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Menogaril, an Anthracycline Compound with a Novel Mechanism of Action: Cellular Pharmacology

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Menogaril, an anthracycline compound possessing a significant antitumor activity after both po and iv administration, has been introduced into clinical trials. However, its mechanism of action has not been clarified yet. This study revealed that its cytotoxicity correlated very well with the inhibition of macromolecular synthesis, indicating the involvement of interaction with DNA. The spectrophotometric study showed a weaker binding of this compound to calf thymus DNA when compared to that of doxorubicin (adriamycin). Despite the lower binding affinity of menogaril to DNA, pronounced DNA cleavage was observed in an intact cell system, indicating that the character of the interaction with DNA is different from intercalation. In contrast to doxorubicin, menogaril is extensively localized in the cytoplasm. The cytoplasmic localization prompted us to study its effect on cytoskeleton proteins. It was found that menogaril inhibited the initial polymerization rate of tubulin, indicating a possible contribution of this process to the overall cytotoxicity of menogaril.

Key words: Menogaril — Interaction with DNA — Microtubular system — Anthracycline compound

Menogaril (7-con-O-methylnogarol, TUT-7) is an anthracycline[1] showing significant antitumor activity against murine tumors[2] and against human tumor clonogenic cells. This compound (Fig. 1) was synthesized by the replacement of neutral sugar with a methyl function and by removal of the carbomethoxy group at the C-10 position of nogalamycin.[1] Unlike nogalamycin, menogaril was reported to have a low binding affinity to DNA,[3] indicating that its cytotoxicity may be mediated through a mechanism other than the binding to DNA. Additional evidence emerged from studies on the intracellular distribution of menogaril, showing the accumulation of this compound in the cytoplasm,[4, 5] as revealed by fluorescence microscopy. This may indicate the importance of the cytoplasm as a site for cytotoxic activity, although a low nuclear fluorescence of anthracycline compounds may be caused by quenching by nuclear DNA,[6] resulting in the lack of fluorescence in the nucleus.

Despite recent clinical trials of this promising agent, its mechanism of action has not been clearly elucidated yet. Our studies were undertaken to evaluate the effect of menogaril on several intracellular processes which may be involved in its cytotoxicity. Cellular pharmacology studies, including the kinetics of macromolecular synthesis inhibition, intracellular distribution, interaction with nucleic acids and microtubular proteins, were also conducted.

MATERIALS AND METHODS

Compounds Menogaril was prepared by Upjohn Co. (Kalamazoo, MI) and etoposide (VP-16) was purchased from Nihon Kayaku Co., Tokyo. Tritium-labeled compounds used in this study were obtained from New England Nuclear. Other chemicals and biochemical reagents were purchased from commercial sources.

Cell lines L1210 leukemia cells were maintained *in vitro* by serial culture in RPMI 1640 medium containing 10% fetal bovine serum and 50 μM mercaptoethanol.

Tubulin preparation and polymerization Tubulin was prepared from mouse brain by a one-step polymerization procedure.[7] The microtubules were separated by ultracentrifugation at room temperature, and the resulting pellets were solubilized in ice-cold MES buffer and used for polymerization experiments. The polymerization mixture, consisting of 1 ml of tubulin preparation and the drug (added in a small volume of DMSO), was preincubated at 0°C for 10 min. The polymerization process was initiated following addition of GTP in 200 μl of MES buffer to a final concentration of 1 mM and an increase of the temperature to 37°C. The polymerization process was monitored by measuring the change of absorbance at 350 nm.

Competitive binding to vinblastine and colchicine binding sites The homogenate (33%) prepared from MX-1 human breast cancer xenografts was used to prepare

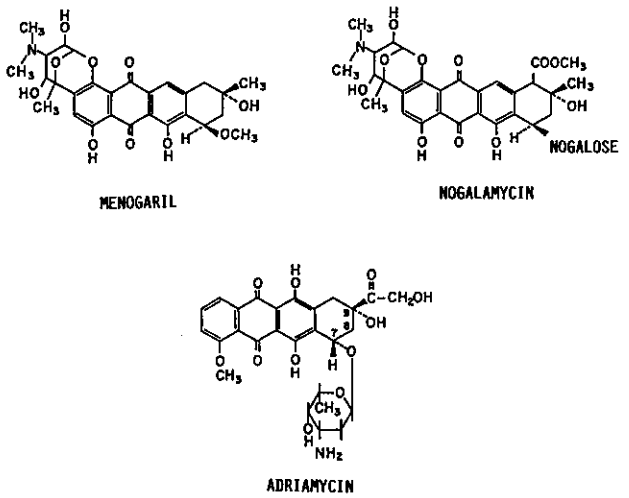

Fig. 1. Chemical structures of menogaril, nogalamycin, and doxorubicin (ADR).

100,000g cytosol. The binding of radiolabeled colchicine and vinblastine to cytosolic proteins in the presence of increasing concentrations of menogaril was measured by using the activated charcoal methods previously reported.[8, 9] The cytosol was incubated for 1 h at 37°C with tracer concentrations (0.2 μCi/50 μl of cytosol) of colchicine or vinblastine alone and in the presence of menogaril (1, 10, and 100 μM). Bound and free fractions of either colchicine or vinblastine were separated by activated charcoal suspension. Tubulin-bound radioactivity was measured in the supernatants obtained after centrifugation.

Inhibition of macromolecular synthesis The synthesis of DNA, RNA, and protein was measured by determination of the amount of radiolabeled precursors (1 μCi of [^{3}H]dThd, 2 μCi [^{3}H]Urd, and 2 μCi [^{3}H]Leu) incorporated into the TCA-insoluble materials derived from treated cells, compared with the untreated control. L1210 cells were suspended in 0.20 ml of the incubation mixture (5×10^5 cells/ml) and were incubated alone or in the presence of menogaril or doxorubicin (ADR) for 3 h at 37°C in a CO_2 incubator. Radiolabeled precursors of macromolecular synthesis (10 μl) were added at 60 min before completion of the 3 h incubation period. TCA-precipitated material was solubilized prior to counting in liquid scintillation fluid. Parallel experiments were performed to measure cytotoxicity by the trypan blue exclusion method.

Binding to DNA-spectrophotometric studies The absorption spectra of menogaril or ADR were measured in a Hitachi spectrophotometer at room temperature. Quartz cells of 5 cm path length were used throughout. A stock solution of calf thymus DNA (Sigma) was prepared (1 mg/ml) in 10 mM Tris-HCl buffer, pH 7.4.[10] Spectrophotometric titrations were performed by adding a constant volume of drug solution to 10 mM Tris-HCl buffer containing various concentrations of DNA. Drug concentration was constant throughout the experiment and was 5 μM. After addition of an interacting agent to DNA solution, 10 min was allowed for equilibration, then the absorption spectra were recorded against the reference solution containing corresponding concentrations of DNA, and the absorbances at 470 and 480 nm due to menogaril and ADR, respectively, were measured. DNA concentration in the stock solution was determined using a molar extinction coefficient at 260 nm of 6600 M^{-1} (nucleotide base) cm^{-1}. Molar extinction coefficients of free and DNA-bound menogaril or ADR were determined spectrophotometrically, assuming that binding is complete when the absorbance of the studied compounds does not change despite further increase of DNA concentration. The binding was estimated according to the direct spectral method described by Byrn and Dolch.[11] The binding parameters were calculated by a non-linear iterative least-squares method.[12]

Measurement of nucleoside triphosphate (dNTP and rNTP) pool L1210 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and 50 μM 2-mercaptoethanol. Cells were treated with 1 μM menogaril and at the indicated time intervals the cells were harvested and washed twice with calcium-, magnesium-free PBS. Cell pellets were extracted with 0.3 M TCA on an ice bath for 30 min and then centrifuged at 15000 rpm for 1 min. The resulting supernatants were neutralized to pH 7–8 by addition of 1.1 volume of Freon-amine solution (0.5 M tri-n-octylamine in 1,1,2-trichlorotrifluoroethane). Aliquots of aqueous phase were used directly to measure ribonucleoside triphosphates (rNTP) by HPLC. The concentrations of deoxyribonucleoside triphosphates (dNTP) were determined following decomposition of rNTP with periodate and methylamine.[13, 14] HPLC determinations were performed using a Whatman-Partisphore SAX column and a mobile phase consisting of 0.4 M $NH_4H_2PO_4$ and 10% CH_3CN buffer, pH 3.5 or 3.25 for rNTP and dNTP, respectively. The flow rate was 1 ml/min, and spectrophotometric detection was done at 254 nm.

Contour-clamped homogeneous electric field (CHEF) electrophoresis of L1210 cell DNA L1210 cells were exposed to various concentrations of menogaril, ADR and VP-16 for 3.5 h at 37°C. After being washed, the cells were fixed in 1% agarose gel and treated with aqueous 0.1 M EDTA, pH 9.0, 1% lauroyl sarcosine, and proteinase K (1 mg/ml) for 48 h at 50°C.[15] Blocks were washed 3 times with 0.2 M EDTA, pH 8.0, and subjected to CHEF electrophoresis, which was performed under

the reported conditions.[15] The gel was stained with ethidium bromide (0.5 μg/ml).

RESULTS

Interaction of menogaril with microtubular proteins of the cell Figure 2 indicates the effect of menogaril on tubulin polymerization to microtubules. Menogaril showed a minimal inhibitory effect on microtubule formation under steady-state conditions in the concentration range of 6.25 to 50 μM. However, there was a concentration-dependent inhibition of the initial polymerization rate of tubulin. The plot of the unpolymerized fraction versus polymerization time shows a clear dependence of the slope upon menogaril concentration (inset in Fig. 2). Thus, in this range of concentration, menogaril interferes with tubulin polymerization. Preliminary experiments showed the binding of menogaril to microtubular proteins using either ultrafiltration or the tubulin fluorescence quenching technique (data not shown).

To explain the specificity of this interaction, an additional experiment to examine the interaction between menogaril and colchicine or vinblastine was performed. The binding of both compounds to 100,000g cytosol prepared from MX-1 human breast cancer xenografts was not affected over a wide range of menogaril concentration (data not shown). The above results indicate that menogaril does not share binding sites with colchicine or vinblastine, which are known to induce conformational changes in tubulin structure.

Inhibition of macromolecular synthesis Menogaril predominantly inhibited DNA synthesis and, to lesser extents, RNA and protein synthesis in L1210 cells (Fig. 3). Approximately one order higher concentration of menogaril was required to inhibit RNA synthesis. At the concentration of 3 μg/ml, the DNA synthesis was inhibited by 66 and 70% following exposure to menogaril and ADR, respectively. In contrast to menogaril, ADR inhibited RNA synthesis to the same extent as DNA synthesis and did not significantly affect protein synthesis.

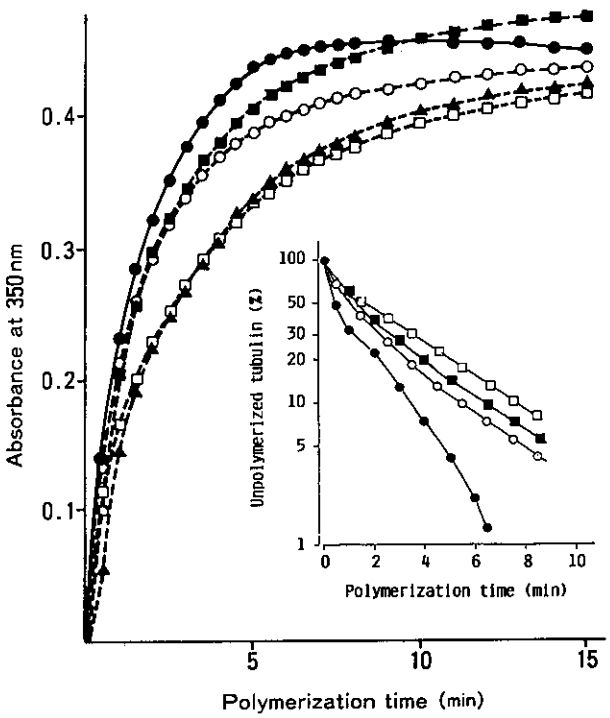

Fig. 2. Polymerization of mouse brain tubulin in the absence (control) of (●), and in the presence of 6.25 (○), 12.5 (■), 25 (□), and 50 μM (▲) menogaril, monitored by measuring the change of absorbance at 350 nm. The inset presents the initial polymerization rate expressed as a time-dependent change of the fraction of unpolymerized tubulin.

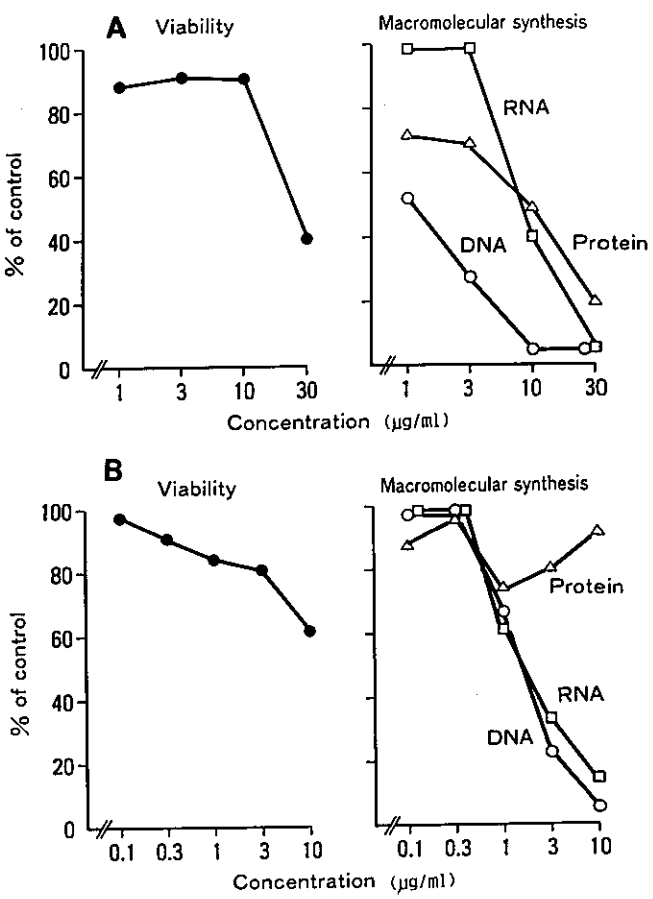

Fig. 3. The effects of menogaril (A) and ADR (B) on macromolecular synthesis and viability of L1210 cells.

On the other hand, there was a parallel inhibition of both RNA synthesis and protein synthesis by menogaril, though higher concentrations were necessary to affect these processes to a significant extent. The inhibition of macromolecular synthesis was not associated with a significant decrease of cell viability in the concentration range of 1–10 μg/ml. However there was a concentration-dependent decrease of cell viability after exposure to ADR. Thus the inhibition of DNA synthesis by menogaril preceded its cytotoxicity, while in the case of ADR these two effects appear to occur at the same time.

Menogaril binding of calf thymus DNA Typical spectral changes associated with menogaril-DNA interaction are presented in Fig. 4. The absorbance change, from completely bound to free menogaril (hypochromic effect), was 0.126 at 470 nm (wavelength chosen for the determination of the binding). There was a shift of the absorption maximum of menogaril from 468 to 478 nm accompanying the binding (bathochromic shift). The presence of the isosbestic points clearly demonstrates the presence of two kinds of molecules, e.g., free and DNA-bound form of menogaril. The inset of Fig. 4 shows the Scatchard plot for the menogaril-DNA interaction. As shown, menogaril possesses two types of binding site on the DNA molecule, though their nature is not clear. Kinetic analysis of the binding isotherm of menogaril presented in Fig. 5 (panel A) showed the presence of one specific binding site and one non-specific binding site. The identification of this binding character is based on the fitting of the experimental data to an appropriate theoretical model described by the equation presented in Fig. 5 (panel A). Based on this model, the number of

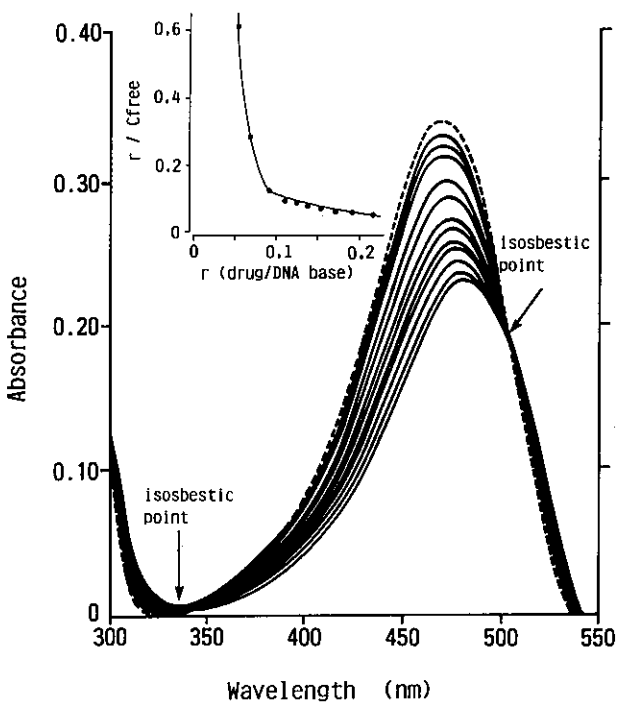

Fig. 4. Changes in the visible spectrum of 5 μM menogaril induced by increasing concentrations of calf thymus DNA (Sigma) and a Scatchard plot for menogaril binding (inset). All solutions were prepared in 10 mM Tris·HCl buffer, pH 7.4, and the spectra were recorded against the reference solution containing corresponding DNA concentrations.

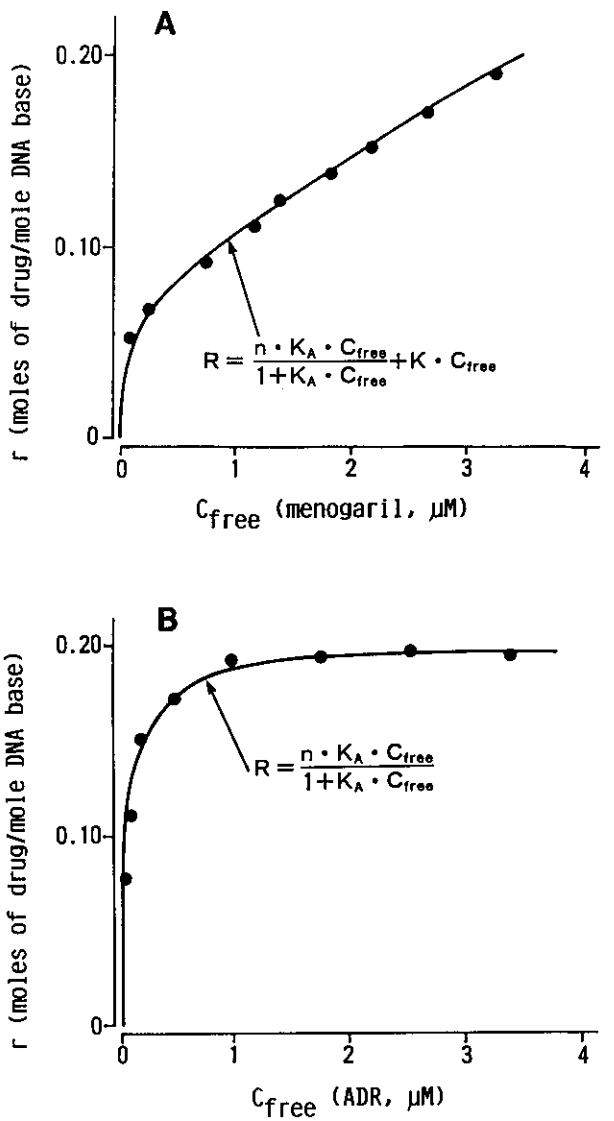

Fig. 5. Binding of menogaril (A) and ADR (B) to calf thymus DNA. The experimental points obtained from spectral changes were fitted to several binding models. The solid lines represent the best-fitted lines to theoretical models describing the binding of these two compounds to DNA (see the text).

binding sites (nP=r), the affinity of binding (KA) and the proportionality constant for non-specific binding (K) were calculated. The estimated binding parameters were as follows: association constant $31.15 \pm 5.03 \times 10^6$ M^{-1}; number of binding sites 0.068 ± 0.002 mol of menogaril per mol of DNA base; proportionality constant for non-specific binding 0.039 ± 0.001. A relatively high value of the association constant would indicate a very specific binding of menogaril to DNA. However, due to inaccuracy in the estimation of the molar extinction coefficient of the bound form of the drug, based on direct measurements of absorbance in the presence of a high DNA concentration, so that a small deviation may cause a significant error, an attempt was made to obtain this value by extrapolation of absorbance to a drug-to-DNA molar ratio of zero. The extrapolated value was used to recalculate the binding parameters of menogaril to DNA. The model assuming the presence of one specific and one non-specific binding sites appeared to be suitable to describe the recalculated experimental data. The binding parameters estimated in this way were as follows: number of binding sites, $r=0.132 \pm 0.045$ mol drug per mol of DNA base; $KA=0.67 \pm 0.23 \times 10^6$ M^{-1}; and $K=0.24 \pm 0.007$.

The same experimental procedure was used to estimate the binding of ADR to DNA for comparative purposes.

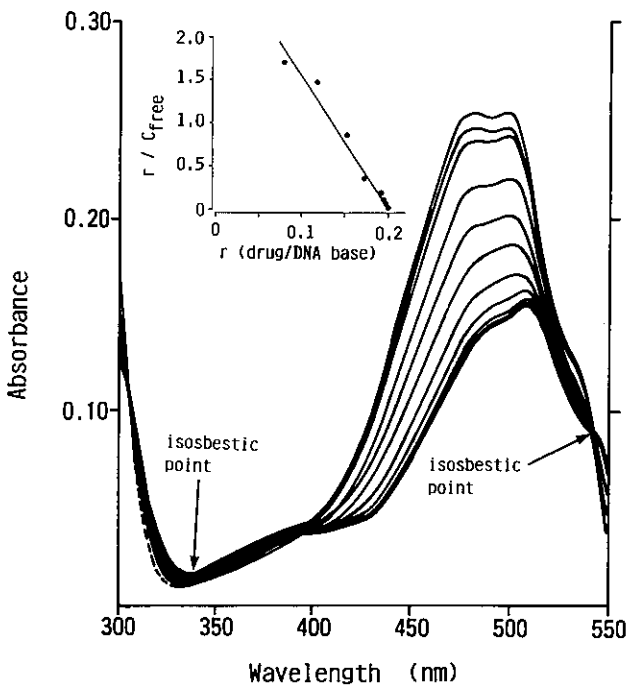

Fig. 6. Changes in the visible spectrum of 5 μM ADR induced by increasing concentrations of calf thymus DNA (Sigma) and a Scatchard plot for ADR binding (inset).

The spectral changes induced during interaction of ADR with calf thymus DNA are presented in Fig. 6. The Scatchard plot presented in the inset of Fig. 6 and the binding isotherm in Fig. 5 (panel B) clearly show the presence of one specific binding site, corresponding to 0.202 ± 0.004 mol of ADR per mol of DNA nucleotide base. The affinity of the binding to DNA is higher and is expressed by the association constant, $KA=15.07 \pm 1.03 \times 10^6$ M^{-1}. Taking into account the binding parameters, it is clear that the affinity of menogaril binding to DNA is lower than that of ADR, as determined under the same experimental conditions.

Intracellular pool of NTP Changes in the size of the nucleoside triphosphate (NTP) pool of L1210 cells following exposure to 1 μM menogaril are presented in Fig. 7. During the incubation period, the pool size of rNTP gradually increased when compared to control values (Fig. 7 panel B). A similar pattern was observed in the dNTP pool during the 3 h incubation period (Fig. 7 panel A). However, after 6 h of incubation, abnormalities in dNTP pool size were observed, namely dCTP and dTTP were increased while the dATP level dropped slightly and the dGTP concentration returned to the control range. Thus, menogaril does not induce significant changes in the molar composition of the rNTP pool despite increased total concentrations of each of the individual rNTPs. In contrast, there is an obvious imbalance between individual dNTPs of L1210 cells.

Cellular DNA cleavage induced by menogaril; comparison with VP-16 and ADR DNA breaks induced by menogaril or by VP-16 and ADR, used as reference compounds, were observed in L1210 cells subjected to electrophoresis (Fig. 8). There was a concentration-dependent DNA fragmentation following a 3.5 h treatment of L1210 cells with menogaril, VP-16 or ADR (1–30 μM). DNA of untreated (control) cells did not show any sign of fragmentation and remained at the origin of the gel. A visual comparison of the effects of the above three compounds indicates that the most pronounced effect is exerted by menogaril. Both ADR and VP-16 treatments resulted in accumulation of heavy DNA fragments located close to the origin. Menogaril treatment, on the other hand, caused fragmentation of DNA leading to the accumulation of smaller fragments (700 to 260 kb and below). These data indicate a high potency of menogaril to induce DNA breaks, which are presumed to be related to its cytotoxic effects.

DISCUSSION

The study presented here provides detailed information on the cellular pharmacology of menogaril, a new anthracycline antitumor antibiotic which has recently been the subject of clinical trials.

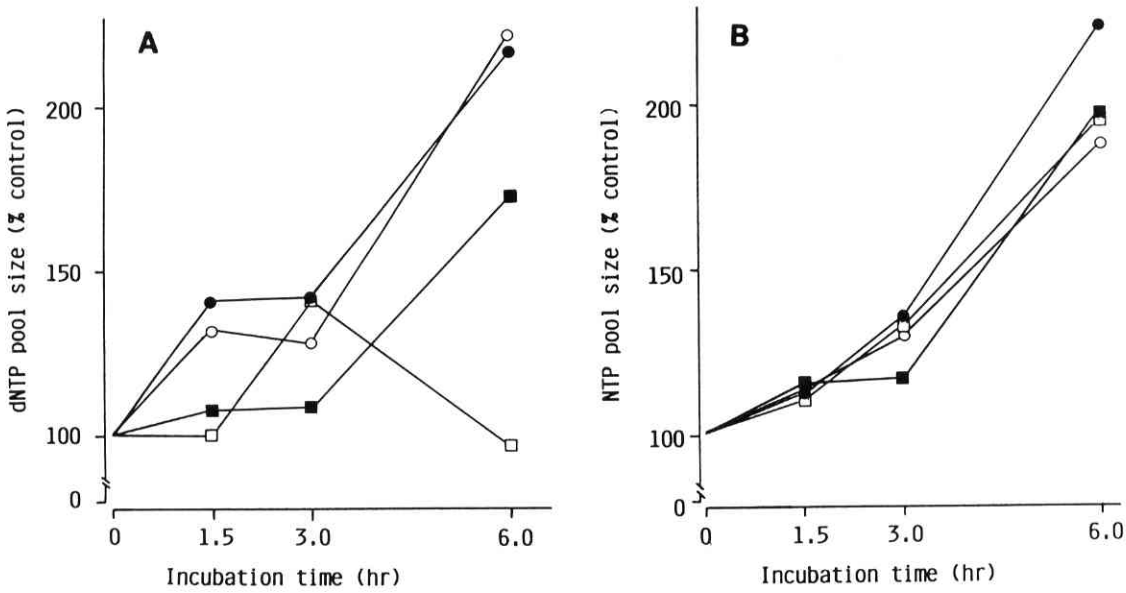

Fig. 7. Changes in concentrations of deoxyribonucleoside triphosphates (A) and ribonucleoside triphosphates (B) in L1210 cells treated with 1 μM menogaril. Panel A: ●, dCPT (13.7); ○, dTTP (32.9); ■, dATP (9.9); □, dGTP (2.4). Panel B: ●, CTP (203); ○; UTP (511); ■, ATP (1717); □, GTP (627). Numbers in parenthesis show concentrations (pmol/10^6 cells) at zero time. Each experimental point represents the mean value of 3 independent determinations. There was no significant variation in control values during the incubation period.

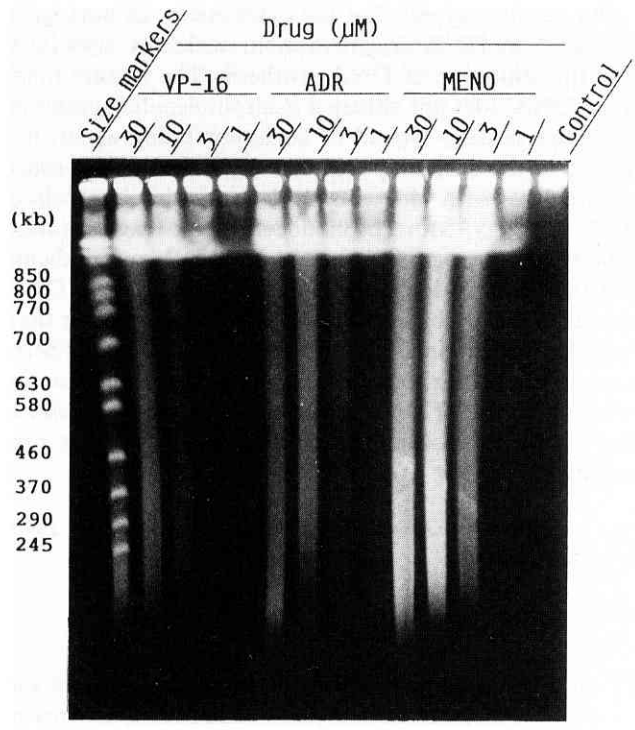

Fig. 8. The cleavage of cellular DNA in L1210 cells induced by ADR, menogaril, and VP-16.

Previous reports[4,5] on intracellular distribution showed a predominant localization of menogaril in the cytoplasm. Observations by light and fluorescence microscopy confirmed this finding. However, a careful examination indicated the presence of compartmentalization of menogaril in the cytoplasm and its ability to penetrate to the nucleus of the intact cell. On the other hand, ADR (used as a reference compound) was mainly accumulated in the nucleus. A weak nuclear localization of menogaril is consistent with the data reported by Egorin *et al.*,[16] who observed the uptake of menogaril by isolated nuclei of L1210 cells.

The localization of menogaril in the cytoplasm prompted us to investigate its effect on the microtubular system of the cell since aclacinomycin, which also accumulates in the cytoplasm was reported to interact with cellular tubulin.[17] Our results revealed a concentration-dependent inhibition of the initial polymerization rate of tubulin, without a significant effect on the total polymerization process over the studied range of concentration (6.25–50 μM). Moreover, menogaril has an ability to bind to the microtubular proteins, as determined by an ultrafiltration method and by measurement of the quenching of tubulin fluorescence (data not shown). Although this binding occurs, it does not involve colchicine or vinblastine binding sites. The interaction with

microtubular proteins requires further evaluation since the intracellular concentration of menogaril observed *in vitro* is higher than that recently used in our experiments.

The controversy over the relation of cytotoxicity and the inhibition of macromolecular synthesis[16, 18] prompted us to re-examine this question. Despite differences in methodological approach, our data are in agreement with those reported by Egorin *et al.*,[16] who observed equivalent activity of menogaril and ADR to inhibit DNA synthesis. There are some interesting differences in the macromolecular synthesis-inhibitory activities of these two compounds. Namely, ADR inhibits to the same extent both DNA and RNA synthesis while its effect on protein synthesis is minimal. On the other hand, the effect of menogaril on the synthesis of RNA is one order of magnitude weaker, but there is also a significant inhibitory effect on protein synthesis. These data indicate the involvement of the interaction of menogaril and DNA in the cytotoxicity. The lack of parallel inhibition of both RNA and DNA syntheses as caused by ADR may result from the different mode of interaction with DNA.

The interaction of menogaril or ADR with calf thymus DNA was confirmed by the spectral changes of both compounds induced by DNA. The Scatchard plot for ADR binding to DNA indicates the presence of one specific binding site while that for menogaril reveals two types of binding sites. Kinetic analysis using several models suggested that one type relates to specific binding and the other to non-specific binding with a proportionality between bound and free concentrations of menogaril. However, recalculation of the binding parameters confirmed that the specific binding of menogaril to DNA is of lower affinity than that of ADR. The binding constant of menogaril is at least one order of magnitude higher than that previously reported. This probably results from the different models used for the calculations of the binding parameters and a different buffer composition. Recent results indicate a significant difference between the interactions of menogaril and ADR with calf thymus DNA, and a detailed analysis of the binding will be presented elsewhere. However, since ionic strength significantly affects the binding of menogaril to DNA and this compound has a low inhibitory effect on DNA polymerase (data not reported here), it seems that menogaril is not a DNA intercalating agent. As a matter of fact, ADR binds to DNA even in the presence of a high NaCl concentration, despite a lowering of the binding affinity.[10] On the other hand, DNA polymerase requires single-stranded templates, the formation of which is strongly inhibited by the stabilization of DNA duplex structure by intercalating agents.[19]

Such a type of weak interaction with DNA raises the possibility of drug accumulation around DNA molecules, possibly leading to interference with the activities of the enzymes controlling DNA topology and repair. The pronounced DNA fragmentation in intact cell systems following exposure to menogaril probably results from the inhibition of the activity of enzymes controlling the topology of DNA, e.g., topoisomerases. It should be stressed that the fragmentation of DNA by menogaril is more severe than that by VP-16 and ADR. Wozniak and Ross[20] have reported a correlation between DNA breakage and cytotoxicity induced by VP-16. VP-16-induced DNA fragmentation is believed to be mediated by the inhibition of topoisomerase II activity.[21–23] Therefore, menogaril-induced DNA fragmentation may be related to the inhibition of the activity of this enzyme, since the menogaril-induced an imbalance in the dNTPs pool, even though obvious, could not contribute to the fragmentation of DNA. Recent investigations have revealed that ADR causes DNA-protein cross links in the complex of DNA and topoisomerase II that has been stabilized by ADR.[24]

Our results suggest that the cytotoxicity of menogaril is related to DNA fragmentation, which is associated with the inhibition of DNA synthesis. The weaker binding to DNA does not induce a macromolecular synthesis-inhibitory pattern typical of an intercalating agent, but enough of the drug may concentrate in the nuclear space to interfere with the enzymatic activities controlling DNA topology. More pronounced DNA fragmentation induced by menogaril than that by ADR may indicate that the contribution of ADR entrapped between DNA strands to the inhibition of topoisomerase II is lower than that of ADR retained close to the DNA surface. Therefore, one may expect that menogaril binding to the external surface of DNA molecules will induce stabilization of the DNA-topoisomerase II complex, thus preventing the sealing of DNA strands.

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