Stimulation of Site-Specific Topoisomerase II-Mediated DNA Cleavage by an *N*-Methylpyrrolecarboxamide–Anilinoacridine Conjugate: Relation to DNA Binding†

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ABSTRACT: The DNA binding properties and effects on topoisomerase II of MePyGA, an anilinoacridine derivative bearing an *N*-methylpyrrolecarboxamide unit at position 1′, have been compared with those of its precursor glycylanilinoacridine and the structurally related antileukaemic drug amsacrine. Electric linear dichroism spectroscopy reveals that MePyGA intercalates its acridine chromophore between DNA base pairs with a preference for GC-rich sequences, whereas both its structural analogue lacking the *N*-methylpyrrole unit and amsacrine intercalate into DNA without any strong sequence preference. The effects of the test drug on the catalytic activities of topoisomerase II were studied *in vitro* using purified calf thymus enzyme and ^{32}P-labeled DNA. MePyGA stabilizes the topoisomerase II–DNA covalent complex and stimulates the cutting of DNA at a subset of preexisting topoisomerase II cleavage sites. The removal of the *N*-methylpyrrole unit abolishes both the GC-preferential binding to DNA and the topoisomerase II-mediated DNA cleavage. MePyGA and amsacrine stimulate the cleavage of DNA by topoisomerase II at different places: cleavage stimulated by amsacrine is consistent with the expected adenine requirement at position +1 whereas the predominant sites of DNA cleavage stimulated by MePyGA contain a cytosine at position ±1. This is the first instance where an anilinoacridine derivative differing only by the nature of the substituent at position 1′ has been found to affect the catalytic activity of topoisomerase II differently. The spectroscopic and biochemical data lead to the conclusion that two functional domains can be identified in MePyGA: its anilino group can be regarded as a skeletal core to which are connected (i) the tricyclic acridine moiety which represents the DNA-binding domain and (ii) the *N*-methylpyrrole moiety which constitutes the topoisomerase II-targeted domain. The structure of the substituent at position 1′ of the anilinoacridine chromophore evidently determines the location of the sites of DNA cleavage by topoisomerase II. These findings provide guidance for the synthesis and development of new topoisomerase II-targeted antitumor anilinoacridine derivatives.

Amsacrine [*m*-AMSA; 4′-(9-acridinylamino)methanesulfon-*m*-anisidide] is an antitumor agent first synthesized 20 years ago (Cain & Atwell, 1974; Cain et al., 1975). In just a few years it has found an established place in the clinic for the treatment of acute myelogenous leukaemia (Cassileth & Gale, 1986). It is commonly accepted that the anticancer activity of amsacrine is connected with its ability to bind to DNA (Waring, 1976; Wilson et al., 1981) and to interfere with topoisomerases (Nelson et al., 1984; Kohn et al., 1987). In addition, amsacrine induces DNA single-strand breaks (Burr-Furlong et al., 1978), interacts with copper so as to produce free radical-induced DNA strand breaks (Wong et al., 1984), and reacts with sulfhydryl groups on peptides (e.g., glutathione) and proteins (Wong et al., 1986). There now exists compelling evidence linking the inhibition of topoisomerase II activities by amsacrine to the cytotoxicity of the drug. For example, (i) the biologically inactive ortho isomer *o*-AMSA is markedly less potent than *m*-AMSA in inhibiting topoisomerase II (Nelson et al., 1984), and (ii) cell lines resistant to amsacrine possess catalytically active but drug-resistant forms of topoisomerase II (Pommier et al., 1986; Per et al., 1987; Mayes et al., 1993). Therefore, for amsacrine as well as for other DNA intercalating antitumor drugs including some anthracycline, anthraquinone, and ellipticine derivatives, it is interference with topoisomerase II which is believed to constitute the critical event in the mechanism of cytotoxicity (Wang, 1985; Riou et al., 1993; Ralph et al., 1994). As a corollary, the design of topoisomerase II inhibitors should lead to the discovery of new anticancer drugs (Kohn et al., 1987; Drlica & Franco, 1988; Liu, 1989).

On the basis of structure–activity relationships, Baguley et al. (1990) have proposed a model that divides amsacrine into two functional domains: the acridine chromophore constitutes the DNA-binding domain, and the anilino group would

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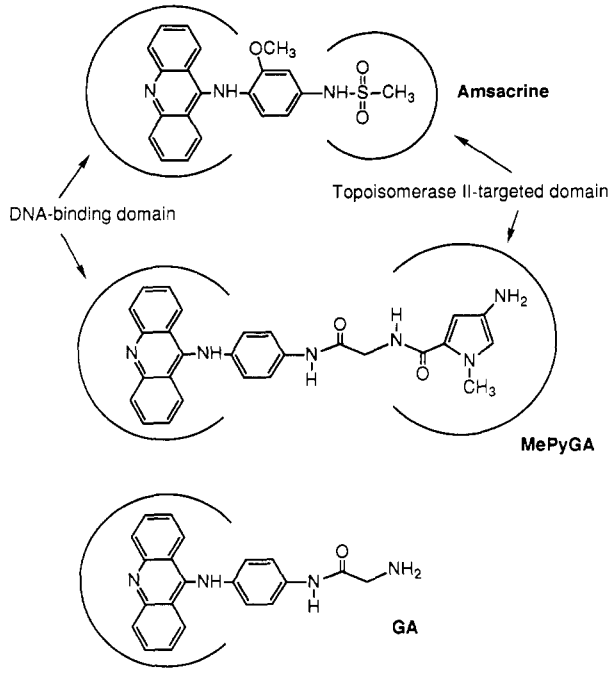

FIGURE 1: Chemical structures of the drugs used in this study.

represent the topoisomerase II-binding domain. The methoxy group is not absolutely required for activity, but it can serve as a switch to enhance (meta position) or to block (ortho position) the activity of the drug. In fact, from recent studies (Zwelling et al., 1992) it has been suggested that the 1′-methanesulfonamide side chain attached to the anilino group is the putative site of interaction with topoisomerase II. Therefore, by varying the nature of this side chain at position 1′, one can expect to change the capacity of the drug to interfere with topoisomerase II so as to modulate its cytotoxicity. Despite its effective antileukemic properties, amsacrine induces severe adverse effects (e.g., bone marrow toxicity) which limit its use. The development of less toxic derivatives of amsacrine would be valuable.

From the many hundreds of anilinoacridine derivatives which have been designed to date [see Denny (1989) and Baguley (1991) for reviews], we noticed that certain derivatives having a sterically demanding substituent at position 1′ remained cytotoxic. For example, the (bis-anilino)acridine derivative SN12489 (NSC 140701) has proved to be as efficient as amsacrine at promoting DNA cleavage by topoisomerase II (Pommier et al., 1987), and the level of DNA lesions produced by the drug in cells correlates well with cytotoxicity. These observations prompted us to examine the issue of DNA binding and topoisomerase II poisoning by an *N*-methylpyrrolecarboxamide–glycylanilinoacridine conjugate, henceforth called MePyGA (Figure 1). This molecule represents a truncated analogue of a netropsin–acridine hybrid ligand which upon binding to DNA locates its netropsin-like bis-pyrrole moiety in the minor groove and intercalates its acridine chromophore (Bailly et al., 1990). Recently, we showed that this antitumor hybrid molecule has the capacity to stabilize the topoisomerase II–DNA complex (Bailly et al., 1992a) which we attributed to the presence of its bis-pyrrole moiety. In this paper we use MePyGA as the corresponding mono-*N*-methylpyrrole carboxamide derivative to examine whether it remains a topoisomerase II inhibitor and whether its capacity to bind preferentially to certain sequences in DNA correlates with the induced sequence-specific cleavage of DNA by the enzyme.

MATERIALS AND METHODS

Drugs. The synthesis of MePyGA, together with complete spectral characterization, was previously described (Bailly et al., 1989). Amsacrine (*m*-AMSA) was obtained by a modification (Hénichart et al., 1982) of the initial procedure (Cain et al., 1975).

Biochemicals. The three natural DNAs from calf thymus (CT DNA, highly polymerized sodium salt), *Clostridium perfringens*, and *Micrococcus lysodeikticus* together with the double-stranded alternating poly(dA-dT)·poly(dA-dT), poly(dG-dC)·poly(dG-dC) and nonalternating poly(dA)·poly(dT), poly(dG)·poly(dC) polymers were purchased from Sigma and were used without purification except for CT DNA which was deproteinized twice with sodium dodecyl sulfate. Their concentrations were determined by applying molar extinction coefficients of 6600, 6600, 6950, 6600, 8400, 6000, 7400 M^{-1} cm^{-1}, respectively (Wells et al., 1970). 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 from Boehringer Mannheim. Reagents for DNA sequencing were from NEN Dupont Inc. [γ-^{32}P]ATP and [α-^{32}P]dATP (3000 Ci/mmol) were purchased from Amersham. SV40 DNA was purchased from BRL (Gaithersburg, MD). Calf thymus DNA topoisomerase II (containing both α and β isoforms), pBR322, and pSP65 DNA were purified according to published procedures (Miller et al., 1981; Halligan et al., 1985; Maniatis et al., 1982).

Electric Linear Dichroism (ELD). The theory and practice for measurements of electric linear dichroism have already been the subject of detailed reports (Houssier, 1981). The construction of the pulse generator and computerized optical measurement system have been previously described (Houssier & O'Konski, 1981). Linear dichroism ΔA is defined as the difference between the absorbance for light polarized parallel ($A_\parallel$) and perpendicular ($A_\perp$) to the applied field at a given wavelength. The reduced dichroism is $\Delta A/A = (A_\parallel - A_\perp)/A$, where A is the isotropic absorbance of the sample measured in the absence of electric field at the same wavelength and using the same pathlength.

In these experiments the DNA molecules are oriented by an electric pulse, and the dichroism in the region of the absorption bands of DNA or of the ligand bound to DNA is probed using linearly polarized light. When DNA solutions are exposed to the electric field pulses, at 260 nm the absorbance of light polarized parallel to the electric field vector is lower than the absorbance of light polarized perpendicularly ($A_\parallel < A_\perp$), indicative of a negative dichroism. Similar negative dichroism signals are observed with intercalator–DNA complexes in the absorption band of the ligand. By contrast, when rectangular electric pulses are applied to a solution of a minor groove binder bound to DNA, the change of the absorption of light in the ligand absorption band is different ($A_\parallel > A_\perp$), indicative of a positive dichroism of the complex. Therefore, on the basis of the sign and amplitude of the observed signals, this technique can reveal the mode of binding of the ligand via an estimation of its orientation with respect to the DNA helix.

For comparative purposes, the reduced dichroism of a ligand–DNA complex measured in the ligand absorption band must be analyzed with respect to the reduced dichroism measured for the same DNA or polynucleotide at 260 nm in the absence of drug (Bailly et al., 1992b). The dichroism ratio is defined as $DR = (\Delta A/A)^{\text{ligand-DNA}}/(\Delta A/A)^{\text{DNA}}$. The numerator in this ratio refers to the reduced dichroism of the

drug–DNA complex measured at the maximum absorption of the DNA-bound ligand. The denominator refers to the reduced dichroism of the same DNA sample at 260 nm in the absence of drug and is always negative under the experimental conditions used. The dichroism ratio is expected to be +1 if the transition moment of the drug chromophore is parallel to the DNA bases, as in the case of intercalative binding. Under our conditions, the DR for any chosen drug–DNA and drug–polynucleotide complexes can be compared with good relative accuracy, independent of the polymer size. A DR value for a given drug–DNA complex is representative of the geometry of the complex but cannot be considered as an indication of the relative affinity of a drug for the DNA used. All measurements of DR values were repeated twice with high reproducibility.

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 base pair DNA fragments. Both fragments were present in the reaction with topoisomerase II. For 5′-end labeling, pBR322 and SV40 DNA were first cut with *Nde*I, treated with alkaline phosphatase, and labeled at the 5′-end using T4 polynucleotide kinase and [γ-^{32}P]ATP prior to 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 test drug at various concentrations in 40 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM DTT, 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 °C. The cleavage reaction was terminated by adding SDS and proteinase K to final concentrations of 0.4% and 0.1 mg/mL, respectively, and the mixture was incubated for another 30 min at 50 °C. Then 5 μL of loading buffer (0.05% bromophenol blue, 50 mM EDTA, 50% sucrose) was added to each reaction mixture (15 μL) prior to electrophoresis.

Experiments with covalently closed circular pSP65 DNA were analyzed on 5% polyacrylamide gels as previously described (Bailly et al., 1992a). After being stained with ethidium bromide, polyacrylamide gels were photographed under UV illumination using Polaroid films. The cleavage products of 3′-end-labeled pBR322 DNA fragments were analyzed on 1% agarose–0.1% SDS gels as previously described (Fossé et al., 1992). The 5′-end labeled *Nde*I–*Ava*I restriction fragment from pBR322 DNA and the *Nde*I–*Pst*I restriction fragment from SV40 DNA were reacted with topoisomerase II in the absence and presence of the test drug in a total volume of 60 μL, digested with proteinase K, and extracted with phenol–chloroform prior to ethanol precipitation. The samples were resuspended in 3 μL of loading buffer (80% formamide, 20 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) and heated at 100 °C for 3 min before being loaded on to an 8% polyacrylamide gel containing 7 M urea in TBE buffer: 89 mM Tris base, 89 mM boric acid, 2.5 mM Na_2 EDTA, pH 8.3. Gels were transferred to Whatman 3MM paper, dried under vacuum at 80 °C, and then subjected to autoradiography at −70 °C with an intensifying screen. Exposure times of the X-ray films (Fuji R-X) were adjusted according to the number of counts per lane loaded on each individual gel.

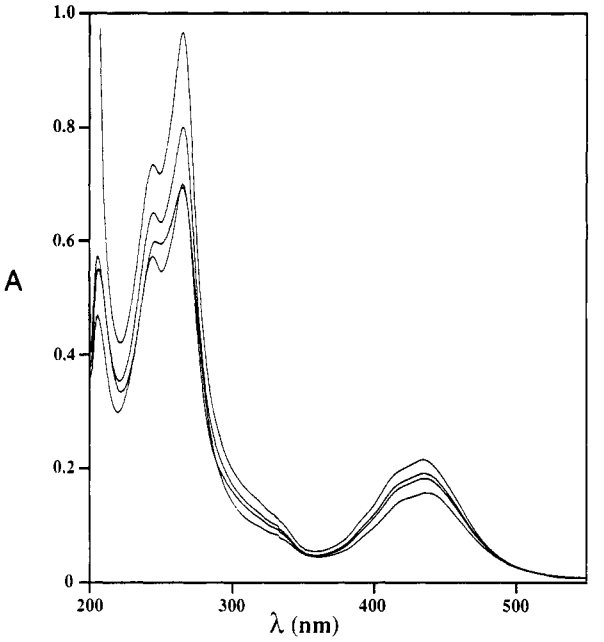

FIGURE 2: Absorption spectra of MePyGA in the absence and presence of calf thymus DNA. To 3 mL of drug solution (20 μM in 1 mM sodium cacodylate buffer, pH 6.5) were added successive amounts of DNA. Spectra were referenced against DNA solutions of exactly the same DNA concentration and were adjusted to a common baseline. The DNA–phosphate/drug ratio increased as follows (top to bottom): 0, 0.4, 0.9, and 1.3. At high P/D ratios (up to 10.0) no further qualitative changes occurred, and the hypochromism at 440 nm plateaued at about 46%.

Mapping and Quantitation 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 (Fossé et al., 1990, 1991). Due to the limited resolution of agarose gels, a minimum distance of 50 base pairs between two cleavage regions is necessary to consider them as distinct. For the sample analyzed on a polyacrylamide gel, the positions of topoisomerase II cleavage sites were determined at nucleotide resolution by reference to sequence markers (Maxam & Gilbert, 1980). pBR322 DNA is numbered according to Watson (1988). 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 area under each well-resolved peak was calculated (Bailly et al., 1992a).

RESULTS

Binding to DNA. Figure 2 displays the spectral changes that occur when MePyGA is added to calf thymus DNA. A marked hypochromism is observed in the 260- and 440-nm bands (up to 46% at P/D = 10). The interaction of the drug with DNA causes a bathochromic shift of 7 nm in the visible absorption maximum (from 434 to 441 nm), owing to the perturbation of the complexed chromophore system upon binding to DNA. The absence of isosbestic points in these spectra suggests that more than a single mode of binding can occur, especially at high ligand/DNA ratios where some sort of nonspecific (external) binding is often seen with drugs.

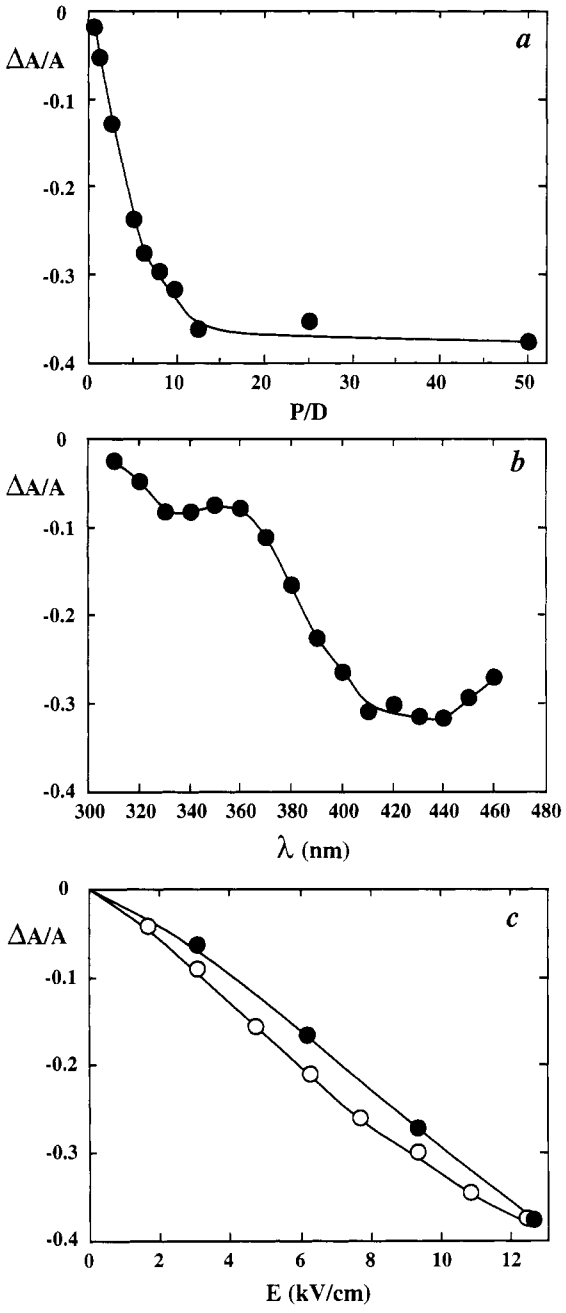

FIGURE 3: Dependence of the reduced dichroism $\Delta A/A$ on (a) the DNA–phosphate to drug ratio (P/D), (b) wavelength, and (c) electric field strength. (●) MePyGA; (○) DNA. Conditions: (a) 440 nm, 12.5 kV/cm, (b) P/D = 10, 12.5 kV/cm, (c) 440 nm for compound MePyGA, 260 nm for DNA, P/D = 10 in 1 mM sodium cacodylate buffer, pH 6.5.

Electric linear dichroism (ELD) experiments were performed to define the orientation of the ligand chromophore with respect to the DNA helix. Figure 3 shows a typical set of experimental data for the dependence of the reduced dichroism $\Delta A/A$ on the DNA–phosphate to drug ratio (P/D), the wavelength, and the electric field strength. The mode of binding of MePyGA was analyzed only on the basis of the highest ELD values, obtained when the drug molecules are fully bound to DNA, i.e., for P/D ratios >10. At lower P/D ratio, the measured ELD values fall significantly due to the appearance of unbound molecules in the solution (Figure 3a). The ELD spectrum of the MePyGA–DNA complex is shown in Figure 3b. The reduced dichroism is always negative in sign, even in the 300–350-nm region where the *N*-methylpyrrole carboxamide unit aborbs the light. This situation differs from that observed previously with the bis(methylpyrrole)–acridine conjugate which exhibited positive reduced dichroism signals around 320 nm (Bailly et al., 1990, 1992a). It is likely that the methylpyrrole substituent of compound MePyGA does not penetrate into the minor groove but probably extends out from the surface of the double helix (so as to engage in contacts with the enzyme). The intensity of the ELD signal is a function of the degree of alignment of the DNA molecules in the electric field. The reduced dichroism depends upon field strength similarly at 260 nm for the DNA bases and at 440 nm for compound MePyGA (Figure 3c). This indicates that the acridine ring is tilted close to the plane of the DNA bases, consistent with an intercalative mode of binding.

Table 1: Dichroism Ratios (DR) of the Drug–DNA Complexes[a]

DNA and polynucleotides	%GC	GA	MePyGA	amsacrine
poly(dG)·poly(dC)	100	0.81	1.76	0.58
poly(dG–dC)·poly(dG–dC)	100	0.98	1.44	1.01
Micrococcus lysodeikticus DNA	72	nd	1.01	1.15
calf thymus DNA	42	0.95	0.85	1.05
Clostridium perfringens DNA	26	nd	0.91	0.72
poly(dA–dT)·poly(dA–dT)	0	1.12	0.67	1.27
poly(dA)·poly(dT)	0	0.70	0.35	0.41

[a] DR = $[(\Delta A/A)^{\text{drug-DNA}}]/[(\Delta A/A^{\text{DNA}}]$, drug/DNA ratio = 0.1, E = 12.5 kV/cm in 1 mM sodium cacodylate buffer, pH 6.5. The reduced dichroism was measured at 260 nm for the polynucleotides and at 440 nm for drug–DNA complexes. nd, not determined.

The binding of MePyGA to nucleic acids has been studied using a variety of synthetic polymers and natural DNAs of widely differing base composition with the aim of obtaining information concerning the sequence dependence of the mode of binding of the drug to DNA. Compounds MePyGA, GA, and amsacrine were studied under identical conditions. To take into account the intrinsic behavior of the DNA and the polynucleotides under the electric pulses, we refer to the comparative dichroism ratio DR, i.e., the reduced dichroism of the drug–DNA complex measured at 440 nm in the acridine band (without any contribution from the DNA molecules) divided by the dichroism of the DNA measured at 260 nm in the absence of drug. The results are summarized in Table 1. In the presence of compound GA, the DR values do not vary markedly from one DNA to another; the acridine moiety retains much the same orientation whatever the DNA composition. This compound is thus a non-sequence-selective DNA-binding agent. However, we note that the DR values corresponding to the complexes of the drug with the homopolymers are consistently lower than those with the alternating copolymers. In the presence of MePyGA the results are very different from those obtained with compound GA which lacks the *N*-methylpyrrole carboxamide unit. The DR values increase with the GC content of the DNAs indicating clearly that the drug exhibits a preference for binding to GC-rich DNA sequences. The DR value obtained with the copolymer poly(dG)·poly(dC) is more than 5-fold higher than that obtained with the copolymer poly(dA)·poly(dT). The fact that MePyGA complexes with the two GC-containing polynucleotides exhibit larger reduced dichroism amplitudes than complexes with DNA (DR > 1) indicates that the drug is likely to induce a local stiffening of the DNA around the intercalation site which enhances the degree of orientation of the DNA molecules in the electric field. The results with amsacrine (Table 1) have been previously discussed (Bailly et al., 1992b) and are consistent with the literature data (Wilson et al., 1981): amsacrine binding to poly(dA-dT)·poly(dA-dT) is slightly tighter than binding to poly(dG-dC)·poly(dG-dC), and the alternating copolymers are much

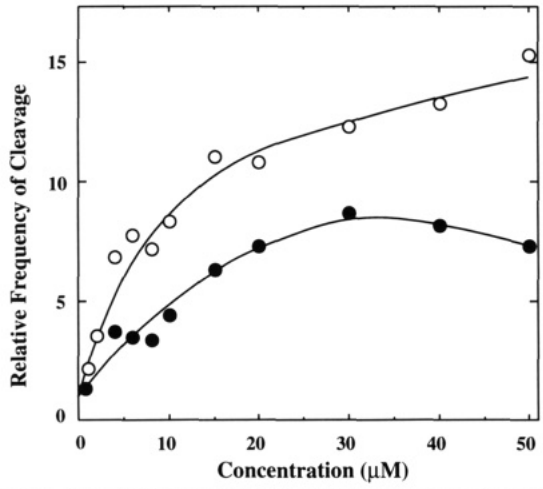

FIGURE 4: Stimulation by MePyGA (●) and amsacrine (○) of DNA topoisomerase II-mediated double-strand DNA cleavage. The extent of cleavage (%) of pSP65 DNA was determined as described under Materials and Methods, and the value is expressed relative to the extent of cleavage observed in the presence of topoisomerase II alone.

preferred over the homopolymers, with the binding to poly(dG)·poly(dC) being favored over binding to poly(dA)·poly(dT).

The results of the ELD studies can be summarized as follows: (i) upon binding to DNA, MePyGA intercalates its acridine nucleus between DNA base pairs, (ii) the removal of the *N*-methylpyrrole–carboxamide unit does not hinder the capacity of the drug to intercalate into DNA, and (iii) unlike amsacrine and compound GA, the pyrrole–anilinoacridine conjugate binds preferentially to GC-rich sequences in DNA.

Drug-Induced Stabilization of the Topoisomerase II–DNA Complex. It is well established that, in the presence of purified topoisomerase II and DNA, amsacrine stabilizes the enzyme–DNA interaction in the form of a so-called "cleavable complex" (Tewey et al., 1984). In this complex the enzyme is covalently linked to the 5′-end of the DNA, and treatment with a detergent (e.g., SDS) results in the formation of DNA strand breaks which can be revealed by gel electrophoresis of the DNA fragments. Figure 4 displays the appearance of DNA double-strand cleavage by topoisomerase II as a function of amsacrine and MePyGA concentration. MePyGA is somewhat less efficient than amsacrine which, as expected, massively stimulates the cleavage of DNA by topoisomerase II. However, MePyGA does have a marked effect on the cleavage reaction whereas compound GA (i.e., its synthetic precursor) does not (Figure 5). Thus, the strong stimulation of topoisomerase II-mediated DNA cleavage by MePyGA is directly attributable to the presence of the *N*-methylpyrrole–carboxamide unit which is the only substituent that distinguishes compounds GA and MePyGA. The pyrrolecarboxamide substituent at position 1′ on the anilinoacridine chromophore must therefore play a decisive role in the stabilization of the topoisomerase II–DNA complex, and in that respect it appears to mimic efficiently the sulfonamide group of amsacrine which is believed to stimulate the enzyme-mediated DNA cleavage.

Mapping of the Drug-Stimulated DNA Cleavage by Topoisomerase II. The next question was whether MePyGA and amsacrine, which are both potent inhibitors of topoisomerase II, do so by stabilizing enzyme–DNA complexes at similar or different sites along the DNA, i.e., whether the modified topoisomerase II-binding domain of the drug has an effect on the intensity and location of the DNA cleavage sites. The 4326 base pair *Eco*RI–*Hin*dIII restriction fragment from

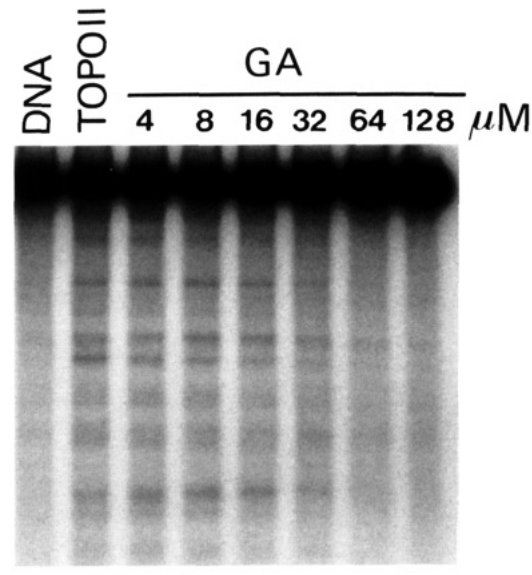

FIGURE 5: Inhibition of topoisomerase II-mediated double-strand cleavage of DNA by compound GA. The 3′-end-labeled 4326 base pair *Eco*RI–*Hin*dIII restriction fragment (lane labeled DNA) was incubated with purified topoisomerase II in the absence (lane Topo II) or presence of various concentrations of compounds GA (4–128 μM), treated with SDS and proteinase K and subjected to electrophoresis on a 1% agarose gel.

pBR322 was radioactively labeled at the 3′-end of the *Eco*RI site and used as a substrate for purified calf topoisomerase II. The DNA cleavage patterns resulting from topoisomerase II-mediated double-strand breaks stimulated by amsacrine and MePyGA were studied over a large range of drug concentrations. As shown in Figure 6A, only a few discrete bands can be seen with topoisomerase II in the absence of drug whereas much stronger bands are produced at several defined positions in the presence of MePyGA or amsacrine. The patterns of DNA cleavage induced by topoisomerase II in the presence of either drug are significantly different. Evidently, despite their structural homology these two anilinoacridine derivatives modulate the catalytic activity of the enzyme differently. Again we observed that, under identical experimental conditions, compound GA did not stimulate DNA cleavage by the enzyme: over the concentration range from 4 to 16 μM GA the cleavage of DNA by topoisomerase II remained practically unaffected. Only at higher concentrations (32–128 μM) did the compound inhibit the background cleavage observed with the enzyme alone (Figure 5). Inhibition of cutting at high concentrations is a common feature of DNA intercalating drugs (Tewey et al., 1984; Fossé et al., 1990). Thus, removal of the *N*-methylpyrrole moiety abolishes all stimulation of DNA cleavage by topoisomerase II. The glycinamidoanilino group provides a suitable connector between the DNA- and topoisomerase II-binding domains but is apparently not involved in the interaction of the drug with the enzyme.

In order to make a quantitative comparison of the specific distribution of cleavage regions produced by topoisomerase II in the absence and presence of drugs, selected lanes from the gel in Figure 6A were scanned with a densitometer. Figure 6B shows the profiles of DNA cleavage generated by topoisomerase II in the absence or presence of amsacrine or MePyGA. Ten major regions of DNA cleavage were identified; their genomic localization and respective intensities are given in Table 2. Both the amsacrine- and MePyGA-stimulated DNA breaks are located at regions which can be detected in the absence of drug. As observed by others with

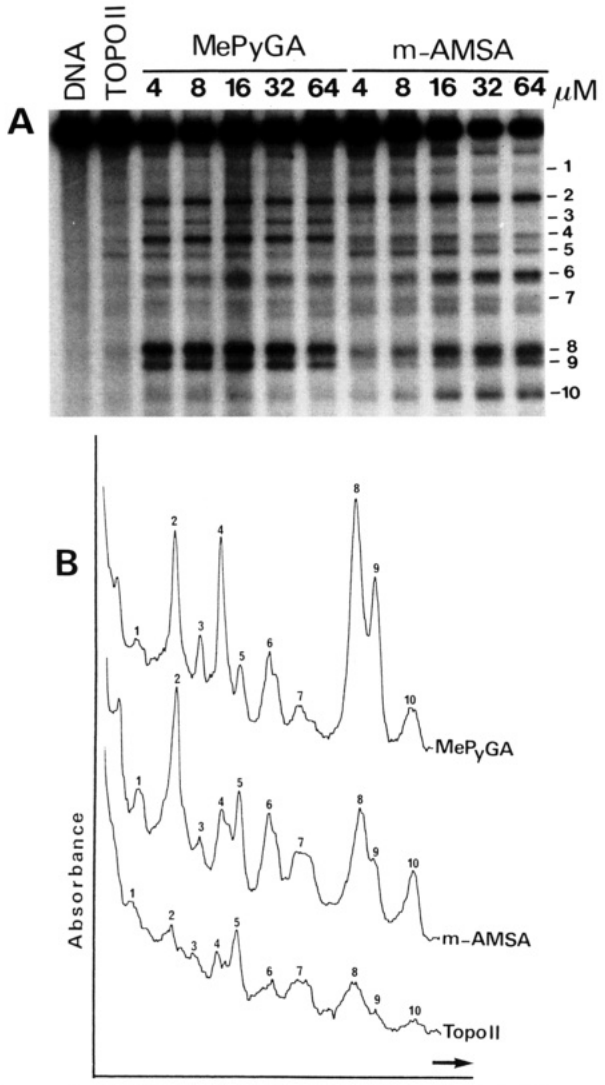

FIGURE 6: Stimulation of topoisomerase II-mediated double-strand cleavage of DNA by amsacrine and MePyGA. (A) The 3′-end-labeled 4326 base pair *Eco*RI–*Hind*III restriction fragment (lane labeled DNA) was incubated with purified topoisomerase II in the absence (lane Topo II) or presence of various concentrations (4–64 μM) of compound MePyGA and amsacrine. After SDS/proteinase K treatment samples were analyzed on a 1% agarose gel. (B) Lanes Topo II, 8 μM MePyGA, and 8 μM *m*-AMSA were scanned. The chief regions of topoisomerase II-mediated DNA cleavage are numbered from 1 to 10. The direction of electrophresis is left to right, as indicated by the arrow.

anilinoacridine derivatives (Nelson et al., 1984; Rowe et al., 1986), there is no qualitative redistribution of the cleavage regions in DNA. Yet the densitometric profiles in Figure 6B and the data in Table 2 clearly indicate that amsacrine and MePyGA exert distinct effects on topoisomerase II. For examples, MePyGA fails to affect cleavage of DNA at the region corresponding to peak 5 whereas cleavage at this region is plainly stimulated by amsacrine. Conversely, MePyGA stimulates the DNA cleavage much more strongly than amsacrine at regions around nucleotide positions 1980, 2225, 3320, and 3395, which correspond to peaks 3, 4, 8, and 9, respectively. It is clear that the pyrrolecarboxamide group attached to position 1′ of the anilinoacridine chomophore effectively serves as a topoisomerase II interacting domain and is critical in determining those sites at which topoisomerase–DNA interactions can take place.

Sequencing of Drug-Stimulated DNA Cleavage Sites. The ability of MePyGA to stimulate cleavage of DNA at certain regions more efficiently than amsacrine may be related to the

Table 2: Location and Extent of DNA Cleavage Induced by Topoisomerase II in the Presence of Amsacrine and MePyGA in the *Eco*RI–*Hin*dIII DNA Fragment from pBR322[a]

band	location of cleavage region[b]	relative band intensity[c] (MePyGA/amsacrine)
1	1135	0.6
2	1640	0.9
3	1980	2.3
4	2225	2.7
5	2425	0.5
6	2725	0.9
7	2985	0.5
8	3320	2.2
9	3395	2.7
10	3555	0.6

[a] pBR322 DNA ^{32}P-labeled at the 3′-end of the *Eco*RI site was reacted with purified calf thymus topoisomerase II in the presence of 8 μM amsacrine and 8 μM MePyGA. See Figure 6. [b] The *Eco*RI 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. [c] Ratio of the relative intensity of a given band in the presence of compound MePyGA to the intensity of the same band in the presence of amsacrine.

nucleotide sequence around these regions. To investigate this possibility, a detailed comparison of the different sequence requirements of amsacrine and MePyGA was performed by studying the topoisomerase II-mediated cleavage of DNA in a sequencing gel system (Figure 7). The cleavage sites were sequenced using a 5′-end-labeled *Nde*I–*Ava*I restriction fragment from pBR322 DNA (870 base pairs in length) which contains a region cleaved specifically in the presence of MePyGA (band 3 in Figure 6 and Table 2). In this region, cleavage in the absence of drugs is barely detectable (lane topoII) and only weakly stimulated by amsacrine (lane m-AMSA). The positions of five and six cleavage sites stimulated by amsacrine and MePyGA, respectively, were accurately determined within the 160 base pair sequence accessible to densitometric analysis (from nucleotide positions 2010–2170 of the pBR322 genome). The topoisomerase II DNA cleavage patterns produced by MePyGA and amsacrine are clearly different (Figure 7).

In order to clarify and extend these observations, additional experiments were carried out with another DNA fragment from SV40 DNA. The amsacrine- and MePyGA-induced sites of DNA cleavage by topoisomerase II were localized at nucleotide resolution within the 80 nucleotide sequence spanning positions 4900–4980 of the SV40 genome. Here also the DNA cleavage patterns induced by topoisomerase II in the presence of amsacrine and MePyGA are different. The location, relative intensity, and sequence surrounding the sites of cleavage determined in pBR322 DNA and SV40 DNA are summarized in Table 3. Each drug-stimulated cleavage site was identified on only one strand of the DNA fragments. It is likely that the cleavage site identified on one strand is paired with the corresponding site, staggered with the expected 5′ overhang of four bases, on the complementary strand. However, we have chosen not to indicate these putative complementary cleavage sites because amsacrine is known to stimulate both single- and double-strand breaks (Zwelling et al., 1981; Pommier et al., 1991). The ratio of single- to double-strand breaks is higher with amsacrine than with most other topoisomerase II inhibitors (Muller et al., 1988). By analogy with amsacrine, we cannot exclude the possibility that some sites of DNA cleavage detected with compound MePyGA are really single-strand breaks.

Recent studies have shown that the drug-induced stimulation of DNA cleavage by topoisomerase II depends on the nature

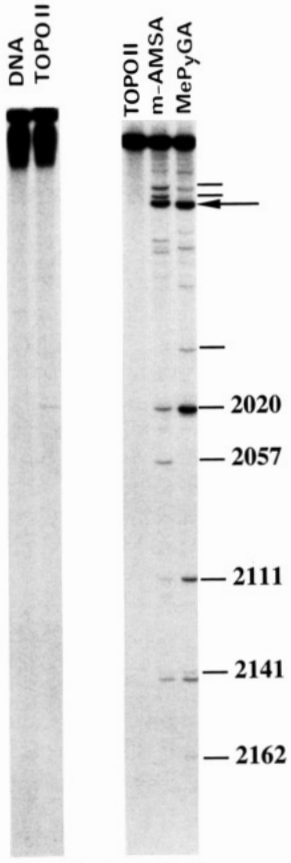

FIGURE 7: Sequence analysis of the cleavage sites stimulated by amsacrine and MePyGA. The *Nde*I–*Ava*I (870 base pairs) fragment of pBR322 DNA (lane labeled DNA), 5′-end-labeled at the *Nde*I site, was incubated in the absence (lane Topo II) or presence of 16 μM *m*-AMSA or 16 μM MePyGA. Topoisomerase II cleavage reactions were analyzed on an 8% denaturing polyacrylamide gel as described under Materials and Methods. Numbers at the right of the gel refer to the nucleotide position of cleavage sites. The band of strong intensity pointed out by the arrow and the two bands above constitute the cleavage region numbered 2 in Figure 6. The intensity of cleavage at region 2 is aproximately the same for amsacrine and MePyGA as indicated in Table 2.

of the bases adjacent to the cleavage site (Pommier et al., 1991; Fossé et al., 1991; Freudenreich & Kreuzer, 1993; Capranico et al., 1994). Accordingly, we compared the nature of the nucleotides on both sides of the identified cleavage sites in order to determine whether the differences in DNA cleavage patterns between MePyGA and amsacrine might be attributable to the nature of the bases at positions −1 and +1. The base sequence preference observed with amsacrine is consistent with the canonical A(+1) and/or T(+4) adjacent base requirement (Pommier et al., 1991; Capranico et al., 1993). Indeed, eight out of the nine amsacrine cleavage sites encompass an adenine residue at position +1 and/or a thymine residue at position +4. We did not observe any strong stimulation of DNA cleavage by *m*-AMSA at sites devoid of A(+1) and T(+4) bases. The adjacent base requirement for MePyGA seems to be quite different from that for amsacrine. For example, amsacrine stimulates the DNA cleavage at position 2057, a site which encompasses the A(+1) and T(+4) bases, whereas MePyGA produces no effect at all. In contrast, MePyGA strongly stimulates topoisomerase-mediated DNA cleavage at position 2020, a site which has neither an adenine at position +1 nor a thymine at position +4. Unlike what is observed with amsacrine, three cleavage sites which have a purine at position −1 and a pyrimidine at position +5 (sites 4957, 2057, and 4913) are very weakly, if at all, stimulated

Table 3: Sequences of the Cleavage Sites Stimulated by MePyGA and Amsacrine

position[a]	sequence	relative cleavage intensity[b] MePyGA	amsacrine
	-6 ↓ +10		
2020_p	AGATGT**C**TGCCTGTTC	4	2
4912_s	AGAAG**C**AAATACCTCA	4	2
2111_p	AGCTG**C**ATGTGTCAGA	3	1
4916_s	GCAAATACCTCAGTTG	3	4
2143_p	CCGCTTACAGACAAGC	2	2
4918_s	AAATA**CC**TCAGTTGCA	2	0
2162_p	GCTTGT**C**TGCTCCCGG	1	0
2141_p	GCTTA**C**AGACAAGCTG	1	0
2036_p	TCGTGAAGCGATTCAC	1	1
4957_s	TTGATGAGCATATTTT	1	4
2057_p	CGGTAAAGCTCATCAG	0	2
4913_s	GAAGCAAATACCTCAG	0	1

[a] Positions of the drug-stimulated cleavage sites within pBR322 DNA (p) and SV40 DNA (s). [b] 0, 1, 2, 3, and 4 correspond to zero, very weak, weak, medium, and strong cleavage, respectively. Arrows point out the cut which occurs between positions −1 and +1. The cytosine residues in −1 and +1 are printed in bold type.

by MePyGA (Table 3). Because only a limited number of topoisomerase II cleavage sites stimulated by MePyGA have been located at nucleotide resolution, it is not possible to determine rigorously the adjacent base requirement, if any, for MePyGA to stimulate DNA cleavage. However, the data appear sufficient to conclude that in contrast to amsacrine MePyGA does not exhibit a preference for an adenine at the 5′ terminus of the cutting site. It is also noteworthy that all sites (positions 2020, 4912, 2111, 4918, 2162, and 2141) where the stimulation of topoisomerase II-mediated DNA cleavage is more pronounced with MePyGA than with *m*-AMSA have a cytosine at positions −1 or +1, namely a G·C base pair adjacent to the cut. Conversely, all sites (positions 2143, 2036, 4916, 4957, 2057, and 4913) where the stimulation of DNA cleavage is identical or more intense with *m*-AMSA than with MePyGA are devoid of a cytosine residue at positions −1 and +1 (Table 3).

DISCUSSION

DNA-binding agents can inhibit topoisomerase II activity by at least three different mechanisms: (i) by stabilizing the

topoisomerase II–DNA cleavable complex; (ii) by suppressing DNA cleavage by impeding access of topoisomerase II to sequences in DNA selectively recognized by the drug; or (iii) by preventing cleavage as a result of binding to the enzyme so as to inhibit its interaction with DNA (Corbett et al., 1993). The DNA minor groove binders distamycin and Hoechst 33258 (Fesen & Pommier, 1989; Woynarowski et al., 1989a,b), the antitumor agents merbarone (Drake et al., 1989), fostriecin (Boritzki et al., 1988), and certain bis(2,6-dioxopiperazine) derivatives (Tanabe et al., 1991) as well as certain intercalating anthracycline derivatives [e.g., aclarubicin (Jensen et al., 1993)] appear to inhibit topoisomerase II by one or other of the latter mechanisms. It is obvious from the results reported in the present paper that the synthetic compound MePyGA belongs to the first category just as does its parent compound amsacrine. By comparison with previous studies (Bailly et al., 1992a), the mono-*N*-methylpyrrolecarboxamide derivative appears as efficient as its bis-*N*-methylpyrrole carboxamide analogue in stabilizing the cleavable complex. This suggests that the extent of the stimulation of DNA cleavage depends on the nature rather than on the size of the topoisomerase II-binding domain.

Comparing the effects produced by compounds MePyGA and GA, we find that the substituent at position 1′ on the anilino group effectively constitutes a putative topoisomerase II-interacting site. By this token amsacrine and MePyGA possess structurally different topoisomerase II-binding sites, which explains why they behave differently as regards interference with the catalytic activity of the enzyme. Clearly the nature of this substituent at position 1′ is of great significance in determining which topoisomerase II–DNA interactions can take place. Removal of the topoisomerase II-targeted domain of compound MePyGA, which evidently results in the abolition of the topoisomerase II-mediated DNA cleavage, is in agreement with previous studies which showed that anilino-9-aminoacridine (i.e., the core of amsacrine without the methoxy and methanesulfonamide substituents) is practically incapable of inducing topoisomerase II-mediated DNA cleavage *in vitro* (Rowe et al., 1986). But the topoisomerase II-mediated DNA cleavage patterns reported to date with anilinoacridine derivatives were always very similar to those produced by amsacrine (Rowe et al., 1986; Pommier et al., 1987). Here we report the first instance where an anilinoacridine derivative differing only by the nature of the substituent at position 1′ affects the catalytic activity of topoisomerase II quite differently.

It is of particular interest that MePyGA always stimulates the DNA cleavage reaction more strongly than amsacrine when a cytosine is present at position ±1, for the sequencing data suggest that the drug favors cleavage of DNA by topoisomerase II at sites adjacent to a GC base pair. Compare this with the ELD data which clearly reveal that the drug intercalates preferentially into GC-rich sequences. Moreover, when the *N*-methylpyrrole moiety is absent (as in compound GA), the drug does not intercalate preferentially into GC sequences and has no effect on topoisomerase II. Therefore, an attractive explanation for all these results is to propose the following structural model of the drug–DNA complex: while the acridine ring is intercalated into DNA, the appended anilino group (which is oriented at about 70° to the plane of the acridine ring; Neidle et al., 1986) causes the *N*-methylpyrrole residue to project from the surface of the minor groove in such a fashion as to permit interaction between the carboxamide group and the 2-amino group of guanine (the only hydrogen bond donor group exposed in the minor groove). The rest of the pyrrole moiety would protrude outside the double helix so as to interact directly with the enzyme. This conjectural model would account satisfactorily for both the topoisomerase and the ELD data.

At first sight, the preferential intercalation of MePyGA into GC-rich DNA sequences may appear paradoxical since the *N*-methylpyrrole–carboxamide unit generally constitutes a structural element used to design AT-specific minor groove binding drugs (Dervan, 1986; Bailly & Hénichart, 1991). However, the ELD spectrum of the drug–DNA complex clearly indicates that in this case the single *N*-methylpyrrole residue does not reside within the minor groove, otherwise we would expect to detect positive reduced dichroism signals at 320 nm as observed with netropsin–anilinoacridine conjugates (Bailly et al., 1990, 1992a). Both experimental and molecular modeling studies have shown that the bis-pyrrole moiety of the netropsin–anilinoacridine hybrid dominates the binding reaction with DNA by penetrating deeply into the minor groove of AT-rich sequences so as to hinder proper insertion of the acridine ring between base pairs and force it to remain tilted at an angle to the base pair plane (Bailly & Hénichart, 1994). In contrast, although the *N*-methylpyrrole moiety evidently participates in the binding reaction, the binding of MePyGA to DNA appears to be dominated by the acridine chromophore. As a result of the intercalation of the acridine (Figure 3) and the rigid conformation of the connector, the mono-*N*-methylpyrrole residue cannot penetrate into the minor groove. However, we suspect that it lies neatly at the surface of the groove where it can engage in contact with the 2-amino group of guanine. In other words, the predominance of either the groove binding mode or the intercalation process can explain preferential binding to AT- or GC-rich sequences, respectively.

Recently, the cytotoxicity of MePyGA toward L1210 murine leukemia cells was evaluated *in vitro*. IC_{50} values of 0.1, 0.5, and 2.5 μM were measured for amsacrine, MePyGA, and compound GA, respectively. These values correlate with the capacity of each drug to stabilize the topoisomerase II–DNA complex (Figure 4), suggesting that interference with topoisomerase II may be responsible for the biological activity. Preliminary experiments *in vivo* reveal that, unlike its analogue GA which lacks the methylpyrrole residue, MePyGA inhibits the proliferation of leukemia in mice with minimal toxic side effects (manuscript in preparation). Therefore, although the present study is focused on experiments *in vitro*, there is every indication that topoisomerase inhibition dependent upon drug–DNA binding reflects the action of MePyGA against its principal target inside cells.

In conclusion, the study supports the original model of Baguley et al. (1990) in showing that the presence of a substituent at position 1′ of the anilinoacridine chromophore is required to permit the drug to interfere with the catalytic activities of topoisomerase II. The nature of this substituent determines the location of the sites of drug-induced DNA cleavage by the enzyme. On the basis of molecular modeling studies, Macdonald et al. (1991) proposed that the pharmacophore of topoisomerase II-trapping drugs is constituted of (i) a planar ring system, (ii) a substituent ring, and (iii) an additional pendant moiety of *heterogeneous structure*. The studies reported here provide experimental support for these computations and indicate clearly that the nature of the side chain is of prime importance in eliciting the observed poisoning of topoisomerase II. There is no doubt that the substituent at position 1′ on the anilino ring strongly affects the catalytic activity of the enzyme. Accordingly, we may tentatively identify the 1′ substituent as the topoisomerase-

targeted domain first proposed by Baguley et al. (1990). However, it behoves us to keep in mind that so far no evidence exists for direct binding of drugs to topoisomerase II. It is also possible that drugs trap enzyme–DNA cleavable complexes by increasing the energy barrier for the religation rather than by directly binding to the enzyme in the cleavable complex. Recently, Capranico et al. (1994) suggested that steric and electronic features rather than the chemical identity of a particular substituent may be critical in determining the positional sequence specificity of drugs, a view with which we fully agree. Our results also correlate with the model proposed by Zwelling et al. (1992) for amsacrine, and, by analogy, the structure of MePyGA can be divided into two functional domains linked by the anilino group which provides the skeletal structure connecting (i) the tricyclic acridine moiety which represents the DNA-binding domain and (ii) the *N*-methylpyrrole moiety which constitutes the topoisomerase II-targeted domain. The existence of two functional domains in amsacrine and MePyGA also accords with the structure–activity analyses on analogues of anthracyclines (Jensen et al., 1993). A similar topoisomerase II–DNA binding model that divides the drug into two functional domains has likewise been proposed for epipodophyllotoxin derivatives (Chow et al., 1988) and several other structurally different topoisomerase II poisons (Capranico et al., 1994). Thus it may turn out that such a model points to a general principle valid for the majority of topoisomerase II inhibitors. In addition, the present results provide clues to the development of new anilinoacridine derivatives more active against drug-resistant cell lines. Alterations in the protein binding domain of amsacrine can indeed lead to compounds with equal cytotoxicity in sensitive and resistant cell lines (Denny, 1989).

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