

Bax/Bcl-2 expression levels of 2-methoxyestradiol-exposed esophageal cancer cells

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ABSTRACT

2-Methoxyestradiol (2ME), an endogenous metabolite of 17 β -estradiol, has been reported to play an active role in the induction of apoptosis in both proliferating endothelial and cancer cells. Since it has been indicated that an increased ratio of pro-apoptotic Bax protein to anti-apoptotic Bcl-2 protein expression can be associated with apoptosis, and since the exact action mechanism of 2ME is still not clearly defined and appears to vary according to cell type, the influence of 1 μ M 2ME was investigated on Bax and Bcl-2 expression levels in squamous esophageal carcinoma cells. 2ME exposure led to statistically significant decreases (0.69 over DMSO controls) in Bcl-2 expression levels. In contrast, no statistically significant effects were observed on Bax expression levels after exposure to 2ME. The Bax/Bcl-2 ratio for 2ME-exposed cells was 1.45, normalised against Bcl-2 levels. Although the exact mechanisms of apoptosis induction in squamous esophageal cancer cells require further investigation, the present study suggests that this altered ratio in favor of Bax could lead to the induction of apoptosis in these cells.

2-Methoxyestradiol (2ME), once considered an inactive end-metabolite of 17 β -estradiol, has been reported to contain antiproliferative effects on various tumor cell lines regardless of the cell's hormone receptor status (10). Early research on 2ME's biological activity stated its effect on mitotic spindles and cell cycle progression. 2ME caused mitotic accumulation and abnormal mitotic spindle formation in both estrogen receptor (ER) positive- and ER negative cells (12). Furthermore, 2ME targeted proliferating endothelial cells as well as tumor cells, culminating into the initiation of apoptosis. As a consequence, this endogenous estradiol metabolite has emerged as a promising anticancer agent (9).

New evidence with regard to 2ME's action mechanism implicates the involvement of both the extrinsic and intrinsic pathways of apoptosis induction. However, the exact mechanism of 2ME is still not clearly defined and appears to vary according to cell type (11). In addition, we have previously shown that degree of differentiation of esophageal carcinoma cells influences the susceptibility of tumor cells to the anti-mitogenic effects of prostaglandinA₂, another endogenous metabolite playing an active role in the induction of apoptosis. More pronounced effects were observed in less differentiated squamous esophageal cancer cell lines, while more differentiated and normal cells appeared to be less affected (5).

Dose-dependent studies revealed that 10⁻⁶ M 2ME was most inhibitory to WHCO3 cell proliferation and decreased cell numbers by almost 60% (13). Light microscopy as well as electron microscopy demonstrated hallmarks of apoptosis in WHCO3 cells. These hallmarks included cell shrinkage,

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membrane blebbing, micro-nuclei formation, condensed chromatin, nuclear fragmentation and intact cell membranes after 24 h of WHCO3 exposure to 10^{-6} M 2ME. In addition, flow cytometry showed that 10^{-6} M 2ME also caused spindle disruption and a G₂/M arrest of WHCO3 cells thereby preventing them from proceeding through the cell cycle (13). Furthermore, it has been revealed that 2ME displays a biphasic pattern on cell proliferation at concentrations ranging from 10^{-8} M to 10^{-5} M, namely a stimulatory effect at low concentrations and an inhibitory effect at the highest concentration. 2ME caused the strongest inhibitory effect at a concentration of 10^{-6} M (7). Since we have also previously observed morphological hallmarks of apoptosis in 2ME-exposed squamous esophageal carcinoma cells (3) and since an increased ratio of pro-apoptotic Bax protein to anti-apoptotic Bcl-2 protein can be associated with the induction of apoptosis (2, 6, 8), the effect of 2ME on Bax and Bcl-2 protein expression levels were investigated in squamous esophageal carcinoma cells in the search for the underlying mechanism(s) leading to the induction of apoptosis.

The WHCO3 cell line (a poorly differentiated non-keratinising squamous esophageal cell carcinoma) was a gift from Professors Thornley and Veale (Department of Zoology, University of the Witwatersrand, Johannesburg, SA). WHCO3 cells were obtained through a biopsy from a patient with squamous esophageal carcinoma. 2ME, Eagle's Minimum essential medium (EMEM) with Earle's salts, L-glutamine and NaHCO₃, Trypsin-EDTA and Trypan blue were supplied by Sigma Chemical Co. (St. Louis, USA). Heat-inactivated fetal calf serum, sterile cell culture flasks and plates were obtained through Sterilab Services (Johannesburg, SA). Phosphate buffered saline (PBS) 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 (H+L) peroxidase conjugate, mouse anti-Bcl-2 (clone Bcl-2 100) antibody and mouse anti-Bax (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 (Munich, 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).

Cells were grown and maintained as monolayer cultures in minimum essential medium, containing

10% heat inactivated fetal calf serum and a 10% mixture of 10 mg/ml penicillin, 10 mg/ml streptomycin and 25 mg/ml fungizone at 37°C in a humidified atmosphere containing 5% CO₂. A Stock solution of 2ME was prepared in dimethyl sulfoxide (DMSO). The solvent concentrations in the media never exceeded 0.05%.

500 000 viable cells were seeded in 25 cm² culture vessels and incubated for 24 h. Cells were harvested after 24 h of exposure to vehicle controls and 1 µM 2ME. Cells were homogenised in saline (150 mM NaCl, pH 7.4). 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 known protein concentration (0.1 mg 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 ml of PBS (pH 7.4) containing 0.5% casein, for 60 min at 37°C. Blocking medium was replaced with cell culture supernatant containing the monoclonal antibody (diluted 1 : 100 in blocking buffer) and incubated at 37°C for 45 min after which the plates were washed three times in blocking buffer and incubated for 30 min 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 ml 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 effect of 1 µM 2ME was evaluated on the expression levels of Bax and Bcl-2 in WHCO3 cells compared to vehicle-treated controls after 24 h of exposure (Figs. 1-2). A statistically significant decrease (indicated by an * on the graph) was observed in Bcl-2 expression levels of 2ME-exposed cells when compared to vehicle-treated controls (*P* < 0.05). Bcl-2 expression levels were decreased 0.69-fold over DMSO controls (Fig. 1). In contrast, no statistically significant effects on Bax expression levels were observed after exposure of WHCO3 cells to 2ME (Fig. 2). The Bax/Bcl-2 ratio for 2ME-

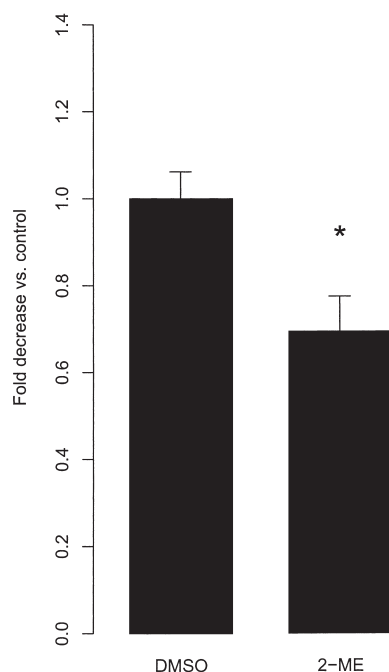


Fig. 1 Bcl-2 expression levels of 2ME-exposed WHCO3 cells and vehicle-treated control cells after 24 h. Bcl-2 expression levels were normalised with regard to vehicle-treated control cells. 2ME exposure led to a statistically significant 0.69-fold decrease (indicated by an * on the graph) in Bcl-2 expression levels over vehicle-treated controls ($P < 0.05$).

exposed WHCO3 cells was 1.45, normalised against Bcl-2 levels.

Several researchers have confirmed biological activities of 2ME in diverse cancer cell types and in endothelial cells. These activities include mitotic accumulation, formation of abnormal mitotic spindles, the influence on cell cycle progression and the effect on the ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2, ultimately leading to initiation of apoptosis (9, 14). Our previous research has demonstrated that 2ME causes significant decreases in cell growth, overexpression of extracellular signal regulated protein kinase (ERK1/2) and subsequent induction of apoptosis (4, 5, 12, 13). It was found that 2ME-exposed human melanoma cells showed higher levels of Bax and undetectable levels of Bcl-2 compared to untreated controls (2). Zhang and Raveche (1998) indicated that an altered Bax/Bcl-2 ratio caused by mitotic spindle inhibitors, can lead to the induction of apoptosis in malignant B-1 cells (15). Similarly to our findings in this paper, El-Rayes *et al.* (1) also reported no significant effect on the level of Bax expression in prostate cancer cells (PC-3) treated with ciprofloxacin, a relatively nontoxic anti-

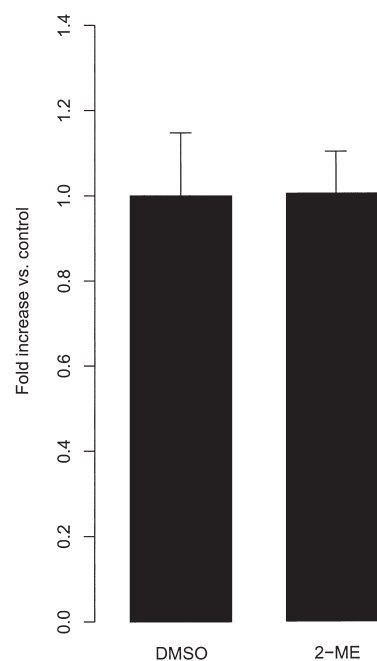


Fig. 2 Bax expression levels of 2ME-exposed WHCO3 cells and vehicle-treated control cells after 24 h. Bax expression levels were normalised with regard to vehicle-treated control cells. No statistically significant effect on Bax expression levels was observed after exposure to 2ME.

biotic which has recently been confirmed to have anti-tumor activity in a variety of human tumor cells. However, although no significant effect on the level of Bax expression was observed, they demonstrated down-regulation of Bcl-2 expression, similarly to our findings in this paper. This effect led to an increase in the ratio of Bax/Bcl-2 (1.8-fold when compared to the control) (1). They also suggested that this altered ratio could contribute to the induction of apoptosis.

2ME is thought to be well tolerated according to data obtained from initial Phase I and II clinical trials and currently being evaluated in multiple tumor types (9). In addition, preclinical studies contribute to the search of clarifying 2ME's diverse cellular effects that terminate in apoptosis induction.

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