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FURTHER STUDIES ON THE GENERATION OF REACTIVE OXYGEN SPECIES FROM ACTIVATED ANTHRACYCLINES AND THE RELATIONSHIP TO CYTOTOXIC ACTION AND CARDIOTOXIC EFFECTS*

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Abstract—The relative ease of generation of reactive oxygen species from a series of reductively activated aglycone and sugar modified anthracyclines was compared by the extents of single strand scission in supercoiled PM2-covalently closed circular (CCC)-DNA. The electrochemical properties of these agents were used as a quantitative measure of the ease of reduction and subsequent reoxidation of the reduced species. The relationship of these processes to various biological properties of the anthracyclines, in particular to the measured cardiotoxicity of the drugs, was examined. An attempt was made to identify those structural changes which ameliorate the cardiotoxic effects measured in other test systems while permitting the expression of antitumor properties.

The antibiotics adriamycin (**2**) and daunorubicin (**3**) (Fig. 1) are among the most promising clinically active drugs for the treatment of a range of neoplasms [1, 2]. Their antineoplastic action correlates with their strong intercalative binding to duplex nucleic acids, with resulting inhibition of DNA replication and RNA synthesis [1–4]. However, their clinical efficacy is severely limited by dose-related cardiotoxicity [5, 6]. Consequently, efforts are being made to understand the molecular basis of the cardiotoxicity so that the anthracyclines may be modified structurally to remove this side-effect. The anthracyclines undergo a cyclic redox reaction *in vivo* giving rise to reactive oxygen species including $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$ (Fig. 2) [7]. There is growing evidence that relates the cardiotoxicity of the anthracyclines to the generation of $O_2^{\cdot-}$ and $OH^{\cdot}$ in heart tissue [8–12] (which is deficient in the protective enzymes superoxide dismutase, glutathione peroxidase and catalase [13–15]) and with the resultant lipid peroxidation and DNA lesions. Since the aglycone moiety of the anthracyclines is the centre of the redox activity, we recently reported [12] on the correlation evident between the lessened superoxide anion generation by reduced 5-iminodaunorubicin (**9**) and its lowered cardiac toxicity when compared with daunorubicin (**3**) for electrocardiographic changes in the rat [11]. The present paper extends this approach and examines the factors that cause lessened redox cycling and the relationship to cytotoxicity and cardiotoxicity in a series of aglycone and sugar modified anthracyclines.

* Studies Related to Antitumor Antibiotics, Part XXV.
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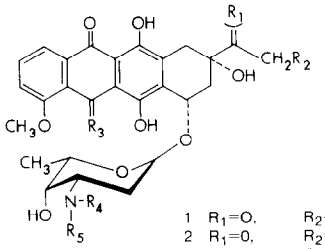

1	R_1=O,	R_2=OH,	R_3=O,	R_4=CH_3,	R_5=CH_3
2	R_1=O,	R_2=OH,	R_3=O,	R_4=H,	R_5=H
3	R_1=O,	R_2=H,	R_3=O,	R_4=H,	R_5=H
4	R_1=O,	R_2=H,	R_3=O,	R_4=CH_3,	R_5=CH_3
5	R_1=O,	R_2=H,	R_3=O,	R_4,	R_5= $-[CH_2]_5$
6	R_1=O,	R_2=H,	R_3=O,	R_4=CH_2Ph,	R_5=H
7	R_1=O,	R_2=H,	R_3=O,	R_4,	R_5= $-[CH_2]_2\,O[CH_2]_2-$
8	R_1=H, OH,	R_2=H,	R_3=NH,	R_4=H,	R_5=H
9	R_1=O,	R_2=H,	R_3=NH,	R_4=H,	R_5=H
10	R_1=O,	R_2=H,	R_3=O,	R_4=CH_2Ph,	R_5=CH_2Ph
11	R_1=O,	R_2=H,	R_3=NH,	R_4=CH_2Ph,	R_5=CH_2Ph

Fig. 1. Structural formulae of adriamycin (**2**), daunorubicin (**3**) and modified anthracyclines.

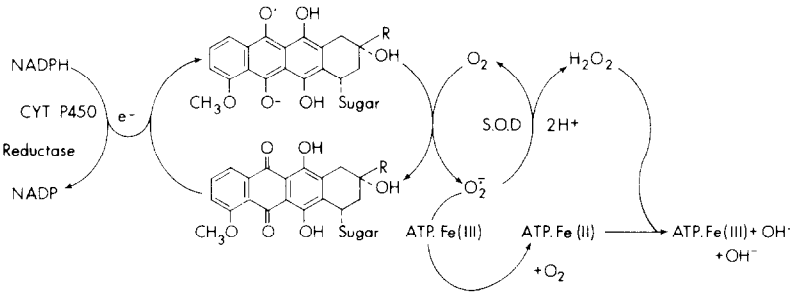

Fig. 2. Schematic representation of microsomal cytochrome P-450-mediated redox cycling of anthracycline chromophore leading to superoxide and, by the intervention of complexed iron, to the hydroxyl radical.

MATERIALS AND METHODS

Daunorubicin (**3**) was purchased from CalBiochem, La Jolla, CA. Adriamycin (**2**) was obtained from the National Cancer Institute, NIH. The additional modified anthracycles were prepared at SRI, Menlo Park, CA. Superoxide dismutase (EC 1.15.1.1) was from the Sigma Chemical Co., St. Louis, MO, and catalase (EC 1.11.16) (beef liver) was from the Aldrich Chemical Co., Milwaukee, WI. PM2-CCC-DNA was prepared as described previously [16]. Other materials used were reagent grade, except for acetonitrile (spectrophotometric grade).

Ethidium fluorescence assay for superoxide anion generation by nicking of DNA by reductively activated anthracyclines. All fluorescence measurements were performed on a G. K. Turner and Associates model 430 spectrofluorometer equipped with a cooling fan to reduce fluctuations in the xenon lamp. Wavelength calibration was performed as described in the manual for the instrument. One centimeter square cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 600 nm. Medium sensitivity (100 scale) was generally used, and water was circulated between the cell compartment and a thermally regulated bath at 22°. The reactions were performed at 37° in a total volume of 200 μl containing 50 μl potassium phosphate buffer (pH 7.0), 1.02 A_{260} units of PM2-covalently closed circular (CCC)-DNA (92% CCC), 9×10^{-5} M anthracycline, and 5.3×10^{-3} M sodium borohydride. The concentrations of other components used are given in the legend to Fig. 3. At intervals, 20 μl aliquots from the reaction mixture were added to 2.3 ml of the ethidium assay mixture, which was 20 mM potassium phosphate (pH 11.8), 0.4 mM EDTA, and 0.5 μg/ml of ethidium bromide. The fluorescence of the diluted solution was measured. The solution was then heat denatured at 96° for 4 min, cooled in ice, and equilibrated in a water bath at 22° for 5 min. The fluorescence of the solution was again measured. The conversion of PM2-CCC-DNA to nicked circular DNA results in a 30% increase in fluorescence in the pH 11.8 assay solution [15]. After heat denaturation and cooling, the CCC-DNA returns to register while the formation of nicked DNA is revealed by complete loss of fluorescence [17]. As additional controls, 20 μl of the reaction mixtures for the two anthracyclines were added to 2.3 ml of a solution of the assay medium without ethidium, which showed no fluorescence at 525 nm.

Electrochemistry. Polarographic studies, both DC and differential pulse (DPP), and cyclic voltammetric studies (CV) were carried out as described previously [12, 18, 19]; DPP pulse height was 5 mV. The temperature was 37.5° throughout, and all potentials were measured and are reported with respect to the aqueous Saturated Calomel Electrode (S.C.E.) at that temperature. The drop time in the polarographic runs was 2 sec; all solutions were deaerated by purified N_2 for at least 10 min prior to study.

Solutions of all compounds were 1.0×10^{-4} moles/l in concentration, made up in aqueous stock solution of 10^{-2} moles/l KCl and 10^{-2} moles/l phosphate buffer (pH 7.1). When, as in most cases, the compounds were not sufficiently soluble in aqueous media, either 30% of 50% acetonitrile by volume was added to take them into solution; the contents of the presaturator were adjusted to the same composition to prevent significant loss of acetonitrile upon deaeration. Since solvent changes vitiate direct comparison of potentials, daunorubicin itself was studied in aqueous solution and each of the partially aqueous mixtures to permit comparison. The 30% and 50% acetonitrile solutions gave essentially identical results.

The ability of the forms of these compounds produced by electroreduction to undergo reoxidation was studied by cyclic voltametry at hanging mercury drop electrodes at scan rates decreasing from 500 mV/sec to 10 mV/sec. The peak current of the cathodic process was measured from the extrapolated almost flat cathodic baseline, virtually identical to the curve obtained in base electrolyte alone. Where, as with the quinones, two inseparable CV cathodic peaks were observed, the cathodic current was taken as the greatest peak current measured from the baseline. Only one anodic process was observed in any case. The initial scan was always toward negative potential and the switching potential always at least 50 mV negative of the peak of the cathodic process or processes. The peak current of the anodic process was measured from the extrapolated curve following the cathodic peak. Under these conditions [20], the ratio of $I_p(A)$, the peak current for the anodic (oxidation) process, to $I_p(C)$, the peak current for the cathodic (reduction) process, is a

Table 1. Comparison of biological test data for anthracyclines with generation of reactive oxygen species

Anthracycline	ΔT_m (°)	Inhibition of synthesis in L1210 cells* DNA ED_{50} (μM)	RNA ED_{50} (μM)	Activity against P388 leukemia† (mice q 4d, 5, 9, 13) %T/C (mg/kg)	Cardiotoxicity Zbinden MCCD in rats min. cum. cardiotoxic dose‡ (mg/kg)	O_2 consumption in rat liver microsomes (% activity of adriamycin)§	$E_{\frac{1}{2}}$, V DC Polarogram 50% CH_3CN	Reoxidation of reduced species CV (500 mV/sec)	Relative generation of reactive oxygen species (75 min, 45 μM drug)
1	17.5	0.62	0.12	186 (2.4)	6	145	−0.65	>95	90
2	13.4	1.5	0.58	159 (8)	11	100	−0.64	63	88
3	11.2	0.66	0.33	132 (8)	14	109	−0.64	67	90
4	15.3	0.67	0.064	167 (10)	11–16	108	−0.64	>95	90
5	16.4	0.62	0.03	191 (6.2)		88	−0.655	‖	87
6	10.2	1.6	0.17	184 (18.8)		87	−0.65	‖	73
7	6.1	0.35	0.0103	187 (0.2)		25	−0.654	75	55
8	3.3	4.0	5.6			11	−0.72	10	43
9	6.25	1.6	1.3	130 (3)	64	8.2	−0.70	13	55
10	1.4	>100	10	208 (38)	125	0.5	−0.65	‖	10
11	−0.8	>1000	160	101 (5–150)			−0.745	‖	0

* ΔT_m and ED_{50} values were taken from Refs. 21 and 22.

† Assays were arranged through the Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute. BDF or CDF mice were injected i.p. on days 1–9 or days 5, 9 and 10 with the specified drug dose. T/C is the ratio of average survival times of treated mice to untreated controls in percent; see Refs. 21 and 22.

‡ Cardiotoxicity in rats [21]; assay described in Ref. 11.

§ Ref. 23.

‖ Compound insoluble in water.

quantitative measure of the reoxidizability of the reduced form under the experimental conditions used. The ratio will be unity, or 100%, if all of the material reduced can be reoxidized.

RESULTS

Daunorubicin (**3**) at a concentration of 4.5×10^{-5} M at pH 7.2, 37°, caused 90% single strand scission of PM2-CCC-DNA in 75 min. 5-Iminodaunorubicin (**9**) under comparable conditions caused only 50% scission, while 13-dihydro-5-iminodaunorubicin (**8**), *N,N*-dibenzyldaunorubicin (**10**), and *N,N*-dibenzyl-5-iminodaunorubicin (**11**) gave 43, 10 and 0% DNA scission under comparable conditions. The *N,N*-dimethyl daunorubicin (**4**) was highly effective, giving 90% DNA scission in 75 min. The results of the other anthracyclines leading to intermediate amounts of generation of reactive oxygen species, as compared by the relative extents of DNA scission, are given in Table 1 and Fig. 3.

All compounds showed an initial reduction around −0.65 V (quinone or modified quinone ring) whose diffusion current was about 0.3 μA for all compounds in all media; the values of the peak currents (DPP) differed because complete separation of proximate peaks (designated by † in Table 2) could not be achieved. A process near −1.15 V, also present in virtually all compounds, had a diffusion current about 15% less than that of the initial reduction. The compounds with substituents upon the sugar moiety had essentially the same reduction potential as daunorubicin, while those involving 5-imino-substituted chromophores were considerably more difficult to reduce than daunorubicin.

Cyclic voltammetric studies of **8** gave results very similar to those of **9**, that is, in neither case was reoxidation of the 5-imino compound possible; the same phenomena was observed in the **10–11** comparison. For the quinone compounds, a single anodic (oxidation) peak was observed in all cases, while either one or two closely spaced cathodic (reduction) peaks were observed depending upon the compound. As the scan rate decreased from 500 mV/sec (at which many of the compounds did not give two distinguishable peaks) to 20 mV/sec, separation of the two cathodic peaks became more distinct. The ratio $I_p(A)/I_p(C)$ was always less than unity, increasing toward unity with increasing rate of scan, except for the imino compounds which exhibited little or no anodic peak. The reduction waves (DC) or peaks (DPP, CV) of the imino compounds were in the approximate ratio A:B:C = 2:1:2.

The cyclic voltammetric results for aqueous solutions are shown in Fig. 4, which includes all compounds soluble in aqueous solution. Those compounds not soluble in aqueous solution were measured in 50% acetonitrile (Fig. 5). For compounds where a good curve could not be obtained

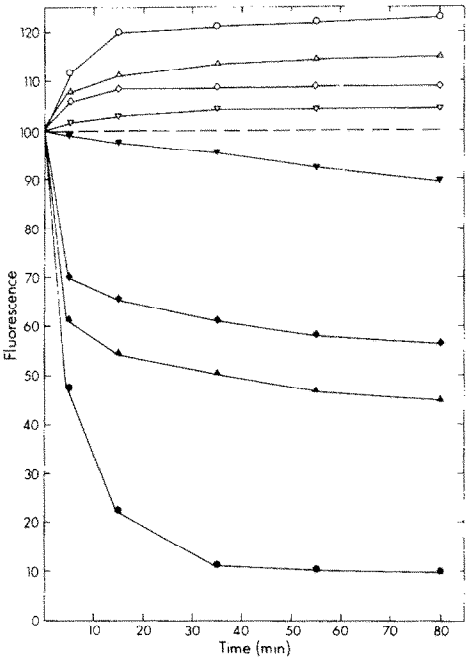

Fig. 3. Relative extents of single strand scission of PM2-CCC-DNA by reductively activated daunorubicin and analogues. Reactions were performed at 37° in a volume of 200 μl buffered at pH 7.0 with 50 mM potassium phosphate containing 1.0 A_{260} units/ml of PM2-CCC-DNA (92% CCC) and 2.6×10^{-3} M $NaBH_4$. Aliquots (30 μl) at the indicated times were added to 2.3 ml of the standard pH 11.8 ethidium bromide assay mixture [15]. The before heat denaturation fluorescence readings are shown with open symbols and the closed symbols are fluorescence readings after the denaturation at 96° for 4 min followed by rapid cooling. Additional components were: (○—○) 4.5×10^{-5} M daunorubicin hydrochloride; (△—△) 4.5×10^{-5} M 5-iminodaunorubicin hydrochloride; (◇—◇) 4.5×10^{-5} M 13-dihydro-5-iminodaunorubicin hydrochloride; (▽—▽) 4.5×10^{-5} M *N,N*-dibenzyldaunorubicin and 10% CH_3CN, and (----) 4.5×10^{-5} M *N,N*-dibenzyl-5-iminodaunorubicin and 10% CH_3CN and the control experiment.

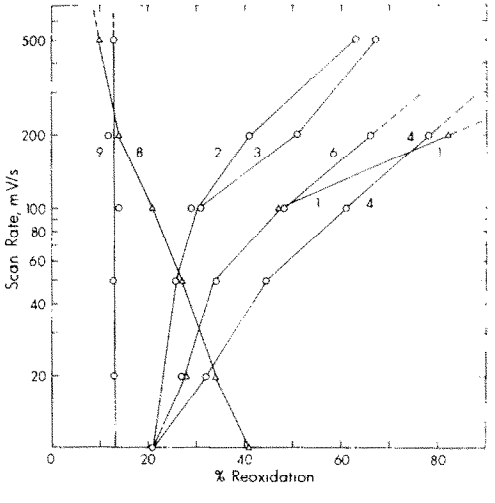

Fig. 4. Reoxidation of compounds in aqueous solution. The concentration of the compounds was nominally 1.0×10^{-4} moles/litre in aqueous phosphate buffer and KCl at pH 7.1. Identification of compounds corresponds to Fig. 1. Each symbol corresponds to one to six separate measurements, generally agreeing ± 3%. Lines for different compounds are merged when within experimental error. Compounds not shown were not soluble in aqueous solution. Temperature, 37.5°.

Table 2. Electrochemical properties of anthracyclines*

Compound	% CH_3CN	A	B	C	D	Method
1	0	−0.585†	−0.670	—	−1.40	DC
1	0	−0.615	−0.660	−1.10	−1.42	E_p, DPP
1	50	−0.650†	†	−1.150		DC
1	50	−0.625†	−0.690†	−1.120		E_p, DPP
2	0	−0.625†	†	−1.240		DC
2	0	−0.640†	−0.672	−1.255		E_p, DPP
3	0	−0.64†		−1.150	−1.34	DC
3	0	−0.64†		−1.15	−1.34	E_p, DPP
3	30	−0.625†		−1.10†	†	DC
3	30	−0.627†		−1.09	−1.34	E_p, DPP
3	50	−0.640		−1.15†	†	DC
3	50	−0.640		−1.14	−1.34	E_p, DPP
4	0	−0.640†		−1.22		DC
4	0	−0.570†	−0.680†	−1.25		E_p, DPP
4	50	−0.640†	†	−1.28		DC
4	50	−0.625†	−0.698†	−1.08	−1.425	E_p, DPP
5†	50	−0.655†	†	−1.085		DC
5†	50	−0.650†	−0.690†	−1.210		E_p, DPP
6	30	−0.650†	†	−1.107		DC
6	30	−0.648†	−0.69†	−1.115		E_p, DPP
7	50	−0.654		−1.151		DC
7	50	−0.655		−1.137		E_p, DPP
8	0	−0.660	−0.830	−1.148		DC
8	0	−0.670	−0.883	−1.180		E_p, DPP
8‡	50	−0.720		−1.195		DC
8‡	50	−0.710	−0.965	−1.285		E_p, DPP
9§	0	−0.657				DC
9	0	−0.67	−0.94	−1.17		DC
9‡	50	−0.700	−0.960	−1.250		DC
9‡	50	−0.701	−0.975	−1.290		E_p, DPP
10	30	−0.650				DC
10	30	−0.650			−1.405	E_p, DPP
10	50‡	−0.650			−1.35	DC
10	50‡	−0.650			−1.41	E_p, DPP
11†	50	−0.745	−0.885	−1.24	−1.565	DC
11‡	50	−0.745	−0.930	−1.310	−1.630	E_p, DPP

* Successive reductions of compound designated A, B, C, D; values are polarographic half-wave potentials measured in volts against aqueous SCE (DC) or peak potentials (E_p, DPP) measured at 5 mV modulation.

† Separate processes observed were distinguishable only by DPP of cyclic voltammetry (while DC polarographic wave appears distorted). Absence of † notation implies symmetric DPP peak.

‡ Apparent process observed near −0.35 V, probably due to adsorption.

§ Triton X-100 was used to suppress anomalous maxima.

at 500 mV/sec, an extrapolated value was obtained from Fig. 4. These values are entered in Table 1. We consider that this value is an appropriate index of comparison for the rapid transfer of an electron to oxygen to generate superoxide. It should be noted that the iminoquinones showed only a small degree of reoxidizability (if any) since the current actually remained cathodic throughout for them, and that they differed markedly from the quinones whose degree of reoxidizability increased with scan rate. Values for **11**, measured in 50% acetonitrile and not shown in Fig. 5, were similar to those of **8**.

A comparison of the extents of interaction of the anthracyclines with DNA, the inhibition of macromolecular synthesis (DNA and RNA) in leukemia L1210 cells, some cardiotoxicity data in rats, relative extents of oxygen consumption by rat liver microsomal activation, the aglycone quinone group redox potentials, and the relative extents of reactive oxygen species generation as measured by the degree of

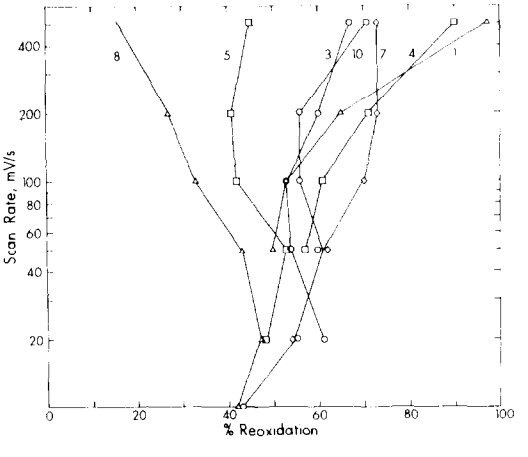

Fig. 5. Reoxidation of compounds in 50% acetonitrile. Conditions as Fig. 4.

DNA scission under standard conditions is given in Table 1.

DISCUSSION

The rationale of this research is that, since anthracyclines accumulate in the heart and since cardiac tissue is deficient in those enzymes designed to protect the cell from oxidative lesions (superoxide dismutase, catalase and glutathione peroxidase [13–15], factors which interfere with the oxygen-cycling capacity of the drug could well lead to the alleviation of cardiotoxic effects. Comparison of measurements of the extent of oxygen consumption by rat liver microsomes [7, 23], the complementary examination of oxidative lesions produced in biomolecules (specifically supercoiled DNA), and electrochemical properties [12] provides valuable information. This permits identification of the structural effects on (i) ease of reduction on the chromophore, (ii) ease of reoxidation or electron transfer from the reduced species, and (iii) secondary effects on conformation as they affect drug binding and/or redox properties.

Examination of Table 1 reveals some trends in the overall effects of specific structural changes in the anthracyclines. Introduction of an imino group at position 5 in ring C of the aglycone renders the drug more difficult to reduce and much more difficult to reoxidize (Figs. 4 and 5) which has, in turn, the effect of drastically reducing the extent of microsomal oxygen cycling and of the generation of reactive oxygen species compared with adriamycin. While the binding of the drug to DNA is reduced, this structural change has a salutary effect in reducing cardiotoxicity (at least from the limited data available). In this group of compounds, prior reduction of the side chain (**8**) has little effect on redox properties of the quinone ring or oxygen cycling as expected since **8** is anticipated as a primary metabolite of **9** [1, 2].

N-Methylation of the sugar group (**1**, **4**) had an adverse effect in permitting both somewhat easier reduction of the chromophore and greater reoxidation with concomitant enhancement of oxygen cycling and a significant increase (for **1**) in cardiotoxicity. At the same time the binding to duplex DNA was not materially affected and the antileukemic activities of **1** and **4** were still relatively high. There is a slight increase in ΔT_m for **1** so the small methyl groups in the quaternary amino group still permit formation of an electrostatic bond to a phosphate on the outside of the helix [1, 2, 24].

When benzyl groups were introduced into the sugar, more profound changes in properties were discernible. Two *N*-benzyl groups in **10** and **11** drastically inhibited the extent of both microsomal oxygen consumption and generation of $O_2^{\cdot-}$ and $OH^{\cdot}$, as measured by DNA scission. The marked amelioration of the cardiotoxicity in **10** clearly suggests more than a coincidence. A possible explanation is steric inhibition to the cytochrome P-450 reductase in the case of microsomal action. Steric hindrance leading to suppression of DNA binding (ΔT_m of 1.4 and 0.8° for **10** and **11**) may account for the observed low extents of DNA scission. The effects of the *N*-benzyl groups in severely inhibiting binding of **10** and **11** to duplex DNA may also account for the marked reduction of inhibition of macromolecular synthesis in leukemia L1210 cells observed for these two compounds. Although **10** was essentially inactive in all *in vitro* tests, it showed enhanced antitumor activity in the mouse.

Removal of one of the *N*-benzyl groups from **10** to give **6** (which is a metabolite of **10**) [25] has the effect of permitting binding to DNA and greater superoxide production and, consequently, a greater degree of microsomal oxygen cycling although considerably less than in the case of the parent daunorubicin. Substitution of the piperidino group in the sugar evidently does not impede binding to DNA and has only a slight effect on the chromophore redox potential and the attendant degree of oxygen cycling.

The subtle dependence of the properties of the anthracycline on the nature of the substituents is indicated by example **7** where a morpholino group, while still permitting strong binding to the DNA, substantially reduced the extent of microsomal oxygen consumption and the concomitant generation of reactive oxygen species.

Of greatest significance was the correlation, apparent in Table 1, between microsomal O_2 consumption and DNA scission by reductively activated drug. These are independent measures of the postulated mechanism involving O_2 cycling (Fig. 2) and the correlation was observed through the series of ten analogs. It is likely that this mechanism is associated with cardiotoxicity. It is also possible, though much less certain at this time, that the mechanism is associated with antitumor effects. Thus, it is essential to compare *in vivo* potencies of antitumor and cardiotoxic effects among analogs to determine eventually if diminished O_2 cycling produces a clinical advantage for any given analog. Future determination of *in vivo* potency of cardiotoxic effects of morpholino analog 7 is of greatest interest, since this is the outstanding example of an analog that shows reduced O_2 cycling yet increased antitumor potency in mice. Continued use of these tests for generation of reactive oxygen species and comparison with other biological tests should clarify the mechanisms of anthracycline action and assist in the design and selection of improved analogs.

In conclusion, it appears to be possible to separate the antitumor and cardiotoxicity effects of these anthracycline drugs by certain specific structural modifications, especially those that directly affect redox potential. In the examples given, a 20-fold decrease in cardiotoxic potency was obtained in the rat as measured by electrocardiographic changes. In addition, the present results appear to substantiate the premise that the cardiotoxicity of the anthracyclines is related to the redox cycling of the chromophore specifically leading to damage of cardiac tissue.

It seems likely that further improvement in the drug therapeutic index, including a reduction of cardiotoxicity, will require both *de novo* synthesis of new aglycone analogues of daunorubicin and adriamycin and incorporation of appropriately substituted sugar moieties to prevent redox cycling entirely while permitting intercalative binding to DNA. Efforts in this direction will be reported subsequently.

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