Effects of Anthracycline Compounds on Transmembrane Redox Function of Cultured Hela Cells

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Introduction

Anthracycline antibiotics are one of the most important agents used in the treatment of human cancer. It has long been thought that DNA is the primary target for the cytotoxic mechanism of this drug on susceptible cells (3,7,11,18). The DNA receptor hypothesis is attractive due to a reasonably high affinity between some of these drugs and nucleic acids. However, the N-substituted derivatives, such as N-acetyl daunomycin and N-trifluoro-acetyl adriamycin (AD 32), have very low affinity toward DNA, yet they are capable of inhibiting cell mitosis (6,7,11). Similarly, significant mitotic inhibition of daunomycin and adriamycin are observed under conditions in which DNA synthesis is unaffected (20,21). Therefore, there are reasons to suspect the anthracycline antitumor drugs might kill cancer cells in more than one way. Anthracycline antibiotics affect microsomal electron transport (1,2,19) and interact with the cytoskeleton (15). Studies of Goormaghtigh (9) have shown that adriamycin inactivates the last oxidation site of the respiratory chain, cytochrome oxidase. Furthermore, plasma membranes interact with adriamycin (12), and anthracycline drug-membrane interaction (8) and adriamycin effects on surface properties of sarcoma 180 ascites cells (14) have also been described. Recently, direct evidence that adriamycin can be actively cytotoxic without entering cells has been presented (22,23); the killing effect is achieved solely by action at the cell surface. To test this hypothesis of drug-plasma membrane interactions, we have, therefore, investigated redox enzymes, and pH changes, which can affect cell functions. From our studies, proton efflux coupled to the transmembrane redox system are observed. It is shown to be modified to a similar degree as redox functions by the action of anthracycline antibiotics. It is concluded that the plasma membrane is a promising target for these anticancer agents.

Materials and Methods

All chemicals were of the highest grade from commercial sources. Anthracycline compounds were obtained from Bristol Laboratories.

HeLa cells were grown in flasks with Eagle's medium containing 10% fetal calf serum, 100 μ u. of penicillin and 170 μg streptomycin per ml at pH 7.4 and maintained in a similar medium containing 2% fetal calf serum. Cells were prepared for study by pelleting the trypsinized suspension cultures at 27,000 g. The pellet was diluted with TD-Tris buffer (NaCl 8g/1, KCl 0.34g/1, Na_2HPO_4 0.1 g/1 and Trizma base 3g/1, pH 7.5) to a final concentration of 0.1 gm cells/ml.

The rate of ferricyanide reduction by HeLa cells was determined in an Aminco DW-2a dual beam spectrophotometer with a linear recorder, a cuvette stirrer, and a 37 °C temperature controlled cuvette chamber. The assay of ferricyanide reduction was performed as described previously (5), except TD-Tris buffer instead of 0.05 M sodium phosphate buffer, pH 7.0, was used. Absorbance changes were measured with the dual beam at 420 nm minus 500 nm. The extinction coefficient for ferricyanide reduction $\Delta A420$ equals 1 $mM \cdot cm^{-1}$.

Ferricyanide induced proton generation was measured in a 2 ml cuvette with an Orion 701 A pH meter and a Corning glass combination electrode. Cells were suspended

in a salt sucrose solution (10 mM KCl, 10mM NaCl, 10 mM $CaCl_2$, 0.1 M sucrose and 5% of TD-Tris buffer), to a final cell concentration of 0.005 gram wet weight per ml (g.w.w./ml). Sample was stirred continuously and air was bubbled through the reaction mixture to remove CO_2. After the pH came to an equilibrium, 0.15 mM ferricyanide was added. The proton generation was measured by the change in pH over the range from pH 7.0 to pH 7.4.

Treatment of cells with drugs was carried out using cells harvested during the exponential growth phase. The cells were suspended in TD-Tris buffer plus 2% fetal calf serum and incubated with various concentrations of drugs at 37°C for 1 hr. with shaking. After incubation the cells were chilled in an ice bath and diluted 10 fold with ice-cold TD-Tris buffer to stop the drug reaction. Surviving cell fraction was measurd immediately.

Cell survival was determined by using the eosin Y exclusion method after treating the cells with drugs. The colorless viable cells were counted. The method of using eosin Y as a vital stain for cells was described by Mishell and Shiigi previously (13).

Results

In order for internal NADH to reduce the impermeable ferricyanide outside the cell a redox system is needed to carry electrons across the membrane. The use of intact cells, therefore, provides an assay of the transmembrane redox enzyme activity. Figure 1 demonstrates two phase kinetics of ferricyanide reduction in HeLa cells (fast rate

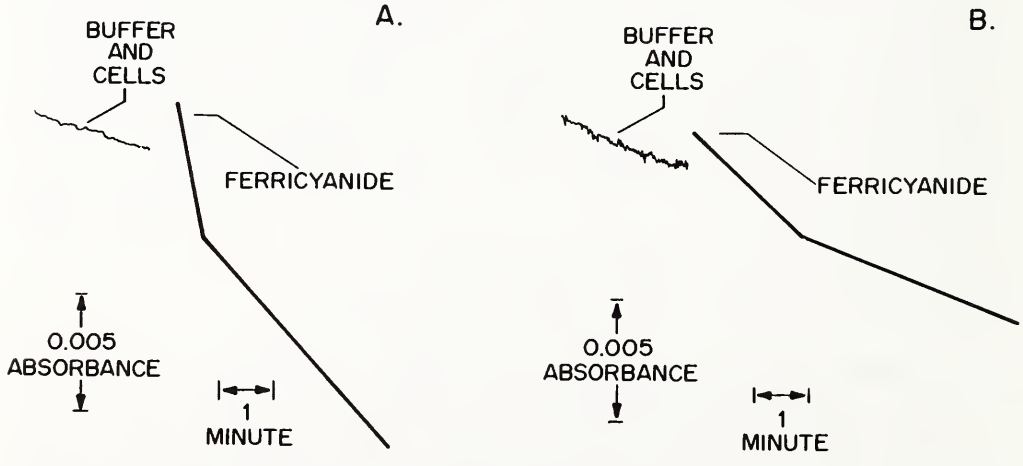

FIGURE 1. *Ferricyanide reduction by HeLa cells with and without the presence of adriamycin. A. without adriamycin. B. with adriamycin (10^{-6}M).*

and slow rate) with and without the presence of adriamycin. The rate of reduction increased in a sigmoidal manner with increasing ferricyanide concentrations (Sun and Crane unpublished). Reciprocal plots of the rate as a function of the ferricyanide concentration showed the aparent Km to be 0.125 mM for the fast rate and 0.24 mM for the slow rate (Figure 2). The maximum ferricyanide reducing activity up to .500 nmoles/min/g cells (wet weight) was observed for the fast rate. However, only about half of this value was reached for the slow rate.

The effect of adriamycin and other anthracyclines on the rate of HeLa cell ferricyanide reduction is shown in Figure 3. All anthracycline antibiotics give a significant inhibition of the fast rate at 10^{-7}M. A stronger maximum inhibition was seen with

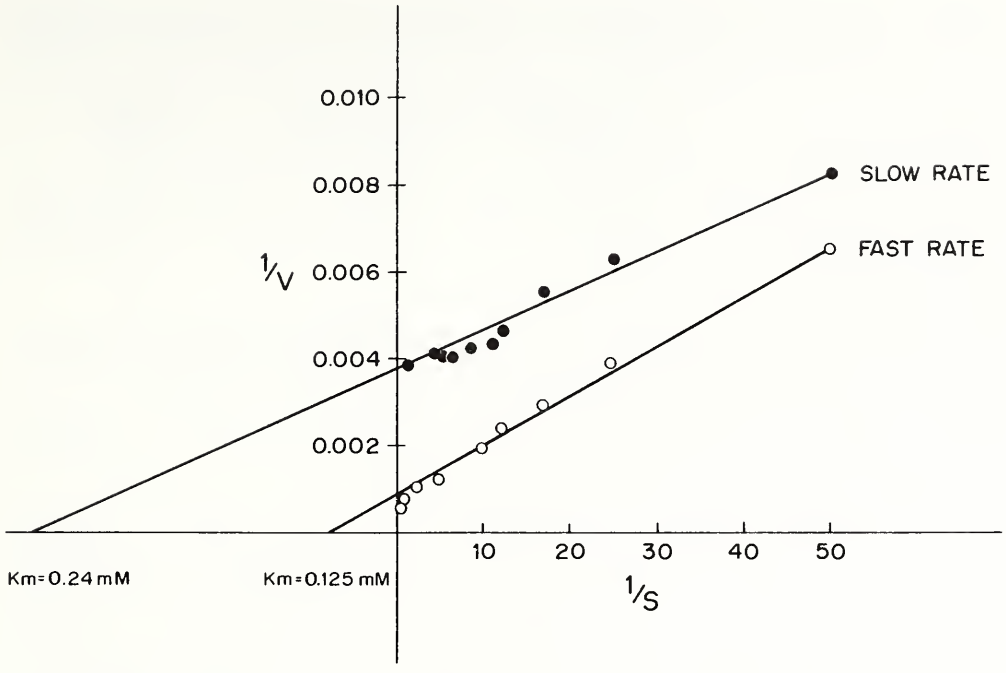

FIGURE 2. *Reciprocal plots of the rate of ferricyanide reduction as a function of ferricyanide concentration.*

adriamycin (90%) that with daunomycin and carminomycin (75%) at 10^{-5}M. Marcellomycin, aclacinomycin and even AD 32, which does not damage DNA, also caused ~50% maximum inhibition at the same concentration. The slow rate of ferri-

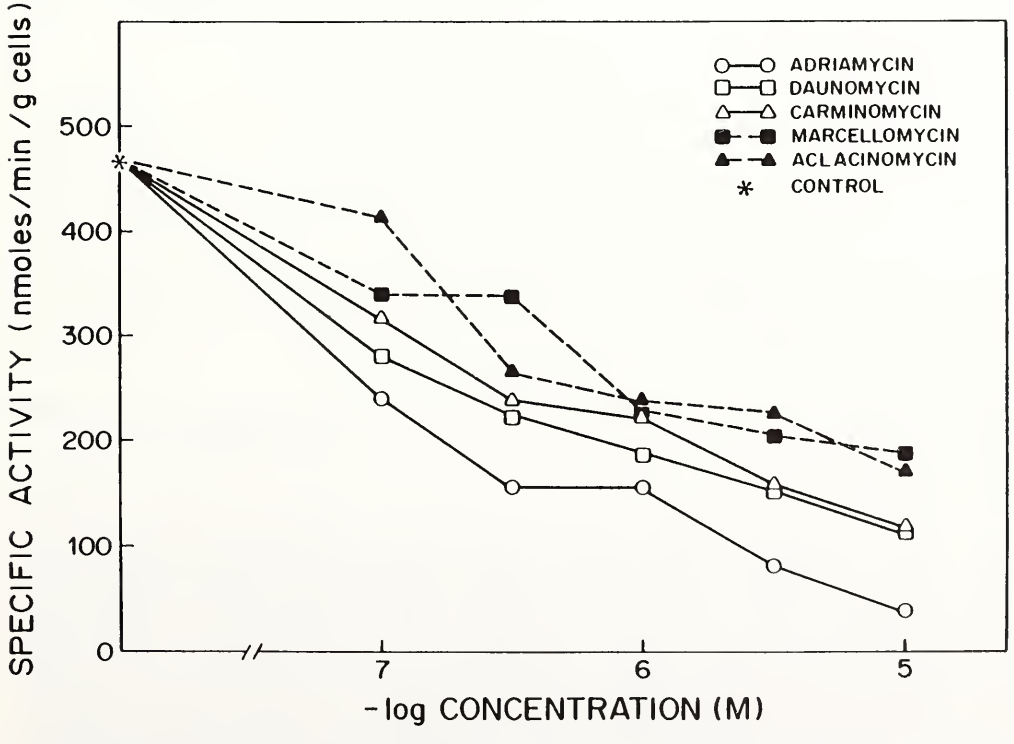

FIGURE 3. *The effect of anthracycline compounds on the fast rate of ferricyanide reduction in HeLa cells.*

TABLE 1. *The effect of anthracycline compounds on the slow rate of ferricyanide reduction in HeLa cells*

Additions	Slow rate without drugs (n moles/min/g.w.w)	Slow rate with drugs (n moles/min/g.w.w.)	% Inhibition
Adriamycin ($1X10^{-7}M$)	265	215	19
Adriamycin ($1X10^{-6}M$)	255	170	32
Aclacinomycin ($1X10^{-7}M$)	250	200	20
Aclacinomycin ($1X10^{-6}M$)	270	185	31
AD 32 ($1X10^{-7}M$)	260	195	25
AD 32 ($1X10^{-6}M$)	260	150	42

Drugs were added after the second phase rate was stabilized.

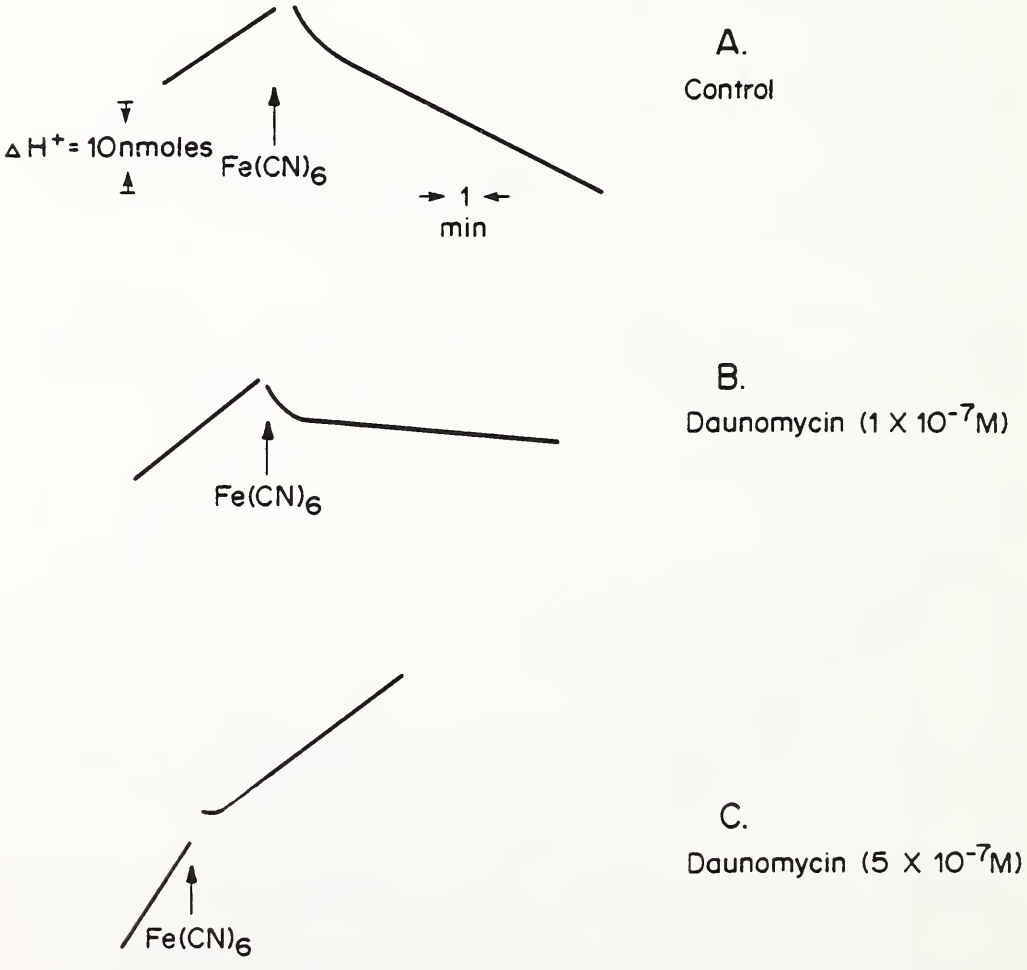

FIGURE 4. *The effect of daunomycin on ferricyanide induced proton release from HeLa cells. Cells were incubated in salt buffered solution at room temperature. The reaction mixture reached a steady rate of proton uptake within 5 minutes without ferricyanide. Addition of ferricyanide to the reaction mixture caused proton release from the cells. The rate of proton release was measured after the addition of ferricyanide and corrected by subtracting the blank rate in the absence of ferricyanide. The relation between pH change and proton increase was standardized by adding known amounts of 0.01M HCl.*

cyanide reduction was also inhibited significantly (31-44%) by the anthracycline antibiotics tested (Table 1). Actually, AD 32, which is the least potent in affecting nucleic acids, appeared to be the strongest cytotoxic drug among those three antibiogics tested in affecting the slow rate of ferricyanide reduction.

Coupling the proton release to the transmembrane redox activity was found in HeLa cells (Figure 4). Under the influence of these cytostatic drugs, a significant decrease (30-77%) in proton generation was demonstrated at a concentration range 1-5 x 10^{-7}M (Table 2).

TABLE 2. *The effect of anthracycline antibiotics on ferricyanide induced proton extrusion in HeLa cells*

Additions	Activity [n moles H^+/min/g cell (w.w)]	% Inhibition
Control	416	—
Adriamycin (1 x 10^{-7}M)	258	38
Adriamycin (5 x 10^{-7}M)	240	43
Daunomycin (1 x 10^{-7}M)	186	55
Daunomycin (5 x 10^{-7}M)	168	60
Carminomycin (1 x 10^{-7}M)	256	39
Carminocycin (5 x 10^{-7}M)	96	77
Marcellomycin (1 x 10^{-7}M)	256	48
Marcellomycin (5 x 10^{-7}M)	144	65
Aclacinomycin (1 x 10^{-7}M)	288	31
Aclacinomycin (5 x 10^{-7}M)	128	69
AD 32 (2.5 μg/ml)	297	30
AD 32 (5.0 μg/ml)	95	77

cell concentration was 0.1 g w.w/ml and $Fe(CN)_6$ was 0.3 mM.
w.w. = wet weight

The cytotoxic activities of anthracycline antibiotics were compared by measuring the surviving fractions of cells treated with various concentrations of drugs for 1 hr. Figure 5 shows the dose-response survival curve. A significantly large portion of cells were sensitive to the three anthracycline compounds tested at a concentration of 1 x 10^{-7}M. However, the curve indicates that cells were a little more resistant to AD 32 than to aclacinomycin and adriamycin. Similar results were previously (10) reported *in vitro* (human leukemia cells). AD 32 appeared to be 10 fold less potent than adriamycin as measured by effects on cell growth and cell survival.

Discussion

In this communication we propose that interaction of the plasma membrane with quinone anticancer drugs is important to their mode of antineoplastic action. The generally accepted mechanism of action for these antibiotics is based exclusively on their interaction with DNA and the inhibition of nucleic acid function (3,7,11,18). This interpretation has been questioned by us. According to the prevailing explanation intercalation of anthracycline compounds with DNA requires a free glycosidic amino group (17,25). In AD 32, the basic amino group has been converted into an amide. However, our results indicate that this compound still inhibits the growth of HeLa cells and induces effects on membranes. Similar results affecting the growth of cells (CCRF-CEM) in culture and producing a highly significant antitumor effect against mouse leukemia (10) by this drug have also been reported.

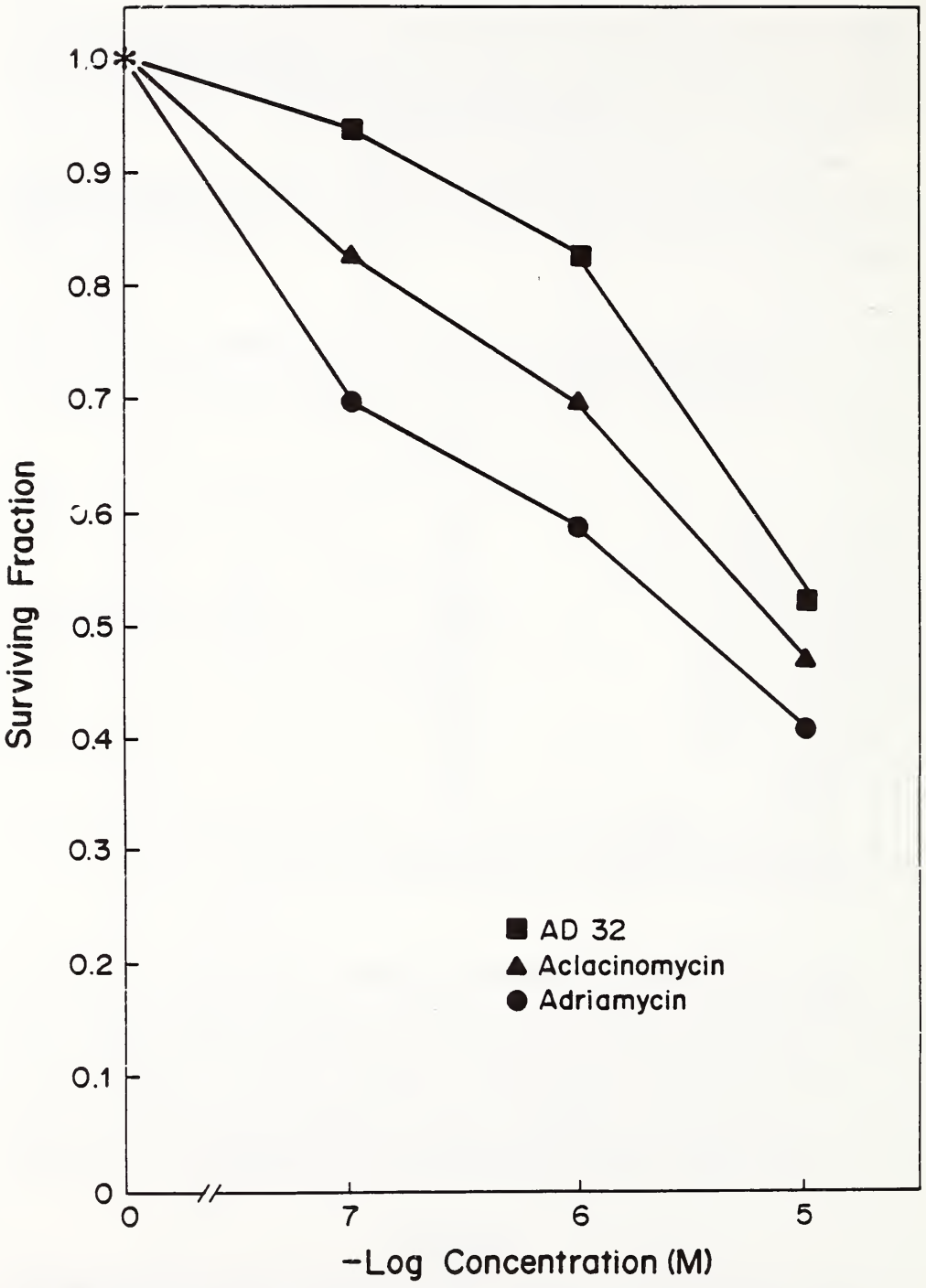

FIGURE 5. *The dose-response survival curve of HeLa cells after one hour drug treatment.*

The reduction of external ferricyanide by intact cells has been attributed to an NADH dehydrogenase which extends across the plasma membrane. Internal NADH is oxidized by external ferricyanide through this enzyme (4,5). The drug effect on ferricyanide reduction by intact cells indicates that the transmembrane dehydrogenase is inhibited. It has been proposed previously that the plasma membrane regulates cell functions (1). The effects of drugs on cell growth which we observe emphasizes that

this plasma membrane dehydrogenase is involved in the control of cell function. These enzymes have been related to selective amino acid transport (16). It is reasonable to assume that the inhibition of membrane redox enzyme activities could contribute to inhibition of cell function and cell damage caused by these drugs.

Tritton and Yee have directly demonstrated that adriamycin is able to destroy murine cancer cells (L1210) by acting on their plasma membranes (23). Adriamycin was chemically attached to insoluble agarose beads that are too large to get into cells. However, the cancer cells die. From a differnt approach, our results also provide direct evidence of drug and plasma membrane interactions as a cytotoxic mechanism for anthracycline antibiotics.

Although our analysis is carried out with a short exposure (3 min) of cells to drugs, the plasma membrane enzymatic and cell proton transfer parameters responded immediately. These changes can be regarded as a direct result of the effect of these antitumor substances. Evidence that the higher doses of drugs are accompanied by more morphological cell damage (granulation and formation of spherical instead of geometrical cells), as well as more pronounced changes in electrophysiological properties and plasma membrane redox activities further support this idea (Sun and Crane, unpublished).

The effects of cytostatic drugs on the surface charge of cultured cells has been reported (24). The results of this study clearly support the idea that redox enzymes and proton export respond rapidly in the plasma membrane and these effects are a sensitive indicator for effects on a great number of cell functions. It is also strongly suggested that it can be used as a parameter in pretherapeutic sensitivity tests for drugs.

Acknowledgment

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