Cytotoxicity and Cellular Uptake of Doxorubicin and its Formamidine Derivatives in HL60 Sensitive and HL60/MX2 Resistant Cells

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Abstract. *Background: In this work a comparison was made of the cytotoxicity and cellular uptake of doxorubicin (DOX) and two of its derivatives containing a formamidino group (–N=CH–N<) at the 3' position with morpholine (DOXM) or hexamethyleneimine (DOXH) ring. All tests were performed in doxorubicin-sensitive HL60 and -resistant HL60/MX2 cells which are known for the presence of altered topoisomerase II. Results: Cytotoxic activity of DOX toward HL60/MX2 cells was about 195 times lower when compared with the sensitive HL60 cell line. DOXM and DOXH were approximately 20 times more active in resistant cells than DOX. It was found that the uptake of DOX was lower in resistant cells by about 16% , while that of DOXM and DOXH was lower by about 36% and 19% , respectively. Thus the changes in the cellular uptake of anthracyclines are not associated with the fact that cytotoxicity of DOXM and DOXH exceed the cytotoxicity of DOX. Experiments in cell-free system containing human topoisomerase II showed that topoisomerase II is not inhibited by DOXM and DOXH. Conclusion: Formamidinoanthracyclines may be more useful than parent drugs in therapy against tumor cells with altered topoisomerase II activity.*

The anthracycline antibiotics are currently widely used as effective anticancer agents. Among numerous series of new anthracyclines synthesized over the past 30 years, some derivatives containing heterocyclic rings are of interest because this class of compounds exhibit lower cardiotoxicity and has a different mechanism of action from those of parent compounds (1). It has been shown that the transformation of the amino group at position 3' of the daunosamine moiety into the formamidino group (–N=CH–N<) resulted in a series of new derivatives exhibiting advantageous biological properties, such as low cardiotoxicity *in vivo* and the ability to overcome multidrug resistance (2-5). Although some features of this novel class of anthracyclines seem to be very promising, there is a lack of biochemical explanation for their low cardiotoxic effects, as well as for their high activity against cell lines with different multidrug-resistance (MDR) phenotypes. Interestingly, many formamidinoanthracyclines were shown to be active against resistant cell lines independently of the mechanism of drug resistance (4, 5). Novel derivatives were highly cytotoxic to cell lines exhibiting overexpression of the *MDR1* gene product P-glycoprotein (Pgp) and to cells with atypical MDR due to the presence of altered DNA topoisomerase II (4, 5). These findings suggest that the presence of a bulky heterocyclic amine bound to the 3' position of an anthracycline *via* a formamidine linker changes the cellular uptake and the mechanism of cytotoxic action of these anthracyclines.

In the present work, we focused on two doxorubicin analogues (Figure 1) with a formamidino group, one containing the cyclic amine moiety morpholine (designated DOXM) and another containing hexamethyleneimine (DOXH). The aim of this study was to determine whether the mechanism by which DOXM and DOXH are able to overcome atypical MDR is due to the enhanced uptake of these drugs or is a result of a different mechanism of cytotoxicity which is not related to topoisomerase inhibition. Both analogues were compared with DOX for their cytotoxicity, cellular uptake and inhibition of DNA topoisomerase II in a cell-free system. All tests were performed on HL60 parental (sensitive) and HL60/MX2 cells. Variant HL-60/MX2 cells are resistant to mitoxantrone due to the lack of the β isoform of topoisomerase II and express a truncated α isoform (6) and references therein.

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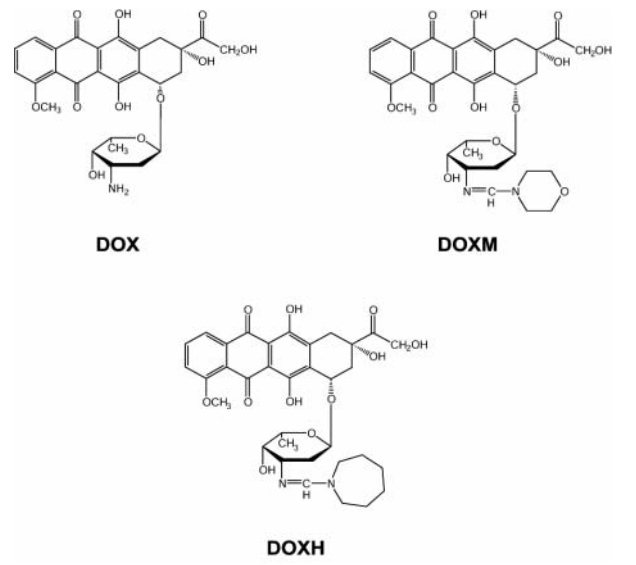

Figure 1. *Structures of tested compounds.*

Materials and Methods

Drugs. The new derivatives of doxorubicin DOXM and DOXH of 97.5 and 97.2% purity respectively, according to an HPLC method, were synthesized at the Institute of Biotechnology and Antibiotics in Warsaw, by treatment of the parent doxorubicin with active derivatives of formylamines (3). Etoposide was purchased from Sigma (St Louis, MO, USA). Anthracycline derivatives and etoposide were dissolved in water and made up fresh for each experiment.

Cell lines and cell culture. The human promyelotic HL60 and HL60/MX2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in RPMI-1640 (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Scotland, UK), 5 mM HEPES buffer (Sigma) in a humidified 5% CO_2 atmosphere at 37˚C.

Cytotoxicity assay. The cytotoxic activity of tested compounds was assayed by measuring their inhibitory effects on HL60 and HL60/MX2 cell proliferation. To measure cytotoxicity of doxorubicin and its analogues, a tetrazolium dye method was used (7). Cells in logarithmic growth phase were seeded at 1×10^4 cells/well in 48-well plates and incubated with different concentrations of antracyclines (in the range of 0.005 μM – 0.5 μM) for 72 hours in triplicates in a final volume of 1 ml. A total of 50 μl of sterile aqueous solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (Sigma) (5 mg/ml) was added to each well for additional 3 hours. The blue formazan precipitate was dissolved in DMSO (Sigma) and the absorbance of solutions was measured at 540 nm. For each experiment, determinations were carried out on three replicates. 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 fit to an exponential equation using X-ACT v. 3 statistical software (SciLab, Hamburg, Germany).

Cellular uptake. Uptake of anthracyclines was estimated by fluorimetric measurements after extraction of tested compounds with ethanol/HCl mixture as described by Riganti *et al*. (8). HL60 and HL60/MX2 cells at 10^7 cells/ml were treated in growth medium with 10 μM DOX, DOXM and DOXH at 37˚C. After treatment, aliquots of cell suspension were mixed with 5 ml of ice-cold phosphate-buffered saline (PBS) and centrifuged at 1,500 rpm for 5 min, then washed twice with ice-cold PBS. The cell pellets were collected and 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. Optimal excitation and emission wavelengths were 485 and 590 nm, respectively. To estimate the drug concentrations, calibration curves in the concentration range of 0.01-0.5 μM were prepared. 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.

Topoisomerase II DNA cleavage assay. The procedure of Lemke *et al*. (9) was used with minor modifications. The reaction mixture of total volume 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, Vilnius, 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 definition). The reactions were stopped by adding sodium dodecyl sulfide and proteinase K, final concentrations 0.35% and 0.3 mg/ml, respectively. After another 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) were added. Samples were loaded on 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. Gel was then destained in distilled water and photographed on Ilford FP4 film (ISO 125, format 6×9.5 cm). Developed film negatives were scanned at 300 dpi, then the digitized images were analyzed by Gel Scan v.1.45 software (Kucharczyk T.E., Poland). The amount of linear DNA in each line was calculated as a percentage of total DNA, assuming that total DNA was the sum of peaks areas corresponding to supercoiled, nicked and linear DNA.

Results

The dose-response cytotoxicity potencies of HL60 and HL60/MX2 cells after 72 hours' exposure to different anthracycline derivatives are presented in Table I. For HL60 cells, of the anthracyclines tested the greatest cytotoxic activity was shown by the parent drug DOX. The IC_{50} for DOX was ~10 nM, whereas its derivatives (DOXM and DOXH) were almost 3.5 times less active. In experiments performed on the HL60/MX2 cell line, the cytotoxic activity of DOX towards these resistant cells was ~200 times lower when compared with HL60. In this case, the resistance index was about ~195 (resistance index is the ratio of the IC_{50} value for the resistant cell line to the IC_{50} value for the drug-sensitive cell line). The formamidine derivatives of DOX

Table I. *Cytotoxicity of anthracyclines toward HL60 and HL60/MX2 cells as determined by MTT assay.*

Compound	Cytotoxicity, IC_{50} (nM)		Resistance index (RI)
	HL60	HL60/MX2	
DOX	9.4±1.8	1823±2*	194.9
DOXM	32.3±6.0	58±3*	1.8
DOXH	34.5±0.8	82±6*	2.4

All values are the means of three independent experiments, each performed in triplicate±S.E.M; *$p<0.05$. Resistance index (RI) is the ratio of the IC_{50} value for the resistant cell line to the IC_{50} value for the drug-sensitive cell line.

Table II. *Uptake of anthracyclines into HL60 and HL60/MX2 cells. HL60 and HL60/MX2 cells were exposed to 10 μM drugs for 1 h at 37˚C in growth medium and then uptake of anthracyclines was measured as described under Materials and Methods.*

Compound	Cellular uptake (pmoles/10^6 cells)		Relative uptake (%)
	HL60	HL60/MX2	
DOX	220±3	185±5	84
DOXM	511±13*	328±4*	64
DOXH	334±15*	273±15*	82

All values are the means of three independent experiments, each performed in triplicate±S.E.M. *Difference in uptake between DOX and its derivatives is significant at $p<0.05$. Relative uptake is the ratio of the drug uptake measured for resistant cells to that of the sensitive cell line ×100%.

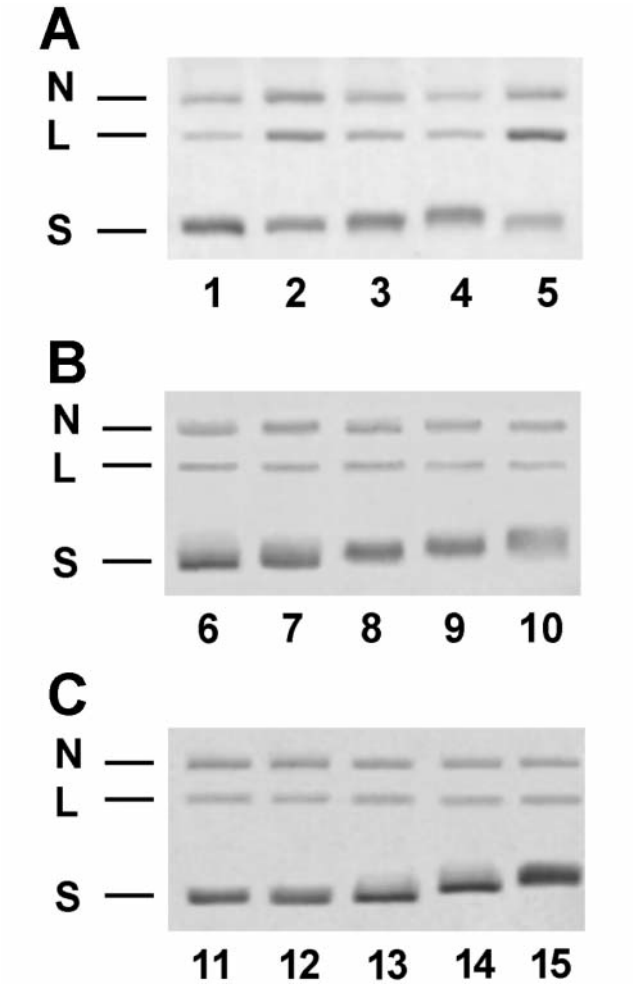

Figure 2. *The effect of DOX and its formamidinoderivatives DOXM and DOXH on DNA cleavage by topoisomerase II. Representative images of agarose gels are shown. Cleavage assay was performed in the absence or presence of drugs as described under Materials and Methods. A, Effect of DOX: lane 1, control (reaction mixture carried out without drug); lanes 2-4, reaction in the presence of 5, 10 and 25 μM DOX; lane 5, 100 μM etoposide. B, Effect of DOXM: lane 6, control; lanes 7-10, reaction in the presence of 5, 10, 25 and 50 μM DOXM. C, Effect of DOXH: lane 11, control; lanes 12-15, reaction in the presence of 5, 10, 25 and 50 μM DOXH. N, nicked DNA; L, linear DNA; S, supercoiled DNA. Under these conditions of electrophoresis, relaxed DNA migrates along with supercoiled DNA and these forms cannot be distinguished.*

also exhibited decreased cytotoxicity toward HL60/MX2 cells as compared to that for HL60 cells but the difference in cytotoxic activity toward resistant and sensitive cells was much lower, with resistance indices of 2.4 and 1.8 for DOXM and DOXH, respectively. Both formamidino derivatives of DOX were approximately 20 times more active toward resistant cells than was the parent drug (Table I). The derivative possessing the morpholine ring (DOXM) exhibited the highest cytotoxic activity against HL60/MX2 cells and was slightly more active than the derivative with the hexamethyleneimine moiety (DOXH).

All studied anthracyclines – DOX and its derivatives – were taken up less efficiently by HL60/MX2 cells when compared with HL60 cells. Cellular uptake levels of these compounds expressed as pmoles/10^6 cells are shown in Table II. Both formamidinoanthracyclines were taken up more efficiently than DOX by the sensitive as well as the resistant cell line. It was found that the uptake of DOX was lower in resistant cells by 16%. The uptake of DOXM and DOXH was also lower by about 36% and 18%, respectively. The most "penetrating" compound of the examined anthracyclines was DOXM.

To examine whether the cytotoxicity of tested anthracyclines is linked to the ability of these drugs to inhibit activity of DNA topoisomerase II, DOX and its derivatives were tested for their effect on DNA cleavage mediated by human DNA topoisomerase II. As shown in Figure 2, among the tested anthracyclines, only DOX was an efficient stimulator of supercoiled DNA cleavage mediated by topoisomerase II. This is indicated by more intense DNA bands corresponding to nicked and linear DNA

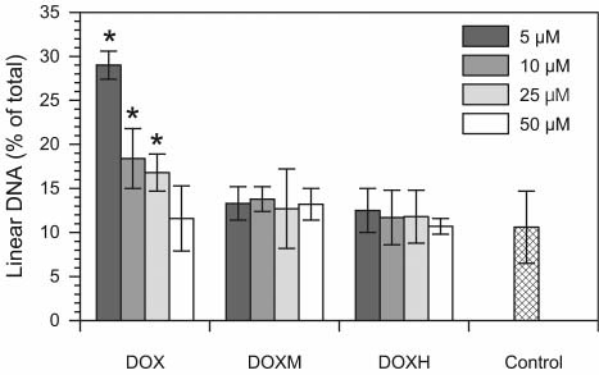

Figure 3. *Stimulation of supercoiled pBR322 DNA cleavage by tested compounds. The amount of linear DNA was estimated by gel densitometric measurements. Data are means of three independent experiments±SD. *Amount of linear DNA formed in the presence of drug was significantly higher than that in the control assay, p<0.05.*

when the assay was carried out in the presence of 5 and 10 μM DOX (Figure 2, lanes 3 and 4). The effect of 5 μM DOX was similar to that of 100 μM etoposide which was used for comparison as a classical topoisomerase II inhibiting drug (Figure 2, lane 6). Neither formamidine derivative affected DNA cleavage by topoisomerase II (Figures 2 and 3). At concentrations exceeding 5 μM, all anthracyclines lowered migration rates of supercoiled DNA (Figure 2, lanes 3-5, 8-10, 11-15. This effect was not related to the topoisomerase II action but reflects DNA unwinding caused by anthracycline intercalation into the DNA double helix. The results of DNA cleavage assays were analyzed by gel densitometry. As shown in Figure 3, DOX increased the amount of linear DNA in a concentration-dependent manner. The highest amount of linear DNA was formed at 5 μM. At higher DOX concentrations, linear DNA was formed much less efficiently and at 50 μM DNA cleavage was not stimulated. DOXM and DOXH were inactive in this process as the amount of linear DNA formed in the presence of tested compounds was close to that in the control assays over the entire range of tested concentrations (Figure 3).

Discussion

DNA topoisomerase II is known to be a critical target of anticancer drugs which are able to stabilize the so-called cleaved complex (a transient complex of topoisomerase II with cleaved DNA). Some important anticancer agents such as etoposide, doxorubicin and mitoxantrone belong to this group of drugs (1, 6, 10). Resistance to topoisomerase inhibitors may be due to several different factors: reduced drug accumulation resulting from overexpression of drug transporting proteins such as P-gp and MRP (11, 12); changes of cytotoxic signaling leading to apoptotic cell death (6); and mutation in or altered expression of DNA topoisomerase II genes (10). As development of resistance to anthracyclines and anthracenediones by cancer cells is a serious problem which reduces the clinical efficacy of these agents, it is necessary to design new drugs which may overcome resistance to commonly used drugs such as doxorubicin, daunorubicin and mitoxantrone. Formamidine derivatives of anthracyclines seem to be a good solution for achieving this goal. Earlier papers on this group of compounds demonstrated that several cell lines resistant to DOX and daunorubicin were sensitive to formamidino-anthracyclines (4, 5). Because formamidine derivatives of anthracyclines were found to be active against cell lines with different phenotypes of MDR, including those whose mechanism of resistance depends on the expression of P-gp and MRP or on the presence of altered topoisomerase II (4, 5), it can be hypothesized that this group of formamidinoanthracyclines was taken up more efficiently by resistant cells and possesses a different mechanism of action when compared with parent compounds.

In this work, we attempted to find a biochemical explanation for the fact that DOXM and DOXH are active against HL60/MX2 cells, which are known for the presence of altered topoisomerase II. Our results provide conclusive evidence that the presence of a formamidine group containing morpholine or hexamethyleneimine ring at the 3' position enhances the uptake of anthracycline drugs by HL60 and HL60/MX2 cells. This effect is especially pronounced in the case of DOXM (Table II). These results agree with data obtained for daunorubicine derivatives possessing an identical structural modification which were tested on non resistant L1210 murine leukemia cells (13). Therefore it can be concluded that addition of a morpholine ring in formamidino-anthracyclines significantly enhances the ability of these drugs to penetrate cell membranes. The reduced uptake of DOXM and DOXH by HL60/MX2 cells may explain why these drugs were approximately twice less cytotoxic against this cell line when compared with HL60 cells. However, changes in the cellular uptake of tested anthracyclines are not clearly associated with the fact that cytotoxicity of both DOXM and DOXH exceeded that of DOX against resistant HL60/MX2 cells. Data provided by the experiments on the effect of DOX and its derivatives on supercoiled DNA cleavage by topoisomerase II in a cell-free system indicated that contrary to the effect of DOX, DOXM and DOXH do not stabilize the cleavable complex of topoisomerase II with DNA (Figures 2 and 3). Thus it may be concluded that topoisomerase II is not the primary target of DOXM and DOXH and that high cytotoxic activity of these compounds toward HL60/MX2 cell line is due to a mechanism of action which is not related to DNA topoisomerase II inhibition.

Conclusion

The introduction of a formamidine group containing morpholine or hexamethyleneimine ring at the 3' position appears to be a useful way to obtain derivatives of anthracyclines which are highly cytotoxic against tumour cells which have developed multidrug resistance resulting from altered topoisomerase II activity. These data support the opinion that the majority of formamidinoanthracyclines may appear to be more useful in therapy than the parent antibiotics due to their reduced adverse effects (cardiotoxicity) and very good antiproliferative activity toward resistant cancer cells.

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