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Research Article

Inhibition of PI3K and mTOR Sensitises Oestrogen Receptor Positive Human Breast Cancer Cells to a Large Fraction of Radiation Dose

Mogammad Hamid¹, Roswita Hamunyela^{1,2}, Antonio Serafin¹ and John Akudugu^{1*}

¹Division of Radiobiology, Department of Medical Imaging and Clinical Oncology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg 7505, South Africa;

²Dr B May Cancer Care Centre, Ministry of Health and Social Services, Private Bag 13198, Harvey Street, Windhoek, Namibia

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Abstract

Resistance to radiotherapy has been attributed to the expression of proteins pertinent to cancer cell survival. Treatment approaches that can effectively target these proteins, to possibly augment the effect of radiotherapy, are lacking. This is partly due to the heterogeneity in cellular expression of potential target proteins like human epidermal growth factor receptor 2 (HER-2), progesterone receptor (PR), and oestrogen receptor (ER). Such heterogeneity can result in an inability to adequately target all cells, and thus treatment failure. Hypofractionated radiotherapy has become a common clinical practice, and developing approaches that can enhance the effect of this regimen may prove beneficial to cancer management. In this study, an inhibitor of HER-2 (TAK-165) and a dual inhibitor of phosphoinositide-3-kinase (PI3K) and mammalian target of rapamycin (mTOR) (NVP-BEZ235) were tested for their radiomodulatory effects, at 6 Gy, in three human breast cell lines (MDA-MB-231, MCF-7, MCF-12A) with low expression of HER-2, and different expression levels of ER and PR. Pre-treatment with TAK-165 or a cocktail of TAK-165 and NVP-BEZ235 yielded a modest or no radiosensitisation in all cell lines. NVP-BEZ235 treatment resulted in a significant radiosensitisation of the ER and PR overexpressing cells (MCF-7), but not in the ER and PR negative cells (MDA-MB-231 and MCF-12A). These results strongly suggest that inhibition of PI3K and mTOR in ER-positive tumours might sensitise them to hypofractionated radiotherapy, and that triple-negative cancers may not benefit from this regimen.

***Correspondence:** John Akudugu, Division of Radiobiology, Department of Medical Imaging and Clinical Oncology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg 7505, South Africa, Tel: +27-21-938-9942; E-mail: jakudugu@sun.ac.za

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Introduction

Radiotherapy is a significant primary treatment for

breast cancer, as it plays an essential role in the local control of the disease [1]. Hypofractionated radiotherapy, which employs large fractional doses, reduces treatment time, decreases the potential of accelerated tumour cell repopulation and improves local tumour control, has become popular in breast cancer treatment [2,3]. Hypofractionated and stereotactic radiotherapy can be given as fractional doses ranging from 5 to 30 Gy [4-9]. Evidence suggests that even at such large fractional doses, the linear-quadratic formalism can be used for estimating the effect of treatment in tumours and normal tissue to the same degree of certainty as in conventional fractionated radiotherapy [10-12].

Despite advances in early diagnosis and treatment efficacy, the mortality rate of breast cancer remains high, and has been attributed to treatment evasion by metastatic lesions and tumour recurrences [13]. Tumour radioresistance is a significant clinical obstacle, as it limits the effectiveness of radiotherapy [13,14]. It has been suggested that targeting the human epidermal growth factor receptor 2 (HER-2) pathway may increase the anti-tumour activity of ionising radiation [15]. Although treatment of HER-2 positive cancers with trastuzumab has yielded positive results, significant levels of resistance to this treatment approach are encountered [16]. A full potential cannot be harnessed for HER-2 targeted therapy, as not all breast cancers express targetable levels of the protein. Besides, it is often not feasible to effectively target all malignant cells with a single therapeutic agent at concentrations that can yield absolute cytotoxicity [17]. Targeting HER-2 alone would also fail in triple-negative breast cancer patients who constitute a significant 20% of the global breast cancer burden [18], and tend to be more prevalent in young Black and Hispanic women [19]. Unfortunately, in addition to exhibiting little or no HER-2 activity, these cancers do not express other potential targets like the progesterone receptor (PR) and oestrogen receptor (ER). However, they tend to overexpress the epidermal growth factor receptor (EGFR) [20]. Therefore, targeting residual HER-2 and downstream signalling components of the EGFR family members may further potentiate the therapeutic benefit of stereotactic radiotherapy for triple-negative breast cancer. It was recently demonstrated that inhibition of phosphoinositide-3-kinase (PI3K) and mammalian

target of rapamycin (mTOR) sensitised breast and prostate cancer cells to 2 Gy of radiation, whilst protecting normal prostate cells [21,22].

This study sought to investigate whether inhibition of HER-2, PI3K and mTOR can sensitise human breast cancer cells to a single fraction of 6 Gy, as may be used in stereotactic radiotherapy. For this, MDA-MB-231, MCF-7 and MCF-12A cells were pre-treated with TAK-165 (a HER-2 inhibitor) and NVP-BEZ235 (a dual inhibitor of PI3K and mTOR) and immediately irradiated. To evaluate the radiomodulatory effect of each inhibitor, cell survival was determined using the colony forming assay. The influence of HER-2, ER and PR expression status on cellular radiosensitivity and the significance of inhibiting the HER-2/PI3K/mTOR pathway in irradiated breast cancer cells are discussed.

Materials and Methods

Cell culture

Two human breast cancer cell lines (MDA-MB-231 and MCF-7) and an apparently normal immortalised mammary epithelial cell line (MCF-12A) were routinely cultured in Roswell Park Memorial Institute medium and Dulbecco's modified Eagle's medium/nutrient F-12 HAM, respectively. The cancer cells were a gift from Professor S. Prince (University of Cape Town, South Africa). The MCF-12A cell line was also a gift from Professor AM Engelbrecht (Stellenbosch University, South Africa). The growth media were supplemented with 10% heat-inactivated foetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cell cultures were routinely incubated at 37°C in a humidified atmosphere (95% air, 5% CO₂). Cells were grown as monolayers and were used for experiments upon reaching 80-90% confluence. The MDA-MB-231, MCF-7 and MCF-12A cell lines were used in all experiments at passage numbers ranging from 15-30, 20-30 and 20-35, respectively.

Specific inhibitors

TAK-165 (C₂₅H₂₃F₃N₄O₂; M_w = 468.47; TOCRIS Biosciences, UK; cat #: 3599) was used to inhibit HER-2 and NVP-BEZ235 (C₃₀H₂₃N₅O; M_w = 469.55; Santa

Cruz Biotechnology, Texas, USA; cat #: 364429) was used to inhibit both PI3K and mTOR. Stock solutions of TAK-165 (21 mM) and NVP-BEZ235 (106 mM) were prepared in dimethyl sulfoxide and stored at 4°C and -20°C, respectively, until needed.

Radiosensitivity modification by TAK-165 and NVP-BEZ235

The colony forming assay was used to measure cellular radiosensitivity. Cell cultures in exponential growth were trypsinised and plated (500-10000 cells per 25-cm² culture flask, depending on the cell line), and incubated for 3-4 h to allow the cells to attach. To investigate the influence of inhibitor exposure on radiosensitivity, cells were treated with 30 nM of TAK-165 and 17 nM of NVP-BEZ235, immediately irradiated at room temperature (22°C) to 6 Gy using a Faxitron MultiRad 160 X-irradiator (Faxitron Biophysics, Tucson, AZ, USA) at a dose rate of 1.0 Gy/min, and re-incubated. These inhibitor concentrations were ~1.0–1.5 times the equivalent concentration for 50% cell kill (EC₅₀) of TAK-165 and ~3.4–4.1 times the EC₅₀ of NVP-BEZ235 in all cell lines [21, unpublished data]. The use of a relatively high NVP-BEZ235 concentration 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 and in combination) 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 cater for inter-experimental variations in inhibitor toxicity, as exposures to predetermined concentrations do not always yield the expected cell kill. After growing for 7-10 days, depending on the cell line, colonies were fixed in glacial acetic acid:methanol:water (1:1:8, v/v/v), stained with 0.01% amido black in fixative, washed in tap water, air-dried, and counted. Three independent experiments were performed and the mean surviving fractions were determined and expressed in terms of the surviving fraction at 6 Gy (SF₆).

The interaction between inhibitors and X-irradiation

(6 Gy) was expressed as a modifying factor (MF), given by the ratio of surviving fractions (SF) in the absence and presence of inhibitors as follows:

$$MF = (SF(6 \text{ Gy})) / (SF([\text{inhibitor}] + 6 \text{ Gy}))$$

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

Results

The intrinsic radiosensitivity of the MDA-MB-231 (PI3K wild-type), MCF-7 (PI3K mutant) and MCF-12A (PI3K wild-type) cell lines at 6 Gy emerged as 0.128 ± 0.027 , 0.096 ± 0.024 and 0.178 ± 0.009 , respectively. The PI3K wild-type cell lines tended to be more radioresistant than their PI3K mutated counterpart.

To investigate whether radiosensitisation of breast cancer cell lines exists at higher fractional doses, as may be encountered in stereotactic radiotherapy, the effect of blocking the activities of HER-2, PI3K and mTOR, and immediately irradiating cell cultures to 6 Gy was assessed. Cell survival data are presented in Figure 1 for the three cell lines. Pre-treatment of MDA-MB-231 cells (PI3K wild-type) with the HER-2 inhibitor (TAK-165) appeared to increase radioresistance, whilst treatment with either the dual inhibitor of PI3K and mTOR (NVP-BEZ235) alone or in combination with TAK-165 resulted in a slight reduction in radiosensitivity ($P > 0.38$). On the other hand, its PI3K wild-type counterpart MCF-12A cells were radiosensitised when pre-treated with TAK-165 and NVP-BEZ235, either singly or as a cocktail, giving radiation modifying factors of 2.34 ± 0.39 , 2.62 ± 0.93 and 3.24 ± 1.54 , respectively. Interestingly, while HER-2 inhibition had no effect on the radiosensitivity of the PI3K mutant MCF-7 cell line, inhibition of PI3K and mTOR yielded a 12-fold radiosensitisation (Figure 1 and Table 1). Pre-treatment with the inhibitor cocktail resulted in a modifying factor of only 2.34 ± 0.68 . The data for the radiomodulatory effects of the specific inhibitors on the 3 breast cell lines are summarised in Table 1.

Figure 1: Clonogenic cell survival at 6 Gy (SF_6) for 3 human breast cell lines after irradiation with X-rays: MDA-MB-231, MCF-7, and MCF-12A cells were irradiated without or in the presence of TAK-165 (HER-2 inhibitor) and NVP-BE2235 (dual inhibitor of PI3K and mTOR), either administered singly or in combination. Bars represent the mean surviving fraction $\pm$ SEM from three independent experiments. In comparison with SF_6 without inhibitors: * $P > 0.05$; ** $0.005 \leq P \leq 0.05$; *** $P < 0.005$.

Discussion

Radiotherapy has become part of standard care in both curative and palliative breast cancer treatment [23,24]. However, inherent or acquired radioresistance is thought to be the reason why many tumours do not respond favourably to radiotherapy [1,14]. It has been shown that the treatment of breast cancer cells with 2-6 Gy of radiation results in the elimination of radiosensitive sub-populations of cells, leaving the radioresistant cells to repopulate [24]. It is assumed that the surviving and repopulating cancer cells are capable of providing molecular protection against the cytotoxic effects of radiation therapy. Developing approaches that can be used in conjunction with radiotherapy to enhance cancer treatment outcome is desirable.

There is evidence to show that inhibition of proteins involved in the PI3K/Akt/mTOR pathway can result in significant radiosensitisation of glioma and breast cancer cells over radiation absorbed doses of up to 8 Gy [15,25,26]. In recent studies, a similar radiosensitisation was demonstrated at 2 Gy in breast and prostate cell lines when pre-treated with inhibitors of HER-2, PI3K and mTOR [21,22]. In this investigation, HER-2 inhibitor (TAK-165) and dual inhibitor of PI3K and mTOR (NVP-BE2235) were evaluated for their radiomodulatory effects at 6 Gy in 3 breast cell lines.

With the exception of the MCF-12A cell line, pre-treatment of cell cultures with TAK-165 did not yield a measurable radiosensitisation at 6 Gy (Table 1). The radiosensitisation of MCF-12A cells seen here cannot be attributed to differences in HER-2 expression, as HER-2 in all cell lines is low and comparable [27-29]. No added benefit was apparent when MCF-12A cells were exposed to a TAK-165 and NVP-BE2235 cocktail. Similarly, neither pre-treatment of MDA-MB-231 cells with NVP-BE2235 nor a cocktail of TAK-165 and NVP-BE2235 resulted in a significant radiosensitisation. Although MDA-MB-231 and MCF-12A cells are PI3K wild-type, they are known to be ER and PR negative [27]. This indicates that tumours with signalling features similar to those of these cell lines might not benefit from adjuvant hypofractionated radiotherapy with inhibitors of HER-2, PI3K and mTOR. However, inhibition of PI3K and mTOR resulted in ~12-fold radiosensitisation in the PI3K mutant MCF-7 cell line (Table 1). The high level of radiosensitisation observed here is at variance with the relatively low sensitisation noted when another dual inhibitor of PI3K and mTOR (PI-103) was used in the same cell line elsewhere [15]. This is likely due to the fact that NVP-BE2235 appears to be a better radiosensitiser than PI-103. When umbilical venous endothelial cells and bladder and laryngeal cancer cells were pre-treated with the two inhibitors, the former consistently showed higher levels of radiosensitisation

Table 1: Summary of radiosensitivity and dose modifying data for three human breast cell lines treated with inhibitors TAK-165 (for HER-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 + TAK-165	0.160 ± 0.025	0.80 ± 0.21
	6 Gy + NVP-BEZ235	0.107 ± 0.020	1.20 ± 0.34
	6 Gy + NVP-BEZ235 + TAK-165	0.093 ± 0.015	1.38 ± 0.37
MCF-7	6 Gy	0.096 ± 0.024	
	6 Gy + TAK-165	0.102 ± 0.004	0.94 ± 0.24
	6 Gy + NVP-BEZ235	0.008 ± 0.002	12.00 ± 4.24
	6 Gy + NVP-BEZ235 + TAK-165	0.041 ± 0.006	2.34 ± 0.68
MCF-12A	6 Gy	0.178 ± 0.009	
	6 Gy + TAK-165	0.076 ± 0.012	2.34 ± 0.39
	6 Gy + NVP-BEZ235	0.068 ± 0.024	2.62 ± 0.93
	6 Gy + NVP-BEZ235 + TAK-165	0.055 ± 0.026	3.24 ± 1.54

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.

at 6 Gy [30,31]. These findings were similar to those shown here.

The marked radiosensitisation demonstrated for inhibition of PI3K and mTOR in the MCF-7 cell line, and elsewhere [26,30,31], cannot be attributed to differences in PI3K status. Using two PI3K wild-type colon cancer cell lines and their mutant derivatives, Prevo and colleagues demonstrated no significant difference in radiosensitisation by the PI3K and mTOR inhibitor, PI-103 [30]. However, a predominantly common feature of the MCF-7 cell line, and those used elsewhere [30,31], is that they are oestrogen receptor (ER) positive [32-34]. The high levels of radiosensitisation seen in these cell lines following NVP-BEZ235 treatment may be attributed to inhibition of an enhanced ER-mediation of PI3K activity. ER-dependent activation of PI3K has been demonstrated in MCF-7 cells [35]. Upregulation of PI3K activity would be expected to render cells more susceptible to radiation-induced death when such activity is inhibited.

In conclusion, these data strongly suggest that inhibition of PI3K and mTOR in ER-positive tumours, regardless of their PI3K status, might sensitise them to radiotherapy regimens that employ large fractional doses. Further studies interrogating the role of the oestrogen receptor in modulating the effects of large fractions of radiation dose when PI3K and mTOR are inhibited may significantly contribute towards improving the outcome of hypofractionated radiotherapy, especially for malignancies that overexpress this receptor. Triple-negative cancers may not benefit significantly from this therapeutic approach.

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Conflicts of Interest

The authors report no conflicts of interest.

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