Effects of Structural Modifications of Daunorubicin on *In Vitro* Antileukemic Activity

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Abstract. *Background/Aim: In the search for new derivatives of anthracycline antibiotics, formamidinodaunorubicins containing in the amidine group either a morpholine moiety (DAUFmor) or a hexamethyleneimine moiety (DAUFhex) were synthesized. The biological effects of daunorubicin (DAU), DAUFmor and DAUFhex were compared. 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 spectrophotometric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay and the electronic Beckman-Coulter method. Results: Temporary changes in the leukemia cell viability, size and count were found. The antileukemic activities of the new DAU analogs were weaker than that of daunorubicin. MOLT-4 cells were more sensitive than ML-1 cells to the action of all agents. Among the formamidinodaunorubicins, DAUFmor appeared to be more active in ML-1 cells than DAUFhex, but there were not differences between the analyzed values in MOLT-4 cells. Conclusion: The structural modifications of daunorubicin were responsible for the different antileukemic potentials of the two formamidinodaunorubicins.*

Anthracyclines play a key role in the treatment of many neoplastic diseases. Structural modifications of currently approved anthracycline antibiotics are an important way to improve their anticancer activity. Many attempts to modify the structure of these antibiotics have been made (1-3). For this reason, amidinoanthracyclines which are new analogs of anthracyclines, were synthesized. In these compounds, the amino group ($-NH_2$) at the C-3 position of the daunosamine moiety was replaced by the formamidine system (–N=C–N<) containing the rest of the cyclic amines with gradually increasing ring size (4, 5).

Daunorubicin is the first anthracycline antibiotic which was approved for clinical use. This agent is still widely used in anticancer therapy. Daunorubicin has activity against acute lymphoblastic and myeloblastic leukemias, pediatric solid tumors and adult solid malignancies (2, 3). In the search for 'a better anthracycline', formamidinodaunorubicins (Figure 1) which contained either a morpholine moiety (DAUFmor) or a hexamethyleneimine moiety (DAUFhex) in the amidine group were synthesized (4, 5).

Cell viability, cell size and count are accepted as being important parameters in determining and characterizing antileukemic activity of various chemotherapeutic agents (6-9). Therefore, the present study was undertaken to compare the *in vitro* effects of daunorubicin, DAUFmor and DAUFhex on human acute lymphoblastic leukemia MOLT-4 cells and human acute myeloblastic leukemia ML-1 cells. Temporary changes in the viability, size and count of the acute leukemia cells exposed to the three anthracycline agents were analyzed.

Materials and Methods

Cells. Human acute lymphoblastic leukemia MOLT-4 cells and human acute myeloblastic leukemia ML-1 cells (European Collection of Cell Cultures, Salisbury, Wiltshire, UK) were maintained in RPMI-1640 medium (Gibco BRL Life Technologies, Warsaw, Poland) supplemented with 10% fetal calf serum (Gibco BRL Life Technologies, Warsaw, Poland), 2 mM L-glutamine (Sigma Aldrich, Poznań, Poland), and antibiotic antimycotic solution (AAS; Sigma Aldrich, Poznań, Poland). AAS contained 20 units of penicillin, 20 μg streptomycin and 0.05 μg amphotericin B. Every third day, the acute leukemia cells were passaged. The cells grew at 37˚C in an atmosphere of 5% CO_2 in air (HERAcell incubator; KendroLab, Warsaw, Poland).

Chemicals. The three anthracycline compounds (Figure 1), daunorubicin and two formamidinodaunorubicins DAUFmor and

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Key Words: Daunorubicin derivatives, human acute leukemia cells, cell viability, cell volume and count, *in vitro* antileukemic activity.

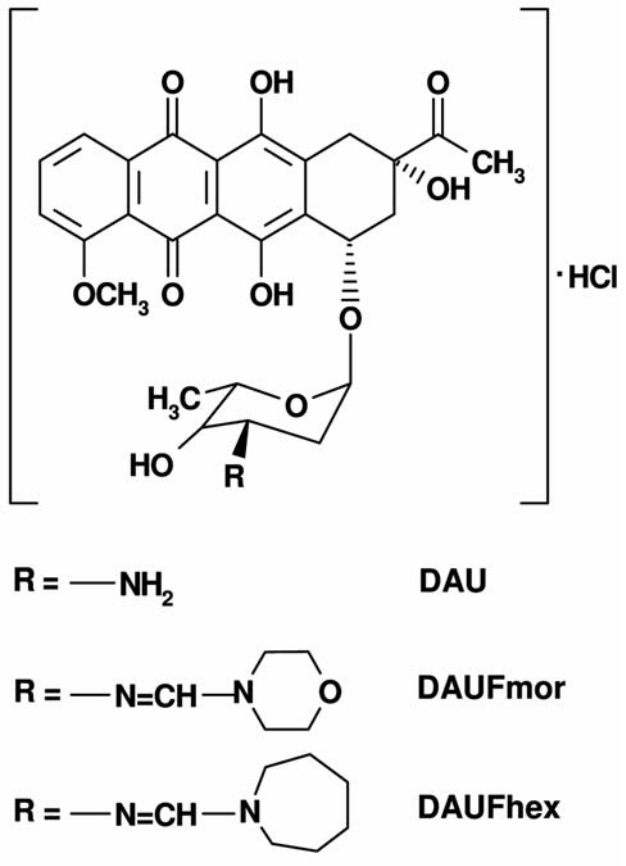

Figure 1. *Chemical structures of daunorubicin (DAU), DAUFmor, and DAUFhex.*

DAUFhex were synthesized at the Institute of Biotechnology and Antibiotics (Warsaw, Poland). The formamidinodaunorubicin compounds were obtained in the reaction of daunorubicin hydrochloride with acetals, derivatives of N-formylamines. The structure of the formamidinodaunorubicins were confirmed by their ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra. According to the high preasure liquid chromatography (HPLC) analysis, the purities of DAUFmor and DAUFhex were 98.8 and 99.2%, respectively (4). Daunorubicin, DAUFmor, and DAUFhex were dissolved in aqua pro injectione (Polpharma, Starogard Gdański, Poland). All solutions were freshly-prepared directly before treatment of the acute leukemia cells.

Anthracycline doses and cell treatment. After a dilution of the cell suspension to a density of 15×10^{4} cells/ml medium, MOLT-4 and ML-1 cells were subjected to the anthracycline agent exposure. MOLT-4 cells were exposed to DAU, DAUFmor, and DAUFhex at concentrations of 25 nM, 50 nM, and 75 nM, respectively, and ML-1 cells to the action of these anthracycline agents applied at the concentrations of 75 nM, 150 nM, and 225 nM, respectively. The controls consisted of untreated MOLT-4 and ML-1 cells. The chosen concentrations of daunorubicin, DAUFmor, and DAUFhex used in the present studies are based on the unpublished data of our previous experiments performed on these two human acute leukemia cell lines.

Analyses of leukemia cells after the anthracycline application. The temporary changes occurring in human acute lymphoblastic leukemia MOLT-4 cells and human acute myeloblastic leukemia ML-1 cells were observed at 24 h, 48 h, and 72 h after exposure to daunorubicin and its two formamidinodaunorubicin derivatives. At these three time intervals, the cell viability, volume and count were assessed.

The assays were conducted using the spectrophotometric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay and the electronic Beckman-Coulter method. In viable, metabolically active cells, the tetrazolium ring of MTT is cleaved, yielding formazan crystals. Changes in the metabolic activity of cell populations result in a concomitant change in the amount of formazan formed. The Beckman-Coulter method of cell sizing and counting is based on the detection of an electrical pulse which results from the passage of each cell through an aperture. The amplitude of the produced electrical pulse depends on the cell volume. The number of pulses indicates the cell count.

In vitro spectrophotometric MTT assay: MTT (Sigma-Aldrich) was dissolved in RPMI 1640 medium, at a concentration of 5 mg/ml, and filtered through a 0.2-μm filter. Subsequently, 100 μl of the yellow MTT solution was added to each well of a 24-well plate, containing 1 ml of the cell suspension. The cells were then incubated at 37˚C with 5% CO_2. A blank solution was prepared according to the above procedure using complete medium without cells. After a three-hour incubation period, the resulting formazan crystals were dissolved with 1 ml of acidified isopropanol (0.05 N HCl in absolute isopropanol), and the absorbance of the obtained solution was measured at wavelength of 570 nm using a Pharmacia Ultrospec III spectrophotometer (Pharmacia LKB Biotechnology, Uppsala, Sweden).

The half maximal inhibitory concentration (IC_{50}) value determination: The half maximal inhibitory concentration is a measure of the effectiveness of a compound in inhibiting biochemical processes and biological functions. According to the *in vitro* MTT assay, the IC_{50} represents the concentration of the tested anthracycline agent that is required for 50% inhibition of the human leukemia cell viability. Based on the obtained data using the *in vitro* MTT assay, the IC_{50} values for daunorubicin and its two analogs, DAUFmor and DAUFhex, calculated separately, at 72 h after the human leukemia cells exposure to the anthracycline agents. To determine the IC_{50} values, the concentration range used of each antracycline was from 10-1,000 nM.

Measurement of cell size and count: Samples of the acute leukemia cell suspension (500 μl) were taken from flasks and immediately diluted in 4.5 ml ISOTON II (Beckman-Coulter filtered electrolyte solution based on 0.9% saline). After the dilution of the leukemia cell suspension, individual cells were measured using a Z2 Coulter counter (Beckman-Coulter, Miami, FL, USA). The volume and count distribution of leukemia cells was obtained using a counter equipped with a 100 μm diameter orifice. The flow rate was 500 μl/12.5 s. The range for MOLT-4 cell measurement was determined as 65-3674 fL and for ML-1 cell measurement as 180-8181 fL. The volume and count of MOLT-4 cells and ML-1 cells were analyzed, respectively, at 402-3674 fL, and at 459-7346 fL. The instrument was calibrated using 10 μm diameter latex beads (Beckman-Coulter CC size standard). The mean cell volume and the cell count were determined using Z2 AccuComp software (Beckman-Coulter, Miami, FL, USA).

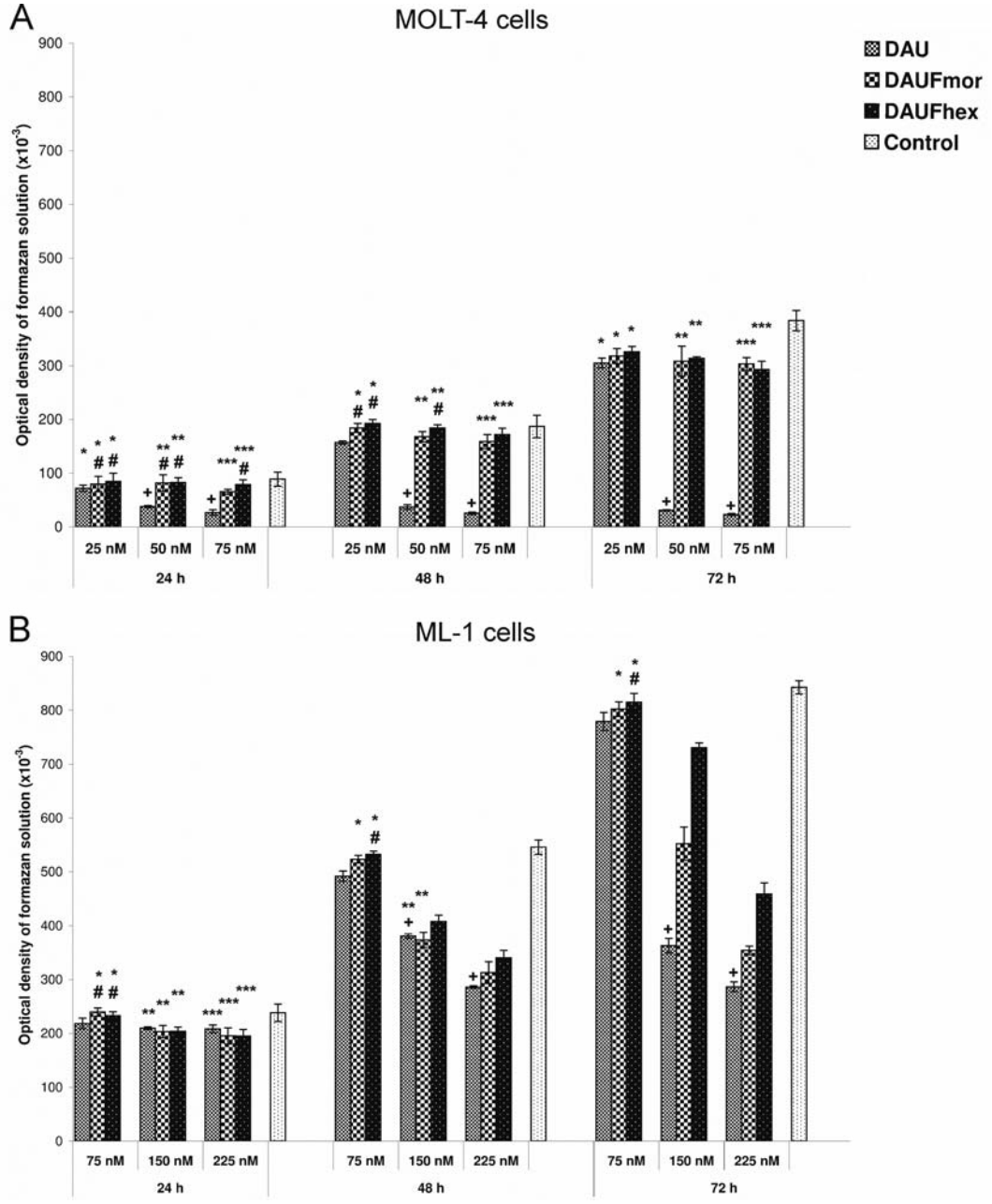

Figure 2. *Effects of daunorubicin (DAU), DAUFmor, and DAUFhex on the viability of MOLT-4 cells (A) and ML-1 cells (B). The extent of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) conversion to formazan in the acute leukemia cells reflects their viability. Values not significantly different at p<0.05 according to the Duncan's test: *, **, *** between the groups of leukemia cells treated with the anthracycline agents; #compared to control; +between the time points.*

Statistical evaluation. Statistical significance of differences in the amount of formazan formed, the IC_{50} value, and the cell volume and count were evaluated by an analysis of variance and the Duncan's new multiple range test. A difference with $p<0.05$ was considered statistically significant. The results were confirmed by three independent experiments carried out in triplicate.

Results

The effects of daunorubicin and its two new derivatives DAUFmor, and DAUFhex on temporary changes in the acute leukemia MOLT-4 and ML-1 cell viability (Figure 2), size

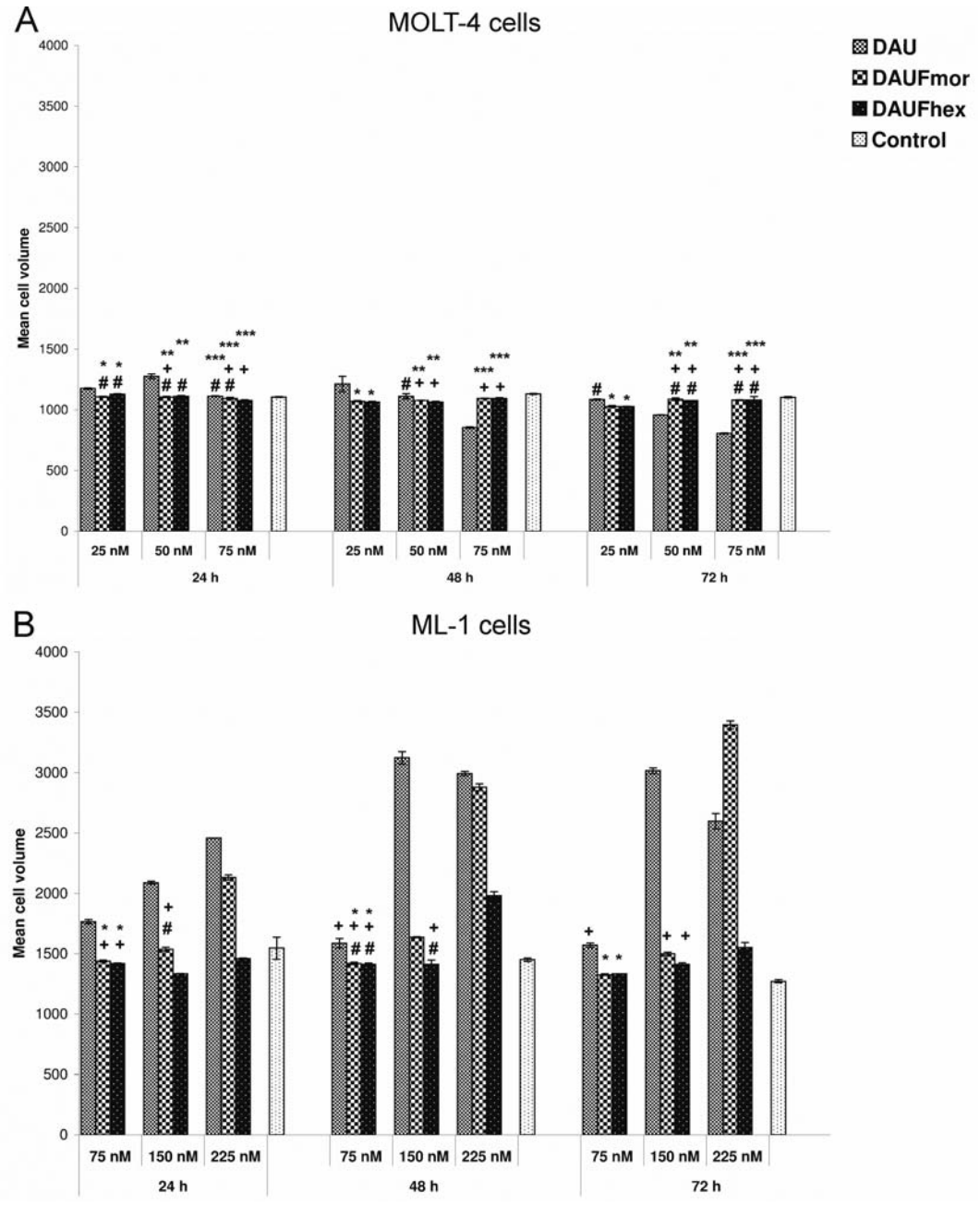

Figure 3. *Effects of daunorubicin (DAU), DAUFmor, and DAUFhex on the mean volume of MOLT-4 cells (A) and ML-1 cells (B). A decrease in the cell volume can indicate that the cell can undergo apoptosis, and an increase of the cell volume can be associated with occurring mitotic catastrophe or necrosis. Values not significantly different at p<0.05 according to the Duncan's test: *, **, *** between the groups of leukemia cells treated with the anthracycline agents; #compared to control; +between the time points.*

(Figures 3 and 4), and count (Figure 5) were compared. On the basis of the obtained results, it can be generally stated that the antileukemic activities of the new DAU analogs were weaker than that of daunorubicin, and MOLT-4 cells were more sensitive than ML-1 cells to the action of all agents (Figures 2-5; Table I). Moreover, between the analogs, DAUFmor appeared to be more active in ML-1 cells than DAUFhex, as shown by the lower IC_{50} value found for DAUFmor than DAUFhex (Table I). DAUFmor affected the viability, volume and count of ML-1 cells to a greater degree than did DAUFhex (Figure 2B, 3B, 4B, 5B). In the case of the MOLT-4 cell exposure to the action of the DAU derivatives,

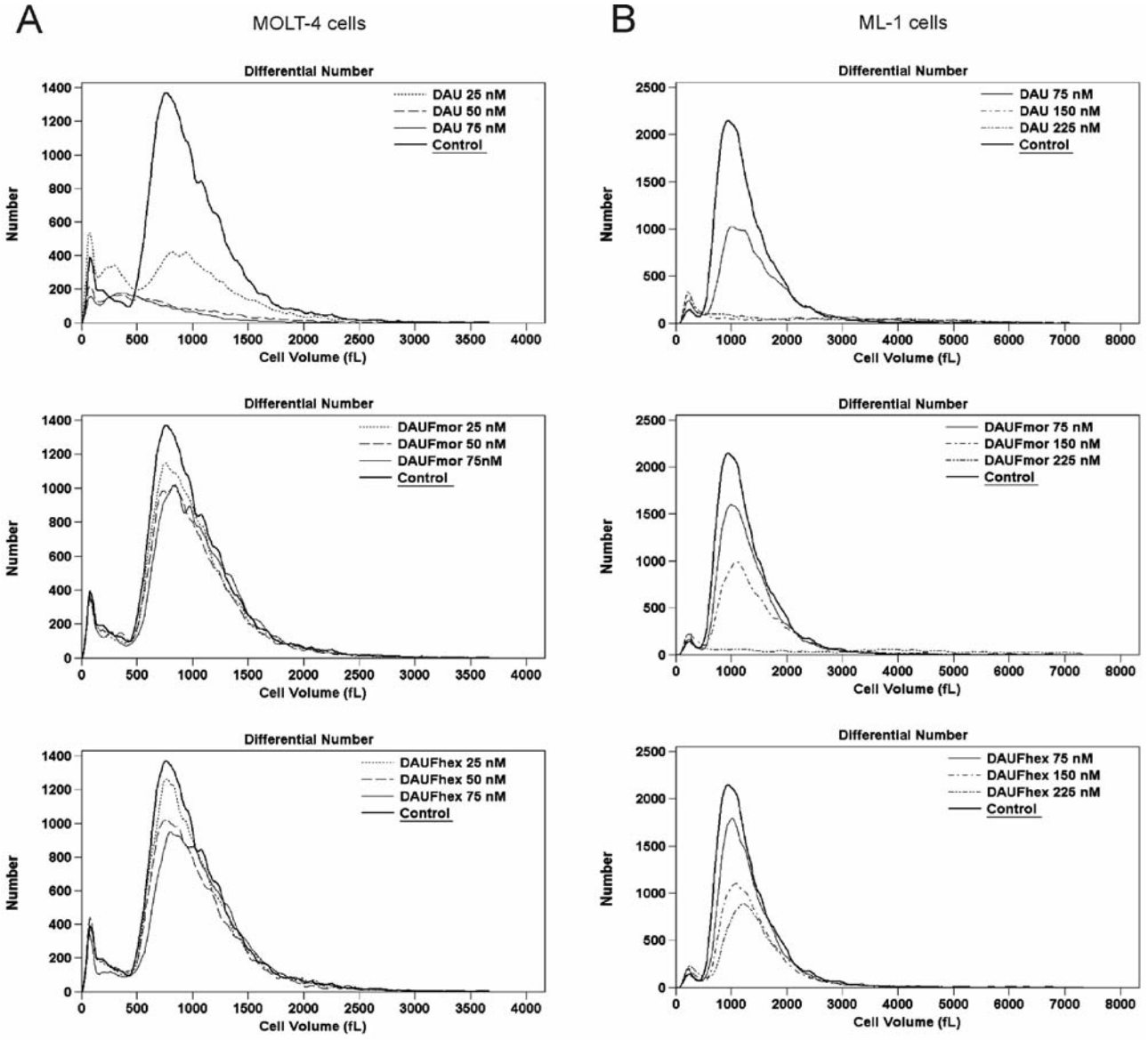

Figure 4. *The mean volume distribution curves of MOLT-4 cells (A) and ML-1 cells (B) recorded 72 h after exposure to the action of daunorubicin (DAU), DAUFmor, and DAUFhex. The peaks on the left represent cellular debris, presumably apoptotic bodies and necrotic cell fragments, which were excluded from the analysis of the MOLT-4 and ML-1 cell volume.*

there was no difference between the IC_{50} values assessed for DAUFmor and DAUFhex (Table I). Differences between the viability, volume and count of MOLT-4 cells after application of DAUFmor and DAUFhex were also not observed (Figure 2A, 3A, 4A, 5A).

The structural modifications of daunorubicin were reflected in the different leukemia cell response to the action of DAU and its two derivatives. The antileukemic potential towards MOLT-4 cells and ML-1 cells depended on the agent tested and its dose, the time interval after the anthracycline application, and the cell line used (Figure 2- 5; Table I).

Discussion

The two tested daunorubicin derivatives, DAUFmor and DAUFhex, differ in the size of the cyclic amine ring in the amidine group. Among the formamidinodaunorubicins, the analog containing a six-membered morpholine ring with heteroatom-oxygen in γ position in the formamidine group appeared to be more active in ML-1 cells, while the analog containing a seven-membered hexamethyleneimine ring with CH_2 group in the γ position was less active. These daunorubicin analogs also differ because the DAUFmor

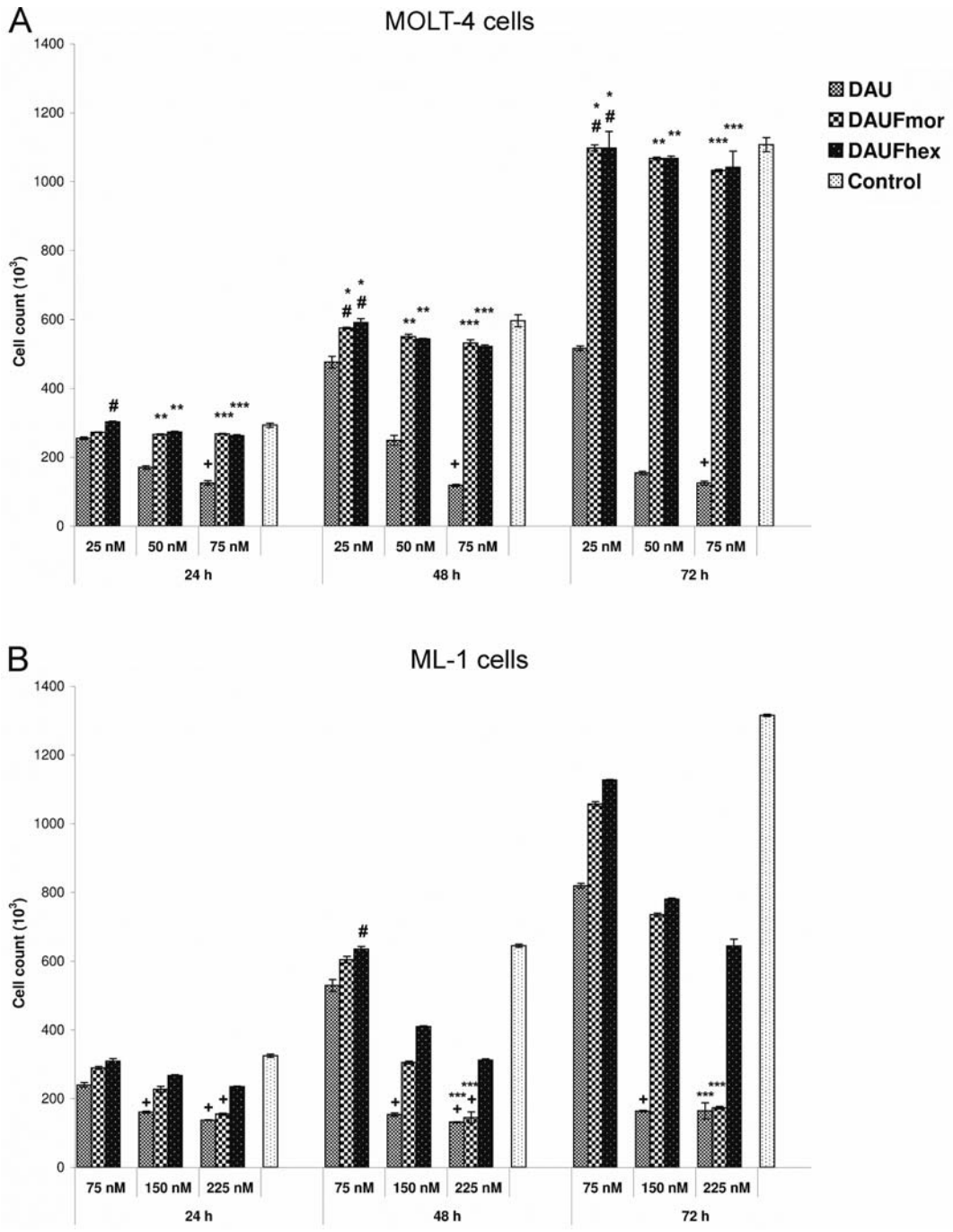

Figure 5. *Effects of daunorubicin (DAU), DAUFmor, and DAUFhex on the count of MOLT-4 cells (A) and ML-1 cells (B). Values not significantly different at p<0.05 according to the Duncan's test: *, **, *** between the groups of leukemia cells treated with the anthracycline agents; #compared to control; +between the time points.*

molecule has an oxygen atom whereas DAUFhex has two CH_2 groups. Moreover, due to the presence of the free electron pairs on the oxygen atom, DAUFmor is capable of forming an additional hydrogen bond with proton-donating sites, which may also considerably alter its biological properties.

The various biological activities of formamidinoanthracyclines and the parent antibiotics were also shown by Wąsowska *et al.* (10-12). The results of these investigations performed on the cancer cell lines, such as A549 (non-small cell lung carcinoma), SW707 (colon adenocarcinoma), T47D

Table I. *The half inhibitory concentration (IC_{50}) values for daunorubicin (DAU) and its two analogs DAUFmor and DAUFhex, determined at 72 h after the exposure of MOLT-4 and ML-1 cells to the action of the three anthracycline agents.*

	Human acute leukemia cell line	
	MOLT-4	ML-1
Agent	IC_{50} ± SD (nM)	
DAU	36.50±2.86	125.00±11.20
DAUFmor	85.17±3.33*	187.50±8.70
DAUFhex	85.58±3.09*	241.25±14.34

*Values not significantly different at $p<0.05$ according to the Duncan's test.

(breast cancer) and HCV29T (urinary bladder cancer) point to the varied action of anthracycline derivatives. It was also demonstrated that among the various formamidinodaunorubicins, the derivative containing a six-membered ring in the amidine group displayed greater activity against the different cancer cell lines (10).

Despite the findings of previous (10-12) and the present investigations, the mechanisms of the biological action of the new derivatives of daunorubicin and other anthracyclines on malignant cells remain unclear.

To summarize, the influence of the structure of DAU, DAUFmor and DAUFhex on the temporary changes occurring in the viability, size and count of MOLT-4 and ML-1 cells was found. These are the first data comparing the activities of DAU and the formamidinodaunorubicins which in the amidine group either contain a morpholine moiety or a hexamethyleneimine moiety, against the human acute lymphoblastic and myeloblastic leukemia cells.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Acknowledgements

The Authors wish to thank Urszula Kłaput for her excellent technical assistance.

The study was supported by the Research Projects K/ZDS/00788, K/ZDS/001720.

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Received August 30, 2012
Revised October 14, 2012
Accepted October 15, 2012