RESEARCH ARTICLE

Roles of the *Bcl-2/Bax* Ratio, Caspase-8 and 9 in Resistance of Breast Cancer Cells to Paclitaxel

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Abstract

 The goal of this study was to establish paclitaxel resistant MCF-7 cells, as *in vitro* model, to identify the molecular mechanisms leading to acquired chemoresistance in breast cancer cells. Resistant cells were developed by stepwise increasing exposure to paclitaxel. Gene expression levels of Bax and *Bcl-2* along with protein levels of caspase-8 and caspase-9 were evaluated in two resistant cell lines (MCF -7/Pac64 and MCF -7/Pac5 nM). Morphological modifications in paclitaxel resistance cells were examined by light microscopy and fluorescence activated cell sorting (FACS). As an important indicator of resistance to chemotheraputic agents, the *Bcl-2/Bax* ratio showed a significant increase in both MCF-7/Pac5nM and MCF-7/Pac 64nM cells (p<0.001), while caspase-9 levels were decreased (p<0.001) and caspase-8 was increased (p<0.001). FACS analysis demonstrated that MCF -7/Pac64 cells were smaller than MCF-7 cells with no difference in their granularity. Our results support the idea that paclitaxel induces apoptosis in a mitochondrial-dependent manner. Identifying breast cancer patients with a higher *Bcl-2/Bax* ratio and caspase 9 level and then inhibiting the activity of these proteins may improve the efficacy of chemotheraputic agents.

Keywords: Breast cancer cells - MCF-7 - paclitaxel - chemoresistance - *Bcl-2/Bax* - caspases

Introduction

 Breast cancer is the second most common form of cancer after lung cancer in human and the most prevalent cancer among women (Moulder and Hortobagyi, 2008). The prevalence of this malignant disease is growing worldwide, more than 1.3 million women worldwide are diagnosed with breast cancer every year (Parkin et al., 2005; Garcia et al., 2007). Different strategies have been developed for diagnosis and treatment of this cancer (Ghanbari et al., 2014; Sabzichi et al., 2014). Chemotherapy is the common strategy for cancer therapy; specially, it is momentous part of the treatment following surgical treatment (Carrick et al., 2009). However, resistance to chemotherapy remains a major obstacle on the way of effective treatment in breast cancer patients.

 Chemoresistance is often observed in the epithelial malignancies including breast, lung, and ovarian cancers and leads to unfortunate clinical outcome (Wild et al., 2004; Seve et al., 2005; Schrohl et al., 2006; Crotzer et al., 2007). Resistance might occurred either in the beginning of chemotherapy, or after a successful initial response to chemotherapy even (Pommier et al., 2004). Consequently, the main purpose of molecular oncology is to understand the basis of chemoresistance procedure. Clarification of drug resistance molecular mechanisms can lead to find novel molecules with provided targets for

chemotherapeutic agents, diagnostic tests or prognostic markers (Tabasinezhad et al., 2013).

 Apoptosis is an important pathway for cell death following various types of chemotherapy. The mitochondrial pathway of apoptosis (or intrinsic) is generally controlled via the interaction between components of the *Bcl-2* protein family (Danial and Korsmeyer, 2004; Chipuk et al., 2010).

 The mitochondrial pathway of apoptosis is dependent upon the *Bcl-2* family of proteins for the efficient release of pro-apoptotic factors from the mitochondrial inters membrane space (Chipuk et al., 2006; Lindsay et al., 2011; Ola et al., 2011). The *Bcl-2* family of proteins is divided into three groups, based on the presence of *Bcl-2* homology domains (BH1-4 domains): anti-apoptotic *Bcl-2* proteins (e.g., *Bcl-2*, Bcl-w, Bcl-xL, A1 and Mcl-1), the effector pro-apoptotic members (e.g., *Bax* and Bak) and the BH3-only proteins (e.g., Bad, Bid, Bik, Bim, Bmf, bNIP3, HrK, Noxa and Puma) (Oltvai et al., 1993; Reed 1998; Kelly and Strasser, 2011). Proteins that stimulate or inhibit apoptosis interact with each other and determine the fate of cells for death or survive. Enhancement in expression of *Bax*, increases sensitivity to apoptotic stimuli and decreases tumor enlargement (Bargou et al., 1996). It has been suggested that the *Bcl-2/Bax* ratio may be more important than either promoter alone in determining apoptosis (Oltvai et al., 1993; Stoetzer et

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al., 1996).

Induction of apoptotic pathways (extrinsic or intrinsic) can cause the activation of caspase proteins (Degterev et al., 2003; Fulda and Debatin, 2006). Caspase-8 is mainly activated in extrinsic apoptotic pathway, while caspase-9 activation is related to the intrinsic pathway (Cryns and Yuan, 1998; Olsson and Zhivotovsky, 2011).

Paclitaxel, a member of taxane family, is one of the most common first-line treatments in breast cancer patients. Paclitaxel is a diterpenoid compound that obtained from Taxus brevifolia. It disturbs depolymerization of microtubules in malignant cells (Gabizon and Papahadjopoulos, 1988; Gabizon et al., 1990); thereby, inhibits cell division and triggers apoptosis (Gabizon, 1992; Ward et al., 2007). Paclitaxel is used as an adjuvant in the standard chemotherapy method for treatment of lymph node positive breast cancer as well as in patients with inflammatory breast cancer (Ring and Ellis, 2005; Ward et al., 2007). Unfortunately, increased resistance and stopped responses to taxanes limit the efficacy of therapy and cause mortality (Saloustros et al., 2008).

MCF-7 cell line is an excellent *in vitro* model for studying the mechanisms of chemoresistance. It is susceptible to apoptosis and applied for various investigations on apoptosis or survival of cancer cells (Simstein et al., 2003; Ghafouri-Fard et al., 2012; Hamedeyazdan et al., 2012; Darakhshan et al., 2013; Seifi-Alan et al., 2013). Developing resistant cancer cells to specific chemotherapeutic agents can provide valuable model systems to investigate molecular mechanisms of chemoresistance (Rastogi and Sinha, 2009; McDermott et al., 2014). The goal of this study was to develop paclitaxel resistant MCF-7 cells as *in vitro* model to shed light on molecular mechanisms leading to acquired chemoresistance in breast cancer cells. This study also studied the expression of principle genes responsible for pro-apoptotic and anti-apoptotic *Bcl-2* family members. Furthermore, the levels of caspase-8 and caspase-9 proteins were compared in paclitaxel resistant and non-resistant MCF-7 cells.

Materials and Methods

Materials

Paclitaxel was purchased from Ebetaxel®, EBEWE Pharma (Unterach- Austuria); RPMI-1640 Medium; 3-(4, 5-dimethyl-2- thiazolyl) -2,5-diphenyl -2H- tetrazolium bromide (MTT) and penicillin–streptomycin were obtained from Sigma-Aldrich (St Louis, MO,USA); Fetal Bovine Serum (FBS) was from Invitrogen; Primers were purchased from MWG Biotech(Ebersberg, Germany); RNX™ -Plus Kit was obtained from CinnaGen (Tehran, Iran); REVERTA-L RT reagents kit was from Central Institute of Epidemiology (Moscow, Russia); SYBR green PCR Master Mix was purchased from Applied Biosystems (Warrington, UK).

Cell culture

The human MCF-7 breast cancer cells were obtained from National cell bank of Iran (Pasteur institute, Iran). The MCF-7 cells were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 mg/ml streptomycin and 100 units/ml penicillin G. Cells were preserved in a humidified atmosphere with 5% CO_2 at 37°C incubator. Cultured cells at 70-80% confluency were washed with pre-warmed phosphate buffered saline (PBS) and detached from the flask using trypsin-EDTA with incubation at 37ºC for 3-5 min, followed by addition of culture media containing 10% FBS to neutralize the excess trypsin activity. The cell suspension was then centrifuged and the cell pellet was re-suspended in fresh culture media to be used in the experiments.

Assessment of cell viability using MTT assay

Cell proliferation and viability of sensitive MCF-7 cells and paclitaxel-resistant cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT, 5mg/ml, Sigma) which evaluates the percentage of viable cells. The cells with about 70% confluency were collected from culture flask with 0.05% trypsin/EDTA solution. Cells were seeded into 96- well plate (200μl/well) with concentration of 4×10^4 cells/cm². MCF-7, MCF-7/Pac5nM and MCF-7/Pac 64nM cells with about 50% confluency were treated with increasing concentrations of the paclitaxel (0.1 - 50nM), (2.5-100 nM) and (50-1000 nM), respectively. Four wells were remained untreated as control. MTT assay was carried out 24, 48 and 72 hours after treatments. To prepare MTT reagent, 2mg of MTT powder was dissolved in 1 ml PBS. The culture medium was exchanged with 150 μl fresh media plus 50 μl MTT reagent (2mg/ml in PBS); the cell-free wells were considered as blank control. Cells were incubated in 37°C with 5% CO_2 and humidified atmosphere for 4 hours. Then the MTT solution was removed and 200μl of DMSO and 25μl Sorenson buffer (0.1M NaCl, 0.1M glycine adjusted to pH: 10.5 with 1M NaOH) was added to each well. The plate was maintained for 15 min at 37°C and then the optical density (OD) of the wells was determined at 570 nm through a spectrophotometric microplate reader (Biotek, EL x 800. USA).

Development of paclitaxel-resistant MCF-7 cells

MCF-7 cells were cultivated as explained above and cells with about 20-30% confluency were treated with the concentrations of paclitaxel. Initial treatment dose was one-tenth of IC_{50} of paclitaxel (0.5 nM) which was determined via MTT assay. Paclitaxel resistant MCF-7 cells were established by treating cells with continues and stepwise paclitaxel doses (0.5 to 64 nM). Culture medium for growth of paclitaxel-resistant MCF-7 cells was enriched with 20% FBS and 10% conditioning medium (Conditioning medium was the supernatant medium of Non-resistant MCF-7 cell line with about 80% confluency). Cells can be subculture when become confluent then dose of paclitaxel was increased to 1.5 time of previous dose and this process continued to reach 64nM paclitaxel concentration.

Generally, cells will tolerate the lower doses of paclitaxel, when the cells look not to have tolerated a drug treatment it is unwise to repeat another round of drug treatment so cells maintained in previous dose for more days. MTT assay was applied to confirm the resistance

cells to each dose of paclitaxel (Coley, 2004).

Evaluation of the morphology of paclitaxel-resistant cells

To study of alteration in morphology of paclitaxel-resistance cells, light microscope was applied to take images from parental MCF-7, MCF-7/Pac5nM and MCF-7/Pac 64nM cells. Fluorescence-activated cell sorting (FACS) was used to examine size and granularity of the cells. The cells were washed and resuspended in 2 ml PBS and then the size and granularity of the cells were evaluated by forward scatter (FSC) and side scatter (SSC) factors, respectively. About 10,000 cells were assessed for each sample.

Assessment of mRNA expression via Real-time RT-PCR

mRNA expression of *Bcl-2* and *Bax* genes involved in apoptosis pathway were investigated in parent MCF-7 cell line and two paclitaxel- resistant MCF-7 cells including MCF-7/Pac5nM and MCF-7/Pac 64nM cells. RNA extraction of the cells was done by RNA extraction kit (RNX-Plus) according to the protocol. Quality of RNA was qualified by agarose gel electrophoresis. Concentration of extracted RNA was evaluated by optical density measurement (A260/A280 ratio) with NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA). REVERTA-L RT reagents kit was applied for conversion of RNA to cDNA. Then the reaction tubes were incubated at 42°C for 60 min. Real time PCR was carried out using the SYBR Green-based PCR Master Mix and analyzed on a Corbett 6000 Rotor-Gene thermocycler (Corbett Research). Beacon Designer™ 5.01 software was used to design Primers for cDNA amplification (Primers sequences are showed in Table 1). Total volume of amplification reactions was 25 μL and each well was included 12.5 μl of SYBR Green PCR Master Mix, 1 μl of cDNA, 70-100 nM of both forward and reverse special primers. The PCR thermal cycling steps were included 10 min at 95°C, 40 cycles of 25 s at 95°C for denaturation step, 25 s at annealing temperature, and 25 s at 72°C for the extension respectively. Final 10 min incubation at 72°C was carried out to completion of amplicons. The 18S rRNA expression was provided as an internal reference gene (housekeeping gene) to normalize the expression of the apoptotic genes. Melting-curve analysis was carried out after amplification to verification the validity of the amplicon. Pfaffl method was applied for reporting gene expression level.

Measurement of Caspase-8 and Caspase-9 protein levels

Caspase-8 and Caspase-9 levels were evaluated in MCF-7/Pac5nM and MCF-7/Pac 64nM cells and compared with their levels in control MCF-7 cell line using enzyme-linked immunosorbent assay (Platinum ELISA; eBioscience©) according to the instructions of the manufacturer.
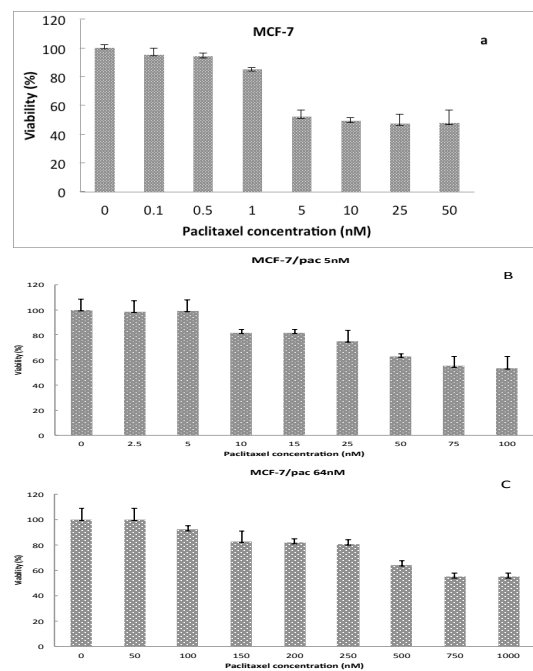
Statistical analysis

All statistical analyses were performed using SPSS 16.0 software. Data obtained from three or more separate experiments were expressed as mean±SD. Data were analyzed by One-Way ANOVA and Tukey post Hoc tests. p-values less than 0.05 were considered as statistically significant.

Results

IC$_{50}$ 0f parent MCF-7 cells and paclitaxel-resistant MCF-7 cells

In order to evaluate the cytotoxicity of paclitaxel, cells were exposed to various concentration of paclitaxel and their viability was evaluated via MTT assay. Our results showed an IC$_{50}$ level of 5nM for MCF-7 cells (Figure 1a). Then, paclitaxel-resistant MCF-7 cells were developed by stepwise increasing concentration of paclitaxel (0.5 to 64nM). Among which, MCF-7 resistant to 5nM (MCF-7/Pac 5nM) and 64nM (MCF-7/Pac 64nM) paclitaxel were selected for further investigation. IC$_{50}$ levels for MCF-7/

Table 1. Applied Primer sequences for Quantitative PCR

Gene name	Primer sequence	Amplicon length (bp)	Tm (°C)
18S rRNA	F: 5'-CGATGCGGCGGCGTTATTC-3'		
	R: 5'-TCTGTCAATCCTGTCCGTGTCC-3'	198	59
Bcl-2	F: 5'-CATCAGGAAGGCTAGAGTTACC-3'		
	R: 5'-CAGACATTCGGAGACCACAC-3'	181	61
Bax	F: 5'- GATGCGTCCACCAAGAAG-3'		
	R: 5'- AGTTGAAGTTGCCGTCAG-3'	163	54

Table 2. *Bcl-2/Bax* Ratio was Calculated and Show that it was Significantly Increased in MCF-7/Pac 5nM and MCF-7/Pac 64nM Campare with Parent MCF-7 Cell Line

Gene	Control	MCF-7/Pac 5 nM	MCF-7/Pac 64 nM
Bcl-2	1	1.243	1.258
Bax	1	0.545	0.132
Bcl-2/Bax	1	2.280	9.486

**Figure 1. Column Charts of MTT Assay Results of Parent MCF-7 Cell Line (a), MCF-7/Pac 5nM (b) and MCF-7/Pac 64nM (c)**

pac 5 nM and MCF-7/pac 64 nM cells were increased from 3.5 nM to 55 nM and 500 nM, respectively (p<0.05) (Figure 1b, c).

Bcl-2/Bax ratio and expression of Bax and Bcl-2 genes in paclitaxel resistance cells

Our results from Real time-RT PCR method showed that *Bax* mRNA level decreased in both resistant cells compared to non-resistant cells. Decrease in mRNA expression level of *Bax* gene was more significant in MCF-7/Pac64 nM cells than MCF-7/Pac5 nM (p<0.001) (Table 2). The mRNA expression level of *Bcl-2* was elevated in both resistant cells. However, these was no significant differences between two different resistant cells (p>0.05) (Table 2). *Bcl-2/Bax* ratio is a key indicator in susceptibility of the cells to apoptosis. Our results revealed a significant increase in *Bcl-2/Bax* ratio in both paclitaxel resistant cells (p<0.001; Table 2).

*Caspase-8 and 9 proteins level in paclitaxel-resistant MCF-7 cells*

ELISA method was used for evaluation of Caspase-8 and 9 protein levels. Level of caspase-8 was significantly increased in MCF-7/Pac5nM (p<0.01) and MCF-7/Pac
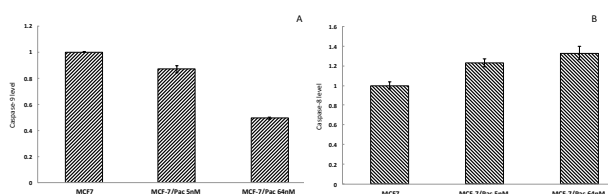
Figure 2. Evaluation of Caspase-8 and 9 by ELISA. a) level of Caspase-9 decreased in both of paclitaxel resistant cell lines compare with parent MCF-7 cell line, MCF-7/Pac5nM (p<0.001), MCF-7/Pac 64nM (p<0.001). **b)** However, level of Caspase-8 rose significantly in MCF-7/Pac5nM (p<0.01) and MCF-7/Pac 64nM (p<0.003) compared with MCF-7 cell line
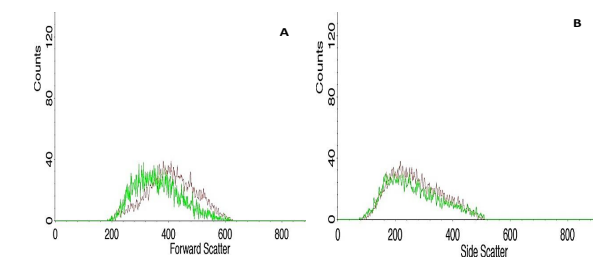
Figure 4. FSC Flow Cytometry. A) MCF -7/Pac64 cell was relatively smaller than MCF-7 parental cells, as revealed by a leftward shift in the mean FSC: B) However SCC flow cytometry showed that no significant difference in complexity between MCF -7/Pac64 cell compared with MCF-7 parental
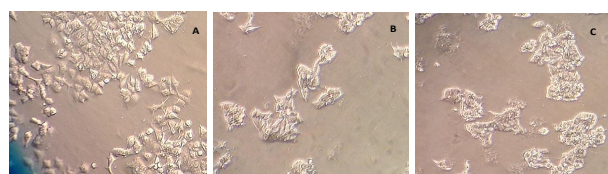
Figure 3. Light Microscopic Image Displayed Morphology of Parental MCF-7 and two Resistant Cell Lines. A) Normal MCF-7 cell line, B) MCF-7/Pac5nM, C) MCF-7/Pac64nM

64nM (p<0.003) compared with control cells. However, there was a significant decrease incaspase-9 level in both resistant cells (p<0.001). There were significant differences in the levels of caspase-8 and caspase-9 between two resistant cells (p<0.001) (Figure 2).

Morphology alteration in MCF-7/Pac5nM and MCF-7/Pac 64nM cells

Light microscopic observations indicated alterations in the morphology of both resistant cells. These resistant cells were more condensed than parent MCF-7 cells. MCF -7/Pac64 cells also showed more condensity than MCF-7/Pac5 cells (Figure 3). Our results from FACS analysis revealed that MCF -7/Pac64 cells were relatively smaller than control cells (Figure 4a) but there was no significant difference in granularity between MCF -7/Pac64 and control cells (Figure 4b). Our results showed no significant differences in size and density between two different paclitaxel-resistant cells.

Discussion

Paclitaxel is an important anticancer drug that is clinically used for patients with various types of cancers; however, tumor cell resistance to this agent remains the main obstacle in successful cancer therapy. Investigation of molecular mechanisms involved in the chemo-resistance can be helpful in designing novel strategies in cancer treatment. Paclitaxel resistant MCF-7 cell line was developed by some of researchers for study about resistance mechanisms and involved molecules in this cell line for example; paclitaxel binding proteins, extracellular matrix proteins, drug transporters and β-tubulin isoforms (Hembruff et al., 2008; Iseri et al., 2010; Zuo et al., 2010; Wang et al., 2014). In this study, we investigated role of some of key apoptotic proteins in paclitaxel resistant breast cancer cells. We first established paclitaxel -resistant breast cancer cells from MCF-7 cell line. After determination of cytotoxicity in resistant and non-resistant cells, the cells resistant to IC_{50} value and the highest dose of paclitaxel were chosen as two resistant cells for mechanistic studies to evaluate mRNA expression levels of *Bax*, *Bcl-2* as well as caspases-8 and -9 protein levels.

Bax is a proapoptotic *Bcl-2* family member that plays a key role in induction of mitochondrial dependent apoptosis. *Bcl-2* is an antiapoptotic member that can neutralize *Bax* function in initiation of cell death. Proteins that stimulate or inhibit apoptosis determine the fate of the cell for death or survive. High *Bax* expression stimulates sensitivity to apoptotic agents and decreases tumor enlargement (Bargou et al., 1996).On the other hand, low expression of *Bax* inhibits apoptotic pathways. Overexpression of anti-apoptotic *Bcl-2* gene occurs in many of cancer cells, and prevents cell death induced by nearly all anticancer drugs and radiation. Our findings demonstrated that the expression of *Bcl-2* was markedly increased in our developed paclitaxel resistant cells, which is in agreement with previous studies (Crawford and Nahta 2011; Shajahan et al., 2012).

The ratio of *Bcl-2/Bax* is a key regulator for regulation of cytochrome c release from the mitochondria and it also

determines the susceptibility of a cell to apoptosis (Oltvai et al., 1993; Yang and Korsmeyer 1996). In addition, many of the components in this pathway are currently being explored as markers for tumorigenesis and potential targets for treatment (Gee et al., 1994; Piche et al., 1998). Our results showed a 2.28 fold increase in *Bcl-2/Bax* ratios for MCF-7/Pac 5nM and 9.48 fold increase for MCF-7/Pac 64 nM compared with non-resistant MCF-7 cells (*Bcl-2/Bax* ratio=1). High *Bcl-2/Bax* ratio can cause inhibition of apoptosis and resistant to chemotherapeutic agents (Leung and Wang 1999; Childs et al., 2002).

The pro and anti-apoptotic members of the *Bcl-2* and their associated proteins are integrated with survival signals and coupled to the release of mitochondrial cytochrome c and the activation of caspases (Chinnaiyan 1999). Stimulation of extrinsic and/or intrinsic apoptotic pathways could leads to the activation of caspase proteins (Degterev et al., 2003; Fulda and Debatin 2006). In the typical cell models, caspases are classified into initiator (for example caspase-8, -9) and effector (caspase-3, -6, -7), according to their role and order of activation in apoptosis (Strasser et al., 1995; Thornberry and Lazebnik 1998). Caspase-8 is involved in receptor-dependent apoptotic pathway, while caspase-9 is a member of mitochondrial-dependent apoptosis (Cryns and Yuan 1998; Ouyang et al., 2012). It was expected that in resistant cells, the level of caspase-8 should be low or identical to parent MCF-7 cell. Interestingly, in both of MCF-7/Pac5nM and MCF-7/Pac 64nM cells, the level of caspase-8 is elevated. This result can be explained by the lack of caspase-3 protein level in MCF-7 cells (Tang et al., 2000; Ferreira et al., 2012). Then, caspase-8 can be the best substitute for caspase-3 in this cell line to proceed apoptosis (Simstein et al., 2003). For activation of caspase-8, participation of activated caspase-3 is crucial. Since MCF-7 cells have some nucleotide deletion incaspase-3 gene (Janicke et al., 1998), increased level of caspase-8 couldn't trigger extrinsic apoptosis pathway in paclitaxel resistance cells; MCF-7/Pac5nM and MCF-7/Pac 64nM.

In line with previous study (Scaffidi et al., 1998) we didn't find the involvement of the extrinsic apoptotic pathway in MCF-7 cells.

Down regulation of caspase-9 is expected to take place in resistant cells. Likewise, decreased level of caspase-9 indicates that resistance to paclitaxel occurs via suppression of intrinsic pathway. These results are compatible with our findings for the *Bcl-2/Bax* ratio, since *Bcl-2* family controls the intrinsic apoptotic pathway.

Based on these results, additional studies on modulators of the *Bcl-2/Bax* pathways such as hormones, chemotherapeutic agents, and genetic background may be necessary to fully understand the potential of this pathway in cancer diagnosis and therapy. Further clarification of the *Bcl-2* regulating mechanism by various signals would be important.

In conclusion, our data suggest that increased in the *Bcl-2/Bax* ratio and decreased in caspase-9 level can play a key role in the resistance of MCF-7 cells to paclitaxel, however further studies are warranted to validate these findings.

Acknowledgements

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