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Analytical and biological inequivalence of two commercial formulations of the antitumor agent bleomycin

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Abstract Bleomycin is an antitumor agent which is a mixture of glycopeptides containing at least 55–75% bleomycin A_2 and 25–32% bleomycin B_2 fractional composition. Two bleomycin formulations, bleomycin sulfate, USP (Blenoxane, Bristol-Myers Squibb Oncology, Princeton, N.J.) and bleomycin HCl (Tianjin Hebei Pharmaceutical, Tianjin, China) were compared analytically and biologically. Reverse-phase high-performance liquid chromatography (HPLC) analyses using the USP methodology showed that Blenoxane contained primarily (69%) bleomycin A_2 and 29.3% bleomycin B_2. In contrast, Tianjin-supplied bleomycin HCl contained 97% bleomycin A_5 fraction. In vitro tumor cell growth inhibition assays showed equivalent activity in human OVCAR-3 ovarian cancer cells and slightly greater potency in murine L-1210 leukemia cells for the Tianjin formulation. In C57/Bl mice bearing B-16 melanoma tumors, Tianjin-supplied bleomycin produced slightly greater tumor growth inhibition at the expense of greater drug-induced lethality at higher dose levels. These studies show there are significant differences in two international bleomycin formulations. These compositional differences lead to altered biologic effects.

Key words Anticancer · Bleomycin · Equivalence

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Introduction

Bleomycin is an antibiotic agent with tumor activity in a number of neoplastic diseases including testicular cancer, non-Hodgkin's lymphomas, head and neck cancer, Kaposi's sarcoma and cervical cancer [1]. The mechanism of action involves iron chelation and the subsequent generation of oxygen free radicals in the presence of molecular oxygen. This causes DNA strand-breaks which produces lethality, primarily in the G_2 phase of cell division [2, 3].

Chemically, bleomycin is a mixture of related glycopeptides obtained from fermentation cultures of *Streptomyces verticillus* [4]. The molecular weight of the mixture is approximately 1400 Da. In the US it is commercially supplied by Bristol-Myers Squibb as the sulfate salt which is water soluble. Bleomycin USP has a stated potency of not less than 1.5 Units and not more than 2 Units/mg [4]. The bleomycin complexes elaborated by *S. verticillus* include a number of related subfractions. There are at least five bleomycin A fractions and two B fractions which differ primarily in their terminal amine moiety (see Fig. 1). To meet USP standards, the content of the bleomycin A_2 fraction should be between 55% and 75% and the content of the bleomycin B_2 fraction should be between 25% and 32%. The content of bleomycin B_4 fraction should not be more than 1% and the combined percentage of A_2 and B_2 bleomycin fractions must not be less than 85%. Since this molecule is known to also chelate copper, resulting in an inactive antitumor agent, the commercial formulation should contain $<0.1\%$ copper. As such, active bleomycin fractions will contain three basic nitrogens with pKa values of 7.3, 4.7 and approximately 2.9.

Because bleomycin is a mixture of active fractions, the pharmacology of the different composite fractions can become clinically important. Specifically, previous toxicology studies suggest that there are substantial differences in the biological activities of the A_2, A_5 and B_2 fractions which together account for over 98% of the

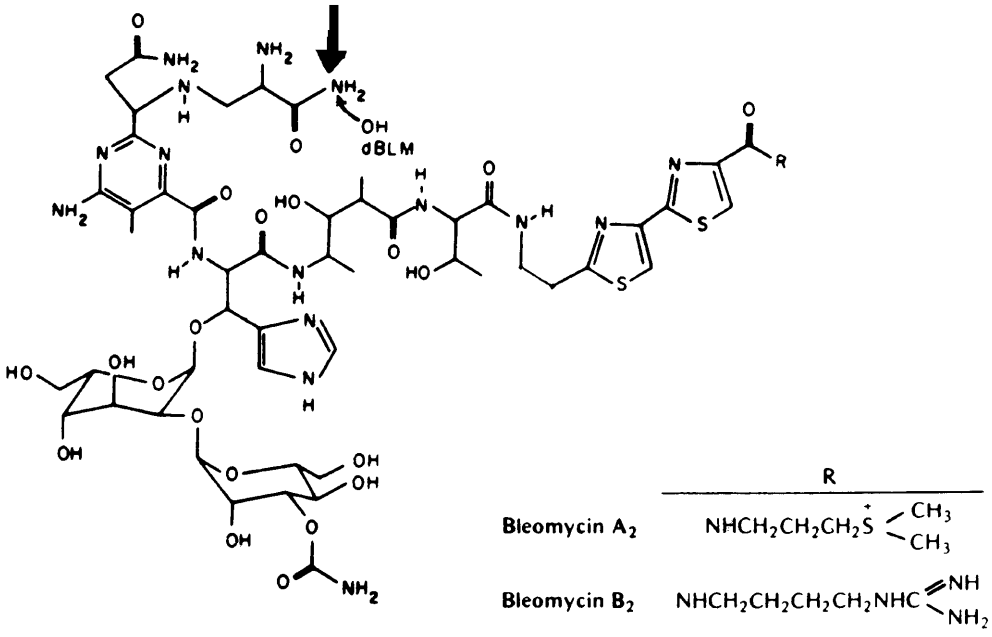

Fig. 1 The structure of the different major bleomycin fractions

standardized USP formulation [5, 6, 7]. Standardization and equivalence of formulations have been shown to be important considerations in evaluating clinical toxicity of generically available anticancer agents, such as cytarabine, from different manufacturers [7]. This would be even more important for antitumor agents comprising of mixtures of active fractions, as is the case with bleomycin, which is currently available worldwide from different manufacturers. In the study reported here two international bleomycin formulations were compared using (1) high-performance liquid chromatography (HPLC) for analysis of fractional bleomycin content, (2) in vitro antitumor assays, and (3) in vivo assays of antitumor activity. The results show that formulation differences may alter the biological antitumor activity of this unique antibiotic agent.

Materials and methods

Drugs

Blenoxane was purchased from Bristol-Myers Squibb Oncology Products and was supplied as 15 Units lyophilized powder in rubber-stoppered glass vials. Each vial contained 15 Units bleomycin and was manufactured by Nippon Kayaku Company, Tokyo, Japan. It was stored at 4 °C prior to reconstitution in 0.9% sodium chloride for injection, USP. Purified bleomycin fractions A_2, A_5 and B_2 were kindly supplied by Nippon Kayako Company.

Bleomycin HCl was supplied in glass ampules containing 15 mg lyophilized white powder. It was manufactured by Tianjin Hebei Pharmaceutical Factory, Tianjin, China. It was exported by BS Medicinal and Biochemical Company, Shenzhen, China, under Import License Number 013290. The batch number was 911101 with a label reading "Use Before: 5/1994" and it was stored at 4 °C prior to reconstitution in 0.9% sodium chloride for injection, USP.

HPLC analytical procedures

Two previously reported HPLC methods were utilized for assaying the composition of the two different bleomycin formulations. For both methods, bleomycin powder was reconstituted with sterile water for injection immediately prior to analysis. A Waters Radial-Pak C-18 stationary phase cartridge was employed in both procedures (Waters Corporation, Milford, Mass.). The flow rate was maintained by a Waters Model 510 Solvent Delivery System. Bleomycin was detected by UV absorbance at 254 nm using a Waters Model 441 UV detector. Samples were injected via a Waters 712 WISP autoinjector. Data were recorded and processed with Perkin Elmer Nelson Analytical Software (Model 2600 Chromatography Software, rev 3.0; Nelson Analytical, Cupertino, Calif.).

Method 1

This reverse phase (C-18) method was based on that of Shiu et al. [8]. The flow rate was 2.0 ml/min using an isocratic mobile phase comprising a 30:70 mixture of acetonitrile/0.2 *M* ammonium acetate. Bleomycin was quantitated by UV absorbance at 254 nm using peak area integration. A 10-µl injection volume was used. The published elution order in this procedure is bleomycin A_2, B_2 and A_1. Bleomycin A_5 is not mentioned in this report, but has been shown to elute between the A_2 and B_2 fractions in the current studies using purified bleomycin A_5.

Method 2 (USP method)

This reversed-phase system also utilizes a C-18 stationary phase (5–10 µm particle size) with a linear gradient elution of 10% to 40% methanol in an aqueous buffer ramped over 60 min [4]. The buffer consisted of 960 mg sodium 1-pentanesulfonate dissolved in 1 l of 0.08 *N* acetic acid and adjusted to pH 4.3 with ammonium hydroxide.

Detection was via UV absorbance at 254 nm. The elution order is bleomycinic acid, bleomycin A_2 (anticipated major peak), A_5, and B_2 (second major peak) and B_4, and desmethylbleomycin A_2.

In vitro tumor growth inhibition studies

These studies were performed in tumor cell lines using either a mitochondrial enzyme (MTT) assay for suspension cultures [9] or a protein dye assay, sulforhodamine B (SRB), for adherent tumor cells [10]. Both assays are currently used by the US National

Cancer Institute for testing antitumor activity of new compounds [10]. Drugs are added to 5×10^3–10^4 cells/well in 96-well 1.0-ml plastic plates containing RPMI-1640 growth medium with 5–10% fetal bovine serum. Cells are exposed continuously to the drugs at a (logarithmic) range of concentrations. After 8–14 days of incubation at 37 °C in a humidified air/CO_2 (95%/5%) atmosphere, the wells are washed and cells harvested for measurement of MTT (tetrazolium) dye reduction by mitochondrial enzymes from viable cells [10]. The colored formazan product is solubilized in DMSO and absorbance is measured at 540 nm on an automated plate reader. The results are expressed in relation to untreated control wells, minus the low background absorbance. Murine L-1210 leukemia cells have been studied using this assay [11].

The SRB assay has been used to assess growth inhibitory effects in an adherent human OVCAR-3 ovarian adenocarcinoma cell line [12]. The method used followed that of Skehan et al. as currently practised by the National Cancer Institute (NCI) [10]. Tumor cells are plated at densities of 1000–2000/well in 96-well plastic microtiter plates. After growth in RPMI-1640 culture medium containing 10% (v/v) fetal bovine serum and exposure to potential cytotoxic agents, the cells are washed and fixed with trichloracetic acid. Total protein in the cells is then stained with 0.4% (w/v) SRB dye for 30 min. Unbound dye is rinsed off using 1% acetic acid and protein is quantitated by UV absorbance at 564 nm [10]. For both assays, bleomycin testing was by the continuous exposure method to take into account the marked schedule dependence of cell killing by this agent [13].

Biological studies in mice

Acute lethality studies

These studies were performed in adult, 20–25 g, female BALB/c mice (Jackson Laboratories, Bar Harbor, Me.). The mice were weighed and injected intraperitoneally (IP) on a daily × 4–5 basis at a daily dose of 100 mg/kg. Two studies were performed: an initial study using a fixed 500 mg/kg (cumulative) dose ($n = 5$ per group), and a subsequent trial comparing 400 mg/kg and 500 mg/kg doses. The mice were weighed two or three times per week and were allowed access to food and water *ad libitum*. They were housed four to a cage (ten mice per treatment group) on a 12-h light/dark cycle. Postmortem examinations were not performed.

Antitumor efficacy in vivo

In vivo efficacy was assessed in adult female C57/Bl mice (Jackson Laboratories) using the B-16 melanoma model [14]. In this model, anesthetized mice are injected with 10^5 freshly harvested B-16 melanoma cells into the right front flank muscle. Intraperitoneal drug dosing is begun 24 h later at a volume of 0.1 ml/10 g body weight using a daily × 9 schedule. Two dose levels were evaluated for each formulation: 4 or 8 mg/kg per days for 9 consecutive days based on the previous studies of Goldin and Kline [15]. Palpable tumors were measured three times per week and bidimensional measurements (made by caliper) were converted to mass using the formula [14]:

$$\frac{\text{Length (mm)} \times \text{Width (mm)}^2}{2} = \text{Tumor mass (mg)}$$

Both studies, toxicity and antitumor efficacy, used the commercially supplied bleomycin formulations, either Blenoxane (Bristol-Myers Squibb), or bleomycin HCl (Tianjin Hebei Pharmaceutical).

Statistical analyses

Survival in normal mice given high dose bleomycin injections was assessed using the Wilcoxon method [16]. Differences in tumor mass between control mice and mice treated with either type of bleomycin were assessed using an analysis of variance (ANOVA). Statistical significance was assumed for P-values ≤0.05.

Results

Bleomycin fraction content

Using the two reverse-phase methods, significant differences were shown between the two formulations. Using method 1, the Tianjin-supplied bleomycin was shown to contain primarily (>95%) bleomycin A_5 with trace amounts of bleomycin A_2 and B_2 (Table 1). Bleomycin sulfate USP (Blenoxane) was shown to contain approximately 70% bleomycin A_2 and 29% bleomycin B_2 (Table 1). In these HPLC assays, the different bleomycin fractions exhibited the predicted elution profiles.

The same approximate bleomycin fractional composition was detected using the USP-specified HPLC procedure. These results are summarized in Table 2. Again,

Table 1 Bleomycin chemical composition by HPLC method 1. Values are mean percentages (SD) (*ND* not detected)

Formulation	Bleomycin fractional content			
	Other	A_2	A_5	B_2
Blenoxane (Bristol-Myers Squibb)	1.40 (0.02)	69.4 (3.9)	ND	29.3 (3.2)
Bleomycin HCl (Tianjin Hebei)	ND	0.24 (0.03)	96.9 (0.49)	2.85 (0.50)

Table 2 Bleomycin chemical composition by USP HPLC method. Values are mean percentages (SD) (*ND* not detected)

Formulation	Bleomycin fractional content			
	Other	A_2	A_5	B_2
Blenoxane (Bristol-Myers Squibb)	3.19 (0.27)	71.2 (1.71)	ND	28.90 (0.69)
Bleomycin HCl (Tianjin Hebei)	ND	1.4 (0.4)	96.54 (0.1)	2.19 (0.10)

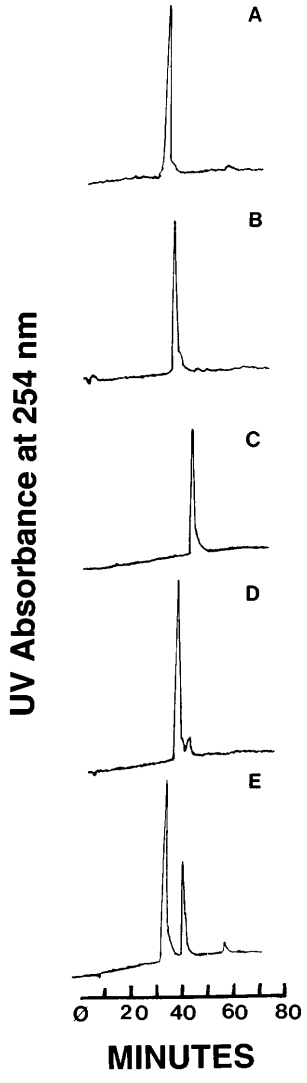

Fig. 2A–E A comparison of HPLC chromatograms (USP method) for the different bleomycins: **A** bleomycin A_2, **B** bleomycin A_5, **C** bleomycin B_2 fraction, **D** Blenoxane bleomycin sulfate USP, **E** Tianjn bleomycin. In these assays, one absorbance unit full scale was equivalent to 10 mV of detector response

the major fraction in the Tianjin-supplied product was bleomycin A_5 with only trace amounts of other bleomycins detected, primarily bleomycin B_2 (2%). Blenoxane was shown to contain primarily the A_2 (71%) and B_2 (29%) fractions. Each of the major bleomycin components was completely resolved as single eluting peaks in the USP method. The retention times for the purified bleomycin fractions were 37.6 min for bleomycin A_2, 39.9 min for bleomycin A_5 and 47.1 min for bleomycin B_2. As Fig. 2 shows, Blenoxane (panel D) was composed of a predominant A_2 peak, a lesser B_2 peak and a tiny late-eluting unknown peak at approximately 57.1 min. No bleomycin A_2 and a very small B_2 fraction were seen in the Tianjin formulation, which was therefore predominantly bleomycin A_5.

Overall, these studies show that the Tianjin-supplied bleomycin HCl formulation does not conform to USP specifications and is primarily composed of the bleomycin A_5 fraction. This was consistent for both HPLC assay procedures.

Table 3 Lethality of high-dose bleomycin in BALB/c mice

Formulation	Dose[a] (mg/kg)	Lethality[b] (%)	Mean day of death (SD)
Blenoxane (Bristol-Myers Squibb)	400	50	9.0 (0)
	500	90	13.5 (1.29)
Bleomycin HCl (Tianjin Hebei)	400	60	14.0 (1.41)
	500	100	10.3 (2.75)

[a] 100 mg/kg IP × 4 or 5 days (400 mg/kg or 500 mg/kg, respectively)
[b] n = 10 mice per group

High-dose survival in mice

Both bleomycin formulations produced significant lethality in normal mice given daily IP doses of 100 mg/kg for 4–5 days (Table 3). There was slightly greater lethality with the Tianjin formulation, although the differences were not statistically significant. The mean day of death in these trials ranged from day 9 to day 14 for both formulations. There was also significant weight loss and lethargy with both bleomycin formulations. This resulted in a mean weight loss of 3–5 g/mouse, representing 15–25% of pretreatment body weight.

In vitro antitumor activity

Growth inhibition studies in the human ovarian carcinoma cell line OVCAR-3 showed a similar dose-response for the two formulations. These studies were performed using the SRB method for staining protein in adherent cell populations. The 50% inhibitory concentration was 0.38 μg/ml for Blenoxane and 0.81 μg/ml for Tianjin-supplied bleomycin (Fig. 3A). This difference was not significant ($P > 0.05$) by ANOVA.

In L-1210 murine leukemia cells, the Tianjin-supplied bleomycin appeared to have greater cytotoxic potency (Fig. 3B). These studies were performed using the MTT dye-reduction methodology. The 50% inhibitory concentrations were estimated to be 0.2 μg/ml for the Tianjin formulation compared with 1.2 for Blenoxane ($P < 0.05$ by ANOVA).

In vivo antitumor activity

Activity was demonstrated for both formulations in the B-16 melanoma model. Both preparations slowed the growth of B-16 tumors in a dose-dependent fashion (Table 4). All treated mice (both formulations) had a significantly reduced tumor mass compared with the saline-treated control mice on each tumor measurement

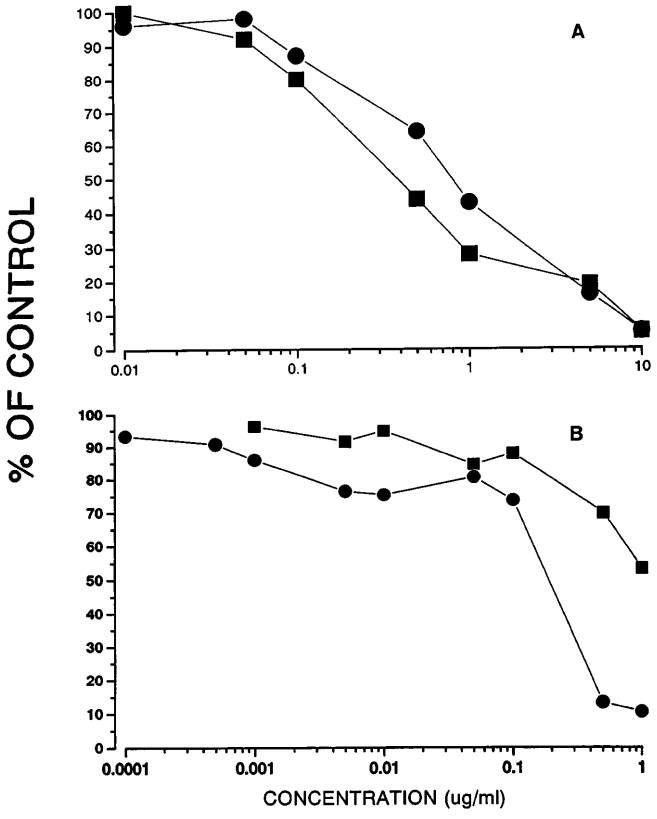

Fig. 3A,B Bleomycin antitumor activity in vitro (continuous exposure) against human ovarian carcinoma cells in the SRB growth-inhibition assay (**A**) or murine L-1210 leukemia cells in the MTT assay (**B**), following treatment with Blenoxane (■) or Tianjin-supplied bleomycin HCl (●)

day ($P \leq 0.05$ by the paired *t*-test). At the highest dose of 8 mg/kg per day × 9 days, the Tianjin formulation produced significantly greater tumor growth inhibition, but at the expense of greater toxicity. This resulted in some early deaths (three of eight) in the high-dose (8 mg/kg) Tianjin bleomycin group. Thus, median survival was only 18 days in the group receiving 8 mg/kg Tianjin bleomycin compared with 22 days in the control group, and ≥25 days in all other treated groups. All surviving mice were humanely sacrificed on day 25 owing to the presence of large tumor burdens.

Discussion

These studies show that compositional variation in bleomycin formulations can produce altered biologic effects. The predominant A_5 fraction in the Tianjin formulation may explain the enhanced growth-inhibitory activity seen with this formulation in the L-1210 leukemia cells in vitro, and in mice with B-16 melanoma. However, this increase in cytotoxic potency was accompanied by enhanced toxicity in the melanoma trial in C57/bl mice. Of interest, this was not noted in the non-tumor-bearing BALB/c mice given high doses of either formulation.

Greater toxicity of bleomycin A fractions has been previously reported. Umezawa et al. observed that daily IP injections of 500 μg (about 25 mg/kg per day) or 250 μg bleomycin A complex (about 12 mg/kg) caused rapid death of mice during the injection [6]. Bleomycin A complex given IP at doses of 400 or 250 mg/kg caused death in two of five mice within 5 days. The remaining three animals died 5–10 days later. Thus, the LD_{50} value in mice given bleomycin A fractions is approximately 150–200 mg/kg. In contrast, lower toxicity is noted with bleomycin B fractions [5, 6, 16].

Other findings also indicate greater potency of A fraction bleomycins. In dogs given 50 mg/kg bleomycin A complex, there were significant increases in hepatic enzymes, which is not noted with other bleomycin species [16]. In other studies [17, 18], significant differences in pulmonary toxicity of different fractions of bleomycin have been shown in mice treated by the intratracheal route. Relative to Blenoxane (mixture), bleomycin A_5 produces greater lethality. This is similar to the current findings in the C57 mouse strain. The previous study also showed that with the bleomycin B_2 fraction, pulmonary toxicity is relatively mild and the severity of pulmonary fibrosis and metaplasia of these bleomycins can be attributed to the terminal bleomycin substituents, spermidine and agmatine. The presence of agmatine on the terminal group of bleomycin B_2 is thought to explain the lower toxicity of this compound. Overall, the

Table 4 Antitumor activity of two bleomycin formulations in mice with B-16 melanoma. Values are mean (SD) tumor mass (g) 0 no palpable tumor in any animal (if no SD is shown, only one or two mice had tumors present). All mice surviving at 25 days were sacrificed

Group[a]	Dose	Days after implantation					
	(mg/kg)	13	15	18	20	22	25
Control	0	0.37 (0.47)	2.39 (0.839)	4.37 (2.35)	6.39 (2.12)	8.8 (3.76)	9.84
Blenoxane	4	0.1 (0.2)	0.41 (0.54)*	0.60 (0.80)*	1.64 (1.76)*	2.37 (2.17)*	2.91 (1.30)*
Blenoxane	8	0	0*	0.254*	0.55 (0.80)*	2.9 (2.14)*	4.06 (1.47)*
Chinese bleomycin	4	0	0.78 (0.90)*	1.065 (1.28)*	3.39 (2.02)	5.87 (3.74)	6.40 (3.39)
Chinese bleomycin (8)	0	0*	0*	0.5*	0.5 (0.47)*,**	2.1 (1.37)*	

*$P < 0.05$ by ANOVA vs control, ** $P < 0.05$ by ANOVA vs equivalent dose of alternate formulation

previous studies show that the A_2 fraction produces similar toxic effects to bleomycin USP in mice. Importantly, the A_5 fraction is associated with significantly greater pulmonary toxicity. This confirms the current finding that differences among the fractional components of bleomycin yield different biologic activities in terms of acute toxicity.

Differences in antitumor efficacy among the bleomycin fractions have also been observed. In the Ehrlich ascites model in the mouse, the bleomycin A complex has been noted to have significant activity with daily IP injection of 500, 250, 125 or 62.5 μg/mouse, representing a range of 25 to 3.125 mg/kg. In this study, the bleomycin A_2 fraction was shown to have significantly greater antitumor potency than the bleomycin A_5 fraction. Again, the B fraction bleomycins appeared to have lower antitumor potency than the A fractions in this Erlich carcinoma model in mice. In another study bleomycin A_2 sulfate, bleomycin A_5 sulfate and bleomycin B_2 sulfate plus parent material (bleomycin complex) were compared for their antitumor activity against Lewis lung carcinoma [15]. All of the bleomycins were active, producing increased lifespans of 68%, 52%, and 70% for the A_2, A_5 and B_2 fractions, respectively. However, none of the individual fractions was superior to the official bleomycin mixture which produced a 70% increased lifespan in the Lewis lung cancer model. Thus, there does not appear to be any therapeutic advantage for any single bleomycin fraction compared with the defined mixture specified as bleomycin sulfate USP.

In summary, a Tianjin bleomycin HCl formulation was shown to significantly differ from the USP specifications for fractional content of different bleomycin species. The predominance of bleomycin A_5 and the trivial proportion of bleomycin B_2 fraction found in the Tianjin formulation support the conclusion that this formulation is not equivalent to Blenoxane. The slightly greater cytotoxic potency of Tianjin bleomycin HCl in vitro and in the B-16 bearing mice did not translate into enhanced therapeutic efficacy since it was accompanied by much greater lethality in vivo. This is compatible with the higher proportional content of bleomycin A_5 fraction which is known to produce greater pulmonary toxicity than other bleomycin fractions [17,18]. Overall, the current data show that the two bleomycin formulations are not equivalent in chemical or biological terms. These findings suggest that caution should be exercised in comparing efficacy or substituting clinically different formulations of antitumor agents in the treatment of cancer.

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