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Exploring the Role of Enzalutamide in Combination with Radiation Therapy: An *In Vitro* Study

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Abstract. *Background/Aim: Androgen receptor plays a key role in prostate cancer development and is a causative agent of its radio-resistance. The present study investigated the potential radio-sensitizing effect of enzalutamide, a second-generation anti-androgen, in human prostate cancer cells. Materials and Methods: The radio-sensitizing effect of enzalutamide was assessed in the androgen-dependent LNCaP cells and the androgen-independent PC3 cells by clonogenic assay and γ-H2AX assay. Results: Enzalutamide-treated LNCaP cells showed a significant decrease of cell survival at all radiation doses tested. An increased number of γ-H2AX-positive nuclei was observed, suggesting a possible impairment of the DNA repair machinery. Conversely, enzalutamide did not exhibit a significant radio-sensitizing effect on PC3 cells. Conclusion: The combination of enzalutamide with ionizing radiation significantly improves radio-sensitivity of hormone-dependent LNCaP cells. Translated in the clinical practice, our results may help to find additional strategies to improve effectiveness of radiotherapy.*

Treatment of prostate cancer (PC) has been rapidly changing in recent years. The available evidence clearly shows the efficacy of the new hormonal drugs such as enzalutamide and abiraterone in the treatment of metastatic, castration-resistant prostate cancer (mCRPC) (1, 2), more recently, clinical data favouring their use for the treatment of metastatic, castration-sensitive disease have been collected (3, 4); Radiotherapy (RT) remains a standard treatment option for non-metastatic PC patients (5). It has been demonstrated that the androgen receptor (AR) axis signalling plays a key role in PC development (6) and is one of the causative agents of the radio-resistance of disease (7). Unfortunately, given the lack of preclinical data in the available literature, molecular bases of the interaction between AR and ionizing radiation (IR) are still not completely understood (8-11). The new hormonal agents may provide a good opportunity to improve the efficacy of radiotherapy.

In this innovative scenario, a multi-modal approach also to metastatic PC patient management, may be suggested in the near future. Further clinical trials are needed, exploiting the advances in metastasis-directed therapies like stereotactic body radiation therapy (SBRT), to perform a focal, ablative treatment of oligometastatic PC (12, 13) and oligoprogressive CRPC (14).

The aim of our study was to investigate, in two different PC culture cell lines (LNCaP and PC3), the potential radio-sensitizing effect of enzalutamide, a second-generation anti-androgen, capable of inhibiting AR signalling pathway at multiple levels, in combination with IR.

Materials and Methods

Cells and cell culture. Human prostate cancer cell lines LNCaP and PC3 were grown in RPMI 1640 with 25 mM HEPES (Sigma-Aldrich, Saint Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies Co., Carlsbad, CA, USA), 2 mM L-glutamine (Gibco), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Gibco). Cells were maintained at 37˚C with 5% CO_2 in humidified incubator.

Drug treatment. Enzalutamide (Selleck Chemicals®, Houston, TX, USA) was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich) to a stock concentration of 10 mM. Cells grown in 35 mm petri dishes were incubated with fresh culture medium containing 10 μM enzalutamide or the corresponding volume of DMSO for 48 h, irradiated and subsequently processed as indicated.

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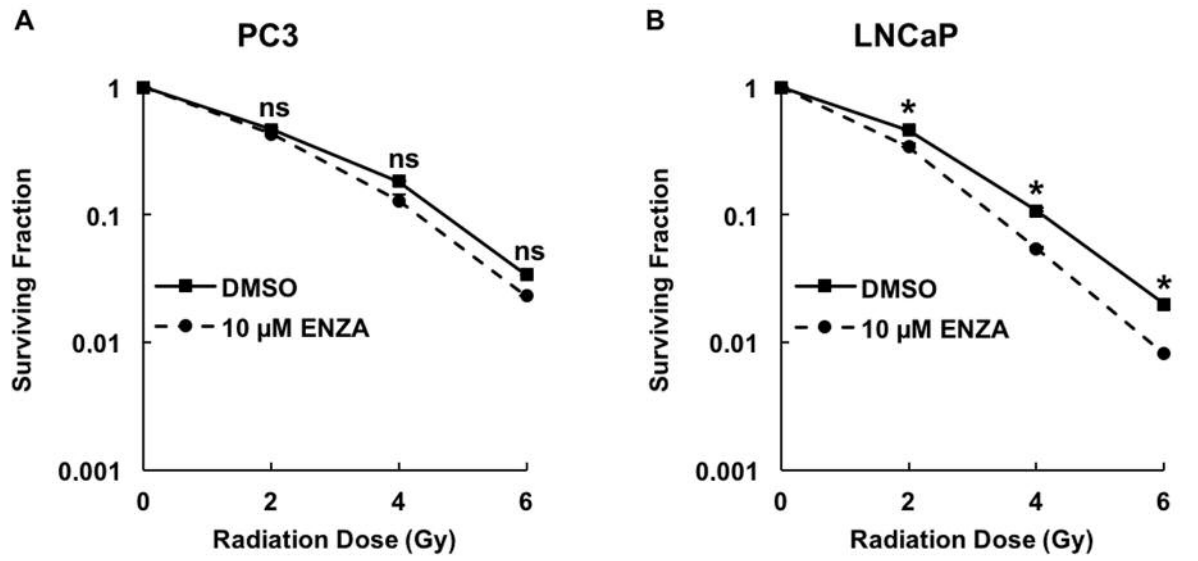

Figure 1. *Enzalutamide sensitizes LNCaP cells to ionizing radiation. PC3 (A) and LNCaP (B) cells were treated with 10 μM enzalutamide (ENZA) for 48 h, exposed to different radiation doses (2, 4 and 6 Gy) and plated for clonogenic assay. Fourteen days after irradiation, cells were fixed and stained with 0.1% crystal violet. The colonies with 50 or more cells were counted. Values represent the average of 3 independent experiments, each one conducted in triplicate. (*p-value<0.05; ns: non-significant).*

Cell irradiation. Cells were irradiated at the Istituto del Radio "O. Alberti" - Department of Radiation Oncology, University and Spedali Civili Hospital, Brescia - using a 6 MV photon beam Linear Accelerator with a dose rate of approximately 4 Gy/min. For clonogenic assay, cells were exposed to a single radiation dose of 2, 4 and 6 Gy. For immunofluorescence analysis, a single dose of 2 Gy was applied. After irradiation cells were incubated and maintained at 37 ˚C until collection or fixation at established time intervals.

Clonogenic cell survival assay. Immediately after irradiation, cells were trypsinized, counted and plated for clonogenic cell survival assay in appropriated cell number in 10 cm dishes. Cells were maintained in RPMI 1640 containing 10% FBS at 37˚C with 5% CO_2 in a humidified incubator. After 14 days, surviving colonies were fixed with 80% ice cold ethanol and stained with 0.1% crystal violet in 20% methanol, all from Sigma-Aldrich. Colonies of 50 cells or more were counted. Survival fraction (SF) was calculated as the ratio between the number of colonies formed in treated dishes and the number of colonies formed in the controls. Cell survival curves were generated for either radiation alone or in combination with enzalutamide.

A cell survival curve describing the relationship between the absorbed dose and the fraction of cells that survived was set up. The survival fraction was obtained *in vitro* as the ratio of the plating efficiency of treated cells to that of untreated control cells, where the plating efficiency is the percentage of seeded cells growing into colonies. The survival fraction was plotted on a log-linear scale with dose on the linear x-axis.

Immunofluorescent staining for γ-H2AX. Cells were seeded onto glass coverslips and treated as indicated above. Following irradiation, cells were gently washed with PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS++) and fixed at established time intervals with 3% paraformaldehyde containing 2% sucrose in PBS++ for 15 min at room temperature. Paraformaldehyde was quenched with 50 mM NH_4Cl in PBS++ for 15 min. Cells were then permeabilized with 0.1% Triton X-100 in PBS++ twice for 5 min and then blocked with 1% BSA in PBS++ three times for 10 min. Cells were incubated for 1 h with rabbit anti-γ-H2AX primary antibody (Cell Signaling, Danvers, MA, USA) diluted 1:100 in 1% BSA in PBS++, washed three times for 10 min with 1% BSA in PBS++ and incubated for 45 min with secondary anti-rabbit Alexa Fluor-555 antibody (Invitrogen, Carlsbad, CA, USA) diluted 1:400 in 1% BSA in PBS++. Finally, specimens were washed three times for 10 min with 1% BSA in PBS++, incubated for 1 min with DAPI (0.1 μg/μl) in PBS++, and mounted using an anti-fade mounting medium (DAKO Agilent, Santa Clara, CA, USA) on a glass slide. Analysis was performed on ZEISS LSM 510 META confocal laser scanning microscope (Carl Zeiss). Images were processed with the use of Image J software.

Statistical data analysis. Data are expressed as the mean±standard deviations (S.D.) of at least 3 independent experiments. Statistical analysis was performed using unpaired Student's *t*-test. *p*-Value<0.05 was considered statistically significant.

Results

Enzalutamide reduces LNCaP cells survival fraction after irradiation. Enzalutamide treatment resulted in a significant reduction of the surviving potential of LNCaP cells (Figure 1B). Of note, already at the clinically relevant dose of 2 Gy the surviving fraction of LNCaP was significantly reduced from 0.46 in control cells to 0.34 in enzalutamide-treated cells. Conversely, treatment with enzalutamide did not result

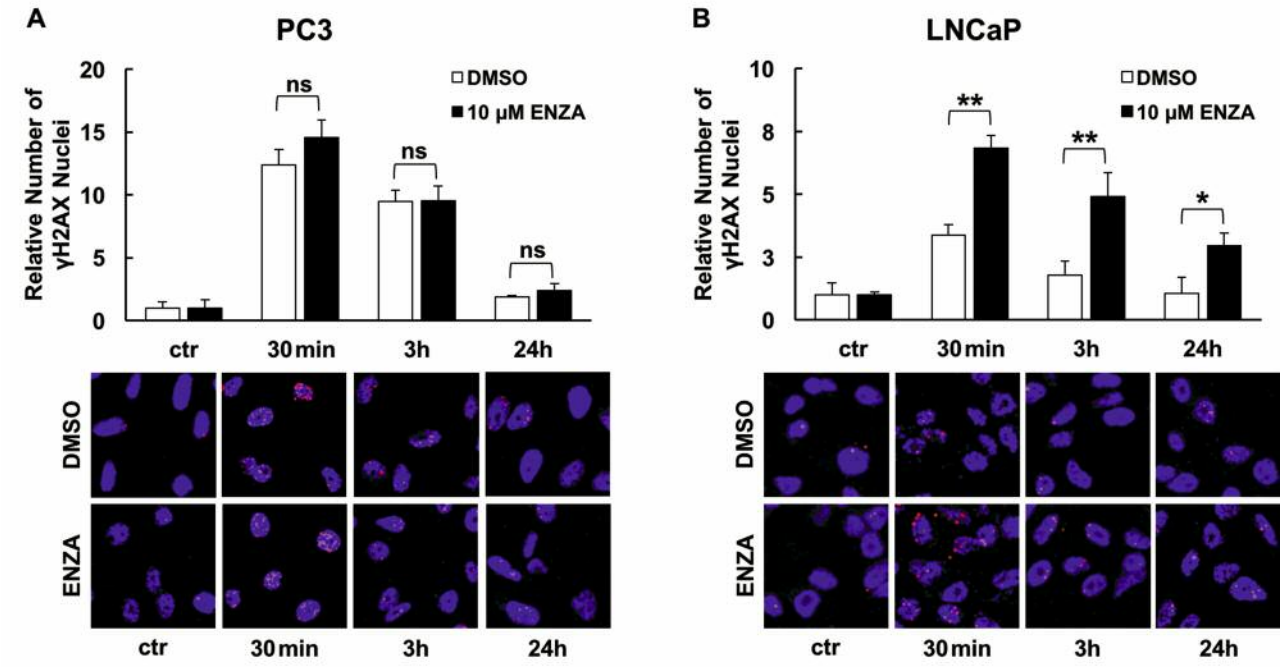

Figure 2. *Enzalutamide impairs DNA-repair machinery in LNCaP cells. PC3 (A) and LNCaP (B) cells were treated with 10 μM enzalutamide (ENZA) for 48 h and exposed to 2 Gy ionizing radiation. Cells were fixed at the indicated time intervals and processed for indirect immunofluorescence with rabbit anti-γ-H2AX primary antibody. Nuclei were counterstained with DAPI. For each experimental condition, at least 5 different fields were analyzed with at least 20 nuclei/field. Results are normalized to control cells (CTR=0 Gy). Quantitative analysis (upper panels) and representative images (lower panels) are shown. The reported values represent the average of 3 independent experiments. (*p-value <0.05; **p<0.01; ns: non-significant).*

in a significant reduction of the surviving fraction of PC3 cells at all radiation doses tested (Figure 1A). All these findings suggest a possible role of enzalutamide in increasing the radiation effects in the AR-sensitive LNCaP cell line.

Enzalutamide impairs DNA-repair machinery in LNCaP cells. An early event after irradiation is represented by the DNA damage, to which cells respond activating different DNA-repair mechanisms. Among these mechanisms, phosphorylation of histone H2AX (γ-H2AX) is a commonly used parameter to detect DNA double strand breaks (DNA-DSB). Thus, detection of γ-H2AX inside the nuclei represents a hallmark of DNA damage. Regardless of the enzalutamide treatment, 30 min after irradiation the number of γ-H2AX-positive nuclei increased both in PC3 cells (Figure 2A) and in LNCaP cells (Figure 2B), as expected. Of note, while in PC3 cells the relative number of γ-H2AX-positive nuclei was not statistically different between enzalutamide treated and control cells, in LNCaP cells the number of positive nuclei was increased by 2-fold in enzalutamide treated cells in comparison with control cells. 3 h and 24 h after irradiation, the relative number of positive nuclei detected in PC3 cells decreased over time and, of note, no difference between treated and untreated PC3 cells were observed. On the contrary, while in control LNCaP cells the relative number of γ-H2AX-positive nuclei decreased with time, the same did not apply for the enzalutamide treated LNCaP cells. Indeed, at all intervals taken in consideration, the number of γ-H2AX-positive nuclei was statistically increased in enzalutamide treated LNCaP cells compared to control cells. These data indicate that enzalutamide interferes with the DNA repair mechanism of the AR-sensitive LNCaP cell line, possibly resulting in a decreased survival potential after IR in comparison with the AR-resistant PC3 cell line.

Discussion

AR plays a key role in prostate carcinogenesis, disease resistance to hormonal therapies and progression (15). RT with or without androgen deprivation therapy (ADT) is the main therapeutic option for non-metastatic PC (5); ADT with or without Docetaxel or Abiraterone systemic therapy is one of the most effective therapeutic strategies for metastatic PC (3, 4, 16, 17), suggesting that androgens and the AR signaling pathway may be closely involved in prostate carcinogenesis. Despite initial clinical and biochemical responses to surgical or pharmaceutical castration, over time

some tumor cell clones may become gradually resistant to ADT, so that progression to CRPC is unavoidable (18). AR seems to have a key role in the mechanisms underlying this event: evidence is available of androgen deprivation inducing overexpression of AR in the tumor cell clones so that their proliferative response is invariably high even for low doses of testosterone of autocrine and paracrine origin (19). Preclinical literature data revealed enzalutamide to inhibit AR signalling pathway by preventing androgens binding to AR, its nuclear translocation, the related DNA activation and subsequent AR transcription (20). Polkinghorn *et al*. reported that AR plays a key role in the transcriptional regulation of a large subset of DNA repair genes enhancing the DNA repair ability, thus promoting PC radio-resistance. In addition, they observed that the antiandrogen molecule ARN-509 produces a significant decrease of DNA repair gene expression in *in vitro* conditions (21). Based on this knowledge, we investigated whether enzalutamide concurrent to RT increases radio-sensitivity of PC cells. The radiosensitizing effect of enzalutamide on hormone-independent PC3 cells and hormone-dependent LNCaP cells was assessed by clonogenic cell survival assay. Our findings demonstrated that pre-treatment with enzalutamide significantly radio-sensitizes LNCaP cells at the different radiation dose levels tested (especially at 2 Gy), while the same effect was not observed in PC3 cells; only a slight, but not statistically significant radio-sensitizing effect at higher radiation doses was demonstrated in these cells. The cellular response to DNA double strand breaks (DSB) is the activation of different kinases, leading to a fast phosphorylation of the histone variant H2AX on serine 139 (γ-H2AX) at the sites of DNA damage, as reported in the literature (22, 23). Immunofluorescence detection of γ-H2AX is a reliable and sensitive technique to monitor DNA damage, in particular DSB itself and its repair (24). Irrespective to the treatment with enzalutamide, the percentage of γ-H2AX-positive nuclei progressively decreased over time in PC3 cells, suggesting that enzalutamide does not affect the DNA repair mechanisms of this cell line. Conversely, a significantly higher number of γ-H2AX positive nuclei (when compared to controls) was detected in enzalutamide treated LNCaP cells at all the time points and this effect seems therefore of long duration. This observation suggests that enzalutamide may alter the DNA repair machinery in LNCaP cells. All these considered, our results demonstrated that enzalutamide can reduce the clonogenic survival ability of irradiated LNCaP cells, probably by impairment of the DNA DSB repair mechanism (as observed in our γ-H2AX immunofluorescence study), resulting in a significant radio-sensitizing effect. Conflicting results come from pre-clinical studies about radiobiological effects of LHRH-analogues and/or first-generation anti-androgens like Bicalutamide (25-28). Our study showed that the *in vitro* effect of combining new hormonal therapies and IR may have some interesting clinical implications. Firstly, pre-clinical, *in vitro* evidence was obtained that enzalutamide has a radio-sensitizing effect over castration-sensitive PC cells. This observation might be useful to consider the combination of enzalutamide and RT to improve local control of disease, especially in high- and very high-risk PC, since intraprostatic recurrence is still one of the main causes of treatment failure (29). Moreover, we have already pointed out that SBRT is feasible and effective in the treatment of oligometastatic and oligoprogressive PC (13, 14). Even in this setting, combining a highly selective, ablative radiation treatment with new hormonal molecules may ameliorate also systemic disease control. Further studies are needed to better understand the clinical relevance of these mechanisms. Since clinical data suggesting a safe association between the new hormonal drugs and RT are actually available in literature (30), the organization of Phase III trials seems worthwhile. Of note, there is a growing attention of the international scientific community towards preclinical and clinical trials combining ionizing radiation with new hormonal drugs, immunotherapy and target therapy, not only for the treatment of PC but also for other malignancies (31).

Conclusion

Despite advances in the treatment of PC in recent years, resistance to available therapeutic options is still observed and the interaction between IR and AR is not completely understood. Our results indicate that combining enzalutamide with IR significantly increases the radio-sensitivity of the hormone-dependent LNCaP cell line suggesting that the molecular mechanism underlying such radio-sensitization may reside in the impairment of DNA repair machinery. Clinical, phase III trials should be encouraged to better understand the clinical impact of these mechanisms, possibly improving RT efficacy.

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