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Radiopotentiation of enzalutamide over human prostate cancer cells as assessed by real-time cell monitoring

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ABSTRACT

Aim: To evaluate the radiopotentiation of enzalutamide in human prostate cancer cells.
Background: While radiotherapy is the first line of treatment for prostate cancer, androgen blockade therapies are demonstrating significant survival benefit as monotherapies. As androgen blockade can cause cell death by apoptosis, it is likely that androgen blockade will potentiate the cytotoxic activities of radiotherapy.
Materials and methods: Here, we tested the potential synergistic effects of these two treatments over two human metastatic prostate cancer cells by real-time cell analysis (RTCA), androgen-sensitive LNCaP cells (Lymph Node Carcinoma of the Prostate) and androgen-independent PC-3. Both cell lines were highly resistant to high doses of radiotherapy.
Results: A pre-treatment of LNCaP cells with IC50 concentrations of enzalutamide significantly sensitized them to radiotherapy through enhanced apoptosis. In contrast, enzalutamide resistant PC-3 cells were not sensitized to radiotherapy by androgen blockade.
Conclusions: These results provide evidence that the enzalutamide/radiotherapy combination could maximize therapeutic responses in patients with enzalutamide-sensitive prostate cancer.

1. Background

Radiotherapy is the most widely used first-line treatment for prostate cancer. Approximately 50% of the patients are treated with radiotherapy alone or in combination with

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other therapies. However, a significant percentage of patients relapse.[1] Androgen receptor (AR)-targeted therapies have been developed to prevent proliferation and induce apoptosis in prostate cancer cells,[2] and these are demonstrating significant survival benefit as monotherapies. This is certainly the case of enzalutamide, a highly selective AR antagonist, preventing its binding to androgen and nuclear translocation. Nevertheless, primary and secondary resistances eventually develop through a series of mechanisms that include AR mutations that hamper binding to enzalutamide.[3] However, preclinical findings suggest that its combination with traditional treatments may achieve additive or synergistic effects. This has been recently corroborated.[4] These effects should most likely reduce the appearance of resistance to AR blockade and augment patient benefit.[5] Hence, a collection of combinatorial treatments with enzalutamide are currently being tested such as mTOR and HER2 inhibition[6] and antagonists of apoptotic inhibitors, amongst other agents.[7]

Radiotherapy is an efficacious inducer of cell death and enzalutamide treatment should further sensitize irradiated prostate cancer cells to the induction of apoptosis. Most of the studies on apoptotic agents employ end-point MTT cytotoxicity assays or clonogenic assays. Impedance-based technologies have been developed to monitor cell growth/viability in real time, such as the xCelligence RTCA technology.[8] This technology has the sensibility to detect changes in cell size, shape and growth, making it a very useful technique for monitoring effects of pharmacological agents on cellular biology.[8,9] Moreover, RTCA integrates the real-time data to plot inhibition curves to accurately calculate inhibitory doses (ID) at any given time.

2. Aim

In this study we have addressed the potential of RTCA technology to test whether a radiotherapy/enzalutamide combinatorial regimen in prostate cancer cell lines may warrant their application in human patients.

3. Material and methods

3.1. Cells

LNCaP and PC3 human prostate cancer cell lines were purchased from the ATCC and grown as recommended by the manufacturer. The LNCaP cell line (Lymph Node Carcinoma of the Prostate) is derived from human prostate adenocarcinoma cells from a lymph node metastasis of a 50-year old Caucasian male in 1977 and they are androgen-sensitive adherent epithelial cells. LNCaP cells were grown in an RPMI-1640 medium. PC-3 cells were grown in an F-12 medium. All culture media were supplemented with 10% FCS, penicillin and streptomycin.

3.2. Enzalutamide and radiotherapy

Enzalutamide was stored at a concentration of 10 mM in DMSO. Enzalutamide was diluted in medium before being added to cell cultures. The final concentrations of DMSO in the cultures ranged from 0.01% to 0.1%. Triplicate prostate cancer cell cultures were subjected to 3 different doses of radiotherapy, 10, 20 and 30 Gy in the Radiation Oncology Department of the Hospital of Navarre in collaboration with the Physics department. Photonic irradiation (6 MeV) was performed with a Clinac 21ex lineal accelerator (Varian). Irradiation dosage was applied in a single fraction for each cell line and each experiment, with an irradiation rate of 600 UM/min. Hence, 10, 20 and 30 Gy correspond to total dosage. The treatment field was 16×12 cm, with a source to surface distance of 100 cm.

3.3. Flow cytometry

Cells were collected and stained using the eBioscience Annexin V apoptosis assay as described by the manufacturer. Data was collected with a FACS CANTO (BD bioscience). Experiments were performed in triplicates.

3.4. Real-time cell monitoring (RTCA)

Cell growth and viability were monitored by XCelligence RTCA (ACEA) as described before[10] for up to two weeks of culture. Inhibition curves were obtained using the in-built ACEA software with RTCA data.

3.5. Statistics

RTCA cultures were carried out in duplicates and experiments repeated thrice. The Cell Index data was highly reproducible and normally distributed. Therefore, the treatment groups were compared with one-way ANOVA using the means from triplicates and data from all replicate experiments, during the last 24 h of culture. Pair-wise comparisons were carried out with Tukey's test. Data on Annexin-V staining was compared using the non-parametric test of Mann–Whitney, as the variances of the groups were significantly different. GraphPad Prism was used to carry out statistical tests.

4. Results

4.1. Estimation of enzalutamide IC50s on two human metastatic prostate cancer cell lines by real-time cell monitoring

We analyzed the sensitivity to enzalutamide of two human prostate cancer cell lines extensively used in preclinical work by real-time monitoring of cell growth (xCelligence RTCA). The LNCaP cell line was derived from a lymph node metastasis of a 50-year old male Caucasian patient. These cells are androgen and estrogen receptor-positive and also sensitive to androgen blockade. PC-3 cells were chosen as androgen insensitive control cells which were obtained from a bone metastasis of a 62-year-old male Caucasian patient with a grade IV prostate adenocarcinoma.

First, we tested the use of real-time cell monitoring to estimate the sensitivity of the two cell lines to enzalutamide, as most of the studies utilize end-point-based assays. Concentrations of enzalutamide ranging from 0 to 10 μM were added to cell cultures and their growth was monitored up to

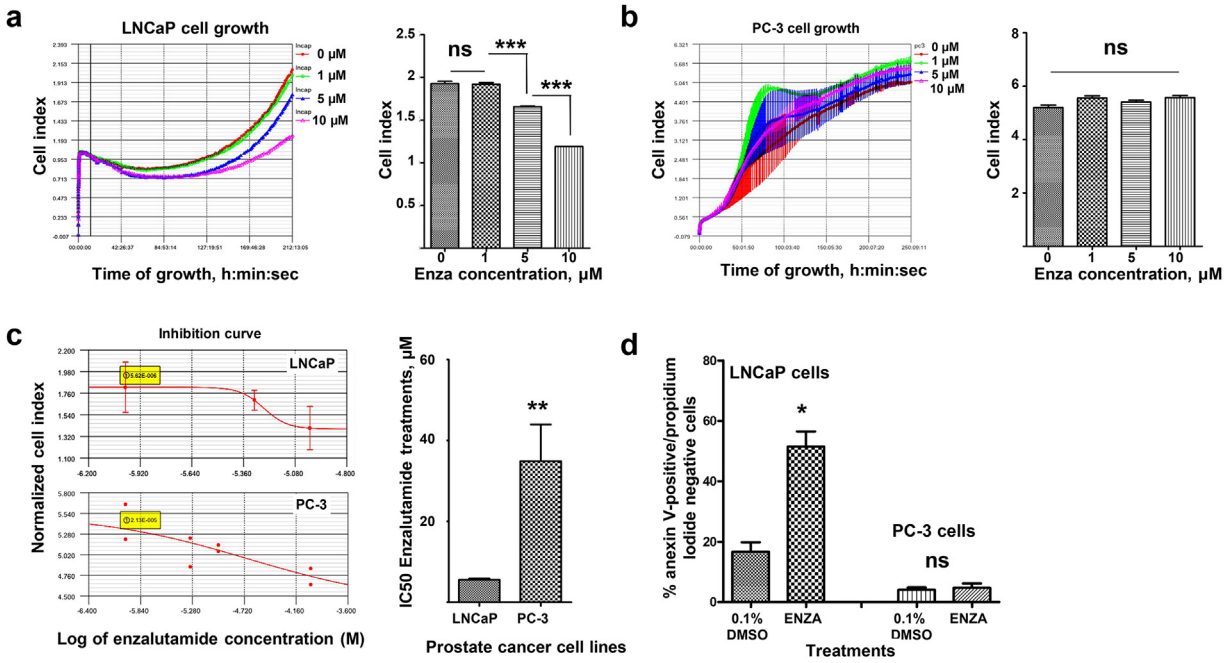

Fig. 1 – Inhibition of cell growth by enzalutamide as assessed by real-time cell monitoring. (a) The graph on the left shows real-time growth kinetics from control-treated LNCaP cells (DMSO carrier) or treated with 1, 5 or 10 µM enzalutamide, as indicated in the graph. On the right, the data from two RTCA experiments with duplicates within each one are plotted as a bar graph, with means (Cell Indexes) and standard deviations as error bars. (b) As in (a) but with PC-3 cells. Cell index is plotted as means from triplicate independent experiments together with the error bar. (c) The graphs on the left show the inhibition plots of either LNCaP cells (top graph) or PC-3 cells (bottom graph) following enzalutamide treatments at increasing concentrations, as calculated by RTCA. The calculated IC50 values are highlighted in yellow within each graph. On the right, column graphs representing the calculated IC50s from three independent experiments, each experiment in duplicates, for LNCaP and PC-3 cells as indicated. Relevant statistical comparisons are shown. (d) Bar graph representing the percentage of annexin V-positive/propidium iodide negative cells in cultures of the indicated cell lines, treated with DMSO or enzalutamide at the IC50 concentrations as calculated in (c). Relevant statistical comparisons are shown within the graphs. *, **, *, indicate significant ($P < 0.05$), very significant ($P < 0.01$) and highly ($P < 0.001$) significant differences, respectively.**

12 days. DMSO was added as a control and treatments were carried out in duplicates and repeated thrice. The growth of LNCaP cells exhibited exponential kinetics which was highly significantly delayed with increasing enzalutamide concentrations, especially from 5 µM onwards(Fig. 1a). In contrast, no significant effects were observed in the growth of PC-3 cells at any of the tested enzalutamide concentrations, in agreement with their intrinsic resistance to enzalutamide (Fig. 1b). Then, IC50 concentrations were estimated with RTCA data for the two cell lines (Fig. 1c). The calculated IC50 for LNCaP cells (5.6 ± 0.8 µM; $n = 4$) was comparable to that published by other authors using end-point cytotoxicity assays (between 1 and 5 µM).[11,12] In contrast, the calculated IC50 value for PC-3 cells had to be extrapolated up to a non-physiological value (34.9 ± 9 µM; $n = 4$). This was consistent with the intrinsic resistance of PC-3 cells to androgen blockade (Fig. 1c).

Real-time cell monitoring is based on impedance readings from cell culture plates on which cells attach and proliferate.[8] Although it can be used to test cell viability, this technique does not directly provide information on cell death. Therefore, we confirmed the induction of apoptosis by enzalutamide in prostate cancer cells by a standard flow cytometry apoptosis assay based on annexin V staining/propidium iodide exclusion. Hence, the two cell lines were treated with a non-cytotoxic concentration of 0.1% DMSO or enzalutamide for 48 h at their corresponding IC50 concentrations (Fig. 1c). Then, the percentage of annexin V-positive cells was quantified by flow cytometry, and it was confirmed that enzalutamide significantly increased apoptosis in treated LNCaP cells (Fig. 1d). As expected, PC3 cells were refractory to apoptosis induction by enzalutamide.

4.2. *Enzalutamide pre-treatment potentiates radiotherapy*

Prostate cancer cell lines were extraordinarily resistant to radiotherapy compared to other non-prostate cancer cell lines (data not shown). Therefore, we tested whether the pro-apoptotic effects of enzalutamide could also sensitize cells to radiotherapy-induced cell death. To test this, LNCaP and PC-3 cells were pre-treated with DMSO or enzalutamide at their IC50 concentrations for 3 days. At these concentrations, cell growth is delayed but cells remain mostly viable. Then, cells were irradiated with 10, 20 and 30 Gy and their growth and viability were monitored by RTCA. As expected from our preliminary studies, doses of 30 Gy had to be used

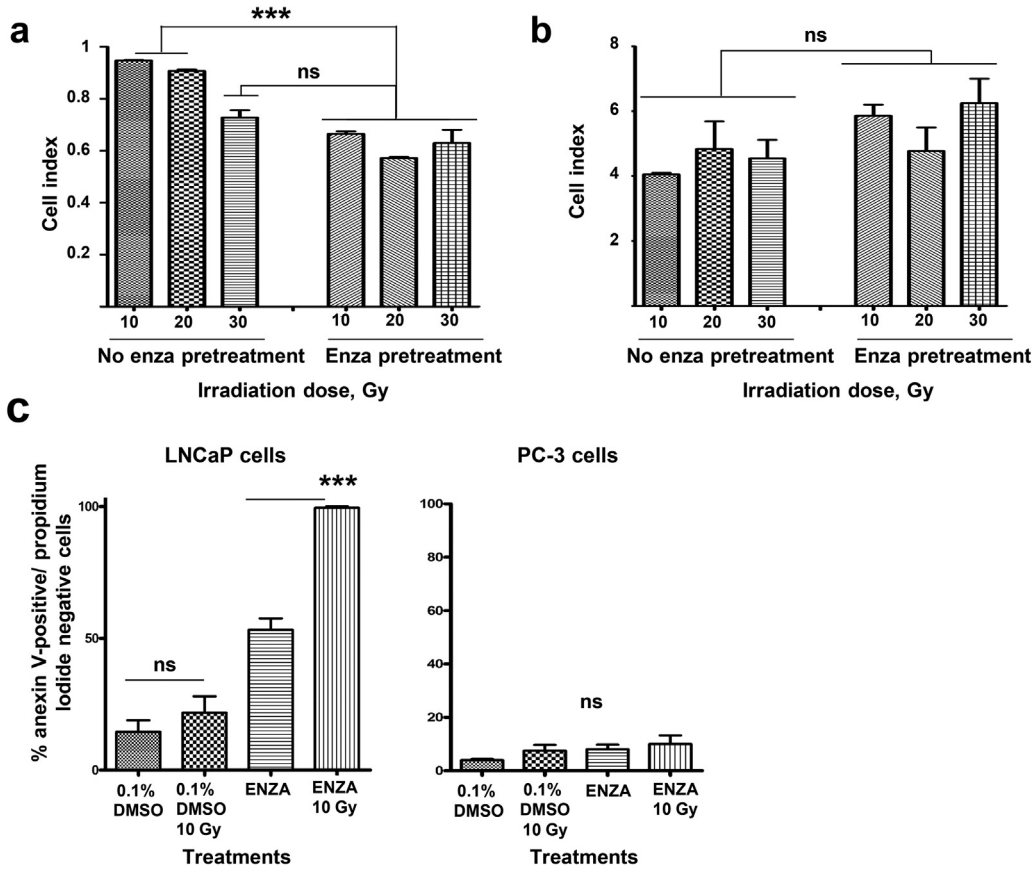

Fig. 2 – Enzalutamide sensitizes LNCaP cells but not PC-3 cells to radiotherapy. (a) RTCA cell growth data of LNCaP cells treated with either DMSO (vehicle control, graph on the left) or enzalutamide (graph on the right) for three days followed by the irradiation doses as indicated. The bar graph represents means of Cell Index with standard deviations as error bars. Treatments with DMSO (no enza) or enzalutamide are indicated, as well as irradiation doses. (b) Same as in (a) but for PC-3 cells. (c) Bar graph representing the percentage of annexin V-positive/propidium iodide-negative cells in cultures of the indicated cell lines, treated with DMSO or enzalutamide at the IC50 concentrations followed by treatments with 10 Gy where indicated. Relevant statistical comparisons are shown within the graphs. *, indicates highly significant differences ($P < 0.001$). ns, indicates non-significant differences ($P > 0.05$).**

to induce measurable cell death in untreated LNCaP cells by RTCA (Fig. 2a). In contrast, pre-treatment of LNCaP cells with enzalutamide strongly sensitized LNCaP cells to irradiation causing rapid cell death at the minimum tested dose of 10 Gy. Enzalutamide-resistant PC-3 exhibited exponential growth kinetics at the three tested irradiation doses, and pre-treatment with 40 μM enzalutamide did not have any effect whatsoever to their growth (Fig. 2b). These results strongly indicated that enzalutamide sensitized prostate cancer cells to radiotherapy as long as they respond to androgen blockade. To evaluate if these synergistic effects were caused by increased apoptosis, annexin V staining followed by flow cytometry was used. As observed by RTCA, LNCaP cells pre-treated by DMSO were highly resistant to radiotherapy-induced apoptosis at a dose of 10 Gy. In contrast, pre-treatment with IC50 concentrations of enzalutamide very highly sensitized cells to radiotherapy-induced apoptosis at a dose of 10 Gy (Fig. 2c). PC-3 cells remained insensitive to enzalutamide, radiotherapy or their combination, suggesting that enzalutamide's apoptotic properties were required for radiopotentiation.

5. Discussion

Prostate cancer therapy heavily relies on androgen deprivation which has improved survival.[13] Nevertheless, disease progression still occurs (castration-resistant prostate cancer), although there is evidence that in this case cancer progression continues to be driven by androgen signaling.[13] To reinforce the suppression of AR signaling, several AR antagonists have been developed. Amongst these, enzalumtamide (MDV3100) is an inhibitor that binds to the ligand-binding domain of AR and blocks its nuclear translocation.[14] Enzalutamide shows also benefit for the treatment of castration-resistant prostate cancer.[15]

As radiotherapy is the most widespread first line treatment for localized prostate cancer, its combination with enzalutamide represents a rather logical choice. Our data agrees with a recently published paper demonstrating the potential of this combination.[4]

In this study we have applied impedance-based real-time cell monitoring to evaluate the potential of the

enzalutamide/radiotherapy combination over two human prostate cancer cell lines; enzalutamide-sensitive LNCaP cells and enzalutamide-resistant PC-3 cells. These two cell lines are extensively used in pre-clinical evaluation of prostate cancer treatments. Our RTCA data confirmed the IC50s of enzalutamide for LNCaP cells estimated by other authors using end-point based assays, as well as the enzalutamide-resistance of PC-3. Enzalutamide increased apoptosis in LNCaP cell cultures, and suggested that this property may further sensitize these cells to radiotherapy. In fact, we found that LNCaP and PC-3 cells were strongly resistant to radiotherapy according to RTCA data. Indeed, PC-3 cells remained viable even at the highest irradiation dose tested in this study (30 Gy), while LNCaP cells only died at this very high dose.

Interestingly, a short pre-treatment of LNCaP cells with enzalutamide at the IC50 concentration strongly sensitized them to radiotherapy, which induced cell death by apoptosis at the lower dose tested in this study (10 Gy). In contrast, PC-3 cells remained insensitive to irradiation in the presence of enzalutamide. However, these results strongly suggested that the pro-apoptotic properties of enzalutamide were absolutely required for its radiopotentiating effects, only over androgen blockade-sensitive cells. Additionally, we demonstrate that RTCA is a simple and straightforward method to evaluate cell death by radiotherapy.

6. Conclusions

Based on our findings and on recently published data, the combination of radiotherapy with enzalutamide represents a good basis for the treatment of locally advanced prostate cancer. Moreover, the irradiation doses could be lowered for patients with enzalutamide-sensitive tumors. Our data is fully in agreement with recently published data and provides the basis for using enzalutamide as an agent to potentiate radiotherapy for prostate cancer.

Data availability statement

Reagents or cell lines derived from this study will be provided when requested.

Conflict of interest

None declared.

Financial disclosure

Astellas Oncology, Inc. provided a research project grant that partially funded this project but did not take any part in designing the experimental plan, data collection or analysis.

Author contributions

MB, IBL, PAN, IV and DE performed experiments and analyzed data. DGS, DE, GK and FA designed the study, performed experiments and analyzed the data. All authors contributed to the writing of the manuscript.

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