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 (–NH₂) 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% CO₂ in air (HERAcell incubator; KendroLab, Warsaw, Poland).

Chemicals. The three anthracycline compounds (Figure 1), daunorubicin and two formamidinodaunorubicins DAUFmor and
DAUFhex were synthesized at the Institute of Biotechnology and Antibiotics (Warsaw, Poland). The formamidinoaunorubicin compounds were obtained in the reaction of daunorubicin hydrochloride with acetals, derivatives of N-formylamines. The compounds were obtained in the reaction of daunorubicin and its two formamidinoaunorubicin 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.

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 anthracycline 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).
Statistical evaluation. Statistical significance of differences in the amount of formazan formed, the IC\textsubscript{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 DAUF\textsubscript{mor}, and DAUF\textsubscript{hex} on temporary changes in the acute leukemia MOLT-4 and ML-1 cell viability (Figure 2), size...
(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 IC₅₀ 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,
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 formamidinodaunorubincins, 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
molecule has an oxygen atom whereas DAUFhex has two CH₂ 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...
(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.

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**References**


**Table I.** *The half inhibitory concentration (IC₅₀) 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.*

<table>
<thead>
<tr>
<th>Human acute leukemia cell line</th>
<th>MOLT-4 IC₅₀ ± SD (nM)</th>
<th>ML-1 IC₅₀ ± SD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agent</strong></td>
<td><strong>36.50±2.86</strong></td>
<td><strong>125.00±11.20</strong></td>
</tr>
<tr>
<td>DAU</td>
<td>85.17±3.33*</td>
<td>187.50±8.70</td>
</tr>
<tr>
<td>DAUFhex</td>
<td>85.58±3.09*</td>
<td>241.25±14.34</td>
</tr>
</tbody>
</table>

*Values not significantly different at \( p<0.05 \) according to the Duncan’s test.

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