Differential Effects of Anthracycline Drugs on Rat Heart and Liver Microsomal Reduced Nicotinamide Adenine Dinucleotide Phosphate-dependent Lipid Peroxidation


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ABSTRACT

Rat liver microsomes have previously been utilized as a model biological system to study the activation of Adriamycin to the semiquinone free radical intermediate and the enhancement by Adriamycin of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation and oxygen consumption. Incubating rat liver microsomes with Adriamycin or other structurally similar benzanthraquinone anticancer drugs resulted in marked stimulation of NADPH-dependent lipid peroxidation. Carminomycin and 4-demethoxydaunorubicin were considerably more potent than Adriamycin, while daunorubicin, deacetyl Adriamycin, N-demethyl-adriamycin, aclacinomycin A, adriamycin 14-nicotinate, and steffimycin were approximately equipotent to Adriamycin in this test system. In contrast, lapachol, anthragallol, alkylaminoanthracenedione, mitomycin C, streptonigrin, 5-iminodaunorubicin, and the Adriamycin:DNA complex potentially inhibited microsomal lipid peroxidation. Methotrexate, cyclophosphamide, 5-fluorouracil, nogalamycin, or rubidazone had little or no effect. α-Tocopherol-deficient rat heart microsomes but not control heart microsomes were susceptible to increased NADPH-dependent lipid peroxidation (up to 10-fold) when incubated with Adriamycin or other anthracycline analogs which stimulated peroxidation in liver microsomes. The rate of NADPH oxidation in the presence of microsomes was enhanced 4-fold by either Adriamycin or daunorubicin; lapachol, anthragallol, aminoanthracenedione, and 5-iminodaunorubicin at concentrations which inhibited lipid peroxidation had no effect on NADPH oxidation. NADPH:citochrome P-450 reductase activity was unaltered by those drugs which either stimulated or inhibited peroxidation. These results suggest that although several Adriamycin-like anthracycline drugs dramatically stimulated NADPH-dependent, reactive oxygen-mediated lipid peroxidation in rat liver and heart microsomes, other quinone-containing anticancer drugs do not enhance lipid peroxidation. Interestingly, there appears to be a direct correlation between the ability of certain anthracyclines to stimulate heart or liver microsomal lipid peroxidation and the effects of these drugs in other cardiotoxicity model systems.

INTRODUCTION

The use of the anthracycline antineoplastic agents Adriamycin and daunorubicin has been restricted because of clinical and histopathological demonstrable dose-limiting cardiotoxicity. The delayed cardiomyopathy that may develop over a lengthy course of Adriamycin therapy is manifested by the onset of insidious, intractable, sometimes fatal congestive heart failure (29). The incidence of cardiotoxicity increases abruptly in patients who have received a total cumulative dose exceeding 550 mg/sq m (7). Recent investigations into the molecular pharmacology of Adriamycin and daunorubicin have revealed that these anthracyclines accept a single electron from microsomal NADPH:cytochrome P-450 reductase (EC 1.6.2.4) to form semiquinone free radical intermediates (2, 13, 32) which in turn transfer electrons to oxygen, reducing molecular oxygen to superoxide anion (3, 13). One of the results of Adriamycin free radical formation and enhanced superoxide production is a dramatic stimulation of NADPH-dependent heart and liver microsomal lipid peroxidation (27). The potential cytotoxic effects of lipid peroxidation include disintegration of endoplasmic reticulum and mitochondrial membranes (15, 31), inhibition of vital enzymes (26, 31), and damage to nucleic acid (36). In comparison, the morphological changes induced by anthracycline antineoplastic drugs in cardiac myocytes include vacuolization, mainly due to sarcoplasmic reticulum swelling, mitochondrial pleomorphism and degeneration, myofibrillar lysis, and transient nucleolar segregation (11, 17). Several reports have implicated lipid peroxidation in vivo as a possible mechanism of Adriamycin myocardial damage in experimental animals (12, 30), and these reports have generated speculation that drug-stimulated lipid peroxidation may be a major toxicogenic event in human anthracycline-induced cardiomyopathy.

Currently, a number of anthracycline drugs are being evaluated clinically with the intention of discovering an Adriamycin analog with both therapeutic superiority and diminished cardiotoxicity. Preclinical toxicity screening of the numerous Adriamycin analogs (>300) in a predictive animal model system would greatly aid oncologists in developing toxicological evaluation strategy prior to using these new drugs in humans. Since lipid peroxidation may be relevant to human anthracycline-induced cardiotoxicity, a comparison of the capability of anthracyclines to stimulated microsomal lipid peroxidation could provide a valuable means to assess their cardiotoxic potential and provide preclinical toxicological predictability. To this end, we have extended our previous studies on Adriamycin-augmented microsomal lipid peroxidation (27) to include selected anthracycline analogs and other quinone-containing compounds (Chart 1).

MATERIALS AND METHODS

Chemicals and Drugs. NADPH (type III), NADP+, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type VII), MgCl2, bovine serum albumin, and 2-thiobarbituric acid were purchased from Sigma...
Adriamycin Analogs and Lipid Peroxidation

Chart 1. Structures of most of the drugs used in this study. Note the structural similarity of many of the drugs to Adriamycin and daunorubicin.
RESULTS

Differential Effects of Quinone-containing Drugs on Liver and Heart Microsomal Lipid Peroxidation. Many compounds used in this study are fermentation products or semisynthetic analogs similar in structure to the parent drugs Adriamycin and daunorubicin; others are synthetic drugs which substantially differ in structure from the parent anthracyclines (Chart 1). Most compounds were selected on the basis that they contain a quinone moiety which could potentially be converted to a semiquinone free radical by microsomal NADPH:cytochrome P-450 reductase and undergo redox cycling resulting in enhanced reactive oxygen production. We have previously established optimum incubation conditions for demonstrating stimulation of reactive oxygen-dependent microsomal lipid peroxidation by redox cycling compounds (27, 42).

Rat liver microsomes are especially rich in NADPH:cytochrome P-450 reductase activity and have therefore been used extensively as an in vitro model biological system to investigate the NADPH-dependent activation of Adriamycin and Adriamycin analogs to semiquinone free radicals (2, 12, 32), Adriamycin-dependent superoxide formation (13), enhanced oxygen consumption (2, 3), and the stimulation by Adriamycin of microsomal lipid peroxidation (12, 27). When liver microsomes were incubated with Adriamycin, daunorubicin, or other benzanthraquinone analogs in the presence of an NADPH-generating system and an oxygen atmosphere, concentration-dependent 4- to 5-fold increases in the endogenous rate of lipid peroxidation were measured (Charts 2 to 4). The drug-mediated enhancement of microsomal peroxidation was maximal at drug concentrations in the range of 100 to 200 μM for most compounds tested. However, 4-demethoxydaunorubicin and carminomycin were noteworthy in that they produced maximal augmentation of peroxidation at lower concentrations (30 to 50 μM) than did Adriamycin (Chart 3). Moreover, the concentration-effect curves for these 2 Adriamycin analogs were sharply

- Adriamycin
- 4-dehydrodaunorubicin
- 4-demethoxydaunorubicin
- Adriamycin O-demethylcarboxylic acid
- Adriamycin O-carboxylic acid
- Adriamycin O-acetic acid
- Adriamycin O-carboxylic acid
- Adriamycin O-acetic acid

Chart 2. Concentration-dependent effects of Adriamycin (A), daunorubicin (D), and N-dimethyladriamycin (C) on rat liver microsomal lipid peroxidation expressed as nmol malonaldehyde equivalents per mg protein per 60 min. Data are expressed as means for 3 to 6 samples. *, values significantly different from endogenous peroxidation rates (p < 0.05).
biphasic and unexpectedly demonstrated potent inhibition of peroxidation at drug concentrations at or above 100 μM. As shown in Tables 1 and 2, the Adriamycin- and daunorubicin-like benzanthraquinones deacetyladriamycin, N-demethyl-adriamycin, aclacinomycin A, adriamycin 14-nicotinate, and steffimycin also augmented liver microsomal lipid peroxidation severalfold (compare chemical structures shown in Chart 1).

When NADPH (2.0 mM) was substituted for the NADPH-generating system, the extent of endogenous lipid peroxidation was less but nonetheless capable of being stimulated as much as 7-fold by Adriamycin, daunorubicin, or several other analogs (see chemical structures shown in Chart 1). The resistance of control rat heart microsomes to undergo lipid peroxidation has been reported previously (21, 27), and the high content of membranous α-tocopherol accounts for the resistance of heart microsomes to peroxidation (21). On the other hand, after lowering the level of α-tocopherol in heart microsomes by feeding animals a diet deficient in this vitamin, considerable lipid peroxidation occurs (27) (also see below).

In contrast to liver microsomes, heart microsomes from control rats did not peroxidize well even in the presence of drugs (Table 1). The resistance of control rat heart microsomes to endure lipid peroxidation has been reported previously (21, 27), and the high content of membranous α-tocopherol accounts for the resistance of heart microsomes to peroxidation (21). On the other hand, after lowering the level of α-tocopherol in heart microsomes by feeding animals a diet deficient in this vitamin, considerable lipid peroxidation occurs (27) (also see below).

Not all quinone-containing drugs stimulated lipid peroxidation in liver microsomes. For example, peroxidation was strongly inhibited by lapachol, anthragallol, and the N-heterocyclic quinones mitomycin C and streptogenin (Charts 3 and 5; Tables 1 and 2). Bisalkylaminoanthracenedione and dihydroxyminoanthracenedione, drugs lacking sugar moieties and having alkyl substituents, inhibited peroxidation at rather low concentrations (1 to 10 μM), as did the imidoquinone 5-imino-

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**Figure 3.** Effects of 4-demethoxydaunorubicin (4-demethoxy daunorubicin) (•), carminomycin (☐), and mitomycin C (☐) on liver microsomal lipid peroxidation. The effects of Adriamycin (☐) are included for comparison. Note the abscissa log scale. Statistical evaluation as in Chart 2.

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**Figure 4.** Effects of deacetyladriamycin (desacetyl Adriamycin) (☐), adriamycin 14-nicotinate (☐), and aclacinomycin A (☐) and lack of effect of methotrexate (☐) and cyclophosphamide (☐) on liver microsomal lipid peroxidation. Note the absence of the pronounced biphasic effect with the first 3 drugs that was especially prominent with Adriamycin and carminomycin. Statistical evaluation as in Chart 2.

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**Figure 5.** Rubidazone and nogalomyacin, 2 Adriamycin analogs with bulky hydrophobic substituents, had little effect on lipid peroxidation. The Adriamycin:DNA complex inhibited lipid peroxidation by about 70%, most likely because the Adriamycin was sequestered from microsomal cytochrome P-450 reductase by intercalation of the drug between the base pairs of DNA. Calf thymus DNA (1.28 mg/ml) added to incubation mixtures just prior to Adriamycin diminished the stimulation of peroxidation by Adriamycin by more than 50%, suggesting that DNA-bound Adriamycin could not redox cycle to generate reactive oxygen or, alternatively, that reactive oxygen preferentially interacted with the DNA instead of lipids.

**Figure 6.** Quelamycin (Adriamycin complexed with 2 to 3 equivalents of iron) appeared to stimulate peroxidation dramatically at concentrations as low as 10 μM; however, it is impossible to assess the amount of peroxidation that may have been due to the presence of free or even bound iron in quelamycin. Exogenous ferrous iron (10 μM) stimulated the initial rate of NADPH-supported rat liver microsomal lipid peroxidation nearly 30-fold. At the same time, Adriamycin (100 μM) had no effect on either the time course or extent of ferrous iron-promoted microsomal peroxidation. Thus, the stimulation of lipid peroxidation by quelamycin is difficult to evaluate.

Several class-representative anticancer drugs that do not contain a quinone group (e.g., methotrexate, cyclophosphamide, and 5-fluorouracil) had no effect on liver microsomal lipid peroxidation (Chart 4; Table 2). It has been reported previously (3) that methotrexate and cyclophosphamide likewise had no effect on NADPH-dependent oxygen consumption catalyzed by rat liver microsomes. These results suggest that as a class of chemical compounds the benzanthraquinones, particularly those with a B-ring hydroquinone which is capable of electron resonance with the adjacent C-ring quinone moiety to form tautomeric isomers, are...
Effects of additional anticancer drugs on rat liver microsomal lipid peroxidation

Drugs were added to microsomes and incubated as described in Table 1. Lipid peroxidation was measured by the thiobarbituric acid method.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control liver</th>
<th>α-Tocopherol-deficient liver</th>
<th>Control heart</th>
<th>α-Tocopherol-deficient heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>19.0 ± 5.6°</td>
<td>88.8 ± 17.4</td>
<td>0.73 ± 0.55</td>
<td>3.86 ± 0.55</td>
</tr>
<tr>
<td>Adriamycin (100 μM)</td>
<td>77.5 ± 7.3°</td>
<td>107 ± 4.0</td>
<td>0.39 ± 0.30</td>
<td>30.9 ± 0.7°</td>
</tr>
<tr>
<td>Daunorubicin (200 μM)</td>
<td>83.4 ± 15.8°</td>
<td>89.9 ± 2.8</td>
<td>1.03 ± 0.77</td>
<td>46.2 ± 7.2°</td>
</tr>
<tr>
<td>Carminomycin (30 μM)</td>
<td>63.1 ± 5.9°</td>
<td>129 ± 3.1°</td>
<td>1.41 ± 0.88</td>
<td>25.2 ± 2.0°</td>
</tr>
<tr>
<td>4-Demethoxydaunorubicin (25 μM)</td>
<td>91.2 ± 2.4°</td>
<td>114 ± 7.5</td>
<td>1.90 ± 1.48</td>
<td>20.8 ± 0.7°</td>
</tr>
<tr>
<td>5-iminodaunorubicin (10 μM)</td>
<td>0.88 ± 0.12°</td>
<td>59.3 ± 10.8</td>
<td>0.47 ± 0.18</td>
<td>6.71 ± 2.11</td>
</tr>
<tr>
<td>Dihydroxyaminoanthracenedione (10 μM)</td>
<td>1.18 ± 0.44°</td>
<td>2.77 ± 0.68°</td>
<td>0.63 ± 0.08</td>
<td>0.0 ± 0.0°</td>
</tr>
<tr>
<td>Mitomycin C (50 μM)</td>
<td>5.36 ± 3.13°</td>
<td>94.7 ± 5.0</td>
<td>0.01 ± 0.01°</td>
<td>6.10 ± 1.07</td>
</tr>
<tr>
<td>Methotrexate (100 μM)</td>
<td>16.8 ± 9.2</td>
<td>73.9 ± 11.7</td>
<td>0.35 ± 0.16</td>
<td>6.01 ± 3.06</td>
</tr>
<tr>
<td>Cyclophosphamide (100 μM)</td>
<td>11.9 ± 7.0</td>
<td>111 ± 8.3</td>
<td>0.39 ± 0.11</td>
<td>4.46 ± 0.88</td>
</tr>
</tbody>
</table>

° Concentrations given are those which produced maximal stimulation or the lowest concentrations which produced marked inhibition.  
° Mean ± S.D.; N = 3 to 6 except for the control (no drug) value where N = 18.  
° Significantly different (p < 0.05) from the control value.  
° Significantly different (p < 0.05) from values without added drugs.

**Table 2**

Effects of additional anticancer drugs on rat liver microsomal lipid peroxidation

Drugs were added to microsomes and incubated as described in Table 1. Lipid peroxidation was measured by the thiobarbituric acid method.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Malonaldehyde equivalents (nmol/mg protein/60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20.8 ± 5.9°</td>
</tr>
<tr>
<td>Deacetyladiamycin (200 μM)</td>
<td>91.2 ± 2.4°</td>
</tr>
<tr>
<td>N-Dimethyladiamycin (200 μM)</td>
<td>71.8 ± 19.5°</td>
</tr>
<tr>
<td>Aclacinomycin A (200 μM)</td>
<td>70.9 ± 4.7°</td>
</tr>
<tr>
<td>Adriamycin 14-nicotinate (200 μM)</td>
<td>58.9 ± 13.7°</td>
</tr>
<tr>
<td>Quelamycin (10 μM)</td>
<td>102 ± 2.0°</td>
</tr>
<tr>
<td>Steffimycin (100 μM)</td>
<td>74.1 ± 18.1°</td>
</tr>
<tr>
<td>Adriamycin:DNA complex (1.28 mg/ml)</td>
<td>7.75 ± 2.08°</td>
</tr>
<tr>
<td>Nogalamycin (100 μM)</td>
<td>15.4 ± 9.8°</td>
</tr>
<tr>
<td>Rubidazole (100 μM)</td>
<td>31.8 ± 18.1°</td>
</tr>
<tr>
<td>Streptonigrin (50 μM)</td>
<td>2.46 ± 1.12°</td>
</tr>
<tr>
<td>Aminoanthracenedione (10 μM)</td>
<td>0.30 ± 0.33°</td>
</tr>
<tr>
<td>Lapachol (5 μM)</td>
<td>0.91 ± 0.14°</td>
</tr>
<tr>
<td>Anthragallol (5 μM)</td>
<td>0.90 ± 0.20°</td>
</tr>
<tr>
<td>5-Fluorouracil (100 μM)</td>
<td>14.8 ± 8.5°</td>
</tr>
</tbody>
</table>

° Concentrations given are those which produced maximal stimulation or the concentrations which produced marked inhibition.  
° Mean ± S.D.; N = 3 to 6 except for the control (no drug) value where N = 18.  
° Significantly different (p < 0.05) from the control value.  
° Significant difference (p < 0.05) from control values.

**Chart 5.** Inhibitory effects of lapachol (●), aminoanthracenedione (○), and 5-iminodaunorubicin (□) on liver microsomal lipid peroxidation. Note the abscissa/log scale. In this experiment, endogenous peroxidation was 15.6 ± 3.8 nmol malonaldehyde equivalents per mg protein per 60 min. It can be seen that lapachol and aminoanthracenedione were approximately 10 times more potent inhibitors than was 5-iminodaunorubicin. Statistical evaluation as in Chart 2.

Capable of strongly stimulating microsomal NADPH:cytochrome P-450 reductase-dependent lipid peroxidation. Structurally simpler naphthoquinones like lapachol and anthraquinones like anthragallol and anthrarufin inhibit lipid peroxidation by a potent yet unresolved mechanism. In further support of this concept, we found that menadione, a naphthoquinone structurally similar to lapachol and a known inhibitor of peroxidation (38), decreased endogenous microsomal lipid peroxidation in our system by 95% when added to incubations at 10 μM (data not presented). It is also possible that the daunorubicin sugar side chain has a role in determining the ability of the quinone-containing drugs to stimulate lipid peroxidation, perhaps by stabilizing the interaction of the anthracycline drugs with the flavin mononucleotide of cytochrome P-450 reductase (19). This provocative hypothesis is currently being investigated further in our laboratory.

**Stimulation of Lipid Peroxidation by Anthracyclines in α-Tocopherol-deficient Rat Heart Microsomes.** Many of the Adriamycin analogs tested in this study stimulated lipid peroxidation severalfold in rat liver microsomes. On the other hand, control rat heart microsomes were refractory to drug-stimulated peroxidation (Table 1). Since rat heart microsomes have from 2 (21) to 4 (39) times as much α-tocopherol as do liver microsomes, it is plausible that the relatively high concentration of α-tocopherol in cardiac microsomes controlled lipid peroxi-
oxidation by virtue of its antioxidant property (47). To test this hypothesis, we isolated heart microsomes from α-tocopherol-deficient rats and found that the endogenous peroxidation in heart microsomes from α-tocopherol-deficient animals was 5 times greater than was the peroxidation measured in heart microsomes from control animals. Moreover, lipid peroxidation in heart microsomes from α-tocopherol-deficient animals was increased as much as 10-fold by adding Adriamycin, daunorubicin, camptothecin, or 4-demethoxydaunorubicin (Table 1).

Methotrexate, 5-iminodaunorubicin, mitomycin C, or cyclophosphamide had no significant effects on peroxidation in α-tocopherol-deficient heart microsomes; dihydroxyaminonanthracenedione was strongly inhibitory (Table 1). Thus, it appears that those drugs which stimulate rat liver microsomal peroxidation are also capable of stimulating rat heart microsomal lipid peroxidation, provided the concentration of the membrane antioxidant α-tocopherol is sufficiently diminished.

Effects of Quinone-containing Drugs on NADPH Oxidation and NADPH-dependent Cytochrome c Reduction. Since aminonanthracenedione, anthragallol, lapachol, and 5-iminodaunorubicin potentely inhibited NADPH-dependent microsomal lipid peroxidation, we investigated the effects of these drugs on the rates of NADPH oxidation and NADPH-dependent cytochrome c reduction (an estimate of NADPH-cytochrome P-450 reductase activity) in the presence of microsomes. Under conditions where Adriamycin or daunorubicin enhanced NADPH oxidation by about 4-fold, lapachol, anthragallol, aminonanthracenedione, or 5-iminodaunorubicin had little effect on microsomal NADPH oxidase activity when added at drug concentrations (10 μM) which inhibited lipid peroxidation by greater than 90% (Table 3). However, higher concentrations (50 to 100 μM) of these drugs slightly stimulated NADPH oxidation (20 to 60%). None of the drugs at the concentrations tested significantly altered the rate of microsomal NADPH:cytochrome P-450 reductase-dependent cytochrome c reduction, even in the presence of superoxide dismutase, which would prevent reduction of cytochrome c by superoxide anion generated during semiquinone autoxidation (Table 3). Superoxide dismutase slightly inhibited (10%) the endogenous cytochrome P-450 reductase activity; however, this inhibition was not enhanced in the presence of Adriamycin, suggesting that most of the electrons passed directly from NADPH to cytochrome c with little reduction of molecular oxygen to superoxide. Cytochrome c is a well-known inhibitor of NADPH-dependent microsomal lipid peroxidation (46), presumably because it limits reactive oxygen production. It should be noted, however, that these experimental results do not rule out the possibility of direct cytochrome c reduction due to drug semiquinone free radicals.

DISCUSSION

The data presented in this study affirm the hypothesis that microsomal NADPH:cytochrome P-450 reductase catalyzes the formation of anthracycline semiquinone radicals and that these radicals are capable of reducing oxygen to superoxide and other species of reactive oxygen which greatly enhance NADPH-dependent microsomal lipid peroxidation. Quinone-containing, Adriamycin-like drugs augmented reactive oxygen-dependent peroxidation in heart microsomes as well as liver microsomes, provided that the concentration of the endogenous membrane antioxidant α-tocopherol was sufficiently diminished. The important role of α-tocopherol in controlling microsomal peroxidation is demonstrated by the fact that heart or liver microsomes from α-tocopherol-deficient rats peroxidize more than 4 times faster than do microsomes from control rats (Table 1). Furthermore, we have observed that heart microsomes from control mice, which apparently have lower α-tocopherol concentrations than do rat heart microsomes (21), undergo considerable endogenous NADPH-dependent lipid peroxidation which is stimulated as much as 10- to 12-fold by Adriamycin and several other anthracyclines (28). Based upon the above and other observations, we believe it is reasonable to suggest that either liver microsomes or α-tocopherol-defi-

<table>
<thead>
<tr>
<th>Lipid peroxidation (nmol malondialdehyde equivalents/mg protein/60 min)</th>
<th>NADPH oxidation (nmol/mg protein/min)</th>
<th>NADPH-cytochrome P-450 reductase activity (nmol cytochrome c reduced/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20.1 ± 8.3 b</td>
<td>7.46 ± 0.64</td>
</tr>
<tr>
<td>Adriamycin (100 μM)</td>
<td>65.9 ± 18.9 c</td>
<td>28.5 ± 2.5 c</td>
</tr>
<tr>
<td>Daunorubicin (100 μM)</td>
<td>79.6 ± 8.4 c</td>
<td>27.3 ± 2.6 c</td>
</tr>
<tr>
<td>5-Iminodaunorubicin (10 μM)d</td>
<td>1.16 ± 0.74c</td>
<td>7.34 ± 0.25</td>
</tr>
<tr>
<td>Lapachol (10 μM)</td>
<td>0.60 ± 0.35c</td>
<td>6.68 ± 0.40</td>
</tr>
<tr>
<td>Anthragallol (10 μM)</td>
<td>0.84 ± 0.25c</td>
<td>7.84 ± 0.41</td>
</tr>
<tr>
<td>Aminonanthracenedione (10 μM)</td>
<td>0.92 ± 0.26c</td>
<td>7.99 ± 0.55</td>
</tr>
</tbody>
</table>

* Superoxide dismutase (4 × 10⁻³ M) had no significant effect on the rate of cytochrome c reduction in the absence or presence of Adriamycin (100 μM). Control activity (nmol cytochrome c reduced per mg protein per min), 331 ± 26; plus superoxide dismutase, 296 ± 34. Activity in the presence of Adriamycin (nmol cytochrome c reduced per mg protein per min) (100 μM), 327 ± 23; plus superoxide dismutase 309 ± 44.

** Mean ± S.D.; N = 4 to 8.

c Significantly different (p < 0.05) from values without the test drug.

d 5-Iminodaunorubicin at 100 μM, however, significantly inhibited (p < 0.05) cytochrome c reduction (cytochrome c reduced, 264 ± 11 nmol/mg protein/min).
ciant heart microsomes provide a convenient, suitable model biological system that can be used to assess the ability of anthracycline drugs to enhance reactive oxygen-dependent membrane lipid peroxidation. In addition, results obtained using rat liver microsomes accurately predict qualitative results for the heart microsomal test system.

Quinoid anticancer drugs which function efficiently as single-electron shunting agents and dramatically stimulate lipid peroxidation in microsomes are, in general, stereochemically similar to Adriamycin and daunorubicin (Chart 1). Furthermore, they retain the lipophilic and electronic character of Adriamycin since they differ from the parent compound by only minor modifications of functional groups; however, they retain the requisite quinone moiety. For example, deacetyladriamycin, N-demethyladriamycin, adriamycin nicotinate, steffimycin, and aclacinomycin A all stimulated peroxidation in similar concentration-dependent fashions with maximal effects in the range of 100 to 200 μM. However, not all quinone-containing analogs of Adriamycin stimulated microsomal lipid peroxidation, revealing that other chemical requirements were obligatory.

Two benzanthraquinone analogs of Adriamycin, nogalamycin and rubidazone, which structurally resemble Adriamycin in the vicinity of the quinone moiety, had little effect on microsomal lipid peroxidation. A likely explanation is that the bulky, lipophilic substituents on nogalamycin and rubidazone sterically hindered the proper interaction of the drugs with microsomal NADPH:cytochrome P-450 reductase and therefore efficient redox cycling of the drugs could not occur. Bachur et al. (4) found that rubidazone and nogalamycin produced only marginal stimulation of oxygen consumption by liver microsomes relative to Adriamycin. Consequently, nogalamycin and rubidazone would not be expected to markedly stimulate microsomal lipid peroxidation given the limited ability of these drugs to enhance the enzymatic reduction of molecular oxygen to superoxide anion.

In contrast to the severalfold stimulation of lipid peroxidation by the Adriamycin-like benzanthraquinone drugs, the structurally simpler naphthoquinones like lapachol and anthraquinones like anthragallol and aminoanthracenedione potently inhibited microsomal peroxidation even though they possess the quinone moiety required for redox cycling. These drugs are structurally and chemically much different from Adriamycin; most conspicuously, they lack glycosidic side chains (daunosamine), and a saturated ring corresponding to the A-ring of Adriamycin. Consequently, nogalamycin and rubidazone would not be expected to markedly stimulate microsomal lipid peroxidation given the limited ability of these drugs to enhance the enzymatic reduction of molecular oxygen to superoxide anion.

The mechanism by which drugs like aminoanthracenedione, lapachol, and anthragallol inhibit microsomal lipid peroxidation remains elusive. These agents had no effect on microsomal catalyzed NADPH oxidation at a drug concentration (10 μM) which potently inhibited peroxidation, suggesting that the transfer of electrons from NADPH to the flavin moiety of cytochrome P-450 reductase was unimpeded by the presence of the drugs. Indeed, lapachol, anthragallol, and aminoanthracenedione at 50 to 100 μM concentrations slightly stimulated NADPH oxidation. Likewise, the rate of cytochrome c reduction by microsomes was not decreased by any of the lip peroxidation-inhibiting drugs, indicating that electron flow from the reductase to the electron acceptor cytochrome c was not blocked. In support of this contention, Bachur et al. (3) found that lapachol had little effect on NADPH-dependent oxygen consumption or NADPH oxidation in the presence of rat liver microsomes. Thus, it appears that the mechanism of drug inhibition of microsomal lipid peroxidation does not involve interruption of electron flow through cytochrome P-450 reductase. The inhibition may be mediated by efficient radical quenching reactions such as drug radical dismutation or dimerization combination or by preventing hydroxyl radical generation by chelating endogenous microsomal iron, since metal ions appear to have an important role in drug-stimulated NADPH-dependent microsomal lipid peroxidation (27, 42).

A final possibility is that the quinone moiety of certain drugs may have been easily reduced by 2 electrons to the quinol form since microsomal NADPH:cytochrome P-450 reductase can catalyze the reduction of 2-electron as well as one-electron acceptor substrates (16). Quinols formed by the 2-electron reduction of quinone-containing drugs could subsequently reduce oxygen preferentially to hydrogen peroxide with little or no production of superoxide anion. Hydrogen peroxide would be quickly scavenged by peroxisomal or adventitious catalase, a ubiquitous contaminant in microsomes (44), thus preventing lipid peroxidation.

The inhibitory effect of 2 other drugs on lipid peroxidation should be briefly discussed. The reduced form of 5-iminodaunorubicin is relatively stable because of intramolecular hydrogen bonding (41) and thereby resists redox cycling, resulting in a diminished ability to generate superoxide anion (24). Therefore, 5-iminodaunorubicin may inhibit lipid peroxidation by impeding the enzyme-catalyzed transfer of electrons from NADPH to molecular oxygen. Likewise, the Adriamycin-DNA intercalated complex apparently does not efficiently redox cycle to generate superoxide in the presence of microsomes and NADPH (18). The addition of DNA to incubations containing microsomes, NADPH, and Adriamycin causes a quenching of the Adriamycin semiquinone-radical electron spin resonance signal (34). Thus, at least in vitro, DNA would be expected to decrease the stimulation of lipid peroxidation by Adriamycin. These observations do not rule out the possibility that intercalative or nonintercalative binding of Adriamycin to DNA could divert electrons or reactive oxygen away from lipids toward the nucleic acid. Indeed, recently, Adriamycin has been shown to produce oxygen-mediated DNA cleavage resulting from the conversion of Adriamycin to a semiquinone free radical by purified rat liver NADPH-cytochrome P-450 reductase (5).

In vivo, a number of pharmacological factors contribute to the antineoplastic activity and toxicity of the anthracycline anticancer drugs. One factor which could have contributed to the differential effects of quinone-containing anticancer drugs on microsomal peroxidation is drug metabolic activation and inactivation. It has been proposed that the semiquinone free radical is the obligatory intermediate in the NADPH-cytochrome P-450-dependent anaerobic deglycosidation of Adriamycin (1). However, in vitro, in the presence of adequate amounts of oxygen, neither Adriamycin nor several other anthracyclines appear to be deglycosidated or otherwise irreversibly biotransformed by microsomal enzymes (2) as they are in vivo (37). On the other hand, some of the other drugs tested in this study may have been oxidized by liver microsomal cytochrome P-450, and it is plausible that such microsomal metabolism may...
have diminished the ability of some quinone-containing drugs to generate reactive oxygen and stimulate lipid peroxidation. We have not attempted to investigate the in vitro microsomal metabolism of the many drugs used in this study, with the exception of Adriamycin, which we found to be unchanged when incubated with microsomes, NADPH, and a 100% oxygen atmosphere (27).

The relative stimulatory effects of anthracyclines on rat liver or heart lipid peroxidation reported in the present study correlate to some degree with what is known about their relative cytotoxicity and activity in other biological systems. It is of special interest to note that carminomycin and 4-demethoxydaunorubicin, drugs which lack the methoxy substituent present in Adriamycin and daunorubicin, were especially potent stimulators of lipid peroxidation. Similarly, carminomycin was the most active of quinone-containing anticancer agents tested for the ability to augment NADPH-dependent oxygen consumption by rat liver microsomes (2). Kirchner et al. (20) estimated the equicardiotoxic dose of carminomycin in rabbits to be 4 times lower than that of daunorubicin. In another study designed to compare to the effects of 10 antitumor anthracycline derivatives on several biochemical parameters of leukemia cell growth inhibition, Schwartz and Kanter (33) found that carminomycin and 4-demethoxydaunorubicin were highly potent cytotoxic drugs, greater than 8 times more effective than was Adriamycin as estimated by inhibition of tumor cell growth and DNA synthesis and by DNA damage. The minimal cumulative cardiotoxic dose of 4-demethoxydaunorubicin has been estimated to be 3 times lower than that of Adriamycin (8), and Zbinden et al. (48) found that 4-demethoxydaunorubicin was the most cardiotoxic anthracycline derivative of 37 tested in the rat electrocardiogram acute cardiotoxicity model. Interestingly, they also found 5-iminodaunorubicin to have low cardiotoxicity. Tong et al. (41) also reported 5-iminodaunorubicin to be less acutely cardiotoxic than Adriamycin in rats, and 5-iminodaunorubicin was less toxic than either Adriamycin or daunorubicin to drug-exposed isolated rat myocytes (23). 5-Iminodaunorubicin, unlike Adriamycin and most quinone analogs, inhibited rather than stimulated microsomal peroxidation. Thus, there are certain similarities in the relative effects of carminomycin, 4-demethoxydaunorubicin, 5-iminodaunorubicin, Adriamycin, and daunorubicin on microsomal lipid peroxidation and the relative effects of these drugs in other biological systems.

The manifestation of lipid peroxidation-mediated anthracycline cardiomyopathy as target organ toxicity would depend upon factors which affect the pharmacological actions of the drugs and the presence and activity in heart tissue of biochemical and enzymatic defenses against reactive forms of oxygen. Relative to other organs, the heart is grossly deficient in 2 important enzymatic defenses. Superoxide dismutase specific activity in rat heart is about one-fifth of the specific activity found in rat liver (9, 43), and heart catalase activity is 1/45 liver catalase activity in rats (40) and 150 times lower than liver activity in mice (10). It appears that in heart muscle, membrane α-tocopherol may be the most important defense against toxic reactive oxygen metabolites and in protecting membranes against lipid peroxidation. It is possible that chronic anthracycline treatment progressively depletes cardiac membrane α-tocopherol to levels which can no longer prevent peroxidation of lipids and, since other defenses against reactive oxygen are lacking, subsequent doses of anthracyclines could stimulate uncontrolled lipid peroxidation resulting in cardiotoxicity.

In conclusion, it should be emphasized that although several Adriamycin-like drugs can stimulate heart and liver microsomal lipid peroxidation as much as 10-fold, not all quinone-containing anticancer drugs enhance microsomal lipid peroxidation. While it is hoped that this abbreviated structure-activity study will help to elucidate the detailed molecular pharmacology and toxicology of anthracycline antitumor drugs, the significance of these findings must await verification of the role of reactive oxygen and lipid peroxidation in human anthracycline-induced cardiotoxicity. Nevertheless, these results may provide some meaningful insight as to the cardiotoxic potential of some anthracycline anticancer agents currently undergoing or awaiting clinical trial and provide a rational basis for designing new anthracycline drugs with decreased cardiotoxicity.

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