rd, Broses LJ, Lorenzatti Hiles G, Liebert M, Rubin JR, Day KC, Hussain M, Neamati N, Tomlins S, Palmbos PL, Grivas P, Day ML (2018) Molecular correlates of in vitro responses to dacomitinib and afatinib in bladder cancer. Bladder Cancer 4: 77–90.
Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP (1995) Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332: 1393–1398.
Tilley WD, Buchanan G, Hickey TE, Bentel JM (1996) Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin Cancer Res 2: 277–285.
Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, Wasielewska T, Welsbie D, Chen CD, Higano CS, Beer TM, Hung DT, Scher HI, Jung ME, Sawyers CL (2009) Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science 324: 787–790.
Wade CA, Kyprianou N (2018) Profiling prostate cancer therapeutic resistance. Int J Mol Sci 19: 904.
Watanuki Z, Kosai H, Osanai N, Ogama N, Mochizuki M, Tamai K, Yamaguchi K, Satoh K, Fukuhara T, Maemondo M, Ichinose M, Nukiwa T, Tanaka N (2014) Synergistic cytotoxicity of afatinib and cetuximab against EGFR T790M involves Rab11-dependent EGFR recycling. Biochem Biophys Res Commun 455: 269–276.
Whang YE, Armstrong AJ, Rathmell WK, Godley PA, Kim WY, Pruthi RS, Wallen EM, Crane JM, Moore DT, Grigson G, Morris K, Watkins CP, George DJ (2013) A phase II study of lapatinib, a dual EGFR and HER-2 tyrosine kinase inhibitor, in patients with castration-resistant prostate cancer. Urol Oncol-Semin Ori 31: 82–86.
Wind S, Schnell D, Ebner T, Freiwald M, Stopfer P (2017) Clinical pharmacokinetics and pharmacodynamics of afatinib. Clin Pharmacokinet 56: 235–250.
Wu J, Wei H, Qu H, Feng Z, Long J, Ge Q, Deng H (2017) Plasma vascular endothelial growth factor B levels are increased in patients with newly diagnosed type 2 diabetes mellitus and associated with the first phase of glucose-stimulated insulin secretion function of beta-cell. J Endocrinol Invest 40: 1219–1226.
Wu SY, Chen TM, Gmeiner WH, Chu E, Schmitz JC (2013) Development of modified siRNA molecules incorporating 5-fluoro-2'-deoxyuridine residues to enhance cytotoxicity. Nucl Acids Res 41: 4650–4659.
Yeh S, Kang HY, Miyamoto H, Nishimura K, Chang HC, Ting HJ, Rahman M, Lin HK, Fujimoto N, Hu YC, Mizokami A, Huang KE, Chang C (1999) Differential induction of androgen receptor transactivation by different androgen receptor coactivators in human prostate cancer DU145 cells. Endocrine 11: 195–202.
Yuan Y, Sheng Z, Liu Z, Zhang X, Xiao Y, Xie J, Zhang Y, Xu T (2020) CMTM5-v1 inhibits cell proliferation and migration by downregulating oncogenic EGFR signaling in prostate cancer cells. J Cancer 11: 3762–3770.
Zhang XQ, Lee MS, Zelivianski S, Lin MF (2001) Characterization of a prostate-specific tyrosine phosphatase by mutagenesis and expression in human prostate cancer cells. J Biol Chem 276: 2544–2550.
Zhu Y, Luo J (2020) Regulation of androgen receptor variants in prostate cancer. Asian J Urol 7: 251–257.