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In vitro effects of 2-methoxyestradiol on cell numbers, morphology, cell cycle progression, and apoptosis induction in oesophageal carcinoma cells

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The influence of 2-methoxyestradiol (2-ME) was investigated on cell numbers, morphology, cell cycle progression, and apoptosis induction in an oesophageal carcinoma cell line (WHCO3). Dose-dependent studies (1×10^{-9}M–1×10^{-6}M) revealed that 2-ME significantly reduced cell numbers to 60% in WHCO3 after 72 h of exposure at a concentration of 1×10^{-6}M compared to vehicle-treated cells. Morphological studies entailing light-, fluorescent-, as well as transmission electron microscopy (TEM) confirmed 2-ME's antimitotic effects. These results indicated hallmarks of apoptosis including cell shrinkage, hypercondensation of chromatin, cell membrane blebbing, and apoptotic bodies in treated cells. Flow cytometric analyses demonstrated an increase in the G_2/M-phase after 2-ME exposure; thus preventing cells from proceeding through the cell cycle. β-tubulin immunofluorescence revealed that 2-ME caused spindle disruption. In addition, increased expression of death receptor 5 protein was observed further supporting the proposed mechanism of apoptosis induction via the extrinsic pathway in 2-ME-exposed oesophageal carcinoma cells. Copyright © 2009 John Wiley & Sons, Ltd.

KEY WORDS — oesophageal carcinoma; 2-methoxyestradiol; metaphase block; apoptosis

INTRODUCTION

2-Methoxyestradiol (2-ME), a 17-beta estradiol metabolite, is a mitogen antagonist and tubulin poison that hinders cell proliferation and induces apoptosis in a large diversity of non-tumor and tumor cells.[1–4] 2-ME implements both its antiangiogenic and antitumor influence regardless of the cell's hormone receptor status and is accountable for abnormal mitotic spindle formation and mitotic accumulation in both estrogen receptor (ER) positive- and ER-negative cells.[5–7] Accordingly, this endogenous estradiol metabolite has manifested as a potential anticancer agent.[5]

Current evidence has suggested that 2-ME is the causative agent leading to an increase in Cdc2 kinase activity, the activation of c-Jun NH_2-terminal kinase signaling, generation of reactive oxygen species and an altered ratio of Bax/Bcl-2 in favor of Bax, ultimately culminating into apoptosis.[8–11] Cell division cycle (Cdc) 2 kinase activity is a cell cycle regulatory component essential for commencement of mitosis, whereas Cdc2 inactivation is needed for mitotic exit. Prolonged Cdc2 activity can sustain the cell in mitosis for an indefinite period until particular conditions are met for mitotic exit.[12] JNK is involved in the phosphorylation and inactivation of Bcl-2, a pro-apoptotic protein, thus contributing to apoptotic induction.[8,9] Nevertheless, apoptotic induction via the extrinsic and intrinsic pathways appears to be dependent on cell type.[13–15]

It is also known that 2-ME displays a dose-dependent biphasic pattern on cell proliferation at concentrations ranging from 10^{-8} to 10^{-5} M. Stimulatory effects have been demonstrated at low concentrations of 2-ME and inhibitory effects were observed at high concentrations.[3,11,16] Correspondingly, *in vivo* studies have demonstrated stimulation and inhibition of tumor growth by 2-ME depending on dosage.[17,18] In concert, these research studies imply a multifaceted nature of the action of 2-ME. Data illustrated that, in addition to the established signaling pathways there may be supplementary pathways that have not been identified contributing to biphasic effects of 2-ME.[13,14,16,19–21] This biphasic effect may also be cell line specific.

Preclinical data illustrated that 2-ME might be considered in the treatment of multiple myeloma, sarcoma and other solid tumors, therefore portraying it as a possible anticancer agent when compared to conventional chemotherapeutic treatments.[5,16,22,23] Phase I and phase II clinical trials with 2-ME revealed its therapeutic potential when administered to patients with metastatic breast cancers and prostate cancers with only minor side-effects in some of the patients namely hot flushes, reversible liver enzyme elevations, fatigue, and

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diarrhoea.[5,16,19,22] Clinical studies employing 2-ME in cancer patients revealed that treatment is linked with clinical advantages including prolonged, stable disease, partial or complete responses, and an exceptional safety profile.

Since the mechanism of action of 2-ME is multifaceted and appears to vary according to cell type[2,5,11,24] the aim of this study was to investigate the mechanism of action of 2-ME in an oesophageal carcinoma cell line by determining its influence on cell numbers, morphology, cell cycle progression, and apoptosis induction.

MATERIALS AND METHODS

Materials

2-ME, Eagles' Minimum Essential Medium with Earle's salts, L-glutamine and $NaHCO_3$ (MEM), Trypsin-EDTA, trypan blue, thymidine, hydroxyurea, anti-human Bcl-2 antibody, mouse monoclonal antibody against human β-tubulin (Clone 2-28-33) biotin-conjugated anti-mouse IgG (Fab-specific, developed in goat), FITC-conjugate diluent and ExtrAvidin®-FITC conjugate were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Hematoxylin, eosin, ethanol, xylol, and Entellan® fixative were purchased from Merck (Darmstadt, Germany). Propidium iodide was supplied by DAKO Chemical Supplies (Glostrup, Denmark). DAKO LSAB Kit was purchased from Dako Corporation (Santa Barbara, CA, USA). The death receptor five antibody and human anti-goat IgG were purchased from Calbiochem (Darmstadt, Germany). Heat-inactivated fetal calf serum (FCS), sterile cell culture flasks and plates were obtained though Sterilab Services (Kempton Park, Johannesburg, SA). Phosphate buffered saline (PBS), penicillin, streptomycin, and fungizone were obtained from Highveld Biological (Sandringham, SA). Quetol, Reynolds' lead citrate, aqueous uranyl acetate, and toludine blue were purchased from Merck Co. (Johannesburg, South Africa). All other chemicals were of analytical grade and supplied by Sigma Chemical Co. (St. Louis, MO, USA).

Cell cultures

WHCO3 cells were a gift from Professors Veale and Thornley (Department of Zoology, University of Witwatersrand, Johannesburg, South Africa). These cells were obtained though a biopsy from a patient with squamous oesophageal carcinoma and are described as a poorly differentiated, non-keratinizing cell line. Cells were propagated as monolayers in MEM at 37°C in a humidified atmosphere containing 5% CO_2. Media were supplemented with 10% heat-inactivated fetal calf serum, penicillin ($100\,\mu g\,L^{-1}$), streptomycin ($100\,\mu g\,L^{-1}$), and fungizone ($250\,\mu g\,L^{-1}$). Non-viable cells were excluded with the trypan blue staining procedure. Stock solutions of 2-ME were prepared in dimethyl sulphoxide (DMSO) at concentrations of 2×10^{-3}M and stored at room temperature. The DMSO content of the final dilutions never exceeded 0.1% (v/v). Controls included showed that 0.1% had no toxic effects on these cells in experiments conducted.

Dose-dependent growth studies

Exponentially growing WHCO3 cells were seeded in 24-well culture plates at a density of 20 000 viable cells per well and exposed to a dilution series of 2-ME with a final concentration of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M respectively for 72 h at 37°C. The experiment was terminated by replacing the growth medium with 300 µl of 1% glutaraldehyde in PBS for 15 min. Crystal violet (1%, in PBS) was added for 30 min. The culture wells were subsequently immersed in running tap water for 15 min. After the plates had dried, 500 µl of 0.2% Triton X-100 was added to each well. Plates were incubated for 90 min and 200 µl of the liquid content was transferred to 96-well plates.[25] The absorbance (measured at 570 nm) of the samples was analyzed using an EL_X800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden SA). Results shown are representative of three independent experiments (each conducted in triplicate).

Morphology studies

Propidium iodide and hoechst 33342 staining. To study the viability and presence of apoptotic cells after 2-ME treatment, 500 000 WHCO3 cells were seeded onto heat-sterilized cover slips and exposed to 1×10^{-6} M 2-ME for 24 h. An exposure time of 24 h was chosen since significant reductions in cell number were visible after 24 h of treatment with 2-ME. Medium was removed, the cells were gently rinsed with PBS and 2 ml of $0.5\,\mu g\,ml^{-1}$ Hoechst 33342 (HO) in PBS was added to each well. Samples were incubated for 20 min at 37°C in a CO_2 incubator whereafter 0.5 ml of propidium iodide (PI) solution ($40\,\mu g\,ml^{-1}$ in PBS) was added. Within 5 min cover slips were mounted on microscope slides with mounting fluid (90% glycerol, 4% N-propyl-gallate, 6% PBS) and examined under a fluorescence microscope. Photographs were taken with 400 ASA film on a Nikon Optiphot microscope (Nikon, Tokyo, Japan) with UV-light and a blue filter. Viable and apoptotic (although having irregular appearances) cells will stain light blue. The latter phenomenon illustrates that these cells have functional cell membranes capable of excluding PI. However, cells with compromised membrane integrity will stain bright red.[26]

Hematoxylin and eosin staining (H and E staining)

WHCO3 cells (250 000) were seeded onto heat-sterilized cover slips in 6-well plates. Cells were exposed to 1×10^{-6} M 2-ME for a period of 24 h at 37°C. Many cells were not adherent to cover slips after exposures of 24–48 h and had disintegrated to become floating debris. Thus, it was not possible to observe morphological changes occurring during this period when studying the influence of 2-ME on WHCO3 cells by means of H and E staining. Cells were

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fixed in Bouin's fixative for 60 min after exposure to 2-ME and stained by standard hematoxylin and eosin staining procedures.[27]

Transmission electron microscopy (TEM)

Exponentially growing cells (500 000) were seeded in 25 cm^2 flasks and exposed to 0.1% DMSO (vehicle control) and 1×10^{-6} M 2-ME for 24 h respectively. Cells were washed with PBS (3x) and scraped off the bottom of the flask. Subsequently, ultra-thin sections of cells were prepared. Cells were fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer, (pH 7.4–7.6) for 1 h and rinsed 3 times for 5 min each with 0.075 M phosphate buffer. Thereafter sections were fixed in 0.25% aqueous osmium tetroxide and rinsed (3x) in distilled water in a fume hood. Samples were dehydrated in ethanol (70, 100%), infiltrated with 30% quetol in acetone for 1 h and furthermore infiltrated with 60% quetol in ethanol for 1 h, and thereafter with pure quetol for 4 h. Sections were polymerized at 65°C for 24–36 h. Ultra-thin sections were mounted on grids, contrasted for 10 min in 4% aqueous uranyl acetate and rinsed in water. Enhancement of contrast was obtained by placing the samples in Reynolds' lead citrate for 2 min and rinsing the samples in water. Samples were cut into 0.5 μm monitor sections, stained with toluidine blue, and immersed in immersion oil for TEM.

Cell cycle progression

WHCO3 cells were seeded into 25 cm^2 flasks as described above. Cell cycle analyses were performed after 24 h of exposure to 1×10^{-6} M 2-ME at 37°C. Cells were trypsinized in equal volumes of trypsin (0.25%) and EDTA (1 mM), fixed in 99.5% methanol and stored at −20°C. Methanol was removed by centrifugation at $200 \times g$ for 10 min. The sediments were resuspended in 1 ml 1% $CaCl_2$ and 50 μg ml^{-1} propidium iodide and incubated for 20 min while shaking gently. Each analysis was based on at least 10 000 events employing a Coulter Epic-XS flow cytometer. The data were analyzed using a multicycle analysis program (MulticycleAV software).

Immunofluorescent detection of β-tubulin

To visualize the effect of 2-ME on spindle formation in WHCO3 cells, indirect immunofluorescence was employed. Cells (500 000) were seeded onto heat-sterilized glass cover slips in 6-well plates. After exposure to 2-ME or DMSO for 24 h at 37°C, cells were fixed in 10% formalin (2 mM EGTA in PBS) for 10 min and permeabilized in ice-cold 97% methanol containing 2 mM EGTA at −20°C for 10 min. Subsequently cells were washed in PBS (3×5 min) before incubation for 1 h with a mouse monoclonal antibody against human β-tubulin (Clone 2-28-33; 1:1000). After washing with PBS, cells were incubated with biotin-conjugated anti-mouse IgG (Fab-specific, developed in goat) in FITC-conjugate diluent as secondary antibody for 1 h (1:100). Following washing, cells were finally incubated with ExtrAvidin®-FITC conjugate (1:200 in FITC-conjugate diluent) for 1 h. The cover slips were mounted with a glycerol-based mounting fluid after the final 3×5-min wash step. The cells were examined with a Nikon Optiphot microscope equipped with an episcopic-fluorescence attachment and an excitation-emission filter with an average wavelength of 495 mm for FITC.

Immunofluorescent detection of death receptor 5 (DR5)

Cells (500 000) were seeded onto heat-sterilized glass cover slips in 6-well plates and exposed to 2-ME or DMSO controls for a period of 24 h at 37°C. The cells were then fixed in 10% formalin (2 mM EGTA in PBS) for 10 min and permeabilized in ice-cold 97% methanol containing 2 mM EGTA at -20°C for 10 min. Cells were subsequently washed in PBS (3×5 min) before incubation for 1 h with a mouse monoclonal antibody against human Death Receptor 5 (Clone 2-28-33; 1:1000). After washing with PBS, the cells were incubated with biotin-conjugated anti-mouse IgG (Fab-specific, developed in goat) in FITC-conjugate diluent as secondary antibody for 1 h (1:100). Following washing, cells were finally incubated with ExtrAvidin®-FITC conjugate (1:200 in FITC-conjugate diluent) for 1 h. The cover slips were mounted with a glycerol-based mounting fluid after the final 3×5-min wash step. Cells were examined with a Nikon Optiphot microscope equipped with an episcopic-fluorescence attachment and an excitation-emission filter with an average wavelength of 495 mm for FITC.

Statistical analysis

Data obtained from three independent experiments were statistically analyzed for significance using the two-tailed Student *t*-test for samples. Means are presented in bar charts, with T-bars referring to standard deviations (SD). p-values < 0.05 were regarded statistically significant.

RESULTS

Dose-dependent growth studies

WHCO3 cell growth was expressed as a percentage of the control after exposure to different concentrations of 2-ME (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M) for 72 h. 2-ME reduced cell numbers to 60% when compared to vehicle-treated controls after exposure to 10^{-6} M 2-ME for 72 h. An * indicates a statistically significant p-value < 0.05 for growth inhibition (Figure 1).

Morphology studies

Propidium iodide and hoechst 33342 staining. PI and HO staining were conducted to determine the presence of apoptotic cells after treatment with 2-ME. Viable and apoptotic cells have intact cell membranes and are stained

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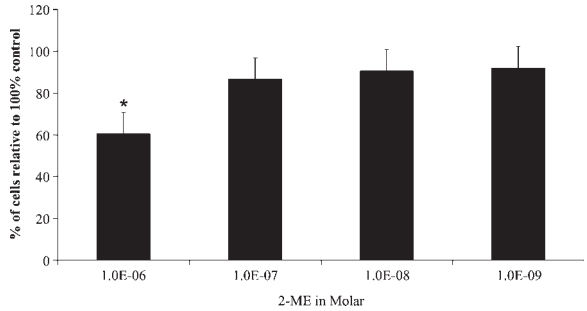

Figure 1. Dose-dependent study of WHCO3 cells treated with a dilution series of 2-ME (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M) for 72 h. Cell numbers are expressed as a percentage of the control. A 40% decrease in cell number was noted at 10^{-6} M of 2-ME WHCO3-treated cells. * indicates p-value < 0.05

light blue, while cells that have lost their membrane integrity are stained bright red. After 2-ME treatment most cells stained light blue (indicated in black and white) and were rounded in appearance due to a metaphase block and showed apoptotic features including cytoplasmic shrinking, membrane blebbing, and apoptotic bodies (Figure 2A, B).

Hematoxylin and eosin staining (H and E staining)

The antiproliferative effect of 2-ME observed above could be attributed to either growth inhibition (cytostatic effect) or induction of cell death. Thus, morphological characteristics of the cytoplasm and nuclear components of cells treated with 2-ME and DMSO respectively were studied by means of hematoxylin and eosin staining to confirm 2-ME's antimitogenic effect (Figure 3A, B). After 2-ME treatment, most cells were rounded in appearance due to a metaphase block and showed apoptotic features including hypercondensed chromatin, cytoplasmic shrinking, membrane blebbing, and apoptotic bodies when compared to their vehicle-treated controls (Figure 3A, B).

Transmission electron microscopy

TEM was employed to view subcellular structures in two dimensions. WHCO3 control cells (Figure 4A) revealed

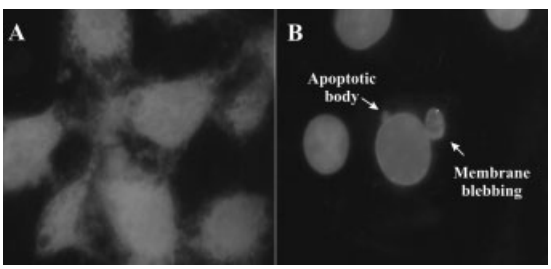

Figure 2. Propidium iodide and Hoecsht 33342 staining of WHCO3 cells exposed to 0.1% DMSO (vehicle) (A) and 1×10^{-6} M 2-ME (B) for 24 h (black and white images; 400 × magnification). 2-ME-treated cells are rounded in appearance due to a metaphase block. Apoptotic features including cytoplasmic shrinking, membrane blebbing, and apoptotic bodies are also visible

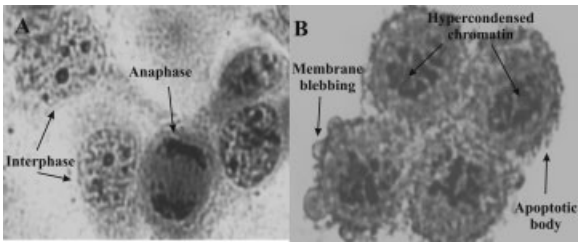

Figure 3. Hematoxylin and Eosin staining of WHCO3 control cells exposed to 0.1% DMSO (vehicle) (A) and 1×10^{-6} M 2-ME (B) for 24 h (400 × magnification). Clusters of rounded cells with hypercondensed chromatin, as well as apoptotic bodies are visible in the treated cells

intact nucleoli in contrast to cells treated with 2-ME that showed condensed chromatin, irregular nuclear membrane, and increased mitochondrial aggregation toward the nucleus (Figure 4B).

Cell cycle progression

Quantitative analysis of DNA content was conducted by means of flow cytometry in order to determine the effects of 2-ME on cycle progression after 24 h of exposure. 2-ME-treated cells revealed an increase in the sub $G_{1/0}$ apoptotic fraction when compared to vehicle-treated cells (Figure 5). An increase in the amount of cells in the G_2/M-phase was also evident in the treated cells.

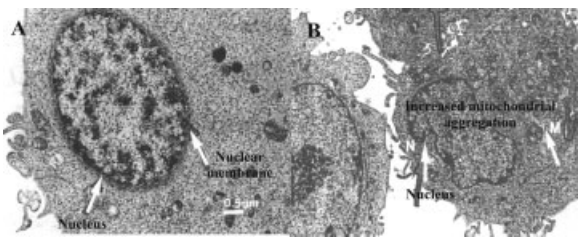

Figure 4. (A) Transmission electron microscopy of WHCO3 control cells exposed to 0.1% DMSO (vehicle) (A) and 1×10^{-6} M 2-ME (B) for 24 h. Hypercondensed chromatin and increased mitochondrial aggregation around the nucleus are visible (B). (Scale bar = 0.5 μm)

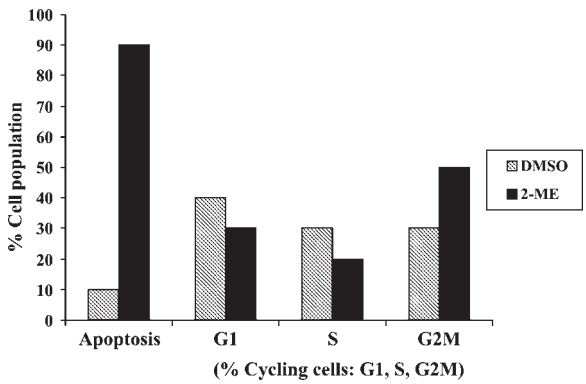

Figure 5. The effect of 2-ME on cell cycle progression after 24 h of exposure. 2-ME-treated cells presented with an increased sub $G_{1/0}$ apoptotic fraction, as well as a G_2/M-phase increase when compared to vehicle-treated cells.

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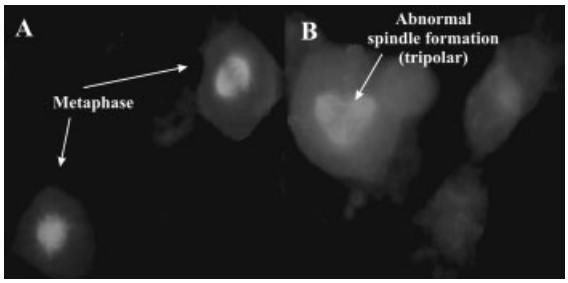

Figure 6. Immunofluorescent staining of β-tubulin in WHCO3 cells. 2-ME-treated cells were rounded, accumulated in metaphase and spindle disruption with fragmented polar formations were evident when compared to vehicle-treated controls (A, B)

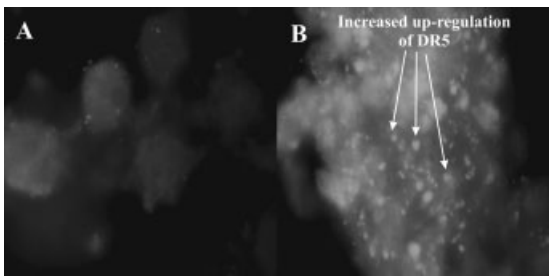

Figure 7. Immunofluorescent detection of DR5 in 2-ME-treated WHCO3 cells. Up-regulation of DR5 expression was observed as an increased occurrence of white spots when compared to vehicle-treated controls (A, B). (Magnification $\times$ 400)

Immunofluorescent detection of β-tubulin

Since previous research has shown that 2-ME induces cell death by causing microtubule disruption and blocking cells in metaphase in other cell lines, the influence of 2-ME was subsequently investigated on spindle formation in WHCO3 cells by means of immunofluorescent staining of β-tubulin. 2-ME-treated cells were rounded, accumulated in metaphase and also showed spindle disruption with fragmented polar formations when compared to vehicle-treated controls (Figure 6A, B).

Immunofluorescent detection of DR5

To investigate whether the extrinsic pathway of apoptosis was activated after treatment with 2-ME, DR5 was chosen as a marker. Immunofluorescent detection of DR5 in 2-ME-treated WHCO3 cells demonstrated an up-regulation of DR5 expression when compared to vehicle-treated controls (Figure 7A, B).

DISCUSSION

Previous research has revealed that 2-ME plays an important role in the induction of apoptosis and especially in actively proliferating cancerous cells.[3,5,16,22,28,29] Pribluda *et al.*[16] accounted of the antiproliferative effects of 2-ME by listing various cell lines that are affected by 2-ME at inhibitory concentrations ranging from 0.08 to 5 μM.

In the present study conducted, 2-ME was shown to exert antiproliferative activity in the WHCO3 oesophageal carcinoma cell line investigated. Morphological changes occurring during apoptosis namely cell shrinkage, membrane blebbing condensation of nuclear chromatin into sharply delineated masses that become marginated against the nuclear membrane, as well as the formation of apoptotic bodies[30] were demonstrated in 2-ME-treated WHCO3 cells.

These data are consistent with previous results from our laboratory where inhibition of cell growth in breast cancer cells (MCF-7) was demonstrated following 2ME treatment. 2ME-treated MCF-7 cells also exhibited abnormal metaphase cells, membrane blebbing, apoptotic bodies, and disturbed spindle formation. However, these observations were either absent, or less pronounced in the non-tumorigenic MCF-12A cells.[11]

In this study, cells treated with 2-ME revealed an increase in mitochondrial numbers aggregating around the nuclear envelope. Mitochondria are important sensors and amplifiers in intracellular death signaling pathways and are core components of the cell death machinery.[14] Changes in mitochondrial membrane structure, either by disruption of the outer membrane or by Bax activation[31] can lead to apoptosis. In addition, up-regulation of DR5 was also demonstrated in 2-ME-treated cells and is consistent with previous data where 2-ME was shown to up-regulate DR5 and sensitize cancer cells to TRAIL-induced apoptosis in pancreatic adenocarcinoma cell lines.[32]

Furthermore, we have previously demonstrated a significant increase in Cdc2 kinase activity in 2-ME-treated cells when compared to vehicle-treated controls in WHCO3 cells.[10] Cdc2 kinase activity was statistically significantly increased (1.7-fold) ($p < 0.005$) after 2-ME exposure when compared to vehicle-treated controls. Our observation contributes to the elucidating of the mechanism of action in WHCO3 oesophageal carcinoma cells and reveals that 2-ME causes a metaphase arrest, disrupts mitotic spindle formation, enhances Cdc2 kinase activity leading to persistence of the spindle checkpoint, and thus prolonged metaphase arrest culminating in the induction of apoptosis in WHCO3 cells. The observed up-regulation of DR5 further supports the proposed mechanism of apoptosis induction via the extrinsic pathway in WHCO3 oesophageal carcinoma cells.

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