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Potentiation of the Cytostatic Effect of Bleomycin on L5178y Mouse Lymphoma Cells by Pepleomycin*

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Abstract—*Bleomycin (BLM) and pepleomycin (PEP) are two chemically related glycopeptide antitumor antibiotics which differ in their terminal residues only. Studying the growth-inhibitory potencies of BLM (clinical mixture), BLM-A_2, BLM-B_2 and PEP in the L5178y mouse lymphoma cell culture system, it was elucidated that the slopes of the dose-response curves at the* ED_{50} *concentration (around 1 μg/ml) were steeper for PEP than for BLM. This result together with cytotoxicity determinations revealed a cytostatic action of PEP within a closer concentration range than BLM. Both drugs inhibit cell proliferation during S- and G_2-phase. Given in combination, BLM and PEP inhibit cell proliferation in a highly significant synergistic way (FIC indexes:* 0.25–0.46). *This* in vitro *result, which might be of therapeutic importance, is correlated with differences on the molecular level. Determinations of the ratio between the number of single- and double-strand breaks in the DNA (the target molecule of the drugs) revealed a considerably lower value for DNA from BLM-treated cells* (1.9:1) *than for DNA from PEP-treated cells* (13:1).

INTRODUCTION

BLEOMYCINS (BLMs) are a group of glycopeptide antitumor antibiotics, discovered by Umezawa *et al.* [1]. The backbone of the BLMs consists of β-aminoalanine:pyrimidinyl propionamide:β-hydroxy-histidine:valerate:threonine:bithiazole and a gulose:mannose side chain [2].

The 300 different BLMs are distinguished by their terminal amine groups attached to the bithiazole moiety. BLM is clinically used in the therapy of squamous cell carcinomas [3], especially of the highly differentiated types [4]. Experimental data show that BLM promotes massive degradation of DNA *in vivo* [5] and *in vitro* [6, 7]. It is suggested that this property determines the cytotoxicity of the antibiotic [8].

While this antineoplastic glycopeptide produces only minor hematological and gastrointestinal toxicity, its clinical usefulness is limited by a dose-related pulmonary toxicity [9]. During an extensive screening program a new and more active BLM analog was discovered [10, 11]. It has a lower pulmonary toxicity [12] than the currently available BLMs, and was termed pepleomycin (PEP). The terminal amino moiety of PEP is 3-[(*S*)-1′-phenylethylamino]propylamine [12].

In the present communication we attempt to demonstrate that PEP potentiates the cytostatic effect of BLM *in vitro*. This unexpected result was obtained both with pure BLM-A_2 and BLM-B_2, and with the clinically used BLM mixture.

MATERIALS AND METHODS

Compounds

The clinical mixture of BLM (containing 55–70% A_2; 25–32% B_2; < 7% A_2'; and < 1% B_4) and PEP were obtained from H. Mack, Illertissen (F.R.G.). Pure BLM-A_2 and BLM-B_2 was prepared by Nippon Kayaku Co., Ltd following the instruction of one of the authors [1].

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Abbreviations: BLM, bleomycin; PEP, pepleomycin or 3-[(*S*)-1′-phenylethylamino]propylaminobleomycin; FIC, fractional inhibitory concentration.

Cell culture

L5178y mouse lymphoma cells were maintained in suspension culture as described previously [13]. For the dose–response experiments the cultures (5 ml) were initiated by inoculation of 5×10^3 cells/ml and incubated at 37°C in roller tubes for 48 or 72 hr; the controls reached cell concentrations of 9.5×10^4 (48 hr) or 5.7×10^5/ml (72 hr) respectively. Synchronization of the cells was performed by the double-thymidine method as described previously [14]. After this procedure more than 90% of the cells accumulate at the end of the G_1-phase of the cell cycle. The duration of the S-phase was determined by incorporation studies with [^{3}H]-thymidine [14] and it was found to be 5.4 hr. Mitosis could be ascertained by determination of cell growth and was located in the period ranging from 7 to 8 hr after removal of the thymidine block. S-phase cells were immediately taken after the release of the thymidine block and G_2-phase cells 5.4 hr post-release.

Cell viability was determined as follows: the cells (5×10^3 cells/ml) were exposed to the drug for 48 hr in spinner cultures (minimal essential medium–spinner modification without carbonate but with 10 mM HEPES; 10% horse serum). Subsequently the drug was removed by 2 washings, followed by dilution and re-inoculation. Viability is expressed in % as the ratio of the growing efficiency of the treated cells to the growing efficiency immediately before addition of the drug.

For determination of the drug effects on cellular DNA synthesis 80-ml spinner cultures were inoculated with 10^5 cells/ml and incubated for 24 hr. Cell proliferation in these experiments had to be given in doubling steps [15], because of the higher accuracy of this method for the documentation of short-term experiments (2 doubling steps), starting with a high cell density (10^5 cells/ml).

Cell concentrations and volume distributions were determined with a Model B Coulter counter with a 32-channel size-distribution plotter; calibration of the counter was performed with paper mulberry pollen (diameter: 13.5 μm; Hollister-Stier, Lab., Coulter).

Statistical methods

The ED_{50} was estimated by logit regression [16]. The slope of the dose–response curve at the ED_{50} was calculated [16].

The characterization of the distribution curves was performed by applying the parameters: mean value, skewness and kurtosis [16]. The mean value (M_1) represents the mean of the distribution calculated according to the equation:

$$M_1 = \frac{\Sigma f X}{N},$$

where X is the channel number, f its frequency and N the number of cells analyzed (routinely 25,000). In the second moment form (M_2) this becomes:

$$M_2 = s^2 = \frac{\Sigma f(X-M_1)^2}{N}.$$

The standard deviation is calculated as follows:

$$s = \sqrt{(M_2)}.$$

For the calculation of the skewness (α_3) and the kurtosis (α_4) the third (M_3) and fourth moments (M_4) are formed by

$$M_3 = \frac{\Sigma f(X-M_1)^3}{N} \text{ and } M_4 = \frac{\Sigma f(X-M_1)^4}{N}.$$

From these equations skewness and kurtosis are given:

$$\alpha_3 = \frac{M_3}{(M_2)^{1.5}} \text{ and } \alpha_4 = \frac{M_4}{(M_2)^2} - 3.$$

α_3 and α_4, obtained from different determinations, were compared after normalization (z) according the equation:

$$z = \frac{X-M}{s};$$

after this transformation a correlation with the standardized normal distribution ($M_1 = 0$, $s = 1$) became possible.

The mathematical evaluation of the fractional inhibitory concentration indexes (FIC indexes) of PEP–BLM combinations was performed according to published equations [17] and experimental procedures [18]. FIC > 1 = antagonism; FIC $\simeq 1$ = additive effects; FIC < 1 = suggestive of synergism; FIC < 0.5 = significant synergism.

DNA extraction and characterization

DNA was extracted from 1.2×10^7 cells according to a previously described procedure [19]. The molecular weight of the DNAs was determined from sedimentation rates, obtained by the velocity–sedimentation method [20]. An analytical ultracentrifuge (Beckman, model E), equipped with u.v.-optics, monochromator, scanner and multiplexer was used. S-values were corrected for standard conditions and extra-

polated to zero concentrations. From $S^{\circ}_{20,w}$ values, determined under neutral and alkaline conditions, molecular weights were calculated using the relations given by Eigner and Doty [21], Hagen [22] and Prunell and Bernardi [23]. The weight-average number of double-strand breaks and single-strand breaks (alkaline-labile damage) was estimated according to Sicard *et al.* [24]. DNA concentration in L5178y cells was determined as described [25].

RESULTS

Cytostatic activity of the individual agents

The ED_{50} concentration in dose–response experiments (72 hr) for BLM (clinical mixture) in the L5178y cell system was determined to be 1.42 ± 0.09 μg/ml. Under identical culture conditions the ED_{50} concentrations of the pure compounds were as follows: BLM-A_2, 0.26 ± 0.02 μg/ml; BLM-B_2, 0.20 ± 0.02 μg/ml; and PEP, 0.82 ± 0.05 μg/ml. The slopes of the dose–response curves at the ED_{50} for the BLMs were nearly identical: BLM (clinical mixture), −1.33; BLM-A_2, −1.34; and BLM-B_2, −1.33. In contrast, the slope of PEP at the ED_{50} was steeper and was estimated to be −1.96. To determine the efficiency of the drugs during the logarithmic growth phase of the cells the ED_{50} concentration was determined after a cultivation period of 48 hr. Under these conditions nearly identical ED_{50} concentrations were evaluated: BLM (clinical mixture), 1.39 ± 0.08 μg/ml; BLM-A_2, 0.24 ± 0.02 μg/ml; BLM-B_2, 0.20 ± 0.02 μg/ml; and PEP, 0.79 ± 0.05 μg/ml. The viability of the cells was not changed significantly when treated for 48 hr with twice the ED_{50} concentration of either the BLMs or PEP. At higher concentrations of BLM and, more pronounced, of PEP the drugs act cytotoxically. At $3 \times ED_{50}$ the cell viability for the respective drugs were as follows: BLM (clinical mixture), $88 \pm 5\%$; BLM-A_2, $85 \pm 5\%$; BLM-B_2, $87 \pm 5\%$; and PEP, $77 \pm 5\%$. At $5 \times ED_{50}$ the differences in the cytotoxicity between the BLMs and PEP were very pronounced: BLM (clinical mixture), $72 \pm 5\%$ cell viability; BLM-A_2, $74 \pm 5\%$; BLM-B_2, $73 \pm 5\%$; and PEP, $46 \pm 4\%$.

Both PEP and BLMs cause 'unbalanced growth' [26] of L5178y cells. After incubation of the cells with the ED_{50} concentration of PEP the volume distribution curve changes significantly (Fig. 1). While the mean size of untreated cells was determined to be 1538 ± 668 μm^3 (mean value: 14.14 ± 6.14), this value increases to 1718 ± 685 μm^3 (15.80 ± 6.29) for the PEP-treated cultures. Together with the shift in the channel number, the distribution curve shows a deviation to the right (skewness from +0.59 to +0.48). As a further criterion for 'unbalanced growth' we determined the kurtosis of the curves. The value for the control was −0.22 and for the PEP-treated cultures −0.57, representing the broader distribution of the curve recorded from PEP-treated cultures. Similar values were calculated from size distribution curves obtained from cultures treated with the ED_{50} concentration of BLM (clinical mixture): mean value, 15.85 ± 6.36 (vol., 1724 ± 692 μm^3); skewness, +0.46; and kurtosis, −0.60.

The effect of PEP on cell proliferation is not restricted to the S-phase only (Fig. 2). Determining the cytostatic potency on S-phase cells, a reduction of the cell number after one division cycle from 185,000 to 142,000/ml was observed; G_2-phase cells were influenced by the same PEP concentration to almost the same extent (182,000 to 135,000/ml). Interesting was the finding that 3.7 μg/ml of BLM (clinical mixture) influences the proliferation of the two cell-cycle populations in the same way: S-phase cells from 192,000

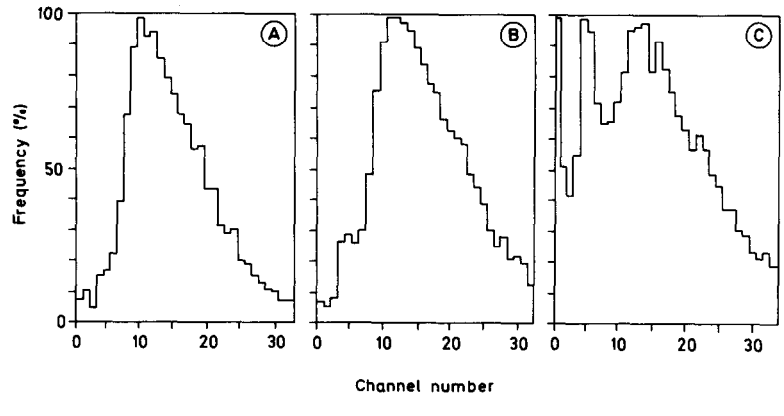

Fig. 1. Volume distribution of L5178y cells not treated with the drug (inoculation, 5000 cells/ml; incubation time, 72 hr) (A); (B) cultures treated with 0.8 μg PEP/ml (inhibition of cell growth, 48%); and (C) cultures treated with a combination of 0.41 μg PEP/ml and 0.13 μg BLM-A_2/ml (inhibition, 77%). The statistical parameters of the distribution curves are as follows: (A) mean value, 14.14 ± 6.14; skewness, +0.59; kurtosis, −0.22; (B) 15.80 ± 6.29, +0.48, −0.57; and (C): 16.36 ± 6.33, +0.50, −0.67.

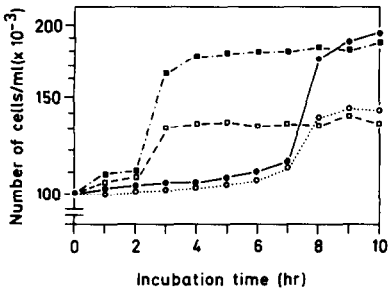

Fig. 2. Effect of pepleomycin (PEP) on the proliferation of S-phase- and G_2-phase L5178y cells. Synchronized cells were obtained as described in Materials and Methods. Fifty-milliliter cultures were inoculated with 100,000 cells/ml in the *presence of* 0 *or* 1.9 μg *PEP/ml. The cell number was* determined as indicated. S-phase cells in the absence (●) *or presence of PEP* (○); *G_2-phase cells in the absence* (■) *or presence of PEP* (□).

(controls) to 146,000 (BLM-treated) and G_2-phase cells from 188,000 to 143,000/ml.

Cytostatic activity of the drugs in combination

In the central part of this study the interaction of PEP with the BLMs was determined. Calculating the FIC indexes for PEP in combinations with BLM (clinical mixture), BLM-A_2 or BLM-B_2 revealed values lower than 0.5, indicating a significant synergism (Table 1). Concentration ratios between PEP and the BLMs were chosen from 0.4:1.0 to 6.4:1.0. Three different concentration ratios were selected for each combination. The lowest FIC index (0.26) was calculated for PEP:BLM (clinical combination), followed by PEP:BLM-A_2 (0.40) and PEP:BLM-B_2 (0.46).

The synergistic interaction of the PEP–BLM combination is also demonstrable on the basis of the evaluation of the size distribution curves. One example is shown in Fig. 1 (C): cells were incubated with the $0.5 \times ED_{50}$ of BLM-A_2 (0.13 μg/ml) and the $0.5 \times ED_{50}$ of PEP (0.41 μg/ml). Assuming an additive effect, a 50% inhibition of the cell growth and a monomodal distribution curve similar to that obtained with $1 \times ED_{50}$ of PEP (Fig. 1B) should be expected. However, the experiments revealed a 77% inhibition and a bimodal distribution curve (Fig. 1C) with the following characteristics for the main curve: mean value, 16.36; skewness, +0.50; and kurtosis, −0.67. These values describe a cytostatically inhibited population of the 'unbalanced growth' type. In addition to the main curve a minor curve with a mean value of 7.2 (vol., 783 μm^3) was recorded. The latter curve represents the cytotoxically affected portion of the total cell population. In the first and second channels of the curve (Fig. 1C) the amount of cell debris is subsumed; this portion was absent both in the untreated population and in the population treated with the ED_{50} of the drug.

Table 1. Fractional inhibitory concentration indexes (FIC indexes) for PEP–BLM combinations on L5178y cells

Drug combination	Concentration ratio	FIC index
PEP: BLM (clinical mixture)	0.4 : 1.0	0.29
	1.6 : 1.0	0.25
	6.0 : 1.0	0.24
PEP: BLM-A_2	0.8 : 1.0	0.39
	3.2 : 1.0	0.42
	6.4 : 1.0	0.38
PEP: BLM-B_2	0.5 : 1.0	0.45
	2.0 : 1.0	0.46
	4.0 : 1.0	0.48

The concentration ratios are based on *x* μg PEP/ml to *y* μg BLM/ml.

Differential fragmentation of DNA by BLM and PEP

To determine the effect of the drugs on DNA in intact cells spinner cultures (100,000 cells/ml) were incubated with increasing BLM-A_2- or PEP concentrations. The 50% inhibition (expressed in reduction of doublings) of cell proliferation under these conditions was obtained with 0.65 μg BLM-A_2/ml or 1.90 μg PEP/ml (Table 2). DNA was extracted from the cultures by a procedure which gives high percentage yields. Determination of the number of double- and single-strand breaks (alkaline-labile sites) in DNA under ED_{50} conditions revealed almost identical values for both BLM and PEP. However, after incubation with $2 \times ED_{50}$- or $3 \times ED_{50}$ concentrations the ratios between the number of single- and double-strand breaks caused by the drugs changed considerably; the ratios for BLM-A_2 were 1.86 and 1.95 respectively and for PEP 15.57 and 11.46. This result shows that at higher concentrations BLM-A_2 preferentially causes double-strand breaks while after PEP-treatment the number of single-strand breaks is increased.

DISCUSSION

It is generally agreed that DNA is the target molecule for the cytostatic and cytotoxic activity of BLM [7, 27] and PEP [11]. The bithiazole- and the terminal residues in their molecules contribute to the binding to DNA [28] and to the degradation of this macromolecule [29]. In the present study it is shown that the mode of actions of BLM and PEP, which are distinguished only by their terminal moieties, towards DNA are different. BLM is known to cause both single- and double-strand breaks in DNA [30, 31], probably

Table 2. Number of single- and double-strand breaks in DNA from PEP- or BLM-treated L5178y cells

Drug	Concentration (μg/ml)	Cell proliferation (doublings)	Yield of DNA (%)	Molecular mass of DNA (Da × 10^{-6}) Native	Denatured	Strand breaks Double	Single
None	—	1.69	93	28.4	14.1	0	0.03
BLM-A_2	0.65	0.84	91	25.5	7.6	0.23	3.5
	1.30	0.62	95	13.5	7.0	2.2	4.1
	1.95	0.39	87	9.5	4.8	4.0	7.8
PEP	1.90	0.81	90	25.5	7.4	0.23	3.7
	3.80	0.49	85	21.0	3.8	0.7	10.9
	5.70	0.27	89	17.0	3.0	1.3	14.9

Cultures of 10^5 cells/ml were incubated in 80-ml spinner cultures (see Materials and Methods). The yield of DNA extracted is given as % on the basis of DNA content of L5178y cells (7.9×10^{-12} g/cell).

by two independent events [30]. Under our conditions the ratio between the number of single- and double-strand breaks of DNA isolated from BLM-treated cell cultures (at concentrations above the ED_{50}) is approximately 2:1. In contrast, PEP caused a 6- to 7-fold higher number of single-strand breaks in intact cells. The reasons for the differences in this ratio are not known; it might be postulated that PEP has lost the ability to form dimers (composed of two PEP-monomers), as has been suggested for BLM [31].

On the cellular level the two drugs BLM and PEP show some common features. Firstly, both compounds cause 'unbalanced growth' of L5178y cells and secondly, they inhibit proliferation of synchronized cells in both the G_2- and S-phases to the same extent. However, the described difference in the molecular mode of action between BLM and PEP is correlated with some other growth-inhibitory potencies of the two anti-cancer drugs. Compared with BLM, PEP inhibits cell proliferation within a closer concentration range, expressed by the steeper slope of the dose–response curve. More important, the two drugs showed a highly significant synergistic interaction in our cell culture experiment using L5178y mouse lymphoma cells. This result was drawn from the determinations of the FIC indexes, as well as from mathematical evaluations of the size distribution curves recorded from drug-treated cell populations. Since both PEP and BLM are clinically used, this finding might contribute to a further improvement in rational combinations of these drugs. In an earlier study [18] a potentiating inhibitory effect of steffimycin B and the benzimidole derivative Hoechst 33258 on cell proliferation by BLM has been described; however, these combinations were not appropriate for clinical use.

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