Differential Poisoning of Topoisomerases by Menogaril and Nogalamycin Dictated by the Minor Groove-Binding Nogalose Sugar†

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ABSTRACT: The effect of DNA binding on poisoning of human DNA TOP1 has been studied using a pair of related anthracyclines which differ only by a nogalose sugar ring. We show that the nogalose sugar ring of nogalamycin, which binds to the minor groove of DNA, plays an important role in affecting topoisomerase-specific poisoning. Using purified mammalian topoisomerases, menogaril is shown to poison topoisomerase II but not topoisomerase I. By contrast, nogalamycin poisons topoisomerase I but not topoisomerase II. Consistent with the biochemical studies, CEM/VM-1 cells which express drug-resistant TOP2α are cross-resistant to menogaril but not nogalamycin. The mechanism by which nogalamycin poisons topoisomerase I has been studied by analyzing a major topoisomerase I-mediated DNA cleavage site induced by nogalamycin. This site is mapped to a sequence embedded in an AT-rich region with four scattered GC base pairs (bps) (at −10, −6, +2, and +12 positions). GC bps embedded in AT-rich regions are known to be essential for nogalamycin binding. Surprisingly, DNase I footprinting analysis of nogalamycin−DNA complexes has revealed a drug-free region from −2 to +9 encompassing the major cleavage site. Our results suggest that nogalamycin, in contrast to camptothecin, may stimulate TOP1 cleavage by binding to a site(s) distal to the site of cleavage.

Camptothecins, a new class of anticancer drugs with a broad spectrum of antitumor activity, are known to inhibit the religation step of the breakage/reunion reaction of TOP1 (*1*, *2*). In addition to camptothecins, a number of new TOP1 poisons have been identified (*3*−*14*). Strikingly, many of them are also dual poisons of topoisomerase I and II. For example, saintopin, intoplicine, protoberberines, morpholinyl doxorubicin, actinomycin D, and nitidine are all dual poisons of topoisomerases I and II (*5*, *6*, *11*, *14*−*17*). Most if not all of these dual poisons are also known to bind DNA (*5*, *6*, *11*, *14*−*17*). These findings suggest that drug−DNA interaction may be a common denominator for poisoning of either topoisomerases. In the case of TOP2 posions, there exists a strong correlation between drug intercalation and enzyme poisoning (*18*). However, much less is known about the DNA binding mode responsible for TOP1 poisoning.

Recent studies have demonstrated that a number of DNA minor groove binding ligands such as bi- and terbenzimidazoles are potent TOP1-specific poisons (*3*, *19*). These results tend to indicate that DNA minor groove binding may be important for TOP1 poisoning. However, the bibenzimidazoles Ho33342 and Ho33258 have been shown to unwind DNA, which is indicative of an intercalative mode of DNA binding (*19*, *20*). On the other hand, the X-ray crystallographic structure of a terbenzimidazole−DNA complex has revealed DNA unwinding associated with terbenzimidazole binding to the DNA minor groove (*21*). These results cloud the issue on minor groove-directed interaction in TOP1 poisoning.

To test whether an interaction with the DNA minor groove is important for poisoning TOP1, we have chosen to study a pair of anthracyclines, menogaril and nogalamycin, which differ only by a nogalose sugar ring which is primarily responsible for binding to the DNA minor groove. The anthracylcine antibiotic nogalamycin, isolated from *Streptomyces nogalator*, exhibits both antitumor and antibacterial activities (*22*). Its derivative menogaril, which lacks the nogalose ring, also exhibits antitumor activity (*22*). However, significant differences between the two drugs in their cell cycle specific cytotoxicity and inhibition of DNA and RNA synthesis have been observed (*22*). Nogalamycin is particularly attractive for studying topoisomerase poisoning because the drug structure has been determined by both X-ray crystallography and two dimensional NMR (*23*−*27*). As shown in Figure 1, the aglycon ring of nogalamycin intercalates into DNA base pairs while the nogalose and bicyclic amino sugars are positioned in the minor and major grooves of DNA, respectively. Unlike other DNA minor groove binders, such as Ho33342, distamycin and netropsin, which prefer binding to AT-rich DNA (*28*), nogalamycin prefers binding to GC base pairs (*27*). However, because of the hook-like structure of intercalated nogalamycin, transient local melting of DNA has been proposed to facilitate nogalamycin binding to the GC base (*24*, *29*). Consequently, it has been proposed that nogalamycin prefers to bind GC base pairs embedded in AT-rich region (*24*). In this

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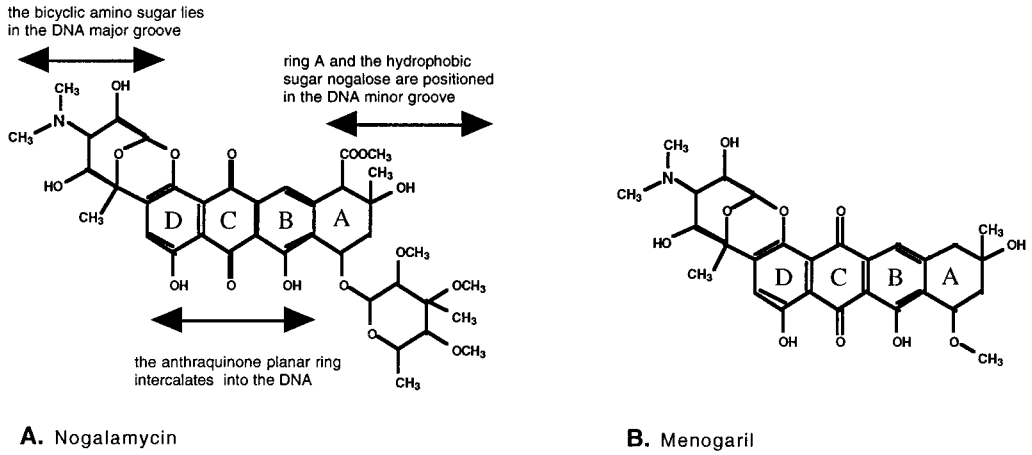

FIGURE 1: Chemical structures of nogalamycin and menogaril. (A) Nogalamycin: the anthraquinone planar ring intercalates into the DNA, with its long axis roughly perpendicular to that of the base pairs. The ring A and the nogalose sugar are positioned in the DNA minor groove. The bicyclic amino sugar lies in the DNA major groove. (B) Menogaril: the derivative of nogalamycin without the nogalose sugar.

communication, we show that the nogalose sugar ring of nogalamycin, which binds to the minor groove of DNA, plays an important role in directing TOP1-specific poisoning. While nogalamycin is a TOP1-specific poison, menogaril, which lacks the nogalose ring, does not poison TOP1 but instead poisons TOP2. The observed switching in poisoning of different topoisomerases by menogaril and nogalamycin suggests the importance of drug interaction with the DNA minor groove in TOP1 poisoning.

MATERIALS AND METHODS

Materials. Nogalamycin and Hoechst 33342 (Ho33342) were purchased from Sigma Chemical Company, while menogaril and *m*-AMSA, camptothecin, and mitoxantrone were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. All drugs were dissolved in DMSO at 10 mg/mL and kept from light at −20 °C. Restriction enzymes, the Klenow fragment of DNA polymerase I, and Vent polymerase were from New England Biolabs, Inc. Wild-type human DNA TOP1 was purified from *Escherichia coli* BL21(DE3) harboring pET1B (*13*).

Mammalian topoisomerases I and II were isolated from calf thymus glands according to published procedures (*30*). All bacterial media were from DIFCO Laboratories (Detroit, MI) and the cell culture media were from Life Technologies, Inc. (Gaithersburg, MD).

Cytotoxicity Assay. IC_{50} was determined by the MTT-microtiter plate tetrazolinium cytotoxicity assay (MTA) as described (*13*). CEM and CEM/VM-1 were kindly provided by Dr. William Beck (Memphis, TN) (*31*). Cells were maintained by regular passage in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (0.1 mg/mL). The cells (2000 cells/well, seeded in 200 μL growth medium) were exposed continuously for 4 days to different drug concentrations, and assayed at the end of the fourth day. Each concentration was repeated at least twice in six replica wells. The results were plotted and the IC_{50} then measured.

Topoisomerase Cleavage Assay. Topoisomerases I and II cleavage assays were performed as previously reported (*1*, *3*). Samples subjected to heat cleavage reversal were incubated at 65 °C for 15 min prior to reaction termination. The plasmid YEpG was linearized by *Bam*HI and ^{32}P labeled at its 3′-ends as described (*3*).

Sequence Determination of Nogalamycin-Stimulated DNA Cleavage Sites. The sequence of a major TOP1 cleavage site band A was determined using a two-step procedure.

(1) Mapping by Agarose Gel Electrophoresis. *Bam*HI-linearized YEpG plasmid was end-labeled at both 3′-ends using Klenow polymerase and [α-^{32}P]dCTP (Amersham), followed by either *Sal*I or *Nhe*I digestion to obtain DNA uniquely labeled at either the upper or the lower strand. These DNA fragments were then reacted with TOP1 in the presence of different concentrations of nogalamycin, and the position of a major cleavage band (band A) was located on the plasmid sequence by comparing with size markers made from the same labeled DNA fragment restricted with various restriction enzymes.

(2) Sequence Determination of the Cleavage Sites. A plasmid region of 1438 base pairs encompassing a major nogalamycin-stimulated cleavage site mapped in YEpG was amplified with Vent polymerase using the primers 5′-CTGACGCTCAGTGGAACG-3′ and 5′-CAACATTAGT-CAACTCCG-3′ and purified with QIAquick PCR purification kit (QIAGEN). The PCR product was then digested with *Eco*RI (an internal site), end-labeled with [α-^{32}P]dATP and Klenow polymerase, and further digested with *Ssp*I at an internal *Ssp*I site to obtain a 191 base pairs of DNA fragment uniquely labeled at one end (the *Eco*R1 end). This 191 bp fragment was then purified away from the other labeled fragments through an 8% nondenaturing polyacrylamide gel and eluted by the "crush and soak" method (*32*). The purified DNA band was reacted with calf thymus TOP1 in the presence of different concentrations of nogalamycin. Aliquots of the reaction products in formamide sequencing buffer were briefly heat-denatured and directly loaded onto a 6% denaturing polyacrylamide gel alongside with the purine ladder (G+A) obtained by the Maxam and Gilbert chemical sequencing reaction (*33*). The gel was dried, autoradiographed, and analyzed to determine nogalamycin-stimulated DNA cleavage sites.

DNase I Footprinting of Nogalamycin−DNA Complexes. DNase I footprinting was carried out in DNase I buffer as described previously (*29*). DNase I was titrated to optimal concentrations for use in the footprinting experiments.

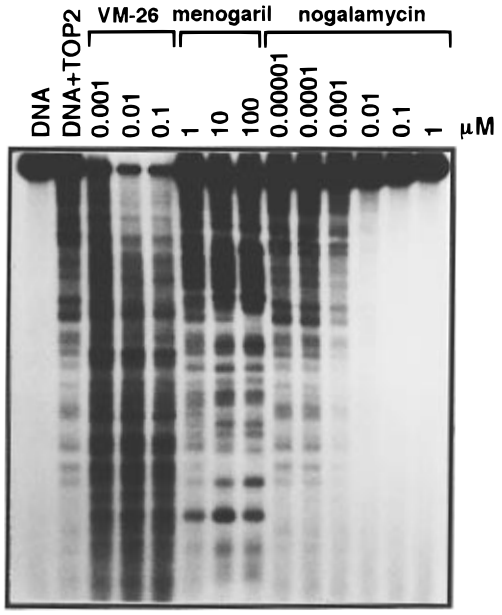

FIGURE 2: Induction of mammalian DNA TOP2-mediated DNA cleavage by nogalamycin and menogaril. TOP2 cleavage assays were performed as described in Materials and Methods. Reactions were terminated by treatment with SDS and proteinase K for 1 h prior to electrophoretic analysis in 1.0% agarose gel with neutral TBE (89 mM Tris borate/8 mM EDTA, pH 8.0) electrophoresis buffer. The drug concentrations were marked above each lane. VM-26 was included as a positive control.

RESULTS

Menogaril, but Not Nogalamycin, Stabilizes TOP2 Cleavable Complexes. We compared the poisoning activity of menogaril and nogalamycin toward mammalian TOP2. As shown in Figure 2, menogaril can induce DNA cleavage in the presence of calf thymus TOP2 in a concentration-dependent manner, consistent with results from the previous report (*34*). The parent compound nogalamycin, however, despite its close structural similarity with menogaril, failed to stimulate TOP2-mediated DNA cleavage in a broad range of concentrations tested. In addition, nogalamycin inhibited TOP2-mediated background DNA cleavage at higher nogalamycin concentrations. This behavior is consistent with the mode and strength of binding of the two compounds to DNA. Menogaril is known to be a weak DNA intercalator (*22*), whereas nogalamycin has a much stronger binding affinity for double-standed DNA (10^6-10^7 M^{-1}). The dose-dependent TOP2−DNA cleavage inhibition observed for nogalamycin in Figure 2 is probably related to its DNA binding properties and is similar to what observed for other strong DNA intercalators (*13*, *18*). Our results clearly indicate that nogalamycin, different from its derivative menogaril, is not a TOP2 poison. Consistent with these results, CEM/VM-1 cells, which are cross-resistant to many TOP2 drugs (*31*) (see resistance ratios for VM-26, *m*-AMSA and mitoxantrone in Table 1), and known to express drug-resistant TOP2 (*35*), are cross-resistant to menogaril but not nogalamycin (Table 1).

Nogalamycin, but Not Menogaril, Stabilizes TOP1 Cleavable Complexes. We tested the ability of the two related drugs, nogalamycin and menogaril, to stimulate DNA cleavage in the presence of purified calf thymus and recombinant human TOP1. As shown in Figure 3, menogaril did not significantly induce TOP1-mediated DNA cleavage in the concentration range tested, consistent with its lack of inhibition of mouse TOP1 catalytic activity (*34*). Quite surprisingly, however, nogalamycin stimulated TOP1-mediated DNA cleavage (Figure 3) in the same concentration range shown to inhibit TOP2-mediated DNA cleavage (see Figure 2 and discussion above). To further characterize the sequences of these cleavage sites, we compared the DNA cleavage pattern induced by the intercalator nogalamycin with that induced by camptothecin, a non-DNA binder whose molecular interactions within the cleavable complex are better characterized (*36*) and to that of the two DNA minor groove binders Ho33342 and distamycin, which poison the mammalian TOP1 at a well-defined consensus sequence (*3*). As shown in Figure 3, the DNA cleavage pattern induced by nogalamycin is different from that induced by either camptothecin or Ho33342. However, some of the cleavage bands induced by nogalamycin appear to be at the same positions as those induced by either camptothecin or Ho33342. DNA cleavage induced by nogalamycin in the presence of TOP1 was shown to represent the cleavable complexes by a heat reversal experiment (Figure 4). Brief heating of the reaction

Table 1: The Atypical Multidrug Resistant CEM/VM-1 Cells Are Cross-Resistant to Menogaril but Not Nogalamycin

	IC_{50} (μM)		
drug/cell lines	CEM	CEM/VM-1	resistance ratio[a]
VM-26	0.04	2.2	55
m-AMSA	0.15	1.8	12
mitoxantrone	0.002	0.06	30
menogaril	0.065	1.2	18
nogalamycin	0.001	0.0008	0.8
camptothecin	0.004	0.005	1.2

[a] Resistance ratio is defined as IC_{50} (CEM/VM-1)/IC_{50}(CEM). IC_{50} is reported as an average from at least two separate determinations.

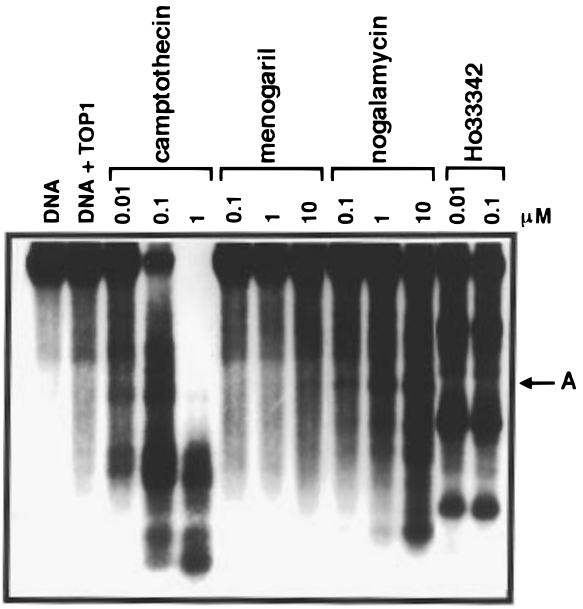

FIGURE 3: Effect of nogalamycin and menogaril on mammalian TOP1-mediated DNA cleavage. TOP1 cleavage assays were performed as described in Materials and Methods. Reactions were terminated by adding SDS and proteinase K. Following 1 h of incubation at 37 °C, the reactions were alkali-denatured prior to loading onto 1.0% agarose gel in neutral TBE electrophoresis buffer. The drug concentration for each reaction was indicated above each lane. Camptothecin was included as a positive control. Calf thymus TOP1 was used in this experiment. Identical results were also obtained using recombinant human DNA TOP1.

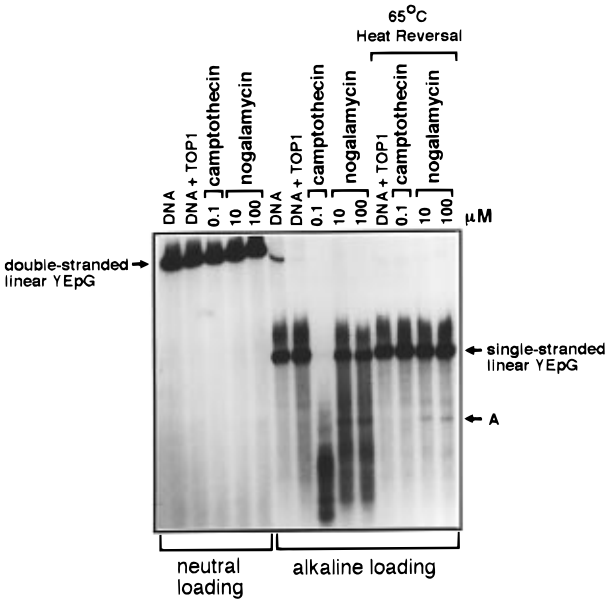

FIGURE 4: Nogalamycin induced reversible single-stranded DNA breaks in the presence of mammalian DNA TOP1. The DNA cleavage reactions were performed as described in Materials and Methods. All reaction products except for those in the first five lanes (indicated as "neutral loading" at the bottom of the gel) were alkali-denatured prior to agarose gel electrophoresis. Samples in lanes labeled 65 °C heat reversal were incubated at 65 °C for 15 min prior to termination of the reactions. The position of one major nogalamycin cleavage site (band A) was marked on the right-hand side of the panel.

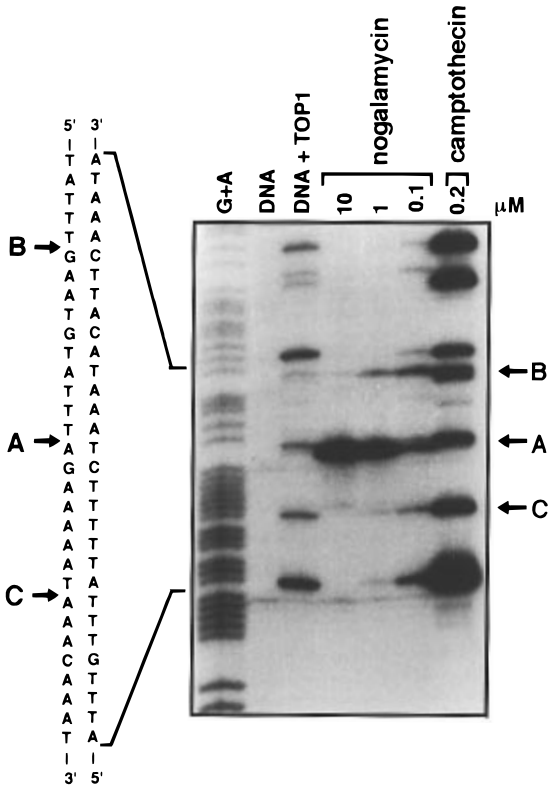

FIGURE 5: Sequencing of cleavage sites induced by nogalamycin in the presence of calf thymus TOP1. The DNA cleavage reactions were performed as described in Materials and Methods on a 191 base pairs PCR amplified fragment of YEpG. The sequence surrounding a major nogalamycin-stimulated cleavage site (band A in Figure 4) was indicated by an arrow. The three marked cleavage bands (band A, band B and band C) represent the three classes of cleavage sites observed in the presence of nogalamycin; Band A: cleavage was stimulated by nogalamycin. Band B: cleavage was stimulated by nogalamycin at low concentrations (0.1 and 1 μM) but inhibited at the highest concentration (10 μM). Band C: cleavage was inhibited at all concentrations of nogalamycin tested.

mixture to 65 °C for 15 min prior to reaction termination with SDS resulted in complete elimination of DNA cleavage induced by camptothecin and partial elimination of DNA cleavage induced by nogalamycin. It is particularly noteworthy that one major DNA cleavage site induced specifically by nogalamycin, labeled band A, remained detectable after brief heating. It is unclear why the reversal of cleavage induced by nogalamycin is incomplete. Nevertheless, one can conclude that at least the majority of the cleavage is due to the formation of reversible cleavable complexes in the presence of nogalamycin and TOP1.

Nogalamycin Can Enhance or Abolish TOP1-Mediated DNA Cleavage at Specific DNA Sequences. On the basis of cleavage mapping using agarose gel, nogalamycin was shown to stimulate DNA cleavage at multiple sites in the plasmid DNA (Figure 3). In order to determine the sequences at the sites of cleavage, we performed sequencing analysis using a 191 bp DNA fragment encompassing the band A cleavage site described above. The 191 bp labeled DNA fragment was reacted with calf thymus TOP1 in the presence of nogalamycin, and the products were analyzed by DNA sequencing. The acrylamide gel showing the DNA region surrounding this nogalamycin cleavage site and its DNA sequence is shown in Figure 5. The DNA sequence is characterized by interspersed GC base pairs embedded in AT-rich regions, a known binding motif for nogalamycin (*37*). A close analysis of the nogalamycin-induced DNA cleavage pattern at the sequence level identified three different types of nogalamycin cleavage sites. The first subset of cleavage sites is represented by band A in Figure 5, which occurs in the sequence 5′-TATTTA-GAAAAATAAA-3′ with the cleavage occurring between the underlined TA step. Nogalamycin strongly stimulated DNA cleavage at this site in a dose-dependent manner. It is interesting to note that DNA cleavage is observed up to 10 μM of nogalamycin, a concentration at which nogalamycin is shown to strongly inhibit TOP2-mediated DNA cleavage (see Figure 2). The second subset of DNA cleavage sites is represented by band B, which is a weak TOP1 cleavage site. Cleavage at band B is stimulated by nogalamycin at low concentrations (0.1 μM), but progressively inhibited at higher nogalamycin concentrations. The third subset of cleavage sites is represented by band C, which is strongly stimulated by TOP1 alone, further enhanced by camptothecin but always inhibited by nogalamycin at concentrations higher than 0.1 μM. This kind of inhibition of TOP1 cleavage reaction is reminiscent of similar inhibition of TOP2 cleavage reactions by strong DNA intercalators (*38*). These sites present the loose specificity found for TOP1 and camptothecin (*39*) since they are characterized by the presence of TA or TG cleavage steps surrounded by A or T runs flanking the cleavage sites.

DNase I Footprinting of Nogalamycin−DNA Complexes. To determine if nogalamycin binds in the vicinity of the major cleavage site (band A), we have footprinted the nogalamycin DNA complexes using the same labeled DNA fragment described above and DNase I. As shown in Figure 6, the footprints of nogalamycin around the major cleavage site were present both upstream and downstream from the site of cleavage (band A) (see the solid line marked to the

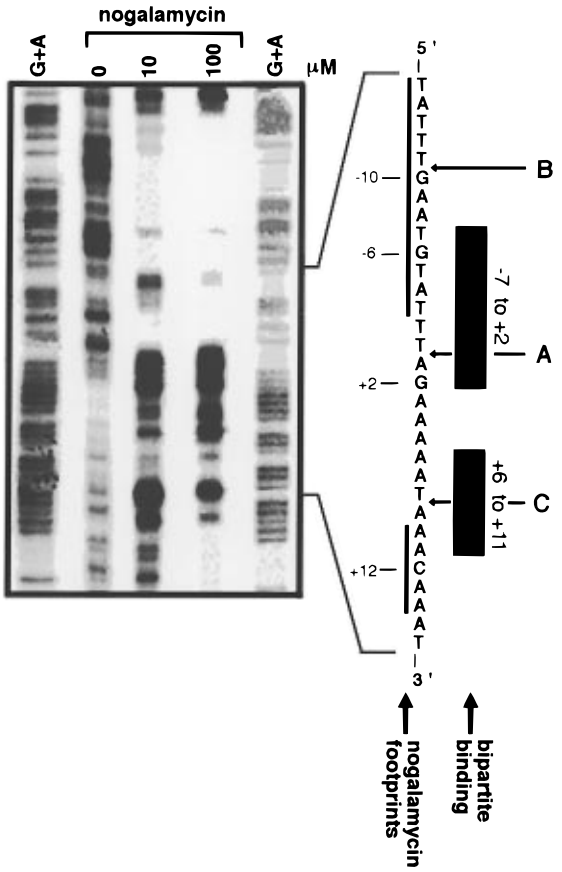

FIGURE 6: DNase I footprinting of nogalamycin DNA complexes. DNase I footprinting was performed as described in Materials and Methods. The two G+A ladders generated by chemical sequencing, shown on either side of the gel, were used as sequencing markers. Nogalamycin concentrations were indicated on top of each lanes (0, 10, and 100 μM, respectively). The DNase I concentration used for samples containing nogalamycin (10 and 100 μM samples) were 10 times higher than the sample without nogalamycin (0 μM). The drug footprints were marked as two solid lines to the left of the sequence. The major cleavage site, band A, and the two minor cleavage sites, bands B and C, were marked to the right of the sequence. The G and C located at -10, -6, $+2$, and $+12$ positions were also marked to the left of the sequence, respectively. The bipartite binding regions (-7 to $+2$ and $+6$ to $+11$) (*56*) were marked by thick bars to the right.

left of the sequence). The downstream footprint (from $+10$ to $+14$) was rather distal to the site of cleavage but still located within the downstream TOP1 binding site (see thick bars marked to the right of the sequence). Surprisingly, the G at the $+2$ position was not footprinted, suggesting the absence of nogalamycin binding immediately downstream from the major site of cleavage (band A).

DISCUSSION

Nogalamycin and menogaril have similar chemical structures, differing only by the nogalose sugar ring. While both drugs have antitumor activity, a number of differences in their cellular activities have been noted. Nogalamycin is specifically lethal to the S-phase cells, while menogaril is cytotoxic to cells in all phases of the cell cycle (reviewed in ref 22). Nogalamycin inhibits RNA synthesis more than DNA synthesis while menogaril inhibits DNA synthesis more than RNA synthesis (*22*). Our current studies, which show differential targeting of the two major DNA topoisomerases by nogalamycin (TOP1 poison) and menogaril (TOP2 poison), may provide an explanation for the differences in their biological activity. First, camptothecin, a prototypical TOP1 poison, has been demonstrated to be exquisitely lethal to S-phase cells (*40*, *41*). A collision between the replication fork and the TOP1 cleavable complexes has been proposed to explain the S-phase-specific cytotoxicity of camptothecin (*42*−*44*). Consequently, the S-phase specific cytotoxicity is expected for all TOP1 poisons including nogalamycin. On the other hand, TOP2 poisons, such as etoposide and *m*-AMSA, are known to kill cells in all phases of the cell cycle, especially at higher drug concentrations (*41*). The lack of cell cycle specific cytotoxicity of menogaril is thus consistent with its being a TOP2 poison. Second, camptothecin is known to be a potent reversible inhibitor of RNA synthesis (*45*). Since TOP1 is primarily localized within the transcribed region and the TOP1 cleavable complexes are known to block elongating RNA polymerases (*46*), the strong inhibitory effect of nogalamycin on transcription could be explained. However, nogalamycin, being a strong DNA binder, may also inhibit transcription nonspecifically by blocking the DNA template. Menogaril, like etoposide, is a TOP2 poison. There is evidence that etoposide strongly inhibits DNA synthesis (*47*). This is consistent with the role of TOP2 in DNA replication and chromosome segregation (*48*, *49*). Our studies with CEM/VM-1 cells, which have demonstrated differential cross-resistance of CEM/VM-1 to menogaril but not nogalamycin, also support the differential poisoning of the two major topoisomerases in cells. The cross-resistance results for menogaril suggest strongly that the major cytotoxic target of menogaril is TOP2α. However, it is less certain whether the major cytotoxic target of nogalamycin is TOP1.

Menogaril stimulates TOP2-mediated DNA cleavage. Careful inspection of Figure 2 has revealed that some of the background cleavage sites are suppressed while others are enhanced by menogaril. This characteristic is consistent with the fact that menogaril is a DNA intercalator. Menogaril binding to DNA may stimulate or inhibit TOP2 cleavage depending on the sequence context surrounding the site of cleavage. By contrast, nogalamycin can only inhibit TOP2-mediated DNA cleavage. The dramatic inhibitory effect of nogalamycin on TOP2-mediated DNA cleavage at concentrations higher than 0.01 μM is consistent with its higher DNA affinity. The lack of a stimulatory effect of nogalamycin on TOP2-mediated DNA cleavage could be due to steric interference of the minor groove binding nogalose sugar with the TOP2 ternary complex.

There appears to be several types of TOP1-mediated DNA cleavage sites which are differentially affected by nogalamycin. The strength of cleavage at some sites is strongly stimulated by nogalamycin (e.g., the site represented by the major cleavage site, band A) while that of others is either weakly stimulated or reduced by nogalamycin. The molecular basis for this differential effect is unclear. One possibility is that the precise location of the drug binding site relative to the site of DNA cleavage may be important in determining the effect of the drug on TOP1-mediated DNA cleavage. TOP1 is known to interact with DNA at regions flanking the site of cleavage (*50*). Drug binding immediately upstream or downstream of the cleavage site may affect the cleavage/religation reaction differentially. The major TOP1-mediated DNA cleavage site (band A) in the presence of nogalamycin occurs in a sequence 5′-TTAGAAAAAT-3′

(cleavage occurs between the underlined TA), which is identical to a known TOP1 binding and cleavage hotspot in rDNA from *Tetrahymena* (*51*). Previous studies of the TOP1-mediated DNA cleavage sites in the presence of Ho33342 have suggested a cleavage consensus sequence of 5′-TCATTTTT-(AT-rich)-3′ where cleavage occurs between the underlined TC sequence (*3*). On the basis of the preference of binding by Ho33342, it has been proposed that Ho33342 binds to the DNA minor groove at the TTTTT sequence and somehow interferes with the TOP1 cleavage/religation cycle (*52*). By comparing the two cleavage sites and knowing the preference of nogalamycin binding to GC base pairs embedded in AT-rich region, we speculated that nogalamycin might intercalate between the AG sequence (G at the +2 position downstream from the site of cleavage) near the site of DNA cleavage. However, our footprinting data show clearly that this region (−2 to +9) is drug free even at very high nogalamycin concentrations (Figure 6). On the other hand, there are prominent footprints present both upstream and downstream of the major cleavage site. Since the closest GC bp is 6 bp upstream (the downstream nogalamycin binding site is 12 bp away) from the site of cleavage, nogalamycin must have exerted its cleavage stimulating effect from a distance. The closest downstream nogalamycin binding site is located between +10 and +14 (note that the C at the +12 position favors nogalamycin binding). Since the DNase I footprint of nogalamycin is known to be 5−6 bp in length (*29*), the +10 to +14 footprint could represent a single nogalamycin binding site. While the putative upstream drug binding site (see G at −6 in Figure 6) is overlapping with the critical TOP1−DNA interaction site (−7 to +2) (*53*), this downstream drug binding site (see G at +12 in Figure 6) is only slightly overlapping with the less critical TOP1−DNA interaction site (+6 to +11). At present, it is not clear which distal nogalamycin binding site (see Gs at −10 and −6, and C at +12 in Figure 6) is responsible for TOP1 poisoning. Mutational analysis to systematically replace the GC base pairs (at positions −10, −6, and +12) with AT base pairs is underway to assess the potential role of distal drug binding in TOP1 poisoning. It should be noted, however, that studies with the TOP1 poison, camptothecin and TOP2 poisons, adriamycin, *m*-AMSA, and etoposide have all led to the suggestion that the drug binds in the vicinity of the site of cleavage (within 1 bp from the cleavage site) (*39*, *54*−*56*). On the other hand, UV-damaged DNA can induce TOP1-mediated DNA cleavage from a distance as far as 10 bp away from the site of cleavage (*57*).

The importance of the nogalose in directing specific poisoning of the two major topoisomerases is rather striking. However, a number of dual poisons of topoisomerases I and II exhibit a similar dual mode of binding. For example, actinomycin D, which is a dual poison of both topoisomerases I and II, is an intercalator with its phenoxazone ring inserted between the GC base pairs, while the pentapeptide ring is interacting with the minor groove of DNA through hydrogen bonding with the bases (*58*, *59*). Morpholinyl doxorubicin, another dual poison, also has a similar dual mode of binding (*16*). Because of this unusual dual mode of DNA binding, it is difficult to evaluate the precise structural perturbation that is necessary for TOP1 poisoning.

The importance of minor groove binding in TOP1 poisoning is perhaps best exemplified by the bi- and terbenzimidazoles (*3*) whose primary mode of DNA binding is believed to be through an interaction with the minor groove (*20*, *21*, *60*). While it is tempting to conclude that it is the binding to the DNA minor groove that is important for poisoning of TOP1, previous studies have demonstrated that the bibenzimidazole Ho33342 can unwind DNA which is characteristic of intercalation (*19*, *20*). On the basis of studies of a number of bi- and terbenzimidazoles, we found no correlation between unwinding and TOP1 poisoning (unpublished results). In addition, the crystallographic study has also revealed unwinding of the duplex DNA in the terbenzimidazole complex (*21*). It is therefore possible that unwinding observed for Ho33342 may be the consequence of drug binding to the DNA minor groove rather than an unknown second mode of drug binding. Further studies are necessary to establish the critical mode of DNA binding and its associated structural changes in selective poisoning of mammalian DNA TOP1.

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