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DNA unwinding and inhibition of T4 DNA ligase by anthracyclines

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ABSTRACT

The ability to alter DNA tertiary structure of ten anthracycline derivatives whose antitumor potency is known was studied by an assay that makes use of nicked circular DNA and bacteriophage T4 DNA ligase. This assay allows the detection of tertiary structure alterations caused by DNA binding of both intercalating and non-intercalating drugs. The determination of these events can be obtained at different temperatures in the range of activity of DNA ligase. The results indicate that anthracyclines alter the DNA tertiary structure but this property does not correlate with their cytotoxic or antitumor activities. An additional interesting finding was that several anthracyclines inhibit T4 DNA ligase. The inhibition can be complete and is a cubic function of drug concentration. The inhibition of DNA ligase does not correlate with the ability of anthracyclines to alter the tertiary structure of DNA but is dependent from the presence of an amino group on the sugar ring.

INTRODUCTION

There is good evidence that a variety of antitumor drugs, including anthracyclines, induce single- and double-stranded breaks on DNA of mammalian cells, both in vivo and in vitro, by interfering with the mechanism of action of DNA topoisomerase II (1-4). We have recently found that the relaxing activity of human DNA topoisomerase II is inhibited by a group of selected anthracycline derivatives but the inhibition does not strictly correlate with their antitumor activity (5). Most probably, as reported for many DNA damaging agents, including simple ones such as UV-radiation and methylmethanesulfonate (6,7) which are known to alter the tertiary structure of DNA, the unwinding of the DNA molecule could be responsible for an altered mode of action of DNA enzymes (8,9) or for their partial inhibition. Thus we have studied the ability of a group of anthracyclines, whose cytotoxic and antitumor activities are known, to alter the DNA tertiary structure. To this purpose we have developed an assay, based on the used of nicked circular DNA and DNA ligase, that allows detection of DNA tertiary structure alterations caused by both intercalating and non-intercalating drugs. The assay has allowed us to prove that anthracyclines not only strongly affect the DNA tertiary structure but also that they interfere with the reaction of ligation. This last property does not correlate with their ability to bind DNA or to

affect its tertiary structure but it is strongly dependent from the structure of anthracyclines.

MATERIALS AND METHODS

Materials: DNA, Drugs and Enzymes

Naturally supercoiled DNA of pAT153 was purified on CsCl and sucrose gradients as described by Maniatis et al. (10).

[^{3}H] poly d(A-T) was prepared by incubating at 37°C 4 ml of the following reaction mixture: 60 mM potassium phosphate buffer, pH 7.4; 6 mM $MgCl_2$; 0.1 mM DTT; 0.5 mM dTTP; 0.6 mM dATP; 50 units of DNA polymerase I large fragment; 5 µg poly d(A-T); 120 µCi [^{3}H] dTTP (30 Ci/mmol) and 0.6 mg bovine serum albumin (BSA). After 25 hours 92% of the labelled material was acid-insoluble and the reaction was stopped by addition of 160 µl of 0.5 M EDTA and heating at 70°C for 20 min. The mixture was then dialysed twice *vs.* 500 ml of 1 M NaCl, 5 mM EDTA, pH 8 and twice *vs.* 500 ml 20 mM Tris-HCl, pH 8, 0.5 mM EDTA. The polymer was then reduced down to the optimal size by digestion with pancreatic DNase (11).

Anthracycline derivatives were kindly supplied by Dr. F. Arcamone, Farmitalia-Carlo Erba. Their structural formulas are shown in Fig. 1.

Ethidium bromide and chloroquine phosphate were purchased from Sigma Chemical Co., St. Louis. Agarose was purchased from Bio-Rad Laboratories, Richmond.

Bacteriophage T4 DNA ligase was purchased either from Toyobo, Osaka, or Bethesda Research Laboratory, Bethesda.

DNA Circle-ligation Assay

Nicked circular pAT153 DNA substrate (100 ng), prepared according to Depew and Wang (12), is exposed to 1 unit of DNA ligase after equilibration at the desired temperature of incubation in the following reaction buffer (20 µl): 66 mM Tris-HCl, pH 7.6; 6.6 mM $MgCl_2$; 10 mM DTE; 0.7 mM ATP and the desired concentration of anthracyclines. After 10 min of incubation the reaction is stopped by addition of EDTA (20 mM final concentration). Before loading onto the gel, added drugs are removed by extraction with 2x10 volumes of butanol saturated with the electrophoresis buffer. To measure the DNA unwinding, samples are boiled for 5 min to denature unligated substrate before loading onto the gel.

[^{3}H] Poly d(A-T) Ligation Assay

Inhibition of ligation revealed by the previous assay was quantified using a modification of the method of Modrich and Lehman (11). Reaction mixture (50 µl) contained 50 mM Tris-HCl, pH 7.6; 10 mM $MgCl_2$; 1 mM ATP; 0.8 µg [^{3}H] poly d(A-T), 27 cpm/pmol; 0.6 µg BSA; 1 mM DTT and an amount of bacteriophage T4 DNA ligase that gives a linear response in the assay. After 30 min incubation at 37°C the reaction mixture was heated in boiling water for 3 min to inactivate DNA ligase and, after chilling, 50 µl of a mixture containing 80 mM Tris-HCl, pH 8; 5 mM DTT; 30 µg BSA and 9 units of exonuclease III (Boehringer) were added. After incubation for 30 min at 37°C, 80 µl samples were spotted onto a Whatman GF/C filters and batch washed with trichloroacetic acid (13). One unit of T4 DNA ligase is the amount of enzyme activity that converts 100 nmol poly d(A-T) to an exonuclease III-resistant

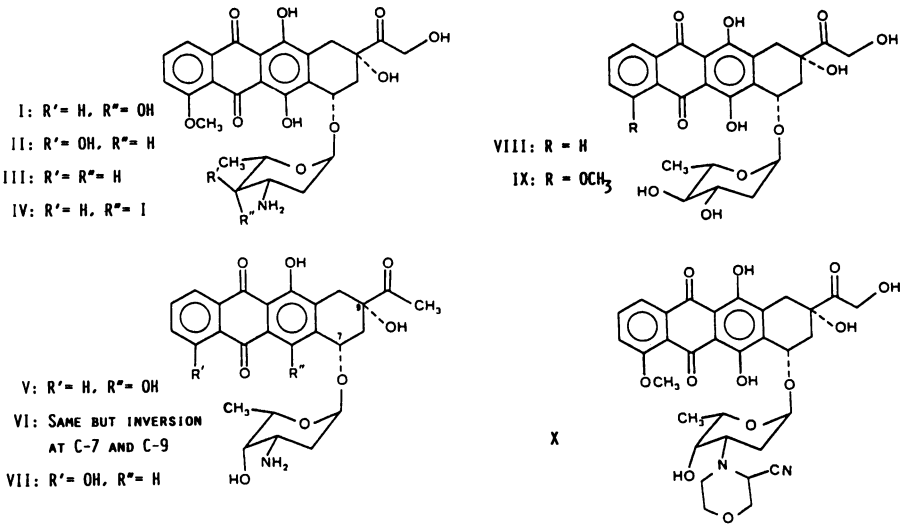

Figure 1: Structures of anthracycline derivatives studied in this work. I, doxorubicin; II, epirubicin; III, esorubicin; IV, 4'-deoxy-4' iododoxorubicin; V, idarubicin; VI, 7(R),9(R)-idarubicin; VII, 4-demethyl-6-deoxydaunorubicin (6-deoxycarminomycin); VIII, 3'-deamino-4-demethoxy-3'-hydroxyepirubicin; IX, 3'-deamino-3'-hydroxyepirubicin; X, 3'-deamino-3'-(3-cyano-morpholin-4-yl)-doxorubicin.

form within 30 min at 30°C, according to Modrich and Lehman (11). Radioactivity in control assay was less than 4 pmol, while the ligation obtained with the amount of DNA ligase used in the experiments corresponded to about 700 pmol of exonuclease III resistant material. At the concentrations used in the present work all tested compounds neither interfered with the action of the exonuclease III nor caused a breakdown of the ligated polymer upon boiling for 3 min.

Agarose gel electrophoresis

One percent agarose gels were made in Tris-Acetate-EDTA (TAE) buffer (0.04 M Tris Acetate, 2 mM EDTA, 18 mM NaCl, final pH 8.0). Gels were run at 1.4 V/cm for 14 h, at 25°C. In the case of the bidimensional electrophoresis, the second electrophoresis was performed exactly as just described for the monodimensional one, except that the gel was soaked for 1 h in 10 μg/ml chloroquine in TAE buffer and a similar concentration of chloroquine was present in the electrophoretic buffer. Gels were stained for 30 min with 1 μg/ml ethidium bromide and washed twice for 15 min with H_2O. The bidimensional gel was first throughly washed in water and then stained as just described. Photographs were taken under UV 312 nm light with Polaroid 55 film.

Calculation of unwinding

Negatives were analysed on a Beckman DU8 densitometer and the average linking number (Lk) of each ligated population of topoisomers was

calculated by a technique similar to the one already described (12), but that method was expanded in order to include in the calculation both positively and negatively supercoiled molecules. In practice, to each single topoisomer identified after bidimensional electrophoresis (14), a positive or negative value was attributed depending on their behaviour in the electrophoretic condition previously described. After microdensitometric scanning of well resolved populations of topoisomers, the average value (Vm) of each population of ligated molecules obtained in each experimental point is calculated in the following way:

$$Vm = \frac{\sum_n (V_n H_n)}{\sum_n H_n} \qquad (1)$$

were V_n is the integer value of nth topoisomer present in the Gaussian distribution under analysis and H_n its area. The ΔLk is then calculated in the following way:

$$\Delta Lk_x = Vm_x - Vm_0 \qquad (2)$$

where Vm_0 is the average value of the control in the absence of drug and Vm_x the average value in the presence of x concentration of the drug.

The equivalent ratio between concentration and unwinding (CU) is then calculated averaging the N ratios $C_n/\Delta Lk_n$, i.e.:

$$CU = \frac{\sum_n C_n/\Delta Lk_n}{N} \qquad (3)$$

were C_n and N are the concentration of nth item and N the total items number, respectively.

RESULTS

Determination of DNA unwinding by the DNA circle-ligation assay

The assay of unwinding utilized in this work is a modification of the one adopted for studying DNA unwinding by RNA polymerase (15). The substrate DNA is a nicked circular molecule that is never under torsional tension, even in the presence of intercalating drugs. In fact intercalating drugs, exactly as temperature (12), modify the twist along the double helix and the consequent change in the number of times one helix wounds around the other is istantaneously achieved by the free rotation at the level of a nick. Upon closure of the last nick, DNA ligase freezes the covalent structure of the circular DNA as it is in that exact moment, generating a population of molecules which have acquired the topological properties typical of relaxed DNA circles (Lk=Tw). When the drug is removed, the linking number (Lk) which is a topological invariant, remains constant, while the twist of the DNA acquires the value typical of the new environmental conditions. To compensate for the resulting torsional stress, the axis of the DNA molecule winds in space giving rise to superhelical turns. The results of the analysis of the negatives of gel picture can therefore be expressed as Lk. In all cases here

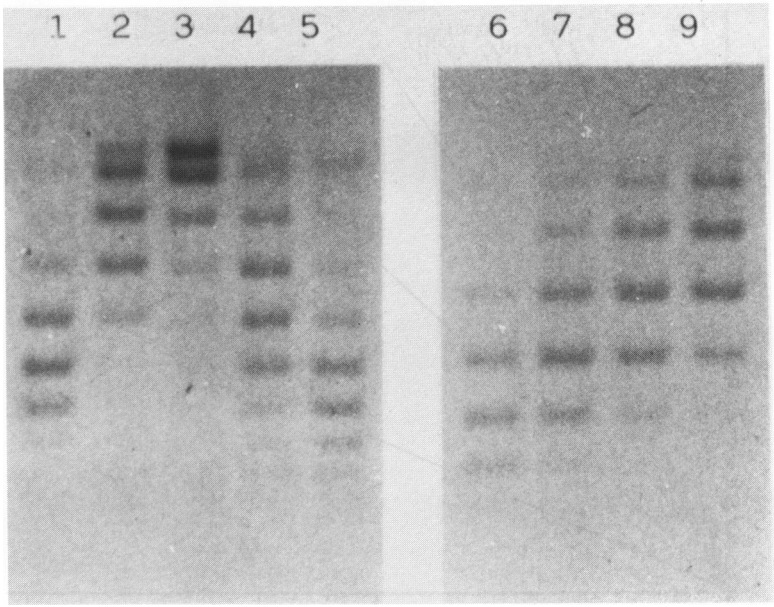

Figure 2: Two examples of determination of unwinding by compounds V and VI using the DNA circle-ligation assay. 1 and 6, controls; 2-5, 0.5, 0.75, 1 and 1.25 μM compound V; 7-9, 1.1, 1.65 and 2.2 μM compound VI. Ligation was performed at 0°C. Reaction was stopped by adding 20 mM EDTA, drugs extracted and samples run on 1.4 % agarose gels in TAE buffer.

reported we have found that the resulting DNA molecules are negatively supertwisted, indicating that all the analysed drugs unwind DNA upon binding.

The assay can be utilized between 0 and 40°C that is in the wide range of efficiency of bacteriophage T4 DNA ligase. Then in accordance with Depew and Wang (12), we have calculated the Δ°C required for an unwinding of 360° that is the Δ°C required to alter by one unit the Lk of our plasmid pAT153 DNA. We have obtained a value of approximately 14°C (data not shown) that is not too different from those previously reported for similar DNAs (12). Then we can express the unwinding values of DNA binding drugs obtained by the DNA circle-ligation assay also as Δ°C equivalents.

Fig. 2 shows the separation on agarose gel of the products of the DNA circle-ligation assay performed in the presence of compounds V and VI. Control DNA molecules appear as a lightly positively supertwisted population of topoisomers (Fig. 2, lane a). Upon ligation in the presence of increasing concentrations of DNA binding drugs, the resulting population of molecules becomes increasingly negatively supertwisted compared to the control. On the gel the topoisomers initially appear more relaxed and then progressively more negatively supercoiled (Fig. 2). In this conditions, the microdensitometric analysis of the gel is possible at relatively low concentrations of drugs or

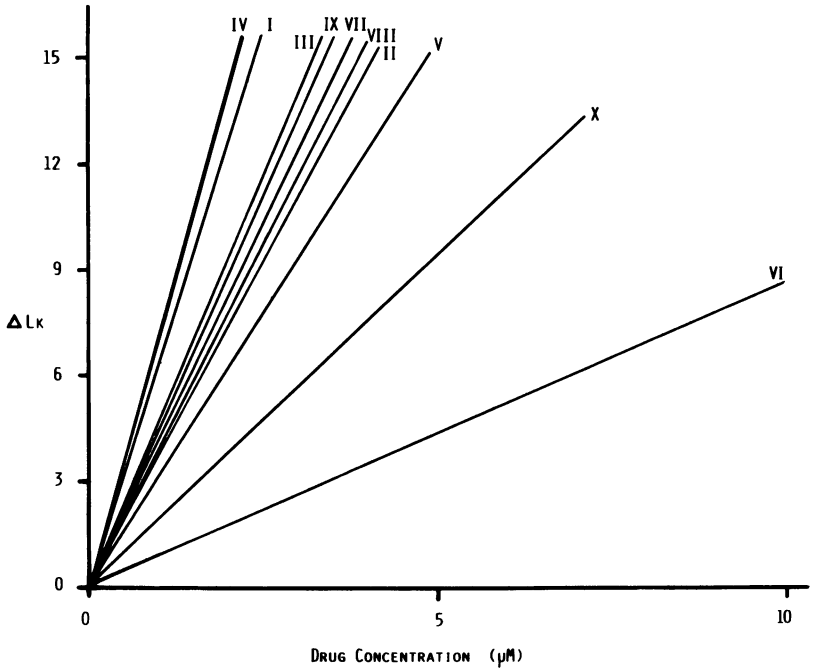

Figure 3: Graphic representation of DNA unwinding produced by anthracyclines derivatives as determined by the circle-ligation assay. Circular, nicked pAT153 molecules were covalently closed at 0°C by an excess of bacteriophage T4 DNA ligase. After extraction of drugs at the end of the incubation and separation by electrophoresis on agarose gel of ligase-generated topoisomers, drug-induced change of linking number (ΔLk) was determined (see Methods).

at concentrations resulting in an unwinding sufficient to allow the resolution of the population of negatively supercoiled DNA topoisomers. However, if informations at intermediate concentrations are required, samples could be electrophoresed in agarose gels and buffers containing 10 μg/ml of chloroquine.

Fig. 3 reports the graphical determination of DNA unwinding measured by the DNA circle-ligation assay in the presence of several anthracyclines. In all cases the $\triangle$ Lk is linearly proportional to drug concentration. All tested anthracycline derivatives alter DNA tertiary structure with 4'-deoxy-4'iododoxorubicin (IV) being approximately 10 times more potent than 7(R), 9(R)-idarubicin (VI).

The low but still well detectable unwinding ability of this last compound, included in the group as internal negative control since i) does posses neither citotoxic or antitumor activity, ii) has an extremely low affinity for DNA and iii) is not an intercalating agent (16), indicates that

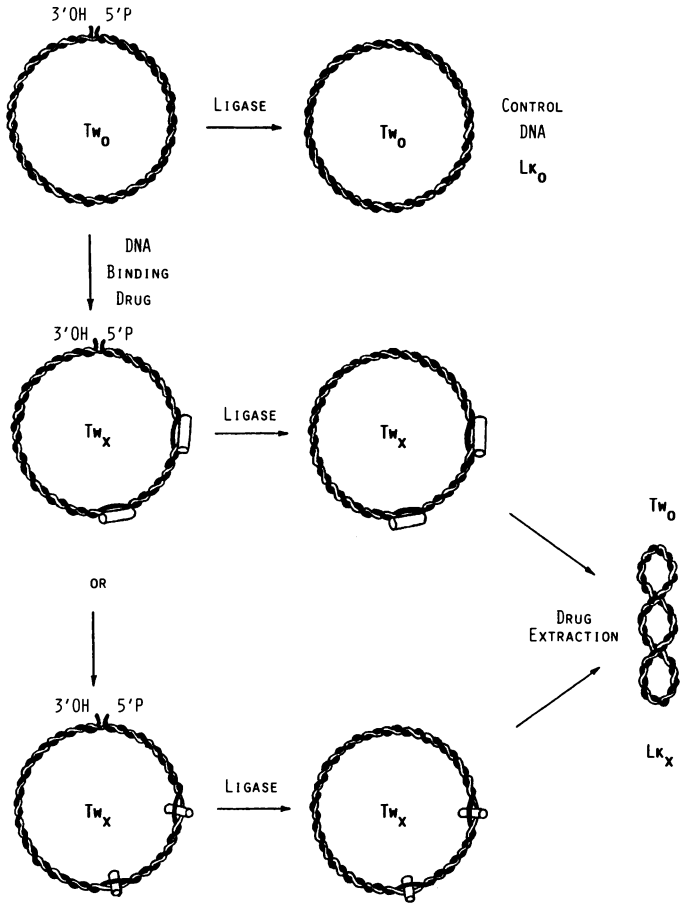

Figure 4: Principle of the circle-ligation assay to detect alterations of DNA structure. Both external and internal binding to DNA result in a change of the twist (Tw) of the douplex helix and therefore in the number of times that the two helices are intertwined. This linking number becomes a topological invariable (Lk) after ligation. Upon drug removal the twist changes back to normal and the linking number remains constant while the axis of the double helix compensate for the ΔLk between the DNA samples ligated in the presence of drug versus the control one.

the DNA circle-ligation assay here described can evidentiate any DNA binding resulting in alterations of the DNA twist angle. This important property of the DNA circle-ligation assay has also been substantiated by positive results (not shown) obtained with 4'-6-diamidino-2- phenylindole (DAPI), an externally binding drug not belonging to the anthracyclines derivatives. The DNA circle-ligation assay can be visualised as in Fig. 4 and depends on a very

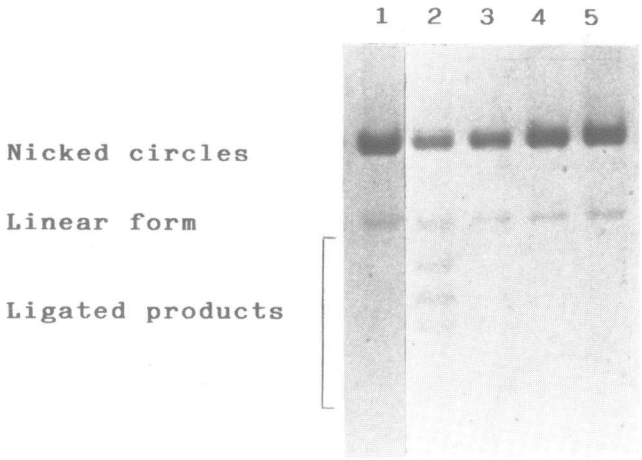

Figure 5: Dependence of inhibition of T4 DNA ligase on doxorubicin (I) concentration. Nicked, circular pAT153 molecules were ligated in the presence of 0.2 units of T4 DNA ligase at 0°C for 10 min in the absence of drug (lane 1) or in the presence of doxorubicin (1.7, 3.4 and 6.8 μM in lanes 2, 3 and 4). Unligated substrate is visible in lane 5.

large excess of DNA ligase. When the amount of enzyme in the reaction mixture is reduced to the minimal amount sufficient to ligate all the DNA substrate, also inhibition of ligation was observed in the presence of some of the anthracyclines. To better utilize the DNA circle-ligation assay for revealing DNA ligase inhibitors, the unligated DNA was denatured at the end of the incubation, allowing its visualisation and determination as the slowliest band under our conditions of analysis. When the inhibition of ligation was in this way tested with doxorubicin (compound I, Fig. 5) and on the other nine anthracyclin derivatives (data not shown), we found that at least six compounds clearly behave as DNA ligation inhibitors. In these cases the inhibition was concentration dependent and was complete at high drug concentration. The inhibition of DNA ligase by anthracyclines was confirmed and better quantified by using the ligation assay based on the circularization of poly d(A-T) (11). The results obtained by this assay, expressed as dose inhibiting 50% of ligation (ID50), are shown in Table I and graphically presented in Fig. 6. The inhibitory potency varies by approximately a factor of 30, a much wider variability than that of unwinding potency. There is no correlation between inhibition of DNA ligase and either DNA unwinding or inhibition of DNA topoisomerase II (5) caused by the anthracyclines under consideration. However a strong correlation between the structure of these anthracycline derivatives and the inhibition of T4 DNA ligase is observed. In fact all compounds possessing an amino group in position 3' of the sugar are

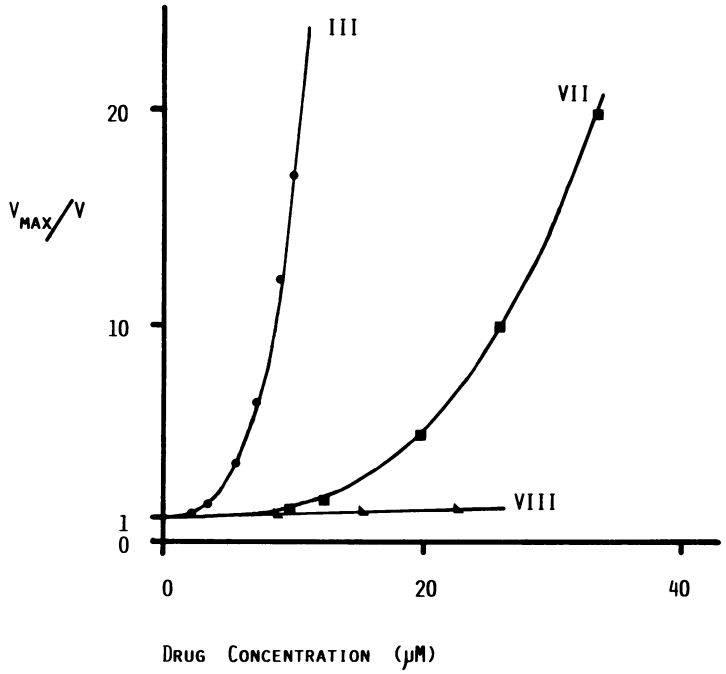

Figure 6: Drug and concentration dependence of the inhibitory effect of 3 anthracycline derivatives upon T4 DNA ligase. Compounds shown in the figure are representative of the three kinds of responce to the assay. [^{3}H]poly d(A-T) was reacted with 0.006 units of enzyme for 30 min at 37°C and ligation values were plotted as Vmax/V against concentrations of drugs. Vmax corresponds to 14×10^3 cpm.

good inhibitors of T4 DNA ligase and an hydroxyl group in the position 6 of the planar structure confers to these molecules a stronger inhibitory potency.

A very striking aspect of the inhibition of the bacteriophage T4 DNA ligase by these DNA binding drugs is that it can be well described by the cubic function:

$$\frac{V_{max}}{V} = K I^3 + 1 \qquad (4)$$

were I is the uM concentration of inhibitor Vmax is the initial velocity in absence of drug, V the initial velocity in the presence of the drug and K is a constant. K values, obtained by least-squares method applied to equation (4), are listed in Table I.

Table I: Comparison of the DNA Unwinding and T4 Ligase Inhibitory Properties of Anthracyclines

Drug	CU (μM)	ΔT (°C)	ID50 (μM)	K $(\mu M)^{-3}$
I	0.16 ±0.01	88	5.1	7.8×10^{-3}
II	0.28 ±0.03	52	2.7	5.1×10^{-2}
III	0.21 ±0.01	68	4.0	1.6×10^{-2}
IV	0.14 ±0.01	100	5.2	7.0×10^{-3}
V	0.33 ±0.04	44	5.2	7.1×10^{-3}
VI	1.1 ±0.09	13	88	1.5×10^{-6}
VII	0.24 ±0.03	60	14	3.7×10^{-4}
VIII	0.25 ±0.02	57	50	8.0×10^{-6}
IX	0.22 ±0.02	67	38	1.8×10^{-5}
X	0.53 ±0.05	27	51	7.7×10^{-6}

CU: Concentration of drug corresponding to 360° of unwinding.
ΔT: Theoretical difference in temperature mimicking the unwinding by 1 μM drug.
ID50: Drug concentration inhibiting 50% ligation of poly d(A-T) obtained from K_3 values
K: Coefficent of I^3 in equation (4); see text.

DISCUSSION

We have shown in this paper that a selected group of anthracycline derivatives strongly affect the tertiary structure of DNA but this property does not correlate with their cytotoxic or antitumor activities. The study included both intercalating and non-intercalating drugs and was possible by an assay that generates DNA topoisomers upon removal of drugs from nicked circular DNA molecules closed by DNA ligase in the presence of the tested compound. The assay allow the determination of reversible DNA alterations of any kind since the measured unwinding potency (Fig. 4) appears as the sum of the distorsions caused by external binding and intercalations. By this assay it is possible to prove that non-intercalating drugs, such as compound VI of Fig. 1, indeed unwind DNA. The total unwinding measured by the assay linearly correlates with the free drug concentrations indicating that in the range of concentration used the DNA substrate is not saturated by the binding drugs. In addition the amount of ligase usually utilized for the assay is in great excess and can ligate all the nicked substrate at time 0 even at 0°C (not shown). Taken together these observations exclude an influence of the inhibitory effects of drugs on the detection of DNA structural alterations. In fact the assay has allowed us to discover that several anthracycline derivatives interfere with the DNA ligation process. The observation that the inibition by anthracyclines is a cubic function of their concentration suggest that, in analogy with the process of intercalation, depends on a cooperative fenomenon. However their

inhibitory effect on DNA ligase, quantified by the poly d(A-T) circularization assay, does not correlate with the ability to alter the tertiary structure of DNA. In addition a structure-function correlation has been observed: only anthracycline derivatives with a free amino group in the 3' position of the sugar inhibit DNA ligase. Substitution of the amino group with a cyano-morpholin (derivative X) or hydroxyl groups (derivatives VIII and IX) leads to a loss of activity. Derivative VI, inactive despite the free amino group, represents an apparent exception because of the opposite steric configuration of the sugar radical (Fig. 1). On the contrary the presence of an hydroxyl group in the chromophore (6 position) confers to the amino derivatives a higher activity. In conclusion we have developed a new technique based on the use of T4 DNA ligase allowing the detection and quantification of DNA unwinding deriving from intercalation and external binding of drugs and conversely shown that non-intercalating drugs can unwind DNA. We have also found that T4 DNA ligase is inhibited by antracyclines derivatives possessing an amino group in the sugar residue, showing a direct structure-function correlation in an in vitro assay.

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