Differential Formation of Hydroxyl Radicals by Adriamycin in Sensitive and Resistant MCF-7 Human Breast Tumor Cells: Implications for the Mechanism of Action

Birandra K. Sinha,* Aspandiar G. Katki, Gerald Batist, Kenneth H. Cowan, and Charles E. Myers
Clinical Pharmacology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892
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ABSTRACT: Adriamycin-stimulated formation of •OH in sensitive and resistant subline of human breast tumor cells (MCF-7) has been examined by electron spin resonance spectroscopy. It was shown that adriamycin significantly stimulated the formation of •OH spin adducts [5,5-dimethyl-1-pyrroline *N*-oxide (DMPO)–OH] in the sensitive cells but not in the resistant cells. By use of spin-broadening techniques and inhibition of •OH with high molecular weight poly(ethylene glycol), which does not enter intact cells, it was shown that 60–65% of adriamycin-induced •OH were located extracellularly and were metal ion dependent since they were decreased in the presence of desferal. Furthermore, superoxide dismutase and catalase, enzymes that detoxify superoxide and hydrogen peroxide, also significantly inhibited adriamycin-induced •OH formation and protected against the cytotoxicity of adriamycin. The differential •OH formation in these two cell lines is not due to diminished activities of flavin-dependent activating enzymes nor decreased accumulation of the drug in the cells but appears to be related to enhanced activities of detoxifying enzymes, particularly, glutathione peroxidases in the resistant cells.

The anthracycline antibiotic adriamycin (ADR) is one of the most effective drugs in the treatment of a number of human cancers (Carter, 1975). However, although there is now a large literature on the biochemical effects of ADR, there is no consensus as to how this drug kills cancer cells. ADR is known to intercalate DNA (DiMarco, 1975), bind to membranes and alter their structure and function (Tritton & Yee, 1982), undergo enzymatic reduction to a free radical species (Bachur et al., 1978; Sato et al., 1977; Doroshow, 1983; Myers et al., 1977; Sinha et al., 1984; Kalyanaraman et al., 1984; Mimnaugh et al., 1985; Winterbourn, 1981), and avidly bind to a range of transition metal ions (Muindi et al., 1984, 1985). In addition, ADR has been shown to alter topoisomerase II activity (Tewey et al., 1984). Several of the above biochemical events have been shown to occur in tumor cells. Moreover, it is now clear that ADR exposure initiates multiple biochemical processes, not all of which may be relevant to cell kill. It has been experimentally difficult to decipher which of these biochemical events are responsible for the impressive cytotoxicity of ADR.

Similar problems have occurred in the study of other anticancer drugs, and examination of the biochemical events associated with the evolution of drug resistance has proved to be of value in such situations. In the case of methotrexate, for example, alterations have been seen in both the amount of dihydrofolate reductase and the affinity of this enzyme for methotrexate (Cowan et al., 1982), thus offering clear confirmation of this enzyme as a target of drug action. With this background, we have examined the biochemical changes associated with the evolution of ADR resistance with the hope of gaining similar insights into the mechanism of ADR tumor cell kill. For several reasons, we have chosen the MCF-7 human breast cancer cell line. ADR is the most active drug in the treatment of breast cancer, and MCF-7 is a well-characterized cell line that preserves many of the biochemical and endocrine characteristics of breast tissue. Peak plasma levels following iv administration of ADR reach 5–10 μM, and ip administration can reach 30 μM (Ozols et al., 1982; Green et al., 1983). Thus, the selection for resistance was continued until the IC_{50} for the resistant cells reached 4.8×10^{-6} M as compared to 2.5×10^{-8} M for the wild-type MCF-7 cell line. This resistance has proved stable for as long as 52 weeks in the absence of drug.

It has been shown that ADR resistance is often associated with altered drug uptake (Inaba & Johnson, 1977, 1978). In this resistant cell line, there was a 50% decrease in steady-state cell association of ADR. This change in drug uptake is clearly not sufficient to explain fully the degree of resistance seen.

A range of flavin reductases can reduce ADR to a semiquinone, which, in turn, can react with oxygen to produde $O_2^{\bullet-}$, H_2O_2, and •OH. Various investigators have proposed that the antitumor activity of ADR resulted from drug-induced free radical formation (Bachur et al., 1978; Sato et al., 1977; Dorsoshow, 1983). Sato et al. (1977) have provided ESR evidence of drug semiquinone radical formation in Ehrlich ascites cells, and Doroshow (1983) has detected $O_2^{\bullet-}$ and H_2O_2 generation from this same murine cell line. In addition, a variety of inhibitors and quenchers of reactive oxygen species including hydroxyl radical have been reported to lessen the cytotoxicity of ADR (Doroshow & de Vries, 1984; Doroshow, 1986a,b). In this study, we have examined the mechanism of formation of these reactive free radicals using electron spin resonance (ESR) and spin-trapping techniques. Furthermore, we have evaluated the role of these oxy radicals in tumor cell killing by examining the effects of specific scavengers of reactive oxygen, e.g., superoxide dismutase and catalase, on ADR toxicity in both sensitive and resistant cells. A part of these data has been published in an abstract form (Sinha et al., 1985).

Experimental Procedures

Materials. Adriamycin was a gift of the Drug Development Branch of the National Cancer Institute, Bethesda, MD. NADPH, NADH, and poly(ethylene glycol) (M_r 8000) were from Sigma Chemical Co., and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Aldrich Chemical Co.

and purified by two vacuum distillations before use.

Methods. The enzyme activities in the wild-type and the resistant MCF-7 cell lines were examined by washing the cells in cold phosphate-buffered saline (PBS) and sonicating (Heat System Ultrasoncis, Inc., New York, Model W-225) on ice for 5 s three times at power settings of 6. The enzyme activities and protein concentration measurements were carried out on the homogenates. Xanthine oxidase was determined by the method of Fried and Fried (1974). The cytochrome NADPH-reductase was assayed according to the method described by Masters et al. (1965) and is expressed as nanomoles of cytochrome *c* reduced per minute per milligram of protein. The cytochrome b_5-reductase was determined as described by Strittmatter and Velick (1957) and is expressed as nanomoles of NADH oxidized per minute per milligram of protein. The quinone reductase (DT-diaphorase) activity was assayed with 2,6-dichloroindophenol (DCIP) or menadione as substrates according the method of Ernster (1967) and is expressed as nanomoles per minuter per milligrams. Superoxide dismutase (SOD) was assayed according to the method of Winterbourn et al. (1975) and is expressed as the amount of protein required to inhibit 50% of NBT reduction. Catalase was determined as described by the method of Beutler (1984) and is expressed as moles per minute per milligram of protein. Glutathione peroxidase was determined according to the method described by Paglia and Valentine (1967) with H_2O_2 and cumene hydroperoxide as the substrates and is expressed as nanomoles of NADPH oxidized per minute per milligram of protein. glutathione was assayed after precipitating proteins with perchloric acid and by use of the supernatant according to the method of Ellman (1959) and is expressed as nanomoles per 10^6 cells.

The MCF-7 cells (WT and ADR^R) were grown in monolayer in modified IMEM medium supplemented with 2 mM glutamine, penicillin–streptomycin (100 units/mL; 10 mL/L), and 5% fetal calf serum (Grand Island Biologicals Co., New York) under an atmosphere containing 5% CO_2. For ESR studies, the media was removed, and the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and suspended in PBS at a cell density of 2.5×10^6/mL. A typical incubation for the determination of •OH radicals contained, in 1 mL, cells, DMPO (100 mM), and ADR, and the mixture was incubated in the presence of air for 10 min. The spectrum was recorded on a Varian E-104 or ER 220 D IBM-Brucker spectrometer (9.5 GHz) with a field modulation of 100 kHz. The effects of inhibitors on the formation of •OH were examined by preincubating them with cells before adding DMPO or ADR.

The relative sensitivity of the WT and the ADR^R cells to ADR in the presence or absence of SOD and catalase was examined by plating the cells in six-well Linbro dishes (30000–60000 cells/well) in 2 mL of IMEM containing 5% FCS. SOD and catalase were preincubated for 30 min before adding the drug. After 7 days, the cells were harvested and counted. SOD was inactivated with H_2O_2 as described previously (Hodgson & Fridovich, 1975; Bagley et al., 1986). Catalase was heat-inactivated (100 °C for 10 min) followed by dialysis against PBS buffer containing ethylenediaminetetraacetic acid (EDTA).

Results and Discussion

Figure 1A shows a typical ESR spectrum obtained from the wild-type MCF-7 cells in the presence of ADR and DMPO, a spin trap. The DMPO adduct spectrum consists of a quartet (1:2:2:1) with hyperfine splitting constants of $a^N = a^H = 14.9$ G. These splitting constants are characteristic for a DMPO–OH adduct (Harbour & Bolton, 1984), which results from trapping of •OH and required the presence of both the cells and ADR. In the absence of ADR, less than 10% of the DMPO–OH was formed. In contrast, only traces of the DMPO–OH adduct were detected in the drug-resistant cells. In order to confirm that the DMPO–OH adducts resulted from a reaction between DMPO and free •OH, ethanol or Me_2SO was added, known scavengers of •OH which react with free •OH to form carbon-centered radicals. These radicals are then trapped by DMPO, resulting in a decline of the DMPO–OH signal and the development of a signal characteristic of the relevalent carbon-centerd adduct. Figure 1B illustrates the result of such an experiment, which confirms the presence of •OH in the MCF-7 cells exposed to ADR.

We have used a line-broadening technique to determine whether ADR-stimulated •OH formation with these cells was

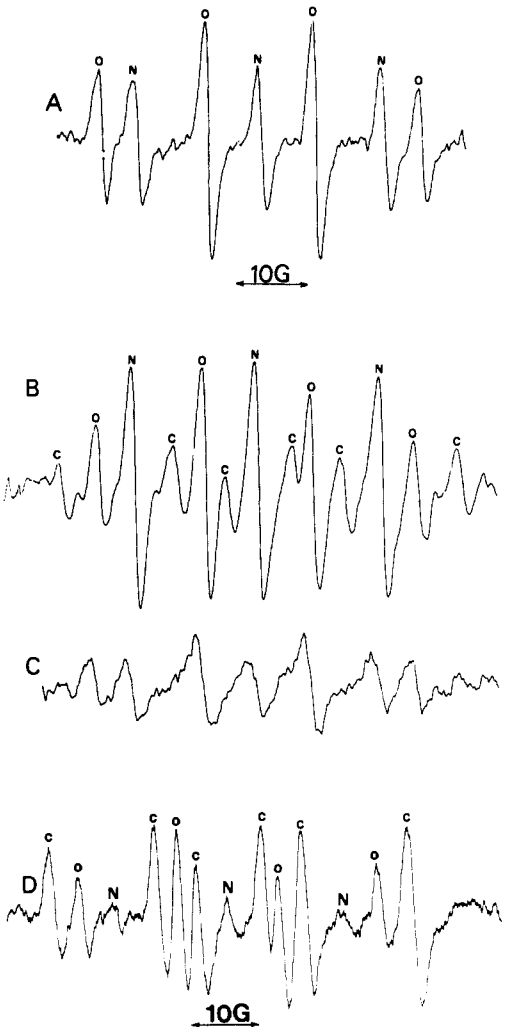

FIGURE 1: (A) ESR spectrum obtained from wild-type MCF-7 cells in the presence of 330 μM adriamycin and 100 mM DMPO. (B) Identical with (A) except the incubation mixtures contained 50 mM DMSO. The hyperfine splitting constants for DMPO–OH adducts (o) are $a^N = a^H = 14.9$ G and for the DMPO–CH_3 (c) adducts are $a^N = 16.5$ G and $a^H = 23.8$ G. Some nitroxide (N) with $a^N = 17.5$ G are also present. The nitroxide signal was not present in the DMPO used in these experiments and thus represents breakdown of DMPO by the cells. (C) Identical with (A) except 50 mM chromium oxalate was added. (D) Identical with (A) except 50 mM poly(ethylene glycol) (M_r 8000) was added. The hyperfine splitting for DMPO–C (PEG) are $a^N = 16.0$ G and $a^H = 22.1$ G. The ESR settings were as follows: field = 3390 G; microwave power = 20 mW; modulation amplitude = 2.0 G; receiver gain was 5×10^4 for (A) and (C) and 1×10^5 for (B) and (D).

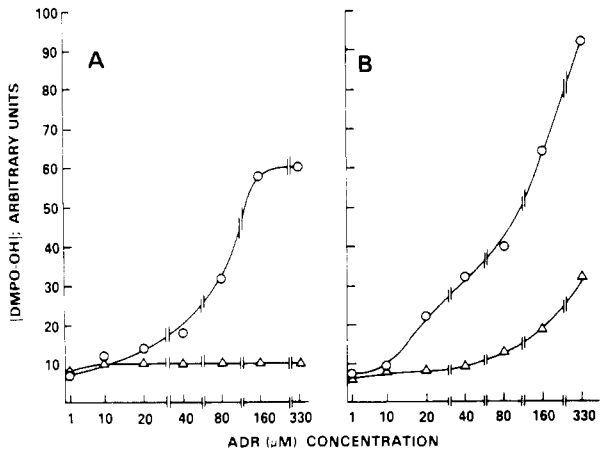

FIGURE 2: (A) DMPO–OH formation from the wild-type MCF-7 cells (○) and the resistant cells (△) as a function of ADR concentration. The relative concentration of DMPO–OH was obtained by monitoring the intensity of the high-field line of the DMPO–OH adduct spectrum. (B) DMPO–OH formation in the wild-type (○) and the resistant cell (△) as a function of ADR concentration and in the psernce of NADPH (1 mM). The ESR settings are identical with those described in Figure 1.

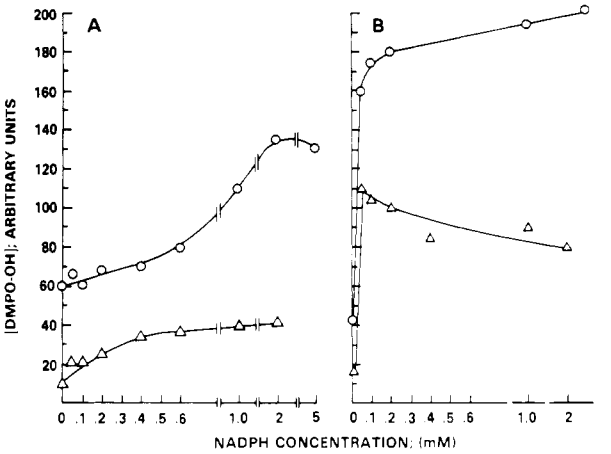

FIGURE 3: (A) Relative DMPO–OH formation in the wild type (○) and the resistant cells (△) in the presence of 330 μM ADR and increasing concentration of NADPH. (B) The relative concentration of DMPO–OH adducts in the sonicated wild-type (○) and resistant cells (△) in the presence of 330 μM ADR and increasing concentration of NADPH.

intracellular or extracellular. This ESR technique involves the use of paramagnetic metal ions, such as ferricyanide, Ni^{2+} and Cr^{3+}, which broaden the ESR spectrum of nitroxides by dipole–dipole interactions (Berg & Nesbitt, 1979; Sinha & Cysyk, 1981; Coan, 1985; Samuni et al., 1986). In addition, due to charge and relatively large molecular size, these metal ions enter cells very slowly. Previous workers have shown that chromium oxalate, in particular, does not enter cells to a significant degree over the time course required for our experiments (10 min). Addition of chromium oxalate to the wild-type cells in the presence of ADR decreased the intensity of the DMPO–OH signal by 60–65% (Figure 1C). This would suggest that 60–65% of the ADR-stimulated •OH formation is extracellular in location and thus is accessible to the Cr^{3+}. Recent studies of Samuni et al. (1986) have also shown that high molecular weight scavengers of •OH like poly(ethylene glycol) (PEG) are excluded from the cells. Additon of PEG (M_r 8000) to the incubation mixtures resulted in immediate scavenging of the •OH radicals, as indicated by a decrease in the DMPO–OH signal intensity and an appearance of DMPO carbon-centered adducts (Figure 1D) formed from the reaction of free •OH with PEG. This then further indicated that ADR-stimulated formation of •OH is, in part, extracellular.

Since ADR is known to bind to membranes (Sinha & Chignell, 1979; Tritton & Yee, 1982), it is possible that ADR treatment may induce alterations in the membrane structure such that the MCF-7 cells became damaged and leaky. In order to test the possibility that cells became damage during our ESR studies, we measured the leakage of two cytosolic enzymes, xanthine oxidase and glutathione peroxidase, under similar conditions to those of the ESR studies in the presence or the absence of ADR and DMPO. In the presence of 330 μM ADR, the highest concentrations used in the ESR studies, no activities of these cytosolic enzymes could be detected in the supernatant from either cell line up to 30 min of incubation, suggesting that cells were not damaged during our ESR studies. These observations clearly indicate that ADR-stimulated •OH formation is, in part, extracellular. However, other mechanisms such as the possible diffusion of H_2O_2 extracellularly and the subsequent formation of •OH are not excluded at this time.

Figure 2 shows the dose-dependent ADR-stimulated DMPO–OH adduct formation with both cell lines. While ADR stimulated the formation of DMPO–OH adducts in a dose-dependent fashion with the wild-type cells, there was little detectable •OH formation with the resistant cells, even at drug concentrations in excess of those likely to be encountered in vivo. Addition of 1 mM NADPH to both cell lines further enhanced the formation of DMPO–OH adducts from ADR (Figure 2B). While the resistant cells showed an increase in the formation of the •OH in the presence of ADR and NADPH, the relative concentration of the •OH formed with the wild-type cell line was always significantly higher (3–4-fold). While NADH also stimulated the formation of •OH with both cell lines, NADPH was a better reductant for the formation of the OH radicals with these cells (data not shown). These observations are of considerable interest since both NADH and NADPH cross cell membranes poorly. This supports the concept that ADR can undergo reduction to a free radical at the cell surface. The hydroxyl radical formation was dependent on the concentration of NADPH (Figure 3), and the relative concentrations of the DMPO–OH with the wild-type cell line was significantly higher than that measured with the resistant cell line.

This difference in free radical formation may result, in part, from decreased net accumulation of the drug in the resistant cells. However, studies in sonicated cell extracts, which eliminates any transport or net drug accumulation problems, also show the same relative differences in hydroxyl radical formation between these two cell lines (Figure 3B). These results clearly show that differences in •OH formation between these two cell lines is not due to differences in drug accumulation. Recently, Floyd (1983) has reported that DMPO–OH adducts may be reduced enzymatically, and therefore, it is possible that the differential •OH formation observed in the sensitive and resistant MCF-7 cells in the presence of ADR may have resulted from the differences in the rate of the reduction of the DMPO–OH adducts by the cell lines. We have, thus, investigated the reduction of DMPO–OH adducts formed from the Fenton system (Fe^{2+} and H_2O_2) catalyzed by the sonicated cells. When the sonicated cells (sensitive or resistant) were added to the preformed DMPO–OH adducts, no difference in the concentration of the DMPO–OH adducts was observed. Furthermore, there was no line broadening of the DMPO–OH signl as shown by Floyd during the reduction of the adducts. These observations then clearly indicate that the difference in ADR-stimulated DMPO–OH adduct for-

Table I: Relative Enzyme Activities in Wild-Type and Resistant MCF-7 Cell Lines (Means ± SEM)[a]

enzyme	wild type	resistant cells
xanthine oxidase	10.0 ± 0.8	7.9 ± 0.7
cytochrome-NADPH reductase	3.86 ± 1.9	3.3 ± 0.9
cytochrome b_5 reductase	129.9 ± 9.2	160.5 ± 13.6
DT-diaphorase (DCPIP)	561.5 ± 14.8	243.5 ± 9.2
superoxide dismutase	44.8 ± 1.3	42.4 ± 5.9
catalase	3.3 ± 0.2	2.4 ± 0.2
glutathione peroxidase		
H_2O_2	4.0 ± 0.9	15.9 ± 2.1
cumene peroxide	2.9 ± 0.7	37.4 ± 5.1
glutathione	9.1 ± 1.7	8.2 ± 2.3

[a] For experimental details, see Methods.

mation is not due to the differences in the rate of reduction of the DMPO–OH adducts.

However, the difference between these cell lines may arise from the changes in ADR activation and/or elimination of toxic free radicals in the resistant cells. At present, the enzymes known to activate ADR to a semiquinone free radical, which then forms superoxide and hydroxyl radicals, include (a) NADPH-cytochrome P-450 reductase, (b) NADH-cytochrome b_5 reductase, and (c) xanthine oxidase (Pan & Bachur, 1980). The detoxifying enzymes that might alter the rate of •OH formation include (a) superoxide dismutase, (b) catalase, (c) DT-diaphorase, and (d) glutathione peroxidase. We have therefore examined the relative activities of these enzymes in both cell lines in an attempt to understand the differential •OH formation. Results presented in Table I show that there were no significant differences in the activities of the flavine-dependent ADR-reducing enzymes in these cell lines. Our results with these enzymes are unexpected in that the activities of reductases measured in these cells are very high and approach that reported for liver (Mimnaugh et al., 1985). It is thus clear that both cell lines are well equipped to carry out enzymatic reduction of ADR to a free radical. Recently, it has been shown that DT-diaphorase, an enzyme responsible for the two-electron reduction of quinones, protects tumor cells from quinone-induced free radical damage (Keys et al., 1985). We have therefore also examined this, and the relative activity of DT-diaphorase was found to be 2-fold lower in the resistant cells, which produce significantly less •OH in the presence of ADR (Table I). Thus, it would appear that DT-diaphorase does not play a role in protecting the resistant cells against ADR-stimulated free radical cell kill.

There was no difference in activities of the enzymes superoxide dismutase or catalase, known to consume $O_2^{\bullet -}$ and H_2O_2, respectively (Table I). However, glutathione peroxidase activity was found to be increased in the resistant cell line. When assayed with H_2O_2 as a substrate, a 4-fold increase was observed in the peroxidase activity in the resistant cells line, and activity against cumene hydroperoxide, a well-characterized substrate for both enzymes, was increased 12-fold in the resistant cell line. The increased activity seen against cumene hydroperoxide as compared to H_2O_2 reflects an increase in detoxification of organic peroxides by glutathione *S*-transferase, an enzyme responsible for detoxification of drugs and other xenobiotics through conjugation with reduced glutathione. We have purified to homogeneity and characterized the glutathione *S*-transferase from the drug resistant cells, and these results have been reported separately (Batist et al., 1986). However, this transferase does possess marked organic peroxidase activity. As shown in Table I, glutathione, a key substrate for these peroxides, was similar in the two cell lines and present in considerable excess over the published K_m's for both glutathione peroxidases.

Table II: Effects of Scavengers of Reactive Oxygen on ADR- (100 μM) Stimulated Hydroxyl Radical Formation in Sensitive and Resistant MCF-7 Cells in the Absence and Presence of NADPH (1 mM) and DMPO[a]

system	addition	% DMPO–OH
WT intact		100
	SOD, 100 μg/mL	100
	SOD, 500 μg/mL	50
	catalase, 500 μg/mL	70
	desferal, 5 mM	55
WT intact	NADPH	100
	SOD, 50 μg/mL, + NADPH	0
	catalase, 50 μg/mL, + NADPH	80
WT lysed	NADPH	100
	SOD, 50 μg/mL, + NADPH	0
	catalase, 50 μg/mL, + NADPH	60
	desferal, 5 mM	50
ADR^R lysed	NADPH	100
	SOD, 50 μg/mL, + NADPH	0
	catalase, 50 μg/mL, + NADPH	23
	desferal, 5 mM	50

[a] The effects of inhibitors on the hydroxyl radical formation were determined as described under Methods. Desferal was preincubated with the cells for 1 h before adding the drug. SOD and catalase were preincubated with cells for 30 min before adding the drug.

In order to directly test the effectiveness of these changes in peroxidase activity, we carried out growth inhibition studies in both cell lines by cumene hydroperoxide. The IC_{50} for the wild type was 140 μM as compared to 1.6 mM for the ADR-resistant cell line. This 11.5-fold difference is an excellent quantitative correlation with the measured 12-fold difference in organic peroxidase activity between the two cell lines. Thus, we can conclude that the measured difference in peroxidase activity translates into an increased capacity by the intact cells to detoxify organic peroxides.

These results indicate that the resistant cells suppress •OH production via rapid clearance of peroxides. This is consistent, with ADR radical chemistry since all known mechisms by which ADR produces •OH are peroxide-dependent (Sinha et al., 1984; Kalyanaraman et al., 1984; Mimnaugh et al., 1985; Muinndi et al., 1984, 1985), as shown:

$$ADR^{\bullet} + O_2 \rightarrow ADR + O_2^{\bullet -}$$

$$2O_2^{\bullet -} \rightarrow H_2O_2$$

$$ADR + Fe^{3+} \rightarrow ADR^{\bullet}\ (\text{oxidized}) + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$$

$$ADR^{\bullet} + H_2O_2 \rightarrow ADR + {}^{\bullet}OH + {}^{-}OH$$

The mechanism of •OH formation in these cells was further evaluated by examining the effects of scavengers of reactive oxygen species. Results in Table II show that SOD at high concentrations inhibited ADR-dependent •OH formation in the intact cells by 50%. Catalase, however, was less effective. It is interesting to note that both SOD and catalase were significantly better in inhibiting the ADR-dependent •OH formation in the presence of NADPH in both intact and sonicated cells (Table II). Since neither SOD nor catalase easily penetrate cellular membranes (Michelson & Puget, 1980), the significant inhibition of •OH formation in the presence or in the absence of NADPH strongly implicate reduction–oxidation of anthracyclines at the cell surface by enzymes requiring NADP/NADPH systems. Since the •OH formation from peroxides is also metal dependent, we examined the effects of desferal, a strong chelator of iron. As shown in Table II, desferal inhibited the ADR-dependent •OH formation by 50% in both intact and sonicated cells. Thus, the formation of •OH

Table III: Effects of Superoxide Dismutase and Catalase on Cytotoxicity of Adriamycin on Sensitive and Resistant MCF-7 Cells

system	addition	% survival
WT + ADR (10^{-8} M)		40
	SOD[a]	
	50 μg/mL	40
	100 μg/mL	46
	SOD	
	50 μg/mL	72
	100 μg/mL	80
	catalase[b]	
	100 μg/mL	50
	250 μg/mL	60
	catalase	
	100 μg/mL	70
	250 μg/mL	80
ADR^R + ADR (10^{-6} M)		60
	SOD[a]	
	50 μg/mL	48
	100 μg/mL	50
	SOD	
	50 μg/mL	80
	100 μg/mL	90
	catalase[b]	
	100 μg/mL	47
	250 μg/mL	52
	catalase	
	100 μg/mL	94
	250 μg/mL	96

[a] SOD was denatured with H_2O_2 as described under Methods. [b] Catalase was denatured by heating at 100 °C for 10 min as described under Methods.

in these cells is, at least in part, iron ion mediated.

To test whether the •OH formed during activation of ADR contributes to the killing of these cells, we also examined the effects of SOD and catalase on the cytotoxicity of ADR. The data presented in Table III clearly show that both SOD and catalase strongly protected MCF-7 cells (sensitive and resistant) against ADR-mediated cell killing. In contrast, denatured enzymes were without any significant protective effects. It is interesting to note that the resistant cells that produce significantly less ESR-detectable •OH were also protected by SOD and catalase. Thus, oxygen radicl formation contributes significantly to ADR-mediated cytotoxicity even in these drug-resistant cells. It must be emphasized that inhibition of •OH formation and protection of toxicity of ADR by SOD and catalase indicate that oxygen radicals formed at or near the cell surface contribute to killing of the MCF-7 human breast tumor cells. Recent observations of Dorshow (1986a,b) support these conclusions.

In summary, our studies have shown that the wild-type MCF-7 cells have an unexpectedly high activity of those flavin enzymes required for ADR free radical formation. In the ADR-resistant cell line, •OH formation is suppressed, not by alteration in the activity of these flavin reductases but rather due to an elevated activity of the glutathione peroxidases present in the resistant cells. Our studies show that ADR-stimulated •OH formation is both exo- and endocellular. The exocellular •OH formation was indicated by (a) paramagnetic line broadening of the DMPO–OH signal by Cr^{3+} located outside the cells, (b) quenching of •OH by high molecular weight poly(ethylene glycol), which does not enter cells, (c) stimulation of •OH formation by NADPH and NADH, and (d) inhibition of ADR-mediated •OH formation and amelioration of ADR toxicity by SOD and catalase, which have been reported not to cross the cell membrane. However, regardless of the site of •OH generation, these results clearly show that free radical formation plays a role in the ability of ADR to kill the MCF-7 human breast cancer cells.

Registry No. Adriamycin, 23214-92-8; glutathione peroxidase, 9013-66-5; hydroxyl radical, 3352-57-6.

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Studies on the Interaction of Cupric Isonicotinohydrazide with DNA†

S. Divakar,‡ M. B. Vasudevachari,§ A. Antony,§ and K. R. K. Easwaran*,‡
Molecular Biophysics Unit and Microbiology & Cell Biology Laboratory, Indian Institute of Science, Bangalore 560012, India
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ABSTRACT: The interaction of cupric isonicotinohydrazide ($Cu^{II}INH$), an antiviral compound, with calf thymus DNA was investigated by circular dichroism (CD) and nuclear magnetic resonance (NMR). Gel electrophoresis of DNA incubated with $Cu^{II}INH$ showed cleavage of DNA to various extents. This cleavage was found to be time and concentration dependent. In the presence of $Cu^{II}INH$ the positive CD band at 274 nm disappeared and the negative band at 246 nm showed a decrease in the mean residual ellipticity value, indicating binding of $Cu^{II}INH$ to DNA. ^{31}P NMR studies indicated that the binding of copper in $Cu^{II}INH$ is to the phosphate oxygen of the DNA backbone. The binding of $Cu^{II}INH$ was also found to be reversible. Addition of ethylenediaminetetraacetic acid to the $Cu^{II}INH$–DNA complex resulted in breaking of the complex and restoring the original structural features of the B family of DNA in the resulting fragments. At the concentration level of $Cu^{II}INH$ employed, both $CuSO_4$ and INH independently did not show any interaction with DNA.

Copper complexes of chelating agents like 1,10-phenanthroline and thiosemicarbazones exhibit antiviral activity by their interaction with the nucleic acid templates and inhibit proviral DNA synthesis (Pillai et al., 1977; Sigman et al., 1979; Downey et al., 1980). Cupric ion is known to exhibit a high affinity for DNA reversibly under appropriate conditions (Eichhorn & Shin, 1968; Pezzano & Pato, 1980). Isonicotinohydrazide (INH),[1] which was found to be effective against tuberculosis (Hillerband et al., 1975), is known to form a complex, $Cu^{II}INH$, with copper ions resulting in a decrease in copper level in blood and liver (Albert, 1956; Sodikov & Gaponko, 1971). It has been reported that $Cu^{II}INH$ inhibits the multiplication of avian myeloblastosis virus by blocking the process of reverse transcription (Vasudevachari & Antony, 1985a). This inhibition was shown to be due to preferential binding of $Cu^{II}INH$ to the enzyme reverse transcriptase (Vasudevachari & Antony, 1982). Recently, it has been shown that $Cu^{II}INH$ cleaves pBR322 form I DNA into smaller fragments (Vasudevachari & Antony, 1985b). We report here evidence to show that $Cu^{II}INH$ interacts with DNA leading to cleavage of DNA based on circular dichroism (CD) and ^{31}P nuclear magnetic resonance (NMR) studies.

Materials and Methods

Materials. Calf thymus DNA (sodium salt, A grade) was obtained from Calbiochem or Sigma Chemical Co. (type I). Tris(hydroxymethyl)aminomethane, isonicotinohydrazide (INH), ethylenediaminetetraacetate (disodium salt), agarose [low electroendosmosis (EEO)], and ethidium bromide were from Sigma Chemical Co. Unless otherwise stated, incubation experiments involving calf thymus DNA (80 μg/mL) dissolved in 20 mM Tris-HCl buffer (pH 7.6) were carried out at 37 °C for 2 h. Horizontal agarose gel electrophoresis was carried out with 0.7% agarose in Tris–acetate–EDTA (pH 8.3) (Hayward & Smith, 1972).

Calf thymus DNA was sonicated as follows. One hundred milligrams of calf thymus DNA (Sigma, type I containing 5.1% Na) was dissolved in 15 mL of degassed sodium cacodylate buffer (10 mM sodium cacodylate, 0.1 M NaCl, pH 7.0) and kept overnight at 5 °C. Nitrogen gas was bubbled into the DNA sample for 15 min, and it was sonicated for 1 h by using a Bronson Model B-15 Sonifier at 0–5 °C. A $^1/_4$-in.

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‡Molecular Biophysics Unit.
§Microbiology & Cell Biology Laboratory.

[1] Abbreviations: INH, isonicotinohydrazide; CD, circular dichroism; NMR, nuclear magnetic resonance; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; kb, kilobase(s).

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