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Specific inhibitors of PI3K, mTOR and AR exhibit strong synergism and radiosensitization in human prostate cell lines

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► ***Original article***

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ABSTRACT

Background: Single agent use of chemotherapy in prostate cancer is very limiting, as large doses are often required for tumour control and can lead to elevated systemic toxicity. Targeting of survival proteins of the epidermal growth factor receptor (EGFR), phosphoinositide 3-kinases (PI3K), mammalian target of rapamycin (mTOR) and androgen receptor (AR) pathways with cocktails of specific inhibitors might yield optimum therapeutic benefit with minimal toxicity. ***Materials & Methods:*** The modes of interaction of the dual inhibitor of PI3K and mTOR (NVP-BEZ235), EGFR inhibitor (AG-1478), and AR inhibitor (MDV3100) in in vitro cultures of four human prostate cell lines (DU145, LNCaP, BPH-1 and 1542N) were evaluated as cocktails, using clonogenic cell survival, subsequent to validation of the androgen dependency. Components of cocktails (Cocktail 1 (AG-1478 and NVP-BEZ235), Cocktail 2 (NVP-BEZ235 and MDV3100), and Cocktail 3 (MDV3100 and AG-1478)) were used at equivalent concentrations for 50% cell killing. Combination indices (CI) for the cocktails were determined and used as descriptors of inhibitor interaction. Radiomodulatory effects of inhibitor cocktails were also evaluated. ***Results:*** Inhibitor cocktails selectively showed strong to very strong synergism and radiosensitization. Concurrent inhibition of PI3K/mTOR and AR could potentially be of better therapeutic benefit than inhibition of EGFR and PI3K/mTOR or AR and EGFR, as the potential benefit of EGFR targeting was found to be limited. ***Conclusion:*** These data may guide the design of potent treatment approaches for prostate cancer.

INTRODUCTION

Crosstalk, a method of cellular pathway communication, may present itself as an important factor during prostate cancer (PCa) progression, giving cells a survival advantage (Zhu and Kyprianou, 2010), and might serve as a potential target for therapy. Crosstalk further emphasises the importance of combination therapy for optimum tumor control.

Chemotherapeutics, such as enzalutamide, abiraterone and prednisone have yielded positive outcomes, however, toxicity to normal tissue remains a challenge [1]. Research has shown that normal tissue can be spared when therapy is administered by dose alterations, or by employing radiation and chemotherapy combination strategies [2]. Such approaches can help tremendously in reducing high dose chemotherapy toxicity [3, 4]. It was also demonstrated in an *in vitro* system that treatment with the dual inhibitor of phosphoinositide 3-kinases (PI3K) and mammalian target of rapamycin (mTOR), NVP-BEZ235, resulted in better cancer cell inactivation than traditional radiotherapy [5]. Targeted therapy looks at the critical pathways involved in the initiation and progression of PCa [6]. The androgen receptor (AR) which is at the epicentre of PCa research, and is involved in the crosstalk between critical pathways is largely neglected [7]. Since the disease is driven by hormones, it should be controlled by hormones, for example, androgen deprivation therapy (ADT), but tumor resistance has been shown to limit therapeutic advantage [8]. Capitalizing on the crosstalk between pathways by targeting the PI3K/mTOR/EGFR pathway might assist in solving the problem of PCa resistance to hormonal therapy [9]. The dual inhibition of PI3K and mTOR (NVP-BEZ235) has been studied in breast, head and neck, and colon cancers [10-12]. Previous investigations have shown that the effects of NVP-BEZ235 may be novel in prostate cancer cells [2, 5].

PCa management is faced with numerous clinical challenges. Although conventional therapy has proved to be successful in the management of localized and early diagnosed cases, clinicians battle to address the cellular resistance seen in late diagnosed cases with metastases. Resistance can be attributed to mutations, over-treatment, and mistreatment, resulting in tumor recurrences [13]. Cells survive through activation of molecular signaling routes such as the AR, EGFR and PI3K/Akt/mTOR pathways [14]. Also, cellular exposure to

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ionizing radiation has been proven to activate these survival pathways [15], leading to treatment resistance. Crosstalk between signaling pathways has also been cited as the main reason for resistances and recurrences [16]. Studies on signaling pathways, such as those involving AR, EGFR, PI3K, Akt, and mTOR [2, 5, 16, 17], might lead to the identification of potentially effective therapeutic interventions for prostate cancer.

In this study, the modes of interaction of the dual inhibitor of PI3K and mTOR (NVP-BEZ235), EGFR inhibitor (AG-1478), and AR inhibitor (MDV3100) in *in vitro* cultures of four human prostate cell lines (DU145, LNCaP, BPH-1 and 1542N) were interrogated using the following cocktails: Cocktail 1 (AG-1478 and NVP-BEZ235), Cocktail 2 (NVP-BEZ235 and MDV3100), and Cocktail 3 (MDV3100 and AG-1478). For this, the cytotoxicities of inhibitors and combination indices were determined from clonogenic cell survival data. To evaluate the capacity of these inhibitors to potentiate the radiotherapy, their radiomodulatory effect was also assessed. Data on inhibitor cytotoxicity, combination indices, and their ability to mediate the effects of ionizing radiation can guide the design of novel treatment modalities for prostate cancer. (Bitting and Armstrong, 2013; Courtney *et al*., 2010).

MATERIALS AND METHODS

Cell lines and culture maintenance

The benign 1542-NPTX (1542N) human prostate epithelial cell line was derived from a primary adenocarcinoma of the prostate and immortalized with E6 and E7 genes of the human papilloma virus 16 and was used to represent normal prostate tissue. The cell line was provided by Professor JRW Masters (Prostate Cancer Research Centre, University College London, UK).

The human prostate cancer cell line, DU145, derived from a metastatic lesion of the central nervous system, was obtained from Professor P Bouic (Synexa Life Sciences, Montague Gardens, South Africa).

The benign prostatic hyperplasia-1 (BPH-1) cell line was established from human prostate tissue obtained by transurethral resection. Primary cell cultures were immortalized with simian virus 40 (SV40) large T-antigen. For this study, even though BPH-1 cell line is not defined as a cancer, it was assumed to be tumor-derived as it has been suggested that it may be a precursor to PCa.

The LNCaP cell line was established from a supraclavicular lymph node metastasis of human prostatic adenocarcinoma. LNCaP has a fibroblastoid morphology, low anchorage potential, is adherent, but grows in aggregates and as single cells in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma-Aldrich, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (HyClone, UK), and penicillin (100 U/ml) / streptomycin (100 mg/ml) (Lonza, Belgium). The cultures require gentle handling at all times because the cells are easily dislodged by tapping, shaking or pipetting. The low anchorage potential is also responsible for the 10-20% cell loss during media changes in long-term experiments. To address this problem, flasks were coated with a mixture of 100 µl of fibronectin (Sigma-Aldrich) and 15 ml of sterile phosphate buffered saline (PBS) (Lonza, USA) and incubated overnight, before use. The cells were obtained from Professor Helmut Klocker (Department of Urology, University of Innsbruck, Austria).

The 1542-NPTX (passage number: 18-31), BPH-1 (passage number: 2-11) and LNCaP (passage number: 4-13) cell lines were grown routinely in RPMI-1640 medium (Sigma-Aldrich, USA). The DU145 cell line (passage number: 7-22) was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Germany). All growth media were supplemented with 10% heat-inactivated FBS (HyClone, UK) and penicillin (100 U/ml) / streptomycin (100 µg/ml) (Lonza, Belgium), unless stated otherwise. Cell cultures were incubated at 37°C in a humidified atmosphere (95% air and 5% CO_2). Cells were grown as monolayers in 75-cm^2 flasks (Greiner Bio-One, Germany), and were used for experiments upon reaching 70 – 90% confluence.

Cellular proliferation arrest by 5a-dihydrotestosterone (DHT)

To confirm the androgen dependence status of the cell lines used in this study, their sensitivity to androgen treatment was determined. For this, 8000-10000 cells were seeded in 24-well multiwell plates with medium containing charcoal-stripped fetal bovine serum (csFBS). By stripping of serum with activated carbon, steroid hormones, specifically 5a-dihydrotestosterone (5a-DHT), endogenous hormones, growth factors and cytokines are removed. This, thus, enables the *in vitro* evaluation of the effects of steroid hormones by addition of 5a-DHT to cell cultures. The medium for the 1542N cell line was supplemented with 0.5% csFBS, the BPH-1 cell line with 10% csFBS, and 5% csFBS for the LNCaP cell line, which served as the positive control. 5a-DHT (Sigma-Aldrich, Germany) was added a day later (2 days later in the case of LNCaP) in concentrations ranging from 0.001-10 nM, for a period of 4 days. The experiment was stopped after 4 days and the cell growth determined by crystal violet assay. Two control flasks were set up, one with charcoal-stripped FBS and one with non-stripped FBS. The optical density (OD) readings, expressed as a percentage of the OD of the positive controls (cultures with non-stripped FBS), were plotted against concentrations of DHT.

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Target inhibitors

NVP-BEZ235 ($C_{30}H_{23}N_{50}$; MW = 469.55; Santa Cruz Biotechnology, Texas, USA) potentially and reversibly inhibits class 1 PI3K and mTOR catalytic activity. AG-1478 ($C_{16}H_{14}CIN_3O_2$; MW = 315.8; Tocris Bioscience, UK) is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor. MDV3100 ($C_{21}H_{16}F_4N_4O_2S$; MW = 464.44; KareBay Biochem, USA) is an androgen receptor (AR) inhibitor. It is a potent oral antagonist to the AR that lacks any agonist activity, and prevents nuclear translocation of AR and its binding to DNA. Stock solutions of NVP-BEZ235 (106 mM), AG-1478 (10 mM) and MDV3100 (10 mM) were reconstituted in dimethyl sulfoxide (Sigma-Aldrich, France) and stored at -20°C until needed.

Target inhibitor toxicity measurements

To assess the influence of the inhibitor concentration on cytotoxicity, single-cell suspensions were seeded (600-100 000 cells per flask, depending on cell line) into 25 cm^2 culture flasks, and incubated for 3-4 h to allow the cells to attach. The cells were exposed to their respective inhibitors; NVP-BEZ235 (0.001-1000 nM), AG-1478 (1-1 000 000 nM) and MDV3100 (0.1-10 000 nM) and incubated for 7-10 days for colony formation. The colonies were fixed, stained, washed in tap water, air-dried and counted. To determine the equivalent concentration of each inhibitor for 50% cell kill (EC_{50}), the surviving fractions (SF) were plotted as a function of log (inhibitor concentration) and fitted to the 4-parameter logistic model in equation (1).

$$SF = B + \frac{T-B}{\{1-10^{[(logEC_{50}-D)HS}\}} \quad (1)$$

Where; B and T are the minimum and maximum of the sigmoidal curve, respectively, D is the log (inhibitor concentration), and HS is the steepest slope of the curve. For each cell line and dose point, three independent experiments were performed.

Determination of combination indices

To test for potential interaction between inhibitors of each cocktail, the toxicity data were fitted to equation (2).

$$log(f_a/f_u) = m \times log(D) - m \times log(D_m) \quad (2)$$

where f_a and f_u are the affected and unaffected fractions of cells, respectively, to generate median-effect plots for each inhibitor. D is the concentration of inhibitor, D_m is the median-effect concentration of inhibitor, and the coefficient m is an indicator of the shape of the inhibitor concentration-effect relationship [4, 18]. The shape parameter m = 1, >1, and <1 are for hyperbolic, sigmoidal, and flat-sigmoidal inhibitor concentration-effect curves, respectively.

The mode of interaction between any two inhibitors was assessed by determining combination indices (CI) for each inhibitor cocktail from the fitted parameters of equation (2), using equation (3).

$$CI = \frac{D_1}{\left\{D_{m1} \times \left(\frac{f_{a1}}{1-f_{a1}}\right)^{\frac{1}{m_1}}\right\}} + \frac{D_2}{\left\{D_{m2} \times \left(\frac{f_{a2}}{1-f_{a2}}\right)^{\frac{1}{m_2}}\right\}} \quad (3)$$

Where; D_1 is the concentration of Inhibitor 1 and D_2 is the concentration of Inhibitor 2. m_1 and m_2 are the respective shape parameters. f_{a1} and f_{a2} are given as: (1 - SF, as defined in equation (2)). D_{m1} and D_{m2} are the corresponding median-effect concentrations. Synergism, additivity, and antagonism are indicated by CI<1, CI=1, and CI>1, respectively. Furthermore, the criteria for very strong synergism, strong synergism, and synergism are CI<0.1, 0.1≤CI≤0.3, 0.3<CI≤0.7, respectively [18].

Determination of radiosensitivity modification by target inhibitors

The effect of inhibitor exposure on radiosensitivity was investigated. Attached cells were treated with their respective inhibitors at the predetermined EC_{50}: 1542N (400 nM of AG-1478 and 53.82 nM of NVP-BEZ235 or 21.36 nM MDV3100); DU145 (6613 nM of AG-1478 and 16.25 nM of NVP-BEZ235 or 22.43 nM MDV3100); LNCaP (302 nM of AG-1478 and 6.10 nM of NVP-BEZ235 or 183.2 nM MDV3100) and BPH-1 (677 nM of AG-1478 and 6.11 nM of NVP-BEZ235 or 20.35 nM MDV3100). For each experiment, sets of cell culture flasks given inhibitors alone (singly and in combination) were immediately irradiated with 2 - 10 Gy, using a Precision MultiRad 160 X-irradiator (Precision X-Ray Inc., Branford, CT, USA), at a dose rate of 1.0 Gy/min. Unirradiated flasks, without inhibitors, served as Controls for cultures irradiated with and without inhibitors, respectively, or a cocktail of both inhibitors at the same concentration. Inhibitor-treated cell cultures were used as Controls for those receiving inhibitors and irradiation to allow for inter-experimental variations in inhibitor toxicity, as exposures to predetermined concentrations do not always yield the expected cell kill. The interaction between inhibitors and irradiation was expressed as a modifying factor ($MF_{survival}$), given as the ratio of surviving fractions at a dose of X-rays (or the mean inactivation dose, $\bar{D}$) in the absence and presence of inhibitor (equation (4)).

$$MF_{survival} = \frac{SF(X-rays)}{SF(inhibitor+X-rays)} \quad or \quad \frac{\bar{D}(X-rays)}{\bar{D}(inhibitor+X-rays)} \quad (4)$$

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

Statistical analysis

The GraphPad Prism computer programme (GraphPad Software, San Diego, CA, USA) was used to

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perform statistical analyses. For associations, linear regression analyses were used. Standard equations were used to fit nonlinear relationships. Data were presented as the mean ± standard error of the mean (SEM) of three independent experiments, as indicated by error bars. For each experiment and data point, 3 replicates were assessed. To compare two data sets, the unpaired *t*-test was used. *P*-values were calculated from two-sided tests. A *P*-value of <0.05 indicated a statistically significant difference between the data sets.

RESULTS

Androgen sensitivity of DU145, LNCaP, BPH-1, and 1542N cells

The crystal violet vital dye staining assay showed that when the DU145 cells were grown in medium with charcoal-stripped fetal bovine serum (csFBS), cell proliferation decreased by about 35% (figure 1A), indicating dependence on essential growth factors including androgen and other hormones. Addition of 5a-dihydrotestosterone (5a-DHT), at concentrations of 0.001 to 1.0 nM, appeared to induce a further concentration-dependent reduction in cell proliferation, but the decrease in proliferation was not statistically significant (P=0.2433). The rate of proliferation at 1.0 nM was found to be approximately half the rate in cultures treated with medium containing non-stripped FBS. However, at higher 5a-DHT concentrations (10 and 100 nM), cell proliferation recovered to levels comparable with those in non-stripped FBS cultures.

Growing the LNCaP cells in csFBS-containing medium resulted in a 38% reduction in cell proliferation (figure 1B). When grown in csFBS-containing medium, spiked with 5a-DHT, a clear 5a-DHT concentration-dependent increase in proliferation emerged, indicating dependence of these cells on androgen availability.

As expected, depriving the BPH-1 cells of essential growth factors when they were grown in medium supplemented with charcoal-stripped FBS, led to a 34% reduction in proliferation (figure 1C). Adding 5a -DHT at concentrations ranging between 0.001 and 100 nM did not result in a recovery in cell proliferation, indicating that the BPH-1 cells are insensitive to androgen treatment.

Interestingly, when the apparently normal 1542N cells were grown in medium supplemented with csFBS, a less than 20% reduction in proliferation was observed (figure 1D). As in the case of the DU145 cells, treatment of the 1542N cells with 5a-DHT (0.001 to 0.1 nM) resulted in an additional concentration-dependent reduction in proliferation, reaching a minimum of ~66% at 0.1 nM. However, the decrease in cell proliferation did not reach statistical significance (P=0.3558). Exposure of cells to higher concentrations of 5a-DHT led to complete recovery in proliferation.

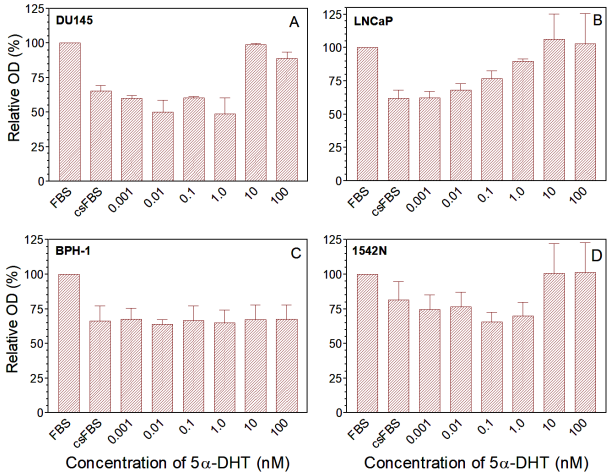


Figure 1. The effect of 5a-dihydrotestosterone (DHT) addition to medium with charcoal stripped fetal bovine serum (csFBS) on the proliferation measured by crystal violet dye staining assay in human prostate cells: **(A)** DU145, **(B)** LNCaP, **(C)** BPH-1, and **(D)** 1542N.

Cytotoxicity of NVP-BEZ235, AG-1478 and MDV3100

Figure 2 shows that the EGFR inhibitor (AG-1478) exhibits a concentration-dependent toxicity in all cell lines, and sensitivity to inhibitor treatment was expressed in terms of equivalent concentration for 50% cell killing (EC_{50}) as the mean (±SEM). Treatment with AG-1478 resulted in the LNCaP showing more sensitivity than the other cell lines, and DU145 showing the most resistance. The EC_{50} of the normal cell line (1542N) emerged as 416±46 nM and was significantly lower than those of the DU145 (7431±1052 nM, P=0.0402, R^2=0.6915) and BPH-1 (708±60 nM, P=0.0055, R^2=0.8815) cell lines (table 1). The EC_{50} of the relatively more sensitive LNCaP cell line did not differ significantly from that of the 1542N cell line (290±31 nM, P=0.0783, R^2=0.5805).

Inhibition of PI3K and mTOR inhibitor with NVP-BEZ235 also resulted in a concentration-dependent cell killing (figure 2). The rank order of resistance to NVP-BEZ235 treatment is BPH-1 ≈ LNCaP<DU145<1542N, with EC_{50}-values of 6.26±0.59, 6.42±0.74, 16.49±2.97, and 51.33±11.81 nM, respectively. All tumour cell lines were significantly more sensitive to NVP-BEZ235 treatment than the normal cell line, as shown in table 1 (P£0.0047).

The cytotoxicity of the androgen receptor inhibitor (MDV3100) was also concentration-dependent (figure 2). The LNCaP cell line was significantly more resistant to androgen receptor inhibition than the rest of the cell lines (P£0.0008), which were similarly sensitive to MDV3100 treatment. The rank order of increasing sensitivity to MDV3100 treatment is LNCaP ® 1542N ≈ BPH-1 ≈ DU145, with EC_{50}-values of 92.73±10.55, 16.92±0.87, 16.64±1.20, and 15.74±0.94 nM, respectively (table 1).

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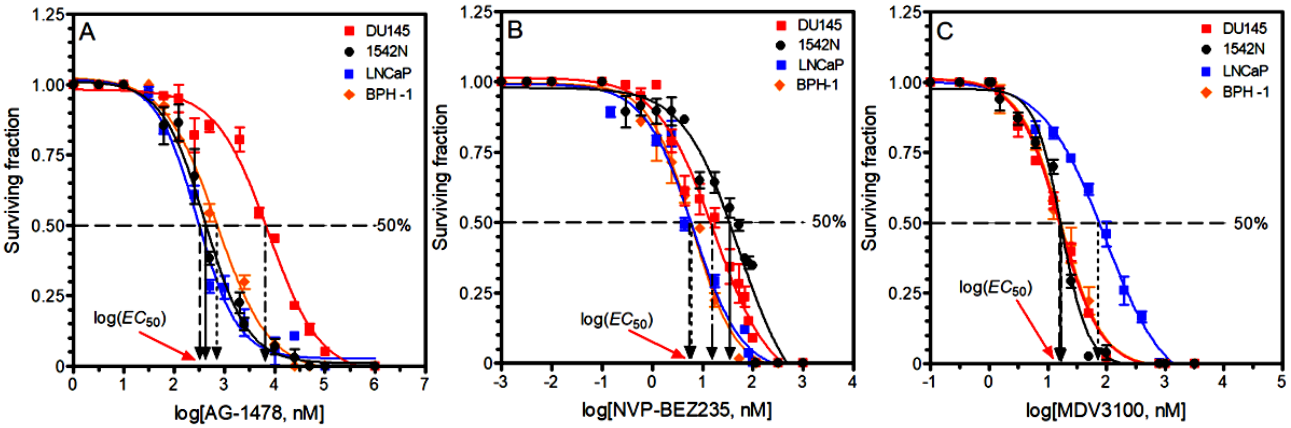


Figure 2. Cytotoxicity curves for PI3K and mTOR inhibitor (NVP-BEZ235: 0.001-1000 nM), EGFR inhibitor (AG-1478: 1 - 1 000 000 nM), and AR inhibitor (MDV3100: 0.1-10 000 nM) for 4 human prostate cell lines 1542N, DU145, LNCaP and BPH-1. Curves were obtained by plotting cell survival as a function of log(inhibitor concentration). Cell survival was determined by the colony assay, and data were fitted to a 4-parameter logistic equation. Data points are means±SEM of 3 independent experiments.

Table 1. Summary of cytotoxicity data for 2 human prostate cancer cell lines (DU145 and LNCaP), a benign prostatic hyperplasia cell line (BPH-1), and a normal prostate cell line (1542N) treated with EGFR inhibitor (AG-1478), PI3K and mTOR inhibitor (NVP-BEZ235), and AR inhibitor (MDV3100). EC50 denotes the equivalent concentration for 50% cell survival. T and B are the maximum and minimum of the concentration-response curve, respectively (figure 2). HS is the steepest slope of the curve.

Cell line	Treatment	EC_{50} (nM)	*T*	*B*	*HS*
DU145	AG-1478	7431±1052	0.98±0.02	-0.03±0.03	-0.84±0.08
	NVP-BEZ235	16.49±2.97	1.02±0.02	-0.08±0.05	-0.80±0.08
	MDV3100	15.74±0.94	1.01±0.01	-0.02±0.01	-1.22±0.08
LNCaP	AG-1478	290±31	1.02±0.02	0.03±0.02	-1.07±0.11
	NVP-BEZ235	6.42±0.74	0.99±0.01	-0.03±0.02	-0.91±0.08
	MDV3100	92.73±10.55	1.00±0.02	-0.10±0.04	-0.84±0.07
BPH-1	AG-1478	708±60	1.03±0.01	-0.02±0.01	-0.83±0.05
	NVP-BEZ235	6.26±0.59	0.99±0.02	-0.03±0.02	-1.02±0.09
	MDV3100	16.64±1.20	1.01±0.02	-0.02±0.02	-1.23±0.11
1542N	AG-1478	414±46	1.01±0.02	0.01±0.01	-1.02±0.10
	NVP-BEZ235	51.33±3.81	0.98±0.02	-0.16±0.09	-0.79±0.11
	MDV3100	16.92±0.87	0.98±0.01	-0.01±0.01	-1.92±0.17

Inhibitor interaction

To determine the nature of cellular response to treatment with the EGFR, PI3K and mTOR, and AR inhibitors, the cell survival data presented in figure 2 were log transformed to obtain median-effect plots for the inhibitors. Figure 3 represents the median-effect plots for the four respective cell lines. For DU145 the curves for EGFR and PI3K/mTOR inhibition are virtually parallel, with slopes of 0.78 and 0.73, respectively (table 2; m<1.0), indicating flat -sigmoidal concentration-effect curves. The median-effect concentrations (D_m) of AG-1478 and NVP-BEZ235 were low, and emerged as 13.28 and 14.27 nM, respectively. On the other hand, androgen receptor inhibition in these cells yielded a much steeper curve with a slope of 1.63 (m>1.0), indicating a sigmoidal response. The corresponding median-effect concentration of MDV3100 was ~370-fold higher than those of AG-1478 and NVP-BEZ235 (table 2).

For the LNCaP cell line the curves for the PI3K/ mTOR and androgen receptor inhibition are parallel and emerge with slopes of 0.81 and 0.83, respectively (table 2; m<1.0), implying that NVP-BEZ235 and MDV3100 display flat-sigmoidal cytotoxic effects in the LNCaP cell population. The corresponding median-effect concentrations were 4.47 and 68.19 nM. Inhibition of EGFR in these cells with AG-1478 resulted in a steeper curve (m=1.27), indicating a sigmoidal cytotoxic response. The median-effect concentration of AG-1478 in the LNCaP cell line was 368 nM.

PI3K/mTOR and androgen receptor inhibition in the BPH-1 cell line resulted in a sigmoidal cytotoxic response, giving shape parameters of 1.22 and 1.54, respectively (figure 3 & table 2). The respective median-effect concentrations were found to be 4.31 and 14.14 nM. Inhibition of EGFR in BPH-1 cells yielded a flat-sigmoidal response, with a shape parameter of 0.95 and a resulting median-effect concentration of AG-1478 of 760 nM.

Inhibition of EGFR, PI3K/mTOR, and AR in the 1542N cell line yielded hyperbolic, flat-sigmoidal, and sigmoidal cytotoxic responses, respectively (figure 3). The shape parameters for treatment with AG-1478, NVP-BEZ235, and MDV3100 were 1.0, 0.67, and 1.70, respectively. The corresponding median-effect concentrations emerged as 517, 41.82, and 11.35 nM.

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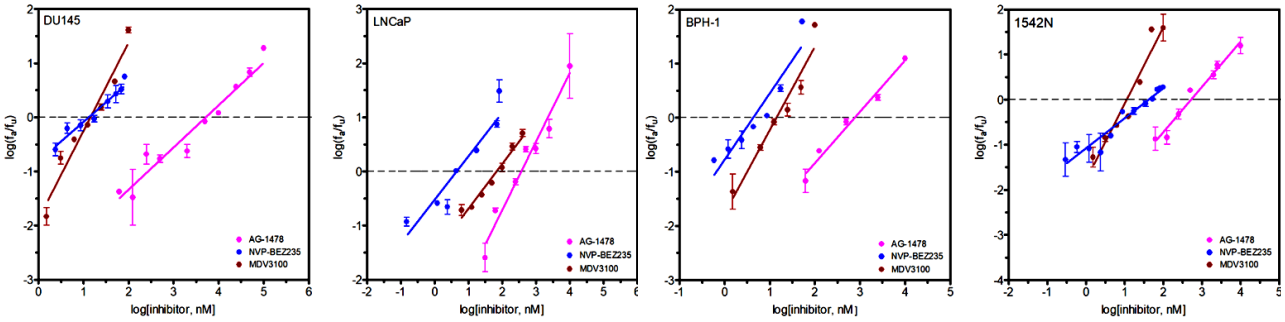


Figure 3. Median-effect plots for 4 human prostate cell lines (DU145, LNCaP, BPH-1, and 1542N), treated with NVP-BEZ235, AG-1478 and MDV3100, from toxicity data presented in figure 2. Transformed data were fitted to the function: log (fa/fu)=m×log (D)–m×log(Dm), where fa and fu are the affected and unaffected fractions of cells, respectively, and the coefficient m is an indicator of the shape of the inhibitor concentration-effect relationship (m=1, >1, and <1 indicate hyperbolic, sigmoidal, and flat-sigmoidal inhibitor concentration-effect curves, respectively), Dm is the median-effect concentration of inhibitor, and D is the concentration of inhibitor [18]. Horizontal dotted lines are the median-effect axes.

Table 2. Summary of parameters of median-effect plots for EGFR inhibitor (AG-1478), PI3K and mTOR inhibitor (NVP-BEZ235), and AR inhibitor (MDV3100) in 2 human prostate cancer cell lines (DU145 and LNCaP), a benign prostatic hyperplasia cell line (BPH-1), and normal prostate cell line (1542N).

Cell line	Treatment	*M*	D_m (nM)	Shape of concentration-effect curve
DU145	AG-1478	0.78 ± 0.06	5162 ± 3	flat-sigmoidal
	NVP-BEZ235	0.73 ± 0.07	13.28 ± 0.46	flat-sigmoidal
	MDV3100	1.63 ± 0.10	14.27 ± 0.27	Sigmoidal
LNCaP	AG-1478	1.27 ± 0.11	368 ± 2	Sigmoidal
	NVP-BEZ235	0.81 ± 0.07	4.47 ± 0.17	flat-sigmoidal
	MDV3100	0.83 ± 0.05	68.19 ± 0.62	flat-sigmoidal
BPH-1	AG-1478	0.95 ± 0.06	760 ± 2	flat-sigmoidal
	NVP-BEZ235	1.22 ± 0.13	4.31 ± 0.17	Sigmoidal
	MDV3100	1.54 ± 0.13	14.14 ± 0.40	Sigmoidal
1542N	AG-1478	1.00 ± 0.08	517 ± 2	Hyperbolic
	NVP-BEZ235	0.67 ± 0.06	41.82 ± 0.74	flat-sigmoidal
	MDV3100	1.70 ± 0.13	11.35 ± 0.30	Sigmoidal

To evaluate potential interactions between the EGFR, PI3K and mTOR, and AR inhibitors in the DU145, LNCaP, BPH-1, and 1542N cell lines, combination indices (*CI*) were determined for two-inhibitor combinations (at EC_{50} concentrations). The inhibitor-inhibitor interaction data are summarized in table 3. Combination of all inhibitors with each other resulted in very strong synergism (*CI*<0.1) in the DU145 cell line.

While antagonism (*CI*>1.0) was observed in the LNCaP cell line when the EGFR inhibitor (AG-1478) was combined with the PI3K/mTOR inhibitor (NVP-BEZ235) or the AR inhibitor (MDV3100), a very strong synergism emerged when cells were concomitantly treated with NVP-BEZ235 and MDV3100.

In the BPH-1 cell line, combining AG-1478 with NVP-BEZ235 or MDV3100 resulted in a very strong synergism (CI<0.1). However, concomitant treatment of cells with NVP-BEZ235 and MDV3100 was only synergistic (0.1≤CI≤0.3).

Similarly, combination of all inhibitors with each other in the apparently normal cell line (1542N) yielded effects that ranged from strong to very strong synergism (*CI*<0.1).

Radiomodulation by inhibitors

To investigate the relationship between the fraction of cells retaining their reproductive integrity and absorbed radiation doses, and the impact of cellular exposure of inhibitors of EGFR (AG-1478), PI3K/mTOR (NVP-BEZ235), and AR (MDV3100) on radiosensitivity, the colony forming assay was used. The clonogenic cell survival data for the cancer cell lines (DU145 and LNCaP) are presented in figure 4. Pre-treatment of the most radioresistant cell line (DU145; SF_2=0.53) with AG-1478, MDV3100, and a cocktail of both inhibitors radiosensitized the DU145 cells. However, the radiosensitization by AG-1478 emerged only at doses higher than 6 Gy (figure 4A), as reflected in the modifying factors not differing markedly from unity (table 4). Although the radiosensitization by NVP-BEZ235 and the cocktail was high, it did not reach statistical significance, except for PI3K/mTOR inhibition when the parameter D^- was used. This significant radiosensitization is in line with the strong synergism seen in this cell line between these inhibitors (table 3). Pre-treatment with NVP-BEZ235 and MDV3100, singly and in combination, resulted in radiosensitization over the entire radiation dose range, with modifying factors ranging from 1.20 to 4.33 (table 4). Only the radiosensitization by pre-treatment with NVP-BEZ235 (based on D^-) or pre-treatment with cocktail NVP-BEZ235 and MDV3100 (based on SF_6 and D^-) emerged statistically significant

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with modifying factors of 1.36 (P=0.0489), 4.33 (P=0.0339), and 1.37 (P=0.0422), respectively. This level of radiosensitization seems to be consistent with the strong synergism seen in this cocktail (table 3).

Table 3. Combination indices for EGFR inhibitor (AG-1478), PI3K and mTOR inhibitor (NVP-BEZ235), and AR inhibitor (MDV3100), when used concurrently at their respective EC50 concentrations in 2 human prostate cancer cell lines (DU145 and LNCaP), a benign prostatic hyperplasia cell line (BPH-1), and normal prostate cell line (1542N).

Cell line	Agent 1	Agent 2		
		AG-1478	NVP-BEZ235	MDV3100
DU145	AG-1478	-	0.0082	0.0784
	NVP-BEZ235	0.0082	-	0.0787
	MDV3100	0.0784	0.0787	-
LNCaP	AG-1478	-	1.1035	1.0830
	NVP-BEZ235	1.1035	-	0.0315
	MDV3100	1.0830	0.0315	-
BPH-1	AG-1478	-	0.0998	0.0741
	NVP-BEZ235	0.0998	-	0.1591
	MDV3100	0.0741	0.1591	-
1542N	AG-1478	-	0.0096	0.1095
	NVP-BEZ235	0.0096	-	0.1028
	MDV3100	0.1095	0.1028	-

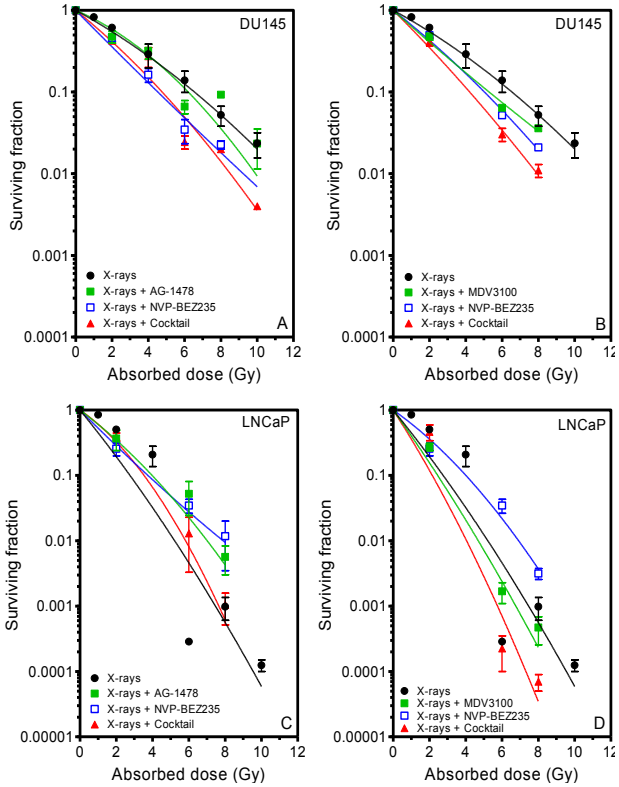


Figure 4. Cell survival curves for the **(A, B)** DU145 and **(C, D)** LNCaP cell lines after X-ray irradiation, following AG-1478, NVP -BEZ235, and MDV3100 treatment. Inhibitors were administered either singly or in combination at EC50 concentrations. Symbols represent the mean surviving fraction ± SEM from three independent experiments.

In general, pre-treatment of the androgen dependent and most radiosensitive cell line (LNCaP; SF_2=0.49) with AG-1478, NVP-BEZ235, or their cocktail either had no effect or tended to enhance cell survival (figure 4C), as can be deduced from the modifying factors presented in table 4. The absence of a significant radiosensitization by the inhibitor cocktail supports the finding that the mode of interaction between AG-1478 and NVP-BEZ235 in this cell line is antagonistic (table 3). However, exposure of the LNCaP cells with the androgen receptor inhibitor, MDV3100, either alone or in combination with NVP-BEZ235 resulted in enhanced radiosensitivity (Figure 4D). In terms of SF_2 and SF_6, the modifying factors were found to range between 2.72 and 21.30, with the cocktail tending to yield high radiosensitization (table 4). The extent of radiosensitization seen in the cocktail pre-treated cell cultures supports strong synergism between NVP-BEZ235 and MDV3100 (table 3). The modifying factors obtained from $\bar{D}$ were lower (table 4).

Table 4. Modifying factors (MF), relative to X-ray treatment alone, derived from SF2, SF6, and values for the DU145 and LNCaP cell lines, irradiated in the presence of AG-1478 (AG), NVP-BEZ235 (NVP), and MDV3100 (MDV), singly or in combination, at EC50 concentrations. Errors in modifying factors were calculated using error propagation formulae for ratios.

Treatment	DU145			LNCaP		
	Parameter	*P-value*	*MF*	Parameter	*P-value*	*MF*
	SF_2			SF_2		
X-ray	0.53 ± 0.10	-	-	0.49 ± 0.16	-	-
X-ray+AG	0.64 ± 0.19	0.6219	0.83 ± 0.29	0.36 ± 0.08	0.4159	1.36 ± 0.54
X-ray+NVP	0.37 ± 0.06	0.1500	1.43 ± 0.36	0.29 ± 0.07	0.2145	1.69 ± 0.69
X-ray+MDV	0.41 ± 0.02	0.1538	1.29 ± 0.25	0.18 ± 0.07	0.0785	2.72 ± 1.38
X-ray+AG+NVP	0.42 ± 0.08	0.3775	1.26 ± 0.34	0.36 ± 0.12	0.4660	1.36 ± 0.64
X-ray+NVP+MDV	0.38 ± 0.06	0.1711	1.39 ± 0.34	0.12 ± 0.01	0.0298 *	4.08 ± 1.37
	SF_6			SF_6		
X-ray	0.13 ± 0.04	-	-	0.02 ± 0.01	-	-
X-ray+AG	0.12 ± 0.02	0.8012	1.08 ± 0.38	0.03 ± 0.01	0.5343	0.67 ± 0.40
X-ray+NVP	0.05 ± 0.01	0.0724	2.60 ± 0.95	0.03 ± 0.01	0.4175	0.67 ± 0.40
X-ray+MDV	0.08 ± 0.01	0.1538	1.63 ± 0.54	0.0027 ± 0.0009	0.0506	7.89 ± 3.62
X-ray+AG+NVP	0.05 ± 0.02	0.1370	2.60 ± 1.31	0.01 ± 0.01	0.4331	2.00 ± 2.23
X-ray+NVP+MDV	0.03 ± 0.01	0.0339 *	4.33 ± 1.97	0.0010 ± 0.0001	0.1152	21.30 ± 7.03
	$\bar{D}$			$\bar{D}$		
X-ray	3.19 ± 0.38	-	-	2.73 ± 0.29	-	-
X-ray+AG	2.94 ± 0.32	0.5692	1.09 ± 0.18	2.26 ± 0.30	0.2703	1.21 ± 0.21
X-ray+NVP	2.34 ± 0.15	0.0489 *	1.36 ± 0.18	1.88 ± 0.21	0.0433 *	1.45 ± 0.22
X-ray+MDV	2.67 ± 0.13	0.1544	1.20 ± 0.15	1.83 ± 0.11	0.0168 *	1.49 ± 0.18
X-ray+AG+NVP	2.51 ± 0.27	0.1867	1.27 ± 0.20	2.21 ± 0.20	0.1396	1.24 ± 0.17
X-ray+NVP+MDV	2.33 ± 0.12	0.0422 *	1.37 ± 0.18	2.40 ± 0.37	0.4466	1.14 ± 0.21

*Statistically significant difference between X-ray treatment alone and irradiation in the presence of inhibitor.

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The radiation dose-response curves of the benign and apparently normal cell lines following inhibitor treatment are shown in figure 5. Overall, pre-treatment of the benign prostatic hyperplasia cell line (BPH-1) to NVP-BEZ235 or a cocktail of NVP-BEZ235 and AG-1478 did not result in a significant change in radiosensitivity (figure 5A; table 5). However, pre-treatment of these cells with AG-1478 alone appeared to lead to enhanced radioresistance, on the basis of the mean inactivation dose (table 5). The absence of a marked enhancement in radiosensitivity in the cocktail pre-treated cultures does not support the strong synergy seen between NVP-BEZ235 and AG-1478 (table 3). Similar results were obtained when the BPH-1 cells were pre-treated with MDV3100 or a cocktail of MDV3100 and NVP-BEZ235 (figure 5B), and do not reflect the synergy demonstrated between these inhibitors (table 3).

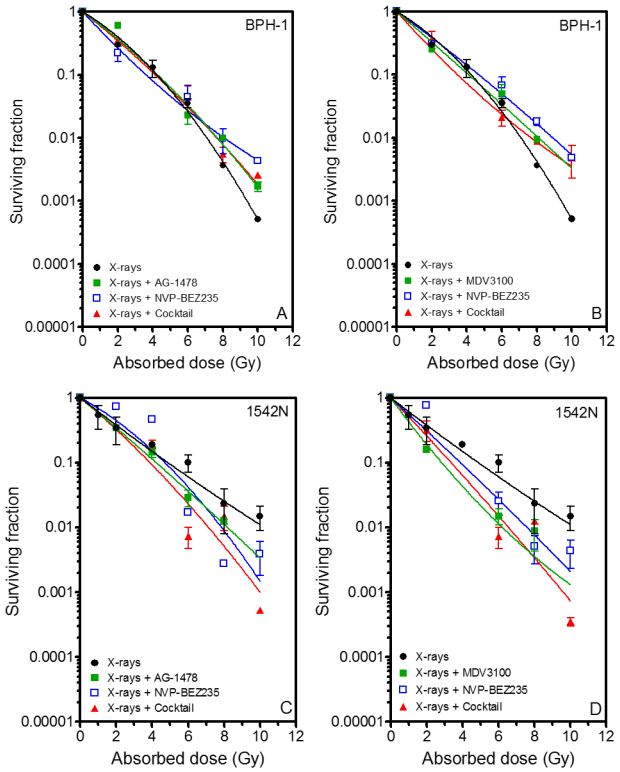


Figure 5. Cell survival curves for the **(A, B)** BPH-1 and **(C, D)** 1542N cell lines after X-ray irradiation, following AG-1478, NVP-BEZ235, and MDV3100 treatment. Inhibitors were administered either singly or in combination at EC50 concentrations. Symbols represent the mean surviving fraction ± SEM from three independent experiments.

Pre-treatment of the apparently normal prostate cell line (1542N) with the PI3K/mTOR and AR inhibitors, singly or in combination, only seemed to induce radiosensitization at radiation doses higher than 4 Gy (figure 5C). In fact, the PI3K/mTOR inhibitor (NVP-BEZ235) showed a radioprotective effect in these cells at lower doses. From the modifying factors presented in table 5, no significant effect was apparent for all treatment permutations. Again, the absence of a marked effect in the cocktail pre-treated cultures does not support the strong synergy shown between AG-1478 and NVP-BEZ235 (table 3). Similarly, pre-treatment of the 1542N cells with MDV3100 or a cocktail of MDV3100 and NVP-BEZ235 did not result in significant radiomodulatory effects (figure 5D; table 5). This finding is also not in line with the strong synergism shown between MDV3100 and NVP-BEZ235 (table 3).

Table 5. Modifying factors (MF), relative to X-ray treatment alone, derived from SF2, SF6, and values for the BPH-1 and 1542N cell lines, irradiated in the presence of AG-1478 (AG), NVP-BEZ235 (NVP), and MDV3100 (MDV), singly or in combination at EC50 concentrations. Errors in modifying factors were calculated using error propagation formulae for ratios.

Treatment		BPH-1			1542N	
	Parameter	*P-value*	*MF*	Parameter	*P-value*	*MF*
	SF_2			SF_2		
X-ray	0.40 ± 0.07	-	-	0.39 ± 0.10	-	-
X-ray+AG	0.39 ± 0.06	0.8196	1.03 ± 0.24	0.34 ± 0.02	0.5568	1.15 ± 0.30
X-ray+NVP	0.29 ± 0.10	0.3718	1.38 ± 0.53	0.45 ± 0.05	0.4857	0.87 ± 0.24
X-ray+MDV	0.34 ± 0.06	0.4570	1.18 ± 0.29	0.20 ± 0.04	0.0720	1.95 ± 0.63
X-ray+AG+NVP	0.40 ± 0.08	0.9944	1.00 ± 0.27	0.33 ± 0.06	0.5345	1.18 ± 0.37
X-ray+NVP+MDV	0.26 ± 0.03	0.0640	1.54 ± 0.32	0.22 ± 0.06	0.1355	1.77 ± 0.66
	SF_6			SF_6		
X-ray	0.03 ± 0.01	-	-	0.07 ± 0.04	-	-
X-ray+AG	0.04 ± 0.01	0.3869	0.75 ± 0.31	0.04 ± 0.01	0.3180	1.75 ± 1.09
X-ray+NVP	0.04 ± 0.02	0.6367	0.75 ± 0.45	0.04 ± 0.01	0.4242	1.75 ± 1.09
X-ray+MDV	0.04 ± 0.01	0.4338	0.75 ± 0.31	0.01 ± 0.01	0.1274	7.00 ± 8.06
X-ray+AG+NVP	0.04 ± 0.02	0.4640	0.75 ± 0.45	0.03 ± 0.01	0.2014	2.33 ± 1.54
X-ray+NVP+MDV	0.02 ± 0.01	0.5175	1.50 ± 0.90	0.02 ± 0.01	0.1303	3.50 ± 2.66
	D^-			D^-		
X-ray	1.94 ± 0.14	-	-	2.09 ± 0.44	-	-
X-ray+AG	2.91 ± 0.06	0.0011 *	0.67 ± 0.05	1.59 ± 0.37	0.4769	1.32 ± 0.46
X-ray+NVP	1.84 ± 0.28	0.7556	1.05 ± 0.18	3.46 ± 0.17	0.0694	0.60 ± 0.13
X-ray+MDV	1.94 ± 0.13	0.9848	1.00 ± 0.10	1.53 ± 0.04	0.3414	1.37 ± 0.29
X-ray+AG+NVP	2.25 ± 0.15	0.1533	0.86 ± 0.08	2.12 ± 0.11	0.9631	0.99 ± 0.21
X-ray+NVP+MDV	2.19 ± 0.31	0.4913	0.89 ± 0.14	2.01 ± 0.33	0.9043	1.04 ± 0.28

*Statistically significant difference between X-ray treatment alone and irradiation in the presence of inhibitor.

DISCUSSION

Prostate cancer presents itself as a heterogeneous disease [19], meaning the same tumour expresses

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different genotypes, giving clinicians a headache in managing the disease, because treatment is not a "one size fits all" approach. Conventional therapy still plays a pivotal role in the management of PCa, however, there is room for improvement as quality of life and survival rate of patients is compromised. Targeted therapy has been investigated in the last 3 decades and promising outcomes have been brought forward [6, 20]. Interestingly, the biological processes leading to the development of PCa might provide the answers in offering tailored medicine. For the purpose of this study, the androgen sensitivity of 4 prostate cell lines was investigated (DU145, LNCaP, BPH-1, 1542N). As mentioned, PCa is a disease of hormones and its progression is driven largely by hormones, and so the majority of prostate cancers are androgen dependent [21]. Consequently, PCa patients benefit from androgen deprivation therapy by surgical or chemical castration, and combination therapy, too, has yielded results in a number of clinical trials [22].

The challenge, then, is the androgen independent stage, where prostate tumour growth and survival does not depend on androgens only, but on other avenues, for example, the PI3K and EGFR pathways. The molecular basis for the switch from androgen dependence to androgen independence in prostate cancer is largely unknown and has been under investigation for decades [23-25]. However, it has been speculated that this may be attributed to a feedback loop, also known also as "crosstalk", between survival pathways [9]. For example, inhibition of AR might activate pro-survival pathways involving PI3K/mTOR or EGFR, increase in proliferation and survival, and result in castration-resistant prostate cancer (CRPC) and hormone-refractory prostate cancer (HRPC) [26].

The androgen sensitivity assay shows that addition of 5a-dihydrotestosterone (5a-DHT), at concentrations of 0.001 to 100 nM, to cell culture medium has no significant effect on BPH-1 cell growth (Figure 1). However, when the LNCaP cells were cultured in charcoal-stripped medium, spiked with 5a-DHT over the same concentration range, proliferation was enhanced and fully restored at 10 nM of 5a-DHT. These findings are consistent with those of Serafin *et al*. demonstrating that BPH-1 and LNCaP cell lines are androgen independent and dependent, respectively [23]. Contrary to the observation that the DU145 and 1542N cell lines are androgen independent [23, 27], this study shows that 5a-DHT (at 0.001 - 1.0 nM) appeared to inhibit cell proliferation in these cell lines. Interestingly, at higher 5a-DHT concentrations (10 - 100 nM), proliferation in the DU145 and 1542N cells recovered to normal levels. These results seem to suggest that the DU145 and 1542N cell line are not androgen independent as initially thought [23, 27]. In other studies, the PC-3 and DU145 cell lines were seen to not express an androgen receptor and were, therefore, not dependent on androgens for growth [28]. The reason for this disparity is not known, but the current finding that cell proliferation recovered in the DU145 and 1542N cell lines at 5a-DHT concentrations of 10 - 100 nM, thus linking them to androgen dependence, seems to suggest that androgen may have a dual effect on these cells. Does this perhaps suggest that these cell lines have switched from an androgen independent status to an androgen dependent status? This result could be beneficial, especially for androgen independent cancers, which are resistant to treatment, and it would open the door to available treatment options. Chiefly, ADT, which is the standard treatment protocol for androgen dependent cancers. As for the LNCaP cell line, defined as androgen dependent, these results concur with findings of other studies [27, 29]. The LNCaP cell line is believed to have a point mutation in the ligand-binding domain of the receptor gene, and, hence, is responsive to androgens [30].

The cytotoxic effects of AG-1478 (EGFR inhibitor), NVP-BEZ235 (PI3K and mTOR inhibitor), and MDV3100 (AR inhibitor) are concentration-dependent (figure 2). For AG-1478, EC_{50} values ranged from 290 - 7431 nM (figure 2 & table 1), which clearly shows that the normal cells (1542N) are more sensitive to EGFR inhibition than their tumour counterparts and the use of AG-1478 for treatment of prostate cancer might lead to undesirable outcomes. Inhibiting EGFR in the androgen-dependent LNCaP cells resulted in a small therapeutic benefit, with a relative sensitivity of 1.52 [5]. The significant level of resistance to EGFR inhibition seen in the DU145 and BPH-1 cell lines (relative sensitivities of 0.06 and 0.59, respectively), relative to the LNCaP cell line, is likely due to the fact that EGFR expression in the former is more than 5-fold times that in the latter [31]. Higher EGFR expression levels would require significantly larger concentrations of inhibitor to achieve a given proportion of cell killing. On the other hand, EGFR expression in the androgen-dependent LNCaP cells is low and comparable to that in the normal 1542N cells [32], consistent with the observed relative sensitivity of 1.43±0.22 [5].

For NVP-BEZ235 treatment, EC_{50} values ranged from 6.26 – 51.33 nM for all cell lines (figure 2 & table 1), and are consistent with those reported for human breast cell lines [8]. Here, the normal cell line (1542N) is clearly the most resistant to PI3K and mTOR inhibition, making the tumor cell lines 3 to 8 times more sensitive [5]. This resistance can be attributed to NVP-BEZ235 being specifically more toxic to malignant cells, as reported elsewhere [33]. NVP-BEZ235 also has radioprotective characteristics when combined with radiation therapy, as reported previously [2]. The sensitivity ranking of the malignant cell lines (DU145 and LNCaP) may be related to the extent to which NVP-BEZ235 inhibits

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the activity of key components of the PI3K/mTOR pathway, such as, PDK1^{Ser241}, Akt^{Thr308}, Akt^{Ser473}, GSK3b^{Ser19}, Foxola^{Ser256}, S6K$^{Ser235/236}$, 4EBP1$^{Thr27/66}$, and MDM2^{Ser166}. On average, inhibition of activity of these components by a dual PI3K/mTOR inhibitor (XL765) has been shown to be about 2-fold more effective in the LNCaP cell line than the DU145 cell line [34]. The clonogenic cell survival data presented here are consistent with this, with the LNCaP cells being 2.6-fold more sensitive than the DU145 cells [5]. However, the similarity in NVP-BEZ235 cytotoxicity in LNCaP and BPH-1 cells (EC_{50} of 6.42±0.74 and 6.26±0.59 nM, respectively) cannot be corroborated by the finding that NVP-BEZ235 is about 10-fold less effective in inhibiting cell proliferation than the latter cell line [34]. This disparity is likely due to differences in experimental design and endpoints. While the clonogenic cell survival assay described here takes about 2 weeks and reflects delayed effects of PI3K and mTOR inhibition, the cell growth assay of Gravina and colleagues lasts only 24 hours and could miss such effects [34].

For MDV3100 treatment, EC_{50} values ranged from 15.74 - 92.73 nM for all cell lines (figure 2 & table 1). Here, the androgen dependent cell line (LNCaP) seems to be the most significantly resistant to MDV3100, with a low relative sensitivity of 0.18. Similar resistance to MDV3100 treatment was also seen in CRPC, and LNCaP and C4-2 cells [35]. Interestingly, the traditional androgen-independent cell lines (DU145 and BPH-1) showed higher sensitivity to MDV3100 treatment. The rank order of increasing sensitivity to MDV3100 treatment is: LNCaP ® 1542N ≈ BPH-1 ≈ DU145, with EC_{50}-values of 92.73, 16.92, 16.64, and 15.74 nM, respectively (Table 1). The significant level of resistance to MDV3100 inhibition seen in the LNCaP cell line relative to the 1542N, BPH-1 and DU145 cell lines, is likely due to the fact that androgen receptor (AR) expression in the LNCaP is higher than in androgen independent cell lines [35]. Higher AR expression levels would require significantly larger concentrations of inhibitor to effectively block receptor activity and result in a given proportion of cell killing.

Systemic toxicity experienced by patients that undergo chemotherapy for prostate cancer is of significant concern in the clinics. Although chemotherapy drugs like enzalutamide, abiraterone, prednisone, and docetaxel have yielded great results in improving prostate cancer patient survival, systemic toxicity remains a key challenge [36]. Therefore, therapeutic approaches combining a number of agents to achieve optimum therapeutic benefit with minimal toxicity have been proposed [37, 38]. Furthermore, it was demonstrated that a cocktail of PI3K/mTOR and human epidermal growth factor receptor 2 (HER-2) inhibitors radiosensitize breast cancer cells which have been shown to be resistant to trastuzumab [39]. Subsequently, it was demonstrated that a strong synergism exists between small molecule inhibitors of HER-2, PI3K, mTOR and Bcl-2 in human breast cancer cells [4]. In CRPC, a combination of an AKT inhibitor (AZD6363) with an anti-androgen (bicalutamide) resulted in synergistic inhibition of cell proliferation and induction of apoptosis [40]. Antibody-drug conjugates (ADC) and potent anti-androgens (enzalutamide and abiraterone) also yielded synergistic effects when anti-proliferative activity was evaluated in LNCaP and C4-2 cell lines [41].

In preparing drug cocktails for combination therapy, it is highly desirable to achieve as low as possible drug doses within the cocktails. An ideal therapeutic cocktail should have the following attributes: (1) constituent drugs should not antagonize each other; (2) adequate synergy should exist between constituent drugs; (3) the safety profiles of constituent drugs should not overlap, and (4) the therapeutic effectiveness of any constituent drug should not worsen adverse event signatures of the others. In this study, the modes of interaction of the dual inhibitor of PI3K and mTOR (NVP-BEZ235), EGFR inhibitor (AG-1478), and AR inhibitor (MDV3100) in *in vitro* cultures of four human prostate cell lines (DU145, LNCaP, BPH-1 and 1542N) were evaluated, as described elsewhere [4, 18]. For this, the following cocktails were used: Cocktail 1 (AG -1478 and NVP-BEZ235), Cocktail 2 (NVP-BEZ235 and MDV3100), and Cocktail 3 (MDV3100 and AG-1478). The combination indices (CI) for each cocktail in the DU145, BPH-1 and 1542N cell lines ranged between 0.0082 and 0.1591 (table 2), indicating strong to very strong synergism for each inhibitor combination.

Dual inhibition of the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MEK) and PI3K/mTOR pathways in DU145, LNCaP and PC-3 cell lines with AZD6244 and GSK2126458, respectively, also showed synergistic effects [42]. Synergistic effects have also been demonstrated for combination therapy castration-resistant prostate cancer with docetaxel and thymoquinone [43]. The very strong synergism observed for Cocktail 1 in DU145, BPH-1 and 1542N cells, Cocktail 2 in DU145 and LNCaP cells, and Cocktail 3 in DU145 and BPH-1 cells is consistent with that observed in human breast cell lines [4]. Clinically, synergisms have also been demonstrated between AZD6363, bicalutamide, ADC, enzalutamide and abiraterone in prostate cancer [40, 41].

Radiosensitization of prostate cancer cells, specifically LNCaP, DU145, and BPH-1 to radiation may be achieved by targeting pathways that are implicated in radioresistance, such as the PI3K/ mTOR, EGFR, and AR pathways [44]. This approach could lead to the enhancement of the efficacy of radiotherapy in CRPC. Many pre-clinical studies and

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clinical trials have tested this hypothesis, and clinically beneficial outcomes in numerous cancers have been documented [45]. The benefit of targeting these pathways in an effort to develop novel treatment approaches for breast and prostate cancer has been extensively demonstrated [2, 4, 5, 39, 46-48]. Research in breast, head and neck, and lung cancer, involving clinical trials which explore targeted and combination therapies, has yielded a wealth of knowledge. Examples of these research efforts are the following: (1) the targeting of EGFR overexpression in these cancers with receptor-blocking monoclonal antibodies (such as, cetuximab), or small molecule EGFR inhibitors (such as, gefitinib) [36]; (2) the inhibition of the PI3K/mTOR pathway with LY294002, NVP-BEZ235 and NVP-BGT226 [11]; and (3) the inhibition of the androgen receptor pathway in triple negative breast cancer with MDV3100 [49]. These studies have demonstrated that such pathway inhibition sensitizes cancer cells to the cytotoxic effect of ionizing radiation. However, the extent of similar studies in prostate cancer, especially CRPC, is minimal or non-existent.

In this study, the ranking of the cell lines in order of increasing radiosensitivity is: DU145 ® BPH-1 ® 1542N ® LNCaP (SF_2; tables 4 & 5), with only the androgen-dependent cell line being relatively more radiosensitive than the normal cell line. This would imply minimal to no therapeutic benefit of conventional radiotherapy for a majority of prostate cancers. On average, pre-treatment of tumor cell lines with the EGFR inhibitor (AG-1478) resulted in minimal or no radiosensitization. However, pre-treatment of the cells with the PI3K/mTOR inhibitor (NVP-BEZ235), showed desirable results, especially at low therapeutic doses (e.g. 2 Gy), where radioprotection was seen in 1542N cells, while the tumor cells were radiosensitized by 38 - 69% (tables 4 & 5). A similar radioprotection was previously demonstrated in this cell line and normal gut tissue [2, 50]. The present data suggest that combining X-rays with AG-1478 or NVP-BEZ235 at high radiation doses may not be appropriate, as the normal cell line shows high levels of radiosensitization compared to the rest of the cell lines. For the tumor cell lines, the results from a combination of radiation and a cocktail of AG-1478 or NVP-BEZ235 do not seem to differ greatly from those obtained from single inhibitor treatment prior to irradiation. This suggests that dual targeting of the EGFR and PI3K/mTOR pathways may not have a therapeutic advantage over single pathway targeting.

To explore the role of the androgen receptor (AR), which has been thought to be the core driving force for prostate cancer progression, the radiomodulatory effect of the AR inhibitor (MDV3100) was also evaluated in conjunction with PI3K/mTOR inhibitor (NVP-BEZ235). In the androgen-dependent cell line LNCaP, MDV3100 showed the greatest radiosensitization of almost 3-fold at low radiation doses and ~8-fold at high doses (table 4). This may be attributed to the high levels of AR expression in these cells [29]. These results are similar to those of the apparently normal cell line (1542N), which was radiosensitized up to 2- and 7-fold at low and high radiation doses, respectively. By definition, the 1542N cell line is androgen-independent. However, the current findings demonstrate that as the concentration of 5α-DHT increases in 1542N cell cultures, so do the cells shift from an androgen-independent state to being androgen-dependent (figure 1). In the traditionally androgen-independent cell lines (DU145 and BPH-1), the radiosensitization by MDV3100 pre-treatment was minimal. This can be expected as these cell lines have been shown to express low levels of the androgen receptor [29]. However, a switch from androgen-independent to androgen-dependent was apparent in the DU145 cell line (figure 1), making the role of the AR pathway in radiation resistance unclear.

For cancers that behave like LNCaP, a cocktail of MDV3100 and NVP-BEZ235 could be an effective adjuvant to radiotherapy, as these cells were radiosensitized by cocktail pre-treatment up to 4- and 21-fold at low and high doses, respectively (table 4). A potential benefit is also apparent in treating tumours that may be MDV3100-resistant, as seen in the DU145 cell line, with MDV3100/NVP-BEZ235 cocktail prior to high doses of radiation. MDV3100-resistant prostate cancers constitute a significant clinical challenge [35].

CONCLUSION

These data demonstrate that increasing the levels of 5α-DHT might redefine the androgen sensitivity of human prostate cell lines, as 5α-DHT was seen to both inhibit and promote cell proliferation in the DU145 and 1542N cell lines. The reason for this disparity is currently not known. However, this observation might be useful in designing therapeutic interventions for both androgen-dependent and independent cancers. As it has been documented that ADT is adversely affected by tumour independence on androgen, switching cells from androgen-independent to androgen-dependent might be of therapeutic benefit when administrating ADT. Combined targeting of the PI3K/mTOR and AR pathways could potentially be an effective therapeutic strategy for androgen-dependent (whether intrinsic or switched on at high androgen levels) cancers. The potential benefit of EGFR targeting is limited, with the four cell lines used in this study. It is also important to note that use of a cocktail of AG-1478 and NVP-BEZ235, or single inhibitors at high radiation doses may result in high levels of radiosensitization of normal tissue, which is

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undesirable. But of interest, the cocktails of NVP-BEZ235 and MDV3100 which resulted in strong to very strong synergism when inhibitor interaction was tested in the cell lines has potential therapeutic advantage, with the exception of the LNCaP cell line which demonstrated antagonistic effects. Furthermore, significant radiosensitization by inhibitors was demonstrated. These findings might assist in the design of more effective treatment approaches for cancers that typically display resistance to radiotherapy and chemotherapy, as well as better inform clinicians on the effective utilization of combination therapy.

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Ethical consideration**:** Only established cell cultures were used in this study. No animals or human participants were involved.

Author contributions**:** S.M. designed and performed experiments, collected data, and prepared the original draft of the article; A.M.S. assisted with design and performance of experiments, data collection, and revision of article; J.M.A. conceptualized study, analysed data, and reviewed the article. All authors read and approved the final version of the article.

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