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RELEVANCE OF INTERSTRAND DNA CROSSLINKING INDUCED BY ANTHRACYCLINES FOR THEIR BIOLOGICAL ACTIVITY

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Abstract—The relevance of interstrand DNA crosslinking induced by anthracyclines for their cytotoxic action was studied in several biological systems with cells differing in sensitivity towards these compounds. It was done by establishing the correlation between DNA crosslinking and cytotoxic activity of anthracyclines. The study showed that there is a strong positive correlation between cytotoxic activity of anthracyclines and their DNA crosslinking potency in HeLa S_3 cells for a group of six Daunomycin derivatives ($r = 0.97$) as well as for all the studied 13 anthracyclines of divergent chemical structure ($r = 0.95$). Similar relationships between cytotoxic activity and DNA crosslinking ability was found for Adriamycin® and Daunomycin in three other cellular systems: (i) in LoVo and about 20-fold Adriamycin-resistant LoVo/DX human colon adenocarcinoma cells, (ii) CHO-K1 and its Adriamycin-hypersensitive mutant CHO-ADR5 Chinese hamster ovary cells and (iii) HeLa S_3 cells sensitized about 3-fold to cytotoxic action of Adriamycin and Daunomycin by lowering intracellular glutathione content, to about 10% of normal level, by buthionine sulfoximine treatment. The presented results show that DNA crosslinking induced by anthracyclines may be responsible for the cytotoxic activity of these compounds.

Key words: anthracyclines; interstrand DNA crosslinking; mechanism of action

We have shown previously in our laboratory that ADRIA†, DAUNO and several other anthracyclines, most of them used clinically, induce interstrand crosslinks in DNA of tumor cells [1 and accompanying paper]. We have also found that metabolic activation within the cell is necessary for DNA crosslinking by anthracyclines.

The current investigations were performed in an attempt to investigate the relevance of ability to induce DNA crosslinks by anthracyclines for their cytotoxic and antitumor activity. For this, we correlated DNA crosslinking induced by anthracyclines with their cytotoxic activity produced in different cellular systems: (i) against HeLa S_3 for anthracyclines of divergent cytotoxic and antitumor activity; (ii) for selected anthracyclines against cell lines differing in sensitivity to these antibiotics. In the latter system, we used cell lines resistant as well as hypersensitive to anthracyclines, and additionally, cells artificially sensitized to anthracyclines by lowering intracellular GSH content.

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† Abbreviations: ADRIA, Adriamycin; DAUNO, Daunomycin; IODO, Iodorubicin; DR 28, 3′-*N*-[α-(L)-alanyl]-Daunomycin; DR 31, *N*,*N*-dibenzyl-α-(L)-alanyl-Daunomycin; GSH, glutathione; BSO, *S*,*R*-buthionine sulfoximine; PBS, phosphate-buffered saline: 0.17 M sodium chloride, 0.27 mM potassium chloride, 8.1 mM disodium hydrogen phosphate, 0.27 mM potassium dihydrogen phosphate, pH 7.2; SSC, sodium citrate-saline solution: 0.15 mM sodium chloride, 0.015 mM sodium citrate, pH 7; RI, resistance index; CI, crosslinking index; F_{CR}, fraction of crosslinked DNA.

MATERIALS AND METHODS

Chemicals. Sodium perchlorate was from Fluka AG (Buchs, Switzerland). *N*-lauroyl sarcosine, *o*-phtaldialdehyde, nuclease S_1, BSO and the reduced form of GSH were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [methyl-^{14}C]Thymidine (40 Ci/mmol) was from Amersham International (U.K.). All other reagents used were of analytical grade.

Drugs. ADRIA, DAUNO, 4′-epi-ADRIA (Epirubicin), 4′-deoxy-4′-iodo-ADRIA (IODO), 4-demethoxy-DAUNO (Idarubicin), 3′-hydroxy-4′-deoxy-4′-amino-ADRIA and 3′-deamino-3′-hydroxy-DAUNO were kindly provided by Dr Fernando C. Giuliani (Farmitalia Carlo Erba Research and Development, Nerviano, Italy). 13-Hydroxy-DAUNO (Daunorubicinol) and 3-oxime-DAUNO, were obtained from the National Cancer Institute (Natural Products Branch, Bethesda, MD, U.S.A.) by courtesy of Dr Matthew Suffness. *N*-acetyl-DAUNO, DR 28, and DR 31 were provided by Dr Maria Dzieduszycka and Dr Barbara Stefańska from this Department.

Cell culture and media. HeLa S_3 cells, the media, glutamine, vitamins (BME vitamin solution X100), and fetal calf serum were from Gibco Europe (Paisley, U.K.). Antibiotics were from Serva (Heidelberg, Germany).

HeLa S_3 cells were grown in spinner culture in Joklik's modified minimal essential medium supplemented with 5% fetal calf serum and antibiotics (streptomycin, 100 μg/mL; penicillin 100 U/mL).

LoVo and ADRIA-resistant LoVo/DX human adenocarcinoma cells were kindly provided by Dr

Maria Grandi (Farmitalia Carlo Erba Research and Development, Nerviano, Italy). The cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum, glutamine (3 mM), 1% BME vitamins, and antibiotics (streptomycin, 100 μg/mL; penicillin 100 U/mL). LoVo/DX cells were grown with 100 ng/mL ADRIA added to the growth medium at every passage; however, all experiments using the LoVo/DX cells were performed with cells that had been passagged without ADRIA for 1 week.

CHO-K1 and its hypersensitive mutant CHO-ADR5 Chinese hamster ovary cells were a generous gift from Dr Ian D. Hickson (Imperial Cancer Research Fund, Oxford, U.K.). CHO-K1 and CHO-ADR5 cells were maintained in Ham's F10 medium supplemented with 10% fetal calf serum, glutamine (1 mM), and antibiotics.

All the cell lines were grown at 37° in a humidified 5% CO_2–air atmosphere.

Cytotoxicity assay. Cytotoxic activities of anthracyclines against all the cell lines were determined after 3-hr treatment as described earlier (see accompanying paper).

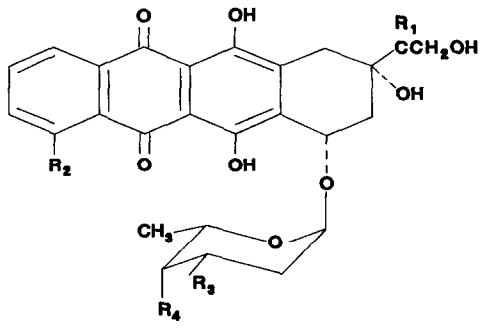
Drug treatment and GSH depletion. The DNA in exponentially growing cells was labeled by adding [methyl-^{14}C]thymidine into the medium to a final concentration of 0.02 μCi/mL. After overnight incubation, cells were washed with growth medium, aliquoted (3×10^6/sample) and incubated with various anthracyclines at 37°. After 3-hr treatment, incubation was stopped by dilution with 10 mL of ice-cold PBS and centrifugation of the cells. The cells were washed twice with PBS and resuspended in 0.4 mL SSC.

In experiments with reduced GSH content, HeLa S_3 cells were pre-incubated with 0.1 mM buthionine sulfoximine for 24 hr, the cells were then washed with growth medium and incubated with the drugs as described above.

DNA crosslinking assay. The procedure applied was a modification of the method described by Parsons [2] and was described in detail elsewhere (see accompanying paper).

Determination of reduced GSH. The fluorimetric method for the determination of reduced GSH was used [3]. Cells were washed twice with cold PBS, resuspended at 1×10^6 cells/mL in PO_4–EDTA buffer (100 mM sodium dihydrogen phosphate-disodium hydrogen phosphate, 5 mM EDTA, pH 8), sonicated and precipitated in 5% phosphoric acid. The resulting supernatant was diluted 1:10 with PO_4–EDTA buffer. The final assay mixture (4 mL) contained 200 μL of the diluted supernatant, 3.6 mL of PO_4–EDTA buffer, and 200 μL of *o*-phtaldialdehyde (1 mg/mL in methanol). After thorough mixing and incubation at room temperature for 15 min, the fluorescence emission at 420 nm was determined following excitation at 350 nm on Perkin-Elmer LS-5B Luminescence Spectrometer. The concentration of GSH was read from a standard curve and expressed in terms of nmol/1×10^6 cells.

Intracellular concentration of anthracyclines. Cells (1×10^6/2 mL of growth medium) were incubated with the compounds for 3 hr. Following incubation with the compounds, the cells were trypsinized and washed twice with 4 mL of ice-cold PBS. Anthracyclines were extracted overnight in the dark at 0° with 3 mL of 1:1 mixture of 0.3 M HCl and 50% ethanol. The suspensions were centrifuged and fluorescence of the supernatants was measured with Perkin-Elmer LS-5B Luminescence Spectrometer at excitation and emission wavelengths of 474 nm and 580 nm for ADRIA, 480 nm and 580 nm for DAUNO and IODO, respectively. The content of the drugs was computed from standard curves prepared from the solutions of anthracyclines in 50% ethanol–0.3 M HCl (1:1).



compound	R_1	R_2	R_3	R_4
DAUNORUBICINOL	OH	OCH_3	NH_2	OH
3-oxime-DAUNO	=NOH	OCH_3	NH_2	OH
N-acetyl-DAUNO	=O	OCH_3	$NHCOCH_3$	OH
DR-28	=O	OCH_3	$NHCOCHNH_2$ (CH_3)	OH
DR-31	=O	OCH_3	$NHCOCHNBzl_2$ (CH_3)	OH
IDARUBICIN	=O	H	NH_2	OH

Fig. 1. Chemical structures of the studied derivatives of DAUNO.

Statistical test. The correlation between cytotoxic activity of anthracyclines studied and their DNA crosslinking properties was analysed by means of the non-parametric Spearman's rank correlation test on the basis of *S*-statistics [4]. The results were statistically evaluated by Student's *t*-test.

RESULTS

(i) Correlation between cytotoxicity and interstrand DNA crosslinking

In this system, we studied a correlation between cytotoxic activity and ability to induce interstrand crosslinks in DNA of HeLa S_3 cells by a group of structurally related DAUNO analogs (for chemical structures see Fig. 1), exhibiting divergent cytotoxic activity towards these cells. Both DNA crosslinking and cytotoxic activity of the studied anthracyclines were determined after 3 hr treatment with the studied compounds. All the DAUNO derivatives, except for biologically inactive DR 31 induced interstrand crosslinks in DNA of HeLa S_3 cells in a dose-dependent manner (Fig. 2, A–F). For each compound

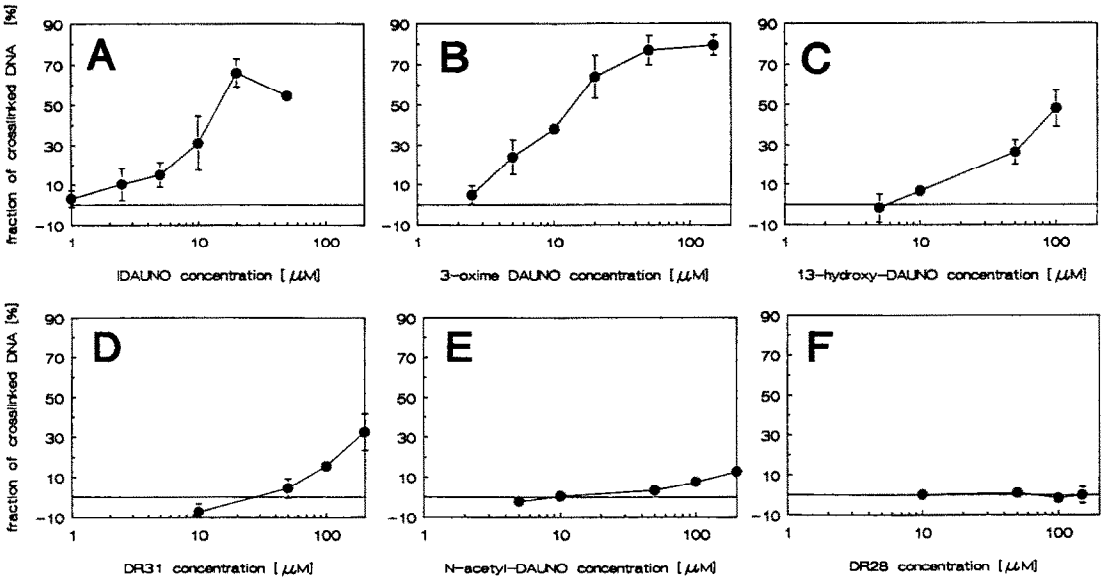


Fig. 2. DNA crosslinking induced by DAUNO derivatives in HeLa S_3 cells. The cells were incubated with the compounds for 3 hr and fractions of crosslinked DNA were then determined as described in Materials and Methods. Points, means from three independent experiments run in duplicate; bars, SD.

Table 1. The comparison between crosslinking potency (C_0) and cytotoxic activity (EC_{90}) of the DAUNO derivatives

	EC_{90} [μM]*	C_0 [μM]†
DAUNO	0.64 ± 0.10	3.7 ± 1
Idarubicin	0.06 ± 0.003	1.3 ± 0.4
13-hydroxy-DAUNO	2.54 ± 0.10	6.1 ± 0.6
N-acetyl-DAUNO	2.82 ± 0.23	13.6 ± 1.6
3-oxime-DAUNO	3.00 ± 0.2	2.41 ± 0.3
DR 31	6.54 ± 0.44	23.2 ± 2.1
DR 28	>20	—

Both C_0 and EC_{90} values were determined after 3-hr incubation with the compounds.

* EC_{90}, concentration required to inhibit growth of HeLa S_3 cells by 90% compared to non-treated cells.

† C_0, concentration at which first DNA crosslink can be detected under experimental conditions applied.

C_0 value, corresponding to concentration at which the first crosslink could be detected (Table 1), was calculated as described earlier (see previous paper in this issue). Similarly as it was found for DAUNO and other anthracyclines studied previously, interstrand DNA crosslinking induced by DAUNO analogs was observed only in cell systems, hence metabolic activation by cellular enzymes is a prerequisite for DNA crosslinking by these compounds (data not shown). The results presented in Fig. 3 show that there is strong positive correlation ($r = 0.97$) between cytotoxicity (EC_{50}) of DAUNO derivatives and their ability to form interstrand DNA crosslinks, expressed as drug concentration C_0 at which the appearance of the first crosslink could be detected. Similar significant positive correlation ($r = 0.95$) between these two determinants could be found when we analysed all the anthracyclines studied by us so far, that is DAUNO analogs as well as the anthracyclines studied previously (see accompanying paper) which comprise ADRIA and its analogs and II class anthracyclines (Aclacinomycin A, Marcellomycin, and Cinerubin A). These least-squares analyses were further strengthened by the results of non-parametric Spearman's rank correlation test of the C_0 and EC_{50} values (cf. Fig. 3).

(ii) DNA crosslinking in LoVo and ADRIA-resistant LoVo/DX cells

LoVo, human colon adenocarcinoma cells, are about 20 times more sensitive to ADRIA and DAUNO than its resistant counterpart LoVo/DX (Table 2). We studied whether there are any differences in DNA crosslinking induced by anthracyclines between the two lines. Figure 4 (panels A–C) presents results of such experiments. In the case of ADRIA and DAUNO, DNA crosslinking produced by these compounds was lower in LoVo/DX than in LoVo cells. For IODO, which is not cross-resistant with the other two anthracyclines and whose sensitivity towards both cell lines is similar, no significant difference could be observed in DNA crosslinking induced by this compound (Fig. 4, panel C). Moreover, the RIs and CIs calculated for all three anthracyclines were comparable (Table 2). The resistant LoVo/DX cells accumulated about 2-fold less ADRIA and DAUNO than the parent LoVo cell line (Fig. 5, panel A and B). As for IODO to which LoVo/DX cells are only marginally resistant (RI = 1.2), there was no significant difference in the level of drug accumulated by LoVo and LoVo/DX cells (Fig. 5, panel C). We addressed the question of whether decreased DNA crosslinking in LoVo/

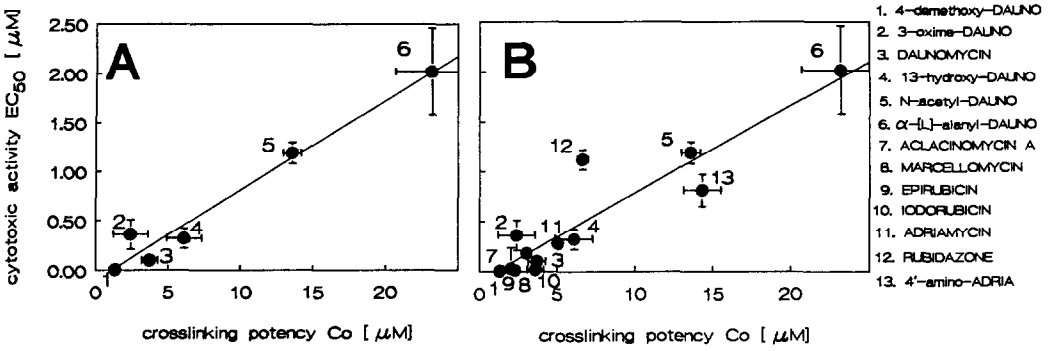


Fig. 3. Correlation between *in vitro* cytotoxic activity of anthracyclines and their interstrand crosslinking potency against DNA of HeLa S_3 cells for DAUNO analogs (panel A) and all the studied anthracyclines (panel B). The data points fit a straight line with linear coefficient $r_{14} = 0.97$ (panel A) and $r_{14} = 0.95$ (panel B) ($\gamma = 0.95$) and non-parametric Spearman's rank correlation evidences a significant correlation ($\gamma = 0.95$) with $r_s = 0.867$ and $S_{14} = 540$ (panel B).

Table 2. Comparison between cytotoxic activity and DNA crosslinking potency of anthracyclines in LoVo and ADRIA-resistant LoVo/DX cells

Compound	$C_{0\,LoVo}$* (μM)	$C_{0\,LoVo/DX}$ (μM)	CI†	RI‡
ADRIA	2.52	56.30	22.5	17.9
DAUNO	2.82	53.60	19.0	20.5
IODO	3.06	6.10	2.0	1.2

Both cytotoxic activity and DNA crosslinking were determined after 3-hr treatment with the drugs.

* C_0, concentration at which first DNA crosslink can be detected.

† CI, crosslinking index, ratio $C_{0\,LoVo/DX}/C_{0\,LoVo}$

‡ RI, resistance index, ratio $EC_{50\,LoVo/DX}/EC_{50\,LoVo}$

DX cells is due to lower accumulation of ADRIA and DAUNO in these cells. When we compared DNA crosslinking induced by these compounds at equal intracellular concentrations there was still a marked difference between LoVo and LoVo/DX cell lines in the level of induced DNA crosslinks (data not shown).

(iii) DNA crosslinking in CHO-K1 and ADRIA-hypersensitive CHO-ADR5 cells

ADRIA was about three times more cytotoxic against CHO-ADR5 than CHO-K1 cells (Table 3). The observed increase in cytotoxicity of ADRIA against CHO-ADR5 cells did not result from enhanced intracellular accumulation of the drug because no difference could be detected in the total amount of ADRIA accumulated after a 3 hr incubation between CHO-K1 and CHO-ADR5 cells (Fig. 6). We also observed the levels of interstrand crosslinks induced by ADRIA in DNA of CHO-K1 and CHO-ADR5 cells (Fig. 7). At ADRIA concentrations higher than 10 μM, the level of DNA crosslinking induced by ADRIA in CHO-ADR5 cells was higher compared to CHO-K1 cells. At

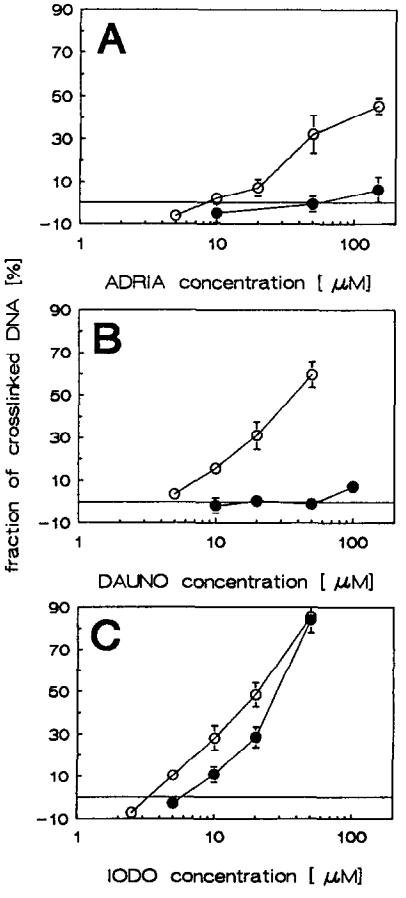


Fig. 4. DNA crosslinking induced by anthracyclines in LoVo (○) and LoVo/DX (●) cells. The cells were incubated with drugs for 3 hr and fractions of crosslinked DNA were determined as described in Materials and Methods. Points, means from three independent experiments run in duplicate; bars, SD.

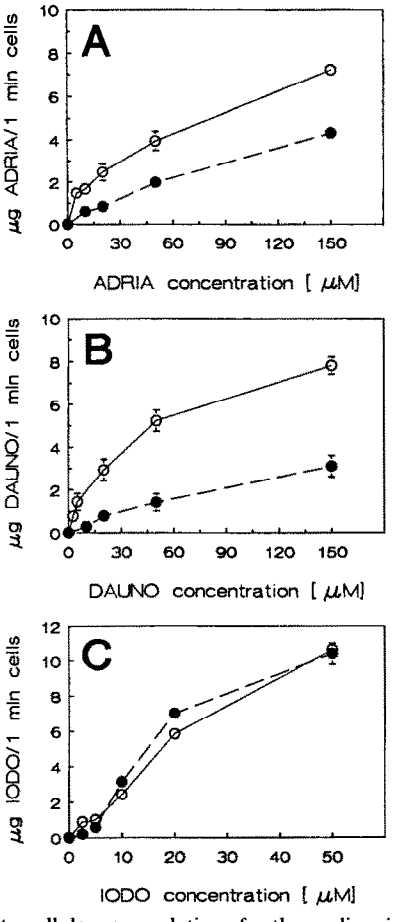


Fig. 5. Intracellular accumulation of anthracyclines in LoVo (○) and ADRIA-resistant LoVo/DX (●) cells following 3-hr incubation with drugs. Points, means from three independent experiments run in triplicate; bars, SD. Occasionally, the confidence intervals were too small to be shown.

Table 3. Cytotoxic activity (EC_{50}) of ADRIA against wild-type CHO-K1 and its hypersensitive mutant CHO-ADR5 cells following 3-hr treatment with the drug

Cell line	EC_{50}* (μ/mL)	Ratio
CHO-K1	0.46 ± 0.06†	3.1
CHO-ADR5	0.14 ± 0.07†	

* EC_{50}, concentration required to inhibit growth of cells by 50% compared to non-treated controls.
† Statistically different ($P < 0.005$).

concentrations below 10 μM, it seems that DNA damage was extensively incurred by the drug which precluded quantitation of DNA crosslinks induced by Adriamycin in this range of concentrations.

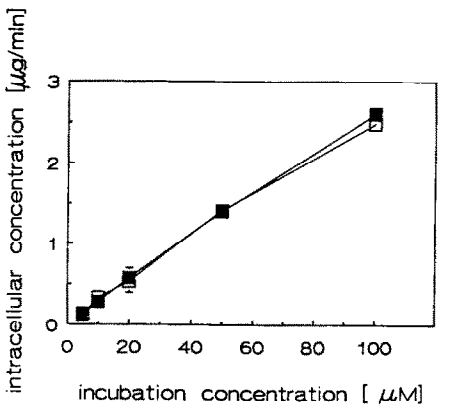


Fig. 6. Intracellular accumulation of ADRIA in CHO-K1 (■) and CHO-ADR5 (□) cells following 3-hr treatment with the drug. Points, means from three independent experiments run in triplicate; bars, SD. Occasionally, error bars were too small to be shown.

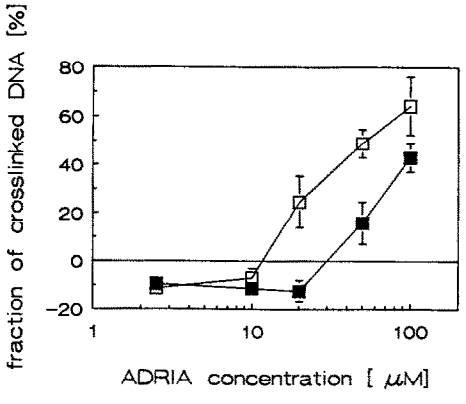


Fig. 7. DNA crosslinking induced by ADRIA in CHO-K1 (■) and CHO-ADR5 (□) cells. The cells were incubated with the drug for 3 hr and fractions of crosslinked DNA were determined as described in Materials and Methods. Points, mean from three independent experiments run in duplicate; bars, SD.

(iv) Potentiation of anthracycline-induced cytotoxicity and interstrand DNA crosslinking by BSO

Addition of BSO (0.1 mM, 24 hr) to exponentially growing cultures of HeLa S_3 cells, produced a decrease in cellular GSH content to about 10% of that observed in control cells. The incubation of cells with BSO had no effect on cell proliferation, nor did it change viability of the cells as determined by Trypan blue exclusion test. Pretreatment with 0.1 mM BSO for 24 hr significantly potentiated the cytotoxic effects of ADRIA and DAUNO (Table 4). The EC_{50} values of ADRIA and DAUNO were, respectively, 5.0- and 5.1-fold lower for cells with reduced GSH content. The enhancement of both ADRIA- and DAUNO-induced cytotoxicity against cells treated with BSO was statistically significant

Table 4. Augmentation of anthracyclines' cytotoxicity by BSO-mediated GSH depletion in HeLa S_3 cells

Compound	EC_{50} (μM) Without (0.1 mM, 24 hr)	With BSO pretreatment (0.1 mM, 24 hr)	Ratio
ADRIA	0.27 ± 0.01*	0.053 ± 0.005*	5.1
DAUNO	0.10 ± 0.01*	0.020 ± 0.003*	5.0

* Statistically different ($P < 0.001$).

Table 5. Increase in DNA-crosslinking potency of anthracyclines (expressed as C_0 values) in HeLa S_3 cells after pre-incubation with BSO

Compound	Crosslinking potency C_0 (μM) Without (0.1 mM, 24 hr)	With BSO pretreatment (0.1 mM, 24 hr)	Ratio
ADRIA	3.71 ± 1.5*	1.01 ± 0.45*	3.7
DAUNO	5.06 ± 1.3†	2.28 ± 0.32†	2.2

* Statistically different ($P < 0.10$).
† Statistically different ($P < 0.05$).

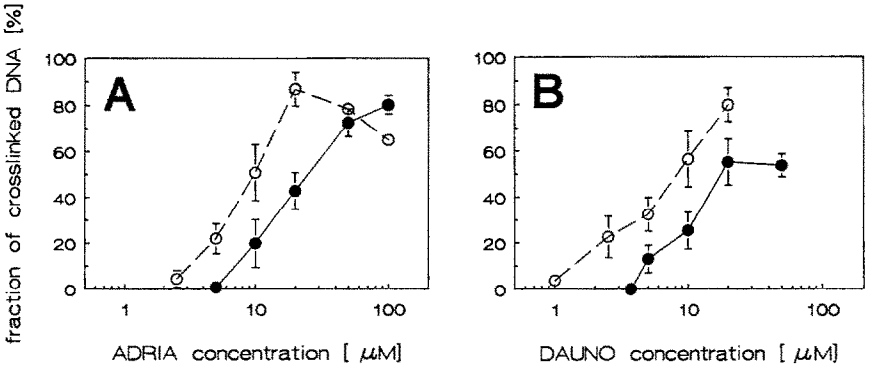


Fig. 8. DNA crosslinking induced by ADRIA (Panel A) and DAUNO (Panel B) in HeLa S_3 cells with normal (solid line) and decreased GSH content (broken line). For GSH depletion HeLa S_3 cells were incubated with BSO (0.1 mM, 24 hr) prior to treatment with the drugs (3 hr) and fractions of crosslinked DNA were then determined as described in Materials and Methods. Points, means from three independent experiments run in duplicate; bars, SD.

($P < 0.001$) when analysed by Student's *t*-test. The BSO treatment did not change intracellular accumulation of the drugs in HeLa S_3 cells (data not shown). DNA interstrand crosslinking induced by both ADRIA and DAUNO was increased in HeLa S_3 cells pretreated with BSO compared to unpretreated controls (Fig. 8, panels A and B). As can be seen in Fig. 8, for both drugs the relationships between drug dose and DNA crosslinking were approximately linear up to 20 μM and, in the case of the cells pre-incubated with BSO, shifted to lower concentration ranges compared to those for cells with unchanged GSH content. The C_0 values calculated for both drugs from these dose-response relationships were lower for cells with reduced GSH content, with factors approximately 2.2 and 3.7 for ADRIA and DAUNO, respectively (Table 5).

DISCUSSION

It was not possible to test directly our hypothesis concerning mechanism of cytotoxic action of anthracyclines. Therefore, we studied a correlation between cytotoxic activity of anthracyclines and their ability to form interstrand DNA crosslinks in different cellular systems.

First, we studied correlation between cytotoxic activity of anthracyclines against HeLa S_3 cells (EC_{50}) and their ability to form interstrand crosslinks in DNA of these cells, expressed as C_0 concentrations. For a group of DAUNO analogs with only limited structural modifications we have demonstrated a significant positive correlation ($r = 0.97$) between these two features (Fig. 3, panel A). It shows the importance of the ability of DNA crosslinking induced by anthracyclines for biological activity of these compounds toward HeLa S_3 cells. This notion is further strengthened by the fact that biologically inactive, both *in vivo* and *in vitro*, DR 31 did not form DNA crosslinks (Fig. 2, panel F). There is also a significant positive correlation between cytotoxic activity and DNA crosslinking ability for all anthracyclines studied by us ($r = 0.95$), including DAUNO analogs mentioned above (Fig. 3, panel B). The studied compounds represent a group of anthracyclines of different chemical structures and, with regard to their inhibition of RNA synthesis, belong to two different classes [5].

We observed that for most anthracyclines concentrations, C_0 determined by us are higher than biologically effective concentrations (Table 1 in this paper and Table 1 in the accompanying paper). The possible source of this discrepancy could reside in the existence of additional effects of anthracyclines'

action diminishing the sensitivity of the method of DNA crosslinking determination used by us. One of such effects could be DNA damage resulting from topoisomerase II inhibition by these compounds that leads to DNA strand breaks in denaturing conditions [6]. If a given compound is able to form DNA crosslinks and produces topoisomerase II-related DNA breaks at the same concentrations, its C_0 value would be increased and, in extreme cases, DNA crosslinking could be completely masked by DNA damage making impossible, determination of DNA crosslinks which is based on increased renaturation of crosslinked DNA. As was found for ADRIA, the relationship between concentration of this drug and the level of double-strand DNA breaks incurred as a result of drug-induced inhibition of topoisomerase II is bell-shaped [7]. It seems that concentrations at which anthracyclines inhibit topoisomerase II–DNA cleavable complex and those at which DNA crosslinking is induced are partially overlapped and it may result in the observed discrepancy between C_0 and EC_{50} values. This effect probably marginally influences correlation coefficient values of the relationship between EC_{50} and C_0 for the studied anthracyclines. However, the possible consequences of topoisomerase II inhibition by anthracyclines for determination of DNA crosslinking induced by these antibiotics need to be established.

In the second system, we studied DNA crosslinking induced by ADRIA, DAUNO and IODO in human colon adenocarcinoma cells both sensitive (LoVo) and resistant (LoVo/DX) to anthracyclines. We have found that lower cytotoxic activity of ADRIA and DAUNO against LoVo/DX compared to LoVo cells was paralleled by decreased crosslinking induced by the two drugs in DNA of LoVo/DX cells (Fig. 4 and Table 2). IODO, which is equally cytotoxic toward both LoVo and LoVo/DX cells, induces a similar level of interstrand crosslinks in DNA of both sensitive and ADRIA-resistant LoVo cells. The different DNA crosslinking in LoVo and LoVo/DX cells can only be partially explained by diminished accumulation of the studied antibiotics in LoVo/DX cells. However, at an equimolar concentration (50 μM), fractions of crosslinked DNA in LoVo cells were 32 and 60% for ADRIA and DAUNO, respectively (Fig. 4), whereas in LoVo/DX cells treated with the two drugs there was no perceptible DNA crosslinking ($F_{CR} = 0$) at 50 μM concentrations. Moreover, DNA crosslinking induced by these compounds was still different in LoVo and LoVo/DX cells when incubated at concentrations at which equal intracellular concentrations were reached.

LoVo/DX cells express MDR-type resistance [8] and the differences in intracellular accumulation clearly have an impact on the overall resistance of these cells, especially to DAUNO. The degree of resistance to anthracyclines of LoVo/DX cells is, however, far greater than predicted from the differences in net drug accumulation which may show that in addition to active outward drug transport and, as a result of it, decreased anthracycline accumulation, other mechanisms of resistance operate in LoVo/DX cells.

In our studies, we have found similar relationships between DNA crosslinking and cytotoxic activity in two cellular systems with enhanced sensitivity to anthracyclines. In the first system, we studied DNA crosslinking by ADRIA in CHO-K1 and its mutant CHO-ADR5 cells hypersensitive to ADRIA. The mutant cell line was found to be about three times more sensitive to ADRIA compared to the parent CHO-K1 cells (Table 3). ADRIA when incubated with CHO-ADR5 cells induced significantly more DNA crosslinks than in DNA of CHO-K1 cells. The increased DNA crosslinking was not a result of the difference in intracellular accumulation of ADRIA between the two cell lines (Fig. 6). Neither could it be a different effect of ADRIA on DNA topoisomerase II in CHO-K1 and CHO-ADR5 cells since both content and activity of topoisomerase II were found to be comparable in these cell lines [9].

In the second system, we have investigated DNA crosslinking in HeLa S_3 cells with reduced levels of cellular GSH. It has been reported that the reduced GSH content sensitizes different cell lines against cytotoxic action of various anticancer drugs, including anthracyclines [10–12]. Following treatment of HeLa S_3 with BSO, GSH levels decrease by about 90% and cytotoxic activity of ADRIA and DAUNO against cells with reduced GSH content is increased about 5-fold compared to HeLa S_3 cells with unchanged GSH level (Table 4). We have found that DNA crosslinking induced by ADRIA and DAUNO in the cells with reduced GSH content was also increased (Fig. 8 and Table 5).

From experiments on DNA crosslinking in HeLa S_3 cells with decreased GSH levels an additional interesting conclusion can be drawn. GSH plays a crucial role in the protection of cells against foreign compounds by acting as a reductant to remove oxygen radicals and by detoxifying reactive drug-related metabolites via GSH conjugate formation [13]. Therefore, a potentiation of anthracycline cytotoxicity towards cells with reduced GSH content as well as increased DNA crosslinking in these cells may be due to decreased GSH-mediated detoxification of anthracycline metabolites capable of covalent binding to cellular macromolecules and forming DNA crosslinks. In this context, our finding is also indirect evidence that metabolic activation in the cell is required for DNA crosslinking by ADRIA and DAUNO.

In summary, our results show that a positive correlation exists between cytotoxic activity of anthracyclines and their DNA crosslinking ability. This conclusion especially concerns ADRIA since this drug was studied in all the employed test systems.

The question remains as to whether the observed positive correlation between cytotoxic activity and DNA crosslinking by anthracyclines may be extended to antitumor activity *in vivo* of these compounds. There is only a general relationship between cytotoxic and antitumor activity and highly cytotoxic compounds have not always proved to possess antitumor activity and clinical usefulness. We have shown, however, that the analog of DAUNO, DR 31, which exerts neither cytotoxic nor antitumor activity is also unable to induce DNA crosslinking in HeLa S_3 cells (Fig. 2, panel F). This may suggest that the relation between cytotoxic activity and DNA

crosslinking by anthracyclines is also true for antitumor activity of these compounds.

Positive results of many tests used to verify the studied hypothesis may support it, but, cannot provide a complete proof, and the hypothesis is true as long as it is not disproved by negative results. This is in agreement with the generally accepted postulate concerning methodology of testing hypotheses [14]. It explains also why several different correlation tests were performed in order to support our hypothesis that interstrand DNA crosslinking ability of anthracyclines is responsible for their cytotoxic activity. In the light of the above-mentioned general rules concerning testing hypothesis, we could ascertain that the hypothesis proposed by us has been strongly supported by the positive results of verifying tests performed by us. We caution, however, that in further studies on DNA crosslinking induced by anthracyclines there is a possibility of obtaining results which may be misinterpreted as negative and such "false negative" results might call into question our hypothesis about relevance of DNA crosslinking induced by anthracyclines for biological activity of these compounds. We would like to point to two such possibilities and these are: (i) the application of inadequate methods for DNA crosslinking measurements and/or (ii) the existence of additional effects of cytotoxic action of anthracyclines which may influence DNA crosslinking determination. Specifically, DNA crosslinking by anthracyclines was not found by generally used methods but alkali and thermal instability of crosslinks induced by these compounds was not taken into account [1]. Moreover, when topoisomerase II inhibition and DNA crosslinking occur within the same range of concentrations, DNA breaks produced in denaturing conditions as a result of inhibition of topoisomerase II–DNA cleavable complex, may mask DNA crosslinking.

The mechanism of cytotoxic and antitumor action of anthracyclines is still uncertain and several alternative mechanisms have been proposed. These include: (i) intercalation between adjacent base pairs in DNA [for review see Refs. 15 and 16], (ii) generation of free radicals [for review see Ref. 17], (iii) disruption of cell membrane functions and integrity [for review see Ref. 18], and (iv) interference with topoisomerase II–DNA cleavable complex [19–21]. None of these mechanisms, however, has been proved unequivocally and this was pointed out by several authors [15, 16, 22].

The mechanism proposed by us of cytotoxic action of anthracyclines, namely induction of interstrand DNA crosslinks, is in agreement, at least in part, with mechanisms (i) and (ii), mentioned above, i.e. intercalation to DNA and free radical generation by anthracyclines. It seems that intercalation to DNA is necessary but not sufficient for biological activity of anthracyclines [23]. The derivatives of DAUNO possessing methoxy instead of hydroxy groups at C-6 and C-11 do not intercalate into DNA and are inactive both *in vivo* and *in vitro* [24]. However, 9-deoxy-DAUNO which is still able to intercalate to DNA lacks any biological activity [25]. It seems reasonable to assume that physico-chemical binding of anthracyclines to DNA by intercalation may be one of the necessary steps leading to covalent binding and interstrand DNA crosslinking induced by these antibiotics. Anthracyclines are involved in reduction-oxidation processes and, apart from redox cycling, may undergo enzyme-catalysed one- or two-electron reduction to form quinone or semiquinone methide and other species which are capable of binding covalently to DNA [for review see Ref. 26]. This feature of anthracyclines is particularly interesting with respect to our hypothesis since it may be one of the pathways leading to alkylation and interstrand crosslinking of DNA by these compounds.

In conclusion, on the basis of our studies we may propose the following sequence of events of cytotoxic action of anthracyclines, especially ADRIA:

(1) Metabolic activation of anthracycline.

(2) Intercalation into DNA of anthracycline molecule (provided it can be metabolized once intercalated) or its metabolite (the actual sequence of these two steps is unknown).

(3) Binding to DNA (apparently covalent) of metabolically activated anthracycline and formation of interstrand DNA crosslinks.

(4) Irreversible arrest of tumor cell cycle progression at G_2 phase due to the formation of DNA crosslinks [for review see Ref. 27].

(5) Death of cells irreversibly arrested in G_2 phase by apoptosis [28].

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