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Topoisomerase II-Mediated DNA Cleavage by Amonafide and Its Structural Analogs

YAW-HUEI HSIANG, JACK B. JIANG, and LEROY F. LIU

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 (Y.-H.H., L.F.L.), and Medical Products Department, E. I. DuPont DeNemours and Company, Wilmington, Delaware 19880 (J.B.J.)

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SUMMARY

Treatment of SV40-infected monkey cells with amonafide (benzisoquinolinedione), an intercalative antitumor drug, resulted in rapid accumulation of linearized intracellular SV40 DNA molecules that were protein linked. Studies using purified mammalian DNA topoisomerase II have shown that amonafide and its structural analogs interfere with the breakage-rejoining reaction of the enzyme by stabilizing a reversible enzyme-DNA "cleavable complex." Denaturation of the cleavable complex with sodium dodecyl sulfate resulted in DNA cleavage and the covalent association of topoisomerase II polypeptides with the cleaved DNA. Unwinding measurements indicate that amonafide is a DNA intercalator. These results suggest that amonafide and its structural analogs (e.g., mitonafide) represent a new class of intercalative topoisomerase II-active antitumor drugs. Different from other topoisomerase II-active antitumor drugs, amonafide and mitonafide induce specific DNA cleavage at a single major site on pBR322 DNA. The strong site specificity of amonafide may allow detailed characterization of the intercalator-stabilized, topoisomerase II-DNA cleavable complex.

Mammalian DNA topoisomerase II is proposed to be the principal cellular target for a number of clinically important antitumor drugs, such as anthracyclines (e.g., adriamycin), anthracenediones (e.g., mitoxantrone), acridines (e.g., amsacrine), actinomycins (e.g., actinomycin D), ellipticines (2-methyl-9-hydroxyl-ellipticinium), and epipodophyllotoxins (e.g., VP-16) (reviewed in Refs. 1–6). Despite their apparent structural diversity, these drugs share a common property in interfering with the breakage-rejoining action of topoisomerase II (7–10). They abort the topoisomerase II reaction by trapping a key covalent reaction intermediate, termed the cleavable complex (7–10). Although the chemical structure of the cleavable complex has not been determined, its name is derived from its known reversibility and sensitivity to strong protein denaturants. Exposure of the cleavable complex to a strong protein denaturant (e.g., SDS or alkali) results in DNA strand breaks (both single- and double-strand breaks), with one topoisomerase II polypeptide covalently attached to each of the revealed 5′ ends (7–10). The drug-stabilized cleavable complex can also be readily dissociated following a brief heat (e.g., 65°) or salt (e.g., 0.5 M NaCl) treatment (7–11). Accumulating evidence suggests that drug-stabilized cleavable complexes are primarily responsible for drug cytotoxicity as well as other cellular events such as sister chromatid exchange and chromosomal aberrations (7–10, 12–14).

Amonafide (benzisoquinolinedione), an imide derivative of 3-nitro-1,8-naphthalic acid, has shown considerable cytotoxic and antitumor activity (15–17). It is currently under phase II clinical trials. Early studies have shown that amonafide or its congeners strongly inhibited nucleic acid synthesis at a concentration where protein synthesis is generally unaffected (15). Although inhibition of RNA synthesis was completely reversible upon drug removal, that of DNA synthesis was only partially reversible (15). More recent studies have shown that amonafide induced both DNA strand breaks and protein-DNA cross-links in cultured mammalian cells (17). Furthermore, DNA fragmentation was reversible upon drug removal (17). These results suggest that amonafide may be a topoisomerase-targeting drug. We provide evidence in our present study that amonafide and its structural analogs represent another chemical class of intercalators that target DNA topoisomerase II.

Materials and Methods

Chemicals. *m*-AMSA (NSC 249992), mitonafide (NSC 300288), and amonafide free base (NSC 308847) as well as its hydrochloride salt, were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. YY-186 and YY-187 were synthesized by DuPont (Wilmington, DE). The chemical structures of amonafide and its structural analogs are shown in Fig. 1.

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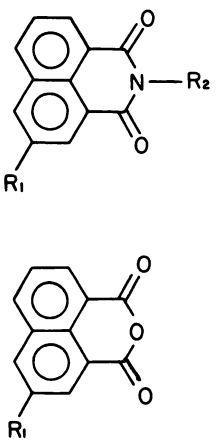
ABBREVIATIONS: SDS, sodium dodecyl sulfate; VM-26, 4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.



1. amonafide ($R_1 = NH_2$, $R_2 = (CH_2)_2N(CH_3)_2$)
2. mitonafide ($R_1 = NO_2$, $R_2 = (CH_2)_2N(CH_3)_2$)
3. YY-186 ($R_1 = NO_2$, $R_2 = H$)

4. YY-187 ($R_1 = NO_2$)

Fig. 1. Chemical structures of amonafide, mitonafide, YY-186, and YY-187.

VM-26 was a gift from Bristol-Myers Co. All drugs except amonafide hydrochloride were dissolved in dimethyl sulfoxide at 10 mM and kept frozen (−70°) in aliquots.

Enzymes and nucleic acids. DNA topoisomerase I and II were purified to homogeneity from calf thymus gland and HeLa cells, using published procedures (18, 19). Dimeric pBR322 DNA was purified by phenol deproteinization of cleared lysates, followed by CsCl/ethidium isopycnic centrifugation and gel filtration through an A-50m column.

Labeling of DNA. *Hind*III-restricted pBR322 DNA was labeled at the 3′ ends using [α-^{32}P]dATP and the large fragment of *Escherichia coli* DNA polymerase I, as described previously (20). The DNA was subsequently digested with *Eco*RI to generate two uniquely end-labeled DNA fragments, 4333 and 31 base pairs in size, respectively.

DNA cleavage assay. Topoisomerase II-mediated DNA cleavage assays were done as described previously (7). Reactions (20 μl) containing 50 mM Tris·HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothreitol, 0.5 mM EDTA, bovine serum albumin (30 μg/ml), 50 ng of end-labeled pBR322 [^{32}P]DNA, and 20 ng of calf thymus DNA topoisomerase II were incubated at 37°. After 30 min, reactions were terminated by the addition of 1 μl of a solution containing 10% SDS and 1.7 mg/ml proteinase K. After an additional 30-min incubation at 50°, the samples were electrophoresed through a 1% agarose gel in TBE (89 mM Tris-borate, pH 8.3, and 2 mM EDTA) buffer. Gels were then dried onto Whatman 3MM chromatography paper and autoradiographed at −80°, using Kodak XAR-5 film and a Dupont lightning plus intensifying screen.

Cells and viruses. Monkey kidney cells (BSC-1) were grown in Eagle's minimum essential medium that was supplemented with 10% heat-inactivated fetal bovine serum. The infection of BSC-1 with SV40 was carried out as described previously (10). Confluent BSC-1 cells were infected with an SV40 viral stock (strain 776) at a multiplicity of infection of 10–30 plaque-forming units/cell. Virus-infected cells were maintained in Eagle's minimum essential medium with 2% fetal bovine serum.

DNA unwinding assay. Reactions (20 μl) containing 50 mM Tris (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml bovine serum albumin, 0.3 μg of supercoiled pBR322 DNA, 7 ng of calf thymus DNA topoisomerase I, and drugs were incubated at 37°. After 30 min, an equal amount (0.3 μg) of supercoiled pBR322 was added, and the reaction was incubated at 37° for another 30 min. The reaction was terminated by the addition of 5 μl of a stop solution (5% sarkosyl, 25% sucrose, 50 mM EDTA, and 0.05 mg/ml bromophenol blue). Electrophoresis (1% agarose gel) was performed at 4° in TBE buffer that was supplemented with 5 mM $MgCl_2$.

Results

Amonafide promotes site-specific DNA cleavage in the presence of topoisomerase II. To investigate whether mammalian DNA topoisomerase II is a target of amonafide, purified calf thymus DNA topoisomerase II and uniquely end-labeled pBR322 DNA were used in an *in vitro* DNA cleavage assay (20). As shown in Fig. 2, amonafide induced a topoisomerase II-mediated DNA cleavage at a major (greater than 80%) site in the pBR322 DNA template (Fig. 2, *lanes J–N*). This cleavage required DNA topoisomerase II (Fig. 2, compare *lanes J* and *P*). DNA cleavage appeared to be slightly reduced at the highest drug concentration, presumably due to drug intercalation (21) (Fig. 2, compare *lanes J* and *K*). In contrast, under identical assay conditions, VM-26 induced extensive DNA cleavage, which was apparently less site specific than that induced by amonafide (Fig. 2, *lanes E–I*). DNA cleavage was rapid and was essentially complete within 5 min (Fig. 3, B).

Amonafide hydrochloride-induced DNA cleavage is reversible. Reversibility is one of the hallmarks of topoisomerase II-mediated DNA cleavage (11). To test whether amonafide-induced DNA cleavage was reversible, drug-stabilized cleavable complexes were briefly heated (65°) or exposed to a higher salt concentration (0.5 M NaCl) before termination with SDS (11, 20). As shown in Fig. 4, both treatments could efficiently reverse amonafide hydrochloride-induced DNA cleavage (Fig. 4A, *lanes E–I* and *lanes J–N*). Heat reversal was especially effective, with 50% reversal in about 30 sec and 95% reversal in 10 min (Fig. 4B). Salt reversal, although relatively less efficient, was still evident (Fig. 4B).

Amonafide hydrochloride induces protein-linked DNA breaks on intracellular SV40 DNA. The ability of amonafide hydrochloride to induce DNA fragmentation and protein-DNA cross-links in cultured mammalian cells suggested that protein-linked DNA breaks might be produced (17). To test this possibility, we examined the effect of amonafide hydrochloride on intracellular SV40 DNA in SV40-infected monkey (BSC-1) cells. As shown in Fig. 5, amonafide hydrochloride and its congener mitonafide induced the accumulation

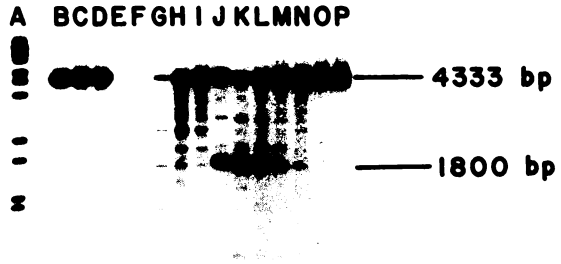


Fig. 2. Amonafide hydrochloride stimulates site-specific topoisomerase II-mediated cleavage of pBR322 DNA. Topoisomerase II-mediated DNA cleavage was done as described in Materials and Methods. *Lane A*, marker DNA with sizes of 8485, 7292, 6369, 5636, 4822, 4324, 3679, 2323, 1929, 1371, 1264, and 702 base pairs, respectively (*top* to *bottom*); *lane B*, DNA only; *lane C*, topoisomerase II added; *lane D*, topoisomerase II and 1% dimethyl sulfoxide (no drug); *lanes E–I*, same as *lane D* except that 125, 25, 5, 1, and 0.2 μM VM-26 was present, respectively; *lanes J–N*, same as *lanes E–I*, respectively, except that amonafide hydrochloride salt was added in place of VM-26; *lane O*, 125 μM VM-26, no topoisomerase II; *lane P*, 125 μM amonafide hydrochloride, no topoisomerase II.

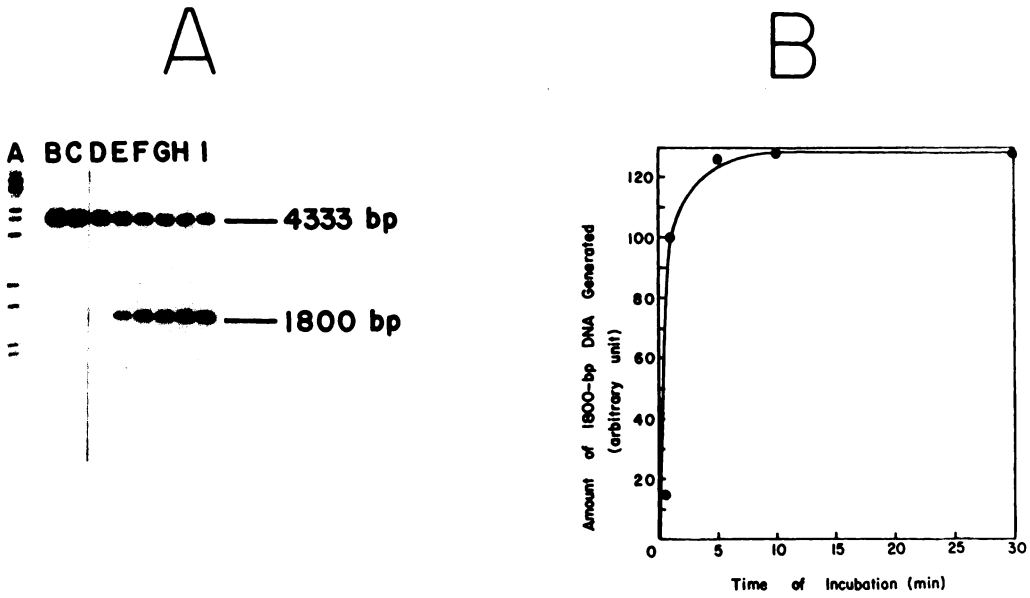


Fig. 3. Rapid induction of topoisomerase II-mediated DNA cleavage by amonafide hydrochloride. A, Time course of topoisomerase II-mediated DNA cleavage. *Lane A*, marker DNA as described in the legend to Fig. 1; *lane B*, DNA only; *lane C*, topoisomerase II and 1% dimethyl sulfoxide (no drug); *lanes D–I*, 25 μM amonafide hydrochloride salt was present in each reaction and the reactions were incubated at 37° for 0.5, 1, 3, 5, 10, and 30 min, respectively. B, The extent of DNA cleavage was quantified by densitometric scanning of the 1800-base pair DNA band. The densitometric scanning was done by scanning the center of each band using a transmittance/reflectance scanning densitometer (Model GS300; Hoeffer Scientific Instruments).

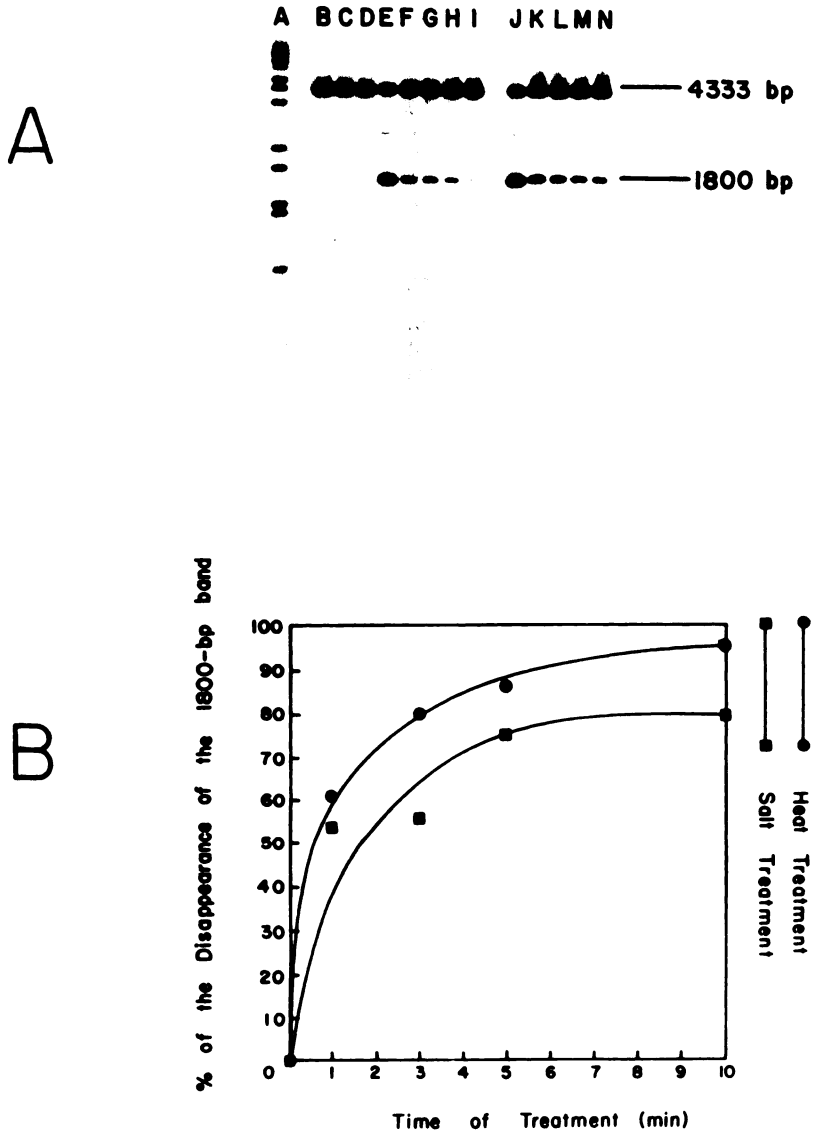


Fig. 4. Rapid reversal of amonafide hydrochloride-induced DNA cleavage following a brief heat or salt treatment. A, Topoisomerase II-mediated DNA cleavage. After a 30-min incubation at 37°, the reaction mixture was either heated to 65° or adjusted to a higher salt concentration (0.5 M NaCl) for specified times before termination with SDS. *Lane A*, marker DNA; *lane B*, DNA only; *lane C*, topoisomerase II added; *lane D*, both topoisomerase II and dimethyl sulfoxide (1%) were present; *lanes E–N*, 25 μM amonafide hydrochloride was present; *lanes E–I*, heat treatment at 65° for 0, 1, 3, 5, and 10 min, respectively; *lanes J–N*, salt (0.5 M NaCl) treatment at 37° for 0, 1, 3, 5, and 10 min, respectively. B, The disappearance of the 1800-base pair band was quantified by densitometric scanning.

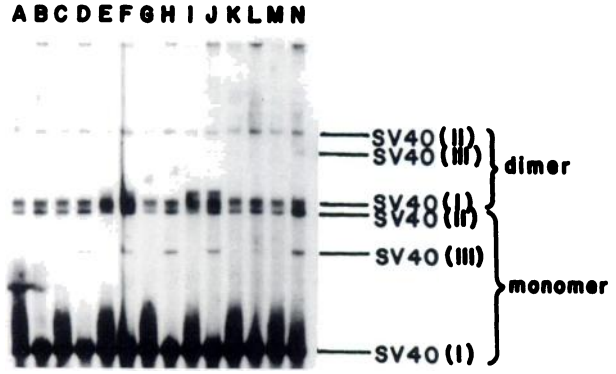


Fig. 5. Amonafide induces protein-linked DNA breaks on intracellular SV40 DNA. The infection of monkey kidney cells (BSC-1) with SV40 virus was carried out as described in Materials and Methods. At 36 hr after infection, viral-infected cells were treated with drugs at 37° for 30 min. Cells were lysed with 1% SDS, 10 mM Tris, pH 8.0, and 20 mM EDTA. *Lanes A*, *C*, *E*, *G*, *I*, *K*, and *M*, cell lysates were extracted with phenol, ethanol precipitated, and electrophoresed in a 0.7% agarose gel. *Lanes B*, *D*, *F*, *H*, *J*, *L*, and *N*, same as *lanes A*, *C*, *E*, *G*, *I*, *K*, and *M*, respectively, except that proteinase K treatment (400 μg/ml at 50° for 3 hr) was done before phenol extraction. *Lanes A* and *B*, control, 1% dimethyl sulfoxide; *lanes C* and *D*, 5 μM amonafide hydrochloride; *lanes E* and *F*, 25 μM amonafide hydrochloride; *lanes G* and *H*, 5 μM mitonafide; *lanes I* and *J*, 25 μM mitonafide; *lanes K* and *L*, 5 μM VM-26; *lanes M* and *N*, 25 μM VM-26.

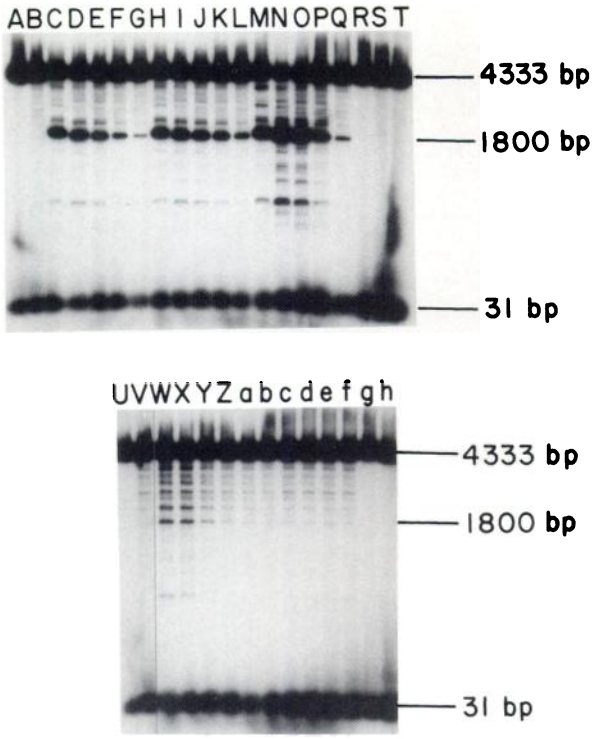


Fig. 6. Topoisomerase II-mediated DNA cleavage by amonafide and its structural analogs. Topoisomerase II-mediated DNA cleavage was done as described in Materials and Methods. *Lanes A* and *U*, control, DNA only; *lanes B* and *V*, topoisomerase II and dimethyl sulfoxide (1%); *lanes C–G*, amonafide hydrochloride was present at 125, 25, 5, 1, and 0.2 μM, respectively; *lanes H–L*, same as *lanes C–G*, respectively, except that amonafide (free base) was added; *lanes M–Q*, same as *lanes C–G*, respectively, except that mitonafide was added; *lanes W–a*, same as *lanes C–G*, respectively, except that YY-186 was added; *lanes b–f*, same as *lanes C–G*, respectively, except that YY-187 was added; *lanes R–T* and *g–h*, no topoisomerase II, 125 μM amonafide hydrochloride, amonafide (free base), mitonafide, YY-186, and YY-187 were added, respectively.

of linearized SV40 DNA (Form III) (Fig. 5, *lanes D*, *F*, *H*, and *J*). The linearized SV40 DNA was protein linked, as evidenced by its specific disappearance after phenol extraction (Fig. 5, *lanes C*, *E*, *G*, and *I*). This finding, in conjunction with those from the DNA cleavage assay in the purified system, suggests that topoisomerase II is responsible for amonafide-induced DNA damage.

R_2 side chain of amonafide is essential for DNA cleavage. It has been shown that, among DNA topoisomerase II poisons from the same chemical class, there is a qualitative correlation between drug-induced DNA cleavage and cytotoxicity (12–14). Because the structure-activity relationships of amonafide derivatives have been studied extensively (16), we tested whether topoisomerase II-mediated DNA cleavage by several amonafide derivatives was also related to their cytotoxicity. Among the four congeners tested (Fig. 1), amonafide and mitonafide, both of which contain two methylene groups in R_2, promoted a significantly greater amount of DNA cleavage than YY-186 or YY-187, which contain no R_2 side chain (Fig. 6, compare *lanes C–Q* and *lanes R–a*). These results are in general agreement with the observation that drug compounds containing two methylene groups in R_2 exhibited the most cytotoxic effect (16).

Correlation between DNA intercalation and topoisomerase II-mediated DNA cleavage. It has been shown in a purified system that DNA intercalation is necessary for topoisomerase II-mediated DNA cleavage among anthracycline congeners.[1] To test whether amonafide and its congeners behave similarly, we carried out a DNA unwinding assay. The unwinding measurement was done in the presence of calf thymus DNA topoisomerase I. To ensure that the plasmid DNA was completely relaxed in the presence of amonafide or its structural analogs, supercoiled plasmid DNA was also added to the reaction mixture that contained topologically relaxed plasmid DNA. The presence of a single Gaussian distribution of topoisomers indicated that the reaction was at its thermodynamic equilibrium, and the linking number changes reflect the equilibrium unwinding angle of amonafide and its structural analogs. As shown in Fig. 7, three of the tested drugs (amonafide, mitonafide, and YY-186) are DNA intercalators (Fig. 7, *lanes C–Q* and *lanes W–a*), and all promoted topoisomerase II-mediated DNA cleavage (Fig. 6, *lanes C–Q* and *W–a*). In contrast, YY-187, the only compound tested that did not intercalate DNA (Fig. 7, *lanes b–f*), was essentially inactive in promoting topoisomerase II-mediated DNA cleavage (Fig. 6, *lanes b–f*). These results lend further support to the hypothesis that DNA intercalation may play a critical role in trapping topoisomerase II-DNA cleavable complexes.

Discussion

Many intercalative antitumor drugs induce protein-associated DNA breaks in cultured mammalian cells and topoisomerase II-mediated DNA strand breaks in a purified system (7–9, 22–24). It has become evident that these drugs interfere with the breakage-reunion reaction of topoisomerase II, resulting in accumulation of topoisomerase II-DNA cleavable complexes and is responsible for the observed "DNA damage" and possibly

[1] A. L. Bodley, L. F. Liu, M. Israel, R. Seshadri, Y. Koeski, F. C. Giuliani, S. Kirschembaum, R. Silber, and M. Potmesil, unpublished results.

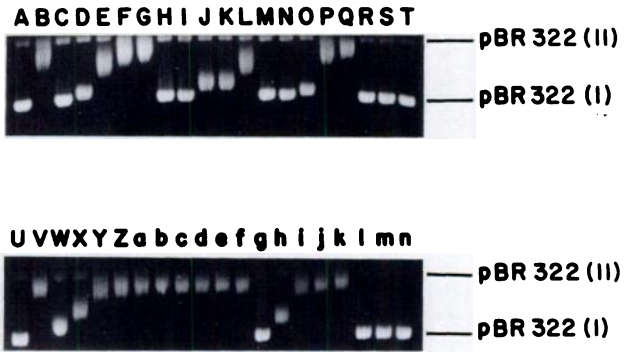


Fig. 7. Unwinding of DNA by amonafide and its structural analogs. DNA unwinding assay was done as described in Materials and Methods. *Lanes A* and *U*, DNA only; *lanes B* and *V*, DNA plus topoisomerase I; *lanes C–G*, amonafide hydrochloride was present at 125, 25, 5, 1, and 0.2 μM, respectively; *lanes H–L*, same as *lanes C–G*, respectively, except that amonafide (free base) was added; *lanes M–Q*, same as *lanes C–G*, respectively, except that mitonafide was added; *lanes W–a*, same as *lanes C–G*, respectively, except that YY-186 was added; *lanes b–f*, same as *lanes C–G*, respectively, except that YY-187 was added; *lanes g–k*, same as *lanes C–G*, respectively, except that *m*-AMSA was added; *lanes R–T* and *l–n*, no topoisomerase I, 125 μM amonafide HCl, amonafide (free base), mitonafide, YY-186, YY-187, and *m*-AMSA were added, respectively.

some of the drug effects such as cytotoxicity and chromosomal aberrations (7–10). Our present results provide strong evidence that amonafide and its structural analogs represent another chemical class of intercalative antitumor drugs that target DNA topoisomerase II.

Our preliminary structure-activity relationship studies of the amonafide analogs have indicated the importance of the R_2 side chain for both the strength of DNA intercalation and the efficiency of topoisomerase II-mediated DNA cleavage. There is a qualitative correlation between the strength of DNA intercalation and the efficiency of topoisomerase II-mediated DNA cleavage. The compounds YY-186 and YY-187, which lack the R_2 side chain, are poor DNA intercalators and apparently are unable to induce extensive topoisomerase II-mediated DNA cleavage in the purified system. The least active compound in terms of topoisomerase II-mediated DNA cleavage and DNA intercalation is YY-187. Under the conditions employed in our assays, YY-187 did not exhibit any detectable DNA unwinding. Neither did YY-187 stimulate topoisomerase II-mediated DNA cleavage to any detectable extent. This result, together with the similar result obtained with the anthracyclines,[1] indicates the importance of DNA intercalation in topoisomerase II-mediated DNA cleavage, at least for intercalative antitumor drugs. However, it is clear from a number of previous studies that DNA intercalation alone is insufficient to determine topoisomerase II-mediated DNA cleavage. Certain structural features of the intercalators are also required for the efficient induction of topoisomerase II-mediated DNA cleavage (7, 8).

Compounds YY-187 and YY-186, which do not have the R_2 side chain, have very similar structures. However, YY-187 does not intercalate DNA under our assay conditions (Fig. 7). YY-186, which differs from YY-187 only at a single position with N substituting for O (see Fig. 1), unwinds DNA significantly, although less so than amonafide or mitonafide. It seems likely that the positive charge at the nitrogen atom is essential for intercalation. The positive charge may stabilize the drug-DNA complex because of the negative charges at the phosphorus atoms in the DNA backbone. The R_2 side chain also enhances drug intercalation, possibly due to the positively charged quaternary nitrogen in R_2. Compounds with the R_2 side chain (amonafide and mitonafide) also appeared to alter the specificity of topoisomerase II-mediated DNA cleavage. The striking cleavage at about nucleotide No.1830 on pBR322 DNA is seen only with amonafide and mitonafide and not with YY-186. One possible explanation is that the R_2 side chain may interact sterically with the enzyme active site when the drug is bound at a particular DNA sequence. The steric interaction may decrease the off-rate of the drug from the enzyme-drug-DNA cleavable complex. The fact that this steric interaction is dependent on the presence of a particular sequence may suggest a tight fit of the drug in the active site of the enzyme.

Regardless of the origin of this striking site specificity of amonafide and mitonafide in the topoisomerase II-mediated DNA cleavage reaction, identification of this unique cleavage site will clearly facilitate biochemical analysis of the putative ternary complex of drug-enzyme-DNA. The high cleavage specificity of amonafide in the presence of topoisomerase II also challenges the validity of using pBR322 DNA for the screening of topoisomerase II-targeting antitumor drugs in the purified system. It is possible that drugs that fail to induce topoisomerase II-mediated cleavage of pBR322 DNA may in fact induce efficient topoisomerase II-mediated DNA cleavage in cultured human cells because of the presence of special sequences in the human genome. Finally, it seems possible to design small molecules that can direct topoisomerase II to cleave DNA at specific sites on the human genome.

Acknowledgments

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Send reprint requests to: Leroy F. Liu, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.