ABSTRACT

Single-strand DNA breaks induced by chromophore-modified anthracyclines related to doxorubicin (including 11-deoxydaunorubicin, 4-demethoxydaunorubicin, 4-demethoxy-11-deoxy-4'-epi-daunorubicin, 4-demethyl-6-O-methylodoxorubicin) in cultured P388 leukemia cells were determined by the filter alkaline elution method. The tested analogues differed markedly in their cytotoxic potency. In the range of cytotoxic concentrations, 11-deoxydaunorubicin produced single-strand DNA break frequency of the same order of magnitude as that produced by doxorubicin, while other derivatives caused much more marked damage on DNA than doxorubicin. Since DNA breaks were found to be protein associated, the type of DNA damage produced by all tested derivatives presumably resulted by action of DNA topoisomerases II, as proposed for doxorubicin and other intercalating agents. Although the "potent" (with respect to DNA damage) derivatives, except 4-demethyl-6-O-methyldoxorubicin, showed an increased cellular drug accumulation as compared to doxorubicin, this did not account for the marked differences in ability to damage DNA. 4-Demethyl-6-O-methyldoxorubicin was the most efficient derivative, producing DNA breaks in a lower range of cellular drug content. A striking biphasic dose-response curve was observed for the 4-demethoxy derivatives, suggesting a complex mechanism of interaction among drug, DNA, and enzyme. A lack of correlation was noted among DNA binding affinity, induction of strand breaks, and cytotoxic activity of these chromophore-modified derivatives. From these observations, it is suggested that multiple actions of anthracyclines at the DNA level are responsible for their cytotoxic activity, which is not simply related to inhibition of a specific DNA-dependent enzyme and/or function.

INTRODUCTION

Anthracycline antibiotics exert a number of biochemical actions at cellular levels (1). DNA intercalation and consequent inhibition of DNA functions are the most extensively studied mechanisms of action (2). Effects on DNA-dependent cell functions have been implicated as the most relevant molecular mechanism by which these antitumor agents inhibit cell growth (1). Evidence for this mechanism came from structure-activity relationship studies. An excellent correlation has been found between DNA binding affinity and relative antitumor activity of a wide range of anthracycline analogues (3–5). Most of these studies were carried out with homogeneous series of derivatives (essentially modified in the amino sugar moiety). Since nonintercalating anthracyclines lack antitumor activity (6, 7), intercalation is probably a necessary but not sufficient condition for antitumor activity of anthracycline antibiotics. Although several intercalating agents are known to be antitumor agents (8), this is not a general observation. For example, ethidium bromide has only marginal antitumor activity (9). In the case of anthracyclines, the correlation between DNA binding affinity and cytotoxic activity was not precise when chromophore modifications were considered (4, 10), in contrast to observations on other sugar-modified series.

Although cellular pharmacokinetics may play a relevant role (11), it is possible that other molecular interactions at the intracellular level (perhaps more specific) are required for optimal activity. Interference with DNA topoisomerase II activity has been recently identified as the molecular mechanism responsible for DNA damage induced by antitumor agents (12). DNA topoisomerase II inhibition, resulting in DNA damage, is also produced by epipodophyllotoxins, without involving DNA binding (13). This enzyme has been proposed as a cytotoxic target for specific antitumor drug design (12). However, the relevance of the inhibition of this enzyme to lethal effects of antitumor intercalating agents remains unclear.

This paper reports a study on structure-activity relationships of a variety of chromophore-modified anthracyclines, with particular reference to DNA damage, DNA binding affinity, and cytotoxic effects.

MATERIALS AND METHODS

Chemicals. Doxorubicin, 4-demethoxy-DNR, 11-deoxy-DNR, 4-demethyl-6-O-methyl-DX, and 4-demethoxy-11-deoxy-4'-epi-DNR (Fig. 1) were obtained from Farmitalia-Carlo Erba (Milan, Italy). Stock solutions of drugs containing 0.5 to 1 mg/ml were prepared in distilled water. Stability of stock solutions was checked before use by high-pressure liquid chromatography.

Cell Line, Drug Treatment, and Cytotoxicity Assay. Murine leukemia P388 cells were cultured in RPMI 1640 medium (Whittaker M. A. Bioproducts, Walkersville, MD) containing 15% inactivated fetal calf serum, 1% 2-mercaptoethanol, and antibiotics at 37°C in 5% CO2/95% air, as described (14). Cells in exponential growth phase at 10⁶/ml cellular density were exposed to drug for 1 h at 37°C. In cytotoxicity assays, after treatment, cells were centrifuged, resuspended, and cultured in drug-free medium for 72 h and then counted in a Coulter Counter (Coulter Electronics, Ltd., Luton, United Kingdom).

Alkaline Elution Technique. Alkaline elution procedures were essentially as reported by Kohn et al. (15) and described elsewhere (14). Cells were labeled with [2-14C]thymidine (0.025 µCi/ml; Amersham International, Amersham, United Kingdom) for 20 h. Cells irradiated on ice with X-rays (Stabilipan, Siemens, 221 nvs/min; or Mevatron 77, Siemens, 1360 rads/min) were kept at 0°C until lysis on the filter.

After drug treatments, 10⁶ labeled cells were layered on polycarbonate membranes of 25-mm diameter and 2-µm pore size (Nucleopore, Pleasanton, CA) and lysed with a solution of 2% SDS, 0.1 M glycine, 25 mM disodium EDTA (pH 10), and protease K (0.5 mg/ml; Merck, Darmstadt, Federal Republic of Germany). The DNAs on the filters were eluted with 0.1% SDS, 20 mM EDTA (acid form), and tetraprolaminium hydroxide (Eastman Kodak, Rochester, NY), pH 12.15 (eluting solution). For the high-sensitivity assay, elution was carried out at 0.03 to 0.04 ml/min for a total of 15 h. For the low-sensitivity assay (used when DNA-SSB frequency was more than 1000 rad-equivalents), elution was at 0.12 to 0.16 ml/min for a total of 30 to 40 min. From retention values of untreated control cells, 300-rad X-irradiated cells (as external standard), and drug-treated cells of the same experiment at a fixed elution time, the DNA-SSB frequency (in rad-equivalents) was calculated according to Kohn et al. (14).

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2 To whom requests for reprints should be addressed.
DNA DAMAGE INDUCED BY ANTHRACYLINE ANALOGUES

Fig. 1. Chemical structures of daunorubicin (DNR) (R^1 = H; R^2 = OH; R^3 = OCH^3; R^4 = H); doxorubicin (DX) (R^1 = OH; R^2 = OH; R^3 = OCH^3; R^4 = H); 4-demethoxy-DNR (R^1 = H; R^2 = OH; R^3 = H; R^4 = H); 4-demethyl-6-O-methyl-DX (R^1 = OH; R^2 = OH; R^3 = OH; R^4 = CH_3); 11-deoxy-DNR (R^1 = H; R^2 = H; R^3 = OCH^3; R^4 = H); 4-demethoxy-11-deoxy-4'-epi-DNR (R^1 = H; R^2 = H; R^3 = H; R^4 = H; this compound is also characterized by 4'-inversion of the hydroxyl group).

Table 1 Growth-inhibitory effects on P388 leukemia cells and DNA-binding constants of anthracycline derivatives

<table>
<thead>
<tr>
<th>Drug</th>
<th>ID_{50} (µg/ml)</th>
<th>K_{a}</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.7</td>
<td>6.5 x 10^{-6}</td>
<td>2</td>
</tr>
<tr>
<td>4-Demethoxy-DNR</td>
<td>0.065</td>
<td>2.4 x 10^{-6}</td>
<td>24</td>
</tr>
<tr>
<td>11-Deoxy-DNR</td>
<td>3.2</td>
<td>0.52 x 10^{-5}</td>
<td>7</td>
</tr>
<tr>
<td>4-Demethyl-6-O-methyl-DX</td>
<td>0.54</td>
<td>0.20 x 10^{-8}</td>
<td>10</td>
</tr>
<tr>
<td>4-Demethoxy-11-deoxy-4'-epi-DNR</td>
<td>0.26</td>
<td>0.31 x 10^{-6}</td>
<td></td>
</tr>
</tbody>
</table>

* ID_{50} drug concentration required for 50% cell growth inhibition. Drug treatments were for 1 h at 37°C. Cells were counted after 72 h of growth in drug-free medium.

Fig. 2. DNA-SSB induced by doxorubicin (•) and 11-deoxy-DNR (O). Cells were treated for 1 h at 37°C in the range of cytotoxic concentrations and lysed on the filter in the presence of proteinase K. Points, mean of at least 3 independent determinations; bars, SD, shown when larger than symbol size.

Fig. 3. DNA-SSB induced by 4-demethoxy-DNR (A), 4-demethoxy-11-deoxy-4'-epi-DNR (O), and 4-demethyl-6-O-methyl-DX (©). Cells were treated for 1 h at 37°C in the range of cytotoxic concentrations and lysed on the filter in the presence of proteinase K. Points, mean of at least 3 independent determinations; bars, SD, shown when larger than symbol size.

on DNA, no detailed structure-activity relationship studies have been reported for anthracyclines. DNA-SSB frequencies produced by a number of chromophore-modified anthracyclines and quantified by the alkaline elution technique are presented in Figs. 2 and 3. In this study, P388 cells were exposed to drugs in the range of pharmacological concentrations for 1 h. Whereas the ability of 11-deoxy-DNR to induce DNA damage was similar to that of doxorubicin (Fig. 2), 4-demethoxy-DNR, 4-demethoxy-11-deoxy-4'-epi-DNR, and 4-demethyl-6-O-methyl-DX produced much higher frequencies of DNA-SSB than did the parent drug (Fig. 3); the magnitude of DNA break frequencies produced by the latter derivatives was comparable to that of acridines and podophyllotoxins (17, 19).

In the concentration range of 0.1 to 1 µg/ml, the demethoxy derivatives produced DNA-SSB up to 5000 rad-equivalents, and 4-demethyl-6-O-methyl-DX had an even greater effect (up to 8000 rad-equivalents in the range of 1 to 10 µg/ml). In addition, a biphasic effect in response to different concentrations of the 4-demethoxy derivatives was observed, since DNA assay, external standard cells received 2000 rads of X-irradiation.

DPC were demonstrated by lysing cells with a solution of 0.2% Sarkosyl (Sigma Chemical Co., St. Louis, MO), 2 M NaCl, and 40 mM disodium EDTA (pH 10), without proteinase K, and eluting without detergent at 0.03 to 0.04 ml/min for 15 h. Polycarbonate filters were also used in these assays.

Cellular Drug Uptake. Treated cells were centrifuged at 0°C and washed twice with cold physiological saline solution. Distilled water and AgNO_3 (final concentration, 3.3%) were then added. Drugs were extracted twice with 1 ml of water-saturated normal butyl alcohol, and fluorescence intensity of extracts was measured by fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT) at excitation and emission wavelengths, respectively, of 500 and 590 nm (doxorubicin), 485 and 565 nm (4-demethoxy-DNR), 425 and 590 nm (11-deoxy-DNR), 455 and 570 nm (4-demethyl-6-O-methyl-DX), and 410 and 565 nm (4-demethoxy-11-deoxy-4'-epi-DNR). Average yield of the extraction procedure was more than 95%. As expected, thin-layer chromatography of cellular extracts showed no detectable amount of metabolites of any anthracycline.

RESULTS

Cytotoxicity Studies. Table 1 compares the growth-inhibitory effects of anthracyclines on P388 leukemia cells after exposure to the drugs for 1 h. 4-Demethoxy-DNR and 4-demethoxy-11-deoxy-4'-epi-DNR produced the greatest lethal effects, since these analogues were about 11- and 3-fold more active than doxorubicin, respectively. 4-Demethyl-6-O-methyl-DX produced inhibitory effects comparable to those of the parent drug, whereas 11-deoxy-DNR was found the least effective.

DNA-SSB Studies. Like other antitumor DNA intercalating agents, doxorubicin induced DNA breaks in exponentially growing cells (16). However, these agents differed in their ability to produce DNA damage (16-18). With respect to this effect...
DNA DAMAGE INDUCED BY ANTHRACYCLINE ANALOGUES

damage was dramatically decreased above 1 μg/ml (Fig. 3).

A comparison of DNA elution kinetics from cells lysed in the presence or absence of proteinase K (Table 2; Fig. 4) showed that, as expected for DNA breaks associated with covalently bound protein (16, 17), enzymatic deproteinization was necessary to detect the breaks. These results documented the presence of DPC in P388 cells treated with tested anthracycline derivatives, as after treatment with doxorubicin and other intercalating agents (Table 2) (16, 17).

Since the extent of DNA damage is expected to be influenced by different cellular pharmacokinetics of the anthracyclines, drug accumulation in P388 cells after 1-h exposure was determined (Fig. 5). As compared with doxorubicin, drug uptake increased for demethoxy derivatives (15- and 2-fold for 4-demethoxy-DNR and 4-demethoxy-11-deoxy-4'-epi-DNR, respectively). For other derivatives cellular drug uptake was comparable to that of doxorubicin.

In Figs. 6 and 7, DNA-SSB frequency is shown as a function of drug uptake. Under these experimental conditions, the DNA damage produced by most of the tested anthracyclines generally occurred in the same range of cellular drug levels. The 4-demethyl-6-O-methyl derivative was the only exception, since this drug was able to induce DNA breaks at more than 10-fold lower cellular levels. The relation between DNA breaks and cytotoxic activity of the chromophore-modified anthracyclines studied is reported in Fig. 8. No correlation was observed between these cellular effects, since equitoxic concentrations of different drugs produced quite different DNA breaks. A lack of

Table 2 Evidence for DNA-protein covalent links produced by anthracycline derivatives

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (μg/ml)</th>
<th>DNA-SSB (rad-equivalents) + Proteinase K</th>
<th>DNA-SSB (rad-equivalents) - Proteinase K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>1</td>
<td>215 ± 1022</td>
<td>6 ± 8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1239 ± 251</td>
<td>117 ± 85</td>
</tr>
<tr>
<td>11-Deoxy-DNR</td>
<td>1</td>
<td>256 ± 163</td>
<td>19 ± 11</td>
</tr>
<tr>
<td>4-Demethyl-6-O-</td>
<td>0.1</td>
<td>795 ± 220</td>
<td>11 ± 16</td>
</tr>
<tr>
<td>methyl-DX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Demethoxy-11-</td>
<td>0.1</td>
<td>338 ± 98</td>
<td>130</td>
</tr>
<tr>
<td>deoxy-4'-epi-DNR</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Cells were exposed to drugs for 1 h; lysis on the filters was carried out in the presence (+) or absence (−) of proteinase K, as described in "Materials and Methods."
* Mean ± SD of 2 to 4 independent determinations.
* Single determination.

Fig. 4. Elution kinetics of DNAs from cells lysed in the presence (open symbols) or absence (closed symbols) of proteinase K. Cells were treated for 1 h at 37°C with 4-demethoxy-DNR, 0.187 μM (0), 4-demethoxy-11-deoxy-4'-epi-DNR, 1.93 μM (●), or 4-demethyl-6-O-methyl-DX, 1.72 μM (2, Δ). Elution kinetics of DNA from control untreated cells is also shown (○).

Fig. 5. Cellular drug accumulation of anthracycline derivatives. Cells were exposed to doxorubicin (●), 4-demethoxy-DNR (△), 11-deoxy-DNR (□), 4-demethoxy-11-deoxy-4'-epi-DNR (○), or 4-demethyl-6-O-methyl-DX (○) for 1 h at 37°C, and then drug uptake was measured as described in "Materials and Methods." Points, mean of 3 to 6 determinations; SDs are lower than 20%.

Fig. 6. DNA lesions induced by doxorubicin (●) and 11-deoxy-DNR (□) as a function of cellular drug uptake. See legend to Fig. 2 for details.

Fig. 7. DNA lesions induced by 4-demethoxy-DNR (△) and 4-demethoxy-11-deoxy-4'-epi-DNR (○), or 4-demethyl-6-O-methyl-DX (○) as a function of cellular drug uptake. See legend to Fig. 3 for details.
ever, this did not correlate with changes in the oxidation-cytotoxic concentrations. This observation does not rule out reduction potential. In addition, the nature of the detected doxorubicin (22). However, DNA breaks not associated with the possibility that a different (free radical-dependent) mechanism (22), were absent or undetectable under our conditions. DPC, probably produced by a free radical-dependent mechanism (2, 7, 10, 24). However, structural modifications lead to appreciable changes in drug affinity for DNA. Nevertheless, the DNA-binding parameters found for these derivatives (Table 1) were apparently unrelated to ability to induce DNA breakage. The presence of the bulky methoxy group at position 4 of the D ring of the chromophore is an important determinant of the geometry of intercalation (7). Chemical modifications involving the 4-demethoxy group are expected to alter the overlap geometry at the intercalation site (10). Since, during interference of intercalating agents with DNA topoisomerase II activity, multiple interactions involving drug, DNA, and enzyme might occur, it is possible that the geometry of intercalation, rather than the strength of intercalation with DNA, is critical for DNA strand scission. This hypothesis is supported by the effects of the 4-demethyl-6-O-methyl-DX. For this derivative, intercalation presumably involves the C and D rings (10), instead of the B and C rings as reported for natural antibiotics characterized by the presence of the C-4 methoxy group (25). Among the tested derivatives, the 4-demethyl-6-O-methyl-DX was the most effective and apparently specific in producing DNA-SSB. The change of the overlap geometry was accompanied by decreased binding affinity. This reduction did not influence cytotoxic activity of the drug, in contrast to previous observations for other derivatives (4, 6). If anthracycline antibiotics exert multiple DNA-dependent effects resulting in lethal damage, it is possible that a reduction of other (less specific) inhibition(s) of DNA functions due to reduced affinity is compensated by enhanced specificity for DNA topoisomerase II.

In general, no correlation was found between DNA-SSB and cell kill induced by anthracycline derivatives, although increased cytotoxic potency of the tested 4-demethoxy derivatives was nearly paralleled by increased production of DNA-SSB (Fig. 3), and an absence of doxorubicin-induced DNA damage was observed in doxorubicin-resistant P388 cells (14). However, a lack of precise relation between protein-associated DNA-SSB and cytotoxicity is expected, since not only production of DNA breaks, but also persistence of drug-induced effects could be relevant to cell lethality. Other authors reported no correlation between cytotoxicity and DNA-SSB using intercalating agents of different classes (18, 20). Recently, it was reported that DNA double-strand breaks correlate more precisely with cytotoxic activity of 4’-(9-acridinylamino)methanesulfon-m-anisidide and 5-imino-daunorubicin than DNA-SSB (26), although this correlation is not a general finding (20, 27).

In addition, this study shows a dramatic reduction of DNA-SSB frequency at the highest concentration of the 4-demethoxy analogues (Figs. 3 and 7). Similar results have been reported for very high concentrations of doxorubicin in hypoxic L1210 cells (22), for an anthracycline bishydrazone derivative in normally aerated L1210 cells (28), and for 2-methyl-9-hydroxyellipticinum in isolated nuclei of L1210 cells (29). Furthermore, this reduction of cellular DNA damage at high drug concentrations parallels results reported by others (30) on interference of intercalating drugs with purified mammalian DNA topoisomerase II in an in vitro system. These results show that DNA-SSB is not always necessary for anthracycline-induced cytotoxicity. However, it is possible that interference with cellular DNA topoisomerase II activity may still occur without resulting in DNA cleavage (30).

These observations suggest that multiple anthracycline-mediated mechanisms resulting in cell death may operate at the DNA level. These include intercalation-dependent aspecific inhibition of DNA functions (4, 31) and interference with specific enzymatic activities (12, 30). This interpretation is also supported by lack of correlation between cytotoxic potency and inhibitory effects on DNA synthesis produced by 4-demethoxy derivatives (31). Relevant to this point is the increased efficacy in DNA damaging of these derivatives reported in Fig. 3, as compared to natural antibiotic.

The results presented in this work support the possibility of modulating the drug interference with topoisomerase activity through structural modification in the chromophore. It is pro-

Fig. 8. Relationship between cytotoxicity and DNA-SSB frequency induced by anthracycline derivatives in P388 cells. Cells were treated for 1 h at 37°C with doxorubicin (■), 11-deoxy-DNR (○), 4-deoxymethoxy-DNR (▲), 4-deoxymethoxy-11-deoxy-4’-epi-DNR (□), or 4-demethyl-6-O-methyl-DX (●).

* S. Penco and V. Malatesta, personal communication.
posed that steric effects imposed by tetracyclic structure produce more selective interference with the enzyme than strength of interaction with DNA.

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Single-Strand DNA Breaks Induced by Chromophore-modified Anthracyclines in P388 Leukemia Cells

Giovanni Capranico, Carla Soranzo and Franco Zunino