

***In Vitro* Induction of Apoptosis and Necrosis by New Derivatives of Daunorubicin**

MARTA STOJAK¹, LIDIA MAZUR¹, MAŁGORZATA OPYDO-CHANEK¹,
MAŁGORZATA ŁUKAWSKA² and IRENA OSZCZAPOWICZ²

¹Department of Experimental Hematology, Jagiellonian University, Cracow, Poland;

²Department of Modified Antibiotics, Institute of Biotechnology and Antibiotics, Warsaw, Poland

Abstract. *Background/Aim:* The comparative effects of daunorubicin, and its new formamidine derivatives containing either a morpholine moiety (DAUFmor) or a hexamethylenimine moiety (DAUFhex) in the amidine group, on induction of programmed cell death were determined. *Materials and Methods:* The experiments were performed on human acute lymphoblastic leukemia MOLT-4 cells and human acute myeloblastic leukemia ML-1 cells. The research was conducted using the flow cytometry annexin V–fluorescein (FITC)/propidium iodide (PI) method and tetramethylrhodamine ethyl ester (TMRE) assay. *Results:* The various patterns of temporary changes of early apoptotic cells, late apoptotic and necrotic cells, and in the frequency of the acute leukemia cells with high values of mitochondrial membrane potential (MMP) were found. Phosphatidylserine externalization, plasma membrane disruption, and changes in MMP occurring in the leukemia cells were dependent on the agent tested, its concentration, the time intervals after daunorubicin, DAUFmor, and DAUFhex application, and on the leukemia cell line used. *Conclusion:* The structural modifications of daunorubicin producing two new analogs, DAUFmor and DAUFhex, induced the different responses of MOLT-4 and ML-1 cells to triggering of programmed death.

Daunorubicin, an anthracycline antibiotic, is widely used in anticancer therapy (1, 2). Nevertheless, its clinical efficacy is limited by cardiotoxicity and cellular drug resistance (3). In the search for new derivatives of daunorubicin with advantageous biological properties, a series of analogs have been synthesized (4, 5). The transformation of the amino

group at position 3' of the daunosamine moiety into a formamidino group (-N=CH-N<) (Figure 1) resulted in a series of new derivatives with different anticancer activity compared to that of daunorubicin (6, 7). It is accepted that anticancer potential is dependent on the effective induction of programmed cell death in cancer cells. However, to our knowledge, there are no published data on induction of programmed cell death by formamidinodaunorubicins containing either a morpholine moiety (DAUFmor) or hexamethylenimine moiety (DAUFhex) in the amidine group.

Apoptosis and necrosis are two major types of programmed cell death (8-10). Changes in the cell membrane are one of the events occurring during apoptotic and necrotic cell death. It is known that loss of phospholipid asymmetry leading to exposure of phosphatidylserine on the outside of the plasma membrane is an early event of apoptosis (11). Moreover, the integrity of plasma membrane is compromised during a late stage of apoptosis and throughout necrosis. Combination staining with fluorescein (FITC)-conjugated annexin V and propidium iodide (PI) allows for the identification of early apoptotic cells, late apoptotic and necrotic cells (12).

Mitochondria play a pivotal role in the regulation of programmed cell death (13, 14). The mitochondrial membrane potential (MMP) is a key indicator of cell viability and death. Changes in MMP directly reflect on cell transition from life to death (15, 16). The MMP plays an important role in maintaining the physiological function of the respiratory chain generating ATP (13). The ATP level is a main determinant of whether apoptotic or necrotic cell death occurs (17-19). Thus, evaluation of the MMP is of critical importance for the assessment of apoptosis and necrosis.

The aim of the present study was to determine and compare the *in vitro* influence of the parent compound daunorubicin and its two analogs DAUFmor, and DAUFhex on human acute lymphoblastic leukemia MOLT-4 cells and human acute myeloblastic leukemia ML-1 cells. The effects of these compounds on triggering phosphatidylserine externalization, plasma membrane disruption, and changes in MMP in the leukemia cells were analyzed.

Correspondence to: Lidia Mazur, Ph.D., D.Sc., Department of Experimental Hematology, Jagiellonian University, Gronostajowa 9, 30-387 Cracow, Poland. Tel: +48 126645237, Fax: +48 126645101, e-mail: lidia.mazur@uj.edu.pl

Key Words: Daunorubicin derivatives, human acute leukemia cells, apoptosis, necrosis, mitochondrial membrane potential.

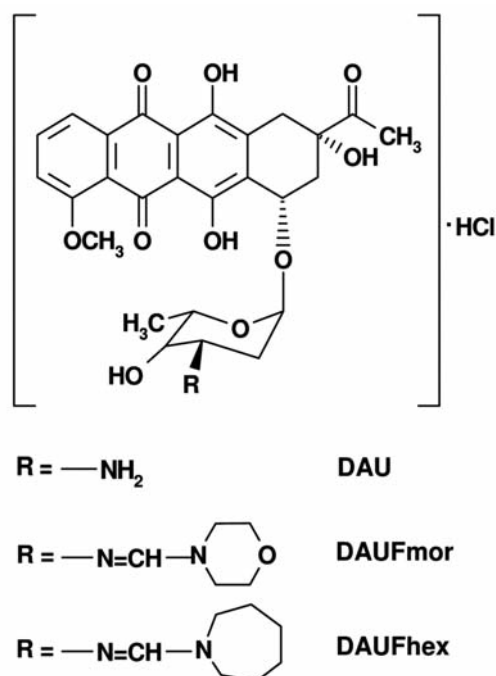


Figure 1. Chemical structures of daunorubicin (DAU) and its new derivatives containing either a morpholine moiety (DAUFmor) or a hexamethyleneimine moiety (DAUFhex) in the amidine group.

Materials and Methods

Cells. Human acute lymphoblastic leukemia MOLT-4 cells and human acute myeloblastic leukemia ML-1 cells were obtained from the European Collection of Cell Cultures (European Collection of Cell Cultures, Salisbury, Wiltshire, UK). MOLT-4 and ML-1 cells were cultured in RPMI 1640 medium (Gibco BRL Life Technologies, Warsaw, Poland) supplemented with 10% fetal calf serum (Gibco BRL Life Technologies), 2 mM L-glutamine (Sigma Aldrich, Poznań, Poland), and antibiotic antimycotic solution (Sigma Aldrich) containing 20 units of penicillin, 20 µg streptomycin and 0.05 µg amphotericin B. Every third day, the acute leukemia cells were passaged. The cells were grown at 37°C in an atmosphere of 5% CO₂ in air (HERAcell incubator; KendroLab, Warsaw, Poland).

Chemicals. Daunorubicin and its two analogs, DAUFmor and DAUFhex, were synthesized (20, 21) at the Institute of Biotechnology and Antibiotics (Warsaw, Poland). The anthracycline compounds were dissolved in "aqua pro injectione" (Polpharma, Starogard Gdański, Poland). Stock solutions of these agents at a concentration of 0.5 mM were stored in the dark at -20°C. All solutions were prepared directly before treatment of the MOLT-4 and ML-1 cells.

Anthracycline doses and cell treatment. After a dilution of the cell suspension to a density of 15×10⁴ cells/ml medium, MOLT-4 and ML-1 cells were treated with anthracycline agents. MOLT-4 cells were exposed to the action of daunorubicin, DAUFmor, and DAUFhex, at concentrations of 25, 50 and 75 nM, and ML-1 cells

were treated with these agents, at the concentrations of 75 nM, 150 nM, and 225 nM (7). The control consisted of untreated MOLT-4 and ML-1 cells.

Analyses of leukemia cells after the anthracycline application. Temporary changes occurring in MOLT-4 and ML-1 cells were recorded at 24 h, 48 h, and 72 h after exposure to anthracyclines. At these three time points, plasma membrane diversity and impairment, and MMP changes were analyzed.

Flow cytometric annexin V-FITC/PI assay. The cell surface exposure of phosphatidylserine and the plasma membrane impairment of cells were assessed using Annexin V-FITC Apoptosis Detection Kit (Calbiochem Merck Millipore, Warsaw, Poland). By staining cells with a combination annexin V-FITC and PI, it was possible to identify non-apoptotic live cells (annexin V-FITC-negative/PI-negative), early apoptotic cells (annexin V-FITC-positive/PI-negative), and late apoptotic and necrotic cells (annexin V-FITC-positive/PI-positive) (12). The procedure of dual staining of cells with annexin V-FITC and PI was performed according to the manufacturer's instruction (Calbiochem Merck Millipore). Briefly, suspension of treated/control leukemia cells, containing 5×10⁵ cells, were washed with PBS (BioMed, Lublin, Poland) and resuspended in 0.5 ml cold binding buffer. Then 1.25 µl of annexin V-FITC was added and the cells were incubated in the dark for 15 min at room temperature. Following incubation, the cells were centrifuged at 100 ×g for 5 min and the supernatant was removed. The cell pellet was resuspended in 0.5 ml cold binding buffer, and 10 µl of the 30 µg/ml PI solution was added. Cell samples were placed on ice, away from light, and FITC and PI fluorescence was immediately measured using FACSCalibur flow cytometer (Becton Dickinson, New Jersey, USA). Data were analyzed using CellQuest Pro software (Becton Dickinson). The frequency of live cells, early apoptotic cells, late apoptotic and necrotic cells was determined.

Analysis of mitochondrial membrane potential. MMP was analyzed by means of the lipophilic cationic dye tetramethylrhodamine ethyl ester perchlorate (TMRE; Sigma Aldrich). TMRE will accumulate within mitochondria in inverse proportion to the MMP according to the Nernst equation. More greatly polarized mitochondria accumulate more cationic dye, whereas depolarized mitochondria accumulate less dye (22).

A TMRE stock solution was prepared at a concentration of 10 mM in dimethyl sulfoxide (Sigma Aldrich) and stored at -20°C. The final concentration of TMRE staining solution used was 100 nM. The suspension of treated/control leukemia cells was washed twice in Hank's balanced salt solution (HBSS; Gibco Life Technologies), resuspended in 100 µl of 100 nM TMRE, and incubated for 20 min at 37°C in the dark. The cells were then washed twice with HBSS, and finally resuspended in a total volume of 0.5 ml HBSS. TMRE fluorescence was detected by FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using the CellQuest Pro software (Becton Dickinson).

Statistical evaluation. All experiments were repeated three times with duplicate or triplicate determinations. All the data are presented as the mean values±standard deviation. Statistical analyses were performed using STATISTICA 10 (StatSoft, Cracow, Poland). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) multiple range test. A difference with *p*<0.05 was considered statistically significant.

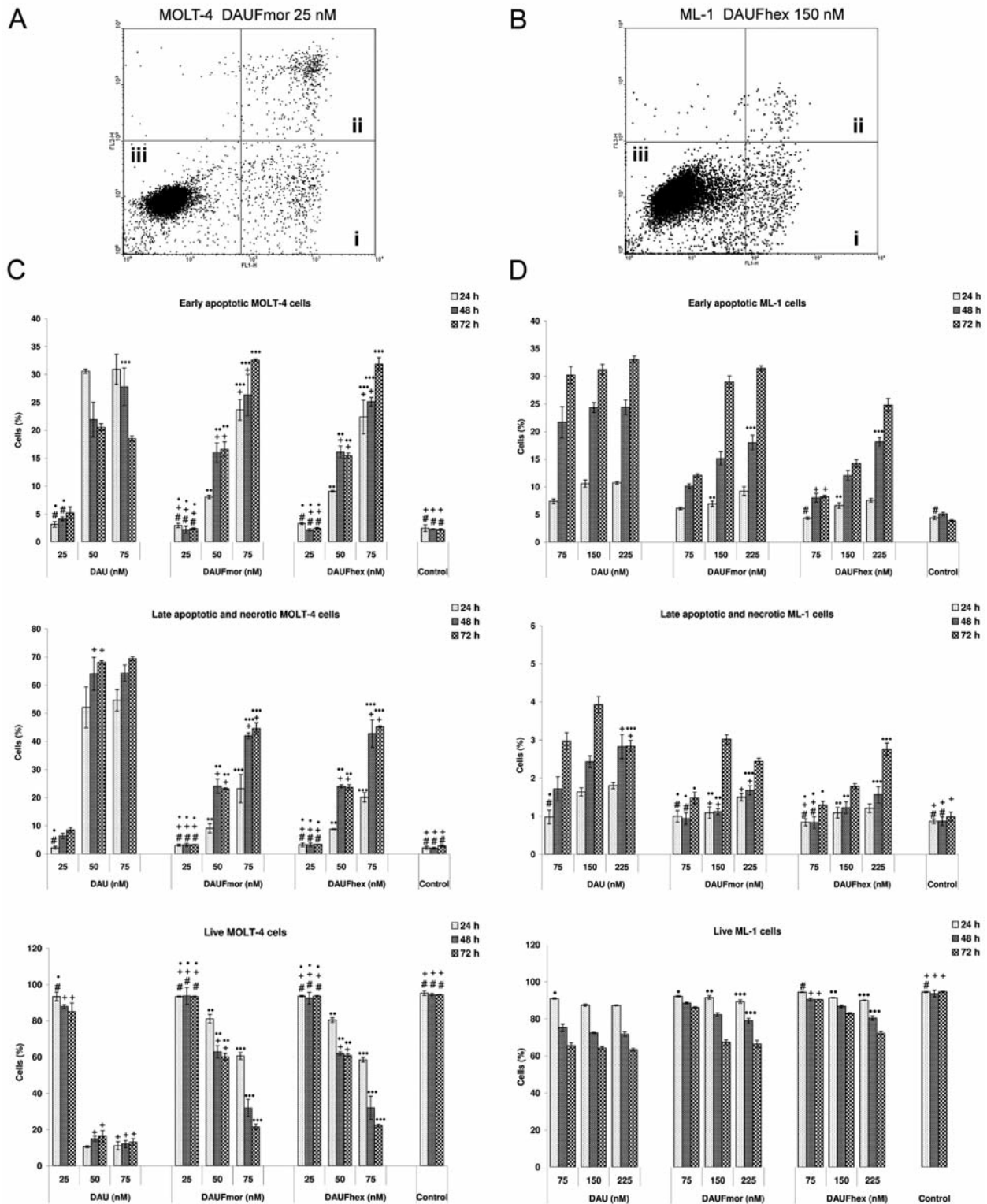


Figure 2. Effects of daunorubicin (DAU), and its new derivatives containing either a morpholine moiety (DAUFmor) or a hexamethyleneimine moiety (DAUFhex) in the amidine group, on induction of apoptosis and necrosis in MOLT-4 and ML-1 cells. Representative dot plots for MOLT-4 cells (A) and ML-1 cells (B): i - apoptotic cells, ii - late apoptotic and necrotic cells, iii - live cells. The frequency of early apoptotic, late apoptotic and necrotic, and live MOLT-4 (C) and ML-1 cells (D). Values not significantly different at $p < 0.05$ according to the Tukey's multiple range test: ●, ●●, ●●● between the groups of leukemia cells treated with the anthracycline agents; # compared to controls; + between the time points.

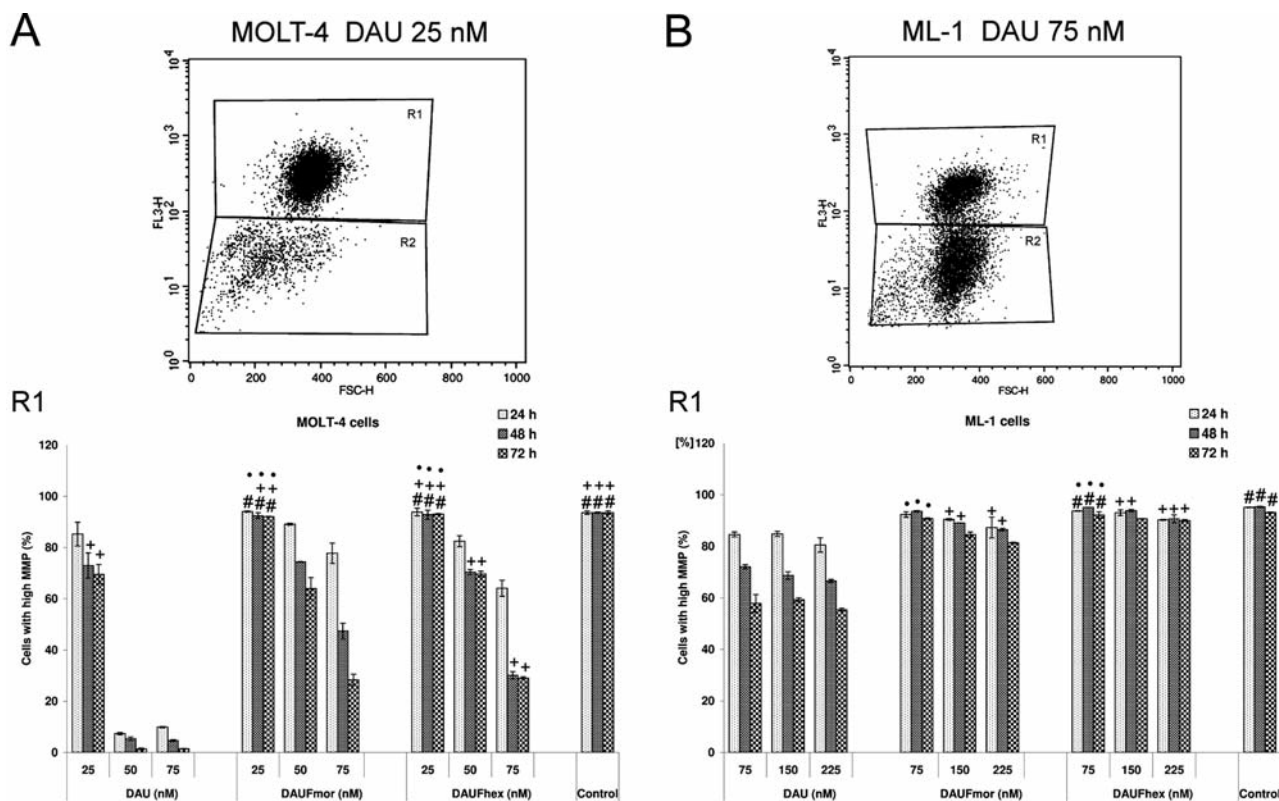


Figure 3. Effects of daunorubicin (DAU), and its new derivatives containing either a morpholine moiety (DAUFmor) or a hexamethyleneimine moiety (DAUFhex) in the amidine group, on the mitochondrial membrane potential (MMP). Representative dot plots showing MOLT-4 cells (A) and ML-1 cells (B) with the high (R1) and low (R2) MMP values. The frequency of MOLT-4 (A) and ML-1 cells (B) with the high MMP values. Values not significantly different at $p < 0.05$ according to the Tukey's multiple range test: ●, ●●, ●●● between the groups of leukemia cells treated with the anthracycline agents; # compared to controls; + between the time points.

Results

The influence of daunorubicin and its two analogs DAUFmor and DAUFhex on alterations in the frequency of MOLT-4 (Figure 2A, 2C) and ML-1 (Figure 2B, 2D) cells undergoing early apoptosis, late apoptosis and necrosis (Figure 2) and the frequency of the leukemia MOLT-4 (Figure 3A) and ML-1 (Figure 3B) cells expressing the high values of MMP (Figure 3) were determined.

Daunorubicin induced apoptotic and necrotic death of human leukemia cells, and affected their MMP to a higher degree than did its analogs DAUFmor and DAUFhex. Moreover, among the daunorubicin derivatives, DAUFmor appeared to be more active in cell death induction and reduction of MMP in ML-1 cells than did DAUFhex. Differences between the effects of DAUFmor and DAUFhex on apoptosis and necrosis, and the loss of MMP triggered in MOLT-4 cells were not found.

MOLT-4 cells were more sensitive than ML-1 cells to triggering programmed cell death. A greater time- and

concentration-dependent decrease of the frequency of cells with high MMP was observed in MOLT-4 cells than ML-1 cells. Throughout the 72 h period after agent application, a distinctly higher frequency of late apoptotic and necrotic MOLT-4 cells was found than ML-1 cells.

Taking into consideration the results obtained, we can generally state that the patterns of changes in early apoptosis, late apoptosis and necrosis, and in the change of MMP depended on the agent tested, its concentration, the time intervals of anthracycline application, and the leukemia cell line used.

Discussion

In the present study, the effects of daunorubicin, and two new analogs were determined. It was demonstrated that the parent anthracycline antibiotic DAU, and the two formamidinodaunorubicins, DAUFmor and DAUFhex, in varying degree caused phosphatidylserine externalization, plasma membrane impairment, and changes of MMP in

MOLT-4 and ML-1 cells. Moreover, it was found that mitochondria were involved in the leukemia cell response to the action of these agents. These are the first data showing that the formamidinodaunorubicin agents trigger programmed cell death of leukemia cells, and comparing their cell death-inducing potential.

The structural modifications of daunorubicin would seem to be responsible for the different antileukemic activities of the two new formamidinodaunorubicins. DAUFmor and DAUFhex differ in the size of the cyclic amine ring in the amidine group. DAUFmor contains a six-membered morpholine ring with an oxygen heteroatom in the γ -position in the formamidine group, while DAUFhex contains a seven-membered hexamethylenimine ring with a CH_2 group in the γ position. The DAUFmor molecule has an oxygen atom, whereas DAUFhex has two CH_2 groups. DAUFmor is capable of forming an additional hydrogen bond with proton-donating sites, due to the presence of the free electron pairs on the oxygen atom. These differences in the molecular structure of the two formamidinodaunorubicin agents, DAUFmor and DAUFhex, surely reflect their different biological properties (4-7).

It is accepted that programmed cell death induction is one mechanism of anticancer therapy (23). However, the relationship between the efficacy of chemotherapy and programmed cell death induction is still an open issue. An understanding of the precise mechanisms of triggering of programmed cell death by new anthracycline derivatives is essential for the development of novel anticancer therapeutic strategies (3).

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Acknowledgements

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