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DIFFERENT MODES OF ANTHRACYCLINE INTERACTION WITH TOPOISOMERASE II

SEPARATE STRUCTURES CRITICAL FOR DNA-CLEAVAGE, AND FOR OVERCOMING TOPOISOMERASE II-RELATED DRUG RESISTANCE

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Abstract—In contrast to the classic anthracyclines (doxorubicin and daunorubicin), aclarubicin (ACLA) does not stimulate topoisomerase II (topo II) mediated DNA-cleavage. This distinction may be important with respect to topo II-related drug resistance, and the aim of this study was to clarify drug-structures responsible for this difference. Various ACLA analogs were tested for: (a) interaction with purified topo II, (b) induction of DNA cleavage in cells, (c) cellular uptake and (d) cytotoxicity. A remarkable distinction was seen between analogs containing the chromophore aklavinone (AKV) (e.g. ACLA) which have a carboxymethyl group ($COOCH_3$) at C-10 and drugs with a β-rhodomycinone (RMN) chromophore with hydroxyl groups at C-10 and at C-11. Thus, RMN-containing analogs, including the aglycone RMN itself, effectively stimulated topo II-mediated DNA cleavage. In contrast, AKV-containing drugs inhibited DNA cleavage and antagonized cytotoxicity mediated by RMN-containing drugs. In OC-NYH/VM cells, exhibiting multidrug resistance due to an altered topo II phenotype (at-MDR), cross-resistance was only seen to the RMN-containing drugs whereas no cross-resistance was seen to the non-DNA cleaving AKV-containing compounds. Thus, our data show that one domain in the anthracycline is of particular importance for the interaction with topo II, namely the positions C-10 and C-11 in the chromophore, and further that at-MDR was circumvented by a $COOCH_3$ substitution at position C-10. These findings may provide guidance for the synthesis and development of new analogs with activity in at-MDR cells.

There is increasing evidence that specific interactions of the "classic" anthracyclines doxorubicin and daunorubicin with the nuclear enzyme topoisomerase II (topo II††) play a role in the cytotoxic activity of these drugs [1–7]. Their mechanism of toxicity appears to be linked to a stimulation in the formation of cleavable complexes between DNA and topo II [1–4]. This is supported by the finding that resistance to the topo II-targeting drugs VP-16 [4′-demethylepipodophyllotoxin-9-(4,6-*o*-etylidene-β-D-glucopyranoside)] and m-AMSA [4′-(9-acridinylamino)-methanesulfon-*m*-anisidide], due to an altered topo II activity, confers cross-resistance to doxorubicin and daunorubicin [5–7]. In contrast, the cytotoxic anthracycline aclarubicin (ACLA) inhibits the formation of cleavable complexes by topo II [4, 8]. This suppressive effect seems to be due to ACLA inhibiting topo II binding to DNA [9]. By inhibiting binding, ACLA effectively antagonizes the cytotoxicity of VP-16, m-AMSA and daunorubicin [4, 8]. Thus, DNA intercalating agents may inhibit topo II activity by two different mechanisms: (a) by trapping the enzyme on the DNA as a cleavable complex and (b) by suppressing DNA cleavage presumably by inhibiting the enzyme from binding to DNA.

The anthracyclines are among the most potent drugs in the treatment of a wide range of human neoplasms. Development of resistance to the drugs is a major problem, and much effort is devoted to the search of new anthracyclines without cross-resistance to the parent drugs. If topo II is the target for some or all of the anthracyclines, then the different interaction mechanisms of the anthracyclines with the enzyme could constitute the basis for differences in sensitivity patterns. We and others have previously demonstrated that ACLA displays a sensitivity pattern different from doxorubicin and daunorubicin [10, 11]. A different pattern is also suggested clinically as ACLA has demonstrated activity in

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†† Abbreviations: ACLA, aclarubicin, aclacinomycin A; AKV, aklavinone; at-MDR, altered topoisomerase multi drug resistance; m-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; MtOH, methanol; RMN, β-rhodomycinone; SCLC, small cell lung cancer; SSB, DNA single strand break; TBE buffer, 90 mM Tris–HCl, 90 mM boric acid, 2.0 mM EDTA (pH 8.0); topo II, topoisomerase II; VP-16, 4′-demethylepipodophyllotoxin-9-(4,6-*o*-etylidene-β-D-glucopyranoside); DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

acute myeloid leukemia refractory to treatment with daunorubicin [12]. There are two well defined mechanisms for resistance to "classic" anthracyclines. The most well known is P-glycoprotein related multidrug resistance. We have previously demonstrated ACLA cross-resistance in daunorubicin resistant small cell lung cancer (SCLC) cells expressing the drug transporter P-glycoprotein [10]. Recently, an alternative drug resistant phenotype was described. Drug resistance as a consequence of an altered or reduced topo II activity was first characterized by Pommier *et al.* [13], Danks *et al.* [14] and Drake *et al.* [15]. This altered topoisomerase multidrug resistance (at-MDR) phenotype may be of particular interest in SCLC as the topo II targeting agents VP-16 and VM-26 are some of the most effective and frequently used drugs in SCLC protocols [16, 17]. The present study demonstrates a lack of cross-resistance to ACLA in an at-MDR SCLC cell line, and aims to disclose which molecular structures in anthracyclines are responsible for the retained activity in at-MDR cells. The activity of two groups of anthracyclines with different chromophores were correlated. Analogs containing the chromophore aklavinone (e.g. ACLA), which has a carboxymethyl group at C-10, were compared to drugs with a rhodomycinone chromophore, which holds hydroxyl groups at C-10 and at C-11. When the drugs were tested for interaction with topo II, a remarkable distinction between the two aglycones was demonstrated. Thus, rhodomycinone in contrast to aklavinone (AKV) effectively stimulated topo II-mediated DNA cleavage. The cytotoxicity of the rhodomycine-holding analogs correlated to their DNA cleavage activity in whole cells. In contrast, aclavinone (AKV) containing derivatives did not cleave DNA *in vivo*. All rhodomycinone derivatives displayed cross-resistance in the at-MDR cell line, in particular the most potent rhodomycinone-containing anthracycline, oxaunomycin. Thus, only minor differences in the chromophores of the anthracyclines appear to separate different mechanisms of action and indicate possible ways to circumvent at-MDR.

MATERIALS AND METHODS

Drugs. Structural formulae of the test drugs are depicted in Fig. 1. The compounds are divided into two groups according to their chromophore. The two chromophores are AKV (D6) which contains a carboxymethyl group at C-10 whereas β-rhodomycinone (RMN) (D5) has hydroxyl groups at C-10 and at C-11. The drugs containing AKV include D4 (aklavin) with the sugar L-rhodosamine and ACLA with the three sugars L-rhodosamine, 2-deoxyfucose and L-cinerulose attached to the chromophore. Drugs containing the rhodomycinone chromophore include D3 (oxaunomycin) in which the sugar is L-daunosamine [18, 19], D2 with the *N,N*-dimethylated sugar L-rhodosamine and D1 which holds three sugar groups identical to ACLA. ACLA was obtained from Lundbeck (Copenhagen, Denmark) and was dissolved in sterile water 1 mg/mL). The compounds D1–D6 were kindly supplied by Dr H. Nishida, Mercian Corporation (Fujisawa, Japan) and were diluted in dimethyl sulfoxide (DMSO) to stock solutions of 1 mg/mL, aliquoted and stored at −20°. In experiments employing cells, all drugs were diluted more than 100-fold with tissue culture medium (RPMI 1640 supplemented with 10% fetal calf serum) before use.

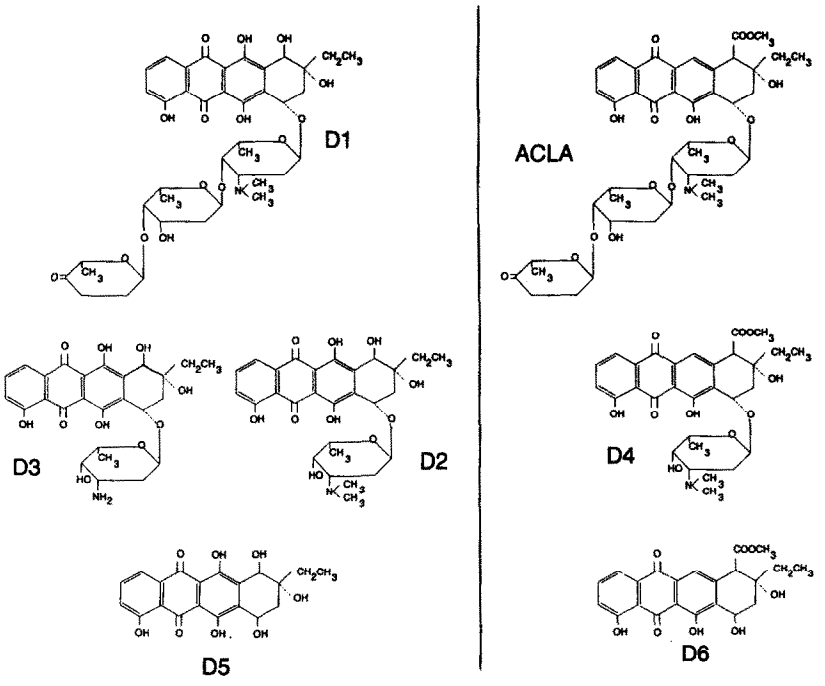
The homogeneity of the anthracyclines was invesigated by TLC on precoated 0.25 mm silica gel 60 plates (Merck, Darmstadt, F.R.G.) employing two different solvent systems: (A) $CHCl_3$–methanol (MtOH)–H_2O (32:13:2, by vol.); (B) $CHCl_3$–MtOH–acetic acid (30:5:2.5, by vol.). As evaluated by TLC, D2 was approximately 90% pure. Further purification was, however, not attempted due to scarcity of the drug. None of the impurities in D2 migrated as the other drugs employed. All the other compounds appeared >90% pure in TLC.

Cell lines. L1210 mouse leukemia cells were grown in suspension culture in RPMI 1640 supplemented with 10% fetal calf serum plus penicillin and streptomycin. The human SCLC cell line used was OC-NYH [20]. Source, relation to therapy, maintenance and monitoring have previously been described [10, 20]. The at-MDR line was selected for resistance to VM-26 (teniposide) by exposing cells continuously with increasing concentrations of drug. After 6 months the cells grew in 0.25 μM VM-26 and the subline (OC-NYH/VM) was maintained for 3 months at this concentration [21]. The doubling time was increased from 22 hr (SD = 0.3 hr, N = 3) in the OC-NYH cells to 32 hr (SD = 4 hr, N = 3) in OC-NYH/VM.

DNA cleavage mediated by purified topo II. Calf thymus topo II was purified according to a previously described procedure [22]. All experiments utilized pUC19 DNA, which was linearized with EcoRI and end-labeled with [α-^{32}P]dATP (Amersham, U.K.) and sequenase, version 2.0 (USB). The DNA fragment was incubated at 30° with 100 U of topo II in a 20-μL reaction volume containing 10 mM Tris–HCl (pH 7.5), 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.2 mM dithiothreitol, 2.5% (v/v) glycerol, in the presence or absence of drug. After 5 min of incubation, the reactions were terminated by the addition of sodium dodecyl sulfate (SDS) to 1%. Following digestion with proteinase K, 1/5 vol. loading buffer (50% glycerol, 0.05% bromphenol blue, 0.03% xylene cyanol and 50 mM EDTA) was added and the samples were loaded on a 1% agarose gel and electrophoresed in TBE buffer [90 mM Tris–HCl, 90 mM boric acid, 2 mM EDTA (pH 8)]. The gel was dried under reduced pressure and subjected to autoradiography.

Drug accumulation. L1210 cells (2 mL, 1×10^6 cells/mL) were incubated with 3 μM of each of the compounds for 60 min at 37°. At the end of the incubation, 10 mL of ice-cold phosphate-buffered saline (PBS) was added. Cells were then spun down at 150 *g* for 5 min and washed twice with 10 mL of PBS at 4°. Drained cell pellets were solubilized with 3 mL of 0.3 N HCL–50% ethanol as described by Skovsgaard [23].

Fluorescence of the extractions was determined in a Hitachi F-4000 spectrofluorometer and the drug concentrations were determined using 1 μM standards (D1, D2, D3, D5 excitation and emission



			Color	Rf value A	Rf value B
D1	Betaclamycin A	RMN-RN-dF-cinA	red	0.90	0.60
D2	β - Rhodomycin 1	RNM-RN	red	0.48	0.23
D3	Oxaunomycin	RMN-DN	red	0.33	0.30
D4	Aklavin	AKV-RN	yellow	0.63	0.38
D5	β - Rhodomycinone	RMN	red	0.90	0.89
D6	Aklavinone	AKV	yellow	0.95	0.93
ACLA	Aclarubicin	AKV-RN-dF-cinA	yellow		

RN: rhodosamine ; dF: deoxyfucose ; Cin A: cinerulose A ; DN: daunosamine

Rf values obtained with TLC using the two solvent systems described in "Materials and Methods".

Fig. 1. Chemical structures and TLC mobility of the anthracycline derivatives studied.

wavelength, 497 and 548 nm, respectively; D4 462 nm, 543 nm; D6 434 nm, 509 nm; ACLA, 434 nm, 520 nm).

Measurement of DNA single-strand breaks (SSB). DNA damage in L1210 cells was quantitated by the alkaline elution filter methods, as described in detail by Kohn *et al.* [24]. Instead of irradiation, the cells were exposed to 100 μM H_2O_2 for 60 min on ice, corresponding to an irradiation dose of 300 rad as described previously [25]. Cells were washed with PBS (10 mL, 0°), and lysed with 5 mL of SDS–EDTA lysis solution (2% SDS, 0.1 M glycine and 0.025 M Na_2EDTA) at pH 10. This was followed by addition of 2 mL SDS–EDTA lysis solution containing 0.5 mg/mL of proteinase K (Sigma Chemical Co., MO, U.S.A.). DNA was eluted from the filters with tetrapropylammoniumhydroxide–EDTA pH 12.1 containing 0.1% SDS at a rate of 0.035 mL/min. Fractions were collected at 20 min intervals for 3 hr. The fractions and filters were then processed as described in Ref. 24. DNA SSB frequency expressed in rad-equivalents was calculated on the basis of first-order elution kinetics [24].

Topo II assay. Catalytic activity of topo II was assayed by decatenation of kinetoplast DNA into free minicircles. Crude nuclear extracts were prepared as described by Woessner *et al.* [26]. Protein concentrations in the extracts were determined by the Bradford protein assay. Nuclear extracts from OC-NYH and OC-NYH/VM were prepared in parallel and topo II catalytic activity was assayed immediately after preparation by decatenation of kDNA as described previously [26]. Briefly, equal amounts of protein from the two cell lines were employed and serial dilutions of nuclear extracts were incubated with 0.5 μg of kDNA in a final volume of 20 μL at 30° for 30 min. Reactions were terminated by the addition of SDS and samples were then electrophoresed through 1% agarose in TBE buffer at 150 V for 1 hr. After staining with ethidium bromide, the gels were photographed under UV illumination.

Immunodetection of topo II. Approximately 2×10^6 exponentially growing cells were washed in cold PBS and lysed in 50–100 μL lysis buffer (9 M urea, 4% v/v NP40 and 2% v/v β-mercaptoethanol; pH 9.5). The cellular DNA was digested with 500

U/mL Benzon nuclease (Alfred Benzon) for 5 min on ice. The lysate was immediately loaded on a 6.5% (w/w) SDS–polyacrylamide gel (80 μg/lane) and transferred to Immobilon™PVDF membrane (Millipore) after electrophoresis. Then the membrane was washed in a solution containing 50 mM Tris–HCl (pH 8.8), 500 mM NaCl and 1% (v/v) Tween 20 and probed for 1 hr at 25° with polyclonal rabbit antibody directed against the C-terminus of the alpha-form of human DNA topo II (ICI, Cambridge, U.K.) at 1:1000 dilution. Alkaline phosphatase-conjugated swine-anti-rabbit antibody (DakoPatts, Copenhagen, Denmark) was used as the secondary antibody and was applied for 1 hr. The blot was developed, after 3 × 5 min washing (50 mM Tris–HCl, pH 8.8; 500 mM NaCl; 0.2% Tween 20), using Nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). Reactions were stopped by transfer to 20 mM EDTA, (pH 7) for 5 min followed by air drying. Controls were performed by omission of the primary antibody. Quantitation of immunoreactive bands was done by densitometric scanning with a Shimadzu CS903 chromatoscanner.

Clonogenic assay. Drug toxicity was assessed by colony formation in soft agar with a feeder layer containing sheep red blood cells as described previously [27]. The cell viability was assessed with dye exclusion in a hemocytometer. Single-cell suspensions (2×10^4 cells/mL) in fetal calf serum-medium were exposed to the drugs for the indicated time at 37°, and washed twice with medium at 37°. In other experiments, the cells were plated directly in agar with the drug (continuous drug exposure). Cells (2×10^4) were plated to obtain 2000–3000 colonies in the control dishes. The colonies were counted after 3 weeks of incubation. DMSO concentrations never exceeded 1% and had no influence on the plating efficiency of the cell lines employed.

RESULTS

DNA cleavage mediated by purified topo II

The effect of the different anthracyclines on topo II-mediated DNA cleavage was studied *in vitro* by reacting highly purified calf thymus topo II with a ^{32}P-labeled DNA fragment. Addition of D1 (Fig. 2, A Lanes 4–8) and D2 (Fig. 2, A lanes 9–13) in the concentration range from 0.01 to 1 μM had no influence on cleavage, whereas 10 and 100 μM inhibited background cleavage obtained with the enzyme alone. In contrast, the addition of D3 (Fig. 2, A lanes 14–18) in the concentration range from 1 to 10 μM induced new topo II-mediated DNA cleavage sites and stimulated preexisting sites when compared to the topo II-mediated DNA cleavage in the absence of drug. The maximum effect was at 10 μM and background inhibition was obtained at 100 μM. Remarkably, the aglycone D5 also stimulated cleavage significantly with a maximum effect at 100 μM (Fig. 2, B lanes 6–10). In contrast, the aglycone of ACLA (D6) did not stimulate cleavage, and at the highest concentration tested (100 μM) some cleavage-inhibition was seen (Fig. 2, B lanes 11–15). D4 inhibited almost all topo II-mediated cleavages at concentrations ≥1 μM and no stimulation of cleavage was observed (Fig. 2, B lanes 1–5). Thus, D4 (aklavin) was a more effective cleavage-inhibitor than D6.

The results with D5 and D6 clearly demonstrate that the chromophore itself is of great significance in determining which topo II-interactions can take place. The cleavage results show that the substitution of the sugar daunosamine in D3 with rhodosamine to D2, i.e. the dimethylation of the amino group in oxaunomycin, abolishes DNA cleavage by topo II. If the dimethylated sugar group is combined with the non-cleaving chromophore (D4) the outcome is a potent cleavage inhibitor.

DNA SSBs

To ascertain whether these effects were accompanied by production of DNA SSBs in cells, the DNA single strand cleaving activity of the drugs was assessed in L1210 cells using an alkaline elution filter method. Experiments were performed after treatment of whole cells with the anthracyclines for 1 hr at 37°. D2, D3 and D5 all produced DNA SSBs in dose-dependent manners (Table 1). D3 (oxaunomycin) was most potent at all tested doses and D3 was the only drug exhibiting important cleavage at the lowest dose tested (0.3 μM). D3 cleavage leveled at 1 μM at approx. 1000 rad-equivalents and D2 cleavage leveled at 3 μM at approx. 500 rad-equivalents. The effect of D5 increased with increasing doses and a maximum effect of the aglycone was not demonstrated, apparently this is above 500 rad-equivalents. In contrast to the cleavage stimulation that was obtained with congeners of oxaunomycin, ACLA as well as D4 and D6 did not stimulate cleavage at any dose tested. Also in these experiments the dimethylation of the amino group in D3 to the compound D2 resulted in a dramatic reduction in DNA cleavage. However, both D1 and especially D2 demonstrated SSBs whereas no cleavage was obtained with these drugs using purified topo II (Fig. 2). Apparently this discrepancy is due to a higher resolution obtained with the alkaline elution assay. Thus, SSBs were also obtained with D2 in isolated L1210 nuclei (data not shown) and an intracellular metabolism to a cleaving compound is thus unlikely. However, it must be emphasized that SSBs in cells could be a result of numerous other causes than topo II. Thus, our data cannot rule out that DNA cleavage by D1 and D2 is due to a direct effect on DNA or an interaction with topo I.

We have previously shown that co-induction with ACLA effectively abolishes VP-16, m-AMSA and daunorubicin-mediated DNA cleavage in cells [4, 8]. This effect appears to be due to ACLA inhibiting topo II binding to DNA. A similar inhibitory effect was obtained by ACLA on D3 cleavage (Table 1). We were then interested to see whether this effect could also be obtained with D4 as this drug inhibited cleavage by the enzyme alone. As depicted in Table 1, D4 was also able to antagonize the cleavage obtained both with VP-16 and D3.

Accumulation of drugs in L1210 cells

The accumulation of the different compounds in

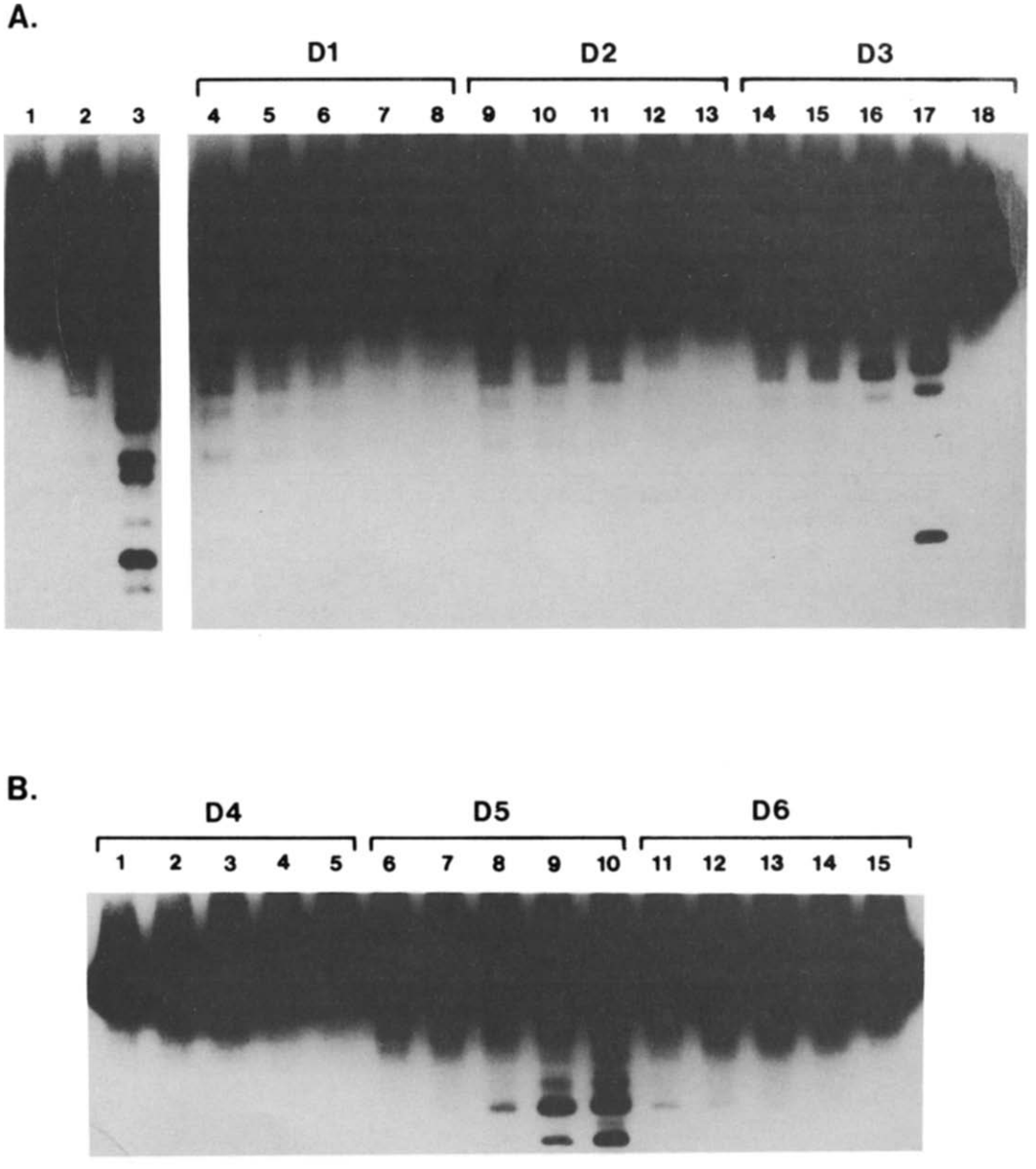


Fig. 2. Drug-effect on topo II mediated DNA cleavage. (A) Lane 1, DNA end-labeled with [α-^{32}P]-dATP (control); lane 2, plus calf thymus topo II (control); lane 3, DNA plus topo II plus m-AMSA (1 μM); lanes 4–8, DNA plus topo II plus D1 (0.01, 0.1, 1, 10 or 100 μM); lanes 9–13, D2 (0.01, 0.1, 1, 10 or 100 μM); lanes 14–18, D3 (0.01, 0.1, 1, 10 or 100 μM). (B) Lanes 1–5, D4 (0.01, 0.1, 1, 10 or 100 μM); 6–10, D5 (0.01, 0.1, 1, 10 or 100 μM); 11–15, D6 (0.01, 0.1, 1, 10 or 100 μM).

L1210 cells was measured to facilitate the correlation of drug effects *in vitro* and in cells. The data after a 1 hr exposure of the cells to 3 μM of each compound are presented in Table 2. Major differences in drug accumulation were observed when comparing the drugs. The highest drug accumulations were obtained with ACLA reaching 2000–2200 pmol/10^6 cells, followed by D1, D2, D4 reaching 1000–1400 pmol/10^6 cells. D3 exhibited an intermediate accumulation at 500 pmol/10^6 cells, whereas the L1210 cells only accumulated very low amounts of the two aglycones D5 and D6, 20 and 60 pmol/10^6 cells, respectively.

Accordingly, differences in drug accumulation do not explain the differences in cleavage activity between drugs with the RMN chromophore and drugs with the AKV chromophore. The low accumulation of D5 may explain why a maximum effect of D5 was not obtained in the alkaline elution assay in L1210 cells exposed to up to 10 μM.

Cytotoxicity in SCLC cells

The cytotoxic potency in the compounds was evaluated by clonogenic assay on the SCLC cell line OC-NYH as described in Materials and Methods.

Table 1. DNA SSB formation in rad-equivalents obtained in L1210 cells incubated for 1 hr with the indicated drugs

Drug		Drug concentration (μM) 0.3	1	3	10
D1		41	67 (65–83) N = 5	38 (0–77) N = 2	0
D2		66	294 (247–348) N = 5	509 (428–590) N = 2	477
D3		540	950 (743–1193) N = 5	1192 (1101–1284) N = 2	1017
D4		0	0 N = 3	0 N = 2	0
D5		0	35 (30–35) N = 3	240 (199–280) N = 2	477
D6		0	0 N = 3	0 N = 2	0
ACLA		0	0	—	—
VP-16				462	
VP-16	+10 μM D4			0	
D3	+1 μM ACLA		663		
D3	+10 μM ACLA		0		
D2	+1 μM ACLA		83		
D2	+10 μM ACLA		0		
D3	+10 μM D4		420		

When more than one experiment was performed, the results are shown as the median followed by the range in parentheses.

Table 2. Summary of experimental findings

Drug	DNA-cleavage *in vitro*	DNA-cleavage in cells 0.3	1	3	10‡	Drug accumulation* (pmol/10^6 cells)	Cytotoxicity† LD_{50} (nM) NYH	NYH/VM	RR
D1		–	(+)	–	–	1200–1500	23	28	1.2
D2		–	+	++	++	1000–1200	11	26	2.4
D3	+++	++	+++	+++	+++	500–540	1.1	3.9	3.5
D5	+++	–	+	++	+++	20–21	630	860	1.4
ACLA	inhib	–	–	–	–	2000–2200	10	10	1.0
D4	inhib	–	–	–	–	1400–1420	220	140	0.6
D6		–	–	–	–	35–80	3200	1700	0.5

RR, relative resistance, OC-NYH/VM:OC-NYH.
* Results from two independent experiments in L1210 cells. Drug concentration 3 μM, 60 min at 37°.
† Results from continuous incubations.
‡ Concentration μM.

The results are shown in Fig. 3 and in Table 2. D3 (oxaunomycin) was the most potent of the drugs and as little as 1.1 nM D3 reduced the number of surviving colonies to 50% of controls (LD_{50}). ACLA was 10-fold less potent than D3 but more potent than all the other agents. Thus, when these data are compared with the SSB frequency of the different compounds, there is no correlation between DNA-cleavage and cytotoxicity. However, within the drugs containing the RMN chromophore there is a clear correlation between SSB frequencies and cytotoxicity (Tables 1 and 2 and Fig. 3). The dimethylation of D3 to D2 resulting in an abolished topo II cleavage-stimulation *in vitro* and a reduced DNA SSB formation in cells, also reduces cytotoxicity in OC-NYH cells by a factor of approx. 10. A similar 10-fold difference in cytotoxicity between D3 and D2 in L1210 cells has been described previously [19]. When two sugar groups are added to D2 to yield D1, the cytotoxicity is decreased by a factor of two in agreement with the reduced DNA cleavage. Interestingly, D4 (aklavin) is approximately 20-fold less cytotoxic than ACLA with three sugar groups; this has been described in Friend leukemia cells [28] and a similar difference is found in the present investigations. Thus, the addition of the two sugar groups increases cytotoxicity in the AKV containing drugs and decreases cytotoxicity in the RMN type.

To investigate further the differences between the agents, we studied the effect of ACLA and D4 on the cytotoxicity of D3. Figure 4A demonstrates the influence of ACLA on D3 toxicity. ACLA at a concentration of 0.1 μM significantly reduces the cytotoxicity of 0.04 μM D3, and at 0.3 μM ACLA nearly full protection of the cells toward D3 was reached. The effect of D4 on the cytotoxicity of D3 is shown in Fig. 4B. It also appears that D4 is an effective antagonist of D3 cytotoxicity. The antagonism is dose-dependent and at the highest D4 dose tested (2.6 μM) the increase in the survival is

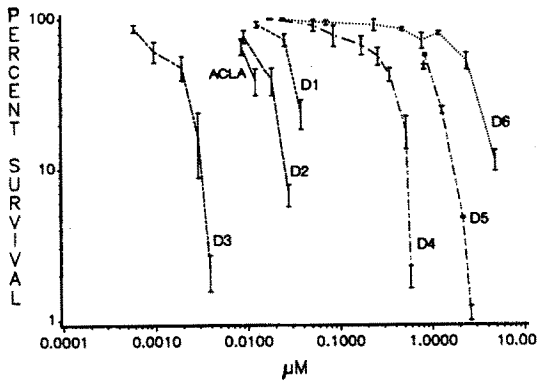


Fig. 3. Dose–response curves to the seven anthracyclines in a clonogenic assay on OC-NYH SCLC cells using continuous drug exposure (see Materials and Methods). Points, mean; bars, SEM obtained in N experiments. ACLA (———) (N = 8); D1 (– – –) (N = 6); D2 (———) (N = 7); D3 (— — —) (N = 7); D4 (—··) (N = 5); D5 (—···) (N = 2); D6 (·····) (N = 3).

above 3 logs. These results are in accordance with the effect of ACLA and D4 on D3 mediated DNA-cleavage in cells. Thus, we have demonstrated that D3 has the same property as we have previously published with VP-16, m-AMSA and daunorubicin [4, 8], and the mechanism of activity of D3 appears to be analogous to the classic topo II targeting agents.

Cytotoxicity in at-MDR cells

Compared to OC-NYH, the VM-26 resistant subline OC-NYH/VM has a 3-fold reduced topo II content (Fig. 5) and a similar reduction is observed in the catalytic activity of nuclear extracts (data not shown). OC-NYH/VM is cross-resistant to all drugs targeting topo II e.g. doxorubicin, VP-16 and m-AMSA but the line is not resistant to vincristine or to ACLA [21, 29]. The cell line does not express P-glycoprotein [29] and resistance is not modulated with verapamil (unpublished observation). Thus, all of our data indicate that resistance in OC-NYH/VM is due to an altered topo II and that the cell line has the characteristics of the at-MDR phenotype [30]. The cytotoxicity of the anthracyclines were compared in OC-NYH and at-MDR OC-NYH/VM cells. The results are depicted in Figs 6 and 7. Importantly, the lack of cross-resistance between ACLA and VM-26 in OC-NYH/VM is confirmed in the present studies (Fig. 6A). In addition there is no cross-resistance to D4 (aklavin) (Fig. 7B). In fact, OC-NYH/VM appears more sensitive to D4 than the "wild type" line. In contrast, there is a clear cross-resistance to D3 (Fig. 6B) (relative resistance RR = 3.5) supporting the notion that this drug has topo II as its cellular target. There is a minor but statistically significant change in sensitivity to D1 (Fig. 7A), with a relative resistance RR = 1.2 (Table 2), and to the aglycone D5 RR = 1.4 (Table 2). Compared to these two drugs, the cross-resistance is more distinct to D2 (RR = 2.4) (Fig. 7A). A summary of the results is depicted in Table 2. As seen in the table there is cross-resistance in OC-NYH/VM to all RMN holding drugs and the degree of resistance is directly

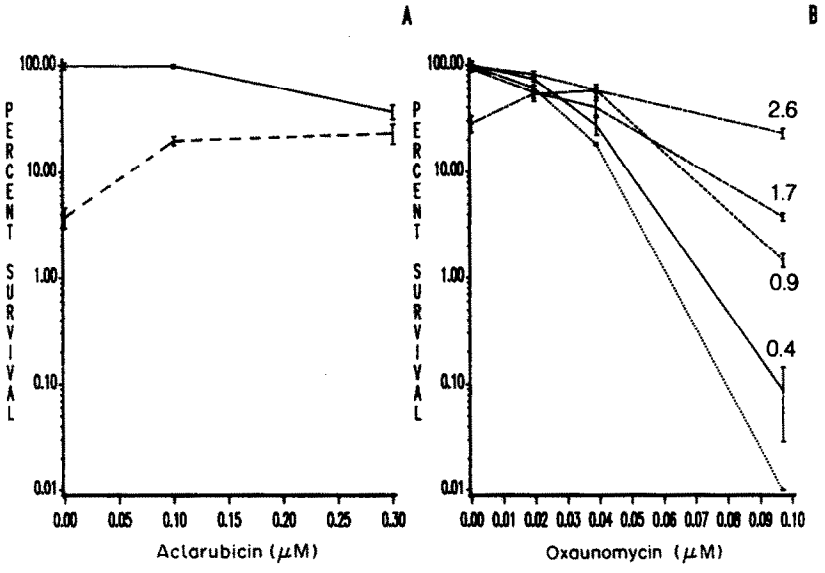


Fig. 4. (A) ACLA dose–response curves obtained in a clonogenic assay on OC-NYH cells incubated for 1 hr. Effect of ACLA in the absence (———) and presence of 0.04 μM D3 (oxaunomycin) (– – –). (B) D3 (oxaunomycin) dose–response curves obtained on OC-NYH exposed for 1 hr. Effect of D3 (oxaunomycin) in the absence (·····) and presence of 0.4 μM D4 (aklavin) (———), 0.9 μM (– – –), 1.7 μM (———), and 2.6 μM D4 (aklavin) (—·—). Points, mean; bars, SE from triplicate cultures.

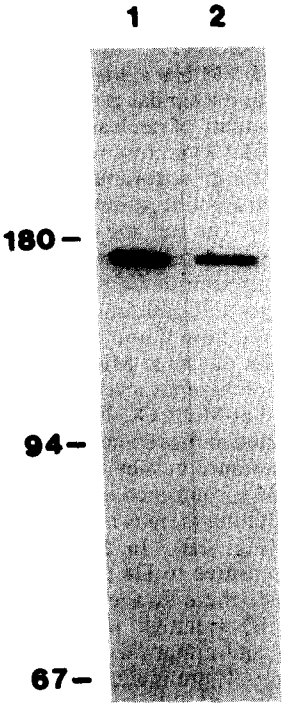


Fig. 5. Western blot of topo II in nuclear extracts from OC-NYH (lane 1) and OC-NYH/VM (lane 2). Numbers to the left of the blot indicate position and size of the molecular mass markers (kDa). Equal loading of protein was confirmed by amidoblack staining of the membrane.

correlated to the DNA cleavage activity in L1210 cells. In OC-NYH cells, D2 almost equals ACLA in potency. In OC-NYH/VM, however, ACLA is 2.6-fold more effective. In OC-NYH/VM, D3 is still the most potent drug, but the difference from ACLA is now only 2.5-fold as compared to a 10-fold difference in the OC-NYH cells.

DISCUSSION

Intercalating agents can inhibit topo II activity by at least two different mechanisms: (a) by trapping/stabilizing the cleaved topo II–DNA complex, or (b) by suppressing DNA cleavage presumably by inhibiting the enzyme from binding to DNA. The clinically active anthracycline ACLA [31] does not stabilize the cleaved complex and appears to inhibit the enzyme only by the latter mechanism. Recently, we have investigated the mechanism of action of ACLA by employing assays which make it possible to separate the DNA binding, cleavage, and religation reactions of topo II. These experiments suggest that ACLA abolishes the DNA cleavage activity of topo II by preventing the initial noncovalent DNA binding reaction of the enzyme [9]. These findings imply that the antagonistic properties of ACLA on the cytotoxicity of cleavage stimulators as VP-16, m-AMSA, daunorubicin [4, 8], and also oxaunomycin as observed in the present paper is mediated via an inhibition of the DNA binding reaction of topo II.

The inhibition of topo II cleavage by ACLA is comparable to the effects of the minor groove binders distamycin and Hoechst 33258 [32], and it has been suggested that ACLA could be regarded as a minor groove binder with the three sugar groups

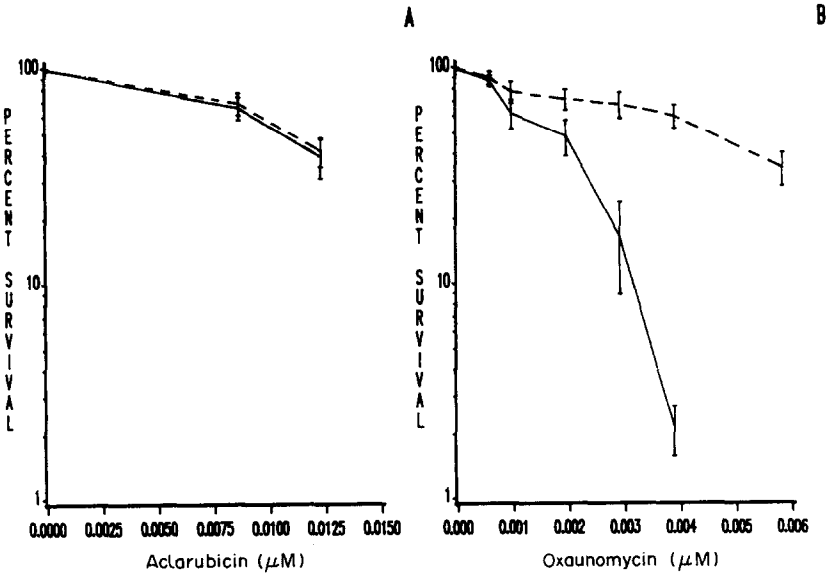


Fig. 6. Dose–response curves obtained in a clonogenic assay with (A) ACLA on cell lines OC-NYH (———) (N = 8) and OC-NYH/VM (----) (N = 6); and with (B) oxaunomycin (D3) on cell lines OC-NYH (———) (N = 7) and OC-NYH/VM (----) (N = 7). Points, mean; bars, SEM from N experiments (continuous drug exposure).

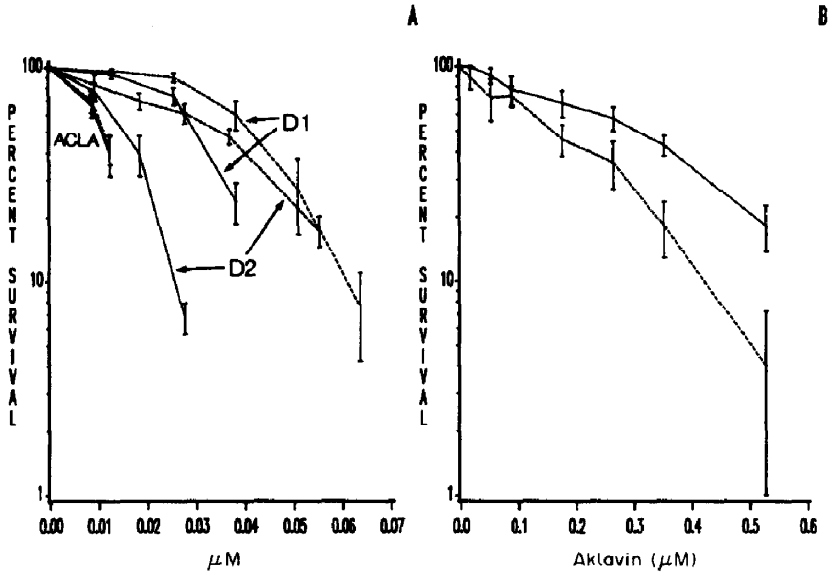


Fig. 7. (A) D1 and D2 dose–response curves obtained in a clonogenic assay. D1 on cell lines OC-NYH (———) (N = 6) and OC-NYH/VM (----) (N = 6). D2 on cell lines OC-NYH (———) (N = 6) and OC-NYH/VM (----) (N = 6). For comparison the ACLA data from Fig. 6A are included. (B) D4 (aklavin) dose–response curves on cell lines OC-NYH (———) (N = 5) and OC-NYH/VM (----) (N = 5). Points, mean; bars, SEM from N experiments (continuous drug exposure).

placed in the minor groove [33, 34]. It is therefore of interest that we find in the present study that topo II cleavage inhibition is obtained not only with ACLA but also with aklavin, with only a single sugar group attached to the aglycone D6. These data indicate that the inhibition does not occur via binding to the minor groove and that changes in the chromophore are of more importance.

The results with D6 and D5 also clearly demonstrate that the chromophore itself is of great significance in determining which topo II-interactions can take place. In contrast to D6, the aglycone D5 effectively stimulated topo II-mediated DNA cleavage. D6 has a carboxymethyl group at C-10 whereas D5 has hydroxyl groups at C-10 and at C-11. The hydroxyl group at position C-11 may have an impact on the level of DNA cleavage. Thus, Capranico *et al.* [35] found that the removal of the C-11 hydroxyl group in daunorubicin to 11-deoxy-daunorubicin resulted in a reduced, but, not in an abolished topo II-mediated DNA cleavage. Analogous to the C-11 position, modifications at C-4 also have a quantitative impact on cleavage. Daunorubicin has a methoxy group at position C-4 and both 4-demethyl-daunorubicin (carminomycin) and 4-demethoxy-daunorubicin (idarubicin) are 4-fold more potent than daunorubicin [35, 36]. Furthermore, 4-demethoxy-daunorubicin is a more potent topo II cleavage stimulator than daunorubicin [35]. Oxaunomycin has a free hydroxyl group at C-4, and in agreement with the results with daunorubicin, Kolar *et al.* [36] recently demonstrated that methylation of the O-4 significantly reduced the cytotoxicity of oxaunomycin. Accordingly, it appears that it is not the removal of a hydroxyl group at C-11, but rather the addition of a carboxymethyl group at C-10 that appears primarily responsible for abolishing the topo II-mediated DNA cleavage. This is in agreement with the results of Bodley *et al.* [3], who found that the cleavage obtained with doxorubicin and daunorubicin was completely inhibited by introducing bulky substitutions at position C-9.

In addition to the C-9 and C-10 substitutions in the chromophore, modifications in the sugar groups are also important. Substitution of the sugar L-daunosamine in D3 with L-rhodosamine to D2 i.e. the dimethylation of the 3′-nitrogen in oxaunomycin, abolishes DNA cleavage by purified topo II and also reduces the SSB formation in cells. Similar to these results, Bodley *et al.* [3] showed that doxorubicin cleavage by topo II was abolished by dimethylation of the sugar aminogroup to *N,N′*-dimethyldoxorubicin (AD280). In agreement with our results with D2, AD280 induced SSBs in cells, and the dimethylation greatly reduced the amount of cleavage when compared to doxorubicin.

Thus, there are at least two domains in the anthracycline molecule which may determine major biological properties of the congeners. The most important seems to be the C-9 and C-10 position of the chromophore. The side chain attached to the chromophore may prevent the formation of a ternary drug-topo II–DNA complex. Consequently, topo II is not stabilized and the cleavable complex is not formed. The second domain, which is important for anthracycline intercalation as demonstrated by Bodley *et al.* [3], is localized at the 3′-nitrogen of the daunosamine moiety. With the sugar(s) of the drug molecule positioned along the minor groove of

DNA, the C-9–10 domain of the chromophore apparently has an effect on the formation of topo II–DNA complexes. The major conclusions of the present investigations are in accordance with the results obtained with structure–activity analysis on analogs of m-AMSA. Studies with resistant cell lines exhibiting altered topo II have shown that while alterations in the acridine "DNA-binding" domain of m-AMSA can increase drug potency, they do not significantly alter the resistance level (LD_{50} ratio) of the resistant line, against which the drugs remain inactive [37]. In contrast, alterations in the anilino "protein-binding" domain can provide compounds with equal cytotoxicity in sensitive and resistant lines [37]. The lack of cross-resistance to ACLA in an at-MDR cell line described in the present paper is in accordance with the *in vivo* results by Johnson and Howard [38]. They demonstrated cross-resistance to daunorubicin but a lack of cross-resistance to ACLA in mice inoculated with m-AMSA resistant P388 cells. The P388/m-AMSA was one of the first cell lines shown to exhibit multidrug resistance due to an altered topo II [15].

Recently, much interest has been focused on new topo II targeting drugs that do not induce cleavable complex formation but inhibit the enzyme via alternative mechanisms [4, 8, 32, 39–43]. For intercalating agents and possibly also minor groove binders, blocking of access of topo II to its substrate seems to be the mechanism involved. This effect is not confined to topo II as the minor groove binders distamycin and Hoechst 33258 [44, 45] also inhibit topo I.

The present structure–activity results may give clues to development of new anthracyclines with activity in tumors exhibiting an at-MDR phenotype. The clear correlation between DNA SSB formation, cytotoxicity and cross-resistance in RMN-containing anthracyclines and the uniform lack of topo II mediated-DNA cleavage with the AKV-holding drugs show that the anthracyclines can be separated into at least two different types. One type has a cytotoxicity linked to topo II activity while the other is independent of topo II activity.

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