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Influence of the Structure of New Anthracycline Antibiotics on their Biological Properties

MALGORZATA WASOWSKA[1], IRENA OSZCZAPOWICZ[1], JOANNA WIETRZYK[2], ADAM OPOLSKI[2], JANUSZ MADEJ[3], STANISLAW DZIMIRA[3] and JANUSZ OSZCZAPOWICZ[4]

[1]*Institute of Biotechnology and Antibiotics, 5 Staroscinska Str., 02-516 Warsaw;*
[2]*Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, 12 Weigla Str., 53-114 Wroclaw;*
[3]*Faculty of Veterinary Medicine Agriculture University, 31 Norwida Str., 50-375 Wroclaw;*
[4]*Department of Chemistry, Warsaw University, 1 Pasteura Str., 02-093 Warsaw, Poland*

Abstract. *In the search for new derivatives of anthracycline antibiotics with advantageous biological properties, particularly with lower toxicity and/or higher activity, a series of new analogs of antibiotics applied in therapy such as daunorubicin, doxorubicin, as well as epidoxorubicin and, for comparison, analogs of epidaunorubicin, have been synthesized. Our results show that the new derivatives have antiproliferative activities similar to or higher than the parent antibiotics. The toxicities of these analogs were significantly lower, with LD_{50} values from 1.8- to 18.4-fold higher than the referential drugs. Cardiotoxicity determinations, using male mice treated with a single dose of 75% of the LD_{50} values of all tested compounds, indicated that the cardiotoxicity of the new analogs is significantly lower than that of the parent drugs.*

Anthracycline antibiotics are very effective anticancer drugs, with proven activity in acute lymphomas, sarcomas and a wide range of carcinomas. Unfortunately, these antibiotics produce a dose-dependent cardiotoxicity, which results in significant cardiomyopathy, thus limiting their clinical usefulness (1). Cumulative doses of daunorubicin, doxorubicin and epidoxorubicin below 600, 550 and 700 mg/m^2, respectively, are considered to be safe. On increasing this dose, there is an increased risk of cardiotoxicity *e.g.* at doxorubicin 550 mg/m^2, the risk of congestive heart failure was estimated to be 7%, whereas at 700 mg/m^2 it increased to 18% (2-5).

Correspondence to: Malgorzata Wasowska, M.Sc., Department of Modified Antibiotics, Institute of Biotechnology and Antibiotics, 5 Staroscinska St., 02-516 Warsaw, Poland. Tel: 4822/8496051 ext. 230 or 260, Fax: 4822/8483332, e-mail: wasowskam@iba.waw.pl

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This cardiotoxicity may be explained by a direct effect of the anthracycline on one or more biochemical processes in the myocytes or by generation of free radicals, which may lead to a variety of subcellular changes in the myocardium, including the slow loss of myofibrils and vacuolization of myocardial cells (1).

There are several ways to reduce the anthracycline-induced cardiotoxicity of anthracycline antibiotics. One of them is a change in administration of these drugs as well as limiting the overall cumulative dose. The other ways to reduce cardiotoxicity are the encapsulation of these antibiotics to liposomes, the use of cardioprotectors, as well as synthesis of modified anthracyclines with better therapeutic properties than the parent drugs (6).

In the search for new derivatives of anthracycline antibiotics with advantageous biological properties, particularly with lower toxicity and/or higher activity, a series of new analogs of antibiotics applied in therapy, such as daunorubicin, doxorubicin and epidoxorubicin and, for comparison, derivatives of epidaunorubicin, have been synthesized. In these analogs, the -NH_2 group in the daunosamine moiety was replaced by the formamidine system (-N=CH-NR^1R^2) containing the rest of the cyclic amines of gradually increased ring size (compounds **1-16**, Figure 1). The new compounds were obtained by treatment of the parent antibiotics with active derivatives of formylamines (7).

The aim of this work was to determine the cardiotoxicity of the new anthracycline derivatives and to compare these data with results of their antiproliferative activity and toxicity. Additionally, the influence of the structure of these analogs on their biological properties was investigated.

Materials and Methods

Antibiotics. Daunorubicin, doxorubicin, epidaunorubicin and epidoxorubicin, as well as their derivatives, denoted as **1-16**, of

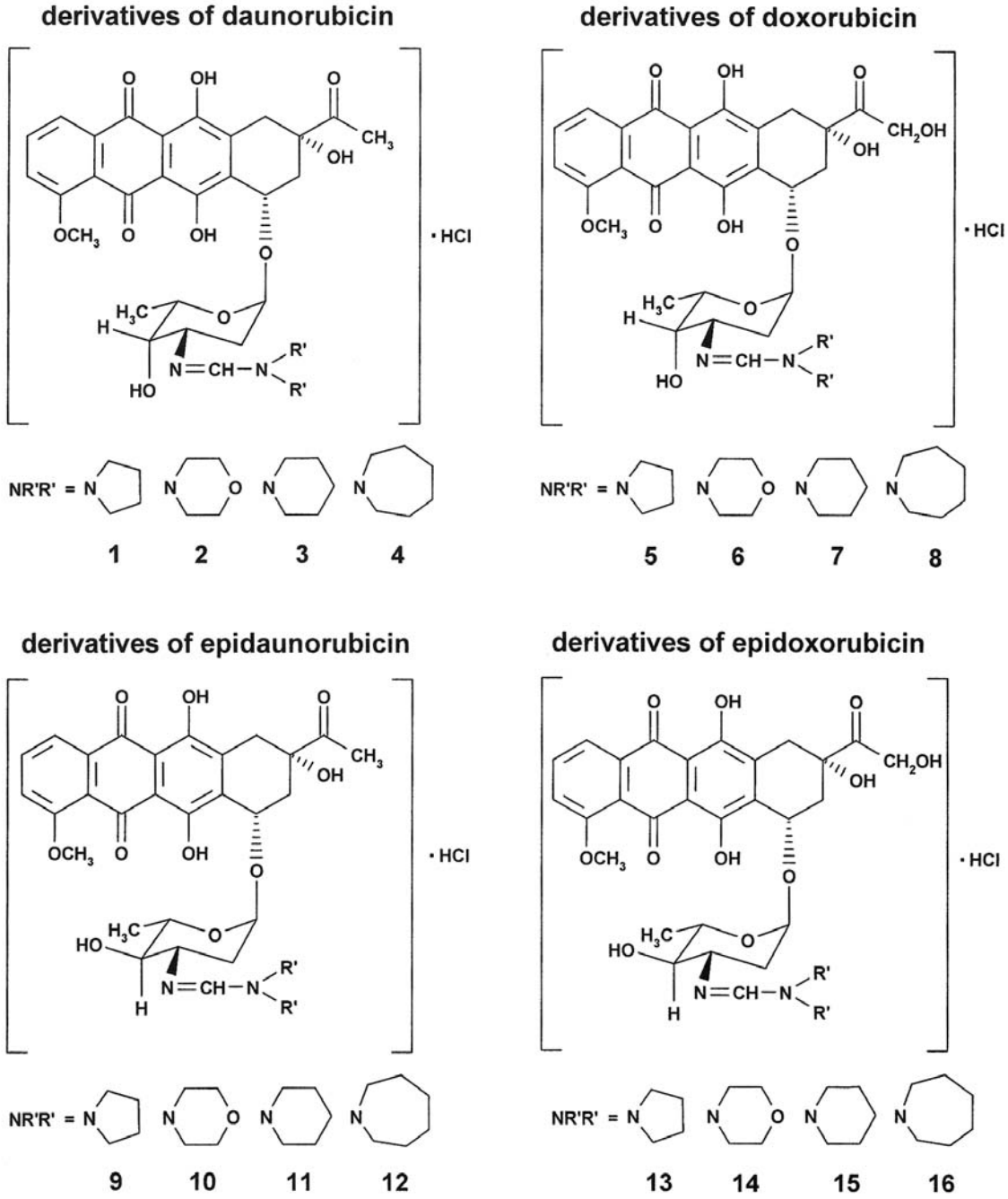

Figure 1. *Structures of new derivatives of anthracycline antibiotics.*

purity ≥97.5% according to HPLC method, were obtained from the Institute of Biotechnology and Antibiotics in Warsaw, Poland.

Cell lines. The cells of the following human cancer lines were used: A549 (non-small cell lung carcinoma), SW707 (colon adenocarcinoma), T47D (breast cancer) and HCV29T (urinary bladder cancer). All lines were obtained from the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The cells were maintained in culture medium (RPMI 1640) containing 10% fetal calf serum (Gibco, Grand Island, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (both from Polfa Tarchomin, Warsaw, Poland) and 2 mM glutamine (Gibco, Warsaw, Poland), under standard cell culture conditions (humidified atmosphere of 95% air and 5% CO_2 at 37˚C).

Antiproliferative assay in vitro. Twenty-four hours before application of the tested compounds, the cells were plated in 96-well plates at

Table I. *Antiproliferative activity in vitro of the tested compounds against the cells of human cancer cell lines.*

Compounds	Cell lines (ID_{50}±SD in ng/ml)			
	SW707	A549	HCV29T	T47D
1	155.2±1.6	562.1±1.2	240.3±2.5	457.1±1.6
2	110.4±1.5	398.2±1.2	62.4±1.9	398.1±1.5
3	115.2±1.4	427.4±1.3	204.3±1.9	372.2±1.3
4	302.3±1.5	1660.1±2.1	355.5±1.5	624.6±2.3
Daunorubicin	*43.0±1.5*	*301.5±1.2*	*275.0±1.1*	*178.1±2.4*
5	6.2±1.8	32.4±1.1	9.9±1.9	7.6±1.9
6	0.6±1.3*	10.6±1.5*	1.0±1.5*	1.1±2.4*
7	3.3±1.9	23.0±1.3	4.5±1.6	4.6±1.6
8	39.2±1.4	164.5±1.2	41.8±1.6	44.3±1.5
Doxorubicin	*16.7±1.8*	*167.6±2.6*	*11.6±1.3*	*33.1±1.6*

Table II. *Antiproliferative activity in vitro of the tested compounds against the cells of human cancer cell lines.*

Compounds	Cell lines (ID_{50}±SD in ng/ml)			
	SW707	A549	HCV29T	T47D
9	580.1±1.9	940.0±2.5	832.5±1.4	205.1±2.0
10	383.8±1.6	631.0±2.3	241.4±1.3	143.0±1.3
11	455.0±1.5	824.0±3.1	131.6±1.7	170.1±2.1
12	698.5±1.4	1031.5±2.0	304.1±1.3	212.9±1.1
Epidaunorubicin	*29.7±1.8*	*54.0±2.5*	*25.5±1.5*	*11.7±1.4*
13	1552.2 ±2.3	4638.5 ±1.6	1141.4 ±2.2	628.0±2.6
14	506.1±2.2	2562.0 ±1.4	354.5±1.3	300.6±1.8
15	1091.2 ±1.6	4722.1 ±1.6	1087.6 ±1.6	460.1±1.4
16	1157.1 ±1.6	6231.7±1.7	1115.4 ±1.2	497.1±1.3
Epidoxorubicin	*46.6±4.7*	*403.8±2.0*	*41.6±3.6*	*18.7±1.9*

*statistically significant ($p<0.05$)

a density of 10^4 cells per well, and cultured at 37˚C in a humid atmosphere saturated with 5% CO_2. Then the cells were exposed for 72 hours to various concentrations of the tested compounds. Test solutions of the compounds **1-16** and the parent anthracycline antibiotics (1.0 mg/ml) were prepared *ex tempore* for each test by dissolving the compounds in water for injection. After that, the tested solutions were diluted in culture medium to reach final concentrations in the range from 10,000 to 0.1 ng/ml. The antiproliferative effect *in vitro* was determined using the SRB method (8). The optical densities of the samples were measured on a Multiskan RC photometer (Labsystem, Helsinki, Finland) at 570 nm. The results were calculated as an inhibitory dose 50% (ID_{50}) – the dose of the tested compound which inhibits proliferation of the cancer cells by 50% as compared to the control untreated cells. The ID_{50} values for analogs **1-16**, calculated separately, and the mean values for each experiment±SD are presented in Tables I and II. Each compound at every concentration was tested in triplicate. Each experiment was repeated 3-5 times.

Statistical evaluation. For independent samples, Student's *t*-test was applied and a *p* value lower than 0.05 was considered significant. The results for cardiotoxicity were subjected to analysis of variance (ANOVA); $p<0.05$ were considered significant (10).

Animals. Male mice [BALB/c x DBA/2] (CD2F1), (weight 19-22 g), supplied from the Animal Breeding Centre of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland, were maintained under standard laboratory conditions.

All experiments to determine LD_{50} as well as cardiotoxicity were performed according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education, issued by the New York Academy of Sciences Ad Hoc Committee on Animal Research and approved by the First Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland.

LD_{50} evaluation. The investigated compounds were dissolved *ex tempore* in physiological saline. Initially, the range of the administered dose was determined (5 male mice per group). Mice (36 animals for every compound) were injected intraperitoneally (*i.p.*) with the tested solutions at a single dose in the range from 2.0 to 128.0 mg/kg. Control animals received injection of physiological saline (equal volume). The animals were observed for 3 weeks. The values of LD_{50} for individual determinations were computed graphically from the curve presenting the relationship between mortality (in %) and logarithm of the administered dose (in mg/kg) (9). The results are summarized in Table III.

Histopathological evaluation. For histopathological studies, male mice (10 mice per group) were used. The tested compounds were dissolved in physiological saline and injected once *i.p.* at a dose about 75% of the LD_{50} values as a single dose. The following doses were applied: for daunorubicin – 2.3 mg/ml; for its analogs **1**, **2**, **3**, **4** – 37.0, 18.0, 38.0 and 37.0 mg/kg; for doxorubicin – 9.5 mg/kg; for its analogs **5**, **6**, **7**, **8** – 17.0, 2.3, 4.0 and 36.0 mg/kg; for epidaunorubicin – 2.0 mg/kg; for its analogs **9**, **10**, **11**, **12** – 36.0, 33.0, 36.0 and 37.0 mg/kg; for epidoxorubicin – 17.0 mg/kg; for its analogs **13**, **14**, **15** and **16** – 67.0, 48.0, 36.0 and 40.0 mg/kg, respectively. Control animals received injection of physiological saline (equal volume). After 3 weeks, any animals that had not already died were sacrificed and dissected. The hearts were collected, fixed with 4% buffered formalin, processed routinely and the preparations were stained with hematoxylin and eosin. The observed histopathological changes (using light microscope Anxiophot, Carl Zeiss Jena), such as atrophy of cross-striations, enlargement of nucleus and hydropic degeneration of cytoplasm, myocardial fibrosis, venostasis, erythrorrhagia and histiocytes infiltration, were denoted as: "0" - in the case of absence or very slight pathological signs; "1" - in the case of mild pathological signs; whereas for distinct and severe pathological signs, as "2" and "3", respectively. The obtained results are presented in Figure 2.

Results

As shown in Table I, the ID_{50} values for derivatives of doxorubicin (**5-8**) and for the parent doxorubicin were in the range of 0.6-164.5 and 11.6-167.6 ng/ml, respectively, but for daunorubicin analogs (**1-4**) and the referential daunorubicin these values were in the range of 62.4-1660.1

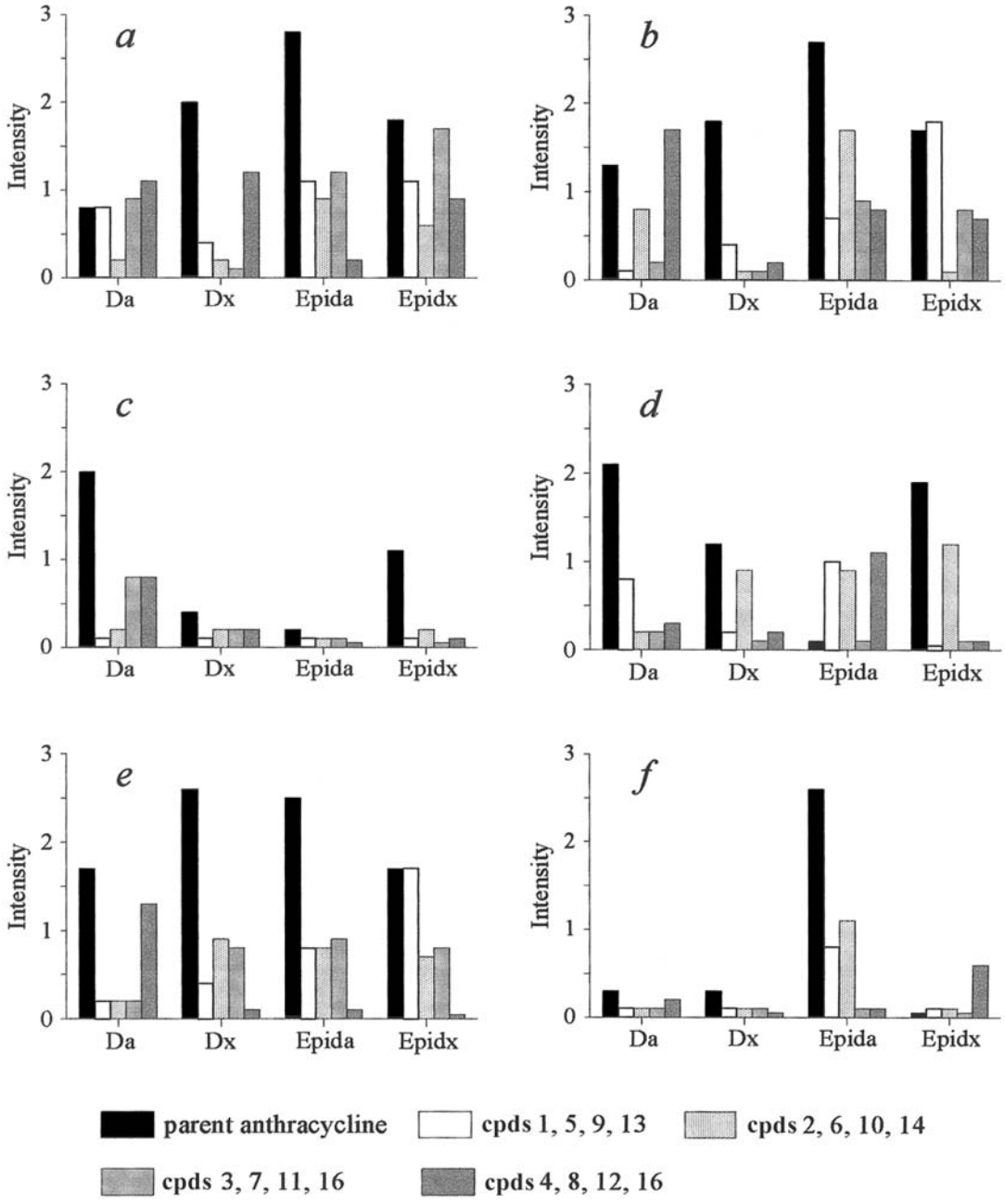

Figure 2. *Intensity of histopathological signs. a - atrophy of cross-striations, b - enlargement of nucleus and hydropic degeneration of cytoplasm, c - myocardial fibrosis, d - venostasis, e - erythrorrhagia, f - histiocytes infiltration.*

and 43.0-301.5 ng/ml, respectively. Among derivatives **1-8**, the highest activity was revealed by analogs **6** and **2** (0.6-10.6 and 62.4-398.2 ng/ml, respectively), but the lowest by derivatives **4** (301.3-1660.1 ng/ml) and **8** (39.2- 164.5 ng/ml).

The differences in activity between analog **6** and the parent drug against all the tested cell lines were statistically significant ($p<0.05$). The antiproliferative activity of analogs **5** (6.2-32.4 ng/ml) and **7** (3.3-23.0 ng/ml) were weaker than that of **6**, but higher than the parent drug. The derivatives of epidaunorubicin (**9-12**) and epidoxorubicin (**13-16**) (Table II) also exhibited activity weaker than that of **6** and, among these derivatives, the highest activity was revealed by compounds **10** and **14,** but the lowest by analogs **12** and **16**.

As shown in Table III, the LD_{50} values of the new analogs (**1-5** and **8-16**) were 1.8- to 18.4-fold higher than those for the parent drugs, except for two derivatives of

Table III. *Lethal dose (LD_{50}) of the tested compounds.*

Compounds	LD_{50}±SD (mg/kg)
1	50.2±2.51*
2	24.0±1.32
3	51.1±2.19*
4	49.2±2.55*
Daunorubicin	*3.1±0.12*
5	22.3±0.78
6	3.0±0.12
7	5.8±2.61
8	48.0±3.07
Doxorubicin	*12.6±0.57*
9	48.0±2.92*
10	44.0±2.02*
11	48.0±2.82*
12	49.8±2.44*
Epidaunorubicin	*2.7±0.16*
13	90.0±5.85
14	64.0±3.71
15	48.0±2.52
16	53.0±2.01
Epidoxorubicin	*22.3±0.83*

*statistically significant ($p<0.05$)

doxorubicin (**6** and **7**) for which these values were lower than that for the parent drug (3.0 and 5.8 *versus* 12.6 mg/kg). Statistically significant ($p<0.05$) differences in LD_{50} values were observed for analogs **1**, **3**, **4** and **9-12**.

Advantageous, statistically significant ($p<0.05$) differences in histopathological changes between the obtained derivatives and the corresponding parent anthracyclines were observed (Figure 2). Signs such as atrophy of cross-striations were revealed by analogs **2**, **5-14** and **16**, enlargement of nucleus and hydropic degeneration of the cytoplasm as well as erythrorrhagia by compounds **1-3**, **5-12** and **14-16**, venostasis by analogs **1-5**, **7**, **8** and **13-16**, myocardial fibrosis by compounds **1-4** and **12-16,** and histiocytes infiltration by derivatives **8-12**, respectively.

Statistically significant but disadvantageous histopathological changes were observed only in the case of venostasis for derivatives of epidaunorubicin (**9-12**), and histiocytes infiltration for analogs of epidoxorubicin (compounds **13-16**).

Discussion

The new compounds (**1-16**) were synthesized to investigate the influence of their structure on such biological properties as antiproliferative activity, acute toxicity, expressed as LD_{50}, and cardiotoxicity. The analogs of daunorubicin (**1-4**) and doxorubicin (**5-8**) differ by substitution at the 14-position (H or OH, respectively), whereas the derivatives of epidaunorubicin (**9-12**) and epidoxorubicin (**13-16**) differ additionally by the position of the hydroxyl group at C-4'. Moreover, in each group of these derivatives individual analogs gradually differ by the ring size of the rest of a secondary cyclic amine, namely pyrrolidine, piperidine, morpholine and hexamethyleneimine (Figure 1).

The antiproliferative activities of the new analogs were higher in the case of derivatives of doxorubicin (**5-8**) and similar or weaker for derivatives of daunorubicin (**1-4**), epidaunorubicin (**9-12**) and epidoxorubicin (**12-16**). Among all the derivatives, the most active appeared to be the analogs containing a morpholine ring in the formamidine group (**2**, **6**, **10** and **14**), while the least were the analogs containing a hexamethyleneimine ring (**4**, **8**, **12** and **16**). The decrease in activity of the epidaunorubicin and epidoxorubicin derivatives in comparison to the analogs of daunorubicin and doxorubicin may be explained by the influence of the equatorial orientation of the hydroxyl group at the C-4'position. Importantly, all the new analogs, except compounds **13**, **15** and **16** against the A549 cell line, satisfied the adopted criterion of new compounds that, in *in vitro* screening, should have LD_{50} values lower than 4,000 ng/ml (11).

A significant decrease of acute toxicity, except for two derivatives of doxorubicin (**6** and **7**) containing in the formamidine group a six-membered ring such as morpholine and piperidine, was observed. There were statistically significant ($p<0.05$) differences in the LD_{50} values for all analogs of epidaunorubicin (**9-12**) and derivatives of daunorubicin (**1**, **3** and **4**). The highest decrease of the LD_{50} was revealed by derivatives containing in the formamidine group the rest of pyrrolidine (**1**, **5**, **9** and **13**) and hexamethyleneimine (**4**, **8**, **12** and **16**).

It should be pointed out that, for comparison of the cardiotoxicity of the new derivatives and the parent antibiotics, we chose a dose equal to about 75% of the lethal dose (LD_{50}) and, for this reason, the tested compounds were administered only as a single dose. This means that, in some cases, we compared very differentiated (1.8- to 18.5-fold) doses *e.g.* 2.3 mg/kg of daunorubicin with 37.0-38.0 mg/kg of derivatives **1-3**. The obtained results showed considerable differences between cardiotoxicity of the new analogs and the parent antibiotics. The animals which received these derivatives, in most cases, revealed almost normal myocardium, sometimes with slight or moderately-marked histopathological signs, whereas among animals treated with the parent anthracyclines, these signs were severe.

Comparing the differences of the six histopathological changes tested between the obtained derivatives (**1-16**) and the corresponding parent anthracyclines, the majority of

these differences were found to be statistically significant ($p<0.05$). Statistically significant and advantageous differences in the case of histopathological signs such as atrophy of cross-striations were revealed by almost all the new analogs, except derivatives **1**, **3**, **4** and **15**, enlargement of the nucleus and hydropic degeneration of the cytoplasm as well as erythrorrhagia, except compounds **4** and **13** and venostasis, except analog **6**, respectively. In the case of myocardial fibrosis (derivatives **5-11**) and histiocytes infiltration (analogs **1-7**), comparing the obtained compounds to the parent antibiotics, small differences were observed which were statistically insignificant ($p>0.05$). In general, these derivatives contained the piperidine or pyrrolidine ring in the formamidine group (**1**, **3**, **11**, **13** and **15**).

Among all the new derivatives, only two groups of compounds showed an increase in pathological changes with respect to the parent drug, such as venostasis for analogs of epidaunorubicin (**9-12**) and histiocytes infiltration for derivatives of epidoxorubicin (**12-16**).

In conclusion, almost all the tested derivatives, obtained by replacement of the amino group in position C-3' of the daunosamine moiety by the formamidine group containing the rest of the cyclic amine, displayed significant decreases of acute toxicity (except **6** and **7**) and cardiotoxicity (**1-16**) with respect to the parent antibiotics. The analogs of doxorubicin (**5-8**) showed the highest activity. Moreover, we found a relationship between the structure of the new derivatives and their biological properties.

Of note, despite the acute toxicity of doxorubicin analogs **6** and **7**, their cardiotoxicity was considerably lower than that of the parent drug, the large differentiation of applied doses, appearing to be a great advantage of these analogs, particularly because of their still high antiproliferative activity and low toxicity.

These data support that the majority of these new derivatives may appear to be more useful in therapy than the parent antibiotics on account of their reduced adverse effects at similar or even higher antiproliferative activity.

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