NVP-BEZ235 Enhances Radiosensitivity of Human Prostate Cancer Cells but Acts as a Radioprotector to Normal Prostate Cells

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Abstract

Targeted therapy for prostate cancer may offer potential improvement over current conventional therapies because of its specificity. Although conventional treatments are effective, they are not always curative, and have several limitations. In prostate cancer, activation of the epidermal growth factor receptor (EGFR) and the phosphatidylinositol-3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathways have been implicated in tumorigenesis, and resistance to both conventional and targeted anticancer therapies. Single-target therapies may fail due to cellular heterogeneity. Concomitant targeting of EGFR and PI3K/mTOR cell signaling components may enhance the efficacy of radiotherapy. In this study, the effect of an EGFR inhibitor (AG-1478) and a dual inhibitor of PI3K and mTOR (NVP-BEZ235) on the radiation response of a human prostate carcinoma cell line (DU145) and a normal prostate cell line (1542N) was investigated, using the colony forming assay. Treatment of DU145 cells with AG-1478 and NVP-BEZ235, either singly or in combination, resulted in a slight radiosensitisation of DU145 cells. Neither AG-1478 nor a cocktail of both inhibitors had an effect on the radiation response of the 1542N cell line. Interestingly, NVP-BEZ235 significantly protected 1542N cells from radiation-induced cell death. These data suggest that a specific inhibitor of PI3K and mTOR (NVP-BEZ235) could potentially be effective as a radio-protector.

Introduction

Prostate cancer is the second most common cancer in men, accounting for almost a million newly diagnosed cases and over two-hundred thousand deaths per year worldwide [1]. Radiotherapy, surgical resection and hormone-based therapy are, thus far, the most effective treatment protocols for patients with localised prostate disease [2]. However, major challenges are that most patients are diagnosed at an advanced stage, and even the approximately 30% of patients who present with early stage disease tend to relapse within 5 years...
[1,2]. Tumor recurrences are partly attributable to developed resistance against treatment. To improve the management of prostate cancer, development of approaches that more effectively target malignant cells is warranted.

Prostate cancer cells utilise multiple molecular pathways to proliferate, survive and invade tissue during the course of tumor progression [3]. The epidermal growth factor receptor (EGFR) and phosphatidylinositol-3-kinase (PI3K) pathways are central in disease progression and prostate cancer cell survival following radiation exposure [4-6]. EGFR is expressed in a variety of epithelial tissues and plays an important role in development, proliferation and differentiation [6,7]. High levels of EGFR have been observed in a variety of cancers, including those of the prostate, breast, colon, bladder, kidney and ovary [2,8]. Activation of EGFR is known to enhance tumor growth and progression [8,9]. Poor response to therapy also correlates with EGFR overexpression [8,9]. Alterations of the PI3K pathway, which involves the mammalian target of rapamycin (mTOR) and serine-threonine protein kinase (Akt), have been reported in over 42% of primary prostate tumors and 100% of metastatic tumors [10]. These include mutations and altered expression profiles of proteins within the pathway, leading to increased PI3K/Akt/mTOR signaling activity through mutations in the phosphatase and tensin homolog (PTEN) gene. Targeting these pathways may potentially improve the management of prostate disease. Preclinical studies have demonstrated that inhibition of EGFR sensitises cancer cells to cytotoxic agents and radiation [11,12]. Blocking PI3K activity has also been shown to yield similar effects [10,13]. Targeting of single signaling components often does not result in optimal cytotoxicity. This is partly due to non-uniform distributions of target antigen expression in cell populations, which may limit the ability to target all cells with toxic levels of therapeutic agents [14-16]. Therefore, developing therapeutic approaches that concomitantly target multiple signaling components of the EGFR and PI3K pathways might significantly sensitisise prostate cancer cells to radiotherapy and improve treatment outcome.

In the present work, studies were conducted to determine if inhibition of EGFR, PI3K and mTOR can preferentially radiosensitise human prostate cancer cells. To achieve this, benign (1542N) and malignant (DU145) human prostate cells were treated with AG-1478 (an EGFR inhibitor) and NVP-BEZ235 (a dual inhibitor of PI3K and mTOR) and concomitantly irradiated, and radiation-induced cell death was assessed using the colony forming assay.

Materials and Methods

Cell lines and culture maintenance

The normal 1542N cell line was derived from normal prostate epithelial tissue of a patient with primary adenocarcinoma of the prostate and immortalised with E6 and E7 genes of the human papilloma virus 16 [17], and was a gift from Prof JRW Masters (Prostate Cancer Research Centre, University College London, UK). Cells were grown in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma-Aldrich, Germany). The malignant DU145 cells were derived from a metastatic lesion of the central nervous system [18], and was a gift from Prof P Bouic (Synexa Life Sciences, Montague Gardens, South Africa). Cells were routinely grown in Minimum Essential Medium (MEM) (Sigma-Aldrich, Germany). Growth media were supplemented with 10% heat-inactivated foetal bovine serum (FBS) (HyClone, UK), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Lonza, Belgium), and cell cultures were incubated at 37°C in a humidified atmosphere (95% air and 5% CO₂). Cells were grown as monolayers in 75-cm² flasks (Greiner Bio-One, Germany, cat # 658170) and were used for experiments (passages 3-12) upon reaching 80-90% confluence.

Target inhibitors

NVP-BEZ235 (Santa Cruz Biotechnology, TX, USA, cat # 364429) is a dual inhibitor of phosphatidylinositol-3-kinase (PI3K) and mammalian target of rapamycin (mTOR), with an inhibitory concentration at 50% (IC₅₀) of 7.5 nM for PI3K and 5 nM for mTOR in highly metastatic human prostate tumor cells [19,20]. AG-1478 (Tocris Bioscience, UK, cat # 1276) is a specific inhibitor of EGFR with an IC₅₀ of 3 nM in non-small cell lung cancer cells [21]. Stock solutions of NVP-BEZ235
(106 mM) and AG-1478 (10 mM) were prepared in dimethyl sulfoxide and stored at -20°C until used.

**Cell survival assay and radiosensitivity**

The colony assay was used to measure intrinsic radiation response in both cell lines. Cultures in exponential growth were trypsinised to give single-cell suspensions and were plated (300-100 000 cells per flask, adjusted for irradiation dose) into 25-cm² culture flasks (Greiner Bio-One, Germany, cat # 690160), and incubated for 4-5 h to allow the cells to attach. Cell cultures were then irradiated to 0-10 Gy with 60Co γ-rays and reincubated. The mean dose rate used in this investigation was 0.83 Gy/min (range: 0.78-0.87 Gy/min). Cultures were irradiated at room temperature (22°C). After growing for 10-14 days, depending on the cell line, colonies were fixed in glacial acetic acid:methanol:water (1:1:8, v/v/v), stained with 0.01% amido black in fixative, washed in tap water, air-dried, and were counted. Three independent experiments were performed for each dose point, and the mean surviving fractions were fitted to the linear-quadratic (LQ) model to generate survival curves. Cellular radiosensitivity was expressed in terms of the surviving fraction at 2 Gy (SF₂).

**Determination of radiosensitivity modification by AG-1478 and NVP-BEZ235**

To investigate the influence of inhibitor exposure on radiosensitivity, 300 to 10 000 cells were seeded in 25 cm² tissue culture flasks and incubated for 4-5 h to attach. Cell cultures were then treated with 17 nM NVP-BEZ235 (~2.3-3.4×IC₅₀ for metastatic human prostate tumor cells), 15 nM AG-1478 (~5×IC₅₀ for non-small cell lung cancer cells), or a cocktail of both inhibitors at the same concentrations 30 min prior to irradiation to 2 Gy. As controls for cell cultures irradiated with and without inhibitors, batches of cell cultures treated with inhibitors alone (singly and in combination) and untreated unirradiated cultures were used, respectively. The interaction between inhibitors and γ-irradiation (2 Gy) was expressed as a modifying factor (MF), which is given by the ratio of surviving fractions (SF) in the absence and presence of inhibitors as follows:

$$MF = \frac{SF(2 \text{ Gy})}{SF([\text{Inhibitor}]+2 \text{ Gy})}$$

The criteria for inhibition, no effect, and enhancement of radiosensitivity by inhibitors are MF<1.0, MF=1.0 and MF>1.0, respectively.

**Data analysis**

Statistical analyses were performed using the GraphPad Prism (GraphPad Software, San Diego, CA, USA) computer program. Standard equations were used to fit nonlinear relationships. Data were calculated as the means (± SE) from three independent experiments. For each experiment and data point, 3 replicates were assessed. To compare two data sets, the unpaired t-test was used. P-values and coefficients of determination, R², were calculated from two-sided tests. A P-value of <0.05 indicates a statistically significant difference between the data sets.

**Results**

**Intrinsic cellular radiosensitivity**

Cell survival data for the human prostate carcinoma and normal cell lines were fitted to the linear-quadratic model, and the corresponding dose-response curves are presented in Figure 1. Intrinsic cellular radiosensitivity was expressed in terms of the surviving fraction at 2 Gy (SF₂). The malignant DU145 cell line emerged as more radioresistant than its normal 1542N counterpart, with SF₂-values of 0.53 ± 0.07 and 0.36 ± 0.09 for DU145 and 1542N, respectively. The difference in radiosensitivity was, however, not statistically significant (P=0.1894).

**Modification of radiotoxicity by inhibitors**

To assess whether blocking the activities of PI3K, mTOR and EGFR, with specific inhibitors results in changes in cellular radiosensitivity, cell cultures were treated with NVP-BEZ235 (against PI3K and mTOR), AG-1478 (against EGFR), or a combination of both inhibitors and subsequently irradiated to 2 Gy. In DU145
**Figure 1:** Clonogenic cell survival curves for the prostate carcinoma cell line (DU145) and normal prostate cell line (1542N) after $^{60}$Co γ-irradiation. Symbols represent the mean surviving fraction ± SE from three independent experiments. Standard errors are not transformed into a logarithmic scale. Survival curves were obtained by fitting experimental data to the LQ model.

**Figure 2:** Clonogenic cell survival at 2 Gy ($S_{2}$) for 2 human prostate cell lines after $^{60}$Co γ-irradiation: (A) malignant DU145 and (B) normal 1542N. Cells were irradiated without or in the presence of NVP-BEZ235 (dual inhibitor of PI3K and mTOR) and AG-1478 (EGFR inhibitor), either administered singly or in combination. Bars represent the mean surviving fraction ± SE from three independent experiments.
cells (Figure 2A), inhibition of PI3K and mTOR with NVP-BEZ235 alone led to a reduction in SF2 from 0.58 ± 0.12 to 0.39 ± 0.08. Although this translated to about 50% increase in radiotoxicity (Table 1), the sensitisation was not statistically significant ($P=0.2432$, $R^2=0.3187$). A larger, but not significantly different radiosensitisation was also observed following inhibition of EGFR with AG-1478, with SF2 decreasing from 0.58 ± 0.12 to 0.32 ± 0.07 ($P=0.1274$, $R^2=0.4794$). When the cells were treated with a cocktail of NVP-BEZ235 and AG-1478, the surviving fraction at 2 Gy emerged as 0.35 ± 0.05 and was not significantly different from those obtained for single inhibitor treatment (Table 1). On average, treating DU145 cells with NVP-BEZ235 and AG-1478, either singly or in combination, yielded a ~2-fold reduction in cell survival. In contrast, treatment with NVP-BEZ235 alone significantly protected the normal 1542N cells against radiation-induced cell death (Figure 2B and Table 1). The surviving fraction at 2 Gy increased from 0.36 ± 0.06 to 0.69 ± 0.11, yielding a dose modifying factor of 0.52 ± 0.12 ($P=0.0413$, $R^2=0.6874$). Pre-irradiation exposure of these cells to AG-1478 or a cocktail of NVP-BEZ235 and AG-1478 resulted in minimal reduction in cell survival. The corresponding dose modifying factors were 1.24 ± 0.12 and 1.13 ± 0.22.

Discussion

Radiation therapy plays an important role in prostate carcinoma treatment. However, the radioresistance of prostate cancer cells limits the outcome of radiotherapy [22]. It is assumed that surviving and repopulating carcinoma cells are capable of providing molecular protection against the cytotoxic effects of radiotherapy. Long-term survival of prostate cancer patients treated with radiotherapy, thus far, appears to benefit a restricted sub-group [23]. Novel therapeutic approaches are, therefore, desirable. To address this need, the effect of inhibiting EGFR, PI3K, and mTOR on the radiation responses of a prostate carcinoma cell line (DU145) and a normal prostate cell line (1542N) was evaluated.

The cell survival data in Figure 1 suggest that the normal 1542N cell line is relatively more radiosensitive than the malignant DU145 cell line, and are consistent with those reported elsewhere [24,25]. Treatment with radiation alone might not be beneficial as it appears to be more toxic in normal cells than in their malignant counterparts. Pre-treatment of DU145 cells with the EGFR inhibitor (AG-1478), dual PI3K and mTOR inhibitor (NVP-BEZ235), and a cocktail of both inhibitors reduced the surviving fractions at 2 Gy, giving average radiation enhancement factors of 1.81, 1.49, and 1.66, respectively (Figure 2A and Table 1). These findings suggest that while AG-1478 and NVP-BEZ235 could potentially be used as individual prostate tumor radiosensitisers in conventional radiotherapy, their combined administration does not seem to be more superior in sensitising prostate cancer cells to radiation exposure. These observations are consistent with preclinical and clinical studies demonstrating enhanced radiosensitivity in a variety of cell lines and tumors following inhibition of EGFR, PI3K and mTOR [26-28]. The radiosensitisation observed in the 1542N cell line at 2 Gy by AG-1478 or a cocktail of AG-1478 and NVP-BEZ235 was much less than that seen in the malignant cell line (Figure 2B and Table 1), indicating that an improved therapeutic benefit may be achieved with less extensive normal tissue toxicity when AG-1478 is used as an adjuvant to radiotherapy.

Interestingly, a significant radio-protective effect emerged when the normal prostate cell line (1542N) was pre-treated with NVP-BEZ235 (Figure 2B and Table 1). In fact, inhibiting PI3K and mTOR with NVP-BEZ235 almost doubled clonogenic survival in 1542N cells. Similar radio-protective properties of NVP-BEZ235 have been demonstrated in normal gut tissue [20]. The radio-protection by NVP-BEZ235 illustrated here for normal prostate cells and its apparent radiosensitisation of malignant prostate cells can have significant ramifications in the radiotherapy of prostatic disease, as well as, in radiation protection.

In conclusion, the current data demonstrate that simultaneously inhibiting PI3K and mTOR plays an important role in EGFR/PI3K/mTOR-mediated radioresistance in prostate cancer. The results show that NVP-BEZ235 treatment predisposes prostate cancer cells to high radiation sensitivity, while their normal counterparts are significantly protected from the cytotoxic effects of ionising radiation. These findings provide the basis for further studies involving a larger panel of cell lines to fully elucidate the malignancy-dependent roles of NVP-BEZ235 in modifying radiation effects.
Table 1: Summary of radiosensitivity and dose modifying data for 2 human prostate cell lines (malignant DU145 and normal 1542N) treated with inhibitors NVP-BEZ235 (against PI3K and mTOR) and AG-1478 (against EGFR).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>$SF_2^*$</th>
<th>$MF_2^*$</th>
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<tbody>
<tr>
<td>DU145</td>
<td>2 Gy</td>
<td>0.58 ± 0.12</td>
<td></td>
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<tr>
<td></td>
<td>2 Gy + NVP-BEZ235</td>
<td>0.39 ± 0.08</td>
<td>1.49 ± 0.43</td>
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<tr>
<td></td>
<td>2 Gy + AG-1478</td>
<td>0.32 ± 0.07</td>
<td>1.81 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>2 Gy + NVP-BEZ235 + AG-1478</td>
<td>0.35 ± 0.05</td>
<td>1.66 ± 0.42</td>
</tr>
<tr>
<td>1542N</td>
<td>2 Gy</td>
<td>0.36 ± 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 Gy + NVP-BEZ235</td>
<td>0.69 ± 0.11</td>
<td>0.52 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>2 Gy + AG-1478</td>
<td>0.29 ± 0.06</td>
<td>1.24 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>2 Gy + NVP-BEZ235 + AG-1478</td>
<td>0.32 ± 0.03</td>
<td>1.13 ± 0.22</td>
</tr>
</tbody>
</table>

$SF_2$ and $MF_2$ denote the surviving fraction and radiation modifying factor at 2 Gy, respectively. *Mean ± SE. #Mean ± error: errors were calculated using appropriate error propagation formulae.

**Author Disclosures**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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**References**


