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COMPARISON OF INTRACELLULAR DRUG RETENTION, DNA DAMAGE AND CYTOTOXICITY OF DERIVATIVES OF DOXORUBICIN AND DAUNORUBICIN IN A HUMAN COLON ADENOCARCINOMA CELL LINE (LoVo)*

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Abstract—Formation of DNA single strand breaks (SSB) was assayed by alkaline elution in LoVo cells treated with doxorubicin, daunorubicin and six derivatives of these drugs modified either in the chromophore or the sugar. Seven compounds showed a biphasic relationship (initial increase and then a decrease) for the formation of DNA-SSB over the concentration range 0.05–10 μg/ml. At a drug concentration in the range causing an increase of DNA damage very fast repair of DNA-SSB was observed for 4′-deoxydoxorubicin and 4-demethoxydaunorubicin; the kinetics of DNA-SSB investigated after drug removal at a drug concentration reducing DNA-SSB showed a time dependent increase of DNA damage for both drugs although with different patterns. 4′-Deoxydoxorubicin reduced the effect of radiations on the rate of elution of DNA in a way resembling the formation of DNA interstrand cross links (ISC) at concentrations at which DNA-SSB were reduced. DNA-ISC were not produced by chemical reactions occurring during sample processing for alkaline elution and this derivative was not metabolized by LoVo cells. The IC_{50} of the anthracyclines were on a several log range, though for most of the derivatives the cytotoxicity curve showed a plateau at growth inhibition of about 15–30% at increasing intracellular drug levels. A relationship between DNA damage and cytotoxicity was observed only in a very small range of DNA-SSB. It is likely that the different effects of these anthracyclines on the formation of DNA-SSB depend on a qualitatively different interaction between drug-DNA and topoisomerase II when the drug concentration is raised.

The clinical importance of Dx§ (Adriamycin®) in the treatment of a wide range of neoplastic diseases is widely recognized, but little is known about the mechanisms through which the anthracyclines exert their cytotoxic and antitumoral activities. Most of these drugs are known to affect DNA through intercalative binding [1], to produce reactive oxygen species by enzymatic mechanisms [2] and to have some action at the cell surface [3].

Anthracyclines have also been shown to induce the formation of DNA strand breaks specifically associated with DNA–protein cross links where the protein involved has been identified as the enzyme topoisomerase II [4–6]. This enzyme cuts reversibly double stranded DNA and becomes covalently linked to the 5′ termini at the break site via phosphotyrosyl bonds; thereafter the enzyme allows the passage of an intact DNA double strand through the cut and closes the break producing a topological modification of the DNA molecule [7]. The DNA–topoisomerase II intermediate called "cleavable complex" has been isolated and shown to be the intermediate in the breaking-rejoining process [8].

In vivo topoisomerase II is involved in the separation of daughter chromosomes at the end of DNA synthesis [9, 10], in the formation of sister chromatid exchange [11] and seems also to have a structural role, being involved in the formation of chromosome scaffold [12] and the nuclear matrix [13].

The enzyme has been proposed as a cytotoxic target for drugs producing DNA–topoisomerase II cross links [14]. However, the connection between inhibition of this enzyme and toxic effects of antitumor agents remains unclear. One of the main questions to be answered is whether there is a quantitative relationship between the extent of DNA cleavage and the cytotoxic effect.

Ellipticine and *m*-AMSA were found to cause a higher frequency of DNA breaks in L 1210 cells than doxorubicin and 5-iminodaunorubicin at equitoxic doses [5, 15]. No correlation was observed between the ID_{50} of some anthracyclines and the formation of DNA-SSB in P388 cells; in this study a decrease of DNA-SSB was observed at high concentrations of the drugs [16] and this biphasic effect was also observed

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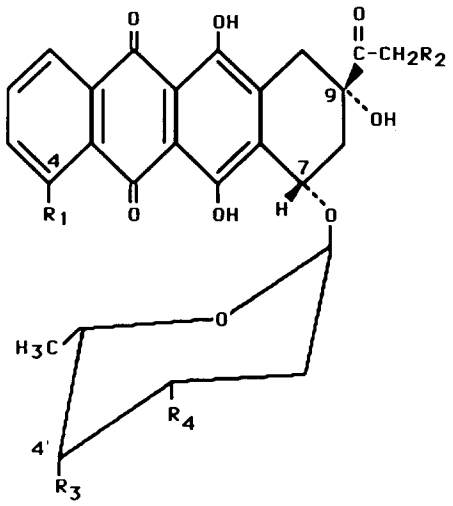
§ Abbreviations used: DNA-SSB, DNA single strand breaks; DNA-ISC, DNA interstrand cross links; Dx, doxorubicin; DN, daunorubicin; 4-DMDx, 4-demethoxydoxorubicin; 4-DMDN, 4-demethoxydaunorubicin; 4′-dDx, 4′-deoxydoxorubicin; 4′-IDx, 4′-iododoxorubicin; iso-Dx, 3′-deamino-3′-hydroxy-4′-deoxy-4′-amino-doxorubicin (isodoxorubicin); 7(R),9(R)-4-DMDN, 7(R),9(R)-4-demethoxydaunorubicin; HPLC, high pressure liquid chromatography; BME, Basal medium Eagle; PBS, Phosphate-buffered saline (0.15 M NaCl with 10.9 mM KH_2PO_4 and 5–6 mM $NaHPO_4$).



ANTHRACYCLINES	R_1	R_2	R_3	R_4
Doxorubicin	OCH_3	OH	OH	NH_2
Daunorubicin	OCH_3	H	OH	NH_2
4-demethoxydoxorubicin	H	OH	OH	NH_2
4-demethoxydaunorubicin	H	H	OH	NH_2
7(R),9(R)-4-demethoxydaunorubicin	H	H	OH	NH_2
4'-deoxydoxorubicin	OCH_3	OH	H	NH_2
4'-iododoxorubicin	OCH_3	OH	I	NH_2
Isodoxorubicin	OCH_3	OH	NH_2	OH

Fig. 1. Chemical structures of anthracyclines.

in L 1210 cells for bisantrene [17] and L 1210 nuclei for ellipticine and 2-methyl-9-hydroxyellipticinium [18]. In an *in vitro* system consisting of purified topoisomerase II and pBR 322-DNA a biphasic relationship was observed for several anthracyclines and other intercalating agents between the drug concentration and the amount of DNA breaks by gel electrophoresis resolution of the DNA fragments [6].

We report here the presence of a biphasic dose–response relationship for the production of DNA-SSB for a series of eight anthracyclines modified in the chromophore or the sugar. The apparent formation of DNA-ISC for 4'-dDx was also observed and we investigated the mechanisms producing these effects in a human colon adenocarcinoma cell line (LoVo).

MATERIALS AND METHODS

Drugs. Dx, DN, 4-DMDx, 4-DMDN, 4'-dDx, 4'-IDx, iso-Dx and 7(R),9(R)-4-DMDN were from Farmitalia-Carlo Erba (Milano, Italy). All the anthracyclines were in the hydrochloride form, and were shown to be 99% pure by HPLC; compounds were dissolved at the concentration of 0.1 mg/ml in sterile distilled water and stored in the dark at −20°. Dilutions were made in growth medium immediately before use.

Cell culture. LoVo cells [19, 20] were maintained at 37° in a humidified CO_2 atmosphere. Culture medium was Ham's F 12 (Gibco, Grand Island, NY) supplemented with 15% fetal calf serum (Flow Laboratories, Irvine, U.K.), 1% vitamins (BME vitamin solution 100X Gibco), 1% glutamine (200 mM, Gibco), 100 U/ml penicillin and 100 μg/ml streptomicin.

Labelling conditions and drug treatment for alkaline elution. Cell monolayers were grown in 25 cm^2 flasks (Costar, Cambridge, MA). Cultures used for experiments were in exponential growth with a doubling time of 24 hr.

Uniform labelling of DNA was obtained with [*methyl*-^{3}H]thymidine (0.05 μCi/ml, 30 mCi/mmol) (New England Nuclear, Boston MA). After 24 hr tritiated thymidine was removed and chasing with fresh medium was performed for about 16 hr. Cells (0.5–1 × 10^6) were treated for 4 hr at 37° by the addition of the appropriate dilution of the stock anthracycline solution. In experiments in which 37° incubation was performed after drug treatment, the cells attached to the flasks were gently washed twice with 5 ml of PBS and reincubated with 5 ml of medium. At the end of drug treatment or recovery the cells were washed twice with 5 ml of cold PBS (4°), scraped off with a rubber policeman and resuspended in 10 ml of PBS.

Alkaline elution. DNA damage was detected by the alkaline elution technique described by Kohn *et al.* [21]. In the assay to detect DNA-SSB, cells were loaded onto 25 mm diameter 0.8-μm pore size polycarbonate filters (Nucleopore Co., Pleasanton, CA). The cells were lysed on the filters at room temperature with 5 ml of lysis solution containing 0.1 M glycine, 0.025 M EDTA and 2% sodium dodecylsulfate (pH 10). The lysis solution was allowed to elute, and the funnels were then connected to the pumps and

Table 1. Association with proteins of DNA-SSB produced by anthracyclines in LoVo cells

Drug	C_M μg/ml	C_i ng/10^6 cells	DNA-SSB (Rad-Equivalents) Protein* associated	Non protein associated
Doxorubicin	1.0	180	145 ± 31†	3 ± 1
Daunorubicin	1.0	275	172 ± 25	ND
4-Demethoxydoxorubicin	0.25	200	90 ± 69	177 ± 2
4-Demethoxydaunorubicin	0.1	42	266 ± 24	189 ± 23
4'-Deoxydoxorubicin	0.25	120	159 ± 30	54 ± 15
4'-Iododoxorubicin	0.25	150	125 ± 34	111 ± 32

C_M, drug concentration in the medium.
C_i, intracellular drug concentration after 4 hr of treatment.
* Protein associated DNA-SSB were calculated by subtracting the non protein associated DNA-SSB in samples without proteinase K from the total DNA-SSB in samples with proteinase K.
† Mean ± SE.
ND, not detectable.

2 ml of lysis solution containing proteinase K (Merck, Darmstadt, F.R.G.) (0.5 mg/ml) were added. The funnels were filled with about 40 ml of a solution containing 0.02 M EDTA, 0.1% sodium dodecylsulfate and tetrapropylammonium hydroxide (Eastman Kodak Co, Rochester NY) 20% in water added to give a pH of 12.2. The pumping rate was 0.04 ml/min and fractions were collected at 60 min intervals for 15 hr. Before counting, fractions were mixed with Aquassure (New England Nuclear) containing 0.7% acetic acid. Acetic acid was used to reduce chemiluminescence produced by the alkali. Untreated cells were also irradiated with 300 rads before loading on the filters. The fraction of DNA remaining on the filter was plotted against time, after normalizing the fractions collected to the first fraction. Preliminary experiments in which the fraction of DNA remaining on the filter was plotted against that of the DNA of untreated cells labelled with [^{14}C]thymidine, used as internal standard, gave the same results. To report DNA breaks as rad equivalents calculations were made according to Kohn *et al.* [21].

To detect DNA-ISC treated and untreated cells were irradiated with 300 rads before the alkaline elution; cells were processed as for DNA-SSB assay.

Cytotoxicity assay. Exponentially growing cultures were harvested with 0.25% trypsin (Gibco) and resuspended in culture medium at the concentration of 4×10^4 cells/ml and seeded in 36 mm plastic dishes (Falcon, 2 ml/dish). The cells were exposed to the drugs at different concentrations in replicate samples for 4 hr, then the medium was withdrawn, cells were rinsed once with PBS and fresh culture medium was added.

After 72 hr cells were harvested with 0.25% trypsin and counted with a model ZBI Coulter Counter (KONTRON). Percent growth was calculated as number of cells in treated samples divided by control cell number (×100); the IC_{50} values were calculated on dose–response curves.

Intracellular drug accumulation and release. Exponentially growing cells were adjusted to the concentration of 3×10^5 cells/ml in 60 mm plastic dishes (Falcon, 4 ml/dish). After 48 hr incubation they were exposed to different concentrations of drugs in replicate samples for 4 hr, then the medium was withdrawn and cells were quickly washed twice with ice-cold PBS.

Samples for drug release evaluation were further incubated for 1 hr in fresh cell culture medium. At the end of the incubation cells were harvested with 0.25% trypsin at room temperature, suspended in 3 ml ice-cold PBS and collected by centrifugation at 1500 rpm at 4°. The intracellular drug was extracted with 2 ml of a mixture of ethanol/aqueous 0.3 N HCl (1:1 v/v) and measured spectrofluorimetrically at the excitation wavelength of 479 nm and the emission wavelength of 593 nm for all compounds. Intracellular drug levels were calculated against a calibration curve and related to 10^6 cells. Cell number was determined with a Coulter Counter on an aliquot of the cell suspension before centrifugation.

Intracellular metabolism. Exponentially growing cells were seeded at the concentration of 3×10^5 cells/ml in 150 cm^2 flask (Falcon, 40 ml/flask). After 48 hr incubation at 37° the growth medium was withdrawn and replaced with culture medium containing 2.5 μg/ml of drug. After 4 hr exposure the medium was withdrawn and the cells were quickly washed twice with ice-cold PBS and harvested with a few drops of 0.25% trypsin at room temperature. Detached cells were collected by low-speed centrifugation at 4°. Drugs and their metabolites were extracted with 0.4 ml of water and 0.1 ml of $AgNO_3$ 33% after vigorous shaking at 4° for 10 min. Two ml of isoamyl alcohol were added and samples were shaken again for 10 min at 4°, then centrifuged at 1000 *g* for 10 min at room temperature. The organic phase was withdrawn and dried over nitrogen. Intracellular metabolites were analysed by HPLC with a Perkin-Elmer (Norwalk, CT) Series 3B liquid chromatograph using a reversed-phase C_{18}-μBondapak column 30 cm × 3.9 mm. The parent drugs and their metabolites were separated by isocratic elution with a mobile phase of 70% KNa_2PO_4 0.05 M and 30% CH_3CN brought to pH 3 with 0.1 M H_3PO_4 with a flow rate of 1.5 ml/min. Compounds were detected by fluorescence using a Perkin-Elmer Model 650/40 fluorescence spectrophotometer with

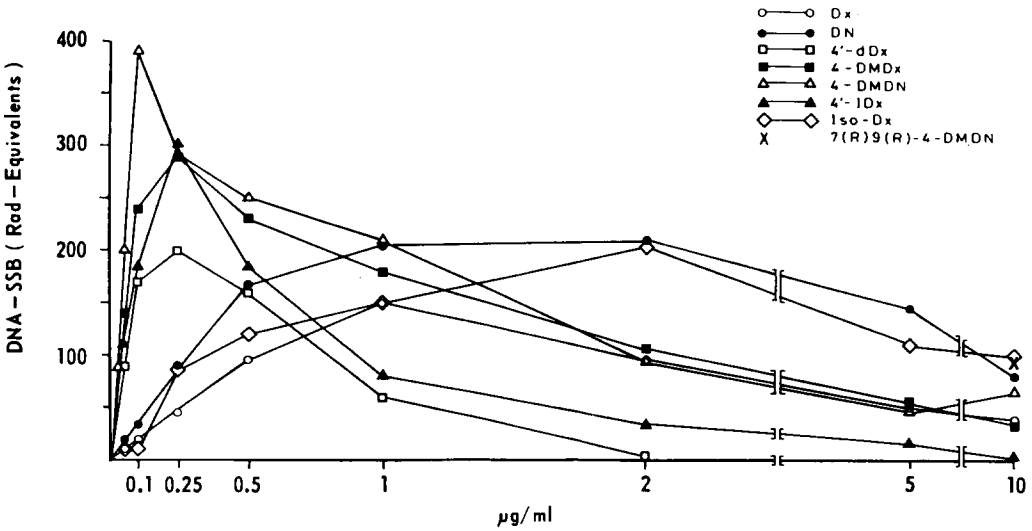


Fig. 2. Effect of drug concentration on DNA-SSB frequency. Cells were exposed to different concentrations of drugs for 4 hr at 37° and then loaded on polycarbonate filters. The cells were lysed in the presence of proteinase K and radioactive DNA was subjected to elution at pH 12.2. Break frequencies were calculated as Rad Equivalents, obtained from the elution slopes. For 7(R),9(R)-4-DMDN the DNA-SSB are reported only for the highest concentration.

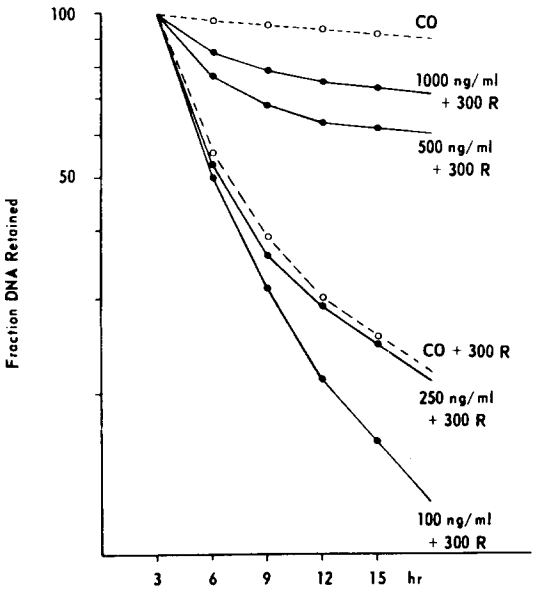


Fig. 3. Formation of DNA-ISC after cell treatment for 4 hr at 37° with different concentrations of 4'-dDx. (CO, untreated cells.)

excitation and emission wavelengths 479 and 593 nm, respectively. Intracellular drugs and their metabolites were identified by comparing the retention time of unknown peaks with those of standards. The percentage of metabolism was determined by counting the cells in the samples and related to 10^6 cells.

RESULTS

Chemical structures of anthracyclines

The derivatives chosen for this study were analogs of Dx and DN modified either in the chromophore or the sugar (Fig. 1).

Modifications in the chromophore involved substitution of the methoxy group in the 4 position with hydrogen and inversion of the steric configuration of the groups at positions 7 and 9 of 4-DMDN. The 4-DMDN has shown good antitumor activity and is now under clinical trial [22, 23]. 7(R),9(R)-4-DMDN has no antitumoral activity (unpublished data) and is weakly cytotoxic.

The modifications in the sugar involved positions 3' and 4'. In position 4' the hydroxy group was substituted with hydrogen and iodine (4'-dDx and 4'-IDx) and the positions of the hydroxy and amino groups were inverted (iso-Dx).

DNA-SSB dose–response relationship

The frequency of DNA-SSB associated with proteins and that obtained in the absence of proteinase K is reported in Table 1. Non protein associated breaks were undetectable for Dx and DN, but their number was quite relevant for the other anthracyclines. For 4-DMDx and 4-DMDN the level of protein associated DNA-SSB was higher than that of protein associated breaks. All the anthracyclines investigated produced DNA-SSB over the concentration range 0.05 to 10 μg/ml after 4 hr incubation and a biphasic dose–response was observed (Fig. 2), 7(R),9(R)-4-DMDN formed DNA-SSB only at the highest concentration.

The production of DNA-SSB did not appear to be related to the intracellular drug levels, in fact the order of potency for the production of total protein and non protein associated DNA-SSB was 4-DMDN>4-DMDx>4'-IDx>4'-dDx>DN>Dx while the intracellular drug concentration at peak level of DNA damage decreased in the order DN>4-DMDx>Dx>4'-IDx>4'-dDx>4-DMDN (Table 1).

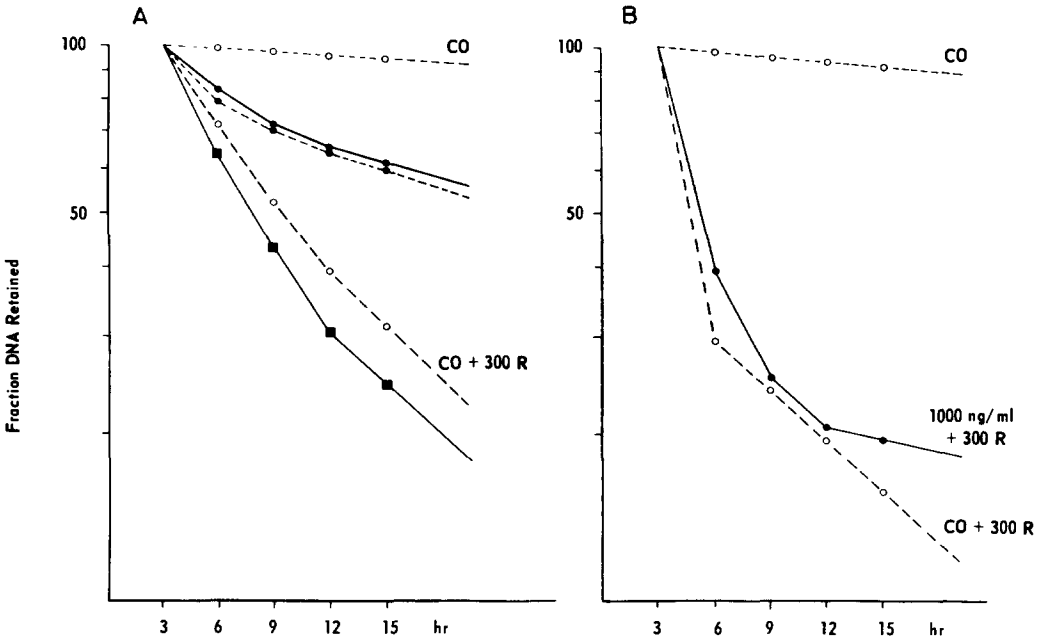


Fig. 4. Experiments showing that DNA-ISC are not due to chemical artifacts (for details see text). Elution curve of cells treated with 4′-dDx (1 μg/ml) for 4 hr at 37° (●——●), elution curve after addition to the previous sample of an equal number of unlabeled and untreated cells (●---●); elution curve of the DNA of labelled but untreated cells to which an equal number of cells treated with 4′-dDx but unlabelled were added before elution (■——■) (panel A). Elution curve of the DNA of cells treated and irradiated on the filter (●——●); elution curve of the DNA of untreated cells irradiated on the filter (○----○) (panel B).

Table 2. Intracellular drug concentrations after 4 hr treatment (C_0); intracellular concentrations after 1 hr incubation in drug free medium (C_1); drug efflux (E). Percentage of drug metabolized in LoVo cells after 4 hr treatment (%met.)

Drug	C_0 ng/10^6 cells	C_1	%E	%met.
Doxorubicin*	121 ± 7	77 ± 7	37	ND
Daunorubicin	335 ± 14	149 ± 12	56	10
4-Demethoxydoxorubicin	917 ± 52	914 ± 66	1	ND
4-Demethoxydaunorubicin	731†	337†	54	12
4′-Deoxydoxorubicin	576 ± 173	350†	39	ND
4′-Iododoxorubicin	766 ± 77	448 ± 46	42	12
Isodoxorubicin	391†	313†	5	2
7(R),9(R)-4-demethoxydaunorubicin	218†	190†	13	5

* Cells were treated with drugs (1.25 μg/ml) for 4 hr, values are the mean ± SE.
† Single determination.
ND, not detectable.

DNA interstrand cross links

Our study on the relationship between the dose and DNA-SSB formation was started with 4′-dDx. The decrease in DNA-SSB occurred at quite a low concentration (0.5 μg/ml) and we wondered whether this was due to the formation of DNA-ISC that could reduce the elution of DNA through the filters. After irradiation of the cells treated with 4′-dDx we did in fact find a drop in DNA elution compared to the irradiated control cells after proteinase K treatment (Fig. 3). This effect occurred at drug concentrations causing a decrease in DNA-SSB (Fig. 2).

As it has been observed that anthracyclines, in the presence of alkali, can form radicals [24], we investigated whether the formation of DNA-ISC might be due to chemical activation of the drug during alkaline elution.

The hypothesis was formulated that the cells could act as a drug sink and the drug released during cell lysis on the filter at pH 10 could react with DNA, causing DNA-ISC. To verify this hypothesis labelled cells treated for 4 hr with 1 μg/ml of 4′-dDx were mixed with an equal number of unlabelled and untreated cells before loading them on the filters. In a complementary experiment unlabelled cells treated with the drug were mixed with an equal number of labelled but untreated cells. If the hypothesis was correct we would observe a decrease in the formation of DNA-ISC for the treated labelled cells and an increase for the labelled untreated cells. Figure 4A reports the

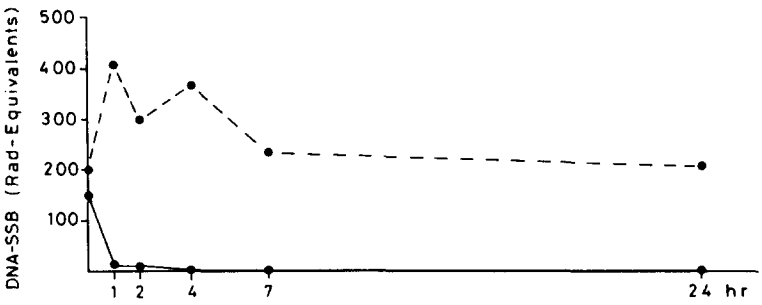


Fig. 5. Kinetic of DNA-SSB repair after cell treatment for 4 hr at 37° with 4-DMDN; 0.05 μg/ml (●——●) and 2 μg/ml (●----●). Values are the mean of two experiments.

results, showing no decrease or increase in the formation of DNA-ISC.

We also checked whether only the fraction of the drug intercalated into DNA could react with the lysis buffer to form reactive intermediates. Untreated cells were loaded on the filters and lysed, the lysate was washed with 10 ml of Tris buffer containing 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA and 15 μg/ml of bovine serum albumin at pH 7.4 and the drug (1 μg/ml) was added in 2 ml of this buffer and incubated in the funnels for 4 hr at 37°. This solution was then eluted and the filters washed with 5 ml of Tris buffer; the filters were removed from the funnels and irradiated on ice in a Petri dish with 300 rads. The filters were put back on the funnels and 5 ml of the lysis solution were added, the funnels were connected to the pumps and proteinase K and the elution buffer were added. As shown in Fig. 4B the DNA elution curves for treated and untreated samples were the same.

No degradation of 4'-dDx, in the presence of the lysis buffer, was observed by HPLC (data not shown) and no metabolites of 4'-dDx were detected in LoVo cells (Table 2). Formation of DNA-ISC was also observed for 4'-IDx but not for the other anthracyclines (data not shown). Therefore the decrease of DNA-SSB is not related to formation of DNA-ISC because the biphasic relationship for DNA breaks is observed for all derivatives.

Kinetics of DNA-SSB and DNA-ISC after drug removal

In the attempt to clarify whether the formation of DNA-ISC affected the kinetic of DNA-SSB after drug removal we studied the recovery of DNA cleavage for 4'-dDx and 4-DMDN. They presented similar kinetics of DNA-SSB formation, but differed in the production of DNA-ISC. DNA-SSB repair was assayed at two concentrations on the increasing and decreasing part of the curves reported in Fig. 2. For 4-DMDN with 0.05 μg/ml about 100% repair was observed after 1 hr (Fig. 5); with 2 μg/ml the amount of DNA-SSB increased rapidly after drug washout, giving a peak comparable to that obtained at the concentration of 0.1 μg/ml after 4 hr treatment (Fig. 2). At 7 hr of recovery a decrease of DNA-SSB was observed, but at 24 hr the amount of DNA-SSB was still almost the same as without recovery. For 4'-dDx at 0.1 μg/ml repair was very fast, 75% of the breaks disappearing after 1 hr (Fig. 6); with 1 μg/ml DNA-SSB steadily increased up to 7 hr. However, whether this increase is related to an unspecific fragmentation of DNA due to the cytolytic process remains to be established. The DNA-SSB produced by treatment with Dx (1 μg/ml) for 4 hr were virtually unrepaired after 2 hr of recovery, but later repair occurred and only about 25% of the breaks were detectable after 24 hr (data not shown). After 1 hr of recovery in drug free medium there is a partial but similar release of 4-DMDN, 4'-dDx and Dx from the cells (Table 2). The effects of drug release on DNA-SSB seems however to be different either with different concentrations of the same drug or with the same concentration of different drugs. In the second case for Dx and 4'-dDx at 1 μg/ml after 2 hr of recovery it is observed no repair or an increase of DNA-SSB respectively at the same rate of drug efflux.

Relationships between cytotoxicity, intracellular drug concentration and DNA-SSB

The IC_{50} (Table 3) span over wide log range, but for most of the compounds growth inhibition reached a plateau on plotting the growth inhibition against the intracellular concentration (Fig. 7). Drugs showing higher uptake are those that reach the plateau at a lower intracellular concentration.

The fact that some cell survived treatment with high drug concentrations (Fig. 7) could suggest that the cells seeded initially are not killed but arrested in a particular phase of the cell cycle. However according to this hypothesis we would observe a plateau at a percentage of growth of 12.5%, taking into account the initial number of cells and the doubling time. The presence of a plateau at percentage of cell growth of about 20%, for some derivatives (Fig. 7), seems instead to indicate that a small number of the cell seeded initially can duplicate at high drug concentrations representing a small population resistant to the effects of anthracyclines. With the colony assay this plateau effect was not observed presumably because the number of resistant cells was too small to form an appreciable number of colonies (data not shown). The relationship between the formation of DNA-SSB and the inhibition of the growth rate is reported in Fig. 8. DN, 4-DMDx, 4-DMDN, iso-Dx and 4'-IDx inhibited cell growth more than 70% at a concentration which caused DNA-SSB in amounts corresponding to about 25 rad equivalents, and the

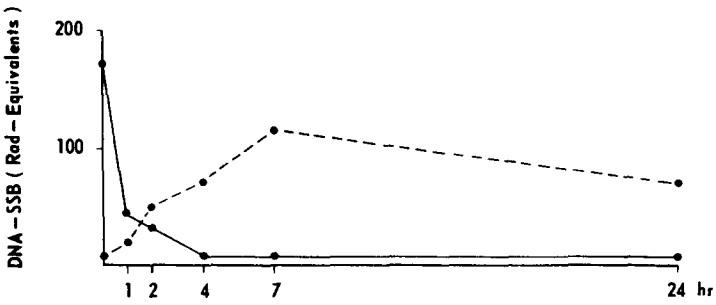


Fig. 6. Kinetic of DNA-SSB repair after cell treatment for 4 hr at 37° with 4'-dDx; 0.1 μg/ml (●——●) and 1 μg/ml (●- - -●). Values are the mean of two experiments.

Table 3. Growth inhibition (IC_{50}) measured after 4 hr treatment and 72 hr recovery in drug-free medium

Drug	IC_{50} (ng/ml)
Doxorubicin	450 ± 33*
Daunorubicin	70 ± 4
4-Demethoxydoxorubicin	6.2 ± 1
4-Demethoxydaunorubicin	9.5 ± 1
4'-Deoxydoxorubicin	44 ± 5
4'-Iododoxorubicin	15 ± 2
Isodoxorubicin	50 ± 4
7(R),9(R)-4-demethoxydaunorubicin	80,000 ± 149

* Mean ± SE.

cytotoxic effect reached a plateau even though the number of breaks continued to rise following the increase of the intracellular concentration (Fig. 9). Reporting the formation of DNA-SSB against the intracellular concentration a biphasic effect is observed at high intracellular drug levels (data not shown), but this effect does not decrease the cytotoxicity. Dx and 4'-dDx also showed a close relationship between DNA damage and cytotoxicity but are on a different curve and show the same IC_{50} as the first group of drugs with a four times higher frequency of DNA-SSB.

DISCUSSION

In this study we clarified the relationships between

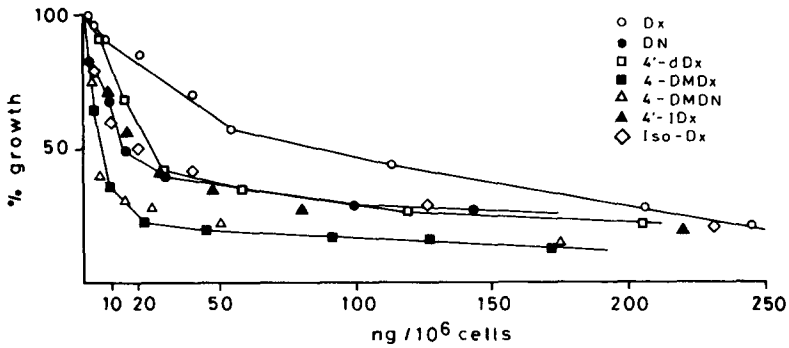


Fig. 7. Relationship between the inhibition of cell growth and anthracycline intracellular accumulation.

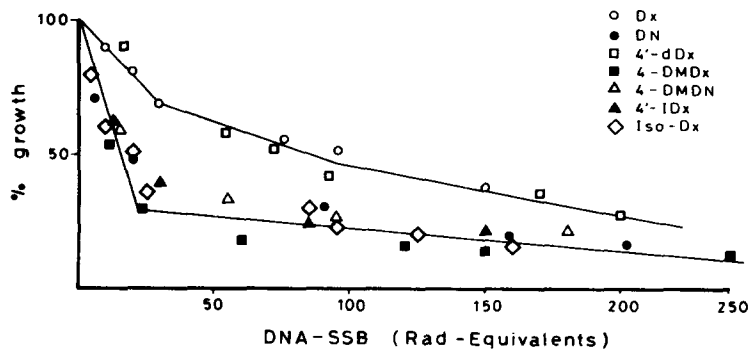


Fig. 8. Relationship between the frequency of DNA-SSB and cell growth inhibition.

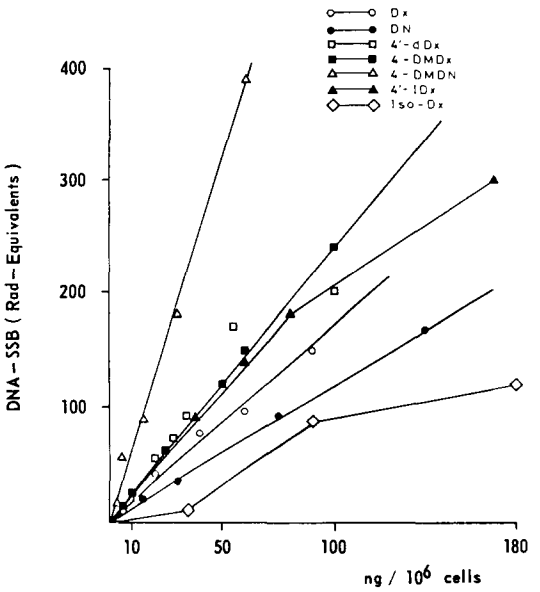


Fig. 9. Relationship between formation of DNA-SSB and anthracycline intracellular accumulation.

intracellular concentration, DNA-SSB formation and cytotoxicity for eight structurally closely related anthracyclines.

In a very small range of concentrations and DNA-SSB two groups of anthracyclines showed a good correlation between DNA damage and cytotoxicity (Fig. 8). However at about 70–80% of growth inhibition the cytotoxicity reached a plateau in relation either to the intracellular drug concentration (Fig. 7) or the frequency of DNA-SSB. This effect could be explained by the presence of a small population of cells resistant to the effect of the drugs. It has in fact been observed that LoVo cells made resistant to Dx show much lower uptake than sensitive cells, and formation of DNA-SSB is observed at higher concentrations than for sensitive cells [25].

The observation that there are two groups of anthracyclines producing about a four times different number of breaks at equitoxic concentrations (IC_{50}) (Fig. 7) and the very low level of DNA-SSB at which a correlation with the cytotoxic effect is observed suggests that a direct quantitative relationship between DNA cleavage and cytotoxicity is unlikely. In a similar study the frequency of DNA-SSB and the cytotoxicity of several anthracyclines did not correlate in P 388 cells [16]. Moreover in these cells 4-DMDN was about six-fold less cytotoxic than in LoVo cells but produced ten times more breaks at comparable intracellular concentrations.

According to the hypothesis that the enzyme topoisomerase II is the target responsible for the cytotoxic effect, modifications of the interaction between DNA and the enzyme and the biological effect produced by this process may well not be related only on the basis of the total number of breaks. The biphasic effect on DNA breaks formation by the intercalating agent 2-methyl-9-hydroxyellipticinium has been recognized as resulting from complete dissociation between DNA and topoisomerase II or from non- covalent binding [26]. Probably this effect is caused by extensive distortion of the DNA structure induced by an excess of drug intercalated into the macromolecule inhibiting recognition by the enzyme of its binding sites. This hypothesis is supported by the fact that for anthracyclines that do not bind to DNA, in an *in vitro* system with PMC 41 plasmid, no biphasic effect has been observed in the cleavage reaction [27]. The kinetics of DNA-SSB formed by 4'-dDx and 4-DMDN after drug removal seems to confirm the hypothesis that an excess of drug intercalated into DNA may inhibit binding of topoisomerase II to DNA causing therefore a lower number of cleavable complexes and DNA breaks after protein denaturation. At low drug concentrations DNA-SSB are repaired very rapidly after drug efflux from the cells (Figs 5 and 6). At drug levels producing a decrease of DNA-SSB their increase, after drug washout, could be due to reduced distortion of DNA structure produced by drug efflux and to better recognition by topoisomerase II of its binding sites on DNA.

An additional peculiar finding of the present study was the formation of DNA-ISC by 4'-dDx. Although formation of DNA-ISC has been reported for Dx and DN with a DNA renaturation technique [28], and attributed to metabolic activation, it is unlikely that they can be produced this way at least in LoVo cells. Anthracyclines do not possess the chemical characteristics for the formation of bifunctional reactive intermediates [29], 4'-dDx as the other derivatives examined is not metabolized by LoVo cells (Table 2) and the mutagenic activity of anthracyclines is lost in V79 cells in the presence of the S9 fraction [30]. Furthermore the repair curve of DNA-ISC produced by 4'-dDx showed a biphasic behaviour with a decrease after 2 hr of recovery and then a further increase of DNA-ISC up to the initial levels at 7 hr after the end of treatment (data not shown). This effect seems to exclude the formation of covalent bonds between the drug and DNA, but could be attributed to drug redistribution between intracellular compartments. For all these reasons it would be better to consider this finding as formation of "apparent" DNA-ISC.

In accordance with the theory of DNA-ISC detection by alkaline elution the failure to separate the two strands of DNA results in a drop in elution after irradiation that is measured as DNA-ISC. An alternative mechanism to the covalent linkage of the two strands could be the formation of concatenated DNA loops tangled together [31] or the separation of DNA strands could be prevented by electrostatic intra or interstrand cross links formed by highly charged drug aggregates [32]. Further studies are needed to elucidate the mechanism for the apparent formation of DNA-ISC in cells treated with 4'-dDx also considering that this compound appeared to possess a different spectrum of antitumor activity compared to other anthracyclines [33].

In conclusion our study shows that there is no quantitative relationship for anthracyclines between the cytotoxic effect and the formation of protein associated DNA-SSB. Nevertheless there appear to be qualitative differences in the modification of the interaction DNA–topoisomerase II and these may be

important to explain the pharmacological properties of these drugs.

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