Influence of 2-methoxyestradiol on cell morphology and Cdc2 Kinase activity in WHCO3 esophageal carcinoma cells

Annie JOUBERT and Sumari MARAIS
Department of Physiology, University of Pretoria, P.O. Box 2034, Pretoria, 0001, South Africa
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ABSTRACT
The influence of $1 \times 10^{-6}$ M exogenous 2-methoxyestradiol (2ME) was investigated on nuclear and cytoplasmic morphology, as well as Cdc (cell division cycle) 2 kinase activity in WHCO3 esophageal carcinoma cells. Mitotic indices after 18 h of 2ME exposure revealed an increase in metaphase cells (9.0%) when compared to the vehicle-treated cells (0.9%). 2ME-treated cells showed apoptotic cells at 5.6% after 18 h of exposure to dimethyl sulphoxide, compared to 0.9% in vehicle-treated cells. Increased morphological characteristics of apoptosis were observed in 2ME-treated cells after 21.5 h of exposure. Twelve percent of cells were in apoptosis when compared to the 1.6% of vehicle-treated cells. Furthermore, 42.4% of cells were arrested in metaphase after 21.5 h of 2ME exposure compared to 2.9% of vehicle-control cells present in metaphase. Cdc2 kinase activity was statistically significantly increased (1.7-fold) ($P < 0.005$) after 18 h of 2ME exposure when compared to vehicle-treated controls. Although the mechanism of 2ME’s action on esophageal carcinoma cells is not yet elucidated, the present study revealed that 2ME caused metaphase arrest, as well as an increase in Cdc2 kinase activity that culminated in the induction of apoptosis in these cells.

2-Methoxyestradiol (2ME) is a naturally occurring physiological estrogen metabolite of 17beta-estradiol and considered as an antimitogenic compound and tubulin poison. Early research on 2ME’s biological activity stated its effect on mitotic spindles and cell cycle progression (32). It has been shown to inhibit cell proliferation and to induce apoptosis in a large variety of tumor cells and is harmless to most normal cells (21). 2ME exerts both antiangiogenic and antitumor effects regardless of the cell’s hormone receptor status (10, 19, 23, 24, 32, 34). 2ME causes mitotic accumulation and abnormal mitotic spindle formation in both estrogen receptor (ER)-positive and negative cells (23, 24, 32).

Recently, researchers have demonstrated the antiangiogenic influence of oral 2ME on a laser-induced murine model of choroidal neovascularization with no side effects of toxicity or weight loss being observed (9). Schumacher et al. (2006) investigated the efficacy on growth inhibition of 2ME on human hepatocellular carcinoma in vitro (31). 2ME caused a reduction in cell growth in these cells and it was concluded that the mechanism of action appears to be induction of apoptosis. SK-Hep1 hepatocellular carcinoma cells were the most sensitive to 2ME and an up-regulation of the p53 and p21 proteins was observed. However, normal human hepatocytes were not influenced when treated with 2ME (31).

Roswall et al. (2006) demonstrated 2ME’s anti-proliferative effects in five human anaplastic thyroid carcinoma cell lines (HTh7, HTh 74, HTh83, C643, and SW1736) after treatment with 2ME. It was revealed that a G2/M-arrest was followed by an increased fraction of cells present in sub-G1 (29).
latter indicates reduced quantity of DNA or hyper-condensed DNA that may represent apoptotic bod-
ies, as others and we have previously illustrated
(15–17). However, 2ME had no influence on the an-
aplastic thyroid KAT-4 cancer cells (29), supporting
the idea that the action mechanism of 2ME is not
clearly defined and appears to vary depending on
cell type (23).

2ME was also shown to augment Fas ligand/tu-
ror necrosis factor-related apoptosis-inducing ligand
induced apoptosis in MIA PaCa-2 human pancreatic
carcinoma cells and it was suggested that oxidative
stress preceded 2ME-mediated c-Jun NH₂-terminal
kinase activation, leading to elevated Fas level (3).
JNK plays a key role in the phosphorylation and in-
activation of the pro-apoptotic protein Bcl-2, thus
contributing to the induction of apoptosis (3, 5).
Furthermore, Joubert et al. (2005) demonstrated that
the Bax/Bcl-2 ratio for 2ME-treated WHCO3 cells
was 1.45 normalized against Bcl-2 levels (14). Ex-
posure of human cervical carcinoma cells (HeLa) to
2ME revealed a Bax/Bcl-2 ratio of 1.87 normalized
against Bcl-2 levels in these cells, also suggesting
that this altered ratio in favor of Bax could lead to
the induction of apoptosis in these cells (13).

2ME has emerged to be highly effective in reduc-
ing tumor growth in vitro and in vivo and renders to
be a promising anticancer agent. 2ME (Panzem™)
has demonstrated good safety in phase I/II trials for
cancer. Preliminary preclinical data demonstrated
that 2ME might be used in the treatment of sarco-
ma, multiple myeloma and other solid tumors, thus
depicting it as a potential antitumor agent when
evaluated against conventional chemotherapeutic
treatments (8, 22, 23, 34, 35).

However, the precise action mechanism of
2ME is multifaceted, still not clearly defined and
appears to vary according to cell type (23, 25).
Therefore, the aim of this study was to determine
the mechanism of action of 2ME in a non-keratinis-
ing squamous esophageal carcinoma cell line by in-
vestigating the effects of 2ME on cytoplasmic and
nuclear morphology, including Cdc (cell division
cycle) 2 kinase activity as a cell cycle regulatory
component.

MATERIALS AND METHODS

Materials. 2ME, minimum essential medium eagle
with Earle’s salts, L-glutamine and NaHCO₃ (MEM),
trypsin-EDTA and trypan blue were supplied by
Sigma Chemical Co. (St. Louis, MO, USA). Penicil-
lin, streptomycin and fungizone were obtained from
Highveld Biological (Sandringham, South Africa).
Heat inactivated fetal calf serum (FCS), sterile cell
culture flasks and plates were obtained through
Sterilab Services (Kempton Park, Johannesburg,
South Africa). Haematoxylin, xylol and Entellan®
fixative were purchased from Merck (Darmstadt,
Germany). The MESACUP Cdc2 Kinase Assay Kit
and HCK-gel suspension were supplied by MBL
Medical & Biological Laboratories (Nagoya, Japan).
Cdc2 reaction buffer, biotinylated MV peptide, POD
(peroxidase)-conjugated streptavidin, wash concen-
trate (10 × PBS), substrate A (o-phenylenediamine),
substrate B (H₂O₂), stop solution (20% H₃PO₄)
and phosphorylation stop reagent (PBS containing
50 mM EDTA) were supplied with the kit. ATP,
Tris-HCl, EDTA, EGTA, Brij35, PMSF, beta-glyc-
erophosphate, Na₃VO₄, 2-mercaptoethanol and the
protease inhibitor cocktail were supplied by Sigma-
Aldrich (St. Louis, MO, USA). NaCl was purchased
from Associated Chemical Enterprises (Southdale,
South Africa), MgCl₂ from Merck and HEPES was
supplied by BDH Laboratory Supplies (Poole, Eng-
land). All other chemicals were of analytical grade
and purchased from Sigma Chemical Co.

Cell culture. The WHCO3 cell line (a poorly differ-
entiated non-keratinising squamous esophageal cell
carcinoma) was a gift from Professors Thornley
and Veale (Department of Zoology, University of the
Witwatersrand, Johannesburg, South Africa). WHCO3
cells were obtained through a biopsy from a patient with squamous esophageal carcinoma.
Cells were grown as monolayers in MEM at 37°C
in a humidified atmosphere containing 5% CO₂. Me-
dia were supplemented with 10% heat inactivated
fetal calf serum, penicillin (100 µg/L), streptomycin
(100 µg/L) and fungizone (250 µg/L). Non-viable
cells were excluded with the trypan blue staining
procedure. Stock solutions of 2ME were prepared in
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 in-
cluded showed that 0.1% had no toxic effects on ex-
periments conducted.

Light microscopy (haematoxylin and eosin cell
staining). In an effort to acquire more insight into
the antimitogenic effect of 2ME observed in cell
proliferation studies previously conducted in our
laboratory (36), haematoxylin and eosin cell staining
was used to determine the morphological character-
istics of cytoplasm and nuclear components of
WHCO3 cells after exposure to 10⁻⁶ M 2ME. The
latter concentration was chosen, since it has been demonstrated that 2ME exhibits 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 a pronounced inhibitory effect at a concentration of $10^{-6}$ M (19). Exponentially growing WHCO3 cells were seeded at 300 000 cells per well in 6-well plates on heat-sterilized coverslips. After a 24 h incubation period at 37°C allowing for cell adherence, cells were exposed to $1 \times 10^{-7}$ M 2ME and incubated for 18 h and 21.5 h respectively at 37°C. Exposure times of 18 h and 21.5 h were chosen to monitor the increase in metaphase cells, since previous results in our laboratory had revealed that peak occurrence of apoptosis occurs after 24 h of exposure to 2ME in WHCO3 cells (36).

Cells were fixed in Bouin’s fixative for 60 min after exposure to 2ME and stained by standard haematoxylin and eosin staining procedures (33). To establish 2ME’s influence on cell proliferation, mitotic indices of vehicle-treated cells, as well as 2ME-exposed cells were also determined on the stained slides by counting 1000 cells on every coverslip.

**Cell cycle checkpoint regulatory molecule—Cdc2 kinase assay.** WHCO3 cells were grown in 75 cm$^2$ flasks until 80% confluency and exposed to $1 \times 10^{-6}$ M 2ME for 18 h. Since activation of Cdc2 kinase is fundamental for the initiation of mitosis and since 2ME caused a prominent block in metaphase after 18 h of exposure, the influence of 2ME was determined on WHCO3 Cdc2 kinase activity after 18 h of exposure. Cells were harvested and washed (three times) with PBS. Subsequently, $2 \times 10^7$ cells were suspended in 1000 μL sample buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 5 mM EDTA, 2 mM EGTA, 0.01% Brij35, 1 mM phenyl methyl sulfonyl fluoride, 25 mM beta-glycerophosphate, 1 mM Na$_2$VO$_4$, 50 mM mercaptoethanol, 0.08% protease inhibitor cocktail) and lysed by sonication on ice. Cell extracts were centrifuged for 1 h at 100 000 $\times$ g. Protein content was confirmed with the Bradford method (4). Twenty μL of the HCK-gel was prepared by washing twice with sample buffer before it was incubated with 300 μL of cell extract for 1 h on ice. After incubation, the HCK-gel was washed three times in sample buffer and twice with 2nd washing buffer (25 mM HEPES buffer, pH 7.5 and 10 mM MgCl$_2$), followed by centrifugation for 5 min at 1 500 $\times$ g. The phosphorylation reaction was prepared on ice by adding 5 μL of the gel sediment, 5 μL 10 $\times$ Cdc2 reaction buffer, 5 μL biotinylated MV peptide and 30 μL distilled water. The reaction was initiated by adding 5 μL 1 mM ATP and incubated for 30 min at 30°C. Consequently the reaction was terminated by the addition of 200 μL phosphorylation stop solution and the reaction solution was centrifuged for 20 s at 11 000 $\times$ g. One hundred μL of reaction mixture was transferred to the microwell strips coated with monoclonal antibody 4A4, provided with the kit. After 1 h incubation at 25°C, the wells were washed five times with wash solution and 100 μL POD conjugated streptavidin was added to each well. After 30 min incubation at 25°C, the wells were washed five times with wash solution and 100 μL POD substrate solution was added to each well. The microwell strips were incubated for 5 min at 25°C before 100 μL stop solution was added to each well and the absorbance read at 450 nm in an ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden, South Africa).

**Statistics.** Data were obtained from independent experiments. Results obtained are shown as the mean ± SD and were statistically analyzed 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 $< 0.05$ were regarded as statistically significant and indicated by an * or number as indicated in the legends.

**RESULTS**

**Light microscopy (haematoxylin and eosin staining)**

The influence of $1 \times 10^{-6}$ M 2ME was investigated on cell morphology in WHCO3 esophageal carcinoma cells and compared to vehicle-treated controls after 18 h (Fig. 1) and 21.5 h (Fig. 2) of exposure. As already mentioned, exposure times of 18 h and 21.5 h were chosen to monitor the increase in metaphase cells, since previous results in our laboratory had revealed that peak occurrence of apoptosis occurs after 24 h of exposure to 2ME in WHCO3 cells (36).

The WHCO3 control showed normal cell division including cells in metaphase after 18 h of exposure to DMSO. Interphase cells were also observed (Fig. 1A). 2ME-Treated cells showed an increase in metaphase cells and revealed hypercondensed chromatin after 18 h of exposure (Fig. 1B). The WHCO3 control revealed interphase cells and normal cell divi-
sion including cells in metaphase and telophase after 21.5 h of exposure to DMSO (Fig. 2A). 2ME-Treated cells showed an increase in cells with hypercondensed chromatin and apoptotic bodies after 21.5 h of exposure (Fig. 2B). Vehicle-treated control cells showed apoptosis in 0.9% of cells after 18 h of exposure to DMSO. Characteristics of apoptosis were observed in 5.6% of the 2ME-treated cells (Fig. 3). In addition, increased morphological hallmarks of apoptosis were observed in 2ME-treated WHCO3 cells after 21.5 h of 2ME exposure. Mitotic indices demonstrated 12.0% of cells in apoptosis when compared to the 1.6% vehicle-treated cells after 21.5 h of exposure to 2ME (Fig. 4). Furthermore, 42.4% of cells were blocked in metaphase after 21.5 h of 2ME exposure compared to 2.9% of vehicle-control cells in metaphase (Fig. 4).

Cell cycle checkpoint regulatory molecule—Cdc2 kinase assay
Since activation of Cdc2 kinase is fundamental for the commencement of mitosis and since 2ME caused a prominent block in metaphase after 18 h of exposure, the influence of $1 \times 10^{-6}$ M 2ME was determined on Cdc2 kinase activity of WHCO3 cells after 18 h of exposure and compared to vehicle-treated controls. Cdc2 kinase activity was statistically significantly increased (1.7-fold) ($P < 0.005$) in 2ME-treated cells when compared to vehicle-treated controls (Fig. 5).

DISCUSSION
Several researchers have demonstrated the biological activities of 2ME in endothelial cells and different tumor cell types. These activities include mitotic
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arrest, abnormal mitotic spindle formation, 2ME’s effects on cell cycle progression and its influence on the ratio of pro-apoptotic Bax to anti-apoptotic Bel-2, ultimately culminating in apoptosis (23, 35). Our previous research has also revealed that 2ME causes significant decreases in cell proliferation, overexpression of extracellular signal regulated protein kinase (ERK1/2) and successive induction of apoptosis in malignant cell lines (12, 32, 36). Multi-drug-resistant tumor cells also appear to be highly sensitive to 2ME with 2ME having negligible reversible side effects on normal tissue (30). Nevertheless, the exact action mechanism of 2ME is complex, still not clearly defined and appears to vary according to cell type.

Fig. 3 Mitotic indices of WHCO3 cells after 18 h of exposure to vehicle and 2ME, expressed as a percentage of a thousand cells counted. An increase in metaphase cells (9.0%) was observed when compared to the 0.9% vehicle-treated cells. Vehicle-control cells showed 0.9% of cells to be in apoptosis, while 5.6% of 2ME-treated cells showed hallmarks of apoptosis.

Fig. 4 Mitotic indices of WHCO3 cells after 21.5 h of exposure to vehicle and 2ME, expressed as a percentage of a thousand cells counted. Treated cells showed an increase in apoptotic cells (12.0%) when compared to the 1.6% vehicle-treated cells. 42.4% of 2ME-exposed cells were blocked in metaphase compared to 2.9% of vehicle-control cells present in metaphase.

Fig. 5 Cdc2 kinase activity of vehicle- and 2ME-treated WHCO3 cells after 21.5 h of exposure. 2ME caused a statistically significant increase (1.7-fold) when compared to vehicle-treated controls. P-values < 0.05 were regarded as statistically significant and indicated by an * on the graph.
In this study we investigated the influence of 2ME on morphology, as well as Cdc2 kinase activity as a cell cycle regulatory component in the WHCO3 esophageal carcinoma cell line. 2ME caused metaphase arrest, as well as an increase in Cdc2 kinase activity that culminated in the induction of apoptosis in these cells. These results were consistent with morphological hallmarks of mitotic arrest and disrupted mitotic spindle formation. Wang et al. (2000) defined that an accumulation of cells with condensed chromatin can be attributed to a characteristic of a mitotic block (38). Hypercondensed chromatin was also observed in the 2ME-treated WHCO3 cells. Likewise Attalla et al. (1996) demonstrated that 2ME caused a metaphase block in Jurkat cells (2). Reiser et al. (1998) showed a pronounced effect of 2ME in normal dividing human umbilical vein cells, as well as in immortal angiotumor-producing rat sinusoidal endothelial cells mediated through a block at G$_2$/M of the cell cycle (27).

It was confirmed that the anti-endothelial and anti-angiotumor influence of 2ME supports its potential as a possible therapeutic agent against solid organ tumors, benign or malignant vascular growths, including other pathologic conditions dependent on angiogenesis.

In this study 2ME-treated WHCO3 cells revealed a high percentage of dividing cells. However, most of these cells were abnormal and blocked in metaphase. Research has shown that cells blocked in metaphase pave the way for apoptosis induction (11, 18, 28). Similar results were obtained with WHCO3 cells where an increase in metaphase cells after 2ME treatment was observed, followed by cells showing hallmarks of apoptosis. Ray et al. (2006) have shown an accumulation of prostate cancer cells in S and G$_2$/M phases after 2ME exposure, with reduced cell numbers in the G$_1$ phase (26). In addition, cyclin B1 was overexpressed and phosphorylation of Cdc2 was induced. These data were consistent with apoptosis induction, growth arrest at the G$_2$/M phase and the accumulation of cells in the S phase. It was suggested that cyclin B1 and Cdc2 phosphorylation might be associated with the mechanism of action of 2ME (26). 2ME also caused a block in G$_2$/M in human melanoma cells in culture followed by apoptosis (10). Nevertheless, Golebiewska et al. (2002) demonstrated that osteosarcoma cells exposed to 1 × 10$^{-6}$M 2ME were arrested in G$_1$, thereby further supporting the hypothesis that the action mechanisms of 2ME are dependent on cell type (11).

In this study, 2ME caused a metaphase arrest in the WHCO3 esophageal carcinoma cells, including an increase in Cdc2 kinase activity that culminated in the induction of apoptosis. An increase in Cdc2 kinase activity, as well as an increase in the amount of apoptotic cells were also observed in the tumorigenic MCF-7 cell line after exposure to 2ME (37). On the contrary, only a slight increase in Cdc2 kinase activity, including a less prominent increase in the amount of apoptotic cells was observed in the 2ME-exposed non-tumorigenic MCF-12A cell line (37). Cdc2 activation is fundamental for the initiation of mitosis, whereas inactivation of Cdc2 is necessary for mitotic exit (1). Western blot analysis of 2ME-treated human melanoma cells showed the involvement of G$_2$/M regulatory proteins including Cdc2 kinase, thus implying that Cdc2 is involved in the cell cycle block. In addition, levels of p53, Bax and p21 were increased, while that of the anti-apoptotic Bcl-2 protein was undetectable in cells treated with 2ME (10). As already mentioned, we have previously demonstrated that the pro-apoptotic Bax/anti-apoptotic Bcl-2 ratio for 2ME-treated WHCO3 cells was 1.45 normalized against Bcl-2 levels, thus favouring induction of apoptosis (14). The increased Cdc2 activity observed in 2ME-treated WHCO3 cells is consistent with the morphological hallmarks of mitotic arrest observed. Previous research has reported an increase in Cdc2 kinase activity during apoptosis (6, 7, 20).

Although the mechanism of 2ME’s action on esophageal carcinoma cells is not yet elucidated and necessitate further investigation, the present study suggests that 2ME causes metaphase arrest, as well as an increase in Cdc2 kinase activity, culminating in the induction of apoptosis in WHCO3 cells.

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