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2-methoxyestradiol: New perspectives in colon carcinoma treatment

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ABSTRACT

Colon carcinoma represents a major problem in oncology, since this type of cancer responds poorly to conventional chemotherapy. Many groups are actively involved in the search of new experimental strategies to bypass this problem. We investigated the effects of 2-methoxyestradiol (2-ME), which derives from the NADPH-dependent cytochrome P450 metabolism of 17 β -estradiol. This compound has raised much interest in the past few decades for its inhibitory effects on the growth of cancer cells of different origin; however, little is known about its use on colon carcinoma-derived cell lines. In the present study, we investigated the effects of 2-ME on cell proliferation and cell cycle of two human colon carcinoma cell lines, namely HCT116 and SW613-B3. Our results showed a net anti-proliferative effect of 2-ME on both cell lines, which is accompanied by cell cycle arrest; moreover, we demonstrated that 2-ME is able to induce apoptosis as well as autophagy. This body of evidence points out that 2-ME could be considered as a promising tool against colon carcinoma.

Keywords

Apoptosis; autophagy; cell cycle; colon carcinoma cells; 2-methoxyestradiol

1. INTRODUCTION

Resistance toward chemotherapy remains one of the principal obstacles to the effective treatment of cancer. Among the different therapeutic strategies adopted to beat cancer, a positive role of estrogens has been reported. Biological activity of estrogens is mainly mediated by their binding to estrogen receptors (ER) α and β , that belong to the steroid/thyroid hormone receptor superfamily of nuclear receptors. They are activated upon binding of the ligand, being able to interact with cis-regulatory elements of target genes either by the direct association to estrogen-response elements (EREs) or through the interaction with other DNA-bound transcription factors, such as activator protein 1 (AP-1) and stimulating protein-1 (SP-1) (Matthews and Gustafsson 2003).

The present research focuses on the use of 2-methoxyestradiol (2-ME) to block colon cancer cell proliferation. Once thought to be an inactive metabolite, 2-ME derives from the NADPH-dependent cytochrome P450 metabolism of 17 β -estradiol and has been described to inhibit cancer proliferation. A pioneering study reported the ability of 2-ME to inhibit cell proliferation of a bovine brain-derived capillary endothelial cell line (Fotsis et al. 1994). Thereafter, a similar effect on various tumor cell lines was described (Pribluda et al. 2000; Qadan et al. 2001; Bu et al. 2002). 2-ME was found to inhibit tubulin polymerization by interacting at the colchicin-binding site and to disrupt microtubule dynamics (Kamath et al. 2006; Bhati et al. 2007). It is generally considered that low (nanomolar range) concentrations of 2-ME could be effective on cell proliferation, depending on cell types, while the anti-mitotic property requires micromolar concentrations.

Many studies have reported the ability of 2-ME to induce apoptosis in various human cancer cell lines, triggering both the extrinsic and intrinsic pathways (Mooberry 2003; Sutherland et al. 2007). 2-ME was also found to exert a strong anti-angiogenic effect (reviewed in Pribluda et al. 2000; Mooberry 2003; Dubei et al. 2009), possibly correlated to its ability to inhibit Hypoxia Induced Factor-1 (HIF-1) expression by altering microtubule assembly dynamics (Mabjeesh et al. 2003; Mooberry 2003). A recent proteomic survey has identified new mechanisms of action of 2-ME in pancreatic cells, involving glucocorticoid receptor and NF- κ B (Basu and Haldar 2010). Taken together, the reported effects allow the identification of 2-ME as a promising anti-cancer drug.

Few studies have been conducted on the effect of 2-ME on colon carcinoma (Carothers et al. 2002; Zou et al. 2006), a type of cancer that responds poorly to therapies, and represents a major problem in clinical oncology. The present investigation aims at depicting the effects of 2-ME on cell proliferation, cell cycle, apoptosis and autophagy of two human colon carcinoma-derived cell lines: the highly tumorigenic HCT116 and the non-tumorigenic/drug-resistant, SW613-B3. Our results demonstrated that 2-ME exerts a strong anti-proliferative activity, promotes cell cycle arrest and induces apoptosis as well as autophagy.

2. MATERIALS AND METHODS

2.1 Cell culture and treatments

Human colon carcinoma HCT116 and SW613-B3 cells, HeLa (from uterine cervix carcinoma), and FO46 normal fibroblasts were grown at 37°C under a 5% CO₂ atmosphere, in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 0.1 mg/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine and 2% sodium pyruvate (all reagents were from Celbio, Milano, Italy). Twenty-four h after seeding, cells were treated for 24 h either with etoposide (Sigma Aldrich, Milano, Italy, stock solution: 50 mM in DMSO) or 2-methoxyestradiol (2-ME, Sigma, stock solution: 10 mM in DMSO), followed by 24-h recovery in drug-free medium. Drug concentrations are specified for each assay. In most experiments, the final concentration of DMSO in culture medium was less than 0.2% (v/v) and it did not have any effect on the activities tested in this study. Under some conditions, a fraction of 2-ME-treated cells tended to detach; this population was analyzed separately or in combination with attached cells, as specified for each assay.

2.2 Morphological analysis

For microscope observation, cells grown in 3.5-cm diameter Petri dishes (5x10⁴/ml) were treated with 10 µM 2-ME for 24 h, followed by 24-h recovery in drug-free medium. At the end of the treatment, cells were observed using an Olympus IX71 microscope equipped with a 10x objective and images were acquired with a digital camera Cool SNAP_{ES} (PhotoMetrics, CA, USA), using the MetaMorph acquisition software; Adobe Photoshop 9.0.2 was used as elaborating software.

2.3 Viability assays

The effect of 2-ME on cell proliferation was evaluated by two different procedures (Giansanti et al. 2009): 1) the MTT metabolic viability assay, which measures mitochondrial activity, and 2) the measurement of the amount of DNA released from cells after alkaline lysis, that is proportional to the cell number. 1) *MTT assay*. Cells were seeded in 96-multiwell plates at a density of 10³/100 µl/well. Twenty-four h later, cells were treated for 24 h with 1 µM or 10 µM 2-ME, followed by 24-h recovery in drug-free medium. In addition to untreated controls, parallel samples were incubated under the same conditions with 0.1% DMSO to evaluate the possible effect of 2-ME solvent. At the end of the incubation, 20 µl of CellTiter 96 AQueous One Solution Cell Proliferation Reagent (Promega Italia, Milano, Italy) were added to each well. The plates were then maintained for 4 h at 37°C and analyzed with a microplate reader (Gio.De Vita, Roma, Italy) at 492 nm. Experiments were performed in quadruplicate and repeated three times. This procedure allows the evaluation of viability of the whole cell population. Data of treated are expressed in comparison to control samples ± SD. 2) *DNA release assay*. Cells were seeded in 6-cm diameter Petri dishes at a density of 5x10⁴ cells/ml and, 24 h later, treated for 24 h with 10 µM 2-ME, and further incubated for 24 h in drug-free medium. Untreated controls, and samples incubated under the same conditions with 0.1% DMSO were also considered. Detached cells were discarded, while attached cells were washed in PBS, trypsinized, centrifuged for 5 min at 1500 rpm and lysed with 1 ml of 0.1 M NaOH; samples were accurately mixed and heated for 30 min at 50°C, then allowed to reach room-temperature and kept at 4°C until the spectrophotometric analysis. The amount of released DNA, which is

proportional to the number of cells, was measured as the absorbance at 260 nm. Optical density of treated samples was compared to the values of control cells. Three independent experiments were carried out. Statistical analysis was performed and data are presented as mean $\pm$ S.D.

2.4 Clonogenic assay

To evaluate colony-forming ability, 2.5×10^2 cells/ml were seeded in 6-cm diameter Petri dishes and, 24 h later, treated for 24 h with 2-ME. Given that this assay implies the administration of 2-ME to single cells, a low concentration, i.e. 5 μ M was used. After drug removal, attached cells were washed with PBS and further grown in complete medium for 10 days, to allow colony formation by surviving cells. Untreated controls and samples incubated with the vehicle 0.05% DMSO were set up in parallel. Colonies were then washed with PBS, fixed for 10 min with 100% methanol, and stained with Coomassie Blue (0.1% Page Blue G-90, 50% methanol and 7.4% acetic acid). Colonies with more than 50 cells were counted. The percentage of colonies formed after treatment relative to untreated cells was determined. Experiments were performed in duplicate and repeated three times.

2.5 Flow cytometry

Cells were seeded in 10-cm diameter Petri dishes (10^6 /dish), grown in complete medium for 24 h and treated with 10 μ M 2-ME for 24 h. After the treatment, detached cells were recovered and added to the attached cells once the latter were trypsinized. The whole cell sample was centrifuged for 5 min at 1500 rpm at 4°C and resuspended with cold 0.9% NaCl (10^6 cells/ml); cold 100% ethanol was added to cell suspension to a final concentration of 70%. Samples were then incubated for 30 min at room temperature with 30 μ g/ml propidium iodide (Sigma) and 2 mg/ml RNase A (Sigma), kept overnight at 4°C and finally analyzed using a Coulter Epics XL II flow cytometer (Beckman Coulter, Milano, Italy). For each sample, 10^4 cells were measured and data were analyzed. The fluorescence intensity was converted in histograms, and the percentage of cells in each phase of the cellular cycle was calculated with XL II software. The applied procedure allows the evaluation of cell cycle distribution of the whole cell population. Experiments were repeated three times.

2.6 Western blotting

HCT116 and SW613-B3 cells were treated for 24 h with 100 μ M 2-ME. Attached (A) and detached (D) cell populations were harvested separately. Protein expression was evaluated by Western blotting according to Donà et al. (2008). After running and transfer of proteins onto nitrocellulose, membranes were incubated overnight at 4°C, or 3 h at room temperature, with MAbs against the following proteins: PARP-1 (C2-10 Alexis Biochemicals, Vinci-Biochem, Firenze, Italy, diluted 1:1000); cyclin A (Cy-A1 Sigma, diluted 1:500); cyclin B1 (GNS1 Santa Cruz Biotechnology, TebuBio, Magenta, Italy, diluted 1:1000); p53 (DO-1 Santa Cruz Biotechnology, diluted 1:2000); caspase-3 (31A1067 Alexis, diluted 1:250); γ -tubulin (GTU-88 Sigma, diluted 1:10000). In some experiments, polyclonal antibodies against the following proteins were used: cyclin D1 (Upstate Biotechnology, Prodotti Gianni, Italy, diluted 1:500); p21 (C-21, Santa Cruz Biotechnology, diluted 1:1000); cleaved caspase-8 (Asp391 Cell Signaling Technology, Celbio, diluted 1:1000); cleaved caspase-9 (Asp315 Cell Signaling Technology, diluted 1:1000). Then, HRP-conjugated anti-mouse or anti-rabbit secondary antibody (Jackson

ImmunoResearch, Suffolk, UK, diluted 1:10000) was applied for 45 min at room temperature. All antibodies were diluted in TBS (140 mM NaCl, 100 mM Tris-HCl, pH 7.5), containing 5% skim milk and 0.1% Tween-20. Visualization of immunoreactive bands was achieved using a chemiluminescent substrate (Immun-Star™ WesternC™ Chemiluminescent Kit, Bio Rad Laboratories, Segrate, Italy). Three independent experiments were performed. Densitometric analysis of immunoreactive bands was achieved with BioRad Quantity One software.

2.7 Internucleosomal DNA degradation

For DNA ladder analysis, control and treated samples containing 2.5×10^6 cells were processed as described in Giansanti et al. (2009). HCT116 and SW613-B3 cells were treated for 24 h with 100 μ M 2-ME. Attached (A) and detached (D) cell populations were harvested separately. HeLa cells treated with 100 μ M etoposide for 24 h (E) or growth factor-deprived (long-term, LT) were used as positive DNA ladder control (Torriglia et al. 1999). Pictures were taken with a photographic digital camera Kodak DC290 (Rochester, NY, USA).

2.8 Immunofluorescence experiments

Cells were seeded on coverslips (5×10^4 cells/ml) and treated 24 h later either with 10 μ M 2-ME or RS2780 (kindly provided by Prof. R. Silvestri, University of Roma La Sapienza, Italy) for 24 h. After treatment, attached cells were fixed with cold paraformaldehyde (2% in PBS) for 20 min and then post-fixed overnight with 70% ethanol at -20°C. The day after, cells were permeabilized on ice with 0.1% Triton X-100 in PBS, incubated for 30 min with PTN (PBS containing 10% newborn calf serum and 0.1% Tween-20) and then for 1 h at room temperature with the polyclonal antibody to AIF (apoptosis inducing factor) (Cell Signaling Technology, diluted 1:50), the MAb to mtHSP70 (JG1 Alexis, diluted 1:50) or with the anti-phosphorylated-H3 (P-H3) polyclonal antibody (Ser10 Upstate, diluted 1:20) in PTN. After washes with PBS, cells were incubated for 1 hour at room temperature with rhodamine- or fluorescein-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Jackson ImmunoResearch) and then stained for 10 min with 0.2 μ g/ml Hoechst 33258 in PBS in the dark. Finally, coverslips were mounted on slides with Anti-fade solution (90% glycerol, 20 mM Tris-HCl pH 7.5, 0.1% DABCO diluted in PBS). Samples were analyzed using an Olympus IX71 microscope equipped with a 63x objective; images were acquired as described above. Three independent experiments were performed.

2.9 Autophagy markers

For the evaluation of autophagy, cells were treated with 100 μ M 2-ME for 24 h, stained with 1 μ g/ml Acridine Orange (AO, Sigma) for 15 min at 37°C, then observed with an Olympus BX51 microscope. Images were taken with an Olympus C4040 camera. The autophagic markers Beclin-1, Atg 5-12, LC3 were monitored by Western blot (as described in section 2.6) by using the “Autophagy antibody kit” (Cell Signaling) under manufacturer’s conditions. *In situ* conversion of LC3 form I to form II was visualized by immunofluorescence with the following protocol: control and treated cells were fixed with cold paraformaldehyde (4% in PBS) for 15 min, washed with PBS and permeabilized with cold acetone for 5 min. After washings with PBS, samples were incubated with bovine serum albumin (4% in PBS) for 10 min and with the polyclonal

antibody to LC3 (see the above kit, diluted 1:100) for 1 h at 37°C. Samples were then processed as in section 2.8 and observed with the above described microscope.

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3. RESULTS

3.1 2-ME treatment affects colon carcinoma cell viability

After setting the experimental conditions using HeLa cells (Supplementary Fig. 1), we analyzed the effects of 2-ME on human colon carcinoma cell lines HCT116 and SW613-B3. As shown in Fig. 1A, HCT116 cells still attached to the plastic support after the 24 h-treatment with 10 μ M 2-ME, were fewer, larger in size compared to control cells and with a rounded morphology; a fraction of cells tended to detach from the Petri dish. After the 24-h recovery (24 h+24 h), the floating cell population increased notably, indicating a stronger and prolonged effect of the drug on this cell line. The other colon carcinoma cells, i.e. SW613-B3, after a 24-h treatment were larger in size compared to control cells and tended to detach, while after an additional 24 h-recovery, cells started to acquire the original cell morphology (Fig. 1B). The response of the two cell lines to 2-ME appeared to be similar, with a marked loss of cell adhesion; however, SW613-B3 cells were less affected during the recovery period and reverted to the starting phenotype.

Then, we addressed the impact of 2-ME on cell viability. Preliminary experiments revealed that the addition of DMSO, which is the vehicle of 2-ME, to cell culture did not affect cell viability. As revealed by MTT assay, the 24 h-treatment of HCT116 cells with 1 μ M 2-ME reduced cell viability to 65.2 ± 0.01 with respect to the untreated cells considered as 100, and to 48.6 ± 0.04 after further recovery in drug-free medium (Fig. 1C). This effect was more pronounced at the higher concentration of the drug (10 μ M), which caused a minimal residual viability of 2.2 ± 0.03 after 24 h-treatment and 11.1 ± 0.01 after further recovery (Fig. 1C). SW613-B3 cell viability was reduced only to 67.5 ± 0.01 at the end of the treatment with 1 μ M 2-ME and to 74.9 ± 0.01 after recovery (Fig. 1D); even after the administration of 10 μ M 2-ME, the values were not increased (60.6 ± 0.02 after 24 h-treatment and 82.5 ± 0.09 after additional 24 h-recovery) (Fig. 1D). On the whole, these results showed a dose-dependent and irreversible response of HCT116 cells to 2-ME treatment (as it occurs for HeLa cells, Supplementary Fig. 1); on the contrary, the effect of the drug on SW613-B3 cells was poor and reversible at both concentrations, thus supporting the general drug-resistant behavior of this cell line.

When evaluated by DNA release measurement, HCT116 cell proliferation was affected after the treatment with 1 μ M 2-ME (82.6 ± 0.08 after 24 h-treatment and 64.3 ± 0.22 after further recovery); the effect increased with 10 μ M 2-ME (41.5 ± 0.08 and 40.8 ± 0.06 after 24 h-treatment and further recovery, respectively) (Fig. 1E). On the contrary, 1 μ M 2-ME did not impair SW613-B3 cell proliferation ($93.7\% \pm 0.01$ and 87.3 ± 0.03 after 24 h-treatment and further recovery, respectively), while the 10 μ M concentration reduced proliferation to 69.2 ± 0.05 after 24 h-treatment and 59.6 ± 0.06 after the additional recovery (Fig. 1F). Consistent with the results from the MTT assay described above, SW613-B3 cells resulted more resistant to 2-ME compared to HCT116 cells.

The inhibitory effect of 2-ME on cell proliferation of the two colon carcinoma cell lines was confirmed by data on the colony forming ability. Taking into account that the assay was applied to single cells, the treatment was performed with 5 μ M 2-ME for 24 h followed by a further growth in fresh medium to allow colony formation (10 days). As illustrated in Supplementary Fig. 2, we observed that the ability of both cell lines to form colonies appeared greatly reduced as an effect of 2-ME. Quantitative data are reported in Fig. 1G, showing a residual ability to form colonies of 9.6% for HCT116

and 14.9% for SW613-B3 cells; the clonogenic potential of the latter cell line appeared to be less affected than that of HCT116 cells.

3.2 Effect of 2-ME on cell cycle

To correlate the anti-proliferative effect of 2-ME with cell cycle disturbance, we analyzed cell cycle distribution by flow cytometry on both cell lines treated with 10 μ M 2-ME. As illustrated in Fig. 2, both 2-ME-treated cell lines showed a higher percentage of cells accumulated in the G₂/M phase of cell cycle after 24 h-treatment compared to control cells (63.6% for HCT116, panel b; 49.4% for SW613-B3, panel f). After further 24 h-recovery in drug-free medium, the HCT116 cell population (panel d) was no longer predominantly accumulated in the G₂/M phase (12.9%) and a 33.2% fraction of the population was characterized by cells containing an amount of DNA <2C, which is a typical marker of apoptotic cells (A₀ region). Also SW613-B3 cells showed a decreased percentage of cells blocked in G₂/M (11.4%) and a concomitant increase in G₁ (26.8%) and S (21.3%) phases (panel h), thus suggesting that this cell line was able to re-enter cell cycle after drug removal. However, these cells also undergo apoptosis, as indicated by the A₀ fraction accounting for 19.2% of cell population. Notably, cells with DNA content >4C were present in both cell lines after treatment with 2-ME, possibly originating through an endoreduplication process; this would account for the cell population showing an increase in size when observed by microscopy (Ting et al. 2010).

3.3 Analysis of cell cycle checkpoints

To clarify whether the effects induced by 2-ME on cell cycle were attributable to an impaired progression through G₂/M phase, the levels of cyclins D1, A, and B1 were analyzed by Western blotting. Cyclin D1 is active in G₁ phase and is required for cell cycle G₁ transition; cyclin A is necessary to S phase progression and S/G₂ transition, and is degraded at entry in mitosis, while cyclin B1 increases from late G₂ to metaphase, and is degraded in anaphase to allow mitotic exit.

Western blot analysis was performed on control cells (C) and on both the attached (A) and detached (D) fractions of cells treated with 100 μ M 2-ME for 24 h (Fig. 3A) followed by the quantitative evaluation of band intensity (Fig. 3B). Cyclin D1 levels decreased in both fractions of HCT116 treated cells (compare 1 a.u. of control cells with 0.2 for attached and 0.4 for the detached population). As shown in the same Figure, cyclin A levels remained unchanged. Cyclin B1 expression increased by 4- and 1.4-times in the detached and attached fraction of 2-ME-treated HCT116 cells, respectively. This increase can be due to a lack of degradation of cyclin B1 and could be responsible for the accumulation of HCT116 cells at the transition between G₂ phase and mitosis, which impairs the ability to conclude cell cycle. In fact, they became floating, possibly undergoing apoptosis.

In SW613-B3 cells, we observed that the levels of cyclin D1 and B1 slightly increased (1.4-times) in the attached fraction and decreased to 0.7 in the detached one; cyclin A remained unchanged in the attached fraction and resulted much lower in the detached one (0.2 a.u.). These data indicate that, after 2-ME treatment, attached SW613-B3 cells remained arrested in G₂ phase, while detached cells were not able to progress through mitosis and, as a cause of this, probably underwent apoptosis, as suggested by the low levels of all cyclins analyzed.

To understand if the different status of p53 (wt for HCT116 and mutated for SW613-B3 cells) could influence the response to 2-ME, we evaluated the levels of p53 and of its

target p21 by Western blotting (Fig. 3). The basal level of p53 appeared to be similar in both cell lines; in 2-ME-treated HCT116 cells, we found that p53 level was reduced to about 60% in both attached and detached fractions compared to control cells; the parallel decrement of p21 levels indicates that these proteins are functionally correlated. SW613-B3 cells showed a lower expression in p53 amount compared to control. Remarkably, p21 level increased by two-times in treated, G₂-arrested, attached cells. The p53-independent modulation of p21 in p53-mutated cells could contribute to the cell cycle arrest in G₂/M observed after 2-ME administration in SW613-B3 cells. In fact, as recently reviewed (Cazzalini et al. 2010), p21 not only mediates cell cycle G₁/S transition but also plays a crucial role in the G₂-phase checkpoint.

To further characterize the 2-ME-induced G₂/M cell cycle arrest, we analyzed the expression of phosphorylated histone H3 (P-H3), which is a good marker of mitosis. As illustrated in Fig. 4, after the treatment with 2-ME, immunofluorescence experiments showed a 2- and 5-times increase of the amount of P-H3-positive HCT116 and SW613-B3 cells, respectively (11.6% and 53.5%, compared to control cells: 6.2% and 11.8%), indicating that 2-ME arrested HCT116 cells prevalently at G₂/M transition, while SW613-B3 cells mainly accumulated during the final step of mitosis, in agreement with the data previously obtained by flow cytometry experiments.

3.4 2-ME induces apoptosis in colon cancer cells

In order to understand whether 2-ME is able to induce apoptosis in HCT116 and SW613-B3 cells, we analyzed several apoptotic markers, i.e. internucleosomal DNA fragmentation (by DNA ladder analysis), PARP-1 proteolysis and caspase-3, -8 and -9 activation (by Western blotting). This set of experiments was performed with 100 μ M 2-ME in order to increase the amount of detached cells as an effect of the treatment, being this population usually characterized by apoptotic features. To avoid the possible interference of DMSO, parallel samples containing the equivalent concentration of DMSO in the culture medium were processed; all the assays revealed no direct effect of the solvent on apoptotic parameters (not shown).

As a positive control of apoptotic DNA fragmentation, according to a standard protocol (Torriglia et al. 1999) we used HeLa cells either treated with 100 μ M etoposide (an inhibitor of DNA topoisomerase II which induces DNA double strand breaks and apoptosis) or growth factor-deprived. In fact, as illustrated in Fig. 5, HeLa cells treated with etoposide (E), or maintained in culture for 9 days (LT, long term) showed a clear DNA ladder. DNA analysis carried out on both attached (A) and detached (D) HeLa cell fractions obtained after 24 h treatment with 100 μ M 2-ME revealed the appearance of DNA ladder, thus demonstrating the ability of 2-ME to induce apoptosis. In the same experimental conditions, HCT116 cells also showed a clear DNA ladder in the detached fraction, while in SW613-B3 samples only a faint DNA degradation was visible. Accordingly, PARP-1 proteolysis, a hallmark of caspase-dependent apoptosis (Soldani and Scovassi 2002), occurred in etoposide (E) and 2-ME-treated HeLa cells, where a total conversion of PARP-1 from full-length (113 kDa) to the 89 kDa fragment was observed (Fig. 6). HCT116 cells also showed a complete PARP-1 degradation, while intact PARP-1 was still visible in SW613-B3 cells together with the cleaved fragment. The analysis of this parameter supported the observation that 2-ME is able to induce apoptosis in colon carcinoma cells.

We then investigated the event upstream PARP-1 proteolysis, i.e. caspase-3 activation. Western blotting analysis revealed in etoposide-treated HeLa cells (E) as well as in the

detached fractions of 2-ME-treated HeLa and HCT116 cells a nearly complete conversion of the 35 kDa pro-caspase-3 to the 17 and 12 kDa active fragments. Caspase-3 activation also occurred in SW613-B3 cells, even if uncleaved caspase-3 was still detectable in the detached fraction (Fig. 6). These results suggest that in SW613-B3 cells, PARP-1 and caspase-3 conversion into proteolytic fragments requires a longer time to occur. This point should be further addressed by a time-course study.

Finally, we evaluated the caspase-8 and -9, which are activated during the extrinsic or intrinsic apoptotic pathway, respectively, and in turn regulate the activation of caspase-3. Western blot analysis revealed the activation of caspase-8 in all 2-ME-treated cells, while the active form of caspase-9 was visible only in HeLa cells (Fig. 6). These results demonstrated that 2-ME was able to activate the extrinsic pathway in colon carcinoma cell lines. Lambert et al. (2004) demonstrated that 2-ME is capable of activating apoptosis in a caspase-independent manner. For this reason, we analyzed the localization of AIF (apoptosis-inducing factor) that translocates from the mitochondria to the nucleus under a caspase-independent paradigm of death; in parallel, we monitored the mitochondrial protein HSP70 (mtHSP70) by double immunofluorescence experiments. The applied procedure was recently developed in our laboratory to visualize AIF translocation in human cancer cells treated with the pyrazolecarboxamide RS2780 (Giansanti et al. 2009). As a positive control of the assay, we included in the experiment HeLa cells treated under conditions leading to AIF release (Giansanti et al. 2009). The results reported in Fig. 7C confirmed that the RS2780 induces a redistribution of mitochondria (as illustrated by the red labeling of the mitochondrial protein HSP70), together with the translocation of a pool of AIF to the nucleus (green fluorescence). On the contrary, the 24 h-treatment with 10 μ M 2-ME caused an altered mitochondrial organization within the cells but AIF localization remained confined to mitochondria in both colon carcinoma cell lines (Fig. 7A, HCT116 cells; 7B, SW613-B3 cells). Thus, under our experimental conditions, 2-ME did not activate the AIF-dependent apoptotic pathway, even if an involvement of mitochondria (and by consequence, of the intrinsic pathway) in 2-ME-induced apoptosis cannot be excluded.

3.5 2-ME induces autophagy

Finally, we investigated the possible occurrence of autophagy in 2-ME-treated cells. This issue was addressed first by staining cells with Acridine Orange (AO), which enters acidic compartments like lysosomes, where it emits orange fluorescence that is suggestive of autophagy. We recorded this phenomenon in HeLa and SW613-B3 cells treated with 2-ME (upper part of Supplementary Fig. 3A and B, respectively). We further provided the *in situ* evidence of the conversion of LC3 (Light Chain 3), into the form LC3-II, that is a marker of the final step of autophagy (HeLa cells, Supplementary Fig. 3A, lower part). To go deeper into the finding that 2-ME could induce autophagy in our experimental conditions, we analyzed in details the LC3 pathway in SW613-B3 cells by evaluating crucial factors such as Beclin-1 and the complex Atg 5-12. Western blot analysis (Supplementary Fig. 3B, lower part) revealed that Beclin-1 increased (about 2-times) in 2-ME-treated SW613-B3 cells. In the same samples, we detected the Atg 5-12 complex as well as the conversion of the form I of LC3 into the form II (Supplementary Fig. 3B, lower part). These results provided the evidence that 2-ME could induce autophagy; more precisely, the process appeared to be regulated by Beclin-1, which, in turn, promotes the formation of the Atg complex responsible for the LC3 conversion into the form II.

4. Discussion

It is widely known that colon carcinoma is extremely resistant to conventional chemotherapy (Majer et al. 2007); for this reason, many investigators are involved in the search of experimental strategies able to improve the efficacy of the treatments. In this respect, our laboratory has recently identified a pyrazolecarboxamide (Giansanti et al. 2009) and an arylthioindole (Giansanti et al. 2010) that proved to be efficient in inhibiting colon carcinoma cell proliferation.

In the present study we addressed the possible use of 2-ME to inhibit the proliferation (and kill) two human colon carcinoma-derived cell lines, i.e. HCT116 and SW613-B3, which differ for a number of properties, including p53 status and tumorigenic potential (Table 1). In cells treated with 2-ME, we analyzed various parameters, including cell proliferation, cell cycle distribution and checkpoints, and apoptosis. Table 1 reports also the features of HeLa cells, which have been used in this study to set up the experimental conditions. The data obtained in this cell line were in agreement with those reported by Li et al. (2004) and enabled us to define the treatment protocol for colon carcinoma cell lines HCT116 and SW613-B3 (see Supplementary Fig. 1).

The results we obtained on colon carcinoma cell lines showed that 2-ME interferes with HCT116 cell viability in a dose-dependent/irreversible manner, while its effect on SW613-B3 is less potent and is reversible. This different impact of 2-ME could be explained by the high intrinsic resistance of the latter cell line to a panel of drugs (Donzelli et al. 1999; Bottone et al. 2003; Gorrini et al. 2003). However, we found that 2-ME has an inhibitory effect on cell viability of both colon carcinoma cells, thus suggesting that it could be a promising cytotoxic, anti-proliferative agent.

Given that 2-ME might produce undesired effects on normal cells, we addressed this issue by analyzing cell proliferation of human FO46 normal fibroblasts after a 24 h-treatment with 1 and 10 μ M 2-ME and also after a further 24 h-recovery in drug-free medium. We did not observe a marked effect of 2-ME on cell viability (data not shown), in line with the original observation by Seegers et al. (1997), who demonstrated that normal human skin fibroblasts are not affected by 2-ME. Accordingly, the comparison between normal human fibroblasts and melanoma cell lines revealed that the former cells were not sensitive to the treatment (Dobos et al. 2004). The loss of effect of 2-ME on normal cells was also supported by the report by Van Zijl et al. (2008), showing that breast cancer MCF-7 cells are sensitive to 2-ME while their normal counterpart (i.e. MCF-12A cells) is not. Also the comparison between primary cultures of normal cells and cancerous ovarian epithelium revealed that only the latter cells are sensitive to 2-ME (Kato et al. 2008).

Next, we demonstrated that 2-ME displays a dose-dependent effect on cell cycle, leading to the accumulation of cells in G₂/M characterized by the presence of phosphorylated-H3 histone. Cell cycle arrest was more evident in HCT116 cells, which were unable to re-enter cell cycle and showed upregulated levels of cyclin B1 (possibly due to the lack of its degradation) leading to cell death and detachment.

Although the effect of 2-ME on cell cycle was more pronounced for HCT116 cells, it occurred irrespectively of the p53 status, which is wt for HCT116 cells and mutated for SW613-B3 cells, where the accumulation in G₂/M phase could be due to a p53-independent upregulation of p21. The comparison between other cancer cell lines characterized by a different p53 behaviour will allow a better definition of the p53-independent role of p21. The effects of 2-ME on cell cycle we observed are in

agreement with previous reports on other cancer cell lines (Kumar et al. 2001; Lin et al. 2001; Dobos et al. 2004; Li et al. 2004; Kar et al. 2008; Maran et al. 2008).

The above observations prompted us to investigate the possible pro-apoptotic effect of 2-ME. Remarkably, we found that 2-ME induces caspase-dependent apoptosis, as supported by the appearance of DNA ladder, PARP-1 proteolysis and caspase-3 activation. In particular, our data indicated an involvement of caspase-8 and not of caspase-9 in the apoptotic cascade proteolysis, thus suggesting the activation of the extrinsic pathway. However, our evidence that mitochondria from 2-ME treated cells have an altered distribution, in line with the reported increase in mitochondria aggregating around the nuclear envelope (Thaver et al. 2009), could support an effect of the drug on mitochondria. In fact, it has been reported that 2-ME promotes the release of cytochrome c and the translocation of Bax protein to mitochondria in pancreatic cancer cells (Qanungo et al. 2002). These aspects could be evaluated in our experimental system. The effect of 2-ME on mitochondrial function and structure could be ascribed to the generation of oxidative stress and/or inhibition of SOD, with the consequent accumulation of superoxide (Huang et al. 2000; Wood et al. 2001; Fulda et al. 2010; Ting et al. 2010). The inhibitory effect of 2-ME on SOD has been exploited to potentiate the efficacy of photodynamic therapy (Golab et al. 2003).

The ability of 2-ME to induce apoptosis is a very important notion. In fact, given that drug resistance of cancer cells is often correlated with the evasion of apoptosis, a major goal in cancer research is to selectively increase the susceptibility of cancer cells to apoptosis-based strategies (Giansanti and Scovassi 2008). Previous studies have reported the ability of 2-ME to induce apoptosis in various human cancer cell lines, by triggering both the extrinsic (Pribluda et al. 2000; LaValle et al. 2003; Mooberry et al. 2003; Shimada et al. 2004; Kato et al. 2008; Thaver et al. 2009) and intrinsic pathway (Yue et al. 1997; Attalla et al. 1998; Schumacher and Neuhaus 2001; Bu et al. 2002; Mooberry 2003; Shimada et al. 2003; Gao et al. 2005; Zou et al. 2006; Fukui and Zhu 2009).

The activation of caspase-independent apoptosis by 2-ME has also been explored. Due to the generation of ROS by 2-ME and the consequent impairment of mitochondrial dynamics, it has been shown that after 2-ME treatment, crucial apoptotic factors, including AIF, are released from mitochondria in rat sarcoma cells (Lambert et al. 2004) and in human ovarian epithelium (Kato et al. 2008). In our experimental conditions, we were unable to see AIF translocation in colon carcinoma cells treated with 2-ME.

A recent body of evidence correlates the effect of 2-ME to the induction of autophagy in different cell lines (Chen et al. 2008; Kirches and Warich-Kirches 2009; Lorin et al. 2009, 2010; Stander et al. 2010). We addressed this point by analyzing different factors involved in the autophagic pathway, that are Beclin-1, the complex Atg 5-12, and LC3. Glick et al. 2010). The procedures we applied allowed the detection of autophagic markers in 2-ME-treated cells, thus suggesting that not only apoptosis but also autophagy are induced by 2-ME. However, the correlation (if any) of these processes in our experimental system needs further investigation; special attention has to be paid to the possible protective effect of autophagy towards apoptosis, which could account for the drug resistance of some cancer cells (Dikic et al. 2010; Moreau et al. 2010).

The aim of our study was to elucidate the effects of 2-ME on human colon carcinoma cells, currently used in our laboratory as model system to test the efficacy of potential anti-cancer agents. A previous report (Carothers et al. 2002) described that 2-ME induces p53-mediated apoptosis in different human colon carcinoma cell lines, i.e.

RKO, SW48 and HCT116. The latter cell line has been used also in the present study. In line with the above cited paper, we found that 2-ME induces apoptotic DNA ladder and PARP-1 proteolysis in HCT116 cells; in addition, we provided the evidence of the activation of caspase-3 and -8 and of the re-localization of mitochondria in 2-ME-treated cells. It has to be taken into account that our experimental design differed from that of Carothers et al. (2002), given that our analysis included the use of a second colon carcinoma cell line, i.e. the SW613-B3, which we found to be extremely drug resistant (Donzelli et al. 1999; Bottone et al. 2003; Gorrini et al. 2003). For this reason, the concentrations of 2-ME we used (1-100 μ M) were higher than those administered by Carothers et al. (2002), which reached a maximum of 2.5 μ M. The rationale of our choice is supported by data on cell viability (not shown), demonstrating that we worked at sub-lethal concentrations of 2-ME. Of note, the highest 2-ME concentration used in the present study (i.e. 100 μ M) is included in the range of human plasma exposure, achieved with the lowest (400 mg daily) and highest (2200 twice a day) administered doses, calculated on the basis of compound clearance (Sweeney et al. 2005; Dahut et al. 2006; Lakhani et al. 2007).

In conclusion, our observations indicating that 2-ME is able to block the growth of colon carcinoma cells and to kill them add new insights into the possible use of 2-ME against this type of cancer. 2-ME administered orally proved to be efficient in a clinical trial against solid tumors (Sweeney et al. 2005; Dahut et al. 2006). More recently, *in vivo* studies concluded that the administration of 2-ME (Panzem®) is a powerful tool for treating various types of cancers (<http://clinicaltrials.gov/ct2/results?term=2-methoxyestradiol>). Of course, some problems correlated to the availability and metabolism of 2-ME exist, given that it is rapidly metabolized to inactive compounds. Technical improvement of 2-ME administration by nanocrystal dispersion was the object of recently set up clinical trials (Tevaarwerk et al. 2009; Harrison et al. 2010). In this respect, an active research aims at finding new methods of delivery, mainly based on the encapsulation of 2-ME (Wang et al. 2009; Du et al. 2010; Mueck and Seeger 2010). Interestingly, the 2-ME analog ENMD-1198 is currently administered orally to oncology patients in a phase I clinical trial (Pasquier et al. 2010; Zhou et al. 2010). Despite the encouraging results about the beneficial effect in 2-ME in contrasting cancer, the mechanisms at the basis of its pharmacological activity are not fully elucidated. Further work is needed to dissect the pathways induced by 2-ME, and to exploit the correlation between apoptosis and autophagy.

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LEGENDS TO FIGURES

Fig. 1. Effect of 2-ME on cell morphology, proliferation and colony forming ability of colon carcinoma HCT116 and SW613-B3 cells. Cells were treated with 10 μ M 2-ME for 24 h, followed by a 24 h-recovery in drug-free medium (24 h+24 h). (A,B) Microscopic observation of HCT116 and SW613-B3 cells, respectively. Bar = 50 μ m. (C,D) Cell viability evaluated by MTT assay that measures metabolic mitochondrial activity of HCT116 and SW613-B3 cells, respectively. Cells were harvested at the end of the 24 h-treatment with 10 μ M 2-ME (white columns) and after a further 24 h-recovery in drug-free medium (grey columns). Data are expressed as percentage of treated cells compared to control cells. Standard deviation (S.D.) was calculated and since it ranged between 0.01 and 0.09, it was not visualizable within the graph. (E,F) Cell proliferation measured by an assay based on DNA release from HCT116 and SW613-B3 cells, respectively. Cells were harvested at the end of the 24 h-treatment with 10 μ M 2-ME (white columns) and after a further 24 h-recovery in drug-free medium (grey columns). Data are expressed as percentage of treated cells compared to control cells. S.D. was calculated and since it ranged between 0.01 and 0.22, it was not visualizable within the graph. (G) Colony forming ability evaluated on cells treated with 5 μ M for 24 h and further grown for 10 days in drug-free medium.

Fig. 2. Effect of 2-ME on cell cycle. Flow cytometry was performed on cells harvested after a 24 h-treatment with 10 μ M 2-ME and after a further 24 h-recovery in drug-free medium. The sample includes both the attached and detached cell populations. The distribution of HCT116 and SW613-B3 cells in the various cycle phases (G_1 , S, G_2/M) was calculated as described in Materials and Methods. A_0 region defines cells with DNA content $<2C$; polyploid cells show DNA content $>4C$. A typical result out of three is shown.

Fig. 3. Analysis of cell cycle checkpoints. (A) Western blot analysis of cyclins D1, A, B1, p53 and p21 was performed on protein extracts from control (C) and 2-ME-treated HCT116 and SW613-B3 cells. Attached (A) and detached (D) cell fractions were analyzed separately. Loading control was checked by γ -tubulin level. A representative result out of three is shown. Antibodies are described in Materials and Methods. (B) The expression level of the various proteins analyzed by Western blots shown in panel A was quantified by measuring band intensity by BioRad Quantity One software. For each band, the expression ratio with the relative tubulin intensity was calculated. Densitometric analysis of immunoreactive bands was expressed by considering control cells (C) as 1.0 arbitrary unit (a.u.). Sample A corresponds to attached cells; sample D to the detached population.

Fig. 4. Labeling of mitotic cells by phosphorylated histone H3 (P-H3). Immunofluorescence analysis was performed on control and 2-ME-treated HCT116 and SW613-B3 cells according to the procedure described in Materials and Methods. Bar = 50 μ m.

Fig. 5. Internucleosomal DNA fragmentation (DNA ladder) in HCT116 and SW613-B3 cells. DNA was extracted from attached (A) and detached (D) cell fractions harvested separately after a 24 h-treatment with 100 μ M 2-ME. Positive controls of apoptosis:

HeLa cells treated for 24 h with 100 μ M etoposide (E) or growth factor-deprived (long-term, LT). M: DNA molecular marker. Gel electrophoresis was detailed in Materials and Methods. A representative experiment out of three is shown.

Fig. 6. Proteolysis of PARP-1, caspase-3, -8 and -9. Protein extracts were prepared from control HeLa, HCT116 and SW613-B3 cells (C) and samples treated for 24 h with 100 μ M 2-ME; attached (A) and detached (D) fractions were processed separately. HeLa cells treated with 100 μ M etoposide for 24 h (E) were used as positive control for apoptotic cells. Loading control was checked by γ -tubulin level. At least three independent experiments were carried out according to the procedure reported in Materials and Methods.

Fig. 7. Distribution of mitochondria and localization of AIF. (A,B) Double immunofluorescence experiments of control cells and treated with 10 μ M 2-ME for 24 h HCT116 (A) and SW613-B3 (B) cells. (C) HeLa cells treated with 10 μ M RS2780 for 24 h. Blue fluorescence: staining with Hoechst 33258; green fluorescence: AIF protein; red fluorescence: mtHSP70; yellow fluorescence: merge. Details of the immunofluorescence procedure are reported in Materials and Methods and in Giansanti et al. (2009). Bar = 50 μ m.

Table 1. Principal features of HCT116, SW613-B3 and HeLa cell lines.

<i>Cell line</i>	<i>Origin</i>	<i>p53</i>	<i>Tumorigenic potential</i>	<i>MMR</i>
HCT116	Colorectal cancer	wt	+	Deficient
SW613-B3	Colon carcinoma	Mutated (codon 273)	-	Proficient
HeLa	Uterine cervix	Inactive	+	Proficient

Fig. 1

Fig. 2

A

B

HCT116	cyclin D1	cyclin A	cyclin B1	p53	p21
C	1.0	1.0	1.0	1.0	1.0
A	0.2	0.8	1.4	0.6	0.5
D	0.4	1.1	4.0	0.6	0.2

SW613-B3	cyclin D1	cyclin A	cyclin B1	p53	p21
C	1.0	1.0	1.0	1.0	1.0
A	1.4	1.0	1.4	0.9	2.1
D	0.7	0.2	0.7	0.7	0.0

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7