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Is the Drug-Responsive NADH Oxidase of the Cancer Cell Plasma Membrane a Molecular Target for Adriamycin?

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Enhanced growth inhibition and antitumor responses to adriamycin have been observed repeatedly from several laboratories using impermeant forms of adriamycin where entry into the cell was greatly reduced or prevented. Our laboratory has described an NADH oxidase activity at the external surface of plasma membrane vesicles from tumor cells where inhibition by an antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984), and by the vanilloid, capsaicin (8-methyl-*N*-vanillyl-6-noneamide) correlated with inhibition of growth. Here we report that the oxidation of NADH by isolated plasma membrane vesicles was inhibited, as well, by adriamycin. An external site of inhibition was indicated from studies where impermeant adriamycin conjugates were used. The EC_{50} for inhibition of the oxidase of rat hepatoma plasma membranes by adriamycin was several orders of magnitude less than that for rat liver. Adriamycin cross-linked to diferric transferrin and other impermeant supports also was effective in inhibition of NADH oxidation by isolated plasma membrane vesicles and in inhibition of growth of cultured cells. The findings suggest the NADH oxidase of the plasma membrane as a growth-related adriamycin target at the surface of cancer cells responsive to adriamycin. Whereas DNA intercalation remains clearly one of the principal bases for the cytotoxic action of free adriamycin, this second site, possibly related to a more specific antitumor action, may be helpful in understanding the enhanced efficacy reported previously for immobilized adriamycin forms compared to free adriamycin.

KEY WORDS: NADH oxidase; adriamycin; adriamycin conjugates; drug conjugate; plasma membrane; cancer; molecular target; anthracyclines; doxorubicin.

INTRODUCTION

Anthracycline antibiotics, adriamycin (doxorubicin), daunomycin, and mitomycin, are among the most important agents used in the treatment of human cancer (Arcomone, 1985). DNA is considered widely to be the primary target for the cytotoxic mechanism of this drug on susceptible cells. Adriamycin has been shown to intercalate between the bases of the DNA molecules and to inhibit further DNA and RNA biosynthesis (Haidle and McKinney, 1986). Interference with DNA and RNA synthesis and taken together with impairment of topoisomerase II activity (Myers *et al.*, 1988) normally are considered to be sufficient to account for the cytotoxicity of adriamycin. However, additional sites of action have been proposed (Rogers *et al.*, 1983; Thornalley *et al.*, 1986). Moreover, strong inhibitions of growth by *N*-substituted anthracycline derivatives such as *N*-acetyl daunomycin and *N*-acetyl adriamycin were observed under conditions in which DNA synthesis was unaffected (Silverstrini *et al.*, 1970, 1973). Therefore, there were reasons to suspect that the

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anthracycline antitumor drugs might affect cancer cells in more than one way. In this regard, adriamycin has been suggested to interact directly with the cell membrane (Goldman *et al.*, 1976; Murphree *et al.*, 1976; Bucher *et al.*, 1983; Doroshow, 1983). Such an interaction has been inferred from effects of adriamycin on the functioning of membrane-bound redox enzymes (Löw and Crane, 1978; Tewey *et al.*, 1984; Rogers and Tökes, 1984). Also, when adriamycin enters the cell, oxygen radicals are produced that cause severe damage to the plasma membrane and interfere with cytoskeletal assembly (Bachur *et al.*, 1979; Deliconstantinos, 1987; Jadot, 1986; McCay *et al.*, 1976; Goormaghtigh *et al.*, 1983; Alegria *et al.*, 1990; Powis, 1989).

The concept of a cell surface site of action for adriamycin is not new. Inhibition of cell growth by adriamycin and other anthracyclines covalently linked to polymers to prevent entrance into cells was first demonstrated by Tritton and Yee (1982) and Tökes *et al.* (1982). Growth inhibition was observed with adriamycin attached to agarose beads (Tritton and Yee, 1982), polyvinyl alcohol (Wingard *et al.*, 1985), polyglutaraldehyde microspheres (Tökes *et al.*, 1982; Rogers *et al.*, 1983), *N*-(2-hydroxypropyl)methacrylamide (Seymour *et al.*, 1990), and diferric transferrin (Yeh and Faulk, 1984). From these studies, it was concluded that the anthracyclines disrupt cellular growth processes without actually entering cells (see also Rogers and Tökes, 1984). An important consideration in arriving at this conclusion was the observation that the anthracycline conjugates often were more effective than was the free drug on a per mole of adriamycin basis. With adriamycin attached to agarose, immobilized adriamycin was 100 to 1,000 times more active than was free adriamycin (Tritton and Yee, 1982). With adriamycin bound to the polyglutaraldehyde microspheres (Tökes *et al.*, 1982; Rogers *et al.*, 1983; Rogers and Tökes, 1984), increased effectiveness on an adriamycin basis also was observed. The concept of transferrin-receptor targeted transferrin conjugates of antitumor drugs introduced by Faulk *et al.* (1980) has led to the development of transferrin–adriamycin conjugates which have proven to be disproportionately more effective than free adriamycin (Faulk *et al.*, 1991). The transferrin–adriamycin conjugates were tested clinically and found to be therapeutic in the treatment of leukemia (Faulk *et al.*, 1990a; Yeh *et al.*, 1984). Additionally, they inhibited the growth of both transformed and adriamycin-resistant cells (Yeh *et al.*, 1984; Faulk *et al.*, 1990a, 1991, Fritzer *et al.*, 1992; Bérczi *et al.*, 1993). At conjugate doses therapeutically effective in patients, cardiotoxicity was not observed (Rogers and Tökes, 1984; see also Adler *et al.*, 1995). With polymer-bound adriamycin, the level of drugs in the heart was reduced 100-fold compared to free drug in mice following administration of the conjugated drug (Seymour *et al.*, 1990). Reduction in toxicity with retention of activity has been reported as well for anadriamycin-*N*-(2-hydroxypropyl)methylacrylamide copolymer conjugate (Seymour *et al.*, 1994). In one example, an inactive anthracycline analog, 4-dimethoxy-7,9-di-epi-daunorubicin, acquired significant cytostatic activity with doxorubicin-resistant and -sensitive L 1210 cells when linked to polyglutaraldehyde microspheres (Rogers and Tökes, 1984).

Adriamycin linked to monoclonal antibodies has proven superior to adriamycin alone in some studies (Adler *et al.*, 1995; present study) but not in others (Dilman *et al.*, 1986; Kaneko *et al.*, 1991). Hydrolysis was shown to be necessary for activity by Kaneko *et al.* (1991). Although polyethylene glycol–adriamycin conjugates containing beta-lactamase-sensitive linkers were found to be relatively stable and less toxic to mice than free adriamycin, enhancement of their activity by co-administration with cell targeted lactamase (Senter *et al.*, 1995) also was taken as evidence for a requirement for hydrolysis for activity.

If at least some forms of immobilized adriamycin are to exert their cytostatic action without actually entering cells, then a plasma membrane target, at or near the cell surface, would be required. Adriamycin was shown by Sun and Crane (1984a,b; 1990) to inhibit the ability of cultured cells to transfer electrons from internal electron donors to external impermeant electron acceptors such as ferric iron associated with ferricyanide or diferric transferrin as a measure of plasma membrane electron transport (see also Sun and Navas, 1986). This inhibition was observed as well with adriamycin conjugated to diferric transferrin but at one-tenth the concentration required for inhibition by unconjugated adriamycin.

The inhibition of NADH-ferricyanide oxidoreductase by adriamycin, however, was a relatively high-dose response and one given by both rat liver and HeLa cell membranes (Sun *et al.*, 1987b). In this report, we describe an NADH oxidase activity of the plasma membrane correlated with growth that was inhibited by both free and conjugated adriamycin in the nanomolar dose range, specifically in cancer but not in normal cells.

MATERIALS AND METHODS

Spectrophotometric Assay

NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN to inhibit any potential mitochondrial oxidase activity, and 150 μM NADH at 37°C with constant stirring. Activity was measured using a Hitachi U3210 or SLM 2000 (Aminco) with continuous recording over 5 or 10 min intervals. A millimolar extinction coefficient of 6.22 was used to determine NADH disappearance.

Purification of Plasma Membranes from HeLa Cells

HeLa cells grown as suspension cultures were collected by centrifugation for 6–15 min at 1,000 to 3,000 rpm (e.g., 6 min at 3,000 rpm or 15 min at 1,000 rpm). The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10–30 min to swell the cells. Homogenization was with a Polytron homogenizer for 30–40 sec at 10,500 rpm using a PT-PA 3012/23 or ST-probe and 7–8 ml aliquots. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 1,000 rpm (175 *g*) to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at 1.4×10^6 *g* min (e.g., 1 h at 23,500 *g*) to prepare plasma membrane-enriched microsome fractions. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of approximately 1 ml per pellet from 5×10^8 cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis as described (Morré and Morré, 1989). The upper phase, enriched in plasma membranes, was diluted 5-fold with 1 mM sodium bicarbonate and the membranes were collected by centrifugation. The purity of the plasma membrane was determined to be >90% by electron microscope morphometry. The yield was 20 mg plasma membrane protein from 10^{10} cells.

Isolation of Plasma Membranes from Rat Livers

Male rats, 200 to 250 g, 50 days old of the Holtzman strain (The Holtzman Company, Madison, Wisconsin) provided with standard diet and drinking water *ad libitum* were killed by decapitation and drained of blood. The livers were removed, weighed, and minced rapidly at room temperature with scalpels or single-edged razor blades. All other operations were at 0 to 4°C. The minced tissue, in lots of approximately 10 g each, were mixed with 20 ml of chilled homogenization medium (37.5 mM Tris-maleate, pH 6.4, 0.5 M sucrose, 1% dextran, and 5 mM $MgCl_2$). The homogenate was squeezed through a single layer of miracloth (Chicopee Mills, New York) to remove unbroken cells and connective tissues. The homogenate was centrifuged for 15 min at $5{,}000 \times g$ using a rotor of the swinging bucket type to concentrate the Golgi apparatus. The lipid on top of the tube and the supernatant fluid were removed by suction. The Golgi apparatus-enriched yellow-brown phase of the pellet (usually upper 1/2 to 1/3) which lies above the red to pink and dark brown layers containing whole cells, nuclei, and fragments of plasma membrane (Morré, 1971) was removed.

The remainder of the $5000 \times g$ pellet after removal of the Golgi apparatus-enriched upper layer was the starting material for isolation of plasma membrane vesicles. Cold 1 mM $NaHCO_3$ (5 ml) was added to each tube and the friable yellow-brown upper part of the pellet was resuspended with a pen-brush, leaving the reddish tightly packed bottom part of the pellet undisturbed. The resuspended material was homogenized in aliquots of 5 ml each in a 30-ml stainless steel (Duragrind) homogenizer 20 times by hand. The homogenates were combined, diluted with cold 1 mM $NaHCO_3$ (1:1 dilution), and centrifuged at $6000 \times g$ in an HB4 rotor for 15 min. The supernatant was discarded and the pellet was used for the two-phase separation (Morré and Morré, 1989).

The two phase system contained 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w) polyethylene glycol 3350 (Fisher), and 5 mM potassium phosphate buffer (pH 7.2) (Morré and Morré, 1989). The homogenate (1 g) was added to the two-phase system and the weight of the system was brought to 8 g with distilled water. The tubes were inverted vigorously 40 times in the cold (4°C). The phases were separated by centrifugation at 750 rpm ($150 \times g$) in a Sorvall HB4 rotor for 5 min. The upper phases were withdrawn carefully with a Pasteur pipette, divided in half, and transferred

into 40-ml plastic centrifuge tubes, diluted with cold 1 mM $NaHCO_3$, and centrifuged at 10,000 × *g* in an HB4 rotor for 30 min. Plasma membrane pellets were resuspended in 50 mM Tris-Mes buffer (pH 7.2) and stored at −70°C. Proteins were determined by using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985) with bovine serum albumin as standard. Yields were approximately 3–5 mg per 10 g liver and purity greater than 80% (Navas *et al.*, 1989).

Isolation of Plasma Membranes from Rat Hepatomas

For isolation of hepatoma plasma membranes (Bruno *et al.*, 1992; Navas *et al.*, 1989) male Fischer 344 rats weighing 100–125 g were purchased from Harlan Animal Supply (Indianapolis, Indiana). Hepatocellular carcinomas induced initially with 2-acetylaminofluorene (designated RLT-28) or with nitrosamine (designated RLT-N) were propagated in syngeneic recipients as described by Kloppel and Morré (1980). In brief, the tumors were harvested immediately after the animal was killed, cleaned in basic salt solution, and trimmed to remove any capsular or necrotic material. Two pieces (approximately 1 mm × 1 mm × 7 mm) were injected subcutaneously with a cancer-implant needle (Popper and Sons, New Hyde Park, New York) on the left mid-lateral surface. The animals were fed *ad libitum* and killed after about 3–4 weeks. The remainder of the procedure was the same as for liver, except that the polymer concentrations were increased to 6.6% (w/w) in the aqueous two-phase partition step.

Source of Adriamycin

The adriamycin source was doxorubicin hydrochloride (98%) from Sigma (St. Louis, Missouri).

Preparation of Conjugates

a. With Transferrin

Transferrin–adriamycin conjugates were prepared by reaction of adriamycin and transferrin with glutaraldehyde and separation of complexes with defined stoichiometry (Faulk, *et al.*, 1990b; Yeh and Faulk, 1984; Sizensky *et al.*, 1992; Bérczi *et al.*, 1993). The complexes used in these studies had an adriamycin:transferrin molar ratio of 3:1 and were prepared with human holotransferrin. The concentrations of transferrin and adriamycin in individual fractions were calculated by successive approximation from standard curves for transferrin and adriamycin determined at both 238 and 495 nm to give the conjugation number. Concentrations used in experiments are based on adriamycin in the conjugate. Moles of conjugate used would be one-third of the adriamycin concentration shown under conjugate.

b. With Cyclodextrin

α-Cyclodextrin (α-CD) was dried at 105°C overnight at atmospheric pressure. From the dry α-CD, 2.7 g (2.8 mol) was dissolved in 50 ml of pyridine (dried over NaOH). To this solution, 25 g of succinic anhydride (250 mmol) in 30 ml of pyridine was added. The reaction was heated overnight at 80°C with stirring. After reaction, 50 ml distilled water was added and the persuccinylated α-CD was washed with excess dichloromethane and dried under vacuum. Persuccinylated α-CD was purified by reversed-phase chromatography (Sephasil C18, 2.5 × 18 cm, Pharmacia Biotech) with a solvent system composed of 5% methanol in dichloromethane (5.07 g, 2.2 mmol, m.p. = 125–130°C uncorrected). The Rf of the product on silica gel TLC was 0.28 while that of the α-CD was 0.54 (*n*-butanol:ethanol:water = 4:3:2). The degree of succinylation of the α-CD was 14.1 as determined by plasma desorption mass spectrometry.

For conjugation, 5.3 mg of doxorubicin hydrochloride (9.1 μmol) was reacted with excess Ag_2CO_3 in 3 ml water for 5 min. The solution was filtered, lyophilized, and the dark red-brown HCl-free doxorubicin was redissolved in 5 ml methanol. To this solution, 0.390 g of persuccinylated α-CD (164 μmol) and excess 2-isobutoxy-1-isobutoxycarbonyl-1, 2-dihydroquinoline (IIDQ) in 5 ml methanol were added. The reaction was run for 3 h. A flaky red-brown precipitate appeared. Methanol was evaporated and the conjugate was extracted with dichloromethane, washed with water. Dichloromethane was evaporated to 1 ml. The conjugate was purified by preparative silicagel TLC (methanol:dichloromethane = 1:20) (18.7 mg, 1.9 μmol).

c. With Antibody

Adriamycin was conjugated to immunopurified peptide antibodies to the major glycoprotein of feline immunodeficiency virus. Affinity columns using peptides against which the antibodies were generated also were prepared and the antibodies were bound to the affinity column to protect the antibody binding site during conjugation with the activated form of the drug. This ensured that the antibody binding site was protected during conjugation and that little or none of the drug was conjugated through the portion of the molecule involved with antigen binding.

For immunoaffinity purification of the antibody, the antigen peptide conjugated to bovine serum albumin (10 mg/ml) was coupled to cyanogen bromide-(CNBr) activated Sepharose 4B (Pharmacia) by incubating the peptide along with swollen Sepharose in 0.1 M $NaHCO_3$, pH 8.3 (coupling buffer) at 4°C for 16 h. The remaining active groups were quenched by transferring the gel to a solution of 0.2 M glycine, pH 8.0, and incubating for 2 h at room temperature. The column was washed with coupling buffer and then with 0.1 M acetate containing 0.5 M NaCl at pH 4.0, and finally with the coupling buffer. The peptide–sepharose conjugate was then incubated with the antisera at 4°C for 16 h. The immunoaffinity-purified antibody was eluted from the gel by using 100 mM triethylamine, pH 11.5, and the eluted fraction was dialyzed against 20 volumes of phosphate-buffered saline (PBS).

The immunoaffinity-purified antibody conjugated to the CNBr-activated Sepharose was incubated with activated drug in the presence of 10 mM of the coupling reagent 1-ethyl-3(3-diethylaminopropyl) carbodiimide (EDAC) (Sigma). The unbound drug was removed by washing 3 times with PBS. The drug conjugated with antibody was then eluted from the Sepharose by using 100 mM triethylamine, pH 11.5, and the eluted fraction was dialyzed against 20 volumes of PBS.

RESULTS

Response of NADH Plasma Membrane Oxidases of Membranes from Normal and Cancer Cells and Tissues

With plasma membranes from rat liver, a normal plasma membrane source, the NADH oxidase activity was resistant to inhibition by adriamycin. Activity was inhibited only at micromolar concentrations of adriamycin with an EC_{50} of about 7 μM (Fig. 1). In contrast, with plasma membranes of rat hepatomas, the NADH oxidase activity was inhibited even at nanomolar concentrations of adriamycin (Fig. 2). Activity was 20% inhibited by 1 nM adriamycin and by 50% at 1 μM adriamycin. With plasma membranes of human cervical carcinoma cells (HeLa) grown in culture, the EC_{50} of adriamycin for inhibition of NADH oxidase was 0.7 nM (Fig. 3).

With hepatoma plasma membranes and, to a lesser extent, with HeLa cell plasma membranes, there appear to be at least two NADH oxidase activities of differing sensitivities to adriamycin. One may correspond to the activity found in liver plasma membrane and require micromolar concentrations of adriamycin to inhibit (Fig. 1). The other, inhibited by nanomolar adriamycin concentration, may be specific to the plasma membrane of transformed cells (Figs. 2 and 3).

There is no evidence to suggest that the growth factor-dependent and -independent activities are catalyzed by the same protein. Also, we do not know if the activity responsive to adriamycin in transformed cells but not in liver represents an altered form of one of these or if it, too, represents a different protein. Likewise, the manner of coupling of the growth factor-dependent activity to the EGF or other growth factor

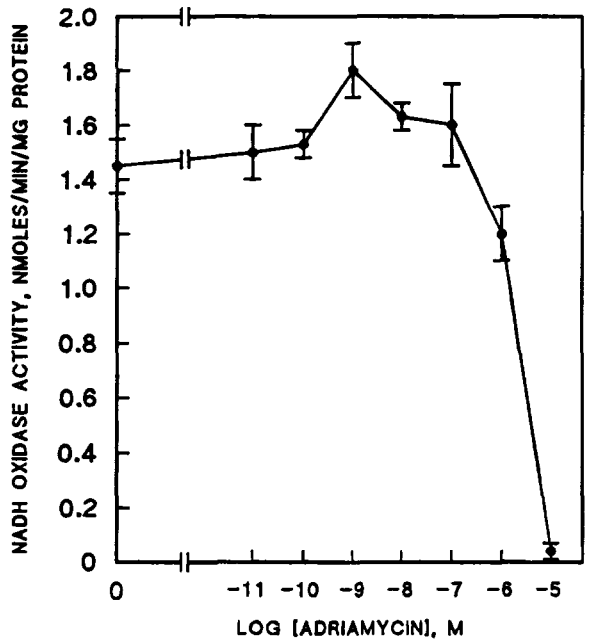

Fig. 1. Inhibition of NADH oxidase activity of plasma membrane of rat liver by adriamycin. The EC_{50} for inhibition was ca. 7 μM adriamycin.

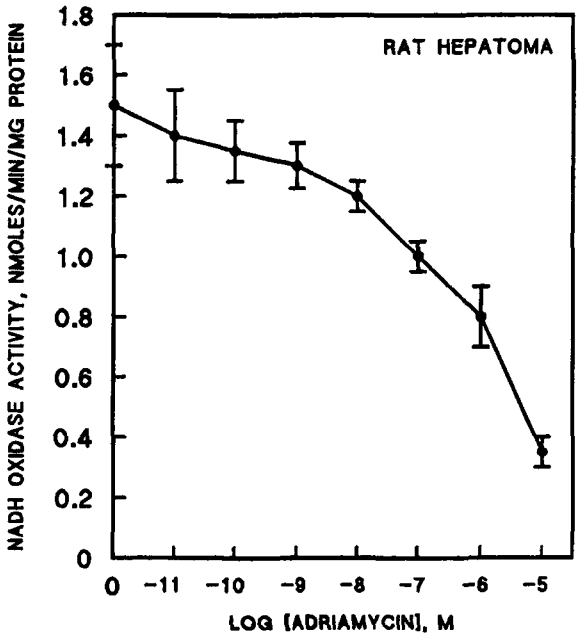

Fig. 2. Inhibition of NADH oxidase activity of plasma membrane of RLT-N hepatoma by adriamycin. The EC_{50} for inhibition was ca. 1 μM.

receptors has not been investigated. The small stimulations of NADH oxidation by nanomolar concentrations of adriamycin may also be of interest in view of stimulations of growth of some lines of cultured cells by low concentrations of the drug (Vichi and Tritton, 1989).

We previously reported that the NADH oxidase activity of plasma membranes of rat hepatomas was constitutively activated and, in contrast to that of rat liver (Brightman *et al.*, 1992), was no longer growth factor- and hormone-responsive (Morré *et al.*, 1991; Bruno *et al.*, 1992). HeLa cells also appeared to contain a constitutively-activated NADH oxidase (Morré *et al.*, 1995e). Since the NADH oxidase activity especially of plasma membranes of HeLa cells was more sensitive to adriamycin than that of liver, a possibility considered was that the growth factor-activated form of the NADH oxidase was inhibited by adriamycin. To test this possibility, responses of the hormone- and growth factor-activated component of the NADH oxidase of rat liver plasma membranes to adriamycin were determined. The basal NADH oxidase activity of liver plasma membranes was unaffected by adriamycin at 1 μM (Fig. 1), but NADH oxidase activity stimulated by epidermal growth factor (EGF) was inhibited completely by 0.1 μM adriamycin (Fig. 4). The activity of NADH oxidase of rat liver plasma membrane stimulated by diferric transferrin was also inhibited completely by adriamycin at concentrations as low as 10^{-8} M (Table I, but at these concentrations the drug was without effect or stimulatory to the NADH oxidase activity of rat liver plasma membranes in the absence of the growth factors (Fig. 1). The NADH: external acceptor reductase of the plasma membrane was either unaffected, or, at most, 50% inhibited at adriamycin concentrations 1000 times greater than those required to inhibit the hormone/growth factor-stimulated oxidation of NADH.

Adriamycin conjugated to diferric transferrin was ineffective in inhibiting NADH oxidase activity of plasma membrane vesicles from rat liver (Fig. 5). However, the conjugate did inhibit the activity with plasma membrane vesicles from rat hepatoma (Fig. 6) and HeLa cells (Fig. 7). The EC_{50} for inhibition of the constitutively activated NADH oxidase was about 30 nM for the conjugate and rat hepatoma plasma membranes (Fig. 6) compared to 1 μM for free adriamycin (Fig. 2), and about 0.3 nM for the conjugate and HeLa plasma membrane (Fig. 7) compared to about 0.7 nM for free adriamycin (Fig. 3). In contrast to results with free adriamycin (Fig. 1), the adriamycin conjugated to diferric transferrin was not inhibitory to the NADH oxidase activity of plasma membranes from normal rat liver (Fig. 5), even at the highest concentration tested of 100 μM. Rather, activity was stimulated.

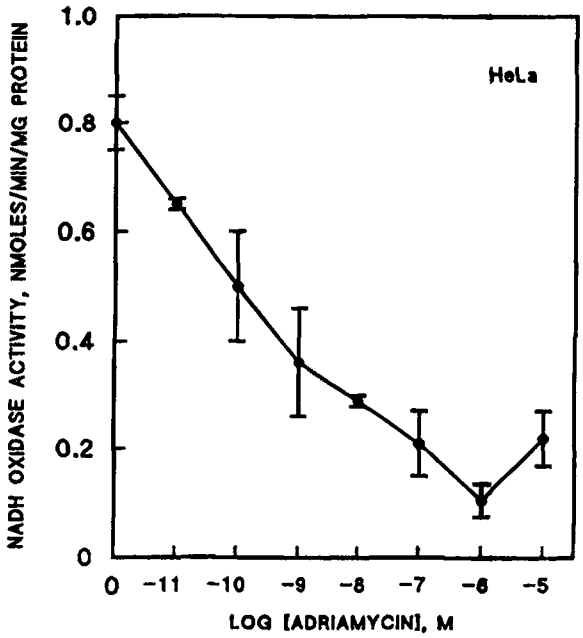

Fig. 3. Inhibition of NADH oxidase activity of plasma membrane of HeLa cells (human ovarian carcinoma) by adriamycin. The EC_{50} was ca. 0.7 nM.

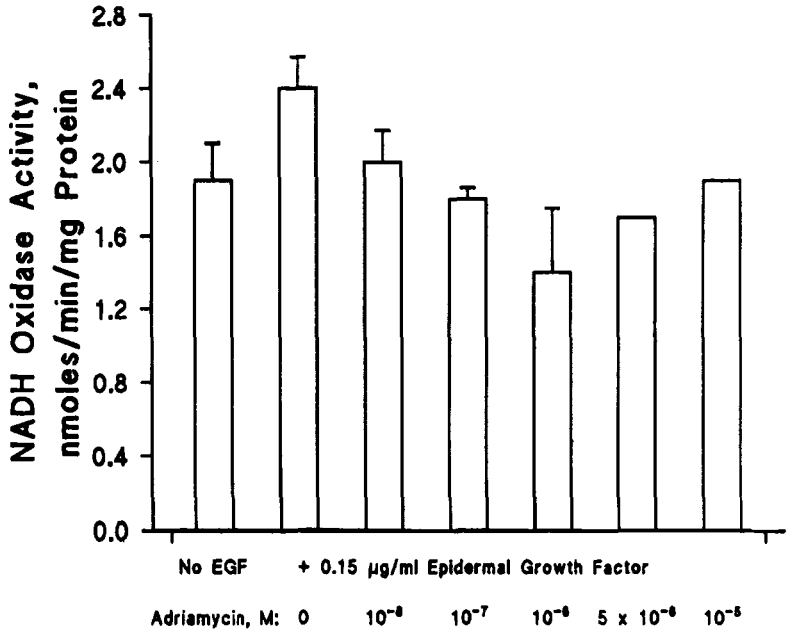

Fig. 4. Inhibition of epidermal growth factor (0.15 μg/ml EFG)-stimulated NADH oxidase activity of rat liver plasma membrane by adriamycin. A blank value of 0.6 nmol/min/mg protein was subtracted. Results are from three determinations ± standard deviations except for the two highest concentrations of adriamycin where determinations were made in duplicate.

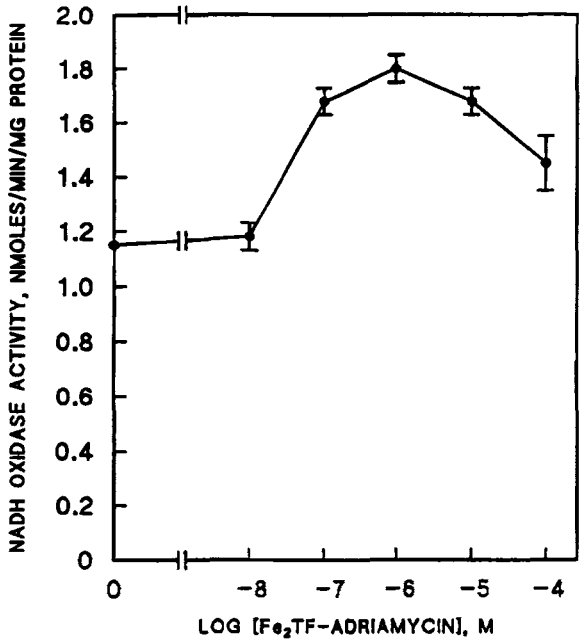

Fig. 5. NADH oxidase activity of rat liver plasma membranes as a function of the concentration of adriamycin conjugated to diferric transferrin. The stimulation may be in response to the transferrin portion of the conjugate.

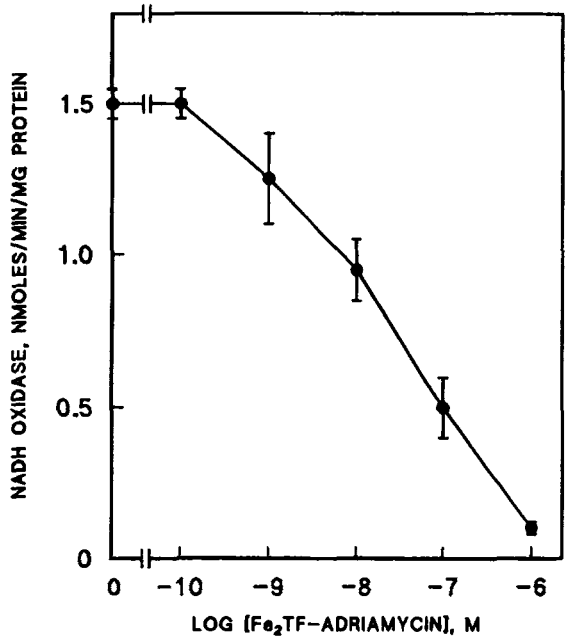

Fig. 6. Inhibition of NADH oxidase activity of plasma membranes of rat hepatoma RLT-N by adriamycin conjugated to diferric transferrin. The activity was inhibited half maximally at about 30 nM.

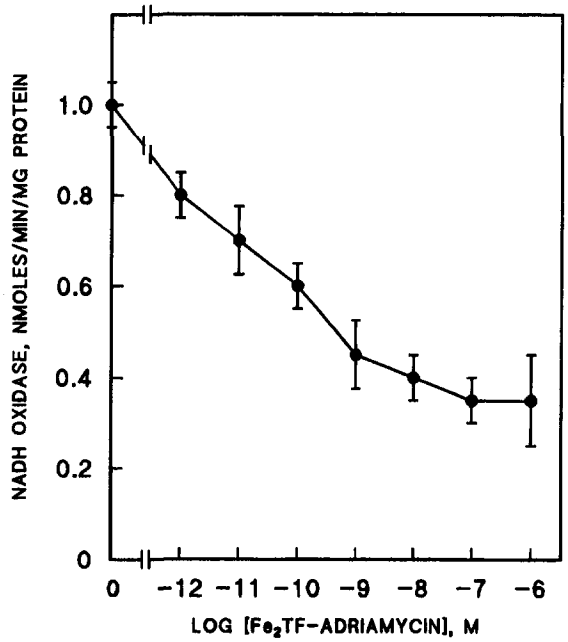

Fig. 7. Inhibition of NADH oxidase activity of plasma membranes of HeLa cells by adriamycin conjugated to diferric transferrin. The activity was inhibited half maximally at about 1 nM adriamycin.

Presumably, the stimulation was in response to the transferrin portion of the conjugate which is known to stimulate the NADH oxidase activity of rat liver plasma membranes (Table I). While conjugate was without effect on the basal activity of rat liver plasma membranes, it did inhibit NADH oxidase activity of rat liver plasma membranes stimulated by diferric transferrin (Table I). Also inhibited was the NADH oxidase activity of HL-60 cells and of HL-60 cells resistant to free adriamycin (Fig. 8).

In addition to the glutaraldehyde-mediated linking of adriamycin to diferric transferrin, additional conjugates with adriamycin were prepared and tested as adriamycin coupled both to antibodies and to α-cyclodextrin. Adriamycin coupled by the method described to affinity-purified peptide antibodies directed against feline immunodeficiency virus (FIV) inhibited the NADH oxidase as well as the growth of Crandall feline kidney cells infected with FIV (Table II). With free adriamycin, growth of both normal (uninfected) and FIV-infected cells was inhibited by 10 μM adriamycin but not by 1 μM adriamycin (Table II). Since the conjugate was effective at about 100 nM final concentration, this suggests that the conjugate was at least 10 times more effective than free adriamycin in its ability to inhibit growth of virus-infected cells when coupled to an antibody directed against the virus glycoprotein expressed at the cell surface of infected cells. Adriamycin linked to cyclodextrin was also effective in inhibiting growth of HeLa cells and

Table I. Effect of Adriamycin Conjugate and Free Adriamycin (ADR) on the Component of NADH Oxidase of Rat Liver Plasma Membrane Stimulated by Diferric Transferrin[a]

Inhibitor concentration	Diferric transferrin-stimulated NADH oxidation (nmol min^{-1} mg $protein^{-1}$)	
	ADR	ADR conjugate
None	0.38 ± 0.09	0.38 ± 0.09
10^{-9}	0.17 ± 0.03	0.17 ± 0.02
10^{-8}	0.04 ± 0.01	0.04 ± 0.01
10^{-7}	0.03 ± 0.01	0.05 ± 0.01
10^{-6}	0.00 ± 0	0.025 ± 0.02

[a] Assay in 0.05 M potassium phosphate buffer at pH 7.4 with 50 μM NADH, 17 μM differic transferrin, and 0.5 mg membrane in 2.8 ml. The absorbance change in response to diferric transferrin was measured at 340 nm with reference at 430 nm.

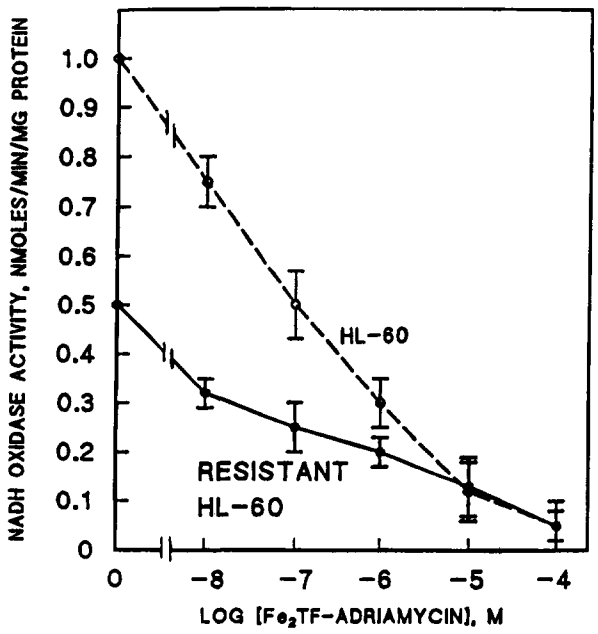

Fig. 8. NADH oxidase activity as a function of the concentration of adriamycin conjugated with diferric transferrin comparing plasma membranes from HL-60 cells susceptible (open circles) and resistant (solid circles) to adriamycin. The adriamycin-resistant cell line was provided by Drs. W. P. Faulk and K. Barabas, Methodist Center for Reproduction and Transplantation Immunology, Indianapolis, Indiana. The growth and electron transport characteristics of the cell line have been reported previously (Morré *et al.*, 1994).

Table II. EC_{50} for Inhibition of Growth Based on Cell Number after 72 h of Culture and Oxidation of NADH by Isolated Plasma Membrane Vesicles of FIV-infected CFK Cells as a Function of Adriamycin–anti FIV Glycoprotein (Adriamycin–Ab) Conjugate Compared to Free Adriamycin

	EC_{50}	
Drug	Growth (cell number)	NADH oxidation
Free adriamycin	>1 μM	1 μM
Adriamycin–Ab conjugate	0.1 μM	~0.07 μM

Table III. Rates of NADH Oxidation in Sera from Normal vs. Tumor-Bearing Rats

	nmol/min/ml serum	
Sera	No adriamycin	+25 μM adriamycin
Normal	1.7 ± 0.1	1.75 ± 0.05
RLT-28 bearing	1.0 ± 0.1	0.35 ± 0.05

in the inhibition of NADH oxidation by plasma membrane vesicles from HeLa cells (data not shown).

The drug-responsive NADH oxidase activity is an ectoprotein shed from the cell surface and appears in culture media conditioned by growth HeLa cells (Morré *et al.*, 1996) as well as in sera from tumor-bearing rats and cancer patients (Morré *et al.*, 1996). In keeping with these observations, the NADH oxidase of sera of rats bearing RLT-28 hepatomas was inhibited by adriamycin whereas that of sera of normal rats was not inhibited (Table III).

DISCUSSION

The NADH oxidase inhibited by adriamycin was first described in mammalian cells as a hormone- and growth factor-responsive activity of plasma membranes of rat liver (Brightman *et al.*, 1992). An NADH oxidase activity subsequently was found to be associated with the plasma membrane of rat hepatomas. However, the activity of rat hepatoma plasma membranes appeared to be constitutively activated and was no longer responsive to hormones and growth factors (Bruno *et al.*, 1992).

These studies were subsequently extended to plasma membranes of HeLa cells which bound the antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984) (Morré *et al.*, 1995a). The binding protein was identified as a ca. 34 kDa protein of the plasma membrane with NADH oxidase activity (Morré *et al.*, 1995b,c). In those cells and tissues where growth was inhibited by LY181984, the NADH oxidase was also inhibited by LY181984 (Moya-Camarena *et al.*, 1995; Morré *et al.*, 1995d) and, where growth was not inhibited by LY181984, neither was the NADH oxidase inhibited (Morré *et al.*, 1995d). Similar results were obtained with the inhibition of plasma membrane NADH oxidation in cancer cells by capsaicin (Morré *et al.*, 1995e). Thus a pattern may be emerging of a small cadre of antitumor drugs with previously undetermined modes of action that inhibit NADH oxidase in parallel to their abilities to inhibit growth.

The observation that an active antitumor sulfonylurea [N-(4-aminophenylsulfonyl)-*N'*-(4-chlorophenuyl)urea (LY237868)] retained tumor specific growth and plasma membrane NADH oxidase inhibitory activity when conjugated to an impermeant support (Kim *et al.*, 1997), adds to the evidence for a site of antitumor drug action at the cell surface.

Previous studies have pointed to the possibility of a site or sites at the external cell surface somehow related to the ability of immobilized adriamycin to inhibit cell growth (Rogers and Tökes, 1984; Barabas *et al.*, 1992). Among the candidates for such sites are NADH-ferricyanide oxidoreductases sensitive to inhibition by adriamycin, bleomycin, cis-platin, and actinomycin D (Sun *et al.*, 1992; Faulk *et al.*, 1991; Sun and Crane, 1985; Sun and Crane, 1984a; Sun *et al.*, 1984b). NADH-driven ferricyanide reduction by HeLa cells is inhibited by the above antitumor drugs at the same concentrations that are cytotoxic (Sun *et al.*, 1992; Sun and Crane, 1984a,b; Sun and Crane, 1985). Inhibition was observed at micromolar concentrations of adriamycin, and the activities of rat liver plasma membranes (Löw *et al.*, 1986) as well as of plasma membranes of HeLa cells were inhibited. This inhibition was observed as well with adriamycin conjugated to diferric transferrin (Sun *et al.*, 1992; Faulk *et al.*, 1991) but at one-tenth the concentrations required for inhibition by unconjugated adriamycin. In contrast to the NADH-ferricyanide oxidoreductase, the NADH oxidase of rat liver plasma membranes described in the present study was not inhibited by adriamycin except at very high concentrations of > 5 μM, whereas the NADH oxidase activity of plasma membranes of rat hepatomas was inhibited with an EC_{50} of 1 nM. Thus the NADH oxidase activity of the plasma membrane

is characterized by a much higher sensitivity and selectivity to adriamycin than that provided by the NADH-ferricyanide oxidoreductases.

The efficacy of adriamycin conjugated to impermeant supports for inhibition growth and NADH oxidase activity, while not specifically implicating NADH oxidase as the target, does point to a cell surface target location. The drug-responsive oxidation of NADH inhibited by the sulfonylureas is at a site at the external cell surface capable of oxidizing NADH (NADH is an impermeant substrate) (Morré, 1995). Since it is unlikely that mammalian cells would ever be exposed to millimolar concentrations of NADH at their surface, an alternative function of the oxidase has been considered. The true function may be that of a protein disulfide–thiol interchange protein capable, as well, of oxidizing NADH with the concomitant reduction of protein disulfides (Morré, 1994). The drug-responsive NADH oxidase of transformed cells is inhibited by thiol reagents and is presumed to have active-site thiols (Morré and Morré, 1995b). Binding of sulfonylurea, for example, affords protection against *N*-ethylmaleimide binding to the putative NADH oxidase protein of HeLa cell plasma membranes (Morré *et al.*, 1995b).

Assuming that our supposition is correct, that the NADH oxidase protein of HeLa cell plasma membrane represents a unique binding site for antitumor drugs leading to growth inhibition and apoptosis of the inhibited cells (Morré and Morré, 1995b; Morré *et al.*, 1995e), then NADH oxidase represents a potentially useful and exploitable target for anticancer drug design. If for no other reason, it may be important that drug conjugates could be prepared that would be clinically effective without entering the cell. Such conjugates could be designed with enhanced specificity, a greater efficacy, and a potentially reduced toxicity. The net result would be a substantially increased margin of safety. This would be especially true for adriamycin where severe systemic toxicity including myelosuppression and cardiotoxicity is the major dose-limiting consideration (Minow *et al.*, 1975).

Targeting of adriamycin to actively growing cells which have increased expression of transferrin receptors by means of transferrin–adriamycin conjugates was introduced by Faulk *et al.* (1980). These conjugates proved to be disproportionately more effective compared to free adriamycin than would be anticipated solely on the basis of increased numbers of transferrin receptors. Efficacy was increased 10-fold and toxicity was not observed even at more than 10 times the usual limiting dose. Thus, compared to an equivalent amount of adriamycin, the margin of safety would appear to be increased by at least a factor of 100. Other nonhydrolyzable conjugates may be equally or even more effective than those prepared with diferric transferrin. Combination of an anticell adhesive synthetic Arg-Gly-Asp-Ser analog and adriamycin resulted in increased antimetastatic activity compared to both peptide and drug (Komazawa *et al.*, 1995). Adriamycin conjugated to monoclonal antibodies to hepatoma-associated antigens by a dextran bridge were active against intrahepatic tumors in athymic mice (Adler *et al.*, 1995). Reduction of systemic toxicity was shown by the absence of adriamycin fluorescence in myocardial tissue in conjugate-treated mice, whereas with free adriamycin or a mixture of adriamycin and antibody, strong myocardial fluorescence of adriamycin was seen. Also since the adriamycin does not need to enter the cell to be effective, the conjugates afford opportunities of overcoming adriamycin resistance based on drug transport across the plasma membrane (Fritzer *et al.*, 1992; Bérczi *et al.*, 1993; Morré *et al.*, 1994). Neither adriamycin–transferrin conjugate nor free adriamycin derived from the conjugate reach cell nuclei in concentrations sufficient to be cytotoxic solely by a mechanism based on DNA intercalation (Barabas *et al.*, 1991, 1992).

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