Biochemical Pharmacology, Vol. 35, No. 8, pp. 1309–1323, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Press Ltd.

REDOX ACTIVITIES OF ANTITUMOR ANTHRACYCLINES DETERMINED BY MICROSOMAL OXYGEN CONSUMPTION AND ASSAYS FOR SUPEROXIDE ANION AND HYDROXYL RADICAL GENERATION*

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(*Received* 4 *March* 1985; *accepted* 13 *September* 1985)

Abstract—To explore the structural characteristics of various derivatives of the anticancer drugs, doxorubicin and daunorubicin, for exhibiting redox activities believed to be associated with toxic radical production, we tested over fifty derivatives in a rapid screening procedure for augmenting oxygen consumption by rat liver microsomes. Measurement of parent drug disappearance and of metabolite appearance for fourteen anthracyclines with a broad range of activities for augmenting oxygen consumption indicated that a single reaction, conversion to the 7-deoxyaglycone, occurred. Multiple tests of selected compounds showed that the liver microsome system exhibited saturation kinetics, and calculated values of V_{max}/K_m gave the same relative order of activities as did the screening test. The liver microsome system was not found to be stereoselective. Measurements of the abilities of a number of the anthracycline derivatives after chemical activation by reduction with sodium borohydride to convert oxygen to superoxide anion, or to the hydroxyl radical, were also made. The reactivities of the anthracyclines in these latter two assays were positively related to the activities obtained in the rat liver microsome screening test, suggesting that all three tests were measuring various steps in the sequence from anthracycline semiquinone radical formation through oxygen activation and radical formation. Superoxide anion generation from chemically reduced anthracyclines was inhibited by the addition of calf thymus DNA, and the extent of inhibition was positively correlated with the measured DNA association constants of the anthracyclines. However, the DNA association constants were unrelated to superoxide anion generation in the absence of DNA or to the augmentation of oxygen consumption in liver microsomes. Half-wave potentials were negatively correlated with both the results of the microsomal oxygen consumption test and the production of superoxide anion in the chemical test system. No relationships were discerned among the DNA association constants, half-wave potentials, or reoxidizabilities of the anthracyclines tested. Comparisons of the relatively low activities of certain of the anthracyclines in the biochemical and chemical tests for oxygen activation with their known high activities against murine tumors *in vivo*, but low cardiotoxicities in animal model systems, suggest that the separation of the cytotoxic antitumor and cardiotoxic actions of these derivatives may have been achieved.

The anthracyclines, daunorubicin (DAU) and doxorubicin (DXR), are among the most widely prescribed drugs in the treatment of a range of human malignancies [1–3]. However, their clinical efficacy is severely limited by a dose-related risk of cardiotoxicity, and numerous anthracycline analogs have been prepared with the objective of separating the cardiotoxic side-effects from the anticancer cytotoxicity [2, 4–7].

The biochemical basis for the cardiotoxicity of the anthracyclines is uncertain but there is growing evidence that it is related to the redox activity of the quinone moiety leading to the production of a semiquinone and reactive oxygen species (Fig. 1) [8–10]. These latter species, which include superoxide anion, hydrogen peroxide, and the hydroxyl radical produced by secondary reactions, can damage nucleic acid [5, 11, 12] or cause lipid peroxidation [13–18]. Lipid peroxides have been shown to induce cardiotoxicity in rats [19], and rat hearts were found to accumulate anthracyclines more than did liver or skeletal muscles [20]. Thus, a linkage between radical production and the cardiotoxic effects of anthracyclines is indicated.

In animal studies, the radical generating reaction has been demonstrated in all tissues examined thus far, including liver [8, 13], heart [21, 22], kidney [23, 24], lung [23], spleen [8], and red blood cells [25, 26]. Microsomes [8, 13], mitochondria [21], and nuclei [17, 24] of mammalian tissues exhibit this activity. Cardiac tissue, however, seems to be particularly susceptible to prolonged oxidative attack because of relatively low levels of antioxidants or enzymes that protect against such attack [27].

We previously demonstrated [5, 6], with a limited number of anthracyclines, that the redox activity, as measured by either activation of the compounds by

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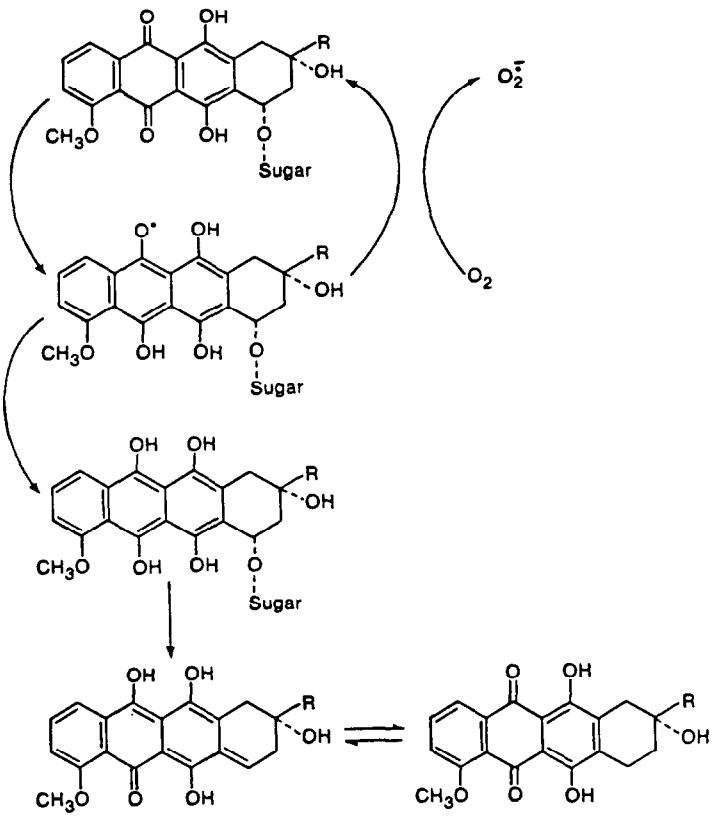


Fig. 1. Enzymatic reduction of anthracycline chromophore and subsequent electron transfer to oxygen and reductive deglycosidation via the ring C dihydroanthracycline.

chemical reduction or by rat liver microsomes, is related to various biological properties of the drugs, in particular to measured cardiotoxicity in animal models. A 20-fold reduction in cardiotoxicity was found with no sacrifice of antileukemic activity [6]. We have now extended these studies to a larger number of anthracyclines and have examined the relationships among the relative activities of the derivatives to augment oxygen consumption by rat liver microsomes. In addition, we examined the capacities of the chemically activated anthracyclines to generate reactive oxygen radicals. Separate assays were applied to determine the relative production of superoxide anion (in the presence and in the absence of DNA) and the generation of the hydroxyl radical by the chemically activated anthracyclines. Electrochemical studies of the derivatives provide additional pertinent information. Relations between the various measured parameters were examined in those cases wherein sufficient (>10) paired observations were available. Finally, the stoichiometry of the conversion of various anthracyclines to 7-deoxyaglycone metabolites by the liver microsomes and the possible stereospecificity for this reaction of a model pair of 13-dihydrodiastereoisomers of an anthracycline were studied.

* Unpublished studies of Acton, Christie, Mosher and Tong, SRI International.

MATERIALS AND METHODS

Chemicals. The compounds employed in these studies are organized in Tables 1–4 by structural similarities. The sources or previous reports of the compounds are as follows: Compounds 1 and 7 were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD; Compounds 12, 14, 18, 19, 21–23, 25, 26, and 28 [28]; Compounds 2–6 and 8–11 were gifts of Drs. F. Arcamone and A. M. Casazza, Farmitalia Carlo Erba, Milan, Italy; Compounds 15–17, 35, and 41–43 [29]; Compounds 20, 24, 31, and 32 [30]; Compounds 13, 27, 36, and 45–50*; Compounds 29, 30, 33, 34, 37, and 38 [31]; Compound 40 [32]; Compound 44 [33]; Compounds 51–56 were gifts of Dr. T. Oki, Pfizer Co., Tokyo, Japan; and Compound 39 was a gift of Dr. M. Israel, Memphis, TN.

Nitro-blue tetrazolium (NBT) was purchased from the Sigma Chemical Co., St. Louis, MO. PM2-CCC-DNA was prepared as described previously [34]. Other materials used were reagent grade, except for acetonitrile (spectrophotometric grade). Calf thymus DNA, which was purchased from Sigma, was dissolved in 0.1 M potassium phosphate buffer, pH 7.2, and sonicated as previously reported [35].

Preparation of liver microsomes. Young adult male Sprague–Dawley rats (225–250 g; Simonsen Laboratories, Gilroy, CA) were fasted overnight and killed by decapitation. The livers were quickly removed and

washed with ice-cold 0.15 M KCl, and the microsomes were prepared as in Step 1 of the method of Omura and Takesue [36]. For our studies, the liver from a single rat was homogenized in an electric blender for 10 sec in 4 vol. of 0.15 M KCl. The homogenate was equally divided into four tubes, and the washing steps were performed as described (*loc. cit.*) with 10 ml of each solution. All procedures were performed at 4°, and the final washed microsomal pellets were stored at −20° no more than 9 days before the oxygen consumption studies.

Microsomal oxygen consumption assay. Anthracycline stimulation of microsomal oxygen consumption was determined using a Clark electrode and a biological oxygen monitor (model 53, Yellow Springs Instrument Co., Yellow Springs, OH). The following modifications of the method of Bachur *et al.* [37] were employed. Frozen microsomes were uniformly suspended in 2% Triton N-101 (Sigma) in 0.1 M, pH 7.5, potassium phosphate buffer at room temperature using gentle strokes (4–5) of an all-glass tissue homogenizer. Protein levels were determined using the dye-binding method of Bradford [38] and the dye concentrate supplied by Bio-Rad Laboratories, Richmond, CA. Human albumin (fraction V, Sigma) served as a protein standard. The standard procedure for assaying each anthracycline at a single concentration consisted of adding 3.6 ml of 0.2 M, pH 8.0, potassium phosphate buffer (gassed at 37° with laboratory air) to the incubation vial. An aliquot of microsomes (0.1 ml; approximately 10 mg protein/ml) was added, the oxygen probe inserted, and the meter of the oxygen monitor set at 100% oxygen saturation. After 5 min at 37°, 0.1 ml of a solution of NADPH (P-L Biochemicals, Inc., Milwaukee, WI; 177 mg/ml in the above pH 8.0 buffer) was added to the chamber and allowed to equilibrate for 1.5 min; then the endogenous oxygen consumption rate was determined for 1 min. After 0.5 min, 0.2 ml of the anthracycline solution that was 2.0 mM in 80% polyethylene glycol 200 (PEG-200, J. T. Baker Chemical Co., Phillipsburg, NJ) in water was added* and allowed to equilibrate for 1 min; then the rate of oxygen consumption was again measured for 1 min. All oxygen consumption rates were determined by coupling the output voltage of the oxygen monitor to an analog-to-digital multimeter (model 3438-A, Hewlett-Packard Co., Santa Clara, CA) and a strip-chart recorder (model 255, Linear Instruments Corp., Costa Mesa, CA). The digital voltage output of the multimeter was coupled to a keystroke programmable calculator (model 9815-A, Hewlett-Packard Co.) that was programmed to determine the oxygen consumption rate for 1 min after the addition and equilibration of the NADPH and of the anthracycline. Utilizing the quantity of microsomal protein added in the assay, the computer program provided the nmoles of oxygen consumed per mg protein per min based upon an initial dissolved oxygen content of 0.93 μmole in the incubation mixture. The activity of all compounds was expressed as a percentage of the activity found for DXR with the same microsomal preparation at the same time. By this means, we always related the activity of the anthracycline derivative to that of DXR. Measurements of the activities of experimental compounds and DXR were made in triplicate. Coefficients of variation for the assays were routinely ≤6%. The mean activity of different microsomal preparations (N = 11) over 2 years for DXR was 122 ± 10 nmoles oxygen per min per mg microsomal protein. Occasionally, repeat analyses of derivatives studied earlier were performed. Agreement within ±10% of the mean of multiple observations was typical.

Determinations of kinetic parameters (K_m and V_{max}) for oxygen consumption were performed using anthracycline concentrations ranging from 0.05 to 0.5 mM. Both the Lineweaver–Burk [39] and the Hofstee [40] plotting techniques were employed to estimate the kinetic parameters. In some studies, we removed samples (10–20 μl) from the incubation flask immediately after the addition of the anthracycline, at intervals during the disappearance of oxygen, and again at intervals after the oxygen had been exhausted. These samples were frozen immediately in dry ice for subsequent analysis for parent anthracycline and metabolites. Upon thawing, the samples were diluted in 0.5 to 1.0 ml of mobile phase and 100μl aliquots were chromatographed using the conditions for the liquid chromatography of DXR described previously [41].

NBT assay for superoxide anion produced from reduced anthracyclines in the presence of oxygen. Superoxide anion reduces NBT to yield diformazan, which exhibits intense absorption at 560 nm [42]. Each mole of NBT reduced corresponds to 4 moles of superoxide anion. To measure the yield of superoxide anion from the anthracyclines, reaction mixtures containing 13 μM anthracycline and 350 μM NBT in 0.1 M potassium phosphate buffer, pH 7.2, were monitored at 560 nm in 1 × 1 cm cells of a spectrophotometer equipped with constant temperature regulation at 37° (model 250, Gilford Instrument Laboratories, Inc., Oberlin, OH) before and after the addition of 660 μM sodium borohydride. Control experiments showed that sodium borohydride had no direct effect on NBT or diformazan. The activities of the anthracycline derivatives were expressed in percent of that of DXR.

The reduction of NBT to diformazan by the drugs in the presence of DNA was carried out in a manner similar to that described above except for the addition of 210 μM sonicated calf thymus DNA. In this case, the values reported are the percent inhibition in the NBT test that resulted when DNA was added.

Assay for hydroxyl radical generation employing nicking of circular DNA by reduced anthracyclines in the presence of oxygen. This assay, which depends on the sensitive detection of single-strand cleavage of supercoiled DNA by hydroxyl radicals and is measured by ethidium fluorescence changes, was performed as described previously [6] using 90 μM anthracycline and 5 μM sodium borohydride.

Electrochemical studies. The association constants of the anthracyclines with DNA were measured by a conditioning potential rapid sweep voltammetric technique as described previously [43]. The values herein were measured on mercury in aqueous acetate

* PEG-200 was an excellent solvent for water-insoluble anthracyclines such as Compound 25 of Table 2. Comparative tests of DXR in water and in 80% PEG-200 in water indicated that the PEG-200 contributed no more than an 8% increase in oxygen consumption.

buffer at pH 5.6 employing calf thymus DNA. Because some of the compounds have low aqueous solubility, we determined these constants in dilute aqueous acetonitrile as indicated in Table 8.

Cyclic voltammetry (CV) was used to determine the reoxidizability. The present values were measured at 500 mV/sec and are comparable with those given in Table 1 of Lown *et al.* [44]. It was possible in the present studies to extend measurements of reoxidizability in aqueous solution to lower concentrations. The values for reoxidizability given in Table 9 are mostly for compounds in aqueous solution and so differ somewhat from values reported previously for some of these compounds measured in 50% acetonitrile solution [6]. The polarographic half-wave potentials are also given with saturated calomel as the potential reference electrode.

Calculations. Correlation tests between various groups of observations were performed by linear regression analysis [45]. Plots of correlation tests not included in this paper are available from the senior author on request.

RESULTS

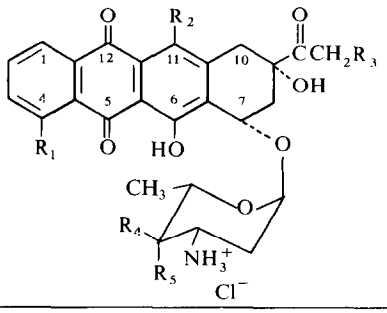
Microsomal oxygen consumption. Table 1 presents results obtained in the rat liver microsomal assay with DXR and DAU and certain derivatives. In the DXR analogs, removal of the methyl group at C_4 and of the hydroxyl group at C_{11} in the same molecule (Compound 3) enhanced, whereas removal of the methoxy group at C_4 (Compound 2) or of the hydroxyl group at C_{11} (Compound 4) reduced, the relative activity. Removal of the hydroxyl at C'_4 (Compound 5) or reversal of the configuration at C'_4 (Compound 6) had little effect on the relative activity, however. In the DAU series, the changes in Compounds 8–10 in the 4, 4′, and 11 positions did not result in a marked change in activity from DAU. As in the DXR series, removal of the hydroxyl of DAU at C_{11} (Compound 11) caused about a 50% reduction in activity.

Table 2 lists a much larger collection of DXR and DAU analogs wherein the major alterations from the parents are at the sugar amino group. The relatively high activities of Compounds 12 and 18 and the low activities of Compounds 13 and 19 through 27 suggest that decreased activities are related to increased size of the substituent on the sugar amino group. By contrast, compounds wherein the amino nitrogen of the sugar is incorporated into a ring (Compounds 14–17; 28–36) exhibited activities in the range of 26% (Compound 33) to 135% (Compound 14) of DXR. Compound 36, wherein sulfur replaces the oxygen in the morpholinyl ring, exhibited a lower activity than any other derivative of this type. Compounds 37 and 38, with cyclic substituents on the basic amino group, exhibited substantial activity. Compound 39, with the long ester group at R_1 and the metabolically stable trifluoroacetyl group at R_2, was relatively inactive.

Because a number of 13-dihydro derivatives have been reported to exhibit antitumor activities nearly equal to their parent anthracyclines [1], we examined several of these analogs (compounds designated with the suffix —OL in Tables 2 and 3). We found that the 13-dihydro derivatives and their parent drugs (Compounds 15 and 16; 22 and 23; 25 and 26; 29 and 30; and 33 and 34 of Table 2; Compounds 41 and 42; 44 and 45 of Table 3) exhibited nearly the same activities.

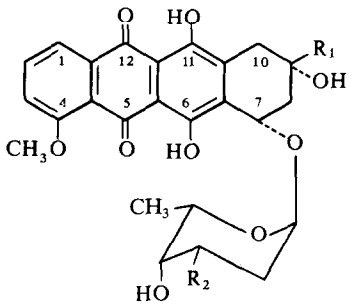
The only chromophore-modified anthracycline compounds reported to date are the 5-imino derivatives (Table 3) of both DXR (Compounds 40–43) and DAU (Compounds 44–46) and the glycosides

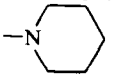
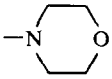
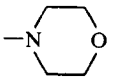
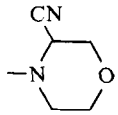
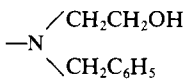
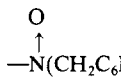
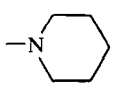
Table 1. Effects of DXR and DAU and their ring-substituted analogs on microsomal O_2 consumption



No.	Anthracycline Name	R_1	R_2	R_3	R_4	R_5	Percent of DXR activity
1.	Doxorubicin (DXR)	—OCH_3	—OH	—OH	—H	—OH	100
2.	4-Demethoxy-DXR	—H	—OH	—OH	—H	—OH	24
3.	4-De-*O*-methyl-11-deoxy-DXR	—OH	—H	—OH	—H	—OH	138
4.	11-Deoxy-DXR	—OCH_3	—H	—OH	—H	—OH	53
5.	4′-Deoxy-DXR	—OCH_3	—OH	—OH	—H	—H	100
6.	4′-Epi-DXR	—OCH_3	—OH	—OH	—OH	—H	88
7.	Daunorubicin (DAU)	—OCH_3	—OH	—H	—H	—OH	109
8.	4-Demethoxy-DAU	—H	—OH	—H	—H	—OH	102
9.	4-Demethoxy-4′-*O*-methyl-DAU	—H	—OH	—H	—H	—OCH_3	107
10.	4.De-*O*-methyl-11-deoxy-DAU	—OH	—H	—H	—H	—OH	120
11.	11-Deoxy-DAU	—OCH_3	—H	—H	—H	—OH	44

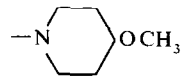
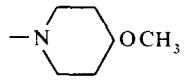
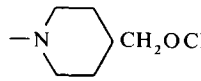
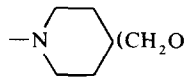
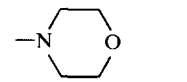
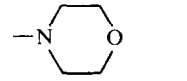
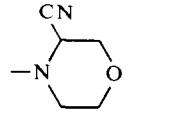
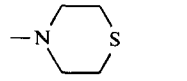
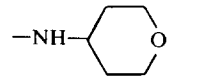
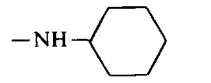
Table 2. Effects of N-alkylated analogs of DXR and DAU on microsomal O_2 consumption



Anthracycline No.	Name	R_1	R_2	Percent of DXR activity
12.	*N,N*-Dimethyl-DXR	—C(=O)CH$_2$OH	—N(CH$_3$)$_2$	145
13.	*N,N*-Dibenzyl-DXR-OL	—CHOHCH$_2$OH	—N(CH$_2$C$_6$H$_5$)$_2$	16
14.	3′-Deamino-3′-(1-piperidinyl)-DXR	—C(=O)CH$_2$OH		135
15.	3′-Deamino-3′-(4-morpholinyl)-DXR	—C(=O)CH$_2$OH		51
16.	3′-Deamino-3′-(4-morpholinyl)-DXR-OL	—CHOHCH$_2$OH		28
17.	3′-Deamino-3′-(3-cyano-4-morpholinyl)-DXR	—C(=O)CH$_2$OH		36
18.	*N,N*-Dimethyl-DAU	—C(=O)CH$_3$	—N(CH$_3$)$_2$	108
19.	*N*-Decyl-DAU-OL	—CHOHCH$_3$	—NH(CH$_2$)$_9$CH$_3$	23
20.	*N*-(2,2-Diphenylethyl)-DAU	—C(=O)CH$_3$	—NHCH$_2$CH(C$_6$H$_5$)$_2$	6
21.	*N*-(*p*-Dimethylaminobenzyl)-DAU	—C(=O)CH$_3$	—NHCH$_2$C$_6$H$_4$N(CH$_3$)$_2$	46
22.	*N*-Benzyl-DAU	—C(=O)CH$_3$	—NHCH$_2$C$_6$H$_5$	12
23.	*N*-Benzyl-DAU-OL	—CHOHCH$_3$	—NHCH$_2$C$_6$H$_5$	30
24.	*N*-Benzyl-*N*-(2-hydroxyethyl)-DAU	—C(=O)CH$_3$		10
25.	*N,N*-Dibenzyl-DAU	—C(=O)CH$_3$	—N(CH$_2$C$_6$H$_5$)$_2$	2
26.	*N,N*-Dibenzyl-DAU-OL	—CHOHCH$_3$	—N(CH$_2$C$_6$H$_5$)$_2$	4
27.	*N,N*-Dibenzyl-*N*-oxide-DAU	—C(=O)CH$_3$		21
28.	3′-Deamino-3′-(1-piperidinyl)-DAU	—C(=O)CH$_3$		73

(*continued*)

Table 2 (*continued*)

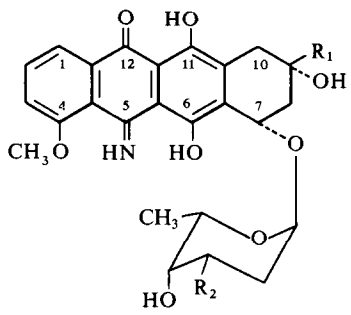
Anthracycline No.	Name	R_1	R_2	Percent of DXR activity
29.	3′-Deamino-3′-(4-methoxy-1-piperidinyl)-DAU	—C(=O)CH$_3$		73
30.	3′-Deamino-3′-(4-methoxy-1-piperidinyl)DAU-OL	—CHOHCH$_3$		64
31.	3′-Deamino-3′-(4-methoxymethyl-1-piperidinyl)-DAU	—C(=O)CH$_3$		84
32.	3′-Deamino-3′-[4,4-bis(methoxymethyl)-1-piperidinyl]-DAU	—C(=O)CH$_3$		50
33.	3′-Deamino-3′-(4-morpholinyl)-DAU	—C(=O)CH$_3$		26
34.	3′-Deamino-3′-(4-morpholinyl)-DAU-OL	—CHOHCH$_3$		29
35.	3′-Deamino-3′-(3-cyano-4-morpholinyl)-DAU	—C(=O)CH$_3$		29
36.	3′-Deamino-3′-(1-thio-4-morpholinyl)-DAU	—C(=O)CH$_3$		17
37.	*N*-(Tetrahydropyran-4-yl)-DAU	—C(=O)CH$_3$		68
38.	*N*-Cyclohexyl-DAU	—C(=O)CH$_3$		57
39.	AD-32	—CH$_2$OC(=O)(CH$_2$)$_3$CH$_3$	—NHC(=O)CF$_3$	4

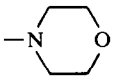
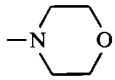
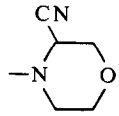
of *cis*-7,9-dihydroxy-9-acetyl-6,7,9,11-tetrahydroxantho[2,3-*g*]tetralin [4, 7, 46]. Both the iminoanthracyclines and the tetralin derivatives exhibited very low activities in the microsome test system.

Aglycones of DXR (Compounds 47 and 48) and of DAU (Compounds 49 and 50) are listed in Table 4. It is interesting that these aglycones exhibited substantial activities although they were less than those of their respective parent drugs. In both series, the C_7-hydroxyl aglycones were more active than the aglycones without the hydroxyl. In the aclacinomycin A series with three, two, and one sugar residues, respectively (Compounds 51, 52, and 53), activity progressively increased as size of the carbohydrate moiety decreased, again suggesting that the bulk of the substituent at C_7 may determine the extent of activity. In addition, as in the DXR and DAU series, we found that replacement of the sugar at C_7 with hydroxyl (Compound 54) reduced the activity of the parent (Compound 53).

Concurrent chromatographic measurement of the disappearance of DXR and the appearance of the 7-deoxyaglycone with the consumption of oxygen in a typical experiment is shown in Fig. 2. No other anthracycline (e.g. 13-dihydro derivatives or other aglycones) was detected and, after a few minutes following depletion of oxygen, no parent DXR was detected. Concurrently, the 7-deoxyaglycone was found in approximately stoichiometric amounts. These results for DXR are also shown on the first line of Table 5, which lists a number of similar test runs with other anthracyclines (Compound 7 of Table 1 and twelve other compounds of Table 2). In almost all cases, very close to theoretical recovery of total compounds as parent and 7-deoxyaglycone was obtained whether those of high (e.g. Compounds 12

Table 3. Effects of 5-imino analogs of DXR and DAU on microsomal O_2 consumption



Anthracycline No.	Name	R_1	R_2	Percent of DXR activity
40.	5-Imino-DXR	$-\overset{O}{\overset{\|}{C}}CH_2OH$	$-NH_2$	8
41.	3′-Deamino-3′-(4-morpholinyl)-5-imino-DXR	$-\overset{O}{\overset{\|}{C}}CH_2OH$		6
42.	3′-Deamino-3′-(4-morpholinyl)-5-imino-DXR-OL	$-CHOHCH_2OH$		5
43.	3′-Deamino-3′-(3-cyano-4-morpholinyl)-5-imino-DXR	$-\overset{O}{\overset{\|}{C}}CH_2OH$		5
44.	5-Imino-DAU	$-\overset{O}{\overset{\|}{C}}CH_3$	$-NH_2$	8
45.	5-Imino-DAU-OL	$-CHOHCH_3$	$-NH_2$	11
46.	*N*,*N*-Dibenzyl-5-imino-DAU	$-\overset{O}{\overset{\|}{C}}CH_3$	$-N(CH_2C_6H_5)_2$	2

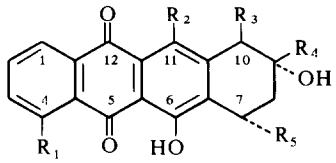
and 18) or low activity (Compounds 22 and 25) were tested. Recoveries of the fourteen compounds including DXR ranged from 80 to 123% of theoretical, with a mean of 102%.

Comparison of Compound 23 with its diastereoisomers (Compounds 23A and 23B, Table 5) indicated that the isomers gave essentially the same extents of formation of aglycone, although the recovery for the mixture was closer to theoretical than that for the isomers. The relative activities for enhancing oxygen consumption in liver microsomes of Compound 23 (the diastereoisomeric mixture) and the separate isomers are shown in Table 6. No difference was noted. In other screening tests employed at SRI for a first assessment of activities of anthracyclines, we found nearly equivalent activities for the inhibition of DNA and RNA synthesis in L1210 cells by the mixture and the isomers of Compound 23 (Table 6). It is apparent that isomerism at C_{13} did not influence activity in these tests. Our earlier studies in rats had demonstrated that only single isomers are formed *in vivo* by reduction of the keto group at C_{13} in Compounds 22, 25, and 44 [41, 47].

More extensive tests of the comparative activities of selected anthracyclines produced the kinetic data of Table 7. All the compounds exhibited saturation kinetics as shown by the assessments of the relation between levels of substrates and activities. V_{max} and K_m values calculated either by the plotting techniques of Lineweaver and Burk or of Hofstee were nearly identical, and the values for V_{max}/K_m are roughly comparable in percent of DXR to the values for the test compounds listed in Tables 2 and 3 in percent of DXR. Especially noteworthy is Compound 44, which was nearly inactive in both the screening and the kinetic tests. Thus, the values in Tables 1–3 are good first approximations of relative activities of these anthracyclines.

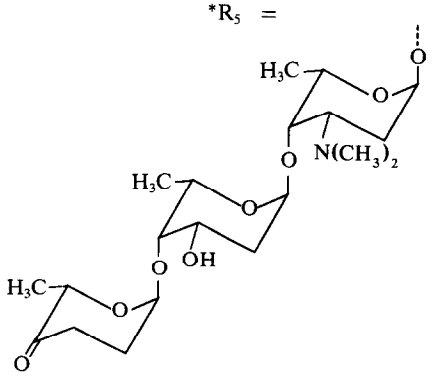
Measurement of reactive oxygen species generated from chemically reduced anthracyclines in the presence of oxygen. The uptake of oxygen observed with anthracyclines in the presence of microsomes involves

Table 4. Effects of DXR and DAU aglycones and aclacinomycin A and its derivatives on microsomal O_2 consumption

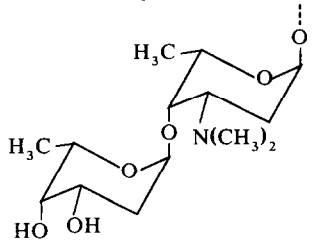


Anthracycline No.	Name	R_1	R_2	R_3	R_4	R_5	Percent of DXR activity
47.	DXR-ONE	$-OCH_3$	$-OH$	$-H$	$-\overset{O}{\overset{\|}{C}}CH_2OH$	$-OH$	68
48.	7-Deoxy-DXR-ONE	$-OCH_3$	$-OH$	$-H$	$-\overset{O}{\overset{\|}{C}}CH_2OH$	$-H$	30
49.	DAU-ONE	$-OCH_3$	$-OH$	$-H$	$-\overset{O}{\overset{\|}{C}}CH_3$	$-OH$	70
50.	7-Deoxy-DAU-ONE	$-OCH_3$	$-OH$	$-H$	$-\overset{O}{\overset{\|}{C}}CH_3$	$-H$	10
51.	Aclacinomycin A	$-OH$	$-H$	$-\overset{O}{\overset{\|}{C}}OCH_3$	$-CH_2CH_3$	—*	34
52.	MA 144 SI	$-OH$	$-H$	$-\overset{O}{\overset{\|}{C}}OCH_3$	$-CH_2CH_3$	—†	107
53.	1-Deoxypyrromycin	$-OH$	$-H$	$-\overset{O}{\overset{\|}{C}}OCH_3$	$-CH_2CH_3$	—‡	136
54.	Aklavinone	$-OH$	$-H$	$-\overset{O}{\overset{\|}{C}}OCH_3$	$-CH_2CH_3$	$-OH$	81
55.	β-Rhodomycinone	$-OH$	$-OH$	$-OH$	$-CH_2CH_3$	$-OH$	113
56.	γ-Rhodomycinone	$-OH$	$-OH$	$-OH$	$-CH_2CH_3$	$-H$	44

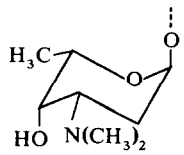
*R_5 =



†R_5 =



‡R_5 =



enzymatic reduction of the drug chromophore and interaction with oxygen to generate reactive oxygen species and the relatively unreactive semiquinone (Fig. 1). These oxygen species can also be generated by interaction of oxygen with the anthracycline activated by reduction with sodium borohydride [5, 6]. To explore the relationship between the enzyme-mediated and the chemically-mediated activation of oxygen, we measured the extent of production of the superoxide anion and the hydroxyl radical by chemically-reduced anthracyclines.

Table 8 presents in column 2 the relative production of superoxide anion by twenty-six anthracyclines (in percent of DXR). Because the formation of super-

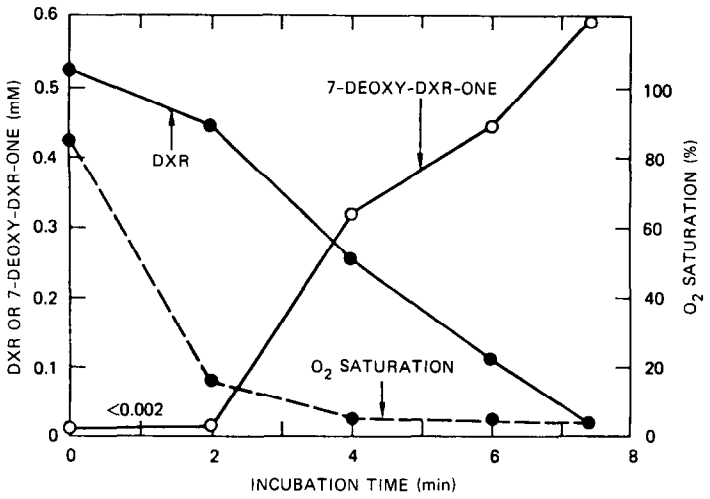


Fig. 2. Typical pattern of the disappearance of the parent anthracycline (DXR), depletion of oxygen, and appearance of the 7-deoxyaglycone with time in incubation media containing rat liver microsomes.

oxide anion from DXR was inhibited by the addition of calf thymus DNA, we also examined this effect on a number of the derivatives (column 3 of Table 8). Also, to examine whether the inhibitory effects of DNA on the generation of superoxide anion by the chemically-activated anthracyclines is a function of their DNA association constant, we also measured their DNA association constants. The value of the association constant given (column 4 of Table 8) is the mean of four measurements made at conditioning potentials of −0.4, −0.6, −0.8, and −1.0 V; these values are in good agreement and do not systematically vary with potential.

Finally, as a measure of hydroxyl radical production in the chemical system, we show in the last column of Table 8 the results of measurements of the extent of single-strand scission of PM2-CCC-DNA by various anthracyclines. The values for Compounds 1, 7, 12, and 18 were reported in an earlier communication [6].

Electrochemical studies. The quinone half-wave potentials reported in Table 9 are confirmed by peak potentials in cyclic voltammetry. However, in few of these measurements does the reduction appear to be a simple reversible two-electron process, so the potential given in Table 9 can be considered only an approximate value of the free energy of reduction. The potentials suggest, however, grouping of the compounds into three classes: (a) most of the compounds with a redox potential near −0.65 V; (b) 11-deoxy Compounds 3, 4, 10, and 11 which, like aclacinomycin A

Table 5. Metabolism of anthracyclines to 7-deoxyaglycones by rat liver microsomes

Anthracycline No.*	Initial concn (nmoles/ml)	Compound found (nmoles/ml)		Recovery	
		Parent	7-Deoxyaglycone	Total nmoles/ml	Percent of initial concn
1	495	16	592	608	123
7	512	23	402	425	83
12	495	5	449	454	92
17	100	<3	98	98	98
18	494	13	480	493	100
20	504	290	140	430	85
22	500	397	4	401	80
23†	99	<3	110	110	111
23A‡	99	<3	63	63	64
23B§	99	6	65	71	72
25	526	485	<3	485	92
28	125	<3	114	114	91
29	125	<3	121	121	97
33	125	37	72	109	87
34	101	48	43	91	90
37	100	12	80	92	92

* See Tables 1 and 2 for the identity of the anthracycline.

† Mixture of 13-dihydro diastereoisomers.

‡ Purified 13-dihydro isomer with chromatographic characteristics of that formed by enzymatic reduction.

§ Purified 13-dihydro isomer formed by chemical reduction.

Table 6. *In vitro* tests of the diastereoisomers of *N*-benzyl-DAU-OL

Isomer tested	Microsomal O_2 consumption (% of DXR)	ED_{50} (μM) for inhibition of nucleic acid synthesis by L1210 cells*	
		DNA	RNA
Diastereoisomeric mixture	30	1.8	0.52
Metabolic isomer†	32	1.8	0.52
Chemical isomer	27	1.7	0.43

* Studies were conducted by D. Taylor, SRI International.
† Chromatographically identical to the isomer produced metabolically from *N,N*-dibenzyldaunorubicin [41].

and related Compounds 51–54, were about 100 mV easier to reduce, and (c) 5-imino derivatives (Compounds 40, 45, and 46), which were about 100 mV more difficult to reduce.

Significant variation of reoxidizability, which is simply the ratio of subsequent anodic peak current to preceding cathodic peak current, was found for all compounds save some whose reoxidizability was high (>90%) or low (<30%) at all sweep rates (Fig. 3). The lowest levels of reoxidizability were, as expected, found for the 5-imino derivatives (Table 9, Compounds 40, 45, and 46). It is noteworthy that Compounds 3 and 10 followed the same pattern, as well as Compounds 4 and 11, suggesting that demethylation at position 4 significantly affects reoxidation.

Correlations between various measured parameters. Possible correlations between the various measurements of the properties of the anthracyclines were examined by linear regression analyses, and the results of tests wherein ten or more pairs of observations were available are shown in Table 10. P values of >0.05 are not considered significant and, therefore, there is *no* correlation between the pairs of observations yielding those values. As shown in the first group of entries, augmentation of microsomal oxygen consumption was positively correlated with superoxide anion production (Fig. 4) or hydroxyl radical production as measured by DNA nicking in the chemical system (Fig. 5). Adding DNA to the chemical system for measuring superoxide anion production caused inhibition but the level of inhibition was unrelated to the microsomal oxygen consumption or to the production of superoxide anion in the chemical system in the absence of DNA (second group of entries). Thus, it is not surprising that the DNA association constants are not related to microsomal oxygen consumption or to superoxide anion production in the chemical system without added DNA as shown in the second group of entries. However, the inhibitory role of DNA in the chemical system was a function of the DNA association constant as shown in the third group of entries. It is most interesting to note that the half-wave potential was negatively correlated with both the microsomal oxygen consumption and the production of superoxide anion in the chemical system. No relationships were discerned among DNA association constants, half-wave potentials, or reoxidizabilities of the anthracyclines tested.

DISCUSSION

Our previous studies suggested a direct relation between the ability of a given anthracycline, such as Compound 44 (Table 3), to augment hepatic microsomal oxygen consumption and *in vivo* cardiotoxicity [5–7]. The present results permit a closer analysis of those structural parameters in anthracyclines that promote or suppress the ability of anthracyclines to augment microsomal oxygen consumption and to activate oxygen after chemical reduction.

It is apparent from the data summarized in Table 1 that the extent of microsome-induced oxygen consumption was relatively insensitive to minor structural changes in the anthracycline. Thus, removal of the 4-methyl and 11-hydroxy groups in the same molecule; or removal of the 4-methoxy of DAU; or removal of

Table 7. Enzyme kinetic constants for selected anthracyclines in the rat liver microsome test system

Anthracycline No.*	Lineweaver–Burk				Hofstee			
	r	V_{max}†	K_m‡	V_{max}/K_m	r	V_{max}†	K_m‡	V_{max}/K_m
1	0.999	401	0.40	1000	0.988	411	0.41	1000
17	0.993	96	0.12	797	0.976	99	0.13	764
28	0.995	260	0.28	928	0.968	251	0.27	930
29	0.998	253	0.27	937	0.972	283	0.31	913
44	0.999	43	0.91	47	0.896	36	0.74	49

* Final concentrations ranged from 0.05 to 0.50 mM; N = 5.
† Values are nmoles O_2 consumed/mg protein/min.
‡ Values are mM.

Table 8. Superoxide production by chemically-activated anthracyclines in the absence and presence of DNA, DNA association constants, and DNA nicking by chemically-activated anthracyclines

Anthracycline No.	Superoxide production: No DNA (% of DXR)	Superoxide production: DNA added (% inhibition)	DNA association constant* [(1/mole) × 10^5]	DNA nicking by ethidium assay (% of DXR)
1	100	84	1.10	100
3	128	45	0.39	—
4	120	42	0.76	—
7	89	81	0.52	102
10	220	48	0.20	—
11	120	42	0.47	—
12	—	—	—	102
13	13	9	0.08†	—
18	—	—	—	102
22	—	—	—	83
25	—	—	—	11
28	90	97	1.47	99
29	8	77	—	—
30	85	97	1.67	—
33	62	92	1.53	62
36	51	90	—	—
37	90	77	0.38	—
39	28	—	—	—
40	21	0	0.38	10
44	—	—	—	62
45	10	37	0.23	49
47	48	21	0.17‡	—
48	32	7	—	—
49	43	18	0.20§	—
50	27	14	0.04§	—
51	25	77	1.26	51
52	167	85	0.73	—
53	187	74	0.52	—
54	133	20	0.08†	—
55	72	54	0.00§	—
56	41	—	—	—

* Determined in aqueous phosphate buffer unless otherwise noted.
† In 3% aqueous acetonitrile.
‡ In 5% aqueous acetonitrile.
§ In 30% aqeuous acetonitrile.

the hydroxy group, inverting the configuration of the hydroxy group or adding a methoxy at the 4'-carbon of the sugar yielded activities within about 20% of the parent compounds. Substantial decreases were only noted when the 11-hydroxy group was deleted from either DXR or DAU. Compound 2, 4-demethoxy-DXR, exhibited an unusually low activity, and we can offer no explanation for this result.

By contrast, introduction of bulky substituents either in the sugar moiety or the C_{13} side chain caused substantial changes in microsomal oxygen consumption, as illustrated in Table 2. The highest activities were found when the sugar amino group was substituted by relatively small groups such as dimethyl in Compounds 12 and 18. The relatively bulky benzyl group invariably reduced activity whether the derivative contained one or two benzyl groups on the nitrogen, the substitution with benzyl was separated by a methylene group from the amino nitrogen, or the benzylated amino group was converted to an N-oxide. Substantial reduction in activity was also observed when the C_{14}-hydroxy of DXR was esterified with a long side chain or when the sugar amino group was masked with the trifluoroacetyl group as in Compound 39. The consistent reduction in activity in the microsome assay as a result of the introduction of bulky substituents in the molecule (and despite undiminished electrochemical redox potential of the chromophore) suggests fairly stringent steric requirements of the metabolizing enzymes involved.

Interestingly, compounds wherein the amino group was substituted with groups such as tetrahydropyranyl or cyclohexyl (Compounds 37 and 38 of Table 2) exhibited activities greater than those of the compounds substituted with aryl groups at the amino nitrogen. However, they also showed diminished activity relative to the parent anthracyclines. Similarly, as a group, the compounds wherein the nitrogen of the amino group had been incorporated into an alicyclic ring (as in the piperidinyl and morpholinyl derivatives) generally yielded compounds with appreciable activities. The most striking exception in this group was the relatively inactive thiomorpholinyl derivative (Compound 36).

The essential requirement of the intact quinone structure for activity in the liver microsome test system was clearly demonstrated by the 5-imino derivatives of Table 3. Without exception, the com-

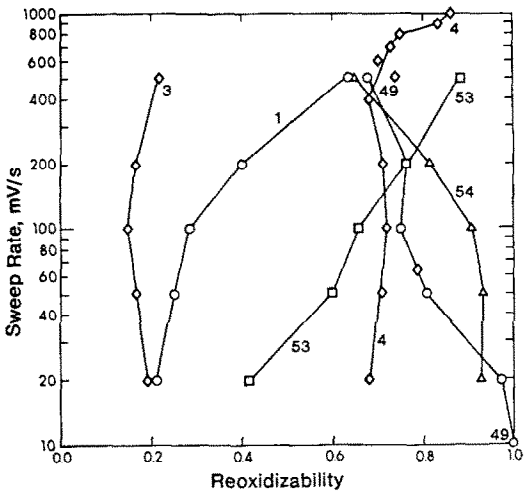


Fig. 3. Reoxidizability of anthracyclines as a function of scan rate. The medium was described in Table 9 with the concentration of compounds indicated. Each symbol corresponds to 1–6 separate measurements using fresh hanging mercury drop electrodes each time, generally agreeing to ±3%. Compound 10 (not shown) follows the same pattern as Compound 3, while Compounds 47, 48 and 50 (also not shown) are on an almost vertical line at 100%.

pounds of this group were relatively inactive. On the other hand, the aglyclones, but not the 7-deoxy-aglycones, of DXR and DAU exhibited relatively high activities (Table 4). The only other class of chromophore-modified anthracyclines reported to date, the glycosides of *cis*-7,9-dihydroxy-9-acetyl-6,7,9,11-tetrahydroxantho[2,3-g]tetralin, which exhibit antileukemic cytotoxicity, are also very low in microsomal activity (1–12% of DXR) [46]. This

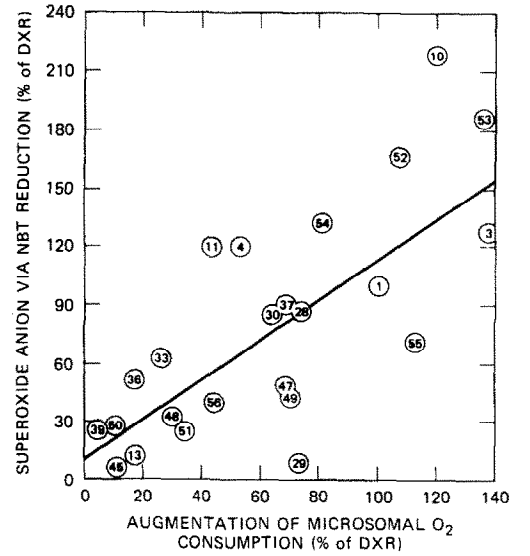


Fig. 4. Correlation of superoxide anion production by anthracyclines chemically reduced in the presence of oxygen with augmentation of oxygen consumption by metabolically-activated anthracyclines. The circled numbers identify the anthracyclines (see Tables 1–4).

Table 9. Half-wave potential and reoxidizability of anthracyclines

Anthracycline	Half-wave potential (V)	Reoxidizability (%)
1	−0.64	63
3	−0.53	22
4	−0.54	73
7	−0.64	67
10	−0.53	26
11	−0.54	63
12	−0.65*'†	98
13	−0.67*	91‡
18	−0.64*'†	92
25	−0.65*	87‡
28	−0.64§	82
29	−0.635	77
30	−0.60	44
33	−0.63§	48
36	−0.62	76
37	−0.64*	81
39	−0.66*	65*
40	−0.72*	25*
45	−0.70‖	40*, 32‖
46	−0.745*	>2*
47	−0.67*	100*
48	−0.72*	100*
49	−0.65‖	68‖
50	−0.72*	100*
51	−0.55, −0.65†'‖	72
52	−0.57, −0.65†	71
53	−0.54, −0.61†	88
54	−0.58	65
55	−0.62, 0.74*'†	93
56	−0.67, −0.78*'†	91

* Measured in 50% aqueous CH_3CN.

† Double wave in classical polarography; appearing as double peak in DPP and double cathodic peak in CV.

‡ Measured at 200 V/sec.

§ Value for aqueous solution; a value for 50% CH_3CN is given in Ref. 6.

‖ Measured in 30% aqueous CH_3CN.

confirms the requirement for a quinone moiety for this latter activity. In addition, increasing the bulk of the substitution at C_7 in aclacinomycin A derivatives with di- and trisaccharides decreased the activity of the parent 1-deoxypyrromycin (Table 4). The 7-deoxyaglycone, γ-rhodomycinone (Compound 56), was also relatively low in activity compared with its 7-hydroxy analog, β-rhodomycinone (Compound 55).

The correlations between the activities in the microsome screening test with both a chemical measure of the production of superoxide anion, the primary reactive oxygen species, and a biochemical measure of the secondarily formed hydroxyl radical are consistent with currently available information on the mechanism of the generation of toxic radicals from DXR and DAU [10–12, 48]. These latter two assays were designed to determine distinctly different species. The assay involving DNA nicking measures the generation of hydroxyl radicals eight chemical steps after the initial reduction of the anthracycline chromophore [6]. The NBT assay, in contrast, measures the formation of superoxide anion generated

Table 10. Tests of correlation of various groups of observations

Comparison	No. of pairs	Correlation coefficient (r)	Level of significance of correlation (P)
Microsomal O_2			
vs superoxide (no DNA)	26	0.738	<0.001
vs superoxide (with DNA)	24	0.281	>0.05
vs DNA nicking	12	0.803	<0.005
vs DNA association constant	21	−0.135	>0.05
vs half-wave potential	30	−0.548	<0.005
vs reoxidizability	30	0.106	>0.05
Superoxide (no DNA)			
vs superoxide (with DNA)	23	0.287	>0.05
vs DNA association constant	21	0.0308	>0.05
vs half-wave potential	26	−0.766	<0.001
vs reoxidizability	26	0.266	>0.05
Superoxide (with DNA)			
vs DNA association constant	21	0.778	<0.001
vs half-wave potential	24	−0.392	>0.05
vs reoxidizability	24	−0.0741	>0.05
DNA association constant			
vs half-wave potential	21	−0.202	>0.05
vs reoxidizability	19	−0.184	>0.05
Half-wave potential			
vs reoxidizability	30	0.0933	>0.05

immediately after the interaction of the anthracycline semiquinone with oxygen [7]. Reduction of the NBT occurred both by reaction with the hydroperoxy radical (superoxide ion) and hydrogen peroxide and directly by the reduced antibiotic. The selective inhibition of NBT reduction by superoxide dismutase and catalase, amounting to 74% in the case of DAU, confirms that in the presence of oxygen the major pathway of reduction of NBT is via the initially formed superoxide anion [7].

Our findings that the liver microsomes produced only 7-deoxyaglycone metabolites of anthracyclines (Table 5) confirms the earlier conclusion of Bachur *et al.* [37] and suggests that this limitation of metabolism by liver microsomes may apply generally to anthracyclines. Also, the parallel results between the single measurements of activities and the multiple measurements needed for kinetic studies (Table 7) suggests that the former can serve as a preliminary guide for assessment of redox activity of anthracyclines. In this regard, the close relationship between results using liver microsomes and using chemically activated anthracyclines to produce superoxide anion (Fig. 4) indicates that either test may be used for screening anthracyclines for this activity.

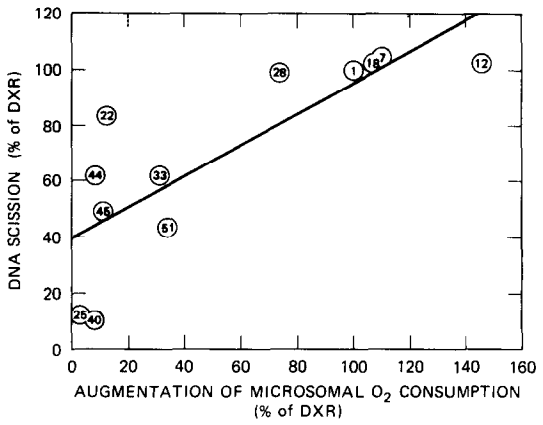


Fig. 5. Correlation of hydroxyl radical production, assayed by DNA scission, by anthracyclines chemically reduced in the presence of oxygen with augmentation of oxygen consumption by metabolically-activated anthracyclines. The circled numbers identify the anthracyclines (see Tables 1–4).

The lack of significant relationships between the measured DNA association constants and the augmentation of microsomal oxygen consumption and the chemical production of superoxide anion in the presence of DNA is in agreement with reports of others [17, 49–51] that intercalation of the anthracyclines with DNA reduces the availability of the compounds for participation in other reactions that depend on activation via metabolic or chemical reduction. Such separation of processes no doubt explains the lack of correlation (Table 10) between the DNA association constants and superoxide anion production without DNA as well as the absence of a correlation between superoxide anion production in the presence and absence of DNA. Finally, consistent with these ideas, is the finding that the DNA association constants and superoxide anion production when DNA was added are highly correlated (Table 10).

While the *in vitro* measurements provide much mechanistic information on the relative activity of various structural variations of the anthracycline

class of antibiotics, we hasten to add that projecting this information to *in vivo* activities must be made with great caution. Given this proviso, certain useful trends may be noted. For example, the imino derivatives (Compounds 40–46, Tables 3, 7, 8) were relatively inactive in all the tests reported in the present paper. Yet, they are effective antitumor agents in murine screens [4, 5]. Others have also reported that Compound 44 (5-imino-DAU) is a very poor substrate for oxygen-radical generating systems [21, 52] and that it is inhibitory in a test system that measures lipid peroxidation as a product of radical generation [22]. We found, in metabolic studies in rats, that Compound 44 is not a prodrug for DAU, nor could we detect the formation of a 7-deoxyaglycone *in vivo* [47]. In addition, available studies in animal models [53] indicate that Compound 44 exhibits substantially less cardiotoxicity than the parent anthracyclines. These observations by us and by others reinforce an earlier suggestion that the imino derivatives provide the first series of anthracyclines wherein cardiotoxicity and anticancer cytotoxicity have been separated [4, 5]. The 9-acetyl-9-hydroxyxantho[2,3-*g*]tetralin chromophore modified analogs, which are virtually devoid of microsomal activity but exhibit antileukemic cytotoxicity [7, 46], provide another example in this class of compounds wherein the quinone moiety is not essential for anticancer activity.

A different example of the contrast between very low *in vitro* activity in the microsome test system and high *in vivo* antitumor activity in murine systems is *N,N*-dibenzyldaunorubicin (Compound 25, Table 2). We [41] found this compound to be acting as a prodrug for more active metabolites in studies in rats. With the dibenzyl derivative, no biochemical mechanistic test *in vitro*, which does not incorporate metabolic alteration of the drug, disclosed this activity. This compound is also another relatively noncardiotoxic anthracycline derivative [54].

The most potent cytotoxic group of anthracyclines to date are the morpholinyl and cyanomorpholinyl derivatives, Compounds 15, 17, 33, and 35 of Table 2 [29]. In the rat microsomal or the chemical test system these compounds did not exhibit high activities. However, tests using P388 tumor cells showed that they are taken up much more readily than was DXR; they are also cytotoxic to P388 cells resistant to DXR [55]. In addition, Compound 17 of this series exhibited increased molar potencies of 150 to 15,000 times that of DXR against human breast and ovarian carcinomas in clonogenic assays [56].

In conclusion, the augmentation of oxygen consumption by rat liver microsomes and related chemical assays can be useful techniques for an initial assessment of one important aspect of the activity of anthracyclines. However, the results must be combined with pharmacological data, especially on drug disposition and metabolism, to provide pertinent basic information for the development of useful chemotherapeutic agents.

Acknowledgements—This work was supported in part by Grant CA-32215 (to J. H. P.) from the National Cancer Institute, USPHS; and by grants from the National Cancer Institute of Canada (to J. W. L.) and from the Natural Sciences and Engineering Research Council of Canada (to J. W. L. and J. A. P.). The authors thank Dr. E. M. Acton of SRI International for providing compounds and his substantial knowledge in the anthracycline field of chemotherapeutic agents.

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