Interactions of Novel Morpholine and Hexamethylene Derivatives of Anthracycline Antibiotics with DNA

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Doxorubicin (DOX), daunorubicin (DRB), epidoxorubicin (EDOX) and their analogues with a 3′-NH_2 group in daunosamine form a covalent bond with a 2-NH_2 group of guanine via a methylene group from formaldehyde (CH_2O). It is assumed that a Schiff base type intermediate is formed between CH_2O and the 3′-NH_2 group in the reaction. This reaction is supposed to occur in the cell. New analogues of anthracyclines with formamidine functionality bound to C-3′ of daunosamine and containing the bulky morpholine (DRBM, DOXM and EDOXM) or hexamethyleneimine rings attached are studied in our laboratory. These substituents decrease the association of the drugs to DNA and potentially hinder the formation of Schiff base-intermediates. Our experiments indicate that the formation of the covalent complexes by DRB, DOX and EDOX under these conditions is confirmed by a high enhancement (17–40x) of the inhibition of overall RNA synthesis by *E. coli* RNA polymerase on T7 DNA. DRBM and DOXM exhibit a lower enhancement of the inhibition by CH_2O (7–13x). The other analogues show a 1.6–3x increase of inhibition. Hence, their covalent binding is lower than that of the parent compounds. These conclusions are confirmed by spectrophotometric estimations following removal of non-covalently associated drugs. Electrophoretic analysis of drug-DNA complexes formed in the presence of CH_2O indicates that DRBM and DOXM as their parent compounds induce labile cross-links in DNA. Comparison of the results obtained at the subcellular level with cytotoxicity estimations indicates that there is a correlation between cytotoxicity of the anthracyclines on L1210 cells and transcriptional template activity of drug-DNA complexes formed in the presence of CH_2O ($r = 0.64$; $n = 9$). These data confirm a notion that covalent attachment of anthracyclines to DNA is an essential event leading to cytotoxicity.

Key words: Anthracyclines, DNA-Interactions

Introduction

Anthracycline antibiotics belong to the anticancer drugs which exert their cytotoxic effects by means of interactions with DNA. Doxorubicin (DOX, also known as adriamycin), daunorubicin (DRB) and epidoxorubicin (EDOX) are currently used in cancer chemotherapy. However, the toxicity, particularly the cardiotoxicity of these drugs, remains one of the clinical problems since severe side effects impose limitations on their applications. Hence, several structural modifications have been proposed to improve the therapeutic properties of this class of compounds (Arcamone and Cassinelli, 1998; Monneret, 2001).

New analogues of anthracycline antibiotics with an amidine group bonded to the daunosamine moiety at C-3′ and containing morpholine and hexamethyleneimine rings (Fig. 1) have been synthesized at the Institute of Biotechnology and Antibiotics (Warsaw). These derivatives reveal an antiproliferative activity against some human cancer

Abbreviations: DOX, doxorubicin, trade name adriamycin; DRB, daunorubicin; EDOX, epidoxorubicin; DRBM, DOXM, EDOXM, amidine morpholine derivatives; DRBH, DOXH, EDOXH, amidine hexamethylene imine derivatives of daunorubicin, doxorubicin and epidoxorubicin; pT7–7SOD, pT7–7 plasmid bearing T7 RNA polymerase promoter *lac* UV 5 (ϕ 10) with human Cu/Zn superoxide dismutase sequence; CT, calf thymus; ss, single stranded; ds, double stranded; DTT, dithiothreitol; IC_{50} and IA_{50}, drug concentrations resulting in a 50% decrease in RNA and pppApU synthesis; ED_{50}, the drug concentration effective to inhibit 50% of the cell growth after a 72-h exposure of L1210 cells to the drug.

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cell lines similar to or even higher than that of the parent antibiotics (Oszczapowicz *et al.*, 1997, 2000). As the 3′-NH_2 group of daunosamine is important for non-covalent (Chaires *et al.*, 1996) and covalent interactions (Cullinane *et al.*, 1994a,b; Leng *et al.*, 1996; Taatjes *et al.*, 1997, 1998) with DNA, these modifications may change the drug properties.

Two lines of experiments were performed to characterize the interactions between anthracyclines and DNA: (i) Studies on non-covalent interactions which are represented by an intercalation of the aromatic rings chromophore system stabilized by interactions of the daunosamine moiety within the minor groove of DNA (Chaires *et al.*, 1996). (ii) Investigations on covalent complexes which form DOX and DRB with DNA in the presence of reagents as different as Fe(III) ions and dithiothreitol (DTT), H_2O_2 or formaldehyde (Cullinane and Phillips, 1990; Cullinane *et al.*, 1994a,b; Taatjes *et al.*, 1997, 1998; Leng *et al.*, 1996). It has been documented that the NH_2 group at position C-3′ of daunosamine binds covalently to DNA in the presence of formaldehyde (CH_2O). These adducts are presumably similar to those formed in the cell (Taatjes *et al.*, 1999; Podell *et al.*, 1999; Cullinane *et al.*, 2000; Burke and Koch, 2001).

The aim of these studies was to compare interactions of the new analogues which carry substantial modifications at the 3′-nitrogen of daunosamine. Non-covalent interactions of the drugs with DNA were characterized by means of fluorescence titration and equilibrium dialysis, and in RNA synthesis *in vitro* system by the assay of total RNA synthesis and the synthesis of the initiating dinucleotide pppApU by *E. coli* RNA polymerase. A covalent adducts formation by anthracyclines with DNA mediated by CH_2O was estimated spectrophotometrically after extraction of unreacted drugs with phenol according to Leng *et al.* (1996). Since the covalent attachment of drugs considerably decreases DNA transcriptional template activity (see Gniazdowski and Cera, 1996 for the review) we reassayed the total RNA synthesis and the abortive initiation following adduct formation with CH_2O. A variety of experimental approaches have shown that interstrand cross-links are induced by anthracycline both in subcellular systems and in the cell (Cullinane *et al.*, 1994b; Składanowski and Konopa, 1994; Taatjes *et al.*, 1998; Zeman *et al.*, 1998; Luce *et al.*, 1999) and this effect is possibly a major mechanism of the action (Podell *et al.*, 1999; Cullinane *et al.*, 2000; Burke and Koch, 2001).

The results presented here indicate that most of the new derivatives exhibit a lower affinity to DNA which is reflected by a decrease of binding constants and lower inhibitory effects on RNA synthesis *in vitro* systems. These substituents decrease but do not abolish the CH_2O- mediated attachment of the new analogues to DNA.

Materials and Methods

Anthracycline antibiotics: doxorubicin (DOX), daunorubicin (DRB), epidoxorubicin (EDOX) and their hexamethyleneimine and morpholine derivatives (Fig. 1) were synthesized at the Institute of Biotechnology and Antibiotics, Warsaw (gifts of Drs. I. Oszczapowicz and M. Wąsowska). The purity of these anthracyclines was at least (≥) 98%. Calf thymus (CT) DNA (Worthington, New Jersey), formaldehyde, ACS reagent and *E. coli* DNA-dependent RNA polymerase (lot 63 H 95201) of the nominal activity 1200 units/mg protein (Sigma, St. Louis) were used. Phage T7 DNA, pT7–7SOD plasmid DNA, phage T7 DNA-dependent RNA polymerase and most other reagents were the same as previously (Piestrzeniewicz *et al.*, 1998a,b). Cell culture reagents were described by Studzian *et al.* (2001).

DNA binding experiments were done with sonicated CT DNA in a buffer containing of 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.185 M NaCl, pH 7.0, at a temperature of 28 °C ± 2 °C. DNA was sonicated, purified by phenol extraction, then fractionated on a Sepharose 4B column (Chaires *et al.*, 1982). The DNA size in the fractions was analyzed by gel electrophoresis using Mass Ruler DNA Ladder, Low Range (MBJ Fermentas, Vilnius). The fractions of the 200–300 bp region were pooled, concentrated and dialyzed. The equilibrium dialysis was performed as described by Chaires *et al.* (1982). The concentration of free drug (c_f) in the dialysate was measured by absorbance at 480 nm and calculated using molar coefficients for the drugs (Table I). The total drug concentration (c_T) in the dialysis bag was calculated from absorbance at the isosbestic point which was determined for each drug (see Table I) and association constants K were computed. Fluorescence titration experiments were conducted on a Perkin-Elmer LS 50 B spectrofluorimeter (Chaires *et al.*, 1982). An excitation wavelength of 499 nm was applied. Titrations were performed by maintaining a fixed DNA concentration of 0.1 mM bp and

changing drug concentrations (2.5, 5, 10, 15, 20, 50 μM) (Chaires *et al.*, 1993).

The estimation of covalent adducts formation was performed essentially according to Leng *et al.* (1996) with sonicated DNA prepared as described above and dialysed against a sodium borate buffer, pH 8.2. Covalent binding reactions were conducted for 2 h in the presence of CH_2O in a solution containing DNA, anthracyclines (at the concentrations specified in Table II), sodium borate buffer, pH 8.2, at 5 mM (0.015%) or 167 mM (0.5%) CH_2O at 24 °C or 37 °C. The reactions were stopped by phenol addition and non-covalently bound drugs were extracted. Then the covalently bound portion was estimated using a molar extinction at the isosbestic points.

The interstrand cross-link formation was assayed using pT7–7 DNA plasmid of 2473 bp linearized with Hind III. Anthracycline-DNA complexes were formed similarly for the RNA polymerase assay for 2 h at 37 °C in the presence of 5 mM CH_2O. DNA and drug concentrations were 0.3 mM and 0.1 mM, respectively. The complexes if indicated were purified by extraction of non-covalently interacting drugs with phenol (Leng *et al.*, 1996; Cullinane *et al.*, 2000; Swift *et al.*, 2003). The controls containing no CH_2O were prepared accordingly. The samples were denatured for 5 min at the indicated temperature in 60% loading dye solution (90% formamide, 6.6 mM EDTA, 0.07% xylene cyanol, and 0.07% bromophenol blue). Then the samples were quenched on ice and loaded onto a 0.8% agarose gel and subjected to electrophoresis in TAE buffer [Tris-(hydroxymethyl)aminomethane-acetate, 40 mM, EDTA 1 mM, pH 8.0] at 45 V. The gels were stained with ethidium bromide and analysed using Image Master VDS (Amersham Pharmacia Biotech, Uppsala).

The inhibition of overall RNA synthesis and abortive initiation was assayed as described by Piestrzeniewicz *et al.* (1998b) and Gniazdowski *et al.* (1988). the quantification of overall RNA synthesis was done by trichloroacetic acid (TCA) precipitation of RNA synthesized by *E. coli* RNA polymerase on phage T7 DNA or CT DNA. *E. coli* RNA polymerase in the presence of ATP and UTP repetitively synthesizes pppApU on phage T7 DNA (abortive initiation). Anthracycline-DNA complexes were formed at a DNA concentration of 200 μg/ml (0.33 mM bp) and various drug concentrations in 40 mM Tris/HCl buffer, pH 8.0, containing KCl (0.1 M), $MgCl_2$ (3 mM) and EDTA (0.1 mM) either in the presence of 5 mM CH_2O (covalent complexes) or without CH_2O (non-covalent complexes) for 2 h at 37 °C. Then the portions containing 2.5 μg of DNA were mixed with the four nucleoside triphosphates (overall RNA synthesis) or ATP and [^{14}C] UTP only (abortive initiation) and other components of the incubation mixture (Gniazdowski *et al.*, 1988), equilibrated for at least 15 min and RNA or pppApU synthesis was initiated by the addition of *E. coli* RNA polymerase (5.3 U) and incubated for 10 min at 37 °C before precipitation with TCA (RNA) or application on a Whatman 3 MM paper and the chromatograms were developed to separate pppApU from the substrates (Piestrzeniewicz *et al.*, 1998b). RNA or initiating dinucleotide syntheses were assayed at 2–3 drug concentrations and expressed as percentage of the controls containing no inhibitor. Drug concentrations resulting in a 50% decrease in RNA synthesis (IC_{50}) or in pppApU synthesis (IA_{50}) were read from the inhibition curves. DNA preincubated with CH_2O, 5 mM, or without CH_2O for 2 h was used in the controls containing no inhibitors for, respectively, covalent and non-covalent complexes. Drug concentrations which are shown here correspond to those which were at the incubation with the enzyme. At the complex formation their concentrations were eigthfold higher.

Cytotoxic effects of the drugs were assayed by measuring the inhibition on murine leukaemia L1210 cells proliferation (Studzian *et al.*, 2001).

Results and Discussion

Non-covalent interactions

Association constants of the anthracycline antibiotics to DNA were estimated by means of equilibrium dialysis experiments and fluorescence titration. They are presented in Table I. The binding constant of DRB to CT DNA obtained by means of equilibrium dialysis at 28 °C ($K = 2.94 \cdot 10^5 M^{-1}$) is consistent with the data reported in other papers [$K = 6.6 \div 7.0 \cdot 10^5\ M^{-1}$ at 20 °C and 0.2 M Na^+ (Chaires *et al.*, 1982, 1993, 1996)]. The binding constant of DOX ($K = 3.8 \cdot 10^5\ M^{-1}$) is about fourfold lower than that obtained by Chaires *et al.* (1993) even taking into consideration different temperatures of the assay. The K value of EDOX is similar to those obtained for DRB and DOX. Essentially similar K values were obtained by the fluorescence titration method and the mean values

Table I. Non-covalent interactions of anthracycline antibiotics with DNA. Binding constants as estimated by means of equilibrium dialysis and spectrofluorimetric assay, effects on DNA-dependent RNA polymerase activity, inhibition of overall RNA and pppApU syntheses.

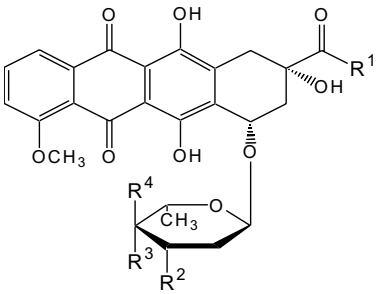
Drug	E^{f}_{480}	E	$K_{(d)} \times 10^{-4}$ [м$^{-1}$]	$K_{(f)} \times 10^{-4}$ [м$^{-1}$]	$K_{(f/d)} \times 10^{-4}$ [м$^{-1}$]	log $K_{(f/d)}$	CT DNA IC_{50} [μм]	T7 DNA IC_{50} [μм]	T7 DNA IA_{50} [μм]
DRB	11500[a]	5100[a]	29.4 ± 2.6 (5)	35.0 ± 2.4 (3)	32.2	5.51	4.8 ± 0.1 (2)	5.8 ± 0.3 (2)	7.5 ± 1.3 (2)
DRBM	10650	3080[c]	1.8 ± 0.85 (3)	1.9 ± 0.39 (3)	1.85	4.27	5.5 ± 0.2 (2)	5.9 ± 0.04 (2)	13.0 ± 1.4 (2)
DRBH	10033	5000	8.7 ± 1.5 (5)	12.23 ± 7.1 (3)	10.7	5.03	8.1 ± 0.2 (2)	7.2 ± 0.8 (2)	8.8 ± 1.1 (2)
DOX	11350	4920[b]	38.2 ± 2.4 (5)	47.5 ± 4.0 (3)	43.3	5.64	3.4 ± 0.5 (2)	3.0 (1)	6.3 ± 0.4 (2)
DOXM	10800	7300	2.15 ± 0.28 (4)	2.15 ± 0.89 (3)	2.15	4.33	5.1 ± 0.2 (2)	4.1 (1)	12.0 ± 0.7 (2)
DOXH	10300	5050	11.1 ± 2.3 (4)	19.1 ± 1.1 (3)	15.1	5.18	5.6 ± 0.2 (2)	4.1 ± 0.1 (2)	7.5 ± 1.4 (2)
EDOX	11400	4310	49.2 ± 2.9 (4)	72.0 ± 2.0 (3)	60.6	5.78	4.3 ± 0.2 (2)	3.8 ± 0.03 (2)	6.5 ± 0.7 (2)
EDOXM	10700	5200	13.2 ± 1.1 (5)	16.9 ± 2.0 (3)	15.1	5.18	5.8 ± 0.4 (2)	3.2 ± 0.1 (2)	5.8 ± 0.4 (2)
EDOXH	10300	5850[d]	37.0 ± 1.08 (3)	39.2 ± 0.06 (3)	38.1	5.59	4.5 ± 0.2 (2)	4.1 ± 0.1 (2)	7.3 ± 1.1 (2)

E^{f}_{480} (mol^{-1} cm^{-1}), extinction coefficients of free drugs at 480 nm; E, extintion coefficients at the isosbestic points at 540 nm, except [b] (560 nm), [c] (510 nm), [d] (520 nm), [a] data of Chaires *et al.* (1982). $K_{(d)}$ and $K_{(f)}$, binding constants from equilibrium dialysis and fluorescence titration experiments, respectively. $K_{(f/d)}$ are average values of $K_{(d)}$ and $K_{(f)}$. IC_{50}, drug concentration inhibiting RNA synthesis on CT DNA or T7 DNA *in vitro* to 50% of the control value. IA_{50}, drug concentration inhibiting pppApU synthesis on T7 DNA to 50% of the control value. The mean values of two or more experiments are given as indicated in parentheses ± range values (n = 2) or standard deviations (n > 2) are shown.

estimated by both methods are considered in further discussion. Anthracyclines form with DNA a complex with a chromophore intercalating between two base pairs and the intercalation is assisted by an interaction of the daunosamine moiety in the minor groove of the DNA helix. The sugar 3′-NH_2 group contributes to the interactions (Chaires *et al.*, 1996). Introduction of the amidine group to the anthracycline structure at position C-3′ of daunosamine increases the basicity. The presence of a bulky hexamethyleneimine or morpholine ring (Fig. 1) may impose a steric hindrance on the groove interactions of daunosamine. The latter effect seems to prevail since morpholine and hexamethyleneimine derivatives show a lower binding affinity to DNA than the parent drugs (Table I). Hence, K values of DRBH and DRBM, $1 \cdot 10^5$ м$^{-1}$ and $1.9 \cdot 10^4$ м$^{-1}$ (Table I), respectively, are lower than those K found for DRB. A similar regularity is observed with other morpholine and hexamethyleneimine analogues and DOX and EDOX. A lower relative decrease in binding is seen with a bulkier hexamethyleneimine ring than with a more rigid morpholine ring since DRBM, DOXM and EDOXM show the lowest K values within the series (Table I). This effect of the substituents is consistent with the observation that the 3′-*N*-acetyl derivative of DRB exhibits a considerably lower affinity than DRB which is attributed both to the loss of the positive charge and to steric hindrance from the *N*-acetyl group (Chaires *et al.*, 1993; Ruggiero *et al.*, 1992).

Inhibition of RNA polymerase

The inhibition of RNA synthesis *in vitro* primarily depends on ligand-DNA binding strength and this effect was occasionally correlated to cytotoxic activity if a structurally related family of drugs was assayed (*e.g.* cf. Atwell *et al.*, 1986; Piestrzeniewicz *et al.*, 1998a). It was demonstrated that anthracycline antibiotics inhibit RNA synthesis *in vitro* catalyzed by bacterial RNA polymerase (see Krakow and Kumar, 1980 for the review). The effects of drugs on the abortive initiation and overall RNA synthesis were assayed. In the presence of ATP



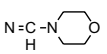
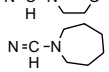
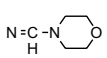
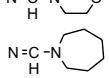
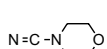
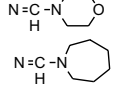
	R^1	R^2	R^3	R^4
DRB	CH_3	NH_2	OH	H
DRBM	CH_3		OH	H
DRBH	CH_3		OH	H
DOX	CH_2OH	NH_2	OH	H
DOXM	CH_2OH		OH	H
DOXH	CH_2OH		OH	H
EDOX	CH_2OH	NH_2	H	OH
EDOXM	CH_2OH		H	OH
EDOXH	CH_2OH		H	OH

Fig. 1. Structures of anthracyclines studied.

and UTP *E. coli* RNA polymerase synthesizes pppApU on A1 and A3 promoters of T7 DNA. The estimation of pppApU at the different anthracycline concentrations allowed determination of IA_{50} values (Table I). The inhibition of overall RNA synthesis does not significantly depend on the template used. IC_{50} values received for calf thymus DNA and phage T7 DNA are close to each other, while the inhibition of pppApU synthesis to 50% is observed at higher concentrations of anthracyclines (Table I). The results are consistent with the data of Kriebardis *et al.* (1987) who found a higher inhibition of RNA synthesis than the initiating dinucleotide with DRB and marcellomycin (Kriebardis and Aktipis, 1988). Although the repetitive synthesis of pppApU is not fully equivalent to the initiation of the polynucleotide chain, there is a coincidence between drugs which were found to inhibit primarily binding of the enzyme to the template and/or initiation of RNA chains and those which inhibit abortive initiation and overall RNA synthesis to similar extent (Piestrzeniewicz *et al.*, 1998a). Hence these results are consistent with a notion that DOX and DRB (Wilmańska *et al.*, 2001) and presumably other anthracyclines which form slowly dissociating complexes with DNA interfere more efficiently with the enzyme moving along the template in the elongation step than at the promoter site where the enzyme is repetitively copying the two-bases fragment.

DOX, DRB and some other anthracyclines in the presence of Fe(III) ions and DTT, H_2O_2 or CH_2O bind to DNA covalently. These adducts are formed between NH_2 groups at C-3′ of daunosamine and C-2 of guanine *via* a methylene bridge from CH_2O either added or spontaneously released in the system (Cullinane *et al.*, 1994b; Leng *et al.*, 1996; Taatjes *et al.*, 1997, 1998; Luce *et al.*, 1999). The amino group at C-3′ is essential for covalent binding to DNA. Its substitutions by a hydroxyl group or a morpholinyl ring abolish the drug propensity to form CH_2O-mediated adducts (Cullinane *et al.*, 1994a; Leng *et al.*, 1996; Taatjes *et al.*, 1997). In our preliminary experiments DRBM and DOXM were susceptible to Fe(III)/DTT activation leading to DNA adducts which induced the termination of RNA chains synthesized by phage T7 RNA polymerase although the effect seemed to be lower than that of the parent drugs (Studzian *et al.*, 2001). Fe(III) and DTT induce apparently lesions in T7 DNA since about a sevenfold decrease was observed in RNA synthesized on DNA preincubated with Fe(III)/DTT (Studzian *et al.*, 2001). This effect is not unexpected since Fe(III) itself and anthracycline-Fe(III) complexes even in the absence of DTT may affect the covalent structure of DNA (Eliot *et al.*, 1984). In this respect CH_2O as a conjugating agent does not seem to induce as multiple lesions to DNA although CH_2O may bind covalently to amino groups of purines forming stable methylene bridges between the bases (Cheng *et al.*, 2003). Actually, when we incubated CT DNA with CH_2O at concentrations of 42 ÷ 333 mM the transcriptional template activity of DNA decreased to a few percent of the controls (not shown). Preincubation of DNA without a drug at 2–10 mM CH_2O in our assay conditions affected pppApU and RNA syntheses no more than 10–12% in respect to the corresponding controls. Hence, we used CH_2O at 5 mM (0.015%) to form the drug adducts in the transcription assay.

Preincubation of CT DNA and T7 DNA with DRB, DOX and EDOX in the presence of CH_2O

Table II. Covalent binding of anthracycline antibiotics to DNA mediated by CH_2O, effect on overall RNA and pppApU synthesis by anthracyclines upon CH_2O-mediated binding to DNA and cytotoxic activity on L1210 cell proliferation.

Drug	Inhibition			Decrease			Covalent binding (c_b, [μM])			Cytotoxicity
	CT DNA IC_{50} [μM]	T7 DNA IC_{50} [μM]	T7 DNA IA_{50} [μM]	IC_{50}	IC_{50}	IA_{50}	5 mM a	167 mM b	167 mM c	ED_{50} [nM]
DRB	0.25 ± 0.0 (2)	0.14 ± 0.025 (2)	2.14 ± 1.07 (2)	19×	41×	3.5×	12.5 ± 0.4 (2)	23.6	27.2 ± 0.3 (2)	22 ± 2 (5)
DRBM	0.76 ± 0.0 (2)	0.45 ± 0.2 (2)	4.93 ± 0.39 (2)	7.3×	13.1×	2.6×	0.0 ± 0.4 (2)	3.1 ± 0.5[d] (2)	1.8 ± 0.5 (2)	75 ± 15 (5)
DRBH	4.25 ± 0.25 (2)	2.18 ± 0.15 (2)	6.23 ± 1.34 (2)	1.9×	3.3×	1.4×	n.d.	0.2[d]	0.4	150 ± 11 (3)
DOX	0.14 ± 0.0 (2)	0.125 ± 0.0125 (2)	1.90 ± 0.63 (2)	27×	24×	3.3×	13.5 ± 0.8 (2)	25.7	24.6 ± 0.8 (2)	18 ± 5 (5)
DOXM	0.75 ± 0.18 (2)	0.45 ± 0.3 (2)	4.43 ± 1.89 (2)	6.8×	10.9×	2.7×	1.25 ± 0.23 (2)	1.3	2.5 ± 0.3 (2)	24 ± 5 (6)
DOXH	1.56 ± 0.25 (2)	1.69 ± 0.05 (2)	3.21 ± 0.25 (2)	3.6×	2.4×	2.3×	n.d.	0.6[d]	0.4	91 ± 3 (4)
EDOX	0.26 ± 0.0 (3)	0.15 ± 0.04 (2)	1.4 ± 0.27 (2)	17×	25.6×	4.6×	4.25 ± 0.7 (2)	15.6	21.7 ± 0.3 (2)	11 ± 2 (3)
EDOXM	3.88 ± 0.6 (3)	2.82 ± 0.38 (2)	3.87 ± 0.21 (2)	1.5×	1.1×	1.5×	n.d.	0.7[d]	0.3 ± 0.3[d] (2)	70 ± 12 (4)
EDOXH	2.00 ± 0.31 (2)	2.58 ± 0.09 (2)	2.89 ± 0.39 (2)	2.3×	1.6×	2.5×	n.d.	0.8[d]	1.1 ± 0.4[d] (2)	198 ± 18 (4)

IC_{50} and IA_{50} as described in the legend of Table I except that the drugs and CT DNA or T7 DNA were preincubated in the presence of CH_2O (5 mM) for 2 h at 37 °C, then RNA or pppApU synthesis was assayed as described in Materials and Methods. Decrease in IC_{50} or IA_{50} is calculated from corresponding data in Table I and Table II. Covalent binding in the presence of CH_2O, 5 mM or 167 mM, as indicated. CT DNA concentration was 0.17 mM (column a), 0.35 mM (column b) and 0.607 mM (column c), and anthracycline concentration at the adduct formation was 25 μM (a) and 35 μM (b and c), except [d], concentration of drug was 70 μM. ED_{50}, the anthracycline concentration effective to inhibit 50% of the cell growth after a 72-h exposure of L1210 cells to the drug. n.d., not detected.

results in a considerable enhancement of the inhibition of overall RNA synthesis (Table II). IC_{50} values in these experiments are the drug concentration at which the adduct formation is sufficient to decrease RNA synthesis by 50%. A ratio of IC_{50} in the absence of CH_2O (Table I) and IC_{50} estimated after preincubation with CH_2O (Table II) corresponds to the enhancement, which somewhat differs depending on the template. The decreases of IC_{50} values for DRB, DOX and EDOX are 19-, 27- and 17-fold, respectively, when CT DNA is used as a template (Table II). DRBM and DOXM exhibit a lower enhancement of the inhibition, about 7-fold on CT DNA and about 13 and 11 times, respectively, with T7 DNA. The other drugs exhibit only an 1.5- to 3-fold increase in inhibition which may indicate that their covalent binding is considerably lower than that of their parent compounds and morpholine derivatives of DRB and DOX (see Tables I and II).

Covalent binding of all the anthracyclines studied here enhances the inhibition of the abortive initiation to lower extent than the overall RNA synthesis and the differences are not high between the members of the series (Tables I and II). It is not surprising as DRB and presumably other anthracyclines react with guanine forming the adduct and guanine is rather underrepresented in the promotor region and absent from the site from which pppApU is transcribed. A low inhibitory effect on pppApU synthesis may be also due to the presence of a relatively greater number of covalent modifications on long templates compared to a few adducts in the short initiation DNA region.

CH_2O-mediated covalent binding

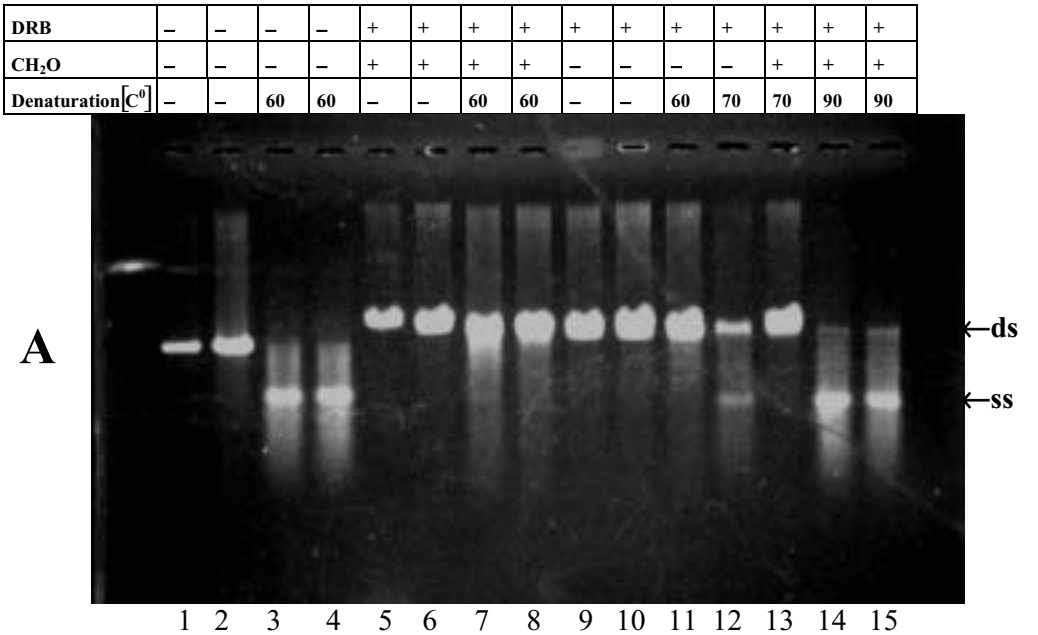
The amount of the adducts was estimated according to Leng *et al.* (1996) after extraction of unreacted drug with phenol. It is assumed that an absorbance of a drug at the isosbestic point wavelength characteristic for a free drug and a drug non-covalently bound to DNA is the same as the absorbance of a covalently attached drug. This assumption is based on optical properties of DRB

cross-linked to DNA with CH_2O (Leng *et al.*, 1996). At 5 mM CH_2O the covalent binding of DRB reached a plateau at 1–2 h (result not shown); so incubation for 2 h was used in our experiments. About 50% of DRB and DOX input (*i.e.* 12.5 μM and 13.5 μM, respectively) are covalently bound to CT DNA in the presence of 5 mM CH_2O while EDOX reacts with lower efficiency (4.25 μM) under these conditions (Table II). DOXM binding is 10-fold lower (1.25 μM) than the binding of DOX. Since covalent adduct formation by other derivatives at 5 mM CH_2O is below the level of detection the yield of anthracycline bonding to DNA was estimated at higher anthracycline (35 μM), DNA (0.35 or 0.61 mM) and CH_2O (167 mM, *i.e.* 0.5%) concentrations. To improve detection some derivatives were used at 70 μM (the data with superscript "d" in Table II). The efficiency of covalent adduct formation under these conditions increased considerably for EDOX from 17% at 5 mM CH_2O to 45% or 60% at 167 mM CH_2O depending on the DNA concentration. The yield of DRB and DOX covalently bound increased from 50% to 70% at 167 mM CH_2O and did not depend on the DNA concentration. DRBM and DOXM are covalently bound at a 10-fold lower level while the yield of the adduct formed by the other derivatives is hardly detected spectrophotometrically. A concentration of 1–2 μM of DRBM covalently bound at 0.35 mM DNA corresponds to about 3–6 drug molecules per 10^3 DNA bp. The transcription assay exhibits higher sensitivity. The bulky adducts at the density 0.5–2 molecules covalently bound per 10^3 DNA base pairs are sufficient to reduce RNA synthesis to 37% (Gniazdowski and Cera, 1996). Therefore, interpreting jointly spectrophotometric estimations and the enhancement of inhibition of RNA synthesis it may be assumed that DRBM and DOXM and, to a lower extent, DRBH and DOXH form covalent adducts with DNA.

Interstrand cross-link formation

CH_2O-mediated covalent binding to guanine occurs preferentially at the self- complementary G-C sites. Aglycone chromophore is intercalated and these interactions are strengthened by hydrogen bonds between the 9-OH group of anthracycline and N-3 and 2-NH_2 of guanine in the complementary strand forming a "virtual" cross-link (Taatjes *et al.*, 1997, 1998). These interstrand cross-links are relatively resistant to denaturation on short incubation at 60–70 °C (Cullinane *et al.*, 1994b; Leng *et al.*, 1996; Swift *et al.*, 2003). We compared complexes of anthracyclines with ds plasmid pT 7.7 and the complexes formed either at 5 mM CH_2O or without CH_2O for 2 h at 37 °C.

DRB-DNA complexes without prior purification from non-covalently interacting drug were subjected to electrophoresis on agarose gel followed by incubation for 5 min at 60 °C, 70 °C or 90 °C in formamide, and quenched on ice (Fig. 2A). Native plasmid DNA (Fig. 2A, lanes 1, 2) migrated as a single band distinctly separated from DNA denatured at 60 °C (Fig. 2A, lanes 3, 4). DRB-DNA complexes either formed at 5 mM CH_1O (Fig. 2A, lanes 5–8) or without CH_2O (Fig. 2A, lanes 9–11), then incubated at 60 °C for 5 min (Fig. 2A, lanes 7, 8, 11) or applied without denaturation (Fig. 2A, lanes 5, 6, 9, 10) migrated in single bands corresponding to native DNA. The difference is visible when DRB-DNA complexes were incubated at 70 °C. The DRB-DNA complex formed in the presence of CH_2O migrates in the ds band (Fig. 2A, lane 13) while a substantial fraction of the non-covalent DRB-DNA complex (Fig. 2A, lane 12) migrated in the ss band. It may be inferred from these observations that a non-covalently interacting ligand occurring in excess effectively stabilizes the ds structure at 60 °C. This covalent complex undergoes denaturation at 90 °C and DNA migrates as the ss band (Fig. 2A, lanes 14, 15). To avoid effects of non-covalent interactions the anthracycline-DNA complexes formed either with or without CH_2O were purified with phenol (Leng *et al.*, 1996) and subjected to electrophoresis followed by denaturation for 5 min at 60 °C (Fig. 2B). DNA preincubated with DRB (see Fig. 2A), DRBM, DOX and DOXM in the absence of CH_2O migrated as ssDNA (Fig. 2B, lanes 5, 9 and 11) and as dsDNA if preincubated with CH_2O for 2 h (Fig. 2B, lanes 4, 6, 10 and 12). The complexes of DRBH, DOXH and EDOX preformed in the presence of CH_2O were resolved into fractions of ds and ssDNA (Fig. 2B, lanes 8, 14, 16). DNA incubated with EDOXM and EDOXH in the presence of CH_2O migrated either in a single band corresponding to ssDNA (Fig. 2B, lanes 18, 20) or a faint band of dsDNA was observed in some experiments (not shown). As with the other anthracyclines complexes formed in the absence of CH_2O they are dissociated at phenol extraction and DNA is denatured under these con-



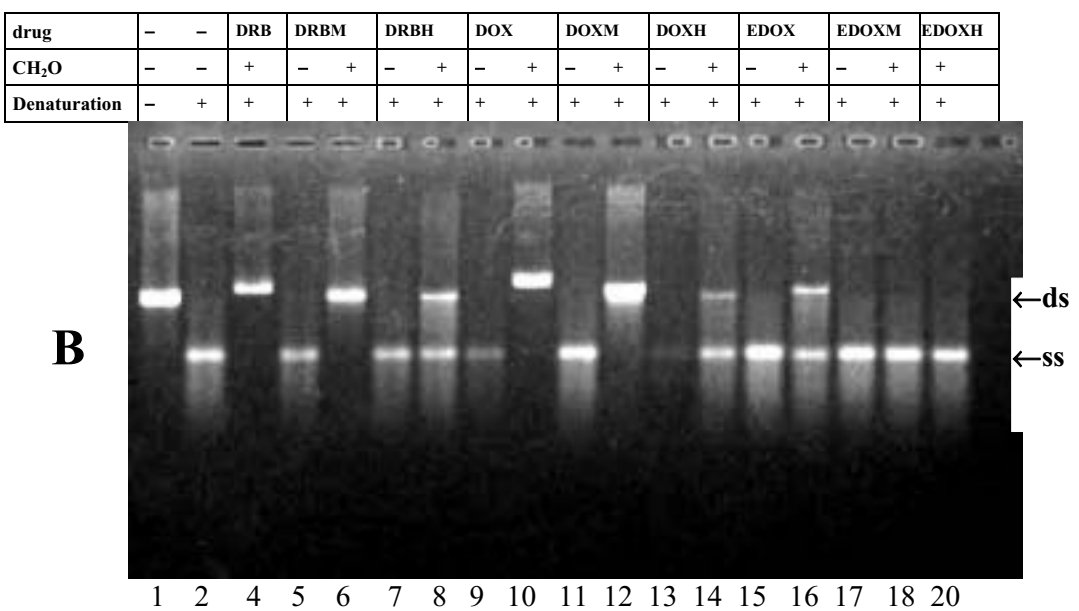


Fig. 2. Electrophoretic analysis of pT7–7SOD plasmid DNA incubated with anthracyclines in the presence and absence of CH_2O. Anthracycline-DNA complexes were formed as described in Materials and Methods in the presence of CH_2O or without it. The samples were heat-denatured in formamide for 5 min, then quenched on ice, loaded onto 0.8% agarose gel and subjected to electrophoresis. Panel A. DNA and DRB-DNA complexes incubated in the presence or absence of CH_2O, heat-denatured at 60 °C, 70 °C and 90 °C or without denaturation as indicated were subjected to electrophoresis without purification. Panel B. Anthracycline-DNA complexes were incubated ± CH_2O, then purified by phenol extraction, denatured at 60 °C as indicated. DRB and EDOXM samples in the absence of CH_2O are missing (lanes 3 and 19) in this gel but they migrated as ssDNA band in other experiments (*e.g.* panel A).

ditions. Except EDOX which reacts with DNA forming covalent complexes of high adduct density (Table I) but shows moderate cross-linking potency (Fig. 2A, B) a cross-linking revealed by gel electrophoresis confirm other data indicating that CH_2O-mediated adducts of amidine derivatives with DNA show the same property of labile interstrand cross-links formed by DRB and DOX.

As it has been pointed out earlier for DRB, DOX and several other anthracycline derivatives there is a correlation between CH_2O-mediated DNA binding and biological effectiveness of these drugs. Our screen on the L1210 murine leukaemia cell line (Table II) shows a similar cytotoxic activity of DOXM (ED_{50} = 24 nM) to those of its parent drug, DOX (ED_{50} = 18 nM), and to DRB (ED_{50} = 20 nM), whereas the other derivatives exhibit a considerably lower cytotoxic effect (ED_{50} ranging from 70 to 200 nM). There are negative correlations between log c_b at 167 mM CH_2O (Table II) and cytotoxicity ED_{50} (r = −0.77, n = 9) as well as between log c_b and IC_{50} estimated on calf thymus DNA in the presence of CH_2O (r = −0.84, n = 9) and a positive correlation between ED_{50} and IC_{50} (r = 0.64, n = 9). On the other hand no correlation is found between the cytotoxicity of the drugs and their non-covalent interactions with DNA. These data are essentially consistent with the notion that the CH_2O-mediated linkage of anthracyclines to DNA is an essential event leading to the cytotoxic effect (Cullinane *et al.*, 1994a,b, 2000; Taatjes *et al.*, 1997; Zeman *et al.*, 1998). It, however, does not seem to be a universal road leading to anticancer activity. It should be noted that the C-3′-morpholine derivatives of DOX and DRB which do not react with DNA upon reductive activation (Cullinane *et al.*, 1994a; Taatjes *et al.*, 1997), hence, presumably do not form CH_2O-mediated adducts, exhibit interesting therapeutic properties (Monneret, 2001). In preliminary studies DRBM and DRBH exhibited cytotoxic effects which were similar or higher than those of the parent compounds. For example for the SW 707 colon cancer cell line ED_{50} was 11 nM for DRBM, 6.6 nM for DRB and 40 nM for DOX (Oszczapowicz *et al.*, 1997, 2000).

It has been found in our experiments that some of the amidine derivatives in the presence of CH_2O form covalently bound adducts which are similar to those formed by the parent compounds. Although the efficiency of binding is lower it does occur with the drugs whose amidine group (Fig. 1) cannot form directly a Schiff base intermediate which is postulated to be transiently formed with DOX or DRB (Leng *et al.*, 1996). We suppose that CH_2O may react with the 2-NH_2 group of guanine forming an imine derivative and subsequently bridge 3′-N at the daunosamine moiety of the amidine derivatives. Alternatively, a Schiff base intermediate is not absolutely required for covalent binding of anthracycline to DNA-mediated by CH_2O.

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