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Effect of combined action of doxorubicin and calcifediol on MCF-7 breast cancer cells

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ABSTRACT

Introduction: Breast cancer is one of the most common cancers in women. Current recommendations for combination therapy in patients with breast cancer are still being developed and new therapies with greater success are sought. A relatively new approach is the administration of cytostatics in combination with vitamins. Hence, the study aimed to clarify whether the combination of calcifediol [25(OH)D3] with doxorubicin affects the response of the MCF-7 breast cancer cell line to the cytostatic.

Material and methods: In the MCF-7 cell line, the authors assessed cytotoxicity using the MTT assay, analysed the cell cycle and cell death mechanism using flow cytometry, and examined the structure of the cytoskeleton and cell morphology.

Results: The results showed that doxorubicin in combination with calcifediol in a 1:1 ratio showed a synergistic effect resulting in a dose-dependent decrease in cell survival. Further studies have shown that this is due to the pro-apoptotic and necrotic effects of the combination of these compounds. There were also changes in the organization of the cytoskeleton and cell morphology. In addition, features of entosis were noted in MCF-7 cells.

Conclusions: The synergistic effect of doxorubicin and calcifediol significantly reduced the viability of MCF-7 breast cancer cells. Inducing the desired effect by lowering the cytostatic dose is of great clinical importance, taking into account the cardiotoxicity of doxorubicin. Another very interesting aspect is the entosis process induced in the present research, which may have a dual nature.

Keywords: breast cancer, doxorubicin, calcifediol, synergistic effect

Introduction

Breast cancer is a threat that has been known for centuries. Currently, it is the most common cancer of women in Poland and the world. After lung cancer, it is the second leading cause of cancer-related deaths in Poland. The incidence of breast cancer continues to increase, as also the number of patients qualified for surgery. These observations are disturbing and may indicate a poor involvement of women in prevention. Resection is usually the first choice for breast cancer treatment. However, it may also be supported with chemotherapy and radiotherapy. The current recommendations for combination therapy in breast cancer patients are still under development and new therapies are being sought to achieve greater success. A relatively new approach is to administer cytostatics in combination with vitamins [1, 2].

Doxorubicin (DOX) is an anthracycline compound that exhibits high cytostatic efficacy. It is used to treat many neoplastic diseases. The action of anthracyclines is based on the inhibition of topoisomerase I and II necessary for DNA synthesis, the formation of abnormal bonds between nitrogen bases in the DNA molecule, and the formation of free radicals that have a destructive effect on DNA. Currently, it is still used in adjuvant therapy and the treatment of metastatic breast cancer. Therapies using a group of these compounds are highly effective. Although other, more modern treatments are available, most patients suffering from breast cancer are treated with anthracyclines at some stage. In breast cancer therapy doxorubicin and its derivative — epirubicin are mostly used. The studies indicate the strongest effect of the drugs in the S and M phases of the cell cycle. In these phases, it can inhibit the action of topoisomerase II by insertion between the DNA double-strand [3].

The synthesis of vitamin D in the body is a series of steps that are initiated in the skin under the impact of ultraviolet radiation. The 7-dehydrocholesterol is then converted to cholecalciferol, a precursor of vitamin D. The enzyme 25 β-hydroxylase converts it in the liver into calcifediol [25-hydroxyvitamin D3, 25(OH)D3], which is a biologically inert compound. The active form is obtained by hydroxylation of this compound in the kidneys to 1β, 25-dihydroxy vitamin D [4, 5]. Scientific studies indicate that not only vitamin D but also its direct precursor, 25(OH)D3, by binding to the nuclear vitamin D receptor (VDR), can regulate the expression of many genes involved in important cellular processes [6].

Mostly they indicate anti-proliferative and anti-cancer properties [7]. The conducted research mainly concerns the effects on breast, prostate, and colon cancer [8]. Vitamin D used in combination with anticancer drugs may show synergistic effects [9, 10].

The study aimed to elucidate whether the combination of calcifediol [25(OH)D3] together with doxorubicin affects the response of the MCF-7 breast cancer cell line to the cytostatic. For this purpose, the authors assessed cytotoxicity using MTT assay, analysed cell cycle and cell death mechanism with flow cytometry and investigated changes in cytoskeletal components with fluorescent labelling.

Materials and methods

Cell culture and treatment

The human breast cancer MCF-7 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in Eagle's Minimum Essential Medium (EMEM, Corning, New York, NY, USA) supplemented with 10% Foetal Bovine Serum (FBS, Corning, New York, NY, USA) and 1% Penicillin-Streptomycin Solution (Corning, New York, NY, USA) in standard culture conditions (5% CO_2, 37°C). For the cell viability analysis (MTT assay) cells were incubated with 100, 250, 500, 750, 1000 nM DOX (TargetMol, MA, USA); 100, 250, 500, 750, 1000 nM calcifediol (TargetMol) or the combination of DOX and 25(OH)D3 in the 1:1 ratio for 24h. Control cells were grown under identical conditions, but without adding active compounds to the culture. Based on the MTT assay, for the other experiments, 750 nM DOX; 750 nM 25(OH)D3 and the combination in the 1:1 ratio were used. The MCF-7 cells were conducted on low passage numbers and regularly tested for mycoplasma contamination using rapid uptake of the DAPI.

MTT assay and interaction between the drugs

The colorimetric MTT test was used to determine the cytotoxic effect of DOX, 25(OH)D3 and their combinations. Cells were grown for 24 h and then treated with 100, 250, 500, 750, 1000 nM DOX; 100, 250, 500, 750, 1000 nM calcifediol, and 1:1 combination of drugs. After 24 hours of cell culture with compounds, cells were washed with PBS and incubated with MTT working solution for 3 hours. The formed formazan crystals were dissolved in 2 mL of isopropanol (10 min, 37 °C; Avantor, Gliwice, Poland) and the absorbance was measured with a spectrophotometer (Spectra Academy, K-MAC, Korea) at a wavelength of 570 nm. After 24 hours of cell culture with compounds, cells were washed with PBS and incubated with MTT working solution for 3 hours. The formed formazan crystals were dissolved in 2 mL of isopropanol (Avantor, Gliwice, Poland) and the absorbance was measured with a spectrophotometer at a wavelength of 570 nm. In these analyses, the dye absorbance of the control cells is assumed to be 100% and was the reference point in assessing the cell viability of the test group.

The Chou-Talalaya median effect principle and CompuSyn software were used to assess potential drug interactions. The method of analysis used enables the determination of combination indexes (CI) in relation to the level of cytotoxicity and indicates: synergism (CI < 1), additimism (CI = 1) and antagonism (CI > 1) [11].

Cell death and cell cycle analysis

An apoptosis detection kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing annexin V Alexa Fluor 488 and propidium iodide (AnnexinV/PI) was used for cell death analysis. In turn, cell cycle analysis was based on the FxCycle PI/RNase Staining Solution (Thermo Fisher Scientific). Cells were performed according to the manufacturer's instructions and analyzed using the Guava® easyCyte™ 6HT-2L system and InCyte software (version 3.3, Merck KGaA, Darmstadt, Germany).

Morphology and actin cytoskeleton staining

Mayer's hematoxylin primary staining was used to evaluate the morphology of the MCF-7 cell line. Control cells treated with DOX, 25(OH)D3 and a combination of these compounds were cultured on glass coverslips. After fixation with 4% paraformaldehyde (Serva, HeidelbergGermany) for 20 min at RT, a series of washes with PBS and deionized water, the cells were stained Mayer's hematoxylin (Aqua-Med, Poland) (3 min, RT) and rinsed 3 × 3 min with running water from the tap. The cells were then washed with distilled water and mounted at Aqua-Poly/Mount (Polysciences Inc., Warrington, PA). Slides were analysed using an Eclipse E800 microscope (Nikon) equipped with a CCD camera DS-5Mc-U1 (Nikon) and NIS-Elements image analysis system (version 3.30; Nikon).
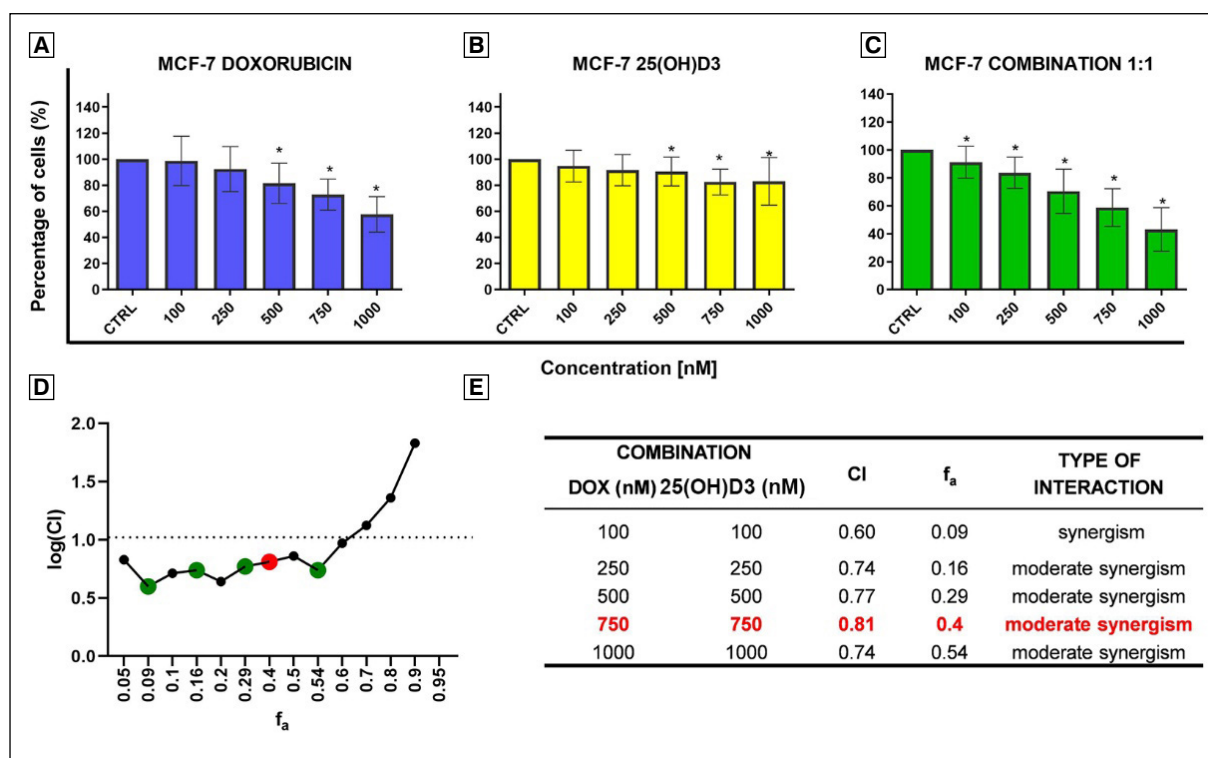
Figure 1. The effect of doxorubicin (DOX) and calcifediol [25(OH)D3] individually and in combined treatment on cell viability of MCF-7 cells. Cell viability analysis was performed based on the MTT test. MCF-7 cells were treated with DOX (**A**) and calcifediol [25(OH)D3] (**B**) at concentrations from 100–1000 nM for 24h and their combination in ratio 1:1 (**C**). The data represent mean values ± SD of 3 independent experiments (n = 3). Statistically significant differences were marked with '*' (p < 0.05; Wilcoxon test). (**D, E**) The combination index plot for DOX and 25(OH)D3 co-treatment in MCF-7 cells in the range of f_a from 0.1 to 0.95. CI < 1 — synergism, CI = 1 — additive effect, CI > 1 — antagonism. For real measuring points, the values have been marked in green. The point marked in red is the selected combination [750 nM DOX and DOX/25(OH)D3]

Cells were cultured and fixed for fluorescent F-actin staining in the same manner. Alexa Fluor 488 phalloidin (dilution 1:40, 20 min, Invitrogen; Thermo Fisher Scientific, Inc.) was used to label microfilaments, and DAPI (dilution 1:20,000, Sigma-Aldrich) to stain cell nuclei. The preparations were analysed using the C1 confocal laser scanning microscope (Nikon). Images were captured and evaluated using EZ–C1 software (version 3.80; Nikon).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). A p < 0.05 value was statistically significant. For the MTT assay, the Wilcoxon test was used where data after treatment with DOX, 25(OH)D3 and their combination in the ratio a 1:1 were compared to the hypothetical value for the untreated cell absorbance estimated as 100%. In apoptosis analysis, the non-parametric Kruskal–Wallis with Dunn's post hoc test was used. In turn, the results obtained in cell cycle analysis were 2-way ANOVA with Dunnet's post hoc test. All data are presented by means ± standard deviation (SD) of three independent experiments (n = 3).

Results

The cytotoxic effect of DOX and 25(OH)D3 individually and in combined treatment on cells viability

MTT assay was used to present the cytotoxic effect of DOX and 25(OH)D3 and their combination in a 1:1 ratio on MCF-7 cell line viability. The use of DOX at doses from 100 to 1000 nM resulted in a dose-dependent decrease in cell viability. As shown in Figure 1A, doses of 500, 750 and 1000 nM induced a statistically significant decrease in the viable cell population compared to the control. A dose-dependent decrease in cell viability was also observed after the application of 25(OH)D3 (Fig. 1B). After treating the MCF-7 cell line with vitamin, as in the case of DOX, statistically significant differences were noted at the doses of 500, 750 and 1000 nM. In turn, the use of a combination of these compounds in a 1:1 ratio generated a much higher decrease in cell viability compared to the use of the compounds alone. All obtained results were statistically significant compared to the control (Fig. 1C). Interaction-type analysis based
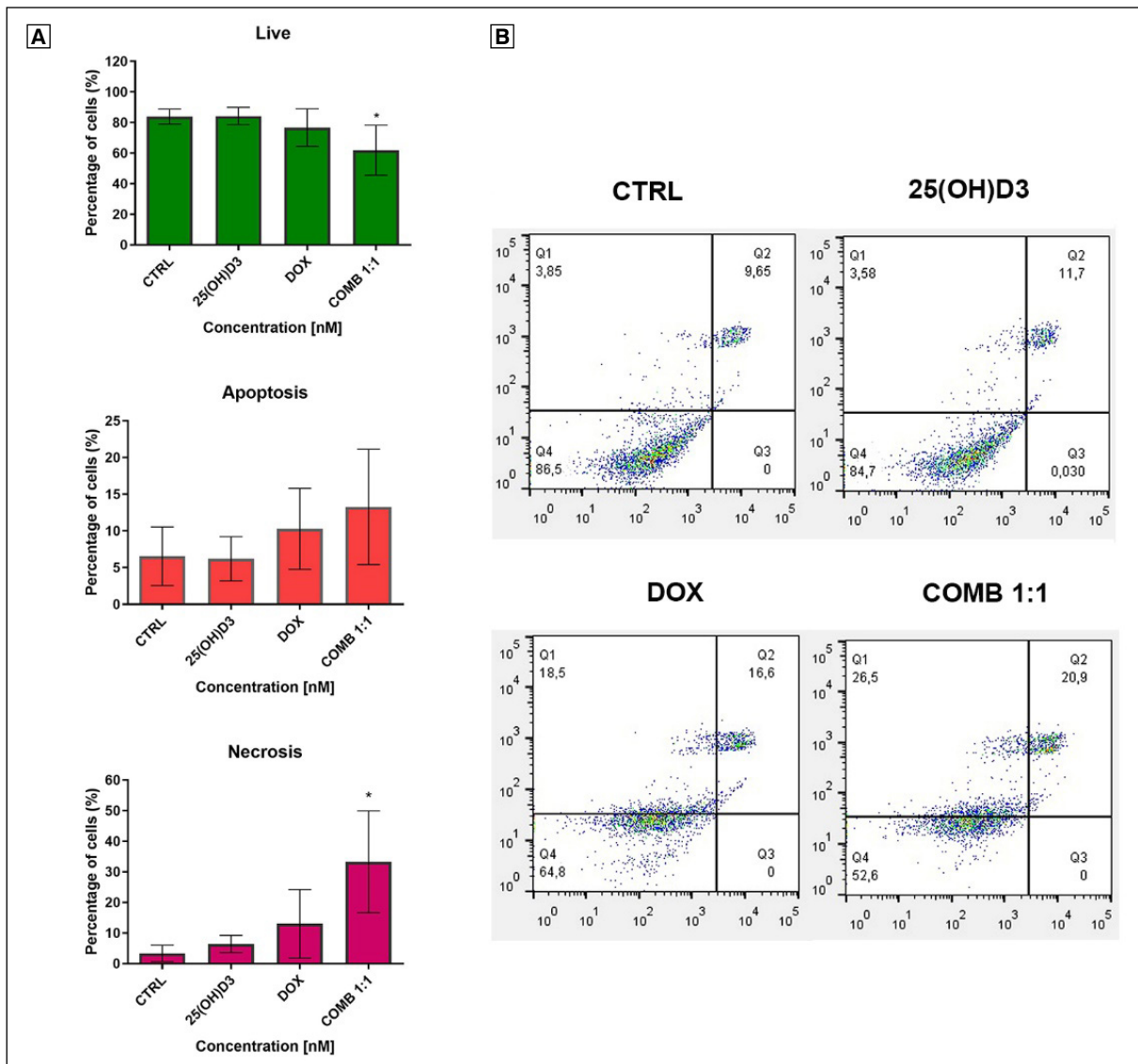
Figure 2. The effect of doxorubicin (DOX) calcifediol [25(OH)D3] individually and in combined treatment on cell death of MCF-7 cells. The cytometric analysis of cell death using Annexin V/PI double staining assay. (**A**) The percentage of live, apoptotic, and necrotic cells. The MCF-7 cells were treated with 750 nM concentration of DOX and 25(OH)D3 for 24h, and the combination of both drugs in ratio of 1:1. '*' indicate statistically significant differences in comparison to control cells ($P < 0.05$; the non-parametric Kruskal–Wallis with Dunn's post hoc test). (**B**) The representative plots

on the Chou-Talalay median effect principle showed a CI < 1 at all concentrations. Thus, synergism was demonstrated [100 nM DOX: 100 nM 25(OH)D3], and moderate synergism for the other combinations (Fig. 1D). Concentrations of 750 nM, DOX, 750 nM 25(OH)D3 and a combination of these compounds were selected for further research, due to the achievement of approximately 50% decrease in cell viability after using the combination of these compounds in a 1:1 ratio (achieving a concentration within the IC50 (half of the maximum inhibitory concentration, $f_a = 0.5$) for 24-hour incubation.

Alterations in cell death and cell cycle following DOX, 25(OH)D3 and their combination exposure

Analysis of cell death after 24-hour treatment of cells with MCF-7 DOX, 25(OH)D3 and their combination in a 1:1 ratio showed a decrease in the percentage of viable cells (AV-/PI-) compared to untreated cells. A statistically significant reduction in the percentage of viable cells of about 50% was observed in the combination of compounds used (Fig. 2A). After 24h incubation with the agents used, an increase in the percentage of apoptotic cells (AV+/PI- and AV+/PI+) was noted, but
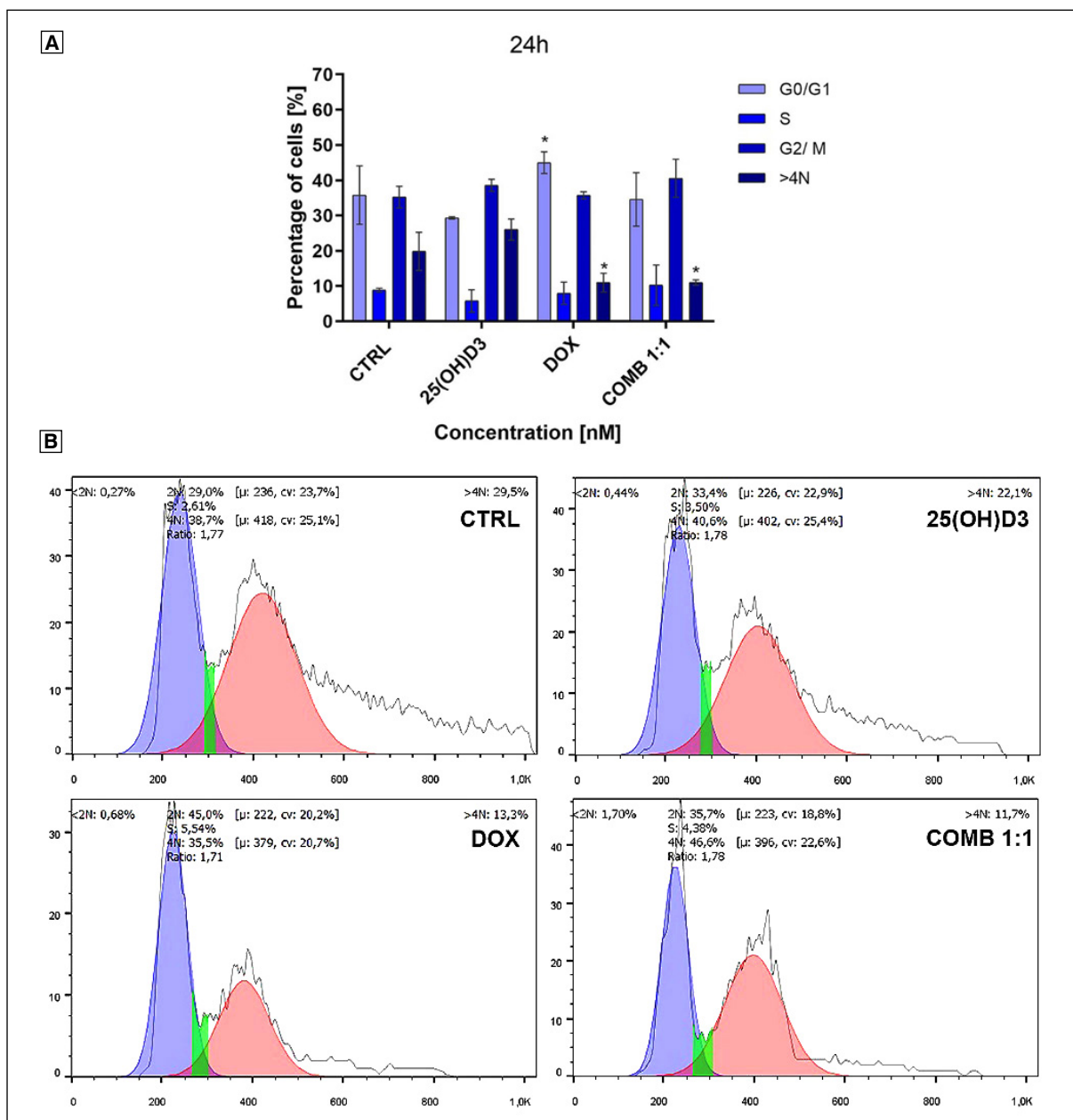
Figure 3. The effect of doxorubicin (DOX) calcifediol [25(OH)D3] individually and in combined treatment on the cell cycle of MCF-7 cells. (**A**) The percentage of cells in G0/G1, S, and G2/M phases and with > 4 N DNA (polyploidy) content. The MCF-7 cells were treated with 750 nM concentration of DOX and 25(OH)D3 for 24h, and the combination of both drugs in ratio of 1:1. '*' indicate statistically significant differences in comparison to control cells (P < 0.05; ANOVA with a Dunnett post hoc test). (**B**) The representative plots

the obtained results are not statistically significant (Fig. 2A). A very interesting result is a statistically significant increase in the percentage of necrotic cells (AV-/PI+) after using the combination of DOX and 25(OH)D3 (Fig. 2A). The representative plots were presented in Figure 2B.

Cell cycle analysis was performed using propidium iodide (PI) as a DNA staining agent and flow cytometry. The results shown in Figure 3 show slight changes in the phase distribution of the cell cycle. After treatment with

25(OH)D3 cells, no statistically significant differences were observed compared to control cells. For cells treated with 750 nM DOX, it statistically significantly increased the percentage of cells in the G0/G1 phase and decreased the polyploid fraction (DNA content > 4 N) (Fig. 3A). In turn, after using the combination of compounds, the only statistically significant difference was the reduction in the percentage of cells in phase > 4N (Fig. 3A). The representative plots were presented in Figure 3B.
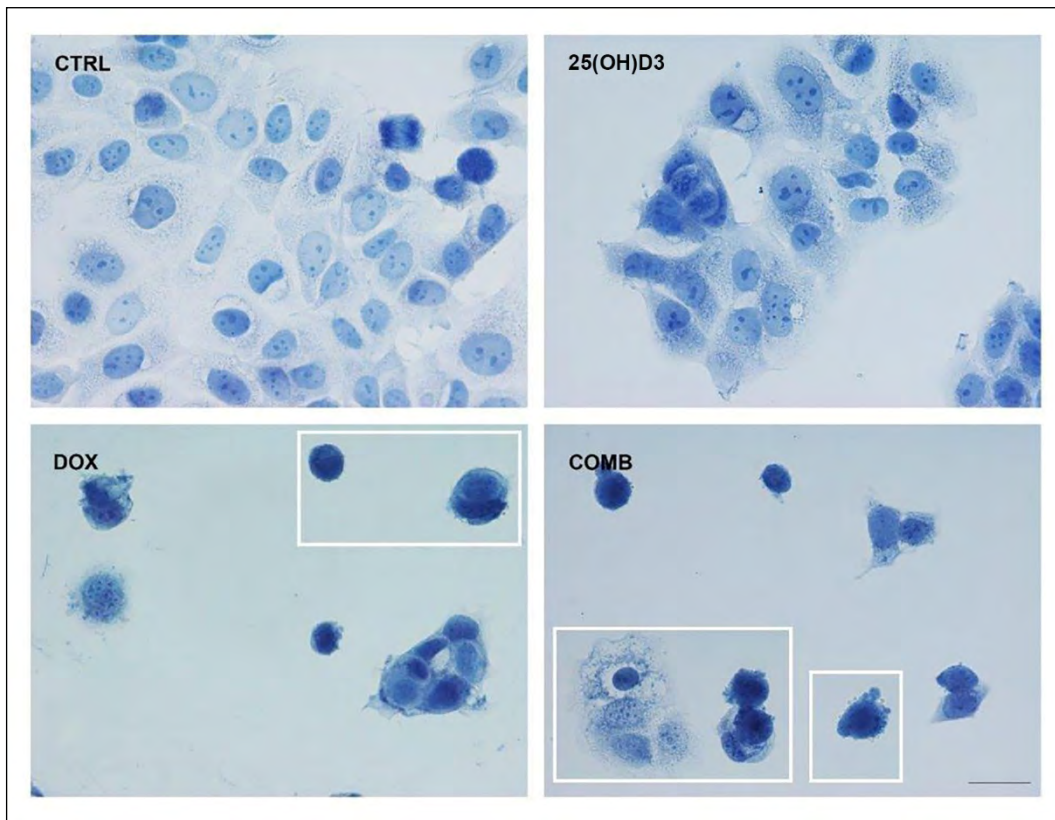
Figure 4. The effect of doxorubicin (DOX) calcifediol [25(OH)D3] individually and in combined treatment on the morphology of MCF-7 cells. The morphology of MCF-7 cells was examined by light microscopy after Mayer's hematoxylin staining. The MCF-7 cells were treated with 750 nM concentration of DOX and 25(OH)D3 for 24h, and the combination of both drugs in the ratio of 1:1. Bar = 50 μm

Morphological and F-actin organization changes of MCF-7 cells after DOX, 25(OH)D3 and their combination treatment

The control cells of the MCF-7 line have epithelial-like morphology. They are an adherent line, growing in a monolayer, where the cells retain. No significant changes compared to controls after treatment with 25(OH)D3. In contrast, cell culture with 750 nM DOX and the DOX/25(OH)D3 combination resulted in a rapid decrease in cell count, reduced cell-to-cell contact, and numerous cells with an apoptotic phenotype. As shown in Figure 4 after treatment with DOX and the combination of compounds, cells were shrunken, nuclear chromatin condensed, and apoptotic vesicles were visible on the surface of the plasma membrane. In addition, what is characteristic of epithelial cells exposed to substances unfavourable for them, areas with binuclear cells are observed, where one of them is shaped like a moon. Such a morphological picture is characteristic of the entosis process.

Changes in the structure and localization of F-actin were analysed using confocal microscopy (Fig. 5). A characteristic distribution of actin filaments extending throughout the cell and cumulating in the cell-cell contact areas was observed in control cells and those treated with 25(OH)D3. In turn, after the use of DOX and the DOX/25(OH)D3 combination, actin filaments depolymerized into short, thin fragments (Fig. 5).

Discussion

Despite significant clinical advances in breast cancer treatment and prevention, it still remains the second cause of cancer-related death in women. Thus, any improvements in the therapeutic protocol are sought. Anthracyclines are one of the most widely used chemotherapeutics used in the treatment of various cancer types. It also includes DOX, which is applied in bladder cancer, lymphoma, leukaemia and also breast cancer therapy. Despite the relatively high effectiveness of the
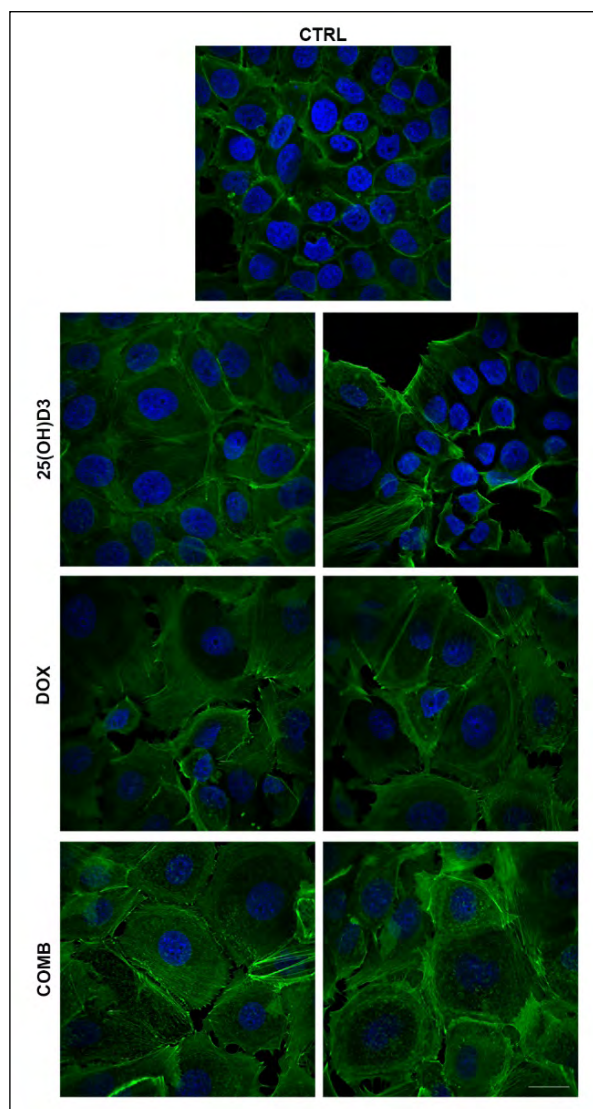
Figure 5. The effect of doxorubicin (DOX) calcifediol [25(OH)D3] individually and in combined treatment on the F-actin organization of MCF-7 cells. F-actin organization in MCF-7 cells was examined under a confocal microscope after fluorescence staining of actin filaments with Alexa Fluor 488 phalloidin. The MCF-7 cells were treated with 750 nM concentration of DOX and 25(OH)D3 for 24h, and the combination of both drugs in ratio of 1:1. F–actin (green), cell nuclei (blue). Bar = 50 μm

drug, it was connected with some side effects of which the most dangerous is cardiotoxicity [12]. Moreover, some recent studies showed, that DOX may enhance EMT and cell migration in the drug-resistant TNBC cells promoting metastasis formation [13]. Thus, the application of DOX in combination therapy with other substances may allow to reduce the dose of chemotherapeutic and limit adverse reactions.

Although the relationship between breast cancer development or progression and vitamin D deficiency remains controversial, a meta-analysis of sixty-eight studies published between 1998 and 2018 suggests a protective effect of circulating vitamin D on breast cancer, but only in premenopausal women [14]. Moreover, the expression of vitamin D receptors in breast cancer cells was confirmed in tissues and established cell lines [15, 16]. In turn, Voutsadakis in 2021, presenting a systematic review and meta-analysis, indicates the prevalence of vitamin D deficiency in patients with early-diagnosed breast cancer. In addition, it suggests that too low levels of vitamin D may affect the development of cancer and its progression [17]. Vitamin D alone reduces the viability of MCF-7 cells [18], but the present goal was to determine the synergistic effect with doxorubicin by reducing the concentration of the cytostatic. The use of doxorubicin alone indicated that the IC50 (drug concentration required to inhibit cell growth by 50%) for the MCF-7 cell line was 4 μM [19]. In their studies, the authors showed that the use of a dose of 750 nM in combination therapy with 25(OH)D3 in a 1:1 ratio reduced cell viability by about 50%. In addition, a moderate synergism of these factors was observed. A similar, also synergistic effect was observed by Marques et al. using 1β, 25, dihydroxy vitamin D3 (1.25 D) in combination therapy with Salinomycin (Sal). The use of both of these compounds shows a synergistic effect, inhibiting the proliferation of MCF-7 cells by activating death at the cellular level [20]. As mentioned earlier, it is very important to reduce the dose of DOX used in therapy due to its cardiotoxicity. The studies presented in the paper indicate such a possibility with vitamin D supplementation. In addition, the results of the present studies are consistent with the experiences presented in the paper by Lee et al. Studies conducted on female Balb/c mice indicated that vitamin D supplementation reduced DOX-induced cardiotoxicity without reducing the anticancer efficacy of the cytostatic. Giving mice vit D3 was effective in reducing DOX therapy-generated levels of reactive oxygen species and mitochondrial damage [21].

An interesting result of the present research was also the observation of entosis. In the case of this process and its pro- or anti-cancer significance, scientists in the world do not agree. Recent reports indicate that entotic figures, or "cell within a cell", are considered a potential prognostic marker in various cancers, including breast cancer. While determining entosis in correlation with classic breast cancer biomarkers HER2, ER, PR and Ki-67, it was noted that in the study cohort, entotic numbers were positively correlated with Ki-67 and HER2, which may indicate a potential diagnostic value [22]. The same research team believes that entosis is

associated with a more malignant phenotype of cancer and a poor prognosis for the patient. And the process itself is an escape mechanism, stimulated by some anti-cancer drugs such as paclitaxel or nintedanib [23, 24]. The entotic cell, hiding from the cytotoxic effect of the drug, remains alive in the host cell, leading to treatment failure and/or disease relapse [25].

In turn, Khalkar et al. indicate the anticancer nature of entosis, in which the inner cell dies. Researchers found that in pancreatic cancer cells of the Panc-1 line, methylselenoesters induce programmed cell death via entosis [26]. Additionally, Su et al. claim that it is the pH of enthotic vacuoles that determines the fate of internalized cells [27].

Conclusions

The synergistic effect of doxorubicin and calcifediol significantly reduced the viability of MCF-7 breast cancer cells. Inducing the desired effect by lowering the cytostatic dose is of great clinical importance, taking into account the cardiotoxicity of doxorubicin. To confirm the research field, studies on other breast cancer cell lines of various invasiveness should be presented. The next step could be to transfer the research to an in vivo model. Another very interesting aspect is the entosis process induced in the present research, which may have a dual nature.

Article information

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