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COMPARATIVE CARDIAC OXYGEN RADICAL METABOLISM BY ANTHRACYCLINE ANTIBIOTICS, MITOXANTRONE, BISANTRENE, 4′-(9-ACRIDINYLAMINO)-METHANESULFON-*m*-ANISIDIDE, AND NEOCARZINOSTATIN*

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Abstract—This study examined the effects of various anthracycline antibiotics and mitoxantrone, bisantrene, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA), and neocarzinostatin on oxygen radical formation by cardiac sarcoplasmic reticulum and submitochondrial particles. Doxorubicin, daunorubicin, rubidazone, and aclacinomycin A stimulated superoxide production by both heart fractions in a dose-dependent fashion that appeared to follow saturation kinetics. The anthracycline drugs also significantly increased hydrogen peroxide production by heart sarcosomes and submitochondrial particles. On the other hand, mitoxantrone, bisantrene, *m*-AMSA, and neocarzinostatin did not significantly enhance cardiac reactive oxygen metabolism. Thus, it is unlikely that the mechanism of the cardiac toxicity produced by mitoxantrone and *m*-AMSA in patients previously treated with anthracycline drugs can be directly related to oxidation–reduction cycling catalyzed by cardiac flavin dehydrogenases.

The anthracycline antibiotics are among the most useful chemotherapeutic agents available for the treatment of a wide range of human malignancies [1]. Unfortunately, these drugs produce a cumulative, dose-dependent form of cardiac toxicity that can seriously compromise their utility in clinical oncologic practice [2].

Recent investigations from several laboratories indicate that anthracycline cardiac toxicity may be related to drug-induced oxygen radical metabolism in the heart; these studies suggest that drug treatment could initiate a free radical cascade capable of overwhelming the limited antioxidant defenses of the myocardial cell [3, 4]. Furthermore, drug-induced alterations in the content of myocardial reducing equivalents, including reduced thiols, may play an important role in the expression of anthracycline cardiomyopathy [5, 6]. It now appears that anthracycline-related free radical production is a consequence of the cyclical oxidation and reduction of the anthracycline quinone catalyzed by certain flavin-containing dehydrogenases [7, 8]. Studies by Thayer [9], by Bachur *et al.* [10], and by our own laboratory [11–13] have identified the NADPH cytochrome P-450 reductase activity of cardiac sarcoplasmic reticulum (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4), the cardiac mitochondrial NADH dehydrogenase complex (NADH:[acceptor]oxidoreductase, EC 1.6.99.3), and cardiac xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) as the flavin enzymes capable of activating anthracycline drugs to free radicals in the heart. Based on these experimental studies, we have postulated that the characteristic pathologic picture of anthracycline cardiac toxicity, which consists of disruption of heart mitochondrial and sarcoplasmic reticular membranes [14], may be explained by drug-induced free radical formation in specific myocardial compartments [13].

In recent years, several anthracycline analogs, as well as new antineoplastic agents with similarities to the anthracycline antibiotics, have been developed as potential, non-cardiotoxic alternatives to doxorubicin and daunorubicin. Many of these drugs are currently undergoing extensive clinical trials in man [15, 16], and several, including the anthracyclines aclacinomycin A and rubidazone [17, 18], the non-anthracycline quinone mitoxantrone [15], and the acridine derivative 4′-(9-acridinylamino)-methane-

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sulfon-*m*-anisidide (*m*-AMSA)* [19], are therapeutically active in the treatment of hematological malignancies and solid tumors. Unfortunately, it has been suggested recently that both mitoxantrone and *m*-AMSA could produce clinical congestive heart failure in patients extensively pretreated with doxorubicin or daunorubicin [20–22]. In particular, a congestive cardiomyopathy which depends on the cumulative dose of *m*-AMSA or mitoxantrone may develop in patients previously treated with less than 200 mg/m^2 anthracycline [23–25]. Because recent studies have shown (1) that mitoxantrone can stimulate reactive oxygen production in an hepatic microsomal system [26], (2) that the metabolism of *m*-AMSA may lead to the formation of a quinoidal diimine as well as to alterations in cellular glutathione status [27], and (3) that the cytotoxic effects of neocarzinostatin are mediated, at least in part, by free radical production [28], we have attempted in this study to compare the effects of several anthracycline and non-anthracycline chemotherapeutic agents on oxygen radical production by heart sarcoplasmic reticulum and mitochondria. Our specific goal was to evaluate the potential of each drug to damage the heart by a free radical mechanism that involved flavin-enzyme catalyzed drug activation.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 180–200 g were obtained from the Simonsen Laboratories, Gilroy, CA; from weaning, these animals had been raised on a diet of Wayne Lab-Blox rat pellets with water available *ad lib*. Fresh bovine hearts were a gift of the Acme Meat Co., Los Angeles, CA. Daunorubicin hydrochloride, rubidazone, aclacinomycin A, and *m*-AMSA were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Neocarzinostatin was a gift of Dr. W. T. Bradner, Bristol Laboratories, Syracuse, NY. Mitoxantrone and bisantrene were supplied by Dr. Robert Pocelinko of the Medical Research Division, Lederle Laboratories, American Cyanamid Co., Pearl River, NY. The anthracycline antibiotics and mitoxantrone were reconstituted in sterile water. *m*-AMSA and bisantrene were completely dissolved in dimethyl sulfoxide; the final concentration of dimethyl sulfoxide in all experiments with these two agents did not exceed 1.4 M, a concentration which we found in preliminary experiments did not alter the rate of superoxide production or oxygen consumption by control preparations of cardiac sarcoplasmic reticulum or submitochondrial particles. After reconstitution, the drugs were protected from light until used. Sodium acetate, acetic anhydride, and ethyl alcohol (99% pure) were purchased from the Fisher Scientific Co., Fair Lawn, NJ. Bovine erythrocyte superoxide dismutase (SOD, 2900 units/mg as assayed by the method of McCord and Fridovich [29]), bovine albumin fraction V, cytochrome *c* (type VI from horse heart), EDTA, NADPH type III, NADH grade III, rotenone, and L-histidine were purchased from the Sigma Chemical Co., St. Louis, MO. Catalase of analytical grade (65,000 units/mg protein) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, and was devoid of SOD activity when assayed by the method of McCord and Fridovich [29]. Only glass-distilled, deionized water was used in these studies.

Rat cardiac sarcoplasmic reticulum was prepared by the method of Martonosi [30], exactly as previously described [12], and, most often, was used on the day of preparation. Occasionally, the heart sarcoplasmic reticulum was stored before use for 1 week or less at −70°; preliminary experiments indicated that this procedure resulted in no loss of enzymatic activity. Bovine heart SMP were prepared from bovine heart mitochondria essentially as described by Beyer [31] for ETPH (EDTA-I) except that the isolation medium contained 0.21 M mannitol, 0.07 M sucrose, and 0.01 M Tris–HCl, pH 7.5, and that the mitochondria were sonicated twice.

Superoxide anion production in the experimental samples was determined by the SOD-inhibitable rate of acetylated cytochrome *c* reduction as previously described [13]. The initial, linear rate of acetylated cytochrome *c* reduction was measured spectrophotometrically at 37° and 550 nm with a Gilford model 260 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) equipped with a circulating water bath. Reactions were initiated by the addition of NAD(P)H; chemotherapeutic drugs were added to the reaction mixtures, where indicated, before initiation with reduced cofactor. The rate of superoxide production was calculated from the extent of acetylated cytochrome *c* reduction inhibited by SOD using an extinction coefficient for cytochrome *c* (reduced minus oxidized) of 19.6 mM^{-1} cm^{-1} [32]. Superoxide production for the anthracyclines was investigated over a 10-fold range of drug concentrations, and kinetic constants were determined from Lineweaver–Burk plots of the data. Superoxide formation for the non-anthracycline chemotherapeutic agents was determined using the drug concentrations indicated in the tables.

Oxygen consumption by heart sarcoplasmic reticulum and SMP was analyzed at 37° with a YSI model 53 oxygen monitoring system (Yellow Springs Instrument Co., Yellow Springs, OH) by our previously published technique [12]. The rate of oxygen consumption was based on a value of 597 nmoles for the total dissolved oxygen content of the experimental sample [33]. Hydrogen peroxide accumulation was determined from the percentage of the total oxygen consumption of the experimental mixture that was released by addition of excess catalase (1500 units/ml) to the reaction vessel using a Hamilton syringe introduced into the test chamber through the access slot of the oxygen electrode plunger.

Protein concentrations were determined by the method of Lowry *et al.* [34] using crystalline bovine albumin as the standard. Data were analyzed by the two-tailed *t*-test for independent means (NS, not significant, $P > 0.05$ [35]).

* Abbreviations: *m*-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; SOD, superoxide dismutase; SMP, submitochondrial particles; and ETPH, electron transport particles.

RESULTS AND DISCUSSION

To evaluate the hypothesis that drug-stimulated oxygen radical metabolism might explain the cardiac toxicity of various anthracycline analogs as well as certain new, non-anthracycline chemotherapeutic agents, we examined the effect of these drugs on oxygen radical production by cardiac sarcoplasmic reticulum and submitochondrial particles. The results of this study, shown in Table 1, are consistent with our previous investigations [12, 13]; the anthracyclines doxorubicin, daunorubicin, rubidazone, and aclacinomycin A all increased superoxide anion production by cardiac sarcoplasmic reticulum in a dose-dependent fashion that appeared to follow saturation kinetics. Measurement of superoxide production in this system required each component of the reaction mixture, including drug, NADPH, acetylated cytochrome *c*, and sarcoplasmic reticular membrane, and was completely abolished when the heart sarcosomes were denatured by heat (data not shown). Furthermore, anthracycline treatment significantly enhanced both the rate of oxygen consumption and hydrogen peroxide production in our sarcosomal system (Table 1).

On the other hand, no increase in superoxide anion production by mitoxantrone or bisantrene was detected in heart sarcoplasmic reticulum over a concentration range of 50–300 μM drug. To confirm these findings, we examined the effect of mitoxantrone and bisantrene on the rate of oxygen consumption by cardiac sarcoplasmic reticulum. We found that neither drug at a concentration of 1 mM (5-fold that used for experiments with the anthracyclines) produced any significant effect on the control rate of oxygen consumption in heart sarcoplasmic reticulum, and neither drug increased hydrogen peroxide formation over control levels (Table 1). In a recent study, Kharasch and Novak [26] showed that mitoxantrone was more than 5-fold less active than doxorubicin in stimulating electron transfer and superoxide formation by purified hepatic NADPH cytochrome P-450 reductase. Our finding that cardiac sarcoplasmic membranes did not support reactive oxygen production by mitoxantrone is not unexpected since the specific activity of this enzyme in heart sarcoplasmic reticulum is less than 10% of that found in liver microsomes [13, 36]. In related experiments, we found that neocarzinostatin and *m*-AMSA did not stimulate superoxide anion or hydrogen peroxide production by cardiac sarcoplasmic reticulum (Table 1). The effect of *m*-AMSA on oxygen radical metabolism by cardiac sarcoplasmic reticulum was specifically investigated in these experiments because recent studies have suggested that certain *m*-AMSA metabolites might be capable of initiating oxygen radical formation catalyzed by flavin dehydrogenases [27]. In fact, we found that *m*-AMSA appeared to reduce sarcosomal oxygen consumption significantly (Table 1). Since the metabolism of *m*-AMSA by cardiac sarcoplasmic reticulum was not investigated in this study, we can only speculate on the mechanism of inhibition of oxygen consumption by *m*-AMSA; however, it is

Table 1. Effect of anticancer agents on oxygen radical metabolism by heart sarcoplasmic reticulum

Drug	Kinetic constants for drug-related superoxide production*		Oxygen consumption† (nmoles O_2/min/mg)	H_2O_2 formation† (% total O_2 consumption released by catalase)
	K_m (μM)	V_{max} (nmoles/min/mg)		
Control			12.14 ± 1.39‡	1.43 ± 0.47
Doxorubicin	112.6	13.3	15.40 ± 3.00§	6.36 ± 1.88§
Daunorubicin	33.9	12.3	15.72 ± 1.87§	3.60 ± 1.38§
Rubidazone	409.3	39.2	15.28 ± 0.33§	1.30 ± 0.52
Aclacinomycin A	479.4	60.6	18.11 ± 1.87‖	5.70 ± 1.73‖
Mitoxantrone¶			8.96 ± 2.21	1.20 ± 0.65
Bisantrene¶			8.92 ± 0.97	0.65 ± 0.43
m-AMSA**			4.26 ± 0.83‖	0.75 ± 0.61
Neocarzinostatin††			13.56 ± 1.38	0.90 ± 0.10

* Superoxide production was determined in quadruplicate for each drug dose tested; for the anthracyclines, kinetic constants were calculated from Lineweaver–Burk plots of the data over a 10-fold concentration range. The paired, 1-ml reaction mixtures contained 150 μmoles potassium-phosphate buffer, pH 7.4, 100 nmoles EDTA, 56 nmoles acetylated cytochrome *c*, 200 μg sarcosomal protein, either 0 or 10 μg SOD, and various concentrations of the antineoplastic agents. Reactions were initiated by the addition of 1 μmole NADPH.

† Oxygen consumption was measured in triplicate in a 3-ml reaction mixture that contained 450 μmoles potassium-phosphate buffer, pH 7.4, 300 nmoles EDTA, 1.5 mg sarcosomal protein, and 200 μM anthracycline or the amount of other chemotherapeutic agents indicated in the footnotes. Reactants were bubbled with air for at least 30 min at 37° before use. Reactions were initiated by the addition of 3 μmoles NADPH.

‡ Mean ± S.D.

§ Significantly different from control, $P < 0.05$.

‖ Significantly different from control, $P < 0.01$.

¶ Superoxide formation was examined over the drug concentration range 50–300 μM; oxygen consumption was assayed at a drug concentration of 1 mM. No superoxide production above control levels was detected with these drugs.

** Superoxide formation was determined over the range of 10–100 μM *m*-AMSA and did not differ from control levels; oxygen consumption was assayed at an *m*-AMSA concentration of 1 mM.

†† Superoxide production was determined over the range of 50–200 units/ml neocarzinostatin and did not differ significantly from control levels; oxygen consumption was assayed at a concentration of 200 units/ml.

possible that these findings may be related to the ability of the parent compound to bind reduced thiols [37] that are known to be at the active site of NADPH cytochrome P-450 reductase [36]. Furthermore, it remains to be determined whether the quinoidal diimine hepatic metabolite of *m*-AMSA could stimulate reactive oxygen production by oxidation–reduction cycling.

In a parallel set of experiments, superoxide production and oxygen consumption were examined using cardiac SMP; all of these studies were performed in the presence of rotenone to block mitochondrial electron flow beyond the NADH dehydrogenase complex. Using this preparation, which contains a flavin enzyme system distinct from NADPH cytochrome P-450 reductase, we obtained findings strikingly similar to those demonstrated with cardiac sarcoplasmic reticulum. All four anthracycline drugs increased the rate of superoxide anion production by heart SMP in a concentration-dependent manner that also appeared to follow saturation kinetics (Table 2); furthermore, the relative activity of these agents followed the same order as that obtained for sarcoplasmic reticulum (Table 1). Drug-stimulated superoxide formation by SMP required NADH and the anthracycline, and was ablated when the cardiac SMP were denatured by heat (data not shown). Doxorubicin, daunorubicin, rubidazone, and aclacinomycin A also significantly increased oxygen consumption and hydrogen peroxide production by SMP (Table 2). Finally, we found that mitoxantrone, bisantrene, *m*-AMSA and neocarzinostatin did not alter significantly the rate of oxygen radical metabolism by cardiac SMP (Table 2).

In these studies, we have demonstrated that various anthracycline analogs, but not mitoxantrone, bisantrene, *m*-AMSA, or neocarzinostatin, are capable of stimulating superoxide anion and hydrogen peroxide production by two cardiac flavin enzyme systems. Thus, it seems entirely possible that aclacinomycin A and rubidazone could eventually be found to produce cardiac toxicity in man. Whereas drug-induced reactive oxygen species may contribute to the cardiac toxicity of the anthracycline antibiotics, the data from these experiments do not suggest that cardiac injury following treatment with mitoxantrone or *m*-AMSA could be directly ascribed to an identical mechanism. As we have shown recently, the range of substrate specificity for quinone activation by NADH dehydrogenase, unlike that for NADPH P-450 reductase, is not broad, and drugs of nearly identical reduction potential (such as doxorubicin and mitomycin C) may produce dramatically different effects on oxygen radical production by the mitochondrial enzyme [12]. Thus, appropriate steric conditions may not exist for electron transfer to the mitoxantrone quinone by at least one of the cardiac dehydrogenases investigated in this study. It is also possible that the half-wave potential of mitoxantrone may not allow substantial reduction of the quinone by flavin-containing enzymes under aerobic conditions [38]. It must be pointed out, however, that our findings do not address the possibility

Table 2. Effect of anticancer agents on oxygen radical metabolism by heart submitochondrial particles

Drug	Kinetic constants for drug-related superoxide production*		Oxygen consumption† (nmoles O_2/min/mg)	H_2O_2 formation† (%total O_2 consumption released by catalase)
	K_m (μM)	V_{max} (nmoles/min/mg)		
Control			6.17 ± 1.56‡	0.26 ± 0.45
Doxorubicin	53.2	58.8	99.93 ± 1.73§	1.34 ± 0.42‖
Daunorubicin	32.3	64.9	42.98 ± 4.31§	1.03 ± 0.33‖
Rubidazone	54.1	66.4	32.84 ± 7.92§	3.35 ± 1.70‖
Aclacinomycin A	119.2	131.5	14.23 ± 5.17‖	2.95 ± 1.70‖
Mitoxantrone¶			6.34 ± 2.75	0.00 ± 0.00
Bisantrene¶			5.90 ± 1.21	0.00 ± 0.00
m-AMSA**			5.53 ± 1.02	0.00 ± 0.00
Neocarzinostatin††			5.50 ± 1.89	0.00 ± 0.00

* Superoxide production was determined in quadruplicate for each drug dose tested; for the anthracyclines, kinetic constants were calculated from Lineweaver–Burk plots of the data over a 10-fold concentration range. The paired, 1-ml reaction mixtures contained 150 μmoles potassium-phosphate buffer, pH 7.4, 100 nmoles EDTA, 56 nmoles acetylated cytochrome *c*, 200 μg SMP protein, 2 nmoles rotenone, 0 or 10 μg SOD, and various concentrations of the antineoplastic agents. Reactions were initiated by the addition of 200 nmoles NADH.

† Oxygen consumption was measured in triplicate in a 3-ml reaction mixture that contained 450 μmoles potassium-phosphate buffer, pH 7.4, 300 nmoles EDTA, 600 μg SMP protein, 6 nmoles rotenone, and 200 μM anthracycline or the amount of other antineoplastic agents specified in the footnotes. Reactants were bubbled with air for 30 min at 37° before use; reactions were initiated by the addition of 3 μmoles NADH.

‡ Mean ± S.D.

§ Significantly different from control, $P < 0.01$.

‖ Significantly different from control, $P < 0.05$.

¶ Superoxide formation was examined over the drug concentration range of 50–350 μM and did not differ significantly from control levels; oxygen consumption was assayed at a drug concentration of 1 mM.

** Superoxide formation was determined over the range of 10–100 μM *m*-AMSA and did not differ from control levels; oxygen consumption was examined at an *m*-AMSA concentration of 1 mM.

†† Superoxide production was determined over the range of 50–200 units/ml neocarzinostatin and did not differ from control levels; oxygen consumption was assayed at a concentration of 200 units/ml.

that mitoxantrone, bisantrene, or *m*-AMSA could damage the heart by a free radical mechanism unrelated to oxidation–reduction cycling or by another cytotoxic mechanism; indeed, cytotoxic effects of the acridines on cell surface membranes have been described recently [39]. In any case, it appears that mitoxantrone, bisantrene, and *m*-AMSA do not share with the anthracycline and various other antineoplastic quinones the capacity to directly undergo an appreciable oxidation–reduction cycle catalyzed by cardiac NADPH cytochrome P-450 reductase or NADH dehydrogenase [40].

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