Impact of Apparent Antagonism of Estrogen Receptor β by Fulvestrant on Anticancer Activity of 2-Methoxyestradiol

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Abstract. *Osteosarcoma is one of the most malignant bone tumors of childhood and adolescence. Interestingly, the presence of estrogen receptors α and β has been reported in human bone cells, including osteosarcoma. Thus, inhibitors of estrogens such as fulvestrant, are considered candidates for novel endocrine therapy in treatment of osteosarcoma. Another anticancer agent that seems to be very effective in treatment of osteosarcoma is a derivative of 17β-estradiol, 2-methoxyestradiol. The aim of this study was to determine the anticancer activities of pure anti-estrogen, fulvestrant and combined treatment of fulvestrant and 2-methoxyestradiol towards highly metastatic osteosarcoma 143B cells. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay was used in order to determine the antiproliferative potential of the compounds, and western blotting for estrogen receptors α and β. Flow cytometry was used in order to determine induction of cell death, cell-cycle arrest, mitochondrial depolarization, and DNA damage. Herein, we showed that fulvestrant has anticancer activity only at high concentrations. We were able to find and expression of estrogen receptor β, while we did not detect estrogen receptor α in osteosarcoma 143B cells. Moreover, fulvestrant down-regulated the expression of estrogen receptor β, and this effect was reversed by 2-methoxyestradiol. Thus, the obtained data suggest that 2-methoxyestradiol may exert part of its anticancer activity through modulation of expression of estrogen receptor β.*

Bone tumors are heterogeneous groups of tumors, with osteosarcoma (OS) being the most common. OS is one of the most malignant bone tumors of childhood and adolescence (1). It has its origin in mesenchymal bone tissues and usually arises around the knees and in the regions of long bones (2-4). Bone tumors commonly develop during maximal somatic growth, correlating with the peak of incidence among patients aged 14-18 years (5). There has been no significant progress in OS therapy since 1970; the 5-year survival rate is 75-80% (2-6).

It has been demonstrated that estrogens exert a significant impact on the skeleton during growth and adulthood (7). Moreover, sex steroids, particularly 17β-estradiol (Figure 1), play an important role in the regulation of cell proliferation of human OS (8). Sex steroids act on their target cells by binding to members of the nuclear hormone receptor superfamily of estrogen receptors (ER) α or β (7, 9-11). ERs are encoded on different chromosomes, and have distinct patterns of distribution, differences in structure and in ligand-binding affinity (12). A high ERα:ERβ ratio leads to enhanced cellular proliferation, while a predominance of ERβ over ERα leads to decreased proliferation (13-15). Moreover, the ratio of ERs in normal and malignant tissues is important for the long-term success of chemoprevention (7). Interestingly, the presence of ERs has been reported in human bone cells, including OS (8, 16).

The role of 17β-estradiol in stimulating poorly metastatic MG-63 OS cells has been reported (8). This pro-cancerogenic effect was suppressed by the addition of fulvestrant (ICI 182.780, Faslodex; Figure 1) (8). Fulvestrant binds competitively to the ER, with high affinity, and down-regulates ER by functional blockade and increased turnover (17, 18). Fulvestrant has been evaluated in extensive pre-clinical and clinical trials, that demonstrated its unique features (17-22). Its unique mode of action and the absence of partial agonist activity made it a candidate for the treatment of advanced breast cancer (17-19, 43). Moreover, fulvestrant was found to reduce the size of lung metastases in OS (44).

Another potent anticancer agent evaluated in ongoing clinical trials is 2-methoxyestradiol (2-ME), a natural 17β-estradiol derivative (Figure 1) (29, 30). *In vitro* studies reported that 2-ME inhibited the growth of various cancer

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cells, including those of the colon, ovarian, kidney, pancreas, breast, lung and stomach, as well as OS (23-28). Moreover, 2-ME (branded as PANZEM) has been evaluated in clinical trials, and is believed to possess potential therapeutic activity against breast, ovarian, and prostate cancer, as well as multiple myeloma (29-33). Serum levels of 2-ME range from 3 pM in men to >30 nM in pregnant women. Previously, we demonstrated that 2-ME at physiologically and pharmacologically-relevant concentrations led to death of 143B OS cells (26), while pharmacological concentrations of 2-ME (1-10 μM) inhibited cancer in various experimental models (25, 26, 28, 41, 42). Moreover, 2-ME may be effectively applied in monotherapy as well as in combination therapies with popular chemotherapeutics including bevacizumab (30), docetaxel (31), paclitaxel (32) and other anticancer agents (33).

It is believed that 2-ME lacks significant affinity for ER and is therefore non-estrogenic (25, 34). However, in contrast to physiological levels, pharmacologically active levels of 2-ME are predicted to have significant levels of binding to ER, given the overlap in the concentration ranges for antiproliferative effects and ER binding (35). However, it is not known whether 2-ME has agonistic or antagonistic properties, or tissue dependency (35).

Thus, the aim of this study was to determine the anticancer effects of co-treatment with 2-ME and fulvestrant on highly metastatic OS 143B cells. We also aimed to determine whether 2-ME at pharmacologically-relevant concentrations exerts anticancer activity in an ER-dependent manner.

Materials and Methods

Materials. 2-ME, fulvestrant, tissue culture media, antibiotic cocktail, fetal bovine serum (FBS), trypsin:EDTA, dimethyl sulfoxide, and antibodies to ERα and ERβ were purchased from Sigma-Aldrich (Poznan, Poland). Cell viability, mitochondrial potential, annexin V-propidium iodide, cell-cycle, and DNA-damage assay kits were obtained from Merck Millipore (Warsaw, Poland).

Cell line and culture conditions. The human OS 143B cell line (ATTC-8303) was purchased from the American Tissue Type Collection (Wesel, Germany. The cells were maintained in monolayer culture using Eagle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, penicillin (100 U/ml)/streptomycin (100 μg/ml) cocktail and 15 μg/ml bromo-deoxyuridine (BrdU) (Sigma Aldrich). Cells were cultured at 37˚C in a humidified atmosphere saturated with 5% CO_2. Twenty-four hours after seeding the medium was aspirated.

Cell treatment. Before each experiment, the cells were prepared as previously described (26, 27). The OS 143B cells were treated with different concentrations of fulvestrant, with/without 2-ME depending on the design of experiments. The cells treated with the combination of fulvestrant and 2-ME were first pre-treated for 2 h with fulvestrant in order to block ER. Subsequently, the cells were treated with combination of fulvestrant and 2-ME according to the experimental design (Materials and Methods). The cells were treated in EMEM with antibiotic cocktail, without L-glutamine and without FBS. The stock solutions of 2-ME and fulvestrant were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in experiments was less than 0.1%.

Cell viability assay [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT assay)]. OS 143B cells were treated with serial dilutions of fulvestrant, 2-ME, or a combination of both (within the range of 1 pM-1 mM) for 24 h. The cells treated with the combination of fulvestrant and 2-ME were first pre-treated for 2 h with fulvestrant in order to block ER. The MTT assay was performed as previously described (27). The results are presented as a percentage of that of the control cells (untreated cells). Each experiment was performed at least three times.

Western blotting. OS 143B cells were seeded at a density of 2×10^6 cells/dish. After 24 h of culture in standard medium, the medium was exchanged for FBS-free medium. OS 143B cells were treated with fulvestrant (10 nM), with/without 2-ME (10 μM) for 24 h. The cells treated with the combination of fulvestrant and 2-ME were first pre-treated for 2 h with fulvestrant in order to block ER. Equal amounts of total OS 143B cell lysates were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The membranes were then incubated with primary antibodies to ERα and -β (Sigma Aldrich) (1:1000) overnight at 4˚C and an analysis was performed as previously described (34). Chemiluminescence was detected using ImageQuant LAS 500 (GE Healthcare, Warsaw, Poland). The protein level was quantified by densitometry using Quantity one 4.5.2 software (Bio-Rad, Warsaw, Poland). The protein levels of ERs, as determined by chemiluminescent signal quantification, were normalized relative to the level of β-actin found in the samples. Each experiment was performed at least three times.

Induction of cell death. Assessment of apoptosis with double annexin V-propidium iodide (PI) staining was carried using Muse™ Cell Analyzer with Muse® annexin V (Merck Millipore, Darmstadt, Germany) and dead cell assay kit. OS 143B cells were seeded at a density of 0.3×10^6 cells/well. After 24 h of culture in standard medium, the medium was exchanged for FBS-free medium. Cells were treated with fulvestrant (10 nM, 1 μM and 50 μM) 2-ME (10 μM), or a combination of both for 24 h. The cells treated with the combination of fulvestrant and 2-ME were first pre-treated for 2 h with fulvestrant in order to block ER. After that time, cells were trypsinized and collected. Staining assay was performed to determine the proportion of apoptotic cells in samples. Each experiment was performed at least three times. Untreated OS 143B cells were used as negative and 2-ME-treated cells as positive controls (26,27).

Phosphorylation of histone H2A variant H2AX (H2AX) at serine 139. Assessments of phosphor-specific ataxia telangiectasia mutated (ATM-PE) and a phosphor-specific histone H2A.x-PECy5 staining was carried out using Muse™ Cell Analyzer using Muse® multicolor DNA damage kit (Merck Millipore). OS 143B cells were seeded at a density of 2×10^6 cells/dish. After 24 h of culture, in standard medium, the medium was exchanged for FBS-free medium. OS 143B cells were treated with fulvestrant (10 nM, 1

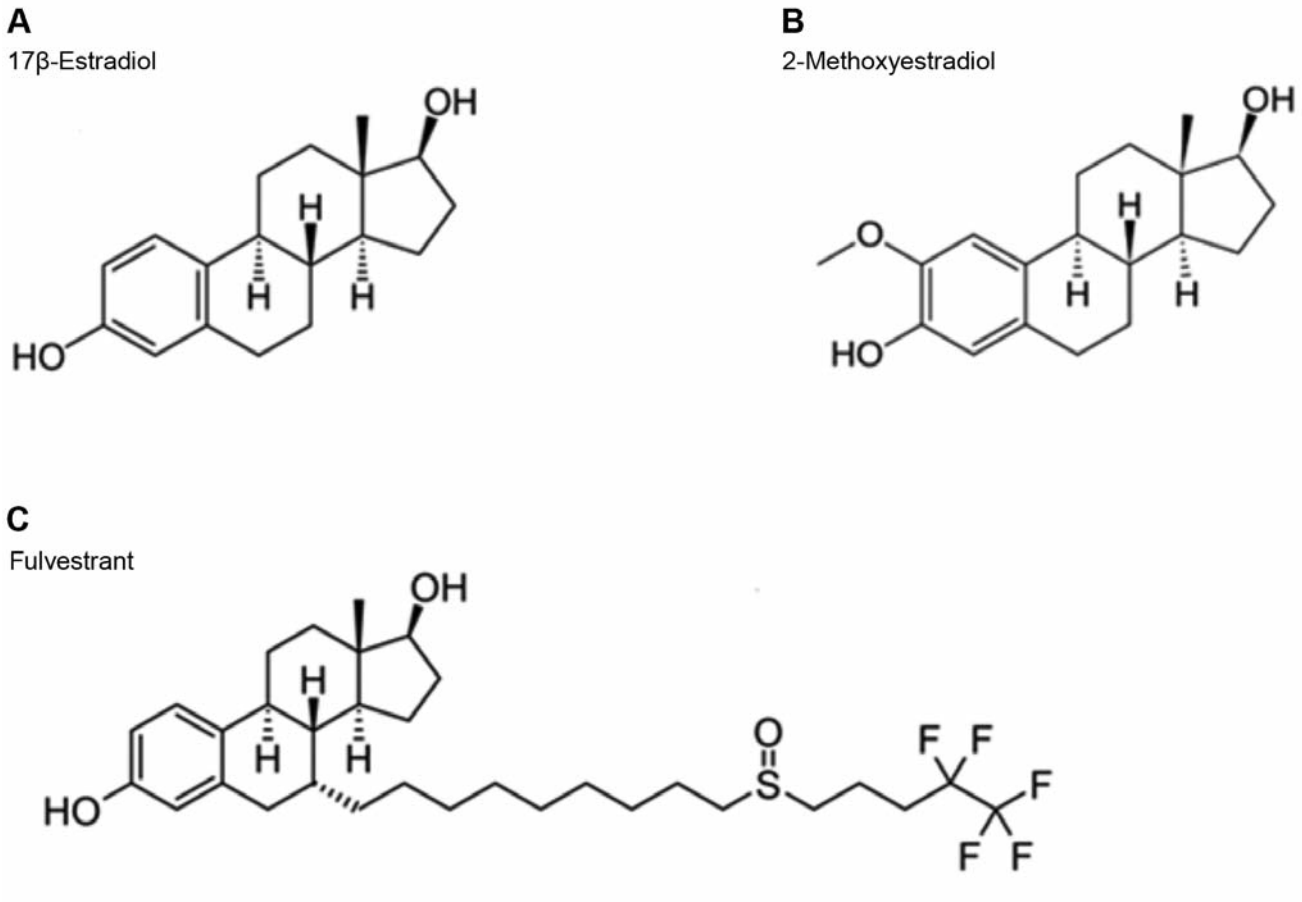

Figure 1. *Chemical structures of 17β-estradiol (A), 2-methoxyestradiol (B), and fulvestrant (C).*

μM), with/without 2-ME (10 μM) for 2 h. The cells treated with the combination of fulvestrant and 2-ME were first pre-treated for 2 h with fulvestrant in order to block ER. Untreated OS 143B cells were used as negative and 2-ME-treated cells as positive controls (26). Each experiment was performed at least three times.

Inhibition of cell cycle. Assessments were prepared by Muse™ Cell Analyzer using Muse® cell-cycle assay kit. OS 143B cells were seeded at a density of 2×10^6 cells/dish. After 24 h of culture in standard medium, the medium was exchanged for FBS-free medium. Cells were treated with fulvestrant, 2-ME, or combination of both for 24 h. The cells treated with the combination of fulvestrant and 2-ME were first pre-treated for 2 h with fulvestrant in order to block ER. Each experiment was performed at least three times. Untreated OS 143B cells were used as negative and 2-ME-treated cells as positive control (26).

Statistical analysis. The results were analyzed with the MuseSoft Analysis (version 1.4.0.0; Merck Millipore). Experimental results are presented as the mean±SD values from at least three independent experiments. Data were analyzed using GraphPad Prism (version 6.03; GraphPad Software, Inc., La Jolla, CA, USA). Significant differences between groups were determined by one-way ANOVA combined with Dunett's multiple comparison test.

Results

Impact of 2-ME and fulvestrant on protein expression of ER in 143B cells. Our first goal was to determine the protein levels of ERα and -β in 143B cells by western blotting. The concentrations used were based on our previous research and literature data (7, 36-39). We used pharmacologically-relevant concentrations of 2-ME, namely 10 μM, at which anticancer efficiency was confirmed in 143B cells (26, 27). We chose to use 10 nM of fulvestrant as at this concentration it was shown to effectively inhibit and down-regulate ER (36, 38, 39). We observed that 143B cells expressed ERβ, while we failed to detect ERα. As shown in Figure 2A, 24-h treatment with fulvestrant down-regulated the protein expression of ERβ by 30%. 2-ME used separately increased the protein level of ERβ by 10%. Interestingly, 2-ME reversed also the down-regulation of ERβ induced by fulvestrant (Figure 2A).

Antiproliferative impact of fulvestrant and 2-ME on 143B cells. We next determined the antiproliferative potential of

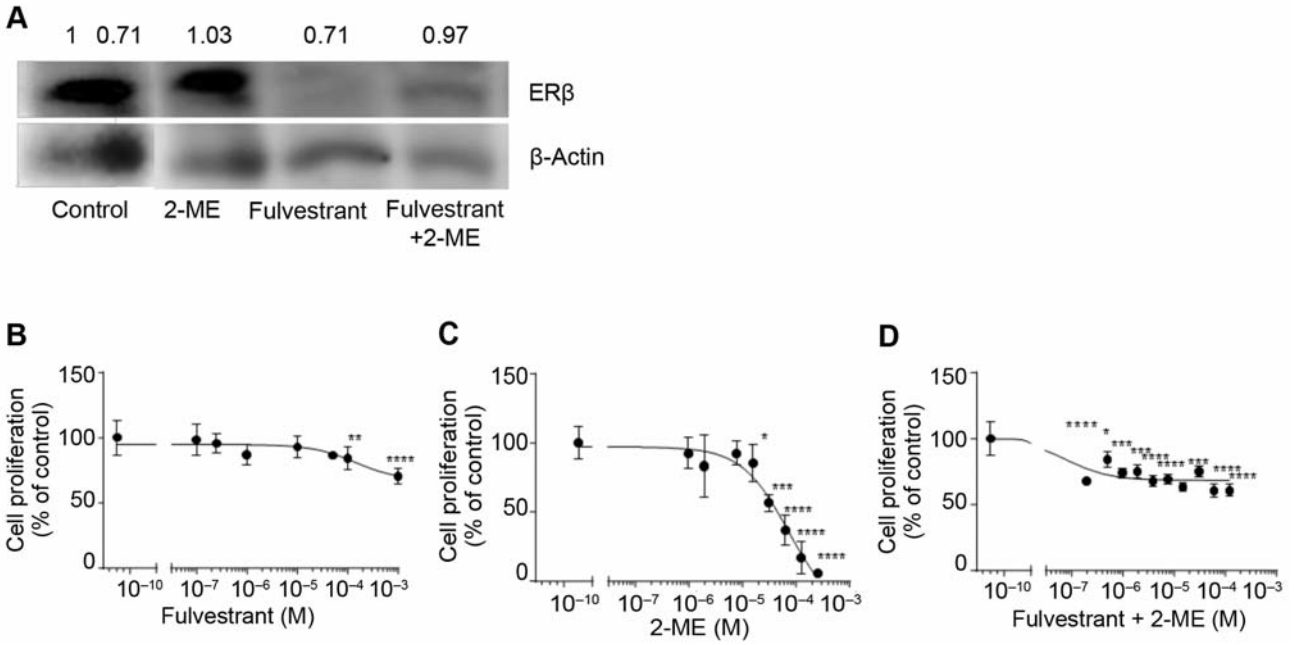

Figure 2. *A: The modulation of estrogen receptor β (ERβ) expression in 143B OS cells by fulvestrant, 2-methoxyestradiol (2-ME), and combined treatment of fulvestrant and 2-ME in osteosarcoma 143B cells. The cells were treated with 10 nM fulvestrant, 10 μM 2-ME, and combined treatment of both for 24 h, the expression of ERβ was subsequently determined by western blotting. B-D: Inhibition of 143B cell proliferation by fulvestrant, 2-ME (27) and combined treatment of 2-ME and fulvestrant. 143B cells were treated with serial dilutions of fulvestrant, 2-ME, and their combination (within the range of 1 pM-1 mM) for 24 h. Cell proliferation was determined by the MTT assay. Data are presented as the mean±SE values from at least three independent experiments. Data were analyzed performing one-way ANOVA combined with Dunnett's multiple comparison test; *significantly different at p<0.01 versus the control. A representative experiment out of three performed is shown.*

fulvestrant and the effect of combined treatment with fulvestrant and 2-ME. Our previous study showed that 2-ME reduced cell viability in a time-dependent manner (26, 27). Herein, the viability of 143B cells significantly diminished from 100% to 81% and 70% in the presence of 10 μM and 1 mM fulvestrant, respectively (Figure 2B). As previously described, we observed-significant decrease in 143B cell proliferation at all concentrations of 2-ME used (1 nM–1 mM) (Figure 2C) (26). Treatment with fulvestrant significantly diminished the antiproliferative effect of 2-ME (Figure 2D). Interestingly, we observed an antagonistic effect of fulvestrant and 2-ME, however, significantly starting from concentrations of approximately 10 μM (Figure 2D).

Impact of fulvestrant and 2-ME on 143B cell death induction. Next, we aimed to determine the impact of fulvestrant and combined treatment with fulvestrant and 2-ME on induction of 143B cell death. In order to determine concentration dependency, in the next studies, we chose concentrations of fulvestrant of 10 nM, 1 μM and 50 μM. As demonstrated in Figure 3A, there were no significant changes in the level of the apoptotic population after 24-h treatment with fulvestrant compared to control cells. We previously demonstrated that the number of apoptotic cells after 24 h incubation with 10 μM 2-ME was significantly increased by up to 31% (27). Moreover, as shown, pretreatment and further treatment with 50 μM fulvestrant significantly reduced 2-ME-induced cell death to approximately 18%, while we did not observe any significant impact of lower concentrations of fulvestrant (10 nM, 1 μM) (Figure 3A). These data are consistent with the antiproliferative potential of separate and combined treatment with fulvestrant and 2-ME (Figure 2B-D).

Impact of fulvestrant and 2-ME on inhibition of cell-cycle progression in 143B cells. Next, we aimed to determine whether fulvestrant and combined treatment with fulvestrant and 2-ME influenced 143B cell-cycle distribution. OS 143B cells were incubated with fulvestrant and combinations of fulvestrant and 2-ME for 24 h. As demonstrated in Figure 3, no significant changes in cell-cycle distribution were observed after treatment with fulvestrant alone (Figure 3B). After 24 h incubation with 10 μM of 2-ME, 66% of the 143B cells were in the G_2 and M phases compared to control cells (16.38%).

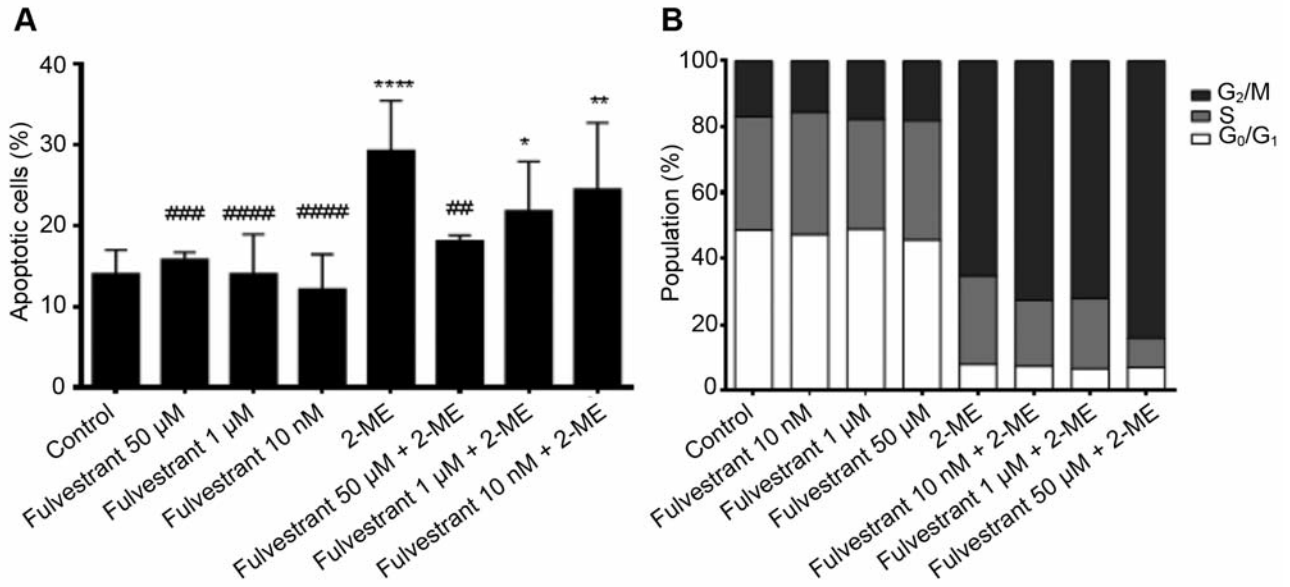

Figure 3. *Impact of treatment with fulvestrant, 2-methoxyestradiol (2-ME), and their combination on osteosarcoma 143B cell death (A) and cell-cycle arrest (B). 143B OS cells were incubated with fulvestrant (50 μM, 1 μM and 10 nM), 2-ME (10 μM), and combination of fulvestrant and 2-ME (50 μM+10 μM, 1 μM+10 μM, 10 nM+10 μM) for 24 h. The cells were harvested and the percentage of apoptotic cells (A) and cell cycle (B) were determined. Values are the mean±SE of three independent experiments (N=6 replicate cultures). Data were analyzed performing one-way ANOVA combined with Dunnett's multiple comparison test; significantly different at: *p<0.01, **p<0.001, ****p<0.00001 versus control; ##p<0.001, ###p<0.0001, ####p<0.00001 versus 2-ME at 10 μM.*

Interestingly, co-treatment with lower concentrations of fulvestrant (1 μM, 10 nM) and 10 μM 2-ME did not significantly change the cell-cycle distribution induced by 2-ME. Co-treatment with 50 μM fulvestrant and 10 μM 2-ME resulted in increased cell-cycle arrest in G_2/M phase (84%) as compared to 2-ME used separately (Figure 3B).

Impact of fulvestrant and 2-ME on mitochondrial depolarization in 143B cells. Our next goal was to determine if separate and combined treatment with fulvestrant and 2-ME influenced the status of the mitochondrial membrane potential. We did not observe any significant changes in mitochondrial potential after 24-h incubation with 1 μM and 10 nM fulvestrant (Figure 4A). However, 24 h treatment with 50 μM fulvestrant or 10 μM 2-ME resulted in a significant increase in the proportion of mitochondrial depolarized cells as compared to the control. Increase in cells with mitochondrial membrane depolarization to 60% and 86% was also observed after 24 h combined treatment with 50 μM fulvestrant with 10 μM 2-ME, and 10 nM μM fulvestrant with 10 μM 2-ME, respectively (Figure 4A).

Impact of fulvestrant and 2-ME on histone H2AX phosphorylation in 143B cells. Previously, we demonstrated that 2-ME used at physiological and pharmacological concentrations led to OS cell death as a result of DNA damage (26). Herein, we determined the impact of fulvestrant and 2-ME used together and separately on H2AX phosphorylation. We recently showed that 2 h incubation is sufficient to observe the induction of double-strand breaks after treatment with 2-ME at physiologically and pharmacologically relevant concentrations (26). Herein, we observed increased phosphorylation of H2AX after 2 h treatment with 10 μM 2-ME (Figure 4B), in agreement with our previous research (26). Interestingly, fulvestrant did not exert any significant effect when used separately nor in combination with 2-ME (Figure 4B).

Discussion

OS is one of the most clinically important sarcomas arising in the osteoskeletal system. The disease has a peak incidence in the first and second decades of life (1-6). Therefore, the possible involvement of sex steroids in the incidence and development of OS has been suggested. The present study was concerned with the determination of the anticancer activity of two potent anticancer agents, pure antiestrogen, fulvestrant, and a derivative of 17β-estradiol, 2-ME, in separate and combined treatments of highly metastatic OS 143B cells.

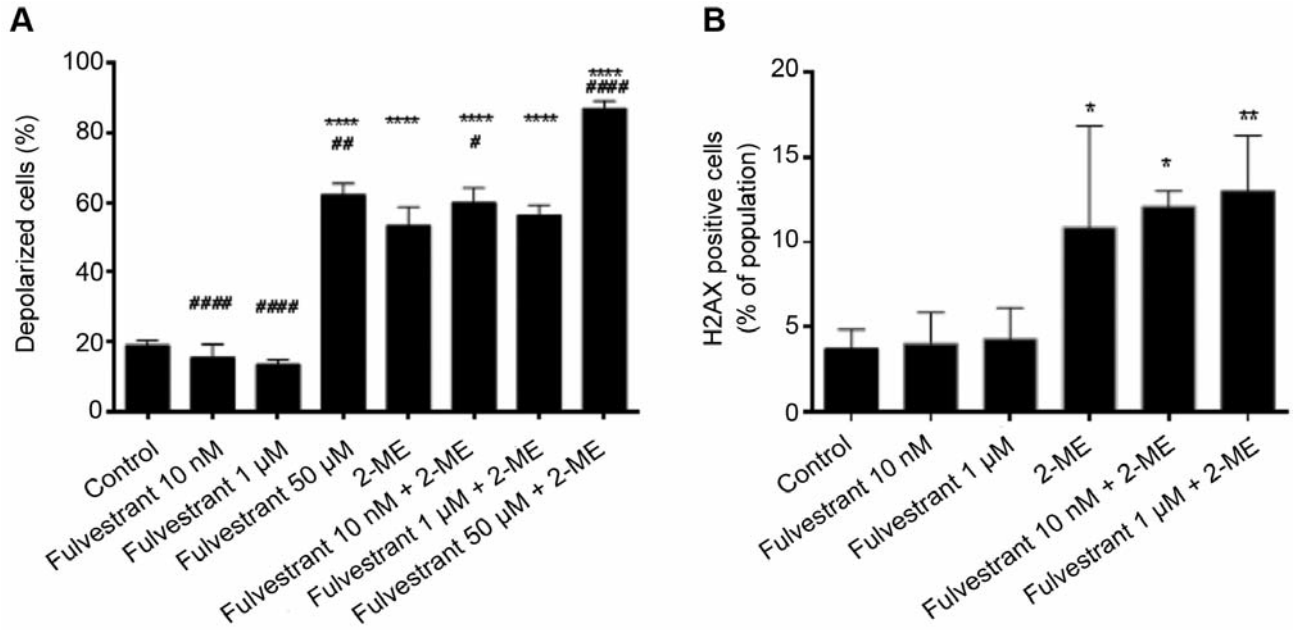

Figure 4. *A: Impact of treatment with fulvestrant, 2-methoxyestradiol (2-ME), and their combination on mitochondria depolarization of osteosarcoma143B cells. 143B cells were incubated with fulvestrant (50 μM, 1 μM and 10 nM), 2-ME (10 μM), and combination of fulvestrant and 2-ME (50 μM+10 μM, 1 μM+10 μM, 10 nM+10 μM) for 24 h. The cells were then harvested and the percentage of depolarized cells was determined. B: Impact of treatment with fulvestrant, 2-ME, and their combination on histone H2A version H2AX (H2AX) phosphorylation in 143B cells. 143B cells were incubated with fulvestrant (1 μM and 10 nM), 2-ME (10 μM), and combination of fulvestrant and 2-ME (1 μM+10 μM, 10 nM+10 μM) for 24 h. The cells were harvested and the percentage of cells with H2AX phosphorylation was determined. Values are the mean±SE of three independent experiments (N=6 replicate cultures). Data were analyzed performing one-way ANOVA combined with Dunnett's multiple comparison test; significantly different at: *p<0.01, **p<0.001, ****p<0.00001 versus control; #p<0.01, ##p<0.001, ####p<0.00001 versus 2-ME at 10 μM.*

In light of the many studies, 2-ME and fulvestrant could be potent and relatively safe agents for treatment of patients with OS (40-42). Although the antiestrogenic activity of fulvestrant is well established, the estrogenic action of 2-ME is still controversial. Fulvestrant is a novel, steroidal, 'pure' antiestrogen and ER down-regulator (17, 18, 19, 43). Previously, Sutherland and co-workers stated that there is little likelihood that endogenous 2-ME has significant physiological actions through binding to ER. In contrast to physiological levels, pharmacologically-active levels of 2-ME are predicted to have significant effects on binding to ER, given the overlap in the concentration ranges for antiproliferative effects and ER binding (35). Thus, in our experimental model we used 2-ME at a pharmacologically relevant concentration (10 μM) (25, 28).

ERs are considered important regulators of human non-neoplastic osteoblast proliferation. Both ERα and -β have been reported to be expressed in human osteoblasts. However, ERα and -β mRNAs have also been detected in OS tissues and cell lines (MG63, SAOS-2) (8, 38). We demonstrated the expression of ERβ in 143B OS cells, while we were not able to detect ERα. However, these data are consistent with previous studies where ERβ was visualized in the great majority of OS cases (44, 47) but ERα was not detected in all the cases (8).

In the present work, consistently with previous studies (19-22), we observed the down-regulation of ERβ expression by fulvestrant. It has been well-established that fulvestrant, after binding to the ER, induces its conformational change which disrupts signaling pathways and accelerates receptor degradation (17, 18, 22). In our experimental model, fulvestrant did exert antiproliferative activity on 143B cells at high concentrations (1 μM-1 mM). As demonstrated by Maran *et al*., 20 μM fulvestrant reduced cell survival to 71% (41). We observed a slightly decreased inhibition of proliferation by fulvestrant as compared with cited work (41). However, this may be explained by the fact MG63 is poorly metastatic cell line in contrast to highly metastatic 143B OS cells (50, 51). Furthermore, OS MG63 express both ERα and -β, while we have shown expression of only ERβ in OS 143B cells. Both ERα and -β may regulate cancer proliferation and cell death (13, 14, 56, 57). However, inhibition of ERα mainly results in cancer cell death (13, 14).

Fulvestrant did not induce cell-cycle arrest or death of 143B cells at any concentration, while we observed mitochondrial depolarization after treatment at high concentration (50 μM) of fulvestrant. Indeed, mitochondrial dysfunction has been shown to participate in the induction of apoptosis. The opening of the mitochondrial permeability transition pore has been demonstrated to induce depolarization of the transmembrane potential, release of apoptogenic factors and loss of oxidative phosphorylation (58). Moreover, in some apoptotic systems, loss of mitochondrial transmembrane potential may be an early event in the apoptotic process (58). However, mitochondrial depolarization may not always result in induction of cell death due to the various mitochondrial defense systems including uncoupling proteins (UCP) (61). In particular, UCP2 has been shown to protect OS cells from the cytotoxic actions of chemotherapeutic drugs (61). Fulvestrant is an alkylosulfonian derivative of estradiol (19, 22) (Figure 1), thus, we might expect that fulvestrant at high concentrations may enter the mitochondria and results in mitochondrial uncoupling. Indeed, 17β-estradiol was reported to promote intrinsic uncoupling of ATP synthase (62). Moreover, it may increase the mitochondrial reactive oxygen species formation by repressing uncoupling proteins (63).

We did not observe any impact of fulvestrant on phosphorylation of H2AX. DNA-damaging chemotherapeutic agents are well known for inducing double-stranded breaks, which rapidly results in the phosphorylation of histone H2A variant H2AX (60). Phosphorylation of H2AX at serine 139 (γ-H2AX) correlates well with double-strand breaks and is the most sensitive marker of DNA damage and subsequent repair of the DNA lesion (60). The mechanisms of protection of ER-positive cancer cells from DNA damage-induced cell death by ER has been reported (59).

Herein, we confirmed also the anticancer activity of 2-ME at pharmacological concentrations towards OS 143B cells (26, 27). We found a decrease in mitochondrial membrane potential induced by 2-ME, consistent with previous studies (64, 65). In our experimental model, 2-ME-induced membrane depolarization was strictly correlated with induction of cell-cycle arrest and cell death. Moreover, the phosphorylation of H2AX, strictly correlated to 2-ME-induced DNA damage, by 2-ME in OS G143B cells was also shown. In previous work, we demonstrated potent anticancer activity of 2-ME towards 143B cells even at low physiologically relevant concentrations due to DNA damage as a result of selective induction of neuronal nitric oxide synthase (nNOS) and nitric oxide generation in nuclei of 143B cells (26, 27).

Interestingly, for the first time, we show that 2-ME may modulate down-regulation of ERβ, which may be a new anticancer mechanism of action for this agent. Yun and co-workers suggested that treatment with 2-ME can activate epigenetically silenced ERβ, leading to prostate cancer cell death (49). The role of ERβ in regulation of apoptosis of malignant cells has recently drawn attention (45, 48, 49). The interaction of ERβ with 17β-estradiol is able to block apoptosis in neurons and promote apoptosis of cancer cells (68). The localization of ERβ was reported in the mitochondria in various types of cells, including OS, suggesting that ERβ may function as a mitochondrial component (45, 48), and its function as such is not cell-specific. Interestingly, in both prostate cancer and breast cancer cells, the introduction of ERβ was shown to induce strong inhibition of cell proliferation (48). It was suggested that down-regulation of ERβ in cancer cells could reduce vulnerability of mitochondria to various oxidative insults and hence reduce apoptosis and promote cancer progression (48).

The impact of 2-ME on ERβ may also result in regulation of mitochondrial biogenesis. Previously, we demonstrated that 2-ME at pharmacological concentrations reduces mitochondrial DNA and regulates mitochondrial mass per cell, a marker of mitochondrial biogenesis (66). ERβ may directly and indirectly regulate mitochondrial biogenesis in response to energy demand (67). From the above, the effect of 2-ME on fulvestrant-induced down-regulation of ERβ probably results in their negative interaction, considering the induction of cell death and inhibition of 143B cell growth. Moreover, one of the reasons 2-ME anticancer activity is counteracted by high concentrations of fulvestrant may be due to the down-regulation of ERβ and perturbation in 2-ME-induced nNOS signaling pathways. It was demonstrated that nNOS may be regulated *via* an ER-dependent manner (52); ERβ in particular is associated with the regulation of nNOS (53).

Interaction between 2-ME and fulvestrant was also presented for human breast tumor cells (54). Fulvestrant counteracted the positive response of vascular endothelial growth factor to 2-ME, suggesting that ER was involved. The study demonstrated a biphasic impact of 2-ME on vascular endothelial growth factor genes mediated through classical ER (54). In other experimental models, co-treatment of MG63 OS cells with 2-ME and fulvestrant did not reduce 2-ME-induced cell death (41). Thus, Maran and co-workers suggested that the anticancer activity of 2-ME is not mediated by conventional ER. It was also reported that 2-ME-mediated inhibition of proliferation and induction of apoptosis in breast cancer cells involves ER-independent pathways (34). However, these claims are based on studies examining the potential for endogenous levels of 2-ME to bind to ER at physiological concentrations (35). Herein, we suggest that 2-ME may regulate the expression of ERβ and thus exert a significant biological anticancer impact.

Conclusion

A combined chemotherapy with well-characterized drugs with distinct mechanisms of action is an important

therapeutic strategy. In addition, combined treatments improve efficacy, allow reduced dosage, and finally result in lowered toxicity and less adverse effects of anticancer therapy. Both 2-ME and fulvestrant are being evaluated in ongoing clinical trials and are considered in the treatment of OS. Fulvestrant might not be effective in treatment of highly metastatic OS. We also found that fulvestrant may have an impact on the mitochondrial chain resulting in mitochondrial uncoupling. The obtained data suggest that fulvestrant and 2-ME should not be used in combined therapy. One reason for interaction between 2-ME and fulvestrant is their opposite effects on the expression of ERβ. We also propose a new mechanism of action of 2-ME, namely the restoration of ERβ expression in OS 143B cells.

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Conflicts of Interest

The Authors declare no conflict of interest.

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