Structure-Activity Relationship of Anthracycline-induced Genotoxicity in Vitro

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ABSTRACT

Anthracycline antitumor antibiotics, such as Adriamycin and daunomycin, are potent genotoxic agents and carcinogens. A variety of anthracycline derivatives was investigated in various in vitro short-term tests, i.e., mutagenesis in Salmonella typhimurium and V79 Chinese hamster cells and induction of unscheduled DNA synthesis in primary rat hepatocytes. Compounds containing a daunosamine sugar moiety (Adriamycin, daunomycin, 4-demethoxydaunomycin, 4-demethoxyadriamycin, and carminomycin) were highly active in both mutagenesis assays. Addition of S9 to the bacteria and cocultivation of V79 cells with rat hepatocytes, in general, decreased the mutagenicity of these compounds. In contrast, anthracyclines with N-alkylated sugar moieties (acilnacolinycin A, marcellomycin, musettamycin, pyrromycin, rudolfomycin, N,N-dimethyladriamycin, N,N-dimethyldaunomycin, N,N-dibenzylaunomycin, N,N-dibenzylaunomycin, 3-deamino-3'-methoxytripiperidinodaunomycin, morpholinodaunomycin, cyanomorpholinodaunomycin, and cyanomorpholinoadriamycin) were weakly mutagenic or not mutagenic at all in both bacterial and mammalian cells. The two latter compounds were weakly active in the Salmonella/microsome assay only after addition of S9. Results obtained in the DNA repair studies did not correlate to these mutagenicity data; while most compounds, including Adriamycin and daunomycin, were either weakly active or inactive at inducing unscheduled DNA synthesis in primary rat hepatocytes, morpholinodaunomycin, cyano-7morpholinodaunomycin, and cyano-7morpholinoadriamycin were extremely active. The results indicate that the mutagenicity of anthracyclines is related more to differences in their sugar moiety than to differences in the chemical structure of their aglycones; N-alkylation of the sugar moiety can abolish or greatly reduce their mutagenic activity. Moreover, induction of unscheduled DNA synthesis, although considered to be due to DNA damage, is not correlated to anthracycline-induced mutations but may possibly indicate covalent DNA interaction.

INTRODUCTION

The long-term toxicity of antitumor agents, i.e., their oncogenicity and their mutagenicity, is a subject of growing concern because of the increased survival of cancer patients which modern chemotherapy has made possible. Therefore, it is of interest to study the structure-activity relationship of toxic/genotoxic activities of antitumor agents in order to possibly separate their cytostatic and genotoxic properties. The anthracycline antitumor antibiotics appear ideally suited for such an investigation: (a) they are among the most powerful cancer chemotherapeutic agents and known to be powerful mutagens and carcinogens (16); and (b) a great variety [about 500 (8)] of derivatives of Adriamycin and daunomycin has been synthesized and is available for an in-depth investigation. The biological activities of anthracyclines are of high complexity. In contrast to the general assumption that carcinogens or their metabolically activated derivatives react covalently with DNA, for anthracyclines, only noncovalent interactions with DNA have been demonstrated (16). In addition, cytotoxic properties of anthracyclines appear to be even unrelated to their physical interaction with DNA; N,N-dibenzylaunomycin is effective as an antitumor agent although it does not interact with DNA (1). In spite of its comparatively very weak interaction with DNA, menogarol is as active against transplantable murine tumors as Adriamycin, which strongly interacts with DNA (5); and cyanomorpholinoadriamycin (2), which has a comparatively low affinity to DNA, is about 600 times more active against P388 leukemia in mice than Adriamycin. Our own preliminary data, too, indicate that, indeed, cytostatic and genotoxic properties of anthracyclines can be separated (17). We now present further results of a more detailed study supporting this suggestion. In this study we used, on the one hand, aglyconederivatives of daunomycin containing a daunosamine sugar moiety and, on the other hand, anthracyclines with N-alkylated sugars (Chart 1; Table 1). Parts of this work were presented previously as an abstract (17).

MATERIALS AND METHODS

Chemicals. Daunomycin, Adriamycin, 4-demethoxydaunomycin, 4-demethoxyadriamycin, and carminomycin were kindly provided by Dr. F. Arcamone, Farmitalia-Carlo Erba, Milano, Italy; and pyrromycin, musettamycin, and rudolfomycin by Dr. G. Lenaz, Bristol-Myers Co., New York, NY. N-Alkylated anthracyclines (N,N-dimethyladriamycin, N,N-dimethyldaunomycin, N,N-dibenzylaunomycin, N,N-dibenzylaunomycin, N,N-dibenzylaunomycin, 3-deamino-3'-methoxytripiperidinodaunomycin, morpholinodaunomycin, cyanomorpholinodaunomycin, and cyanomorpholinoadriamycin) were received from Dr. E. Acton, Stanford Research Institute, Menlo Park, CA. Stock solutions of these compounds containing 1 to 2 mg/ml were prepared in aqueous medium. The stability of the stock solutions was checked immediately before use by thin-layer and high-pressure liquid chromatography. 2-Aminoantracene, 7,12-dimethylbenz(a)anthracene (Sigma, Munich, Federal Republic of Germany), 2-aminofluorene, N-methyl-N'-nitro-N-nitrosoguanidine (EGA-Chemie, Steinheim, Federal Republic of Germany), 8-azaguanine (Calbiochem, San Diego, CA), dimethyl sulfoxide (Merck, Darmstadt, Federal Republic of Germany), tritiated thymidine (specific activity, 70 Ci/mmol), tritiated 3-deoxycytidine (specific activity, 19 Ci/mmol; Amersham, Braunschweig, Federal Republic of Germany), Kodak D10 developer, and NTB photo emulsion (Eastman Kodak, Rochester, NY) were purchased from the indicated companies. Tissue culture media and Araclor-induced rat liver S9 were obtained from Grand Island Biological Co. (Karlruhe, Federal Republic of Germany) and Fresenius.
Salmonella-Microsome Assay. The bacterial mutagenesis assay with Salmonella typhimurium was performed as described previously (3). The tester strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538 were kindly provided by Dr. B. Ames (Department of Biochemistry, University of California, Berkeley, CA).

Mutagenesis in V79 Chinese Hamster Cells. The mutagenesis assay with mammalian cells was carried out as described previously (10). V79 cells were kindly provided by Dr. E. H. Y. Chu (Department of Human Genetics, University of Michigan, Ann Arbor, MI) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin-streptomycin. Cytotoxicity was measured by plating 10^5 cells into 60-mm dishes containing 8-azaguanine-free medium. The test compounds were added 18 hr later; 3 hr after that, the contents of the dishes were replaced with fresh 8-azaguanine-free medium. After incubation for 6 to 8 days, the cultures were fixed and stained, and colonies were counted. The average plating efficiency of control dishes was about 90%. Mutagenicity was measured by plating 2.5 x 10^4 cells into 60-mm dishes containing 8-azaguanine-free medium. The cell number was determined by trypan blue exclusion. The medium was then replaced with fresh 8-azaguanine-free medium, and the cells were incubated for an additional 5 days. The period of 5 days was found to be the optimal expression time for the induction of 8-azaguanine-resistant V79 mutants by anthracyclines (15). Thereafter, the dishes were refed every 2 days with medium containing 8-azaguanine (20 μg/ml). At 10 to 20 days after the addition of 8-azaguanine, the dishes were fixed and stained, and the number of 8-azaguanine-resistant colonies was determined. The mutation frequency was calculated per 10^6 survivors; the background spontaneous mutation frequency in controls was about 60%; the background spontaneous mutation frequency of controls was about 10^-5 colonies/10^5 survivors.

The cocultivation of V79 Chinese hamster cells with primary Wistar rat hepatocytes was performed as described by Langenbach et al. (9). Initially, 2 x 10^6 V79 cells were plated into 25-cm tissue culture flasks; 24 hr later, 5 x 10^6 freshly prepared primary rat hepatocytes suspended in Williams Medium E supplemented with 10% fetal calf serum were added. Two hr later, medium was replaced with fresh Williams Medium E containing test compounds. These cultures were incubated for 48 hr and, thereafter, trypsinized and replated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, at 200 V79 cells/flask, to determine the plating efficiency as described above, and 10^6 cells/flask to determine the yield of 8-azaguanine-resistant mutants as described above (V79 cells and hepatocytes can easily be distinguished by size and morphology). The average plating efficiency of control dishes was about 60%; the background spontaneous mutation frequency of controls was about 15 colonies/10^5 survivors. In these experiments, the optimal expression time of 4 days was chosen.

Induction of Unscheduled DNA Synthesis. This assay was performed in primary Wistar rat hepatocytes according to procedures described by Williams (19). Briefly, freshly prepared hepatocytes were suspended in Williams Medium E supplemented with 10% fetal calf serum and penicillin-streptomycin. Cells (5 x 10^6) were plated into 60-mm dishes containing round plastic coverslips (diameter, 2.5 cm). Two hr later, cultures were refed with medium containing test compounds and tritiated thymidine, 10 μCi/ml. After 18 hr of treatment, coverslips were removed from the dishes, dipped 3 times in Williams medium without serum and once for 10 min in sodium citrate solution (1%), and then fixed with ethanol:acetic acid (3:1). Thereafter, coverslips were mounted on glass slides, dipped in NTB photo emulsion in the dark, and exposed for 10 days. The slides were then developed with Kodak D10, fixed, air-dried, and stained with Harris hematoxylin. Silver grains in over 20 nuclei and 40 surrounding areas of equal size were counted with an automatic colony counter (Biotronic C112; New Brunswick Scientific Co.). “Net grains per nucleus” were calculated by subtracting the mean of grains per cytoplasm from the mean of grains per nucleus. The statistical significance (Student’s t test) was determined. The result is considered positive when p < 0.001 and “net grains per nucleus” were >3.

Isopycnic Cesium Chloride Gradient Centrifugation. This method to determine DNA repair was performed as described by Andrae and Greim (4). Briefly, 5 x 10^6 freshly prepared primary Wistar rat hepatocytes were plated into 60-mm tissue culture dishes in Williams Medium E supplemented with 10% fetal calf serum. Three hr later, medium was replaced with fresh medium containing 5-bromodeoxyuridine (200 μM) and 5-fluorodeoxyuridine (20 μM). The cultures were incubated for 1 hr; thereafter, medium was replaced with fresh medium containing 5-bromodeoxyuridine, fluorodeoxyuridine, tritiated 3-deoxycytidine (5 μCi/ml), and the test compounds. Cultures were incubated for additional 18 hr. Thereafter, medium was removed, cells were washed with standard saline citrate (0.15 M NaCl:0.015 M sodium citrate, pH 7.0)-buffer (supplemented with 0.01 M EDTA), harvested by trypsinization, centrifugated.
Table 1

Various anthracyclines derived from daunomycin and Adriamycin

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<th>Compound</th>
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<th>R3</th>
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<td>-NH2</td>
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<td>-OH</td>
<td>-NH2</td>
</tr>
<tr>
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<td>-OH</td>
<td>-NH2</td>
</tr>
<tr>
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<td>-OH</td>
<td>-NH2</td>
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<td>-H</td>
<td>-N(CH3)2</td>
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<td>-NC</td>
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* DNM, daunomycin; ADM, Adriamycin.

RESULTS

Our results on the mutagenicity of various anthracycline derivatives in the bacterial cell assay are shown in Table 2; only those anthracyclines which exhibited mutagenic activity were included in the table. It can be seen that N,N-dimethyladriamycin and rudolfomycin were weakly mutagenic only in strain TA 98 and pyrromycin only in strain TA 1537 of *S. typhimurium* with and without addition of external metabolic activation system. Musetamycin, N,N-dimethyladriamycin, N-benzyladriamycin, N,N-dibenzyladriamycin, 3'-deamino-3'-methoxyxypiperidinoadriamycin, and morpholinolodaidamycin were found to be nonmutagenic in experiments with the tester strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538 with and without addition of S9-mix. We had reported previously: (a) that some anthracyclines with a daunosamine sugar moiety, i.e., Adriamycin, daunomycin, 4-demethoxyadriamycin, 4-demethoxydaunomycin, and carminomycin, are potent mutagens in the bacterial assay (10, 16); and (b) that some anthracyclines with an N-alkylated sugar moiety, i.e., marcellomycin and aclacinomycin A, are nonmutagenic in *S. typhimurium* (15). Table 2 also shows that, in contrast to the general finding with anthracycline derivatives that their mutagenicity is reduced by addition of an external metabolic-activation system (16), cyanomorpholinolodaidamycin (only in TA 98) and cyanomorpholinolodaidamycin (only in TA 100) were weakly mutagenic only after addition of S9-mix.

Our results with V79 Chinese hamster cells are shown in Table 3; only compounds which showed mutagenic activity were included in the table. The results are in good agreement with those found in the bacterial assay. Musetamycin, pyrromycin, rudolfomycin, N,N-dimethyladriamycin, N,N-dimethyladriamycin, N-benzyladriamycin N,N-dibenzyladriamycin, and 3'-deamino-3'-methoxyxypiperidinoadriamycin were devoid of mutagenic activity; morpholinolodaidamycin, cyanomorpholinolodaidamycin, and cyanomorpholinolodaidamycin exhibited weak mutagenicity. We had reported previously: (a) that anthracyclines with a daunosamine sugar, i.e., Adriamycin, daunomycin, 4-demethoxyadriamycin, 5-iminoadaunomycin, and 7-con-O-methyladenosine (menogarol) are potent mutagens in V79 cells (10, 16) and (b) that anthracyclines...
Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µg/ml)</th>
<th>% of plating efficiency</th>
<th>8-Azaguainine-resistant colonies/10⁶ survivors</th>
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<td>21.1</td>
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*MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.

Table 4

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<th>Compound</th>
<th>Concentration (µg/ml)</th>
<th>% of plating efficiency</th>
<th>8-Azaguainine-resistant colonies/10⁶ survivors</th>
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Table 5

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Our results with those anthracycline derivatives which induced unscheduled DNA synthesis as an indication of DNA damage in primary Wistar rat hepatocytes are shown in Table 5. Adriamycin, carminomycin, pyrromycin, musettamycin, rudolfomycin, N,N-dibenzyladriamycin, and 3'-deamin-3'-methoxyxipiperidinodaunomycin were inactive; we had reported previously that aclacinomycin A is active and marcellomycin is inactive in this assay.

with an N-alkylated sugar moiety, i.e., aclacinomycin A and marcellomycin, are nonmutagenic in this assay (15). Since V79 cells possess a limited metabolic capacity only, with N,N-dimethyladriamycin, morpholinodaunomycin, and cyanomorpholinodaunomycin, the assay was performed in the presence of freshly prepared Wistar rat hepatocytes. However, as shown in Table 4, in these experiments the 3 anthracyclines did not exhibit mutagenic activity even though their cytotoxicity appears to be affected by the hepatocytes. Parenthetically, the extremely high cytotoxic potency of, particularly, Cyanomorpholinodaunomycin should be noted from Table 3.

Our results with those anthracycline derivatives which induced unscheduled DNA synthesis as an indication of DNA damage in primary Wistar rat hepatocytes are shown in Table 5. Adriamycin, carminomycin, pyrromycin, musettamycin, rudolfomycin, N,N-dibenzyladriamycin, and 3'-deamin-3'-methoxyxipiperidinodaunomycin were inactive; we had reported previously that aclacinomycin A is active and marcellomycin is inactive in this assay (15). Thus, our results on the activity of various anthracyclines to induce, on the one hand, mutagenesis and, on the other hand, DNA repair, are not correlated; while most derivatives tested including particularly the potent mutagens were inactive or only weakly active at inducing unscheduled DNA synthesis, morpholinodaunomycin, cyanomorpholinodaunomycin, and cyanomorpholinodaunomycin were among the most active compounds known with this assay. To exclude the possibility that these observations were due to semiconservative instead of unscheduled DNA synthesis, DNA repair induced by cyanomorpholinodaunomycin was determined, in addition, by isopycnic cesium-chloride-gradient centrifugation. This method permits to discern between semiconservative and unscheduled DNA synthesis. As shown in Chart 2, cyanomorpholinodaunomycin-induced radioactively labeled DNA bands with DNA of normal density, clearly indicating that the compound affects repair and not replicative DNA synthesis.

**DISCUSSION**

The present data in conjunction with previously published results clearly demonstrate that, in the case of anthracycline antitumor antibiotics, cytotoxic and mutagenic activities can be
separated. Thus, the anthracycline oligosaccharides (6, 7, 11) and the N-alkylated anthracyclines (1) show good antitumor activity, although we now report them to be either nonmutagenic or very weakly mutagenic. Similarly, as pointed out in “Introduction,” cytotoxic properties of anthracyclines appear to be unrelated to their capacity to interact with DNA. However, it must also be emphasized that, for anthracyclines, activity to interact with DNA and to induce mutagenesis are not correlated either. Thus, in spite of a similar and strong capacity to intercalate into DNA (3, 6, 7), Adriamycin, daunomycin, and 4-demethoxydaunomycin are potent mutagens in contrast to N,N-dimethyladriamycin, N,N-dimethyldaunomycin, N,benzyladriamycin, N,N-dibenzyl- daunomycin, 3'-deamino-3'-methoxy-2-piperidinosdaunomycin, mor- pholino- and cyanomorpholino-derivatives can be metabolically activated to generate genotoxic intermediates which react covalently with DNA and that induction of DNA repair may indicate capacity to covalently interact with DNA rather than mutagenicity of chemicals. The carcinogenic potential of these compounds is of great interest; such studies are presently under way in our laboratory. The present data confirm our earlier conclusion (15, 16) that, in some instances, poor correlations among different short-term tests as well as between in vivo tumorigenicity and activity in in vitro short-term tests must be anticipated and emphasize the necessity of using a battery of complementary short-term assays to detect genotoxic agents.

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ANTHRACYCLINE-INDUCED GENOTOXICITY IN VITRO


Structure-Activity Relationship of Anthracycline-induced Genotoxicity in Vitro

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