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ACTIVITY OF BLEOMYCIN IN IRON- AND COPPER-DEFICIENT CELLS

SUZANNE LYMAN, PAMELA TAYLOR, FRANK LORNITZO, ALAN WIER, DAVID STONE, WILLIAM E. ANTHOLINE* and DAVID H. PETERING†
Department of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, WI 53201, U.S.A.

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Abstract—Three models were used to examine the requirement of bleomycin (Blm) for iron (Fe) to carry out its antitumor or cytotoxic activity. Mice were made iron deficient by dietary means. Animals with depressed iron stores in liver and low plasma and ascites fluid iron supported Ehrlich tumor growth as well as mice maintained on a control diet. Bleomycin was equally effective against this tumor in iron-deficient mice as it was against the tumor in iron-sufficient controls. Likewise, nutrient copper deficiency did not change the efficacy of the drug. Ehrlich cells in culture were treated with a non-growth inhibiting concentration of the chelating agent, 1,10-phenanthroline before or during their exposure to bleomycin. Again, the treated cells were as sensitive to drug as controls, despite the fact that this ligand reduces cellular iron and zinc and can extract iron from Fe(II)Blm. Lastly, it was demonstrated that iron-depleted *Euglena gracilis* cells growing at reduced rates were as sensitive to growth inhibition by bleomycin as control cells.

It has long been thought that the biological mechanism of action of the antitumor agent, bleomycin (Blm‡) involves the strand cleavage of DNA [1]. With the finding *in vitro* that Fe^{2+} is necessary to activate the drug in this oxygen-dependent reaction [2, 3], it has frequently been assumed that iron–bleomycin is the active form of the drug *in vivo*.

Other studies have shown that under some conditions Cu^{+} plus bleomycin, Co(III) bleomycin and light, and Mn(II) bleomycin in the presence of H_2O_2 can cause *in vitro* DNA damage [4–6]. However, it is the metal-free drug that is administered to patients and used in most animal experimentation. Thus, if a metallobleomycin is the pharmacologically active species, one recognizes that the metal-free drug must sequester an activating metal from the host in order to carry out its cytotoxic activities.

In chemical experiments metal ions such as Fe^{2+} are readily available to react with Blm to initiate DNA strand breakage. However, in the organism the drug must compete with other ligands for metals. Indeed, in humans, cancer patients may present themselves for treatment with varying degrees of malnutrition due to cachexia. It is possible, for example, that they are deficient in one or more of the metals implicated in the action of bleomycin [7]. Therefore, it is practically important as well as mechanistically interesting to know whether the activity of this drug can be compromised by metal deficiencies.

One experimental approach to test the role of metals in the cytotoxic properties of metal binding compounds has been to study their antitumor activity in metal-deficient animals or in cell culture systems in which the medium is metal deficient [8, 9]. In the present study, the sensitivity of three iron-limited cellular systems to Blm has been investigated: Ehrlich cells in iron-deficient host mice, Ehrlich cells in culture containing a metal-chelating agent, and *Euglena gracilis* maintained in an iron-depleted, defined growth medium. In addition, the effects of dietary copper deficiency on the activity of bleomycin against the Ehrlich tumor were ascertained.

METHODS

Female HSD/ICR mice were purchased from Harlan Sprague–Dawley, Indianapolis. The mice were provided with either a complete diet or a specific metal deficient diet and glass-distilled water *ad lib*. The complete semipurified diet and diets deficient in either copper or iron were prepared by Zeigler Bros. (Gardner, PA). The complete diet has been described previously [10]. Metals are provided as metal salts which can be deleted to make the metal-deficient diets. The complete diet contained 35.0 μg Fe/g diet, 6.2 μg Cu/g, and 20.0 μg Zn/g. According to analysis, deficient diets contained 6.3 μg Fe/g or less than 0.5 μg Cu/g. The stainless steel mouse cages were pretreated for 1 hr in an EDTA/NaOH bath and rinsed with deionized water. The water bottles were acid washed, rinsed and fitted with silicone stoppers.

Antitumor studies in metal-deficient mice. In the first animal experiment, 11- to 12-week-old adult mice were used. Groups of animals were placed either on a copper-deficient diet or the complete, semipurified diet for 12 weeks. Groups on each diet were then inoculated with 5×10^6 Ehrlich ascites tumor cells. After 24 hr some members of each group were treated for 4 days with 3 mg Blm/kg body weight, previously shown to be a low, effective dose of the drug in this tumor model [11]. Twenty-four

* Department of Radiation Biology and Biophysics, Medical College of Wisconsin, Milwaukee, WI 53233.
† To whom correspondence should be addressed.
‡ Abbreviations: Blm, bleomycin; Co, cobalt; Cu, copper; Fe, iron; Mn, manganese; Zn, zinc; 1,10-phen, 1,10-phenanthroline; and Tris, Trizma base.

Table 1. Characteristics of mice on iron normal, Fe(+), and iron-deficient, Fe(−), diets*

		Week 17	Week 19
1.	Animal weights§ (g)		
	Fe(+)	36.9 ± 4.0	
	Fe(−)	39.2 ± 3.0	38.2 ± 2.4
2.	Hematocrit—erythrocytes as % of total blood volume		
	Fe(+)	44 (2)	44 ± 1
	Fe(−)		44 ± 2
3.	Hemoglobin content of erythrocytes (g/dl)		
	Fe(+)	14.0 (2)	15.4 ± 0.9
	Fe(−)		16.0 ± 1.4
4.	Liver weight (g)		
	Fe(+)		1.85 ± 0.24†
	Fe(−)		1.43 ± 0.29†
5.	Liver ferritin in cytosol (μg Fe/g wet weight liver)		
	Fe(+)		44.7 ± 14.6‡
	Fe(−)		2.0 ± 1.4‡
6.	Serum Fe, pooled‖ (μg/ml)		
	Fe(+)		2.1
	Fe(−)	1.7 (2)	1.4
7.	Ascites fluid Fe (μg/ml)		
	Fe(+), T¶	3.1 ± 0.1	
	Fe(−), T	1.6 (2)	1.7 ± 0.9 (3)
8.	Total Ehrlich cells suspension		
	a. Total cells ($\times 10^8$)		
	Fe(+), T	13.0 ± 4.2	
	Fe(−), T		8.7 ± 2.9
	b. Ascites suspension volume (ml)		
	Fe(+), T	18.9 ± 2.0	
	Fe(−), T		5.7 ± 2.1
9.	Ehrlich cell Fe (μg/10^8 cells)		
	a. Total		
	Fe(+)	2.52 ± 0.84	
	Fe(−)		1.80 ± 0.59
	5. Cytosol**		
	Fe(+)	1.32 ± 0.39	
	Fe(−)		0.68 ± 0.09 (3)

*–‡ There were four animals per group unless noted in parentheses. Means being compared are given the same symbol. Probabilities of less than 0.1 are indicated: (†) $P < 0.05$; and (‡) $P < 0.01$.

§ Group averages at initiation of special diets (3.5 weeks) and at 5.5 and 14.5 weeks. Fe(+): 17.0, 27.2 and 33.5 g respectively; Fe(−): 17.5, 27.3 and 35.7 g respectively.

‖ Pooled plasma from four mice.

¶ Measurements on tumor bearing mice (T) at age 17 weeks were made on day 9 after injection and at 19 weeks were made on day 15 after injection.

** Cytosolic Fe is ferritin-bound.

hours after the last treatment with drug, some of the animals on metal-deficient diets were placed on the complete diet for the remainder of the experiment. Weight changes and appearance of the animals were followed over time as indications of tumor growth and toxicity of the drug. In some cases observations were continued until the animals died; in others, mice were killed so that their metal and tumor status could be evaluated.

A second experiment utilized 3- to 4-week-old weanling mice and investigated only the effects of iron deficiency upon tumor growth and drug efficacy. The animals were kept on the semipurified diets for 13 weeks prior to injection of Fe(+) (iron normal) groups with 5×10^6 cells/mouse for a control anti-tumor experiment and 15 weeks before the larger Fe(−) (iron deficient) antitumor study was started by injection of 5×10^6 cells/mouse. In the latter experiment animals were administered tumor cells from two Fe(−) mice given the tumor 2 weeks earlier, in order to lower intracellular iron stores which could support drug action even in iron-deficient test animals. The averages of their final serum iron and ascites fluid iron were 1.7 and 1.6 μg/ml respectively. This procedure was employed so that residual iron in cells injected into iron-deficient mice would be minimized. Several doses of Blm were used to test the dose–response activity of the drug.

Analyses of metal status in animals. Hematocrit, hemoglobin and serum iron levels were determined

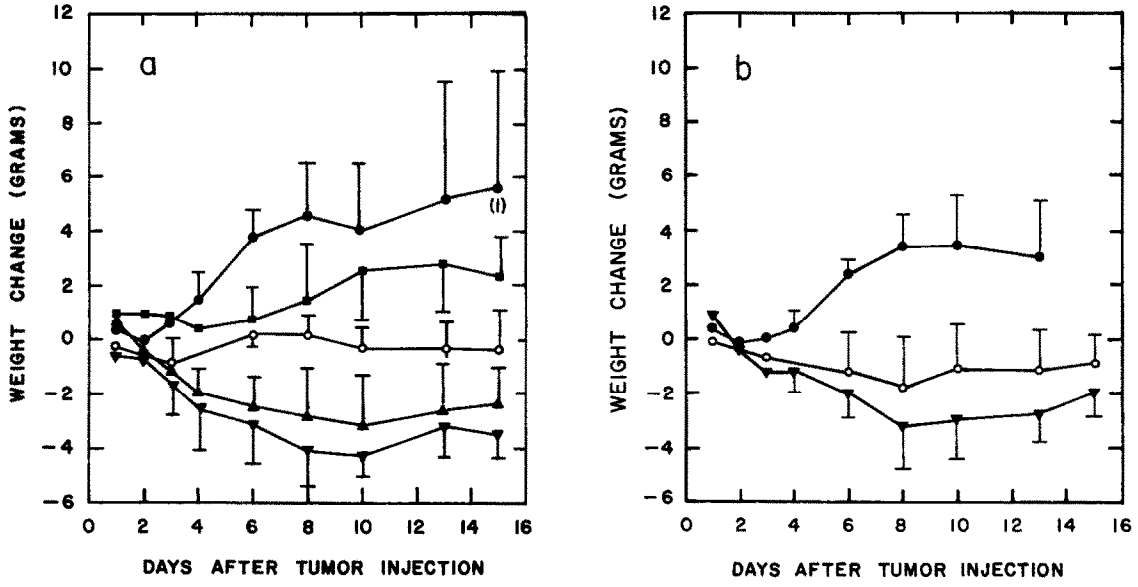

Fig. 1. Effect of Blm on tumor growth in Fe(−) mice. On day 0, 5×10^6 cells/animal (5 mice per group) were injected, and on days 1–4 intraperitoneal injections of Blm were administered. Panel a: All groups were placed on Fe(+) diet on day 5. Key: (●) control tumor; (○) no tumor; (■) 1 mg Blm/kg body weight; (▲) 3 mg Blm/kg; and (▼) 6 mg Blm/kg. Panel b: All groups were maintained on Fe(−) diet. Key: (●) control tumor; (○) no tumor; and (▼) 6 mg Blm/kg body weight. Values are means ± SD.

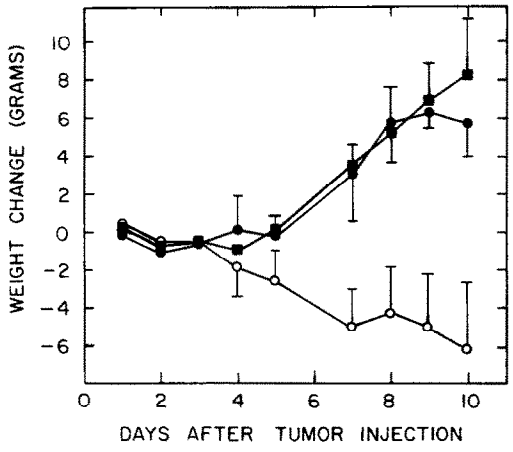

Fig. 2. Effect of Blm on tumor growth in Cu(−) mice. On day 0, 5×10^6 cells/animal (5 animals per group) were injected. Key: (●) Cu(−) control tumor; (■) control tumor of animals changed to Cu(+) diet on day 5; and (○) tumor treated with 3 mg Blm/kg body weight on days 1–4. Values are means ± SD.

in mice maintained on the control and iron-deficient diets. Serum copper was determined in copper-normal and copper-deficient mice. The mice were anesthetized with ether, and blood was collected suborbitally into a heparinized capillary tube for the hematocrit. The serum was removed from the capillary tube after centrifugation, and iron or copper levels were determined by atomic absorption spectrophotometry. Hemoglobin concentration was determined by mixing 0.02 ml of blood with 5 ml of Hycel Cyanmet hemoglobin reagent and measuring the absorbance of the solution at 540 nm. Ferritin iron levels were measured in the cytosol of livers of mice fed complete and iron-deficient diets to obtain an indication of the iron stores of the host during the course of the experiment. After animals were killed by cervical dislocation, their livers were dissected out and weighed. The whole liver was homogenized in 5 ml of ice-cold 5 mM Tris buffer containing 0.25 M sucrose and 10 mM 2-mercaptoethanol, pH 7.5. The homogenate was centrifuged at 20,000 rpm (48,000 *g*) for 40 min, and the supernatant fraction was passed over a G-75 column (2.5 × 60 cm) equilibrated with 5 mM Tris, pH 7.8, to separate ferritin iron from contaminant hemoglobin iron. Fractions (5 ml) were collected, and the amount of iron contained in the ferritin peak was determined by atomic absorption spectrophotometry.

The metal contents of ascites fluid and tumor cytosol were also determined. The ascites suspension was withdrawn from the peritoneum. Then the cells were separated by centrifugation, and the ascites fluid was saved for metal analysis. The tumor cells were washed in 0.01 M phosphate-buffered saline, pH 7.2, and red blood cells were lysed by incubating the cell population with 0.01 M Tris containing 0.83% ammonium chloride for 20 min. Cold glass-distilled water (2 ml) was added to 3×10^8 cells; the suspension was sonicated for 1 min and then centrifuged at 20,000 rpm (48,000 *g*) for 20 min. The resultant supernatant fraction was passed over a G-75 column (1.5 × 30 cm) equilibrated with 5 mM Tris, pH 7.8, and 2-ml fractions were collected. Metal was measured by atomic absorption spectrophotometry.

Cytotoxicity studies in Ehrlich cell culture. Exposure of Ehrlich cells in culture to 1,10-phenanthroline markedly reduces cellular concentrations of iron and zinc at concentrations of the reagent which only modestly affect proliferation. Thus, experiments were designed to see whether Blm remains active against proliferating Ehrlich tumor cells in culture that contain a reduced level of iron or have a chelating agent present that can effectively compete with Blm for Fe(II). Cells were either pretreated with 1,10-phen, washed, and then exposed to Blm or pretreated with 1,10-phen and then exposed to drug in the presence of the ligand. A concentration of 1,10-phenanthroline was chosen at the upper end of the range which did not depress cell growth (see Table 4, footnote). Cells were grown at 37° in spinner flasks for metal measurements or culture plates for growth inhibition studies. The medium contained Eagle's Minimal Essential Medium plus Earle's salts supplemented with 2.5% fetal calf serum as described previously [12]. Metal contents of the cytosol and whole pellet from 2×10^8 cells were measured after mechanical homogenization and centrifugation using atomic absorption spectrophotometry.

Cytotoxicity studies using Euglena gracilis. The unicellular organism, *Euglena gracilis*, strain Z, was purchased from the Culture Collections of Algae, University of Texas, Austin, TX, and was grown on defined medium at pH 3.6 [13]. The iron requirement was supplied by addition of 36 μM $FeSO_4$. Omission of $FeSO_4$ produced an iron-deficient medium with residual iron concentration of 0.1 μM. To reduce the iron concentration further, traces of metal were removed by filtering this nutrient medium through $Mg(OH)_2$ gel and by using phosphoric acid to adjust the pH of the medium, which was made by distilling phosphorus oxy-chloride into water. The distillation was carried out so that HCl produced in the reaction was also distilled from the phosphoric acid solution.

The gel of magnesium hydroxide was prepared by mixing 2.5 g $MgSO_4$ in 20 ml of water with 20 ml of 10% NH_4OH, pH 10.5. About half of the gel was deposited on a Whatman No. 40 ashless filter paper contained in a Buchner funnel. Filter paper covered it, and the rest of the precipitated $Mg(OH)_2$ was added and protected with a third filter paper. This "sandwich" was washed with water and then used. Medium was passed twice through this filter followed the first time by 25 ml H_2O and the second by 50 ml H_2O. After this treatment the iron content of the medium was 3 nM as measured by flameless atomic absorption spectrophometry utilizing a graphite furnace.

To test the sensitivity of the proliferation of *Euglena gracilis* to availability of nutrient iron and the presence of Blm, the inhibition of cell proliferation by Blm in iron-normal and iron-deficient *Euglena gracilis* was assessed after incubation with the drug. In the experiment shown in Fig. 4, *Euglena* were suspended in either iron-sufficient or -deficient medium at 1×10^4 cells/ml in a flat-bottom cell well plate flooded with fluorescent light, and were exposed to drug concentrations ranging from 0.01 to 16 nmol/ml for 96–120 hr at 25°. In the second experiment summarized in Fig. 5, dark adapted cells were incubated in the absence of light in 25-ml culture flasks at an initial concentration of about 2×10^3 cells/ml.

Statistical analysis. Where the statistical significance of results was compared, Student's *t*-test of significance was used for two sample means with unpaired variates.

RESULTS

Antitumor effects of bleomycin in iron-deficient mice. Weanling mice, 3 weeks of age, were placed on the semipurified iron-sufficient and -deficient diets. Liver cytosolic ferritin iron from individual Fe(+) and Fe(−) animals was measured during the course of the experiments. The young mice began the study with little ferritin iron. Between ages 4 and 11 weeks, a sampling of mice showed that in the Fe(+) groups cytosolic ferritin iron increased from 20 to 46 μg/g wet weight of liver, while those in the Fe(−) groups declined from 7 to 1 μg/g. This large difference persisted at 19 weeks (Table 1).

Table 1 demonstrates that the indications of iron deficiency extended beyond liver iron status. Liver weight was affected. Serum iron was also depressed. More importantly for interpretation of the antitumor studies, ascites fluid iron was much lower in iron-deficient mice. This was also reflected in reduced total cytosolic ferritin iron in Ehrlich cells. Thus, both the host and its ascites tumor receiving iron from the Fe(−) diet had less iron according to several measurements.

It was interesting to find in mice that an extended period of iron-deficiency did not lead to reduction in growth or decreased hematocrit or hemoglobin levels (Table 1). Apparently, mice are resistant to these features of severe iron-deficiency, for in another study carried out about the same time, rats given nearly the same Fe(−) diet for 12 weeks grew much slower than the control animals and had severely depressed hematocrit and hemoglobin levels.*

The effects of Blm on tumor growth in Fe(−) animals restored to the complete diet on day 5 are portrayed in Fig. 1a. To be sure that the dose schedule of Blm placed the drug in its effective range, 1, 3, and 6 mg Blm/kg body weight were tested [11]. The tumor grew when treated with the low dose level, but upon intraperitoneal examination no tumor was found in animals treated with 3 and 6 mg/kg drug at the end of the experiment. Similar results were obtained when animals remained on the Fe(−) diet throughout the experiment (Fig. 1b). The results are in accord with data not shown from Fe(+) mice and a larger, previous study in which the lower dose was partially effective and the larger concentrations destroyed the Ehrlich tumor with increasing host toxicity as measured by weight loss [11]. In the present experiment it was noted that the tumor did not cause large weight changes in control animals receiving the iron-depleted diet. This was due to a decrease in ascites fluid (Table 1).

There was little difference in actual tumor cell

* Stemmer K, Petering H G, Lyman S, Krezoski S and Petering D H, manuscripted submitted for publication.

Table 2. Copper levels in mice fed a copper-deficient diet*

	Plasma† (μg/ml)	Ascites fluid† (μg/ml)	Ehrlich cytosol (μg/10^8 cells)
Cu(+)	0.68 ± 0.21‡	0.67 ± 0.10‡	1.0 ± 0.2‡ (4)
Cu(−)	0.29 ± 0.06‡§	0.31 ± 0.04‡§	0.3 ± 0.2‡ (2)
Cu(−) → Cu(+)‖	0.64 ± 0.18§	0.62 ± 0.09§	

* Plasma Cu levels were measured in control mice without tumor; ascites fluid Cu levels were measured in control mice with tumor on day 14 after tumor injection. All groups contained five mice except where indicated by parentheses.

† Averages ± standard deviations are listed. Pairs of means in each column were compared for statistically significant differences. Means being compared are given the same symbol, (‡§) $P < 0.01$.

‖ Cu(−) animals were replaced on the Cu(+) diet on day 6.

Table 3. Effect of 1,10-phenanthroline on the concentration of cytosolic Fe and Zn in Ehrlich cells*

Concentration of 1,10-phenanthroline (nmol/10^5 cells)	Fe† (% of control)	Zn‡ (% of control)
0	100 ± 36§	100 ± 14§
0.05	30 ± 13‖	80 ± 20
0.12	34 ± 7‖	38 ± 1¶
0.25	18 ± 6‖	50 ± 2¶

* Cells were incubated with 1,10-phen for 24 hr followed by chromatographic separation of cytosol over Sephadex G-75.

† Ferritin Fe.

‡ Total cytosolic Zn.

§ Percent of untreated control for 2–3 experiments. Untreated control values: 0.70 ± 0.25 μg Fe/10^8 cells and 3.80 ± 0.50 μg Zn/10^8 cells.

‖ Statistically different from 0 nmol 1,10-phenanthroline, $P < 0.01$.

¶ Statistically different from 0.05 nmol 1,10-phenanthroline, $P < 0.01$.

proliferation between mice fed the complete diet and those on the Fe(−) diet, as shown by results in Table 1. Indeed, in another experiment, cells were passaged three times in Fe(−) mice without noticeable effects on tumor viability. Hence, nutritional iron deficiency had no effect on tumor cell proliferation.

Antitumor effects of Blm in mice fed a copper-deficient diet. Similar experiments were also conducted in 3-month-old mice fed a copper-deficient diet for 12 weeks before tumor injection. This dietary regimen had no effect on tumor growth or the efficacy of Blm against the tumor at a dose of 3 mg/kg animal weight/day for 4 days (Fig. 2). Table 2 summarizes findings about the copper status of animals in this experiment. There is evidence that the animals fed the Cu(−) diet had significantly less Cu than Cu(+) control animals in both plasma and ascites fluid. These levels returned to normal when copper was restored to the diet. Finally, the cytosol of Cu(−) Ehrlich cells had demonstrably less copper than did Cu(+) controls.

Cytotoxic effects of Blm against cells treated with 1,10-phenanthroline. Means have been sought to control the level of transition metals in Ehrlich cells in culture. We found that 1,10-phen substantially reduced cellular levels of iron and zinc. Table 3 shows that when approximately 1×10^6 cells/ml were incubated with various concentrations of 1,10-phen, the cells lost both cytosolic iron and zinc.

Table 4 summarizes the results of several experiments in which cells preincubated with 1,10-phen were treated with Blm in the presence or absence of 1,10-phen. As in the whole animal, there was no reduction in cytotoxicity of Blm relative to normal controls in any of the experiments.

Besides reducing total cellular iron potentially available for the formation of Fe(II)Blm, this ligand minimizes the possibility of formation of this complex in another way. According to Fig. 3, 1,10-phenanthroline stoichiometrically and quantitatively removed iron from Fe(II)Blm in a direct ligand exchange reaction. The reaction was nearly complete within the time of mixing as seen by the formation of the characteristic $Fe(II)phen_3$ spectrum and thus was both thermodynamically and kinetically favorable. It is also known that 1,10-phenanthroline is readily taken up by cells such that 20% of a concentration of 10 nmol/10^5 cells/ml will rapidly associate with cells [12]. In contrast, less than 0.1% of 40 nmol Blm/5×10^5 cells/ml binds to cells over a 1-hr period [14]. It is estimated, therefore, that about 50 times as much 1,10-phen as Blm associated with Ehrlich cells under the conditions of the experiments in Table 4. Should Fe(II)Blm form, intracellular 1,10-phen may also be able to bind this iron through a direct ligand substitution process.

Cytotoxicity of bleomycin towards Euglena gracilis. It was desirable to find a model to test the requirement of Blm for iron in its cytotoxic actions, which permitted simple, direct control of the iron content of the cells. *Euglena gracilis* can be grown in culture on a completely defined medium, which includes $FeSO_4$ as the sole added source of iron. Cells grown in the presence of 36 μM iron contained about 0.22 μg Fe/10^6 cells in their cytosol. Their cytosol contained two large pools of cytosolic iron, separable by Sephadex G-75 chromatography. When such cells were placed in Fe(−) medium (*ca.* 0.1 μM iron), they grew as well as control cells in the complete medium. They continued this rate of growth even after cytosolic iron was exhausted, according to flame atomic absorption analysis. Under these con-

Table 4. Effects of 1,10-phenanthroline on growth inhibition induced by bleomycin*

Pretreatment with 1,10-phen	Treatment		Cell/ml × 10^{-5}†	
	1,10-phen	Blm	(1)	(2)
			4.9 ± 1.1	3.8 ± 0.6
●			4.4 ± 0.6	4.0 ± 0.3
		●	1.9 ± 0.6‡	1.2 ± 0.1‡
	●		4.5 ± 1.1	3.6 ± 0.3
	●	●	2.2 ± 0.7‡	1.2 ± 0.1‡
●	●		4.5 ± 0.7	4.3 ± 0.2
●		●	1.6 ± 0.6‡	1.5 ± 0.1‡
●	●	●	1.4 ± 0.5‡	1.3 ± 0.1‡

* Concentrations used: 10^5 cells/ml, 1.75 nmol 1,10-phen/10^5 cells/ml, and 4 nmol/10^5 cells/ml Blm. Dose response of Ehrlich cell to growth 24-hr treatment with 1,10-phen: at 0 nmol/10^5 cells, 100% of untreated control growth after 24 hr (0, 100), (0.5, 116), (1.2, 105), (2.5, 35), (5.0, −2).

† In (1), pretreatment period was 24 hr; in (2), 48 hr. The treatment period for (1) and (2) was 48 hr, starting with 10^5 cells/ml in each case. Results are presented as average ± standard deviation. (1) The averages of four experiments, each done in triplicate, were averaged. (2) Averages from a single experiment done in triplicate.

‡ Significantly different from the growth of untreated control cells: $P < 0.05$.

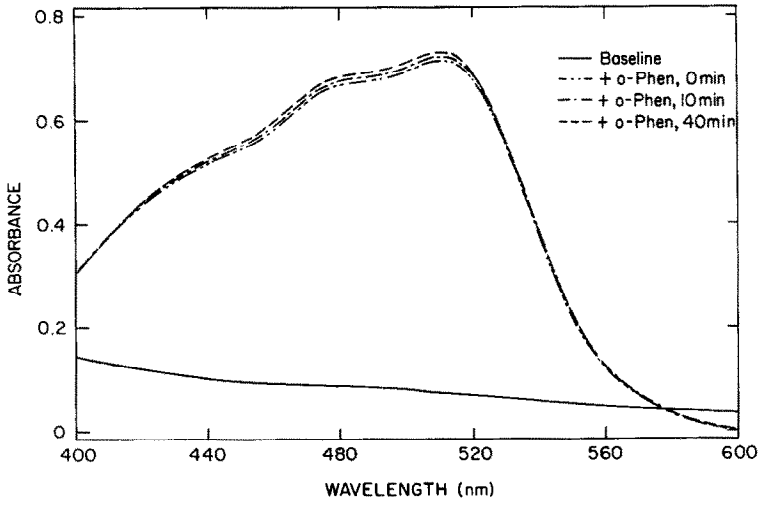

Fig. 3. Reaction of Fe(II)Blm with 1,10-phenanthroline. Fe^{2+} (0.1 mM) and Blm (0.12 mM) were mixed anaerobically with 0.3 mM 1,10-phen in 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesuifonic acid (HEPES) buffer and 0.1 M NaCl at pH 7.3 and room temperature. Spectra were taken before addition of 1,10-phen, immediately after mixing, and over time.

ditions iron deficiency had no effect on the dose response of *Euglena gracilis* proliferation to Blm (Fig. 4). As can be seen in this figure, cells proliferating in the control medium showed a 50% reduction in proliferation in the presence of 0.03 μM Blm. The drug was at least as effective against cells depleted of measurable cytosolic iron as it was against iron-normal control cells. Cell counts included all cells which appeared intact under the microscope. Some were devoid of chlorophyll and may not have been viable. An interesting observation about this experiment is that *Euglena gracilis* is two orders of magnitude more sensitive to Blm than are Ehrlich tumor cells.

Procedures were developed to limit further the iron in the culture medium (Methods), allowing for a more rigorous test of the sensitivity of Blm activity to the iron-status of cells. According to Fig. 5A at 3 nM Fe in the medium, there was nearly no growth of the *Euglena gracilis* population over a 7-day period. Control proliferation rates returned as iron was increased to 50–85 nM. At the lowest concentration, intracellular iron was lowered to less than 5% of that for cells grown in 36 μM Fe.

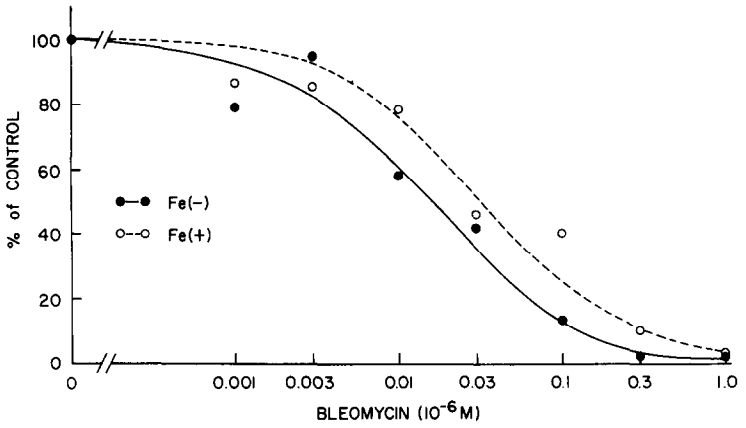

Fig. 4. Effect of Blm on *Euglena gracilis*. *Euglena* were incubated [1×10^4 cells/ml of either Fe(+) (○) or Fe(−) (●)] in their respective medium with 1 nM to 1 μM Blm for 96 hr and counted for cell growth inhibition. The plotted values are averages of results for two duplicate samples with an average range of ± 10%. Fe(−) medium contained 0.1 μM Fe.

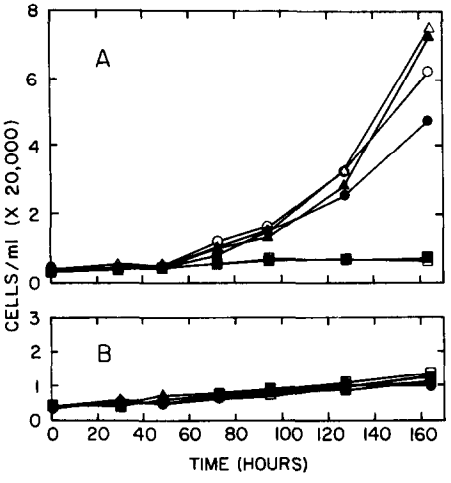

Fig. 5. Dose–response curves for growth of *Euglena gracilis*. Panel A: Cells were incubated initially in 3 nM Fe and then grown in 3 (■), 13 (□), 23 (●), 33 (○), 48 (▲), and 88 (△) nM Fe. Panel B: Same experiment, except that all cultures contained 260 nM Blm.

Cells depleted of iron in the medium containing 3 nM Fe were treated with 260 μM Blm under conditions of various added concentrations of iron as in Fig. 5A. As seen in Fig. 5B, the concentration of iron in the medium was without effect on the growth-inhibiting effect of the drug.

This concentration of 260 nM Blm is nearly 100 times that of Fe^{3+} in the medium. Thus, it is not possible to form extracellular stoichiometric amounts of FeBlm. Moreover, the medium has a pH of 3.6 at which Fe(III)Blm is partially dissociated in solution [15]. In the normal growth medium, formation of Fe(III)Blm cannot be detected spectrophotometrically at pH 3.6, but is observed when the pH is adjusted to 7.0. It is evident that, if significant concentrations of FeBlm are to form, they must do so within the cells and that this must occur even when the cells are stressed for iron to the point that their growth rate is reduced.

DISCUSSION

Since the discovery of Blm and its antitumor properties two decades ago, there have been indications that metals are involved in its mechanism of cytotoxic action. Thus, Blm was isolated from *Streptomyces verticillus* as a complex mixture containing up to 10% CuBlm [16]. The early tests of its antitumor activity utilized CuBlm [17]. Later, after it had been shown that exposure of cells to Blm leads to strand breakage of its DNA, studies of the interaction of Blm with isolated DNA revealed that Fe^{2+} enhances strand cleavage and that Cu^{2+}, Zn^{2+}, and Co^{2+} inhibit the reaction [1–3]. It is now evident that other metals in conjunction with Blm may cleave DNA under special conditions [4–6]. Nevertheless, it has generally been assumed that FeBlm is the active metallodrug. A set of elegant studies has eludicated how Fe(II)Blm activates oxygen to attack the backbone of DNA [18–20]. However, it has not been demonstrated that FeBlm can form in cells from the chelation of cellular iron by the drug to carry out strand cleavage or that such chemistry is responsible for the biological effects of the drug.

Previous studies have tested the requirement of biologically essential metals for the activation of other antitumor or cytotoxic agents. First, it was shown that 3-ethoxy-2-oxobutyraldehyde-bis(thiosemicarbazone) is only active in copper-sufficient rats [8]. Copper deficiency completely inhibits its activity. Indeed, the copper complex is substantially more active in copper-normal animals than the metal-free drug, presumably because it already has a full complement of metal, whereas the ligand finds relatively little copper in the organism available for chelation [21]. Furthermore, among a whole series of metal ions, only Cu^{2+} activates this bis(thiosemicarbazone) to be cytotoxic against tumor cells in cell suspensions maintained for short times in a

metal-depleted medium [22]. In another study, using cells held in a metal-deficient medium during their exposure to α-*N*-heterocyclic carboxaldehyde thiosemicarbazones and their copper and iron complexes, the metal-free ligands were essentially inactive in comparison with their copper and iron complexes [9].

Recently, the antitumor and cytotoxic properties of Blm and its cobalt, iron, copper, and zinc complexes were assessed in animals fed a normal stock diet and in culture in which drugs were incubated with cells in complete or metal-deficient medium, washed free of unbound drug, and then placed in the complete medium [11]. In contrast to the other examples cited above, Fe(III)Blm, the immediate precursor of the presumed active form Fe(II)Blm, is less active in animals than Blm, CuBlm, and ZnBlm at the same dose level. In culture Blm is approximately as active as its iron, copper and zinc complexes even when cells and drugs are incubated in the metal-restricted medium. Such experimental conditions assure that there will be little metal complex formation or metal exchange between complexes in the external medium. Although these experiments did not determine which species of Blm exist intracellularly, it could be argued that, if metal exchange reactions occurred, they take place principally within the cell. For example, Blm, which is accumulated by cells, might chelate cellular iron to form an active species, FeBlm, or CuBlm might exchange its metal for iron.

In the present investigation, the growth inhibitory effects of Blm were examined under conditions which limit both the external and internal concentrations of iron available to the drug. In each case the agent was still completely active. In particular, in the animal study, the drug was effective when injected intraperitoneally among tumor cells, already grown in iron-deficient mice to deplete them of mobilizable iron (Fig. 1). Table 1 shows that the animals were deficient in iron stored in liver and in circulating iron in plasma. Furthermore, the tumor cells maintained in iron-deficient mice had a clearly reduced iron content as did the ascites fluid, which is the direct nutrient source for the cells.

Second, Blm remained active against cells in culture that had been pretreated with 1,10-phen, which substantially reduced cellular iron, as well as the zinc content of the cell pellet (Tables 3 and 4). Further, it was active in such cells even when, 1,10-phen remained in the suspension with Blm. This bidentate ligand is readily taken up by Ehrlich cells, while little Blm enters cells [12, 14]. Thus, the ligand exchange reaction (1) is favored both by

$$3\ \text{phen} + \text{Fe(II)Blm} \rightleftarrows \text{Fe(II)(phen)}_3 + \text{Blm} \quad (1)$$

the large excess of 1,10-phen relative to Blm in cells (calculated as about 50-fold), as well as by the rapid kinetics of the reaction (Fig. 3). The ligand, 1,10-phenanthroline, has large thermodynamic affinity for Fe^{2+} (log apparent stability constant, pH 7, log K = 21.13), Cu^{2+} (log K = 17), and Zn (log K = 17.1), forming *tris* complexes with each metal [23]. Thus, when 1,10-phen interacts with Ehrlich cells, it might perturb the distribution of iron, copper and zinc. Indeed, experiments show that cytosolic iron and zinc were diminished in these cells (Table 3). As such, hypothetical effects of 1,10-phen on Blm activity cannot be specifically ascribed to its interaction with iron. Nevertheless, because it reduces cellular zinc and iron, one infers that it competes with Blm for these metals.

In a recent publication, Takahashi *et al.* [24] also examined the effects of 1,10-phenanthroline and 2,2′-dipyridyl, both Fe^{2+} chelating agents, on the inhibition of DNA synthesis and cell growth caused by Blm and FeBlm. Inspection of their results reveals that there is relatively little capacity of 1,10-phenanthroline to prevent the inhibition of HeLa S_3 cell growth by these compounds: at 2.5 μM 1,10-phen rate of growth increases from 47 to 70% of control for 0.5 μM Blm; 23 to 30% for 1 μM Blm; and 2 to 10% for 2 μM Blm.

Lin and coworkers [25] also examined the effects of chelating agents on the cytotoxicity of Blm. Desferioximine, diethylenetriamine, and pencillamine were without significant effect. The first of these is a particularly effective multidentate ligand for Fe^{3+} which is used in humans to treat iron overload [26]. In this study as well, chelating agents which can compete with Blm for transition metals do not alter drug activities.

The third model investigated in the present study is distinctly different. It uses *Euglena gracilis*, a photosynthetic microorganism, as a system in which to test the requirement of iron for the activity of Blm. Having been isolated from a microbe, Blm may well function naturally as an antibiotic. For example, it has been shown to be cytotoxic to *Escherichia coli* [27].

According to Fig. 4, control cultures of *Euglena gracilis* were very sensitive to Blm. Even when the iron concentration in the acidic medium was reduced from 36 to 0.1 μM and intracellular storage pools of iron were depleted, there was no discernible effect of iron on the dose–response curve for inhibition of growth of the cells by Blm.

Under these conditions of iron-deficiency, *Euglena gracilis* continued to grow at normal rates. Thus, despite the loss of intracellular storage forms of iron, enough iron can be acquired by the cells to satisfy their needs for proliferation.

By suitably treating the growth medium as described in Methods, one can lower the iron concentration to the point that it severely limits cell growth (3 nM Fe), as seen in Fig. 5A. As the iron concentration was raised to 85 nM, control proliferation rates were restored. Over this range of iron concentrations, Blm was active and apparently unaffected by the level of iron in the culture system.

The accumulation of results indicates that nutrient or ligand-imposed iron-deficiency attained in three models has no effect on the growth inhibitory activity of bleomycin. From this it is suggested that host iron-insufficiency or -deficiency will probably not affect the antitumor activity of this drug in humans.

Finally, an experiment was conducted on the effects of a copper-deficient diet on the antitumor properties of Blm (Fig. 2). None was observed even though clear evidence of deficits in host plasma and ascites fluid and cytosolic copper was obtained (Table 2). Thus, even though Blm was isolated from its

microbiological source containing copper and might be inferred to have activity as the copper complex, there is no indication that copper status is a critical factor in the efficacy of the drug [15, 16].

The results provide a useful baseline for future studies inquiring into the role of metals in the antitumor activity of bleomycin. In the past, clear distinctions in biological efficacy of thiosemicarbazones and their copper, iron, and in some cases zinc complexes have been drawn in cellular models or animal studies, which compared the activity of these agents under normal and metal-deficient conditions [21, 22]. In contrast, iron, copper and zinc complexes of bleomycins are not more active than metal-free Blm in animals or cell culture [11, 14]. Nor have the present experiments been able to demonstrate a sensitivity of the cell proliferative effects of Blm to changes in the iron or copper content of target organisms or cells.

The present experiments do not prove or disprove that iron or copper is required for the mechanism of action of bleomycin. No matter how little metal remains in the system, one can always argue that it is sufficient to activate the drug. Nevertheless, they do cast a new light on the question. For example, if one believes that iron is needed, then identification of the mechanism of cellular iron acquisition by Blm becomes an important problem because the obvious sources of stored iron can be substantially depleted at least in *Euglena gracilis* without losing drug activity. According to this view, these results indicate that Blm is a remarkably good cellular chelating agent for iron, and suggest that experiments be designed to elucidate this characteristic of the structure.

The observations about tumor growth in control populations of mice provide new information about the effect of metal deficiencies on tumor growth. Previous reports demonstrated the profound, rapid effect that host zinc-deficiency has on Ehrlich cell proliferation [28, 29]. Similar results have been seen in other tumor systems [30, 31]. In contrast, prolonged feeding of an iron-deficient diet to mice had little effect on the growth of the Ehrlich tumor, despite the evidence that the tumor cells, themselves, had reduced concentrations of iron (Table 1). There have been scattered reports in the literature that cell proliferation in culture is inhibited by agents, such as α-picolinic acid and desferrioxamine, thought to act as iron-chelating agents [32, 33]. It is also known that the iron-transport protein, transferrin, is a ubiquitous growth factor for mammalian cells grown in defined culture. However, the present results indicate that in the whole animal even extended iron-deficient conditions imposed on young growing mice are not sufficient to affect Ehrlich tumor growth.

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REFERENCES

1. Nagai K, Suzuki H, Tanaka N and Umezawa H, Decrease of melting temperature and single strand scission of DNA by bleomycin in the presence of hydrogen peroxide. *J Antibiot (Tokyo)* **22**: 624–628, 1969.
2. Sausville EA, Peisach J and Horwitz SB, Effects of chelating agents and metal ions on the degradation of DNA by bleomycin. *Biochemistry* **17**: 2740–2745, 1978.
3. Sausville EA, Stein RW, Peisach J and Horwitz SB, Properties and products of degradation of DNA by bleomycin and iron(II). *Biochemistry* **17**: 2746–2754, 1978.
4. Ehrenfeld GM, Shipley JB, Heimbrook DC, Suziyama H, Long EC, VanBoom JJ, Van der Marel GA, Oppenheimer NJ and Hecht SM, Copper dependent cleavage of DNA by bleomycin. *Biochemistry* **26**: 931–942, 1987.
5. Chang C-H and Meares CF, Light-induced nicking of deoxyribonucleic acid by cobalt(III) bleomycins. *Biochemistry* **21**: 6332–6334, 1982.
6. Suzuki T, Kuwahara J, Goto M and Suguira Y, Nucleotide sequence cleavages of manganese-bleomycin induced by reductant, hydrogen peroxide and ultraviolet light. Comparison with iron- and cobalt-bleomycins. *Biochim Biophys Acta* **824**: 330–335, 1985.
7. Moldawer LL, Marino MA, Wei H, Fong Y, Silen ML, Kuo G, Manogue KR, Vlassara H, Cohen H, Cerami A and Lowry SF, Cachectin/tumor necrosis factor-α alters red blood cell kinetics and induces anemia *in vivo*. *FASEB J* **3**: 1637–1643, 1989.
8. Petering HG, Buskirk HH and Crim JA, The effect of dietary mineral supplements of the rat on the antitumor activity of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone). *Cancer Res* **27**: 1115–1121, 1967.
9. Saryan LA, Ankel E, Krishnamurti C, Petering DH and Elford H, Comparative cytotoxic and biochemical effects of ligands and metal complexes of α-*N*-heterocyclic carboxaldehyde thiosemicarbazones. *J Med Chem* **22**: 1218–1221, 1979.
10. Stemmer KL, Petering HG, Murthy L, Finelli VN and Menden EE, Copper deficiency effects on cardiovascular system and lipid metabolism in the rat; the role of dietary proteins and excessive zinc. *Ann Nutr Metab* **29**: 332–347, 1985.
11. Rao EA, Saryan LA, Antholine WE and Petering DH, Cytotoxic and antitumor properties of bleomycin and several of its metal complexes. *J Med Chem* **23**: 1310–1318, 1980.
12. Krishnamurti C, Saryan LA and Petering DH, Effects of ethylenediaminetetraacetic acid and 1,10-phenanthroline on cell proliferation and DNA synthesis in Ehrlich ascites cells. *Cancer Res* **40**: 4092–4099, 1980.
13. Price CA and Vallee BL, *Euglena gracilis*, a test organism for study of zinc. *Plant Physiol (Lancaster)* **37**: 428–433, 1962.
14. Lyman S, Ujjani B, Renner K, Antholine W, Petering DH, Whetstone JW and Knight JM, Properties of the initial reaction of bleomycin and several of its metal complexes with Ehrlich cells. *Cancer Res* **46**: 4472–4478, 1986.
15. Antholine WE and Petering DH, Reaction of FeBlm with DNA: Fe(II)Blm-NO. *Biochem Biophys Res Commun* **91**: 528–533, 1979.
16. Umezawa H, Maeda K, Takeuchi T and Okami Y, New antibiotics, bleomycin A and B. *J Antibiot (Ser A)* **19**: 200–209, 1966.
17. Ishizuka M, Takayama H, Takeuchi T and Umezawa U, Activity and toxicity of bleomycin. *J Antiobiot (Ser A)* **20**: 15–24, 1967.
18. Burger RM, Peisach J, Blumberg WE and Horwitz SB, Iron–bleomycin interaction with oxygen and oxygen analogues. *J Biol Chem* **254**: 10906–10912, 1979.
19. Burger RM, Peisach J and Horwitz SB, Activated bleomycin: a transient complex of drug, iron, and oxygen that degrades DNA. *J Biol Chem* **256**: 11636–11644, 1981.

20. Burger RM, Peisach J and Horwitz SB, Effects of O_2 on the reactions of activated bleomycin. *J Biol Chem* **257**: 3372–3375, 1982.
21. Crim JA and Petering HG, The antitumor activity of Cu(II)KTS, the copper(II) chelate of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone). *Cancer Res* **27**: 1278–1285, 1967.
22. Van Giessen GJ, Crim JA, Petering DH and Petering HG, Effect of heavy metals on the *in vitro* cytotoxicity of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone) and related compounds. *J Natl Cancer Inst* **51**: 139–146, 1973.
23. Sillen LG and Martell AE, *Stability constants of metal–ion complexes*, pp. 664–665. The Chemical Society, Burlington House, London 1964.
24. Takahashi K, Takita T and Umezawa H, Effects of *o*-phenanthroline, 2,2′-dipyridyl, and neocuproine on the activities of bleomycin to inhibit DNA synthesis and growth of cultural cells. *J Antibiot (Tokyo)* **39**: 1473–1479, 1986.
25. Lin P-S, Kwock L, Hefter K and Misslbeck G, Effects of iron, copper, cobalt, and their chelators on the cytotoxicity of bleomycin. *Cancer Res* **43**: 1049–1053, 1983.
26. Anderson WF and Heller MC, *Development of Iron Chelators for Clinical Use*. DHEW Publication No. (NIH) 76-994. DHEW, 1975.
27. Cohen SL and I J, Synthesis and the lethality of bleomycin in bacteria. *Cancer Res* **36**: 2768–2774, 1976.
28. Petering DH and Saryan LA, Control of Ehrlich cell division by zinc. *Biol Trace Elements Res* **1**: 87–100, 1979.
29. Kraker A and Petering DH. Tumor-host zinc metabolism: the central role of metallothionein. *Biol Trace Element Res* **5**: 363–374, 1983.
30. Fenton MR, Burke JP, Tursi FD and Arena FP, Effect of a zinc deficient diet on the growth of an IgM secreting plasmacytoma (TEPC-183). *J Natl Cancer Inst* **65**: 1271–1272, 1980.
31. DeWys W and Pories W, Inhibition of a spectrum of animal tumors by dietary zinc deficiency. *J Natl Cancer Inst* **48**: 375–381, 1972.
32. Fernandez-Pol JA, Siderophore-like growth factor synthesis by SV-40-transformed cells adapted to picolinic acid stimulates DNA synthesis in cultured cells. *FEBS Lett* **78**: 345–348, 1977.
33. Basset P, Zwiller J, Revel MO and Vincendon G, Growth promotion of transformed cells by iron in serum-free culture. *Carcinogenesis* **6**: 355–359, 1985.