# *In vitro* effects of 2-methoxyestradiol on MCF-12A and MCF-7 cell growth, morphology and mitotic spindle formation

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Short title: *In vitro* effects of 2-methoxyestradiol on MCF-12A and MCF-7 cells

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## ABSTRACT

The influence of 2-methoxyestradiol (2ME) was investigated on cell growth, morphology and spindle formation in a tumorigenic (MCF-7) and non-tumorigenic (MCF-12A) epithelial breast cell line. Inhibition of cell growth was more pronounced in the MCF-7 cells compared to the MCF-12A cells following 2ME treatment. Dose-dependent studies ($10^{-5}$ – $10^{-9}$ M) revealed that $10^{-6}$ M 2ME inhibited cell growth by 44% in MCF-12A cells and by 84% in MCF-7 cells (*P*-value < 0.05). 2ME-treated MCF-7 cells showed abnormal metaphase cells, membrane blebbing, apoptotic cells and disrupted spindle formation. These observations were either absent, or less prominent in MCF-12A cells. 2ME had no effect on the length of the cell cycle between S-phase and the time a mitotic peak was reached in either cell line but MCF-7 cells were blocked in mitosis with no statistically significant alterations in the phosphorylation status of Cdc25C. Nevertheless, Cdc2 activity was significantly increased in MCF-7 cells compared to MCF-12A cells (*P*-value < 0.05). The results indicate that 2ME disrupts mitotic spindle formation and enhances Cdc2 kinase activity, leading to persistence of the spindle checkpoint and thus prolonged metaphase arrest that may result in the induction of apoptosis. The tumorigenic MCF-7 cells were especially sensitive to 2ME treatment compared to the normal MCF-12A cells. Therefore, differential mechanism(s) of growth inhibition are evident between the normal and tumorigenic cells.

**Keywords:** 2-methoxyestradiol, metaphase block, mitotic spindle formation, spindle checkpoint, multipolar spindles, Cdc2 kinase

## INTRODUCTION

Breast cancer is regarded as the most common neoplastic disease in females.[1,2] Although various factors may contribute to the development of breast cancer, experimental data strongly suggest that estrogens and their metabolites may play a role in its initiation and promotion.[2,3] In contrast, 2-methoxyestradiol (2ME), an endogenous 17ß-estradiol metabolite, exhibits antiproliferative and antiangiogenic properties,[4-9] especially in actively proliferating cancerous cells.[7] These properties have been confirmed by various *in vitro* and *in vivo* studies[3,4,7,10-12]. In addition, phase I and phase II clinical trials with 2ME are currently being conducted in the United States[4]. 2ME showed therapeutic potential when administered to patients with metastatic breast cancers and prostate cancers with only minor side-effects in some of the patients such as hot flushes, reversible liver enzyme elevations, fatigue, diarrhoea and hand-foot syndrome.[4] Nevertheless, the mechanism of action of 2ME is not yet fully elucidated.

Several possible mechanisms have been suggested including the upregulation of death receptor 5,[13] induction of p53-dependent or p53-independent apoptosis,[4,14-16] interaction with tubulin resulting in faulty spindle formation and cell cycle arrest,[4,6,11,17,18] upregulation of cyclin-dependent kinase 2 (Cdk2) activity,[19] overexpression of cyclin B1[20] and increased cell division

cycle 2 (Cdc2) kinase activity.[20,21] There is no evidence for a universal mechanism of action operative in cells sensitive to 2ME[13] and therefore it has been suggested that multiple mechanisms are involved that may be dependent on cell type.[4,8,9,11] In general, the differential sensitivity of 2ME towards different cell lines appears to be tumor-specific, since very little or no toxicity has been observed in some of the studies using normal tissues and in *in vivo* experiments.[5,7,8,14,16] The antiproliferative and spindle disruption properties of 2ME have been described in MCF-7 cells[4,10,12], however, its effect on normal MCF-12A cells are not well documented.

The aim of this study was to investigate and compare the possible differential effects of 2ME on cell growth, morphology and the activity of relevant cell cycle kinases in the normal MCF-12A and tumorigenic MCF-7 breast epithelial cell lines.

## MATERIALS AND METHODS

### Materials

Dulbecco's Modified Eagle's Medium with glucose, sodium pyruvate and L-glutamine (DMEM), Ham's F12 medium with L-glutamine, fetal calf serum (FCS), penicillin, streptomycin, fungizone and trypsin/versene were obtained from Highveld Biological (Pty) Ltd. (Sandringham, South Africa). 2ME, epidermal growth factor (EGF), cholera toxin, insulin, hydrocortisone, dimethyl sulphoxide (DMSO), 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), mouse monoclonal antibody against human ß-tubulin (Clone 2-28-33), biotin-conjugated anti-mouse Fab-specific immunoglobulin G developed in goat, fluoroisothiocyanate (FITC)-conjugate diluent, ExtrAvidin®-FITC conjugate and hydroxyurea (HU) were supplied by Sigma-Aldrich Co. (St. Louis, USA). Phosphate buffered saline (PBS) was purchased from GIBCO BRL (USA). Haematoxylin and eosin were purchased from Merck (Darmstadt, Germany). The CycLex® Checkpoint Kinase Assay/Drug Discovery Kit-1, HCK-gel suspension, MESACUP Cdc2 Kinase Assay Kit and C-TAK positive control were purchased from MBL Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). Chemicals that were not supplied with the kits were of analytical grade and supplied by Sigma Chemical Co. (St. Louis, USA), Associated Chemical Enterprises (Southdale, South Africa), Merck (Darmstadt, Germany) and BDH Laboratory Supplies (Poole, England). MCF-7 (human breast epithelial carcinoma cell line) was supplied by Highveld Biological (Pty) Ltd. (Sandringham, SA). MCF-12A (non-tumorigenic breast epithelial cell line) was a gift from Professor Parker (Division of Medical Biochemistry, University of Cape Town, Cape Town, SA).

### Cell cultures

Cells were grown in 75 $cm^2$ tissue culture flasks at 37 $^oC$ in a humidified atmosphere containing 5% $CO_2$. The maintenance growth medium of the MCF-12A cells consisted of a 1:1 mixture of DMEM and Ham's F12 medium, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10

µg/ml insulin and 500 ng/ml hydrocortisone, while the MCF-7 cells were grown in DMEM. The growth media were supplemented with 10% heat-inactivated FCS, penicillin (100 µg/l), streptomycin (100 µg/l) and fungizone (250 µg/l). A stock solution of 2 x $10^{-3}$ M 2ME dissolved in DMSO was prepared and diluted with medium to the desired concentrations prior to exposure of the cells. The medium of all control cells was supplemented with an equal concentration of DMSO (vehicle). The DMSO content of the final dilutions never exceeded 0.1% (v/v).

## Determination of cell growth

The effect of 2ME exposure on cell growth was spectrophotometrically determined by means of the MTT assay[22].

Exponentially growing MCF-7 and MCF-12A cells were seeded at a cell density of 5 000 cells per well in 96-well tissue culture plates. Cells were exposed to concentrations ranging between $10^{-5}$ M and $10^{-9}$ M of 2ME for 72 h. This concentration range was chosen, since Lippert *et al.* reported that 2ME displayed a biphasic pattern on cell proliferation at concentrations ranging from $10^{-8}$ to $10^{-5}$ M (2ME showed a stimulatory effect at low concentrations and inhibitory effect at the highest concentration).[23] Twenty µl MTT (5 mg/ml in PBS) were added to each well containing 200 µl medium and incubated for 4 h at 37 $^{o}$C. The plates were centrifuged at 2000 rpm for 10 min and the supernatant removed. The pellets were washed with 150 µl PBS and centrifuged at 2000 rpm for 10 min. After removal of the supernatant, 100 µl DMSO were added to each well and plates were shaken for 1 h on a shaker before the absorbance was read at 570 nm (reference 630 nm), using an $EL_X$800 Universal Microplate Reader from Bio-Tek Instruments Inc. (Vermont, USA).

## Morphology study

In an attempt to obtain more insight into the antiproliferative effect of 2ME observed in the cell growth studies, haematoxylin and eosin cell staining was used to qualitatively compare the morphological characteristics of cytoplasm and nuclear components of MCF-12A and MCF-7 cells after exposure to 2ME.

Cells were seeded at 300 000 cells per well in 6-well plates on heat-sterilized coverslips and exposed to $10^{-6}$ M 2ME, since the differential effect of 2ME on cell growth between the normal and tumorigenic cell line was most prominent at this concentration. Cells were incubated for 24 h at 37 $^{o}$C, fixed in Bouin's fixative for 60 min and stained by standard haematoxylin and eosin staining procedures.[24] In order to obtain quantitative data from the morphological study, mitotic indices were determined on the stained slides by counting 1000 cells on every coverslip and expressing it as the percentage of cells in mitosis.

## Immunofluorescent detection of ß-tubulin

Several studies suggested that 2ME interacts with tubulin, resulting in faulty spindle formation and cell cycle arrest. [4,6,11,17,18] Thus, the effects of 2ME was investigated on mitotic spindle formation by means of immunofluorescent detection of ß-tubulin.

Cells were seeded on coverslips and exposed to 2ME as described for the morphology study. The cells were fixed in 10% formalin (containing 2 mM EGTA in PBS) for 10 min and permeabilized in ice-cold 97% methanol (containing 2 mM EGTA, dissolved by adding a few drops of 1mM sodium hydroxide) at –20 $^{o}$C for 10 min. The coverslips were washed in PBS (3 x 5 min) between successive 1 h incubations with each of the following: mouse monoclonal antibody against human ß-tubulin (Clone 2-28-33; 1:1000), biotin-conjugated anti-mouse IgG (Fab-specific, developed in goat) in FITC-conjugate diluent as secondary antibody (1:15) and ExtrAvidin®-FITC conjugate (1:200 in FITC-conjugate diluent). Following three 5 min washes with PBS, the coverslips were mounted with a glycerol-based mounting fluid. 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 nm for FITC (Nikon, Japan).

## Cell cycle length determination

The influence of 2ME treatment on the length of the cell cycle and the mitotic peak in MCF-7 and MCF-12A cells was determined by monitoring synchronized cells over a period of 24 h at 2 h intervals.

Cells were seeded as for the morphology study. Two mM HU was added to the sub-confluent cultures for 20 h to block the cells in early S-phase.[25] Although it has been reported that HU is toxic to some cells, preliminary studies indicated that HU was not toxic to either MCF-7 or MCF-12A cells. Cells were rinsed with PBS (3 x 10 min, 37 $^{o}$C) to wash out the HU and to allow the synchronized cells to continue through S-phase. This point was considered as time zero (0 h). Unsynchronized and synchronized slides served as controls at 0 h. Half of the remaining wells were exposed to $10^{-6}$ M 2ME. At 2 h intervals exposed and vehicle-treated coverslips were fixed and stained with haematoxylin and eosin and their mitotic indices determined in order to investigate the effect of 2ME on the length of the cell cycle.

## Checkpoint kinase assay

Since 2ME blocked MCF-7 cells in metaphase, the CycLex Checkpoint Kinase Assay kit was used to determine the effects of 2ME treatment on the activities of checkpoint kinases. A phospho-specific monoclonal antibody used in the assay kit recognizes the phospho-serine 216 residue in Cdc25C which is phosphorylated by checkpoint kinases.

MCF-7 and MCF-12A cells were grown in 75 $cm^2$ flasks until 80% confluent. Cells were exposed to 2 mM HU for 20 h to block the cells in early S-phase. The HU was washed out with PBS (3 x 10 min, 37 $^oC$) and half of the cells were exposed to $10^{-6}$ M 2ME. The cells were allowed to progress through the cell cycle until the mitotic peak was reached. The cells were harvested and enzyme extracts prepared as suggested by the manufacturer of the kit. Protein content was determined by the Bradford method.[26] The enzyme fractions were added to microtiter wells coated with recombinant Cdc25C provided with the kit. A positive control (0.01 units/well C-TAK positive control), inhibitor control (10 µM staurosporine added to the positive control), ATP minus control (no kinase reaction buffer) and a no enzyme control were included. Kinase activity was determined according to the manufacturer's instructions and quantitated by spectrophotometry by measuring the absorbance at 450 nm using an $EL_X800$ Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden, South Africa).

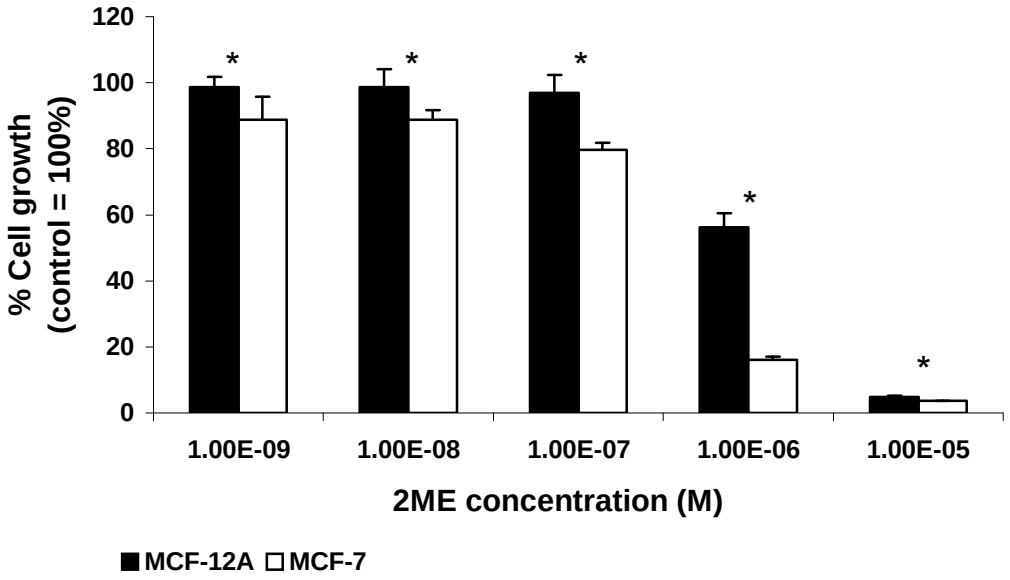


*Figure 1. MCF-12A and MCF-7 cell growth expressed as a percentage of the control after exposure to different concentrations of 2ME ($10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$, $10^{-9}$ M) for 72 h. 2ME reduced cell proliferation in both cell lines, but to a significantly greater extent in the tumorigenic MCF-7 cells compared to the normal MCF-12A cells An * indicates a statistically significant P-value < 0.05 for growth inhibition between cell lines.*

## Cdc2 kinase assay

Given that inactivation of Cdc2 kinase is necessary for mitotic exit and continued Cdc2 activity can maintain cells in the mitotic state for a prolonged period of time[27], its effect on Cdc2 kinase activity was determined by making use of the MESACUP Cdc2 Kinase Assay Kit.

Cells were prepared and harvested as described in the checkpoint kinase assay. Cells (2 x $10^7$) 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 PMSF, 25 mM beta-glycerophosphate, 1 mM $Na_3VO_4$, 50 mM

mercaptoethanol, 0.08% protease inhibitor cocktail) and lysed by sonication on ice. Cell extracts were centrifuged for 1h at 100 000 x g and the protein content determined with the Bradford method.[26] Cdc2 was precipitated from the cell lysates with HCK-gel and kinase activity determined with an enzyme-linked immunosorbent assay according to the manufacturer's instructions. A synthetic peptide serves as a substrate for Cdc2 kinases and a monoclonal antibody recognizing phosphorylated form of the peptide substrate. Absorbance was read at 450 nm in order to quantify Cdc2 activity.

### Statistical analysis of data

Cell growth studies were repeated three times, with sample size (n) = 6 in each experiment and analysed by means of an analysis of variance (ANOVA). Cell cycle kinase assays were repeated twice, with n = 3 in each experiment and analysed with the Wilcoxon two-sample rank test. A *P*-value of less than 0.05 was accepted as significant. Means of quantitative experiments are presented in bar charts, with T-bars referring to standard deviations (SD). All experiments included a set of appropriate controls.

## RESULTS

### Determination of cell growth

2ME reduced cell proliferation in both the non-tumorigenic MCF-12A and the tumorigenic MCF-7 cell line in a dose-dependent manner (Figure 1). ANOVA revealed a statistically significant difference (*P*-value < 0.05) in growth inhibition between the normal and tumorigenic cell line at all the concentrations. MCF-7 cell growth was reduced at all the concentrations tested, while cell growth of MCF-12A cells was only inhibited at $10^{-6}$ M and higher concentrations. Therefore, it was evident from this study that exposure to 2ME inhibited cell growth at much lower concentrations and to a greater extent in the tumorigenic MCF-7 cell line than in the normal MCF-12A cell line.

2ME ($10^{-6}$ M) caused a particularly noticeable effect by inhibiting cell growth by 84% in MCF-7 cells compared to only 44% in MCF-12A cells, suggesting that the tumorigenic cell line is more susceptible to 2ME treatment than the normal cell line. Similar results were obtained in this study using crystal violet as a DNA dye where cell numbers were determined as an indicator of cell growth (data not shown).[28]

### Morphology study

The morphological effects of 2ME on the two cell lines were investigated at $10^{-6}$ M where the differential effect of 2ME on cell growth was most prominent. Qualitative results were obtained by evaluating the haematoxylin and eosin-stained slides. MCF-12A (Figure 2A) and MCF-7 cells (Figure 2D) exposed to 0.1% DMSO control showed dense cell populations including cells in

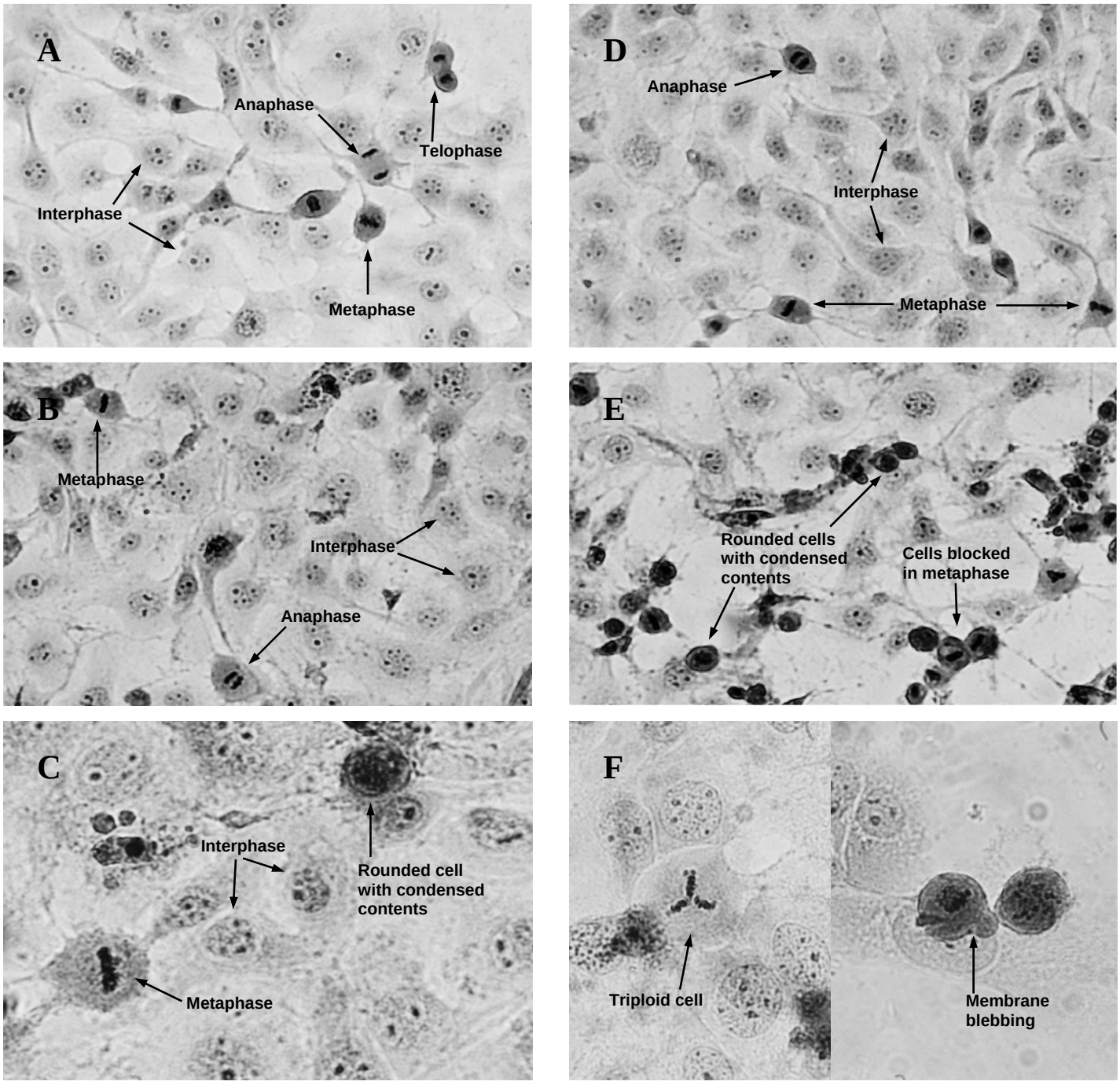


*Figure 2. Haematoxylin and eosin staining of MCF-12A (A,B,C) and MCF-7 (D,E,F) cells exposed for 24 h to 0.1% DMSO control (A,D) and $10^{-6}$ M 2ME (B,C,E,F). MCF-12A (A) and MCF-7 (D) control cells showed dense cell populations and cells in various stages of mitosis were observed. 2ME-treated MCF-12A cells (B)(100x magnification) revealed dense cell populations and many normal mitotic cells were observed (interphase, metaphase and anaphase). 2ME-treated MCF-12A cells (C)(400x magnification) showed some rounded cells with condensed contents. MCF-7 cells exposed to 2ME (E)(100x magnification) indicated less dense cell populations with many rounded cells with condensed contents. MCF-7 cells exposed to 2ME (F)(400x magnification) presented with membrane blebbing and some triploid cells were also observed.*

various stages of mitosis. 2ME-treated MCF-7 cells revealed abnormal metaphase cells, membrane blebbing, triploid cells, as well as apoptotic cells. Cell populations were less dense than in the control cells (Figure 2E and 2F). However, these observations were not as prominent in the 2ME-treated MCF-12A cells (Figure 2B and 2C).

Mitotic indices were determined in order to obtain quantitative data. Of a thousand cells counted, 22% of the 2ME-treated MCF-7 cells were blocked in metaphase and 8.2% showed membrane blebbing compared to only 1.4% of cells in a metaphase block and 0.1% cells with membrane blebbing in treated MCF-12A cells. Treated MCF-7 cells also showed a higher percentage of apoptotic cells than the MCF-12A cells (Table 1).

| | Prophase | Metaphase | Anaphase | Telophase | Metaphase block | Membrane blebbing | Apoptotic cells |
|---|---|---|---|---|---|---|---|
| **MCF-7 cells** | | | | | | | |
| DMSO control | 3.7 | 1.6 | 0.5 | 0.8 | 0.2 | 0 | 0.6 |
| 2ME($10^{-6}$ M) | 1.1 | 0 | 0 | 0 | 22 | 8.2 | 9.3 |
| **MCF-12A cells** | | | | | | | |
| DMSO control | 2.6 | 1 | 0.3 | 0.3 | 0.2 | 0 | 0.1 |
| 2ME($10^{-6}$ M) | 2.3 | 0.4 | 0 | 0.1 | 1.4 | 0.1 | 3.7 |

*Table 1: Cells in prophase, metaphase, anaphase and telophase, cells blocked in metaphase, cells presenting with membrane blebbing and apoptotic cells expressed as percentage of a thousand cells counted after 24 h exposure to 2ME.*

On each slide a thousand cells were counted and the mitotic indices were calculated and expressed as the percentage of cells in mitosis. Distinction was made between normal mitotic cells and abnormal mitotic cells which included cells in metaphase block, cells with abnormal chromosome distribution and cells presenting with membrane blebbing (Figure 3). The 2ME-treated MCF-7 cell line had the highest percentage of dividing cells (31.3%), however, most of these cells (30.2%) were abnormal and were blocked in metaphase.

## Immunofluorescent detection of ß-tubulin

Since mitotic indices indicated that 2ME blocked MCF-7 cells in metaphase, its effect on the mitotic spindle was investigated by making use of immunofluorescent detection of ß-tubulin. Vehicle-treated MCF-12A and MCF-7 cells presented with normal spindles (Figure 4A and

Figure 4C). The 2ME-treated MCF-12A cell line also showed normal spindles (Figure 4B), but the treated MCF-7 cells presented with multipolar spindles (Figure 4D).

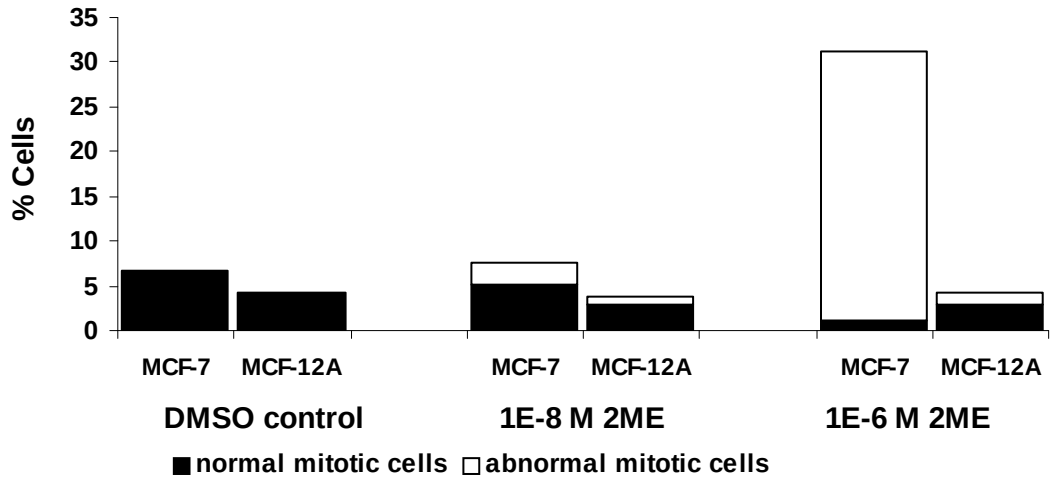


*Figure 3. Mitotic indices of MCF-12A and MCF-7 cells after exposure to 2ME expressed as a percentage of a thousand cells counted. Distinction was made between normal mitotic cells and abnormal mitotic cells that included cells in metaphase block and cells that presented with membrane blebbing.*

## Cell cycle length determination

Figure 5A and Figure 5B illustrates that 2ME treatment had no effect on the length of the cell cycle between early S-phase and the time a mitotic peak was reached in either of the cell lines investigated. In the MCF-12A cell line the peak mitosis occurred at 20 h for both the exposed and the vehicle-treated cells (Figure 5A) and in the MCF-7 cell line peak mitosis occurred at 13 h (Figure 5B). After peak mitosis was reached in the 2ME-treated MCF-7 cell line, the percentage of cells in mitosis remained high, indicating that the cells were blocked in mitosis (Figure 5B).

## Checkpoint kinase assay

The CycLex Checkpoint Kinase Assay was used to determine the effects of 2ME treatment on the activities of checkpoint kinases. No significant difference in the activity of kinases that phosphorylate Cdc25C between the control cells and cells exposed to $10^{-6}$ 2ME was detected in this assay (Figure 6).

## Cdc2 kinase assay

The effect of 2ME treatment on Cdc2 kinase activity was determined by making use of the MESACUP Cdc2 Kinase Assay Kit from MBL. 2ME enhanced Cdc2 kinase activity in 2ME-treated cells compared to the vehicle-treated cells in both cell lines. It is important to note that Cdc2 kinase activity was increased to a significantly greater extent in the MCF-7 cells compared to the MCF-12A cells (*P*-value < 0.05) (Figure 7).

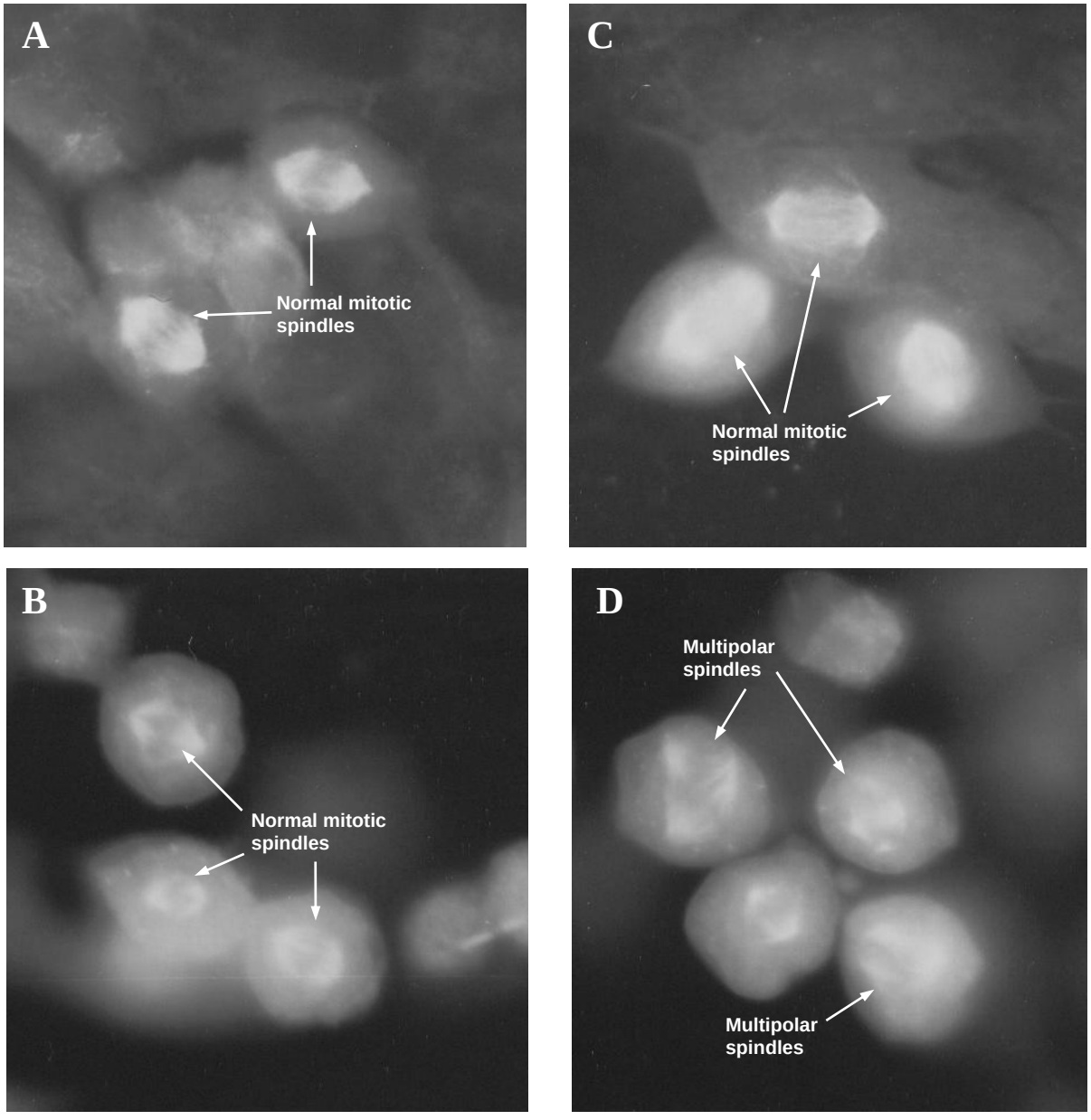


*Figure 4. Detection of β-tubulin in MCF-12A (A,B) and MCF-7 (C,D) cells for 24 h to 0.1% DMSO (vehicle) (A,C) and $10^{-6}$ M 2ME (B,D)(400x magnification). Control cells presented with normal spindles (A,C). 2ME-exposed MCF-12A cells also presented with normal spindles (B), however, MCF-7 cells presented with multipolar spindles, indicating abnormal spindle formation.*

## DISCUSSION

The aim of this study was to investigate and compare the possible differential effects of 2ME on cell growth, morphology and the activity of relevant cell cycle kinases in a normal (MCF-12A) and tumorigenic (MCF-7) breast cell line.

2ME inhibited cell growth at much lower concentrations and to a greater extent in the tumorigenic MCF-7 cell line, compared to the normal MCF-12A cell line, indicating that the tumorigenic cell line is more susceptible to 2ME treatment than the normal cell line. 2ME ($10^{-6}$ M) showed increased selectivity for the tumorigenic MCF-7 cells.

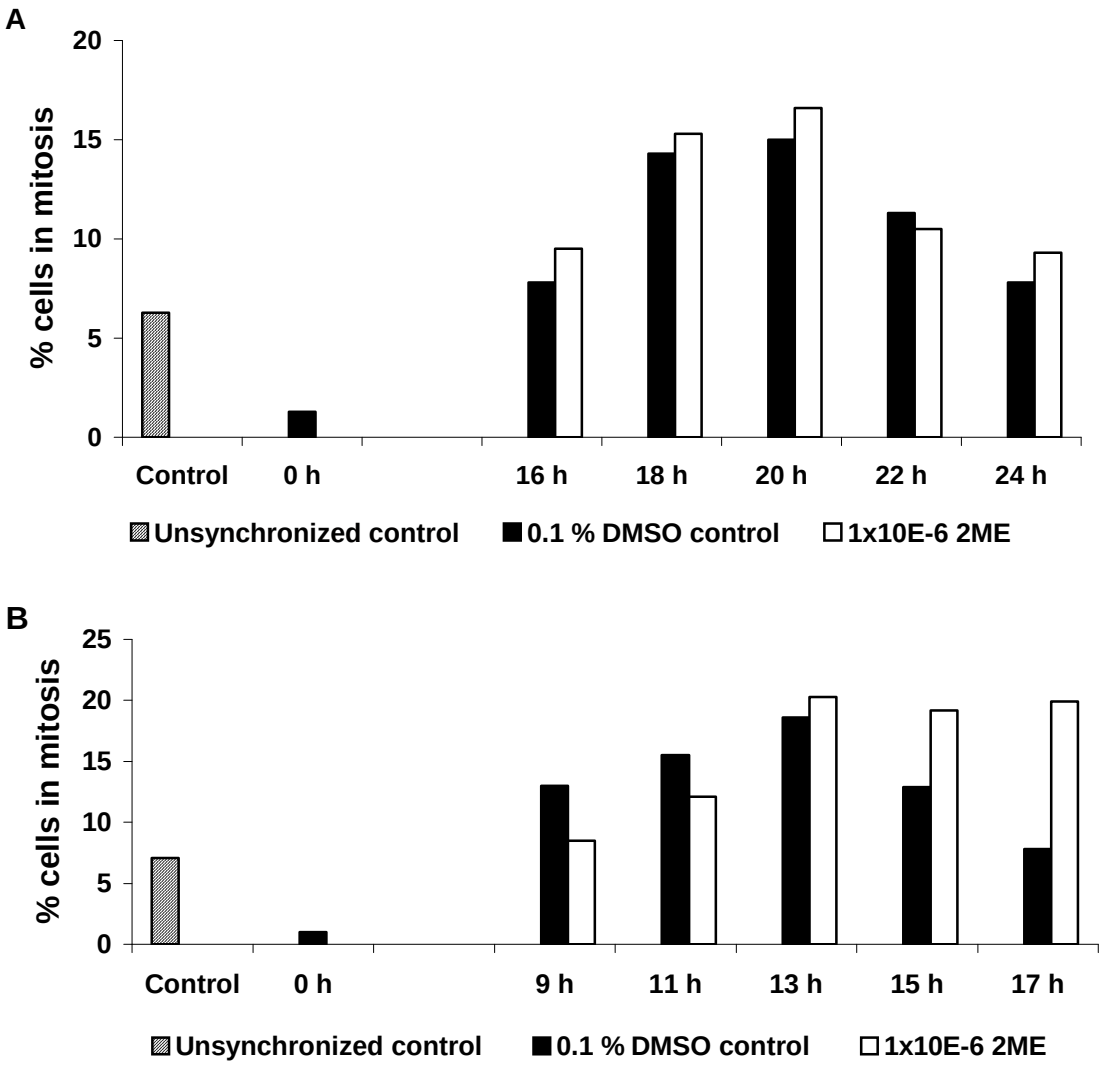


*Figure 5. Percentage of synchronized MCF-12A (A) and MCF-7 (B) cells in mitosis at 2 h intervals. Peak amount of cells in mitosis occurred at 20 h for exposed and vehicle-treated cells in MCF-12A cells (A). Peak amount of cells in mitosis occurred at 13 h for vehicle-treated MCF-7 cells, but mitotic indices of exposed MCF-7 cells remained high, indicating a mitotic block (B).*

Consistent with our results, Pribluda *et al*. reported on the antiproliferative effect of 2ME by listing various cell lines that are inhibited by the compound.[12] Their inhibitory concentrations ranged from 0.08 μM to 5 μM.[12] Less dense cell populations, abnormal metaphase cells, membrane blebbing and apoptotic cells observed in the 2ME-treated MCF-7 cells were consistent with the inhibition of cell growth. Cell growth was inhibited to a lesser extent in treated MCF-12A cells and confirmed by the observation that these cells were morphologically less affected than the tumorigenic cells. Similar to our results obtained with MCF-7 cells, Kumar *et al.* reported rounding of cells, condensation of cell content, induction of apoptosis and detachment of cells from dishes after medulloblastoma-derived DAOY cells were treated with 0.5 and 1 μM 2ME respectively.[5] Wang *et al.* reported that an accumulation of cells with condensed chromosomes is characteristic of a mitotic block[29] as was observed in the 2ME-treated MCF-7 cells in this study. Although treated MCF-7 cells revealed the highest percentage of dividing cells, most of these cells were abnormal and were blocked in metaphase. Similar observations were reported by Joubert *et al.* in SNO oesophageal carcinoma cells.[21] Mitotic arrest may lead to the induction of apoptosis.[30,31] This could explain the increase in apoptotic cells along with the increase in cells blocked in metaphase in 2ME-treated MCF-7 cells. Gui *et al*. also reported an increased amount of cells in mitosis after 2ME treatment of human smooth muscle cells and determined that the increased mitotic cells induced by 2ME were apoptotic.[32] In contrast, 2ME had no effect on cell viability, morphology or the induction of apoptosis in the normal human skin fibroblast strain HSF43.[16]

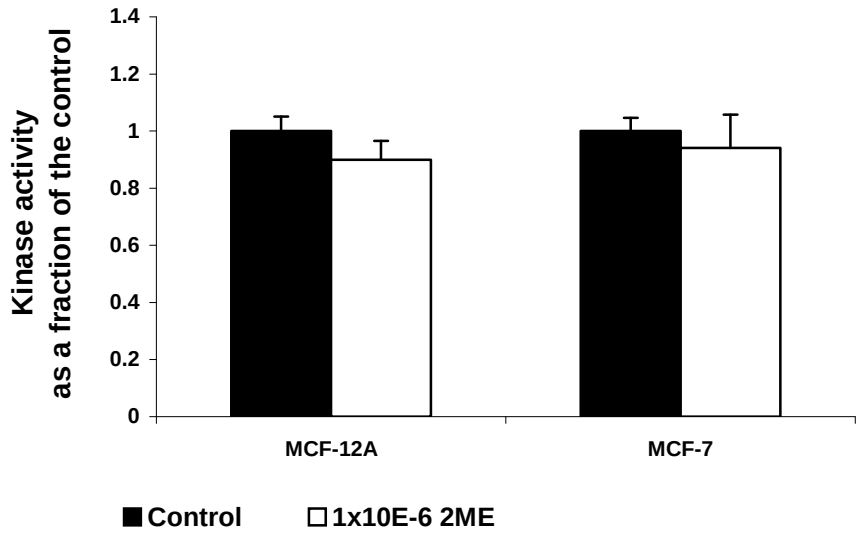


*Figure 6. The effect of 2ME treatment on the activity of kinases that phosphorylate Cdc25C in MCF-12A and MCF-7 cells. There was no statistically significant difference between control cells and 2ME-treated cells.*

These observations are consistent with the data obtained in the present study that normal MCF-12A cells were morphologically less affected than MCF-7 cells. Contrary to results obtained with the MCF-7 cells, 2ME did not block MCF-12A cells in metaphase, suggesting that 2ME may have a differential influence on spindle formation between the two cell lines.

Fluorescent microscopy confirmed that 2ME disrupted spindle formation and induced multipolar spindles in the MCF-7 cells, but not in MCF-12A cells. Cell cycle length determination studies also confirmed that 2ME blocked MCF-7 cells in metaphase, but not MCF-12A cells. Originally it was thought that cancerous cells are more susceptible to drugs that disturb the cell cycle, because they replicate more quickly and more frequently than normal cells.[33]

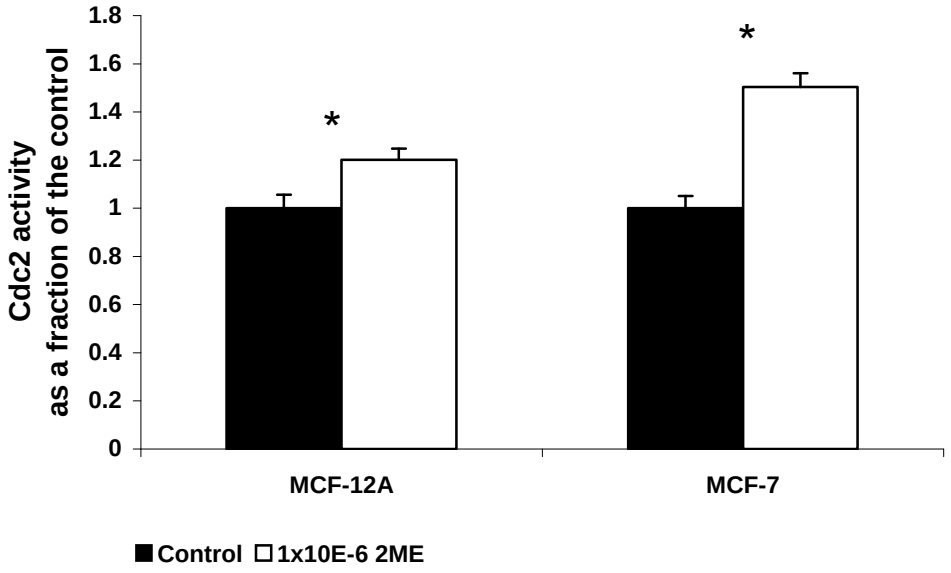


*Figure 7. The effect of 2ME treatment on Cdc2 kinase activity in MCF-12A and MCF-7 cells. Cdc2 kinase activity was significantly enhanced in 2ME-treated cells compared to vehicle-treated cells and in MCF-7 cells compared to MCF-12A cells. An * indicates a statistically significant P-value < 0.05 for kinase activity between control and 2ME-treated cells.*

However, there are many normal cell populations that replicate at least as rapidly as cancerous cells and some slow-growing cancer cells are more sensitive to conventional antitumor treatments than fast-growing cells.[33] Therefore, although MCF-12A cells have a longer cell cycle (and therefore take longer before they are affected by disrupters of the cell cycle than MCF-7 cells), other factors besides the difference in cell cycle length may contribute to the differential susceptibility of the two cell lines to 2ME treatment.One possibility is that mitotic checkpoint functions that are lost in cancerous cells are again activated by antitumor drugs (in this case 2ME) to selectively target tumor cells.[34]

Although there were no significant alterations in the phosphorylation of Cdc25C after 2ME treatment, Cdc2 activity was increased to a greater extent in the MCF-7 cells compared to the MCF-12A cells. In contrast, Kumar *et al.* reported phosphorylation of Cdc25C without any significant alterations in the expression of Wee1 kinase, cyclin B1 or Cdc2 after 2ME treatment in medulloblastoma cells.[5] These contrasting results give further support to the hypothesis that the action mechanisms of 2ME are dependent on cell type.

Activation of Cdc2 is necessary for the initiation of mitosis, whereas inactivation of Cdc2 is required for mitotic exit.[27] Cdc2 kinase activity is responsible for chromosome condensation, cytoplasmic reorganization to convert the interphase microtubule array to the mitotic spindle[35] and nuclear envelope breakdown.[35-37] Continued Cdc2 activity can maintain the cell in the mitotic state for a prolonged period until certain conditions are met for mitotic exit.[27] Furthermore, Gallant *et al.* showed that expression of a non-destructable cyclin B2 protein caused HeLa cells to arrest in a pseudo-mitotic state characterized by condensed chromatin, a disassembled nuclear envelope, and, in a high proportion of cells, multiple mitotic spindles.[38] Cdc2 is localized to spindle microtubules and kinetochores,[27] and both Cdc2 and cyclin B have also been identified at the level of the centrosome,[38,39] suggesting a role for these proteins in the regulation of mitotic spindle formation.[40] Therefore, the increased Cdc2 activity in the 2ME-treated MCF-7 cells is consistent with the morphological indicators of mitotic arrest and disrupted mitotic spindle formation observed in these cells. Similarly, Joubert *et al*. reported an elevation in Cdc2 kinase activity, in conjunction with mitotic arrest and disrupted mitotic spindle formation in SNO oesophageal carcinoma cells treated with 2ME.[21] In contrast to the MCF-7 cells, Cdc2 kinase activity in the non-tumorigenic MCF-12A cell line was only slightly elevated. This is consistent with the normal spindle formation observed in these cells. Several studies also reported an increase in Cdc2 kinase activity observed during apoptosis,[41-43] consistent with the increased amount of apoptotic cells observed after 2ME treatment in the tumorigenic MCF-7 cell line.

In conclusion, exposure to 2ME disrupted mitotic spindle formation, enhanced Cdc2 kinase activity that lead to persistence of the spindle checkpoint and thus prolonged metaphase arrest and resulted in the induction of apoptosis. The tumorigenic MCF-7 cells were especially sensitive to 2ME treatment compared to the normal MCF-12A cells. Therefore, selecting the concentration of 2ME that has maximum inhibitory effect on tumorigenic, but minimal effect on normal cells is crucial in its possible application as an antitumor agent. Although, in general, 2ME seems to target tumorigenic cells, its action mechanisms are dependent on cell type and the basis for selectivity and mechanisms of action are not yet fully understood. Further research concerning the differential action mechanisms of 2ME is essential to create a better understanding regarding the treatment of cancer and may possibly contribute to the development and/or improvement of novel chemotherapeutic agents.

## ACKNOWLEDGEMENTS

This research was supported by grants from the Medical Research Council of South Africa (AG374, AK076), the Cancer Association of South Africa (AK246) and the Struwig-Germeshuysen Cancer Research trust of South Africa (AJ038).

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