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Brief Note

Influence of 2-methoxyestradiol on MCF-7 cells: An improved differential interference contrasting technique and Bcl-2 and Bax protein expression levels

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ABSTRACT: Proteins of the B-cell lymphoma 2 family are crucial for the regulation of apoptosis. B-cell lymphoma 2-associated X is a pro-apoptotic protein, while B-cell lymphoma 2 protein opposes apoptosis. The influence of 1 μM 2-methoxyestradiol was investigated on the expression levels of these two proteins in MCF-7 cells. 2-Methoxyestradiol exposure did not influence B-cell lymphoma 2 protein expression levels after 24 h of exposure. In contrast, B-cell lymphoma 2-associated X protein levels were significantly reduced. An improved differential interference contrasting technique revealed compromised cell density and the presence of a mitotic block in exposed cells. The study proposes that the influence of 2-methoxyestradiol on the expression of these proteins may be time- and cell type dependent and thus not evident during the mitotic block observed. Investigation of the regulation of the B-cell lymphoma 2 family will allow researchers to consider signaling pathways for diseases where apoptosis can potentially be controlled.

Proteins of the B-cell lymphoma 2 family are important regulators of apoptosis. B-cell lymphoma 2-associated X protein is a pro-apoptotic protein, while B-cell lymphoma 2 protein opposes apoptosis. Induction of mitochondrial membrane permeability transition has been associated in the cascade of events involved in the induction of apoptosis (Isenberg and Klaunig, 2000). Cytochrome *c* release from mitochondria is controlled by the B-cell lymphoma 2 family of proteins. Pro-apoptotic proteins namely B-cell lymphoma 2-associated X protein promote release of cytochrome *c* from the mitochondria resulting in apoptosis. Inhibition of apoptosis is orchestrated by B-cell lymphoma 2 and B-cell lymphoma-xL proteins by inhibition of cytochrome *c* release from the mitochondria (Fletcher *et al.*, 2008).

It has recently been shown in our laboratory and by other researchers that an increased ratio of pro-apoptotic B-cell lymphoma 2-associated X protein to anti-apoptotic B-cell lymphoma 2 protein may be associated with apoptosis (Joubert *et al.*, 2005a, b; Kang *et al.*, 1998; Xiao and Zhang, 2008). Liu *et al*. (2004) also reported that an increased mitochondrial B-cell lymphoma 2-associated X protein/B-cell lymphoma-xL protein ratio led to induced B-cell lymphoma 2-associated

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X protein activation in the human leukemic K562 cell line.

Mitotic spindle inhibitors can also induce apoptosis by altering the B-cell lymphoma 2-associated X/B-cell lymphoma 2 ratio (Zhang and Raveche, 1998). Exposure of human melanoma cells to 2-methoxyestradiol, another anti-mitotic drug and disruptor of tubulin polymerization, revealed higher levels of B-cell lymphoma 2-associated X protein and undetectable levels of B-cell lymphoma 2 protein when compared to controls (Ghosh *et al.*, 2003; Tinley *et al.*, 2003).

Since it has previously been reported by our laboratory and other researchers that 2-methoxyestradiol plays an important role in the induction of apoptosis and especially in cells showing carcinogenic properties (Joubert *et al.*, 2005a, b; Joubert and Marais, 2007a, b; Kar *et al.*, 2008) the influence of 2-methoxyestradiol on B-cell lymphoma 2-associated X protein and B-cell lymphoma 2 protein expression in human breast adenocarcinoma cells was visualized by means of PlasDIC, which is an improved method for polarization-optical transmitted light differential interference contrast where, unlike conventional Smith-Nomarski's method, linearly polarized light is only generated after the objective, giving images of outstanding quality.

2-Methoxyestradiol, Dulbecco's Modified Eagle's Medium with glucose, sodium pyruvate and L-glutamine, Trypsin-EDTA and Trypan blue were purchased from Sigma Chemical Co. (St. Louis, USA). Heat-inactivated fetal calf serum, sterile cell culture flasks and plates were supplied by Sterilab Services (Johannesburg, SA). Phosphate buffered saline was purchased from Gibco BRL through Laboratory Specialist Services (Johannesburg, SA). Penicillin, streptomycin and fungizone were obtained from Highveld Biological (Pty) Ltd. (Sandringham, SA). Goat-anti-mouse IgG (heavy and light chain) peroxidase conjugate, mouse anti-B-cell lymphoma 2 (clone Bcl-2 100) antibody and mouse anti- B-cell lymphoma 2-associated X (clone 2DC concentrate) antibody were provided by Sterilab Services (Johannesburg, SA). The Bio-Rad Dye Reagent Concentrate protein assay was purchased from Bio-Rad Laboratories (München, Germany) and supplied by S.A. Scientific Inc. (Midrand South Africa). All other chemicals were of analytical grade and supplied by Sigma Chemical Co. (St. Louis, USA).

The MCF-7 (human breast epithelial carcinoma) cell line was purchased from Highveld Biological (Pty) Ltd. (Sandringham, SA). Cells were propagated and maintained as monolayer cultures in DMEM supplemented with 10% heat inactivated fetal calf serum and a 10% mixture of 10 µg/mL penicillin, 10 µg/mL streptomycin and 25 µg/mL fungizone at 37ºC in a humidified atmosphere containing 5% CO_2. Stock solutions of 2-methoxyestradiol were prepared in dimethyl sulfoxide. Solvent concentrations in the media never exceeded 0.1% (v/v).

Viable cells (5 x 10^5) were seeded in 25 cm^2 culture flasks. PlasDIC images were obtained using the Zeiss Axiovert-40 microscope (Göttingen, Germany) after 24 h of exposure to vehicle controls or 1 µM 2-methoxyestradiol respectively. Subsequently, cells were

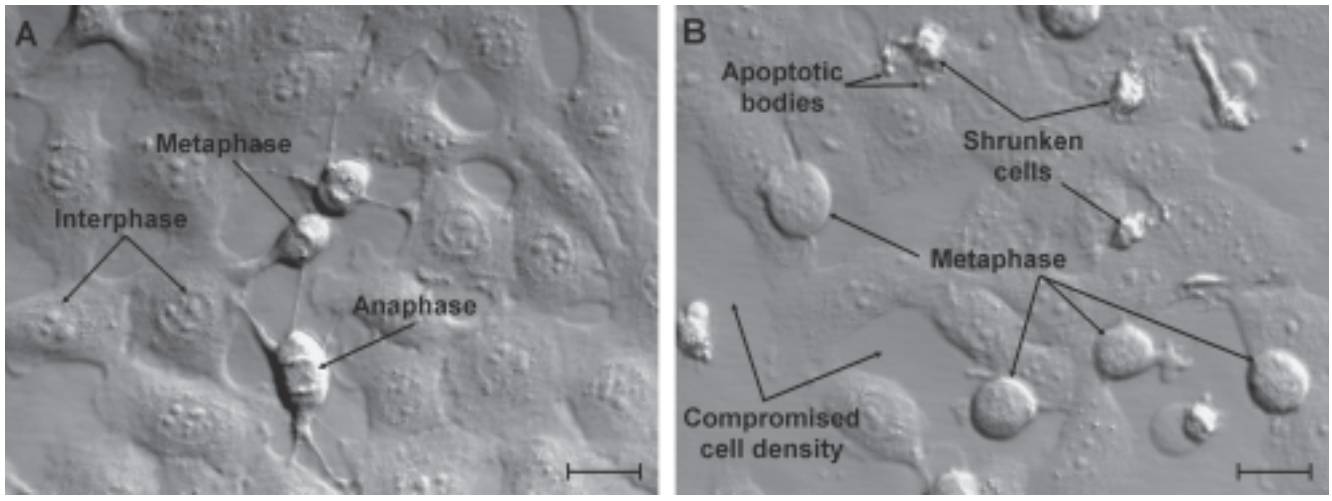

FIGURE 1. PlasDIC images of MCF-7 cells exposed to dimethyl sulphoxide control (a) and 1µM 2-methoxyestradiol (b) after 24 h of exposure (scale bar indicates 10 µm).

harvested, homogenized in saline (150 mM NaCl, pH 7.4) and total protein concentrations of each cell extract were determined by means of the Bio-Rad Dye Reagent Concentrate protein assay according to the manufacturer's instructions. Samples of total protein concentration (0.1mg total protein per well) were coated onto a 96 well microtiter plate, dried under a 150 W lamp in a stream of air generated by an electric fan and subsequently blocked in 300 µl of phosphate buffered saline (pH 7.4) containing 0.5% casein, for 60 minutes at 37ºC. Blocking medium was replaced with cell culture supernatant containing the monoclonal antibody (diluted 1:100 in blocking buffer) and incubated at 37ºC for 45 min after which the plates were washed three times in blocking buffer and incubated for 30 minutes with goat-anti-mouse IgG (heavy and light chain) peroxidase conjugate at a 1:500 dilution with blocking buffer. After a second washing step, 100 µl of developing buffer (10 ml citrate, 10 mg o-phenylene diamine and 8 mg hydrogen peroxide, pH 4.5) was added and the reaction monitored at 450 nm with a SLT 340 ATC scanner (SLT Labinstruments, Austria).

Data obtained from independent experiments are shown as the mean ± SD and were statistically analysed for significance using the analysis of variance (ANOVA)-single factor model, followed by a two-tailed Student's t-test. Means are presented in bar charts, with T-bars referring to standard deviations. *P*-values of <0.05 were regarded as statistically significant.

The influence of 1 µM 2-methoxyestradiol was visualized by means of PlasDIC as differential contrasting technique (Fig. 1a, b) and evaluated on the expression levels of B-cell lymphoma 2-associated X protein and B-cell cymphoma 2 protein in MCF-7 cells compared to vehicle-treated controls after 24 h of exposure (Fig. 2a, b). PlasDIC of treated cells showed compromised cell density when compared to vehicle controls. An increase in the amount of cells in mitosis (rounded cells) indicative of a mitotic block and previously demonstrated in our laboratory (Van Zijl *et al.*, 2008), was also evident. A significant decrease in 2-methoxyestradiol-exposed cells was observed in B-cell lymphoma 2-associated X protein expression levels when compared to vehicle-treated controls ($P<0.05$) (Fig. 2a). In contrast, no statistically significant effects on B-cell lymphoma 2 protein expression levels were observed after exposure of MCF-7 cells to 2-ME (Fig. 2b).

Our previous research has shown 2-methoxyestradiol's antiproliferative effects, overexpression of extracellular signal regulated protein kinase and subsequent induction of apoptosis (Joubert *et al.*, 2005a, b; Joubert and Marais, 2007a, b) in cancerous cells. These results also suggested that the increased ratio of pro-apoptotic B-cell lymphoma 2-associated X protein to anti-apoptotic B-cell lymphoma 2 protein appear to be associated with 2-methoxyestradiol-induced apoptosis observed in HeLa and esophageal carcinoma cells (Joubert *et al.*, 2005a, b). However, the present study proposes that the influence of 2-methoxyestradiol on these protein expression levels may be cell type- and time-dependent and therefore not evident during the mitotic block observed after 24 h of exposure to 2-methoxyestradiol in MCF-7 cells. Investigation of the regulation of the B-cell lymphoma 2 family will contribute to considering signaling pathways for diseases where apoptosis can potentially be controlled.

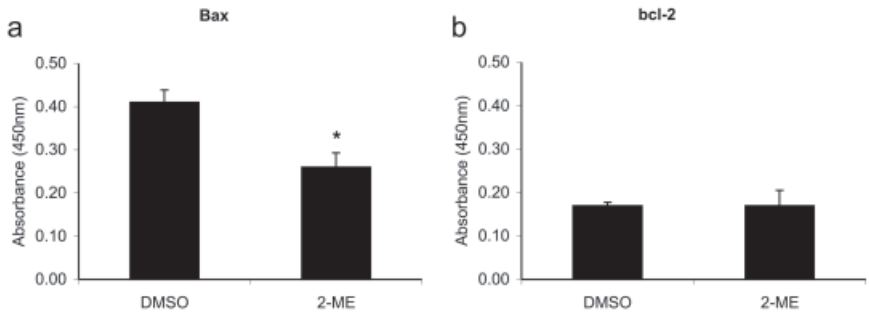

FIGURE 2. B-cell lymphoma 2-associated X (indicated as Bax on the graph) (a) and B-cell lymphoma 2 (indicated as bcl-2 on the graph) (b) protein expression levels of MCF-7 cells after 24 h of exposure to dimethyl sulphoxide and 2-methoxyestradiol respectively (asterisk indicates a statistically significant difference, $P<0.05$).

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