Method D. 5-Chloro-3-[2-(4-piperidinyl)ethyl]indole (4). A mixture of 36 g (0.14 mol) of **26** and 1.8 g of PtO_2 in 360 mL of AcOH was hydrogenated at room temperature and atmospheric pressure until TLC control [MeOH–Me_2CO–Et_2NH, 45:50:5, silica gel GF] indicated that **26** had completely reacted. The catalyst was filtered off and washed with AcOH. The combined filtrate and washings were evaporated to a residue, which was dissolved in 300 mL of H_2O. The solution was alkalinized at pH 9 and the resultant precipitate was extracted with CH_2Cl_2. The organic extract was dried ($MgSO_4$) and evaporated to give 31.3 g of a brown crystalline solid. Recrystallization from Me_2CO gave 22.5 g (60%), mp 160 °C.

Method E. 1-Methyl-3-[2-(4-*N*-methylpiperidinyl)-ethyl]indole (14). To 12.1 g (0.05 mol) of 1-methyl-3-[2-(4-piperidinyl)ethyl]indole (**2**) in 25 mL of toluene was added 6.1 mL (0.075 mol) of ethyl formate in 20 mL of toluene. After 4 h at 75 °C, the mixture was evaporated to dryness. The oily residue was added dropwise under N_2 to 2.85 g (0.075 mol) of $LiAlH_4$ in 100 mL of anhydrous Et_2O. After stirring and refluxing for 3 h, the mixture was cooled to 0 °C and decomposed with 3.4 mL of H_2O, 2.5 mL of 5 N NaOH, and 11 mL of H_2O. The solid material was filtered off and washed thoroughly with Et_2O, and the filtrates were concentrated to dryness to give an oil, which was turned into its hydrochloride and crystallized from 80 mL of CH_3CN: yield 10.35 g (71%); mp 100 °C.

Biological Methods. Uptake of NA, DA, and 5-HT into Rat Brain Synaptosomes. DL-[*methylene*-^{14}C]Noradrenaline bitartrate (53 mCi/mmol), [1-*ethylamine*-^{14}C]dopamine hydrochloride (52 mCi/mmol), 5-hydroxy[side chain 2-^{14}C]tryptamine creatinine sulfate (58 mCi/mmol) were purchased from the Radiochemical Centre, Amersham. Experiments were carried out according to a previously published method[21] using immature female rats (19–21 days). Brain synaptosome preparations were incubated for 5 min at 37 °C with the labelled biogenic amines at a concentation of 10^{-7} M. Four to six concentrations of the drugs were used in duplicate to determine the IC_{50} values (concentrations of drug that inhibit the uptake by 50%).

Uptake of 5-HT into Human Blood Platelets. This was studied by the method described elsewere.[18] Platelet-rich plasma (PRP) was prepared and aliquots were incubated at 37 °C. The drugs and ^{14}C-labeled 5-HT (10^{-7} M) were added after 2 min, and incubation was continued for an additional 1 min. After cooling in an ice bath, the samples were centrifuged. Radioactivity was counted on PRP and supernatant, and the uptake of ^{14}C-labeled 5-HT was calculated by the formula of Buczko et al.[37]

Potentiation of 5-HTP-Induced Tremor in Mice. Groups of eight mice were used. The animals were injected intraperitoneally with a 100 mg/kg dose of L-5-HTP (in 0.9% NaCl solution). The test substances were administered subcutaneously 30 min after 5-HTP injection.

Tremor was evaluated at 75 min in each animal using the following rating scale: 0 = no tremor; 1 = moderate tremor; 2 = severe tremor. The ED_{50} (doses that produced 50% of the maximum effect, i.e., a mean score of 1) were determined from the global scores obtained for each group.

Antagonism of Tetrabenazine-Induced Ptosis in Rats. The test substances were administered orally to rats 0.5 h before tetrabenazine (10 mg/kg, sc). Assessment was made 1 h later, based on the absence or presence of ptosis. ED_{50} values were the doses that prevented ptosis in 50% of animals.

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Cytotoxic and Antitumor Properties of Bleomycin and Several of Its Metal Complexes[1]

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This study examines the concentration-dependent cytotoxicity and antitumor activity of bleomycin (Blm) and Cu-, Zn-, Fe(III)-, and CoBlm using Ehrlich cells in culture and the Ehrlich ascites tumor. The order of activity in culture under several conditions is CuBlm ≈ Blm ≈ ZnBlm > Fe(III)Blm ≫ CoBlm ≈ control. Short exposures of cells to drugs in the presence or absence of serum produced effects on cell proliferation similar to 48-h incubations. With Blm and CuBlm there was no obvious relationship between cytotoxicity and the modest short-term inhibition of DNA synthesis by the drugs. The antitumor experiments produced qualitatively similar results with the order of antitumor potency being CuBlm > Blm > ZnBlm ≈ FeBlm ≫ CoBlm ≈ control tumor. The host toxicity produced by these drugs as measured by weight loss had the opposite ordering: CoBlm ≪ FeBlm ≪ ZnBlm < Blm < CuBlm. At therapeutically effective concentrations, FeBlm was significantly less toxic relative to the other active agents.

The antitumor glycopeptide bleomycin (Blm) is isolated from *Streptomyces verticillus* as a 1:1 copper complex.[2a] The early studies by Umezawa and co-workers demonstrating the antitumor properties of the antibiotic utilized this metal complex.[2b] Later, the metal-free bleomycin was shown to be equally active and to be less toxic to the host.[3] For a number of years thereafter, the interest in metal ions and bleomycin was confined to the demonstration that Cu^{2+}, Zn^{2+}, and Co^{2+} can inhibit the DNA-strand scission reaction carried out by bleomycin in the presence of thiols.[4] The resurgence of the study of metal bleomycins followed the demonstration of Horwitz and co-workers that the addition of Fe^{2+} to the strand-scission assay in the presence or absence of thiols led to a remarkable enhancement of cleavage of the DNA backbone.[5,6] Although biochemical

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(2) (a) H. Umezawa, K. Maeda, T. Takeuchi, and Y. Okami, *J. Antibiot. Ser. A.*, **19**, 200 (1966). (b) M. Ishizuka, H. Takayama, T. Takeuchi, and H. Umezawa, *ibid.*, **20**, 15 (1967).
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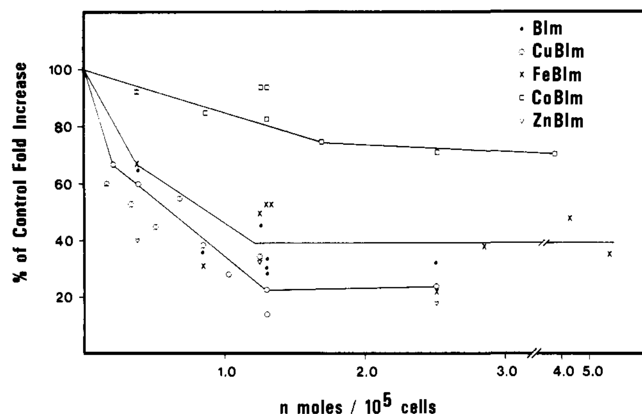

Figure 1. Cytotoxicity of Blm and its metal complexes following 60-min incubation of Ehrlich cells and drugs in the absence of serum. Cell proliferation was measured after 48 h.

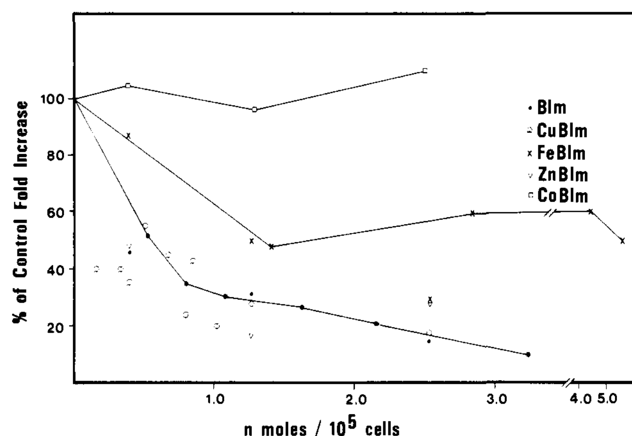

Figure 2. Cytotoxicity of Blm and its metal complexes following 60-min incubation of Ehrlich cells and drugs in the absence of serum. Cell proliferation was measured after 48 h in the presence of serum.

and structural studies on a number of metal bleomycins are now being vigorously pursued, the work has not been grounded in an understanding of the pharmacological properties of these molecules. Thus, this report provides the results of a broadly based inquiry into the cytotoxic properties of metal-free bleomycin in comparison with Cu-, Zn-, Fe-, and CoBlm.[7]

Results

Cell Culture Studies. Figures 1 and 2 present the effect of different concentrations of metal-free bleomycin and its Cu(II), Fe(III), Zn(II), and Co(II) complexes upon Ehrlich cells in culture. The cells were exposed to the drugs in the presence as well as the absence of serum for 60 min, then washed free of drug, and incubated for 48 h in the culture medium. The use of the serum-free incubation medium permitted the exposure of cells to drugs in the absence of competing metals from serum. After these treatments, the extent of cell proliferation was examined. With the exception of CoBlm, all of the compounds tested, including free bleomycin, inhibited cell proliferation. The magnitude of the inhibition was not influenced by the presence or absence of serum during the preincubation period.

(6) E. A. Sausville, R. W. Stein, J. Peisach, and S. B. Horwitz, *Biochemistry*, 17, 2746 (1978).
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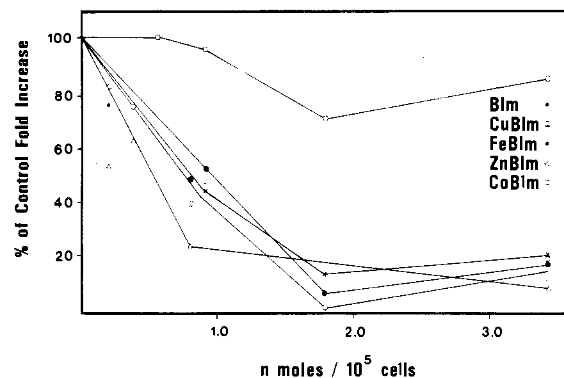

Figure 3. Cytotoxicity of Blm and its metal complexes following continuous exposure of cells and drugs for 48 h.

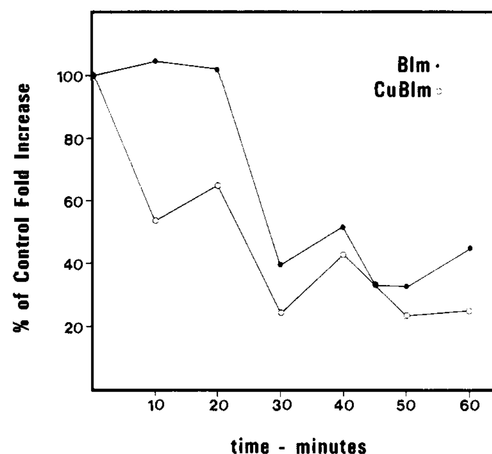

Figure 4. Time course of cytotoxic response of Ehrlich cells to drugs. Ehrlich cells were incubated with 0.8 nm/10^5 cells for varying times in MEM plus 5% fetal calf serum. Cells were then washed and placed in culture. Cell proliferation was measured 48 h later after growth in Eagles MEM and 5% fetal calf serum at 37 °C.

In all cases, the concentration dependence of inhibition is biphasic with a break point near 1 nmol of drug/10^5 cells. At this concentration, Blm, CuBlm, and ZnBlm depressed cell division by 70–80% relative to control. FeBlm was noticeably less effective. Just as the initial 60-min incubation of drugs with Ehrlich cells led to no decrease in cell permeability to Trypan blue, the concentrations of drugs up to 1–1.5 nmol/10^5 cells did not decrease the permeability of cells present after 48 h. However, higher drug to cell ratios produced cell kill. Generally, CuBlm was more effective in this activity than Blm. Interestingly, at concentrations as large as 4–5 nmol of FeBlm/10^5 cells, dead or permeable cells were never detected.

Figure 3 shows that even shorter exposures of cells to CuBlm or Blm are sufficient to exert the significant, prolonged inhibition of growth demonstrated in Figures 1 and 2. Continuous exposure of cells to drugs for 48 h, as summarized in Figure 4, was no more effective than that observed in the short-term experiments (Figures 1–3). Only FeBlm appears to be more cytotoxic under these conditions. The biphasic form of the concentration dependence shown in Figure 4 is similar to those described in Figures 1 and 2.

Effect of Drugs on DNA Synthesis. Table I presents data on the concentration-dependent effects of free bleomycin and copper bleomycin on uptake of radiolabeled thymidine into Ehrlich cells and its incorporation into DNA. Although there is some effect of Blm and CuBlm on both processes relative to control, there is little concentration dependence to the effects over a 20-fold range

Table I. Concentration Dependence of Inhibition of DNA Synthesis by Bleomycin and Copper Bleomycin in Vitro[a]

nm of drug/mg of cell protein	DNA synthesis, mean % of control ± SD		thymidine uptake, mean % control ± SD	
	blenoxane	Cu-blenoxane	blenoxane	Cu-blenoxane
4[b]	67.9 ± 2.5	86.8 ± 3.2	86.0 ± 2.2	94.9 ± 5.6
10	66.1 ± 5.3	61.5 ± 4.3	79.1 ± 1.9	83.5 ± 5.5
40	56.4 ± 2.8	55.3 ± 4.6	69.8 ± 2.6	71.2 ± 4.5
80	40.1 ± 0.9	51.3 ± 5.5	61.9 ± 6.3	68.4 ± 5.2

[a] Cells removed from a donor animal were suspended in fresh MEM and incubated at 37 °C with drug and then exposed to [^{3}H]thymidine (final thymidine concentration approximates 8.6 × 10^{-8} M) for 20 min. Data are presented as percent plus or minus standard deviation for three trials with respect to control values. The absolute incorporation of [^{3}H]dThd into control cells is 17 pmol/5 × 10^6 cells. [b] From Figure 5 using cells from culture.

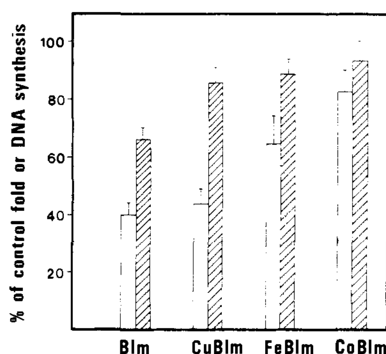

Figure 5. Comparison of short-term incubation of DNA synthesis with cytotoxicity of drugs. Ehrlich cells were incubated for 60 min with 1 nm of drug/10^5 cells (4 nmol/mg of cell protein) in the presence of serum: (▨) incorporation of [^{3}H]dThd into DNA measured at end of 60 min; (□) relative proliferation at end of 48-h incubation of cells in drug-free cell culture media.

in drug concentration. This result differs from observations made with other ligands and metal complexes which inhibit thymidine metabolism and do so with a sharp concentration dependence.[8,9]

A direct comparison of the effects of bleomycin and several of its metal complexes on the metabolism of dThd related to DNA synthesis and the proliferative capacity of Ehrlich cells was carried out using 1 nmol of drug/10^5 cells. As seen in Figure 5, proliferation was inhibited about 60% by Blm and CuBlm, about 35% by FeBlm, and less than 15% by CoBlm. However, only Blm significantly depressed the incorporation of dThd into DNA. CoBlm inhibited neither DNA synthesis nor cell proliferation. Thus, under directly comparable conditions, there is no significant correlation between cytotoxicity and the mild, short-term inhibition of DNA synthesis.

Antitumor Studies. The antitumor activity of all compounds was tested in mice by administering the tumor cells on day 0 and following this with six injections of drugs at 24-h intervals, beginning on day 1. The results are presented in Figures 6–10.

Blm given at the rate of 1 (mg/kg)/day for 6 days has no antitumor effect. All the animals developed tumors at the same rate as the controls. The survival profiles were

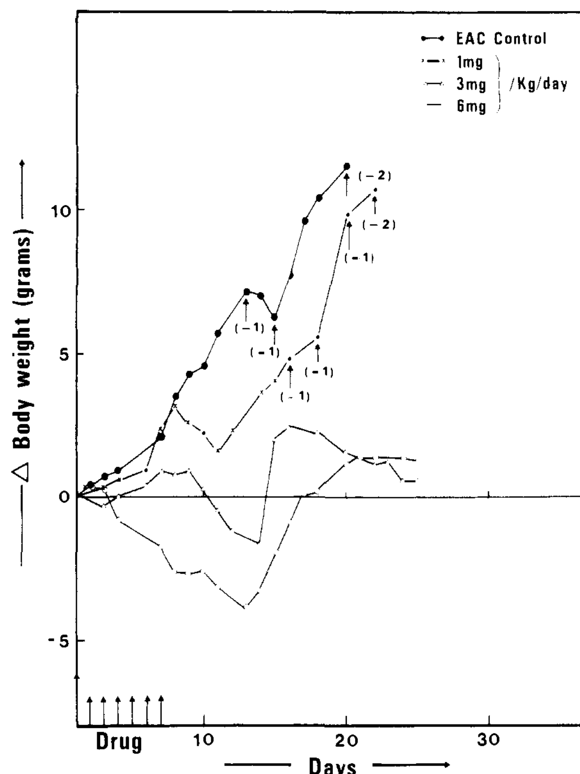

Figure 6. Antitumor activity of Blm. Drug was given on days 1–6. Deaths are noted in parentheses. All five animals survived with a 3 and 6 (mg/kg)/day dose and none with a 1 (mg/kg)/day dose. The illustrative untreated control tumor was for the 6 mg/kg group.

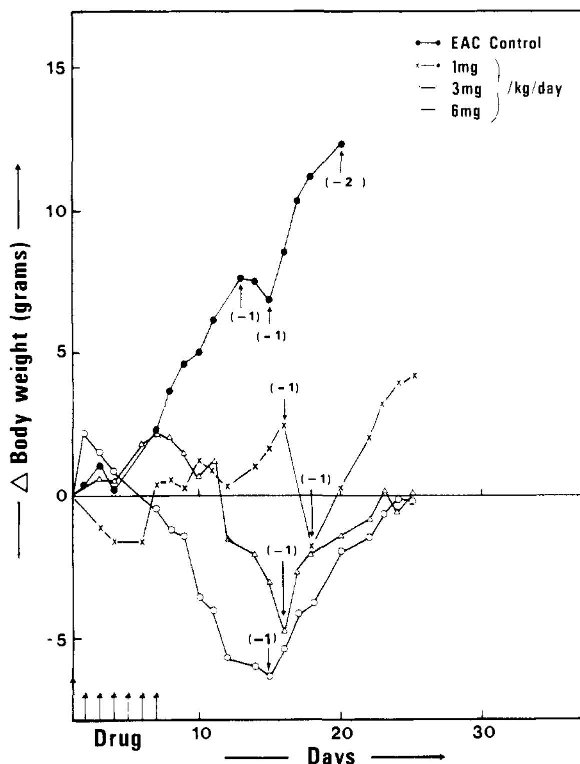

Figure 7. Antitumor activity of CuBlm. Drug was given on days 1–6. Deaths are noted in parentheses. 4/5 animals survived with a 6 (mg/kg)/day dose, 4/5 with a 3 (mg/kg)/day dose (the animals which died had no evidence of tumor), and 3/5 with a 1 (mg/kg)/day dose. The illustrative untreated control tumor was for the 6 mg/kg group.

similar to those that were not treated with any drug. In contrast, at a dose (6 mg/kg)/day for 6 days the animals

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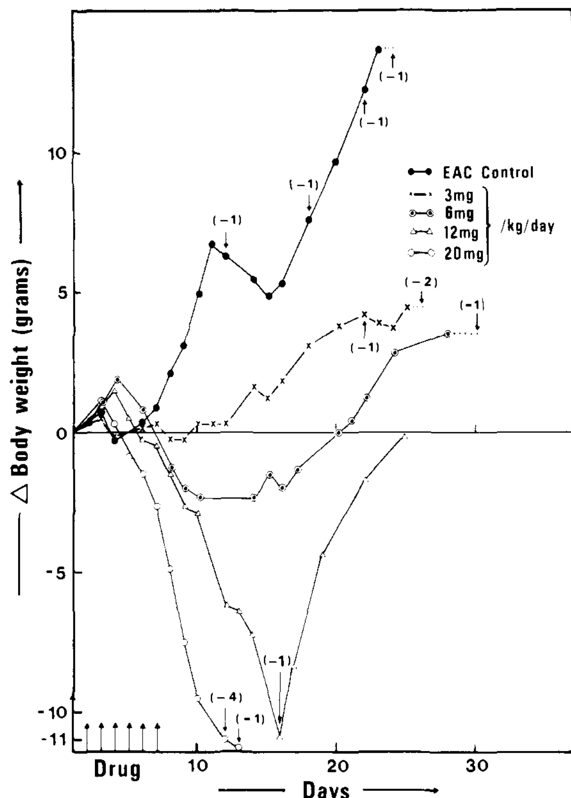

Figure 8. Antitumor activity of ZnBlm. Drug was given on days 1–6. Deaths are noted in parentheses. All animals survived with a 12 (mg/kg)/day dose, 2/5 with a 3 (mg/kg)/day dose, and none with a 20 (mg/kg)/day which was severely toxic. The illustrative untreated control tumor was for the 3 mg/kg group.

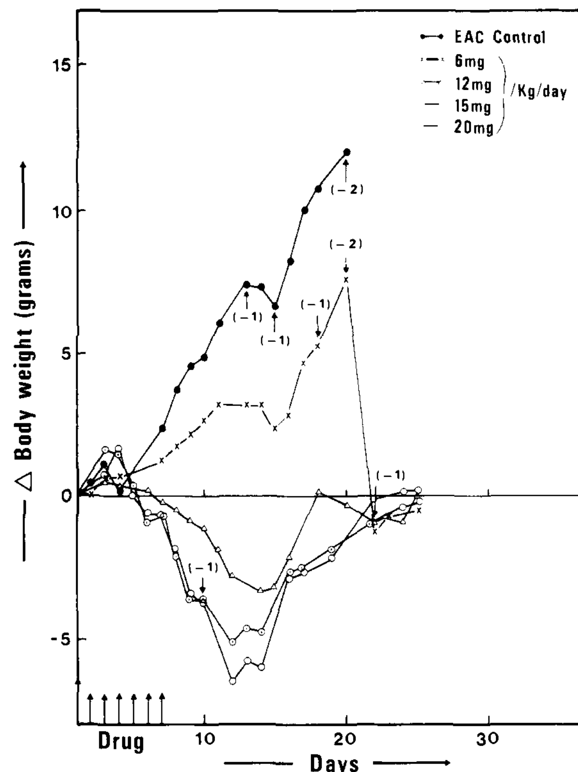

Figure 9. Antitumor activity of Fe(III)Blm. Drug was given on days 1–6. Deaths are noted in parentheses. All animals survived with 15 and 20 (mg/kg)/day doses, 4/5 with a 12 (mg/kg)/day dose, and 2/5 with a 6 (mg/kg)/day dose. The illustrative control tumor was for the 6 mg/kg group.

showed severe toxic symptoms, including loss of body weight, lethargy, diarrhea, and roughening of toe pads. The loss of body weight was noticed on day 2 of drug administration. This continued the next week, reaching a maximum by day 13 well after the completion of Blm injections (Figure 6). Between days 16 and 17 the animals showed quick recovery to normal body weight. All these animals survived for 50 days without any apparent tumor development. The most effective dose schedule tested was 3 (mg/kg)/day for 6 days. Animals in this group showed a 100% survival. The loss of body weight was far less compared to the 6-mg dose and was not accompanied by other toxic symptoms.

CuBlm exerted essentially similar kinds of effects in this model, except the toxicity was much higher at each level of drug tested (Figure 7). For example, one out of five animals died in the group given the 6 mg/kg dose. This was probably due to toxicity, for its body weight had shrunk from 26.5 to 16.5 g. All the other toxic symptoms were common to both Blm and CuBlm. Similarly, in the group receiving the 3 mg/kg dose, there was also a single mortality. CuBlm and Blm could be distinguished in mice given the doses 1 (mg/kg)/day for 6 days. Unlike Blm in which this level of drug had no effect on the progress of the tumor, CuBlm was still moderately effective, leaving two survivors with no evidence of the ascites tumor after 50 days.

Both FeBlm and ZnBlm also have strong antitumor effects as measured in this Ehrlich model. The lowest concentration tested for ZnBlm (Figure 8) was 3 (mg/kg)/day for 6 days. Two out of five animals survived at this level. Four out of five animals survived in the groups treated with 6 and 12 (mg/kg)/day for 6 days. The latter group exhibited significant toxicity symptoms for the first

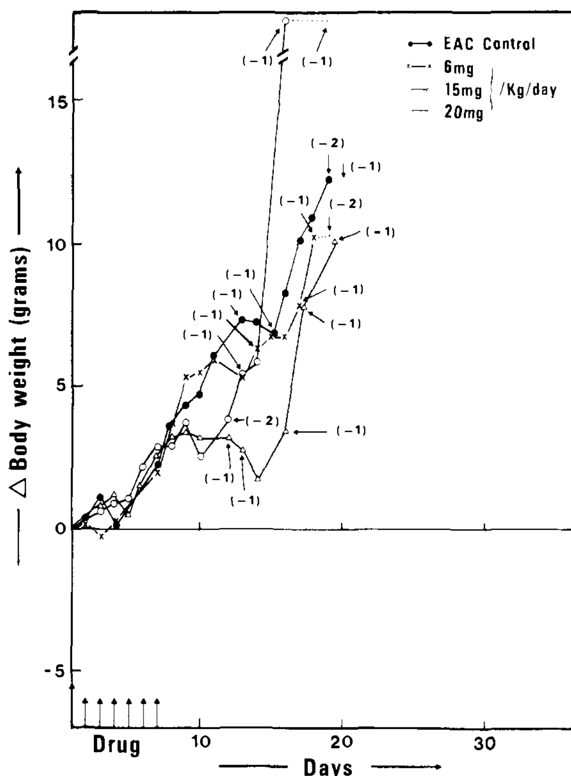

Figure 10. Antitumor activity of CoBlm. Drug was given on days 1–6. Deaths are noted in parentheses. No groups had animals surviving longer than controls. The illustrative control tumor was for the 6 mg/kg group. There was no evidence of toxicity in any drug-treated group.

10–12 days. The former seem free of the symptoms of toxicity noted with Blm and CuBlm. Animals treated with

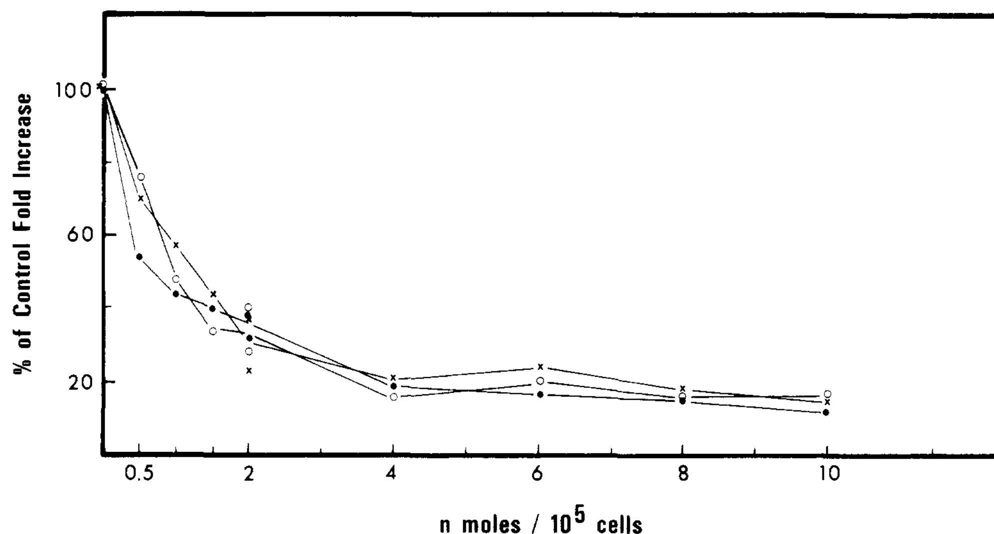

Figure 11. Cytotoxicity of Blm and two preparations of Fe(III) following continuous exposure of cells and drugs for 48 h.

20 (mg/kg)/day showed high toxicity symptoms and died within 12 days.

FeBlm (Figure 9) was the least toxic of all the compounds tested. Animals on dose levels of 15 and 20 (mg/kg)/day for 6 days showed loss of body weight but no diarrhea or lethargy. All of them recovered from this toxicity in the same time interval as Blm and CuBlm. There was 80–100% survival in the groups without any apparent development of tumor. At 12 (mg/kg)/day for 6 days FeBlm was also extremely effective with only mild toxicity in terms of loss of body weight. However, at the 6 mg/kg dose only two out of five animals in this group survived.

CoBlm (Figure 10) behaved the same way in animals as it did in tissue culture experiments. No antitumor activity was observed at any of the three concentrations tested, i.e., 6, 15, and 20 (mg/kg)/day for 6 days. None of the animals in any of these groups exhibited toxic symptoms. Thus, the pattern of antitumor activity of these drugs in animals seems to follow the same course as the cytotoxic effects observed in the tissue culture system: at equivalent doses, CuBlm > Blm > ZnBlm > FeBlm ≫ CoBlm = control.

Comparative Properties of Fe(III)Blm Prepared in Different Ways. The Fe(III)Blm used in the previous experiments was prepared by the air oxidation of Fe(II)-Blm. Because reduced oxygen radicals which damage DNA are generated in this reaction, the question arises whether such radicals also damage the bleomycin ligand.[10] Thus, Fe(III)Blm made in this way was compared with Fe(III)Blm constituted from the ligand and Fe^{3+} as described under Experimental Section.

Extent of complex formation is similar, except that the latter reaction is slower. The final absorbance and EPR spectra are identical. Figure 11 shows a study of the concentration dependence of inhibition of Ehrlich cell proliferation by Blm and the two sources of Fe(III)Blm. The data closely resemble those shown in Figure 3. No difference in the two Fe(III)Blm curves are noted. Figure 12 indicates that this identity of biological activity extends to the antitumor experiment of Figure 9. The drug levels of 6 and 12 (mg/kg)/day for Fe(III)Blm were chosen to provide data in the concentration range of the original experiment in which Fe(III)Blm exerted partial and nearly complete control of tumor development.

(10) R. M. Burger, J. Peisach, W. E. Blumberg, and S. B. Horwitz, *J. Biol. Chem.*, **254**, 10906 (1979).

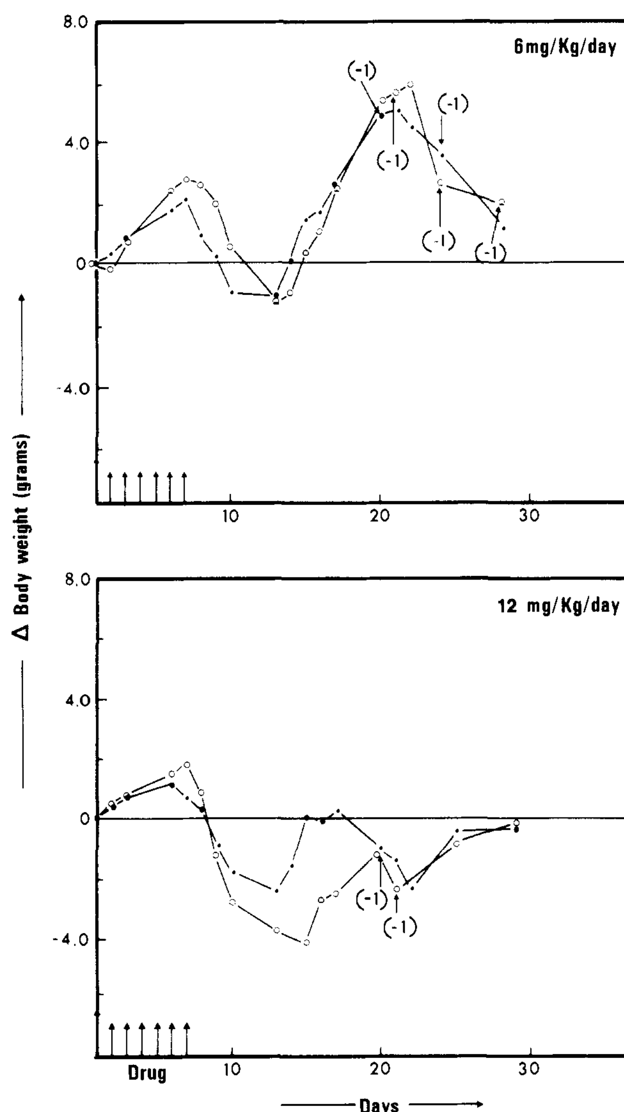

Figure 12. Antitumor activity of Fe(III)Blm's. Drug was given on days 1–6. Deaths are noted in parentheses. Upper panel: 6 (mg/kg)/day. Lower panel: 12 (mg/kg)/day. (●) Air-oxidized Fe(III)Blm; (○) Fe(III)Blm made from ferric ammonium sulfate.

Thirdly, the biochemical efficacy of the two samples of Fe(III)Blm is identical in the DNA strand-scission reaction (Table II). Excess Fe^{2+} is used as the reductant for Fe-

Table II. DNA-Blm Strand-Scission Reaction

A. DNA Degradation by Blm and Fe-Blm Complexes by Acid-Soluble Radioactivity Release

additions as specified,[a] in order	range of % of acid-soluble counts at t = 15 min, %
buffer, DNA, Blm	0.5–0.9 (4)[b]
buffer, DNA, Fe(II)	1.5–3.1 (4)
buffer, DNA, Blm, Fe(II)	40–57 (4)
buffer, DNA, Fe(III)Blm A[c]	0.9–1.3 (2)
buffer, DNA, Fe(III)Blm B	0.7–1.1 (2)
buffer, DNA, Fe(III)Blm A Fe(II)	42–54 (2)
buffer, DNA, Fe(III)Blm B Fe(II)	42–57 (4)
buffer, DNA, Blm, 2-ME	2.7–3.8 (2)
buffer, DNA, Blm, Fe(II)	87–89 (2)
buffer, DNA, Fe(III)Blm A, 2-ME	79–84 (2)
buffer, DNA, Fe(III)Blm B, 2-ME	84–88 (2)

B. Malonaldehyde Production by Bleomycin and Fe-Bleomycin Reaction with DNA

additions as specified,[d] in order	amt of malonaldehyde produced per 200 μL of incubation at t = 15 min, nmol
buffer, DNA, Blm	<0.05
buffer, DNA, Fe(II) 2.5 × 10^{-4} M	<0.05
buffer, DNA, Blm, Fe(II) 2.5 × 10^{-4} M	4.14
buffer, DNA, Fe(III)Blm A[c]	<0.05
buffer, DNA, Fe(III)Blm B	<0.05
buffer, DNA, Fe(III)Blm A, Fe(II)	1.95
buffer, DNA, Fe(III)Blm B, Fe(II)	1.78

[a] Experimental conditions (ordered assays contained some of the following): phosphate buffer (5 × 10^{-2} M); ^{14}C-labeled DNA (8 × 10^{-5} M in thymine bases, 1.6 × 10^{-4} M in total bases); Blm or Fe(III)Blm c (4 × 10^{-5} M), preincubated at 37 °C for 1 min; ferrous ammonium sulfate (1 × 10^{-4} M); 2-mercaptoethanol (0.12 M), incubated at 37 °C for 15 min; stop reaction. [b] Number of assays in parentheses. [c] Fe(III)-bleomycin complexes were prepared as specified elsewhere in this paper: Fe(III)Blm A is prepared by air-oxidation of Fe(II)Blm; Fe(III)Blm B by adding Fe(III) to Blm. [d] Experimental conditions (ordered assays contained some of the following): phosphate buffer (6 × 10^{-2} M); chicken erythrocyte DNA (2.5 × 10^{-4} M in total bases); Blm or Fe(III)-Blm c (2.5 × 10^{-5} M), preincubated at 20 °C for 3–5 min; ferrous ammonium sulfate (1 × 10^{-3} M, or as specified), incubated at 20 °C for 25 min with timed removal of aliquots.

(III)Blm. Not only are the two Fe(III)Blm's identical in activity in Table IIA, but they are similar to Blm in the presence of Fe^{2+}, which is the standard reaction mixture used in this assay.[12] One interesting difference between preformed Fe(III)Blm and Blm + Fe^{2+} is seen in the strand-scission reaction measured by malonaldehyde formation. Here, Fe(III)Blm + Fe^{2+} does not appear as efficient as Blm + Fe^{2+}. The basis for this difference is being examined.

Finally, the ^{1}H NMR spectrum of Fe(II)Blm–CO, prepared by reaction of air-oxidized Fe(III)Blm with $Na_2S_2O_4$, and that of metal-free Blm have been compared. The iron complex is diamagnetic.[11] They are identical and will be described in detail elsewhere. However, it is evident from the work of Oppenheimer et al. that repeated air-oxidation–reduction of FeBlm by $Na_2S_2O_4$ does not alter the NMR spectrum of Fe(II)Blm–CO.[12]

Discussion

The studies reported here demonstrate the importance of complexes involving biologically essential metals in bleomycin pharmacology but do not resolve the fundamental question of whether there are several distinct pharmacological agents among bleomycin and its complexes or whether there is a single proximate agent in vivo. Thus, there is confirmation of the early work of Umezawa and co-workers that Blm and CuBlm have similar antitumor properties against the Ehrlich ascites tumor.[3] For the first time the excellent antitumor properties of Zn- and Fe(III)Blm are demonstrated. Furthermore, it is shown with Fe(III)Blm that the severe host toxicity accompanying the antitumor effects of Blm and CuBlm can be partially avoided so that cytotoxic specificity for tumor is significantly enhanced. Finally, the differing concentration dependence of antitumor and host effects of Zn- and FeBlm relative to CuBlm and metal-free bleomycin clearly show that there is not a simple rapid conversion of all these compounds into Blm in vivo. However, other interconversions between metal complexes involving "available" metals from the host could occur and will be discussed below.

A major conclusion from the animal studies is that in the search for new improved analogues of bleomycin, such as tallysomycin, the simplest approach is to make metal complexes of the drug.[13] It has been known since the early studies of Umezawa that CuBlm is about as active as Blm but distributes itself in mice much differently than Blm and thus may have different in vivo properties.[3] It is shown here clearly with FeBlm and possibly with ZnBlm that the host toxicity of the parent compound can be modified without loss of antitumor activity.

The cell culture studies substantially enrich the conclusions drawn from the antitumor experiments in animals. First, in general, the pattern of cytotoxicity of the compounds in animals and in culture is the same. Only the decreased antitumor effects of ZnBlm in vivo distinguish qualitatively the results in animals and in culture. In addition, however, metal-free bleomycin was active in the model developed to attempt to distinguish whether ligands must form metal complexes to become activated (Figure 1 and ref 9, 14, and 15). In the present adaptation of this model, cells are exposed to drugs in metal-free Eagles minimal essential medium, then washed free of excess drug, and added to normal medium containing fetal calf serum for long-term incubations. The extents of proliferation are then compared with those obtained in similar experiments, in which fetal calf serum with its complement of zinc, copper, and iron is also present in the initial preincubation of cells and drugs. The activity of bleomycin alone agrees with an earlier study in which preincubation and washing was followed by injection of cells into mice to assay viability.[16] However, it is distinctly at variance

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with work on mono- and bis(thiosemicarbazone) and their complexes, which demonstrated clear requirements of metal complexation to activate ligands.[9,14]

The simple interpretation of this result is that the free ligand may be independently active or must chelate its activating metal within the Ehrlich cells. However, there are complicating factors. As described under results, continuous exposure of cells to drugs for 48 h does not progressively increase the inhibitory effects over those for 60 min (Figures 1–3). In fact, even shorter exposure times for Blm and CuBlm produce similar cytotoxic responses in cells (Figure 4). Yet there is no clear cytotoxic lesion produced during these short incubation periods—cells remain impermeable to Trypan blue, and DNA synthesis is not markedly inhibited. If the drugs must interact with cells over longer periods to inhibit proliferation, then once placed in culture for the 48-h incubation, the cell-bound ligand and complexes are then in a medium containing transition metals from fetal calf serum. Because of this, the discrimination in metal exposure provided in the initial preincubation may be lost.

The biphasic concentration dependence of cytotoxicity for all compounds has the appearance of a titration curve. Along with the other results cited above, this is suggestive of the interaction of drugs with a limited number of sites to set in motion the cytotoxic process. Further exposure of cells to drug would not enhance cytotoxicity. The results and hypothesis are consistent with findings that bleomycin inhibits cell-cycle progression in late G_2; yet relatively short exposure of cells to drug can bring about this blockage.[17,18] That is, the inhibition of proliferation is cell-cycle dependent and, hence, time dependent. The effects of an initial binding step or reaction between drugs and cells would only become apparent over a number of hours as cells accumulate in G_2.

Besides the data which relate the cytotoxic effects of bleomycin to inhibition of the cell cycle, several studies have focused on the inhibition of DNA synthesis by bleomycin as an important lesion.[19,20] As noted in previous studies, the present experiments do not support a causal relationship between cytotoxicity and the inhibition of DNA synthesis measured by a decreased incorporation of dThd into DNA.[16,21]

A third site of reaction thought to be related to bleomycin cytotoxicity is DNA itself. In the presence of thiols, bleomycin participates in the strand cleavage of DNA.[22] Recently, these studies have been stimulated by the finding that Fe^{2+} greatly enhances the rate of this reaction.[2a,b] It has thus been proposed that Fe(II)Blm is the proximate cytotoxic agent in the cell, having chelated available iron from the host or the tumor cell.[22] Because Cu^{2+} inhibits this reaction, recent papers have concluded that these ions interfere with the cytotoxicity of bleomycin.[23] Nevertheless, the present studies show clearly that Cu- and ZnBlm are effective cytotoxic and antitumor agents. Umezawa has suggested that Cu(II) is reduced by thiols and dissociated to Cu(I) and free bleomycin in tumor cells to generate the active species bleomycin and then perhaps

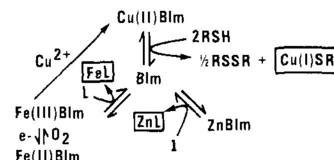

Figure 13. Possible interrelationships between Blm and Cu-, Fe-, and ZnBlm. L represents a cellular ligand.

FeBlm.[24,25] However, studies of the kinetics of reaction of CuBlm with thiols indicate that these reactions are very slow. They are slow enough to explain how Cu^{2+} binding to Blm in the DNA strand-scission reaction can inhibit this process despite the presence of thiols in the reaction medium.[26,27] Thus, this mechanism of conversion of CuBlm to Blm may not be operative in Ehrlich cells. In fact, a recent comparison of the cytotoxic and in vivo DNA strand-scission properties of Blm and CuBlm shows that CuBlm and Blm are equally cytotoxic.[23] However, no cleavage of DNA by CuBlm is noted at toxic concentrations which produce strand scission by bleomycin.

Another recent study of the strand-scission reaction suggests that the methods of preparation of Fe(III)Blm which we have used inactivates the complex toward the strand scission of DNA.[10] In a direct comparison of Fe(III)Blm's made from Fe^{2+} and Blm or Fe^{3+} and Blm, no biological, biochemical, or chemical differences have been discerned. In particular, both samples were equally active in the strand-scission reaction. Past work has indicated that once Blm reacts aerobically with an excess of Fe^{2+} in the absence of DNA, it is no longer an effective reagent in the presence of additional Fe^{2+} in the strand cleavage of DNA.[10] Apparently, those results and their interpretation do not apply here.

The question of the interrelationship of bleomycin and its complexes is raised again here. The general pattern of in vitro cytotoxic effects of Blm and Cu-, Zn-, and FeBlm is similar. Their lack of effect on DNA synthesis is a common property. The lack of significant uptake of Blm and Cu- and FeBlm into Ehrlich cells is also similar (unpublished information). A common intermediate form is suggested from these comparisons. Figure 13 diagrams the plausible interconversions which have been suggested already or may likely exist. For example, Cu^{2+} displaces both Zn^{2+} and Fe^{3+} from Blm. However, chemical and biochemical characterization of these compounds and reactions are incomplete, so that current studies leave a confusing picture. Thus, CuBlm is both thermodynamically stable and kinetically inert in model substitution and redox reactions.[26-28] Its breakdown is not expected on the basis of its known chemistry. Whether iron is available in biological systems for stable chelation to activate Blm is a major question which must be addressed before FeBlm can be considered the complex which mediates bleomycin cytotoxicity. The intriguing position of ZnBlm in this picture must also be considered. Of the three essential metals Zn^{2+}, Cu^{2+}, and Fe^{2+} Fe^{3+}, Zn^{2+} is by far the most readily available. For example, in plasma much of the zinc is

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loosely coordinated to albumin and amino acids.[29,30] Finally, in Figure 13, one cannot omit from consideration the possible cytotoxicity of metals in boxes liberated from bleomycin. In the study of bis(thiosemicarbazonato)copper complexes, cytotoxicity is correlated with the deposition of copper in Ehrlich cells.[31,32]

Experimental Section

Materials. Blenoxane (Blm), the outdated clinical mixture of bleomycin A_2 (~70%) and B_2 (~30%), was used throughout these studies and was a gift to us from Bristol Laboratories, Syracuse, N.Y. Eagles minimal essential medium plus Earle's salts, bovine fetal calf serum, and the trypsin–EDTA solution were obtained from GIBCO. Female Swiss HA/ICR mice were purchased routinely from ARS/Sprague–Dawley, Mad̲ison, Wis.

Tissue Culture Studies. Ehrlich ascites cells [illegible]ly adapted to cell culture by Dr. Edvardas Kaminskas were used for in vitro tissue culture cytotoxicity experiments. The cells were maintained in Eagle's minimum essential medium (MEM) with Earle's balanced salts and penicillin, streptomycin, and 5% bovine fetal calf serum. The pH was maintained at 7.2–7.4 with the addition of $NaHCO_3$. The cells were grown routinely in spinner flasks at 37 °C in incubators without CO_2.

Cytotoxicity Studies. Short-Term Exposure. Cells growing exponentially were collected and suspended in fresh MEM ± 5% serum (3–4 × 10^5 cells/mL) and incubated in sterile disposable 15-mL centrifuge tubes with caps in a water bath at 37 °C with gentle shaking. Drugs were added just before the incubation. At the end of the incubation period, the cells were collected by centrifugation, washed with fresh MEM, resuspended in fresh MEM + 5% serum, and plated in 20-mL disposable plastic monolayer flasks. All flasks were incubated in an atmosphere of 5% CO_2 at 37 °C.

Continuous Exposure. Exponentially growing cells were suspended in fresh MEM + 5% serum (2–3 × 10^5 cells/mL) and were plated in multiwell plates. Drugs were added to the cells either immediately or after 24 h of adaptation. The plates were incubated in 5% CO_2 atmosphere incubators at 37 °C.

Cell Harvesting, Counting, and Processing of the Data. The cells were harvested at the end of the incubation period, usually 48 h, by decanting all the used medium, centrifuging to collect any cells that might have been easily detached, trypsinizing the cells in flasks for 10–15 min, and suspending all the pooled cells in a known volume of MEM for counting.

Cell counting was done manually with a hemacytometer. Cell viability was determined by the Trypan blue dye exclusion method.

Although the ratio of the number of cells to the drug is constant during incubations of cells and drugs for all the samples, because of the subsequent processing, which included two centrifugations, the final cell suspensions showed variable cell counts. Therefore, cell counts were made on all samples before plating and taken as "day 0" count. Counts done after 48-h incubation were "final" count. The ratio of "final/day 0" is defined as the fold increase in cells. All the samples were run at least in triplicates, and standard errors ranged between 2 and 10% control fold increase for all samples. These ratios were normalized as the percentage fold increase of the control cell populations. In this way a number of experiments could be directly compared by plotting normalized fold increase in cell proliferation and the concentration of drug in nmol/10^5 cells.

Animal Maintenance and Antitumor Experiments. Female Swiss mice 8–10 weeks old and weighing 25–30 g were used in these experiments. All the animals were maintained ad libitum on normal purina laboratory chow and tap water throughout the experimental period.

Each group consisted of four to five weight-matched animals, and animals from each group were housed in a single stainless-steel cage. The Ehrlich ascites tumor was maintained routinely by transplanting 2–5 × 10^6 cells into healthy normal mice intraperitoneally from a freshly collected cell suspension of the stock tumor. To begin the antitumor experiments, 5 × 10^6 cells were injected into the groups of mice.

The drug administration was started 24 h after the tumor cell injections. All the drugs were given intraperitoneally once a day for 6 days at a regular interval of 24 h. The individual animal body weights were recorded for bleomycin next 4 weeks, and the survival was followed for a total of 50 days.

Preparation of Bleomycin and Bleomycin–Metal Complexes. Blenoxane was used without any further purification. It contains approximately 70% bleomycin A_2 and 30% belomycin B_2. Solutions of blenoxane (referred to as Bleomycin or Blm throughout this paper) were made in 0.1 M NaCl and quantitated by titrating with a standard $CuCl_2$ solution.

Cu-, Zn-, and CoBlm. Equimolar quantities of Blm (molecular weight 1400) and 0.2 M metal chloride solution were mixed, and the pH was adjusted to 7–7.5.

FeBlm. Equimolar amounts of Blm and ferrous ammonium sulfate or ferrous chloride in 0.1 M NaCl maintained under bubbling N_2 were mixed, and the pH was adjusted immediately to 7.2–7.5. The final form of the complex was Fe(III)Blm, as Fe(II)Blm oxidizes on exposure to air. Alternatively for comparison in Figures 11 and 12 and Table II, Fe(III)Blm was made by mixing equimolar Blm and ferric ammonium sulfate at pH 3 and slowly titrating the solution up to pH 7. In this procedure, the oxidation state of iron is always +3.

All the solutions were sterilized by filtering through a Gelman Acrodisc filter, pore size 0.2-μm, and kept frozen until and after every use.

DNA Synthesis. DNA synthesis was measured by following [^{3}H]thymidine incorporation into a perchloric acid precipitable substance using a modified procedure reported earlier by Saryan et al.[8,9] Bleomycin and its Cu, Fe, and Co complexes were added as solutions in MEM at identical concentrations to suspensions of 5 × 10^6 cells/mL and incubated for 60 min at 37 °C with gentle shaking. The cells were washed once with MEM after 60 min and resuspended in fresh MEM, and the radioactive precursor incorporation was followed for the next 20 min. The radioactivity was estimated in a Unilux III liquid scintillation system (Nuclear Chicago Co.) using an 8:2 (v/v) toluene–ethanol cocktail, which provided improved counting efficiency.

DNA Strand-Scission Reaction. DNA Degradation by Measurement of Acid-Soluble Radioactivity. Blm and Fe–Blm complexes were tested for the ability to degrade DNA into acid-soluble fragments using a modified assay of Horwitz et al.[12] Synthetic ^{14}C-labeled DNA alternating copolymer (poly(dA-[2-^{14}C]dT)·poly(dA-[2-^{14}C]dT); 4.2 μCi/μmol of thymine bases, 5.7 μCi/mL; P. L. Biochemicals Inc., Milwaukee, Wis.) served as an excellent substrate. Assay reactions (total volume 50 μL) were assembled in polystyrene tubes previously rinsed with 0.25 M phosphate buffer, pH 7.0, and exhaustively with glass-distilled water, and contained some of the following additions: 5 × 10^{-2} M sodium phosphate buffer, pH 7.00; 8 × 10^{-5} M ^{14}C-labeled DNA (as thymine bases); 4 × 10^{-5} M bleomycin; 1 × 10^{-4} M Fe^{2+}; and 0.13 M 2-mercaptoethanol. After a 15-min incubation at 37 °C, the reaction tubes are immersed in ice slush and 50 μL of chilled 0.1 M EDTA with 10 mg/mL bovine serum albumin is added and mixed with the assay incubation. Chilled 5 M perchloric acid (10 μL) is then added and the mixture allowed to precipitate on ice for 15 min. The precipitate is harvested by centrifugation at 6000*g* for 25 min and radioactivity determined in the supernatant by liquid scintillation counting as performed above.

Working solutions of ferrous ammonium sulfate and 2-mercaptoethanol were freshly prepared immediately before use and assayed for [Fe^{2+}] or [R–SH], respectively, at the end of the reaction. Ferrous ion concentration was determined by reaction of ferrous ammonium sulfate with excess 1,10-phenanthroline and measured at 510 nm [ϵ_{510} for $(1,10\text{-phen})_3Fe(II)$ = 9700 M^{-1}]. R–SH concentration was determined by reaction of 2-mercaptoethanol with DTNB [5,5′-dithiobis(2-nitrobenzoic acid), Aldrich] and measured at 412 nm (ϵ_{412} for TNB anion = 13 000 M^{-1}). Working solutions of both substances were stable to oxi-

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dation for much longer than the duration of the incubations.

DNA Degradation by Measurement of "Malonaldehyde" Production. Blm and Fe–Blm complexes were tested for the production of malonaldehyde-like products upon reaction with native DNA, using a modified assay of Burger et al.[19] based on the method of Waravdekar and Saslaw.[33] Highly polymerized chicken erythrocyte DNA (P. L. Biochemicals, Inc., Milwaukee, Wis.) served as the substrate. Aldrich Chemical Co. (Milwaukee, Wis.) supplied 2-thiobarbituric acid (4,6-dihydroxy-2-mercaptopyrimidine) and malonaldehyde bis(dimethyl acetal), used as standard. Reaction mixtures (total volume 2.0 mL) were assembled in polystyrene tubes previously rinsed with 0.25 M phosphate buffer, pH 7.0, and exhaustively with glass-distilled water and contained some of the following additions, in order: 60 mM sodium phosphate buffer, pH 7.00; 2.5×10^{-4} M DNA (as total bases); 2.5×10^{-4} M bleomycin [or Fe(III)–Blm complexes). After a 3- to 5-min preincubation at room temperature, Fe^{2+} (1×10^{-3} M) is added as ferrous ammonium sulfate to some incubations. The incubation is mixed briefly and timed aliquots of 0.2 mL are taken up to 25 min. Aliquots are mixed with 1.0 mL of 2-thiobarbitaric acid reagent (4×10^{-2} M 2-thiobarbituric acid, 1×10^{-3} M EDTA) and heated in capped test tubes for 30 min at 75 °C to develop the color. Malonaldehyde standards are prepared simultaneously. After cooling, the absorbance at 532 nm is measured using a Cary 16 spectrophotometer equipped with 1.0-mL cuvettes. Production of malonaldehyde from DNA reactions is quantitated using authentic malonaldehyde produced by acid hydrolysis of malonaldehyde bis(dimethyl acetal), which when reacted with 2-thiobarbituric acid yields a product having an $\epsilon_{532} = 1.6 \times 10^5\ M^{-1}$. The intensely pink-colored products of the reaction between 2-thiobarbituric acid and malonaldehyde and 2-thiobarbituric acid and Blm–Fe^{2+} reactions were indistinguishable by visible absorption spectrophotometry.

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Synthetic and Preliminary Hemodynamic and Whole Animal Toxicity Studies on (*R*,*S*)-, (*R*)-, and (*S*)-2-Methyl-3-(2,4,5-trihydroxyphenyl)alanine

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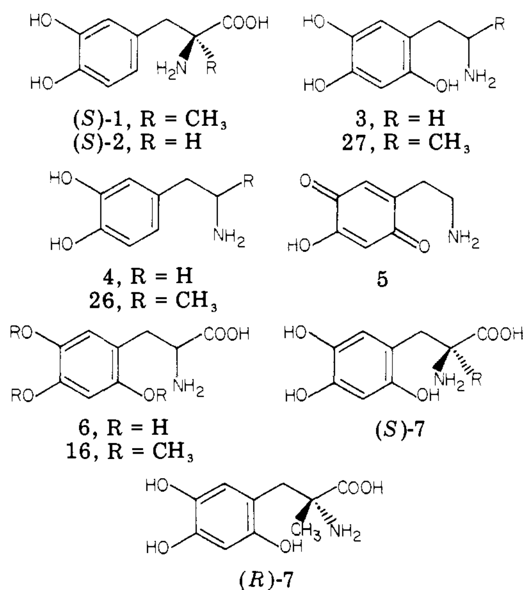
The synthesis, resolution, and absolute configuration assignment of 2-methyl-3-(2,4,5-trihydroxyphenyl)alanine (6-OH-α-Me-Dopa) are reported. Hemodynamic studies in the rat have shown that this structural analogue and potential metabolite of the clinically useful drug (*S*)-α-Me-Dopa possesses weak hypotensive activity which resides in the *R* enantiomer. LD_{50} studies in mice have established that 6-OH-α-Me-Dopa is over four times more toxic than α-Me-Dopa. Chronic exposure to 6-OH-α-Me-Dopa leads to renal and hepatic lesions. The ease of oxidation of this hydroquinone to the electrophilic quinone species may contribute to its enhanced toxicity compared to α-Me-Dopa.

(*S*)-α-Me-Dopa [(*S*)-α-MD, (*S*)-1] is a clinically useful



antihypertensive agent.[1] Although this drug is relatively nontoxic,[2] a number of side effects has been reported. These include depression,[3] a Parkinsonian-type syndrome,[4] psychotic behavior,[5] and a positive antiglobulin test sometimes associated with hemolytic anemia.[6] Perhaps the most dramatic and clinically serious potential side effect of (*S*)-α-MD is its hepatotoxicity, which may lead to severe liver damage and death.[7] The well-known susceptibility of catecholamines to autoxidation[8,12] has led to the proposal that the liver abnormalities associated with the use of (*S*)-α-MD may result from the formation of electrophilic oxidation products that covalently bind to biomacromolecules.[9] Evidence to support this hypothesis is the NADPH-dependent binding of tritium-labeled (*S*)-α-MD to hepatic microsomal macromolecules and the isolation of an (*S*)-α-MD glutathione adduct.[9] Similar sulfhydryl adducts of the endogenous catechol amino acid (*S*)-Dopa [(*S*)-2] have been characterized in the urine of melanoma patients.[10] Adduct formation presumably

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