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Sequence specificity of formaldehyde-mediated covalent binding of anthracycline derivatives to DNA

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Abstract

Daunorubicin (DRB) and doxorubicin (DOX) in the presence of formaldehyde (CH_2O) form covalent adducts with DNA. A G-specific adduct is formed by producing an aminal bridge between the C-3′ of daunosamine and the C-2 of guanine. New derivatives of DRB, DOX and epidoxorubicin (EDOX) with an amidine group bonded to the C-3′ of the daunosamine moiety, with either a morpholine or hexamethyleneimine ring attached to the amidine group, were studied in this paper. DNase I footprinting and analyses with restriction endonucleases were applied to compare the specificity of adduct formed by the amidine derivatives and their parent compounds. These approaches provide consistent results, proving that a GC pair is required for covalent binding of anthracycline derivatives to DNA and that different flanking sequences are able to modify the sequence preference of the drugs. The 5′-GC-3′, 5′-CG-3′ and 5′-TC-3′ sequences were protected most efficiently by the parent compounds and their morpholine derivatives and some increased protection of 5′-TC-3′ sequence was observed for morpholine analogues. Hexamethyleneimine derivatives bind to DNA with much lower efficiency. Finally, the sequence specificity of anthracycline derivatives was correlated with their ability to inhibit binding of transcription factors Sp1 and AP-1 to their DNA recognition sequences. The anthracycline derivatives were more potent in inhibiting Sp1 binding to its cognate GC box than in preventing AP-1 from binding to its mixed A·T and G·C site. Overall, the results indicate that the amidine derivatives of anthracyclines show similar, but not identical sequence specificity as parent compounds, though they exert their effect at a higher concentration.

Keywords: Anthracycline antibiotics; Covalent binding; Footprinting; Transcription factors; Drug specificity

1. Introduction

Daunorubicin (DRB), doxorubicin (DOX) (also known as adriamycin) and epidoxorubicin (EDOX) belong to the anthracycline group of anticancer drugs that exert their cytotoxic effect by interactions with DNA. More specifically, it has been shown previously that DRB and DOX, in the presence of reagents such as Fe(III) ions and dithiothreitol (DTT), H_2O_2 or CH_2O [1–3], form covalent adducts with DNA. In the case of interaction with CH_2O in vitro studies have shown that a Schiff base-type intermediate is formed with the NH_2 group at the position C-3′ in the daunosamine moiety. The intermediate then reacts with exocyclic NH_2 at C-2 of deoxyguanosine forming an aminal bridge. This covalent bonding to the double helical DNA structure is strengthened by additional interactions: intercalation between DNA base pairs and hydrogen bond formation between the 9-OH group of the anthracycline and G in the complementary strand [2,4,5]. These adducts are postulated to correspond to cross-links induced by anthracyclines in cellular DNA [6].

Characterization of the sequence specificity of drug–DNA interactions is essential for understanding of the drug action mechanism. By elucidating the molecular determinants of that specificity, it will be possible to develop principles for the design of new drugs with enhanced anticancer potency. New analogues of anthracycline antibiotics with a formamidine group bonded to the daunosa-

Abbreviations: DRB, daunorubicin; DOX, doxorubicin; EDOX, epidoxorubicin; DRBM, morpholine derivative of daunorubicin; DOXM, morpholine derivative of doxorubicin; EDOXM, morpholine derivative of epidoxorubicin; DRBH, hexamethyleneimine derivative of daunorubicin; DOXH, hexamethyleneimine derivative of doxorubicin; HUVEC, human umbilical vein endothelial cells; TNF-α, tumor necrosis factor alpha; EMSA, Electrophoretic Mobility Shift Assay; DTT, dithiothreitol; oc, open circular plasmid DNA; ccc, covalent closed circular plasmid DNA; ds-oligonucleotide, double-stranded oligonucleotide

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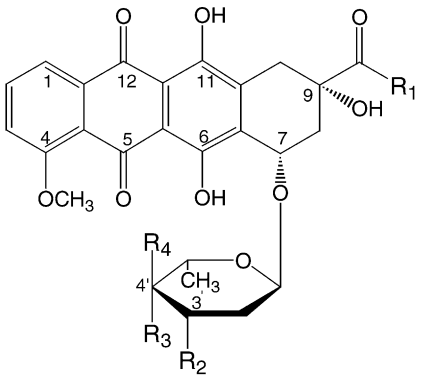

DRB	$R_1 = CH_3$	$R_2 = NH_2$	$R_3 = OH$	$R_4 = H$
DRBM	$R_1 = CH_3$	R_2 = morpholine	$R_3 = OH$	$R_4 = H$
DRBH	$R_1 = CH_3$	R_2 = hexamethyleneimine	$R_3 = OH$	$R_4 = H$
DOX	$R_1 = CH_2OH$	$R_2 = NH_2$	$R_3 = OH$	$R_4 = H$
DOXM	$R_1 = CH_2OH$	R_2 = morpholine	$R_3 = OH$	$R_4 = H$
DOXH	$R_1 = CH_2OH$	R_2 = hexamethyleneimine	$R_3 = OH$	$R_4 = H$
EDOX	$R_1 = CH_2OH$	$R_2 = NH_2$	$R_3 = H$	$R_4 = OH$
EDOXM	$R_1 = CH_2OH$	R_2 = morpholine	$R_3 = H$	$R_4 = OH$

Fig. 1. Chemical structures of anthracycline derivatives.

mine moiety, with either morpholine or hexamethylene rings attached to this group have been synthesized (Fig. 1) [7]. These derivatives have shown antiproliferative activity on some cancer cell lines that was similar to, or higher than the parent compounds [8]. The lack of two hydrogen atoms in the N at position C-3′ of daunosamine does not allow formation of the Schiff base with an aldehyde. However, morpholine derivatives of anthracyclines, DRBM and DOXM, exhibit substantial inhibition of RNA synthesis in vitro and induction of transcription arrests upon preincubation of DNA with these drugs in the presence of Fe(III) and DTT [8] or CH_2O. The complexes formed with DNA in the presence of CH_2O are resistant to extraction with phenol and thermal denaturation (manuscript in preparation), which indicates the covalent nature of these complexes.

The aim of this study was to compare the sequence specificity of CH_2O-mediated DNA binding of the morpholine or hexamethyleneimine derivatives and their parent compounds DRB, DOX and EDOX by means of the DNase footprinting technique and the analysis with restriction endonucleases. The results provide clear evidence that the new anthracycline derivatives and their parent compounds recognize similar, but not identical DNA binding sites. One of the consequences of different affinity of novel anticancer drugs for specific DNA sequences could be their effect on recognition of DNA by transcription factors. In this paper, we have examined the ability of anthracycline derivatives to interfere with the binding of well-characterized transcription factors, Sp1 and AP-1, to their recognition sequences.

2. Materials and methods

2.1. Reagents

Anthracycline antibiotics were synthesized in the Institute of Biotechnology and Antibiotics, Warsaw [7] and kindly provided by Dr. Irena Oszczapowicz and Mrs. Malgorzata Wasowska. The compounds were prepared as stock solutions in water at a concentration 1 mM and stored in the dark at −20 °C and diluted immediately before use.

Oligonucleotides were synthesized and purified in the laboratory of W.T. Markiewicz (Institute of Bioorganic Chemistry, Polish Academy of Sciences). [α-^{32}P]dATP, 6000 Ci mmol^{-1} and T7 Sequenase v.2.0 were purchased from Amersham. DNase I, *Sma*I and *Pvu*II were from Boerhinger. *Hin*dIII, *Sac*I, *Bam*HI, *Eco*RI were from Promega. Plasmid pBluescript II SK$^+$ was from Stratagene and pSKαvHX was constructed previously [9]. Recombinant human tumor necrosis factor alpha (TNF-α) used for endothelial cells activation was purchased from Genzyme Inc. The remaining chemicals were purchased from Sigma.

2.2. Isolation of plasmid DNA

The plasmids used in this study, pBluescript II SK$^+$ and pSKαvHX, were isolated from *Escherichia coli* strain DH5α by the alkaline lysis method (HiSpeed Plasmid Maxi Kit, Qiagen). Densitometer scans (GelDoc System 2000, BioRad) of DNA samples run in agarose gel showed that almost 100% of the plasmids were in covalent closed circular form.

2.3. DNase I footprinting reactions

A 264 bp fragment of pSKαvHX (*Hin*dIII–*Sac*I, −522 to −258 fragment of the promoter region of αv subunit of vitronectin receptor gene) [9], or 288 or 160 bp (*Hin*dIII–*Pvu*II) fragments of pBluescript II SK$^+$ were used in experiments. Plasmid DNA was first digested to completion at the site *Hin*dIII, labeled by filling in the overhangs with Sequenase 2.0 in the presence of [α-^{32}P]dATP, and then digested either with *Sac*I or *Pvu*II. The DNA probes were subsequently purified by 7% (w/v) polyacrylamide gel electrophoresis in 0.5 × TBE. Approximately 50 pmol of the probe were preincubated with anthracyclines at a final concentration of 50 μM in the presence of 5 mM CH_2O in buffer containing 7.5 mM Tris–HCl, pH 8.0, 0.75 mM EDTA. The incubation was carried out for 2 h

in the dark at 37 °C and the unbound drugs were extracted with phenol and DNA precipitated with ethanol. The covalently modified DNA was incubated with DNase I (0.05 U) for 30 s in a total volume of 200 μL in reaction buffer (10 mM Hepes–KOH, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, 2 μg/mL poly[dI-dC]·poly[dI-dC]). About 50% of full-length DNA fragment remained under these conditions. Control experiments were performed in the absence of DNA-binding drugs either with or without 5 mM CH_2O. The products were precipitated and analyzed on a 6% (w/v) polyacrylamide/8 M urea gel along with Maxam–Gilbert sequencing reaction (G). Visualization was performed using autoradiography on Kodak XAR-5 film. Densitometric scans (GelDoc System 2000, BioRad) were used to analyze the autoradiograms.

2.4. Digestion of drug–plasmid DNA complexes with restriction endonucleases

Aliquots containing 0.6 μg of DNA were incubated in the presence of CH_2O (5 mM) with anthracyclines at a concentration 20 μM in the buffer containing 7.5 mM Tris–HCl, pH 8.0, 0.75 mM EDTA for 2 h in the dark at 37 °C. DNA was precipitated with ethanol and resuspended in TE buffer, pH 8.0. DNA incubated with 5 mM CH_2O was used as a control. The drug–DNA complexes and control DNA were digested with restriction endonuclease in appropriate buffers and then they were subjected to 1% (w/v) agarose electrophoresis for 2 h at 80 V in 1 × TBE buffer. The gels were stained with ethidium bromide and photographed under UV lamp. Densitometry (BioRad, GelDoc 2000) of the linear DNA fraction versus circular DNA fractions (covalent closed circular (ccc) and open circular (oc)) was performed to estimate affinity of the drug towards DNA sequences recognized by the enzymes.

2.5. Electrophoretic Mobility Shift Assay (EMSA)

Endothelial cells were isolated from human umbilical vein [10]. Cells from the second passage were used for preparation of the nuclear extract. Extracts from confluent human umbilical vein endothelial cells (HUVECs) either unstimulated or TNF-α treated (10 ng/mL) were prepared as described by Dignam et al. [11] with some modification [12]. They were frozen as aliquots and stored at −80 °C. The double-stranded oligonucleotides (ds-oligonucleotides) containing either two Sp1 binding sites (5′-AATT**CCCCGCCCCCGCCCCC**-3′) or AP-1 consensus sequence (5′-AATTGGAACA**TGAGTCA**TCTATTT-3′) flanked by 13 bp from PAI-1 promoter [13] were labeled by filling in the overhangs with [α-^{32}P]dATP and Sequenase 2.0. They were purified by 7% (w/v) polyacrylamide gel electrophoresis in 0.5 × TBE. Binding sites for transcription factors are shown in bold type. Probes were used within 2 weeks of labeling. The labeled ds-oligonucleotides were preincubated with anthracyclines at the indicated concentrations in the presence of 5 mM CH_2O in the buffer containing 7.5 mM Tris–HCl, pH 8.0, 0.75 mM EDTA. The incubation was carried out for 2 h in the dark at 37 °C. Binding reactions were set up in binding buffer (20 mM Hepes/KOH, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol) in the presence of 2 μg of non-specific competitor poly[dI-dC]·poly[dI-dC] and 50,000 cpm of covalently modified or control ds-oligonucleotide, in a total volume of 20 μL.

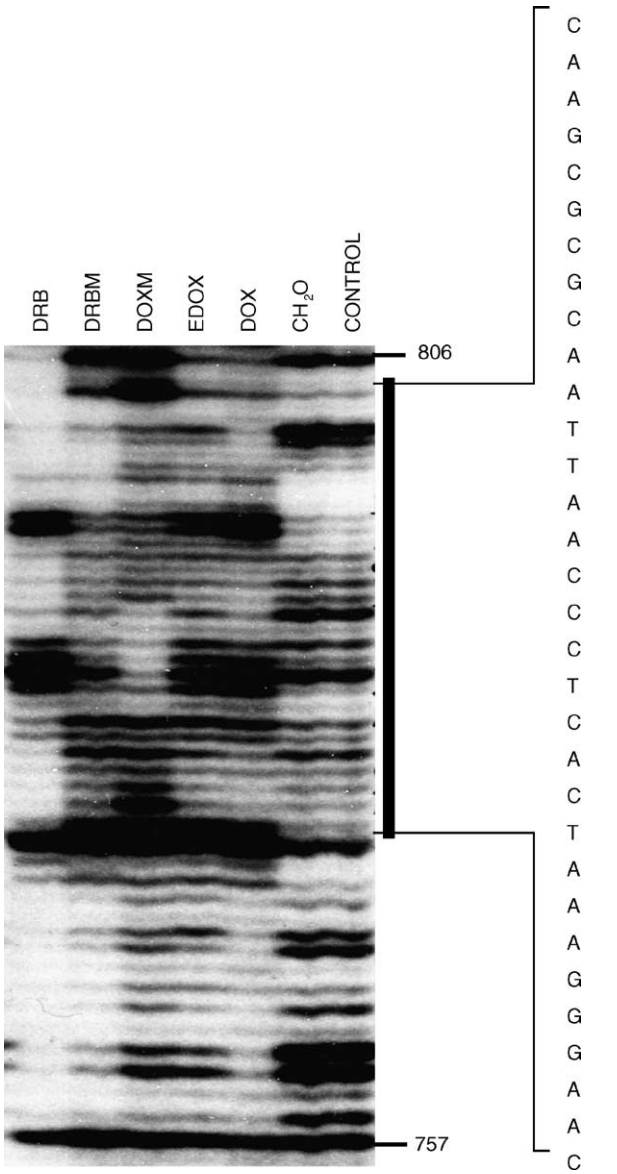

Fig. 2. Effect of anthracycline derivatives on DNase I footprinting. DNase I footprinting of anthracycline derivatives (50 μM), was performed with the *Hin*dIII end-labeled fragment of pBluescript SK$^+$. Only part of *Hin*dIII–*Pvu*II fragment (from 757 to 806) is shown. The control DNA cleavage after treatment with 5 mM formaldehyde is labeled as CH_2O. The position of the sequence has been deduced from G Maxam–Gilbert sequencing reaction (not included). The filled box indicates the location of the sequence listed on the right, for which densitometric analysis is shown in this figure.

Six micrograms of nuclear extract from unstimulated HUVECs or TNF-α-treated HUVECs were used for Sp1 or AP-1 binding site, respectively. After 15 min of incubation at room temperature, samples were subjected to electrophoresis at constant voltage (150 V for 1.5 h) under low ionic strength conditions (0.5 × TBE buffer) on 6% (w/v) polyacrylamide gels. Gels were dried and exposed with intensifying screens at −20 °C.

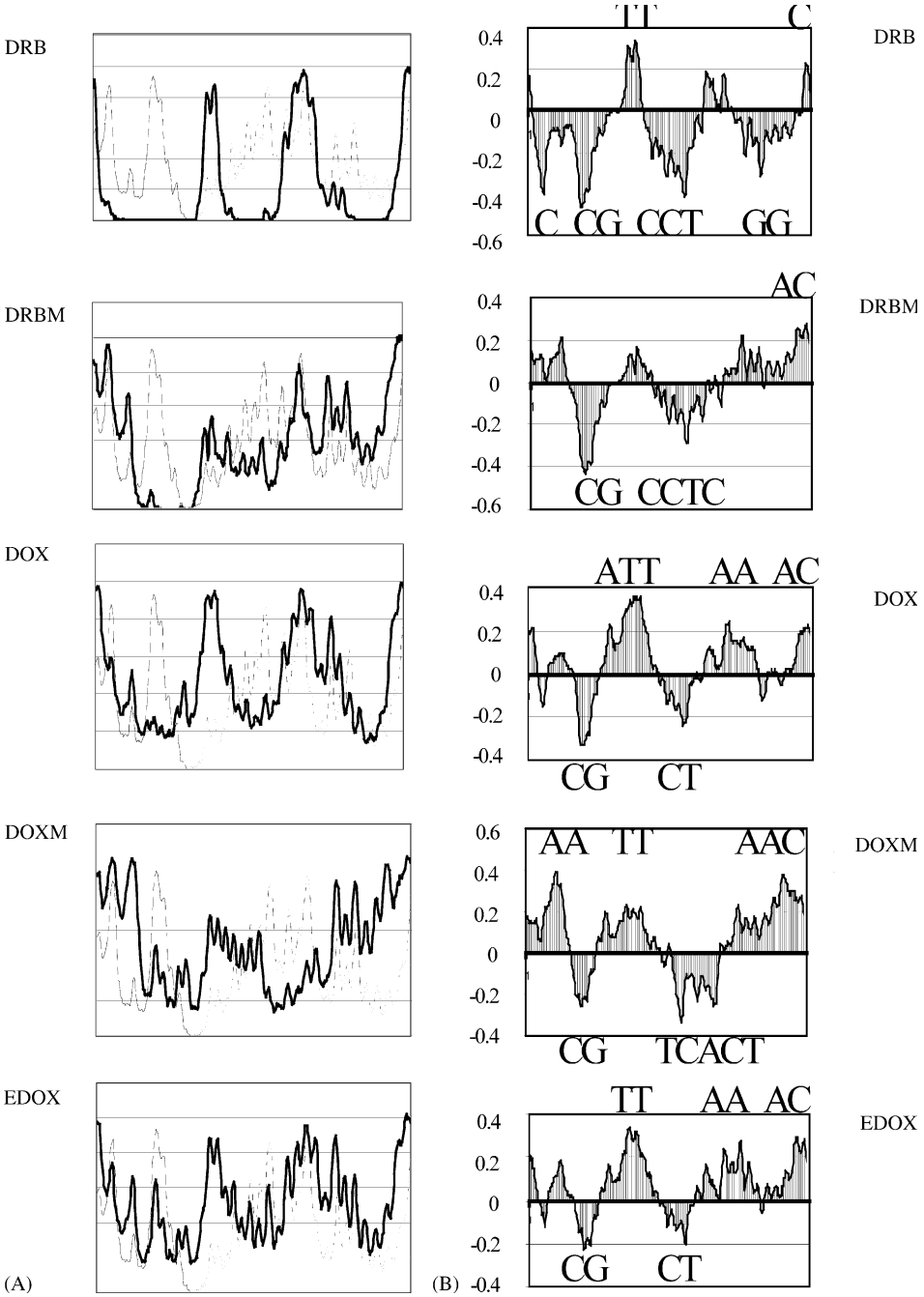

Fig. 3. Densitometric analysis of the DNase I footprinting data. (A) Densitometric scans from DNA digestion patterns performed on the sequence corresponding to the part of *Hin*dIII–*Pvu*II fragment (from 773 to 804) of pBluescript SK$^+$, which is shown in the Fig. 2 (filled box and the sequence on the right). The dashed lines show the scans obtained with DNA incubated with 5 mM CH_2O and the bold lines correspond to the scans obtained with anthracycline-modified DNA. (B) Difference spectra obtained from densitometric scans (A) by subtracting the intensities of the control bands from the intensities of the bands representing DNA covalently modified by anthracyclines. The positive peaks show the sequences where DNA digestion was enhanced, and the negative peaks show the sequences protected from enzyme digestion by anthracyclines. The sequences corresponding to the bands that were reduced or enhanced in intensity by more than 0.2 on the Y scale are listed below or above the peaks, respectively. (C) The DNA sequences (5′-3′) affected by the anthracyclines. Summary of quantitative analysis of DNase I footprinting data obtained for different parts of *Hin*dIII-end labeled DNA fragments of pBluescript II SK$^+$ and pSKαvHX. The solid part of the bars represents the percentage of the dinucleotide sequences, listed below the bars, which were protected from DNase I digestion by more than 50% in the presence of anthracycline derivatives. The grey part of the bars represents the percentage of the sequences that were affected up to 50%. The white part indicates the percentage of the sequences that were either unaffected or even became hypersensitive sites in DNase I digestion.

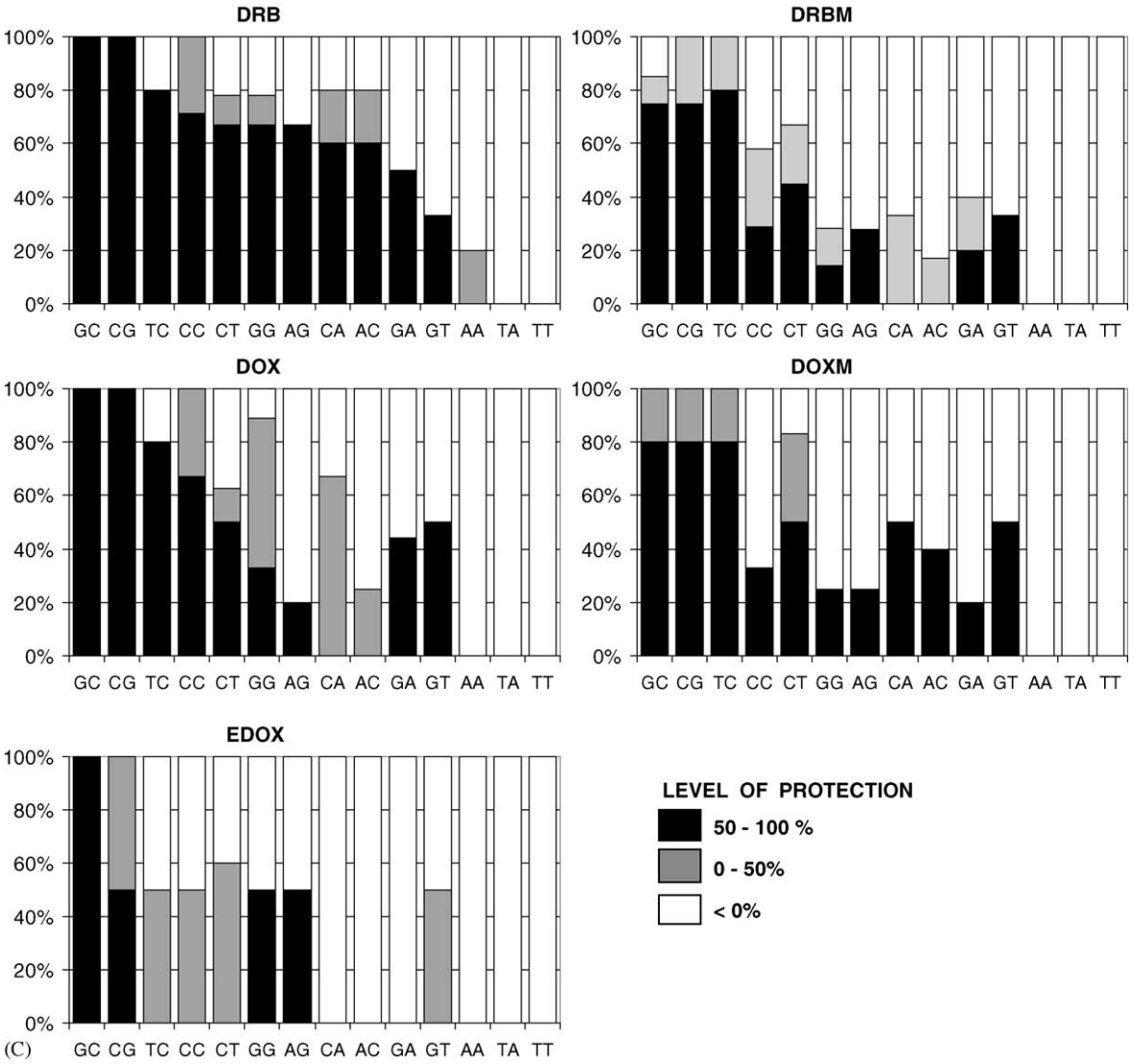

Fig. 3. (*Continued*).

3. Results

The DNA sequence-specific binding ability of anthracycline derivatives was investigated using both DNase I footprinting and restriction endonuclease protection techniques. DNA was incubated with the drugs in the presence of 5 mM CH_2O for 2 h as described in Section 2. To separate the drug–DNA complexes from the unbound drug, phenol extraction and ethanol precipitation was performed prior to the enzymatic digestion. This purification step eliminates the possibility that endonuclease cleavage inhibition is mediated by the direct drug interactions with the enzymes rather than with DNA.

3.1. DNase I footprinting

Footprinting studies were carried out using DNase I digestion of a 264 bp (*Hin*dIII–*Sac*I) fragment of pSKαvHX [10], or 288 or 160 bp (*Hin*dIII–*Pvu*II) fragments of pBluescript II SK^+, *Hin*dIII end-labeled with [α-^{32}P]dATP. A footprinting gel using part of the *Hin*dIII–*Pvu*II fragment covalently modified by DRB, DOX, EDOX and morpholine derivatives of the first two drugs (DRBM, DOXM), is shown as an example of the studies in Fig. 2. A densitometric analysis of the upper part of the autoradiogram is shown in Fig. 3A. The densitometric scans were converted to difference spectra by subtracting the control DNase I digestion band intensity from the intensity of the bands obtained by digestion after covalent complexes between DNA and anthracyclines were formed (Fig. 3B). The positive peaks show the sequences where DNA digestion is enhanced, and the negative peaks show the sequences that were protected by anthracyclines from enzyme digestion. The sequences corresponding to the bands that were reduced or enhanced in intensity by more than 50% are shown below or above the peaks, respectively. Similar analysis was performed for all anthracycline derivatives using *Hin*dIII labeled DNA fragments, both

obtained from pSKαvHX and from pBluescript SK$^+$. Quantitative analysis of the DNase I footprinting data is summarized in Fig. 3C. The levels of protection for particular dinucleotide sequences present at different positions of analyzed DNA fragments were calculated and are collectively represented by one bar in Fig. 3C. The results indicate that DRB, DOX and their morpholine derivatives, at a concentration of 50 μM display a clear preference for dinucleotide sequences containing guanine, either on labeled or unlabeled strand. More than 75% of the sequences: 5′-GC-3′, 5′-CG-3′ and 5′-TC-3′ were protected from DNase I digestion. The intensity of the bands representing these sequences was decreased by at least half (Fig. 3C, black part of the bars), whereas the intensity of the bands for the sequences with no guanine (5′-AA-3′, 5′-TA-3′, 5′-TT-3′) was significantly increased (Fig. 3C, white part of the bars). Not all of the 5′-TC-3′, 5′-CT-3′, 5′-AG-3′, 5′-CA-3′, 5′-AC-3′, 5′-GT-3′ and 5′-GA-3′ sequences, which also contain guanine in the labeled or in the complementary strand, were protected by the anthracyclines. It is likely that this is due to the presence of different flanking sequences that are able to modify the sequence preference of the drug. Some increased protection of 5′-TC-3′ sequence was observed for morpholine analogues, as compared to the sequences of 5′-GC-3′ and 5′-CG-3′ and in comparison to the results obtained for the parent compounds. At 50 μM, EDOX efficiently protected the sequences 5′-GC-3′ and 5′-CG-3′ and had only a modest effect on other sequences containing guanine. Its morpholine derivative (EDOXM) showed a small decrease in the amount of some DNase I digestion products and hexamethyleneimine analogues of DOXH and DRBH showed no detectable changes when compared to the amount of digestion products obtained with the control DNA incubated with formaldehyde alone (results not shown).

3.2. Restriction endonuclease protection

Four different restriction enzymes were used to estimate specificity of the binding of anthracyclines and their derivatives. All of them contain G·C base pairs in their recognition sequences, but differed in the composition of the cutting sites (Table 1, Fig. 4). *Sma*I recognizes the sequence: 5′-CCC/GGG-3′ and cuts the phosphodiester bond between C and G and *Sac*I recognizes the sequence 5′-GAGCT/C-3′ and cuts between T and C. These two dinucleotide sequences 5′-CG-3′ and 5′-TC-3′ were protected from DNase I digestion to a very high degree by DRB and DOX and their morpholine derivatives (DRBM, DOXM), whereas the dinucleotide sequences corresponding to the cutting sites of *Bam*HI (5′-G/G-3′) and *Eco*RI (5′-G/A-3′) were significantly (more than 50%) reduced in intensity in only 20–50% of the sequences available for footprinting assay (Fig. 3C).

Two concentrations of anthracyclines were used during covalent modification of plasmid DNA, 20 and 50 μM. Better discrimination between different anthracycline derivatives and different restriction endonucleases was achieved with 20 μM and only these results are presented. A sample gel is shown in Fig. 4. In most cases, in addition to the DNA linear band, two bands corresponding to open circular and covalent closed circular plasmid DNA were observed. Both uncut bands (oc and ccc) can be treated as fractions of DNA that were protected from digestion with restriction enzymes by anthracyclines and they could be used to measure the affinity of the drugs towards DNA sequences recognized by these enzymes. The quantitative analysis of the restriction endonuclease protection data obtained by densitometry of the gels is summarized in Table 1. The data are consistent with the results obtained in the footprinting assay. The highest decrease in cutting capacity was observed when the DNA modified by DRB and DOX was cut by *Sma*I (C/G). The cutting capacity of *Sma*I was reduced by about 90% (ccc). For DRBM and DOXM, the highest inhibition was observed in the case of *Sac*I (T/C) activity. About 35% of plasmid DNA modified by DRBM was present in the ccc fraction and 20% in the oc fraction, and for DOXM-modified DNA 40% was present in the ccc fraction and 20% in the oc fraction. Interestingly, *Sac*I and *Eco*RI produced a large fraction of open circular plasmid DNA when covalently modified DNA was cut by these enzymes (Table 1). For all anthracyclines, showing protection capability, this fraction is much larger in the case of *Sac*I and *Eco*RI than for *Sma*I and *Bam*HI. *Sac*I

Table 1
The influence of different anthracycline derivatives on cutting capacity of restriction enzymes (percentage of protection)

	CCC/GGG, *Sma*I		GAGCT/C, *Sac*I		G/GATCC, *Bam*HI		G/AATTC, *Eco*RI	
	oc	ccc	oc	ccc	oc	ccc	oc	ccc
DRB	0	86.5 ± 2.1	18.5 ± 2.1	58.5 ± 3.5	8.0 ± 4.2	37.5 ± 10.6	31.5 ± 2.1	11.0 ± 2.8
DRBM	16.0 ± 1.4	34.0 ± 1.4	19.5 ± 3.5	35.0 ± 7.0	0	0	20.6 ± 9.5	0
DRBH	0	2.0 ± 2.8	5.3 ± 4.6	0	0	0	0	0
DOX	4.0 ± 5.6	92.5 ± 10.6	22.5 ± 9.1	60.5 ± 14.8	4.0 ± 5.6	50.5 ± 12.0	29.5 ± 0.7	24.0 ± 2.8
DOXM	6.5 ± 3.5	35.5 ± 0.7	19.5 ± 4.9	39.5 ± 6.4	1.5 ± 2.1	6.5 ± 2.1	14.5 ± 4.9	0
DOXH	4.0 ± 0.0	13.0 ± 9.6	6.5 ± 0.7	5.5 ± 0.7	0	0	3.5 ± 4.9	0
EDOX	2.5 ± 3.5	17.5 ± 3.5	43.0 ± 4.2	0	0	11.5 ± 10.6	26.5 ± 8.5	0
EDOXM	0	0	4.0 ± 4.0	0	0	0	0	0

The percentage of plasmid DNA in the open circular (oc) and close circular (ccc) fractions. The experiment was performed in the conditions where control plasmid DNA was cut completely in the absence of drugs. Each experiment was repeated at least three times and standard deviation was calculated.

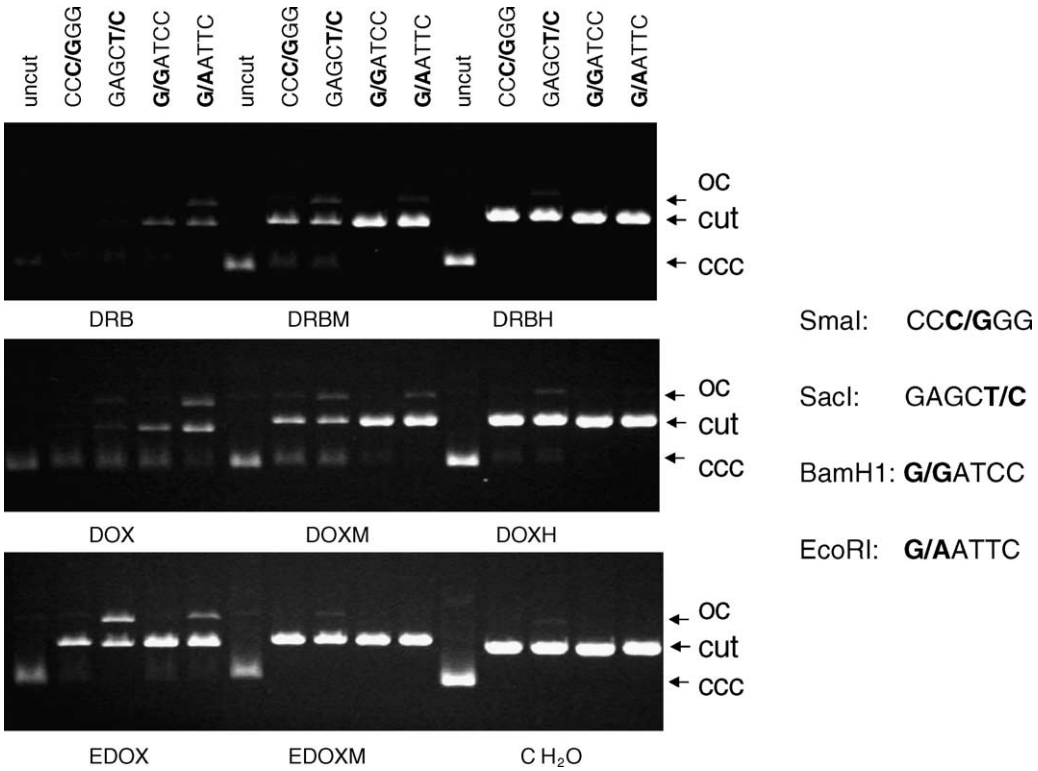

Fig. 4. Restriction endonuclease protection. Electrophoresis in 1% agarose gel following the digestion with *Sma*I, *Sac*I, *Bam*HI and *Eco*RI of pBluescript SK^+ DNA covalently modified with anthracyclines. Drugs with concentration of 20 μM and CH_2O of 5 mM were used in these studies. In control experiments, plasmid DNA was incubated in the absence of drugs but in the presence of 5 mM formaldehyde. Results of densitometry of the cut DNA fraction vs. uncut DNA fractions (ccc and oc) are summarized in Table 1.

produced approximately 43% of the oc fraction when DNA modified by EDOX was used. It is worth noting that these two enzymes have complementary cutting sequences, T/C and G/A, respectively. Restriction enzymes may recognize the modified sequences and cleave one strand, but they frequently dissociate and do not cleave the second strand. Hexamethyleneimine analogues did not inhibit the cutting capacity of restriction enzymes used in these experiments. The same negative results for these analogues were obtained when 50 μM was used (results not shown).

3.3. Electrophoretic Mobility Shift Assay

Since anthracycline derivatives displayed selectivity towards G·C pairs, the effect of the interaction between the transcription factor Sp1 and its doubled binding site consisting of the sequence: 5′-CCCCGCCCCCGCCCCC-3′ was analyzed by means of Electrophoretic Mobility Shift Assay. The AP-1 binding site: 5′-TGAGTCA-3′ was also considered, since it has a mixed A·T and G·C composition and contains the sequence 5′-TC-3′, which is important for the binding of AP-1, and at the same time was recognized by morpholine derivatives as efficiently as the sequences 5′-CG-3′ or 5′-GC-3′ (Table 1 and Fig. 3C). HUVECs were chosen as a source of nuclear proteins able to bind the consensus Sp1 binding site [14] and after stimulation with TNF-α also to bind the consensus AP-1 binding site [13]. DNA–anthracycline complexes were formed in the presence of 5 mM CH_2O. No influence of preincubation with formaldehyde on transcription factor-DNA complex formation was observed (Fig. 5A, lane 11 and control lane 2 and Fig. 5C lane 5 and control lane 2).

For the Sp1 binding site, the greatest inhibition was obtained when ds-oligonucleotides modified by 20 μM DRB, DOX, EDOX and EDOXM were used (Fig. 5A, lanes 3, 6, 9 and 10, respectively). Hexamethyleneimine analogues of DRB or DOX inhibited the binding of Sp1 by less than 10% (lanes 5 and 8, respectively). Moreover, the inhibition of the Sp1 binding to its consensus site by covalent DNA–anthracycline complexes was anthracycline concentration-dependent (Fig. 5B). DRB, DOX, EDOX, DRBM and DOXM at a concentration of 100 μM almost completely abolish DNA–protein complex formation. Some anthracyclines caused the retention of the drug-modified DNA probe at the top of the gel. This was observed for DOX and EDOX at the concentration of 20 μM (Fig. 5A, lanes 6 and 9, respectively). For that reason, their influence on Sp1 binding to its recognition site could be evaluated only at lower concentration. DOX and EDOX at 10 μM inhibited the binding of Sp1 by approximately 50% (Fig. 5B, lane 9) and 30% (Fig. 5B, lane 13), respectively. Interestingly the increased inhibition of DNA–protein complex formation correlated with the augmented occupation of the binding site by the drug, which

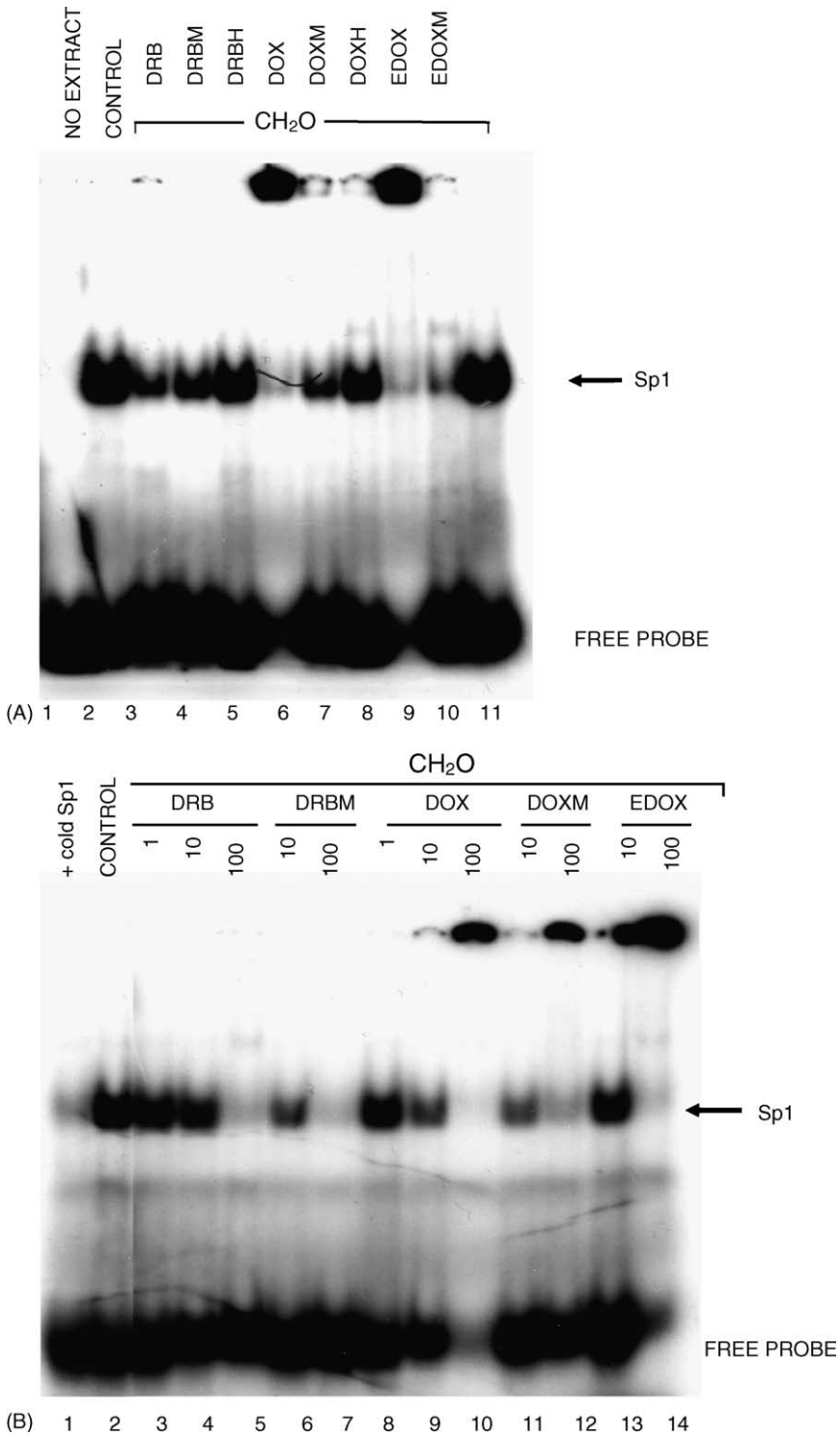

Fig. 5. Effect of anthracycline derivatives on the binding of transcription factors Sp1 and AP-1 to their consensus sequences. EMSA was performed after the incubation of drug-modified ds-oligonucleotides with a nuclear extract from HUVEC. (A) Labeled oligonucleotide representing two Sp1 binding sites was incubated with 20 μM anthracycline derivatives in the presence of 5 mM CH_2O. The modified ds-oligonucleotides were then incubated with 6 μg of nuclear extracts from HUVEC for 15 min. Electrophoretically retarded bands, indicated with an arrow, denote Sp1–DNA complexes. Control samples were prepared in the absence of the drug with (lane 11) or without (lane 2) CH_2O (5 mM). (B) The experiment was performed as in part (A) but the concentrations of the drugs were from 1 to 100 μM, as indicated. The specificity of the Sp1–DNA complexes was confirmed by using "cold" ds-oligonucleotide in the incubation mixture (lane 1). (C) Labeled oligonucleotide containing AP-1 binding site was incubated with anthracycline derivatives (100 μM) as in part (A). Nuclear extract from HUVEC stimulated with TNF-α was used as a source of AP-1 transcription factor. Competition experiment showing the specificity of the protein–DNA complexes is included (lanes 3 and 4). Supershift experiment proving the presence of AP-1 (c-Jun/c-Fos) in the complex was published elsewhere [13].

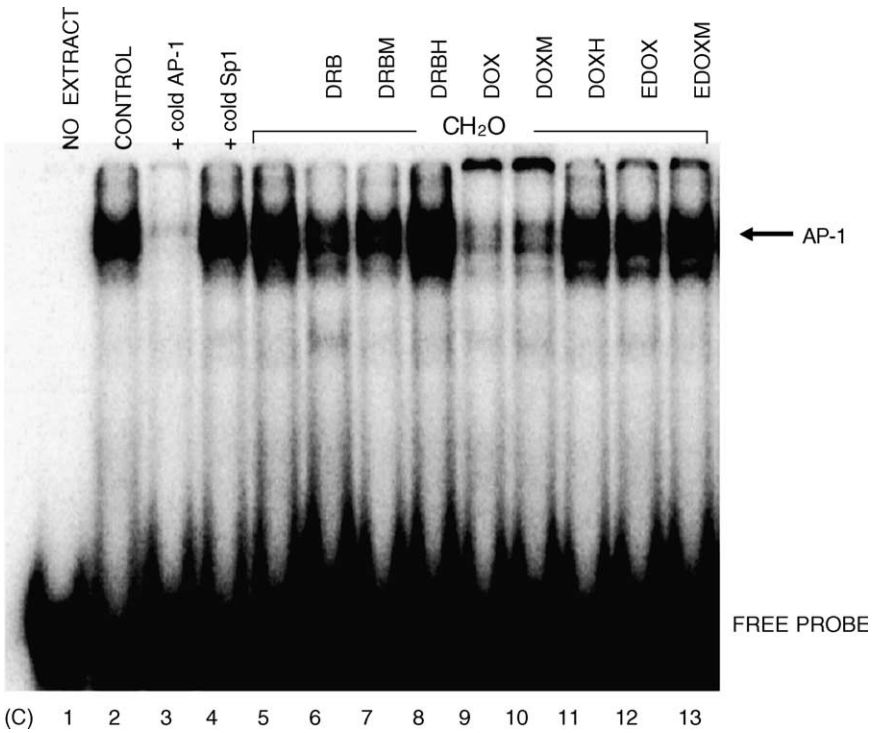

Fig. 5. (*Continued*).

can be seen by a small retardation of the free probe (Fig. 5A and B). The distance between DNA–protein complex and the free probe decreased with increasing concentration of anthracycline derivatives and in the following order: hexamethyleneimine analogues < morpholine analogues < parent compounds (Fig. 5A and B).

Anthracycline derivatives were less potent against AP-1 binding than against Sp1 binding. DRB and its morpholine derivative inhibited AP-1 binding by 50% at the highest concentration used, 100 μM (Fig. 5C, lanes 6 and 7). DOX and DOXM were much more efficient (lanes 9 and 10) but DRBH, DOXH and EDOXM had no effect on AP-1 binding (lanes 8, 11 and 13). No differences between parent compounds and their morpholine derivatives were observed in their ability to inhibit AP-1 binding to its consensus site. DRBH, DOXH, EDOX and EDOXM did not inhibit AP-1 binding (lanes 8, 11, 12 and 13). Interestingly EDOX, which inhibited the binding of Sp1 by approximately 30% at 10 μM (Fig. 5B, lane 13) and caused retention of the Sp1 probe at the top of the gel at 20 μM (Fig. 5A, lane 9), was completely inefficient in preventing AP-1 from binding to its consensus site at 100 μM and also did not cause the retention of the AP-1 probe at the top of the gel at this concentration (Fig. 5C, lane 12). It is likely that this is because EDOX recognizes and binds efficiently only GC and CG sequences which are not present in AP-1 binding site. DOX showed lower sequence specificity than EDOX and bound both Sp1 and AP-1 sequences. This observation is in agreement with the results obtained for DOX and EDOX in footprinting analysis (Fig. 3C). In general, the EMSA data confirm the results obtained in footprinting and restriction analyses.

4. Discussion

Adduct formation by anthracycline antibiotics with DNA mediated by formaldehyde in cell free systems, provides new perspectives in the search for novel and potent analogues. The anthracycline–formaldehyde conjugates as more effective drugs were synthesized [2]. Enhancement of the parent drugs effect by simultaneous application of a low-toxic prodrug releasing formaldehyde was proposed [15]. Attempt to characterize the structure of anthracycline adduct formation led to the concept of ''virtual cross-links'' [16]. A complex between DOX and a double stranded oligonucleotide containing 5′-GC-3′ sequence was stable on denaturing sequencing gel, which proved the existence of the interstrand cross-link [17]. No cross-linking was observed with a similar oligonucleotide containing only a single G·C base pair [18]. Moreover, transcription mapping of adducts indicated that they were mainly formed at the 5′-GC-3′ sequence [1,19].

In this study, we compared the sequence specificity of new amidine derivatives forming CH_2O-mediated adducts to DNA with their parent compounds, i.e. DRB and DOX, whose specificity was well characterized. This study used EDOX, which also forms a covalent complex with DNA [20] and its amidine analogue, EDOXM. The lack of two hydrogen atoms at N at C-3′ of daunosamine in morpholine

Sp1: 5'-AATTCCCCGCCCCCGCCCCC-3'

AP-1: 5'-AATTGGAACATGAGTCATCTATTT-3'

Binding sites for transcription factors are underlined.

Scheme 1. Oligonucleotides used in EMSA.

or hexamethyleneimine derivatives does not allow forming a Schiff base with an aldehyde. There is evidence, however, of morpholine derivatives covalent binding to DNA mediated by CH_2O. This notion is supported by the results presented here which were obtained with the DNA pre-incubated with the drugs in the presence of CH_2O, and then purified from the non-covalent interacting portion with phenol according to Leng et al. [21].

DRB, DOX, EDOX, DRBM and DOXM adducts modified the digestion pattern of DNA by DNase I, protected plasmid DNA from cutting by restriction nucleases and abolished the interaction of transcription factor Sp1 and, to a lesser extent AP-1 with their recognition sequences. Preincubation of DNA with DRBH or EDOXM did not protect DNA from DNase I (not shown) or from restriction nucleases. DOXH showed some protective effect on the activity of restriction nuclease and EDOXM inhibited complex formation of Sp1 to DNA. Furthermore, in recent preliminary studies we have found that DRBM and DOXM irreversible binding to calf thymus DNA was about tenfold lower than that seen with parent compounds, while the DRBH, DOXH and EDOXM binding fell below the detection level (manuscript in preparation).

The DNase I footprinting technique was used to characterize non-covalent interactions of anthracycline antibiotics [22,23]. Our study is the first footprinting approach to look at the specificity of covalent adducts formed by anthracycline derivatives. Specific, reproducible protection patterns were obtained for each DNA fragment. The protected sequences comprise the "classic" anthracycline binding site 5′-GC-3′. In addition, there was a strong protection of 5′-TC-3′, 5′-CT-3′ and 5′-CC-3′ sequences. The protection of these sequences is probably due to the adduct occurring at complementary sequences i.e. 5′-GA-3′, 5′-AG-3′ and 5′-GG-3′, respectively. This assumption is supported by a comparable protection of these sequences, with the exception of the 5′-GA-3′ sequence, which was protected to a lower extent by DRBM and DOXM than 5′-TC-3′. Differences between the drugs were minor. DRB exhibited a broader spectrum of protected sequences, while DOX and especially EDOX showed higher specificity. Relatively high protection of 5′-TC-3′ and 5′-CT-3′ sequences by DRBM and DOXM was observed. As shown with other G·C interacting ligands [24], the enhancement of DNase I activity occurred at the A·T rich regions. An advantage of DNase I footprinting is that a large number of sequences can be screened in one experiment. A disadvantage of using DNase I footprinting is the sequence selectivity inherent in the enzyme itself. This enzyme does not cleave the control DNA evenly, which means that some sequences remain uninformative. The restriction endonucleases are able to cut the recognized site to completion and, if it is a single site in the plasmid DNA, the process can be easily monitored by agarose gel electrophoresis. We have used a set of restriction endonucleases, whose recognition sequences contain G in different sequential contexts and positions versus the sequence of cutting. As mentioned before, DRBH and EDOXM did not give any degree of protection to any sequence. The other anthracyclines tested generally inhibited endonuclease activity to different extents, which is consistent with the notion that they covalently bind to G. It has been found previously [21], that the adduct completely inhibits endonuclease *Nae*I, whose recognition site is 5′-GCGCGC-3′, while it does not inhibit endonuclease *Dra*I, whose recognition site is 5′-TTTAAA-3′. Recognition sequences of the enzymes we have used contain the "classic" anthracycline cross-link sites i.e. 5′-CG-3′ (*Sma*I) and 5′-GC-3′ (*Sac*I). They may contain a drug molecule covalently bound to one G anchoring with hydrogen bonds to guanine in the opposite strand [2]. The *Sma*I recognition site contains exclusively the G·C base pairs. The *Sac*I and *Bam*HI sites contain four G·C base pairs and two A·T base pairs, but the latter site does not contain the high affinity 5′-GC-3′ sequence, hence is unlikely to form the "classic" virtual cross-link. The *Eco*RI site contains two G·C base pairs separated by four A·T base pairs. The inhibition of restriction nucleases by DRB and DOX depended both on the content of G·C base pairs and their sequential arrangements. The highest inhibition of cleavage by DRB and DOX was observed for *Sma*I, and then for *Sac*I and *Bam*HI. Inhibitory effects of EDOX, DOXM and DRBM were considerably lower. These effects of DRBM and DOXM were similar at *Sma*I and *Sac*I, and thus different from the range observed for the parent compounds. DRBM and DOXM were less efficient in forming adducts with DNA. Their structure may be different from the structure of adducts formed by DRB and DOX.

Transcription factors that perform their functions through contacts with specific sequences of DNA either in the promoter region or distant regions enhancing or attenuating the initiation of RNA synthesis are considered as targets of the anticancer drugs. These contacts may be affected by the DNA interacting drugs [14,25,26]. Anthracycline antibiotics were assayed on their propensity to inhibit interactions of several transcription factors with their recognition sequences. It has been shown previously that the base or sequence specificity of the drugs plays a role in this inhibition. Intercalating DRB, DOX and bis intercalating anthracycline derivative WP 631 were found to compete with Sp1 for its binding site [27]. To our knowledge, no data on interactions between transcription factors and DNA covalently modified by anthracyclines are available, except the transcription factor Oct 1, which did not bind to its recognition site modified by covalently

bound DOX [28]. In this study, we have examined the effect of anthracycline derivatives on the recognition of DNA by well-characterized transcription factors, Sp1 and AP-1. These two transcription factors differ in their binding sites. Sp1 recognizes GC-box, whereas AP-1 has a mixed A·T and G·C binding site (Scheme 1). Covalent modifications of Sp1 binding sites lead to the inhibition of DNA–protein complex formation and correlated with previous data on covalent adduct formation: parental compounds > morpholine derivatives > hexamethylene deri- vatives (from the most to the least effective inhibition). The ability of anthracycline to interfere with the AP-1 binding to its DNA recognition sequence showed a similar pattern: parental compounds = morpholine derivatives > hexamethylene derivatives, though a higher concentration of the drug was necessary to achieve DNA–drug complex formation. The parental anthracycline and their morpholine derivatives therefore prevent transcription factors Sp1 and AP-1 from binding to their cognate sequences with different efficiency, which could lead to the diverse gene targeting to achieve their action.

The observations presented in the paper are consistent with our data on cytotoxicity of these compounds on L1210 culture and confirm other results [2–4,16,17], which indicate the contribution of CH_2O-mediated covalent binding to the biological effects of anthracycline. It does not seem, however, to be an absolute requirement, since at least in some cell cultures these analogues show similar or even higher cytotoxicity than the parent compounds [8]. Moreover, another derivative of DOX, which has the 3′-NH_2 group substituted for morpholine ring and does not covalently bind to DNA in the presence of CH_2O [1], exhibits high cytotoxicity and therapeutic properties [29].

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