**Relationship between Effects on Nucleic Acid Synthesis in Cell Cultures and Cytotoxicity of 4-Demethoxy Derivatives of Daunorubicin and Adriamycin**

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**SUMMARY**

Four new derivatives of daunorubicin and two new derivatives of Adriamycin characterized by the absence of the methoxyl groups at the C-4 position have been studied in cell cultures in vitro to establish structure-activity relationships. 4-Demethoxydaunorubicin was 27 to 100 times more active than was daunorubicin when inhibiting the cloning efficiency of exponential-phase HeLa cells and, like daunorubicin, was slightly active on early plateau-phase cells. DNA synthesis in mouse embryo fibroblasts stimulated by fetal calf serum was inhibited equally by the two compounds, although 4-demethoxydaunorubicin was slightly more active than was daunorubicin when inhibiting RNA synthesis. The β anomer of 4-demethoxydaunorubicin showed a reduced activity on HeLa cells compared to its α anomer, but it was equally active on DNA synthesis. The stereoisomers of 4-demethoxydaunorubicin bearing the inverted configuration in positions 7 and 9 were devoid of significant cytotoxic activity and were only slightly active on DNA synthesis at the doses tested. 4-Demethoxyadriamycin and 4-demethoxy-7,9-di-epi-adriamycin were 65 to 500 times more active than was Adriamycin on HeLa cell cloning efficiency and about 10 times more active on DNA synthesis in mouse embryo fibroblasts. Cell uptake in mouse embryo fibroblasts was also investigated for all the new derivatives tested.

**INTRODUCTION**

Previous papers from our laboratory have shown that the absence of the methoxyl group from position C-4 of the anthracycline ring of daunorubicin brings about important alterations in its biological activity in vivo (1) and minor modifications in the inhibition of DNA-dependent polymerases in cell-free systems (25). The stereoisomers of Adriamycin (4-demethoxydaunorubicin, 4′-epi-daunorubicin, and 4′-epi-adriamycin) have also been previously investigated for antitumor activity (1, 3) and biochemical properties (8, 25). Compounds with an inverted configuration in C-1′ (β anomers) had a reduced activity compared to those bearing the amino sugar in the natural configuration (α anomers). Furthermore, α and β anomers of 4-demethoxy-7,9-di-epi-daunorubicin were definitely less active than was the parent compound in cell-free systems (25) and in mice (1); their ability to bind to DNA was also strongly reduced (25). The availability of such a large group of daunorubicin and Adriamycin derivatives prompted us to investigate their effects on nucleic acid synthesis and on cell viability in in vitro cell cultures. The cellular uptake of these compounds was also investigated.

**MATERIALS AND METHODS**

**Materials.** [3H]TdR (specific activity, 56 Ci/m mole) and [3H]UdR (specific activity, 3.3 Ci/m mole) were purchased from The Radiochemical Centre, Amersham, England. Eagle's basal medium, nonessential amino acid solution, calf serum, and FCS were supplied by Grand Island Biological Co., Grand Island, N. Y. BALB/c mice were obtained from Charles River Breeding Laboratories, Calco, Italy.

**Drugs.** Daunorubicin and Adriamycin derivatives (Chart 1) were synthesized by Arcamone and coworkers (1), Farmitalia Research Laboratories, Milano, Italy. The antibiotics were stored in the dark at −20°C. Solutions in distilled water were freshly prepared immediately before use.

**Colony Inhibition Test on HeLa cells.** The colony inhibition test was carried out on HeLa cells (American Type Culture Collection Certified Cell Line 2) as previously described (3). Dose-response curves for the various drugs were obtained for cells at 2 days after (exponential phase) or 5 to 6 days after (early plateau phase) inoculation into 60-mm Falcon plastic dishes (4 × 10⁵ cells/plate) containing 5 ml of Eagle's basal medium supplemented with 1% nonessential amino acid solution, 10% calf serum, and antibiotics (100 μg each of streptomycin and kanamycin per ml and 100 units of penicillin per ml) (5, 23). Medium was changed daily from Day 2, and drugs were added directly to the growth medium approximately 24 hr after the previous medium change. At the end of the drug exposure period (2, 8, and 24 hr), the medium was removed, the monolayer was rinsed 3 times, and the cells were trypsinized from the surface. After resuspension, cell count, and dilution, the cells were plated on Falcon plastic dishes (200 cells/plate) and incubated for 6 days at 37°C in 5% CO₂. At the end of this period, the dishes were fixed and stained, and colonies containing more than 50 cells were counted.

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1 On leave from Farmitalia Research Laboratories, Milano, Italy. Received March 21, 1977; accepted September 21, 1977.

2 The abbreviations used are: [3H]TdR, [methyl-3H]thymidine; [3H]UdR, [G-3H]uridine; FCS, fetal calf serum; MEF, mouse embryo fibroblast.
Incorporation Studies in MEF's. The methods for establishment of primary cultures of BALB/c embryos (MEF's) and for stimulation by FCS have been previously described (8, 21). Cells were treated at different times after change of medium with the compounds under study and were labeled with [³H]TdR or [³H]UdR after various periods of treatment. The methods used for nucleic acid extraction and determination of radioactivity have been reported in detail previously (8, 21).

Uptake in MEF's. The intracellular uptake of the drugs was investigated in MEF cultures treated with the compounds (1.8 μM) 22 hr after medium change. At different times (15 min, 30 min, 1 hr, and 2 hr) after treatment, cells were washed twice with a 0.89% NaCl solution, trypsinized (0.2% trypsin), collected by low-speed centrifugation, and resuspended in 2 ml of a 0.89% NaCl solution. Protein content was determined by the method of Lowry et al. (12) on a 0.2-ml aliquot. For the extraction of the antibiotic, 1 ml of 1-butanol was added to 1.8 ml of cell suspension and thoroughly mixed twice. The uptake of antibiotic was determined by measurement of the pooled aliquots of 1-butanol on a spectrofluorometer at the excitation wavelength of 485 nm and the emission wavelength of 589 nm.

RESULTS

Colony Inhibition Assay on HeLa Cells. The activity of the compounds on the colony-forming ability of HeLa cells in exponential growth is shown in Table 1. For all the active compounds, the activity increased with the increase in time of cell exposure to the drugs. 4-Demethoxydaunorubicin was 27 to 100 times more active than was daunorubicin. Its β anomer showed a significantly reduced activity and was only twice as active as daunorubicin. The compounds bearing the inverted configuration in positions 7 and 9 (α and β anomers) were devoid of any activity at the doses tested. 4-Demethoxyadriamycin was 65 to 200 times more active than was Adriamycin; 4-demethoxy-4'-epi-adriamycin was 150 to 500 times more active than was Adriamycin. After a 24-hr exposure, the inhibition of HeLa cell cloning efficiency by the 4-demethoxy derivatives of daunorubicin and Adriamycin bearing the amino sugar in the natural configuration was of the same order of magnitude.

Early plateau-phase cells were much less sensitive than were cells in exponential growth. A comparison of the effect of the drugs on exponentially growing HeLa cells and on early plateau-phase HeLa cells is shown in Charts 2 and 3. Daunorubicin, Adriamycin, and the derivatives tested did not give inhibitions higher than 50% on early plateau-phase HeLa cells after 24 hr of exposure at the doses tested.

Inhibition of [³H]TdR Incorporation in MEF's. The effects of the compounds on the incorporation of [³H]TdR into...
DNA were investigated in MEF cultures stimulated by FCS. This experimental system was chosen because of the high rate of DNA synthesis induced by the addition of fresh FCS to quiescent MEF's. The highest incorporation of \(^{3}H\)TdR was reached between 20 and 26 hr after the addition of fresh serum (22). Previous studies have shown that, under our experimental conditions, DNA synthesis starts 18 hr after the addition of FCS (21).

The activities of daunorubicin, Adriamycin, and their 4-demethoxy derivatives are compared in Charts 4 and 5. In the cells exposed to the drugs and to \(^{3}H\)TdR from 18 to 24 hr after FCS stimulation, \(^{3}H\)TdR incorporation was inhibited to the same degree by daunorubicin, 4-demethoxy-daunorubicin, and the \(\beta\) anomer of 4-demethoxydaunorubicin.

In contrast, 4-demethoxy-7,9-di-epi-daunorubicin and its \(\beta\) anomer were 10 to 20 times less active. As previously reported (8), Adriamycin was less active than was daunorubicin in this experimental system when such a treatment schedule was used.

4-Demethoxyadriamycin and 4-demethoxy-4'-epi-adria-

mycin were 10 times more active than was Adriamycin; their activity was similar to that of 4-demethoxydaunorubicin (described above).

For analysis of the time dependency of the inhibiting effect on DNA synthesis, MEF cultures were treated for 8 hr with daunorubicin and 4-demethoxydaunorubicin, starting at 0, 8, and 16 hr after the addition of FCS. \(^{3}H\)TdR was added at 20 hr, and \(^{3}H\)TdR incorporation into DNA was measured 6 hr later. Table 2 shows that, as previously observed (21), daunorubicin was more active when cells were exposed to the drug for an 8-hr period starting immediately after FCS stimulation or 8 hr later than it was when treatment started 16 hr later. 4-Demethoxydaunorubicin showed a similar pattern of inhibition. The inhibition of DNA synthesis, measured at 26 hr, was slightly higher in cell cultures treated with 4-demethoxydaunorubicin from 0 to 8 hr or from 8 to 16 hr than it was in cultures similarly treated.
Effect of daunorubicin and 4-demethoxydaunorubicin on \( ^{3}H \)TdR incorporation of MEF cultures stimulated by FCS

<table>
<thead>
<tr>
<th>Time of treatment (hr)</th>
<th>Compound</th>
<th>DNA (( \mu )g)</th>
<th>cpm/( \mu )g of DNA</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-8</td>
<td>Daunorubicin</td>
<td>108,220 ± 4,453</td>
<td>27.2 ± 2</td>
<td>4,047 ± 254</td>
</tr>
<tr>
<td>4-Demethoxydaunorubicin</td>
<td>34,134 ± 1,391</td>
<td>22.0 ± 4</td>
<td>1,657 ± 389</td>
<td>41</td>
</tr>
<tr>
<td>8-16</td>
<td>Daunorubicin</td>
<td>15,490 ± 1,917</td>
<td>22.0 ± 0.5</td>
<td>706 ± 103</td>
</tr>
<tr>
<td>4-Demethoxydaunorubicin</td>
<td>115,207 ± 2,354</td>
<td>28.0 ± 0.5</td>
<td>4,114 ± 10</td>
<td>100</td>
</tr>
<tr>
<td>16-24</td>
<td>Daunorubicin</td>
<td>21,512 ± 185</td>
<td>24.0 ± 0.5</td>
<td>887 ± 15</td>
</tr>
<tr>
<td>4-Demethoxydaunorubicin</td>
<td>11,982 ± 2,451</td>
<td>26.0 ± 0.5</td>
<td>468 ± 109</td>
<td>11</td>
</tr>
<tr>
<td>0-8</td>
<td>Daunorubicin</td>
<td>108,892 ± 1,914</td>
<td>30.0 ± 1.5</td>
<td>3,669 ± 151</td>
</tr>
<tr>
<td>4-Demethoxydaunorubicin</td>
<td>71,371 ± 2,771</td>
<td>24.7 ± 3</td>
<td>2,949 ± 199</td>
<td>80</td>
</tr>
</tbody>
</table>

*Mean ± S.E.

**DISCUSSION**

Anthracyclic antibiotics showing antitumor activity, such as daunorubicin and Adriamycin, bind to DNA and inhibit nucleic acid synthesis (7). In cell-free systems, a strict relationship is evident between the inhibition of DNA and RNA polymerases and the DNA-binding ability, measured as changes in melting temperature of double-helical DNA (\( \Delta T_m \)) or by calculation of the affinity to DNA (\( K_{ap} \)) (7). Processes such as \( ^{3}H \)TdR incorporation into DNA, \( ^{3}H \)UdR incorporation into RNA in cultured cells, and cell viability are sensitive to lower drug concentrations than those in cell-free systems (6). This poses the question of whether the inhibiting effect on nucleic acid synthesis in vivo and cell killing may be simply related to each other and to the template inactivation.

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4-Demethoxy Derivatives of Daunorubicin and Adriamycin

Previously reported structure-activity studies showed that derivatives of daunorubicin and Adriamycin that had a reduced ability to bind to DNA were less active than were the parent compounds in vitro and in vivo (6). From the analysis of structure-activity relationships of the 4-demethoxy analogs of daunorubicin and Adriamycin, it appears that cell killing is more strictly related to DNA-binding ability than it is to the effect on DNA synthesis. In fact, daunorubicin, 4-demethoxydaunorubicin and its β anomer, 4-demethoxyadriamycin, and 4-demethoxy-4'-epi-adriamycin, added when DNA synthesis had already started, exerted a similar quantitative inhibition of DNA synthesis in MEF’s stimulated by FCS. These results do not correlate with a previous report that 4-demethoxydaunorubicin (α and β anomers) exerts a greater effect than does daunorubicin in stabilizing the DNA double helix to heat denaturation (Δ Tm), nor do they correlate with the higher activity of daunorubicin and 4-demethoxydaunorubicin versus the β anomer of 4-demethoxydaunorubicin on nucleic acid-polymerizing enzymes (25). On the contrary, with regard to inhibition of colony formation by HeLa cells treated for 24 hr in the exponential-growth phase, the following order of decreasing effect was observed: 4-demethoxy-4'-epi-adriamycin > 4-demethoxyadriamycin > 4-demethoxydaunorubicin > 4-demethoxydaunorubicin (β anomer) > daunorubicin > Adriamycin. Therefore, the greater killing effect of 4-demethoxy derivatives on HeLa cells in vitro, which correlates well with the previously reported biological activity in mice (1), cannot be explained on the basis of the observed inhibition of nucleic acid synthesis in cell cultures. 4-Demethoxydaunorubicin was slightly more active than was daunorubicin on DNA synthesis when added soon after the addition of serum; the quantitative difference observed, although statistically significant, was not very pronounced. This difference was in agreement with the higher inhibition of RNA synthesis and suggests that the cell killing effect may be ascribed to an early event not coincident with DNA synthesis that is expressed later in the cell cycle also as inhibition of this process. These findings could be related to the fact that these drugs are not strictly phase specific (11), and they support the hypothesis that 1 of the main biochemical effects of anthracycline antibiotics is to promote changes in DNA structure. In fact, daunorubicin and Adriamycin cause early and severe chromosomal aberrations (13, 20, 24) and produce extensive fragmentation of cellular DNA (18, 19).

That the ability to bind to DNA is an important mechanism for these antibiotics is shown by the results obtained with the 7R-9R stereoisomers of 4-demethoxydaunorubicin. Both the α and β anomers showed a reduced ability to bind to DNA, a reduced effect on nucleic acid polymerases in cell-free systems (25), a reduced inhibition of DNA synthesis, no effect on cell viability, and no antitumor activity at all (1), even if cellular uptake was very pronounced.

The cell uptake of anthracyclines has been considered in an effort to explain their biological activity. (4, 6). The 4-

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Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>1-Butanol/Tris-HCl buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daunorubicin</td>
<td>16.2</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>6.4</td>
</tr>
<tr>
<td>4-Demethoxydaunorubicin</td>
<td>32.3</td>
</tr>
<tr>
<td>4-Demethoxydaunorubicin (β anomer)</td>
<td>11.5</td>
</tr>
<tr>
<td>4-Demethoxy-7,9-di-epi-daunorubicin(*)</td>
<td></td>
</tr>
<tr>
<td>4-Demethoxy-7,9-di-epi-daunorubicin (β anomer)</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Drug concentrations were determined spectrophotometrically (see "Materials and Methods").
demethoxyderivatives of daunorubicin that showed a higher affinity for lipids, as substantiated by their 1-butanol/buffer partition coefficient, were taken up in a great amount by cells in vitro, probably as a consequence of this property. In parallel, 4-demethoxy derivatives of Adriamycin also reached higher intracellular concentrations than did Adriamycin.

These data show that 1 of the pharmacological properties of anthracyclines may be relevant to biological activity, such as cell uptake, can be markedly changed by modifications in positions 4, 7, 9, and 1' of the molecule, in addition to positions 13 and 14, which have been previously investigated (2, 4). The data regarding the uptake of the drug by the cell in vivo give only partial information, inasmuch as daunorubicin and Adriamycin have a different intracellular distribution (17). 4-Demethoxy derivatives, on the basis of their physicochemical properties, could very well have an intracellular distribution markedly different from that of the parent compounds, particularly Adriamycin, that could lead to different effects.

The inhibition by anthracyclines of processes not directly connected with DNA synthesis, but essential for cell life and replication, has been described. It has been recently shown that Adriamycin is active on Na⁺-K⁺-dependent ATPase (9), inhibits coenzyme Q₁₀ enzymes (10), alters the level of cardiac cell membrane sites to which ouabain binds (16), and causes a marked increase in the number and total extension of the gap junctions (15). These findings should be taken into account when investigating the antimicrobial activity and toxic effects of these drugs.

REFERENCES


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