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Original Article

STL127705 synergize with olaparib in castration-resistant prostate cancer by inhibiting homologous recombination and non-homologous end-joining repair

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Abstract: Therapeutic resistance to androgen-deprivation therapy is a major challenge for prostate cancer therapy. The present study aims to explore the effects of poly (ADP-ribose) polymerase (PARP) inhibitor olaparib and STL127705 on castration-resistant prostate cancer. Cell lines including PC-3 and enzalutamide-resistant LNCaP (erLNCaP) cells were treated with enzalutamide, enzalutamide plus olaparib, enzalutamide plus STL127705, or the combination of olaparib, STL127705, and enzalutamide. Cell viabilities and cell apoptosis were determined using the sulforhodamine B (SRB) assay and Annexin V/propidium iodide staining, respectively. Flow cytometry assay was applied to determine γH2AX intensity and the percentage of homologous recombination and non-homologous end-joining. Besides, a tumor-bearing animal model was established and treated with drugs as for cell lines. STL127705 and olaparib enhanced cytotoxicity of enzalutamide on erLNCaP and PC-3 cells. Furthermore, STL127705 and olaparib promoted enzalutamide-induced cell apoptosis and enhanced γH2AX intensity. *In vitro* study also showed that the combination of STL127705, olaparib, and enzalutamide inhibited homologous recombination and non-homologous end-joining repair systems in PC-3 cells. *In vivo* study demonstrated that the combination of STL127705, olaparib, and enzalutamide exhibited a significant anti-tumor effect. STL127705 combined with olaparib have a potential therapeutic effect on castration-resistant prostate cancer through inhibiting homologous recombination and non-homologous end-joining repair.

Keywords: Prostate cancer, castration-resistant prostate cancer, PAPR inhibitor, olaparib, STL127705

Introduction

Prostate cancer occurs in prostate land and is one of the most common types of cancer in males [1]. According to a global cancer statistics report, there were 1,276,106 cases of prostate cancer and 358,989 prostate cancer-induced cases in 2018 [2]. Prostate cancer can be caused by inherited or acquired gene mutations [3]. Besides, some risk factors including smoking, age, obesity, and chemical exposure raise the risk of developing into prostate cancer [3]. A growing body of evidence supports that androgen plays an important role in the pathogenesis of prostate cancer [4]. Androgen can promote tumorigenesis and activate the androgen signaling pathways in the development of prostate cancer [4]. In addition, both pre-clinical and clinical studies have demonstrated that androgen deprivation is an effective strategy for the treatment of prostate cancer [5, 6].

Nowadays, there are several therapeutic strategies including chemotherapy, surgery, hormone therapy, and immunotherapy that have been developed against prostate cancer [6]. Hormone therapy, also called androgen deprivation therapy, is used for the patients who are in early-stage prostate cancer with a high risk of recurrence, or are in advanced-stage with cancer metastasis [7]. Although androgen deprivation therapy is effective for the treatment of prostate cancer, castration resistance

is still observed in some of the patients [7, 8]. In order to overcome prostate cancer castration resistance, several medications have been developed. Those medications include autologous cellular immunotherapy (Sipuleucel-T), androgen synthesis inhibitor (Abiraterone), androgen receptor blocker (Enzalutamide), and microtubule inhibitor (Cabazitaxel) [9, 10]. However, there are still some limitations for the application of these medications. For instance, not all patients respond to enzalutamide, and some patients eventually develop into acquired resistance. Therefore, it is important to develop alternative medications to overcome castration-resistant prostate cancer.

Poly (ADP-ribose) polymerase (PARP) is an enzyme playing an essential role in DNA repair [11]. PARP is associated with a series of cellular events including cell proliferation, differentiation, and apoptosis [11, 12]. Besides, PARP is also implicated to play important roles in the occurrence and development of cancers [11]. Therapeutic inhibition of PARP has been applied in the treatment of a variety of cancers [13, 14]. Interestingly, a recent study has demonstrated that the PARP inhibitor olaparib is effective for prostate cancer patients who are not responding to standard treatments [15]. The therapeutic effects of olaparib on prostate cancer are in part due to its role in DNA-repair. The treatment of olaparib is beneficial to DNA-repair by homologous recombination. In addition to homologous recombination, non-homologous end-joining is another repair mechanism in the DNA repair [16]. Herein, the current study was designed to explore the effects of olaparib combined with non-homologous end-joining inhibitor (STL127705) on castration-resistant prostate cancer.

Materials and methods

Cell lines and culture

LNCaP and PC-3 cells were purchased from ATCC (Manassas, VA). LNCaP cells were cultured in RPMI-1640 medium supplied with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) and 1% Penicillin-Streptomycin solution. PC-3 cells were cultured in F-12K medium supplied with 10% FBS and 1% Penicillin-Streptomycin solution. Enzalutamide resistant LNCaP (erLNCaP) cells were constructed according to a previously reported method [9, 17]. In brief, the LNCaP cells were cultured in the presence of enzalutamide (10 μM) for 6 months to acquire the erLNCaP cells.

The sulforhodamine B (SRB) assay

The cells were seeded in a 96-well microplate and incubated overnight. After that, the cells were treated with vehicle alone (Control), enzalutamide, enzalutamide combined with olaparib, enzalutamide combined with STL-127705, or the combination of enzalutamide, STL127705, and olaparib for 5 days. After that, the SRB assay was applied to determine the cell viabilities according to the instructions of the manufacture (Abcam, Cambridge, MA). The median inhibition concentration (IC50) values were calculated.

Cell apoptosis

Annexin V and propidium iodide (PI) double staining were applied to analyze cell apoptosis. The cells were seeded in a 6-well microplate and incubated overnight. After that, the cells were treated with vehicle alone (Control), enzalutamide, enzalutamide combined with olaparib, enzalutamide combined with STL-127705, or the combination of enzalutamide, STL127705, and olaparib for 48 hours. The cells were then harvested and cell suspensions were prepared. The cell suspensions were stained with Annexin V for 15 mins at room temperature. The cell suspensions were then stained with the PI solution for another 15 mins in the dark. The populations of apoptotic cells were measured using flow cytometry.

Determination of γH2AX intensity

The cells were treated with vehicle alone (Control), enzalutamide, enzalutamide combined with olaparib, enzalutamide combined with STL127705, or the combination of enzalutamide, STL127705, and olaparib for 72 hours. After that, the cell suspensions were prepared and stained with primary antibody against γH2AX. Alexa Fluor 488-conjugated secondary antibody was then added and the cell suspensions were measured using flow cytometry. The results were analyzed using FlowJo.

Homologous recombination and non-homologous end-joining repair analysis

To qualify the percentage of homologous recombination and non-homologous end-joining

in the cells, we constructed the homologous recombination-non-homologous end-joining reporter PC-3 cell line according to a previously reported method [18]. In brief, the PC-3 cell line was transfected with homologous recombination-non-homologous end-joining reporter cassettes using electroporation. After 48 hours, puromycine selection was performed to screen cells expressing homologous recombination-non-homologous end-joining reporter.

After the homologous recombination-non-homologous end-joining reporter cells were treated, cell suspensions were prepared and the flow cytometry protocol was constructed to simultaneously analyze the percentage of homologous recombination and non-homologous end-joining in the reporter cell line. The results were analyzed using FlowJo.

Animal studies

Male nude mice were purchased from Shanghai Model Organisms (Shanghai, China) and kept under temperature (22-24°C)- and humidity-controlled (60 ± 5%) condition. All animal protocols were approved by the ethical committee of LONGHUA Hospital. This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985).

After one week of acclimation, the tumor-bearing animal model (n = 5 for each group) was established by the implantation of PC-3 cells (5×10^5 cells/mice) under axillary. Next, the mice were treated with enzalutamide (oral gavage, 30 mg/kg), olaparib (oral gavage, 3 mg/kg), STL127705 (oral gavage, 3 mg/kg), or a combination of them every five days. The experimental period lasted 50 days. Tumor volume was measured every ten days. At the end of the experimental period, the animals were sacrificed and the tumor tissues were collected.

Western blot

Radioimmunoprecipitation assay buffer supplemented with protease inhibitor was applied to lyse the tumor tissues. The protein concentrations of the sample were measured by the bicinchoninic acid protein kit. The equal number of proteins was loaded into the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel followed by transferring the proteins into the polyvinylidene fluoride membrane. The membrane was then blocked in 5% bovine serum albumin solution, and the primary antibody against γH2AX (Abcam) was added and incubated overnight at 4°C. Next, appropriated secondary antibodies were applied and incubated 2 hours at room temperature. GADPH (Abcam) was used as an internal control. An ECL detection kit was applied for chemiluminescence development and the imaging system was applied to qualify the protein expression of γH2AX.

Statistical analysis

Data were presented as mean ± standard error of mean (SEM). Statistical analysis was performed with GraphPad Prism8 (San Diego, CA). A Student's t-test or two-way analysis of variance (ANOVA) was performed. A *p*-value less than 0.05 was defined as a statistical significance between the two groups.

Results

STL127705 and olaparib enhance the cytotoxic effects of enzalutamide on erLNCaP and PC-3 cells

We determined the effects of enzalutamide on cell viabilities of erLNCaP and PC-3 cells in the presence of STL127705, olaparib, or STL127705 plus olaparib. The IC50 values of enzalutamide for erLNCaP and PC-3 cells were 117.5 μM and 4.542 μM respectively (**Figure 1A** and **1B**). Interestingly, both olaparib and STL127705 promoted the cytotoxic effects of enzalutamide on erLNCaP and PC-3 cells. The IC50 values of enzalutamide combined with olaparib for erLNCaP and PC-3 cells were 1.33 μM and 1.112 μM, respectively (**Figure 1A** and **1B**). The IC50 values of enzalutamide combined with STL127705 for erLNCaP and PC-3 cells were 4.867 μM and 2.211 μM, respectively (**Figure 1A** and **1B**). Furthermore, the results showed that the IC50 values of the combination of enzalutamide, STL127705, and olaparib for erLNCaP and PC-3 cells were 0.1524 μM and 0.1468 μM, respectively (**Figure 1A** and **1B**). These results support that STL127705 and olaparib exhibit synergistic effects with enzalutamide on the inhibition of cell proliferation of erLNCaP and PC-3 cells.

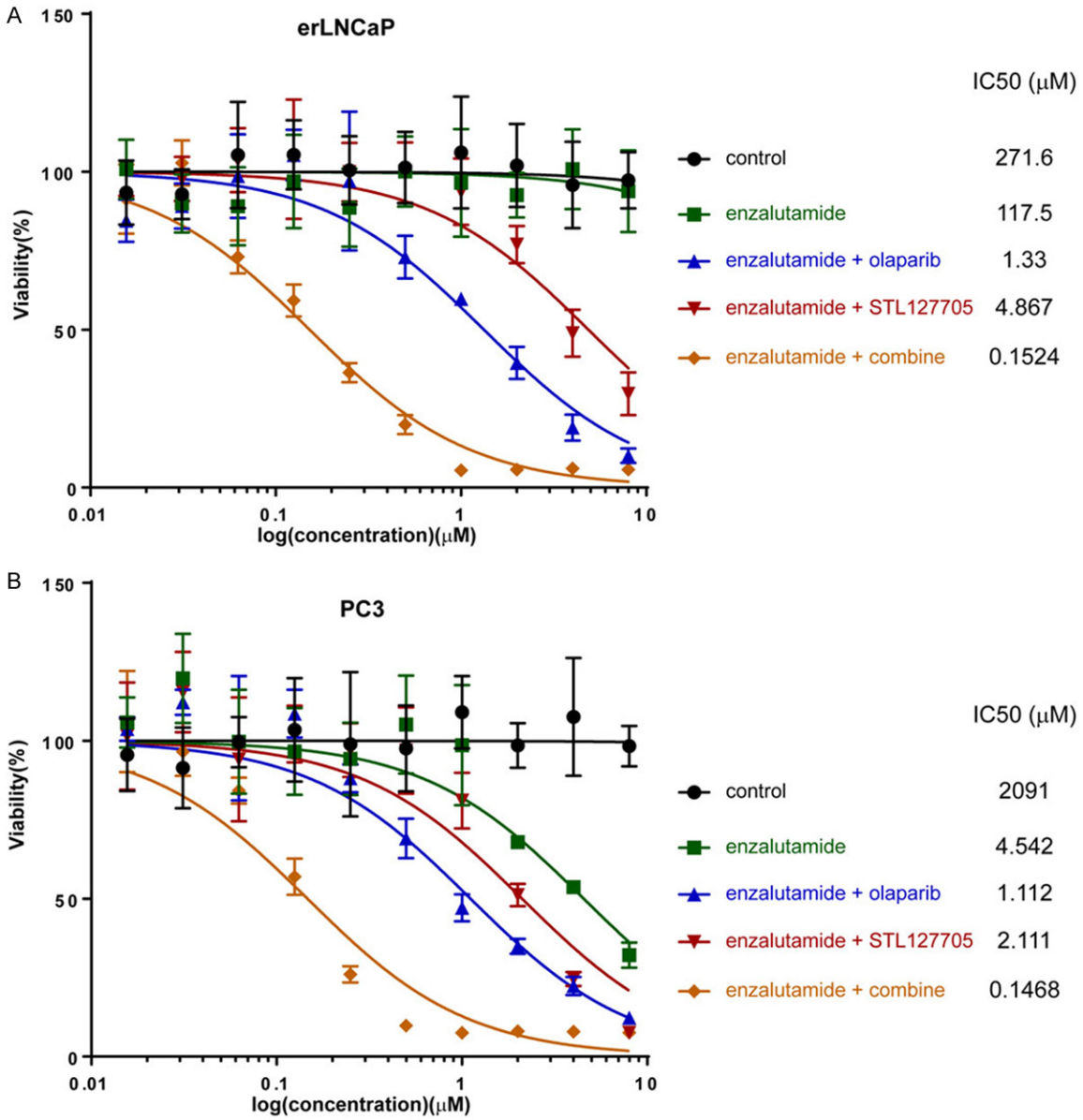

Figure 1. STL127705 and olaparib enhanced the cytotoxic effects of enzalutamide on erLNCaP and PC-3 cells. Enzalutamide resistant LNCaP (erLNCaP) (A) and PC-3 cells (B) were treated with enzalutamide, enzalutamide combined with olaparib, enzalutamide combined with STL127705, or a combination of enzalutamide, olaparib, and STL127705 for 5 days. Next, the cell viabilities were determined and the IC50 values were calculated (n = 5). The data were shown as means ± SEM.

STL127705 and olaparib promote enzalutamide-induced cell apoptosis in erLNCaP and PC-3 cells

We further determined the effects of enzalutamide (10 μM) on cell apoptosis of erLNCaP and PC-3 cells in the presence of STL127705 (1 μM), olaparib (1 μM), or STL127705 (1 μM) plus olaparib (1 μM). The results showed that the treatment of enzalutamide increased the populations of apoptotic cells to 5.74% in comparison to the control group (4.92%) (**Figure 2A** and **2C**). However, the treatment of enzalutamide combined with olaparib, enzalutamide combined with STL127705 significantly increased the populations of apoptotic cells to 49.2% and 25.6%, respectively, whilst the combination of enzalutamide, STL127705, and olaparib dramatically increased the populations of apoptotic cells to 76.0% (**Figure 2A** and

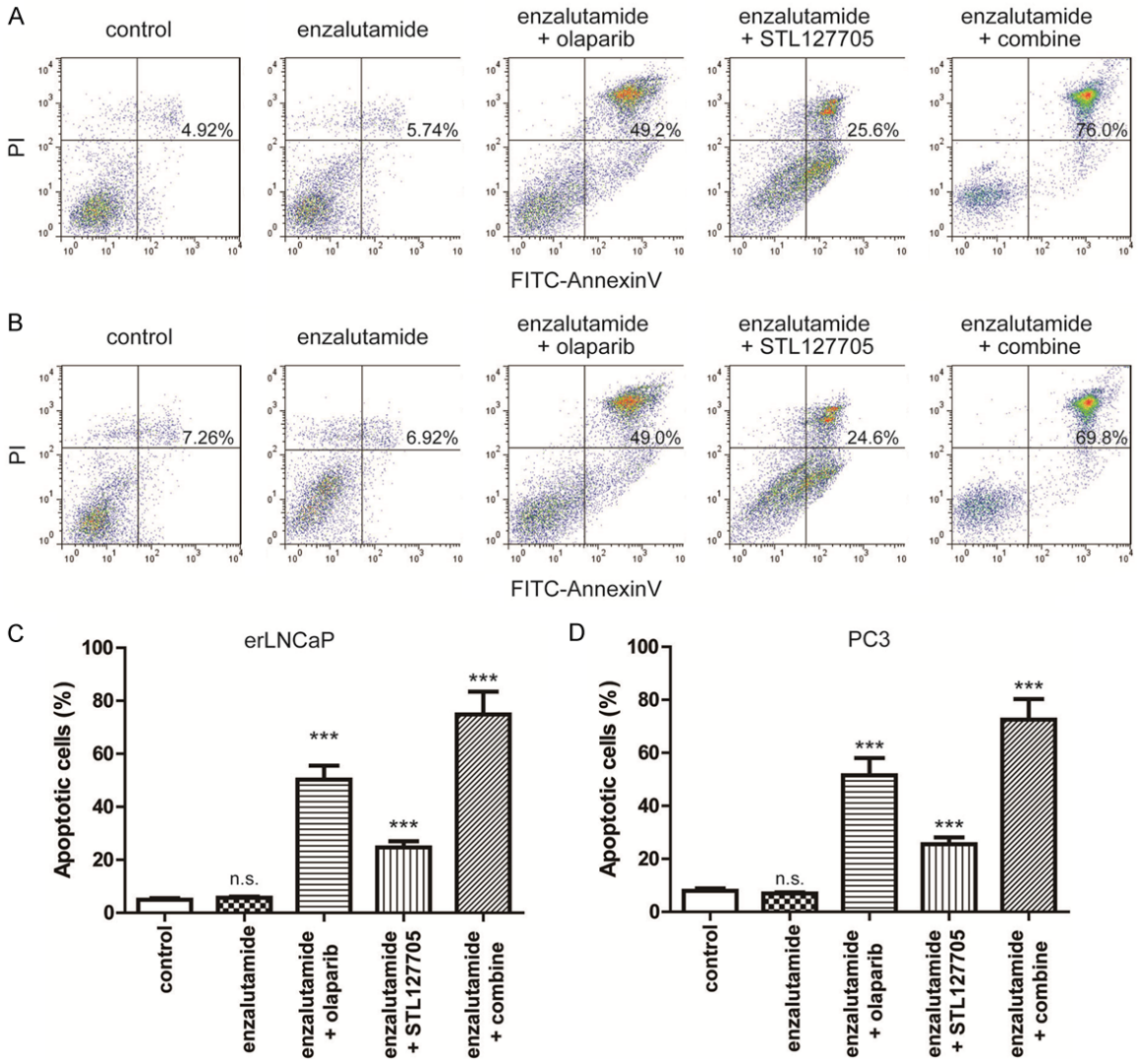

Figure 2. STL127705 and olaparib promoted enzalutamide-induced cell apoptosis in erLNCaP and PC-3 cells. A, B. The erLNCaP or PC-3 cells were treated with enzalutamide (10 μM), enzalutamide (10 μM) combined with olaparib (1 μM), or enzalutamide (10 μM) combined with STL127705 (1 μM) or a combination of enzalutamide (10 μM), olaparib (1 μM), and STL127705 (1 μM) for 48 hours. The cell suspensions were prepared and then stained with buffer containing Annexin V and PI. Flow cytometry was applied to analyze the percentage of apoptotic cells in each group. C, D. The percentage of apoptotic cells was qualified in each group (n = 5). The data were shown as means ± SEM. n.s. indicates $P > 0.05$, *** indicates $P < 0.001$.

2C). Similarly, both STL127705 and olaparib promoted enzalutamide-induced cell apoptosis in PC-3 cells. The results showed that the treatment of enzalutamide combined with olaparib, enzalutamide combined with STL127705 significantly increased the populations of apoptotic cells to 49.0% and 24.6%, respectively, whilst the combination of enzalutamide, STL127705, and olaparib dramatically increased the populations of apoptotic cells to 69.8% (**Figure 2B** and **2D**). Taken together, these results demonstrate that STL127705 and olaparib promote enzalutamide-induced cell apoptosis in erLNCaP and PC-3 cells.

The treatment of STL127705 and olaparib enhance γH2AX intensity in erLNCaP and PC-3 cells

We further determined the levels of γH2AX in the erLNCaP and PC-3 cells that were treated with enzalutamide (10 μM), enzalutamide (10 μM) combined with olaparib (1 μM), enzalutamide (10 μM) combined with STL127705 (1

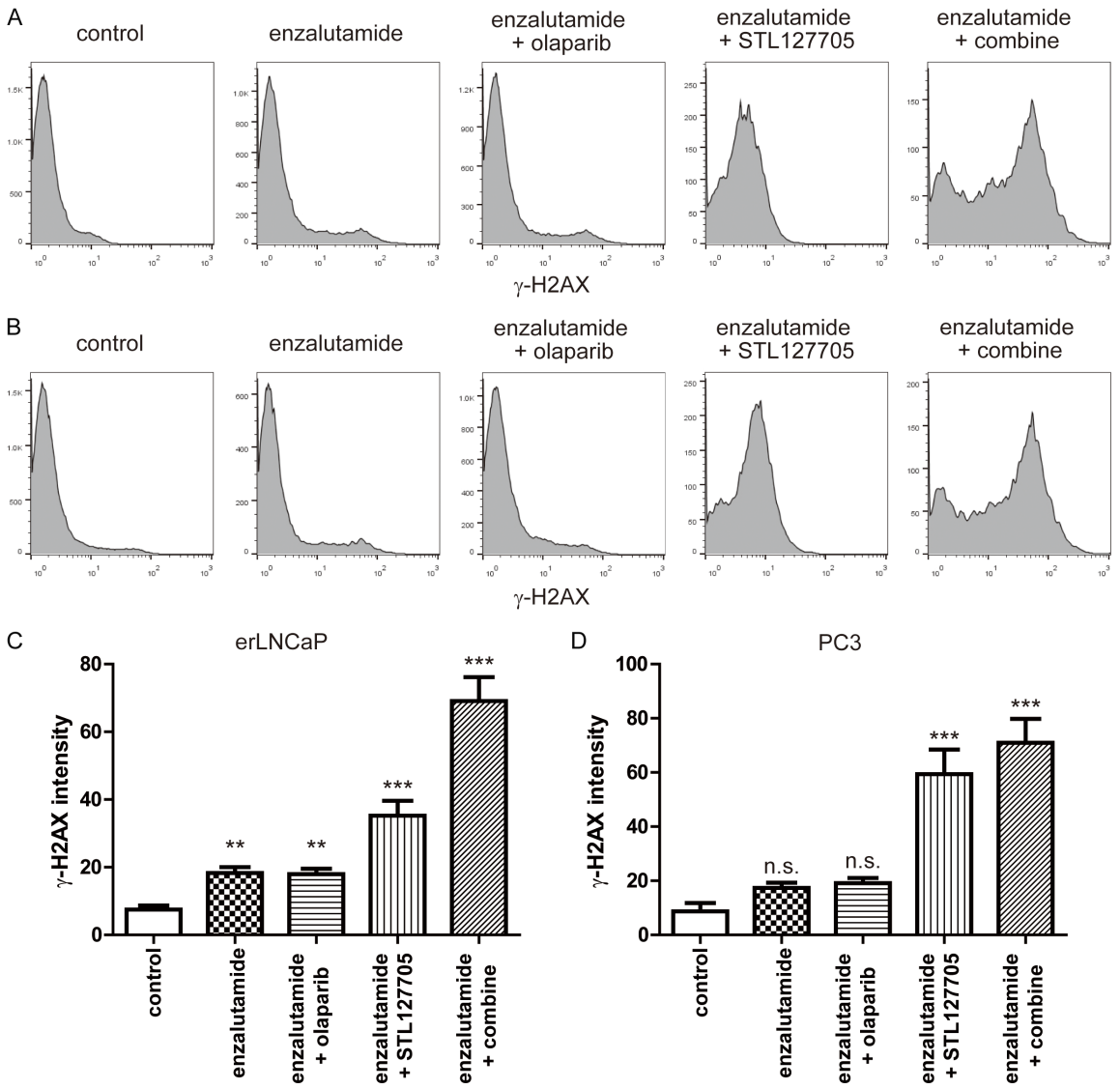

Figure 3. The treatment of STL127705 and olaparib enhanced γH2AX intensity erLNCaP and PC-3 cells. A, B. The erLNCaP or PC-3 cells were treated with enzalutamide (10 μM), enzalutamide (10 μM) combined with olaparib (1 μM), or enzalutamide (10 μM) combined with STL127705 (1 μM) or the combination of enzalutamide (10 μM), olaparib (1 μM), and STL127705 (1 μM) for 72 hours. The cell suspensions were prepared and then stained with primary antibody against γH2AX. FITC-conjugated secondary antibody was added and the cell suspensions were analyzed using flow cytometry. C, D. The γH2AX intensity was qualified in each group (n = 5). The data were shown as means ± SEM. n.s. indicates $P > 0.05$, *** indicates $P < 0.001$.

μM), or the combination of enzalutamide (10 μM), STL127705 (1 μM), and olaparib (1 μM). The results showed that the treatment of enzalutamide or enzalutamide combined with olaparib significantly increased γH2AX intensity in the erLNCaP cells in comparison to the control group ($P < 0.01$, **Figure 3A** and **3C**), whilst the treatment of enzalutamide combined with STL127705, or the combination of enzalutamide, STL127705, and olaparib further increased γH2AX intensity in the erLNCaP cells in comparison to the control group ($P < 0.001$, **Figure 3A** and **3C**).

In addition, we also investigated γH2AX intensity in the enzalutamide-induced PC-3 cells in the presence or absence of olaparib, STL127705, or STL127705 plus olaparib. Interestingly, the results demonstrated that the treatment of enzalutamide combined with STL127705 significantly increased γH2AX intensity in the PC-3 cells in comparison to the

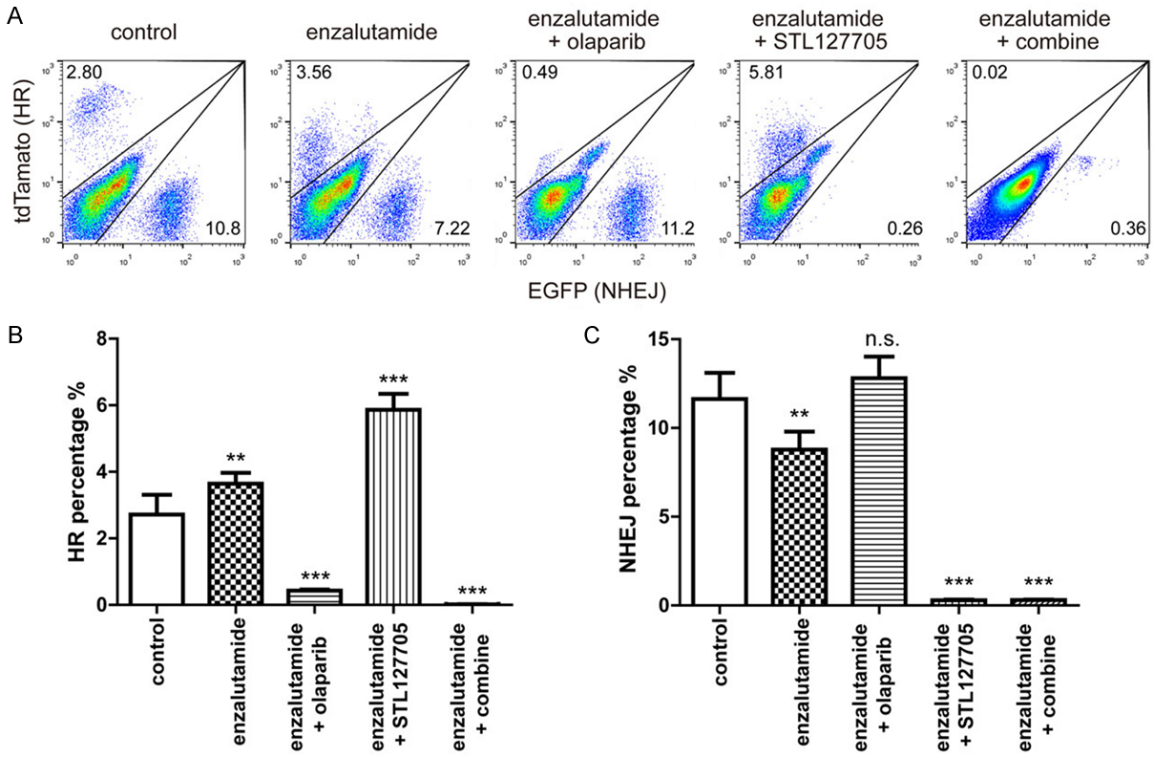

Figure 4. The combination of STL127705, olaparib, and enzalutamide inhibited the homologous recombination and non-homologous end-joining repair systems in the PC-3 cells. A. Calibration of FACS protocol was established for the simultaneous analyze homologous recombination and non-homologous end-joining efficiency in the PC-3 cells by harboring a single copy of the homologous recombination-non-homologous end-joining reporter cassette. B, C. The percentage of homologous recombination and non-homologous end-joining was qualified in each group (n = 5). The data were shown as means ± SEM. n.s. indicates $P > 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

control group ($P < 0.001$, **Figure 3B** and **3D**). Besides, the treatment of the combination of enzalutamide, STL127705, and olaparib also significantly increased γH2AX intensity in the PC-3 cells in comparison to the control group ($P < 0.001$, **Figure 3B** and **3D**). Taken together, these results suggest that enzalutamide enhances γH2AX intensity in the presence of STL127705 plus olaparib in the erLNCaP and PC-3 cells.

The combination of STL127705, olaparib, and enzalutamide inhibits either homologous recombination or non-homologous end-joining repair system in the PC-3 cells

We investigated the effects of enzalutamide on homologous recombination and non-homologous end-joining efficiency in the presence of STL127705, olaparib, or STL127705 plus olaparib. First, we successfully constructed the homologous recombination-non-homologous end-joining reporter PC-3 cell line. Next, flow cytometry was applied to evaluate the homologous recombination and non-homologous end-joining efficiency in different groups. The results showed that the treatment of enzalutamide enhanced homologous recombination efficiency but suppressed the non-homologous end-joining efficiency (**Figure 4A** and **4B**). Interestingly, the treatment of enzalutamide combined with olaparib significantly inhibited homologous recombination efficiency in comparison to the control group ($P < 0.01$, **Figure 4A** and **4B**). However, the treatment of enzalutamide combined with STL127705 enhanced homologous recombination efficiency but inhibited non-homologous end-joining efficiency in comparison to the control group ($P < 0.001$, $P < 0.001$, **Figure 4A-C**). In addition, the treatment of the combination of enzalutamide, STL127705, and olaparib significantly suppressed both homologous recombination efficiency and non-homologous end-joining efficiency in comparison to the control group ($P < 0.001$, $P < 0.001$, **Figure 4A-C**).

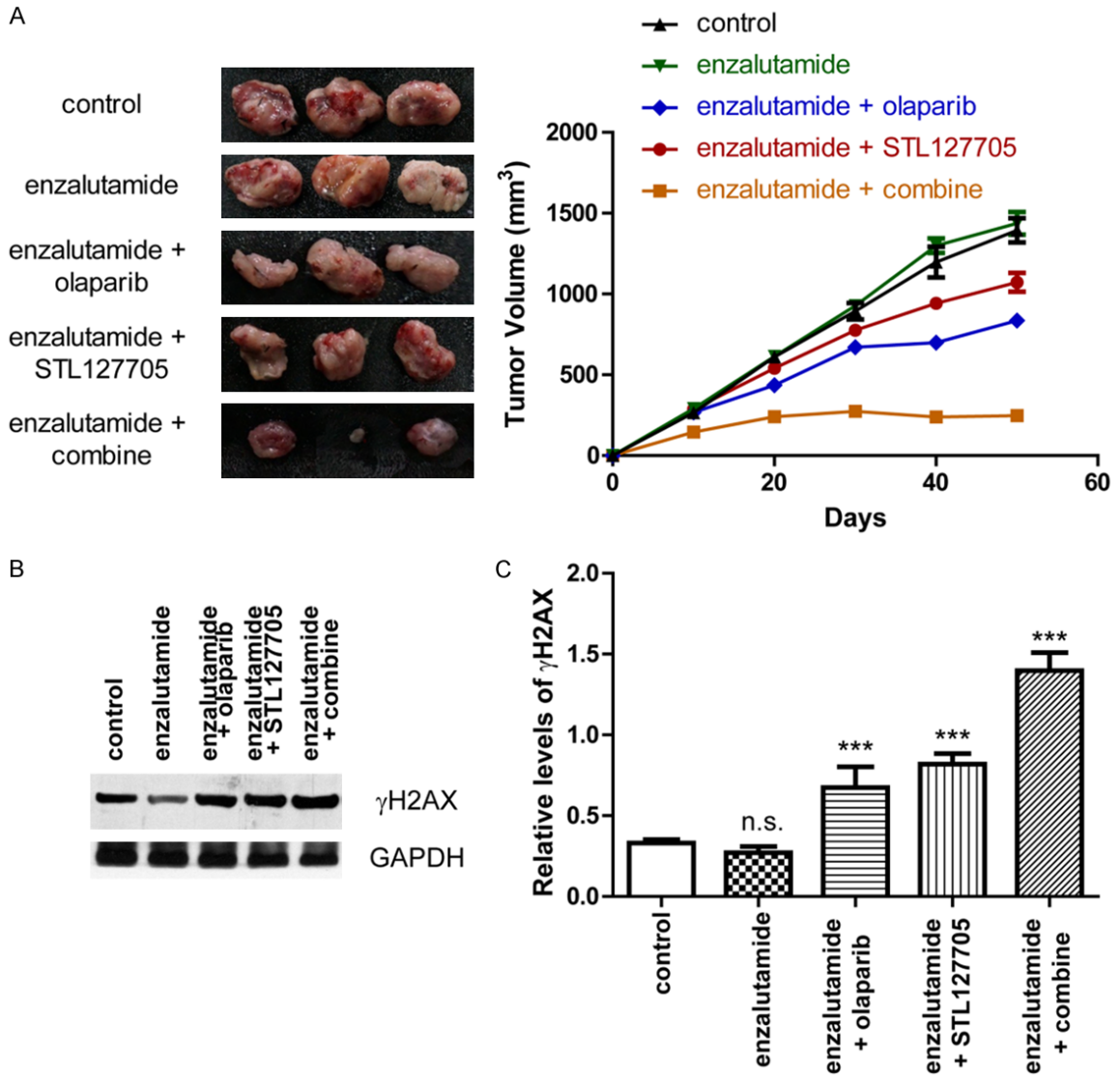

Figure 5. The combination of enzalutamide, STL127705, and olaparib exhibited significant anti-tumor effects on the PC-3 tumor-bearing animal model. A. The tumor-bearing animal model was established by the implantation of PC-3 cells under axillary. Next, the mice were treated with enzalutamide (oral gavage, 30 mg/kg), olaparib (oral gavage, 3 mg/kg), STL127705 (oral gavage, 3 mg/kg), or a combination of them every five days. The experimental period lasted 50 days. Tumor volume was measured every ten days. At the end of the experimental period, the animals were sacrificed and the tumor tissues were collected. B, C. The protein expressions of γH2AX were determined using Western blot and the relative protein levels of γH2AX were qualified in each group.

The combination of enzalutamide, STL127705, and olaparib exhibits significant anti-tumor effects on PC-3 tumor-bearing mouse model

Finally, we determined the anti-tumor effects of different combinations *in vivo* using the PC-3 tumor-bearing mouse model. Interestingly, we found that both STL127705 and olaparib enhanced the anti-tumor effects of enzalutamide, whilst the combination of enzalutamide, STL127705, and olaparib exhibited the strongest anti-tumor effects (**Figure 5A**), as evidenced by the tumor volume.

We then determined the expressions of γH2AX in the tumor tissues. The results showed that both STL127705 and olaparib enhanced the expressions of γH2AX in the tumor tissues in comparison to the control group ($P < 0.001$, **Figure 5B** and **5C**). Additionally, the combination of enzalutamide, STL127705, and olaparib significantly enhanced the expressions of

γH2AX in the tumor tissues in comparison to the control group ($P < 0.001$, **Figures 5B**, **5C** and S1).

Discussion

In the present study, for the first time, we explored the therapeutic potential of the combination of olaparib and non-homologous end-joining inhibitor (STL127705) on castration-resistant prostate cancer. Our results showed that the combination of olaparib and STL-127705 promoted the anti-tumor effects of enzalutamide through 1) enhancing the cytotoxic effects of enzalutamide, 2) enhancing the levels of γH2AX, and 3) inhibiting homologous recombination and non-homologous end-joining repair systems. The combination treatment of olaparib and STL127705 also enhanced the anti-tumor effects of enzalutamide in the PC-3 tumor-bearing animal model by inhibiting homologous recombination and non-homologous end-joining repair. Our results support that a combination of olaparib and STL127705 might be a good strategy for boosting the anti-tumor effects of enzalutamide on castration-resistant prostate cancer.

Androgen deprivation therapy is used for the treatment of early-stage prostate cancer with a high risk of recurrence, or advanced-stage prostate cancer with metastasis [7, 19]. Although it is effective for the treatment of prostate cancer, castration resistance, the major challenge in the treatment of prostate cancer, is still observed in some of the patients [20]. Enzalutamide, also called Xtandi, is an androgen receptor inhibitor for castration-resistant prostate cancer therapy by blocking the effects of androgen [21, 22]. However, prostate cancer eventually develops into enzalutamide resistance through various mechanisms [21, 22]. Therefore, to overcome enzalutamide resistance, the present study was designed to explore an effective strategy by combining PARP inhibitor with non-homologous end-joining inhibitor (STL127705). PARP inhibitor olaparib is effective for prostate cancer patients who are not responding to standard treatments. The mechanistic study showed that its anti-tumor effects may be through, at least in part, the regulation of DNA-repair. In addition, we also applied a combination of olaparib and STL-127705 in the present study.

Firstly, we constructed an enzalutamide-resistant cell line. The current results showed that erLNCaP was less sensitive for the enzalutamide. The IC50 value of enzalutamide for erLNCaP was 117.5 μM, indicating that enzalutamide resistant cell line was successfully constructed. Interestingly, the IC50 value of the combination of olaparib, SLT127705, and enzalutamide for erLNCaP cells was 0.1524 μM. We also applied PC-3 cell line, which is an androgen insensitive control cell line. Similarly, the results demonstrated that the IC50 value of enzalutamide for PC-3 cells was 117.5 μM whereas the IC50 value of the combination of olaparib, SLT127705, and enzalutamide for PC-3 cells was 0.1468 μM. These results suggest that the combination treatment significantly promotes the cytotoxic effects of enzalutamide on erLNCaP and PC-3 cells.

Previous studies have demonstrated that enzalutamide promoted cell apoptosis in the LNCaP cells [23, 24]. In the present study, we found that enzalutamide didn't significantly promote cell apoptosis in erLNCaP and PC-3 cells. Interestingly, the treatment of enzalutamide along with olaparib, STL127705, or olaparib plus STL127705 dramatically promoted cell apoptosis in erLNCaP and PC-3 cells.

Furthermore, we sought to explore the molecular targets of the combination of enzalutamide, olaparib, and STL127705 on cell apoptosis. Both olaparib and STL127705 play important roles in DNA repair [25, 26]. In addition, the levels of γH2AX are altered when a DNA double-strand break occurs [27, 28]. Therefore, we determined the levels of γH2AX in each group. The results showed that the treatment of the combination of enzalutamide, STL127705, and olaparib exhibited significant promoting effects on γH2AX intensity in the erLNCaP and PC-3 cells. These results supported that the combination of enzalutamide, STL127705, and olaparib regulated cell apoptosis by the regulation of γH2AX. We next sought to explore the molecular mechanisms of the combination of enzalutamide, olaparib, and STL127705 on cell apoptosis. Homologous recombination and non-homologous end-joining are two major pathways in DNA repair [29]. As discussed, olaparib is beneficial to DNA repair by regulating the homologous recombination pathway

whilst STL127705 is an non-homologous end-joining inhibitor [26, 30]. As we observed the effects of different combinations on cell apoptosis, we then evaluated the effects of these combinations on homologous recombination and non-homologous end-joining repair. As expected, the combination of enzalutamide and STL127705 significantly decreased the percentage of non-homologous end-joining but increased the percentage of homologous recombination. In addition, the combination of enzalutamide, STL127705, and olaparib inhibited both homologous recombination and non-homologous end-joining in the homologous recombination-non-homologous end-joining reporter PC-3 cell line. It is safe to conclude that the combination of enzalutamide, STL127705, and olaparib leads to DNA repair deficiency, thereby inducing cell apoptosis.

Finally, to confirm the *in vitro* data, we evaluated the combination of enzalutamide, STL-127705, and olaparib on the PC-3 tumor-bearing mouse model, a widely used prostate cancer animal model [31]. The results showed that both STL127705 and olaparib enhanced the anti-tumor effects of enzalutamide, whilst the combination of enzalutamide, STL127705, and olaparib exhibited the strongest anti-tumor effects. Interestingly, the combination of enzalutamide, STL127705, and olaparib significantly enhanced the expressions of γH2AX in the tumor tissues, which are consistent with the *in vitro* data.

Conclusion

In summary, treatment of STL127705 combined with olaparib exhibited potential therapeutic effects on castration-resistant prostate cancer in animal model by increasing the expression of γH2AX and inhibiting homologous recombination and non-homologous end-joining repair.

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Disclosure of conflict of interest

None.

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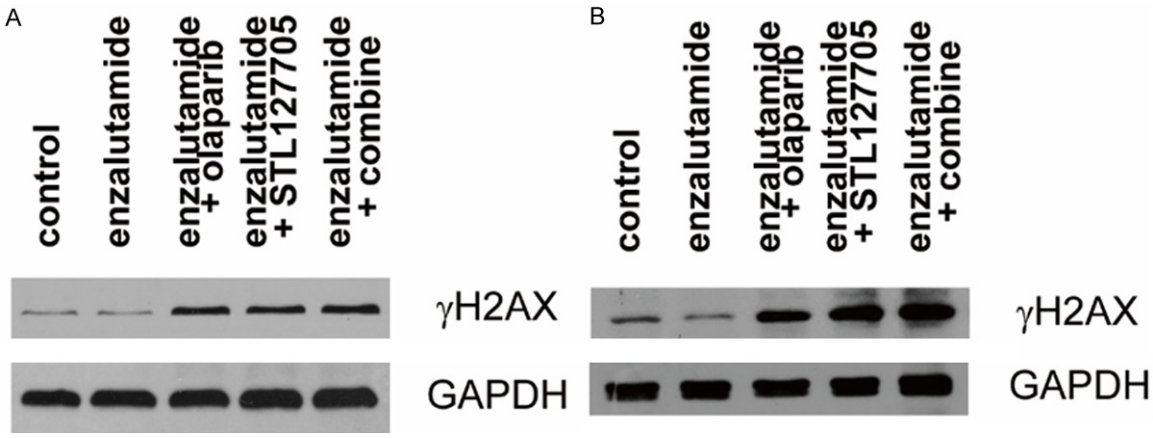

Figure S1. The protein expression of γH2AX in the other two repeated experiments (A, B). GAPDH was used as a loading control.