# Formamidinodoxorubicins Are more Potent than Doxorubicin as Apoptosis Inducers in Human Breast Cancer Cells

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**Abstract.** *Background/Aim: The ability of five formamidinodoxorubicins to induce apoptosis of MCF-7 breast cancer cells was tested. All these compounds were modified at C-3' and contain a formamidine group (–N=CH–NRR), with the rest of the cyclic secondary amine (HNRR) of a gradually increasing ring size. Materials and Methods: Cytotoxicity was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. To analyze apoptosis, double staining using fluorescence probes Hoechst 33258/propidium iodide (PI) and annexin V- Fluorescein isothiocyanate/PI was carried-out. Additionally, the TdT-mediated dUTP nick-end labelling test and activity of caspase 3 were determined. Results: The four tested derivatives displayed a significant increase in antiproliferative activity in comparison to doxorubicin. All of the tested derivatives induced caspase-dependent apoptosis of MCF-7 cells. Conclusion: DOX-F MOR and DOX-F PAZ analogs are more potent apoptosis inducers than doxorubicin.*

Breast cancer is the most common malignancy among women worldwide. In developed countries, the incidence of this kind of cancer ranges between 25 to 30% of all cancer among women (1). Anthracyclines are a group of antibiotics successfully used in chemotherapy in the treatment of many types of tumors, including breast cancer.

Doxorubicin was one of the first anthracycline antibiotics introduced into cancer therapy, and has been used in numerous cancer chemotherapies for over 60 years (2). In spite of its high therapeutic effectiveness, the clinical use of doxorubicin, however, is limited because of its acute toxicity. The most serious side-effect is cardiotoxicity, which can finally lead to severe and irreversible cardiomyopathy (3). For this reason, anthracyclines are still very often studied. The presence of the free amino group in the daunosamine moiety of anthracyclines allows many chemical modifications. Numerous studies have shown that derivatives with modifications in sugar moiety demonstrate the most promising results (4-6).

Therefore, the aim of the present study was to compare the efficacy of doxorubicin and its five derivatives (compounds 1-5 Figure 1) against MCF-7 breast cancer cells. All tested derivatives at the position C-3' of daunosamine moiety contain a formamidine group (-N=CH-N<) with pyrrolidine (DOX-F PYR), piperidine (DOX-F PIP), morpholine (DOX-F MOR), N-methylpiperazine (DOX-F PAZ) or hexamethyleneimine (DOX-F HEX) ring. Attempts were made to evaluate the anti-proliferative activity of all the tested derivatives and to assess their ability to induce apoptosis in breast cancer cells. Analyses were performed at three levels: the cell membrane, nuclear morphology and DNA breaks.

## Materials and Methods

*Drugs and cell culture*. Doxorubicin as well as compounds 1-5 were synthetized at the Institute of Biotechnology and Antibiotics (Warsaw, Poland) through the reaction of doxorubicin chloride with the dimethyl acetal of the respective amine in methanol (7). MCF-7 (human breast adenocarcinoma) cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were grown as a monolayer with a RPMI growth medium supplemented with 10% fetal bovine serum in standard conditions at 37˚C, with an atmosphere of 5% carbon dioxide and 95% air, and with 100% humidity.

*3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay*. The anti-proliferative activity was tested with MTT, as described previously and the half-maximal inhibitory concentration ($IC_{50}$) value determined (8).

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Table I. *The half-maximal inhibitory concentration ($IC_{50}$) values following exposure to doxorubicin (DOX) and compounds 1-5 for human breast cancer cell line MCF-7. *Significantly different from DOX at p<0.0001. The values are the mean±SD of five independent experiments.*

| Compound | DOX | DOX-F PYR | DOX-F PIP | DOX-F MOR | DOX-F PAZ | DOX-F HEX |
|---|---|---|---|---|---|---|
| $IC_{50}$ [nM] | 36.6±6.0 | 18.0±4.0* | 26.0±5.0* | 18.9±3.0* | 21.1±3.0* | 32.1±6.0 |

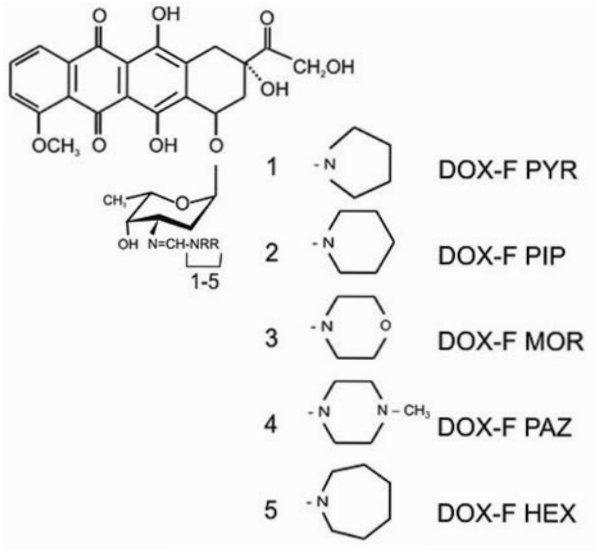


Figure 1. *Chemical structures of new derivatives of doxorubicin tested in the study.*

*Drug treatment*. After analysis of the $IC_{50}$, one concentration (15 nM) was used for the subsequent experiments. The cells were incubated with the drugs at this concentration for different periods of time (4, 48, 72 h). At selected time points, the cells were evaluated for analysis.

*Double staining with Hoechst 33258-propidium iodide (PI)*. Morphological changes typical of apoptosis and necrosis in cells treated with the tested compounds were evaluated by using fluorescence microscopy, according to the methodology described previously (8).

*Analysis of phosphatidylserine (PS) externalization*. PS externalization was determined by flow cytometry using the annexin V-Fluorescein isothiocyanate FITC/PI double staining according to the method described previously (8).

*Measurement of caspase-3 activity*. The activity of caspase-3 was evaluated using fluorimetric assay kits in accordance with the protocol supplied by the manufacturer (Invitrogen, USA) according to the procedure described previously (9). The basis for the assay was rhodamine 110 *bis*-(*N*-CBZ-L-aspartyl-Lglutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110).

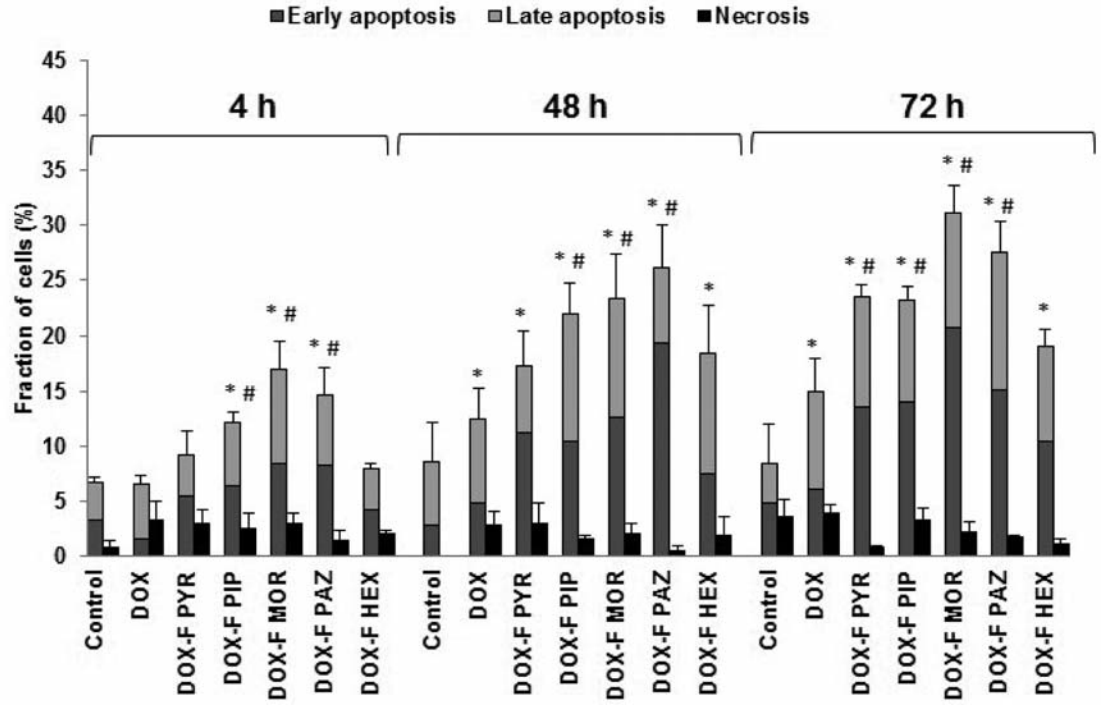


Figure 2. *The fraction of early apoptotic, late apoptotic and necrotic cells after 4, 48 and 72 h exposure to doxorubicin (DOX) and compounds 1-5 obtained after double staining with Hoechst 33258 and propidium iodide. Statistically significant difference in comparison to *control cells, #to the probes with doxorubicin at p<0.05. The values are the mean±SD of five independent experiments.*

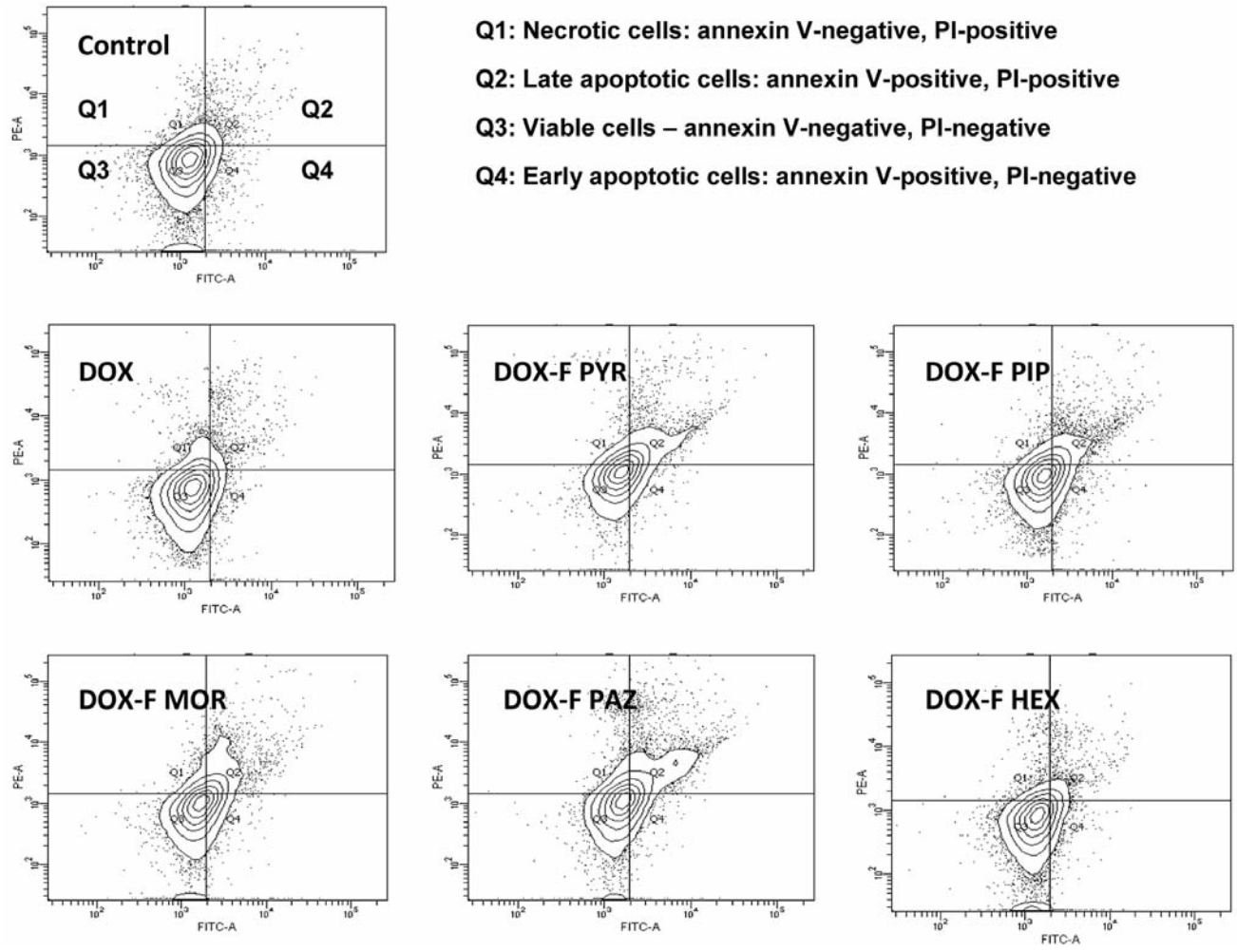


Figure 3. *Representative cytograms after double staining with Annexin V Fluorescein isothiocyanate and propidium iodide (PI) of MCF-7 cell line after incubation with doxorubicin (DOX) and compounds 1-5 for 72 h.*

*TdT-mediated dUTP nick-end labelling (TUNEL) assay*. The TUNEL assay was applied in order to assess DNA damage during apoptosis. The measurements were made according to the procedure described previously (10).

*Statistical analysis*. The data are presented as the 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 less than 0.05 was considered significant.

## Results

*MTT assay*. Table I shows the $IC_{50}$ value calculated for doxorubicin and compounds 1-5. The results obtained show that of the five tested derivatives, four were markedly more cytotoxic than doxorubicin. The least potent derivative was DOX -F HEX ($IC_{50}$=32.10±6.00 nM).

*Analysis of type of cell death by fluorescence microscopy: double staining with Hoechst 33258-PI*. The quantitative data presented in Figure 2 show that doxorubicin as well as all tested derivatives induced both apoptosis and necrosis, but necrosis occurred in only a small percentage of cells. After a short incubation time (4 h) with the derivatives DOX-F PIP, DOX-F MOR and DOX-F PAZ, a relatively large fraction of apoptotic cells (presented as a sum of early and late apoptosis) was observed. The changes were statistically significant in comparison to those for doxorubicin. DOX-F HEX was the least effective derivative. After 72 h of incubation, the highest percentage of apoptotic cells was obtained for DOX-F MOR (31.08%), but values for DOX-F PYR, DOX-F PIP, DOX-F PAZ were significantly higher than for doxorubicin. The number of apoptotic cells was two-fold greater for DOX-F MOR than for DOX. The least potent derivative was DOX-F HEX (apoptosis: 19.04%).

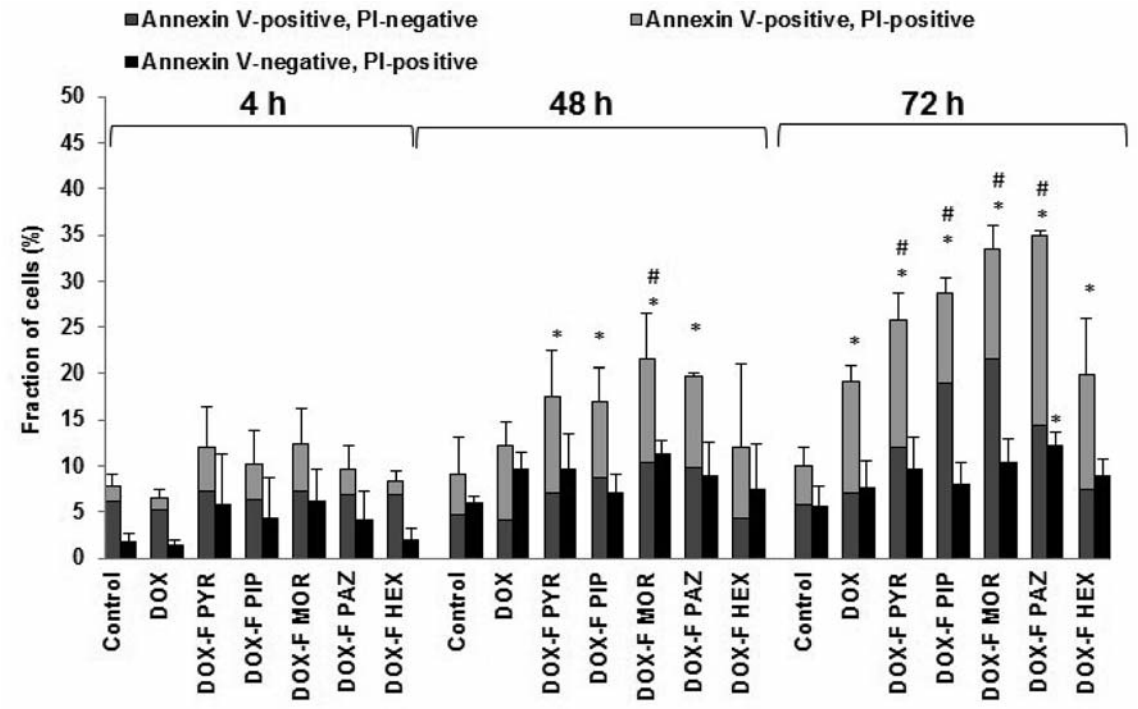


Figure 4. *Flow cytometric analysis of apoptosis and necrosis in MCF-7 cells (double staining with annexin V Fluorescein isothiocyanate and propidium iodide (PI). Statistically significant difference in comparison to *control cells, #to the samples with doxorubicin (DOX). p<0.05 The values are the mean±SD of five independent experiments.*

*Analysis of PS exposure*. The cytometric data (presented as the percentage of counted cells) confirmed that new analogs, as well as doxorubicin, are able to induce apoptosis. Figure 3 presents the cytograms and Figure 4 the quantitative data of control cells and cells exposed to doxorubicin and derivatives 1-5. In the drug-treated cells, the most remarkable increase in green fluorescence (annexin-V-FITC), denoting the PS externalization, was observed after 72 h of exposure to DOX-F PAZ (34.93%). A high fraction of apoptotic cells was also observed after exposure to DOX-F PYR, DOX-F PIP and DOX-F MOR (25.68%, 27.78% and 33.60%, respectively). For all incubation times, the percentage of apoptotic cells obtained on treatment with doxorubicin was lower than after exposure to derivatives DOX-F PYR, DOX-F PIP, DOX-F MOR and DOX-F PAZ.

*Changes in the activity of caspase-3 after incubation with doxorubicin and its derivatives*. All tested compounds caused an increase in the activity of this enzyme as early as after 4 h of incubation time. A strong increase was noted after 72 h of incubation and for this time period, the changes were statistically significant not only in comparison to the control but also when compared to a cells incubated with doxorubicin.

*TUNEL assay*. This assay was used to determine whether apoptotic changes induced by formamidinodoxorubicins and the reference drug doxorubicin are associated with DNA fragmentation. Quantitative data obtained from the flow cytometric analysis are presented in Figure 6. Treatment with the tested formamidinodoxorubicins generated a higher level of TUNEL-positive cells in comparison to untreated (control) cells, as well as doxorubicin-treated cultures. The maximum level of apoptotic cells with DNA strand breaks after 72 h of treatment with the tested drugs, was observed for DOX-F PYR (68.59±5.86%). A slightly lower fraction of MCF-7 TUNEL-positive cells was also noted after treatment with DOX-F PIP, DOX-F MOR and DOX-F PAZ. DOX-F HEX was the least potent in inducing DNA damage, with an effect comparable with that observed for doxorubicin.

## Discussion

The obtained results confirm the beneficial anticancer properties of the tested analogs measured by their ability to induce apoptosis. These data also reveal the structure-activity relationship of the tested derivatives. Following the MTT assay results, the most cytotoxic were analogs with a five-

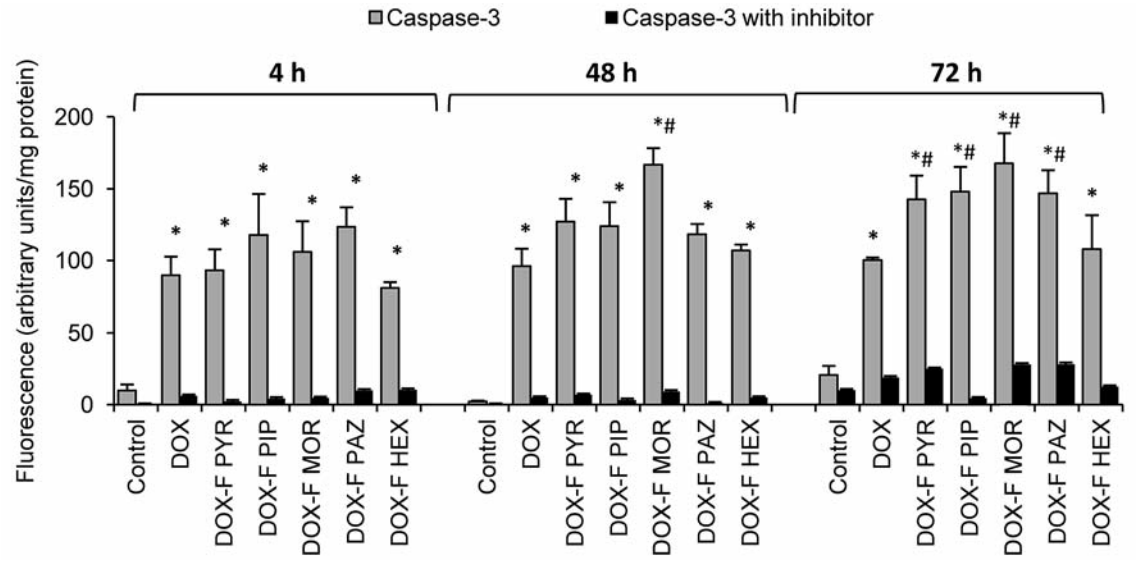


Figure 5. *Caspase-3 activity in MCF-7 cells after incubation with doxorubicin (DOX) and compounds 1-5. Statistically significant difference in comparison to *control cells and #probes with doxorubicin at p<0.05. The values are the mean±SD of five independent experiments.*

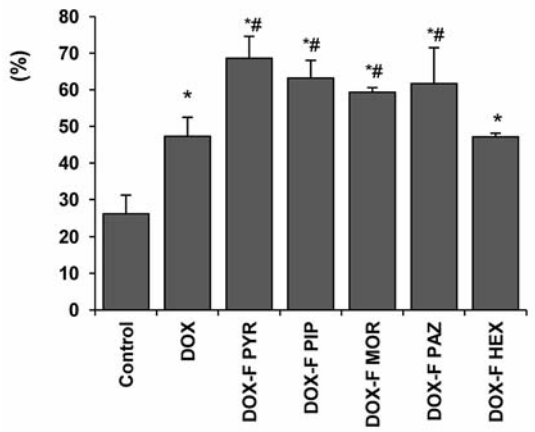


Figure 6. *The effect of doxorubicin (DOX) and compounds 1-5 on the level of TUNEL-positive cells after 72 h exposure to drugs. Statistically significant difference in comparison to *control cells and #probes with DOX at p<0.05. The values are the mean±SD of three independent experiments.*

membered ring (DOX-F PYR) and six-membered ring containing one heteroatom (DOX-F MOR). High antiproliferative activity was also displayed by derivatives containing a six-membered ring with two heteroatoms (DOX-F PAZ) and six-membered rings without the heteroatom (DOX-F PIP). The DOX-F HEX analog containing a seven-membered ring without heteroatom had the lowest activity. It has been demonstrated that the antiproliferative activities of DOX-F PYR, DOX-F PIP and DOX-F MOR were higher in comparison to that of doxorubicin (4). Moreover, almost all derivatives are characterized by a reduced cardiotoxicity (4) (in comparison to doxorubicin), and are able to completely overcome the drug resistance barrier *in vitro* (6). It can be assumed that the presented results would enable one of the analogs to be used to in therapy instead of doxorubicin.

This thesis is further confirmed by the fact that these compounds predominantly induce apoptosis, and necrosis slightly. It is well-known that cells can die by one of two major mechanisms: necrosis or apoptosis. Necrosis is cell death caused by external damage, usually mediated *via* the destruction of the plasma membrane. The necrotic cell exhibits a specific morphology (swollen, enlarged cells) and the plasma membrane undergoes lysis. This release of the internal components attracts inflammatory cells, leading to the tissue destruction characteristic of inflammation. These phenomena are detrimental to the organism. Apoptosis, also called programmed cell death, is favorable for the elimination of damaged cells (11, 12). We herein showed that all the tested derivatives mainly induce apoptosis. Typical morphological hallmarks of programmed cell death, such as chromatin condensation, apoptotic body formation and cell shrinkage, were observed (data not shown). The fraction of necrotic cells after incubation with the tested derivatives remained at a low level. These results were also confirmed by double staining with Hoechst 33258/PI and Annexin-V-FITC/PI and the determination of DNA breaks in the TUNEL assay. We also observed caspase-3 activation, which is a typical event in the apoptotic cascade. All examined compounds induced the activation of executive caspase-3 at a very high level in a time-dependent manner.

The results obtained in this study, as well as the previously published data, indicate that the modifications applied in the daunosamine moiety are quite promising, providing a chance to introduce new analogs into chemotherapy. The tested derivatives demonstrate much better anticancer properties in comparison to doxorubicin. Assuming that a measure of the efficacy of drugs is their ability to induce apoptosis, it may be considered that the new compounds will be effective in the treatment of breast cancer. These new compounds induced apoptosis more intensely than did doxorubicin; moreover, apoptosis was induced much earlier using the analogs than by using doxorubicin.

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