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INTERSTRAND DNA CROSSLINKING INDUCED BY ANTHRACYCLINES IN TUMOUR CELLS

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Abstract—Using a new mild method it is shown for two anthracyclines, Adriamycin® and Daunomycin, that these compounds are able to form DNA crosslinks in HeLa S_3 cells. It was also found that other anthracyclines: Epirubicin, Rubidazone, Iodorubicin, 3′-deamino-3′-hydroxy-4′-amino-Adriamycin, Aclacinomycin, Marcellomycin, and Cinerubin A, induced crosslinks in the DNA of HeLa S_3 cells in a concentration-dependent manner. DNA crosslinks formed by five anthracyclines studied, excluding Iodorubicin, were both alkali and thermally unstable. No DNA crosslinking could be detected when the compounds were added to cell lysates in which cellular enzymes had been inactivated. This implies that metabolic activation is prerequisite for DNA crosslinking by anthracyclines. The kinetics of DNA crosslinks formation by Adriamycin as well as their removal from cellular DNA were also studied. The presented results indicate that all biologically active anthracyclines studied induce DNA crosslinks, and for two of them DNA crosslinking was observed at growth inhibitory concentrations.

Key words: anthracyclines; interstrand DNA crosslinking; metabolic activation; mechanism of action; topoisomerase II

In previous studies on the mode of action of the potent cytotoxic and antitumour 1-nitro-9-aminoacridines, we showed that it is not intercalation to DNA [1] but interstrand DNA cross-linking after metabolic activation [2] which is responsible for the high cytotoxic and antitumour activity [3, 4] of these compounds. We hypothesized that other drugs believed to act through intercalation to DNA and exhibiting high biological activity, are able to induce interstrand DNA crosslinking following their metabolic activation within the cell. DNA crosslinks formed by these compounds could not be detected by the techniques generally used for measuring DNA crosslinking due to the instability of the bonds formed in alkaline pH and/or elevated temperature, conditions which are used in these techniques to denature the DNA. Therefore, we developed a mild method of DNA crosslink determination and this method has been employed to demonstrate that ADR† and DAU induce interstrand DNA crosslinking in HeLa S_3 cells [5]. For both drugs, their previous intracellular metabolic activation was prerequisite for DNA crosslinks formation, and crosslinks formed by these drugs were found to be both alkali and thermally unstable [5].

The aim of this study was to confirm the ability of ADR and DAU to form DNA crosslinks in tumour cells by a novel procedure developed in the course of the study. Additionally, we wondered whether other anthracyclines, especially those introduced recently to clinical use, were also able to form covalent interstrand crosslinks in DNA of tumour cells. The kinetics of DNA crosslinks formation by ADR and their persistence in the cellular DNA after removal of the drugs were also determined. A part of these results was presented in a preliminary form [6, 7].

MATERIALS AND METHODS

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

Drugs. ADR, DAU, EPI, IODO, 4ADR were kindly provided by Dr Fernando C. Giuliani, Farmitalia Carlo Erba Research and Development (Nerviano, Italy). ACLA was from Boehringwerke GmbH (Marburg, Germany). MARC, RUBI, CINE were obtained from the National Cancer Institute (Natural Products Branch, Bethesda, MD, U.S.A.) by courtesy of Dr Matthew Suffness.

Cell culture and media. Hela S_3 cells, the media, glutamine, vitamins (BME vitamin solution 100 X), and foetal calf serum were from Gibco Europe Ltd. (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

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† Abbreviations: ADR, Adriamycin; DAU, Daunomycin; EPI, Epirubicin; IODO, Iodorubicin; 4ADR, 3′-deamino-3′hydroxy-4′-amino-Adriamycin; RUBI, Rubidazone; ACLA, Aclacinomycin A; MARC, Marcellomycin; CINE, Cinerubin A; PBS, phosphate-buffered saline: 0.17 M NaCl, 0.27 mM KCl, 8.1 mM Na_2HPO_4, 0.27 mM KH_2PO_4, pH 7.2; SSC, sodium citrate-saline solution: 0.15 mM NaCl, 0.015 mM sodium citrate, pH 7.

supplemented with 5% foetal calf serum and antibiotics (streptomycin, 100 μg/mL; penicillin 100 U/mL). The cells were grown at 37° in a humidified 5% CO_2–air atmosphere.

Cytotoxicity assay. The cytotoxicity of anthracyclines against HeLa S_3 cells was determined after 3 hr treatment. Exponentially growing cells were seeded at a concentration of 1.5×10^5/mL (4 mL/tube) and incubated at 37° for 24 hr before treatment. The culture medium was then replaced with drug-containing medium and the cells were incubated for 3 hr at 37°, the medium was withdrawn, attached cells were washed twice, 4 mL fresh medium was added to each tube and incubation was carried out for an additional 72 hr. The concentrations of anthracycline required for 90% growth inhibition comparing to control non-treated cells were determined as in Ref. 4.

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

DNA crosslinking assay. The procedure (method B) applied was a modification of the method described by Parsons *et al.* [8]. DNA crosslinking of ADR and DAU was determined additionally by the procedure described earlier [5] (method A). Both techniques rely on the fact that heat denaturation/renaturation of DNA results in a loss of double-stranded DNA. If a drug is able to covalently crosslink DNA then the fraction of renatured, double-stranded DNA is increased compared to the residual, spontaneous renaturation of DNA from control cells (about 10–15% of total DNA).

Cell suspension in SSC (0.4 mL) (3 mL/sample) was added to 3.6 mL of the lysing solution (6.8 M sodium perchlorate, 1 mM EDTA, 0.2% lauroyl sarcosine, 20% v/v methanol, pH 7) with gentle mixing so as to minimize mechanical shearing of DNA. After 3 hr lysis at room temperature, DNA was denatured by heating in a water bath at 50° for 30 min then renatured by rapid dilution with 20 mL of ice-cold acetate buffer (0.04 M sodium acetate, 0.4 M sodium chloride, 5 mM zinc acetate, pH 4.4) and cooling in methanol–ice mixture (−18°, 1 min). The fractions of renatured DNA were determined using nuclease S_1 assay as described previously [5]. The fraction of crosslinked DNA was calculated according to the formula:

$$F_{CR} = \frac{RF_{treat} - RF_{contr}}{100 - RF_{contr}} * 100\%$$

where RF_{treat}, RF_{contr} are renatured double-stranded DNA from treated and control cells, respectively.

To exclude the possibility that the observed increased renaturation of DNA from cells treated with anthracyclines was due to formation of DNA–topoisomerase II cleavable complex, the same procedure for measuring DNA crosslinks was used except that protein digestion before DNA denaturation/renaturation was included. Briefly, on completion of the incubation time, 3 mL of HeLa S_3 cells either control or treated with drugs were washed with PBS, resuspended in 200 μL of SSC and mixed with 200 μL of lysing solution (50 mM Tris–HCl, 10 mM EDTA, 1% lauroyl sarcosinate, pH 8 containing 1 mg/mL proteinase K). The lysates were then incubated at 37° for 2 hr with intermittent mixing. Following cell lysis and proteinase K treatment, 3.6 mL of denaturing solution (6.8 M sodium perchlorate, 1 mM EDTA, 0.1% lauroyl sarcosinate, 20% methanol, pH 7) was added and mixed thoroughly. The lysates were then left for 1 hr, cellular DNA was then denatured and renatured and fractions of crosslinked DNA were determined as described above.

For cell-free system studies, labelled cells were washed and lysed as described above. The drugs were added to the cell lysates to give a final concentration of 50 μM and incubated, protected from light, for 1 hr at room temperature. The cellular DNA was then denatured and renatured and fractions of crosslinked DNA determined as described above. For the thermal denaturation samples were heated at 95° for 15 min and cooled in methanol–ice bath for 2 min. For alkaline denaturation, 0.12 mL 1 M sodium hydroxide was gently added to give 0.03 M final concentration and after 1 hr incubation in the dark at room temperature the samples were neutralized with 0.12 mL 1 M hydrochloric acid. In both cases, fractions of crosslinked DNA were determined as described above.

All DNA crosslinking measurements were performed in duplicate, and means were calculated on the basis of the data from at least three independent experiments. The precision of the method used (method B) calculated as a standard deviation of the method was consistently within ±10% of the means.

RESULTS

Using method B (Fig. 1), ADR and DAU were shown to form interstrand DNA crosslinks in HeLa S_3 cells in a concentration-dependent manner (Fig. 2A and B). For comparison, the data obtained previously by method A are shown as taken from Ref. 5. Fractions of crosslinked DNA determined for both drugs by procedure B are higher than those obtained by procedure A, with formamide as a denaturing agent. This is probably due to less mechanical sharing of cellular DNA in method B, which requires considerably fewer manipulations with cell lysates. The ability of ADR to induce DNA crosslinks in HeLa S_3 cells was confirmed by method B modified in such a way that fractions of crosslinked DNA were assayed by fluorescence, using ethidium bromide homodimer, instead of nuclease S_1 assay. The results obtained with this modification of method B are comparable to those obtained by method B with nuclease S_1 assay.*

We also found that the other antitumour

* A manuscript detailing this assay is in preparation

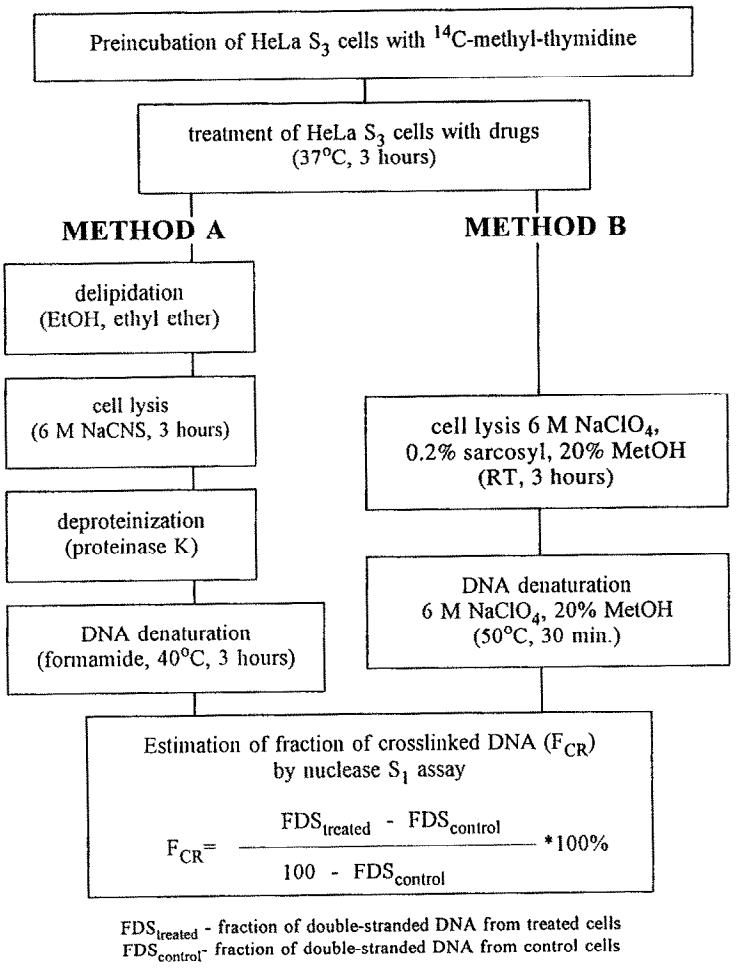


Fig. 1. Scheme describing the experimental procedures for DNA crosslinking quantitation.

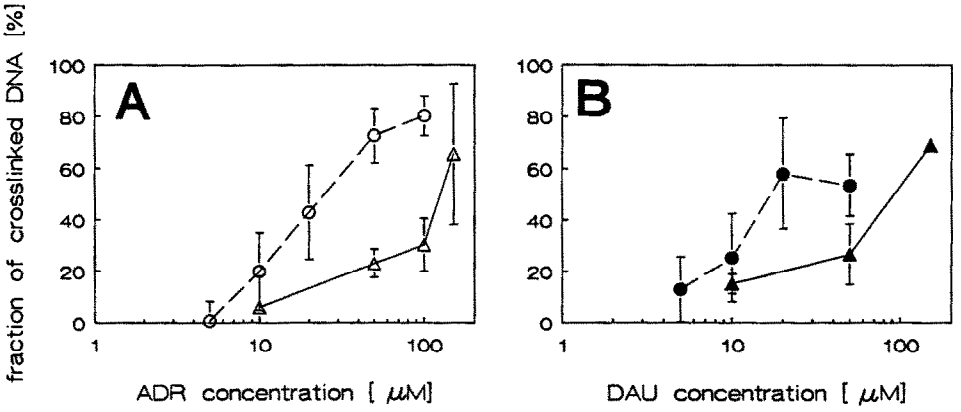
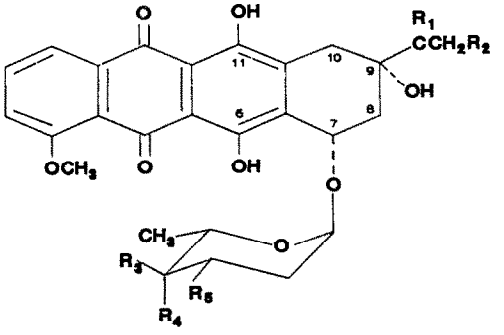
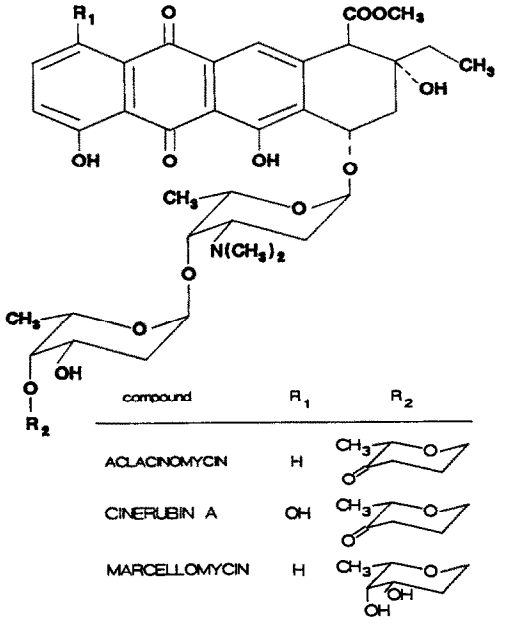


Fig. 2. DNA crosslinking induced by ADR (panel A) and DAU (panel B). HeLa S_3 cells were treated with the drugs for 3 hr and fraction of crosslinked DNA was determined by method A (solid line) (data taken from Ref. 5) and method B (broken line) (see Materials and Methods). Points, means of three independent experiments; bars, SD.



compound	R_1	R_2	R_3	R_4	R_5
ADRIAMYCIN	=O	OH	H	OH	NH_2
DAUNOMYCIN	=O	H	H	OH	NH_2
EPIRUBICIN	=O	OH	OH	H	NH_2
IODORUBICIN	=O	OH	H	I	NH_2
4'-amino-ADRIA	=O	H	H	NH_2	OH
RUBIDAZONE	$=NNHCC_6H_5$	H	H	OH	NH_2

Fig. 3. Chemical structures of anthracyclines studied (Class I).



compound	R_1	R_2
ACLACINOMYCIN	H	
CINERUBIN A	OH	
MARCELLOMYCIN	H	

Fig. 4. Chemical structures of anthracyclines studied (Class II).

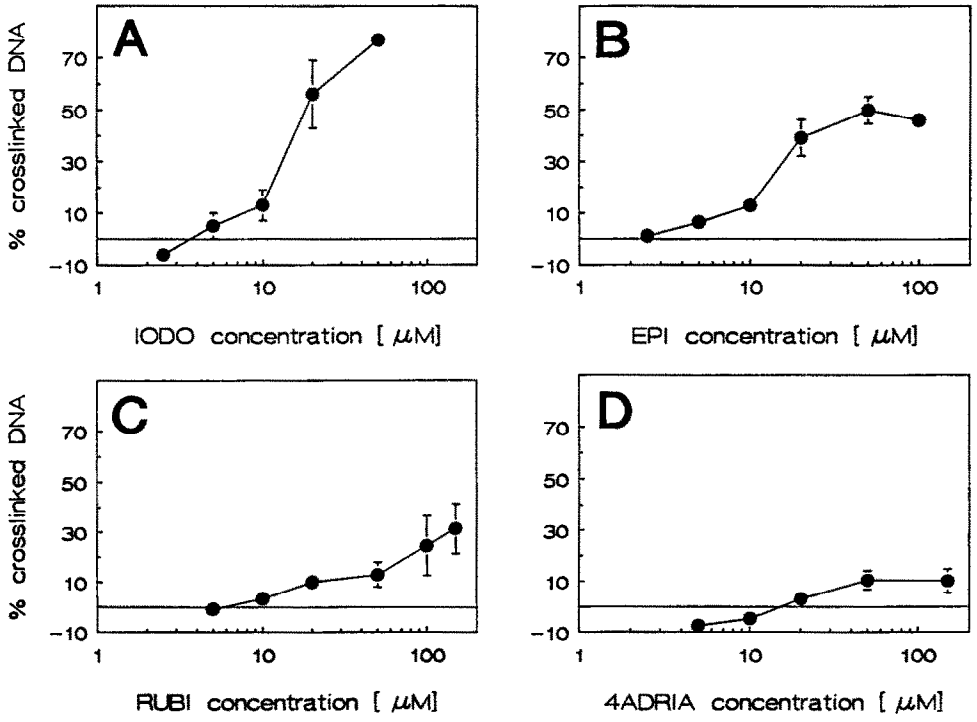


Fig. 5. DNA crosslinking induced by EPI (panel A), RUBI (panel B), 4ADR (panel C), and IODO (panel D). HeLa S_3 cells were incubated with the anthracyclines for 3 hr and fractions of crosslinked DNA were determined as described in Materials and Methods. Points, means from three independent experiments; bars, SD.

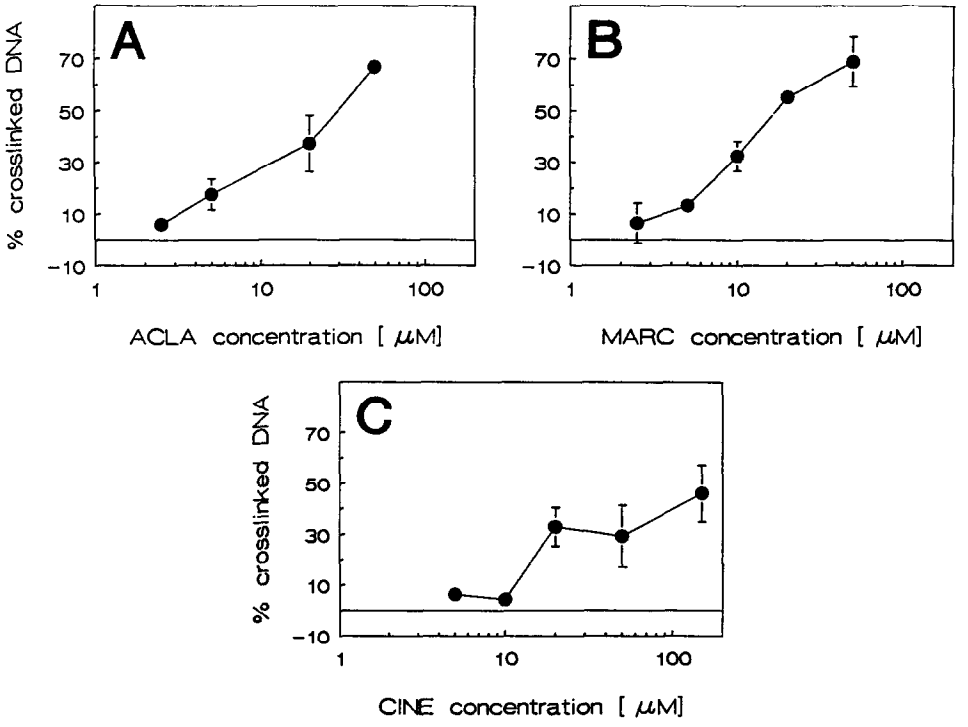


Fig. 6. DNA crosslinking induced by Class II anthracyclines: ACLA (panel A), MARC (panel B), and CINE A (panel C). Points, means from three independent experiments; bars, SD.

anthracyclines, namely EPI, RUB, IODO, 4ADR, ACLA, MARC and CINE (for chemical structures see Figs 3 and 4), similarly to ADR and DAU, induce interstrand crosslinks in DNA of HeLa S_3 cells in a dose-dependent manner (Fig. 5A–D and Fig. 6A–C). For each compound studied, the relationship between drug concentration and fraction of crosslinked DNA was fitted by least-squares to a linear equation and the extrapolated intercepts C_0, at 0% of crosslinked DNA, were calculated. We assumed that C_0 corresponds to concentration at which the first crosslink could be detected and this concentration is a measure of the DNA crosslinking potency of a given compound studied. The C_0 values calculated for all anthracyclines under study are summarized in Table 1.

We also tested whether the increased renaturability of DNA from HeLa S_3 cells treated with anthracyclines, which is a basis for detection of interstrand DNA crosslinks in the assay, stems from strong physicochemical binding of anthracyclines to DNA by intercalation or is caused by DNA crosslinking. The fractions of crosslinked DNA, determined for cell lysates from non-treated cells incubated with studied anthracyclines in cell-free conditions, were very low when compared with fractions of crosslinked DNA from cells treated with anthracyclines at the same 50 μM concentration (Fig. 7). Moreover, the increased DNA renaturation did not result from the inhibition of topoisomerase II by anthracyclines since digestion of proteins in cellular lysates either from control cells or anthracycline-treated ones, before DNA denaturation/renaturation, did not change the level of DNA renaturation (data not shown).

Table 1. The comparison between crosslinking potency C_0 and cytotoxic activity EC_{90} of the anthracyclines studied

Compound	EC_{90}[μM]*	C_0[μM]†
ADR	0.98 ± 0.08	4.7 ± 0.30
DAU	0.64 ± 0.10	3.7 ± 1
EPI	0.88 ± 0.20	3.0 ± 0.6
IODO	0.09 ± 0.01	3.6 ± 0.9
4ADR	6.45 ± 0.90	14.3 ± 2
RUBI	10.14 ± 2.6	6.6 ± 2
MARC	3.00 ± 0.8	2.3 ± 0.7
ACLA	0.40 ± 0.10	2.0 ± 0.6
CINE	0.04 ± 0.10	3.4 ± 1

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

* EC_{90} concentration inhibiting HeLa S_3 growth by 90% comparing to control non-treated cells.

† C_0 concentration at which the first crosslink could be detected under experimental conditions.

We observed only marginal crosslinking of DNA when DNA from cells treated with anthracyclines was denatured at 100°, 15 min or at pH 12 for 1 hr (Table 2). In the case of ADR and DAU, our previous observations concerning thermal and alkaline instability of DNA crosslinks induced by these two compounds [5] were confirmed by the new method, described in this paper. For IODO, however, the fraction of crosslinked DNA induced could still be measured after alkaline denaturation (pH 12, 1 hr) although this amounted to about 50% of that observed in the absence of alkali treatment.

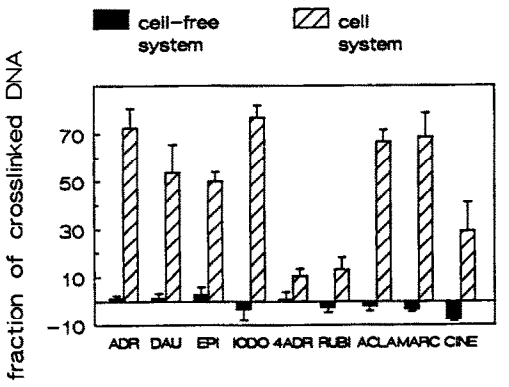


Fig. 7. Fractions of crosslinked DNA determined in HeLa S_3 cells and cell lysates (cell-free system) treated with anthracyclines. The cells and cell lysates were incubated with anthracyclines for 3 hr at 37° and 1 hr at room temperature, respectively, and fractions of crosslinked DNA were determined as described in Materials and Methods.

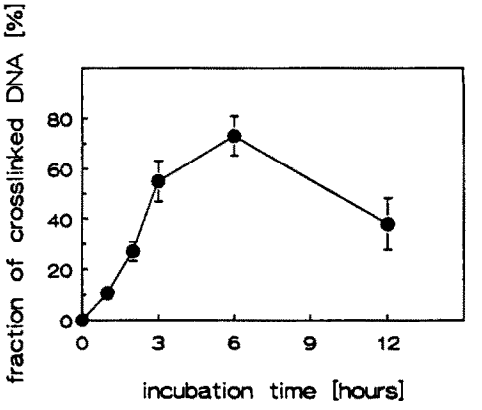


Fig. 8. Time course of formation of interstrand DNA crosslinks in the DNA HeLa S_3 cells treated with ADR. The cells were exposed to 20 μM ADR and the amount of DNA crosslinks was determined by method B as described in Materials and Methods. Points, means of three independent experiments; bars, SD.

Table 2. Fractions of crosslinked DNA induced by studied anthracyclines in HeLa S_3 cells and determined by method B described in Materials and Methods with various denaturation conditions

Compound	Fraction of crosslinked DNA F_{CR} [%]		
	Method of DNA denaturation		
	Thermal*	Alkaline†	Method B‡
ADR	−1.01 ± 0.3	−1.08 ± 0.6	72.6 ± 8
DAU	−0.92 ± 0.5	−0.72 ± 0.3	53.6 ± 12
EPI	0.35 ± 0.5	0.25 ± 0.5	50.0 ± 4
ACLA	−0.20 ± 0.5	−4.20 ± 0.4	66.7 ± 5
IODO	0.40 ± 0.3	37.3 ± 8	76.9 ± 5

The cells were treated with anthracyclines for 3 hr at 37°.
* Thermal denaturation of DNA (100°, 15 min).
† Alkaline denaturation of DNA (pH 12, 1 hr).
‡ Denaturation in 6 M $NaClO_4$, 0.2% sarcosyl, 1 mM EDTA, 20% (v/v) methanol, pH 7 (50°, 30 min).

The studies on the kinetics of DNA crosslinking induced by ADR showed that the level of DNA crosslinks increased during the first 6 hr of incubation of HeLa S_3 cells, with the drug and then decreased slowly (Fig. 8). However, about 50% of the maximal level of DNA crosslinking was still present after a 12 hour treatment. To measure the persistence of DNA crosslinks induced by ADR, i.e. their removal from the cellular DNA by repair processes, HeLa S_3 cells were treated with ADR for 3 hr and fractions of crosslinked DNA were determined at various times of incubation in a drug-free medium. The results of these studies are presented in Fig. 9. The removal of DNA crosslinks, induced by this drug, seems to be biphasic with an initial, rapid decrease during the first 2 hr, followed by almost no further change in the level of DNA crosslinking between the third and sixth post-incubation hours. There was no perceptible DNA crosslinking after a 12 hour recovery period in a drug-free medium.

DISCUSSION

In this study we established a new method by which we confirmed that ADR and DAU induced crosslinks in the DNA of HeLa S_3 cells. Method B, used to determine DNA crosslinking, differed significantly from method A used in previous studies [5], mainly in the conditions of DNA denaturation. Interstrand DNA crosslinking, by ADR, was also shown by a further modified method B, in which the determination of fractions of crosslinked DNA was performed fluorimetrically with ethidium bromide homodimer, a compound which selectively binds to double-stranded DNA and eliminates the possibility of error due to the use, both in methods A and B,

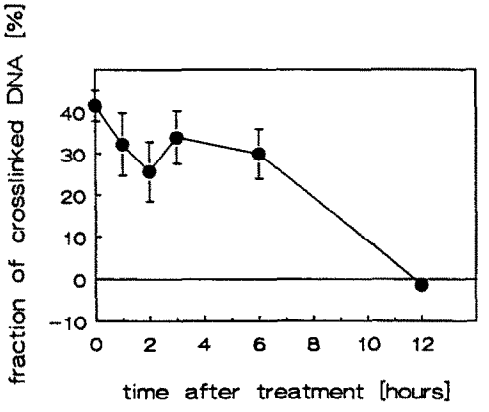


Fig. 9. Removal of interstrand crosslinks from DNA of HeLa S_3 cells treated with ADR. The cells were incubated with 20 μM ADR for 3 hr and fraction of crosslinked DNA was quantitated at various times of post-incubation in drug-free medium. Points, means of three independent experiments; bars, SD.

of nuclease S_1. Thus, interstrand DNA crosslinking, induced by ADR and DAU, gained additional support from the experiments, in which completely independent methods for determining the crosslinks were used.

In the method which we developed for DNA crosslink determination, DNA is denatured under mild conditions, thus avoiding both high temperature and alkalization. Moreover, this procedure obviates the need to purify DNA before denaturation and renaturation. Method B is a modification of that of Parsons [8], who applied the denaturing conditions (6.8 M sodium perchlorate solution, 70°, 10 min) proposed by Geiduschek [9] in studies on DNA crosslinking by merchlorethamine. The most important change introduced was to lower the temperature of DNA denaturation to 50°, by adding 20% v/v of methanol to the denaturing solution.

Fractions of crosslinked DNA for ADR and DAU, determined by method B, are higher than those obtained using method A. However, both methods are based on the measurement of the enhanced renaturation of crosslinked DNA, and the degree of DNA renaturation (and DNA crosslinking determination) is influenced, above all, by the different mechanical shearing of cellular DNA caused by the experimental procedures. This is why the same results can not be expected.

We have found that the other anthracyclines, most of them used clinically, similarly to ADR and DAU, form interstrand crosslinks in the DNA of HeLa S_3 cells. It is interesting that there is no qualitative difference in DNA crosslinking between Class I anthracyclines (e.g. ADR, DAU) and Class II anthracyclines (ACLA, MARC, CINE). According to the proposition of Crooke *et al.* [10], Class I anthracyclines inhibit DNA and RNA synthesis at the same concentrations, whereas Class II anthracyclines preferentially inhibit RNA synthesis. This reported difference is not reflected in the DNA crosslinking induced by these two groups of antibiotics.

All the studied anthracyclines did not form crosslinks when cell lysates, in which cellular enzymes had been deactivated by denaturing agents, were incubated with the studied compounds. When we included protein digestion in method B, we still observed an increase in the fraction of renaturable DNA from treated cells, similar to the results obtained when protein digestion was omitted. Method A, by which DNA crosslinking for ADR and DAU was found for the first time [5], also includes protein digestion. Additionally, we observed increased renaturation of DNA treated with anthracyclines, and DNA crosslinking by these compounds, at concentrations at which anthracyclines only marginally inhibit topoisomerase II activity. It follows that the increased ability to renature DNA from cells treated with the studied anthracyclines cannot be attributed to strong physicochemical DNA binding of these antibiotics by intercalation. Neither is it an effect of the inhibition caused by anthracyclines of topoisomerase II–DNA cleavable complexes which may have survived the mild conditions of DNA denaturation. These results also emphasize the need for metabolic activation of anthracyclines within the cell for DNA crosslink formation. Anthracyclines are readily metabolized in the cell (for review see Ref. 11) and the formation of adducts of anthracyclines with DNA, most probably monofunctional, following chemical and enzymic activation has been well documented both *in vitro* and in cellular systems [12–18]. If we exclude intercalation and stabilization of the DNA structure, by topoisomerase II–DNA complexes, as a source of the increased renaturation of DNA treated with anthracyclines, and take into account the necessity for metabolism for DNA crosslinking by these compounds which, according to what has been shown by other authors, leads to covalent binding, it is safe to suppose that anthracyclines form covalent interstrand crosslinks in cellular DNA. At this point, we would like to stress that, although the features of DNA crosslinking induced by anthracyclines are consistent with the notion that these crosslinks are covalent, we have no direct evidence for this; the exact nature of DNA crosslinks induced by anthracyclines, as well as their chemical structures, requires further studies.

Anthracyclines could be metabolized in many ways, and Moore and Czerniak [19, 20] postulated the possibility of bioreductive activation of anthracyclines yielding quinone methide and semiquinone methide; these suggestions have recently been confirmed experimentally [21, 22]. The formation of quinone methide may explain, however, only monofunctional covalent binding of anthracyclines to DNA. The studies on the relationship between the chemical structure and the biological activity of anthracyclines may shed some light on possible sites of metabolic transformation and DNA binding of these compounds. The strict stereochemical requirement, as for position of the substituents at C-7 and C-9 of the anthraquinone moiety and also for the presence and orientation of hydroxyl groups at C-9, are essential for the biological activity of

anthracyclines. Morever, ring A of the aglycone has to be in a half-chair conformation and any substitutions at position C-8 and/or C-10, changing the conformation of ring A, lead to inactive compounds [23]. It would be safe to suppose that ring A plays an important role in the metabolic activation of anthracyclines and may be directly involved in DNA binding. It may also be proposed that divergent biological activity of anthracycline derivatives, as well as their tissue and organ specificity, can be explained, at least in part, by the different modes of metabolic activation which they may undergo what, in turn, stems from the differences between the chemical structures of these compounds.

There is a distinct qualitative difference between DNA crosslinking induced by anthracyclines and the similar properties of cyanomorpholino derivatives of ADR and DAU, determined by other authors [24–28]. Cyanomorpholinyl analogues of anthracycline bind covalently to cellular DNA and crosslink DNA without any metabolic activation [28], contrary to the anthracyclines which we studied, for which metabolic activation was found to be a prerequisite for DNA crosslinking to occur.

ADR, DAU, and other anthracyclines indeed form DNA crosslinks which are alkaline and thermally unstable and, as we proposed earlier [5], that is why such crosslinks could not be detected by generally used methods for the measurement of DNA crosslinks e.g. alkaline elution [29, 30]. The same was also found for mitoxantrone and ametantrone [6, 7].

The stability of DNA crosslinks differs depending on the type of compounds by which these crosslinks are induced. It was found that DNA crosslinking induced by Nitracrine (Ledakrin), which was confirmed by several independent methods [3], could not be detected when studied by alkaline elution [31] despite the fact that DNA crosslinks formed by Nitracrine withstand a short exposure (1 hr) to alkaline conditions [3]. Contrary to this, DNA crosslinks induced by derivatives of nitrogen mustard, a group of the most extensively studied DNA crosslinking agents, become more stable with increasing pH, although they are eventually destroyed in the extreme alkaline conditions [32]. Instability of DNA crosslinks at high pH was also reported for Mitomycin C [33]. DNA crosslinks formed by nitrogen mustard are also sensitive to heating at neutral pH, whereas those induced by carcinophilin break down spontaneously within several hours [34]. The thermal stability of DNA crosslinks formed by cyanomorpholinyl-ADR has recently been found to range from 63 to 70° [35]. All these facts, and our own results, indicate that methods involving exposure to heat and alkaline conditions may give inaccurate information on DNA crosslinking for some agents.

We have found that DNA crosslinks produced by IODO are partially resistant to alkali. Independently, Belvedere *et al.* [36] have shown, by an alkaline elution technique, that IODO and Esorubicin produce interstrand DNA crosslinks, whereas other anthracyclines, including ADR and DAU do not, although these results were interpreted by these authors with caution. It is interesting to note that both IODO and Esorubicin lack the 4′-OH group in the daunosamine and this similarity in chemical structure may suggest that the 4′-OH group influences the stability of one of the possible covalent bonds between anthracycline molecules and DNA, or that it is involved in the formation of alkali unstable bonds between DNA and anthracycline molecules.

When we compared the capability of inducing interstrand DNA crosslinks and cytotoxic activity of the studied anthracyclines, determined under the same conditions, following a 3 hr treatment of HeLa S_3 cells with antibiotics, for only two anthracyclines, RUBI and MARC, DNA crosslinking was induced at biologically relevant concentrations, i.e. the C_0 values and the EC_{90} concentrations were comparable (Table 1). It follows that the induction of DNA crosslinking by all the other studied anthracyclines, except RUBI and MARC, occurs at concentrations higher than those required to inhibit cell growth. A possible explanation of the discrepancy, between the concentrations at which anthracyclines crosslink DNA and those required to inhibit the growth of HeLa S_3 cells, could be the inhibition of DNA topoisomerase II activity by anthracyclines (for review see Ref. 37). The inhibition of cleavable complexes between DNA and topoisomerase II complexes leads, after exposure to strong denaturants such as detergents or alkali, to protein-associated DNA strand breaks. In most of the methods used to quantify DNA crosslinks, including the methods used in our studies, the crosslinked DNA is observed subsequent to having been returned from denaturing to non-denaturing conditions. The principle of such methods is to distinguish the relative proportions of double and single stranded DNA after the denaturing/renaturing cycle. When a given anthracycline derivative is able to crosslink DNA and produces topoisomerase II-related DNA damage at the same concentrations, the renaturable fraction of the damaged DNA will be diminished due to the preferential loss of "single stranded tails" that will occur when the reannealed DNA is damaged. In an extreme case, DNA degradation produced by topoisomerase II action can fully mask the anthracycline-induced DNA crosslinking. The relationship between the ability to inhibit cleavable DNA–topoisomerase II complexes by ADR (and probably by other anthracyclines) and its concentration is a bell-shaped curve [38], i.e. there is a threshold concentration above which topoisomerase II-inhibiting activity of anthracyclines decreases due to an as yet unknown mechanism. Preliminary results have shown that for the anthracyclines which we studied, concentrations at which DNA crosslinking is induced and those required to inhibit topoisomerase II partially overlap.* This is probably why we were able to quantify DNA interstrand crosslinks induced by anthracyclines at concentrations higher than those biologically relevant.

It is worth noting that DNA crosslinking by anthracyclines explains the reported ability of these compounds (ADR, DAU (for review see Ref. 39), RUBI [40, 41], and ACLA [42]) to induce an accumulation of cells in the G_2 phase of the cell

* A. Skladanowski and J. Konopa, unpublished results.

cycle. As it has been reviewed elsewhere [39], almost all the DNA crosslinking compounds arrest cells in the G_2 phase and this appears to be the first effect of their biological action. Even very few DNA crosslinks, which may not be sufficient to effectively block DNA replication, can probably prevent fully replicated cellular DNA molecules from separation during mitosis, thereby arresting cells in the G_2 phase, which eventually leads to cell death [39].

Our preliminary studies concerning the kinetics of DNA crosslink formation showed that their level, induced by ADR, increased during the 6 hr incubation with the drug. The increase was followed by a decline over 6–12 hr although about 50% of the maximal level of DNA crosslinking was still observed after a 12 hr incubation with the drug. ADR-induced crosslinks were removed within 12 hr from cellular DNA during incubation in a drug-free medium. However, the loss of crosslinks at longer incubation times may be related, not only to extensive repair of DNA lesions incurred by ADR, but also to ADR-induced DNA degradation, which is probably associated with cell death and explains the apparent decline in DNA crosslinking after drug removal. The kinetics curves obtained for ADR are similar to those of other DNA crosslinking compounds e.g. cisplatin [43].

In conclusion, we have shown that nine anthracyclines induce interstrand DNA crosslinking in HeLa S_3 cells. The metabolic activation of these antibiotics within the cell was required for DNA crosslinking to occur. The presented studies also confirmed our hypothesis, put forward previously [5], that highly cytotoxic and antitumour compounds, believed to act by intercalation to DNA, may in fact induce interstrand DNA crosslinks in tumour cells and this property may be responsible for their high biological activity.

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