Biochemical Pharmacology, Vol. 43, No. 3, pp. 615–623, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
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PROPERTIES OF REDOX-INACTIVATED BLEOMYCINS

IN VITRO DNA DAMAGE AND INHIBITION OF EHRLICH CELL PROLIFERATION

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(*Received* 11 *February* 1991; *accepted* 6 *September* 1991)

Abstract—Blenoxane, bleomycin A_2, bleomycin B_2, and demethyl bleomycin A_2 and products of their reactions with Fe^{2+} and oxygen were used to explore the relationship between their capacity to carry out *in vitro* DNA strand scission and their growth inhibitory activity against Ehrlich cells. Reaction of Fe^{2+}, bleomycin and O_2 in the absence of DNA decreased the subsequent effectiveness of various bleomycin congeners to degrade DNA in the presence of Fe^{2+} and oxygen. In comparison with controls, this loss of strand scission activity was not paralleled by equivalent decreases in growth inhibition. Demethyl bleomycin A_2 retained full biological activity relative to bleomycin A_2, despite being only 30% as effective as bleomycin A_2 in its ability to cleave DNA *in vitro*. Prior reaction of bleomycins with Fe^{2+} did not alter their capacity to reduce oxygen or affect their ability to generate the activated intermediate which, for native bleomycin structures, is competent to cleave DNA *in vitro*.

Bleomycin, a glycopeptide antitumor and metal chelating agent, is activated to cleave the backbone of DNA *in vitro* by Fe^{2+} in the presence of oxygen [1, 2]. It is commonly believed that DNA damage is central to the mechanism of action of bleomycin. Nevertheless, recent publications have also suggested that, among congeners of bleomycin, there is not a strong positive correlation between quantitative ability to cause cellular single-strand DNA damage and capacity to cause growth inhibition or cytotoxicity [3–6]. In a well-studied redox reaction, oxygen is reduced by ferrous iron bound to Blm§ to a species which directly attacks the deoxyribose moiety of DNA [7–9]. Several reports have indicated that in the absence of DNA the active species generated in this reaction reacts instead with the organic structure of the drug itself, and, thereby, decreases its subsequent ability to carry out Fe^{2+}-dependent DNA strand scission [10, 11].

The present study is directed toward the question of whether forms of bleomycin with less inherent capability to damage DNA *in vitro* than the clinical mixture of the drug also causes less growth inhibition in cells. To address this issue, the properties of the clinical mixture of bleomycins were compared with those of its principal components, bleomycin A_2 (Blm A_2) and B_2 (Blm B_2), and with demethyl bleomycin A_2 (dMBlm A_2), and redox-inactivated forms of all these reagents. Demethyl bleomycin A_2 was included because it possesses much less *in vitro* DNA strand scission activity than bleomycin A_2 [12]. A brief report related to this work has appeared previously [13].

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§ Abbreviations: Blm, generic abbreviation for all bleomycin species; Blm A_2, bleomycin A_2; Blm B_2, bleomycin B_2; Blx, blenoxane, the clinical mixture of bleomycins; dMBlm A_2, demethyl bleomycin A_2; RIBlm, redox-inactivated bleomycin; FeRIBlm, iron-containing redox-inactivated bleomycin; MBlm, metallobleomycins where M is iron or copper; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MDA, malondialdehyde; and DTC, diethyldithiocarbamate.

EXPERIMENTAL PROCEDURES

Materials. Outdated blenoxane (Blx), the clinical mixture of bleomycins, was a gift of the Bristol Myers Co., Syracuse, NY. Fetal bovine serum and Eagle's minimal essential medium plus Earle's salts were purchased from Gibco, Grand Island, NY. Calf thymus DNA and bovine serum albumin were obtained from the Sigma Chemical Co., St. Louis, MO, and 2-thiobarbituric acid was from the Aldrich Chemical Co., Milwaukee, WI. RPI Products (Mt. Prospect, IL) provided [2-^{14}C]thymidine (sp. act. 50 mCi/mmol). Pronase (grade B) was from the Calbiochem Co., La Jolla, CA, and RNase from Pharmacia, Milwaukee, WI. [^{3}H]Blm A_2 was made as previously described [14]. All other chemicals were of reagent or equivalent grade.

Preparation of redox-inactivated bleomycin. Blm A_2, Blm B_2, and dMBlm A_2 were separated by chromotography of Blx over carboxymethyl-Sephadex [15]. Following the procedure of Burger *et al.* [9], Blx and these isolated components were allowed to react aerobically with various concentrations (1:1, 1:2, 1:4, 1:8) of Fe^{2+} as ferrous ammonium sulfate for 30 min in 20 mM Tris or

phosphate buffers at pH 7 and room temperature. Much of the ferric iron which formed upon oxidation immediately precipitated from solution and was removed by centrifugation. The soluble product is designated iron-containing redox-inactivated bleomycin (FeRIBlm).

To extract Fe^{3+} from the FeBlm complex, a 10-fold excess of diethyldithiocarbamate (DTC) was added, which reacted with uncomplexed Fe^{3+} and Fe(III)Blm to form the neutral complex, $Fe(III)(DTC)_3$. This was readily extracted with ethyl acetate leaving metal-free forms of bleomycin in the aqueous phase. Isolation of the metal-free drug was complete when the UV spectrum of the sample showed no evidence of contaminating DTC. In some cases, the mixture of Blm and $Fe(III)(DTC)_3$ was separated on carboxymethyl-Sephadex [15], which binds the drug but not the tris-dithiocarbamato–iron complex, or by Sephadex G-10 gel permeation chromatography.

In separate experiments to assess the effects of iron extraction on the properties of redox-inactivated bleomycin (RIBlm), samples of Fe(III)Blm at the same concentration used above were made by reacting the drug with 1-, 2-, 4- or 8-fold excesses of $FeCl_3$. Ferric hydroxide precipitate formed and was removed by centrifugation.

Characterization of FeRIBlm. Electron spin resonance (ESR) spectra were taken with a Varian E line Century Series spectrometer operating at 77°K. For some of these spectra, high *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer concentrations (0.5 M) were used to carefully control the pH of the solutions. Oxygen consumption studies utilized a Yellow Springs Instruments model 53 Biological Oxygen Monitor equipped with a standard bath assembly and a model 5331 Oxygen Probe. Experiments were run in 20 mM phosphate buffer, pH 7.0, at 25°, that had been equilibrated with air. The oxygen concentration was assumed to be 0.22 mM.

Thiobarbituric acid/malondialdehyde assay for production of malondialdehyde-like product during DNA strand scission. The method used was based on the procedure of Waravdekar and Saslaw [16] as applied by Burger *et al.* [10] to quantitate the release of base propenals from degraded DNA, which in the assay behave like malondialdehyde (MDA). Briefly, samples prepared by the redox-inactivation procedure or from addition of Fe^{3+} to Blm were mixed at room temperature with stock solutions of phosphate buffer (pH 7.0) and calf thymus DNA to make solutions with final concentrations of 20 mM phosphate, 0.2 mM Blm, and 1.4 mM DNA bases as determined spectrophotometrically by measuring the absorbance at 260 nm and assuming a molar absorptivity of $6600\,M^{-1}cm^{-1}$. Reactions were initiated subsequently by the addition of 0.02 mL of 5 mM $Fe(NH_4)_2(SO_4)_2$ to make a 0.2 mM final concentration of Fe^{2+}. All reactions had pH values of 7.0 to 7.4. At 0.5, 1, 2, and 5 min thereafter, a 0.1-mL aliquot of each reaction mixture was added to 0.9 mL of a 40 mM 2-thiobarbituric acid solution containing 1 mM EDTA to stop the reaction. Resultant solutions were covered with aluminum foil, heated at 90° for 30 min, cooled, and then assayed for malondialdehyde-like product, which forms an intensely colored adduct with thiobarbituric acid, having a molar absorptivity at 532 nm of $16{,}000\,M^{-1}cm^{-1}$.

Acid solubility assay for [^{14}C]DNA strand scission. Radiolabeled DNA from mouse Ehrlich cells grown in spinner cultures was prepared as follows [17]. Cells (8×10^5/mL, 250 mL) were incubated in Eagle's minimal essential medium plus Earle's salts for 24 hr with 0.02 μCi/mL [2-^{14}C]thymidine to label the DNA. Then cells were washed with fresh growth medium without fetal bovine serum, resuspended in 10 mM Tris–1 mM EDTA (pH 7.4) and lysed by the addition of 0.5% Sarcosyl. Following digestion for 4 hr at 37° with 500 μg/mL pronase, the digest was extracted successively with phenol, 1:1 phenol/chloroform, and chloroform, and dialyzed against Tris/EDTA/NaCl. The sample was then further digested with RNase, extracted with 1:1 phenol/chloroform, and dialyzed against 50 mM sodium phosphate, pH 7.4. The DNA was concentrated by ethanol precipitation and resuspended in the phosphate buffer. The product possessed an absorbance 260 nm/280 nm ratio of 1.77 and specific activity of 0.27 μCi/μmol DNA bases.

For the assay of strand cleavage activity, Blm samples were mixed with water, buffer, and [^{14}C]-DNA to yield solutions (0.4 mL) with final concentrations of 20 mM phosphate buffer, pH 7.0, 0.32 mM DNA base (1.90×10^5 dpm/mL) and 0.040 mM Blm. Reactions were started by the addition of 0.02 mL $Fe(NH_4)_2(SO_4)_2$ to give 0.10 mM Fe^{2+}. The final pH in all mixtures was 7.0 to 7.4. After a 15-min incubation at room temperature, reaction tubes were immersed in an ice slush and mixed with 0.4 mL of chilled 0.1 M EDTA containing 10 mg/mL bovine serum albumin. Macromolecules were precipitated over a 15-min period by the addition of 0.08 mL of 5 M $HClO_4$. The precipitate was removed by centrifugation and the solubilized radioactivity was measured with a Beckman LS 3801 liquid scintillation counter using Beckman Ready Solv scintillation fluid. The precipitate was dissolved at pH 7 and also counted. Relative DNA damage was calculated from the radioactivity in the original reaction mixture and in the subsequent supernatant and precipitate.

Assay for growth inhibition. Cultured murine Ehrlich ascites tumor cells were placed in 24-well cell plates at a concentration of 10^5 cells/0.95 mL in Eagle's minimal essential medium plus Earle's salts and 2.5% fetal bovine serum [18]. Sterilized drugs were added (0.05 mL) to duplicate wells to give final concentrations of 1, 2, 4, or 10 nmol/mL. Plates of cells were incubated at 37° in a 5% CO_2 atmosphere for 48 hr in a Bellco Glass Inc. incubator. Attached cells were removed from the wells after reaction with 1 mL of 10% trypsin (15 min) and counted using a Particle Data, Inc., cell counter (Elmhurst, IL). The growth of control cell populations was also determined so that relative drug-induced inhibition of proliferation could be assessed.

HPLC separation of redox-inactivated Blm. Various forms of Blm A_2 were separated by HPLC using a Waters Associates dual pump instrument as follows. Samples containing about 0.5 mM drug were

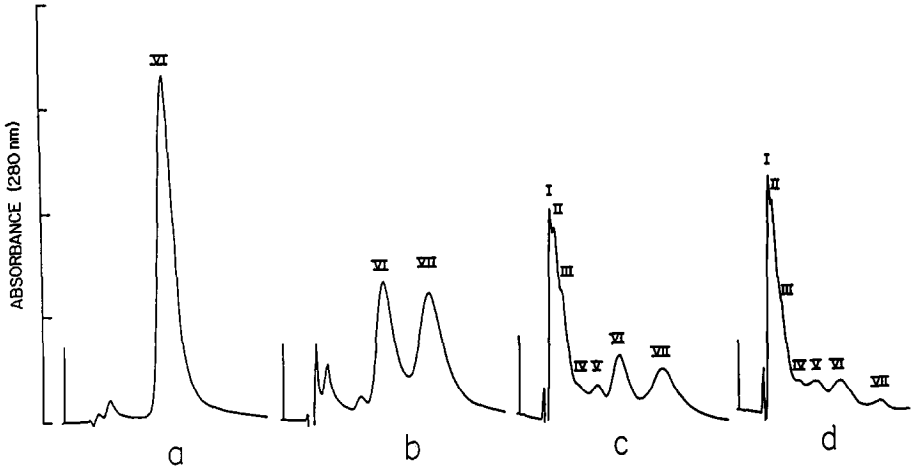

Fig. 1. HPLC separation of redox-inactivated Blm A_2. (a) Blm A_2; (b) Fe(III)Blm A_2 made from 1:1 Blm A_2 to Fe^{3+}; (c) FeRIBlm A_2, 1:1 Blm A_2 to Fe^{2+}; and (d) FeRIBlm A_2, 1:8 Blm A_2 to Fe^{2+}. Bands are numbered in order of elution after injection in plots of absorbance vs time. Band VI, Blm A_2 eluted at 17 min.

injected onto a Waters Nova Pak™ C-18 Steel column (0.6 cm × 16 cm). Components were separated with a solvent system of 60% (0.04 M heptanesulfonic acid sodium salt with 0.10 M triethylamine and 0.025 M glacial acetic acid, pH 6.0) (pump A) and 40% methanol (pump B) at a flow rate of 0.25 mL/min under a pressure of 800–900 psi.

RESULTS

Properties of the reaction of Fe^{2+}, O_2 *and Blm.* During the reaction of Fe^{2+} with Blm and oxygen, ferric hydroxide forms and precipitates out of solution. When this reaction was performed with 1:1 to 1:8 ratios of Blm to Fe^{2+}, using [^{3}H]Blm A_2 prepared as previously described [14], 8–16% of the drug was found to coprecipitate with the iron hydroxide. With the radioactivity to quantitate the total concentration of all forms of Blm in solution, an average molar absorptivity at 290 nm of $1.3 \times 10^4 \pm 0.2\ M^{-1}cm^{-1}$ was calculated for the sum of the bleomycin species present after reaction at all Blm/iron ratios. This is indistinguishable from the accepted value of 1.4×10^4 for native forms of metal-free Blm [19]. Similarly, the extinction coefficients for Fe(III) complexes of Blm or RIBlm were each found to be $2200\ M^{-1}\,cm^{-1}$ at 375 nm, using iron measured by atomic absorption spectrophotometry to quantitate chelated iron.

HPLC analysis of the product mixture of the reaction of Fe^{2+}, Blm A_2, and O_2 showed that it contained at least seven detectable species (Fig. 1, c and d). The first five bands (I–V) were considered to be altered bleomycins; bands VI and VII eluted at times corresponding to the native, metal-free drug (Fig. 1a) and Fe(III)Blm-A_2 (Fig. 1b). Interestingly, the chromatographic profile of Fe(III)Blm A_2 also contained a peak analogous to V and two rapidly moving bands similar to ones seen in the redox-inactivation mixture, migrating in positions corresponding to I and II. It was also noted that at pH 7, Blm A_2 could only be partially saturated with Fe^{3+} (Fig. 1, b through d). The relative sizes of these bands changed as the ratio of ferrous iron to Blm increased in the incubation mixture, with peaks I–V growing at the expense of VI and VII (Fig. 1, c and d). The average percentage of fractions (I–V) ±1 SD (four experiments) in the HPLC eluates after 1:1, 1:2, 1:4, and 1:8 Blm to Fe^{2+} redox-inactivation were, respectively, 47 ± 8, 62 ± 16, 62 ± 16, and 72 ± 5. HPLC analyses of redox-inactivated Blx yielded similar values. It is possible that the multiple bands of RIBlm A_2 contain pairs of iron-containing and iron-free species.

Characteristics of the redox-inactivated product. Fe(III)RIBlm has a spectrum at wavelengths greater than 300 nm which is identical to that for the complex made from $FeCl_3$ and Blm [10]. The EPR spectra of Fe(III)Blx and Fe(III)RIBlx were also identical (Fig. 2, a and b). As shown in Table 1, these two forms in the presence of Fe^{2+} reduced oxygen with comparable initial rates, no matter how much Fe^{2+} had been used to inactivate Blm. Indeed, these are the same as rates measured in the reaction of Fe^{2+} and O_2 mediated by metal-free Blm (Table 1).

Spectra c through g of Fig. 2 show that in both the absence and presence of DNA, Fe(III)Blm and Fe(III)RIBlm achieved similar concentrations of the species associated with the EPR signal at g values of 2.26, 2.17, 1.94. This signal (noted with stars) is also generated during the oxidation of Fe(II)Blm by O_2 or by the interaction of H_2O_2 with Fe(III)Blm [9]. It is thought that this signal represents the activated form of the drug which participates in the degradation of DNA [9]. Thus, according to several assessments of the structure and chemistry of the iron-binding site of RIBlm, it behaves similarly to Blm.

DNA strand scission activity of various bleomycins. The DNA strand cleavage reaction of Blm in the

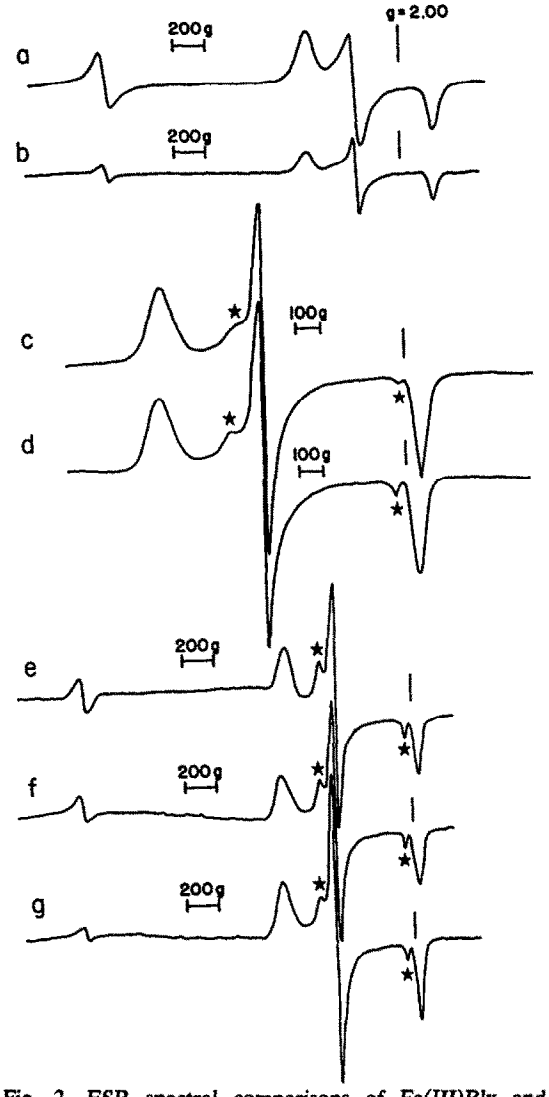

Fig. 2. ESR spectral comparisons of Fe(III)Blx and Fe(III)RIBlx. (a) Fe(III)Blx (1 mM Blx, 1:8 Blx to Fe^{3+}) in 20 mM phosphate, pH 7.4; (b) Fe(III)RIBlx (0.7 mM RIBlx, 1:8 Blx to Fe^{2+}) in 20 mM phosphate, pH 7.4; (c) 1 mM Fe^{2+} plus 1 mM FeRIBlx in 0.5 M Hepes buffer, pH 7.3; (d) 1 mM Fe^{2+} plus 1 mM Blx in 0.5 M Hepes buffer, pH 7.3; (e) 1 mM Fe^{2+} plus 1 mM Blx and 7 mM DNA (bases) in 0.5 H Hepes buffer, pH 7.2; (f) 1 mM Fe^{2+} plus 1 mM FeRIBlx and 7 mM DNA (bases) in 0.5 M Hepes buffer, pH 7.2; and (g) 1 mM Fe^{2+} plus 1 mM Fe(III)Blx and 7 mM DNA (bases) in 0.5 M Hepes buffer, pH 7.2. Spectra a and b, c and d, and e–g were, respectively, obtained at the same instrumental settings. Starred features of spectra c–g stem from activated Fe(III)Blx or Fe(III)RIBlx. Samples including Fe^{2+} were frozen at 77°K within 30 sec of mixing.

presence of Fe^{2+} and O_2 was monitored conveniently by observing the rate and extent of formation of base propenal as the deoxyribose moiety is degraded. To start the reaction, Fe^{2+} was added to a mixture of the drug and DNA. Typical kinetics of the reaction of Blx, Fe(III)Blx, and FeRIBlx, previously inactivated with a series of Fe^{2+} concentrations, are shown in Fig. 3. Compared with the drug which had

Table 1. Rates of oxygen consumption by species of bleomycin in the presence of Fe^{2+}*

Reaction mixture	Preincubation† Blm:Fe	Initial rate of O_2 consumption‡ (μmol/min/mL)
Buffer		0.025 ± 0.003
Blx		0.094 ± 0.015
FeRIBlx	1:1	0.094 ± 0.022
	1:8	0.084 ± 0.008
Fe(III)Blx	1:1	0.087 ± 0.017
	1:8	0.083 ± 0.012

* 0.10 mM Blm species or buffer reacted with 0.10 mM $Fe(NH_4)_2(SO_4)_2$.
† Conditions for formation of FeRIBlm or Fe(III)Blm.
‡ Values are means ± SD for three experiments.

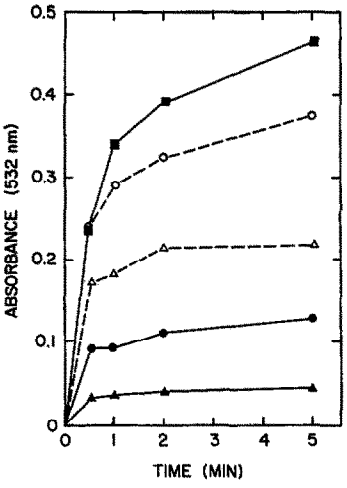

Fig. 3. Kinetics of formation of malondialdehyde-like product from the reaction of Fe^{2+} with various bleomycins. Key: (■) Blx; (●) FeRIBlx, 1:1 Blx to Fe^{2+} redox-inactivated; (▲) FeRIBlx, 1:8 redox-inactivated; (○) Fe(III)Blx, 1:1 Blx to Fe^{3+}; and (△) Fe(III)Blx, 1:8.

not been reacted with ferrous iron, the extent of base-propenal formation progressively decreased as the ratio of Fe^{2+} to Blx in the previous inactivation reaction increased. Most of the inactivation occurred with the stoichiometric addition of Fe^{2+} to the drug, which caused approximately a 60–70% diminution in the capacity of the product to cause strand breakage.

Experiments with redox-inactivated Blm A_2 and Blm B_2 yielded closely similar reductions in DNA strand scission. Each non-redox-inactivated native form produced equivalent amounts of base propenal product upon reaction with Fe^{2+}, DNA, and O_2 (not shown). Both the attenuated effectiveness of Fe^{2+}-treated bleomycins in subsequent DNA strand scission assays and the HPLC results are evidence that bleomycin had been altered during its mediation of the redox reaction between Fe^{2+} and O_2.

The DNA strand cleavage activity of Fe(III)Blm

Table 2. Acid solubilization of radioactivity from [^{14}C]-DNA treated with various bleomycins and Fe^{2+}*

Sample	Percent acid-soluble DNA†	Percent of Blx control
Control: Blx	75	
FeRIBlx (1:1)	30	40
FeRIBlx (1:2)	23	31
FeRIBlx (1:4)	18	24
FeRIBlx (1:8)	16	21
Fe(III)Blx (1:1)	67	89
Fe(III)Blx (1:2)	55	73
Fe(III)Blx (1:4)	57	76
Fe(III)Blx (1:8)	51	68
Control: no drug or Fe^{2+}	0.4	0.5
Control: no drug	0.6	0.8

* All samples were incubated with DNA and Fe^{2+} except where noted. Results are an average of two experiments.
† Initial concentration of DNA bases: 0.32 mM.

prepared from Fe^{3+} and Blm was also examined. Generally, as indicated in Fig. 3, it was somewhat reduced relative to that of the metal-free control. To ensure that the decreased activity of FeRIBlm toward DNA was not related in some way to the presence of Fe^{3+} from the redox-inactivation reaction, samples of the drug were freed of iron by extraction with diethyldithiocarbamate into ethyl acetate solvent. Strand scission activity did not return when iron was removed from the drug (see Fig. 4 described below).

The possibility that FeRIBlx might damage DNA by another mechanism leading to altered products not detected by the assay for base-propenal formation was tested by examining its ability to degrade [^{14}C]-DNA in the presence of Fe^{2+} and O_2. This assay will detect either base release or strand cleavage of DNA. As shown in Table 2, results of this assay confirmed that the initial reaction of Fe^{2+}, O_2, and Blx greatly reduced the capacity of the product to cause DNA damage. Indeed, in both Table 2 and Fig. 3 one sees the same pattern of loss of strand scission activity of FeRIBlm as a function of the preincubation ratio of Blm to Fe^{2+}. The decline in cleavage efficiency of Fe(III)Blm after incubation of different ratios of Fe^{3+} and Blm was also qualitatively similar in Table 2 and Fig. 3.

Relationship of redox-inactivation product distribution to reduction in DNA strand scission activity. According to HPLC analysis, the product of redox-inactivation contains four to five species (Fig. 1). When bands I–IV were collected together from the HPLC eluates and tested for DNA strand scission activity, the combination displayed no capacity to

Table 3. Inhibition of cell proliferation by various forms of blenoxane

Form of Blx*	Preincubation ratio of Fe^{2+} or Fe^{3+} to Blx*†	Percent of control proliferation‡ Treatment (nmol) drug form/(10^5 cells/mL)§ 1	2	4	10
Fe^{2+} + Blx	0‖	72 ± 9¶	61 ± 15	29 ± 6 (12)	20 ± 4 (12)
(FeRIBlx)	1	87 ± 5	72 ± 6	61 ± 8	41 ± 12
	2			56 ± 14	43 ± 14
	4			64 ± 11	43 ± 14
	8			51 ± 10	51 ± 10
Fe^{2+} + Blx,	0‖	78 ± 8	61 ± 7	31 ± 10	26 ± 10
Fe removed**	1	77 ± 4	64 ± 2	57 ± 10	36 ± 9
(RIBlx)	2			59 (2)	46 (2)
	4			60 (2)	46 (2)
	8	80 ± 11	76 ± 13	72 ± 13	48 ± 9
Blx + Fe^{3+}	1	81 ± 8	67 ± 4	43 ± 13	30 ± 3
(FeBlx)	2			46 ± 10	39 ± 9
	4			38 ± 7	24 ± 6
	8	87 ± 11	65 ± 6	42 ± 11	30 ± 4
Blx + Fe^{3+}	0‖	69 ± 11	60 ± 3	36 ± 8 (5)	29 ± 6 (5)
Fe removed**	1	72 (2)	70 ± 10	47 ± 17	30 ± 6
(Blx)	2			40 ± 15	29 ± 8
	4			49 ± 20	50 ± 11
	8	84 ± 8	65 ± 10	50 ± 11	33 ± 8

* Treatment of Blx prior to exposure of cells. Form present after treatment.
† Ratio of Fe^{2+} or Fe^{3+} to Blx in treatments prior to exposure of cells.
‡ (Cell number-treated)/(cell number-untreated control) ×100. Historically, the 48-hr cell number of untreated control samples averaged 4×10^5 cells.
§ Concentration of forms of Blx used to treat cells.
‖ Metal-free drug.
¶ Mean ± 1 SD. There were three incubations of cells and drugs at 0, 1, 2 and 4 preincubation ratios of iron to Blx or six incubations at a ratio of 8 except where noted in parentheses.
** Iron was removed with diethyldithiocarbamate and ethyl acetate.

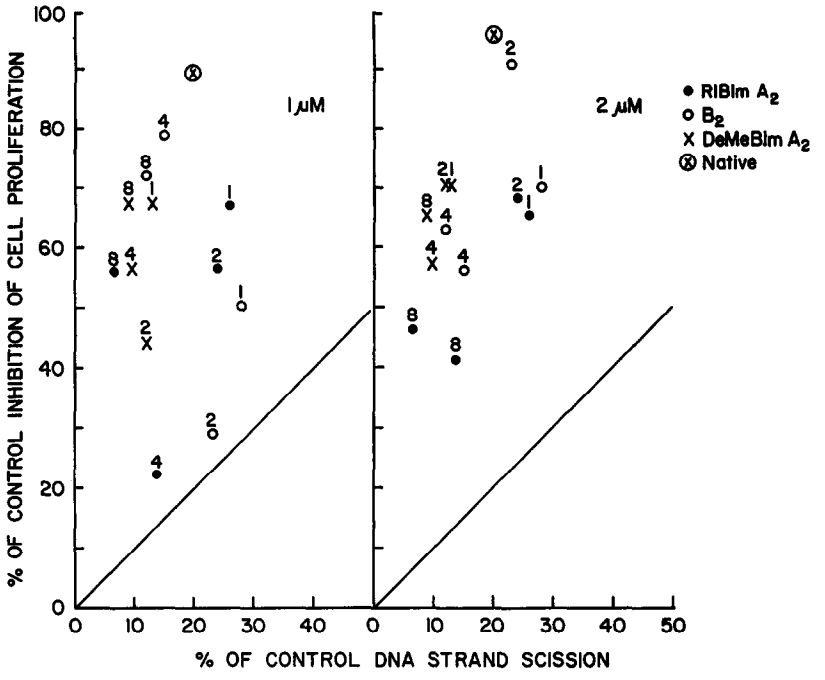

Fig. 4. Growth inhibition by forms of bleomycin as a function of their *in vitro* DNA strand scission activities measured as base-propenal release. Key: (●) FeRIBlm A_2; (○) FeRIBlm B_2; (×) FeRIdMBlm A_2; and (⊗) metal-free native dMBlm A_2. Left panel: growth effects of 1 μM reagent relative to native control samples; right panel: 2 μM. Native dMBlm A_2 was compared with native Blm A_2. Numbers next to data points indicate the ratio of Fe^{2+} to drug used to inactivate bleomycin samples.

cause strand breaks as measured by malondialdehyde-like product formation. Thus, the residual strand scission activity of FeRIBlm can be attributed solely to the presence of native Blm and FeBlm in the mixture of species called FeRIBlm.

Effects of Blx, Blm A_2, and Blm B_2 on Ehrlich cell growth. Native Blx is growth inhibitory to Ehrlich cells grown in culture [18, 20]. Therefore, the growth inhibition by redox-inactivated Blx was examined to see how the chemical inactivation reaction affects its biological activity. Table 3 lists the concentration-dependent effects of various forms of Blx upon cell proliferation. Results are expressed as percentages of untreated cell proliferation. In the absence of drug, cells doubled in number approximately every 24 hr. A perusal of the table shows that generally there was some reduction in growth inhibition by bleomycin after redox inactivation. It was noted in these experiments that Fe(III)Blx was as active as the metal-free drug. Furthermore, extraction of iron from FeRIBlx had no effect on its growth inhibitory properties and also did not affect the activity of Blx, to which Fe^{3+} had been added and then removed.

The relative effects of redox inactivation on growth inhibition and *in vitro* DNA strand scission were next compared graphically by plotting percent of control inhibition of cell proliferation versus percent of control MDA formation for a series of redox inactivations of bleomycins including FeRIBlm A_2, FeRIBlm B_2 and FeRIdBlm A_2 (Fig. 4). If the two parameters are linearly related such that completely redox-inactivated Blm exhibits zero inhibition of cell proliferation and the untreated controls are 100% effective in each test, the experimental behavior would be described by the straight line passing through the origin. Because the drug concentration dependence of growth inhibition was biphasic (Fig. 5), the effect of redox inactivation was only examined

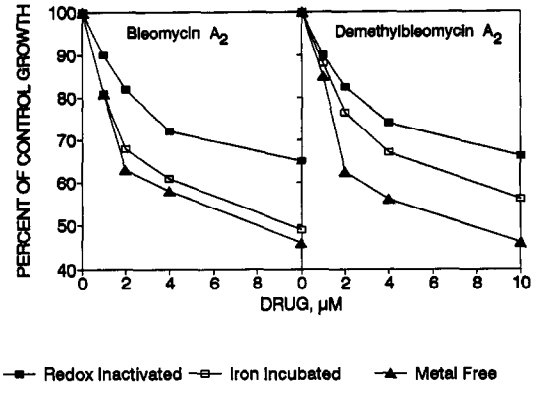

Fig. 5. Comparison of growth inhibition by forms of bleomycin as a function of drug concentration. Key: (▲) bleomycin A_2 or demethyl bleomycin A_2; (□) bleomycin A_2 or demethyl bleomycin A_2 preincubated with an 8-fold excess of Fe^{3+}, and (■) bleomycin A_2 or demethyl bleomycin A_2 preincubated with an 8-fold excess of Fe^{2+} to generate redox-inactivated forms. Incubation time: 24 hr.

in the range of the larger change in cell proliferation rate per unit of bleomycin, between 0 and 2 μM. Because iron remained in the redox-inactivated mixture used during assays of growth inhibition or DNA strand cleavage, percentages in the figure were calculated using native forms of iron-containing drugs as controls. In experiments not shown, iron was removed from redox-inactivated and control samples before testing for growth inhibition and *in vitro* DNA strand scission activity. Plots of those results were qualitatively the same as that shown in Fig. 4.

One can see in Fig. 4 that with few exceptions the experimental points are grouped well above the theoretical line for all concentrations of drugs and for all ratios of Fe^{2+} to Blm originally used in the redox-inactivation reactions. Notably, Blx and its major components, Blm A_2 and Blm B_2, behaved similarly both in terms of control levels of growth inhibition and DNA strand cleavage activities (data not shown) and in their response to redox inactivation.

Effect of redox inactivation on the properties of dMBlm A_2. Included in this study was the examination of a minor component of Blx, dMBlm A_2. According to published results this form of Blm does not bind to DNA and consequently has reduced ability to cleave DNA in the presence of Fe^{2+} and O_2 [21, 22]. In this work it displayed approximately 20% of the DNA strand scission activity of Blx, Blm A_2, or Blm B_2. Nevertheless, according to Figs. 4 and 5, it retained complete ability to inhibit cell proliferation when compared with Blm A_2. Redox inactivation further reduced the capacity of this agent to cause DNA damage and decreased its ability to inhibit cells from growing. It is evident both with the parent dMBlm A_2 species and the redox-inactivated forms of dMBlm A_2 that biological activity can be retained without maintenance of much capacity to damage DNA as measured *in vitro*.

Association of FeRIBlm with cells. Using 1:8 ratios of [^{3}H]Blm A_2 to Fe^{3+} or Fe^{2+}, radiolabeled Fe(III)Blm A_2 and FeRIBlm A_2 were prepared. Following a previous protocol, 10^7 Ehrlich cells/mL were incubated with a 40 μM concentration of either of these species or with [^{3}H]Blm A_2 for 60 min in complete medium, washed three times, and counted for bound drug [20]. Levels of uptake were 19, 21, and 30 pmol/10^7 cells for Blm A_2, FeRIBlm A_2, and FeBlm A_2, respectively. Previous experiments had indicated that Fe(III)Blm accumulates somewhat better than Blm [20]. Importantly, the present results show that the uptake of FeRIBlm A_2 was similar to unmodified forms of Blm A_2. Thus, retention of the growth inhibitory activity of FeRIBlm A_2 in comparison with its loss of reactivity toward DNA *in vitro* was not due to enhanced cellular uptake in comparison with non-redox-inactivated controls.

DISCUSSION

Shortly after it was established that bleomycin and its copper complex were effective antitumor agents in animals, the metal-free material was shown to degrade DNA in cells and to cause DNA strand scission in the presence of mercaptoethanol and oxygen or hydrogen peroxide [21–23]. These observations have become the basis for most of the studies which followed on the mechanism of action of bleomycin [24]. In particular, Horwitz, Peisach, and others were able to rationalize these early results in terms of the *in situ* formation of Fe(II)Blm, which reacts with O_2 and DNA to degrade the polymer [1, 2, 7–9].

To link the biological activity of Blm and its *in vitro* biochemical role in DNA strand cleavage, one must demonstrate at least that some version of the *in vitro* reaction occurs in cells and does so with a dependence on Blm concentration consistent with its involvement in the growth inhibitory response [3–6]. It has now been shown that Blx, CuBlx, and FeBlx decrease the growth of Ehrlich cell populations in a similar dose-dependent fashion [18, 20]. Indeed, they degrade intracellular DNA in similar ways, generating three classes of damage [6]. Only one of these classes, however, may be related mechanistically to inhibition of proliferation for it is produced over the same concentration range of drugs as are the effects on proliferation. This class, F1, comprises 10–20% of the total DNA and appears to result from double-strand cleavage or closely aligned single-strand breaks on opposite strands.

In other studies, it has been shown that F1, but not the other classes of damage, is resistant to rapid cellular repair [25]. This finding further supports F1 DNA damage as a potentially important contribution to toxicity. FeRIBlx and dMBlm A_2, which have lower *in vitro* strand scission activity than FeBlx, generate F1 as well as FeBlx. However, F2 and F3 are depressed markedly at concentrations of Blx and its iron and copper complexes which cause growth inhibition [6]. These findings suggest that F2 and F3 are due to reactions similar to that observed in *in vitro* DNA strand scission, but that F1 may have a different origin.

These studies are continued here. Summarizing, it is shown that the efficacy of Blm in achieving *in vitro* DNA strand scission, varied by altering the conditions of redox inactivation, was not closely coupled to its biological effects on cell proliferation. Indeed, both redox-inactivated Blm and dMBlm A_2 retained much of the growth inhibitory potential of native Blm and dMBlm A_2. Viewed in conjunction with the alkaline elution results [6], these findings indicate that random, single-strand DNA damage inflicted on cells by Blm and probably related mechanistically to *in vitro* single-strand damage was not positively correlated with the inhibition of growth of cell populations seen at low concentrations of Blm.

These observations and conclusions resemble, in some respects, those set forth by Hecht and coworkers [3, 4]. Using several bleomycin congeners including Blm A_2, Blm B_2, and dMBlm A_2, they showed that drug-induced growth inhibition of human KB cells does not correlate with the variable amounts of single-strand breaks caused by these forms of Blm measured by alkaline sucrose gradient centrifugation of cellular DNA [15]. Furthermore, dMBlm A_2 is much less effective in at least single-strand degradation of intracellular DNA than Blm A_2 or B_2 [3, 4]. In contrast, the current results do

not reveal significant differences in the growth inhibitory properties of Blx, Blm A_2, Blm B_2, or dMBlm A_2. Nor do they show differences in base-propenal formation among Blx, Blm A_2 or Blm B_2.

In detail, the present experiments show that exposure of forms of Blm to various levels of Fe^{2+} can decrease subsequent DNA strand scissions activity by 80–90% relative to Blm, as measured by the MDA assay for base propenal formation or by release of acid-soluble radioactivity from [^{14}C]DNA (Figs. 3 and 4 and Table 2). Most of the inactivation occurred at a 1:1 stoichiometry of Fe^{2+} to Blm as shown by a 60–70% loss in DNA strand scission activity relative to FeBlm and a 47% conversion of Blm to new structural forms according to HPLC analysis. Assuming that inactivation occurs when activated FeBlm attacks itself instead of DNA, two electrons are needed during the aerobic reaction of ferrous bleomycin with O_2 to produce this species according to the overall reaction,

$$2\text{Fe(II)Blm} + O_2 \rightarrow \underset{\text{(activated)}}{O_2^{2-}\text{-Fe(III)Blm}'} + \text{Fe(III)Blm} \quad (1)$$

The precise nature of the activated form is unknown [9, 26]. Nevertheless, from the stoichiometry of activation one would predict that at 1:1 Fe^{2+} to Blm, the maximal inactivation would be 50% if it results solely from an intramolecular reaction of the activated intermediate. According to this interpretation and the known 47% conversion of Blm into altered forms, redox-inactivation is highly efficient. Since the activation reaction above also generates stoichiometric amounts of Fe(III)Blm, the addition of increasing concentrations of Fe^{2+} to the reaction mixture can only raise the level of inactivated product asymptotically toward 100%. This, too, was observed in Table 2 and the summary of HPLC results related to Fig. 1.

At present the nature of the modifications of Blm which occur during redox inactivation is unknown, though previous workers have indicated that the pyrimidine or bithiazole moiety is modified [26–28]. According to HPLC separation of redox-inactivated Blm A_2, there are seven resolvable components, which include five altered bleomycins (peaks I–V in Fig. 1), native Blm A_2, and Fe(III)Blm A_2. It is these latter two species which give the redox-inactivated products its residual strand scission activity, as a mixture of peaks I–IV had no *in vitro* capacity to cleave DNA.

Studies of the redox chemistry of FeRIBlx showed that in the presence of the reducing agent, Fe^{2+}, it reacted with oxygen as rapidly as Blx or Fe(III)Blx (Table 1). In the process, according to ESR spectral observations, it also generated the activated intermediate required for *in vitro* degradation of DNA (Eq. 1) by Blx or its ferric complex (Fig. 2c–g). Thus, the reduction in activity of FeRIBlx does not seem to result from the loss of its ability to react with molecular oxygen. Studies to define the structures of the altered materials are continuing. In the present experiments, it is the functional relationship between DNA strand scission and growth inhibitory activities which is investigated.

At all ratios of Fe^{2+} or Fe^{3+} to Blm, HPLC analysis of the products revealed the presence of both Fe(III)Blm and Blm. This is due to the metal-ligand equilibrium properties of the drug, which will be detailed elsewhere.* At pH 7, where $Fe(OH)_3$ readily forms and precipitates from solution, the drug is only about 70% saturated with iron. Interestingly, the Fe(III) complexes of Blx, Blm A_2, and Blm B_2 all displayed less *in vitro* DNA cleavage capacity in the presence of Fe^{2+} than the iron-free drugs. Nevertheless, they were as active as Blm in inhibiting cell proliferation.

In another study, it was shown that 5 and 10 μM FeRIBlx induces no detectable single-strand DNA damage in Ehrlich cells [6]. As described above, this cannot be explained by depressed association of the altered drug with cells in comparison with the parent, unaltered compound; both FeRIBlx and FeBlx are similarly taken up by Ehrlich cells. Since all of these cellular studies were carried out on one cell type, it is not likely that these cells respond differentially to the various forms of native and inactivated drugs causing different rates of repair of the DNA damage. When redox-inactivated FeBlx was tested for biological activity, substantial, though reduced, inhibition of growth was observed at all levels examined (Table 3). Figure 4 clearly shows that relatively more of this property remained after redox inactivation of Blx, Blm A_2, or Blm B_2 than inherent capacity to cause single-strand cleavage of DNA. Together with the lack of cellular single-strand damage caused by FeRIBlx, these data support the view that growth inhibitory activity of bleomycin is not tightly coupled to its ability to cause single-strand breaks in DNA.

Similar conclusions were reached from the examination of dMBlm A_2 and its redox-inactivated products (Fig. 4). In this case the starting form was only about 20–30% as effective as Blx, Blm A_2 or Blm B_2 in the *in vitro* DNA strand cleavage reaction. This was due to the absence of the positively charged dimethylsulfonium group, which stabilizes the binding interaction of Blm A_2 with DNA [12]. Nevertheless, it was completely active in inhibiting cells from proliferating in comparison with Blm A_2 (Fig. 5). Redox inactivation further depressed its DNA strand scission properties relative to Blm A_2 and modestly reduced its ability to inhibit proliferation of Ehrlich cells. Like FeRIBlx, dMBlm A_2 displayed little ability to cause single-strand DNA damage in cells while retaining the capability to inhibit cell proliferation.

Although redox-inactivated bleomycin and dMBlm A_2 display reduced single-strand cleavage activity in cells, they all retain substantial capability to produce apparent double-strand breaks [6]. Thus, double-strand breakage of DNA may be causally related to the growth inhibitory properties of the drug. The fact that single- and apparent double-strand breakage can be uncoupled in cells raises the question of whether double-strand cleavage results from a

* Byrnes RW, Xu R, Antholine WE and Petering DH, manuscript to be submitted.

fundamentally different mechanism than single-strand damage.

Acknowledgements—The authors appreciate the support of NIH Grant CA-22184 and American Cancer Society Grant CH-466.

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