RESEARCH ARTICLE

Histological Subtype of Ovarian Cancer as a Determinant of Sensitivity to Formamidine Derivatives of Doxorubicin - *in Vitro* Comparative Studies with SKOV-3 and ES-2 Cancer Cell Lines

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Abstract

Background: Development of new apoptosis-inducing drugs is a promising trend in anticancer therapy. For this purpose several formamidinoderivatives of doxorubicin were synthesized. The aim of our study was to investigate effects of the five formamidinodoxorubicins in the ES-2 human ovarian clear cell carcinoma line, for comparison with data obtained previously for SKOV-3 human ovarian adenocarcinoma cells, to answer the question of whether and to what extent the histological cell type is a possible determinant of sensitivity to tested anthracyclines. Materials and Methods: In our experimental work the following methods were used: spectrophotometric assays with MTT; fluorimetric assays - double staining with Hoechst 33258 and propidium iodide (PI), measurement of caspase-3, -8, -9 activity, intracellular accumulation of DOX and analogues, estimation of drug uptake, mitochondrial transmembrane potential; flow cytometry - phosphatidylserine (PS) externalization with annexin V-FITC and PI fluorochromes. Results: Effects of the derivatives of doxorubicin were partially linked with the specific type of cancer cell although intracellular accumulation and cellular uptake of DOX and derivatives were similar in both. All of the investigated derivatives were considerably more cytotoxic than DOX. Formamidinodoxorubicins were able to induce caspase-dependent apoptotic cell death in both cell types. Conclusions: All new formamidine derivatives of DOX were able to induce caspase - dependent apoptosis in human ovarian cancer cell lines SKOV-3 and ES-2. Obtained results suggested that formamidine derivatives of DOX may be promising candidates for the prospective chemotherapeutic agents for the two different histological subtypes of ovarian cancer.

Keywords: Apoptosis - caspases - cytotoxicity - formamidine anthracyclines - necrosis

Asian Pac J Cancer Prev, **17 (9)**, 4223-4231

Introduction

Epithelial ovarian cancers may be grouped based on their genetic profile into two varieties. Type I, the slowly developing tumours, accounts for 25% of ovarian malignancies and cause 10% of deaths. This group includes low grade serous, endometrioid, mucinous, and a subclass of clear cell carcinomas. The more aggressive Type II accounts for 75% of ovarian cancers and cause 90% of deaths. Type II includes the high-grade serous, high-grade endometrioid, and undifferentiated carcinomas. These tumours are diagnosed in their advanced stage because of rapid growth (Sun et al., 2013). The aim of this article is to compare the sensitivity of two cancer cell lines (SKOV-3 and ES-2) on formamidine derivatives of doxorubicin (Table 1). The cell lines proposed in this article are derived from different histological subtypes of ovarian cancer, what may determine their sensitivity to various compounds including anticancer drugs. SKOV-3 human ovarian adenocarcinoma cell line is the most commonly used cell line model of ovarian cancer. Although the exact histological origin of SKOV-3 cells is not specified, the cells are commonly used as models for serous ovarian cancer (Domcke et al., 2013). ES-2 cells were derived from human ovarian clear cell carcinoma (CCC), which is biologically distinct from the other EOC subtypes with unique biology and behaviour, and poorer overall survival rate in comparison to non-CCC patients, regardless of the illness stage, so there is the need for a new treatment strategies (Anglesio et al., 2011; Lee et al., 2011).

Doxorubicin (DOX) is widely used in treatment of many types of cancer, including solid tumours and haematological disorders. Unfortunately, DOX is responsible for cardiomyopathy and myelosuppression, which severely limits its therapeutic utility (Dubbelboer et al., 2014). There is an urgent need to develop new

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effective anticancer drugs.

Marczak et al., 2014 research showed that the investigated formamidine derivatives were considerably more cytotoxic to the SKOV-3 cell line than DOX. The predominant type of cell death induced by the anthracycline analogues was apoptosis (Marczak et al., 2014). To better understand the molecular events occurring in ovarian cancer cell lines in response to new formamidine derivatives of DOX, the current study investigated intracellular accumulation of DOX and analogues, estimation of drugs uptake, measurements of caspase-8 and caspase-9 activities and membrane potential analysis.

Materials and Methods

Chemicals

Doxorubicin as well as all its derivatives designated as compounds 1-5 (Table 1) were synthesized at the Institute of Biotechnology and Antibiotics (Warsaw, Poland) All derivatives were obtained by reaction of doxorubicin hydrochloride dimethyl acetal of respective amine in methanol (Oszczapowicz I et al., 2005). Roswell Park Memorial Institute (RPMI) growth medium, Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS), trypsin-EDTA, penicillin and streptomycin were purchased from Lonza (Basel, Switzerland). Annexin V binding Assay Kit was supplied by Invitrogen (Carlsbad, USA). Caspase-3 Activity Assay was from Calbiochem (San Diego, USA). Caspase-8 Fluorimetric Assay Kit and Caspase-9 Fluorimetric Assay Kit was supplied by BioVision Inc. (Milpitas, California, USA). All other chemicals and solvents were of high analytical grade and were obtained from Avantor S.A. (Gliwice, Poland).

Cell culture

SKOV-3 (*human ovarian adenocarcinoma*) as well as ES-2 (*human ovarian clear cell carcinoma*) cells were obtained from American Type Culture Collection (ATCC, Rockville, USA). Cells were grown as a monolayer with RPMI (SKOV-3) or DMEM (ES-2) growth medium supplemented with 10% foetal bovine serum, penicillin (10 U/ml) and streptomycin (50 μg/ml), in standard conditions (Marczak et al., 2014).

Intracellular accumulation of DOX and analogues, estimation of drugs uptake

Our study of compounds accumulation and uptake were carried out following the method described by Szwed et al. (Szwed et al., 2014) Intracellular DOX or derivatives accumulation were evaluated at λex = 488 nm and λem = 590 nm. The intensity of fluorescence were measured on a fluorescent multiwell plate reader (Labsystem Fluoroskan Ascent FL, Inc.).

MTT assay

The effects of DOX and derivatives 1-5 on the cells viability were evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, described previously (Marczak et al., 2014).

Drug treatment

To complete the analysis of double staining with Hoechst 33258-propidium iodide assays, PS externalization and caspases-3, -8 and -9 activity the ovarian cancer cells were removed from monolayer by trypsinization, resuspended in fresh complete medium, centrifuged for 5 min at 200 x g and plated into appropriate dishes at the optimal density. After 24 h the compounds in 80 nM (SKOV-3) or 15 nM (ES-2) were added to the dishes and incubated with the drugs in CO^2 incubator for different period of time (4–72 h) depend on the method.

Analysis of the type of cell death by fluorescence microscopy

Morphological changes typical for apoptosis and necrosis in cells treated with DOX and derivatives were assessed by described previously method with double staining Hoechst 33258 and propidium iodide (PI) (Marczak et al., 2014).

Analysis of phosphatidylserine (PS) externalization

PS externalization was determined using the Annexin V-FITC staining kit (Invitrogen, Carlsband, USA). Briefly, the cell suspension (1 x 10^5 cells) was stained with a solution containing a mixture of annexin V-FITC and PI in 0.01 ml of binding buffer provided by the manufacturer.

Measurement of caspases activity The activity of caspases -3, -8 and -9 was evaluated using the fluorimetric assay kits following the manufacturer's protocol. SKOV-3 and ES-2 cells extracts were mixed with the reaction buffer and incubated with the adequate caspase substrate. Additionally in the control all cell lysates were preincubated for 1 h with the proper reversible caspases inhibitors at 0.04 mM concentration.

Mitochondrial transmembrane potential

At the end of the compounds treatment, the medium was removed and the cells were incubated with 5 M JC-1 in HBSS for 30 min, at 37ºC, in the dark. Then the fluorescence of both JC-1 monomers and dimers was measured on a Fluoroskan Ascent FL microplate reader (Labsystems, Sweden) using filter pairs adequate for dimers (530/590 nm) and monomers (485/538 nm). The results in the figures are shown as a ratio of dimer to monomer fluorescence in relation to the control fluorescence ratio, assumed to be 100%.

Statistical analysis

The data was presented as a mean ± S.D. An analysis of ANOVA variance with a Tukey post hoc test was used for multiple comparisons. All statistics were calculated using the STATISTICA program (StatSoft, Tulsa, OK, USA). A p-value of <0.05 was considered significant.

Results

Intracellular accumulation of DOX and analogues, estimation of drugs uptake

Graphs showing time-dependence of the quantity of drug taken up (U) and excluded from the cells (E) at the

same time point, are presented in Figure 1 (SKOV-3 cell line) and Figure 2 (ES-2 cell line). Table 2 (SKOV-3) and Table 3 (ES-2) show the comparison of other kinetics parameters, such as influx and efflux rate constant, initial influx and efflux (Kin; Kout; It=0; Et=60, respectively), drug taken up and removed by cells within 60 min (Ut=60; Et=60, respectively). No significant differences have been found between the reference compound and all tested derivatives as well as individual compounds in both cell lines. Thus, it can be concluded that intracellular accumulation and cellular uptake of DOX and derivatives are similar regardless of the ovarian cancer cells subtype.

Cytotoxicity assay

The cytotoxic potential of DOX and derivatives in both human ovarian cancer cell lines was measured by a standard microplate MTT colorimetric method. The reference drug, as well as all formamidine derivatives, showed evident concentration-dependent cytotoxic effect. Figure 3 shows the IC_{50} parameters. Striking differences in IC_{50} parameters between DOX and analogues have been noted especially for DOX-F PYR, DOX-F PIP, DOX-F MOR in ES-2 cells. In the SKOV-3 cells the presence of heteroatom in the six-membered rings, for example in morpholine moiety, resulted in their improved activity. The most sufficient were the analogs DOX-F MOR and DOX-F PAZ, but the IC_{50} values obtained for all the compounds were significantly lower than IC_{50} calculated for DOX (Marczak et al., 2014). The IC_{50} parameters calculated for reference drug (DOX) were higher in SKOV-3 cells in comparison to ES-2 cells (352.79 ± 25.04 nM for SKOV-3 cells; 172.00 ± 24.10 nM for ES-2 cells). The opposite situation occurred after incubation of both lines cells with DOX-F PAZ (82.42 ± 8.47 nM for SKOV-3 cells and 133.01 ± 18.47 nM for ES-2 cells), and also after DOX-F MOR exposition (81.37 ± 32.49 nM for SKOV-3 cells and 118.68 ± 11.21 nM for ES-2 cells). Similar values in both cell lines were obtained for DOX-F PYR and DOX-F HEX (DOX-F PYR: 112.48 ± 11.29 nM for SKOV-3 cells and 103.16 ± 12.29 nM for ES-2 cells; DOX-F HEX: 251.27 ± 19.30 nM; 234.77 ± 14,77 nM, respectively). After ES-2 cells exposure on DOX-F PIP, the IC_{50} parameters were about 2-fold lower than those obtained for SKOV-3 cells (61.85 ± 7.17 nM; 112.30 ± 9.55 nM, respectively).

The effect of DOX and formamidine derivatives was also investigated by morphological analysis using microscopy. The morphology of cells treated for 72 h with concentration 15 nM of tested anthracyclines, and examined under an inverted microscope (Olympus IX70, Japan) is shown in Figure 4. In both cell lines the least modifications were observed in cells exposed to DOX. The

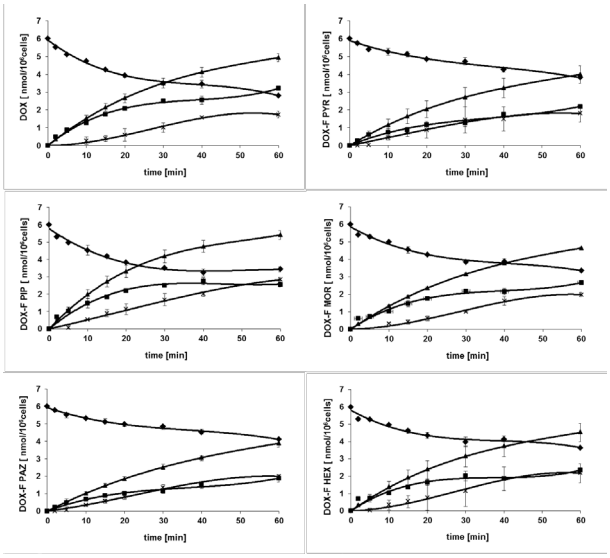


Figure 1. Transport Kinetics of DOX and Formamidine Analogs to SKOV-3 Cells. (■): Amount of drug in external medium; (♦): amount of drug associated with SKOV-3 cells. Drug uptake (▲) and efflux (x). Data are expressed as the mean ± SD of three independent experiments

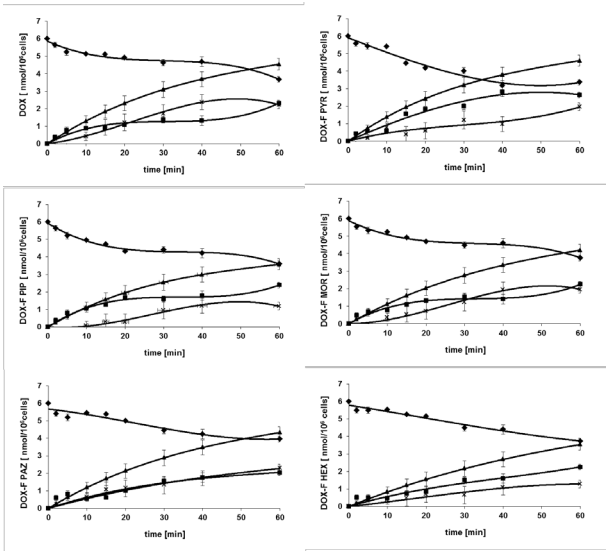
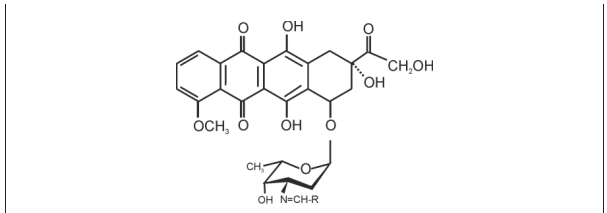


Figure 2. Transport Kinetics of DOX and Formamidine Analogs to ES-2 Cells. (■): Amount of drug in external medium; (♦): amount of drug associated with ES-2 cells. Drug uptake (▲) and efflux (x). Data are expressed as the mean ± SD of three independent experiments



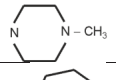
No.	Analogues	Abbreviation	R
1	Pyrrolidine	DOX-F PYR	
2	Piperidine	DOX-F PIP	
3	Morpholine	DOX-F MOR	
4	N-methylpiperazine	DOX-F PAZ	
5	Hexamethyleneimine	DOX-F HEX	

Table 1. Chemical Structures of Tested Formamidine Derivatives of DOX

reduced amount of cells was observed after exposure to all tested analogues. In ES-2 cells DOX-F PIP and DOX-F PYR treatment caused cell elongation and slight increase in the cell size. After the exposure on DOX-F MOR, DOX-F PAZ and DOX-F HEX cells became significantly smaller, and the substantial changes in their shapes were detected. Separation of the cells from the surface of the monolayer was observed after SKOV-3 cells exposition on DOX-F MOR and DOX-F PYR treatment.

Analysis of the type of cell death by fluorescence microscopy: double staining with Hoechst 33258-propidium iodide (PI)

Morphological alterations typical for apoptosis and necrosis in the cells ES-2 were detected after the treatment with DOX and derivatives. After a prolonged incubation time (72 h), not only profound chromatin condensation, cell shrinkage and apoptotic bodies formation were observed, but also cells with condensed chromatin with red fluorescence, indicating loss of membrane integrity (Figure 5). Quantitative representation of a number of apoptotic and necrotic cells observed after double staining with Hoechst 33258 and propidium iodide (PI) are presented in Figure 6. The main difference between SKOV-3 and ES-2 cell line is associated with apoptotic and necrotic cell fractions obtained after 48 h and 72 h exposure to analogues. After SKOV-3 cells incubation with DOX-F PYR, DOX-F PIP, DOX-F MOR, DOX-F PAZ, the apoptotic cells fraction was noted at 15% (48 h) or 20% (72 h) level. Necrotic cell fraction was very low (<5%) (Marczak et al., 2014). In ES-2 cells the necrotic cells fractions are similar to apoptotic or dominated over the apoptotic cells percentage. 48 hour incubation results in a similar percentage of apoptotic and necrotic cells (6-9% apoptotic cells and 7-9% of necrotic cells after exposure on DOX and analogues, with the exception of DOX-F PIP). After the 72 h incubation time an increase was observed in necrotic cells percentage for all tested compounds. The higher increase was denoted for DOX, DOX-F PIP, DOX-F HEX (about 2-fold increase).

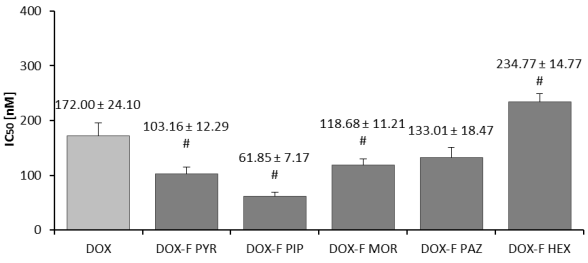


Figure 3. The Values of IC_{50} Parameter Following Exposure to DOX and Compounds 1-5 of ES-2 Human Ovarian Cancer Cell Line. Survival was assessed by MTT Assay. **(#) p<0.0001, changes between IC50 for compounds 1-5 and DOX. The values are the mean ± SD of five independent experiments**

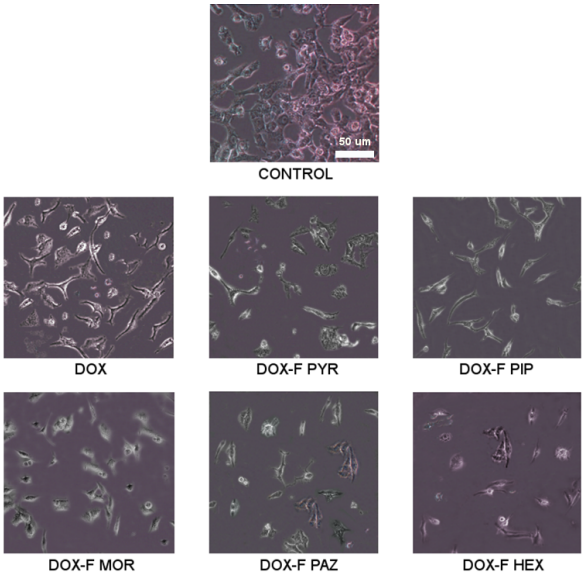


Figure 4. The Morphology of the Control Cells and the Cells Treated with DOX and Compounds 1–5 (15 nM, 72h). Pictures were taken using an inverted microscope equipped with a camera, in a phase contrast (Olympus IX70, Japan; bar 50 μm)

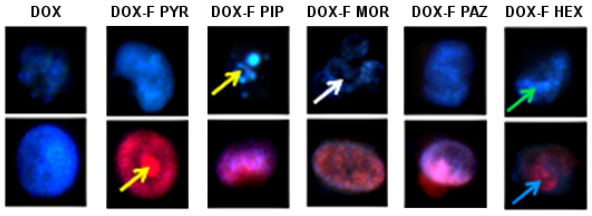


Figure 5. Morphological Changes of Human ES-2 Ovarian Cells at 72h after DOX and Compounds 1–5 Treatment. The cells were stained with Hoechst 33258 and propidium iodide and visualized by fluorescence microscopy (Olympus IX70, Japan; bar 50 μm). Apoptosis-like morphological features: changes in the structure, size and shape, cell shrinkage (green arrow), chromatin condensation (yellow arrows); apoptotic body formation (white arrow); polyploid cells with two nuclei (blue arrow). (For interpretation of the references to colour in figure legend, the reader is referred to the web version of the article)

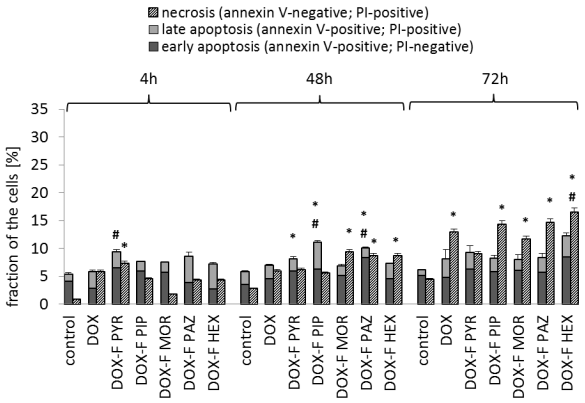


Figure 6. Fraction of the Early Apoptotic, Late Apoptotic and Necrotic Cells After 4, 48 and 72 h exposure on DOX and Compounds 1–5 (15 nM). *p<0.05 statistically significant differences in comparison to control cells, (#) statistically significant differences between probes incubated with compounds 1–5 in comparison to the effect after treatment with DOX. The values are the mean ± SD of five independent experiments

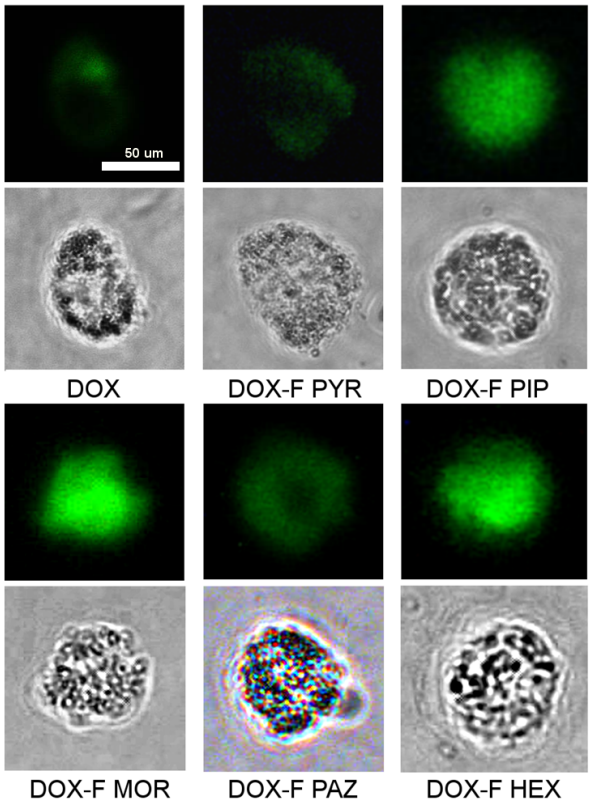


Figure 7. Fluorescence Images of SKOV-3 Cells. Cells were treated with concentration of 15 nM of tested compounds for 48 h and double stained with Annexin V-FITC and PI before examination under fluorescence microscopy (Olympus IX70, Japan), bar 50 μm

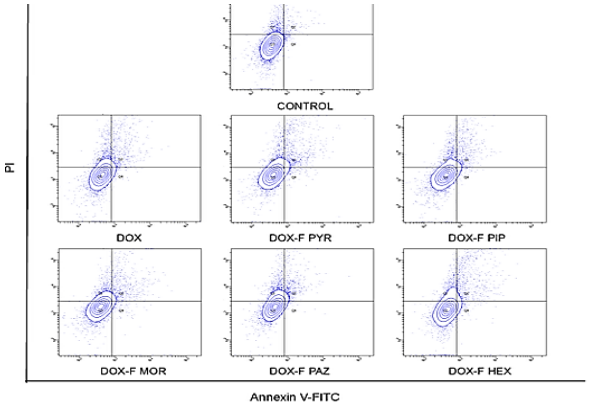


Figure 8. Flow Cytometric Analysis of Apoptosis and Necrosis in ES-2 Cells Measured by Using Annexin V - FITC Apoptosis Detection Kit. Representative cytometric dot-plots images of ES-2 cells obtained after incubation with DOX and compounds 1–5 (15 nM; 72 h). Each specimen presents: viable cells - Annexin V-negative, PI negative (left down corner) Q3; apoptotic cells - sum of early apoptosis (Annexin V-positive, PI negative - right down corner Q4) and late apoptosis (Annexin V-positive, PI positive - right upper corner Q2) and necrotic cells: Annexin V-negative, PI positive (Q1 - left upper corner)

Measurements of phosphatidylserine (PS) externalization

PS externalization was monitored cytometrically and also under a fluorescence microscope. Figure 7 shows the morphological changes in ES-2 cell line after 48 h

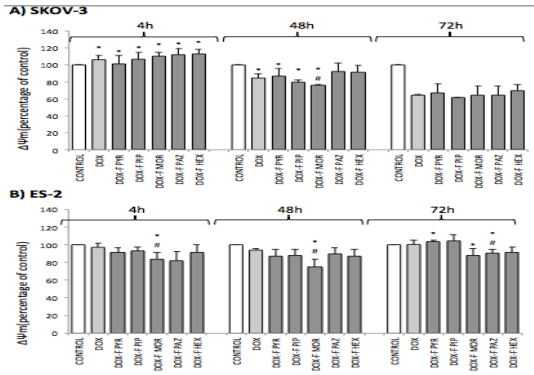


Figure 9. PS Externalization after Double Staining with Annexin-V and PI in Human ES-2 Ovarian Cells. The graph shows fraction of the apoptotic and necrotic cells after 4, 48 and 72 h exposure on DOX and compounds 1–5 with 15 nM concentration. (*)p<0.05 statistically significant differences between fraction of apoptotic or necrotic cells observed in probes incubated with DOX and compounds 1–5 in comparison to control cells, (#) statistically significant differences between samples incubated with compounds 1–5 in comparison to the effect after treatment with DOX. The values are the mean ± SD of five independent experiments

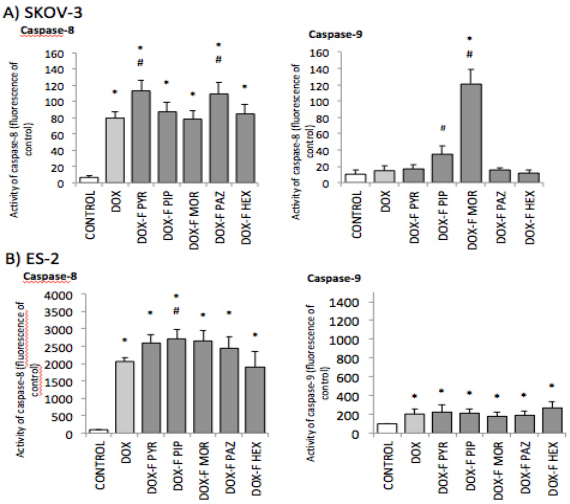


Figure 10. Changes in the Activity of Caspase-8 and Caspase-9 After SKOV-3 (A) and ES-2 Cells (B) Exposure (48h) on DOX and Derivatives. (*)p<0.05 statistically significant differences in comparison to control cells, (#) statistically significant differences between probes incubated with compounds 1–5 in comparison to the effect after treatment with DOX. The values are the mean ± SD of five independent experiments

incubation time with DOX and compounds 1-5. Control cells stained negative for annexin V-FITC (data not shown). In cells exposed to analogues and DOX, a large increase in the green Annexin V-FITC fluorescence was visible. Additionally, simultaneous staining cells with propidium iodide gives the ability to detect necrotic cells. Representative cytometric dot-plots images of ES-2 cells gained after exposure on DOX and compounds 1-5 (72 h) are presented in Figure 8. The quantitative results are presented in Figure 9. For all incubation times (4, 48, 72h) in ES-2 cell line necrotic cell death dominates the apoptotic. The higher number of cells with necrotic

morphology was observed after 48h treatment with DOX and analogs. 72h exposure to DOX and derivatives resulted in a slight decrease of necrotic cells fraction. According to the results, DOX, as well as all tested compounds, induced both apoptotic and necrotic cell death. For SKOV-3 cells, DOX and all tested compounds induced both, apoptotic and necrotic cell death, but contrary to ES-2 cells, the predominance of the first process was significant (Marczak et al., 2014).

Caspase-3, -8, -9 activity measurements

Changes in caspase-3 activity in ES-2 cells exposed to formamidine derivatives, as well as DOX, are presented in Figure 10. ES-2 cells incubation with tested compounds results in moderate increase in caspase-3 activity in comparison to SKOV-3 cell line (Marczak et al., 2014). For DOX and formamidine derivatives the moderate increase in caspase-3 activity was observed after 72h incubation time. Values obtained in ES-2 cells for all derivatives were over 1.5-fold higher than those gained for reference drug (211.76 ± 27.12). Results noted for all analogs were similar and they ranged from 312.23 ± 62.41 (DOX-F MOR), to 385.68 ± 56.46 (DOX-F PIP). In SKOV-3 cells, the observed values for all investigated drugs were from 1.5-fold (for DOX-F PYR, DOX-F MOR, DOX-F PAZ) to 2-fold higher (for DOX) in comparison to ES-2 cell line. In both cell lines the caspase-3 activity after incubation with DOX-F HEX was similar, and the DOX-F PIP was the most potent caspase-3 activator (Marczak et al., 2014).

The current study investigated the measurements of caspase-8 and caspase-9 activities to better understand the molecular events occurring in SKOV-3 and ES-2 cells in response to new formamidine derivatives of DOX (Figure 11). In SKOV-3 cells all tested compounds caused an increase in the activity of this enzyme. The strong increase in the activity of caspase-8 (represented as a percentage of control) was noted after 48h of incubation; for this time the changes were statistically significant not only in comparison to the control cells, but also as compared to the probes incubated with DOX. Whereas activity for DOX was 1216.49 ± 126.30, the percentage of control, for the formamidinodoxorubicins were1724.06 ± 200.22 and 1679.21 ± 207.71 for DOX F PYR and DOX F PAZ respectively. The activity of caspase-8 for DOX-F PIP and DOX-F MOR was similar to those obtained for DOX (1332.23 ± 184.50 and 1190.30 ± 164.30 respectively). The strong increase in caspase-8 activity was also observed in ES-2 cells after 48h exposure to all tested compounds. The caspase-8 activity was most strongly activated after cells incubation with DOX-F PIP (2715.53 ± 262.81). ES-2 cells exposure on DOX-F PYR, DOX-F MOR, DOX-F PAZ resulted in caspase-8 activation remaining at a similar level. The least potent analogue was DOX-F HEX (1905.16 ± 429.84). Taking into account the results of caspase-8 activity obtained for SKOV-3 and ES-2 cells, it can be concluded that all

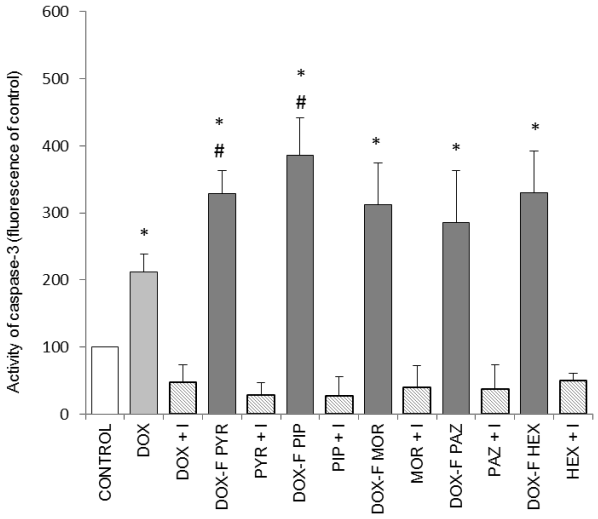


Figure 11. Changes in the Activity of Caspase-3 after ES-2 Cells 72h Exposure on DOX and Derivatives. (*) p <0.05 statistically significant differences in comparison to control cells, (#) statistically significant differences between probes incubated with compounds 1–5 in comparison to the effect after treatment with DOX. The values are the mean ± SD of five independent experiments

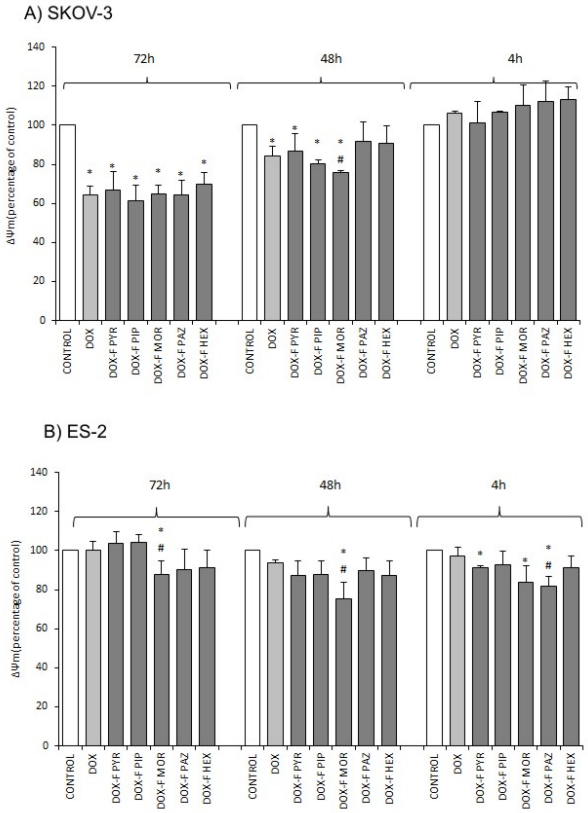


Figure 12. Changes in mitochondrial membrane potential (Δψm) of SKOV-3 and ES-2 cells seeded into black 96-well titration microplates and incubated with DOX and formamidinoantracyclines for 4, 48 and 72h. Mitochondrial membrane potential was estimated with a fluorescence dye JC-1 at the end of incubation directly in the cell monolayers. Fluorescence ratio of JC-1 dimers/JC-1 monomers of control was assumed as 100%. Results are presented as mean ± S.D. of 3-4 independent experiments. (*)p<0.05, significant differences between drug-treated and untreated, control cells (taken as 100%); (#)p<0.05, significant differences between DOX-treated cells and cells exposed on derivatives 1-5

formamidinodoxorubicins induce a strong increase in the activity of caspase-8 in both cell lines.

Caspase-9 is required in most scenarios of apoptotic cell death. As observed previously (Marczak t al., 2014), in SKOV-3 cell line 48h exposure to DOX-F PIP as well as DOX-F MOR resulted in maximal activation of caspase-9. For DOX-F MOR the activation of caspase-9 was over 8-fold higher than those obtained for DOX (1175.11 ± 168.28 and 158.43 ± 53.37 percentage of control cells, respectively). Statistically significant differences were observed also for DOX-F PIP (360.35 ± 91.25) in comparison to DOX. Caspase-9 activity in ES-2 cells, observed after 48 hours of incubation with the all tested compounds, has been frequently weaker than that seen for previously described caspase-8. In the ES-2 cells the 48h exposure to DOX and derivatives induced the maximal caspase-8 activation for DOX-F HEX (273.69 ± 64.39). The results obtained with the other compounds remained at the level of 200% of control cells. Summarising, the results of caspase-9 activity in both cell lines were similar after exposition on DOX, DOX-F PYR, DOX-F PAZ and DOX-F HEX. The main difference between cell lines was observed after cells incubation with DOX-F MOR.

Mitochondrial transmembrane potential

The influence of DOX and formamidinodoxorubicins on mitochondrial membrane potential ($\Delta\Psi m$) was assessed using fluorimetric analysis after staining with the fluorescent dye JC-1. This probe has the unique property of spontaneous formation of red-fluorescent dimers under high mitochondrial potential, whereas its monomeric form, prevalent in cells with low Ψm, shows green fluorescence. In both, SKOV-3 as well as ES-2, cells all tested drugs induced time-dependent changes in mitochondrial membrane potential. Their quantitative representations are presented in Figure 12. In SKOV-3 cells after the 4h incubation time, the JC-1 fluorescence ratio remains at the control cells level (defined as 100%) for all DOX and formamidinodoxorubicins, and the differences between DOX and analogues were not noticed. The JC-1 fluorescence ratio decreased after the prolonged incubation time (48h and 72h). After the 48h, the maximal decrease of the JC-1 fluorescence ratio was observed for DOX-F MOR (about 25% less than in the control samples). In the presence of other analogues, the decrease ranged from 10% to 20% of the control probes. The maximal decrease was observed after 72h. The ratio of JC-1 fluorescence was markedly lower (by about 40%) and was similar after exposure on DOX and derivatives.

The opposite relation occurred in ES-2 cell line. The maximal differences between control samples and derivatives were observed after 4h incubation time. Statistically important changes in comparison to control probe were noted after DOX-F PYR, DOX-F MOR and DOX-F PAZ presence. Additionally, after DOX-F PAZ exposure the differences were statistically significant in comparison to DOX. After the 48h exposure, the differences between control cells and cells treated with DOX and analogues were not noticed, except from DOX-F MOR. Similarly, after the 72h incubation time only DOX-F MOR caused a significant decrease in comparison to DOX.

Discussion

SKOV-3 cell line is the most commonly used cell line model of ovarian cancer. ES-2 cell line was isolated by the Sikic laboratory from a surgical tumour, which was described as a poorly differentiated, highly aggressive and drug resistant variant ovarian cancer with mixed serous and clear cell carcinoma histopathological features (Moisan et al., 2014). Within epithelial ovarian cancers, clear cell carcinoma represents a distinct medical challenge with unique biology and behaviour. Despite its low incidence (3–10 % of EOC cases) clear cell carcinoma is highly mortal, when the disease has extraovarian extension. As between 31-60% of CCC present as stage I of the illness, it is important to understand the determinants of prognosis and the effectiveness of postoperative treatment (Anglesio et al., 2011). It was found that CCC has a poorer overall survival rate in comparison to non-CCC patients, regardless of the illness stage (Lee et al., 2011). Contrary to other histological types in which chemoresistance is an acquired phenomenon, in OCCC chemoresistance is an intrinsic feature. Hence, OCCC recurrent illness after surgery, and advanced illness stage at diagnosis, do not respond to the platinum-based conventional therapy; and this chemo-resistance is associated with a lower response rate to chemotherapy and a poor prognosis (Lopes et al., 2015). Patients who have little or no response to first-line therapy are also resistant to second-line therapy. Hence, in tumour recurrence and when diagnosed at an advanced illness stage, OCCC is an incurable disease (Lopes et al., 2015).

The aim of our present study was to investigate the biological effect of the five formamidinodoxorubicins in ES-2 human ovarian clear cells carcinoma, and compare it with the effect obtained previously for SKOV-3 human ovarian adenocarcinoma cell line, to answer a question whether, and to what extent, a histological cell type is a possible determinant of sensitivity to tested anthracyclines.

The first stage of this study focused on the evaluation of intracellular accumulation of DOX and analogues, and also on the estimation of drugs uptake. The cytotoxic effect of all tested derivatives as well as DOX depends on the drug accumulation in the cell. The drug diffusion through the cell membrane is one of the factors limiting the penetration rate into cells. The quantity of DOX, as well as all formamidine derivatives, taken up by cells within 60 min of incubation was similar within the tested ES-2 and SKOV-3 cell lines.

No important differences in intracellular distribution were observed. The situation was similar for the initial influx and efflux, but also influx and efflux rate constants. This may be explained by the lack of substantial differences in size between the DOX and derivatives. Assessment of other values obtained for both cell lines suggests that drug uptake and kinetic drugs parameters for ES-2 cell line and SKOV-3 cell line are analogous.

The next step of this study was to assess the cytotoxicity of formamidine derivatives in ES-2 cells, and their ability to induce apoptosis. In the SKOV-3 cells the presence

of heteroatom in the six-membered rings improves the compound activity. The most sufficient analogues were DOX-F MOR and DOX-F PAZ (Marczak et al., 2014). Comparison of IC_{50} values obtained for both cell lines suggests that ES-2 cell line is more sensitive to a reference drug and DOX-F PIP than SKOV-3 cells. The opposite situations were denoted after both cell lines exposure to DOX-F MOR and DOX-F PAZ. Additionally, these parameters were almost equal on the ES-2 and SKOV-3 cell lines after incubation with DOX-F MOR and DOX-F PAZ. It suggests that the derivative with 6-membered ring with additional heteroatoms has similar cytotoxicity for each tested ovarian cancer cell line. The least potent analogue in ES-2 cell line was DOX-F HEX, which supports results obtained previously for SKOV-3 cells. The values for this derivative in both cell lines were similar.

Apoptosis plays an important role in the cytotoxicity of many anticancer drugs (Shan et al., 2012; Sun et al., 2015). There are two routes of apoptosis, extrinsic and intrinsic. Extrinsic is associated with the outer membrane receptors. In the intrinsic pathway of apoptosis, mitochondria are involved (Goldar et al., 2015). Numerous changes in cell morphology, typical either of apoptosis or necrosis, were detected after examination of cells stained simultaneously with the two dyes: DNA-intercalating Hoechst 33258, which are able to penetrate the intact cell membrane and propidium iodide (not permeant to live cells, commonly used to detect necrotic cells in a population). Basing on the obtained results under these experimental conditions, it has been shown that formamidine derivatives of DOX can induce both apoptosis and necrosis in ES-2 cancer cells. Compared to SKOV-3 cells, ES-2 displayed a lower level of apoptosis. The dominance of apoptotic cells fraction was noted after SKOV-3 cells incubation with derivatives. Necrotic cell fraction was less than 5%. In ES-2 depending on the incubation time cells the necrotic and apoptotic cells fractions were similar or necrosis dominated over apoptotic cell fractions. This may be caused by the clear cell adenocarcinoma being less responsive to chemotherapy than the ovarian serous adenocarcinoma (Tamada et al., 2007).

Partial confirmation of apoptosis and necrosis induction was also obtained by Annexin V/PI staining. Annexin V, a protein which is able to bind to phosphatidylserine exposed on the surface of the cells. Phosphatidylserine (PS) externalization, which is a consequence of the breakdown in plasma membrane lipid asymmetry, is regarded as an early event in apoptosis (Aubry et al., 1999). Transfer of PS into the outer monolayer is a signal for the cell elimination by phagocytic macrophages (Zwaal et al., 2005). Evidence for the necrotic type of death induced by the anthracycline antibiotics in tumour cells has been provided. The increased fraction of necrotic cells, extended with the length of incubation with the drugs time, might suggest that some amount of the apoptotic cells are able to switch to necrotic mode of death (Koceva-Chyla et al., 2005). It could be another reason why, compared to SKOV-3 cells, ES-2 displayed a higher percentage of necrotic cells.

After the confirmation the DOX and derivatives ability to induce apoptosis the third step of this study focused on determining the pathway of apoptosis (internal or external). For this purpose the activity of activity of 3 types of caspases (caspase-8-involved in the extrinsic pathway, caspase-9, which is part of the intrinsic pathway of apoptosis and caspase 3 - executive enzyme involved in both pathways) was noted. Moreover, the effects of tested compounds on mitochondria (organelles involved in the intrinsic pathway) were investigated by assessing changes in the mitochondrial membrane potential. The extrinsic apoptotic pathway is characterized by activation and cleavage of procaspase-8. The intrinsic apoptotic pathway is mediated by caspase-9 activation, resulting in the release of mitochondrial cytochrome c and the formation of the apoptosome complex (Xue et al., 2014). It was previously proven, that DOX generates apoptosis through caspase-3-dependent process in A2780 human ovarian cancer cells, as well as in the other cancer cell lines (Bellarosa et al., 2001; Gajek et al., 2015). In SKOV-3 cells the strong increase in the activity of caspase-3 was noted after 72h of incubation and for this time the changes were statistically significant not only in comparison to the control but also to the probes incubated with DOX (Marczak et al., 2014). In both cell lines the use of the caspase-3 inhibitor ZVAD-fmk inhibited apoptosis induced by either DOX or derivatives, which suggested a participation of caspase-3 in this process. In SKOV-3 and ES-2 cell lines the increased activity of caspase-8 and -9 was observed. It should be noted that after exposure to DOX-F PYR, DOX-F PIP, DOX-F PAZ and DOX-F HEX, the caspase-8 activity was considerably higher than caspase-9, which suggests a dominance of the extrinsic apoptotic pathway. Only in the case of DOX-F MOR in SKOV-3 cell line the similar activity of both caspases was demonstrated. Thus obtained results supported the mitochondrial membrane potential changes. After the 48h exposure SKOV-3 cells to tested compounds, the maximal decrease of the JC-1 fluorescence ratio was observed for DOX-F MOR (about 25%). It might indicate that in the SKOV-3 cells DOX-F MOR induces both pathways with similar intensification.

Ovarian clear cells cancer is currently managed in the same manner as other EOCs. However, the mechanisms of drug resistance, tumour development, progression and poor clinical outcomes in CCC remain unknown. Molecular studies suggested that CCC is biologically distinct from the other EOC subtypes, so there is the need for a new strategy for treating CCC (Lee et al., 2011). The first step in developing tools to improve CCC therapy is to recognize that CCC represents a distinct from other EOC clinical problem. Results from the current study suggest that all tested formamidine derivatives of DOX were able to induce caspase-dependent apoptosis in both tested human ovarian cancer cells subtypes SKOV-3 and ES-2. The cancer cells type influenced the cell death type dominance, but not the intracellular accumulation and cellular uptake of DOX and derivatives. In each cell line all of the investigated derivatives were considerably more cytotoxic than DOX. Obtained results suggest that formamidine derivatives of DOX may be a promising candidate for the prospective chemotherapeutic agents, independently of type of ovarian cancer.

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