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Inhibition of transplasma membrane electron transport by transferrin-adriamycin conjugates

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Transplasma membrane electron transport from HeLa cells, measured by reduction of ferricyanide or diferric transferrin in the presence of bathophenanthroline disulfonate, is inhibited by low concentrations of adriamycin and adriamycin conjugated to diferric transferrin. Inhibition with the conjugate is observed at one-tenth the concentration required for adriamycin inhibition. The inhibitory action of the conjugate appears to be at the plasma membrane since (a) the conjugate does not transfer adriamycin to the nucleus, (b) the inhibition is observed within three minutes of addition to cells, and (c) the inhibition is observed with NADH dehydrogenase and oxidase activities of isolated plasma membranes. Cytostatic effects of the compounds on HeLa cells show the same concentration dependence as for enzyme inhibition. The adriamycin-ferric transferrin conjugate provides a more effective tool for inhibition of the plasma membrane electron transport than is given by the free drug.

Introduction

Adriamycin is an effective antitumor drug which is used extensively in clinical practice. Its cytostatic effects are attributed primarily to its ability to intercalate with DNA to prevent replication [1,2]. It has also been proposed to generate free radicals [3–5] and inhibit specific enzymes [6–8]. Its effectiveness is decreased by development of cellular resistance based on mechanisms which include active extrusion, decreased uptake or increased detoxification [9–11].

Several laboratories have developed evidence that adriamycin can affect functions in the plasma membrane in addition to interaction with DNA [12–17]. The plasma membrane electron transport system which induces growth in transformed cells is very sensitive to adriamycin and other active anthracycline compounds [18,19]. Since this response is observed after three minutes of incubation of cells with adriamycin and adriamycin takes more than 90 min to reach half-maximum concentration in the nucleus [20], the inhibition of plasma membrane activity must be direct. Inhibition of redox activity in isolated plasma membranes also shows direct action of the drug at the membrane [21,22]. Inhibition of the electron transport by analogs such as 9-deoxyadriamycin, which inhibits growth but does not intercalate with DNA, indicates that adriamycin may inhibit growth by reaction with the plasma membrane dehydrogenase [19].

Targeting of adriamycin to actively growing cells which have increased transferrin receptor expression by the use of adriamycin-transferrin conjugates was introduced by Faulk et al. [23] in 1980. Conjugates of adriamycin and diferric transferrin with glutaraldehyde are therapeutic in treatment of leukemia [24,25] and inhibit growth of both transformed [24–27] and adriamycin-resistant HL60 cells. These conjugates do not introduce adriamycin into the nucleus of viable cells since there is no quenching of adriamycin fluorescence [28]. In this study we show that the adriamycin transferrin conjugate is even more effective than free adriamycin in inhibition of transplasma membrane electron transport in HeLa cells. In addition, the conjugate inhibits the NADH–ferricyanide reductase and holotransferrin stimulated NADH oxidase activity in isolated liver plasma membrane.

Abbreviation: BPS, bathophenanthroline disulfonate.

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Methods

HeLa cells were cultivated in αMEM media with 10% fetal calf serum, 100 U penicillin, 100 μg/ml streptomycin (pH 7.4). Cells were harvested with trypsin treatment followed by addition of 2% serum and pelleted at 150 × *g* [29]. 1 g wet weight (gww) of cells is equivalent to $200 \cdot 10^6$ cells.

Ferricyanide reduction by cells was measured using the dual wavelength mode on the DW2a spectrophotometer by subtracting the change in absorbance at 500 nm from the change at 420 nm [30]. Cells (0.02–0.05 gww) were suspended in 2.8 ml TD Tris buffer (pH 7.4) (140 mM NaCl, 2.5 mM KCl, 0.5 mM $Na2HPO_4$ and 25 mM Tris Cl (pH 7.4) [31]. NADH–ferricyanide reductase by rat liver plasma membrane was measured at the same wavelengths. Membrane (0.05–0.2 mg protein) was in 2.8 ml 0.5 mM phosphate buffer (pH 7.0), with 25 μM NADH and 0.1 mM potassium ferricyanide [21].

Reduction of iron in diferric transferrin by cells was measured by formation of ferrous bathophenanthroline disulfonate (BPS) subtracting absorbance change at 600 nm from change at 535 nm in 2.8 ml TD Tris buffer (pH 7.4), with 10 or 17 μM diferric transferrin and 10 μM BPS [32–34]. Diferric transferrin stimulated NADH oxidase in membranes was measured at 340 minus 430 nm with 0.1 to 0.3 mg membrane protein in 2.8 ml 25 mM Tris chloride (pH 7.4), 1 mM KCN, 25 μM or 50 μM NADH and 17 μM diferric transferrin [35]. Cells and membranes were incubated with drugs for 3 to 5 min before starting the reaction with substrate. Controls were run without cells or membrane [19].

Stimulation of proton release by diferric transferrin was measured by change in pH of the cell suspension within the range of 7.4 to 7.3 in 140 mM NaCl solution plus 100 ml TD Tris for a weak buffering effect [36], using 0.01 gww cells and 17 μM diferric transferrin to start the proton release after equilibration of the cells in the buffer. Calibration was with 100 nmol standard HCl.

Viability studies were in the standard growth media [21,29] by eosin Y exclusion. Cells were trypsinized and counted on a coulter counter with viability determined by eosin Y penetration [29]. Unattached dead cells were removed prior to trypsinization.

Plasma membrane was prepared by aqueous two phase partition from rat liver [37]. Mouse liver plasma membrane was prepared by sucrose gradient centrifugation [38]. Membrane purity was controlled by morphometry and marker enzyme analysis [37,38].

Transferrin-adriamycin conjugates were prepared by two similar procedures by interaction of adriamycin and transferrin with glutaraldehyde and separation of complexes with defined stoichiometry [26,39,53]. 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. Molars of conjugate used would be one-third of the adriamycin concentration shown under conjugate.

Results

Ferricyanide reduction by HeLa cells

Adriamycin inhibits ferricyanide reduction by HeLa cells with half-maximum inhibition at 10^{-7} M and 90% inhibition at 10^{-5} M. Conjugates of adriamycin with ferric transferrin inhibit at lower concentration. Half-maximum inhibition is at 10^{-8} M and 90% inhibition is at $5 \cdot 10^{-7}$ M. The conjugate gives inhibition equivalent to free adriamycin at a 10-fold lower concentration (Fig. 1). The conjugate shows similar inhibition of both the initial fast rate of ferricyanide reduction and the slow rate.

Reduction of diferric transferrin by HeLa cells

The reduction of iron in diferric transferrin by transplasma membrane electron transport from HeLa cells is inhibited by adriamycin and the transferrin-adriamycin conjugate. The conjugate is about ten times more effective as an inhibitor (Table I).

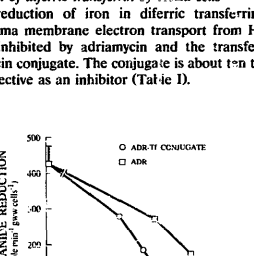

Fig. 1. Effect of adriamycin and adriamycin diferric transferrin conjugate on ferricyanide reduction by HeLa cells. Spectrophotometric assay of decrease in ferricyanide starting at 0.1 mM ferricyanide as in methods with 0.02 gww cells. Concentration of adriamycin in conjugate expressed on the basis of three adriamycin per transferrin. Blank rates without cells and without ferricyanide have been subtracted. Error bar for control is standard deviation for four assays. Similar results in the 10^{-8} to 10^{-6} range have been observed in three separate experiments.

TABLE I

Comparison of adriamycin and adriamycin transferrin conjugate inhibition of diferric transferrin reduction by HeLa cells

Assay with 17 μM Fe_2Tf in TD Tris (pH 7.4). Rates shown with standard deviation and number of assays in parenthesis based on cells from four different cultures.

Concn. (M)	Rate of ferrous BPS formation (nmol ferrous BPS min^{-1} (gww cells)$^{-1}$)	
Inhibitor:	adriamycin	conjugate
None	9.9 ± 2.1 (6)	9.9 ± 2.1 (6)
10^{-8}	9.2 ± 0.9 (5)	5.1 ± 2.4 (6)
10^{-7}	6.3 ± 1.8 (6)	3.8 ± 0.5 (5)
10^{-6}	4.7 ± 2.5 (6)	1.2 ± 1.1 (3)

TABLE II

Comparison of effects of adriamycin and adriamycin ferric transferrin conjugate on the rate of diferric transferrin stimulated proton release from HeLa cells

17 μM Fe_2Tf added to activate proton release in 140 mM NaCl buffered with 100 μl TD Tris (pH 7.4) in 3.0 ml with 0.01 gww cells. Data are a representative experiment from four experiments. The average inhibition at 10^{-7} M was 38 ± 8% with adriamycin and 70 ± 6.7% with conjugate.

Concn. (M)	Proton release rate after Fe_2Tf addition (nequiv. min^{-1} (gww cells)$^{-1}$)	
Inhibitor	adriamycin	conjugate
None	424 ± 101 (3)	424 ± 101 (3)
10^{-8}	330	146
10^{-7}	263	113
10^{-6}	212	64
10^{-5}	95	–

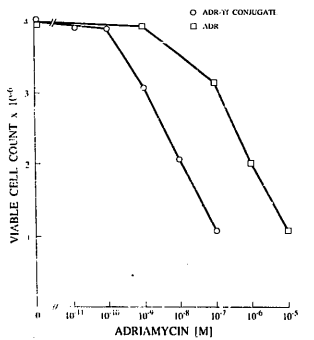

Fig. 2. Effect of adriamycin and adriamycin diferric transferrin conjugate on HeLa cell growth after 24 h in αMEM culture media with 10% FCS. Viable trypsin released cells obtained per 25 cm^2 flask. Similar results have been obtained in one other experiment.

TABLE III

Effect of transferrin adriamycin conjugate and adriamycin on Fe_2Tf-stimulated NADH oxidase by rat liver plasma membrane

Assay in 50 mM Tris Cl at pH 7.4 with 50 μM NADH, 1 mM KCN, 17 μM diferric transferrin and 0.2 mg membrane in 2.8 ml. Absorbance change measured at 340–430 nm [35]. Rates are expressed with standard deviation and number of assays.

Concn (M)	NADH oxidation (nmol min^{-1} (mg protein)$^{-1}$)	
Inhibitor	ADR conjugate	ADR
None	3.7 ± 0.8 (3)	3.4 ± 0.9 (4)
10^{-9}	2.3 ± 1.1 (3)	3.1 ± 1.0 (3)
10^{-8}	0.9 ± 0.8 (3)	1.4 ± 0.9 (4)
10^{-7}	0.5 ± 0.05 (3)	0.3 ± 0.2 (4)
10^{-6}	0.3 ± 0.2 (3)	0.2 ± 0.2 (4)

Diferric transferrin induced proton release

Addition of diferric transferrin to HeLa cells induces proton release from the cells. Both adriamycin and the transferrin-adriamycin conjugate inhibit the induced proton release. As with electron transport activity the complex gives equivalent inhibition to adriamycin at one-tenth the concentration (Table II).

inhibition of proliferation

Both adriamycin and transferrin-adriamycin complex inhibit HeLa cell growth. The decrease in viable cell count after one day in complete media is shown in Fig. 2. Once again the complex is at least ten times more effective in control of cell growth than the free adriamycin.

Diferric transferrin stimulated NADH oxidase of rat liver plasma membrane

The plasma membrane also contains a unique cyanide-insensitive NADH oxidase that is stimulated by diferric transferrin [35,37,40,41]. This activity is inhibited equally well by adriamycin and transferrin-adriamycin conjugate (Table III). The equal effectiveness of free adriamycin and conjugate on the oxidase in the isolated plasma membrane stands in contrast to more effective inhibition of diferric transferrin reductase of whole cells by conjugate.

Discussion

The transplasma membrane electron transport from cells to external electron acceptors is clearly very susceptible to inhibition by the transferrin-adriamycin complex. It is unlikely that the inhibition is based on competition for the transferrin binding site on the receptor by the complex since the inhibition is at concentrations below the affinity of transferrin for the high affinity binding site [42]. It is clearly effective at concentrations much below levels that would affect the

low affinity site which is involved in diferric transferrin reduction [43,44]. Selective binding of conjugate to cells with transferrin receptors has been demonstrated [47]. Bound conjugate is 80% displaced by transferrin at 4°C (Faulk, unpublished data). The inhibition of ferricyanide reduction by the complex shows that the adriamycin in the complex acts at a site other than directly on the transferrin receptor. In contrast, the monoclonal antibody B3/25 for the transferrin receptor gives only 20 percent inhibition of ferricyanide reduction [45].

The inhibition of plasma membrane activities with whole cells shows a similar pattern of concentration required for inhibition by both the conjugate and adriamycin. The conjugate requires less than one-tenth the adriamycin concentration as free drug to inhibit activity with whole cells. If calculated on the basis of moles of conjugate required for inhibition, the conjugate is even more effective. The more effective inhibition by the conjugate with whole cells suggests either than the transferrin targets the drug to the transferrin receptor site (high affinity) or that the conjugate cannot be diluted by penetration into the cell with consequent loss of adriamycin to other sites in the cell such as the nucleus. The failure of adriamycin from the conjugate to reach the nucleus of K562 cells has been demonstrated [28]. The adriamycin and conjugate inhibit the diferric transferrin stimulated NADH oxidase in isolated plasma membrane with almost equal facility, so the transferrin in the conjugate does not appear to give a strong targeting advantage with isolated plasma membranes. A similar equivalence of inhibition of NADH–ferricyanide reductase with isolated membranes has been shown [47]. This would argue for a sparing effect to account for the lower concentration of conjugate giving inhibition with cells.

The use of BPS to detect the formation of ferrous iron from diferric transferrin by the membrane electron transport system has been questioned on the basis that BPS raises the redox potential of the ferric transferrin to allow reduction at a high potential site on the membrane [46]. This is correct and means that the reduction observed measures transmembrane electron transport without giving evidence that transferrin is a natural electron acceptor. The NADH oxidase can be a physiologically significant activity [41]. The stimulation of the oxidase by transferrin does not require BPS so it can demonstrate a natural function of diferric transferrin interaction with the transferrin receptor [45].

In related studies with adriamycin-insensitive HL60 cells (Faulk, W.P., unpublished data), the LD50 for resistant cells was $4.2 \cdot 10^{-7}$ M for transferrin-adriamycin conjugate. Under the same conditions free adriamycin only gave 35% loss of cells. The difference indicates that the conjugate can bypass the multidrug transport system which removes adriamycin from the cell [9,48,49]. Other forms of impermeable adriamycin conjugates with high molecular weight material have proven active in control of proliferation and a site of action at the membrane has been proposed [12–15,28,50]. These agents may also act to inhibit the electron transport but have not been tested in that regard.

The basis for inhibition of the transplasma membrane electron transport by adriamycin or the conjugate is unknown. It could be based on known effects of adriamycin on membranes such as changes in lipid fluidity [14,55], changes in lipid orientation [54] or destruction of sensitive parts of the oxidoreductase (e.g., thiols) by free radicals produced by autooxidation of the drug [51,52]. The small amount of drug required would indicate a direct effect at a specific site.

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