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ORIGINAL ARTICLE

Inhibition of telomerase potentiates enzalutamide efficiency of androgen-sensitive human prostate cancer cells

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Summary

Purpose: *Androgen deprivation therapy (ADT) is one of the main strategies to treat prostate cancer (PCa) at various stages of its development. Androgen receptor (AR) antagonists such as enzalutamide are mainstay treatments for castration-sensitive prostate cancer. Though, a majority of patients initially respond to ADT, most will eventually progress to castrate-resistant, due to the development of different mutations on the AR. PCa cells express high telomerase activity, and there is a correlation between the total activity of telomerase and the Gleason score. Therefore, we hypothesized that the combination of enzalutamide plus a telomerase inhibitor could be more effective than enzalutamide alone in decreasing cell survival.*

Methods: *In this study MTT test, RT-qPCR and image-based cytometry were used to investigate cell viability, apoptosis and cell cycle progression of androgen-responsive human prostate cancer LNCaP cells. The cells were treated with 5 μM enzalutamide and 40 μM telomerase inhibitor BIBR 1532, or with their combinations for 72 hrs.*

Results: *Enzalutamide and BIBR 1532 alone inhibited cell proliferation in a dose-dependent manner. The combinations of the two agents could synergistically induce apoptotic and necrotic cell death. Either inhibition of telomerase by BIBR 1532 or AR blockages by enzalutamide decreased prostate-specific antigen (PSA) and the catalytic component of telomerase, hTERT, expression.*

Conclusion: *These results suggest that telomerase inhibition therapy may contribute to the efficacy of enzalutamide in the androgen-sensitive PCa model.*

Key words: *BIBR 1532, enzalutamide, LNCaP cells, prostate cancer, telomerase*

Introduction

PCa is one of the most common cancers among men of all races, and it is the second leading cause of cancer-related mortality in men [1]. Chemotherapy, radiotherapy and androgen deprivation have been the mainstay of therapy for metastatic PCa. Even though most patients with metastatic PCa initially respond to ADT therapy, the majority of patients develop castration-resistant prostate cancer (CRPC) due to the emergence of different mutations on the AR [2]. On the other hand, therapy options for castration-sensitive and CRPC remain limited. Enzalutamide (Xtandi®) is an AR signaling inhibitor which has been developed for PCa treatment [3]. Enzalutamide blocks binding of androgens to AR, nuclear translocation of AR as well as AR-mediated DNA binding blocks the growth of CRPC, and was shown to increase the lifespan after chemotherapy by a median of 4.8 months compared with placebo [4]. However, patients on androgen deprivation with enzalutamide or other anti-androgen agents eventually develop resistance, and AR may still remain active in these patients with CRPC [5]. Therefore, there is an urgent need to develop and identify novel and efficient therapeutic approaches for PCa.

Telomeres are composed of noncoding nucleoprotein complexes of about 1000-2000 TTAGGG tandem base pair repeats which protect the ends

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of linear chromosomes [6, 7]. With each new cell division, telomeric repeats are incompletely replicated, and therefore, their ends are gradually shortened [8]. Telomere length is regulated by the cellular reverse transcriptase telomerase, a ribonucleoprotein complex, and its maintenance contributes to normal human cellular aging and human diseases [9]. The telomerase holoenzyme is composed of the catalytic subunit telomerase reverse transcriptase (TERT), telomerase RNA component (TERC) and telomerase complex-associated proteins [9]. Previous reports have shown that the vast majority of human carcinomas have high telomerase activity, therefore their telomere lengths are not shortened [7,10]. It has been demonstrated that there is a significant correlation between the total telomerase activity and the volume of Gleason score [10]. On the contrary, none of normal prostate tissues express telomerase activity [11].

2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid (BIBR 1532) is a mixed-type non-competitive inhibitor of telomerase, and has been shown to have little effect on several mammalian DNA and RNA polymerases [12,13]. It specifically targets the telomerase reverse transcriptase catalytic subunit, TERT [14]. Through its effects on telomerase, BIBR 1532 induces senescence or apoptosis in cancer cells [13].

The objective of this study was to investigate whether the combination of enzalutamide with BIBR 1532 would promote CRPC cell proliferation and reveal the underlying molecular mechanisms.

Methods

Cell line and cell cultures

An androgen-sensitive human prostate cancer cell line LNCaP was initially purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in complete phenol-free DMEM/F-12 medium (DMEM/Ham's F-12 Mix 50/50, Winsent, Quebec Canada) with 2 mM L-glutamine and 1500 mg/L sodium bicarbonate, 10 % fetal bovine serum (FBS; Life Technologies, USA), and 1% penicillin–streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2. BIBR 1532 (Cayman,USA) and enzalutamide (Cayman,USA) were dissolved in 100% DMSO (Sigma Aldrich, Hybri-Max) and stored at -20 °C until use. The final concentration of DMSO did not exceed 0.1%, and controls received the same volume of vehicle.

Cell proliferation tests

LNCaP cells were treated with different concentrations of enzalutamide and BIBR 1532 (0-100 μM) for 72 hrs. After the incubation period, the medium was removed and the cells were washed with PBS. Then, the cells were incubated with a 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (MTT,Sigma) for 3 hrs. The medium was removed, MTT tests were performed, and absorbance at 570 nm was measured using a plate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, USA). For the calculation of cell viability, the following equation was used: optical density (OD) sample/OD blank control x 100. Each treatment was repeated at least three times. For morphological comparison, cells were photographed using an inverted microscope (Zeiss Axio Vert. A1, Germany).

Annexin V/propidium iodide (PI) binding assay

The degree of apoptosis was determined by using the Tali image-based cytometer apoptosis kit - annexin V Alexa Fluor 488 and PI, according to the manufacturer's instructions (Invitrogen/Life Technologies, Carlsbad, CA, USA). In brief, the cells were incubated at a density of $2.5x10^5$ in a 6-well plate for 16 hrs and then treated with the agents. The cells were collected 72 hrs post-treatment and washed twice with ice-cold PBS. They were then resuspended in annexin binding buffer (ABB) and incubated with Annexin V Alexa Fluor 488 at room temperature (RT) in the dark for 15 min. After subsequent centrifugation at 300 g for 5 min, the cells were again resuspended in ABB and incubated with PI at RT in the dark for 5 min. The cells were evaluated by Tali image-based cytometer (Invitrogen/Life Technologies, CA, USA) within 30 min.

Cell cycle analysis

LNCaP cells were seeded and treated as described in the previous assay section. The cells were then collected by centrifugation and fixed with 70% ethanol in PBS overnight at -20 °C. After LNCaP cells were harvested, cell cycle progression was evaluated by quantification of the cellular DNA content using PI staining following the manufacturer's instructions (Tali Cell Cycle Kit, Life Technologies, CA, USA). The results were reported as percentages of G1, S and G2/M cells.

RNA isolation, cDNA synthesis, and RT-qPCR

Total RNA was purified using a kit as per the manufacturer's instructions (Thermo Fisher Scientific). RNAs (1 μg) were then subjected to a reverse transcription cDNA synthesis kit (Life Technologies, USA), and the resultant cDNAs were used for real-time PCR analysis with gene-specific primer pairs. mRNA expression analyses were performed on a Step One Plus Real-Time PCR System (Applied Biosystems, CA, USA). Primers used in this study were caspase-8 (F:5'-CTGCTGGGGATGGCCACTGTG-3', R:5'-TCGCCTCGAGGACATCGCTCTC-3'), caspase-3 (F:5'-CAAACTTTTTCAGAGGGGATCG-3', R:5'-GCATACTGTTTCAGCATGGCA-3'), bax (F:5'-CCCGAGAGGTCTTTTTCCGAG-3', R:5'-CCAGCCCATGATGGTTCTGAT-3'), p21Cip1 (F: 5'-GGCGTTTGGAGTGGTAGAAA-3', R:5'-GACTCTCAGGGTCGAAAACG-3'), p27Kip1 (F:5'-CCGGCTAACTCTGAGGACAC-3', R:5'-TGGATCCAAGGCTCTAGGTG-3'), PSA (F:5'-GCGTGATCTTGCTGGGTCGG -3', R:5'-CCTTCTGAGGGTGAACTTGCG-3'), AR (F:5'-AGGATGCTCTACTTCGCCCC-3', R:5'-ACTGGCTGTACATCCGGGAC-3') and hTERT (F:5'-AGAGT-

GTCTGGAGCAAGTTGC-3', R:5'-TCTCCTCCACCTCTGAC TGCTCC-3'. GAPDH (F:5'-TTGGTATCGTGGAAGGACT-CA-3', R:5'-TGTCATCATATTTGGCAGGTTT-3') expressions were used as an internal reference, and all reactions were run in triplicate, and performed three times on separate occasions using different RNA. The qPCR cycling conditions included an initial denaturation at 95 ºC for 5 min followed by 35 cycles at 95 ºC for 15 sec and 60 ºC (58 ºC for MDK) for 60 sec. The fold enrichments of the targets were calculated using the relative quantification according to the $2^{-\Delta\Delta Ct}$ method [15]. The oligonucleotide primers were synthesized by PZR Biotech (Ankara,Turkey). GAPDH expression was used as an internal reference, and all reactions were run in triplicate and were performed three times on separate occasions using different RNA.

Analysis of drug combinations

The interaction between BIBR 1532 and enzalutamide was determined by the isobologram method and the median effect method described by Chou and Talalay [16]. CompuSyn software was used for analysis of combination data in the synergistic studies.

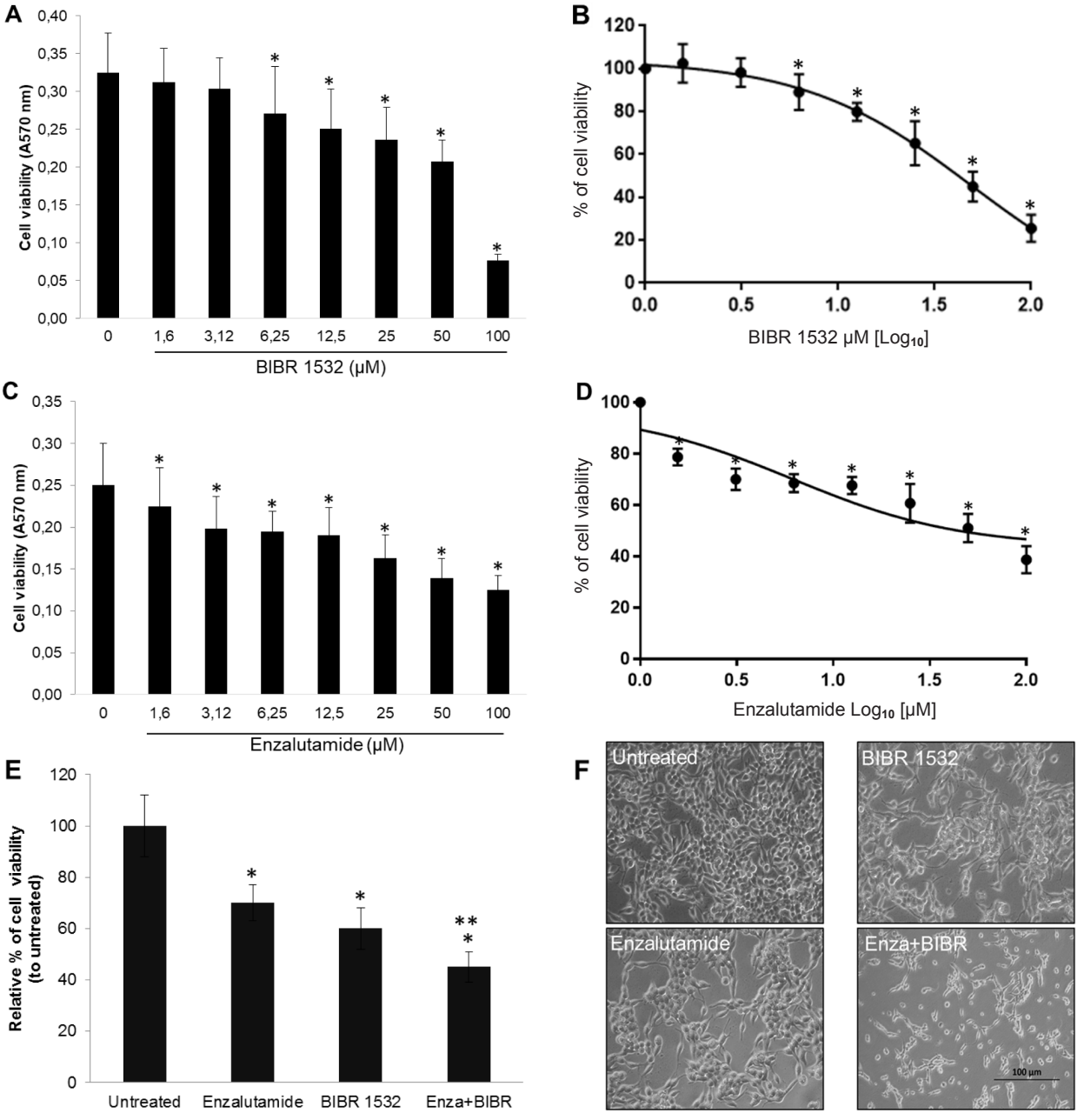

Figure 1. BIBR 1532 and enzalutamide induces LNCaP cell death in a dose-dependent manner, and combined therapy synergistically inhibits the cell proliferation. Human PCa cells were seeded onto 96-well plates with fresh complete medium or the same medium containing BIBR1532 **(A,B)** or enzalutamide **(C,D)** or a combination **(E)** of both at 40 μM and 5 μM, respectively for 72 hrs. Viable cells compared to controls (defined as relative cell viability 1.0) were measured by MTT assay. The data are expressed as mean ± SD of at least three independent experiments. *p<0.05 vs. untreated, **p<0.05 vs. enzalutamide and BIBR 1532. The combination of BIBR 1532 with enzalutamide markedly changed the morphology and the intensity of PCa cells as evaluated by inverted microscopy **(F)**.

Statistics

All experiments were repeated at least twice in triplicate; each data point represented the mean±SD. Statistical significance was compared between the various treatment groups and controls using one-way ANOVA, followed by Duncan's multiple range test for multiple comparisons. P<0.05 was considered to be statistically significant. Statistical analyses were performed using the statistical software SPSS for Windows (19.0; SPSS, Chicago, IL). The IC_{50} values were calculated using GraphPad Prism (7.0; GraphPad Software, Inc., La Jolla, CA, USA).

Results

Combination therapy enhances the antiproliferative effects of enzalutamide

Enzalutamide and BIBR 1532 alone inhibited cell proliferation in a dose-dependent manner (Figure 1A-D). The half maximal inhibitory concentration (IC_{50}) values of enzalutamide and telomerase inhibitor BIBR 1532 were estimated equal to 5 μM and 40 μM, respectively. Enzalutamide in combination with BIBR 1532 significantly increased the cytotoxic effects in LNCaP cells at IC_{50} values (Figure 1E). The combination of the two agents resulted in a synergistic (combination index value at ED50: 0.79) inhibitory effect on cell survival. Microscopic evaluation of monolayer cells showed signs of apoptotic and necrotic cell death due to enzalutamide plus BIBR 1532 treatment (Figure 1F).

Combined treatment significantly increased apoptosis and non-apoptotic cell death

To determine whether BIBR 1532 combination sensitizes LNCaP cells to AR-agonist enzalutamide treatment, cells were treated with 5 μM enzalutamide and 40 μM BIBR1532 or their combination for 72 hrs. Apoptosis and cell death were analyzed by Annexin V and PI. The combination therapy induced cell death and apoptosis to a significantly greater extent than treatment with either agent alone (Figure 2A). To clarify the mechanism under the induction of cell apoptosis in LNCaP cells we analyzed the apoptosis-related gene expressions by RT-qPCR. Such combination therapy did significantly upregulated the mRNA expression of caspase 8 but pro-apoptotic bax expression was downregulated (Figure 3A).

Enzalutamide and BIBR1532 increased cell cycle arrest in G1 phase

To determine the mechanisms underlying the growth inhibitory effect of BIBR 1532, we investigated cell cycle progression and the expression levels of several genes related to cell cycle progression. As shown in Figure 2B, enzalutamide and BIBR 1532 significantly inhibited cell growth by blocking transition at the G1 phase. Treatment with enzalutamide and BIBR 1532 significantly increased the cell number at the G1 phase from 50% to 89% and 79%, respectively, and decreased the cell population at the S and G2/M phases. However, combining the two drugs did not enhance the cell cycle arrest activity of the individual agents. Given the essential role of p21 and p27 in growth arrest after DNA damage, we examined the influence of BIBR 1532 on the induction of p21 and p27 performing RT-qPCR. mRNA expression analysis revealed that the combination therapy led to upregulation of p21 (2.8-fold) and p21 (2.3-fold) compared to controls (Figure 3A).

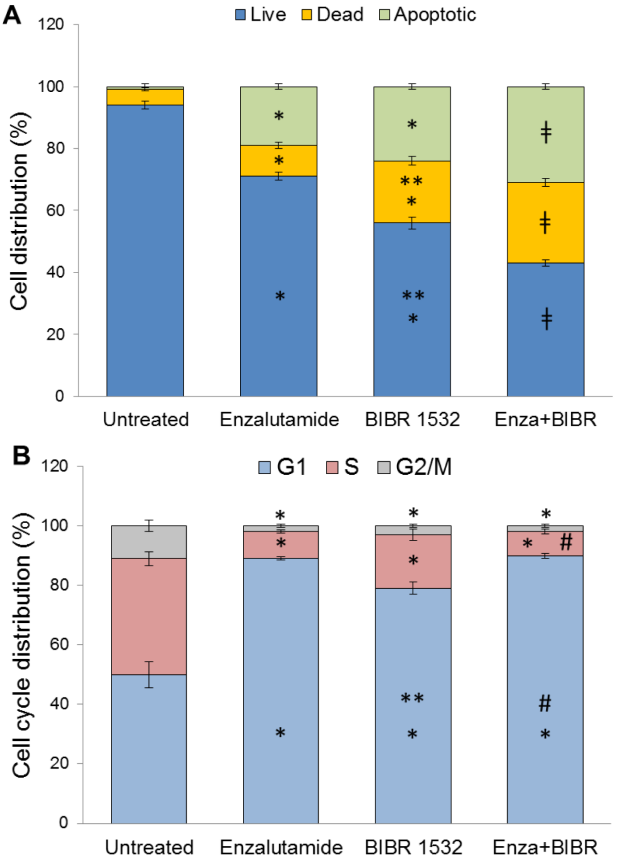

Figure 2. The effect of BIBR 1532, enzalutamide or their combination on apoptosis **(A)** and cell cycle progression **(B)**. The combination of BIBR1532 with enzalutamide significantly promotes apoptosis in LNCaP cells **(B)**. Cells were treated with 40 μM BIBR1532, 5 μM enzalutamide, or a combination of both for 72 hrs. Then, apoptosis was measured using Tali image-based cytometry after the cells were stained with annexin-V/PI. *p<0.01 vs. control, **p<0.01 vs. enzalutamide, ‡p<0.01 vs. untreated, enzalutamide, BIBR 1532 groups. DNA content analyses of LNCaP cells were stained and analyzed by Tali image-based cytometer using commercial cell cycle kit **(B)**. Cells were treated and incubated as explained at **A**. *p<0.01 vs. control, **p<0.01 vs. enzalutamide, #p<0.01 vs. BIBR 1532. The data are expressed as mean ± SD of at least two independent experiments.

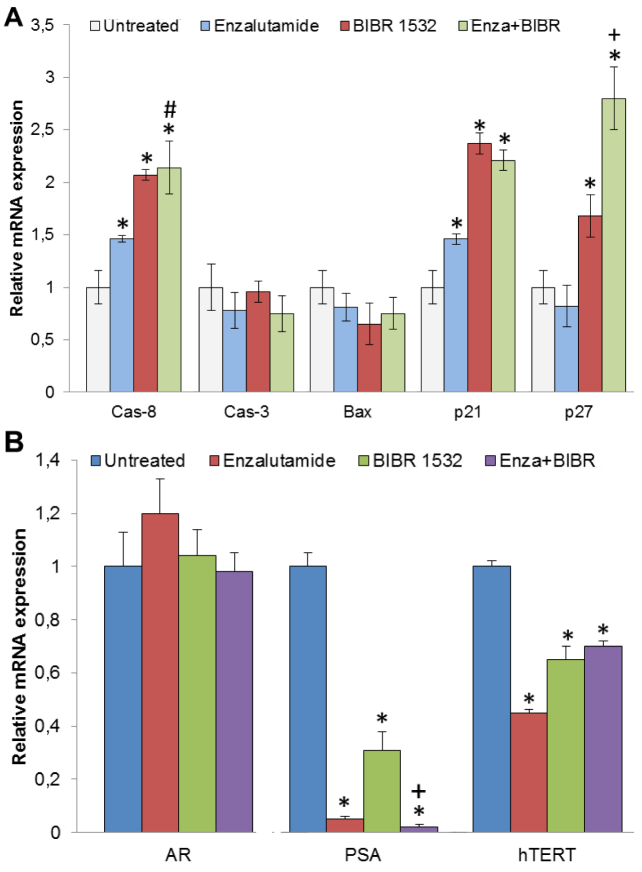

Figure 3. BIBR 1532, enzalutamide or combination therapies regulate gene expressions. LNCaP cells were treated with 40 μM BIBR1532, 5 μM enzalutamide or a combination of the two agents for 72 hrs. mRNA expressions were determined by RT-qPCR. **A:** Caspase-8 (Cas-8), Cas-3, bax, p21 and p27. *p<0.05 vs. untreated, #p<0.05 vs. enzalutamide, +p<0.05 vs. enzalutamide, BIBR 1532. **B:** AR, PSA and hTERT. *p<0.001 vs. untreated, +p<0.05 vs. enzalutamide, BIBR 1532. Data represent the mean ± SD of triple experiments.

Combined therapy decreased androgen receptor and PSA expressions in LNCaP cells

As shown in Figure 3B, enzalutamide treatment significantly decreased the mRNA expressions of PSA and hTERT, while AR expression remained unchanged. Inhibition of telomerase by BIBR 1532 significantly downregulated the mRNA expression of PSA and hTERT, but AR expression appeared unaltered. The combination of enzalutamide with BIBR 1532 resulted in a significant decrease of PSA and hTERT expressions (Figure 3B).

Discussion

High telomerase activity provides cancer cells with unlimited proliferation ability and is one of the hallmarks of cancer. PCa cell lines have relatively short telomeric length and maintain endogenous telomerase activity; hence, they are potential targets for telomere-targeting agents [17]. Indeed, it has been reported that inhibition of telomerase activity would decrease the cell survival [18]. The telomerase inhibitor BIBR 1532 selectively targets the telomerase reverse transcriptase catalytic subunit, TERT [14], and results in senescence or apoptosis of cancer cells [13]. It has been demonstrated that various growth factors, cytokines and kinase pathways increase AR signaling, thus endorsing progression to CRPC in a ligand-independent manner [19]. Enzalutamide is a second-generation antiandrogen that inhibits AR signaling pathway and has been used for the therapy of CRPC patients, however one in the four patients gradually develop drug resistance within 24 months of initial exposure [4]. Therefore, new therapeutic strategies are needed to improve treatment effects.

We attempted to investigate whether the combination of telomerase inhibition and AR-blockage therapy would produce a more effective treatment than with individual treatment in CRPC LNCaP cells. Enzalutamide (5 μM) and BIBR 1532 (40 μM) inhibited the proliferation of LNCaP cells in a dose-dependent manner (Figures 1A-D). Our results are similar to the findings of previous reports that BIBR 1532 induces cytotoxic effect in leukemia [20], breast cancer [21], and endometrial cancer cells [22]. Enzalutamide suppress AR-positive LNCaP cell survival [23,24], induces tumor regression in a mouse model [25], and enhances overall survival up to 4.8 months in patients with PCa [4]. The combination of the two agents caused a significantly synergistic decrease in cell viability (Figure 1E). Feldser and co-workers [26] have indicated that telomerase inhibition can be eroded down to a critical length, followed by initiation of DNA injury response pathways, replicative senescence, or apoptosis. To clarify the synergistic mechanism of BIBR1532 and enzalutamide, we evaluated the level of apoptosis by cytometer, studied the expression levels of apoptosis-related genes, and we then analyzed the cell cycle progression (Figure 2B). Image-based cytometer analyses have revealed that enzalutamide in combination with BIBR 1532 exhibited a much more potent activity than either agent alone to promote LNCaP cells undergoing apoptosis and necrosis (Figure 2A) which supports the previous reports of Feldser and colleagues [26].

RT-qPCR evaluations have shown that either BIBR 1532 or enzalutamide induce apoptosis through extrinsic pathway (i.e. caspase-8), but did not change mitochondrial pathway (Figure 3A). The combinations of the agents did not significantly elevate caspase-8 expression, which demonstrates that the combined therapy might induce apoptosis

via a different mechanism. Indeed, previous studies reported that elevated expression of hTERT potentiates cell growth via several signaling pathways including EGFR, Wnt/β-catenin, c-Myc, NF-κB and PI3K/Akt signaling [27,28]. AR signaling regulates the expression of hTERT and the activity of telomerase [29]. Supportive to this, enzalutamide as well as BIBR 1532 and their combination significantly inhibited AR and hTERT expressions (Figure 3B). Our data therefore indicate that BIBR 1532 in combination with an antiandrogen could represent a viable strategy to improve the therapeutic outcome of androgen ablation therapy.

BIBR 1532 exerts a direct short-term growth inhibition effect in a leukemia cell line through induction of p21 coupled with downregulation of hTERT transcription [30]. Shi et al. [21] have demonstrated that antitelomerase therapy by telomerase inhibitors GRN163L [31] and BIBR 1532 caused cell cycle arrest at G1 phase in bladder and breast cancer cell lines, respectively. Similar to previous results, we have also found that BIBR 1532 treatment retained LNCaP cells in G1 phase (Figure 2B). Furthermore, BIBR 1532 induced the expressions of cell cycle inhibitors p21 and as well as p27 genes (Figure 3A). Our results are in good agreement with previously reported results that BIBR 1532 leads to continuous telomere erosion in carcinoma cell lines such as lung, breast and PCa through p21-induced senescence [13]. The significance of telomerase inhibition in immortal human cells is telomere shortening and subsequent growth arrest or apoptosis [32]. However, the combination of BIBR1532 with enzalutamide did not strengthen the cell cycle arrest effect of both substances alone.

Androgens bind to the AR to transmit their biological function. Mutations or enhanced AR expression may contribute to the progression of PCa, and is commonly associated with poor survival [5]. Scher and colleagues [4] have reported that AR-blockage therapy by enzalutamide resulted in about 50% reduction of serum PSA and extension of survival up to 4.8 months. Another study has shown that the antiandrogen bicalutamide significantly downregulated the expressions of PSA and telomerase [29]. We have checked whether the combined therapy would decrease PSA expression in LNCaP cells. Indeed, we found that the combination of BIBR 1532 with enzalutamide produced robust downregulation of PSA expression, which is in concordance with previous findings [4,33] (Figure 3B).

In conclusion, our results suggest that telomerase inhibitor BIBR 1532 inhibits the proliferation of AR-sensitive LNCaP cell line through cell cycle arrest and apoptosis. The combination of BIBR 1532 with enzalutamide synergistically increases the inhibition of PCa cell proliferation. These data might expand our insights into the efficacy of the combination of telomerase inhibition and current chemotherapy on the therapy of PCa.

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Conflict of interests

The authors declare no conflict of interests.

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