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BLEOMYCIN CONTROL OF TRANSPLASMA MEMBRANE REDOX ACTIVITY AND PROTON MOVEMENT IN HeLa CELLS

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Abstract—Bleomycin, tallysomycin A, tallysomycin $S_{10}b$ and copper–bleomycin have been tested for their capacity to inhibit the transplasma membrane electron transport and associated proton release by HeLa cells. Transplasma membrane redox activity is measured using reduction of external ferricyanide by the cells. At 75 μg/ml bleomycin, tallysomycin A and tallysomycin $S_{10}b$ gave a maximum of 65% inhibition of the ferricyanide reduction rate; half-maximum inhibition was observed at 30 μg/ml. The copper–bleomycin complex was slightly more effective as an inhibitor with half-maximum inhibition at 20 μg/ml. Survival of cells after 1 hr of drug treatment was 50% at 25 μg/ml for bleomycin and copper–bleomycin and at 75 μg/ml for tallysomycin A. Tallysomycin A and tallysomycin $S_{10}b$ gave 75 to 83% inhibition of ferricyanide-induced proton extrusion, respectively, at 50 μg/ml, whereas bleomycin and copper–bleomycin appeared to be slightly less effective with 50 to 60% inhibition, respectively, at 50 μg/ml. In all aspects studied, which included transplasma membrane ferricyanide reduction, ferricyanide-induced proton release, and cell survival, there were significant effects by these compounds on HeLa cells in the range of 25–50 μg/ml.

Bleomycin and tallysomycin A are antibiotics obtained from *Streptomyces verticillus*, which are effective against several malignant diseases, including squamous cell carcinoma and testicular neoplasm [1–3]. Bleomycin also sensitizes lungs to damage at oxygen concentrations normally free from toxic effects [4, 5]. The receptor site for drug action within the cell is generally believed to be DNA, and evidence has been presented for binding of bleomycin to DNA leading to destruction of the DNA [1, 6]. Inhibition of DNA synthesis in cells has been reported [7]. The structurally similar tallysomycin A appears to have a similar mechanism of action [8]. Damage to DNA by bleomycin *in vitro* requires the presence of iron II and oxygen. The iron complex has been shown to generate hydroxyl radicals [9, 10], but the identity of the active agent remains unclear [11, 12]. On the other hand, copper ions prevent damage to DNA by bleomycin [9]. This protective effect has been related to a conformational change in bleomycin as a result of copper chelate formation or to interference with electrostatic binding between DNA and bleomycin [13]. The copper chelate also acts to dismutate superoxide radicals produced by iron catalyzed oxidations [9]. Since the copper–bleomycin complex is more cytotoxic than free bleomycin [14, 15] but does not damage DNA *in vitro* [16], a mechanism of antitumor action based only on the interaction with DNA and inhibition of nucleic acid synthesis has been questioned. It has also been found that tallysomycin is relatively more effective in antitumor activity than bleomycin but causes less breakdown of DNA [17].

There is growing evidence that agents involved in tumor production act at the plasma membrane [18]. Neoplastic transformation has also been related to enzymatic changes in the plasma membrane [19–20], and oncogenes produce membrane associated proteins [22]. Hormones which act at the plasma membrane can be mutagenic [23]. There is also growing evidence that some antitumor drugs, such as diacridines [24], polylysine [25], and anthracyclines [26, 27], act at sites on the cell membrane.

It has been proposed that plasma membrane redox enzymes can regulate cellular function through modification of hormone response [28]. These enzymes may also transduce energy for special amino acid transport [29]. In this paper we report bleomycin and tallysomycin inhibition of transplasma membrane electron transport and associated proton excretion by HeLa cells at concentrations which inhibit the growth of these cells. Bleomycin has been shown previously to inhibit growth of HeLa cells [30].

MATERIALS AND METHODS

HeLa cells were grown in flasks with Eagle's medium containing 10% fetal calf serum, 100 units penicillin and 170 μg streptomycin per ml at pH 7.4. Cells were grown in monolayer cultures and released by treatment with 0.1% trypsin for a few minutes. The cells were collected by centrifugation at 1200 *g*. The pellet was weighed by difference and diluted with TD buffer to a final concentration of 0.1 g cells/ml (NaCl, 8 g/l; KCl, 0.38 g/l; Na_2HPO_4, 0.1 g/l; and Trizma base, 3 g/l; 2% fetal calf serum, final pH 7.5). Cells were harvested in the exponential growth phase. The rate of ferricyanide reduction by HeLa cells was determined in an Aminco DW2a dual

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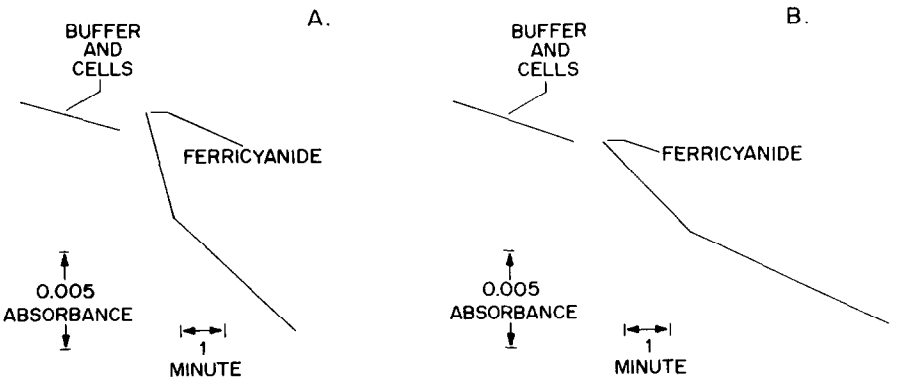

Fig. 1. (A) Ferricyanide reduction by HeLa cells. Cells (200 mg wet wt in 3 ml buffer) were incubated for 3 min before addition of potassium ferricyanide to a final concentration of 0.3 mM. (B) Inhibition of ferricyanide reduction by bleomycin. Bleomycin (50 μg/ml) was incubated with the cells for 3 min before the addition of ferricyanide.

beam spectrophotometer with a linear recorder, a cuvette stirrer, and a 37° temperature-controlled cuvette chamber. The reduction was measured as described previously [31], except that TD buffer was used instead of phosphate. Absorbance changes were measured with dual wavelength, using 420–500 nm. NADH-ferricyanide reductase activity of isolated plasma membranes was also measured using the 420–500 nm absorbance difference in the dual beam mode of the Aminco spectrophotometer [32, 33]. Mouse liver plasma membranes [32] and pig erythrocyte plasma membranes [34] were prepared as previously described.

Proton release was measured with cells suspended in a sucrose-salts solution (10 mM KCl, 10 mM NaCl, 10 mM $CaCl_2$ with 0.1 M sucrose). The suspension was continuously aerated to remove carbon dioxide. The pH was measured with an Orion model A pH meter with a Corning glass combination electrode. Calibration for proton equivalents was with standard HCl. Drugs were preincubated with cells or isolated membranes in the assay buffer for 3 min before the addition of ferricyanide to start the assay. Cell survival was determined using the eosin Y exclusion method [35] and colonogenic assay [36]. Treatment of cells with drugs was carried out during the exponential growth phase. Cells were suspended in TD buffer plus 2% fetal calf serum and incubated with various concentrations of drugs for 1 hr at 37° with shaking. After incubation, the cells were chilled in an ice bath and diluted 10-fold with ice-cold TD buffer to stop the drug reaction. The surviving fraction was measured immediately.

All chemicals were the highest grade from commercial sources. Bleomycin, tallysomycin A and tallysomycin $S_{10}b$ were obtained from Dr. W. T. Bradner, Bristol Laboratories, Syracuse, NY. The copper–bleomycin complex was prepared from equimolar bleomycin and cupric sulfate [9].

RESULTS

The time course of ferricyanide reduction by HeLa cells is shown in Fig. 1. There was a rapid initial reduction, followed by a steady rate over several minutes. If the cells were preincubated for 3 min with bleomycin (50 μg/ml), before addition of ferricyanide, there was a significant inhibition of both the rapid initial and slow ferricyanide reduction rates (Fig. 1). The effect of bleomycin concentration on the rate of ferricyanide reduction is shown in Fig. 2. Maximum inhibition equal to 50% of the control activity was observed at 50 μg/ml. Tallysomycin A and tallysomycin $S_{10}b$ showed similar effectiveness as inhibitors. The copper–bleomycin complex was effective at a lower concentration. The addition of equivalent concentrations of copper chloride or

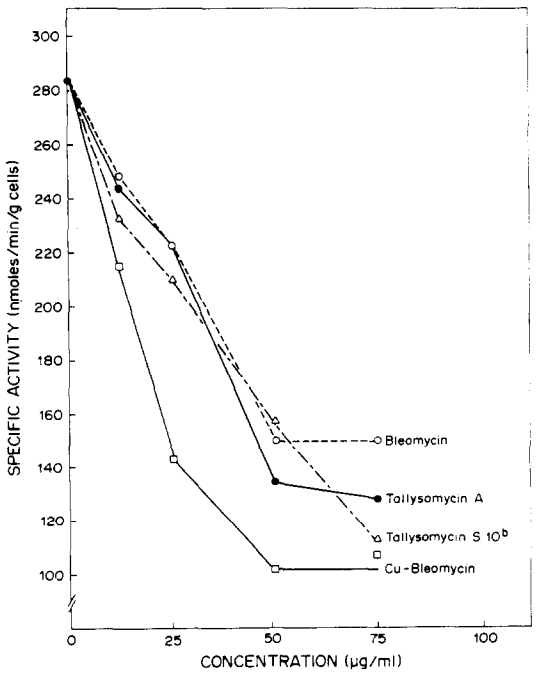

Fig. 2. Effects of bleomycin and tallysomycin on the rate of ferricyanide reduction by HeLa cells. Cells were incubated with the drugs for 3 min before the addition of ferricyanide to start the reaction.

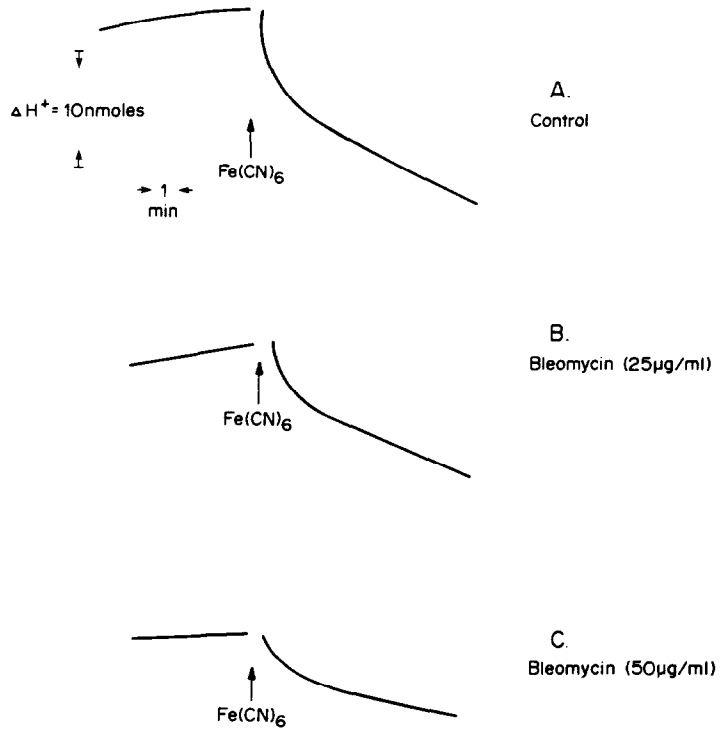

Fig. 3. Effect of bleomycin on ferricyanide-induced proton extrusion by HeLa cells. The assay was performed as described in Methods.

copper EDTA (35 μM) did not inhibit ferricyanide reduction.

To determine any effect of the trypsin treatment used to release cells, the ferricyanide reduction by a line of cells, obtained from Dr. J. Dixon, Purdue, and grown in suspension culture, was tested. HeLa cells S-3 grown in Jocklick Minimum Essential Medium with 5% horse serum and 5% calf serum were assayed. The ferricyanide reduction rates were (fast) 490 ± 25 and (slow) 246 ± 18. Inhibition by bleomycin followed a concentration curve similar to that of trypsin-treated cells. Treatment of the suspension cells with 0.1% trypsin did not change the response to bleomycin.

Proton release was also observed when ferricyanide was added to HeLa cells (Fig. 3). There was an initial rapid release of protons, followed by a slower rate of release, which continued for several minutes. The proton release experiments were done in different media than was used for ferricyanide reduction, so that the relative rates of ferricyanide reduction and proton release are not comparable. Bleomycin inhibited the ferricyanide-induced proton release at concentrations which inhibited ferricyanide reduction (Fig. 3). Similar inhibition of proton release was observed with tallysomycin A and the copper–bleomycin complex (Table 1).

It has been proposed that the transmembrane

Table 1. Effects of bleomycin and its analogs on ferricyanide-induced proton extrusion in HeLa cells*

Additions	Activity (nmoles H^+/min/g wet wt)	% Inhibition
None	403	
Bleomycin (25 μg/ml)	345	14
Bleomycin (50 μg/ml)	180	55
Copper–Bleomycin (25 μg/ml)	315	22
Copper–Bleomycin (50 μg/ml)	150	63
Tallysomycin S_{10}b (25 μg/ml)	220	45
Tallysomycin S_{10}b (50 μg/ml)	70	83
Tallysomycin A (25 μg/ml)	320	21
Tallysomycin A (50 μg/ml)	100	75

* All assays were done with 100 mg cells and 0.3 mM potassium ferricyanide in sucrose-salts medium.

Table 2. Effects of bleomycin and its analogs on NADH–ferricyanide reductase activity of mouse liver plasma membrane and pig erythrocyte plasma membrane*

Membrane source	Compounds added	% Control
Mouse liver plasma membrane	Control	100
	Bleomycin (50 μg/ml)	57
	Copper–Bleomycin (50 μg ml)	48
	Tallysomycin A (50 μg ml)	72
	Tallysomycin $S_{10}b$ (50 μg/ml)	69
Pig erythrocyte membrane	Control	100
	Bleomycin (75 μg/ml)	68
	Copper–Bleomycin (75 μg/ml)	64
	Tallysomycin A (75 μg/ml)	69
	Tallysomycin $S_{10}b$ (75 μg/ml)	63

* The assay was essentially performed as described in Ref. 32; 0.045 μmole NADH was added to each assay. There was a 3-min preincubation of membrane with drugs before starting the assay.

ferricyanide reduction the intact cells is catalyzed by a transplasma membrane NADH dehydrogenase [28]. To study the activity of the transmembrane dehydrogenase, it is necessary to use plasma membranes, which have a minimum contamination by other intracellular membranes. For these studies we used plasma membranes from erythrocytes [28] and mouse liver [32]. Bleomycin inhibited the NADH-ferricyanide reductase activity of isolated plasma membranes (Table 2). The concentration required for inhibition of NADH–ferricyanide reductase activity of mouse liver membranes was similar to the concentration required for inhibition of ferricyanide reduction by cells. Bleomycin was less effective as an inhibitor of NADH–ferricyanide reduction of erythrocyte plasma membrane. Tallysomycin A and the copper–bleomycin complex also inhibited the NADH–ferricyanide reductase activity at concentrations similar to those required for bleomycin. The cytotoxic activities of bleomycin, tallysomycin and the copper complex on HeLa cells were examined by measuring the surviving fraction of cells treated with the drugs for 1 hr. The effect of drug concentration on cell survival is shown in Fig. 4. Bleomycin and the copper complex were equally effective in growth inhibition and were somewhat more effective than tallysomycin. Similar toxicity has been reported for cultured cells [37, 38] and for mice *in vivo* [39]. Assay of cell survival by a colonogenic method was also performed. Bleomycin at 25 μg/ml caused a 63% lethal effect; at 50 μg/ml of bleomycin, the survival value obtained was 17% which is twice as lethal as the value obtained by the dye exclusion method.

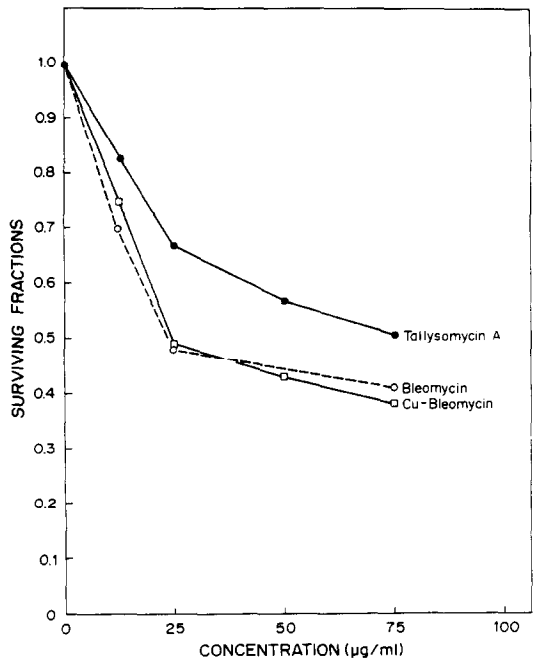

Fig. 4. Survival of HeLa cells after a 1-hr treatment with bleomycin and tallysomycin. Cells were incubated in culture medium with added drugs as indicated. Incubation time was 1 hr.

DISCUSSION

Transmembrane electron transport has been shown to stimulate the growth of melanoma cells [40], and we have found similar stimulation of HeLa cell growth (I. L. Sun and F. L. Crane, unpublished observation). There is also evidence that this redox system is involved in the L system of amino acid transport [29, 41]. We have shown previously [42] that transmembrane ferricyanide reduction by transformed liver cells is inhibited by adriamycin at concentrations which inhibit growth of the cells. The inhibition of the transmembrane ferricyanide reduction, which we observed with bleomycin, may also be involved in the inhibition of cell growth. Both bleomycin and the copper-bleomycin complex have been shown to inhibit cell division [7, 9], and both of these inhibit the transmembrane electron transport. This is in contrast to the fact that copper–bleomycin

does not generate free radicals which react with DNA [8–10]. The effect of the bleomycin and its copper complex on transmembrane redox activity is more consistent with the cell growth inhibition effects of these compounds. It is, of course, reasonable to consider that these antitumor agents may act at sites on the plasma membrane [43], as well as on DNA.

The inhibition of the redox-induced proton release by bleomycin provides a possible basis for the inhibition of growth. It has been shown that an increase in cytosolic pH is correlated to an increase in mitosis [44, 45]. If the transmembrane redox is stimulated, it would contribute to the movement of protons out of the cell. On the other hand, inhibition of electron transport by bleomycin inhibits proton release, which would tend to decrease intracellular pH and inhibit mitosis.

Tallysomycin A and tallysomycin $S_{10}b$ inhibited transmembrane ferricyanide reduction and ferricyanide-induced proton release similar to bleomycin. Higher concentrations of tallysomycin A tended to give a higer inhibition of proton release than bleomycin, whereas the bleomycin tended to be more cytotoxic. The basis for these slight differences may be shown by further study of the binding of these drugs to the plasma membrane.

The similar inhibition produced by bleomycin and the copper–bleomycin indicates that the inhibition of the redox system is not based on free radicals generated by iron–bleomycin complexes at the membrane, since copper bleomycin does not generate these oxygen radicals [9] and only causes a slight increase in lipid peroxides in lung fibroblasts [30]. If the bleomycin induces the formation of superoxide radicals which lead to peroxide and hydroxyl radical formation, then the rate of ferricyanide reduction would be increased by the production of superoxide which reduces ferricyanide. The ferricyanide would also tend to prevent further radical formation by oxidative removal of superoxide.

We proposed that bleomycin reacts with a transmembrane electron transport enzyme on the cell surface to inhibit the activity of this enzyme. The effect on the enzyme requires only a few minutes of incubation so it would not involve internalization of the bleomycin [46]. The inhibition of the enzyme could contribute to the cytotoxic effects of bleomycin.

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