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In Vitro Activity of Bleomycin, Tallysomycin S10b, and Liblomycin against Fresh Human Tumor Cells[1]

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ABSTRACT

The objective of this study was to compare the relative *in vitro* cytotoxicity of bleomycin to that of two newer-generation analogues, tallysomycin S10b and liblomycin. The latter compound is of particular interest as it has recently been shown in preclinical studies to be free of a potential to cause pulmonary injury and yet to possess only a minor potential to produce myelotoxicity. Using the adhesive tumor cell culture system, we evaluated the activity of these three drugs against a panel of 13 human tumors of various types. The range of concentrations chosen was determined and normalized using a nonleukemic permanent mouse hematopoietic progenitor cell line.

Those drug concentrations achieving 90% inhibition of growth (IC_{90}) against the murine cell line were: 6.11 μM bleomycin; 7.53 μM tallysomycin S10b; and 0.6 μM liblomycin. When tested against fresh human tumors at equally myelotoxic IC_{90} concentrations, bleomycin and tallysomycin S10b (nonmyelotoxic compounds) both achieved 90% growth inhibition of all tumors, while liblomycin (a myelotoxic compound) produced an IC_{90} inhibition in 69% of all tumors. A comparison of drug IC_{90} values against individual fresh tumors indicated a correlation between bleomycin and its structurally related analogue tallysomycin S10b. No such correlation, however, was seen with liblomycin in comparison to either bleomycin or tallysomycin S10b. The relative activity of liblomycin *versus* that of bleomycin and tallysomycin S10b varied with individual tumors tested. The response rate of liblomycin, a myelotoxic compound within this normalized range, appears promising. These data represent the first comparison of liblomycin to bleomycin against a spectrum of fresh human tumors using a stem cell assay technique.

INTRODUCTION

For the past 15 yr, Blenoxane has been a major component of many multidrug regimens, especially those used in the treatment of germ cell tumors, lymphomas, and a variety of squamous cell carcinomas (1). The mechanism of action of this agent has been extensively investigated and is well understood in terms of DNA binding and DNA damage (Refs. 2–5). Unfortunately, bleomycin has the potential to produce pulmonary injury, which is characterized in its severest form as a progressive and sometimes fatal pulmonary fibrosis. Much effort has therefore been devoted to developing bleomycin analogues with hopes of demonstrating greater efficacy, an expanded spectrum of clinical activity, and reduced adverse reactions (5).

With the capacity to manipulate the structure of bleomycins through semisynthetic methods, an analogue of bleomycin, tallysomycin S10B, was obtained (6). It differs from bleomycin by an additional amino-sugar (4-amino-4,6-dideoxy-L-talose), a longer peptide chain, and an altered terminal amine moiety (Fig. 1). Tallysomycin S10b was found to be more potent than bleomycin in preclinical studies (7), and its toxicity was comparable to that of bleomycin in animal models (7–9). Tallysomycin S10b was introduced into the clinic as a Phase I agent in 1984.

More recently, with the capacity for the total synthesis of bleomycin derivatives, another analogue, liblomycin, has been chosen for development (5). This agent is structurally less similar to bleomycin than is tallysomycin S10b, exhibiting a bulky lipophilic group at the end of the terminal amine (Fig. 1). In preclinical studies, liblomycin was found to produce little pulmonary injury; however, unlike other bleomycins, it was myelotoxic in dogs (5, 10).

In the present study, we investigated the relative cytotoxicity of bleomycin and these two analogues *in vitro*, against fresh human tumors by means of the ATCCS[4] (11).

A nonleukemic PMHP cell line (12, 13) was used with the MTT assay (14) to evaluate the toxicity of these drugs on "normal" tissue and derive equitoxic concentration ranges to be used against fresh tumors. The concept and rationale in using equitoxic drug concentrations *in vitro* have been discussed previously (15, 16). The relative toxicities of these drugs on the fresh tumors and on this cell line are discussed.

MATERIALS AND METHODS

These investigations were performed after approval from the institutional Human Subjects (Surveillance) Committee.

Drugs. Bleomycin (Blenoxane) and tallysomycin S10b were obtained from Bristol-Meyers Company (Wallingford, CT). Liblomycin was generously provided by Dr. T. Takita (Tokyo, Japan). Drugs were prepared and diluted in saline and stored in polypropylene tubes at −70°C until used.

PMHP Cell Line. Both the 32DC1-23 and WEHI-3B cell lines were kindly provided by Joel S. Greenberger, M. D., University of Massachusetts Medical Center, Worcester, MA. The PMHP cell line 32 DC1-23 is well characterized (12, 13, 17). Interleukin 3, necessary for its growth, is found in the conditioned medium of murine WEHI-3B myelomonocytic leukemia cell lines. Its extraction has been described elsewhere (12, 13). Briefly, murine WEHI-3B cells were grown in RPMI 1640 medium without glutamine plus 10% FBS at 37°C for 10 to 14 days. When roller cultures reached a density of approximately 10^6 cells/ml, cells were removed by centrifugation, and the supernatant-conditioned medium was passed through a 0.2-mm Millipore sterilization filter. The filtrate was dialyzed for 48 h in distilled water and then concentrated 5-fold by an Aquacide dehydration procedure (Calbiochem, San Diego, CA). The concentrated conditioned medium was then added to McCoy's Medium 5A supplemented with 10% FBS (Flow Laboratories, Rockville, MD). The final concentration of conditioned medium was 15%. The 32 DC1-23 cell line was incubated at 37°C in an atmosphere of 5% CO_2 and air.

ATCCS for Fresh Tumors. As previously described (18), solid biopsy specimens of human tumors were minced to 1-mm^3 pieces and disaggregated to single cells by incubation with 0.75% collagenase type III

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[4] The abbreviations used are: ATCCS, adhesive tumor cell culture system; PMHP, permanent murine hematopoietic progenitor; MTT assay, colorimetric tetrazolium assay; IC_{90}, 90% inhibitory concentration; FBS, fetal bovine serum; α-MEM, α-minimal essential medium; LD_{50}, 50% lethal dose.

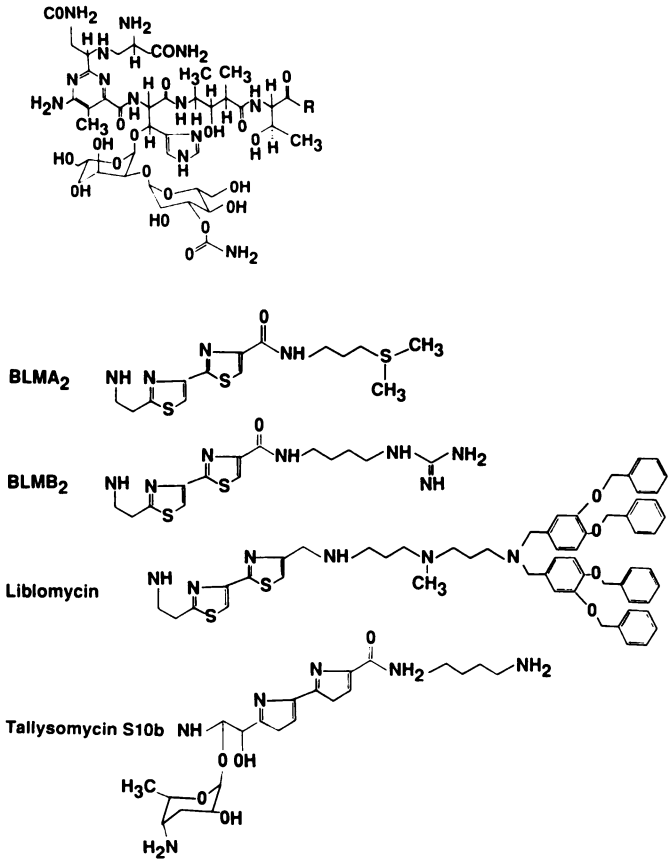

Fig. 1. Structures of bleomycins A_2 and B_2, tallysomycin S10b, and liblomycin.

(Cooper Biomedical, Malvern, PA) and 0.005% DNase (Sigma, St. Louis, MO), in 20 ml of supplemented α-MEM with 10% FBS. Samples were digested for 16 h with constant stirring. α-MEM consists of: Eagle's modification of MEM (K. C. Biological, Lenexa, KS); 2.7 mg/ml of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Sigma); 10% horse serum (J. R. Scientific, Woodland Hills, CA); 100 μg/ml of penicillin and 100 μg/ml of streptomycin (GIBCO, Grand Island, NY); 10 μg/ml of transferrin, 0.5 μg/ml of hydrocortisone, 5 ng/ml of epidermal growth factor, and 0.27 μg/ml of estradiol (Sigma); and 5 μg/ml of insulin (Collaborative Research, Lexington, MA). The median yield of viable cells was 3 million/g of biopsy material, determined by hemocytometer count after trypan blue staining. Viability ranged between 30% and 70%. The cells were resuspended in α-MEM containing 0.6% methylcellulose and inoculated in 24-well plates at 2000 cells/well. After 24 h of incubation, the medium was aspirated, the adherent cells were washed with phosphate-buffered saline, and drugs, at various concentrations, were added to triplicate wells. Control cells were fed with α-MEM only. All cultures were refed with 100% medium after 6 days of incubation, and the cultures were incubated for an additional 6 days, resulting in a total incubation period of 13 days. At the end of the incubation period, the cultures were fixed with 70% ethanol for 20 min and stained with 0.05% crystal violet.

The colony-forming efficiency of the ATCCS is 2%, with the use of α-MEM culture (19). This frequently results in confluent cultures in the control wells, and the cell growth was quantitated by measuring the crystal violet cell-staining density by an image analysis computer. The nonproliferating cell population was assessed by incubating cultures inoculated with 2000 cells/well with high levels of tritiated thymidine (5 μCi/ml) (11). This background value was subtracted from the control and treated wells.

Because fresh human tumors have an unknown and variable plating efficiency, the degree of confluence was determined from a cell inoculum titration consisting of cells plated at 0, 250, 500, 1000, and 2000 cells per well. The staining density of the cells in these wells was plotted against the inoculum, and the linearity of the cell inoculum titration was assessed. In cultures which were overgrown in the control wells as evidenced by a plateau of the inoculum titration curve, the control value was determined by an extrapolation from the linear portion of the inoculum titration. Assays that were excessively overgrown (extrapolated control value greater than 4 times the actual control value) were rejected.

Drug Survival Curves. Drug dose-response data were obtained by adding drugs to the cultures after 24 h of incubation. Drugs were removed during the refeeding at 6 days of incubation, resulting in a 5-day exposure. A 6- to 8-fold drug concentration range was used. This range was determined by cytotoxicity against the PMHP cell line (*vida intra*). Drug survival curves were generated by plotting surviving fractions against drug concentrations using a semilogarithmic scale. In overgrown assays, the cultures treated with low doses of drug that showed absorbance values at the plateau level were excluded from the survival curve, and only cultures that had absorbance values that were below the plateau values were used to construct the survival curve. Thus in all cases survival curves were constructed from subconfluent cultures using a minimum of two points plus the control. In optimally plated assays of moderate sensitivity, all four dose points were commonly used to construct the survival curves. In sensitive assays where significant kill is observed in the low doses of drug, the higher dose drug wells achieving greater than two logs of cell growth inhibition were also excluded. Drug sensitivity was measured by determining the IC_{90} from the drug dose-response curve. The data were fitted to a second order quadratic equation, and the IC_{90} values were calculated using coefficients of the fitted curve (11).

The MTT Assay. The 32 DCL-23 cells were harvested during exponential growth. Cell counts were performed using a hemocytometer. The MTT assay was performed as previously described (14, 20). Briefly, 400 cells were plated in each well of a 96-well microwell flat bottomed plate (Nunc). This seeding density was chosen to ensure that the cells would be in an exponential growth phase at the end of the 5-day incubation period. Cells were inoculated with 0.18 ml of McCoy's Medium 5A, supplemented with 10% FBS and 15% conditioned medium to which 0.02 ml of 10× concentrated drug were added. Each drug was tested at 5 to 10 concentrations, covering a 1- to 2-log concentration range, and chosen to encompass the granulocyte-macrophage colony-forming cell IC_{90} previously determined for bleomycin, which is 2.5 μg/ml (15). After 5 days of incubation, 0.1 mg (50 μl of 2 mg/ml) of MTT (Sigma Chemical Co., St. Louis, MO) was added to each well and incubated at 37°C for an additional 4 h. Plates were then centrifuged at 450 × *g* for 5 min. The medium was then aspirated gently from all plates, taking care not to disturb the formazan crystals at the bottom of the wells. Dimethyl sulfoxide (150 μl) (Sigma) was finally added to each well, and the plates were placed on a shaker for 10 min to solubilize the formazan crystals. The plates were read immediately thereafter, at 550 nm on an MR 580 Micro Elisa reader. Absorbance levels from drug-tested cells were compared with untreated control absorbance values. Each test incorporated a cell dose inoculum, and the true control was determined by an extrapolation, as described in the evaluation of the ATCCS assay (18).

The IC_{90} value was defined as that concentration of drug which achieved 90% reduction of growth (absorbance) in drug-treated cells with respect to the controls. The data were fitted to a second-order quadratic equation, and the IC_{90} value was calculated using the coefficients of the fitted curve as described above.

RESULTS

Drug Cytotoxicity against the PMHP Cell Line. Using the MTT assay with the PMHP cell line, the IC_{90} values were determined for all three drugs. For bleomycin and tallysomycin S10b, the IC_{90}s were similar (6.11 μM and 7.53 μM, respectively). It was lower by an order of magnitude for liblomycin (0.6 μM).

Drug Cytotoxicity against Fresh Human Tumors Using the ATCCS. A panel of 15 fresh primary tumor biopsies was processed using the ATCCS assay. All tumors were obtained from untreated patients, with the exception of one ovarian

tumor. According to our criteria (11), 13 specimens (86.6%) were evaluable for drug sensitivity (4 lung, 3 ovarian, 1 breast, 2 melanoma, 2 cervical, and 1 osteosarcoma) (Table 1), leaving 2 tumors inevaluable because of low growth in the control.

The response rate (the relative number of specimens where an IC_{90} concentration of drug was observed) for liblomycin was estimated *in vitro* against human tumor cells at 5 different concentrations, ranging up to the IC_{90} achieved against the PMHP cell line. Of the 13 human tumors, 9 (69%) were sensitive to the drug within the range of concentrations tested.

Bleomycin and tallysomycin S10b, known to be nonmyelotoxic at cytotoxic concentrations, were tested *in vitro* in a normalized concentration range of 5 different concentrations up to ⅓ their IC_{90} values achieved against the PMHP cell line. At these doses, an IC_{90} was reached in 100% of the tumors. The median tumor IC_{90} value of these drugs (Table 1), determined using the normalized concentration range, show that tallysomycin S10b was 7 times more potent than bleomycin against the panel of human tumors.

As an alternative method of expressing these results, we analyzed the ratio of the PMHP IC_{90} value to the median tumor IC_{90} value. This *in vitro* "therapeutic index" was much higher for tallysomycin S10b (97.4) than it was for bleomycin (10.9), suggesting that tallysomycin S10b might be superior to bleomycin despite their similar structure. To further identify if the pharmacodynamic profiles of these drugs were related, correlation coefficients were established between the individual IC_{90} values of bleomycin and its analogues achieved against the fresh tumors. Bleomycin and tallysomycin S10b had a significant overall correlation ($r^2 = 0.73$), whereas liblomycin correlated poorly when compared to bleomycin ($r^2 = 0.37$) or to tallysomycin S10b ($r^2 = 0.39$) (Fig. 2).

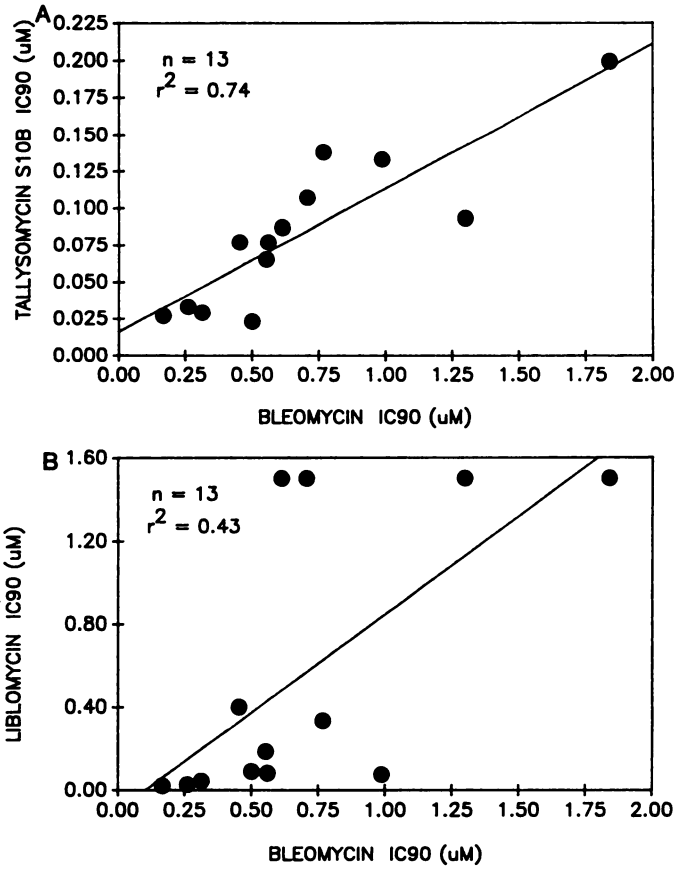

Fig. 2. IC_{90} values (μM) of bleomycin compared to tallysomycin S10b (*upper*) and liblomycin (*lower*) against fresh human tumor biopsies. *n*, number of specimens; r^2, correlation coefficient; ●, best-fitted curve.

DISCUSSION

We assessed the *in vitro* toxicity of bleomycin and two analogues against fresh human lung tumor cells using the ATCCS. This method of screening for cell sensitivity to drugs has previously demonstrated a high rate of successful tests performed *in vitro* on fresh tumors. Its efficacy for the analysis of new chemotherapeutic agents *in vitro* as well as for the comparison of new analogues to classical drugs was also demonstrated (16, 21, 22).

For compounds with little or no pharmacological information, the optimal concentrations to be tested *in vitro* are difficult to determine. Since many chemotherapeutic drugs are myelotoxic, a biologically based approach is to normalize the concentrations at which drugs should be tested *in vitro*, potentially avoiding false-positive results by eliminating the testing of irrelevant concentrations. As a rapid and more uniform alternative to the human bone marrow model (16), the PMHP cell line was used to determine a range of concentrations which was subsequently selected for the drug tests against fresh tumors.

Table 1 *IC_{90} values of bleomycin, tallysomycin S10b, and liblomycin on fresh human tumor cells using the ATCCS assay*

Tumor type	IC_{90} values (μM)		
	Bleomycin	Tallysomycin	Liblomycin
Lung (squamous)	0.313	0.029	0.044
Lung (squamous)	0.613	0.087	>1.500
Lung (squamous)	0.987	0.133	0.075
Lung (adenocarcinoma)	1.840	0.199	1.500
Ovarian	0.260	0.033	0.027
Ovarian	0.167	0.027	0.021
Ovarian	0.767	0.138	0.335
Cervical	0.453	0.077	0.400
Cervical	0.500	0.023	0.090
Breast	0.707	0.107	>1.500
Melanoma	0.553	0.065	0.185
Melanoma	0.560	0.077	0.082
Osteosarcoma	1.300	0.093	>1.500
Median	0.560	0.077	0.185

Bleomycin and related analogues are among a limited number of clinically active drugs in cancer therapy. Their potential for producing dose-limiting pulmonary toxicity has always been a major problem in their clinical use. The development of active and particularly less toxic analogues has therefore been considered essential. Peplomycin, a second generation bleomycin, exhibited a similar tendency as did the parent compound to produce pulmonary toxicity when tested in the clinic (23). Tallysomycin S10b and liblomycin are new synthetic analogues selected by preclinical studies for clinical trials.

Tallysomycin S10b differs from bleomycin principally by addition of a talose sugar attached to the (aminoethyl)bithiazole moiety. This sugar is believed to play a role in determining both the binding affinity and the specificity of drug-mediated DNA scission (3). Tallysomycin had similar IC_{90} activity to bleomycin against the PMHP cell line, yet was 7.2 times more active against fresh tumors. This increased activity for tallysomycin S10b is in agreement with preclinical *in vivo* studies which showed that tallysomycin S10b was, at LD_{50}, up to 4 times more active than bleomycin (7). Tallysomycin S10b, being structurally more related than liblomycin to the parent compound and exhibiting a similar mode of action and cross-resistance (5), showed not unexpectedly a close parallel of *in vitro* response with bleomycin. The expected clinical activity of these compounds is difficult to evaluate because the normalized

in vitro range used could be an inappropriate high dose for nonmyelotoxic drugs. However, we think it valid to compare drugs with similar extramedullary toxicity within this normalized range.

We have previously demonstrated that clinically active myelotoxic drugs tested *in vitro* in the ATCCS achieve an IC_{90} in 30% or more primary tumor samples within the IC_{90} determined against human marrow cells (16). Liblomycin, a relatively myelotoxic drug in preclinical evaluation, achieved in this study a 90% inhibition of growth in 69% of the tumor samples in the normalized concentration range defined by the 32 DCL-23 PMHP cell line. The IC_{90} concentration of liblomycin on the PMHP cell line, however, was 10-fold less than that of bleomycin, indicating the greater toxicity of this new bleomycin on a derivative weight basis. Because of the differing toxicities of liblomycin and bleomycin, conclusions cannot be reached concerning the most active drug clinically. However, as mentioned previously, a response rate of 69% for a myelotoxic drug within this normalized range is encouraging. Interestingly, preclinical data also demonstrated a higher potency for this drug compared to bleomycin both *in vivo* and *in vitro* (4, 5, 10). Besides its potential to produce bone marrow toxicity, this drug is different in many aspects from bleomycin and probably from tallysomycin S10b. It shows little cross-resistance to other members of the bleomycin family including bleomycin and tallysomycin (5). It exhibits an unusual lipophilic capacity and is probably the object of a different metabolism. It causes very little if any pulmonary toxicity (5, 10, 24) and appears to be resistant to bleomycin hydrolase.[5] These differences are most likely due to the unusual multiring system associated with the terminal amine moiety. This study suggests that liblomycin is active *in vitro* against a wide spectrum of human tumor samples. The particular cytotoxicity against ovarian, cervical, and melanoma tumors is especially intriguing. Whether the spectrum of antitumor activity is also different from bleomycin and from tallysomycin S10b depends, of course, on Phase II trials.

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