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The 1′-Substituent on the Anilino Ring of the Antitumor Drug Amsacrine Is a Critical Element for Topoisomerase II Inhibition and Cytotoxicity

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SUMMARY

The mechanism of action of the antitumor drug amsacrine involves intercalation of the acridine chromophore into DNA and inhibition of topoisomerase II. The substituent at position 1′ on the aniline is believed to be essential to the formation of the topoisomerase II/DNA cleavable complex and therefore to the cytotoxicity of the drug. To further delineate the role of the 1′-substituent, we investigated the effects on topoisomerase II activities of three anilinoacridine derivatives that differ only by the nature of the substituent at position 1′. The results of the cytotoxicity assays performed with cells sensitive (DC-3F) and resistant [DC-3F/9-hydroxy-ellipticine (9-OH-E)] to topoisomerase inhibitors are correlated with the effects of the drugs on topoisomerase II-mediated DNA cleavage *in vitro*. The influence of topoisomerase IIα on the mechanism of action of the drugs was examined using resistant DC-3F/9-OH-E cells transfected with a plasmid carrying a wild-type human topoisomerase IIα cDNA. Depending on the nature of the 1′-substituent of the drugs, the restoration of normal topoisomerase IIα catalytic activity in human topoisomerase IIα cDNA-transfected DC-3F/9-OH-E cells either does not modify the susceptibility of the cells to the drug or partially reverses the resistance phenotype. The molecular and cellular studies reveal that topoisomerase IIα is implicated in the cytotoxicity of amsacrine and confirm that the substituent at position 1′ on the anilino ring of amsacrine governs the interaction with topoisomerase II.

Over the span of several decades, pharmacological studies have drawn attention to the fact that most antitumor drugs bind to DNA, suggesting a direct relationship between the effects of the drugs on nucleic acids and their anticancer activity (1–3). The central role of DNA in controlling cell function and growth and the observation that certain antibiotics (e.g., actinomycin, daunomycin, bleomycin) recognize selectively defined nucleotide sequences have contributed to the speculation that the anticancer activity of these drugs occurs at the level of particular genes. However, during the past few years, studies have indicated that the antitumor activity of DNA-binding drugs and antibiotics depends in most cases on their capacity to interfere with the catalytic activities of topoisomerases rather than on their ability to bind to DNA *sensu stricto* (4–6).

This information has been exploited by medicinal chemists to create new categories of antitumor drugs. On the one hand, hundreds of sequence-selective ligands derived from the antiviral antibiotics netropsin and distamycin (the lexitropsins) have been synthesized (7, 8). A few distamycin conjugates containing an alkylating group show promise as antitumor drugs (9, 10). On the other hand, a growing variety of topoisomerase inhibitors have been developed, providing tumor-active compounds (5, 11, 12). We devised a strategy based on both topoisomerase inhibition and sequence targeting to design anticancer drugs. The approach consists of synthesizing sequence-reading pseudopeptides linked to an intercalating chromophore capable of inducing topoisomerase inhibition (13, 14). Recent work in this field (15, 16) opens the exciting possibility that such hybrid molecules, named combilexins, will be efficient molecular tools with which to control tumor cell growth via DNA recognition and topoisomerase inhibition. The first success of this strategy and the increasing need for better cancer therapy agents dictate that special attention be paid to deciphering how combilexin molecules exert their effects on malignant cells.

In the present study, we investigated the effects on topoi-

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ABBREVIATIONS: amsacrine, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; bp, base pair; NMHE, 2-*N*-methyl-9-hydroxyellipticinium; SDS, sodium dodecyl sulfate; hTOP2, human α topoisomerase II cDNA; NetGA, netropsin-glycyl-anilinoacridine; MePyGA, *N*-methylpyrrole-glycyl-anilinoacridine.

somerase II at the molecular and cellular levels of two combilexin molecules (MePyGA and NetGA) that contain an anilinoacridine chromophore (Fig. 1). Compound MePyGA is a DNA intercalator and displays a preference for GC sequences (17), whereas compound NetGA interacts with DNA via a bidentate mechanism involving intercalation of the acridine ring and minor groove binding of the netropsin moiety, which confers a noticeable selectivity for AT-rich sequences (18). The structure of the drugs is derived from that of the antitumor drug amsacrine (19). Although the effect of amsacrine on topoisomerase II was first demonstrated 10 years ago (20), the exact mechanism of interaction between this drug and the enzyme is not yet fully understood. Molecular modeling studies have indicated that the methanesulfonamide group on the anilino ring may constitute the topoisomerase binding domain (21–23). Recently, we showed that the original model of Baguley *et al.* (24) is correct: the presence of a substituent at position 1′ of the anilinoacridine chromophore is required to permit the drug to interfere with the catalytic activity of topoisomerase II (17).

To answer the question of how the modification of the 1′-substituent alters the sensibility of cells to the topoisomerase inhibitors, we studied the effects of the drugs shown in Fig. 1 toward sensitive (DC-3F) and resistant (DC-3F/9-OH-E) Chinese hamster lung cells in which topoisomerase II is normally and deficiently expressed, respectively (25). The involvement of topoisomerase IIα in the resistance of DC-3F/9-OH-E cells to the tested drugs was also determined. The results provide definite evidence that the nature of the 1′-substituent on the anilino ring of amsacrine is essential for interference with topoisomerase II and suggest that this substituent plays a critical role in determining the topoisomerase II isoform preferentially targeted by the drug. The results also provide useful information for future design of antitumor combilexin molecules targeting topoisomerases.

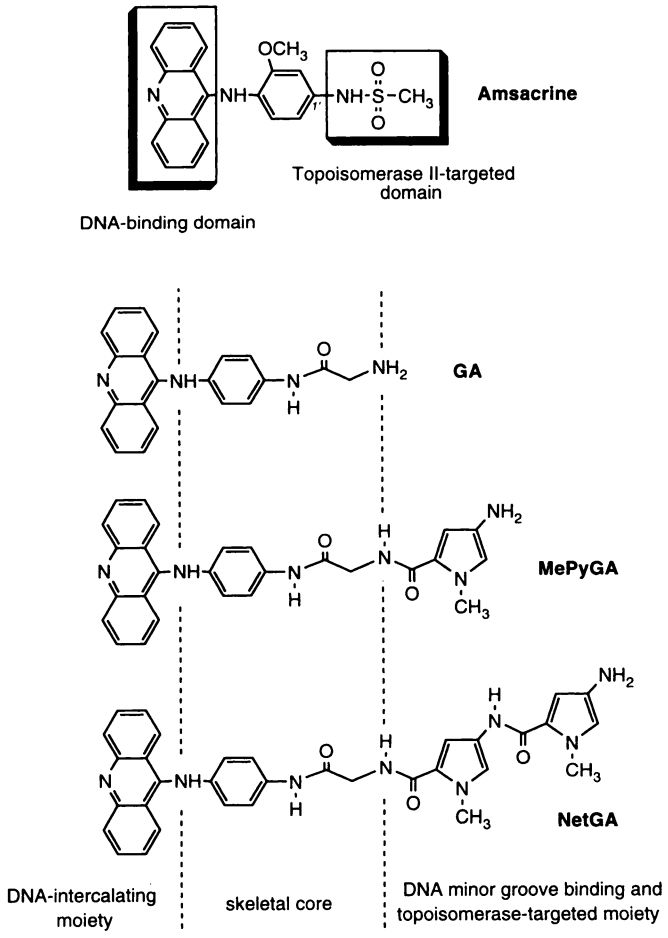

Fig. 1. Chemical structures of the drugs.

Materials and Methods

Drugs and biochemicals. Compounds GA, MePyGA, and NetGA were synthesized as previously described (26). Amsacrine was obtained through a modification (27) of the initial procedure (28). All other chemicals were analytical-grade reagents. Restriction endonucleases and T4 polynucleotide kinase were purchased from New England Biolabs. Calf alkaline phosphatase, proteinase K, and *Escherichia coli* DNA polymerase I (Klenow fragment) were obtained from Boehringer Mannheim. Reagents for DNA sequencing were purchased from DuPont-NEN. [γ-^{32}P]ATP and [α-^{32}P]dATP (3000 Ci/mmol) were purchased from Amersham. Calf thymus DNA topoisomerase II, pBR322 DNA, and pSP65 DNA were purified according to published procedures (29–31).

End-labeling and isolation of the DNA restriction fragments. For 3′-end labeling, pBR322 DNA was linearized with *Eco*RI and labeled with [α-^{32}P]dATP in the presence of the Klenow fragment of DNA polymerase I. The labeled DNA was then digested to completion with *Hin*dIII to generate two (4326 and 27 bp) DNA fragments. Both fragments were present in the reaction with topoisomerase II. For 5′-end labeling, pBR322 was first cut with *Nde*I, then treated with alkaline phosphatase, and labeled at the 5′-end with T4 polynucleotide kinase and [γ-^{32}P]ATP before digestion by the second restriction enzyme. The uniquely 5′-end-labeled DNA fragment was purified by electrophoresis on a 6% polyacrylamide gel and isolated by electroelution followed by ethanol precipitation.

Topoisomerase II-mediated DNA cleavage. The DNA was incubated with topoisomerase II in the absence or presence of the tested drug at various concentrations in 40 mM Tris·HCl buffer, pH 7.5, containing 0.5 mM dithiothreitol, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM ATP, and 30 μg/ml bovine serum albumin for 15 min at 37°. The cleavage reaction was terminated by the addition of SDS and proteinase K to final concentrations of 0.4% and 0.1 mg/ml, respectively, and the mixture was incubated for an additional 30 min at 50°. Then, 5 μl of loading buffer (0.05% Bromophenol blue, 50 mM EDTA, 50% sucrose) was added to each reaction mixture (15 μl) before electrophoresis. Experiments with covalently closed circular pSP65 DNA were analyzed on 0.8% agarose gels at 2 V/cm in a 89 mM Tris-borate buffer, pH 8.0, with 2 mM EDTA, containing 0.5 μg/ml ethidium bromide. Gels were destained and photographed under UV light. The cleavage products of 3′-end-labeled pBR322 DNA fragments were analyzed on 1% agarose/0.1% SDS gels as previously described (32). The 5′-end-labeled *Nde*I/*Ava*I restriction fragment from pBR322 was incubated with topoisomerase II in the absence and presence of the tested drug in a total volume of 60 μl, digested with proteinase K, and extracted with phenol/chloroform before ethanol precipitation. Samples were then analyzed on a 8% polyacrylamide gel containing 7 M urea.

Mapping and quantification of DNA cleavage. The size of the cleaved DNA fragments separated on agarose gels and therefore the localization of the cleavage regions stimulated by drugs were determined by using size markers as previously described (33, 34). Due to the limited resolution of agarose gels, a minimum distance of 50 bp between two cleavage regions is necessary to consider them as distinct. The intensities of the drug-stimulated DNA cleavage regions on the 3′-end-labeled DNA fragments were quantified by densitometry of the corresponding autoradiograms using a Joyce-Loebl Chromoscan 3 (Gateshead, England) interfaced to a microcomputer to

store and analyze the data. To quantify the extent of drug-induced double-strand cleavage in circular pSP65 DNA, negatives of the films were scanned with the densitometer, and the peak areas of linearized DNA (form III) were calculated (35).

Cell lines and culture conditions. The Chinese hamster lung cell lines DC-3F and DC-3F/9-OH-E have been described (36–38). Clone 24 is a subline of DC-3F/9-OH-E transfected with an eukaryotic expression vector containing hTOP2 (39). Monolayer cultures were maintained in Eagle's minimal medium supplemented with 7% fetal calf serum, penicillin (100 IU/ml), and streptomycin (50 μg/ml). The resistant subline DC-3F/9-OH-E was permanently grown in the presence of 9-hydroxy-ellipticine (0.6 μg/ml). Before each experiment, DC-3F/9-OH-E was grown for three passages in the absence of drug.

Chemosensitivity testing. To determine the sensitivity of DC-3F and DC-3F/9-OH-E to the drugs, 16-mm wells of a 24-well dish (Falcon, Becton-Dickinson) containing 1 ml of Eagle's minimal medium supplemented with graded drug concentrations were inoculated with 1 ml of cell suspension containing either 1×10^4 DC-3F cells or 4×10^4 DC-3F/9-OH-E cells. Duplicate assays were carried out for each drug concentration. Assays were performed at least three times with each drug and each cell line. After incubation with the test drug for 72 ± 2 hr, the medium was removed, and cells were counted with a Coulter Counter. The IC_{50} was defined as the drug concentration that inhibits cell growth by 50% compared with untreated controls. The IC_{50} value is obtained by plotting the percentage of residual cells versus the drug concentration on a semilogarithmic scale and is accurately determined from a computer-derived linear regression.

Cell survival. An *in vitro* colony formation assay was used to determine survival fractions after 3-hr drug exposure. Five hundred exponentially growing cells were grown in duplicate into 60-mm-diameter Petri dishes (Falcon, Becton-Dickinson) for 18 hr at 37° before the drug treatment. After 3-hr incubation with the drug, the medium was replaced, and the plates were incubated at 37°. Colonies were stained and counted 8–10 days later. The plating efficiency of the drug-treated plates was normalized to untreated controls. Assays were performed at least three times with each drug and each cell line. Plating efficiencies of control cells were in the range of 45–56% for DC-3F cells, 50–55% for DC-3F/9-OH-E cells, and 50–56% for clone 24 cells.

Results

Cytotoxicity and drug resistance. We studied the effect of the drugs on the growth of DC-3F Chinese hamster lung cells and the variant DC-3F/9-OH-E that has been selected for resistance to the DNA intercalating agent 9-hydroxy-ellipticine (36). DC-3F/9-OH-E cells are cross-resistant to a variety of antitumor agents, including topoisomerase II inhibitors such as amsacrine, VP-16 (etoposide), and the ellipticine derivative NMHE. The resistance to these topoisomerase II inhibitors is associated with reduced topoisomerase II activity and reduced topoisomerase II-mediated DNA cleavage (40). For each drug, the IC_{50} value was determined by the 72-hr assay procedure, and the level of resistance of the

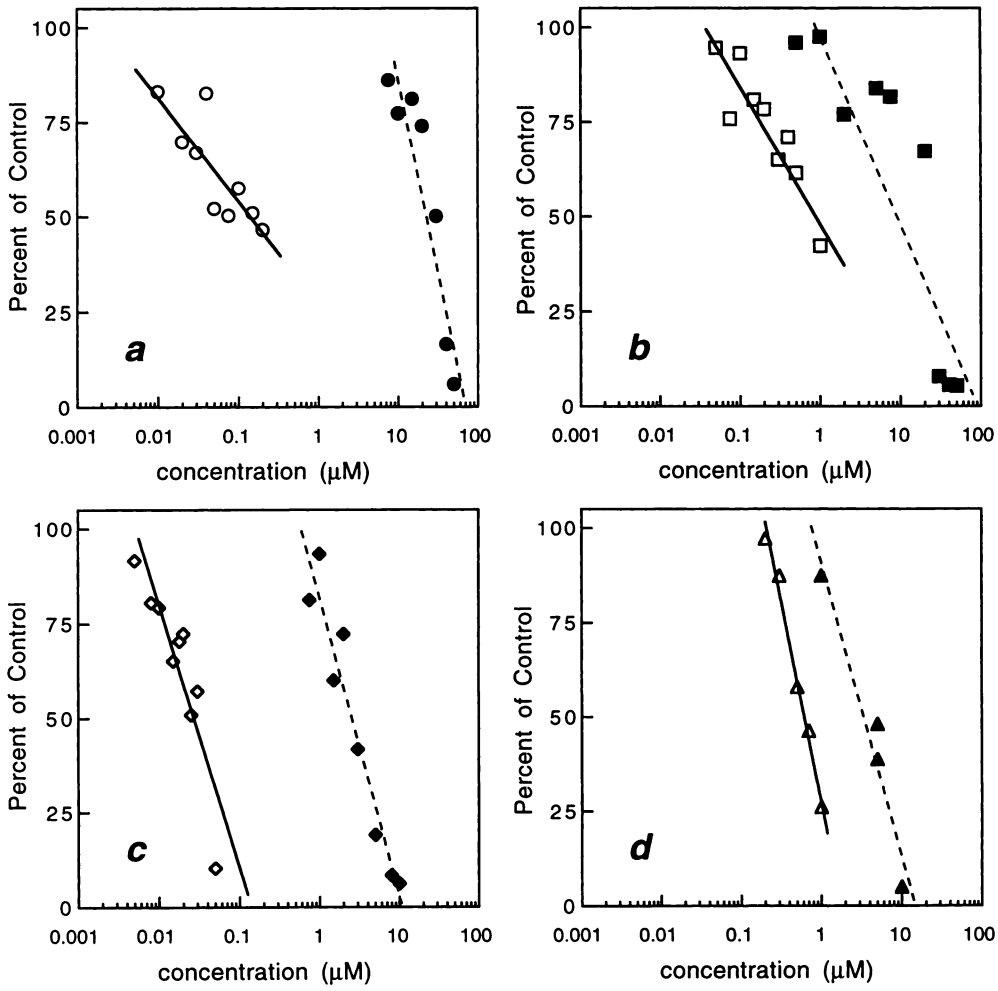

Fig. 2. Effect of MePyGA (a), NetGA (b), amsacrine (c), and GA (d) on the growth of sensitive DC-3F (*open symbols*) and resistant DC-3F/9-OH-E (*filled symbols*) cells. Cells were exposed to increasing drug concentrations for 72 hr. *Points*, average of two independent experiments performed in duplicate.

TABLE 1

Cytotoxic properties of the drugs toward DC-3F-sensitive and DC-3F/9-OH-E-resistant Chinese hamster lung cells

	IC_{50}[a]		Relative resistance index[b]
	DC-3F	DC-3F/9-OH-E	
	μM		
9-OH-Ellipticine	0.15	1.53	10
Amsacrine	0.025 ± 0.03	2.6 ± 0.1	105
GA	0.65 ± 0.03	3.5 ± 0.35	5
MePyGA	0.13 ± 0.05	22 ± 2	170
NetGA	0.9 ± 0.3	10 ± 4	10

[a] Drug concentration that inhibits cell growth by 50% after incubation in liquid medium for 72 hr. Each drug concentration was tested in duplicate (± standard error).

[b] The relative resistance index is the ratio between the DC-3F IC_{50} value and the DC-3F/9-OH-E IC_{50} value.

DC-3F/9-OH-E subline was defined by the following ratio: IC_{50}-resistant subline/IC_{50}-sensitive cell line. The three anilinoacridine derivatives are less cytotoxic than amsacrine (Fig. 2). The cytotoxicity of MePyGA toward sensitive DC-3F cells is comparable to that of 9-OH-E, whereas compound NetGA and GA are much less cytotoxic (Table 1). The extent of cross-resistance of the DC-3F/9-OH-E subline to the tested molecules varies considerably. The relative resistance index measured with compound GA lacking the 1′-substituent is very weak; this is most likely connected to the lack of effect of this compound on topoisomerase II-mediated DNA cleavage (17). Conversely, the cross-resistance to MePyGA is extremely high. It is interesting to note that although compound MePyGA is ~10-fold less cytotoxic than amsacrine, the relative resistance index for MePyGA is much higher than that calculated for amsacrine. The effect of MePyGA on the stabilization of the topoisomerase II/DNA complex (*vide infra*) plus the high resistance ratio strongly support the involvement of topoisomerase II in the mechanism of cytotoxicity of this conjugate molecule. Cross-resistance to NetGA is identical to that determined for 9-OH-E used to established the resistant cell line, suggesting that topoisomerase II contributes equally to the cytotoxic activities of these two compounds. Although both NetGA and MePyGA apparently exert their cytotoxic effects via a topoisomerase II-dependent mechanism, it is clear that topoisomerase II has a much larger impact on the action of the later GC-selective intercalating drug than on the former AT-selective hybrid compound. These observations led us to raise the possibility that the two drugs can interfere differently with topoisomerase II and/or that the activities of the two cellular forms of the enzyme, topoisomerases IIα and β, can be differently responsive to stimulation by the drugs. Both aspects were examined.

To investigate directly the later possibility, we determined the cytotoxic effects of the drugs toward DC-3F/9-OH-E cells transfected with a plasmid carrying a wild-type hTOP2. The clone 24 derived from hTOP2-transfected DC-3F/9-OH-E-resistant cells was isolated and characterized recently (39). These cells have recovered their original topoisomerase IIα catalytic activity. The sensitivity to MePyGA, NetGA, and

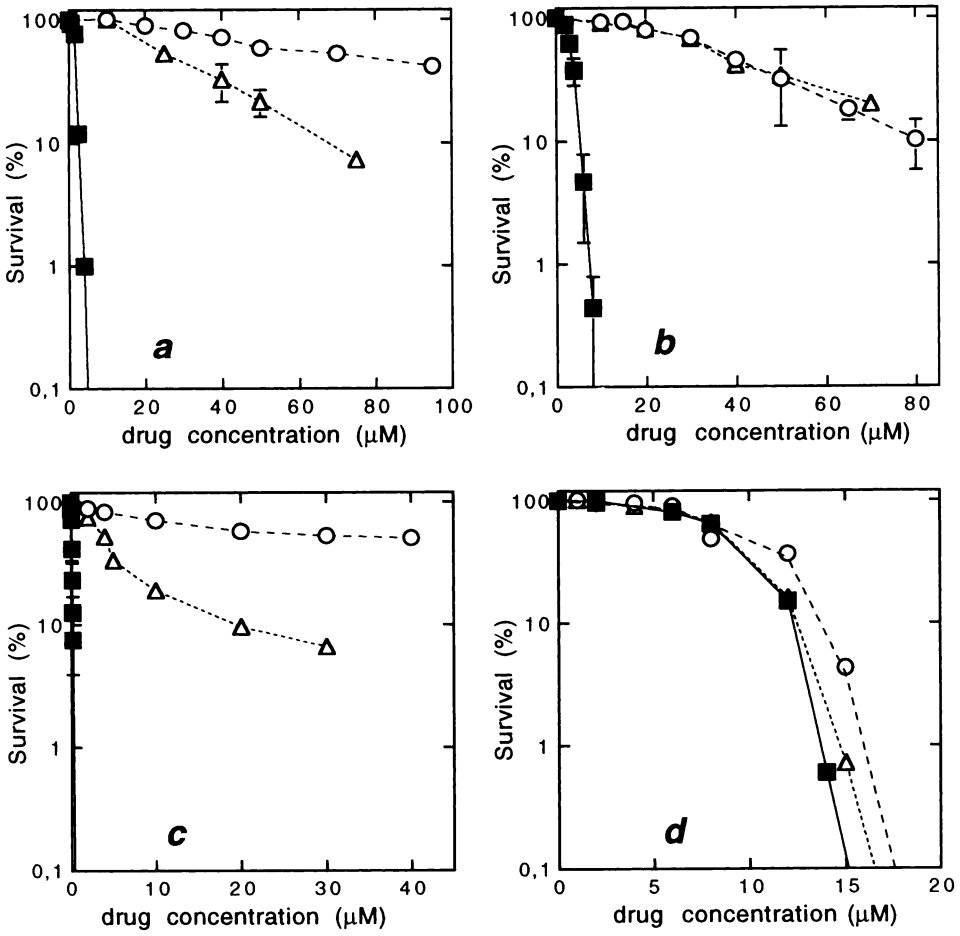

Fig. 3. Cytotoxicity of MePyGA (a), NetGA (b), amsacrine (c), and GA (d) in DC-3F cells (■), drug-resistant DC-3F/9-OH-E cells (○), and hTOP2-transfected clone 24 (△). Cells were incubated with the drugs for 3 hr at 37°. Colonies were stained and counted 8–10 days later. *Points*, average of at least two independent experiments performed in duplicate. *Error bars*, standard error for at least two independent determinations.

the control drugs amsacrine and GA of hTOP2-transfected cells as well as untransfected DC-3F/9-OH-E cells and sensible DC-3F cells was determined with an *in vitro* colony assay after 3-hr incubation with the drug. The restoration of normal topoisomerase IIα activity in clone 24 does not alter the resistance to NetGA (Fig. 3b) or GA (Fig. 3d). Similar results were obtained with 9-OH-E (39). In contrast, the cross-resistance to amsacrine (Fig. 3c) and, to a lesser extent, to MePyGA (Fig. 3a) is reduced in cells from clone 24, indicating that the restoration of the topoisomerase IIα activity can partially restore the sensibility of the transfected cells to these two drugs. These data indicate that topoisomerase IIα is strongly implicated in the mechanism of resistance of DC-3F/9-OH-E cells to amsacrine and MePyGA but not to NetGA.

Effects of the drugs on topoisomerase II *in vitro*. Our next goal was to determine whether compounds MePyGA and NetGA, which exhibit different cytotoxicities, distinct sequence selectivities, and slightly different mechanisms of binding to DNA, produce similar or different effects on topoisomerase II *in vitro*. Fig. 4 is a comparison of the stimulation of double-strand DNA cleavage induced by purified calf thymus topoisomerase II (containing both α and β isoforms) in the presence of amsacrine, MePyGA, and NetGA. The strand breaks observed after dissociation of the homodimeric subunits of topoisomerase II with the detergent (e.g., SDS) reflect the drug-induced stabilization of the DNA/topoisomerase II complex. Although the three drugs strongly stabilize the enzyme/DNA cleavable complex, the extent of inhibition of the strand passing reaction is different for each drug. At low concentrations (<10 μM), the stimulation of double-strand DNA cleavage is more pronounced with MePyGA and NetGA than with amsacrine. However, at higher concentrations (10–30 μM), the three drugs MePyGA, NetGA, and amsacrine promote DNA cleavage to approximately the same extent. A marked inhibition of cleavage is observed with NetGA at concentrations of ≥40 μM. Therefore, MePyGA stimulates DNA cleavage more efficiently and in a wider range of concentrations than does NetGA.

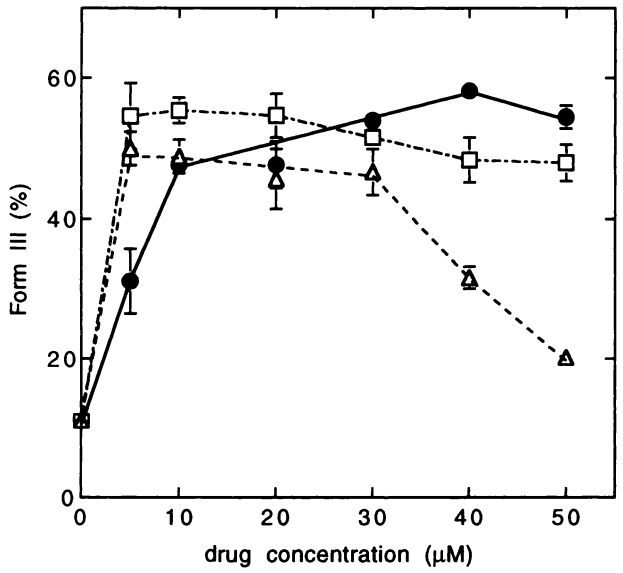

Fig. 4. Stimulation by MePyGA (□), NetGA (△), and amsacrine (●) of topoisomerase II-mediated double-strand DNA cleavage. The stimulation of the cleavable complex formation was determined by measuring the conversion of pSP65 DNA form I (supercoiled) to form III (linear) after incubation with purified topoisomerase II in the presence of increasing drug concentrations. *Error bars*, standard error for at least two independent determinations.

To determine whether the drugs stabilize the enzyme/DNA complex at similar or different sites along the DNA, we studied the topoisomerase II-mediated cleavage sites on a 4326-bp *Eco*RI/*Hin*dIII restriction fragment from pBR322. An autoradiograph of a typical agarose gel obtained after treatment of the ^{32}P-labeled DNA with purified calf thymus topoisomerase II in the absence and presence of various concentrations of NetGA and amsacrine is shown in Fig. 5A. Electrophoresis profiles of three selected lanes are shown in Fig. 5B. Topoisomerase II produces minimal DNA cleavage in the absence of drug, whereas in the presence of amsacrine the enzyme induces a high level of DNA cutting at multiple sites within the pBR322 DNA fragment. The bis-(*N*-methylpyrrolecarboxamide)-anilinoacridine conjugate NetGA significantly enhances DNA cleavage at selected sites, as does its mono-*N*-methylpyrrole counterpart (Fig. 6). It is worth remembering that compound GA lacking the substituent at position 1′ does not stimulate but rather inhibits the topoisomerase II-mediated DNA cleavage (17). Eleven major regions of DNA cleavage were identified within the pBR322 DNA fragment. The genomic localization and respective intensities of the cleavage regions produced by topoisomerase II in the presence of amsacrine and NetGA are indicated in Table 2. Both anilinoacridine derivatives stimulate DNA breaks at regions that can be detected (albeit very weakly) in the absence of drug. There apparently is no redistribution of the cleavage regions in DNA. However, NetGA and amsacrine distinctly affect the cutting activity of the enzyme. For example, amsacrine potentiates DNA cleavage around position 1135 (peak 2), whereas cleavage at this region is minimal in the presence of NetGA. The converse is observed at regions 2225 and 3395 corresponding to peaks 5 and 10. The DNA cleavage patterns resulting from topoisomerase II-mediated double-strand breaks stimulated by NetGA and MePyGA are similar (Fig. 6). However, the stimulation of cleavage is more pronounced with MePyGA than with NetGA. The different levels of DNA strand breaks produced by NetGA and MePyGA may be due to their distinct sequence selectivities and/or to their specific effects on each topoisomerase II isoform.

A few topoisomerase II-mediated DNA cleavage sites stimulated by NetGA were sequenced. We used the 870-bp *Nde*I/*Ava*I restriction fragment from pBR322 DNA previously used to compare the sequence requirements of MePyGA and amsacrine (17). The position of 14 cleavage sites stimulated by amsacrine, MePyGA, and NetGA were compared (data not shown). The topoisomerase II DNA cleavage patterns produced by NetGA and amsacrine are clearly different, whereas those produced by NetGA and MePyGA were found to be similar. However, we observed that the intensity of the cleavage at a defined position is frequently slightly higher with MePyGA than with NetGA, in accordance with the results shown in Fig. 6. Caution must be exercised in interpreting the sequencing data due to the limited number of cleavage sites identified. However, we are inclined to believed that despite their distinct sequence selectivities of binding to DNA, compounds NetGA and MePyGA exhibit the

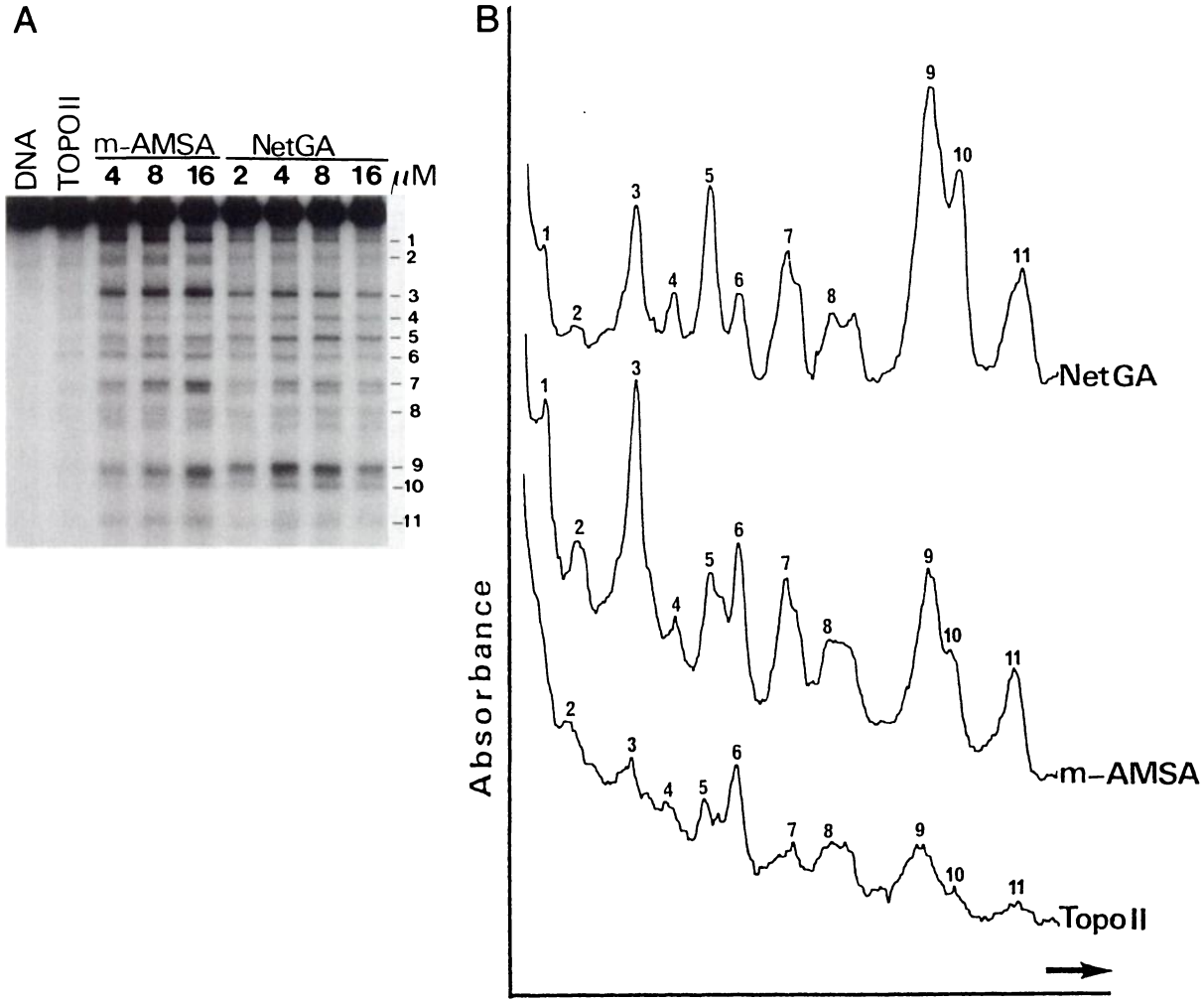

Fig. 5. Stimulation of topoisomerase II-mediated double-strand cleavage by amsacrine and NetGA. A, The 3′-end-labeled 4326-bp *Eco*RI/*Hind*III restriction fragment (*DNA*) was incubated with purified topoisomerase II in the absence (*Topo II*) or presence of various concentrations (2–16 μM) of amsacrine or *NetGA*. After SDS/proteinase K treatments, samples were analyzed on a 1% agarose gel. B, Lanes *Topo II*, 8 μM *m-AMSA* (amsacrine), and 8 μM *NetGA* were scanned. *1–11*, chief regions of topoisomerase II-mediated DNA cleavage. *Arrow*, indicates direction of electrophoresis (left to right).

same adjacent base requirements for stimulation of DNA cleavage by topoisomerase II.

Discussion

The comparison of the cytotoxicity of drugs toward a series of variant cell lines provides a suitable method to assist in identifying their potential cellular targets. It has been clearly established that DC-3F/9-OH-E-resistant cells present altered topoisomerase II activities compared with DC3-F cells (38–41). Recently, we showed that DC-3F/9-OH-E cells contain ~5-fold less topoisomerase IIα than DC-3F cells and that topoisomerase II β is undetectable in the resistant cell line (25). This cell line is more resistant to drugs known to act at the level of DNA topoisomerase II, such as amsacrine, VP-16, and ellipticines, than to drugs that do not interfere with topoisomerase II, such as camptothecin (42). By analogy, the observations (Fig. 2 and Table 1) that DC-3F/9-OH-E cells are significantly less susceptible to the cytotoxic effects of MePyGA and, to a lesser extent, of NetGA than the parental cell line led us to put forward the hypothesis that these two drugs exert their cytotoxic effect via a topoisomerase II-dependent mechanism. At least two lines of evidence support this hypothesis: (i) DC-3F and DC-3F/9-OH-E cells are equally sensitive to compound GA, which does not stimulate topoisomerase II-mediated DNA cleavage *in vitro*, and (ii) the restoration of normal topoisomerase IIα activity in hTOP2-transfected DC-3F/9-OH-E cells (clone 24) does not alter the resistance to compound GA but partially reverses the cellular resistance to compounds such as MePyGA and amsacrine, which are both potent topoisomerase II inhibitors.

The introduction of the hTOP2 gene into the DC-3F/9-OH-E cells increases the cellular content of topoisomerase IIα and therefore restores topoisomerase IIα catalytic activity to the original level (39). However, as shown in this study, the recovery of normal topoisomerase IIα activity in DC-3F/9-OH-E cells does not necessarily reverse the resistance phenotype of these cells. hTOP2-transfected cells are noticeably more sensitive than untransfected resistant cells to amsacrine and MePyGA but not to compounds NetGA and GA, suggesting that these drugs interfere differently with the topoisomerase II isoforms α and β. Recent studies have indicated that the α and β isoenzymes can exhibit different

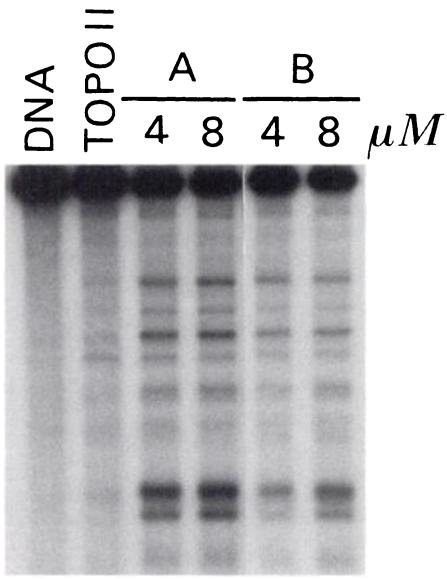

Fig. 6. Stimulation of topoisomerase II-mediated double-strand cleavage by MePyGA (A) and NetGA (B). The 3′-end-labeled 4326-bp *Eco*RI/*Hind*III restriction fragment (*DNA*) was incubated with purified topoisomerase II in the absence (*TOPO II*) or presence of 4 or 8 μM compounds MePyGA (A) and NetGA (B). After SDS/proteinase K treatments, samples were analyzed on a 1% agarose gel.

TABLE 2

Location and extent of topoisomerase II-induced DNA cleavage in the presence of NetGA in the EcoRI/HindIII DNA fragment from pBR322

Band	Location of cleavage region[a]	Relative band intensity[b] (NetGA/amsacrine)
1	1000	0.5
2	1135	0.4
3	1640	0.6
4	1980	1.5
5	2225	1.7
6	2425	0.5
7	2725	1.0
8	2985	0.8
9	3320	1.7
10	3395	2.1
11	3555	1.0

[a] pBR322 DNA ^{32}P-labeled at the 3′-end of the EcoRI site was reacted with purified calf thymus topoisomerase II in the presence of 8 μM drug. The EcoRI restriction site was taken as position 0 of the pBR322 genome. The determination of the genomic position of the cleavage regions was accurate to within ±50 nucleotides.

[b] Ratio of the relative intensity of a given band in the presence of compound NetGA to the intensity of the same band in the presence of amsacrine.

sensitivities to topoisomerase II inhibitors (43–45). The specific effects of amsacrine, MePyGA, and NetGA on DC-3F, DC-3F/9-OH-E, and hTOP2-transfected cells (i.e., on cells expressing different levels of topoisomerases IIα and β) suggest that the nature of the 1′-substituent on the anilino ring is critical for the drug to act distinctly on topoisomerase II isoforms α and β.

The weaker cytotoxicity of NetGA toward DC-3F cells compared with MePyGA is certainly not due to different capacities of penetration into cells because NetGA can easily cross the cell membranes and accumulates preferentially in the nuclei (46). It also cannot be attributed to differing sequence selectivity of topoisomerase II-induced DNA cleavage stimulation because the two drugs promote DNA cleavage at similar sites in DNA (Fig. 6). The difference may be attributable to the fact that MePyGA stabilizes topoisomerase II-DNA covalent complexes over a larger range of concentrations than NetGA. The bis-pyrrole moiety of NetGA attached at position 1′ on the anilino connector represents a very bulky group that resides in the minor groove of DNA (18). Topoisomerase II can apparently mediate DNA cleavage despite the presence of such sterically demanding substituent, prompting the conclusion that the identity of the 1′-substituent can be tuned to a considerable extent to modulate the catalytic activity of topoisomerase II and therefore the cytotoxicity of the drug. We showed recently that the removal of the pyrrole unit linked to the anilinoacridine residue of MePyGA (i.e., compound GA) abolishes all stimulation of DNA cleavage by topoisomerase II (17). In the present study, we showed that although replacement of the *N*-methylpyrrole residue with a longer bispyrrole moiety retains the same DNA-intercalating domain, the extent of topoisomerase II-mediated DNA cleavage is decreased at high drug concentrations (Fig. 4). Because the drugs GA, MePyGA, and NetGA differ only by the nature of the 1′-substituent, we can conclude that the chemical identity of the 1′-substituent is of great significance in determining the interaction of anilinoacridine derivatives with topoisomerase II. In this respect, the cellular and molecular results of the present study are totally consistent with our previous *in vitro* studies (17).

The demonstration that the 1′-substituent on the anilino ring of amsacrine is directly responsible for the interference (and probably for the interaction) with topoisomerase II provides useful information for future design of combilexin molecules. There apparently is no correlation between DNA sequence selectivity and cytotoxicity, whereas there is a correlation between topoisomerase inhibition and cytotoxicity. The compounds MePyGA and NetGA are, respectively, GC and AT selective but display similar effects on topoisomerase II. The drug NetGA, which exhibits a higher level of sequence selectivity than MePyGA, is ~7-fold less cytotoxic than MePyGA toward DC-3F cells [and also toward L1210 leukemia cells (26)]. In the same way, we showed recently that a distamycin/ellipticine combilexin presenting a very high specificity for AT-rich sequences in DNA showed practically no effect on topoisomerases and was not cytotoxic, whereas a sequence-neutral analogue proved to be a potent dual topoisomerase I and II inhibitor and exhibited high cytotoxic properties (16). The topoisomerase II-targeted domain in the series of anilinoacridine combilexins is clearly identified as being the 1′ substituent. We envisage synthesis of anilinoacridine drugs with varying topoisomerase binding domain at position 1′, e.g., a hydroxy flavone chromophore derived from the topoisomerase II inhibitor genistein. However, it must be kept in mind that in addition to the interaction between the drug and its potential targets, the design of tumor-active drugs would ideally demand consideration of a wide range of interdependent parameters, including cellular uptake and efflux, drug metabolism, and drug distribution.

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