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Research Article

Combined Inhibition of PI3K, mTOR and Bcl-2 Significantly Radiosensitises Progesterone and Oestrogen Receptor Negative Breast Cancer Cells

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Abstract

Patients with triple-negative breast cancers (TNBC) constitute about one-fifth of all breast cancer patients. TNBC is an aggressive and heterogeneous disease entity in comparison with other types of breast cancer and, therefore, tends to be resistant to existing treatment regimens, such as, targeted and hormone therapies. There is evidence to suggest that proliferative and survival pathways of triple-negative tumours are still poorly understood, which could be the reason for the observed treatment resistance. Novel treatment approaches are, therefore, needed to overcome the challenges in the treatment of triple-negative breast cancers. Three human breast cell lines (MDA-MB-231, MCF-7 and MCF-12A) were pre-treated with inhibitors of phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), and the pro-survival gene (Bcl-2), and their radiosensitivities were evaluated using the clonogenic cell survival assay. Inhibition of PI3K, mTOR, and Bcl-2 with a cocktail of small molecule inhibitors NVP-BE2235 and ABT-263 resulted in a 4- to 14-fold radiosensitisation of human breast cell lines with features similar to those of triple-negative cancers. These findings suggest that inhibition of PI3K, mTOR, and Bcl-2 can significantly enhance the sensitivity of breast cells devoid of progesterone and oestrogen receptor expression. This approach may have therapeutic potential for breast cancer management.

Keywords: Small molecule inhibitors, radiosensitisation, breast cancer

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Introduction

Triple-negative breast cancers (TNBC) are devoid of expression of progesterone receptor (PR) and oestrogen receptor (ER), and do not overexpress the human epidermal growth factor receptor 2 (HER2). This

subtype constitutes about 20% of women with breast cancer [1], the majority of whom are young Black and Hispanic women [2]. Inadequate expression of these antigens that could potentially be therapeutic targets may be responsible for the apparent resistance of triple-negative breast cancer to existing treatment regimens like radiotherapy. Inhibition of residual HER2, phosphoinositide 3-kinase (PI3K), and mammalian target of rapamycin (mTOR) radiosensitises tumour cells [3-7]. Therefore, it was hypothesised that concomitant inhibition of phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), and the pro-survival gene (Bcl-2) may sensitise TNBC to radiotherapy to a larger extent. To test this hypothesis, the effect of concurrently inhibiting PI3K, mTOR and Bcl-2 on the radiosensitivity of three human breast cell lines with a wide range of HER2, ER and PR expression was assessed. The potential of this combined modality as an effective therapeutic approach for TNBC is discussed.

Materials and Methods

Cell lines and culture maintenance

The human breast cancer cell lines (MDA-MB-231 and MCF-7) and an apparently normal immortalised mammary epithelial cell line (MCF-12A) were chosen for this study because they markedly differ in expression of potential molecular targets [8,9]. While the MCF-7 cell line is ER and PR positive, and expresses low levels of EGFR [10,11], MDA-MB-231 and MCF-12A cells are ER and PR negative and express higher levels of EGFR [8,9]. Furthermore, MDA-MB-231 and MCF-12A cells express wild-type PI3K, whereas the MCF-7 cells are PI3K mutant [12,13].

MDA-MB-231 cells (passage number: 15-30) and MCF-7 cells (passage number: 20-30) were routinely cultured in Roswell Park Memorial Institute medium, RPMI-1640 (Sigma-Aldrich, USA; cat #: R8758). MCF-12A cells (passage number: 20-35) were cultured in Dulbecco's modified Eagle's medium nutrient mixture, F-12 HAM (Sigma-Aldrich, USA; cat #: D8437). All growth media were supplemented with 10% heat-inactivated foetal bovine serum (FBS) (HyClone, UK; cat #: SV30160.30IH), and penicillin (100 U/ml)/

streptomycin (100 µg/ml) (Lonza, Belgium; cat #: DE17-602E). 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 upon reaching 70-90% confluence.

Target inhibitors

NVP-BEZ235 (C₃₀H₂₃N₅O; M_w = 469.55; Santa Cruz Biotechnology, Texas, USA; cat #: 364429) potentially and reversibly inhibits class 1 PI3K and mTOR catalytic activity. ABT-263 (C₄₇H₅₅ClF₃N₅O₆S₃; M_w = 1047.52; gift from the Chemotherapeutic Agents Repository of the Drug Synthesis and Chemistry Branch, National Cancer Institute, USA) disrupts Bcl-2/Bcl-xL interactions with pro-death proteins. Stock solutions of NVP-BEZ235 (106 mM) and ABT-263 (955 µM) were constituted in dimethyl sulfoxide (DMSO) and stored at -20°C until needed.

Determination of radiosensitivity modification by NVP-BEZ235 and ABT-263

To investigate the influence of inhibitor exposure on radiosensitivity, single-cell suspensions were plated (1000-4000 cells per flask) into 25 cm² culture flasks, and incubated for 3-4 h to allow the cells to attach. Attached cells were treated with 17 nM of NVP-BEZ235 or 97 nM ABT-263, or with a cocktail of two inhibitors at the same concentrations. Batches of cell cultures were immediately irradiated with 2 and 6 Gy, representing typical doses per fraction in conventional and hypofractionated radiotherapy, respectively. The use of relatively higher concentrations of NVP-BEZ235 was to ensure adequate inhibition of the dual targets (PI3K and mTOR), as these would be expected to present a larger number of binding sites. For each experiment, sets of cell culture flasks given inhibitors alone (singly or as a cocktail) and unirradiated flasks without inhibitors served as controls for cultures irradiated with and without inhibitors, respectively. 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 at 2 and 6 Gy was expressed as a modifying factor (MF_2 or MF_6), which is given by the ratio of surviving fractions (SF) in the absence and presence of inhibitors as follows:

$$MF_2 = \frac{SF(2 \text{ Gy})}{SF([\text{inhibitor}] + 2 \text{ Gy})} \text{ or } MF_6 = \frac{SF(6 \text{ Gy})}{SF([\text{inhibitor}] + 6 \text{ Gy})}$$

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

Statistical methods

Statistical analyses were performed using the GraphPad Prism (GraphPad Software, San Diego, CA, USA) computer program. Data were calculated as the means ($\pm$ SEM) from at least three independent experiments. For each experiment and data point, three 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

Modulation of radiosensitivity by NVP-BEZ235 and ABT-263 at 2 Gy

At 2 Gy, the rank order of decreasing intrinsic radiosensitivity for the cell lines was MCF-7 ($SF_2 = 0.23 \pm 0.01$), MDA-MB-231 ($SF_2 = 0.57 \pm 0.02$) and MCF-12A ($SF_2 = 0.63 \pm 0.05$). Therefore, the former is deemed radiosensitive while the latter two cell lines are deemed radioresistant. Pre-treatment of radioresistant MDA-MB-231 cells with Bcl-2 inhibitor, ABT-263, alone or a cocktail of ABT-263 and NVP-BEZ235 resulted in significant radiosensitisation, with the latter yielding the highest effect (Figure 1). The corresponding radiation modifying factors emerged as 1.96 ± 0.15 and 4.39 ± 1.69 (Table 1). In this cell line, ABT-263 was marginally more radiosensitising than NVP-BEZ235. Exposure of MCF-7 cells to ABT-263 did not affect their radiosensitivity (Figure 1). This was also reflected in the observation that addition of ABT-263 had no

added benefit in the radiosensitisation seen with NVP-BEZ235 alone. For the radioresistant MCF-12A cell line, inhibition of Bcl-2 alone was more radiosensitising than when both PI3K and mTOR were inhibited. The corresponding radiation modifying factors were 1.85 ± 0.22 and 1.31 ± 0.13 ($P=0.06$), respectively. Pre-treatment of these cells with both inhibitors yielded a 7-fold radiosensitisation (Table 1).

Modulation of Radiosensitivity by ABT-263 and NVP-BEZ235 at 6 Gy

The ranking in intrinsic radiosensitivity seen at 2 Gy was essentially retained at 6 Gy, *albeit* over a very narrow range, with surviving fractions emerging as 0.10 ± 0.02 , 0.13 ± 0.03 , and 0.18 ± 0.01 , for the MCF-7, MDA-MB-231 and MCF-12A cell lines, respectively. To assess whether blocking the activities of PI3K, mTOR and Bcl-2, with specific inhibitors results in changes in cellular radiosensitivity at relatively large fractional radiation absorbed doses, cell cultures were treated with NVP-BEZ235, ABT-263, or a combination of both inhibitors, and immediately irradiated to 6 Gy. In MDA-MB-231, MCF-7 and MCF-12A cell lines, inhibition of Bcl-2 with ABT-263 alone led to a significant radiosensitisation (Figure 2). The radiation modifying factors for the cell lines were similar and emerged as 4.57 ± 1.02 , 4.57 ± 1.16 , and 4.34 ± 0.57 , respectively (Table 2). While NVP-BEZ235 significantly radiosensitised MCF-12A ($MF_6 = 2.62 \pm 0.93$) and MCF-7 ($MF_6 = 12.00 \pm 4.24$) cells, its radiomodulatory effect on MDA-MB-231 cells was minimal with a modifying factor of only 1.20 ± 0.34 . Pre-treatment of cell cultures with a cocktail of ABT-263 and NVP-BEZ235 yielded a very large radiosensitisation of ~14-fold in the more radioresistant MCF-12A and MDA-MB-231 cell lines, while a two-fold radiosensitisation was seen in the MCF-7 cell line (Figure 2 and Table 2).

Discussion

Patients with triple-negative breast cancer may not benefit from existing targeted therapies, as the malignancies do not present with target antigens of relevance to these treatment approaches. Hypofractionated and stereotactic radiotherapy employ large fractions of radiation, and could yield significant

Figure 1: Clonogenic cell survival for three human breast cell lines irradiated to 2 Gy with or without ABT-263 and NVP-BEZ235. Bars represent the mean surviving fraction $\pm$ SEM from at least three independent experiments. Bars represent the mean surviving fraction $\pm$ SEM from three independent experiments. (*P*-values): inhibitor treated in comparison with SF_2 without inhibitors. **P* > 0.05; **0.005 $\leq$ *P* $\leq$ 0.05; ****P* < 0.005.

tumour control with minimal tissue toxicity. Identifying potential components of pro-survival signalling pathways that can be concurrently targeted may further enhance the curative effects of radiotherapy.

Here, it is shown that while inhibition of Bcl-2 has no effect on the radiosensitivity of the MCF-7 cell line at 2 Gy, moderate radiosensitisation was seen in the MDA-MB-231 and MCF-12A cells (Figure 1 and Table 1). These data for the latter cell lines are consistent with those reported elsewhere demonstrating that Bcl-2 inhibitors are potent in Herceptin resistant breast cells [14], and can radiosensitise small-cell lung carcinomas [15]. It is not clear why no radiomodulatory effect was seen in the MCF-7 cell line, given that these cells express as high as 4.5-fold Bcl-2 in comparison with the MDA-MB-231 cells [16,17]. The modest radiation modifying factors seen in all cell lines after pre-treatment with the PI3K and mTOR inhibitor (Table 1) are consistent with those reported previously [6], and do not seem to depend on PI3K status, whereas the MCF-7 cells are PI3K mutant [12,13].

It is further demonstrated that as high as a 7-fold

radiosensitisation can be achieved at 2 Gy when MCF-12A cells, which are devoid of HER2, ER and PR expression [8,9], are pre-treated with inhibitors of PI3K, mTOR and Bcl-2 (Table 1). Similarly, a 4-fold radiosensitisation was seen in the MDA-MB-231 cell line which is also a low expresser of HER2, and is ER and PR negative [8,9]. However, in the ER and PR positive MCF-7 cell line [10,11], inhibition of PI3K, mTOR and Bcl-2 results in less than 2-fold radiosensitisation. These findings are consistent with the radiosensitisation reported elsewhere for pre-treatment of cell cultures with a cocktail of HER2, PI3K and mTOR inhibitors [6]. The marginal radiosensitisation seen in the MCF-7 cells can be attributed to the fact that they are PI3K mutated, in contrast to their PI3K wild-type counterparts, MDA-MB-231 and MCF-12A [12,13]. Inhibition of the mutant PI3K in the former should not be expected to yield significant levels of radiosensitisation. It is also worth noting that the MCF-7 cell line is a low expresser of EGFR [10,11], while the MDA-MB-231 and MCF-12A cells express higher levels of EGFR [8,9]. In fact, the current data and those reported previously [6] suggest a strong correlation between radiosensitisation and EGFR expression, with cells expressing higher levels

Figure 2: Clonogenic cell survival for three human breast cell lines irradiated to 6 Gy with or without ABT-263 and NVP-BEZ235. Bars represent the mean surviving fraction $\pm$ SEM from at least three independent experiments. Bars represent the mean surviving fraction $\pm$ SEM from three independent experiments. (*P*-values): inhibitor treated in comparison with SF_6 without inhibitors. **P* > 0.05; **0.005 $\leq$ *P* $\leq$ 0.05; ****P* < 0.005.

of EGFR tending to be more radiosensitised.

At a larger dose of 6 Gy, however, no difference exists in the radiomodulatory effect of ABT-263, with all cell lines showing a ~4-fold radiosensitisation (Figure 2 and Table 2). This cannot be explained by the marked differences in total Bcl-2 expression in these cell lines [16-18]. However, similarity in radiosensitisation at 6 Gy is consistent with comparable levels of phosphorylated Bcl-2 demonstrated elsewhere [18]. The 6-fold radiosensitisation observed in the MCF-12A cells relative to their MDA-MB-231 and MCF-7 counterparts following pre-treatment with NVP-BEZ235 cannot be attributed to PI3K functionality, given that the former and the MDA-MB-231 cell lines are both PI3K wild-type [12,13]. The large modifying factors seen in the MDA-MB-231 and MCF-12A cell lines when treated with an ABT-263/NVP-BEZ235 cocktail can be attributed to the interrelationship between Bcl-2 and PI3K activities. There is evidence to suggest that inhibition of PI3K blocks Bcl-2 expression [19]. A high level of synergy has also been demonstrated for concomitant inhibition of PI3K and Bcl-2 [20]. Therefore, it can be expected that the concurrent inhibition of PI3K and Bcl-2 would lead to significant radiosensitisation even if suboptimal concentrations of the Bcl-2 inhibitor are used. It is also worth noting that the rank order in radiosensitisation observed at 2 Gy for the ABT-263/NVP-BEZ235

cocktail appeared to be retained at 6 Gy, although the corresponding levels of radiomodulation were much higher (Tables 1 and 2). This further supports the suggestion that the activity of this inhibitor cocktail may be mediated by EGFR. It may be recalled that EGFR expression is low in the ER- and PR-positive MCF-7 cell line [10,11], and the ER- and PR-negative cell lines (MDA-MB-231 and MCF-12A) are high expressers of EGFR [8,9]. An interesting observation is that the NVP-BEZ235/ABT-263 cocktail yielded a significantly lower level of radiosensitisation in the MCF-7 cells at 6 Gy than when cells were pre-treated with NVP-BEZ235 and ABT-263 alone (Table 2). This finding may be attributed to the fact that radiosensitisers can act as antagonists against each other, and their net radiosensitising effects as cocktails often fall below the sum of the individual effects [21]. It is possible that in the MCF-7 cell system, the presence of ABT-263 negates the high radiosensitising effect of NVP-BEZ235. Nonetheless, the data presented here suggest that concomitantly targeting Bcl-2, PI3K and mTOR may be clinically beneficial in some cancers.

In conclusion, it is demonstrated that inhibition of Bcl-2, PI3K and mTOR, using NVP-BEZ235 and ABT-263, either singly or in combination, can significantly radiosensitise human breast cancer cell lines with differing HER2, ER, and PR expression levels to small

Table 1: Summary of radiosensitivity and dose modifying data for three human breast cell lines treated with inhibitors ABT-263 (for Bcl-2) and NVP-BEZ235 (for PI3K and mTOR).

Cell line	Treatment	SF ₂ [*]	MF ₂ [†]
MDA-MB-231	2 Gy	0.57 ± 0.02	
	2 Gy + ABT-263	0.29 ± 0.02	1.96 ± 0.15
	2 Gy + NVP-BEZ235	0.32 ± 0.04	1.78 ± 0.23
	2 Gy + NVP-BEZ235 + ABT-263	0.13 ± 0.05	4.39 ± 1.69
MCF-7	2 Gy	0.23 ± 0.01	
	2 Gy + ABT-263	0.24 ± 0.01	0.96 ± 0.06
	2 Gy + NVP-BEZ235	0.14 ± 0.02	1.64 ± 0.25
	2 Gy + NVP-BEZ235 + ABT-263	0.12 ± 0.02	1.92 ± 0.33
MCF-12A	2 Gy	0.63 ± 0.05	
	2 Gy + ABT-263	0.34 ± 0.03	1.85 ± 0.22
	2 Gy + NVP-BEZ235	0.48 ± 0.03	1.31 ± 0.13
	2 Gy + NVP-BEZ235 + ABT-263	0.09 ± 0.02	7.00 ± 1.65

SF₂ and MF₂ denote the surviving fraction and radiation modifying factor at 2 Gy, respectively. *Mean ± SEM.

†Mean ± error: errors were calculated using appropriate error propagation formulae.

and large fractional radiation doses. These findings suggest that a cocktail of NVP-BEZ235 and ABT-263 may have the potential for rendering triple-negative breast cancer cells more susceptible to radiotherapy. Further evaluation of these targets in a broader panel of cell lines might assist in validating these findings.

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Conflicts of interest

The authors report no conflicts of interest.

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Table 2: Summary of radiosensitivity and dose modifying data for three human breast cell lines treated with inhibitors ABT-263 (for Bcl-2) and NVP-BEZ235 (for PI3K and mTOR).

Cell line	Treatment	SF ₆ [*]	MF ₆ [†]
MDA-MB-231	6 Gy	0.128 ± 0.027	
	6 Gy + ABT-263	0.028 ± 0.002	4.57 ± 1.02
	6 Gy + NVP-BEZ235	0.107 ± 0.020	1.20 ± 0.34
	6 Gy + NVP-BEZ235 + ABT-263	0.009 ± 0.001	14.22 ± 3.39
MCF-7	6 Gy	0.096 ± 0.024	
	6 Gy + ABT-263	0.021 ± 0.001	4.57 ± 1.16
	6 Gy + NVP-BEZ235	0.008 ± 0.002	12.00 ± 4.24
	6 Gy + NVP-BEZ235 + ABT-263	0.045 ± 0.004	2.13 ± 0.57
MCF-12A	6 Gy	0.178 ± 0.009	
	6 Gy + ABT-263	0.041 ± 0.005	4.34 ± 0.57
	6 Gy + NVP-BEZ235	0.068 ± 0.024	2.62 ± 0.93
	6 Gy + NVP-BEZ235 + ABT-263	0.013 ± 0.005	13.69 ± 5.31

SF₆ and MF₆ denote the surviving fraction and radiation modifying factor at 6 Gy, respectively. *Mean ± SEM. †Mean ± error: errors were calculated using appropriate error propagation formulae.

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