Cancer Chemother Pharmacol (1984) 12: 111–115

Cancer Chemotherapy and Pharmacology

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A comparison of the pulmonary toxicity and chemotherapeutic activity of bleomycin-BAPP to bleomycin and pepleomycin

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Summary. *The pulmonary toxicity and antitumor activity of a new bleomycin analog butylamino-3-propylamino-3-propylamine (Blm-BAPP) was investigated and compared with bleomycin and pepleomycin. Blm-BAPP was significantly more pulmonary toxic than bleomycin and had no greater activity against B_{16} melanoma than either bleomycin or pepleomycin. Although pepleomycin was as equitoxic as bleomycin in producing pulmonary fibrosis, doses of pepleomycin greater than 5 mg/kg were more lethal than bleomycin. Not only did the three drugs function similarly in vivo, but they behaved similarly in two in vitro test systems: microsome-catalyzed drug-mediated DNA deoxyribose cleavage and binding to DNA.*

Introduction

One of the major problems associated with cancer chemotherapeutic agents is the existence of dose-limiting organ-specific toxicities [16]. For example, the clinical usefulness of bleomycin, an antibiotic which exhibits a significant therapeutic effect against certain squamous cell carcinomas, Hodgkins disease, and testicular carcinomas [2, 3, 6] is limited by the development of life-threatening pulmonary fibrosis [11]. In contrast to a number of other antineoplastic agents, bleomycin does not depress the bone marrow to a significant extent, thus making it an attractive candidate when designing combination chemotherapy regimens. Several approaches have been utilized to circumvent or minimize the organ-specific toxic reactions elicited by cancer chemotherapeutic agents. These include: (a) the co-administration of an agent(s) capable of preventing a potentially toxic reaction, as illustrated by the administration of sulfhydryl-containing compounds or α-tocopherol with adriamycin [14, 15]; (b) the scheduling of a drug, such as by continuous infusion, in order to reduce the exposure of susceptible host tissues [18]; and (c) to isolate or to synthesize an analog of the parent compound which exhibits either an equal or enhances therapeutic effect but less toxicity, namely, a higher therapeutic index.

Matsuda et al. [12] reported that the bleomycin analog, bleomycin-BAPP (Blm-BAPP), exhibited higher antitumor activity than bleomycin against two transplantable tumor systems and a chemically induced gastric carcinoma. More important perhaps is that Blm-BAPP elicited, as indicated by light microscopy, both a lower incidence and a lower grade of pulmonary fibrosis relative to bleomycin. Interestingly, Blm-BAPP was similar in both its therapeutic and toxic actions to another bleomycin analog, pepleomycin, which had previously been shown by Sikic et al. [20] to exhibit less pulmonary toxicity, as indicated in this case by lung hydroxyproline content, an index of pulmonary collagen. In contrast, Raisfeld [17] reported that the pulmonary fibrosis elicited by both Blm-BAPP and pepleomycin either equalled or exceeded that of bleomycin and that Blm-BAPP was a much more potent stimulator of pulmonary metaplasia. It must be noted, however, that in these latter studies, the drugs were administered directly into the lung by intratracheal instillation. Interestingly, Matsuda et al. [12] demonstrated that when Blm-BAPP was administered to animals SC that lower concentrations of drug accumulated in the lung when compared to bleomycin, but in contrast pepleomycin was distributed to the lung in equal or even higher concentrations than bleomycin. These observations suggest that Blm-BAPP has the potential to initiate toxic reactions in lung cells but suggest that when given systemically, the concentration achieved in the lung may not be sufficient to evoke toxicity in lung cells. Thus, it was of interest to evaluate both the therapeutic effects and pulmonary toxicity of Blm-BAPP relative to bleomycin and pepleomycin given systemically (Fig. 1).

Materials and methods

Animals. Male BDF_1 mice weighing 15–20 g were obtained from Harlan-Sprague-Dawley, Madison, Wis., and were fed Purina chow and water ad libitum.

Chemicals. NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, calf thymus DNA, and 2-thiobarbituric acid were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of the highest purity available.

Drug doses and administration. Bleomycin (NSC 125066), pepleomycin (NSC 276382), and Blm-BAPP (NSC 294979) were obtained from the Natural Products Section, Developmental Therapeutics Program, National Cancer Institute. The drugs were given at the following doses in both the toxicity and therapy studies: bleomycin 5, 10, 15 mg/kg; pepleomycin 5, 6.5, 8, 10, 15 mg/kg; and Blm-BAPP 5, 7.5, 10, 12.5, 15 mg/kg. All drugs were dissolved in 0.9% NaCl solution and administered SC in the left flank. Control mice received SC injections of 0.9% NaCl.

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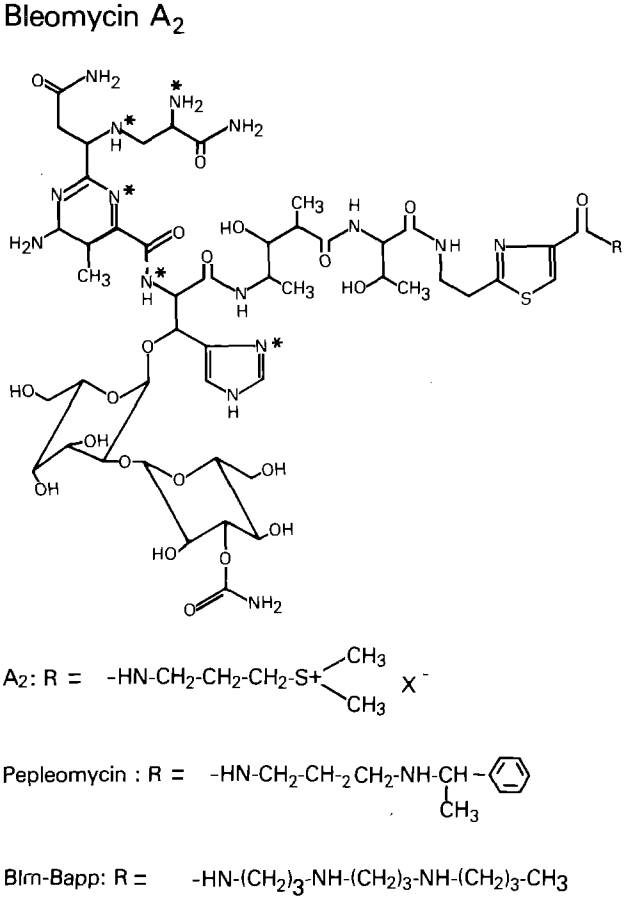

Fig. 1. Chemical structure of bleomycin A_2, pepleomycin, and Blm-BAPP; * denotes metal-binding sites

Pulmonary toxicity studies. For measurements of pulmonary toxicity, the drugs were injected twice weekly for 4 weeks and the mice killed 8 weeks after the initial treatment. The mice were killed by cervical dislocation, the lungs removed, blotted, and their weights recorded. The lungs were transferred to Pyrex hydrolysis tubes (Preiser Scientific, Inc., Charleston, WVa), five volumes of 6 N HCl were added, and the tubes were sealed and placed in an oven at 130° C for 3 h. The sample was then allowed to cool and titrated to pH 6–7 and diluted to a final volume 400 times the original wet lung weight with glass distilled water. Two-milliliter aliquots were assayed in duplicate for whole-lung hydroxyproline content as described by Woessner [26]. Tissue blanks consisted of a sample aliquot with perchloric acid added before chloramine-T. Data are expressed as micrograms of hydroxyproline/whole lung.

Therapeutic studies. B_{16} melanoma was originally obtained from EG & G Mason Research Institute, Worcester, Mass. For transplantation into additional mice, B_{16} melanoma was prepared by careful mincing and passing the tumor through sequentially smaller-gauge needles to form a homogenous suspension of cells in Hanks' buffered salt solution. Cell viability was determined using trypan blue exclusion, and 10^6 cells in 0.1 ml were injected SC into the right flank of each animal. Treatments began on day 4 after implantation with daily SC injection of the drug for 10 days. The length and width of the tumors were measured at 5-day intervals, and the survival time of tumor-bearing animals was recorded.

Microsomal preparation. Mice were killed by cervical dislocation and control lungs (30 lungs/pool), or B_{16} melanoma were quickly excised and placed in ice-cold 150 mM KCl-50 mM Tris-HCl, pH 7.4. The tissues were homogenized using a Potter-Elvehjem-type homogenizer fitted with a Teflon pestle. Microsomes were prepared by differential centrifugation as previously described [9]. The microsome pellet was resuspended in KCl-Tris buffer and assayed for protein content [10]. Microsomes were diluted to 3.5 mg protein/ml in O_2 saturated KCl-Tris buffer.

Assay for drug-mediated DNA deoxyribose cleavage. DNA exposed to an activated bleomycin intermediate results in the cleaving of deoxyribose, resulting in an aldehyde group which reacts with 2-thiobarbituric acid, yielding a chromophore which can be read spectrophotometrically at 533 nm [4]. Microsomes (final concentration 0.5 mg protein/ml) were incubated with drug [100 μM bleomycin A_2 (NSC 146842), pepleomycin, or Blm-BAPP], 0.25 mg/ml DNA, and an NADPH regenerating system (1.9 mM NADP, 20 mM glucose-6-phosphate, 1.1 IU/ml glucose-6-phosphate dehydrogenase, 4.3 mM magnesium chloride) in the absence or presence of 100 μM Fe^{3+} [22]. All incubations were conducted at 37° C in a Dubnoff metabolic shaker under an O_2 atmosphere (flow rate 5 l/min) for 60 min. The reactions were stopped by the addition of 0.75 ml 2.0 M trichloroacetic acid – 1.7 N HCl, and the tubes were centrifuged at 1,000 *g* for 10 min to pellet the denatured protein. The supernatant (0.5 ml) was reacted with 2.0 ml of 1% (w/v) 2-thiobarbituric acid (TBA) at 90° C for 10 min. After cooling, the absorbance was read at 533 nm, and the nmoles TBA-reacting product mg protein/60 min was calculated using an extinction coefficient of 1.6×10^5 $M^{-1} \cdot cm^{-1}$. The basis of this assay has been described elsewhere [4, 24, 25].

Fluorescence quenching by DNA. Chien et al. have used the quenching of bleomycin fluorescence by DNA to study the interaction between bleomycin and DNA [5]. Fluorescence was measured in an Aminco SPF-500 spectrofluorometer in a quartz cuvette. The reaction mixture (2.0 ml) consisted of 2.5 mM Tris-HCl and 1.2 mM NaCl, pH 8.4 [5, 8]. Drugs were studied at a concentration of 14 μM and DNA at 0.875 mg/ml final concentration. Fluorescent quenching was measured at 360 nm with an extinction of the excitation set at 300 nm.

Statistics. Statistical analysis was performed using the Student's *t*-test and results were considered significant if $P < 0.05$ [21].

Results

Comparison of the therapeutic activity of Blm-BAPP, pepleomycin and bleomycin against B_{16} melanoma. The administration of bleomycin to tumor-bearing mice has previously been shown to reduce the growth rate of B_{16} melanoma significantly [20]. While both Blm-BAPP and pepleomycin markedly inhibited tumor growth (Fig. 2), neither analog was more effective than bleomycin at any of the doses tested, although each drug treatment was significantly different from untreated animals. Twenty-four days after tumor implantation, 100% of the animals were still alive following the administration of Blm-BAPP or pepleomyin at 5 mg/kg, while only 75% of the animals were alive at this time point with bleomycin at 5 mg/kg;

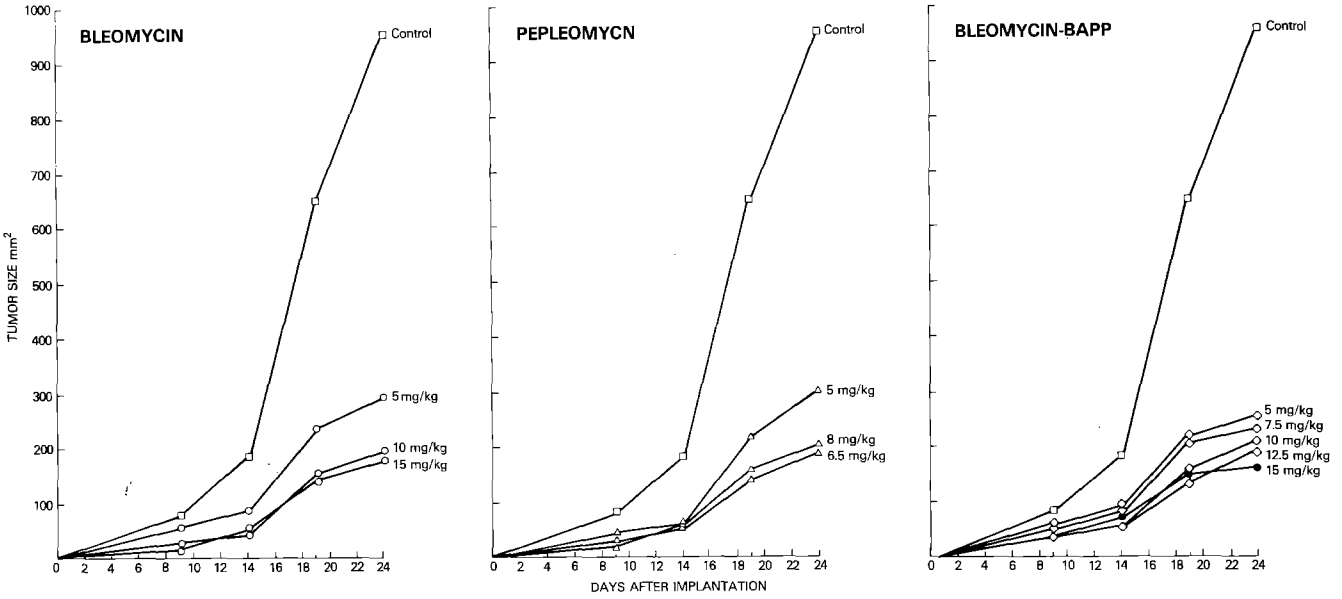

Fig. 2. Therapeutic effect of bleomycin, pepleomycin and Blm-BAPP on tumor size, 0–24 days after implantation. Four days after implantation, animals were injected daily for ten days. Tumors were implanted, measured and recorded as described in Materials and Methods. $n = 8-16$ for each dose. The doses tested were not significant from each other, but were significant from control at $P < 0.001$

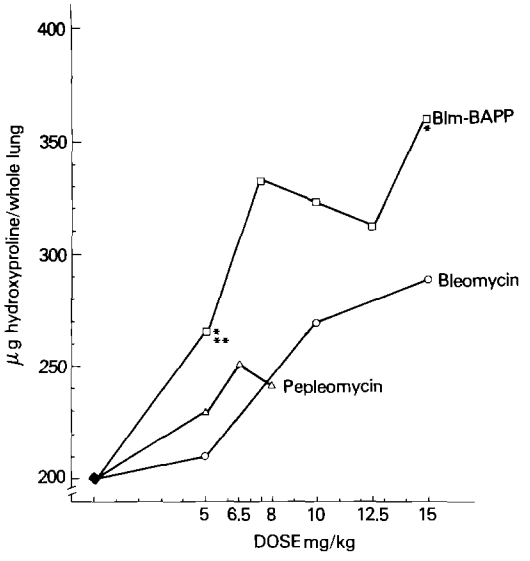

Fig. 3. Toxicity of bleomycin, pepleomycin, and Blm-BAPP at varying doses. Whole-lung hydroxyproline content was measured as described in Materials and methods. Animals were injected twice weekly for 4 weeks, * $P < 0.01$ from bleomycin; ** $P < 0.05$ from pepleomycin. All data points are significant from control at $P < 0.01$

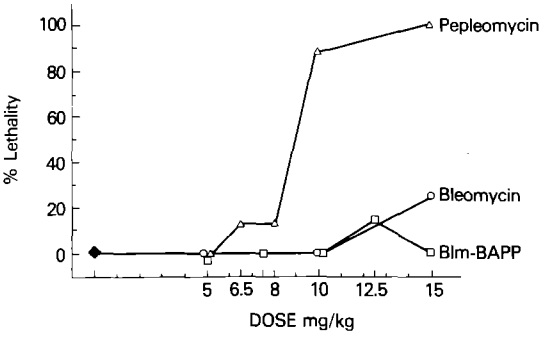

Fig. 4. Percentage lethality of bleomycin, pepleomycin, and Blm-BAPP at varying doses. Animals were injected as in Fig. 3. At 15 mg/kg bleomycin, four of 16 animals died. At 12.5 mg/kg Blm-BAPP, two of 16 animals died

at higher doses of the three compounds there was even lower survival which can be attributed to lethal drug toxicity. Thus, although there is a reduction in tumor growth and hence a therapeutic effect, drug-induced host toxicity appears to be a contributing factor in the survival of tumor-bearing animals, which is what appears to happen clinically.

Comparison of the pulmonary toxicity of Blm-BAPP, pepleomycin and bleomycin. Whole-lung collagen, as measured by hydroxyproline content, has previously been shown to be a reliable index of drug-elicited pulmonary toxicity [20]. Of the three drugs and several doses studied, only bleomycin at 5 mg/kg did not produce a significant increase in lung hydroxyproline content (Fig. 3). Blm-BAPP was 1.5 times more potent than bleomycin in producing pulmonary toxicity even at the lowest dose, while on the other hand, pepleomycin was not significantly more pulmonary toxic than bleomycin at 5 mg/kg. Pepleomycin could not, however, be compared to bleomycin at the higher doses (10 and 15 mg/kg) because of the marked lethality produced by this compound, even at 8 mg/kg (Fig. 4). In general, bleomycin and Blm-BAPP were about equilethal while pepleomycin produced about five times greater lethal toxicity at all doses (Fig. 4).

In vitro interaction of bleomycin A_2, Blm-BAPP and pepleomycin with DNA. Although the exact mechanism by which bleomycin exerts its action on tumor cells is not presently understood, drug-mediated alterations in DNA are believed to contribute to this process [13]. When bleomycin binds to DNA, the fluorescence of the bithiazole moiety of bleomycin is quenched [5, 8]. The data in Table 1 demonstrate that both Blm-BAPP and pepleomycin bind to DNA as indicated by a

Table 1. Effects of DNA on the fluorescence of bleomycin A_2 ($BlmA_2$), pepleomycin (Pep) and Blm-BAPP

	Relative fluorescence[a]
Blm A_2	0.731 ± 0.003
Blm A_2, DNA	0.662 ± 0.010*
Pep	0.660 ± 0.003
Pep, DNA	0.577 ± 0.006*
BAPP	0.783 ± 0.003
BAPP, DNA	0.640 ± 0.016*

[a] Expressed in arbitrary units, mean ± SD, $n = 3$
* $P < 0.001$ from no DNA

Table 2. Comparison of bleomycin A_2 (Blm A_2), pepleomycin (Pep), and Blm-BAPP in mediating DNA chain breakage catalyzed by microsomes from control lung or B_{16} melanoma

Source of microsomes	Additions	Drug-mediated-DNA deoxyribose cleavage[a]
Control lung	Blm A_2	37.2 ± 2.9
	Blm A_2, Fe^{3+}	80.2 ± 8.0
	Pep	38.2 ± 1.3
	Pep, Fe^{3+}	84.8 ± 3.5
	BAPP	44.5 ± 4.3
	BAPP, Fe^{3+}	81.9 ± 3.3
B_{16} Melanoma	Blm A_2	16.7 ± 3.7
	Blm A_2, Fe^{3+}	52.9 ± 10.9
	Pep	24.9 ± 5.3*
	Pep, Fe^{3+}	46.1 ± 23.7
	BAPP	17.9 ± 3.3
	BAPP, Fe^{3+}	58.7 ± 8.4

[a] Expressed as TBA reacting product/mg protein/60 min; mean ± SD, $n = 3-10$
* $P < 0.05$ from Blm A_2

quenching of their fluorescence and, in fact, their fluorescence was quenched by DNA to a greater extent than bleomycin. This suggests that these two compounds may have a greater affinity for this potential target molecule than bleomycin.

Even though bleomycin can bind to DNA, this action in itself does not result in DNA-damage such as base release or deoxyribose cleavage [4]. Studies in cell-free chemical systems have indicated that these actions are facilitated by an activated bleomycin intermediate [4] utilizing iron and molecular oxygen as cofactors. Previous studies in this laboratory have established that the microsomal mixed-function oxidase system is an efficient biological system capable of catalyzing the formation of this DNA-damaging bleomycin intermediate [24, 25]. The data in Table 2 demonstrate that the interaction of bleomycin A_2, Blm-BAPP, and pepleomycin with microsomes isolated from either the lung, the organ of toxicity, or B_{16} melanoma results in significant deoxyribose cleavage. Moreover, the addition of exogenous ferric iron as a cofactor resulted in a significant enhancement of deoxyribose cleavage [22, 23].

Discussion

In contrast to the results of Matsuda et al. [12] and Sikic et al. [20], which suggested that the bleomycin analogs Blm-BAPP and pepleomycin may be more useful clinically because they produced less pulmonary toxicity, the results of this study do not support this contention. Neither Blm-BAPP nor pepleomycin was less pulmonary toxic than bleomycin and, in fact, Blm-BAPP was significantly more pneumotoxic than bleomycin (Fig. 3). This observation is in agreement with Raisfeld [17] with Blm-BAPP and demonstrates that both the systemic and intratracheal administration of this compound results in damage to lung cells which ultimately results in pulmonary fibrosis. In agreement with the report of Sikic et al. [20], we also observed that pepleomycin was substantially more lethal than bleomycin. This response may be related to the unique toxic effect of this compound on the nervous system, as previously described by Sikic et al. [20].

Although many of the morphological and physiological changes associated with bleomycin pulmonary toxicity are well characterized [1, 19], the underlying biochemical and molecular events by which bleomycin initially damages lung cells and thereby sets into motion a complex sequence of cellular events, which ultimately results in the deposition of collagen, is not well understood. Since DNA is generally considered to be the cellular target by which bleomycin causes cytotoxicity and exerts its antiproliferative activity, it is possible that bleomycin-mediated damage to DNA could also contribute to the pulmonary toxicity of bleomycin [24, 25], since DNA damage has been implicated in the pulmonary toxic effects of radiation [7]. The in vitro results presented in this study demonstrate that not only bleomycin A_2 but that also two of its analogs can produce a similar lesion in DNA, that is, deoxyribose cleavage. This iron-dependent reaction was catalyzed by the mixed-function oxidase system of microsomes isolated from both tumor and lung.

The fact that all three compounds responded identically in this in vitro system was not surprising since their structures are identical in the metal-binding site (Fig. 1), which may also account for the fact that all three compounds responded similarly in vivo from both a therapeutic and pneumotoxic standpoint. In this context it is interesting to note that the fluorescence of Blm-BAPP was quenched to the greatest extent by DNA, which may allow it to elicit a pulmonary toxic response at lower doses than bleomycin.

References

1. Bedrossian CWM, Luna MA, MacKay B, Lichteger B (1973) Ultrastructure of bleomycin toxicity. Cancer 32:44
2. Blum RH, Carter SK, Agre K (1973) A clinical review of bleomycin – a new antineoplastic agent. Cancer 31:903
3. Bonnadonna G, DeLena M, Monfardini S, Bartali C, Bajetta E, Beretta G, Fossati-Bellani F (1972) Clinical trials with bleomycin in lymphomas and in solid tumors. Eur J Cancer 8:205
4. Burger RM, Peisach J, Horwitz SB (1981) Mechanism of bleomycin action: In vitro studies. Life Sci 28:715
5. Chien M, Grollman AP, Horwitz SB (1977) Bleomycin-DNA interactions: fluorescence and proton magnetic resonance studies. Biochem 16:3641
6. Einhorn LH, Donohue J (1977) cis-Diamminedichloroplatinum, vinblastine, and bleomycin combination chemotherapy in disseminated testicular cancer. Ann Intern Med 87:293
7. Gross NJ (1982) The pathogenesis of radiation-induced lung damage. Lung 159:115
8. Huang C-H, Galvan L, Crooke SL (1980) Interactions of bleomycin analogues with deoxyribonucleic acid and metal ions studied by fluorescent quenching. Biochem 19:1761
9. Litterst CL, Mimnaugh EG, Reagan RL, Gram TE (1975) Comparison of in vitro drug metabolism by lung, liver and kidney of several common laboratory species. Drug Metab Dispos 3:259

10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193: 265
11. Luna MA, Bedrossian CWM, Lichteger B, Salem PA (1972) Interstitial pneumonitis associated with bleomycin therapy. Am J Clin Pathol 58: 501
12. Matsuda A, Yoshioka O, Ebihara K, Ekimato H, Yamashita T, Umezawa H (1978) The search for new bleomycins. In: Bleomycin: Current status and new developments. Academic Press, New York, p 299
13. Miyaki M, Morohasi S, Ono T (1973) Single strand scission and repair of DNA in bleomycin sensitive and resistant rat ascites hepatoma cells. J Antibiot (Tokyo) 26: 369
14. Myers CE, McGuire W, Young R (1976) Adriamycin: amerlioration of toxicity by α-tocopherol. Cancer Treat Rep 60: 961
15. Olson RD, MacDonald JS, van Boxtel CJ, Boerth RC, Harbison RD, Slonim AE, Freeman RW, Oates JA (1980) Regulating role of glutathione and soluble sulfhydryl groups in the toxicity of adriamycin. J Pharmacol Exp Ther 215: 450
16. Perry MC (1982) Chemotherapy, toxicity, and the clinician. Semin Oncol 9: 1
17. Raisfeld IH (1980) Pulmonary toxicity of bleomycin analogs. Toxicol Appl Pharmacol 56: 326
18. Sikic BI, Collins JM, Mimnaugh EG, Gram TE (1978) Improved therapeutic index of bleomycin when administered by continuous infusion in mice. Cancer Treat Rep 62: 2011
19. Sikic BI, Young DM, Mimnaugh EG, Gram TE (1978) Quantification of bleomycin pulmonary toxicity in mice by changes in lung hydroxyproline content and morphometric histopathology. Cancer Res 38: 787
20. Sikic BI, Siddik ZH, Gram TE (1980) Relative pulmonary toxicity and antitumor effects of two new bleomycin analogs, pepleomycin and tallysomycin A. Cancer Treat Rep 64: 659
21. Snedecar GW, Cochran WG (1972) Statistical methods. The Iowa State University Press, Ames
22. Trush MA (1983) Studies on the interaction of bleomycin A_2 with rat lung microsomes: III. Effect of exogenous iron on bleomycin-mediated DNA chain breakage. Chem Biol Interact 45: 65
23. Trush MA (1982) Demonstration that the temporary sequestering of adventitious iron accounts for the inhibition of microsomal lipid peroxidation by bleomycin A_2. Res Commun Chem Pathol Pharmacol 37: 21
24. Trush MA, Mimnaugh EG, Ginsburg E, Gram TE (1982) Studies on the interaction of bleomycin A_2 with rat lung microsomes: I. Characterization of factors which influence bleomycin-mediated DNA chain breakage. J Pharmacol Exp Ther 221: 152
25. Trush MA, Mimnaugh EG, Ginsburg E, Gram TE (1982) Studies on the interaction of bleomycin A_2 with rat lung microsomes: II. Involvement of adventitious iron with reactive oxygen in bleomycin-mediated DNA chain breakage. J Pharmacol Exp Ther 221: 159
26. Woessner JF (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Arch Biochem Biophys 93: 440

Received December 6, 1982/Accepted October 9, 1983