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Cytotoxicity and inhibitory properties against topoisomerase II of doxorubicin and its formamidine derivatives

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This work was undertaken to compare cytotoxicity, DNA damaging properties and effect on DNA cleavage by topoisomerase II of the anthracycline drug doxorubicin (DOX) and its two derivatives with a formamidino group containing a cyclic amine moiety such as morpholine (DOXM) or hexamethyleneimine (DOXH). The tetrazolium dye colorimetric assay was used to determine the cytotoxic activity of anthracyclines toward L1210 leukemia cells. DNA damage was measured by alkaline elution technique. The effect of anthracyclines on DNA cleavage was studied in a cell-free system containing supercoiled pBR322 DNA and purified human topoisomerase II. The cytotoxicity data and the results of studies on the mechanism of DNA break formation by anthracyclines at the cellular level and in the cell-free system showed that the presence of the formamidino group in the doxorubicin molecule reduced its ability to stimulate DNA cleavage by DNA topoisomerase II. Conclusion: DNA topoisomerase II is not a primary cellular target for DOXM or DOXH. An advantageous feature of formamidinoanthracyclines is their mechanism of cytotoxic action which is not related to the inhibition of DNA topoisomerase II. Therefore this class of anthracyclines seems to be a good source for selection of an anticancer drug directed toward cancer cells with the developed multidrug resistance attributed to the presence of altered DNA topoisomerase II.

Keywords: doxorubicin, formamidinodoxorubicins, topoisomerase II

INTRODUCTION

The anthracycline drug doxorubicin (DOX) is known to have a broad spectrum as well as high antineoplastic activity and is one of the most effective and widely used anticancer drugs (Minnoti *et al*., 2004). Its clinical efficacy is limited by cardiotoxicity and the development of multiple mechanisms of cellular drug resistance (Lehne, 2000; Renes *et al*., 2000; Elliott, 2006). Therefore there is a necessity to develop new doxorubicin derivatives whose structural modifications may change the mechanism of cytotoxic action and circumvent these disadvantageous features of the parent drug.

Among the many possible strategies for improving the therapeutic effectiveness of anthracycline antibiotics one of the most important is synthesis of new derivatives with modified structure (Minnoti *et al*., 2004). In the search for new derivatives with advantageous biological properties many structural modifications, mainly in the daunosamine moiety, have already been described (Danesi *et al*., 1993; Bakker *et al*., 1997; Nadas & Sun, 2006; Battisti *et al*., 2007). Recently it has been shown that as a result of transformation of the amino group at position 3′ of daunosamine into the formamidino group (–N=CH–N<) a series of new derivatives of anthracycline antibiotics useful in therapy can be obtained

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Abbreviations: DOX, doxorubicin; DOXM, doxorubicin containing the formamidino group with morpholine ring; DOXH, doxorubicin containing the formamidino group with hexamethyleneimine ring; ICRF-187 (Cardioxane), *meso*-2,3-bis(3,5-dioxopiperazinyl)butane; SSB, DNA single strand break.

(Oszczapowicz *et al*., 2005). Such modification appears to be a promising way for improvement of biological properties in comparison with those of the parent antibiotics, because some of those formamidinoantracyclines exhibited lowered cardiotoxicity *in vivo* (Wąsowska *et al*., 2005) and ability to induce differentiation of tumor cells (Jakubowska *et al*., 2007; Szuławska *et al*., 2007) and were found to be highly cytotoxic to several neoplastic cell lines with developed multidrug resistance (Wąsowska *et al*., 2006; Wąsowska-Łukawska *et al*., 2007). Although several papers have reported many advantageous features of this novel class of anthracyclines, a biochemical explanation for these findings is lacking. In this work we attempted to answer the question whether the presence of the formamidino group at position 3′ of the daunosamine moiety affects the biological properties of anthracycline, such as the mechanism of cytotoxic action and cellular uptake. To answer this question we compared DOX and its two derivatives (Fig. 1) with a formamidino group containing a cyclic amine group such as morpholine (DOXM) or hexamethyleneimine (DOXH). Both analogues were compared with DOX for their cytotoxicity, cellular uptake, DNA damaging properties and inhibition of DNA topoisomerase II in a cell-free system.

MATERIALS AND METHODS

Drugs. The new derivatives of doxorubicin DOXM and DOXH of purity according to HPLC analysis of 97.5 and 97.2%, respectively, were synthesized at the Institute of Biotechnology and Antibiotics (Warszawa, Poland) by treatment of the parent doxorubicin with active derivatives of formylamines (Oszczapowicz *et al*., 2005). ICRF-187 (Cardioxane) was purchased from Chiron B.V. (Amsterdam, Netherlands). Etoposide was purchased from Sigma (St Louis, MO, USA). The anthracycline derivatives, ICRF-187 and etoposide were dissolved in water and made up fresh for each experiment.

Cytotoxicity assay and anthracyclines cellular uptake. Mouse leukemia L1210 cells were obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw, Poland). The cells were grown in RPMI1640 (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL), 5 mM Hepes buffer (Sigma, St Louis, MO, USA) in a humidified 5% CO_2 atmosphere at 37°C. The cytotoxic activity of the drugs was assayed by measuring their inhibitory effects on L1210 cell proliferation. To measure the cytotoxicity of doxorubicin and its two analogues, the tetrazolium dye (MTT) method was used (Carmichael *et al*., 1987). Cells in logarithmic growth phase were seeded at 2×10^4 cells/well in 24-well plates and incubated with different concentrations of anthracyclines for 72 h in triplicates in a final volume of 2 mL. Then 0.13 mL of sterile aqueous solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma, St Louis, MO, USA) (5 mg/mL) was added to each well for additional 3 h. The blue formazan precipitate formed was dissolved in dimethyl sulfoxide (Sigma, St Louis, MO, USA) and the absorbance of resulting solutions was measured at 540 nm. For each experiment the determinations were done in three replicates. The IC_{50} values (the anthracycline concentration inhibiting the cell growth by 50% after 72 h exposure of cells to the drug) were read from survival curves which were fitted to an exponential equation using X-ACT, a statistical software by SciLab (Germany).

The uptake of anthracyclines was estimated by fluorimetric measurements after extraction of cells with an ethanol/HCl mixture as described by Riganti *et al*. (2005). L1210 cells at 10^7 cells/mL were treated in growth medium with 10 μM anthracycline solutions at 37°C. Aliquots of cell suspension (0.2 mL) were mixed with 5 mL of ice-cold PBS and centrifuged at $1000\times g$ for 5 min, then washed twice with ice-cold PBS. Then cell pellets were resuspended in 2 mL of a 1:1 mixture of ethanol/0.3 M HCl and the fluorescence intensity of these suspensions was measured at 20°C by a Perkin-Elmer LS 55 spectrofluorimeter. The excitation and emission wavelengths were 495 and 590 nm, respectively. To estimate the drug concentrations calibration curves in the concentration range of 0.01–0.5 μM were prepared. At this range of concentrations the fluorescence intensity was proportional to the molar concentration of the anthracyclines. Because the fluorescence was slightly quenched in the presence of cells, each point of the calibration curves was measured in the presence of 2×10^6 cells suspended in the mixture of ethanol/HCl as this number of cells was used in the drug uptake assays.

Measurements of DNA breaks. Cells were incubated for 48 h the presence of 740 Bq/mL of [^{14}C]thymidine (Chemapol, Prague, Czech Republic) and then treated with the studied drugs for 1 h at 37°C. Cell suspensions were centrifuged, pellets were washed with cold PBS and alkaline elution was performed essentially as described by Kohn (1991), except that the pumping rate was 0.1 mL/min and tetrapropylammonium hydroxide in the elution buffer was replaced by tetraethylammonium hydroxide (Sigma, St Louis, MO, USA). Lysates from cells treated with anthracyclines were deproteinized prior to alkaline elution by 30-min digestion at 20°C with 0.5 mg/mL of proteinase K (Sigma, St Louis, MO, USA) dissolved in 10 mM EDTA of pH 10.

Topoisomerase II DNA cleavage assay. The procedure of Lemke *et al*. (2004), was used with mi-

nor modifications. The reaction mixture of a total volume of 20 μL contained 20 mM Tris/HCl, pH 7.5, 7.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 150 mM KCl, 1 mM ATP, and 200 ng of pBR322 (MBI Fermentas, Lithuania) DNA. The reaction was started by the addition of 5 units of DNA topoisomerase II (TopoGEN, Inc, Port Orange, Fl, USA) and carried out at 30°C for 10 min. One unit of enzyme activity was defined as the amount of enzyme decatenating 0.2 μg of kinetoplast DNA in 30 min at 37°C (the producer's definition). The reactions were stopped by adding sodium dodecyl sulfate and proteinase K to final concentrations of 0.35% and 0.3 mg/mL, respectively. After an additional 60 min of incubation at 37°C, 5 μL of gel loading buffer (0.05% bromophenol blue, 2.5 mM EDTA, 25% glycerol, final concentrations) was added. Samples were loaded on a 1% agarose gel containing 0.5 μg/mL ethidium bromide and run for 18 h in TBE buffer (100 mM Tris/borate, 1 mM EDTA, pH 8) at 0.5 V/cm. The gel was then destained in distilled water and photographed. Then digitized images were analyzed by Gel Scan v. 1.45 software (Kucharczyk T.E., Poland). Lanes in the gel images were set manually and optical density of each lane was read. Peaks corresponding to different DNA bands were detected automatically by GelScan software and corrected manually if needed. Optical density of each lane was corrected by subtraction of background level which was set for each lane separately. The amount of linear DNA in each lane was calculated as percentage of total DNA, assuming that total DNA is the sum of peak areas corresponding to supercoiled, nicked and linear DNA.

Statistical analysis. The data were analyzed using SigmaStat v. 3.5 (Systat Software Inc, London, UK) statistics package. All the values in this study were expressed as mean ± S.D. from at least three independent experiments. If no significant differences between variances were found, as assessed by Snedecor–Fisher test, the differences were compared by the two-tailed Student's *t*-test. For data which did not pass normality test the significance of differences was compared by Mann–Whitney *U* test. Results were considered significant when $P<0.05$. The IC_{50} values (the anthracycline concentration effective in inhibiting the cell growth by 50% after 72 h exposure of cells to the drug) were read from survival curves which were fitted to an exponential equation.

RESULTS

Cytotoxicity and uptake of DOX and its derivatives

DOX and its formamidine derivatives DOXM and DOXH exhibited high antiproliferative activity toward L1210 cells. All the anthracyclines tested were significantly more cytotoxic than etoposide, a DNA topoisomerase II trapping agent which was used as a reference compound (Fig. 1 and Table 1). It was found that DOX and DOXM were similar in their cytotoxic potencies whereas DOXH was less cytotoxic. The decrease of cytotoxicity did not seem to be caused by the reduction of the cellular uptake of this novel derivative. As seen in Table 1, the most cytotoxic anthracycline DOX and the least cytotoxic one DOXH, which differ in the IC_{50} values by a factor of 5, were transported into L1210 cells with similar efficiency. The highest uptake was found for DOXM and was about 3-fold higher than that of DOX and DOXH. However, the higher cellular uptake of this derivative did not translate into an enhancement of its cytotoxic potency against L1210 cells.

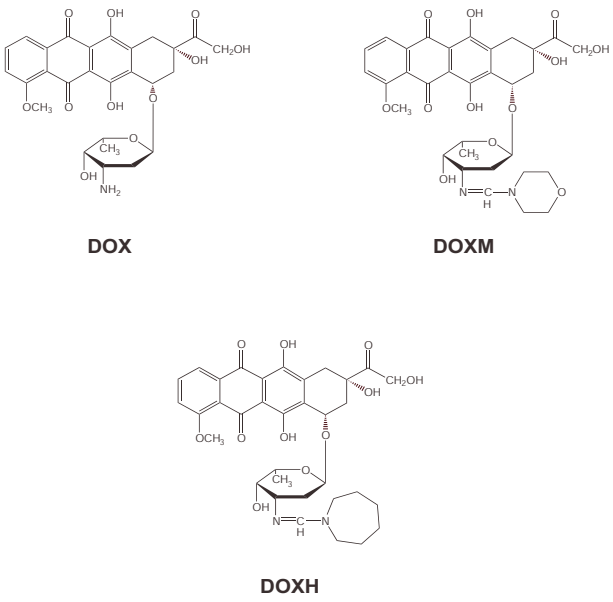

Figure 1. Structures of compounds tested in this study.

To establish the role of the stabilization of DNA topoisomerase II in the mechanism of cell killing by the novel anthracyclines, the effect of ICRF-187, a catalytic inhibitor of the enzyme, on the cytotoxicity of anthracyclines was studied. A 15 min preincubation of cells with 100 μM ICRF-187 significantly reduced the cytotoxicity of the parent anthracycline DOX and etoposide which was used as a reference DNA topoisomerase II cleavage complex stabilizer. In contrast, the cytotoxic activities of DOXM and DOXH were not affected by ICRF-187 (Table 1).

DNA strand breakage

It is known that the production of DNA breaks by trapping DNA topoisomerase II is one of the several mechanisms by which DOX exerts its cytocidal action (Zwelling *et al*., 1981; Minnoti *et*

Table 1. Cytotoxicity and cellular uptake of tested compounds

Compound	Assay without ICRF-187		Assay in the presence of 100 μM ICRF-187		Cellular uptake (pmol/10^6 cells)	
	IC_{50} (nM)	*P*	IC_{50} (nM)	*P*		
DOX	18.3±7.0	N.A.	31.4±4.4	0.049[b]	76.2±3.6	*P*
DOXM	24.4±3.9	0.254[a]	26.0±5.9	0.716[b]	247.5±15.3	<0.001[c]
DOXH	94.0±6.8	<0.01[a]	90.8±7.4	0.603[b]	83.8±4.6	0.05[c]
Etoposide	329±12	N.A.	585±9.3	<0.001[b]	Not tested	

Cells were preincubated for 15 min in the absence or presence of 100 μM ICRF-187 and then incubated for 72 h with anthracyclines or etoposide. Cytotoxicity was determined by MTT assay. All IC_{50} values are means of three independent experiments ±S.D. The significance of differences was analyzed by Student's *t*-test. [a]Cytotoxicity of DOX *vs* cytotoxicity of DOXM and DOXH. [b]Cytotoxicity of tested compounds measured in the absence of ICRF-187 *vs* cytotoxicity measured in the presence of ICRF-187. For uptake studies L1210 cells were exposed to 10 μM drugs for 1 h at 37°C in growth medium and then the amount of anthracyclines accumulated in cells was measured as described under Materials and Methods. Uptake data are means ±S.D. of 7 independent experiments. [c]Uptake of DOXM and DOXH *vs* uptake of DOX. The comparison of uptake of DOX and uptake of DOXM was done by Mann–Whitney *U* test.

al., 2004; Dal Ben *et al*., 2007). To explain whether DOXM and DOXH kill cells by a mechanism involving DNA topoisomerase trapping, we measured DNA strand breaks frequency induced in L1210 cells by alkaline elution of DNA. All the anthracyclines tested produced DNA breaks in a concentration-dependent manner but exhibited significant differences in their DNA breaking potencies (Fig. 2). DOXM appeared to be the most potent DNA breaking agent. The DNA break frequency produced by this compound reached a maximum level in the range of 5–40 μM. At higher DOXM concentrations, the DNA break frequency decreased. The parent anthracycline DOX was also an active DNA breaker but the breaks produced by this drug peaked at a much narrower range of 5–10 μM. When the DOX concentrations exceeded 10 μM, the frequency of DNA breaks decreased. DOXH exhibited a similar dependence between DNA break frequency and the drug concentration as was observed for DOXM, but DOXH was poorly active and produced much less DNA breaks (Fig. 2).

The mechanism of DNA break formation was further investigated by testing whether a catalytic inhibitor of DNA topoisomerase II affects the DNA breakage process induced by anthracyclines. As shown in Fig. 2, ICRF-187 very effectively protected L1210 cells against DNA breaks induced by DOX. In the presence of this drug the DNA break frequency produced by DOX was 8–10 times lower than that measured in the absence of ICRF-187. A protective effect of ICRF-187 against DNA break induced by DOXM was also observed, but the protection was much weaker and the DNA break frequency was lowered only by 10–50% (Fig. 2). A very weak effect of ICRF-187 on the DNA break frequency induced by DOXH was observed (Fig. 2).

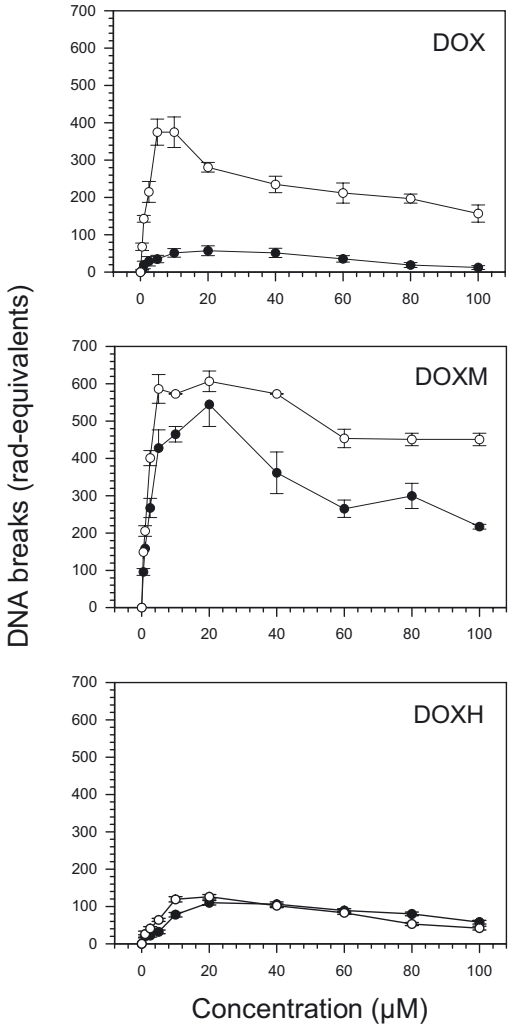

Figure 2. Effect of ICRF-187 on DNA breakage by DOX. L1210 cells were incubated for 15 min in growth medium at 37°C with 100 μM ICRF-187 followed by treatment for 1 h with DOX. The cells were then subjected to alkaline elution. (●) Drug treatment in the presence of ICRF-187. Data are means ±S.D. of three experiments. Statistically significant differences ($P<0.05$) between DNA break frequencies induced by anthracyclines in the absence and in the presence of 100 μM ICRF-187 were found for DOX and DOXM for entire range of concentrations. For DOXH, significant difference ($P<0.05$) was only found for 5 and 10 μM.

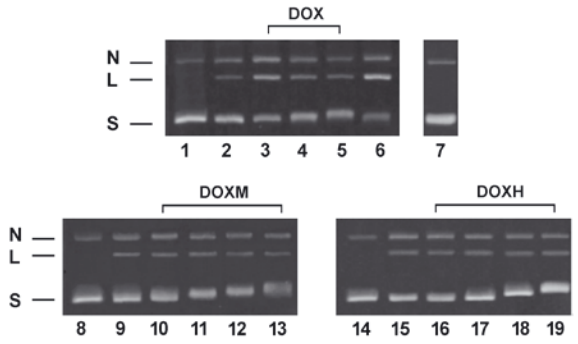

Figure 3. The influence of DOX and its formamidine derivatives on DNA cleavage by topoisomerase II.
Representative images of agarose gels are shown. Supercoiled pBR322 DNA was incubated with 5 units of topoisomerase II in the absence or presence of drugs. *Lanes* 1, 8 and 14, DNA substrate; *lanes* 2, 9 and 15, reaction mixture contained enzyme but no drug; *lanes* 3–5, reaction in the presence of 5, 10 and 25 μM DOX; *lane* 6, 100 μM etoposide; *lane* 7, DNA + 5 μM DOX without enzyme, *lanes* 10–13, reaction in the presence of 5, 10, 25 and 50 μM DOXM; *lanes* 16–19, reaction in the presence of 5, 10, 25 and 50 μM DOXH. **N,** nicked DNA; **L,** linear DNA; **S,** supercoiled DNA.

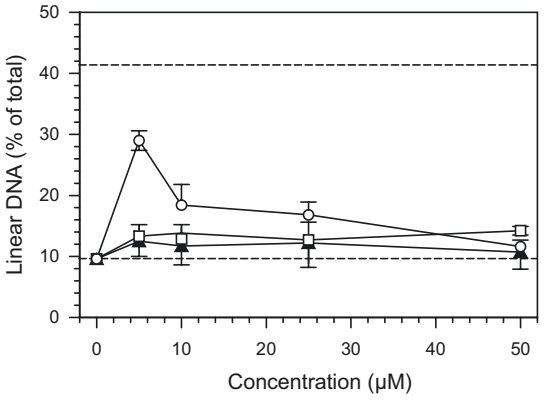

Figure 4. Formation of linear DNA quantified by gel densitometric measurements.
Data are means of three independent experiments ± S.D. Lower dashed line shows average amount of linear DNA in controls without drugs. Upper dashed line shows average amount of linear DNA formed in the presence of 100 μM etoposide. (○) DOX, (□) DOXM, (▲) DOXH. The significance level of differences as assessed by Student's *t*-test: *P* values for control *vs* 5, 10, 25 and 50 μM DOX were 0.002, 0.045, 0.054 and 0.566, respectively. The differences between amount of linear DNA induced by DOXM and DOXH were not statistically significant in the entire range of concentrations, *P* values 0.161–0.867.

The effect of anthracyclines on DNA cleavage by topoisomerase II

The anthracycline derivatives were tested for their effect on DNA cleavage mediated by human DNA topoisomerase II. As demonstrated by the cleavage assay, only DOX enhanced the ability of DNA topoisomerase II to form double and single strand breaks in DNA. DNA bands corresponding to nicked and linear DNA were more intense when the assay was carried out in the presence of 5 or 10 μM DOX (Fig. 3, lanes 3 and 4). The effect of DOX on DNA cleavage was weaker than that of the reference compound etoposide (Fig. 3, lane 6). DOX did not stimulate DNA cleavage itself when incubated with DNA in the absence of topoisomerase II (Fig. 3, lane 7). DOXM and DOXH did not affect DNA cleavage by topoisomerase II (Fig. 4). At higher concentrations of the anthracyclines some changes in the migration rates of supercoiled DNA were visible (Fig. 3, lanes 5, 10, 11, 12, 13, 18, 19). This effect is not related to the action of topoisomerase II, as such an effect was also observed without the enzyme (lane 7).

The results of DNA cleavage assays were quantified by gel densitometry. As shown in Fig. 4, DOX elevated the amount of linear DNA in a concentration-dependent manner. The highest amount of linear DNA was formed at 5 μM. At higher drug concentrations linear DNA was formed much less efficiently. DOXM and DOXH were inactive in this process as the amount of linear DNA formed in the presence of these drugs was similar to that in the control assays (Fig. 4).

DISCUSSION

In this work doxorubicin (DOX) and its formamidine derivatives DOXM and DOXH were studied. It was well known that some compounds containing the amidino group exhibit interesting biological activities (Graut, 1975). Therefore it could be assumed that the transformation of the amino group in the parent doxorubicin to the formamidino group attached to the 3′ position of the daunosamine moiety in the anthracyclines would result in new anthracycline derivatives with better biological properties. Our earlier studies have confirmed this hypothesis (Wąsowska *et al*., 2005; 2006; Wąsowska-Łukawska *et al*., 2007). One of the most interesting features of formamidinoanthracyclines is their ability to circumvent multidrug resistance (MDR). It has been demonstrated for a large group of formamidine derivatives of doxorubicin, epidoxorubicin and daunorubicin that these compounds are able to overcome multidrug resistance of several cell lines resistant to the parent anthracyclines (Wąsowska *et al*., 2005; Wąsowska-Łukawska *et al*., 2007). However, no explanation has been proposed yet for this interesting finding. Multidrug resistance is a multifactorial phenomenon and several drug properties may contribute in its overcoming. We hypothesized that an increase of cellular uptake and/or change in the

mechanism of cytotoxic action (e.g., different cellular targets) might contribute in overcoming MDR by the novel derivatives.

The aim of the current study was to answer the question whether structural changes introduced to the DOX molecule affected the mechanism of cytotoxic action and cellular uptake of the novel anthracyclines. Therefore we compared cytotoxicity, cellular uptake, DNA breakage and inhibitory properties against DNA topoisomerase II of DOX and its two derivatives DOXM and DOXH (Fig. 1).

The mechanism of the anticancer activity of anthracyclines is very complex and despite many years of extensive research in this field it is still not fully understood. It has been proposed in the literature (Minotti *et al*., 2004; Nadas & Sun, 2006; Dal Ben *et al*., 2007), that one of the important mechanisms by which anthracyclines exert biological activity is their interaction with DNA and stabilization of topoisomerase II-DNA cleavable complex, leading to drug induced cell death. As the induction of DNA single strand breaks (SSB) is a typical effect of action of anthracycline topoisomerase II poisons (Zwelling *et al*., 1981; Dal Ben *et al*., 2007), we assayed SSB formation by the anthracyclines tested in L1210 cells. The obtained results showed that among the anthracyclines tested only DOX cytotoxicity is related to its ability to poison topoisomerase II. This is indicated by the results of experiments on the effect of ICRF-187 (Fig. 1) on the cytotoxicity and DNA break frequency produced by DOX and its derivatives. ICRF-187 (Cardioxane) is used in the clinic as a cardioprotective agent and is also a catalytic inhibitor of DNA topoisomerase II (Cvetkovic & Scott, 2005). This compound modulates the action of DNA topoisomerase II-trapping drugs by reducing their DNA breaking and cytotoxic activity (Larsen *et al*., 2003; Lemke *et al*., 2004). ICRF-187 significantly reduced the cytotoxic activity of DOX and etoposide, which was used as a reference compound, but had no effect on the cytotoxicity of DOXM and DOXH (Table 1). This result as well as the much smaller effect of ICRF-187 on DNA break formation by DOXM and DOXH than that caused by DOX (Fig. 2) leads to the conclusion that the introduction of the formamidino group at position 3′ of the daunosamine moiety significantly decreases the affinity of anthracycline for the cleavable complex of topoisomerase II with DNA.

An additional support for the above observation is provided by the data presented in Fig. 4, illustrating the effect of DOX and its derivatives on supercoiled DNA cleavage by purified human topoisomerase II. Only in the presence of DOX was DNA cleavage by topoisomerase II stimulated, as evidenced by a significantly increased amount of linear DNA (Fig. 4). DOX produced a bell-shaped dependence of the linear DNA content on the drug concentration which peaked at 5 μM and then decreased due to autoinhibition. This type of DNA cleavage dependence on drug concentration is a characteristic of DNA topoisomerase II inhibitors which are both intercalators and cleavable complex stabilizers. Intercalating agents used at high concentration unwind DNA and cause that such DNA is not recognized by topoisomerase II as a substrate. For this reason lower concentrations of DOX and other intercalating topoisomerase II poisons are more effective in inducing DNA cleavage. Such an effect has also been described for triazoloacidone and acridine derivatives (see Lemke *et al*., 2004 and references therein).

The formamidine derivatives of DOX were found to be poorly active in stimulating DNA cleavage by topoisomerase II (Figs. 3 and 4). The only change visible in agarose gel images is reduced migration of supercoiled DNA substrate (Fig. 4). This effect is not related to the action of topoisomerase II but seems to be due to the higher weight of DNA–anthracycline complexes when compared to DNA–ethidium bromide complex as molecular weight of DOX and its derivatives is significantly greater than that of ethidium bromide.

Thus the results of experiments performed at the cellular level (cytotoxicity assays and DNA breakage measurements) and the results of DNA cleavage assay are consistent and prove that the stabilization of cleavable complex of topoisomerase II with DNA does not participate in the cytotoxic action of DOXM and DOXH. Therefore the fact that DOXM caused more DNA breaks than DOX, and ICRF-187 could barely reverse this effect can be explained by a new mechanism of DOXM action unrelated to topoisomerase II rather than by an inadequate ICRF-187 amount.

Structural changes in the anthracycline molecule may influence the cellular uptake of the drugs which is important for cell killing action of anticancer drugs. The formamidine derivative (DOXM) was taken up over 3 times more efficiently than DOX, but its cytotoxicity was slightly lower (Table 1). The uptake of DOXH was very similar to that of DOX, but this drug was very feebly active in DNA break formation and also was the weakest cell growth inhibitor (Table 1). These results indicate that changes in the cellular uptake cannot explain the observed differences in the cytotoxic activities of DOX, DOXM and DOXH.

Taking into considerations all biological properties of the tested drugs, a decreased affinity of DOXM and DOXH for the DNA-topoisomerase II cleavable complex seems to be the main reason of their lower cytotoxicity against L1210 cells when compared with DOX. Similar changes in the cyto-

toxic potency of formamidinoanthracyclines was described earlier for daunorubicin derivatives with an identical structural modification as that in DOXM and DOXH (Ciesielska *et al*., 2005). Thus it may be concluded that the structure of the 3′ substituent plays an important role in the recognition of the DNA–topoisomerase II cleavable complex and the presence of the formamidino group reduces the ability of anthracycline antibiotics to poison DNA topoisomerase II. This conclusion is in agreement with the finding of Capranico and his collaborators, who found that topoisomerase II inhibition by anthracyclines depends on the structure of the substituent in the sugar residue (Capranico *et al*., 1997; Dal Ben *et al*., 2007).

The mechanism of cell killing by DOXM and DOXH as well as by other compounds with cyclic amines linked to daunosamine is not fully understood. However, it seems possible that an active metabolite yielded by the reductive metabolism of DOXM and DOXH is responsible for the cytotoxic action of these drugs because they produce DNA breaks by an unknown mechanism not related to topoisomerase II inhibition (Figs. 2–4). It can be assumed that some active metabolites of the anthracyclines tested form DNA adducts by the mechanism proposed by the Phillips group (Cutts *et al*., 2005) and DNA breakage may be a secondary DNA lesion produced by DNA repair. Such a possibility seems to be reasonable as morpholine derivatives of anthracycline have indeed been found to be activated by cellular oxidoreductases (Pawłowska *et al*., 2000) and are able to react with DNA in the presence of the reducing agent formaldehyde (Piestrzeniewicz *et al*., 2004; Szuławska *et al*., 2005).

CONCLUSIONS

Differences in the biological properties of formamidinoantracyclines, such as cytotoxicity, DNA damaging activity and cellular uptake, in comparison to the parent drugs are due to the presence at the 3′ position of the formamidino group, containing a cyclic amine. An advantageous feature of the formamidinoanthracyclines is their mechanism of cytotoxic action which is not related to the inhibition of DNA topoisomerase II, the primary target for the parent drugs. Therefore this class of anthracyclines seems to be a good source from which to select an anticancer drug directed toward cancer cells with the developed multidrug resistance not attributed to the overexpresion of P-glycoprotein but to the presence of altered DNA topoisomerase II.

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