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Anticancer Potential of Oleuropein, the Polyphenol of Olive Oil, With 2-Methoxyestradiol, Separately or in Combination, in Human Osteosarcoma Cells

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Abstract. *Background/Aim: Oleuropein belongs to the potent polyphenols of olive oil. Notably, it is considered as a potentially active anticancer agent. Herein, the anticancer efficiency of oleuropein, when used separately and in combination with the chemotherapeutic agent, 2-methoxyestradiol (2-ME), was investigated in highly metastatic osteosarcoma (OS) cells. Materials and Methods: Human OS cells (143B OS cell line) were incubated with oleuropein and 2-ME, alone or in combination. Cell viability was determined by the MTT assay. Cell migration assays were used in order to determine the anti-migratory potential of the compounds, while their impact on autophagy was evaluated via the LC3-antibody-based detection assay. The interaction between oleuropein and 2-ME was determined via the CalcuSyn software. Results: Both anti-migratory and anti-proliferative effects of oleuropein were demonstrated on human OS cells. Anticancer effects of oleuropein were significantly enhanced after 2-ME addition. Treatment of 143B OS cell with oleuropein, alone or in combination with 2-ME resulted in induction of autophagy. Conclusion: The obtained data suggest an anticancer effect of oleuropein, alone and in combination with 2-ME, on highly metastatic 143B OS cells. Notably, a synergism between oleuropein and 2-ME towards 143B OS cells was detected. The exact mechanism of this synergism needs to be further investigated; nonetheless, induction of nitro-oxidative stress and/or induction of autophagy are suggested.*

Osteosarcoma (OS) is one of the most common bone tumors of childhood and adolescence (1, 2). The main clinical problem with OS is its malignancy and metastatic potential (1-3). The treatment of osteosarcoma involves surgical procedure in combination with chemotherapy, including mainly doxorubicin and cisplatin (3). Unfortunately, the 5-year survival rate of 60-70% has reached a plateau two decades ago, and no further advance with current chemotherapy has been observed (1-3).

Herein, we determined the anticancer potential of the polyphenol of olive oil, oleuropein (Figure 1A), using an OS cellular model. Oleuropein belongs to the secoiridoids, a group of coumarin-like compounds, and is present in the olive leaves and fruits (4). It is the ester of hydroxytyrosol (3,4-dihydroxyphenylethanol) and elenolic acid (5). Numerous studies have indicated that phenolic compounds from olive oil, especially oleuropein, exert anticancer activity in many types of malignancies including breast, pancreatic, and prostate cancers (6-11). Moreover, oleuropein has a variety of biological activities such as antimicrobial (12), hypoglycemic and anti-oxidant (13), antiviral (14), anti-hypertensive (15), hepatoprotective (16) and neuroprotective (17).

To address the question about the anticancer potential of oleuropein in combination with a potent chemotherapeutic, we included in our studies 2-methoxyestradiol (2-ME). 2-ME (Figure 1B) is a physiological metabolite of 17β-estradiol, known for its anticancer properties (18, 19). Branded as Panzem, 2-ME is currently evaluated in ongoing clinical trials focusing on treatment of solid tumors (20-23). Anticancer activity of 2-ME is strictly related to the inhibition of angiogenesis and induction of cell death pursuing actively proliferating cells (21-24). Therefore, quiescent cells are less affected to 2-ME (24-27). Moreover, induction of nitro-

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oxidative stress is involved in the antitumoral activity of 2-ME against various cancer cell models (28-35). We have previously demonstrated that 2-ME, at both physiological and pharmacological relevant concentrations increased the nuclear fraction of neuronal nitric oxide synthase (nNOS) in OS 143B cells (28). Interestingly, nNOS has been suggested as a molecular messenger of 2-ME that leads to the nitric oxide molecule release, resulting in DNA strand breaks and subsequently, in cell death (28). Previously, we have also demonstrated induction of autophagy by 2-ME, suggesting its role in cancer cell death induction or chemoresistance development (34). Under normal conditions, autophagy plays a major role in homeostasis *via* providing protein and organelle quality control. In cancer, autophagy has context-depended roles, including both cancer suppression and promotion (36-38). It has been reported to play an important role in the regulation of carcinogenesis and cancer survival; while, on the other hand, it has been strictly associated with cancer cell death induction (36-38).

Thus, the present study aimed to explore the anticancer potential of oleuropein, separately and in combination with 2ME, in the highly metastatic 143B OS cellular model, analyzing the impact on cell proliferation, migration and induction of autophagy.

Materials and Methods

Reagents. The 143B OS cell line (human osteosarcoma; ECACC 91112502), tissue culture media, antibiotic cocktail (penicillin/streptomycin), heat-inactivated fetal bovine serum (FBS), trypsin:EDTA, dimethyl sulfoxide (DMSO), oleuropein, 2-ME, and MTT were purchased from Sigma-Aldrich (Poznan, Poland). The Muse autophagy LC3-antibody based kit was purchased from Merck (Warsaw, Poland). Cell Émigré Cell Migration assay kit was purchased from Biocolor Life Science Assays (Carrickfergus, UK). Cell culture conditions. The 143B OS cells were cultured in EMEM medium, supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 10% FBS, and 1% antibiotic cocktail. The cell line was incubated under standard conditions at 37˚C and 5% CO_2.

Cell treatment. 2-ME and oleuropein solutions were prepared in DMSO (≥99.9%) and methanol (≥99.8%), respectively, which were further diluted to appropriate concentration with EMEM medium supplemented with 1% antibiotic cocktail. The final concentration of solvents in the incubation medium was less than 0.1%. In order to avoid the impact of the solvents, for the further studies, control cells were treated with an equal volume of the solvent used to prepare oleuropein and 2-ME solutions.

Cells were seeded at the appropriate densities according to the experimental design. After 24 h of culture in standard medium, the cells were incubated with oleuropein and 2-ME separately or oleuropein and 2-ME in combination, at different concentrations for different time periods (24 h, 48 h or 60 h) depending on the design of experiment.

MTT cell proliferation assay. Test was prepared at 96-well flat-bottom plates. 143B OS cells were seeded (8×10^3 cells/well) for 24 h. Then cells were treated with serial dilutions of oleuropein, 2-ME, or a combination of both (within the range of 1 μM - 250 μM) for 24 h and 48 h. Cells treated with vehicle were used as control (considered as 100% viability). The MTT assay was performed as previously described (33). The results are presented as a percentage of the control cells. The logEc50 was calculated and then transform to EC_{50} (μM) using GraphPad Prism v.6.01 software (USA, v.6.01).

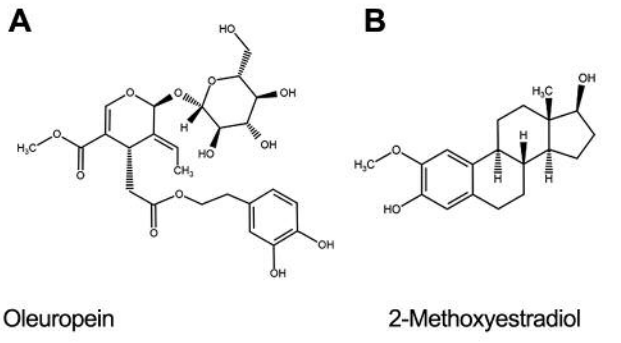

Figure 1. *Structure of examined substations, oleuropein (A) and 2-methoxyestradiol (2-ME) (B).*

CalcuSyn software 2.11 (Biosoft). The analysis of interaction between oleuropein and 2-ME was performed as previously described using a general equation for the dose-effect relationship: $f_a/f_u=(D/D_m)^m$, where D is the dose of drug, Dm is the median-effect dose signifying the potency (see below), fa defines the fraction affected by the dose, fu defines the fraction unaffected (where fu=1-fa), and m is an exponent signifying the sigmoidicity of the dose-effect curve (34).

Cell migration assay (scratch/ wound healing assay), microscopy visualization. 143B OS cells were seeded on 6-wells plate at density 1×10^6 cells/well. The assay was performed as previously described (35). Cells were treated with oleuropein (100 μM) and 2-ME (10 μM) separately or in combination for 60 h. The concentrations of the compounds were based on the results obtained from MTT assay. The wounds (scratch lines) were consequently observed using a phase contrast inverted microscope at 0 and 60 h of incubation.

Cell Migration Assay Kit (BioVendor). Cell migration chips (Cell Émigré Cell Migration assay kit) were used following manufacturers' instructions. The experiment was performed as previously described (34). Briefly, the cells were loaded into the pre-filled chips at a density of 9×10^5 cell/ml in the appropriate medium containing 100 μM oleuropein, 10 μM 2-ME, or their combination. Vehicle-treated cells were used as control cells. The migration of cells was then observed. The post-migration cell morphology was determined by fixation with 10% formalin and staining with crystal violet. The migration distances were observed using a phase contrast inverted microscope after 0 h and 48 h of incubation. Afterward, the migration rate (μM/h) was calculated (magnification 40×).

Autophagy LC3-antibody-based detection assay. The assay using Muse® Autophagy LC3-antibody based kit was performed as

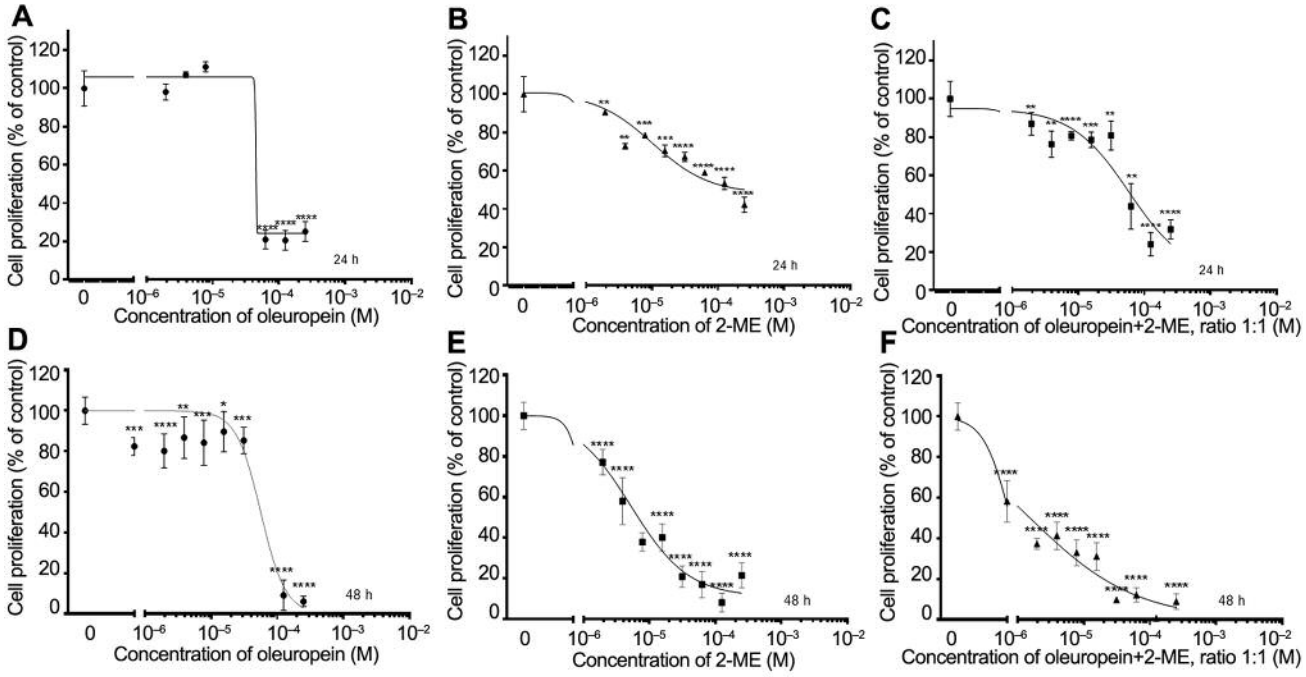

Figure 2. *MTT assays evaluated the 143B osteosarcoma cell proliferation after treatment with oleuropein, 2-methoxyestradiol (2-ME), or both for 24 h (A-C) and 48 h (D-F). Statistical significance level between each experimental group and control cells is indicated in the figure. *p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001.*

previously described (34). Briefly, the cells were incubated with 100 μM oleuropein and/or 10 μM 2-ME according to the experimental design. The cells were incubated with antibody detection reagents and stained with Anti-LC3 Alexa Fluor® 555, clone 4E12 according to manufacturer's protocol. Afterward, the cells were analyzed (5,000 events/sample), and the signals were detected using the Muse Cell Analyzer (Merck Millipore, Poland). The results were then analyzed with the Muse 1.4 analysis software. Autophagy induction ratios were calculated for each test sample relative to control (control was labeled as ratio 1).

Statistical analysis. Results of experiments are presented as the mean from at least three independent experiments. According to cell proliferation assay, the obtained absorbance was converted into % ±standard deviation (SD) values. Data were analyzed using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA). Parameter EC_{50} was determined during fitting. Differences between groups were calculated using one-way ANOVA followed by Dunnett's multiple comparison test or Student's *t*-test. Statistically significant differences were considered when $p<0.1$.

Results

Antiproliferative effects of oleuropein, alone or in combination with 2-ME. The first goal was to examine the antiproliferative potential of oleuropein, when used separately or in combination with 2-ME, on 143B OS cells. The cells were treated with oleuropein, 2-ME, or a combination of both compounds (ratio 1:1), at concentrations ranging between 1 μM and 250 μM, for 24 h and 48 h.

As demonstrated in Figure 2A, only 24 h treatment with oleuropein at 250 μM, 125 μM, 62.5 μM concentrations significantly inhibited 143B OS cell proliferation by 75%, 80% and 79%, respectively ($p<0.00001$). On the other hand, 24-h treatment with 2-ME at all concentrations significantly inhibited proliferation of 143B OS cells by 10%-58% (Figure 2B). In order to perform analysis of interaction, the cells were treated with both compounds. Notably, combination of oleuropein with 2-ME inhibited 143B OS cell growth at all used concentrations. Specifically, 24 h treatment with oleuropein in combination with 2-ME at 250 μM, 125 μM, 31.25 μM, 15.6 μM, 7.8 μM, 3.9 μM, 1.9 μM, and 1 μM (ratio 1:1) resulted in inhibition of proliferation by 68% ($p<0.00001$), 76% ($p<0.00001$), 56% ($p<0.001$), 19% ($p<0.001$), 21% ($p<0.0001$), 19% ($p<0.001$), 23% ($p<0.00001$), 13% ($p<0.001$), respectively (Figure 2C). The calculated EC_{50} values for 24 h treatment of 143B OS cells with oleuropein, 2-ME, and their combination were 111 μM, 10 μM and 58 μM, respectively (Figure 3A).

As demonstrated in Figure 2D, 48-h treatment with oleuropein resulted in potent inhibition of 143B OS cell growth. Specifically, oleuropein used at concentrations of 250 μM, 125 μM, 31.25 μM, 15.6 μM, 7.8 μM, 3.9 μM,

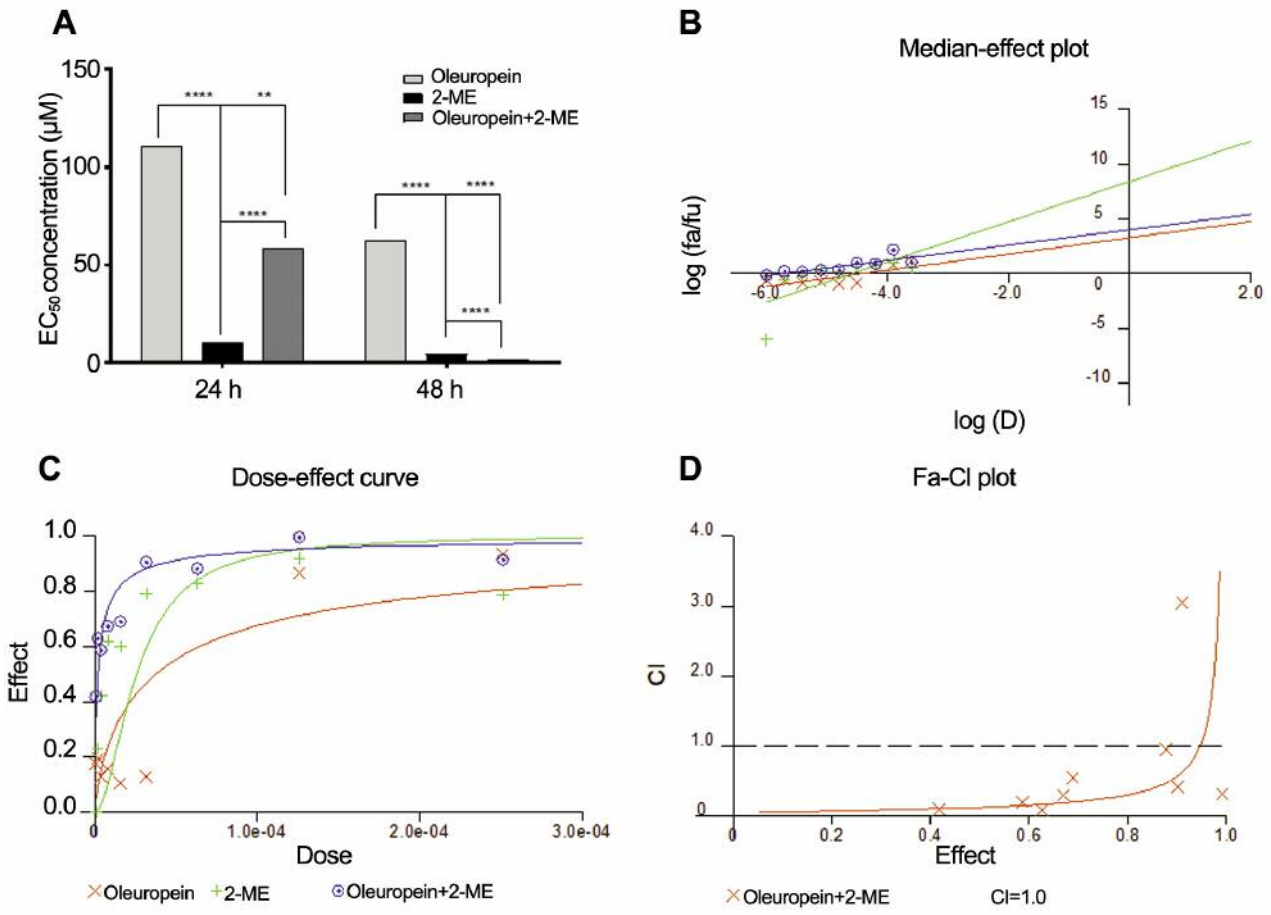

Figure 3. *Synergistic effect between oleuropein and 2-methoxyestradiol (2-ME). EC_{50} values of the drugs are graphically presented (A). Statistical significance level between various experimental pairs is indicated in the figure (**p<0.001, ****p<0.00001 vs. control cells). The data obtained from the MTT were analyzed using the CalcuSyn software version 2.11 (Biosoft) and presented in the form of a Median-Effect Plot (B), a Dose-Effect curve (C), and a Fa-CI plot (D).*

1.9 μM, and 1 μM inhibited 143B OS cell proliferation by 94% ($p<0.00001$), 91% ($p<0.00001$), 15% ($p<0.0001$), 10% ($p<0.01$), 16% ($p<0.0001$), 13% ($p<0.001$), 20% ($p<0.00001$) and 18% ($p<0.0001$), respectively (Figure 2D). Notably, 48 h treatment will 2-ME at the same concentrations inhibited cell proliferation by 78%, 92%, 83%, 79%, 60%, 62%, 42%, and 23% ($p<0.00001$), respectively (Figure 2E). Importantly, 48 h treatment with oleuropein and 2-ME at the aforementioned concentrations resulted in significant inhibition of 143B OS proliferation by 91%, 99%, 88%, 90%, 69%, 67%, 59%, and 63% ($p<0.00001$), respectively (Figure 2F). The calculated EC_{50} values for 48 h treatment of 143B OS cells with oleuropein, 2-ME and their combination were 62 μM, 4 μM and 1.5 μM, respectively.

Synergistic effect of oleuropein and 2-ME. The data from the MTT assay were further analyzed using the CalcuSyn software (Biosoft) to estimate the combination index (CI), in order to evaluate the interaction between oleuropein and 2-ME (Figure 3B-D). Due to the stronger anticancer effect of the used compounds at 48-h incubation as compared to 24 h, the values obtained from the 48-h treatment were chosen for CalcuSyn analysis of interaction between the compounds. The interaction between compounds is presented in Figure 3 as a Median-effect plot (B), a Dose-effect curve (C), and a Fa-CI plot (D) (Materials and Methods section). Notably, the estimated CI value at EC_{50} was 0.11957, at EC_{75} was 0.24102, while at EC_{90} 0.55514, suggesting a synergism between oleuropein and 2-ME (Figure 3B-D, Table I).

Antimigratory effects of oleuropein, alone or in combination with 2-ME, on 143B OS cells. Wound healing (scratch) assay was used to evaluate the migration of oleuropein and/or 2-ME-treated 143B OS cells. Notably, after 60 h of incubation,

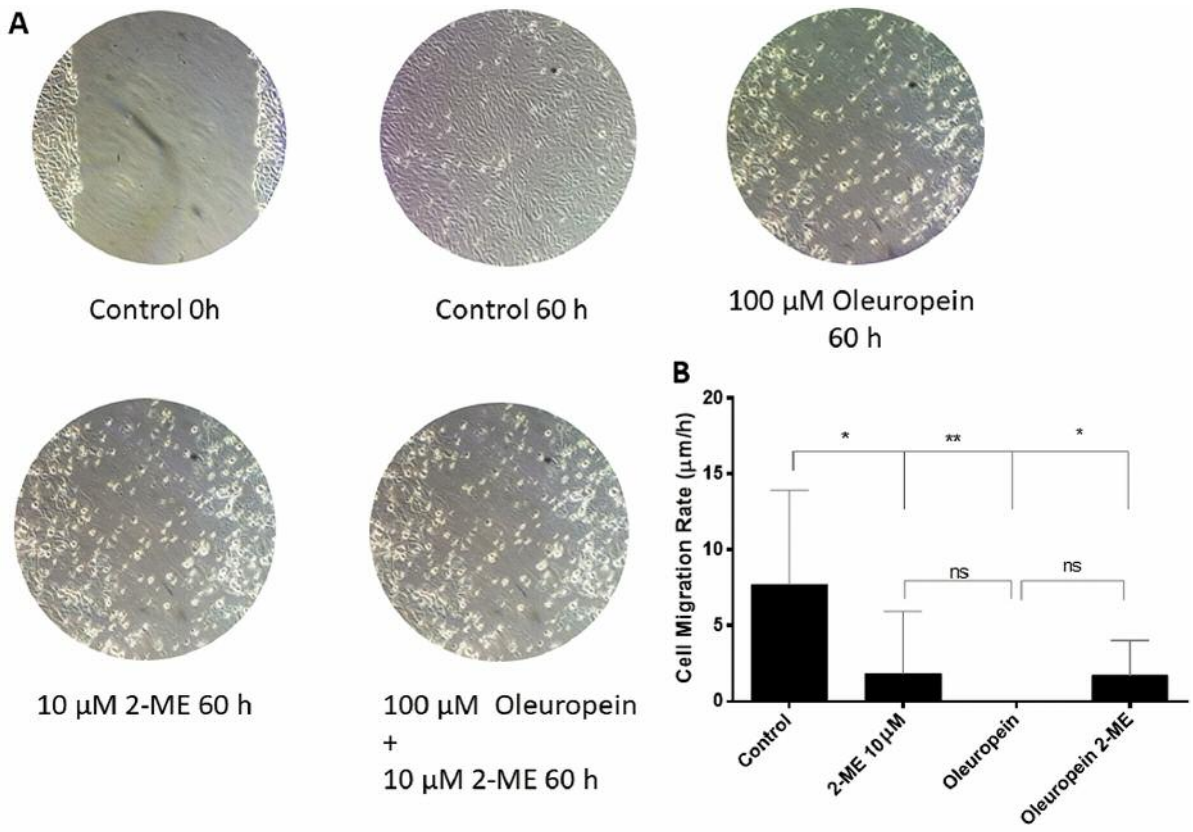

Figure 4. *Wound healing (scratch) migration assay in 143B OS cells treated with 100 µM oleuropein, 10 µM 2-methoxyestradiol (2-ME), both drugs combined, or medium (control). Representative images of microscopy visualization in scratching test after 60 h (A). The migration rate was calculated using chip migration assay (B). Statistical significance level between various experimental pairs is indicated in the figure (*p<0.01, **p<0.001 vs. control cells).*

Table I. *Synergistic effect between oleuropein and 2-ME. The data obtained from the MTT assay after 48h incubation with oleuropein and 2-ME, separately and in combination were analyzed using CalcuSyn software version 2.11 (Biosoft).*

Drug	CI* values at				
	ED_{50}	ED_{75}	ED_{90}	Dm	m
Oleuropein	N/A	N/A	N/A	3.7458e-005	0.74112
2-ME	N/A	N/A	N/A	2.5563e-005	1.83167
Oleuropein+2-ME (1:1)	0.11957	0.24102	0.55514	1.8168e-006	0.69872

CI, Combination index; Dm, the median-effect dose or concentration, represented by the ED_{50} or EC_{50}; m, measurement of the sigmoidicity of the dose-effect curve (m=1, >1, and <1 indicates hyperbolic, sigmoidal, and negative sigmoidal shapes, respectively). *CI is a quantitative measure of the degree of drug interaction in terms of additive effect (CI=1), synergism (CI<1), or antagonism (CI>1) for a given end-point of the effect measurement.

the wound was completely filled with migratory cells from both sides of the scratched area in the vehicle-treated (control) group (Figure 4A). Based on the EC_{50} values obtained from MTT assay, we chose oleuropein concentration at 100 µM and 2-ME at 10 µM for further studies. Notably, 100 µM oleuropein, 10 µM 2-ME, as well as the combination of the two compounds reduced the migration of 143B OS cells as compared with the control (Figure 4A). To further quantify the antimigratory effect of the compounds, the cell migration chips were employed in the study. The 48-h migration rate of 143B OS control cells was specified as 7.1±5.9 µm/h (Figure 4B) while treatment

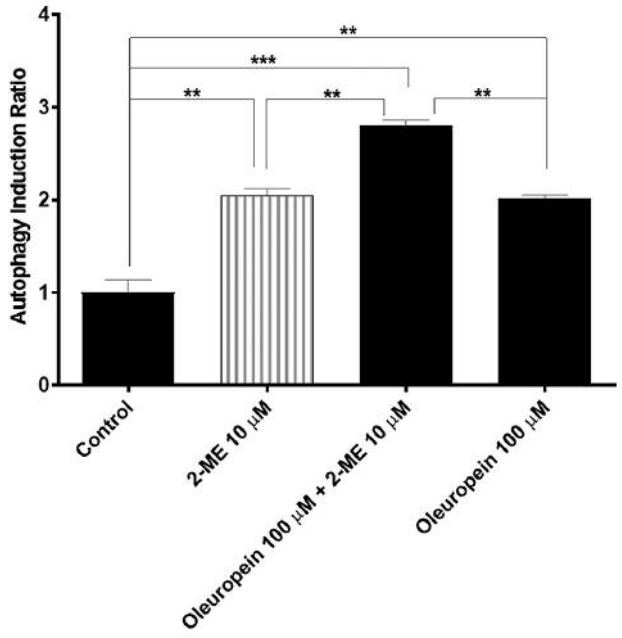

Figure 5. *Induction of autophagy in human osteosarcoma 143B OS cells after treatment with oleuropein, 2-methoxyestradiol (2-ME), or both drugs for 48 h. Statistical significance level between various experimental pairs is indicated in the figure (**p<0.001, ***p<0.0001 vs. control cells).*

with 10 μM 2-ME for 48 h reduced the migration rate to 12.0±4.0 μm/h (p<0.01), in accordance with our previous study (34). Treatment with oleuropein (100 μM) completely inhibited 143B OS cell migration (p<0.001), while in the case of combined treatment, the migration rate was reduced to 1.7 μm/h±2.4 μm/h (p<0.01); however, it was not significantly different from the migration rate of the oleuropein-treated group used separately (Figure 4B).

Autophagy induction in 143B OS cells after treatment with oleuropein, alone or in combination with 2-ME. Finally, the impact of the agents on induction of autophagy was determined. Interestingly, we observed that oleuropein induced autophagy to the same extent as 2-ME alone (2.02 and 2.05 ratio, respectively; p<0.001 *vs.* control) (Figure 5). Autophagy induction ratio in the group treated with the two drugs combined was 2.8, which was significantly increased compared to 2-ME and oleuropein alone groups (p<0.001) (Figure 5).

Discussion

Current medicine aims to determine more effective, personalized therapies, based on the biological profile of each patient. Therefore, evaluating novel chemotherapeutics and elucidating the mechanisms of action of drugs already used in clinical practice are pivotal for cancer management.

Herein, we investigated potent antiproliferative and antimigratory effects of oleuropein, separately and in combination with 2-ME towards highly metastatic 143B OS cells. The anticancer potential of oleuropein has been previously described in different cellular models, including breast and pancreatic cancers (7-11, 39-41). Antiproliferative effects of oleuropein have been reported both in *in vitro* and *in vivo* models (7-11, 42-44). While, antimigratory effects of oleuropein have been previously observed in breast cancer and glioma cellular models, as well as in *in vivo* melanoma models (7, 40, 43). However, up to date, only Morana *et al.* indicated plausible antiproliferative potential of oleuropein towards low metastatic MG63 and highly metastatic SaoS2 OS cell lines, although without investigating antimigrative potential of the compound (10).

In the present study, oleuropein was used in combination with the novel, potent anticancer agent, 2-ME, in order to explore their possible synergism in the OS cellular model. Previously, beneficial effects of oleuropein combined with thymol or doxorubicin in *in vivo* cancer models have been reported (42, 44). Notably, herein, the combined treatment of OS cells with oleuropein and 2-ME demonstrated synergism concerning both antiproliferative and antimigratory potential. However, the mechanism of synergism between the two compounds remains to be elucidated.

One of the hallmarks of human cancer is altered oxidative state compared to the normal tissues (45) and, thus, one of the suggested mechanisms of synergism between oleuropein and 2-ME is the selective activity of the compounds to induce nitro-oxidative stress. Using *in vitro* models, previous studies have reported oleuropein to induce cancer cell death *via* generation of reactive oxygen species (7, 46). The anticancer potential of 2-ME towards OS cells has been widely investigated by our group (28, 29, 33-35). Our previous studies have proven the unique ability of 2-ME to induce nuclear translocation of nNOS associated with local nitric oxide production and oxidative DNA damage, which finally results in cancer cell death (28).

Another plausible mechanism of the observed synergism between the compounds may be regulation of autophagy. Previously, we have shown that 2-ME induces autophagy in 143B OS; however, the same study also suggested that 2-ME may be associated with induction of cancer cell death or, on the other hand, with chemoresistance development (34). Up to date, there are no data regarding the impact of oleuropein on autophagy in cancer cells. Concerning oleuropein, it has been reported to induce autophagy and exert protective effects in neurodegeneration (47, 48). Thus, the role of autophagy as anticancer target of oleuropein needs to be further eludicated.

Conclusion

The obtained data suggest a potent anticancer potential of oleuropein, alone and in combination with 2-ME in highly metastatic OS cells. Moreover, the synergism between the compounds was defined. The exact mechanism of synergism between oleuropein and 2-ME in 143B OS cellular model needs to be further investigated; nonetheless induction of nitro-oxidative stress and/or induction of autophagy are suggested. Due to the fact that both 2-ME and oleuropein are potent anticancer agents currently studied *in vitro* and *in vivo* models, the obtained data may have not only cognitive, but also clinical potential.

Conflicts of Interest

The Authors declare no conflict of interest.

Authors' Contributions

Conceived and designed the study: Magdalena Gorska-Ponikowska; cell culture: Paulina Przychodzen, Roksana Wyszkowska, Magdalena Gorska-Ponikowska; performed the experiments: Paulina Przychodzen, Roksana Wyszkowska; Monika Gorzynik-Debicka; Magdalena Gorska-Ponikowska; graphical part: Tomasz Kostrzewa, Magdalena Gorska-Ponikowska; analyzed the data: Magdalena Gorska-Ponikowska; Database collections: Magdalena Gorska-Ponikowska, Alicja Kuban-Jankowska, Paulina Przychodzen, Roksana Wyszkowska; Manuscript corrections: Magdalena Gorska-Ponikowska, Alicja Kuban-Jankowska, Paulina Przychodzen, Roksana Wyszkowska; Wrote the article: Magdalena Gorska-Ponikowska.

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