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Menogaril, an Anthracycline Derivative, Inhibits DNA Topoisomerase II by Stabilizing Cleavable Complexes

Katsuhiro Ono,[1,3] Yoji Ikegami,[1] Miwako Nishizawa[2] and Toshiwo Andoh[2,4]

[1]*Meiji College of Pharmacy, Department of Hygienic Chemistry, 1-22-1 Yato-cho, Tanashi, Tokyo 188 and* [2]*Laboratory of Biochemistry, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464*

Menogaril, an anthracycline derivative, has been shown to possess antitumor activity in experimental animal systems, and is now under phase II clinical studies. However, its mechanism of action has not been elucidated. We have found that it inhibits the decatenation activity of purified DNA topoisomerase II using kinetoplast DNA from *Crithidia fasciculata*, its IC_{50} being 10 μM, which is comparable to that of etoposide. It does not, however, inhibit topoisomerase I activity at concentrations of up to 400 μM. Binding of topoisomerase II with DNA is not affected, but cleavable complex formation is stimulated by the drug. Cleavage site specificity differes from that of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide. Menogaril was shown to possess a weak double-helix unwinding activity. These findings allow us to classify menogaril as a cleavable complex-stabilizing topoisomerase II inhibitor.

Key words: Menogaril — Anthracycline — Topoisomerase II — Cleavable complex — DNA unwinding activity

DNA topoisomerase II catalyzes an alteration of topological state of DNA by transiently breaking and rejoining of the double strands of DNA, and thereby plays an important role in various aspects of genetic processes.[1-3]

It is now well established that many DNA intercalating agents (acridines, ellipticines and anthracyclines) and non-intercalating agents (epipodophyllotoxins) interact with mammalian topoisomerase II by trapping the enzyme in a covalently linked intermediary complex with DNA, named cleavable complex. Treatment of the complex with strong denaturants such as SDS[5] leads to strand breakage of DNA.[4]

Adriamycin and its congeners are typical anthracyclines exhibiting a wide spectrum of antitumor activity.[5,6] Menogaril (7-con-O-methylnogarol, TUT-7) is an anthracycline derivative which shows a significant antitumor activity against murine tumors *in vivo* and various human tumor cells *in vitro*.[7] Menogaril was reported to have low binding affinity to DNA[8] and to accumulate predominantly in the cytoplasm.[9,10] Wierzba *et al.* found that its cytotoxicity was well correlated with the inhibition of macromolecular synthesis.[11] Despite a low binding affinity of menogaril to DNA a pronounced DNA cleavage was induced in treated cells.[11] In order to determine the mechanism of action we attempted to determine its action on DNA topoisomerases. Results show that menogaril inhibits DNA topoisomerase II and leads to cleavable complex formation with DNA. Menogaril, however, did not affect topoisomerase I activity.

MATERIALS AND METHODS

Chemicals The following chemicals were obtained from the companies in parentheses: menogaril (Taiho Pharmaceutical Co., Ltd., Tokyo), etoposide (VP-16, Bristol-Myers Co., Wallinford, CT), amsacrine (m-AMSA, National Cancer Institute, NIH, Bethesda, MD) and camptothecin (Yakult Honsha Co., Ltd., Tokyo). All drugs were dissolved in dimethylsulfoxide and diluted with appropriate buffer solution before use.

Topoisomerases Topoisomerase I was purified from mouse Ehrlich ascites tumor cells and the enzymatic activity was measured as described by Ishii *et al.*[12] Topoisomerase II was purified from calf thymus essentially according to the procedure described by Halligan *et al.*[13] Catalytic activity was measured in terms of the ATP-dependent decatenation of kinetoplast DNA (k-DNA) as described previously.[14,15]

Binding of topoisomerase II with DNA Nitrocellulose filter binding assay of topoisomerase II complexed with DNA was performed according to McConaughy *et al.*[16]

[3] Present address: Medical Development Department, Taiho Pharmaceutical Co., Ltd., 1–27 Kanda-Nishiki-cho, Chiyoda-ku, Tokyo 101.

[4] To whom correspondence should be addressed.

[5] Abbreviations: SDS, sodium dodecyl sulfate; m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; VP-16, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside); k-DNA, kinetoplast DNA from *Crithidia fasciculata;* IC_{50}, drug concentration inhibiting the activity of the enzyme by 50%.

ColEl DNA was linearized by digestion with *Eco*RI and labeled at its 3′ end with large fragment of *E. coli* DNA polymerase I and [α-^{32}P]dATP. Five ng of 3′ end-labeled linearized colEl DNA was incubated at 0°C for 10 min with various concentrations of topoisomerase II in 40 μl of binding buffer in the presence or absence of drugs. The reaction mixture was diluted 10 times with the binding buffer and filtered through a nitrocellulose filter (Schleicher & Schuell, Dassel, Germany). Trapped radioactivity was measured in a liquid scintillation spectrometer and expressed as ng DNA per filter.

Topoisomerase II-mediated DNA cleavage Assay of cleavable complex formation was performed essentially as described by Liu *et al.*[17] Three ng of 3′ end-labeled linear colEl DNA was incubated at 37°C for 30 min with topoisomerase II in the presence or absence of drugs in 20 μl of reaction buffer. The reaction was stopped by addition of SDS and EDTA. Cleavable complex was precipitated by KCl, washed, solubilized in water and analyzed for radioactivity in a liquid scintillation spectrometer. For cleavage site specificity analysis the cleavable complex trapped by SDS was treated with proteinase K, 0.2 mg/ml, for 1 h at 37°C. Reaction products were analyzed by electrophoresis using a 1% agarose gel in a buffer containing 0.089 *M* tris-borate and 2 m*M* EDTA, pH 8.3. The cleavage pattern was visualized by autoradiography.

DNA unwinding activity assay DNA unwinding activity of drugs was estimated as described by Hsiang *et al.*[18] ColEl DNA was linearized with *Eco*RI restriction endonuclease and then ligated in a reaction mixture (20 μl) with T4 DNA ligase in the presence of 1 m*M* ATP and various concentrations of drugs. The reaction was terminated by the addition of 5 μl of a stop solution (5% sarkosyl, 25% sucrose, 50 m*M* EDTA and 0.05 mg/ml bromphenol blue). Gel electrophoresis was performed in a cold room (4°C) using 1.0% agarose gel and TBE electrophoresis buffer supplemented with 5 m*M* $MgCl_2$.

RESULTS

Menogaril inhibits catalytic activity of topoisomerase II, but not that of topoisomerase I Decatenation of k-DNA is a specific assay for topoisomerase II.[15] In agarose gel electrophoresis, k-DNA stays at the origin (Fig. 1, lane 1) and the decatenated monomer circle produced in the reaction migrates into the gel. As shown in Fig. 1 lanes 1 and 2, complete reaction converted all the substrate into monomer circles. As the concentration of menogaril was increased, the decatenation reaction was inhibited (Fig. 1, lanes 3–6), half-maximum inhibition (IC_{50}) being at 10 μM. m-AMSA and VP-16 used as positive controls at 5 and 20 μM, respectively, completely inhibited the reaction (Fig. 1, lanes 7 and 8), the IC_{50}s being 2 and 10 μM, respectively. Thus, the potency of menogaril in topoisomerase II inhibition is less than that of m-AMSA and equivalent to that of VP-16. Relaxation of supercoiled colEl DNA by topoisomerase I, however, did not seem to be inhibited by the drug, as shown in Fig. 2, although a bizarre profile was obtained at a high concentration range of the drug (lanes 3–5). This could

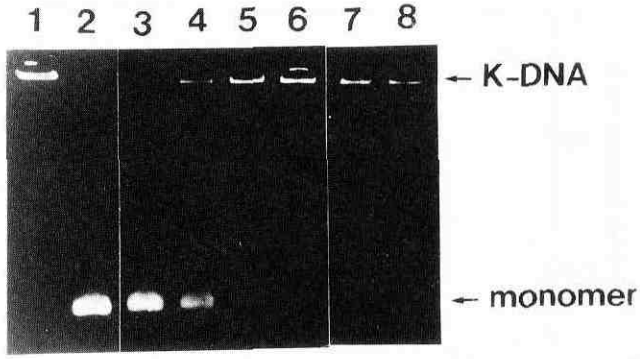

Fig. 1. Inhibition of decatenation of k-DNA by monogaril. Activity of topoisomerase II (1 ng) was assayed by decatenation of k-DNA (150 ng) as described in "Materials and Methods" in the presence of 0, 5, 10, 20 and 30 μM menogaril for lanes 2 to 6, respectively. m-AMSA at 5 μM (lane 7) and VP-16 at 20 μM (lane 8) were used as positive controls. No enzyme was added to the reaction mixture in lane 1. Positions of k-DNA and monomer circles are shown by arrows.

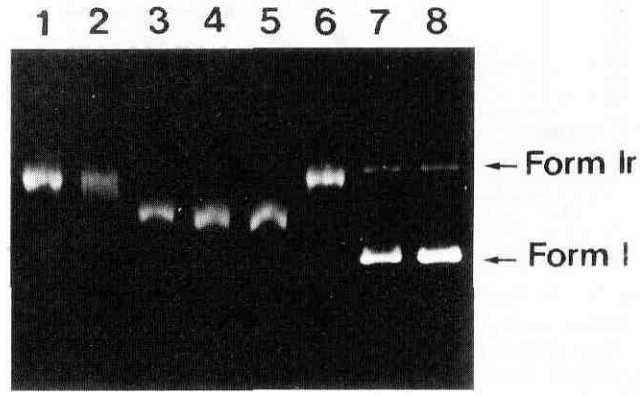

Fig. 2. Inhibility of menogaril to inhibit relaxation of colEl DNA by topoisomerase I. The activity was assayed as described in "Materials and Methods" in the presence of 0 (lanes 1 and 6), 100 (lane 2), 200 (lane 3), 300 (lane 4) and 400 μM (lane 5) monogaril and analyzed by agarose gel electrophoresis. Camptothecin at 10 μM was used as a positive control (lane 7). No enzyme was added to the reaction mixture in lane 8. Form I, supercoiled closed circular DNA; Form Ir, relaxed closed circular DNA.

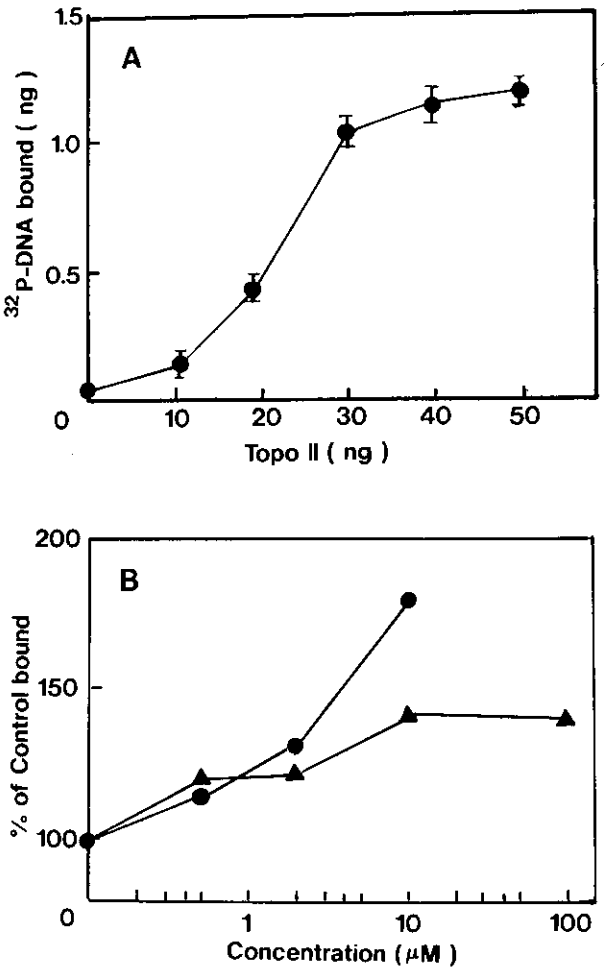

Fig. 3. A. Nitrocellulose filter binding of topoisomerase-DNA complex as a function of topoisomerase II concentration. 3′ End-labeled [^{32}P]colEl DNA (5 ng) was incubated with increasing amounts of topoisomerase II and filtered under the conditions described in "Materials and Methods." B. Effect of menogaril on the binding of topoisomerase II to DNA. Binding of the enzyme (40 ng) to DNA in the presence of increasing concentrations of menogaril (▲) or m-AMSA (●) was estimated as described in part A and in "Materials and Methods."

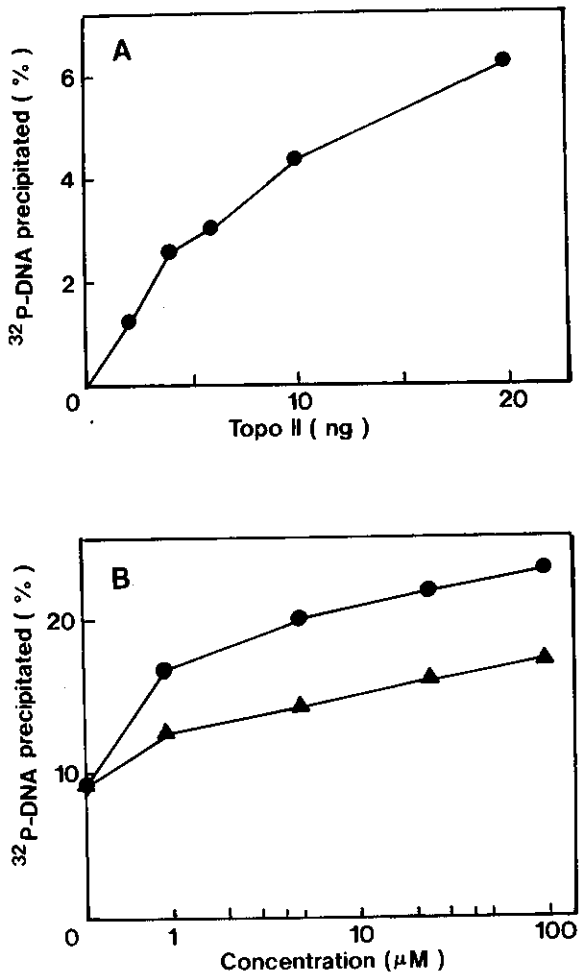

Fig. 4. A. Topoisomerase II-DNA cleavable complex formation as a function of the enzyme concentration. 3′ End-labeled [^{32}P]colEl DNA was incubated with uincreasing amounts of topoisomerase II and covalently linked enzyme-DNA complex was precipitated with SDS-potassium as described in "Materials and Methods." B. Effects of menogaril (▲) and m-AMSA (●) on the formation of topoisomerase II-DNA cleavable complex under the conditions described in part A and in "Materials and Methods." Forty ng of the enzyme was used.

have resulted from its moderate unwinding activity, as will be discussed later in more detail (Fig. 6).

Menogaril stimulates the binding of topoisomerase II to DNA Topoisomerase II initiates the reaction by binding to the substrate DNA, followed by cleavage to form the covalently linked cleavable complex. By simple filtration through nitrocellulose membrane, the two types of complexes, noncovalently and covalently linked, are trapped and thus the total amount of complexes can be estimated. Fig. 3A shows the complex formation between ^{32}P-labeled DNA and topoisomerase II as a function of the amount of the enzyme. The amount of complexes bound to the filter in the presence of menogaril increased up to 10 μM and plateaued at higher concentrations (Fig. 3B). The complex formation seemed to be more efficient with m-AMSA. These results clearly indicate that menogaril does not inhibit the binding of the enzyme to DNA, but rather stimulates the binding.

Menogaril stabilizes the cleavable complex Most of the topoisomerase II-targeting antitumor agents stabilize

the cleavable complex, leading to its accumulation.[4] Menogaril also appeared to fall into this category (Fig. 4). Cleavable complex is formed as a function of the amount of topoisomerase II (Fig. 4A). The amount of the cleavable complex increased as the concentration of menogaril was increased, indicating that the drug stabilizes the complex, although the extent of stimulation seemed to be less than that of m-AMSA (Fig. 4B).

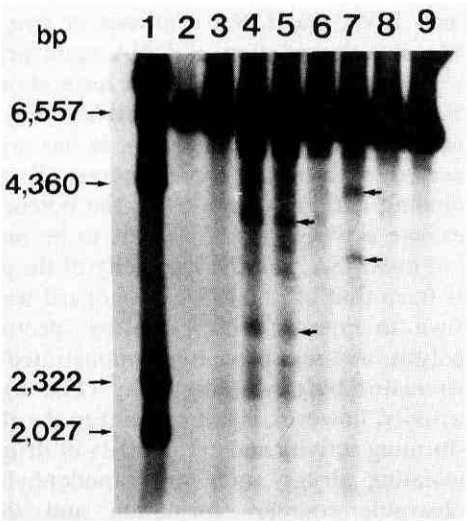

Fig. 5. Drug-stimulated topoisomerase II-mediated DNA cleavage. Five ng of 3′ end-labeled [^{32}P]colEl DNA was incubated with 40 ng of topoisomerase II, and the cleavable complex formed was digested with proteinase K in the presence of SDS, then subjected to electorophoresis followed by autoradiography. Lane 1, molecular weight markers; lane 2, no enzyme and no drug; lane 3 enzyme only; lanes 4–6, enzyme, DNA and m-AMSA at 100, 10 and 1 μM, respectively; lanes 7–9, same as lanes 4–6 except that menogaril was used at 100, 10 and 1 μM, respectively.

Sequence preference of cleavage of menogaril differs from that of m-AMSA Topoisomerase II produced minimal DNA cleavage in the absence of drug (Fig. 5, lane 3). As reported previously,[19] m-AMSA produced topoisomerase II-mediated cleavage at multiple specific sites within colEl DNA (Fig. 5, lanes 4–6), as indicated by arrows. With increasing concentrations menogaril induced cleavage at different sites from those induced by m-AMSA (Fig. 5, lanes 7–9), two prominant sites being indicated by arrows in the figure. The cleavage-inducing potency of menogaril was less than that of m-AMSA.

Menogaril has a weak unwinding activity Adriamycin and its congeners are strong intercalators and their intercalating activity was correlated with their inhibitory activity towards topoisomerase II and hence with their cytotoxicity.[20] A DNA intercalator inserts itself between the stacked bases of DNA, and thus unwinds the double helix of DNA. We performed an unwinding assay of menogaril as described in "Materials and Methods." In principle, a linear DNA molecule is unwound when interacted with an intercalator. When the linear underwound molecules are sealed by ligase, followed by removal of the drug, the DNA molecules assume negative supercoils. As an anthracycline derivative, menogaril is expected to have at least weak DNA-unwinding activity (Fig. 6). Starting with linear DNA, supercoiled circular DNA was formed with menogaril at concentrations of

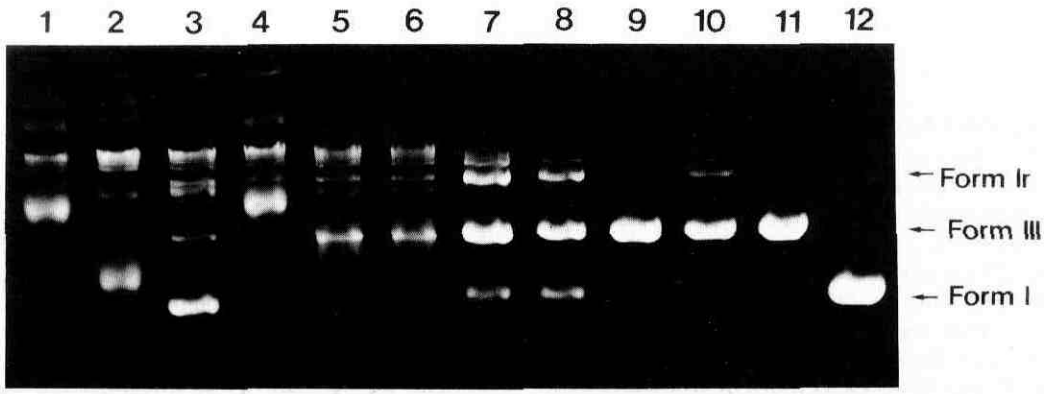

Fig. 6. Unwinding activity of menogaril. *Eco*RI-digested linear colE1 DNA (1.0 μg) (Form III) was incubated with T4 ligase in the presence of m-AMSA at 0 (lane 1), 100 (lane 2) and 300 μM (lane 3), or menogaril at 50 (lane 4), 100 (lane 5), 200 (lane 6), 300 (lane 7) and 400 μM (lane 8). The linear colE1 DNA (1.0 μg) was incubated with drugs alone, m-AMSA at 300 μM (lane 10), or menogaril at 400 μM (lane 11). Reaction mixtures were extracted with phenol and the product DNA was precipitated with ethanol, then dissolved in TE buffer. Finally, 0.5 μg equivalent of the product DNA was analyzed by agarose gel electrophoresis. In lanes 9 and 12 linear and supercoiled closed circular colE1 DNAs, respectively, were run as reference markers.

300 μM and higher (Fig. 6, lanes 7, 8), indicating that menogaril possesses unwinding, i.e. intercalating activity. However, the activity appeared to be much weaker than that of m-AMSA (Fig. 6, lanes 1–3). This was confirmed by another method[21] in which relaxed closed circular DNA was converted to negatively supercoiled DNA in the presence of menogaril and topoisomerase I (data not shown). However, this intercalating activity may not have any relevance to its topoisomerase II-inhibiting activity, since non-intercalating drugs such as epipodophyllotoxins are strong topoisomerase II inhibitors, and strong intercalators such as ethidium bromide are not specific topoisomerase II inhibitors.

DISCUSSION

It has been demonstrated that a number of antitumor drugs affect the breakage-reunion reaction of mammalian DNA topoisomerase II by stabilizing the covalently linked DNA-protein complex, called cleavable complex, which produces DNA breaks upon treatment with strong protein denaturants such as SDS.[4] Bodley *et al.* studied the structure-activity relationship of doxorubicin and daunorubicin congeners.[20] They have shown that there is a positive correlation between DNA intercalation and cytotoxicity with various congeners. However, N-substitution of the daunosamine moiety of anthracyclines tended to inhibit their activity of topoisomerase II-mediated cleavage of DNA.

Menogaril is a novel derivative of anthracyclines devoid of daunosamine and with bulky substitution at C-1 and C-2 of ring D.[11] It shows significant antitumor activity against murine tumors in experimental animals and human tumors in clonogenic assays.[7] Menogaril is thus presumed to exert its cytotoxic effect through interaction with DNA topoisomerase II, forming a cleavable complex. Menogaril was shown to be predominantly localized in cytoplasm of treated cells in contrast with adriamycin, which is localized primarily in the nucleus.[9, 10] It was shown to inhibit tubulin polymerization *in vitro*, and RNA and DNA synthesis *in vivo*, and to induce extensive degradation of DNA upon prolonged exposure.[11] In the present study we have shown that menogaril inhibits topoisomerase II activity (Fig. 1) and stabilizes cleavable complex (Fig. 4B), as has previously been observed with other topoisomerase II-targeting drugs including anthracyclines.[4] But the potency of its DNA cleavage activity (Fig. 5) seems to be much less than that of m-AMSA, and the specificity of the cleavage site differs from that of m-AMSA. Menogaril was previously shown to interact with DNA by spectrophotometric analysis, and was presently demonstrated to be a weak intercalator by unwinding assay (Fig. 6). Intercalating activity, however, is not relevant to the cleavable complex-forming activity and cytotoxicity of drugs, since non-intercalating drugs such as epipodophyllotoxins induce cleavable complex formation and thus are cytotoxic, whereas a strong intercalator such as ethidium bromide does not induce cleavable complex and is not cytotoxic.

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