

2-Methoxyestradiol, an Endogenous 17 β -Estradiol Metabolite, Induces Antimitogenic and Apoptotic Actions in Oli-Neu – Oligodendroglial Precursor Cells and Triggers Endoreduplication via p53 Pathway

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Article

2-Methoxyestradiol, an Endogenous 17 β -Estradiol Metabolite, Induces Antimitogenic and Apoptotic Actions in Oli-Neu—Oligodendroglial Precursor Cells and Triggers Endoreduplication via p53 Pathway

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Abstract: Abnormal growth of oligodendrocyte precursor cells (OPCs) importantly contributes to the progression of glioblastoma tumors. Hence, molecules that block OPC growth may be of therapeutic importance in treating gliomas. 2-methoxyestradiol (2ME), an endogenous tubulin interacting metabolite of estradiol is effective against multiple proliferative disorders. Based on its anti-carcinogenic and anti-angiogenic actions it is undergoing phase II clinical trials. We hypothesize that 2ME may prevent glioma growth by targeting OPC growth. Here we tested this hypothesis by assessing the impact of 2ME on growth of OPC cell line “Oli-neu” and dissected the underlying mechanism(s). Treatment with 2ME inhibited OPC growth in a concentration-dependent manner and accompanied with significant upregulation in expression of p21 and p27, negative cell cycle regulators. Moreover, treatment with 2ME altered OPC morphology from multi-arm processes to rounded cells. At concentrations of 1 μ M and greater 2ME induced apoptosis with increased expression of caspase 3, PARP and caspase-7 fragments, externalized phosphatidylserine staining / apopercentage and increased mitochondrial activity. Flow-cytometry and microscopic analysis demonstrated that 2ME triggers endoreduplication in a concentration-dependent fashion. Importantly, 2ME induced cyclin E, JNK1/2 and p53 expression as well as OPC cell fusion, key mechanisms driving endoreduplication or whole-genome duplication. Importantly, inhibition of p53 with pfithrin- α rescued 2ME-induced endoreduplication. The pro-apoptotic and endoreduplication actions of 2ME were accompanied with upregulation of survivin, cyclin A, Cyclin B, Cyclin D2, ppRB. Taken together our findings provide evidence that 2ME not only inhibits OPC growth and triggers apoptosis, but also activates OPCs in a survival - fight or flight mode leading to endoreduplication. This inherent survival characteristic of OPCs may, in part be responsible for drug resistance in gliomas as observed for many tubulin interacting drugs. Importantly, the fate of OPCs after 2ME treatment may depend on cell-cycle status of the individual cells. Combining tubulin-interfering molecules with drugs, such as pfithrin- α , that inhibit endoreduplication may help inhibit OPC/glioma growth and limit drug resistance.

Keywords: 2-methoxyestradiol; p53; glioblastoma tumor; endoreduplication; gliomas; apoptosis

1. Introduction

Oligodendrocytes represent a specialized brain cell population which play a key neuroprotective role by myelinating neurons/axons and facilitating optimal electrical conductance[1,2]. However, abnormal growth of oligodendrocytes is also responsible for aggressive oligodendroglial neuronal/brain tumors, and constitute 5% of all neuroepithelial tumors and an incidence rate of thousand new cases per year in USA [3–6]. Gliomas, brain tumors of

oligodendroglial or astroglial origin, are extremely difficult to treat due to their low response to DNA alkylating agents as well as the activation of pro-survival pathways. The myelinating oligodendrocytes evolve from oligodendrocyte precursor cells (OPCs) and are morphologically distinct with multiple tentacle or arm like processes [7–10]. These oligodendrocyte processes wrap around neurons/axonal segments to maintain cell-cell contact and regulated by both intracellular tubulin (microtubules) and actin filaments [1,2]. Functionally, they regulate oligodendrocyte function of cell migration as well as active cell debris clearing processes [1,2]. Targeting microtubule with microtubule interfering agents has long been a therapeutic target against oligodendroglial cancer [11,12]. However, resistance to these agents has also been well documented and the underlying mechanisms remain unclear [13,14]. Since OPCs are involved in both good (neuroprotective) and bad (cancer/tumor) pathophysiology [1,2,10], a better understanding the mechanisms driving their activity is required. Moreover, role of endogenous molecules that modulate oligodendrocyte activity may be of clinical importance.

2-methoxyestradiol (2ME), an anticancer drug in phase II clinical trials, has been documented to have antiproliferative and antiangiogenic effects both in vitro and in vivo [15,16]. Although it is generally accepted that 2ME interacts with tubulin, resulting in faulty spindle formation and cell cycle arrest, there is no evidence for a universal mechanism of action and the mediators of apoptotic signalling by 2ME might even be cell-type dependent. In this regard, 2ME protects against multiple proliferative disorders such as atherosclerosis, injury induced neointimal thickening, glomerulosclerosis, pulmonary hypertension, endometriosis, and multiple cancers by targeting mechanisms driving cell growth and death [15,16]. For example, 2ME induced apoptosis in ovarian carcinoma cells via p38 and phospho-Bcl2 pathway [17]. In prostate cancer cells, cyclin B1 and cdc2 phosphorylation and its upstream regulatory molecular networks may be associated with 2ME-induced apoptosis and inhibition of ERK, JNK and p38 abrogated apoptosis in these cells [18]. In breast cancer cells, 2ME-induced apoptosis was associated with JNK activation and increased phosphorylation of the antiapoptotic proteins Bcl-2 and Bcl-xL [19]. In sarcoma cells, the pro-apoptotic protein BAX as well as formation of ROS were involved in the initial apoptotic event and furthermore 2ME treatment caused upregulation of death receptor ligands FasL and TNF- α and induced caspase-8 activation [20].

Taken together, the pleiotropic antitumor activities of 2ME, together with the fact that OPCs contribute to glioblastoma growth, suggests that 2ME might be a promising agent for the treatment of glioma and warrants in-depth investigation of 2ME actions and underlying mechanisms in modulating OPC growth. The fact that 2ME was found to have moderate side effects in clinical phase I/II studies [21] and is effective in an orthotopic rat glioma model highlights its potential in treating gliomas [22]. It is therefore of therapeutic importance for future drug development to understand the mechanism behind 2-ME actions, also in oligodendrocyte cells of the brain.

Hence, the aim of our study was to assess the effects of 2-methoxyestradiol on an oligodendroglial cell line (Oli-neu cells), and to dissect the underlying mechanisms. Oli-neu cells were established from O2A progenitor cells [23]. We chose this cell line as the model system since OPCs are implicated in glioblastoma growth and retain most of OPC phenotypical and electrophysiological characteristics even after several passages. Since 2ME is an investigational cytostatic drug for future, the overall goal of our study was to investigate its impact on oligodendrocyte/OPC health, particularly at pharmacological concentrations of 2ME. To attain our goals we assessed whether: 2ME inhibits OPC growth; 2ME influences OPC morphology; 2ME induces apoptosis in OPCs; 2ME triggers endoreduplication in OPCs; and molecular mechanisms driving the growth inhibitory, apoptotic and endoreduplication actions of 2-ME. Our results show that in OPCs, 2ME induced abnormalities in the cell cycle (endoreduplication) and mitosis eventually lead to cell death. Additionally, we show that 2ME induced endoreduplication in OPCs involves p53.

2. Materials and Methods

2.1. Cell Culture

Oli-neu Cells (OPCs): Culture dishes and flasks used to culture OPCs were coated with poly-D-lysine solution (5 mg/ml H₂O). After coating the surface for 30 min with poly-D-lysine solution, the solution was removed, the coated surface rinsed with sterile water prior to addition of culture medium to the flasks or plates. Freshly defrosted OPCs were plated in 6 cm culture dish and grown to confluence. Subsequently the cells were dislodged by trypsinization and transferred to a T 75 flask. OPCs were plated at a density of 1 or 1.3 million cells in 10 cm plates or in T75 flasks, respectively. OPCs were grown in SATO medium (DMEM containing 1% FCS, 25 g/mL gentamicin, 10 g/mL transferrin, 0.5 M L-thyroxine, 222 nM Na-selenite, 10 g/mL insulin, 16.1/mL putrescine, 62 ng/mL progesterone, 2 mM L-glutamine, 1 mM pyruvate, and 340 ng/mL tri-iodo-thyrodine) or in DMEM/F12 medium supplemented with B27 (DMEM/F12 containing 1% Penicillin/Streptomycin, 2% B27 supplement [with vitamin A], and 1% glutamine).

2.2. Protein Concentration Assay

Protein concentrations were determined by colorimetric assay using bichinonic acid (BCA) protein assay kit (Pierce) according to the manufacturer's protocol. The absorbance was measured with a SpectraFluor Plus (Tecan) and analyzed with the Magellan 6 or 7 software.

2.3. Western Blotting

Western blot analysis was performed with whole-cell lysates. Cells were grown on 6 cm dishes, washed twice with HBSS with Ca²⁺ and Mg²⁺, and lysed by the addition of 100 µL lysis buffer (Cell Signaling Technology) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% triton X-100, 2.5 mM sodium pyrophosphate, 1 mM -glycerophosphate, 1 mM sodium vanadate (Na₃VO₄), 1 g/mL leupeptin, 0.287mM phenylmethanesulfonyl fluoride (PMFS), and 0.2% sodium dodecyl sulphate (SDS). The cell-lysate were scraped from the dishes and homogenized by sonication (thrice for 3 seconds each). The protein concentration was determined spectrophotometrically using bichinonic acid protein assay kit from Pierce and SpectraFluor Plus (Tecan).

Equal amounts of proteins (20-25 g per lane) were diluted with 5X loading buffer (0.313 mM Tris/HCl pH 6.8, 10% SDS, 0.05% bromphenol blue, 50% glycerol; Fermatas, Hanover, MD, USA (R0891) and 0.1 M dithiotheritol (DTT) was added. Thereafter samples were denatured at 95° C for 5 min. After separation in 10% SDS-polyacrylamide gel, the proteins were transferred to a nitrocellulose membrane. Successful transfers were visualized by Ponceau S staining. For this the Ponceau S solution (Sigma) was added to the membrane. After two seconds, the Ponceau S solution was transferred back to the vial and the membrane was washed in PBS (phosphate buffered saline) until the red staining disappeared. When necessary, the membranes were cut at the appropriate molecular weight before the Ponceau staining was washed out. Membranes were blocked in 5% nonfat dry milk in PBS/0.2% Tween 20 either over night at 4° C or at RT for 1 hour. After blocking, the membrane was incubated with the primary antibody (1-4 hours at RT or overnight at 4° C). Primary and secondary antibodies were dissolved in 1% nonfat dry milk in PBS containing 0.2% Tween 20. To remove unbound primary antibodies, the membrane was washed three times for 10 min each, with 1% milk in PBS/0.2% Tween 20. Incubation with peroxidase-conjugated secondary antibodies was again performed either for 1-4 hours at RT or over night at 4 °C. The membrane was washed one time with 1% nonfat dry milk in PBS/0.2% Tween 20 and two times with PBS/0.2% Tween 20. Peroxidase activity was detected using ECL (Pierce) and the membranes were exposed to X-OMAT LS films. When the membranes were analyzed with the LI-COR system, all steps were performed as described above, except that the membranes were blocked in 5% nonfat dry milk in PBS without Tween and incubated with the secondary antibody (IRDye 680/800 conjugated goat-rabbit IgG) for 45 min, and thereafter washed with PBS without Tween 20. For successive detection

of different proteins on the same membrane, the membrane was washed with PBS/0.2% Tween 20 after analysis of the first protein, incubated for 20 min with stripping buffer 1 (0.1 M glycine in PBS, pH 2-3) and subsequently shortly washed in stripping buffer 2 (1M NaCl in PBS). Subsequently the membranes were washed three times with PBS/0.2% Tween-20.

2.4. Growth Studies

Studies were conducted using phenol-red free medium. When not specified, cells were growth arrested for 24 hours in medium containing 0.1% steroid-free FCS. Pretreatment of cells was done by adding the compound of interest during starvation. Cell growth was induced by addition of 2.5% steroid-free FCS in the presence or absence of 0-10 μ M of 2ME. In all cell-growth experiments, cell growth was analyzed by ³H-thymidine incorporation and by counting cell number. For ³Hthymidine incorporation experiments, cells were treated with 2.5% steroid-free FCS plus treatments for 20 hours and subsequently fresh treatment medium was added with 1.0 μ M Ci/well ³H-thymidine for 4 hours. Aliquots from 4 different wells per treatment were mixed with 10 mL scintillation fluid and counted in a liquid scintillation counter. For cell number experiments, sub-confluent cells were growth arrested for 24 hours and subsequently treated for 24 hours as mentioned above. After 24 hours the cells were dislodged by trypsinization and counted in a coulter cell-counter.

2.5. FACS Analysis

For cell cycle distribution analysis, 0.4 million cells were plated in growth medium into 10 cm dishes. After attachment overnight, the cells were washed once with PBS and the medium was replaced medium containing different 2ME concentrations (0-10 μ M). After incubation at 37° C and 5% CO₂ for two days cells were washed twice with HBSS without Ca²⁺ and Mg²⁺ and harvested by trypsinization. After addition of an equal amount of medium, cells were centrifuged for 10 min at 1250 rpm and 4° C. The pellet was re-suspended in 1 mL sample buffer (1 g/L glucose in PBS). Cells were counted and sample buffer was added until the cell number reached a concentration of 1 million cells per mL. The cell suspension (1ml) was centrifuged in a FACS tube for 10 min at 1350 rpm and 4° C. After removal of the supernatant, cells were fixed by drop-wise addition of ice-cold 70% ethanol and stored at 4° C until analysis. For DNA staining with propidium iodide (PI), the fixed cells were centrifuged for 7 min at 2600 rpm and 4° C. Subsequently, the pellet was resuspended in 0.5-1 mL PI-staining solution (50 μ g/mL propidium iodide, 100 U/mL RNase A, sample buffer) and the tubes were shaken for 0.5-1 hour at RT in the dark. The DNA amount was analyzed by flow cytometry.

2.6. Metabolic Activity Using MTT Assay

Cells were grown over night in a 96 well plate (10'000 cells per well). After the treatment with the test compound the medium was replaced by fresh media containing 0,5 mg/mL MTT and the test compound. After incubation for 3-4 hours, the medium was aspirated and the cells were lysed by the addition of 200 μ L DMSO. The absorbance was analyzed by the Spectrofluorometer Plus reader (Tecan Group Ltd.) at 540 nm.

2.7. Apoptosis Studies

To assess the apoptotic impact of 2ME in OPCs, cells plated in culture dishes were grown to sub-confluence and subsequently treated with or without 0 to 10 μ M of 2ME. The OPC lysates were analyzed by western blotting for caspase mediated mechanism by assessing caspase-3 and caspase-7 fragmentation. Moreover, involvement of PARP was assessed by measuring PARP fragmentation. Staining with Apopercantage dye, a specific marker for apoptosis which stains externalized phosphatidylserines was used to assess whether 2ME has apoptotic or necrotic actions on OPCs.

2.8. Microscopy (Bright Field, Fluorescence and Live)

For the analysis of DAPI stained OPCs, the cells were grown on coverslips coated with poly-D-lysine. After the treatment with 2ME for the indicated times, cells were fixed by slowly adding fresh

ethanol plus acetic acid mixture to the cell (7.5 mL EtOH 100 puriss, 2.5 mL acetic acid) and waiting until it evaporates. Thereafter, 10 μ L of DAPI counterstain was added on top of the cells, and the fluorescence was analyzed using the Olympus Microscope BX61.

For live microscopy, cells treated with or without 2ME and observed and/or live images recorded for subsequent analysis using Olympus IX81 microscope. Time-lapse phase contrast imaging of the effects of 2ME on OPN cell activation and fusion. Live videos were recorded following 48 hour treatment of cultured OPCs with 2ME (5 or 10 μ M) and using Olympus IX81 microscope. To analyze the effects of 2ME on OPCs, cells were plated on pre-coated 6 well plates at a density of 200,000 cells /well and allowed to attach and grow for 24 hours. Subsequently, the cells were treated with 2ME for 24 or 48 hours, in presence of complete OPN growth medium and the effects observed by employing phase contrast microscopy.

2.9. Statistical Analysis

Data was analyzed using ANOVA and statistical significance ($p < 0.05$) was calculated using Fisher's Least Significant Difference test.

3. Results

3.1. Cultured OPCs

Cultured oligodendroglial cell line "Oli-neu" which represents OPCs and established from O2A progenitor cells [23] were used in the present study. We chose this cell line as the model system to investigate growth effects of 2ME as it retains most of the phenotypical and electrophysiological characteristics even after several passages. As shown in Figure 1A OPCs grew robustly in culture and retained their characteristic arm/tentacle like processes (Figure 1B).

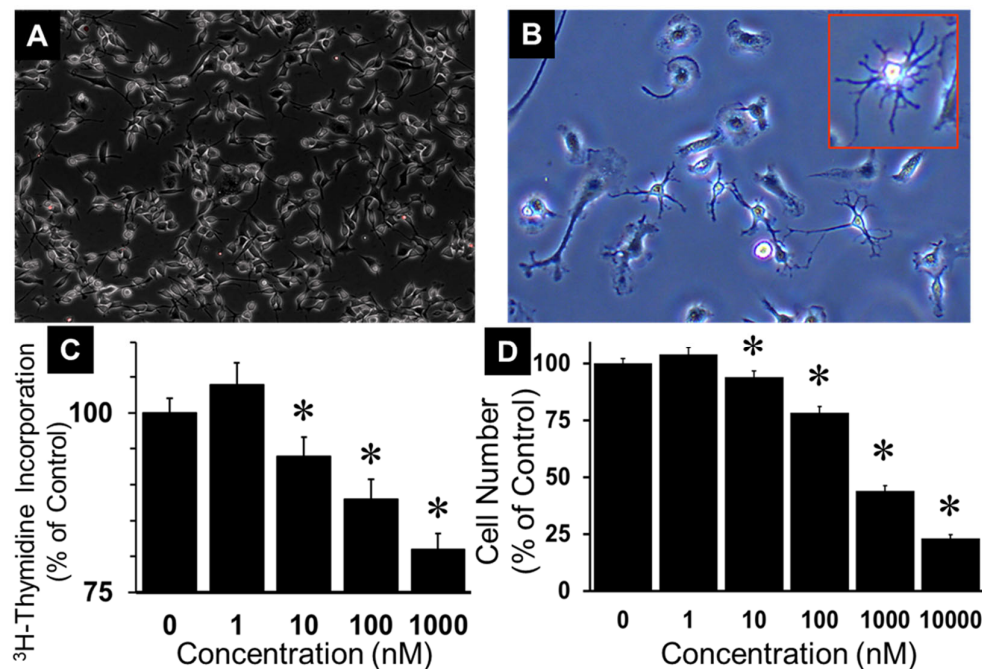


Figure 1. Growth inhibitory effects of 2-methoxyestradiol (2ME) in Oli-neu (OPCs) cells. (A) Representative photomicrograph showing cultured OPCs (4x mag); (B) Representative photomicrograph depicting OPCs with characteristic oligodendrocyte morphology at a higher magnification (400 x); (C) Bar graph showing the concentration-dependent inhibitory effects of 2ME on FCS (2.5%) induced DNA synthesis measured by assessing ³H-thymidine incorporation; (D) Bar graph depicting the inhibitory actions of 0-10 μ M 2ME on 2.5% FCS induced change in cell (OPC)

number. Each data point represents mean \pm SEM from 3 separate experiments, each conducted in triplicate or quadruplicate; * $P < 0.05$ vs vehicle treated control.

3.2. 2ME Inhibits Oli-Neu Cell Growth

To investigate whether 2ME, an major endogenous E2-metabolites, is able to regulate OPC growth, we assessed the concentration-dependent effects of 2-ME on 2.5% SF-FCS induced DNA synthesis and cell number. Treatment with 1-10000 nM 2ME significantly and concentration dependently inhibited DNA synthesis in a concentration dependent fashion. At concentration of 10, 100 and 1000 nM, 2ME abrogated FCS stimulated growth from $100 \pm 0.8\%$ to $91.1 \pm 1.1\%$ ($p < .05$), $86.8 \pm 1.4\%$ ($p < .05$), and $76.3 \pm 1.7\%$ (Figure 1C) ($p < .05$). Similarly, treatment with 10, 100, 1000 and 10000 nM 2ME significantly decreased cell number in a concentration dependent manner from $100 \pm 1.5\%$ to $97.0 \pm 0.8\%$, $90.0 \pm 0.4\%$, $70.7 \pm 0.5\%$, $44 \pm 1.5\%$, and $23 \pm 1.8\%$ (Figure 1D) ($p < .05$).

3.3. 2ME Modulates p21 and p27 Expression

To determine the mechanisms by which 2ME inhibits cell proliferation, we examined the expression of p21 and p27, which negatively regulate cell cycle progression and inhibit growth. Treatment with 10, 100, and 1000 nM 2ME significantly increased p21 expression from $100 \pm 9.4\%$ to $169 \pm 2.1\%$, $250 \pm 30.6\%$, and $129.8 \pm 6.2\%$ (Figure 2) ($p < .05$). Similar to p21, treatment with 1, 10, and 100 nM 2ME significantly increased p27 expression from $100 \pm 5.1\%$ to $228.4 \pm 22.7\%$, $239.8 \pm 9.5\%$, and $196.8 \pm 19.6\%$ (Fig.2) ($p < .05$). Interestingly treatment with 1000 nM 2ME, which induce pro-apoptotic actions, significantly decreased p27 expression to $30.8 \pm 5.9\%$ ($p < .05$). Similar to p27, at concentrations higher than $1 \mu\text{M}$, 2ME lowered the magnitude of p21 expression from 250% at 100nM to 130% and control levels.

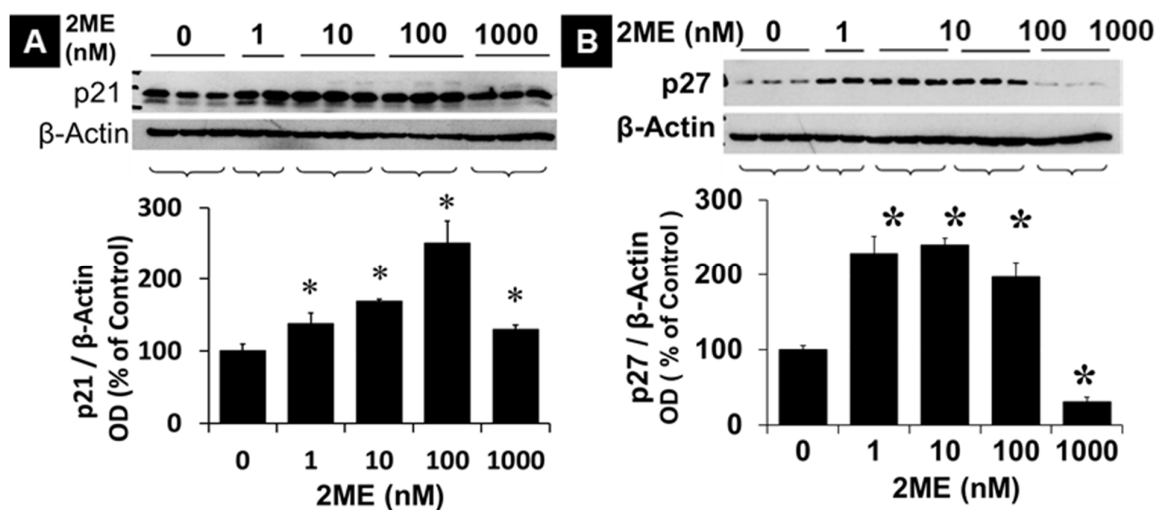


Figure 2. Modulatory effects of 2ME on p21 and p27 expression. Representative Western blot showing the effects of 2ME on the expression of p21 (A) and p27 (B) in Oli-neu cells. Cells were serum-starved for 24 hours in SATO medium without phenol red, without progesterone, and containing 0.25% steroid-free FCS. Subsequently cells were treated with 1-1000 nM 2ME for 48 hours in SATO without phenol red, without progesterone, and containing 1% steroid-free FCS. The bar graphs show the densitometric analysis of the changes observed and normalized to the internal standard, β -actin. The results are presented as mean \pm SEM ($n=3$). * $P < 0.05$ vs control.

3.4. 2-ME Modulates OPC Morphology

Cultured OPCs have specific morphology with tentacle or arm like processes (Figure 1B). Since these processes play a key role in regulating OPC function, we assessed how 2ME impacts this characteristic. As shown in representative phase contrast and immunofluorescence

photomicrographs in Figure 3A–C, treatment of OPCs with 2ME influenced OPC morphology and contracted the arm/tentacle processes in a concentration dependent fashion (Figure 3D). At a concentration of 5 μM of 2ME, most cells lost their elongated arm like processes and became rounded (Figure 3C).

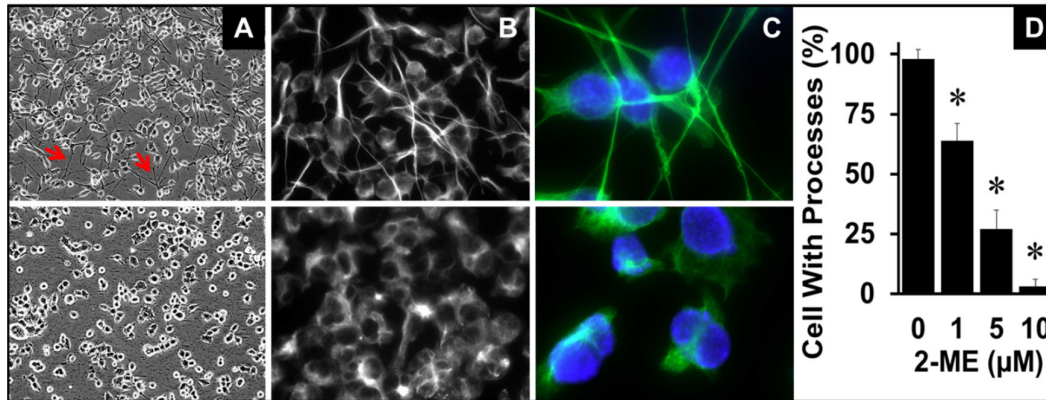


Figure 3. Morphological changes induced by 2ME in Oli-neu cells (OPC). Representative phase contrast (A) and bright field fluorescent (B and C) images showing retraction of OPC arms/tentacles in OPCs treated with 2ME (5 μM) for 24 hours. DAPI was used for nuclear staining (blue). Panel D depicts bar graph depicts the concentration-dependent effects of 2ME on OPC arm retraction. Cells at 10 different location of the photomicrograph were observed and counted to assess the effects of 2ME on cell rounding or loss of tentacles. * $P < 0.05$ vs vehicle treated control.

3.5. 2ME Treatment Induces Apoptosis

Since 2ME inhibited OPC growth, we next assessed its impact on OPC viability. We first measured the viability of Oli-neu cells after 2ME treatment using the MTT assay, which detects mitochondrial metabolic activity as a measure for viable cells. For this, cells were treated with 5 μM 2ME in normal growth medium for 18 hours. Thereafter, MTT was added to the medium and 2 hours after MTT addition the medium was removed and the cells were lysed with DMSO. The metabolic activity was assessed by measuring the absorbance at 540 nm. In parallel, the cell number after treatment with 5 μM 2ME was assessed by cell counting. Although 2ME treatment decreased cell number (data not shown), 5 μM 2ME significantly increased the calculated metabolic activity per cell from $100 \pm 10.0\%$ to $181.1 \pm 10.3\%$ (Figure 4A) ($p < .05$).

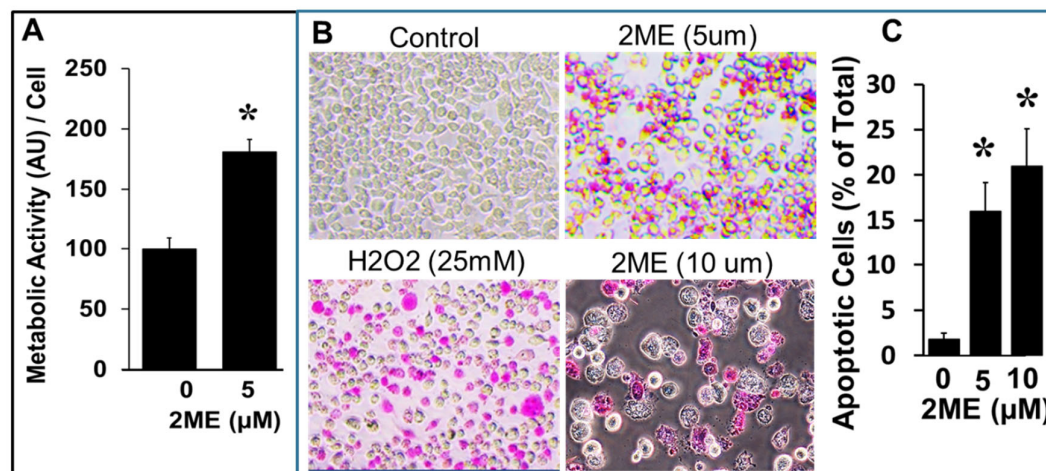


Figure 4. Activation of metabolic activity and apoptosis in Oli-neu (OPC) cells by 2ME. (A) Bar graph depicting the effects of 5 μM 2ME on OPC metabolic activity measured by MTT assay and normalized

to OPC numbers counted after treatment with vehicle or 5 μ M 2ME. The graph represents the metabolic activity per cell. Data represents mean \pm SEM (n=3 each in triplicates). * $<.05$ vs control. (B) Representative photomicrographs stained with the Apopercantage dye, a specific marker for apoptosis, which stains the externalized phosphatidylserines. OPCs treated with H₂O₂ (25mM) served as positive control for apoptosis. (C) Bar graph depicting percentage of apoptotic cells (Apopercantage) in response to 2ME (mean \pm SEM n=3). * $P<.05$ vs control.

To assess, whether 2ME induced cell death by apoptosis or necrosis, 2ME treated cells were stained with the Apopercantage dye, which stains externalized phosphatidylserines. As shown in Figure 4B, treatment with 2ME (5 and 10 μ M) significantly increased the number of cells positively stained for externalized phosphatidylserines from $100 \pm 1.0\%$ to $470 \pm 13.3\%$ and $617.3 \pm 14.5\%$ ($p<.05$).

To assess the role of caspases 3 and 7 in 2ME mediated apoptosis in OPCs we analyzed their fragmentation by Western blotting. Treatment with 0-10 μ M 2ME significantly induced the fragmentation of caspase 3 from $100 \pm 12.2\%$ to $192.2 \pm 8.5\%$, $234.3 \pm 26.9\%$, and $271.3 \pm 26.1\%$ (Figure 5) ($p<.05$). Similar to caspase 3, caspase 7 fragmentation was significantly induced by treatment with 1.25, 2.5, and 5 μ M 2ME from $100 \pm 27.3\%$ to $316.5 \pm 28.8\%$, $319.6 \pm 35.4\%$, and $303.5 \pm 35.2\%$ (Figure 5) ($p<.05$). Furthermore, treatment with 1.25, 2.5, and 5 μ M 2ME significantly increased PARP fragmentation from $100 \pm 40.0\%$ to $1167.9 \pm 228\%$, $655.4 \pm 106.8\%$, and $622.8 \pm 159.5\%$ (Figure 5) ($P<.05$). Consistent with the activation of pro-apoptotic mechanisms, in OPCs incubated with 5 μ M 2ME for 48 hours, DNA staining with PI (propidium iodide; a general marker for cell death) followed by FACS analysis of the PI stained cells showed that 2ME induces cell death in OPC cells. As shown in Figure 7, treatment with 2ME led to a shift and decrease in number of cells with 2N DNA content, probably due to nuclear fragmentation.

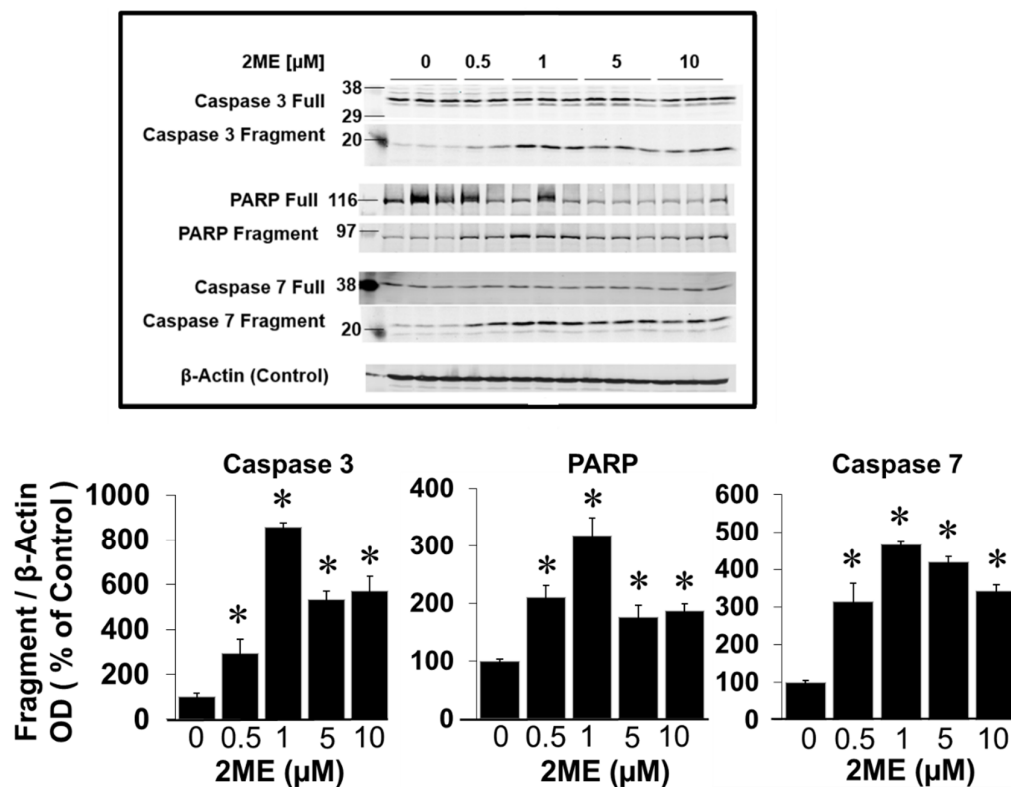


Figure 5. Western blots showing the modulatory effects of 2ME on Caspase 3 and Caspase 7 cleavage as well as on PARP fragmentation in Oli-neu cells. The bar graphs depicts the densitometric analysis of the changes observed in cleavage products or fragments, normalized to the total protein amount. The results are presented as mean \pm SEM (n=3). * $P<.05$ vs control.

3.6. 2ME Treatment Induces Endoreduplication

As a next step we assessed DNA quantity per cell by measuring PI incorporation as well as by DAPI staining. To this end, cells were grown to subconfluency in normal SATO medium. Thereafter, the cells were treated with 0-10 μ M 2ME for 48 hours and PI incorporation was assessed by FACS analysis. As shown in Figure 6, treatment with 2ME > 1 μ M concentration resulted in a high percentage of cells containing a DNA content of >4N, indicating that cells skipped mitotic cell division, i.e. underwent endoreduplication. To confirm this, cells treated with and without 2ME for 48 hours were examined by fluorescence microscopy after DAPI staining. As shown in Figure 6, 2ME treated cells were rounder and bigger than non-treated cells and they contained multiple nuclei (see arrows).

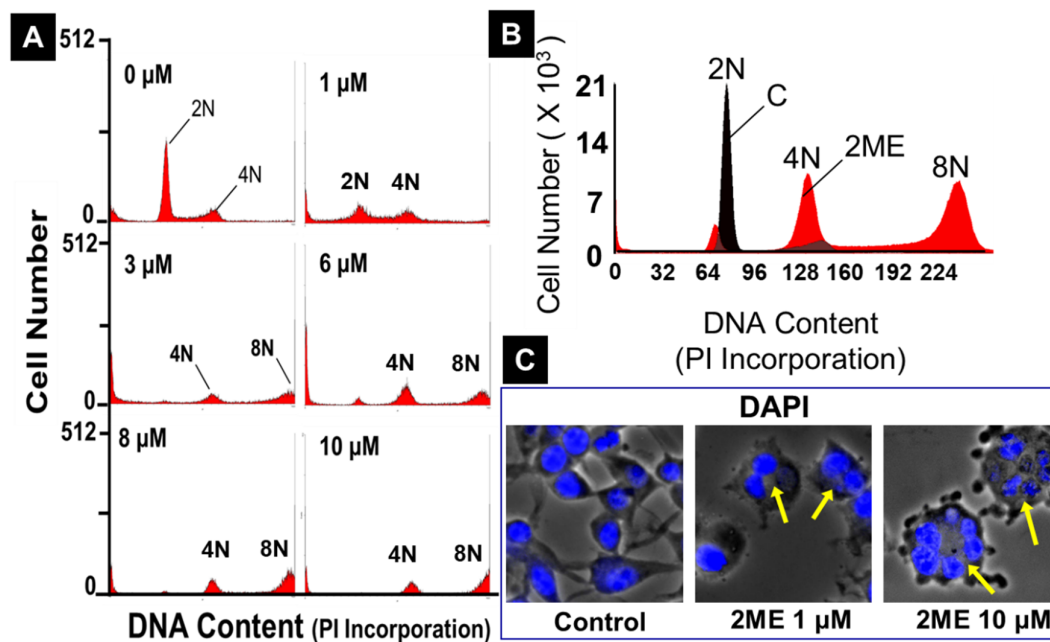


Figure 6. Endoreduplication promoting effects of 2ME in Oli-neu (OPCS) cells. FACS analysis of PI stained OPCS (A and B) and fluorescent microscopy of DAPI stained cells (C) showing that treatment with 2ME induces endoreduplication in OPCS. Panel A depicts OPCS treated with 1 - 10 μ M 2ME for 48 hours and the DNA was stained with propidium iodide prior to FACS analysis. Appearance of 8N cell population is evident in representative FACS of 2ME treated OPCS. Panel B compares normal and 2ME treated OPCS by overlapping untreated OPCS (black) and 2ME treated OPCS (red). Panel C depicts DAPI stained multinucleated OPCS following 2ME treatment.

3.7. Mechanism Underlying 2ME-Induced Endoreduplication

As shown above, 2ME treatment induced endoreduplication and this was accompanied by an increased metabolic activity per cell. To identify the molecular changes induced by 2ME treatment, we assessed the phosphorylation status of Rb and MAPK, which are both known to be involved in proliferation. To this end, Oli-neu cells were grown to subconfluency and treated with or without 10 μ M 2ME for 48 hours. Protein phosphorylation was assessed by Western blot analysis. Rb acts as a tumor suppressor by providing a cell cycle checkpoint between G1 and S (synthesis) phases. The antibody used in this study binds to phosphorylated as well as hyperphosphorylated Rb. The active, underphosphorylated form (pRb) is primarily found in resting or fully differentiated cells, while the hyperphosphorylated form (ppRb) is primarily found in proliferating cells. pRb inactivation by phosphorylation is a critical step leading to S-phase commitment at the G1 checkpoint of the cell cycle. As shown in Figure 7, less pRb was observed in cells treated with 2ME compared to control.

Therefore, the ratio between pRb and ppRb increased due to treatment with 2ME, indicating that treatment with 10 μ M 2ME promoted proliferation. Another protein which is known to play a key role in proliferation is MAPK. Phosphorylation of MAPK, however, was not influenced by treatment with 2ME (Figure 7). Importantly, 2ME downregulated pAkt expression from $100 \pm 3.65\%$ to $73 \pm 1.7\%$ ($P < .05$).

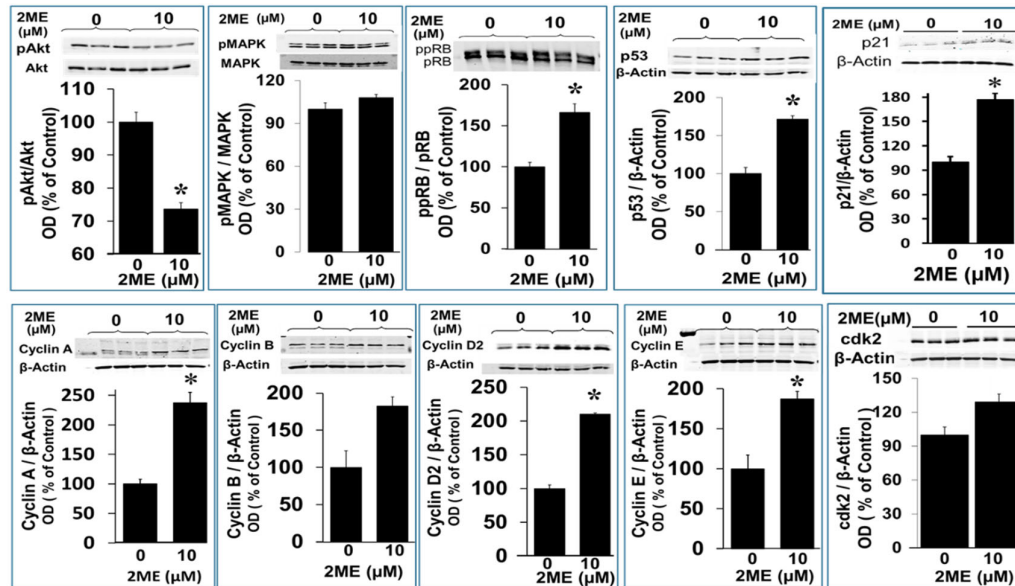


Figure 7. Western blots depicting the modulatory effects of 2ME on various cell cycle and signal transducing proteins associated with endoreduplication. OPCs were treated with 10 μ M 2ME for 48 hours and the changes in pAkt, pMAPK, ppRb, p53, p21, Cyclin A, Cyclin B, Cyclin D2, Cyclin E and cdk2 assessed in the lysates. Representative blots and bar graphs depict the effects of 2ME as compared to vehicle treated control. The modulatory actions are presented as mean \pm SEM (n=3). * $P < .05$ vs control.

Furthermore, the cyclins are a family of proteins which control the progression through the cell cycle by activating Cdk enzymes. There are several cyclins which regulate Cell cycle progression is regulated by several cyclins and are active in different phases. We assessed the impact of 2ME on G1/S cyclins which control cell cycle progression at the G1/S transition by activating Cdk enzymes. Cyclin A is active during G1 phase, while cyclin D and E regulate the transition from G1 to S phase. G2/M cyclins are essential for the control of the cell cycle at the G2/M transition, where cyclin B regulates the progression from the S to G2 phase. Treatment with 10 μ M 2ME for 48 hours resulted in an increase of the expression of cyclin A from $100 \pm 7.7\%$ to $237.8 \pm 17.5\%$, of cyclin B from $100 \pm 22.4\%$ to $182.7 \pm 12.3\%$, of cyclin D2 from $100 \pm 5.5\%$ to $209.0 \pm 2.1\%$, and of cyclin E from $100 \pm 17.2\%$ to $187.4 \pm 9.1\%$ (Figure 7) ($P > .05$) as well as a minor change in cdk2 from 100 ± 4.1 to $127 \pm 3.45\%$ (Figure 7).

Finally, p53 which is known to growth arrest cells at the G1/S regulation point in the case of DNA damage. When DNA repair proteins are able to overcome the DNA damage, the cell continues the cell cycle. In case of irreparable DNA damage, p53 initiates apoptosis. The expression of p53 was analyzed by Western blot analysis after 48 hours with or without treatment with 1.25 to 10 μ M 2ME. As shown in Figure 7, p53 expression was significantly upregulated by 2ME from $100 \pm 8.0\%$ to $171.5 \pm 4.5\%$ ($p < .05$) at 10 μ M, moreover the upregulation was $\approx 300\%$ at 1.25 to 5 μ M of 2ME (Figure 8).

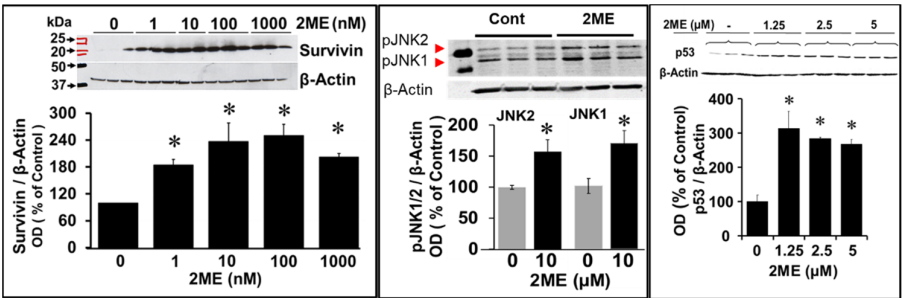


Figure 8. Western blots depicting the modulatory effects of 2ME on various signalling proteins associated with endoreduplication. OPCs were treated with 0-10 μ M 2ME for 48 hours and the changes in JNK1, JNK2, Survivin and p53 expression assessed in the lysates. Representative blots and bar graphs depict the effects of 2ME as compared to vehicle treated control. The bar graphs depict modulatory actions of 2ME and presented as mean \pm SEM (n=3). * P<.05 vs control.

Next we assessed the impact of 2ME on survivin, JNK1 and JNK 2. As Shown in Figure 8, treatment of OPCs with 1-10 μ M 2ME significantly induced survivin expression in a concentration dependent fashion with a maximal increase of \approx 250% at 0.1 μ M concentration. Moreover, treatment with 2ME (10 μ M) significantly induced phosphorylated JNK1 and JNK2. Similarly, treatment with 0-5 μ M 2ME induced p53 expression in OPCs by \approx 300% (Figure 8).

We further analyzed the role of p53 signaling in 2ME driven endoreduplication. We pretreated Oli-neu cells for 2 hours with 20 μ M Pifithrin- α , a reversible inhibitor of p53, and subsequently treated them with 5 μ M 2ME in presence of 20 μ M Pifithrin- α for 24 hours. Subsequently, the cells were fixed, stained with PI, and the DNA content was assessed by FACS analysis as well as phase contrast and bright field microscopy. As shown in Figure 9A, B, treatment with 2ME induced the typical morphological changes in Oli-neu cells, which changed from an elongated and branched structure to a larger and more rounded shape. Treatment with 5 μ M 2ME induced endoreduplication (Figure 9B; second row) with generation of 8N cell population, whereas, pretreatment with Pifithrin- α prevented the 2ME induced endoreduplication (Figure 9B, third row), although the typical morphological changes induced by 2ME were not reversed (Figure 9, third row). The abrogatory effects of Pifithrin- α on 2ME induced endoreduplication was also evident in cells evaluated microscopically. Treatment with 2ME as well as Pifithrin- α inhibited cell proliferation (Figure 9C) and the inhibitory actions were greater in OPCs treated with both 2ME and Pifithrin- α . Interestingly the inhibitory actions of Pifithrin- α on endoreduplication were accompanied with downregulation of 2ME induced p21 and p53 expression (Figure 9D).

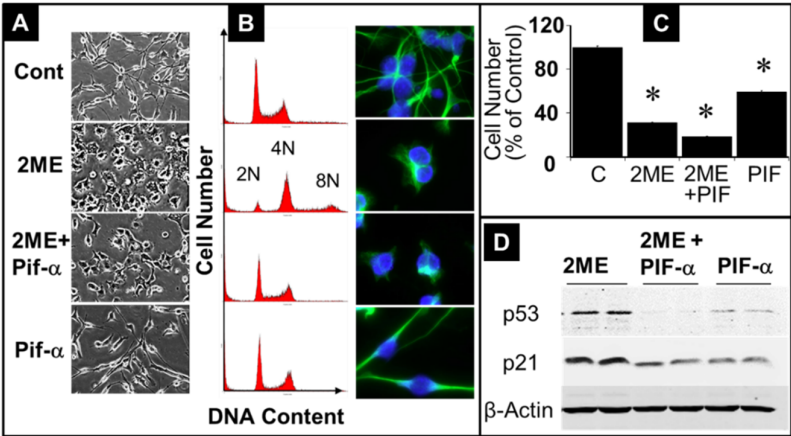


Figure 9. Inhibition of p53 with Pifithrin- α prevents 2ME-induced endoreduplication, but not arm process retraction, in Oli-neu cells. Bright-field microscopy (A) and FACS analysis (B) of Oli-neu cells

treated with 5 μ M 2ME for 18 hours after pretreatment with or without Pifithrin- α 20 μ M for 2 hours. For FACS analysis the DNA was stained with propidium iodide. Representative fluorescent images show 2-ME induced changes in Oli-neu morphology in presence and absence of Pifithrin- α . Bar graph depicts changes in Oli-neu cell number (C) and change in expression of p53 and p21 (D) in presence and absence of Pifithrin- α . All experiments were repeated thrice and values in bar graph represent mean \pm SEM (n=3). * P<.05 vs control.

Microscopic assessment of OPCs also showed cells with multinucleated clusters (marked with red circles) prevalent in 2ME treated group, whereas this clustering was lost in cultures treated with 2ME in presence of Pifithrin- α (Figure 10).

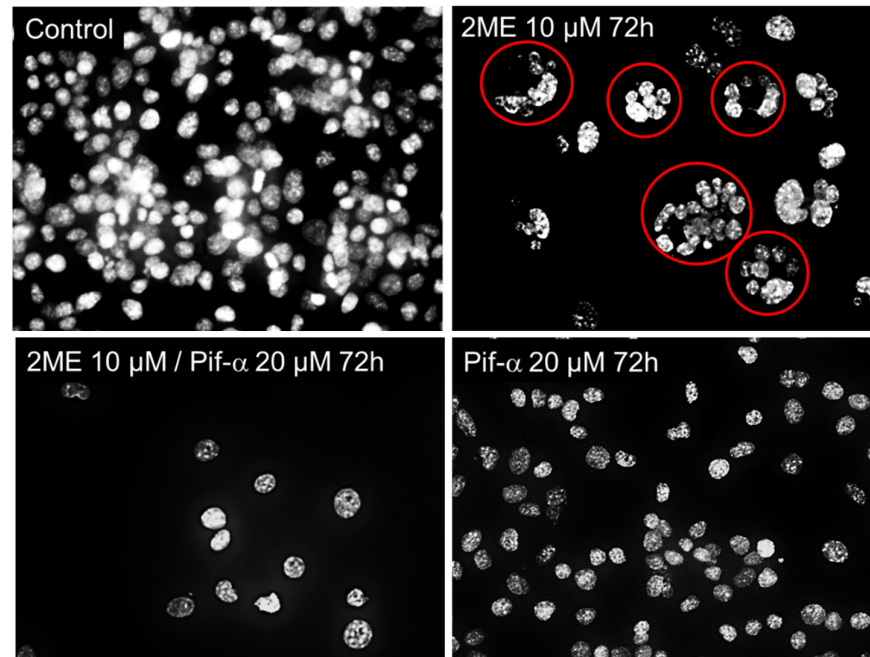


Figure 10. Representative fluorescent photomicrographs showing the of Oli-neu clustering pattern following treatment with 5 μ M 2ME for 72 hours after pretreatment with or without Pifithrin- α 20 μ M for 2 hours. Inhibition of p53 prevented cell prevented 2ME-induced clusters of multinucleated cells.

3.8. 2ME Trigger Cell Fusion in OPCs

Apart from the intracellular mechanisms, fusion of cells may also contribute to multinucleated cells. It is well known that cell-fusion process in cancer cells contributes to drug resistance, as has been observed for microtubule interfering drugs. Since 2ME interacts with tubulin it may also trigger cell fusion. As shown in Figure 11, in live microscopy experiments treatment of Oli-neu cells with 5 and 10 μ M triggered cell activity with some actively migrating and interacting with neighboring cells and undergoing cell fusion. Interestingly, treatment with 2ME upregulated survivin in Oli-neu cells (Figure 8) suggesting that 2ME triggers cell survival activities. The cell fusion activity was restricted to cells that were hyperactive or under duress and capable to move/migrate, suggesting that under crisis for existence OPCs can fuse to survive and resist.

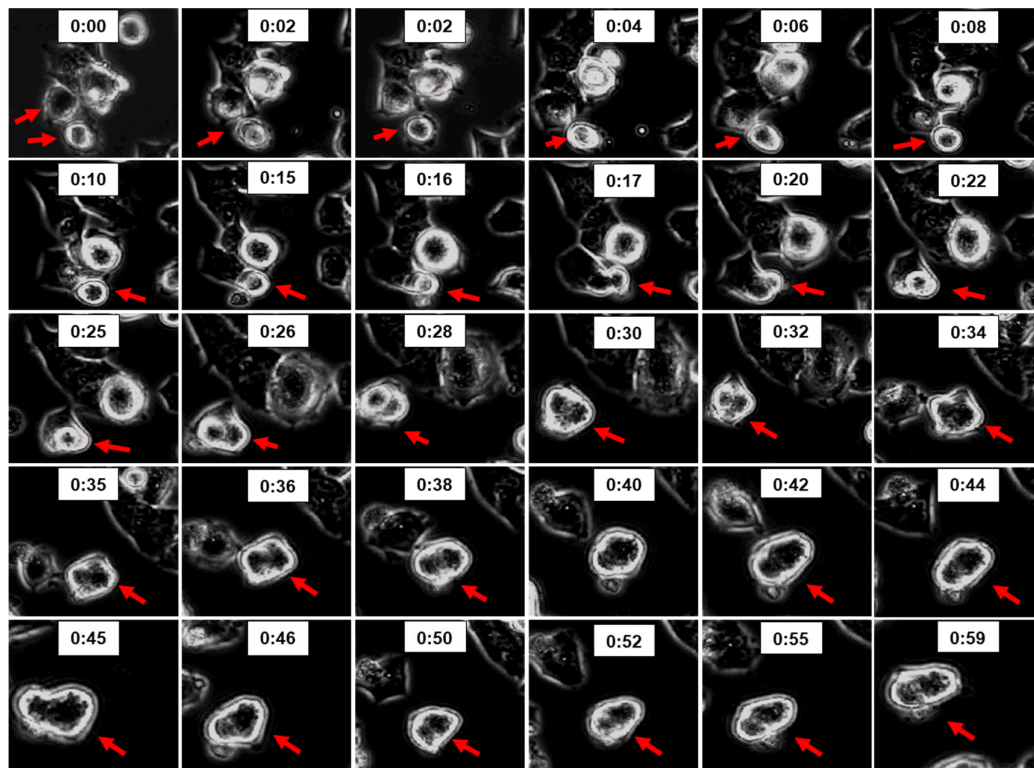


Figure 11. Time-lapse phase contrast imaging showing 2ME induced cell fusion in Oli-neu. Photomicrographs depicts time-sequence of two Oli-neu cells undergoing cell fusion (indicated by red arrows) following treatment of cultures with 5 μ M 2ME. Similar effects were observed in four different experiments.

4. Discussion

2-Methoxyestradiol and endogenous estradiol metabolite is effective against multiple proliferative disorders including various cancer cells. In the present study we demonstrate that 2ME-treatment induced OPCs to be metabolically more active than control cells and caused an accumulation of cells with a DNA content $>4N$, indicating that cells underwent endoreduplication. Our in vitro findings show that 2ME inhibits OPC growth, in part, by inducing apoptosis and up-regulation of pro-apoptotic markers (caspase 3, caspase 7 and PPAR fragmentation; and phosphatidylserine externalization). Importantly, 2ME altered OPC morphology by reducing their characteristic arm processes, moreover at concentrations of $>1\mu$ M 2ME induced endoreduplication in OPCs. The growth inhibitory actions of 2ME were accompanied with upregulation of p21 and p27 (negative regulators of cell cycle) whereas, endoreduplication was accompanied with significant upregulation of many cell-cycle proteins (cyclin A, cyclin B, cyclin D2, cyclin E) as well as p53 a tumor suppressor known to cooperate with cyclin E and drive endoreduplication. At signal transduction level, 2ME failed to influence MAPK phosphorylation; inhibited Akt phosphorylation; and induced pRb as well as JNK1/JNK2 phosphorylation. Interestingly, 2ME upregulated the expression of survivin and triggered OPC cell fusion. Importantly, we demonstrate that inhibition of p53 with pifithrin- α prevented 2ME induced endoreduplication, but not morphological alterations (retraction of cell processes), suggesting that 2ME mediates endoreduplication via p53, whereas its morphological actions involve other mechanism. Our findings provide the first evidence that 2ME not only inhibits OPC growth but also triggers apoptotic and cell survival processes leading to endoreduplication. We hypothesize that combining p53 inhibitors with MTIs may help prevent MTI induced endoreduplication in cancer cells and be of clinical relevance.

Endoreduplication has been observed in pathological evaluation of various cancers including myomas. More it is prevalent in cells treated with MTIs, such as colchicine and nocodazole [24,25].

Our finding that 2ME, a tubulin interfering agent, induced endoreduplication in OPCs is consistent with these observations. This supports the concept that MTIs can switch on a new replication cycle without anaphase chromosome segregation. Additionally, our data shows that several cell cycle proteins are regulated by 2ME treatment and that inhibition of p53 abrogated endoreduplication, indicating that microtubule inhibition leads to endoreduplication by a p53-dependent mechanism.

Although polyploidy in mammals is evident in different mammalian cells under normal conditions (e.g. in trophoblast or myocardial cells) studies indicate that aneuploidy, genomic instability, and defects in cell cycle checkpoints can contribute to tumorigenesis [24]. In this regard, it has been shown that aneuploidy is a characteristic of the great majority of human tumors [26]. Furthermore, it is linked to the progressive development of high-grade, invasive tumors [27,28] and it is a necessary intermediate in the formation of many solid human tumors [29]. Additionally, a high degree of aneuploidy correlates with poor patient prognosis [30,31]. Although polyploidy is observed in tumors and is linked to its progression, a cause and effect relationship remains unclear. Many MTIs known to induce polyploidy also inhibit tumor progression and cancer cell growth, hence it is crucial to understand the mechanisms contributing to endoreduplication, and whether this leads to aneuploidy and tumorigenesis.

In the present study we show that at high concentrations 2ME induces deleterious effects in OPCs. Considerable effort has been made to investigate whether 2ME could be used therapeutically to treat brain cancers, called gliomas, that originate from glial cells such as astrocytes and oligodendrocytes [32,33] by targeting their growth. Since gliomas as they are extremely difficult to treat and often fatal, new therapeutic molecules targeting their growth are required. Chemotherapy often fails in patients with gliomas due to their resistance to the commonly used DNA alkylating agents and due to the activation of pro-survival pathways. The reasons why 2ME is a promising agent for the treatment of gliomas are the following: 1) 2ME is a MTI [34], and chemotherapy regimes that use MTIs such as vincristine are already used to treat gliomas [35]. 2) 2ME has pleiotropic antitumor activities, that include both, inhibition of angiogenesis and cytotoxic effects on tumor cells. 3) Its mechanism does not rely on DNA alkylation. 4) It has been shown to be effective in an orthotopic rat glioma model. 5) It has moderate side effects in clinical phase I/II studies.

Although 2ME has been shown to inhibit growth of tumors and cancer cells, our findings that 2ME induces polyploidy in OPCs. These findings, together with the fact that endoreduplication is linked to tumorigenesis, suggests that a better understanding of the cause and effect relationship between tumor/cancer growth and polyploidy/endoreduplication is required. Moreover, it is important to explore ways to reduce the risks of polyploidy development in response to drugs that interfere with microtubule dynamics. At a molecular level endoreduplication results from an erroneous exit from mitosis due to a failure of the checkpoints, which normally regulate cell cycle arrest at specific stages when previous events have not been completed. Although most cells sense the disturbance of the microtubule assembly after MTI treatment, some cells arrest only transient and thereafter exit mitosis without chromosome segregation and become tetraploid.

Consistent with our observation, 2ME has been shown to induce endoreduplication in both non-cancerous [36], and cancer cells [19,37,38]. The mechanism underlying 2ME-induced endoreduplication remains unclear, although its interaction with tubulin and inhibition of polymerization seems to be involved. Indeed, 2ME is known to be a MTI molecule [34] and endoreduplication has been observed in response to MTIs in cells lacking p53, pRb and CdkIs p21 or p16 [39–43]. Moreover, in vacular smooth muscle cells, 2ME induced endoreduplication by a Cdk2-dependent pathway [37].

Our results showed that p53 expression was regulated by 2ME. However, most studies so far indicate that p53 plays a major role in preventing endoreduplication by being a component of a spindle checkpoint that ensures the maintenance of diploidy. Data from several studies support the role of p53 in this mechanism: 1) p53 was shown to participate in a mitotic checkpoint that inhibits the formation of polyploid cells, since fibroblasts in cultures of p53-deficient mouse embryos exposed to spindle inhibitors formed tetraploid and octaploid cells [43]; 2) p53- and pRb-deficient fibroblasts re-replicate after treatment with the nocodazole, a strong MTI, whereas normal cells arrest in G1

[39,44]; 3) Abrogation of p53 in cooperation with Bcl-xL allowed rapid and progressive polyploidization following mitotic spindle damage [41].

In contrast to the above findings on p53, we observed that 2ME induced endoreduplication was accompanied with p53 upregulation and this was blocked by the p53 inhibitor pifithrin- α . The reasons for abrogation of endoreduplication by p53-inhibitor in OPCs remains unclear. We hypothesize that p21 action may be responsible for this effect as p53-dependent inhibition of endoreduplication has been shown to induce p21 [40,42]. Moreover, upregulation of p21 is known to negatively regulate cell cycle progression at G1 and inhibit Oli-neu progression [45]. In oligodendrocytes, however, p21 has a controversial role as p21 levels increase during S-phase entry [46]. We speculate that the increase in p53 in response to 2ME leads to the expression of p21. Hence, instead of inhibiting the cell cycle as in other mammalian cell types, this induces cells into S-phase entry, resulting in endoreduplication. This contention is supported by the fact that inhibition of p53 by Pifithrin- α inhibited cell cycle progression, moreover treatment with Pifithrin- α alone decreased the percentage of cells in the S-phase compared to control.

Consistent with our findings that p53 may be driving 2ME mediated endoreduplication in OPCs, it has been recently reported that cyclin E driven replicative stress promotes p-53 dependent mitotic bypass and whole-genome duplication (WGD) in p53 proficient cells [47]. This implies that tumor suppressor p53 can directly contribute to cancer evolution. Indeed, many genomics studies have shown that approximately half of the WGD events in cancer happen with wild-type p53 background. Moreover, p53 has been shown to promote mitotic bypass after genotoxic or oncogene stress. However, how WGD happens in p53 proficient cells is still unclear. Zeng and colleagues [47] proposed that elevated cyclin E1 in concert with p53 and its downstream target p21, inhibits mitotic Cdk activity to promote mitotic bypass and complete endoreduplication. Consistent with this contention we observed significant increase in both p53 and p21 and a minor change in cdk2.

Another potential mechanism leading to WGD is cell fusion. Interestingly, we observed increased metabolic and cell fusion activity in OPCs that were treated with 2ME. It is feasible that treatment with 2ME creates a survival crisis in OPCs and the vulnerable cells find partners to escape resist and escape cell death. This may also contribute to MTI drug resistance observed in cancer cells. Interestingly, in OPCs, 2ME induced expression of survivin, which is a member of chromosomal passenger complex implicated in cytokinesis and modulate microtubule dynamics as well as nucleation [48]. Survivin plays an important role in the surveillance mechanism called mitotic spindle assembly checkpoint (MSAC) which regulates metaphase to anaphase transition during mitosis potentially by interacting with p53 [49,50]. Interestingly p53 is also linked to mitochondrial metabolic activity in cancer cells and regulates the balance between cell survival and death [51]. Finally, it is also possible that there are different population of OPCs that express and react differently as has been documented for microglial cells [52].

Our findings could have clinical relevance in deciding whether a glioma should be treated with a MTI or not. Moreover, it might be crucial to determine whether a glioma originates from oligodendrocytes or from another cell type in the brain, such as astrocytes. If the glioma was of oligodendroglial origin, chemotherapy with high concentrations of MTIs could lead to genomic instability and polyploidy. Our results suggest that polyploidy might be inhibited by Pifithrin- α , however, it is obvious that inhibition of a major tumor suppressor protein in vivo might not be the solution to treat cancer cells.

Apart from tumor suppressor proteins, cyclins critically regulate cell cycle progression. One of the cyclins, cyclin B, is a direct target of the anaphase promoting complex (APC). The APC plays an important role in the transition of cells from metaphase to anaphase [53]. The APC acts as a ubiquitin ligase and is required to label cyclin B for subsequent proteolysis, which in turn initiates the transition to the anaphase. It has been shown that 2ME enhances the amount of cyclin B in a tubulin-dependent manner, since the cyclin B enhancement was absent in a tubulin-mutant clone [14 (55)]. The authors suggest that 2ME could induce mitotic arrest by inhibiting APC action. In our experiments, cyclin B was upregulated in response to 2ME. Although, this should lead to a cell cycle arrest, in 2ME treated cells we did not observe a G1 arrest. However, the possibility that 2ME treated

cells were arrested in a pseudo G1 phase with a 8N DNA content cannot be excluded as cell arrest in this phase could be accompanied with an increase in cyclin B expression.

Based on the above findings, 2ME treatment could result in cell cycle arrest even in the presence of induction of both cyclin B and p53 expression. Importantly upregulation of p53 and p21 may occur in non-arrested endoreduplicating cells, as observed here. Our data also showed that 2ME treated cells had less pRb than control cells but similar amounts of ppRb. Therefore the percentage of pRb compared to total Rb was less in 2ME treated cells than in control cells. It is known that pRb binds to E2F, thereby hindering the initiation of gene transcription. Since endoreduplication was associated with less pRb in Oli-neu cells, we suggest that the absence of pRb induced E2F to initiate S-phase transition despite tetraploidy. Our findings indicate that pRb is an important factor in the cellular response to MTIs, particularly in the context of regulation of DNA replication. Indeed, several studies have shown that pRb plays an important role in the cell cycle arrest after tetraploidy and that a functional pRb is crucial for ensuring appropriate cell cycle arrest [39,40,44,54]. Interestingly, we see that 2ME upregulates of JNK1, an important up-stream activator of cyclin B1 and cdc2, and is responsible of 2ME induced endoreduplication in human breast cancer cells [19].

We also observed that 2ME treatment regulated the expression of cyclin E which is a regulatory subunit of Cdk2 and drives cells from G1 to S phase. Increased cyclin E expression is frequently observed in human malignancies and is associated with poor prognosis in various cancers. Under quiescent conditions, cyclin E forms a complex with Cdk2, which inhibits the phosphorylation of pRb and prevents the release of E2F and thereby inhibiting the entry of cells into the S-phase. 2ME has been shown to induce endoreduplication in human smooth muscle cells, and a role for Cdk2 was proposed [37]. In our experiments, however, we observed a minor increase in Cdk2 expression. In another study on MTI induced endoreduplication, it was shown that p21 inhibition of cyclin E/Cdk2 activity prevented endoreduplication after mitotic spindle disruption and, similar to our finding the Cdk2 levels remained constant [42]. In that study, the expression of cyclin E was also investigated and, after an initial decline, probably due to loss of cells in the G1 state, cyclin E levels increased, consistent with the presence of cells in a pseudo G1-state. We speculate that cells undergo endoreduplication after contact with MTIs either by upregulating Cdk2 or by increasing cyclin E expression. Additionally, the expression of cyclin E could be linked to the expression of p21, since increased expression of p53 transactivates p21, which binds to the cyclin E/Cdk2 complex and thereby inhibits the progression to the S-phase.

In addition to cyclin B and E, 2ME treatment also increased expression of cyclin A, a suggested target for oncogenic signals, and is associated with E2F transcription factor. A study by Pagano et al. [55] demonstrated that cyclin A has two distinct kinase activities, one appearing in S-phase, the other in G2. Cyclin A was found to bind to both Cdk2 and Cdc2 and cyclin A complexes with p21 and PCNA. However, no connection between cyclin A and endoreduplication has been found in mammalian cells so far [56] and could potentially be ruled out.

2ME treatment also upregulated cyclin D2 expression. In nocodazole (another strong MTI) treated HCT116 cells [42], cyclin D expression was analyzed in a time-dependent manner. A small decrease in cyclin D1 protein levels was observed after 18 hours of treatment, consistent with the loss of G1 cells and accumulation of cells in G2/M. Nevertheless, the cyclin D1 protein levels increased again after 32 hours of treatment, which was consistent with the presence of cells in a G1-like biochemical state. In our FACS experiments, we found an accumulation of cells with 8N after 48 hours of treatment with 2ME. The 8N peak in the FACS diagram tails on the right towards zero, indicating that cells might be arrested in a pseudo-G2 state. Interestingly, in our experiments we observed that cells pretreated with Pifithrin- α and treated with 2ME showed the typical morphological alterations of MTI treatment, such as retraction of cell processes and rounding-up. Interestingly, these cells did not undergo endoreduplication. Since 2ME treatment is known to induce spindle aberrations [33,57], we speculate that the round morphology of 2ME-treated cells was due to a destruction of the microtubule network, which causes the failure of the spindle apparatus. We speculate that there is a mechanism which senses the failure of spindle function in the 2ME and Pifithrin- α treated cells and

forces the cells to arrest. However, when p53 is not inhibited the mechanism is abrogated or overridden by the presence of p53.

5. Conclusions

Our findings provide evidence that at concentrations greater than 1 μ M 2ME not only induces apoptosis, but also activates OPCs and triggers endoreduplication potentially via p53. Hence therapeutic use of 2ME as an antitumor/anticancer agent may face the same fate as other microtubule interfering drugs. Since Pfifithrin- α , a p53 inhibitor, prevented 2ME induced endoreduplication, combining MTI treatments with p53 inhibitors may prevent endoreduplication and potentially MTI drug resistance in gliomas. Our results provide important leads, however, future in depth studies are required to confirm these possibilities.

Limitations: The focus of our study was to assess the inhibitory potential of 2ME in OPCs, which contribute glioma pathology. Our finding that 2ME inhibits OPC growth, triggers endoreduplication and OPC arm retraction at pharmacological concentrations in vitro would need to be confirmed in an in vivo setting as 2ME pharmacokinetics may differ between in vitro and in vivo conditions and needs to be confirmed in an in vivo setting.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Materials used.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, R.K.D. and S.A.S.; methodology, R.K.D. and S.A.S.; validation, R.K.D. and S.A.S.; formal analysis, S.A.S.; investigation, R.K.D., and S.A.S.; resources, R.K.D.; writing—original draft preparation, R.K.D. and S.A.S.; writing—review and editing, M.S., M.R., G.A., and B.L.; visualization, S.A.S., M.A., M.R.; supervision, R.K.D.; project administration, R.K.D.; funding acquisition, R.K.D. All authors have read and agreed to the published version of the manuscript.”.

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