

# PI3K and mTOR inhibitor, NVP-BEZ235, is more toxic than X-rays in prostate cancer cells

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## ABSTRACT

**Background:** Radiotherapy and adjuvant androgen deprivation therapy have historically been the first treatment choices for prostate cancer but treatment resistance often limits the capacity to effectively manage the disease. Therefore, alternative therapeutic approaches are needed. Here, the efficacies of radiotherapy and targeting the pro-survival cell signaling components epidermal growth factor receptor (EGFR), phosphoinositide 3-kinase (PI3K), and mammalian target of rapamycin (mTOR), with their respective inhibitors are compared. **Materials and Methods:** The cytotoxic effects of inhibitors of PI3K and mTOR (NVP-BEZ235) and EGFR (AG-1478), and X-rays, were evaluated in prostate cell lines (LNCaP: cancer; DU145: cancer; BPH-1: benign prostatic hyperplasia; 1542N: apparently “normal”) using a colony forming assay. The cells were exposed to a range of X-ray doses or varying concentrations of the inhibitors, to obtain cell survival curves from which relative sensitivities (RS) of the tumor cell lines were derived as the ratio of their sensitivities to that of the “normal” cell line. **Results:** The LNCaP cells trended to be more sensitive to X-rays and AG-1478 exposure than 1542N cells, with RS-values of  $1.65 \pm 0.48$  ( $P=0.1644$ ) and  $1.37 \pm 0.22$  ( $P=0.0822$ ), respectively. NVP-BEZ235 emerged as very cytotoxic in all tumor cell lines, yielding RS-values of  $3.69 \pm 0.83$  (DU145;  $P=0.0025$ ),  $8.80 \pm 1.73$  (LNCaP;  $P<0.0001$ ), and  $8.76 \pm 1.70$  (BPH-1;  $P=0.0011$ ). **Conclusion:** These findings demonstrated that targeted therapy, specifically that using NVP-BEZ235, might result in a more effective treatment modality for prostate cancer than conventional radiotherapy.

**Keywords:** Radiotherapy, targeted therapy, prostate cancer, PI3K, mTOR, EGFR.

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## INTRODUCTION

In addition to androgen therapy, radiotherapy is a first line option for the treatment of prostate cancer. Radiotherapy is a highly effective treatment option for localized prostate cancer with manageable treatment-related side effects<sup>(1,2)</sup>. A major challenge in radiotherapy is normal tissue toxicity and patients failing to achieve long-term tumor control. On the other hand, androgen therapy does not benefit patients with androgen-independent cancers as these tumors do not respond to treatment<sup>(3)</sup>. The prognosis for localized and regional prostate disease is good. However, with almost one million new cases of

prostate cancer (PCa) and over a quarter of a million prostate cancer-related deaths recorded per year worldwide, PCa remains the second most common cancer in men, with increasing incidences and mortality rates globally, and also in sub-Saharan Africa<sup>(4,5)</sup>. With the abovementioned problems, new treatment strategies are needed to address these therapeutic dilemmas.

Targeted therapies have emerged as alternative treatment modalities to overcome the issue pertaining to treatment resistance and normal tissue toxicity<sup>(6,7,8,9)</sup>. The use of different omics techniques has led to progress in the molecular classification of both early and late stage prostate cancer which may manifest itself

in targeted, personalized therapies. NVP-BEZ235, a dual inhibitor of PI3K and mTOR, is cytotoxic and yet has the potential to protect normal tissue<sup>(10-12)</sup> and sensitize cancer cells to radiotherapy<sup>(10,11,13,14,15,16,17)</sup>.

PI3K pathway-mediated cross-talk between the androgen receptor (AR), which plays a pivotal role in prostate malignancy, and EGFR, has been demonstrated, and underpinned by preclinical models<sup>(18-20)</sup>. This cross-talk may present itself as an important mechanism during PCa progression, giving cells a survival advantage, and might serve as a potential target for cancer therapy<sup>(21)</sup>.

In search of alternatives to radiotherapy, this study compares the efficacies of radiotherapy and targeting PI3K, mTOR, and EGFR with specific inhibitors NVP-BEZ235 and AG-1478 using four human prostate cell lines (DU145, LNCaP, BPH-1 and 1542N). The potential therapeutic benefit of each agent (radiation or inhibitor) is discussed.

## MATERIALS AND METHODS

### Cell lines and culture maintenance

The apparently “normal” 1542N cell line (passage number: 18-25) was derived from the normal prostate epithelial tissue of a patient with primary adenocarcinoma of the prostate, and immortalised with the E6 and E7 genes of the human papilloma virus 16<sup>(22)</sup>. The cells were a gift from Prof JRW Masters (Prostate Cancer Research Centre, University College London, UK). The epithelial cell line, BPH-1 (benign prostatic hyperplasia-1) (passage number: 2-7), was established from human prostate tissue obtained by transurethral resection. Primary cell cultures were immortalised with simian virus 40 (SV40) large T-antigen<sup>(23)</sup>. The cells were obtained from Professor SW Hayward (Department of Urology, University of California, USA). Although the link between BPH and PCa remains largely controversial, there is ample evidence to suggest that the former is a precursor of the latter<sup>(24,25,26,27)</sup>. Therefore, the BPH-1 cell line is considered as “malignant” in

the current study. The LNCaP cell line (passage number: 4-9) was established from a supraclavicular lymph node metastasis of human prostatic adenocarcinoma<sup>(28)</sup>, and was obtained from Professor Helmut Klocker (Department of Urology, University of Innsbruck, Austria). These cell lines were grown in Roswell Park Memorial Institute medium, RPMI-1640 (Sigma-Aldrich, USA; cat #: R8758) supplemented with 10% (5% for LNCaP) heat-inactivated foetal bovine serum (FBS) (HyClone, UK; cat #: SV30160.30IH), penicillin (100 U/ml) and streptomycin (100 mg/ml) (Lonza, Belgium; cat #: DE17-602E). The malignant DU145 (passage number: 7-18) cells were derived from a metastatic lesion of the central nervous system<sup>(29)</sup>, and were 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; cat #: SV30160.30IH), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Lonza, Belgium; cat #: DE17-602E). All cell cultures were grown as monolayers in 75-cm<sup>2</sup> flasks (Greiner Bio-One, Germany; cat #: 658170) and were maintained by incubation at 37°C in a humidified (relative humidity: 84%) atmosphere (95% air and 5% CO<sub>2</sub>). Cell cultures were used for experiments upon reaching 70-90% confluence.

### Inhibitors

NVP-BEZ235 (C<sub>30</sub>H<sub>23</sub>N<sub>5</sub>O; M<sub>w</sub> = 469.55; Santa Cruz Biotechnology, TX, USA, cat # 364429) is a dual inhibitor of phosphoinositide-3-kinase (PI3K) and mammalian target of rapamycin (mTOR), with an inhibitory concentration at 50% (IC<sub>50</sub>) of ~5 nM for PI3K and 6 nM for mTOR, and shown to have IC<sub>50</sub>-values ranging from ~12 to 17 nM for inhibiting *in vitro* proliferation and survival of prostate cancer cell lines, PC3M, PC3, and DU145<sup>(11,30)</sup>. AG-1478 (C<sub>16</sub>H<sub>14</sub>ClN<sub>3</sub>O<sub>2</sub>.HCl; M<sub>w</sub> = 352.22; Tocris Bioscience, UK, cat # 1276) is a specific inhibitor of EGFR with an IC<sub>50</sub> of 3 nM, and has been shown to have an IC<sub>50</sub> of 1 µM for inhibiting *in*

*in vitro* proliferation of a non-small cell lung cancer cell line, NCI-H2170<sup>(31)</sup>. Stock solutions of NVP-BEZ235 (106 mM) and AG-1478 (10 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C until used.

### Cell culture irradiation and inhibitor treatment

Monolayer cell cultures of DU145, 1542N, LNCaP, and BPH-1 in exponential growth were trypsinized to give single-cell suspensions and were plated for X-ray exposure (300-100000 cells per flask, adjusted for irradiation dose), and for inhibitor treatment (1000-4000 cells per flask, adjusted for inhibitor concentration) into 25-cm<sup>2</sup> culture flasks (Nest Biotechnology, China; cat #: 707001). The cell cultures in 10 ml of growth medium were incubated for 4-5 h (4-7 h for LNCaP) to allow the cells to attach. The LNCaP cell line has altered adhesion properties (low anchoring potential) which explains why the cells are left to settle for a longer period of time<sup>(28)</sup>. The attached cells were then irradiated with X-rays or treated with inhibitors of PI3K and mTOR (NVP-BEZ235) and EGFR (AG-1478), respectively. For X-ray exposure, cell cultures were irradiated at room temperature (22°C) to 0-10 Gy using a Faxitron MultiRad 160 X-irradiator (Faxitron Bioptics, Tucson, AZ) at a dose rate of 1 Gy/min. Cell cultures were treated with inhibitors, without media replacement. Cells were exposed to NVP-BEZ235 (0.001-1,000 nM) and AG-1478 (1-100,000 nM) after appropriate dilution of stock solutions in cell culture medium.

### Radiosensitivity and inhibitor toxicity measurement

The colony assay was used to measure intrinsic cellular radiation response, and cytotoxicity of inhibitors. Briefly, irradiated and inhibitor-treated cells were incubated for 10 days (BPH-1 and DU145) and 14 days (LNCaP and 1542N) to form colonies. To test for possible inhibitor solvent toxicity, two sets of control (untreated) cultures were prepared for each experiment. One set was exposed to DMSO at a final concentration corresponding to that of the highest inhibitor concentration, and the

resulting plating efficiencies of the control culture sets compared. The experiments were stopped by decanting the growth medium, washing with phosphate buffered saline, and fixing with glacial acetic acid:methanol:water (1:1:8, v/v/v). The colonies were stained with 0.01% amido black in fixative, washed in tap water, air-dried, and counted using a stereoscopic microscope (Nikon, Japan; Model #: SMZ-1B). Three independent experiments were performed for each radiation dose and inhibitor concentration, and the mean surviving fractions were calculated. Surviving fractions (*SF*) were calculated according to the formula,  $SF = n_{col}(t) / \{ [n_{col}(u) / n_{cell}(u)] \times n_{cell}(t) \}$ , where  $n_{cell}(t)$  and  $n_{cell}(u)$  represent the number of cells plated in treated (irradiated or inhibitor treated) and untreated (control) cultures, respectively.  $n_{col}(t)$  and  $n_{col}(u)$  are the corresponding number of colonies counted. No inhibitor solvent related toxicity was observed in control (untreated) cultures containing the highest concentration of DMSO (0.000066% for NVP-BEZ235 at 1:10000 dilution of stock; and 0.1% for AG-1478 taken directly from stock).

Cell survival data for X-ray exposure were fitted to the linear-quadratic (LQ) model to generate survival curves (equation (1)), and cellular radiosensitivity, expressed in terms of the absorbed radiation dose at which 50% cell killing occurred ( $D_{50}$ ), was determined.

$$S = \exp [ -\alpha D - \beta D^2 ] \quad (1)$$

where *S* is the surviving fraction,  $\alpha$  and  $\beta$  are the linear and quadratic coefficients, respectively, and *D* is the absorbed dose in Gy.

To determine the equivalent concentration of each inhibitor for 50% cell killing ( $EC_{50}$ ), the surviving fractions were plotted as a function of log (inhibitor concentration) and were fitted to a 4-parameter logistic equation describing a sigmoidal curve (equation (2))<sup>(16,33,34)</sup>.

$$SF = B + \frac{T-B}{\{1-10^{[(\log EC_{50}-D)HS]}\}} \quad (2)$$

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.

To determine whether a treatment agent (X-rays or inhibitor) had a potential therapeutic benefit, a relative sensitivity (*RS*) was derived by comparing the  $D_{50}$  and  $EC_{50}$  of the “normal” prostate cell line, (1542N), with those of the tumor cell lines (DU145, LNCaP, BPH-1) as follows:

$$RS = \frac{D_{50}(\text{“normal”})}{D_{50}(\text{tumor})} \text{ or } \frac{EC_{50}(\text{“normal”})}{EC_{50}(\text{tumor})} \quad (3)$$

The criteria for no potential benefit with possible undesirable effects, no potential benefit, and potential therapeutic benefit of each agent are  $RS < 1.0$ ,  $RS = 1.0$  and  $RS > 1.0$ , respectively.

### Data analysis

Statistical analyses were performed using the GraphPad Prism (GraphPad Software, San Diego, CA, USA) computer program. The relationship between cell survival and X-ray dose was described using a linear-quadratic equation. A 4-parameter logistic equation describing a sigmoidal curve was used to describe the relationship between inhibitor cytotoxicity (cell survival) data and inhibitor concentration. Standard equations were used to fit nonlinear relationships. All data presented in figures and used in curve fitting were calculated as the means ( $\pm$  SEM) 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,  $R^2$ , were calculated from two-sided tests. A *P*-value of  $< 0.05$  indicated 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 radiation dose at which a cell survival of 50% (50% cell killing) was obtained ( $D_{50}$ ), and was presented as the mean ( $\pm$ SEM). The androgen-dependent cell line, LNCaP, emerged as more radiosensitive than its androgen-independent counterparts (1542N, BPH-1, DU145). The rank order of radioresistance in the cell lines was found to be LNCaP < 1542N < BPH-1 < DU145, with  $D_{50}$ -values of  $0.93 \pm 0.19$ ,  $1.53 \pm 0.32$ ,  $1.65 \pm 0.36$ , and  $2.25 \pm 0.54$  Gy, respectively. No statistically significant differences emerged between the radiosensitivity of the “normal” cell line when compared with those of the tumor cell lines as presented in table 1 ( $0.1644 \leq P \leq 0.7797$ ). This translated to relative sensitivities (*RS*) that do not differ significantly from unity. The corresponding *RS*-values for the DU145, LNCaP, and BPH-1 cell lines were  $0.68 \pm 0.21$ ,  $1.65 \pm 0.48$ , and  $0.93 \pm 0.28$ , respectively (table 1).

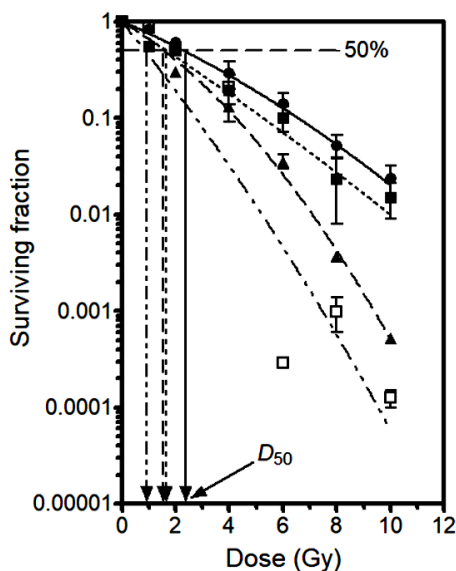
### Cytotoxicity of EGFR inhibitor (AG-1478)

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 ( $\pm$ SEM). Treatment with AG-1478 yielded the same sensitivity ranking, as observed for X-ray exposure (figure 2), with the LNCaP showing more sensitivity than the other cell lines. The  $EC_{50}$  of the “normal” cell line (1542N) emerged as  $400 \pm 38$  nM and was significantly lower than those of the DU145 ( $6613 \pm 1510$  nM,  $P = 0.0147$ ,  $R^2 = 0.8088$ ) and BPH-1 ( $677 \pm 41$  nM,  $P = 0.0079$ ,  $R^2 = 0.8588$ ) cell lines. The  $EC_{50}$  of the relatively more sensitive LNCaP cell line did not differ significantly from that of the 1542N cell line ( $302 \pm 19$  nM,  $P = 0.0822$ ,  $R^2 = 0.5712$ ). This resulted in very low relative sensitivities of  $0.06 \pm 0.02$  and  $0.59 \pm 0.07$  for the DU145 and BPH-1 cell lines (table 2), as determined from equation (3), respectively. The relative sensitivity of the LNCaP cell line was  $1.33 \pm 0.15$ .

**Cytotoxicity of PI3K and mTOR inhibitor (NVP-BEZ235)**

Inhibition of PI3K and mTOR inhibitor with NVP-BEZ235 also resulted in a concentration-dependent cell killing, as shown in figure 3. The rank order of cytotoxicity following NVP-BEZ235 treatment is LNCaP≈BPH-1<DU145<1542N, with  $EC_{50}$ -values of  $6.10 \pm 0.40$ ,  $6.11 \pm 0.64$ ,  $16.25 \pm 4.72$ , and  $53.82 \pm 2.95$

nM, respectively. All tumor cell lines were significantly more sensitive to NVP-BEZ235 treatment than the “normal” cell line as shown in table 2 ( $P \leq 0.0025$ ). The tumor cell lines, DU145, LNCaP, and BPH-1, were found to be 3- to 8-fold more sensitive than the “normal” cell line (1542N) with relative sensitivities of  $3.31 \pm 0.98$ ,  $8.82 \pm 0.75$ , and  $8.81 \pm 1.04$ , respectively (equation (3), table 2).



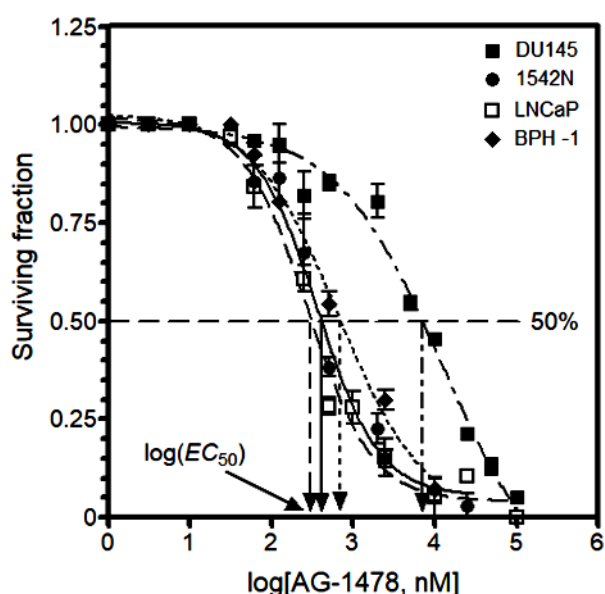
**Figure 1.** Clonogenic cell survival curves for 4 human prostate cell lines [DU145 (●), LNCaP (□), BPH-1 (▲), 1542N (■)] after X-ray irradiation. Survival curves were obtained by fitting experimental data to the linear-quadratic model. Symbols represent the mean surviving fraction ± SEM from 3 independent experiments. Standard errors are not transformed into a logarithmic scale. The dose at which 50% of cells survive ( $D_{50}$ ) is the dose at which each survival curve intersects the horizontal dashed line.

**Table 1.** Summary of cytotoxicity data for 4 human prostate cell lines (“normal”: 1542N; cancer: DU145, LNCaP; benign prostate hyperplasia: BPH-1) and relative radiosensitivity determined by clonogenic cell survival after exposure to X-rays.  $D_{50}$  denotes the absorbed radiation dose required to yield a 50% cell killing (figure 1). The 95% confidence intervals of the  $D_{50}$ -values are in parentheses.  $P$ -value indicates the level of significance in the difference between the  $D_{50}$  of the “normal” cell line (1542N) relative to those of the tumor cell lines (DU145, LNCaP, BPH-1). Relative sensitivity ( $RS$ ) is the ratio of the  $D_{50}$  of the “normal” cell line to those of the tumor cell lines.  $\alpha$  and  $\beta$  are the linear and quadratic coefficients of the respective cell survival curves obtained from the LQ-model (equation (1)).

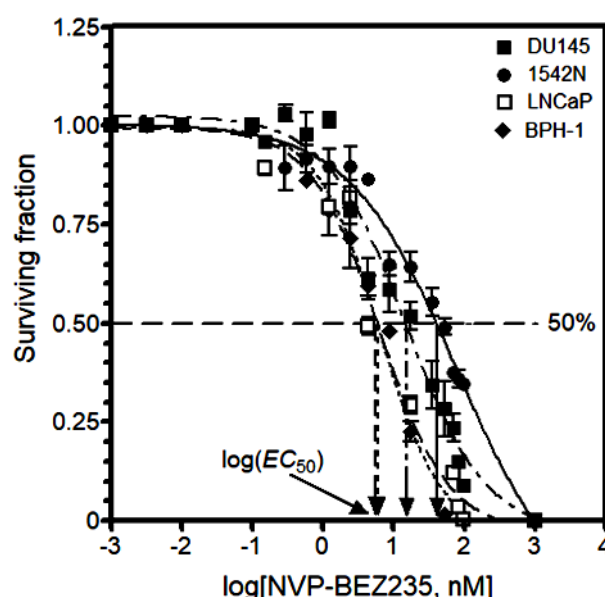
Cell line	$\alpha$ ( $Gy^{-1}$ )	$\beta$ ( $Gy^{-2}$ )	$D_{50}$ (Gy)	$P$ -value	$RS$
1542N	$0.49 \pm 0.11$	$0.00 \pm 0.00$	$1.53 \pm 0.32$ (1.17-2.17)	–	–
DU145	$0.28 \pm 0.06$	$0.01 \pm 0.01$	$2.25 \pm 0.54$ (0.59-3.90)	0.2261	$0.68 \pm 0.21$
LNCaP	$0.78 \pm 0.24$	$0.02 \pm 0.02$	$0.93 \pm 0.19$ (0.37-1.50)	0.1644	$1.65 \pm 0.48$
BPH-1	$0.39 \pm 0.05$	$0.04 \pm 0.01$	$1.65 \pm 0.36$ (0.57-2.73)	0.7797	$0.93 \pm 0.28$

**Table 2.** Summary of cytotoxicity data for 4 human prostate cell lines (1542N, DU145, LNCaP, BPH-1) treated with EGFR inhibitor (AG-1478) and PI3K and mTOR inhibitor (NVP-BE235).  $EC_{50}$  denotes the equivalent concentration for 50% cell survival (figures 2 and 3). The 95% confidence intervals of the  $EC_{50}$ -values are in parentheses. *P*-value indicates the level of significance in the difference between the  $EC_{50}$  of the “normal” cell line (1542N) relative to those of the tumor cell lines (DU145, LNCaP, BPH-1). Relative sensitivity (*RS*) is the ratio of the  $EC_{50}$  of the “normal” cell line to those of the tumor cell lines.

Cell line	Treatment	$EC_{50}$ (nM)	<i>P</i> -value	<i>RS</i>
1542N	AG-1478	400 ± 38 (237-563)	–	–
	NVP-BE235	53.82 ± 2.95 (41.13-66.50)	–	–
DU145	AG-1478	6613 ± 1510(116-13110)	0.0147	0.06 ± 0.02
	NVP-BE235	16.25 ± 4.72 (11.83-36.57)	0.0025	3.31 ± 0.98
LNCaP	AG-1478	302 ± 19 (220-384)	0.0822	1.33 ± 0.15
	NVP-BE235	6.10 ± 0.40 (4.39-7.81)	<0.0001	8.82 ± 0.75
BPH-1	AG-1478	677 ± 41 (499-855)	0.0079	0.59 ± 0.07
	NVP-BE235	6.11 ± 0.64 (3.96-14.17)	0.0011	8.81 ± 1.04



**Figure 2.** Cytotoxicity curves for EGFR inhibitor (AG-1478) treatment of 4 human prostate cell lines (DU145, 1542N, LNCaP, 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 sigmoidal equation. Data points are means ± SEM of 3 independent experiments. The concentration at which 50% of cells survive ( $EC_{50}$ ) is that at which each survival curve intersects the horizontal dashed line.



**Figure 3.** Cytotoxicity curves for PI3K and mTOR inhibitor (NVP-BE235) treatment of 4 human prostate cell lines (DU145, 1542N, LNCaP, 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 sigmoidal equation. Data points are means ± SEM of 3 independent experiments. The concentration at which 50% of cells survive ( $EC_{50}$ ) is that at which each survival curve intersects the horizontal dashed line.

## DISCUSSION

Using the clonogenic survival assay, it is demonstrated for 4 human prostate cell lines that there is no significant therapeutic advantage when  $D_{50}$ -values of the tumor cell lines (LNCaP, BPH-1, DU145) were compared with that of the “normal” cell line (1542N)

(figure 1, table 1). The ranking order, from most radiosensitive to least radiosensitive using X-ray irradiation, is LNCaP>1542N>BPH-1>DU145, and is in close agreement with radiosensitivity data reported previously for these cell lines following cobalt-60  $\gamma$ -ray exposure<sup>(35)</sup>. The absence of a therapeutic benefit may be explained by the fact that the  $D_{50}$ -values are

derived at 50% cell survival which coincides with the shoulder region of the survival curves, and may not differ markedly. For instance, a comparison of doses at the level of 10% survival, yields relative sensitivities of 1.77, 1.18, and 0.78, for the LNCaP, BPH-1, and DU145 cell lines, respectively, indicating a potential benefit for the former two. However, the rationale for choosing  $D_{50}$  and  $EC_{50}$  was to enable comparison of the relative sensitivities ( $RS$ ) of the cell lines at the same level of survival, when as low as possible radiation dose or inhibitor concentration is administered. It should also be acknowledged that molecular assays have been developed applying multigene expression profiles to predict tumor radiosensitivity by comparisons with clonogenic survival data from established cell lines<sup>(36,37)</sup>, that likely have the most potential for clinical implementation. Such methods have been shown to be statistically predictive of tumor response in esophageal and rectal cancers, and of locoregional control in head and neck cancers<sup>(37)</sup>. As such, there is the need to explore avenues like the inhibition of EGFR, PI3K, and mTOR, described here.

The cytotoxic effects of AG-1478 (EGFR inhibitor) and NVP-BEZ235 (PI3K and mTOR inhibitor) are concentration-dependent (figures 2 and 3). For AG-1478,  $EC_{50}$  values ranged from 302–6613 nM (figure 2, table 2), giving relative sensitivities of less than 1.0 for the androgen-independent DU145 and BPH-1 cells. This clearly shows that the “normal” cells (1542N) are more sensitive to EGFR inhibition than their tumor counterparts and use of AG-1478 for treatment of prostate cancer might lead to undesirable outcomes. However, inhibiting EGFR in the androgen-dependent LNCaP cells showed a small therapeutic benefit, with a relative sensitivity of 1.33 (table 2). 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 over 5-fold that in the latter<sup>(38,39,40)</sup>. 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<sup>(41)</sup>, consistent with the observed relative sensitivity of  $1.33 \pm 0.15$ .

For NVP-BEZ235 treatment,  $EC_{50}$  values ranged from 6.10–53.82 nM for all cell lines (figure 3, table 2), and are consistent with those recently reported for human breast cell lines<sup>(16)</sup>. 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 (table 2). This resistance can be attributed to NVP-BEZ235 being specifically more toxic to malignant cells, as reported elsewhere<sup>(42)</sup>. The sensitivity ranking of the malignant cell lines (DU145 and LNCaP) may be related to the extent to which NVP-BEZ235 inhibits the activity of key components of the PI3K/mTOR pathway, such as, PDK1<sup>Ser241</sup>, Akt<sup>Thr308</sup>, Akt<sup>Ser473</sup>, GSK3b<sup>Ser19</sup>, Foxola<sup>Ser256</sup>, S6K<sup>Ser235/236</sup>, 4EBP1<sup>Thr27/66</sup>, and MDM2<sup>Ser166</sup>. 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<sup>(43)</sup>. The clonogenic cell survival data presented here are consistent with this, with the LNCaP cells being 2.7-fold more sensitive than the DU145 cells (Table 2). However, the similarity in NVP-BEZ235 cytotoxicity in LNCaP and BPH-1 ( $EC_{50}$  of  $6.10 \pm 0.40$  and  $6.11 \pm 0.64$ , 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<sup>(43)</sup>. 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 *et al.* lasts only 24 hours and could miss such effects<sup>(43)</sup>.

Use of an immortalized “normal” prostate cell line instead of normal cells derived from radiation dose limiting organs, such as the bladder and rectum, can significantly influence potential therapeutic benefit. Nonetheless, it is worth noting that unmodified normal cell lines

are limited in their capacity to successfully complete intended clonogenic assays, as described in this study.

In conclusion, these data demonstrate that concomitant inhibition of PI3K and mTOR may have a higher therapeutic benefit in the treatment of androgen-dependent and -independent prostate cancers, compared to conventional radiotherapy or EGFR-targeted therapy. The findings might assist in the design of more effective treatment approaches for cancers that typically display resistance to radiotherapy and chemotherapy.

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**Conflicts of interest:** Declared none.

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