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NADPH cytochrome *P*-450 reductase activation of quinone anticancer agents to free radicals

(adriamycin/mitomycin C/streptonigrin/"site-specific free radicals")

NICHOLAS R. BACHUR*, SANDRA L. GORDON*, MALCOLM V. GEE*, AND HIDEO KON†

*Laboratory of Clinical Biochemistry, Baltimore Cancer Research Program, Division of Cancer Treatment, National Cancer Institute, 9-021 Howard Hall II, 655 West Baltimore Street, Baltimore, Maryland 21201; and †Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **With NADPH as the electron donor, rat liver NADPH cytochrome *P*-450 reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4) catalyzes the single-electron reduction of several quinone antibiotics to a semiquinone or free radical state. The benzanthraquinones adriamycin, daunorubicin, carminomycin, 7-*O*-methylnogalarol, and aclacinomycin A and the *N*-heterocyclic quinones streptonigrin and mitomycin C are activated to free radical intermediates which can transfer their single electron to molecular oxygen to form superoxide. The overall K_m range for this electron transfer is 0.4 to 42.1 × 10^{-4} M. We postulate that the formation of the "site-specific free radical" intermediate is central to the cytotoxic action of these antibiotics.**

An unusually high proportion of anticancer agents contain quinone groups. Such quinone-containing anticancer agents include adriamycin, daunorubicin, mitomycin C, streptonigrin, lapachol, and analogs of these agents. Although investigators have identified several potential biochemical sites of action for these drugs, preponderant evidence indicates that the agents act primarily by interfering with DNA and RNA replication (1–4).

We have established that these quinone agents are catalytically activated to a free radical state by a microsomal system requiring NADPH as an electron donor (5, 6). Normal microsomes as well as microsomes from murine leukemia cells catalyze augmented oxygen consumption with the quinone antibiotics, indicative of free radical formation. By using effectors of this microsomal system we obtained indirect evidence that a flavoprotein catalyst of the microsomes was involved and that cytochrome *P*-450 was not. Iyanagi and Yamazaki (7) have shown that NADPH cytochrome *P*-450 reductase (NADPH: ferricytochrome oxidoreductase, EC 1.6.2.4) reduces quinone substrates to semiquinones; and Goodman and Hochstein (8) reported that NADPH cytochrome *P*-450 reductase catalyzes production of superoxide from adriamycin. In the present paper, we describe and characterize the catalysis of drug free-radical formation by homogeneous, soluble, NADPH cytochrome *P*-450 reductase from rat liver microsomes and propose a mechanism for the cytotoxic action of these agents.

METHODS AND MATERIALS

Adriamycin, *N*-dimethyladriamycin, daunorubicin, *N*-acetyldaunorubicin, 7-iminodaunorubicin, aclacinomycin A, mitomycin C, streptonigrin, and lapachol were kindly provided by the Drug Synthesis and Chemistry Branch and the Natural Products Branch (Division of Cancer Treatment, National Cancer Institute, National Institutes of Health). Daunorubicinol (9) and adriamycin $(Fe^{3+})_4$ (10) were prepared as described. Carminomycin was provided by G. F. Gause (Institute of New Antibiotics, Moscow). Rubidazone was supplied by Rene Maral (Rhone-Poulenc, Vitry-Sur-Seine, France). Nogalamycin, 7-*O*-methylnogarol, and steffimycin were provided by G. Neil (Upjohn Co.); and *N*-trifluoroacetyladriamycin-14-valerate (AD-32) and *N*-trifluoroacetyladriamycin (AD-41) were supplied by M. Israel, (Farber Cancer Center, Boston, MA).

NADPH cytochrome *P*-450 reductase was prepared from rat liver microsomes according to the method of Omura and Takesue (11) and further purified by calcium phosphate gel adsorption. The enzyme obtained from the DEAE-cellulose column step was dialyzed against 1 mM KPO_4 buffer (pH 7.5), adsorbed onto calcium phosphate gel, and eluted in a batch with 0.4 M KPO_4 buffer (pH 7.5). The homogeneity of the enzyme was established by disc gel electrophoresis at pH 9.5, 8.0, and 4.5.

Oxygen consumption was determined with a Clark type electrode in a model 53 (Yellow Springs Instrument) instrument. The 1.0-ml reaction mixture contained 0.2 M KPO_4 buffer (pH 8.0), 5 mM NADPH, 0.5–4.0 mg of enzyme protein, and the quinone drugs. The reaction solution was aerated at 37°C for 3 min, enzyme was added, and the system was allowed to equilibrate for 2 min. NADPH was added followed by drug to determine the oxygen consumption. The 1.0-ml reaction mixture contained 0.158 μmol of dissolved oxygen. NADPH oxidation was measured at 340 nm in a Cary model 14 spectrophotometer.

Electron paramagnetic resonance observations were made at room temperature with a Varian E-9 (Century) spectrometer at 100-KHz field modulation. The microwave frequency was measured by a resonant cavity wavemeter calibrated at the National Bureau of Standards. The magnetic field was measured by the proton resonance signal and a frequency counter. The reaction mixtures were the same as for oxygen measurements and the drug concentration was 0.5 mM.

RESULTS

NADPH cytochrome *P*-450 reductase catalyzes a slow, NADPH-dependent oxygen consumption, about 2.2 nmol/min per mg of protein. Addition of a quinone anticancer agent such as adriamycin (0.5 mM) stimulates oxygen consumption to about 11 nmol/min per mg of protein. This enhancement of NADPH cytochrome *P*-450 reductase-catalyzed oxygen consumption is absolutely dependent on pyridine nucleotide cofactor, with NADPH being the most effective (Table 1). Saturation of the reaction occurred at 0.2 mM NADPH and no increase in oxygen consumption was seen up to 2 mM NADPH.

The oxygen consumption had a linear dependence on

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Table 1. Cofactor requirement for NADPH cytochrome *P*-450 reductase quinone-augmented oxygen consumption

Cofactor (20 mM)	Oxygen consumption, nmol/min
NADPH	32.9
NADH	3.0
NADP	0.0
NAD	0.6
FMN	0.3
FAD	0.7

Adriamycin (0.5 mM) was used to augment oxygen consumption.

NADPH cytochrome *P*-450 reductase concentration, and boiled enzyme was inactive. The enzyme showed a maximum for augmented oxygen consumption in phosphate buffer at pH 8.0, whereas Tris buffer was less supportive of the reaction (Fig. 1). Dependence on ionic concentration appeared to be another characteristic of the enzyme; it showed an optimum at 0.2 M phosphate (data not shown).

In addition to augmenting oxygen consumption, the quinone anticancer agents also stimulated NADPH oxidation by NADPH cytochrome *P*-450 reductase (Table 2).

Using the optimal conditions for oxygen consumption by the purified enzyme, we assayed quinone anticancer agents from the benzanthraquinone, *N*-heterocyclic quinone, and napthoquinone classes for activity as substrates. All the quinone agents stimulated oxygen consumption, and the characteristics

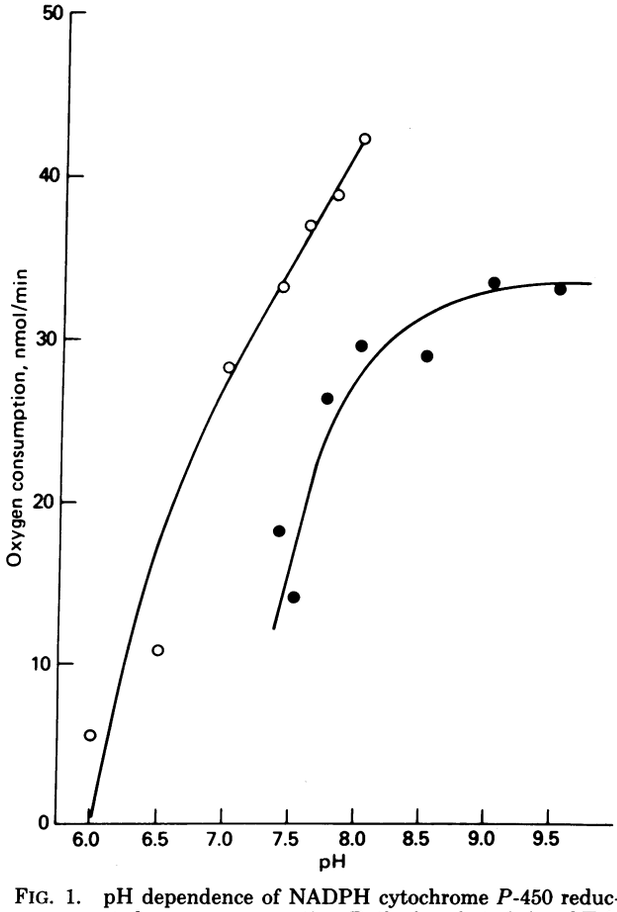

FIG. 1. pH dependence of NADPH cytochrome *P*-450 reductase-augmented oxygen consumption. Both phosphate (○) and Tris (●) buffers were 0.2 M; adriamycin, as the stimulating drug, was 0.5 mM.

Table 2. Antibiotic enhancement of NADPH oxidation

Addition (0.1 mM)	NADPH oxidation, μmol/min/mg protein
Adriamycin	1.12
Daunorubicin	0.89
Mitomycin C	0.24
Streptonigrin	0.85
Lapachol	0.12
None	0.01

The 1-ml reaction mixture contained 200 μmol of KPO_4 buffer (pH 8.0), 0.7 mg of NADPH cytochrome *P*-450 reductase, and 5 μmol of NADPH. The reaction was started by the addition of drug.

of their stimulation fit Michaelis–Menten kinetics, yielding K_m values ranging from 4×10^{-5} M to 4×10^{-3} M (Table 3). The anthracycline antibiotics adriamycin and daunorubicin and their semisynthetic analogs were quite active. In addition to those agents listed in Table 3 for which kinetic constants were measured, the agents AD 32, AD 41, and lapachol also stimulated oxygen consumption but no kinetic constants were determined because of low solubilities of these substances.

Because we previously determined that the anthracycline antibiotics (5) as well as other quinone antibiotics (6) are converted to free radical forms by microsomes, we analyzed the NADPH cytochrome *P*-450 reductase reaction with these agents for free radical formation by electron paramagnetic resonance spectrometry. In the presence of enzyme, NADPH, and quinone substrate, we observed the appearance of distinct and characteristic free radical signals for each quinone (Fig. 2). Enzyme, NADPH, and the quinone were necessary for free radical formation; boiled enzyme was inactive. The observed *g* factors fell in a range of 2.002 to 2.003. Due to the transient nature of the signals, a high modulation amplitude was usually required and, consequently, hyperfine structure was not resolved except in the case of streptonigrin (data not shown). Free radical signals obtained from aclacinomycin A and streptonigrin persisted whereas the signal from mitomycin C was quite small and transient. The observed signals were similar to those obtained in reaction mixtures containing microsomes (6).

Table 3. Apparent kinetic parameters for NADPH cytochrome *P*-450 oxygen consumption*

Agent	K_m, M × 10^4	V_{max}, mol/min/mg protein × 10^7
Benzanthraquinones:		
7-*O*-Methylnogarol	0.4	1.6
Carminomycin	0.7	13.4
N-Acetyldaunorubicin	1.1	3.2
Steffimycin	1.2	10.7
Aclacinomycin A	1.2	10.7
Rubidazone	1.6	8.1
Nogalomycin	2.1	5.4
7-Iminodaunorubicin	3.1	6.4
Daunorubicinol	5.7	19.2
Daunorubicin	8.3	24.2
Adriamycin	9.6	26.9
N-Dimethyladriamycin	10.8	43.3
Adriamycin $(Fe^{3+})_4$	42.1	104.2
N-Heterocyclic quinones:		
Streptonigrin	0.7	14.3
Mitomycin C	2.0	7.1

* Also active agents but no kinetic data available: AD 32; AD 41; lapachol.

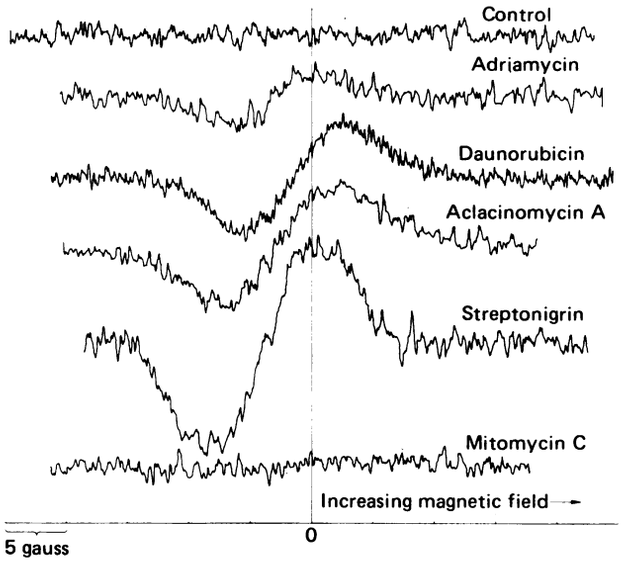

FIG. 2. Electron paramagnetic resonance spectra of NADPH cytochrome *P*-450 reductase-catalyzed formation of quinone anticancer agent free radicals. Reaction mixtures were the same as for oxygen measurements but drug concentration was 0.5 mM. The reactions were run in a stoppered quartz aqueous flat cell. The reactions were started by the addition of enzyme, and the cells were stoppered and scanned over a 50-gauss range. The control reaction mixture contained no drug. Zero reference line corresponds to 3380 gauss.

DISCUSSION

Nishibayashi *et al.* (12) postulated that NADPH cytochrome *P*-450 reductase catalyzes the one-electron reduction of quinones that Iyanagi and Yamazaki (7) showed occurred by a single-electron transfer through the flavoprotein to yield a semiquinone product. We have shown that quinone antibiotics of the benzanthraquinone, *N*-heterocyclic quinone, and napthoquinone families also function as single-electron receptors for NADPH cytochrome *P*-450 reductase electron transfer to yield free radical intermediates. The free radical forms of the antibiotics readily transfer their single electrons to molecular oxygen.

In previous investigations, Schwartz *et al.* (13) found that mitomycin C required microsomal reduction to become cytotoxic. Later, Iyer and Szybalski (3) confirmed the need for reduction before mitomycin C could crosslink DNA. White and White (14, 15) have shown that streptonigrin also is activated by cellular reduction. Investigators have suggested that superoxide radical, peroxide, or hydroxyl radical formed from semiquinones of mitomycin C (16), daunorubicin or adriamycin (8, 17), or streptonigrin (15, 18, 19) may be responsible for the toxic action of these agents. However, these same cytotoxic products of oxygen reduction result from the biochemical reduction of other quinones. Such quinones as vitamin K and coenzyme Q are normal constituents of cells but can yield toxic oxygen products (20). To protect the cellular machinery from superoxide and other reduced forms of oxygen, the mammalian cell contains elaborate defense mechanisms such as superoxide dismutase, glutathione reductase, catalase, peroxidase, and reactive structural cellular components.

The quinone antibiotics we have examined not only have the property to form free radicals, but they also bind with high affinity to DNA and other macromolecules. Through this binding alone, quinone agents produce biochemical consequences. For example, the anthracycline antibiotics adriamycin and daunorubicin can effectively inhibit DNA and RNA synthesis and other biochemical DNA processing by binding to template DNA (21). This binding and distortion of the DNA occurs *in vitro* in purified assay systems but never results in damage to or breakage of the DNA strands. On the other hand, when living cells are treated with adriamycin or daunorubicin, they rapidly show signs of DNA damage (22, 23). Apparently, cellular processes are necessary for damage to be inflicted on the DNA.

Because free radicals are highly reactive, we think that the free radical forms of the quinone antibiotics may act as the damaging agents to DNA and possibly to other macromolecules. We propose that the quinone agents are activated to free radical forms intracellularly by NADPH cytochrome *P*-450 reductase and other quinone reductases and bind to DNA as the free radical (Fig. 3). Thereby, the highly reactive semiquinone form of the antibiotic is brought into close proximity to the double-stranded DNA. The semiquinone form of the antibiotic then may either react directly with the DNA strands or produce reactive oxygen species within the DNA helix which could damage the DNA. It is possible that the quinone antibiotics have affinities for other critical macromolecules (RNA, protein) which may be reactive sites for the free radical forms of the quinone antibiotics. Lown and his coworkers (24) have recently reported chemical evidence supporting this hypothesis. Chemically reduced adriamycin produces breaks in viral circular DNA. The chemireduction of intercalated anthracycline antibiotic produced less damage than did free prereduced quinone antibiotic. This supports the stability of the semiquinone form to react with the DNA. In addition, aglycone forms of the antibiotic that have low binding affinity for DNA but retain the quinone structure produce less DNA damage. Although Lown *et al.* implicated oxygen in the mechanism for DNA damage, it is possible that the semiquinone form of the drug reacts with the DNA. In our laboratory we have detected free radical intermediate forms in the chemically reduced anthracycline antibiotics (unpublished observation). Murakami (25) has proposed reduction of mitomycin C through a semiquinone form as necessary for the action of that quinone agent.

There are several lines of evidence that support the interaction, binding, and consumption of the anthracycline antibiotics in tissues and cells. In our early studies on the fate of tritiated daunorubicin administered to animals, we noted the acid-stable binding of tritiated daunorubicin to mouse tissues (26). This suggested a covalent linkage of the nonextractable anthracycline antibiotic to tissue components. In addition, when animals or humans are given adriamycin or daunorubicin, only 50–60% of the parent compound and metabolites are recovered in the urine and bile (27, 28). This indicates a binding and retention of the drug by the tissues. We also demonstrated that daunorubicin incubated with tissue slices interacted irreversibly so that only a portion of the parent drug and metabolites was re-

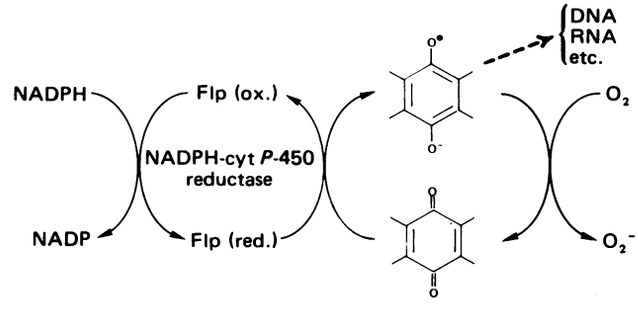

FIG. 3. Proposed scheme for the electron transfer sequence and production of semiquinone intermediates. Flp, flavoprotein.

covered by nondestructive extractions (29). This again suggested a covalent binding of the antibiotic to the tissues.

Data from the laboratory of Myers and coworkers (30) have shown that adriamycin produces clear evidence of *in vivo* free radical formation by the production of the lipid peroxidation product malonaldehyde in adriamycin-treated mice. In addition, deoxyaglycone metabolites of adriamycin, daunorubicin, and other anthracycline antibiotics result from an autoreductive cleavage of the unstable semiquinone free radical intermediate (unpublished data). Therefore, the appearance of the deoxyaglycone metabolites *in vivo* proves the *in vivo* formation of free radical intermediates of these quinone agents. Evidence from previous experimentation supports the theses that (*i*) anthracycline antibiotics form free radicals *in vivo* and *in vitro* and (*ii*) the anthracycline antibiotics apparently bind covalently to tissue components *in vivo* and *in vitro*.

Two characteristics seem necessary for cytoxocity of these drugs. First, the drug must be capable of biochemical activation to a free radical state. Second, the drug must have an affinity for an essential macromolecule, such as DNA, RNA, or other molecules. The quinone antibiotics and perhaps other agents comprise a class of xenobiotic substances that require activation by the intracellular machinery to produce a reactive intermediate state of "site-specific free radicals."

We have shown that NADPH cytochrome *P*-450 reductase, a component of mammalian microsomes, catalyzes the reduction of quinone anticancer agents to free radical forms; and we propose that these site-specific free radicals provide the mechanism for selective damage of essential cellular macromolecules and thereby cause cell damage and death.

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