RESEARCH ARTICLE

Rapamycin and PF4 Induce Apoptosis by Upregulating Bax and Down-Regulating Survivin in MNU-Induced Breast Cancer

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Abstract

Background: To elucidate the role of rapamycin and PF4 on apoptosis regulation via Bax (pro-apoptosis), Bcl-2 (anti-apoptosis) and survivin activation on the growth in the 1-methyl-1-nitrosourea -induced invasive breast carcinoma model. Materials and Methods: Thirty five female Sprague Dawley rats at age 21-day old were divided into 4 groups; Group 1 (control, n=10), Group 2 (PF4, n=5), Group 3 (rapamycin, n=10) and Group 4 (rapamycin+PF4, n=10). MNU was administered intraperitionally, dosed at 70mg/kg body weight. The rats were treated when the tumors reached the size of 14.5±0.5mm and subsequently sacrificed after 5 days. Rapamycin and PF4 were administered as focal intralesional injections at the dose of 20 µg/lesion. The tumor tissue was then subjected to histopathological examinations for morphological appraisal and immunohistochemical assessment of the pro-apoptotic marker, Bax and anti-apoptotic markers, Bcl-2 and survivin. Results: The histopathological pattern of the untreated control cohort showed that the severity of the malignancy augments with mammary tumor growth. Tumors developing in untreated groups were more aggressive whilst those in treated groups demonstrated a transformation to a less aggressive subtype. Combined treatment resulted in a significant reduction of tumor size without phenotypic changes. Bax, the pro-apoptotic marker, was significantly expressed at higher levels in the rapamycin-treated and rapamycin+PF4-treated groups compared to controls ($p<0.05$). Consequently, survivin was also significantly downregulated in the rapamycin-treated and rapamycin+PF4-treated group and this was significantly different when compared to controls (p). Conclusions: In our rat model, it could be clearly shown that rapamycin specifically affects Bax and survivin signaling pathways in activation of apoptosis. We conclude that rapamycin plays a critical role in the induction of apoptosis in MNU-induced mammary carcinoma.

Keywords: Breast cancer - apoptosis - Bax - Bcl-2 - survivin - rapamycin

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Introduction

Breast cancer is the commonest malignancy in women and the second leading cause of cancer deaths. The latest report from Ma et al, in 2012, an expected 226,870 new cases of invasive breast cancer and 39,510 breast cancer deaths are predictable to arise among U.S. women (Hassan et al., 2013). While the incidence and mortality of breast cancer rates over the past 20 years, have been rising speedily in economically less developed regions. Based on the report from GLOBOCAN in 2008, half of the new worldwide breast cancer cases (1.38 million) and 60 % of breast cancer deaths (458,000) occurred in developing countries (GLOBOCAN 2008 (IARC) 2008).

In Malaysia, breast cancer is the most common cancer in women. About 3,242 cases of breast cancer were diagnosed in women in 2007, making up 18.1% of all reported cancer cases and 32.1% of all women-related cancer (National Cancer Registry Report 2007) which affects all Malaysian principal ethnicities (Yip et al., 2006).

Normal breast development is controlled by a balance between cell proliferation and apoptosis, and there is a strong evidence that tumor growth is not just a result of uncontrolled proliferation, but also of reduced apoptosis (Hahm, 1998; Hengartner, 2000). The balance between cellular proliferation and apoptosis is crucial in determining the overall growth or regression of the tumor in response to cancer therapies (Signoreet al., 2013; Wan et al., 2014).

Apoptosis or programmed cell death is an active, energy-dependent process of cell death, which occurs during normal morphological development, in response to certain physiological stimuli, and secondary to cell injury

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and stress (Burz et al., 2009). This type of cell death occurs for the purpose of regulating the orderly removal of cells in discrete tissues which is critical in embryonic development and normal tissue homeostasis. It differs from necrosis by the absence of inflammatory reaction and the naturalness of the process, making it an efficient process in maintaining cellular homeostasis.

Bax and Bcl-2 are Bcl-2 family proteins that regulate programmed cell death and apoptosis (Reed, 1996). The expression of certain types of these proteins results in apoptotic suppression whilst others functions as promoters of apoptosis (Hengartner, 2000). Survivin, a member of the inhibitor of apoptosis (IAP), is overexpressed in almost all malignancies but is rarely detected in normal differentiated adult tissues. Survivin has been shown to inhibit apoptosis, promote cell proliferation and play a main role in cancer progression (Tanaka et al., 2000).

Rapamycin was originally used as an antifungal and recently as an anticancer agent (Ruria Namba et al., 2006). Functioning as a suppressor of the immune system, it has been shown to inhibit the growth of breast cancer and is currently being tested in clinical trials as a novel highly-targeted anticancer agent (Noh et al., 2004). Mammalian target of rapamycin (mTOR) is a protein kinase that involves in cell growth, proliferation and survival (Shor, Gibbons et al., 2009). The mTOR signaling cascade is upregulated in cancer (Ballou and Lin, 2008) and there is a great interest in the role of rapamycin as an inducer of apoptosis in breast cancer (Zhu et al., 2004). Besides that, Platelet factor 4 (PF4) is also an abundant platelet alpha-granule protein and a founding member of the C-X-C chemokine family, which has been recently shown to have an antitumor effect on breast cancer cells (Maione et al., 1991).

The 1-methyl-1-nitrosourea (MNU) murine model for mammary carcinogenesis was firstly discovered by Gulliano et al. (1975) about 30 years ago. Tumors formed are mostly mammary carcinomas (Russo and Russo, 1995) that has a lot of histopathological and biological resemblance of estrogen receptor-positive breast cancer in women (Russo and Russo, 2000). Almost all animals develop mammary tumors after a relatively-short latency (3-4 months) in this model. In addition, the morphology, the originals (terminal end buds of the terminal ductal lobular unit), and the pre-invasive stage (hyperplasia, ductal carcinoma in situ) of the tumors seems similar to human breast cancer (Russo and Russo, 1995; 2000). This model has been used extensively as a preclinical in vivo model for evaluating the potential efficiency of an agent in breast cancer treatment and/or prevention. Based on these favourable characteristics, MNU-induced murine breast cancer model has been chosen for this study.

This study aims to analyse the expression of apoptosis proteins Bax, Bcl-2 and survivin after treatment with rapamycin, PF4 and rapamycin+PF4 in MNU-induced murine breast cancer model.

Materials and Methods

Animal procedures

Thirty five females (35) Sprague Dawley (SD) rats were obtained from the Animal Research and Services Centre of Science University of Malaysia (ARACS). The ethical clearance for using experimental animals was obtained from the USM's Animal Ethics Committee [PPSG/07 (A) /044/(2010) (56)]. The rats were housed and maintained at the Animal House Unit. Caging and rat handling were performed in accordance with good laboratory practice criteria set by the Animal House. The rats were caged in groups of three and were fed with a standard laboratory diet and water (Jaafar et al., 2009; Thompson et al., 1995). The SD rats were divided into 4 groups and each group was given different interventions: a control (untreated) group 1 [n=10], rapamycin-treated group 2 [n=10], Platelet Factor 4-treated group 3 (PF4) [n=5] and combined rapamycin and PF4-treated group 4 (rapamycin+PF4) [n=10].

MNU, rapamycin &PF4 preparation and tumor induction

MNU was provided by Sigma Aldrich. The crystallized form of MNU was dissolved in freshly-prepared 0.9% normal saline prior to tumor induction. The MNU was injected intraperitoneally at a dose of 70 mg/kg body weight into 21-day-old rats (Jaafar et al., 2009). The rats were weighed and palpated for mammary tumor lesions weekly. The onset of tumors was monitored by daily visual inspection and palpation of the mammary regions. The increase in tumor size was monitored by measuring the subcutaneous mass using vernier caliper. Rapamycin was dissolved in absolute ethanol and diluted in mixtures of 10% polyethylene glycol (PEG) -400,8% Ethanol: 10% Tween-80 to a final concentration of 20 μg/0.2ml, while PF4 was dissolved in physiological saline to the same final concentration of rapamycin. From 30 days post-MNU administration until study termination, rats were palpated twice weekly and measured using a digital vernier calliper for tumor size quantification. Normal mammary glands were obtained from virgin female Sprague Dawley rats (n=5) whose age were approximately around 13 weeks old-the average age of tumor-bearing animals at excision.

Experimental design

Malignant mammary gland lesions were monitored for 90 days post-MNU injection. All rats were randomly assigned to four groups after lesions reached a mean tumor diameter of 14.5±0.5 mm. The rats in group 1 (n=10) served as control cohort. Physiological normal saline was injected by a single intralesional injection for the vehicle into each lesion (n=10) at size 14.5±0.5 mm in the control group. Meanwhile, the rats in group 2 (n=10), group 3 (n=5) and group 4 (n=10) were treated with rapamycin, PF4 and rapamycin+PF4, respectively. Rapamycin was dissolved in absolute ethanol and diluted in mixtures of 10% PEG-400, 8% Ethanol: 10% Tween-80 of 20 μg/0.2ml, while PF4 was dissolved in physiological saline to the same final concentration of rapamycin (R. Namba et al., 2006). Each solution was freshly prepared prior to injections. Rats were anesthetized intraperitoneally with a mixture of ketamine-HCl and xylazine (100 and 10 mg/kg, respectively) immediately before injecting allotted interventions. Both groups received a focal intralesional injection at the dose of 20 μg/lesion twice daily for

consecutive days until lesions consistently decreased in 5 days. In synergistic tumor suppression arm, the rats in group 4 (n=10) received a focal intralesional injection of rapamycin followed by PF4 on consecutive days at the dose of 20 μg/lesion for each injection. The injections were again given on a daily basis until lesions reach their final sizes at day 5 post injection. In the negative control [group 5 (n=5)], all rats received no treatment and they represent the normal physiology of rats' mammary pad. All rats were subsequently sacrificed by euthanization via exposure to carbon dioxide in a closed chamber.

Tumor sample collection

Breast tumor specimens were collected when the tumor reached the size (14.5±0.5mm) at which the rats were sacrificed and grouped accordingly into control and intervention groups. Rats were sacrificed using gaseous CO2 inhalation, provided by the ARACS. At necropsy, the rats were skinned and the dissected skins with the tumors intact were photographed to record tumor location and size. All palpable tumors were carefully excised and fixed in 10% formalin for Hematoxylin and Eosin (H&E) and immunohistochemistry (IHC) stainings.

Immunohistochemical analysis

The IHC staining was performed to demonstrate the expression of each apoptotic marker separately: monoclonal Bax (diluted at 1:200 (µl); Santa Cruz Biotechnology), polyclonal Bcl-2 (diluted at 1:100 (µl); Santa Cruz Biotechnology) and monoclonal survivin(diluted at 1:50 (µl); Santa Cruz Biotechnology). A standard-labelled streptavidin biotin (Dako, Glostrup, Denmark) method was used on formalin-fixed paraffin-embedded tissue sections. The tissue blocks were trimmed and sectioned with microtome (Leica, Wetzlar, Germany) to obtain 3-5-µm thick sections which were later deparaffinised in xylene and dehydrated. Slides were then pretreated with Tris EDTA buffer (10mM, pH 9.0) for 14 min and heated in a microwave oven. Subsequently, all the sections were treated for 10 min with peroxidase blocking reagent (DAKO Glostrup, Denmark) to quench the endogenous peroxidase activity, and then incubated with primary antibodies, followed by rinsing with Tris buffered saline (pH 7.2). The sections were incubated for 30 min with optimally diluted biotinylated secondary antibody and for 30 min with horseradish peroxidase before it is ready for use. For visualization, the slides were immersed in diaminobenzidine (DAB) (DAKO, Glostrup, Denmark) substrate for 5 min, followed by washing in distilled water. The slides were then counterstained with Harris hematoxylin, dehydrated and mounted. To assess the specificity of the reactions, gastric adenocarcinoma, tonsillar tissues and colon CA were used as positive controls for Bax, Bcl-2 and survivin expressions, respectively. Negative controls (incubation without the primary antibody) were also used for this purpose.

Immunohistochemistry Scoring System (ISS)

The light microscope (Nikon, Japan) was used to examine the immunohistochemistry stained breast cancer slides. We first scored the expression and immunostaining of Bax, Bcl-2 and survivin according to the procedures as recommended by Assanuma et al. (2005). Bax, Bcl-2 and survivin immunoreactivities were evaluated semiquantitatively according to the percentage of cells showing distinct diffuse cytoplasmic immunohistochemical reaction. Cytoplasmic immunoreactivities were assessed in at least five high-power fields at ×400 magnification and assigned to one of the following categories: 0=<5%; 1=5% to 20%; 2=>20%. Since tumor cells showed heterogeneous staining, the dominant pattern was used for scoring. However, we further grouped the scores obtained into two categories.

Staining intensity was scored on a positive scale (positivity), ranging from 0 to 2 where, 0 was equivalent to no staining, 1+ weakly stained, and 2+ moderately to strongly stained.

Statistical analysis

Data was descriptively presented in mean (SD) / median (IQR) and frequency (percentage). The differences in terms of immunohistochemical expression across all experimental groups were determined by the Kruskal–Wallis test followed by Mann-Whitney test with Bonferroni correction for multiple comparisons. All statistical analyses were carried out using Statistical Product and Service Solutions (SPSS) version 20 (IBM, New York USA).

Results

Type of tumor in intervention groups

The histopathology of the untreated control cohort showed that the severity of the malignancy worsens as the mammary tumor growth. Tumors developed in untreated groups behaved more aggressively [Infiltrating ductal carcinoma, Not otherwise specified (IDC, NOS) and papillary carcinoma] whilst tumor in treated groups transformed to a less aggressive (cribriform carcinoma) subtype. Combined treatment resulted in significant reduction of tumor size without phenotypic tumor changes (Table 1).

Histology of MNU-induced mammary carcinoma

The majority of the carcinoma induced were the cribriform type in all experimental groups. The cribriform carcinoma displayed epithelial clusters surrounded by intense desmoplastic reaction and lymphocytic infiltration (Figure 1A). The other phenotype was papillary carcinoma that displayed numerous papillary projection with thin fibrovascular core (Figure 1B). IDC, NOS infiltrated the breast tissue diffusely and in cords and clusters (Figure 1C). The IDC, NOS carcinoma cells have

Table 1. The Tumor Types in the Intervention Groups

Groups	Tumor types (%)		
	Cribriform	IDC	Papillary
Control, n=10	70	30	0
Rapamycin, n=10	70	10	20
PF4, n=5	40	20	40
Rapamycin+PF4, n=10	40	0	60

large, pleomorphic nuclei with prominent nucleoli and moderate cytoplasm. Mitotic figures were easily seen. The surrounding stroma showed desmoplasia (Figure 1D).

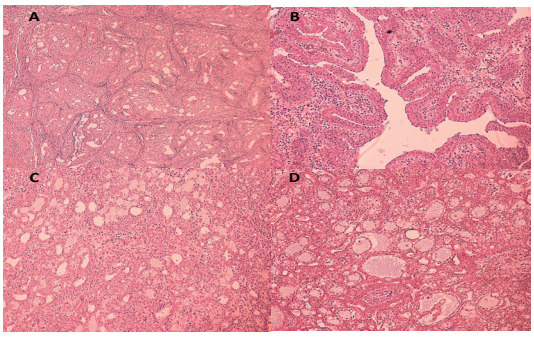

Figure 1. Representative Histology Staining Analysis of Normal Mammary Gland and Invasive Tumor in MNU-Induced Breast Carcinoma. Cribriform carcinoma was from control untreated group displaying the epithelial clusters are surrounded by intense desmoplastic reaction and lymphocytic infiltration **A)**. Papillary carcinoma was from the rapamycin-treated group when the tumor regressed displaying numerous papillary projections with thin fibrovascular core **B)**. Invasive Ductal Carcinoma (IDC), NOS pattern, was from control untreated group displaying tumor cells invaded to the adjacent tissues **C & D)**. (H&E, original magnification x100)

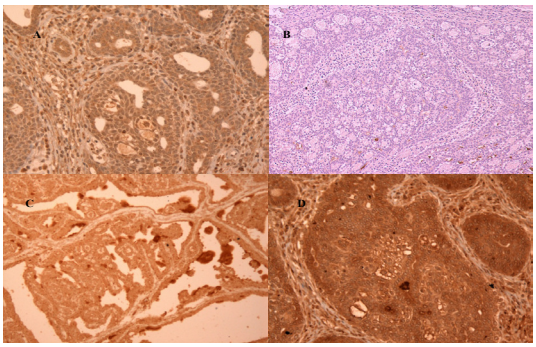

Figure 2. Immunohistochemical Expressions of Representative Markers on Tumor Specimens. A) Cribriform carcinoma positive for Bax, **B)** Negative staining of Bax in cribriform carcinoma. **C)** Papillary carcinoma positive for Bcl-2 **D)** positive staining of survivin in cribriform carcinoma. (Original magnification x200)

Expression of Bax, Bcl-2 and survivin between intervention and control groups (Table 2)

There were significant increases in Baxexpression in both rapamycin and rapamycin+PF4 groups compared to control (p=0.001 and 0.049, respectively) (Table 2). Bax expression was also significantly higher in all PF4-treated groups (p=0.007 and p=0. 003, respectively). On the other hand, Bcl-2 showed similar expression in both groups (p>0.05).

Expression of Bax, Bcl-2 and survivin amongst the intervention groups (Table 3)

Survivin expression was significantly reduced in all groups. There was a significant median difference in terms of survivin expression score between PF4 and rapamycin-treated groups (p=0. 003) (Table 2.). However, Bcl-2 and Bax expression was not significantly different between both groups (p value>0.05) (Table 3).

The addition of PF4 to rapamycin causes a significant increase in Bax expression compared to rapamycin group (rapamycin+PF4 vs rapamycin: 80.5 *vs* 62, p value

Table 4. Intensity of Bax, Bcl-2 and Survivin Immunostaining Expression in Control and Intervention Groups

	Control (%)[b]	Rapamycin (%)[b]	PF4 (%)[b]	Rapamycin +PF4(%)[b]
Baxa				
Strong	2/10 (20)	9/10 (90)	4/5 (80)	8/10 (80)
Moderate	7/10 (70)	1/10 (10)	1/5 (20)	2/10 (20)
Weak	1/10 (10)	0/10 (0)	0/5 (0)	0/10 (0)
Bcl-2a				
Strong	4/10 (40)	5/10 (50)	3/5 (60)	4/10 (40)
Moderate	5/10 (50)	4/10 (40)	1/5 (20)	6/10 (60)
Weak	1/10 (10)	1/10 (10)	1/5 (20)	0/10 (0)
Survivina				
Strong	8/10 (80)	2/10 (20)	0/5 (0)	2/10 (20)
Moderate	2/10 (20)	7/10 (70)	1/5 (20)	3/10 (30)
Weak	0/10 (0)	1/10 (10)	4/5 (80)	5/10 (50)

*[a]The immunointensity among immunopositive cases. In all cases, immunostained nontumor cells such as residual non-neoplastic prostate epithelium or lymphocytes were present which served as an internal control for assessing immunointensity. bPercentage of all cases (none excluded) with moderate or strong immunoreactivity

Table 2. The Expression of Bax, Bcl-2 and Survivin between Intervention and Control Groups

Group	No. of cases (n)	Cytoplasmic localization								
		Bax[a]			Bcl-2[a]			Survivin[a]		
		Median (IQR)		p valueb	Median (IQR)		p-value[b]	Median (IQR)		p-value[b]
Rapamycin vs Control	20	85.5 (11.3)	50 (23.8)	0.001	54.5 (33.5)	51 (36.5)	0.496	62 (27)	79 (26)	0.049
PF4 vs. Control	15	0 (19.0)	50 (23.8)	0.007	67 (43)	51 (36.5)	0.327	42 (17)	79 (26)	0.003
Rapamycin/PF4 vs control	20	80.5 (9.5)	50 (23.8)	0.001	71 (12.3)	51 (36.5)	0.075	49.5 (29)	79 (26)	0.002

*[a]p valueBax <0.001, p value$_{Bcl2}$=0.237, p value$_{Survivin}$=0.001 (Kruskall-Wallis test); [b]Mann-Whitney test, alpha was corrected using Bonferroni correction (0.05/3)=0.017

Table 3. The Expression of Bax, Bcl-2 and Survivin Amongst the Intervention Groups

Group	No. of cases (n)	Cytoplasmic localization								
		Bax[a]			Bcl-2[a]			Survivin[a]		
		Median (IQR)		p-value[b]	Median (IQR)		p-value[b]	Median (IQR)		p-value[b]
PF4 vs Rapamycin	20	90 (19.0)	85.5 (11.3)	0.459	67 (43)	54.5 (33.5)	0.624	42 (17)	62 (27)	0.023
Rapamycin+PF4 *vs* Rapamycin	20	80.5 (9.5)	85.5 (11.3)	0.042	67 (43)	54.5 (33.5	0.096	49.5 (29)	62 (27)	0.058
Rapamycin+PF4 *vs* PF4	20	80.5 (9.5)	90 (19.0)	0.049	67 (43)	67 (43)	0.713	49.5 (29)	42 (17)	0.759

*[a]p valueBax <0.001, p valu$_{eBcl2}$=0.237, p value$_{Survivin}$ = 0.001 (Kruskall-Wallis test); [b]Mann-Whitney test, alpha was corrected using Bonferroni correction (0.05/3) =0.017

=0.042) (Table 2). However, there was no significant difference with regard to Bcl-2 and survivin expression between both groups (Table 3).

Intensity Bax, Bcl-2 and Survivin immunostaining in control and intervention groups

Bax expression was higher in rapamycin group, where 9 out of 10 (90%) specimens showed strong immunostaining compared to control group (20%). On the other hand, survivin was more highly overexpressed in controls (80%) than those in rapamycin (20%) or rapamycin+PF4 groups (20%). The expression of Bcl-2 is similar in all groups.

Discussion

Apoptosis is a highly controlled process in both normal and cancerous breast tissue. Apoptosis inhibition depends partly on the balance of Bax and Bcl-2 protein expression. Our study demonstrates that Bax expression was higher in all breast cancer specimens treated with rapamycin, PF4 and combined groups when compared against a control group (p value=0.001, 0.007 and 0.001, respectively). Survivin expression was markedly reduced in all the intervention groups compared to that in the control group whilst Bcl-2 expressions were fairly similar amongst all groups. Our findings are in accordance with Purcell et al, (2010) and Zhou et al, (2013) who reported that the restitution of Bax-alpha expression, which was originally downregulated in the breast cancer models of SCID and nude mice, restored tumor sensitivity to different apoptotic signals resulting in the reduction of tumor size. Moreover, Jaafar et al, (2012) also corroborated our findings by showing an increase of apoptotic activities in both tumor and intratumoral endothelial cells with stronger Bax expression. Apart from that, our findings on survivin expression was also in tandem with Lu et al, (2009) who reported that higher survivin expression was associated with higher histological grade, poor disease-free and overall survivals and diminished chemosenstivity by modulating P-glycoprotein (PGP) turnover or transport by PGP.

The increase in Bax expression coupled with the attendant reduction of survivin expressions might be ascribed to the pro-apoptotic activity of rapamycin, which enhances Bax expression, a key pro-apoptotic protein in breast cancer pathway (Jurgensmeier et al., 1998; Hassan et al., 2013). Apoptosis or programmed cell death is a physiological cell suicide program that is vital for the growth and preservation of healthy tissues. Deregulation of this pathway occurs in cancer, autoimmune diseases, and neurodegenerative disorders. Two major apoptotic pathways have been identified thus far, the death receptor-mediated and mitochondria-mediated pathways (Tanet al., 2009). Our result showed that rapamycin and PF4 produce anti-tumor effects on mammary tumor progression by promoting cellular apoptosis. This was in line with Shirouzu et al, (2010) and Huynh, (2010) who both demonstrated that rapamycin inhibits the proliferation, migration and growth of premalignant lesions and invasive tumors through induction of apoptosis in hepatocellular carcinoma cells in vitro.

Since we found that tumor apoptosis is an important determinant of mammary tumor invasiveness and progression, the effects of rapamycin and PF4 either alone or in combination was thus evaluated. Interestingly the tumor invasiveness are higher in control groups compared to those treated with rapamycin and PF4. Here we discovered that treatment with rapamycin alone significantly inhibited tumor progression and resulted in the promotion of tumor apoptosis in cell. Rapamycin alone significantly induced Bax and suppressed the Bcl-2 expression which lead to significant tumor regression. Apart from that, histological findings and protein expressions also showed rapamycin-activated Bax expression resulted in induced differentiation of tumor cells, leading to more differentiated mammary tumor morphology, thus reversing the aggressive phenotype of the lesion when compared to untreated controls (Ruria Namba et al., 2006).

Survivin was found to be highly expressed in almost all malignancies, but was rarely detected in normal differentiated adult tissues. Our result shows that survivin was highly expressed in treated groups. In contrast, Jha et al, (2012) theorized that survivin inhibits apoptosis, promotes cell proliferation and enhances angiogenesis, cementing its key role in cancer progression. In neuroblastoma cancer model, the expression of survivin was increased when induced by rapamycin (Samkari et al., 2012). However the relationship between both proteins is one of antagonistic. Bax promotes apoptosis whilst survivin inhibits apoptosis (Huang et al., 2012). Nevertheless, different survivin isoforms may have different effects on apoptosis. For example, (Verdecia et al., 2000) reported that while survivin- ΔEx3 retained its anti-apoptotic function in transfected renal cancer cells, survivin-2B had reduced capacity for inhibiting apoptosis. On the other hand, obligatory expression of survivin-2B was found to inhibit cell growth and sensitize leukemic cells to doxorubicin-induced apoptosis (Zhu et al., 2004). From these findings, the authors recommended that the 2a form of survivin may also be pro-apoptotic or at least be able to attenuate the anti-apoptotic effects of survivin from other isoforms.

In conclusion, our study provides compelling experimental evidence that the Bcl-2-family, pro-apoptotic protein Bax and survivin play a critical role in mediating the mechanism of cell death induced by rapamycin and PF4. Our data support the hypothesis that rapamycin and PF4 up-regulates pro-apoptotic programmed cell death in cells that are unable to undergo Bax/Bcl-2-mediated apoptotic cell death and that apoptosis activity can be increased by attenuating Bax expression in breast cancer cells. This can be further enhanced by the direct promotion of apoptosis using the rapamycin+PF4 alone or in combined. This is obviously a novel addition to the existing literature since for the first time rapamycin was shown to exert its anti-tumor effects on another pathway that is independent of mTOR in breast cancer model. These strategies, which are highly focusing on and targeting the apoptotic pathways deserve further investigation in advanced animal models of breast cancer,

which may provide new modalities in the breast cancer armamentarium.

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