

Comparative Genotoxicity of Adriamycin and Menogarol, Two Anthracycline Antitumor Agents

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ABSTRACT

Adriamycin and menogarol are anthracyclines which cause more than 100% increase in life span of mice bearing P388 leukemia and B16 melanoma. Unlike Adriamycin, menogarol does not bind strongly to DNA, and it minimally inhibits DNA and RNA synthesis at lethal doses. Adriamycin is a clinically active drug, and menogarol is undergoing preclinical toxicology at National Cancer Institute. In view of the reported mutagenicity of Adriamycin, we have compared the genotoxicity of the two drugs. Our results show that, although Adriamycin and menogarol differ significantly in their bacterial mutagenicity (Ames assay), they have similar genotoxic activity in several mammalian systems. Adriamycin is strongly mutagenic in the Ames assay with TA98 and TA100. Menogarol is nonmutagenic to TA98 and TA100. For the mammalian cell culture systems, V79 (Chinese hamster) cells are exposed for 2 hr to drug, following which cell survival, induction of sister chromatid exchanges, chromosome damage, and production of mutants resistant to 6-thioguanine are measured. The percentage of survival obtained with the two drugs ranges between 25 and 50% at 0.15 μ g/ml and 5 to 15% at 0.3 μ g/ml. At 0.15 μ g/ml, Adriamycin and menogarol increase the percentage of cells with chromosome damage from a background level of 8.8 to 30 and 22.5%, respectively. The same drug concentration causes a small but significant increase in sister chromatid exchange rate. Both drugs are equally active (increase mutation frequency about 3- to 6-fold above background) in producing 6-thioguanine-resistant mutants. The induction of micronuclei in polychromatic erythrocytes of rats is the most sensitive assay system. Both drugs cause 10- to 15-fold increase in micronuclei at nontoxic doses.

INTRODUCTION

Recent improvements in cancer therapy have been possible through the use of chemotherapeutic agents in conjunction with surgery and radiation. However, many of these chemotherapeutic agents not only have serious toxic side effects but are also mutagenic, carcinogenic, and clastogenic (15). Therefore, it is essential that new drugs be tested for their possible genotoxic effects. We compare here the genotoxicity of 2 anthracyclines, Adriamycin and menogarol.

Menogarol, previously reported as 7-con-O-methylnogarol, is a new anthracycline (19) which has significant activity against several murine tumors. Since Adriamycin is another anthracycline with potent antitumor activity in animals and in humans, its biological activity was compared with those of menogarol. Both drugs were significantly active against several transplantable

murine tumors such as P388 and L1210 leukemias and B16 melanoma (13). However, the biochemical activity of Adriamycin and menogarol were markedly different in the following respects. (a) at cytotoxic doses, Adriamycin inhibited RNA synthesis much more than DNA synthesis in L1210 cells in culture (10). In contrast, menogarol caused very little inhibition of RNA or DNA synthesis at cytotoxic doses (10); (b) Adriamycin interacted strongly with DNA, in contrast to the weak interaction seen with menogarol (10); (c) cells in S phase were most sensitive to Adriamycin as compared to maximum toxicity of menogarol to cells in G₁ (5). These results collectively suggested that menogarol acts through some mechanism other than the intercalative DNA binding proposed for Adriamycin.

Adriamycin has been shown to be mutagenic (3, 11, 12) and clastogenic (3) to cells in culture and tumorigenic in animals (11). Therefore, we compared the genotoxic effects of these 2 anthracyclines, and the results are presented in this paper. Parts of this paper were presented previously as an abstract (16).

MATERIALS AND METHODS

Drugs

Adriamycin (NSC 123127) was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Menogarol, previously reported as 7-con-O-methylnogarol, was prepared by Wiley *et al.* (19) at The Upjohn Co. Menogarol and Adriamycin were dissolved in 0.1 M glucuronic acid at 1 mg/ml and diluted in medium to the appropriate concentration. The structure of these compounds is shown in Chart 1.

Salmonella Mutagenicity (Ames) Assay

The protocol for the *Salmonella typhimurium*-microsome assay was that described by Ames *et al.* (1). Bacteria (TA98 and TA100) were grown overnight in nutrient broth (Difco) to a cell density of about 2×10^9 /ml. The composition of the media used and the S9 mix have been described by Ames *et al.* (1). To 4 ml of top agar at 45°, 0.2 ml of *S. typhimurium* (about 4×10^8 bacteria), 0.1 ml of drug in dimethyl sulfoxide, and 1.0 ml of S9 mix were added. The resulting mixture was then dispersed rapidly with a 5-ml disposable pipet, and 2 ml were plated on top of 20 ml agar in each of 2 plates. The revertant count for each point was the average of duplicate plates, scored on a modified Artek Model 870 colony counter (Artek Systems, Farmingdale, N. Y.).

Aroclor 1254-induced mouse liver S9² was purchased from Litton Bionetics, Kensington, Md. S9 was prepared from HA/ICR, CD-1 mice.

Cell Culture and Drug Exposure Protocols

V79 cells were maintained as monolayer cultures in Eagle's basal medium, supplemented with 5% fetal calf serum, penicillin (100 units/ml), streptomycin (0.05 mg/ml), and L-glutamine (2 mM). For all experi-

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² The abbreviations used are: S9, 9000 \times g supernatant; SCE, sister chromatid exchange; TG^R, thioguanine resistant; LD₅₀ or LD₆₀, drug concentration lethal to 50% or 80% of cells.

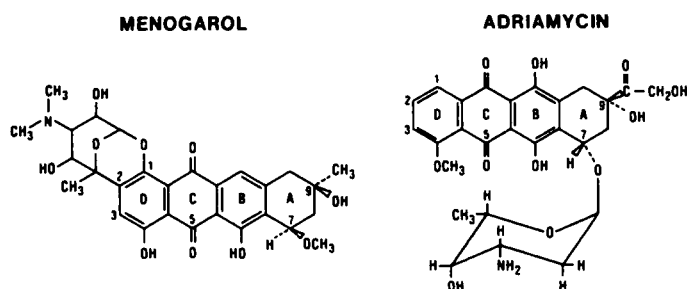


Chart 1. Structures of Adriamycin and menogarol.

ments, stocks were maintained in exponential growth through harvesting and replanting at appropriate intervals. Cells were harvested by treating with trypsin (0.1%) and EDTA (0.02%). All cultures were established 24 hr prior to an experiment in order to assure exponential growth during the experiment. In all experiments with V79 cells (SCE, chromosome damage, and TG^R assays), exponentially growing monolayer cultures were exposed to different levels of drug for 2 hr.

V79 Mutation Assay to Determine TG^R Colonies

Frozen Stock Cells. Each experiment was done with a freshly thawed preparation of 10^{-5} M hypoxanthine: 10^{-6} M aminopterin: 5×10^{-6} M thymidine-treated V79 cells stored in liquid N₂. V79 cells were treated with 10^{-5} M hypoxanthine: 10^{-6} M aminopterin: 5×10^{-6} M thymidine for 7 days in order to reduce the number of TG^R cells in the stock culture. Cells from the frozen stock were thawed and planted at 5×10^5 /75-sq cm flask. These cells were grown for 5 days, with 2 subcultures during that period, before being used in an experiment.

Experimental Protocol. The cells were planted at 2×10^6 /150-sq cm flask and incubated for 24 hr before a 2-hr drug exposure. The drug was then removed, and the cells were rinsed and incubated for 24 hr in fresh medium. Then the cells were subcultured and planted at 3×10^6 /150 sq cm, incubated for 2 days, subcultured again, and planted (10^6 /150 sq cm) and incubated for 3 more days. The cells were then harvested and planted in growth medium (10^3 , or 200 cells/100-mm plate) to determine plating efficiency, and in mutant selection medium (20 μ g/ml 6-thioguanine, 2×10^5 cells/100-mm plate) to determine the mutation frequency. After 7 days, the colonies were stained with 0.2% methylene blue in 70% ethanol and counted. We used 3 to 4 (150-sq cm) flasks per concentration of drug, 3 flasks for vehicle control and 1 flask for positive control (1-methyl-1-nitrosourea, 50 μ g/ml) compound. The plating efficiency (PE) is defined as colonies formed per cells planted.

$$\% \text{ of survival} = \frac{\text{PE of treated} \times 100}{\text{PE of vehicle control}}$$

Mutants/ 10^6 survivors

$$= \frac{\text{Mutants}/10^6 \text{ cells planted}}{\text{PE at time of 6-thioguanine addition on Day 7}}$$

The expression time, defined as the interval between drug exposure and mutant selection, for obtaining maximum number of mutants after treatment with Adriamycin or menogarol, was determined to be 6 days.

Statistical Analysis of V79 Mutation Assay. Large variation in mutation frequency between replicate flasks was seen, similar to that reported by Irr and Snee (9). These authors suggested that the mutation frequencies be transformed to $(1 + \text{mutants}/10^6)^{0.15}$, in order to obtain homogeneous variances so that parametric statistical methods can be applied. The mean response at each dose is compared to the mean control (no treatment) response using Dunnett's *t* test (6). The mean square error from a 2-way analysis of variance with experiments as blocks and doses as treatments is used for a variance estimate. In addition, using a covariance analysis with experiments as treatments, an *F* test is used to test for an increasing linear relationship between log

dose and the transformed response. In order to use control dose data in this test, one-tenth of the lowest noncontrol dose was added to the zero control doses before taking the log of the control dose.

SCE Assay

The method for determining SCE was essentially that of Goto *et al.* (8) as modified by Trzos *et al.* (17). After a 2-hr drug exposure, the cells were gently rinsed and incubated in the dark in medium containing 2.5 μ g of bromodeoxyuridine per ml for 2 cell generations (24 hr). The cells were then treated with colchicine (4 μ g/ml) for 4 hr and harvested. Cells were treated with a hypotonic (0.075 M KCl) solution and then centrifuged and fixed (methanol:glacial acetic acid, 3:1). An air-dried slide preparation was stained (50 μ g Hoechst 33258 per ml for 10 min) and rinsed in H₂O. A coverslip was mounted, using Sorensen's buffer (0.2 M, pH 7.0), and was then exposed to two 15-watt black-light bulbs (GE, F 15 T8-BC) at 2 cm for 50 min. The cells were counterstained with fresh 3% Giemsa in Sorensen's buffer (pH 6.8) for 18 min. The slides were air dried, cleared in xylene, and mounted permanently. Four flasks were used for each drug dose and vehicle control. Mitomycin C was used as a positive control compound. One slide was prepared from each flask and was assigned a letter code. After all of the slides had been evaluated in alphabetical order, the code was deciphered. Metaphase spreads were located at $\times 120$ and 10 well-dispersed, well-stained spreads were counted per slide at $\times 1200$. Statistical analysis showed that this protocol was reproducible, and we could detect a 2-fold increase in SCE over the background SCE level (17).

Chromosome Damage Determination

After a 2-hr drug exposure, the cells were rinsed with medium and then incubated at 37° with medium and harvested after 6 or 24 hr. Colchicine (4 μ g/ml) was added 4 hr prior to harvest. Cells were harvested and treated with 0.075 M KCl solution and then fixed (7). An air-dried slide preparation was stained with 5% Giemsa (in Sorensen's pH 6.8 buffer) for 22 min. The slide was rinsed, air dried, cleared in xylene, and permanently mounted. The slides were coded and evaluated as described for the SCE assay above.

Four flasks were used per dose of drug and for vehicle control, and one slide was prepared per flask. Twenty metaphase spreads were evaluated per slide, according to the method described by Au *et al.* (4). All aberrations were converted into number of chromatid breaks as follows: chromatid gaps, chromatid breaks, and single fragments were counted as one break each; isochromatid breaks, chromatid exchanges, rings, and dicentric were counted as 2 breaks each (4). The results were expressed both as number of chromosome aberrations per metaphase, and as percentage of metaphase with aberration. For the latter expression the number of lesions per metaphase were not taken into account. We assumed that even one chromosome aberration was deleterious to the cell.

Micronucleus Test

For each dose of the drug and the vehicle control, 5 male (Sprague-Dawley, 140 to 180 g) rats were used. The total dose was injected in 2 equal doses given 24 hr apart. The animals were sacrificed at 30 hr after the first dose, and the bone marrow was removed from the 4 long bones of the hind quarters and dispersed in 0.3 ml fetal calf serum. The cell suspension was processed as described by Frank *et al.* (7). For each rat, 500 polychromatophilic erythrocytes and, as a control for artifacts, normochromatophilic erythrocytes were examined for micronuclei. Cyclophosphamide was used as a positive control.

RESULTS

Bacterial Mutagenicity. The mutagenicity of menogarol and Adriamycin for TA98 (to detect frame-substitution mutants) and TA100 (to detect base-substitution mutants) in the Ames plate

assay is shown in Chart 2. Adriamycin is a strong mutagen for TA98, both with and without activation, but is much less mutagenic to TA100. Menogarol is essentially nonmutagenic to either TA98 or TA100, both with or without activation.

Mammalian (V79) Cell Mutagenicity. Chart 3 shows that Adriamycin (LD_{50} 0.12 $\mu\text{g/ml}$) is slightly more lethal to V79 cells than is menogarol (LD_{50} 0.21 $\mu\text{g/ml}$). The dose response for induction of TG^a mutants by 2-hr exposure to the drugs is shown in Chart 4A. When mutation frequency is expressed as mutants/ 10^6 survivors, a large difference in variance is seen at different doses of menogarol and Adriamycin (Chart 4A). In order to aid statistical analysis by making the variances more homogeneous, the mutation frequencies are expressed as transformed $[(1 + \text{mutants}/10^6)^{0.15}]$ values, as described by Iir and Snee (9). The transformed values of the mutation frequency are plotted against the dose in Chart 4B. In each case, the dose-response relationship was statistically significant, based on a one-sided t test for a positive slope. After adjusting for survival by a covariance

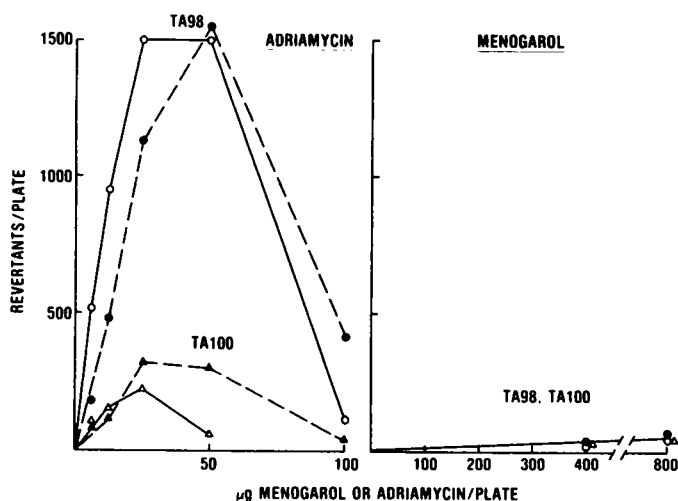


Chart 2. Mutagenicity of Adriamycin and menogarol for *S. typhimurium* TA98 and TA100. ○, ●, TA98; △, ▲, TA100; —, not activated; ---, activation by S-9.

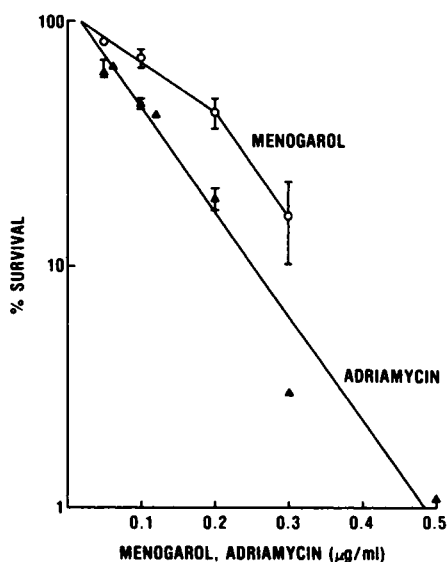


Chart 3. Lethality of menogarol and Adriamycin for V79 cells. Cells were exposed to different levels of the drugs for 2 hr, following which the percentage of surviving cells was determined by cloning.

analysis, there was no statistically significant difference in the transformed values for the 2 drugs. Thus, we conclude that menogarol and Adriamycin are equimutagenic at equitoxic doses.

Chromosome Damage. Chromosome aberrations induced by 2-hr exposure to the drugs are shown either as aberrations/metaphase (Chart 5A), or as percentage of metaphase with chromosome damage (Chart 5B). Using t test (Chart 5A) and normal approximations test (Chart 5B) for proportions, the 2 indicators show significantly greater damage by menogarol and Adriamycin as compared to the control at both 6 and 24 hr. For example, at 6 hr the background level of 8.8% damaged cells was elevated to 30 and 37.5% for the 2 doses of Adriamycin, and to 22.5 and 31.3% for the 2 doses of menogarol. The p value for percentage of cells with chromosome damage (Chart 5B) showed more significance than the p value for aberrations/metaphase (Chart 5A).

Table 1 lists the types of chromosome damage observed at 6 and 24 hr after exposure to the 2 drugs. The results can be summarized as follows: (a) For both drugs, the number of chromatid breaks decreases significantly between 6 and 24 hr. For example, at 0.3 $\mu\text{g/ml}$, both Adriamycin and menogarol caused 41 chromatid breaks at 6 hr, which decreased to 10 and 22

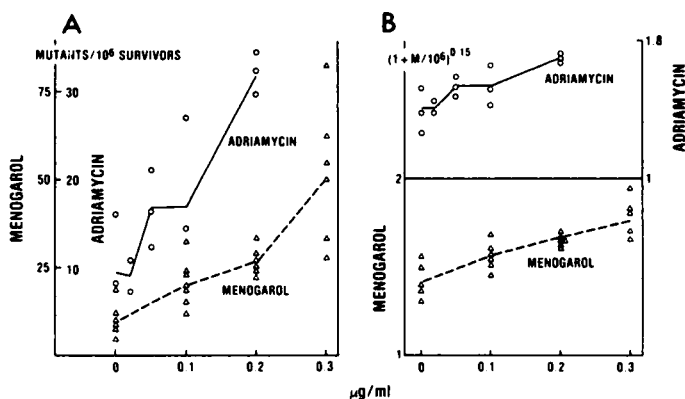


Chart 4. Mutagenicity of different concentrations of Adriamycin and menogarol for V79 cells. A, mutant frequency is expressed as mutants/ 10^6 survivors on the Y axis. B, transformed mutation frequency $[(1 + \text{mutants}/10^6)^{0.15}]$ (Y axis). This procedure reduces the variance seen in A.

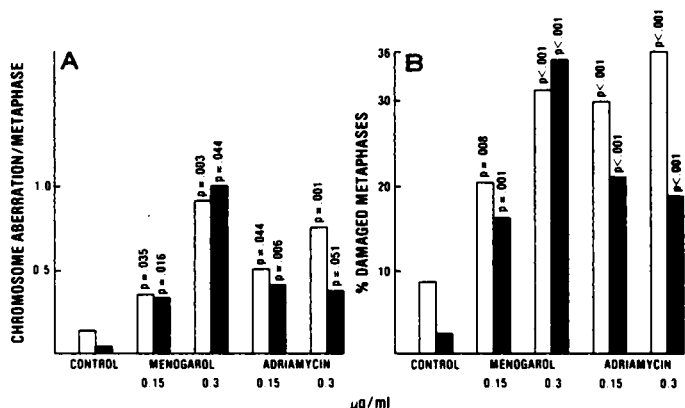


Chart 5. Chromosome aberrations induced in V79 cells by menogarol and Adriamycin. Cells were exposed to drug for 2 hr, following which the cells were washed and incubated in fresh medium. Cells were harvested 6 hr (□) and 24 hr (■) after drug exposure to determine chromosome damage; p values indicate the significance of the difference between the test sample and the control. A, chromosome aberrations per metaphase at different drug concentrations. All aberrations were expressed as chromatid breaks (see text). B, percentage of metaphase cells with damaged chromosomes.

breaks, respectively, at 24 hr. (b) With Adriamycin (0.3 $\mu\text{g/ml}$), the same number of chromosome breaks are seen at 6 and 24 hr; whereas with menogarol (0.3 $\mu\text{g/ml}$), the number of chromosome breaks increases significantly between 6 and 24 hr. (c) There is significant difference in the types of chromosome damage caused by the 2 drugs. Adriamycin causes mostly chromosome and chromatid breaks with few chromosome rearrangements. In contrast, a significant percentage of the damage caused by menogarol is expressed as chromosome rearrangements.

SCE. The SCEs induced by 2-hr exposure to menogarol and Adriamycin are shown in Table 2. Menogarol increased the number of SCEs per cell from the background level of 7.75 to 10.61 for 0.15 $\mu\text{g/ml}$ and to 17.71 for 0.3 $\mu\text{g/ml}$. The significance of the increase was determined by the *t* test. Adriamycin significantly increased the SCEs per cell to 12.34 at 0.15 $\mu\text{g/ml}$ and to 16.4 at 0.3 $\mu\text{g/ml}$.

Micronucleus. Since all of the above tests were conducted *in vitro*, the micronucleus test was included as an *in vivo* genotoxicity end point (Chart 6). Both menogarol (1.56 mg/kg) and Adriamycin (1.25 mg/kg) significantly increased the number of micronuclei per 500 polychromatic erythrocytes.

DISCUSSION

Bacterial Mutagenicity. Adriamycin was more mutagenic to TA98 than to TA100, with or without activation. Similar results have been previously reported (12, 14). Under similar conditions, menogarol was not mutagenic with or without S9 activation. Since menogarol was nonmutagenic in the standard Ames assay, it was retested by preincubating the drug with TA98 or TA100

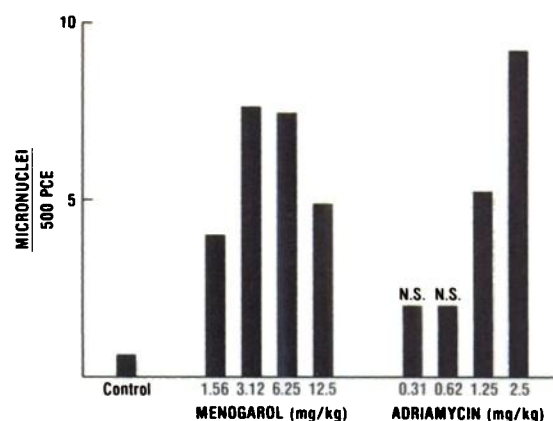


Chart 6. Induction of micronuclei by menogarol or Adriamycin. The *p* value was 0.01 to 0.05 in all cases, except when labeled N.S. (not significant). PCE, polychromatic erythrocytes.

bacterial suspension for 2 hr before planting onto agar plates. Under these conditions, menogarol was not mutagenic to TA100 and was weakly mutagenic (3-fold increase over spontaneous mutation frequency) to TA98.

Adriamycin and daunomycin, both of which contain a free amino group in the anthracycline glycoside, are strong mutagens in the Ames assay. Umezawa *et al.* (18) found that analogues in which the amino group has been methylated, such as *N*-dimethyl-daunomycin, is not mutagenic. Aclacinomycin (containing a dimethylamino group) was not mutagenic, whereas *N*-demethyl-aclacinomycin, with a free amino group, was mutagenic. Menogarol and its parent compound, nogalamycin, do not contain a free amino group, and neither compound (nogalamycin data not shown) was mutagenic in the Ames assay. These results corroborate the findings of Umezawa *et al.* (18) that the amino group of anthracycline glycosides may be essential for bacterial mutagenesis.

V79 Mutagenesis. In contrast to the differences in bacterial mutagenesis seen between Adriamycin and menogarol, both compounds were equally mutagenic to V79 cells at equitoxic doses. Adriamycin has been reported to be mutagenic to V79 cells (11). In our experiments, neither Adriamycin nor menogarol were strong mutagens at significantly toxic doses. Thus, at LD₅₀ for both drugs, the mutation frequency increased only about 3-fold, whereas 1-methyl-1-nitrosourea raised the mutation frequency 50-fold at less than 50% lethal dose (data not shown).

We confirm the variation in mutation frequency between replicate flasks previously reported by Irr and Snee (9). They observed variation both in spontaneous mutation frequency (0 to 16.8 mutants/10⁶ survivors) and in ethyl methanesulfonate-treated flasks (348 to 510 mutants/10⁶ survivors). This variation probably arises from the sampling during subculture of cells through expression time and could be reduced by subculturing greater numbers of cells during this period. However, this would have made the experiments unfeasibly large and was therefore not used. The variance seen between replicate flasks was reduced, but not completely eliminated, when the mutation frequency was expressed as a transformed value, which enabled us to apply standard statistical analysis to these data.

SCE. Au *et al.* (3) reported that Adriamycin causes SCE in mammalian cells. In our experiments, Adriamycin and menogarol were about equipotent in causing SCEs.

Chromosome Damage. Since all chromosome aberrations

Table 1

Chromosome aberrations caused by Adriamycin and menogarol

Cells were exposed to drug for 2 hr and then incubated in fresh medium. Chromosome damage was determined 6 or 24 hr after drug exposure. A total of 80 metaphases were counted for each sample.

Drug	Concentration ($\mu\text{g/ml}$)	Chromatid breaks	Chromosome breaks	Rings	Dicentric	Translocations
6 hr after drug exposure						
None (control)	0	7	2	0	0	0
Adriamycin	0.15	27	7	0	0	2
Adriamycin	0.3	41	9	1	0	1
Menogarol	0.15	16	5	2	0	1
Menogarol	0.3	41	17	5	4	0
24 hr after drug exposure						
None (control)	0	3	0	0	0	1
Adriamycin	0.15	10	10	1	1	0
Adriamycin	0.3	10	10	0	3	0
Menogarol	0.15	10	6	0	3	0
Menogarol	0.3	22	26	6	7	2

Table 2

Sister chromatid exchanges induced by menogarol and Adriamycin

Experiment	Treatment	Mean SCE/21 chromosomes	<i>p</i> ^a
1	Control	7.75 \pm 1.02 ^b	
	Menogarol (0.15 $\mu\text{g/ml}$)	10.61 \pm 2.35	0.034
	Menogarol (0.3 $\mu\text{g/ml}$)	17.71 \pm 2.89	<0.001
2	Control	7.07 \pm 0.61	
	Adriamycin (0.15 $\mu\text{g/ml}$)	12.34 \pm 1.53	<0.001
	Adriamycin (0.3 $\mu\text{g/ml}$)	16.46 \pm 3.36	<0.001

^a Calculated on the basis of null hypothesis that SCE rate of the 0.15- $\mu\text{g/ml}$ -treated and that of control group are equal.

^b Mean \pm S.D.

are thought to arise from initial chromosome or chromatid breaks, they were converted into breaks as suggested by Au *et al.* (2). This enabled better quantitation of the results. Au *et al.* (3) reported that 0.1 μg Adriamycin per ml increased the aberrations per metaphase in Chinese hamster ovary cells from a background level of 0.07 to 1.93, *i.e.*, a 27.5-fold increase. In our experiments with V79 cells, 0.15 μg Adriamycin per ml increased the aberrations per metaphase about 5-fold. This difference could be due to the difference in sensitivity and/or to the repair capability of the 2 cell lines (V79 *versus* Chinese hamster ovary) or to the difference in drug exposure. Au *et al.* (3) measured aberrations after 5 hr of treatment with Adriamycin, whereas our cells were exposed to drug for 2 hr and aberrations were determined at 6 or 24 hr posttreatment. It is possible that repair of chromosome damage or lysis of severely damaged cells during the posttreatment period would decrease the number of aberrations per metaphase.

Our results are also expressed as percentage of metaphase with chromosome damage, which implies that even a single lesion is deleterious and does not make any assumptions regarding the relative effects of single *versus* multiple lesions. To illustrate, suppose Drug X causes 10 aberrations in one metaphase, whereas Drug Y causes one aberration in 10 metaphases. If the results are expressed as aberrations per metaphase, then Drugs X and Y are equally active. However, Drug Y damages 10 times as many cells as does Drug X. Therefore, we consider that expressing the results as percentage of metaphase with chromosome damage may be a better indicator of damage to the cell population than aberrations per metaphase.

Our preliminary comparison of Adriamycin and menogarol in the Ames' bacterial mutagenicity assay showed that Adriamycin was a strong mutagen, whereas menogarol was not mutagenic. This difference was accepted, since Adriamycin interacts strongly with DNA whereas menogarol interacts weakly with DNA. However, subsequent experiments showed that Adriamycin and menogarol are equally genotoxic to mammalian cells. These results clearly point to the discrepancy between bacterial and mammalian cell mutagenicity assays and suggest the necessity of using a battery of complementary short-term assays to increase the chance of detecting genotoxic agents.

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