SYNERGISTIC EFFECT OF PEPLOMYCIN IN COMBINATION WITH BLEOMYCIN ON L5178y MOUSE LYMPHOMA CELLS *IN VIVO*

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Studying the treatment of NMRI mice with ip injections of bleomycin (BLM) for 5 days we found an approximate LD_{50} of 35 mg/kg; the toxicity of peplomycin (PEP) was slightly higher (LD_{50}: approximately 25 mg/kg). The effect of the two drugs on growth of L5178y mouse lymphoma cells in NMRI mice was examined. BLM alone caused at a concentration of 2.5 mg/kg an almost complete inhibition of tumor cell growth; the same effect was determined with 1 mg PEP/kg. At these concentrations the drugs caused an increase of the survival time of 110% (BLM) or 104% (PEP). Given in combination, one-sixth of the optimal doses yielded an 100% increase of the median survival time. These results indicate a significant synergistic activity of the PEP-BLM combination on L5178y cell growth *in vivo* (FIC index: 0.34).

Bleomycins (BLMs) are glycopeptide antibiotics derived from *Streptomyces verticillus*; they were discovered by UMEZAWA[1]. Biochemical studies revealed, that BLMs are members of a new class of DNA-modifying agents, the quasi-enzymes[2,3], which induce intracellular breakage of DNA[4]. They are clinically used as a mixture (BLM-A_2, BLM-B_2, BLM-A'_2) to treat certain human tumors, such as squamous cell carcinomas and malignant lymphomas[5,6]. In 1977, UMEZAWA and coworkers[7] succeeded in developing a new analogue of BLM, which had less pulmonary toxicity than BLM[8]. This antibiotic was named peplomycin (PEP).

In a recent study we demonstrated[9], that the two chemically related antibiotics BLM and PEP inhibit proliferation of L5178y mouse lymphoma cells *in vitro* in a highly significant synergistic way. The present work was undertaken to evaluate the combination effects of BLM and PEP on the growth of the same tumor *in vivo*, using NMRI mice.

Materials and Methods

Chemotherapeutic Agents

PEP and BLM (containing 55~70% A_2, 25~32% B_2, <7% A'_2 and 1% B_4) were obtained from H. Mack, Illertissen (Germany).

Tumor and Animals

The L5178y leukemic cells[10] were maintained by serial transplantation as an ascitic tumor in 32~35 g male outbread NMRI mice[11,12]. The appropriate concentration of leukemic cells for intraperitoneal inoculation (1.2×10^6 cells) was obtained from ascites fluid by dilution in 0.9% NaCl solution. Under these conditions, 95~100% of the mice developed a palpable ascites and 50% of the animals died between day 18 and 20 post inoculation. The increase of body weight after this period of time was 143±15%.

The subacute toxicity tests were performed with 5 mice each. The drugs were applied by daily intraperitoneal injection for 5 days. Both normal mice and mice bearing L5178y cells (eight days after tumor inoculation) were used for this study.

The evaluation of the antitumor effects of BLM and PEP in L5178y leukemia was performed as follows: Mice were made leukemic by ip injection of 1.2×10^6 L5178y cells. Eight days later, the animals were divided into groups of 25 mice. One group was used as control, whereas the other groups were treated ip with BLM or PEP for five consecutive days. During and after the treatment, the body weight as well as the number of survivors were determined.

Statistical Evaluation

T-test to determine the significance of the growth inhibition effects of different concentrations of the drugs was performed according to Student[13]. The mathematical evaluation of the fractional inhibitory concentration index (FIC index) of PEP-BLM combinations was performed according to published equations[14] as applied earlier[9]. The ED_{50} is that effective dose, which causes approximately a 50% reduction of tumor growth.

Results

Toxicity

In analogy to the protocol of ISHIZUKA *et al.*[15] and KANAZAWA *et al.*[16], subacute toxicity tests with 5 NMRI mice each were performed by daily ip injection of the clinical mixture of BLM and of the pure PEP for 5 days. Using normal mice, injection of 35 mg BLM/kg (25 mg PEP/kg) caused death of 1 animal among 5 (3/5) during the first 5 days and two others (0/5) died between days 6 and 10. A similar toxicity was determined with L5178y cell bearing mice: Injection of 35 mg BLM/kg (25 mg PEP/kg) caused death of 2 animals among 5 (2/5) in five days after administration of the drugs, and 2 (1/5) more died before day 10. Injection of 15 mg BLM/kg (10 mg PEP/kg) caused no deaths both for normal mice and L5178y mice.

Mono Chemotherapy

Using L5178y bearing NMRI mice, both BLM and PEP caused a marked and significant inhibition of L5178y cell growth *in vivo* (Fig. 1). The drugs were administered for 5 days (day 8~12 after tumor inoculation). At day 20, BLM caused at a dose of 1.25 mg/kg a reduction of tumor growth from $144 \pm 17\%$ (based on body weight) to $137 \pm 16\%$, at 2.5 mg/kg to $125 \pm 16\%$ ($P<0.005$) and at 5 mg/kg to $119 \pm 14\%$ ($P<0.005$) (Fig. 1A). Using PEP (Fig. 1B) a reduction from $145 \pm 18\%$ to $132 \pm 15\%$ ($P<0.01$) at 0.5 mg PEP/kg, to $127 \pm 14\%$ ($P<0.005$) at 1 mg/kg and to $121 \pm 14\%$ ($P<0.005$) at 2.5 mg/kg was measured. From these results, the therapeutic index (ratio of the approximate values of LD_{50} and of ED_{50}) for BLM was calculated to be 28 (35 mg/kg to 1.25 mg/kg). For PEP the therapeutic index was estimated to be 50 (25 mg/kg to 0.5 mg/kg).

The inhibition of tumor growth in the ascites by the drugs resulted in a marked prolongation of the median survival period (Fig. 2). Untreated animals showed a survival time of 18.6 days. Treatment of the L5178y mice with 1.25 mg BLM/kg produced a 52% increase in survival, with 2.5 mg BLM/kg an 110% increase and with 5 mg/kg an 84% increase (Fig. 2A). PEP treatment at doses of 0.5 mg/kg, 1 mg/kg or 2.5 mg/kg resulted in a 67%, 110% or 39% increase of median survival time (Fig. 2B). These data show, that the optimal doses of the drugs are for BLM 2.5 mg/kg and for PEP 1 mg/kg, under our experimental conditions chosen. A further increase of these doses reduced the median survival time, very likely as a result of drug toxicity.

Fig. 1. Increase of body weight of L5178y bearing NMRI mice.

The body weight of the animals at day 1 was 32~35 g; this value is set to 100%. Eight days after inoculation of L5178y cells, the animals were treated for five days (bars) with BLM (A) or PEP (B) at the following doses; BLM: 1.25 mg/kg (○), 2.5 mg/kg (■) and 5 mg/kg (△) or PEP: 0.5 mg/kg (○) 1 mg/kg (■) and 2.5 mg/kg (△). ●=Untreated tumor mice.

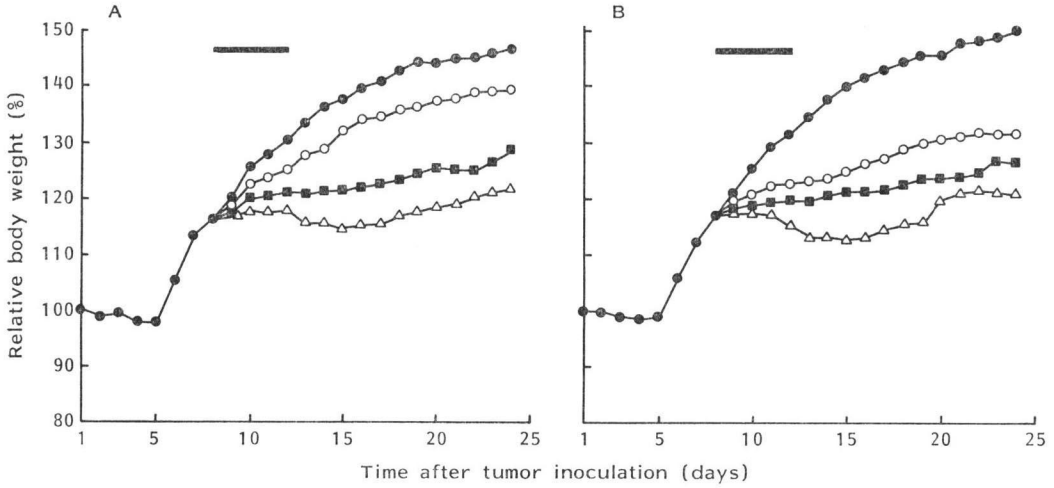
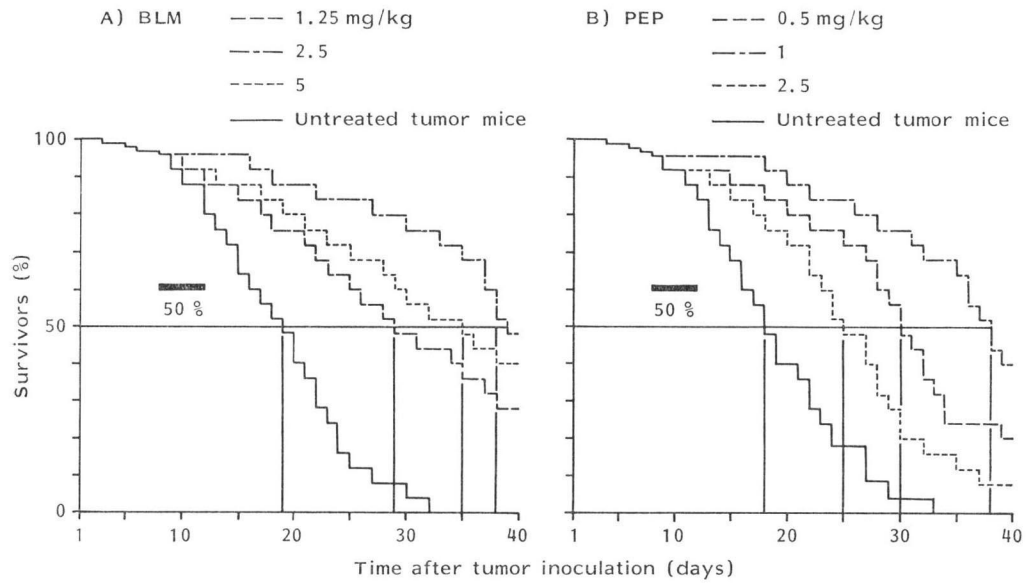


Fig. 2. The effect of BLM (A) and PEP (B) on survival of tumor bearing mice.

Various doses of the compounds were administered as described in Fig. 1.



Combination Chemotherapy

In the central part of the study, the antitumor activity of BLM and PEP given in combination was determined (Table 1). Administration of one-fourth of the optimal dose of the drugs in combination (0.63 mg BLM/kg and 0.25 mg PEP/kg) revealed only a slight chemotherapeutic effect (18% increase of survival time), while a combination of one-sixth of the optimal doses resulted in a 100% increase of the median survival time. Thus, the combination of BLM+PEP provided a significant, synergistic

Table 1. Carcinostatic effects of BLM and PEP on mouse L5178y cells *in vivo*.

Drug combination		Median survival time (days)	Survival time (treated/control) (%)
BLM (mg/kg)	PEP (mg/kg)		
0	0	18.6	100
2.5	—	39.0	210
1.25	—	29.0	152
—	1	38.0	210
—	0.5	30.0	167
0.63	0.25	22.0	118
0.42	0.17	37.0	200
0.21	0.09	29.0	156
0.11	0.05	21.0	113

tumor inhibitory effect (FIC index: 0.34). Even one-tenth of the two optimal doses showed a remarkable tumor inhibitory effect (56% increase of survival). The results also suggest, that the slight life-prolongation rate of this two-drug-combination at one-half of the optimal doses may too be attributed to drug toxicity.

Discussion

If included in human polychemotherapy, BLM is administered in combination with methotrexate[17], with cyclophosphamide, vincristine and methyl-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea[18] and vinblastine[19]. These combinations are plausible also from the molecular point of view, because of the different molecular modes of action of these drugs. However, the main finding in this paper that PEP potentiates the antitumor effect of its structural analog BLM in the L5178y cell system *in vivo* came unexpectedly. We were stimulated to study this combination by our previous observations which showed that PEP potentiates the BLM-cancerostatic effect on L5178y cells *in vitro*[9]. Moreover, it was already known[9] that on molecular level PEP causes preferentially single-strand breaks in DNA, while BLM degrades DNA by double- and single-strand breakages.

The degree of synergism caused by the BLM-PEP combination on L5178y cell growth *in vivo* was highly significant (FIC index: 0.34). Since we have not determined the combination effects in normal mice, the possibility exists that only a potency synergism has been elucidated. Future studies must clarify on the basis of maximum tolerated doses (performed both with normal and tumor-bearing mice), whether the combination effect, described in the present investigation, reflects a therapeutic- or a pharmacological synergism[20].

PEP produced a higher subacute toxicity on mice than BLM. This effect correlates with the *in vitro* data[9] which also revealed a higher cytotoxic effect of PEP. However, PEP has been demonstrated to cause a lower pulmonary toxicity at therapeutic doses than BLM[7]. Consequently, the following two modes of application of PEP in cancer treatment might be deduced; firstly, as substitution for BLM and secondly, in the combination chemotherapy with BLM. However, the clinical utility of the PEP-BLM combination depends on the results of experimental studies which have to investigate, whether or not PEP "synergizes" also the pulmonary toxicity of BLM.

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