BIOLOGICAL STUDIES ON THE DEGRADATION PRODUCTS OF 3-[(S)-1′-PHENYLETHYLAMINO]PROPYLAMINOBLEOMYCIN: A NOVEL ANALOG (PEPLEOMYCIN)

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Pepleomycin (PEP), 3-[(S)-1′-phenylethylamino]propylaminobleomycin has potent activity and is less pulmonary toxic than bleomycin (BLM). Biological activity and toxicity of the following degradation products of PEP have been studied in detail: the product of carbamoyl migration (ISO), the product of decarbamylation (DC), the product of ring closure of the side chain on the pyrimidine moiety (RC), the depyruvamide product (DP) and the product of an enzymatic inactivation (DA).

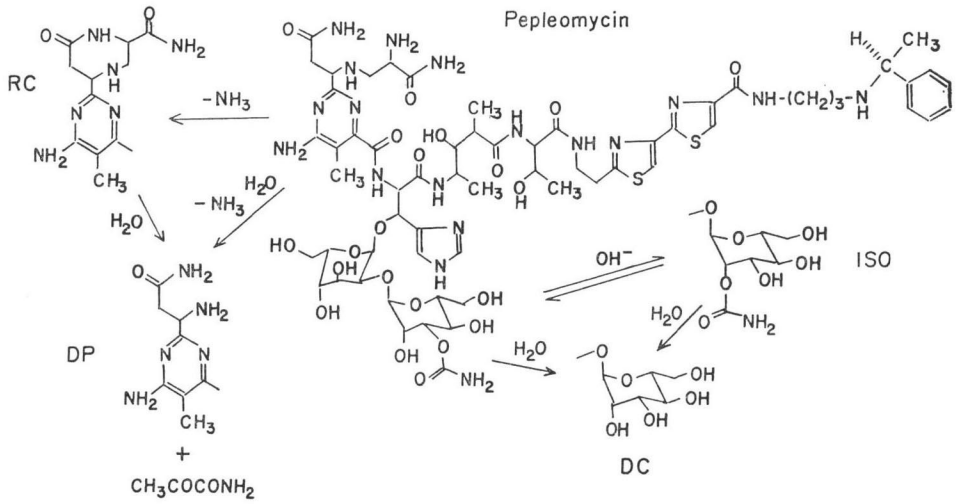
These degradation products showed much lower activity than PEP *in vitro*: antimicrobial and anti-HeLa activities, inhibition of DNA synthesis in AH66 cells and the DNA strand cleavage. Acute toxicity and pulmonary toxicity were tested in mice. Results indicated much lower acute toxicity corresponding to the decreased *in vitro* activity when compared to PEP. DP and RC did not cause lung fibrosis in mice, while ISO and DC showed 1/2.6 and 1/5.7 degree of pulmonary toxicity, respectively, in comparison with PEP.

Pepleomycin (PEP: 3-[(S)-1′-phenylethylamino]propylaminobleomycin)[1] has been developed as a novel analog of bleomycin (BLM) which has less pulmonary toxicity than BLM mixture clinically in use today in the treatment of cancer.

Although PEP is very stable at room temperatures, several degradation products were detected in its heated aqueous solutions of about pH 6. They were confirmed to be due to the carbamoyl migration[2] (ISO), decarbamylation[3] (DC), depyruvamide formation[4] (DP) and ring closure of the side chain on the pyrimidine moiety[4,5] (RC). BLM is inactivated to the deamido compound (DA) by BLM hydrolase[6]. Since there are no systematic informations of biological and toxicological properties of the degraded BLM, such studies seem to be important from the drug-safety aspect.

Studies on the action mechanism of BLM have revealed the complex formation between BLM and Fe (II) followed by the coordination of oxygen to it in the action of BLM against DNA[7,8]. The most probable structure of the complex has been proposed on the basis of the structure of BLM[8]. The α-amino and the secondary β-amino groups of the β-aminoalanine moiety (V), the pyrimidine ring N-1, the imidazole ring N of the β-hydroxyhistidine moiety (IV) and the amino group of IV are involved in the complex formation to produce a square pyramidal coordination. The first group occupies the apical position. The carbamoyl group has been suggested to be in the vacant 6th coordination site. The α-amino group of V is absent in DP and blocked and restrained by the intramolecular amide bonding in RC. The carbamoyl group is absent in DC, and that in ISO is not involved in coordination. In DA, the free carboxyl group of V may be involved in coordination instead of the α-amino group. All these degradation products have a weaker ability in the complex

Fig. 1. Degradation reactions of pepleomycin.
RC, the product of ring closure; DP, the depyruvamide product; ISO, the product of carbamoylmigration; DC, the product of decarbamylation.



formation. Therefore, the investigation of their *in vitro* activities such as DNA strand cleavage and their toxicity is thought to elucidate the mechanism of the antitumor action and structure-activity relationships.

In this paper, we discuss the results of comparative studies on the following biological and toxicological properties of the degradation products: antimicrobial and anti-HeLa activities, inhibition of DNA synthesis in AH66 cells, DNA strand cleavage, acute toxicity and pulmonary toxicity in mice.

Materials and Methods

Bleomycins

Pepleomycin sulfate and the bleomycin mixture were prepared by Nippon Kayaku Co., Japan. The Fe(II) complexes of PEP and its degradation products were prepared by adding an equimolar quantity of ferrous sulfate to their aqueous solutions at 0°C immediately before use.

Preparation of the degradation products

Two g (1.27 mmoles) of PEP sulfate were dissolved in 200 ml of a pH 6.0 (0.064 M) imidazole-hydrochloric acid buffer, followed by heating in the presence of nitrogen gas at 65°C for 7 days. The residual potency against *Mycobacterium smegmatis* ATCC 607 was 48.4%. A chromatography was made on a column (200 ml) packed with CM-Sephadex C-25 (Pharmacia Fine Chemicals Co., Sweden) pretreated with a pH 6.8 (0.05 M) phosphate buffer[4] by elution with the buffer supplied with sodium chloride. The eluate at 0.2 M of sodium chloride gave 21 mg (0.014 mmoles) of an amorphous powder of RC hydrochloride after desalting and lyophilization. The eluate at 0.4 M was applied to a column (200 ml) of SP-Sephadex C-25 pretreated with a pH 2.5 (0.05 M) citrate buffer[4]. The elution was made by increasing the sodium chloride concentration in the same manner. The eluate at 0.55 M gave 695 mg (0.476 mmoles) of DP hydrochloride. The eluate at 0.65 M contained the intact PEP, DC and ISO. After desalting, an excess amount of cupric acetate was added to transform the compounds to copper complexes for the next step. A chromatography was made on CM-Sephadex C-25 (200 ml) pretreated with a pH 4.5 (0.05 M) acetic acid-sodium acetate buffer by analogous elution as described above. After the intact PEP was eluted off with a 0.5 M buffer, both DC and ISO appeared in the eluate at 0.65 M, and were further chromatographed on a silica gel column (Kieselgel 60C; E. Merck, West Germany) by elution with a solvent: 10% ammonium acetate – acetic acid – methanol – ethanol (25: 1: 125: 350 v/v). ISO appeared slightly faster than DC. Since an overlapping was observed, only pure fractions were collected, desalted and lyophilized to give 60 mg (0.037 mmoles) of ISO (Cu)

hydrochloride and 120 mg (0.08 mmoles) of DC (Cu) hydrochloride. They were transformed into copper-free forms by the EDTA method[9].

Authentic samples for identification were prepared by the reported methods: treating PEP with dilute acetic acid[4], aqueous ethanol containing triethylamine[2] or tris-buffer[3]. Identification was performed by analysis of their amino acid compositions and sugar moiety[10], thin-layer chromatographies and cochromatographies with authentic samples by using liquid column chromatographies described above.

Deamido PEP (DA) was prepared enzymatically by using a rat liver BLM amidohydrolase[6].

Chemicals

[6-^{3}H]-Thymidine (5 Ci/mmole) and [methyl-^{3}H]-thymidine (41 Ci/mmole) were purchased from the Radiochemical Centre (England), EAGLE's MEM medium from Nissui Seiyaku Co. (Japan), calf serum from Flow Laboratories (Australia). [^{3}H]-DNA was prepared from rat ascites hepatoma AH66F cells exposed to [methyl-^{3}H]-thymidine by the method of MARMUR[11].

Animals

Male ICR-SLC mice (S.P.F.) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory's Animals (Japan).

Determination of antimicrobial potency

Antimicrobial activities of PEP and its degradation products against *Mycobacterium smegmatis* ATCC 607 were determined by an agar diffusion cylinder method. The potency of PEP free base was defined as 1,000 μg potency/mg.

Determination of growth inhibition of HeLa S_3 cells

HeLa S_3 cells were cultured at the initial density of 2×10^4 cells/2 ml/plate with EAGLE's MEM medium supplemented with 10% calf serum at 37°C for 48 hours in a CO_2 incubator. The medium was changed by 2 ml of fresh medium containing various concentrations of PEP and its degradation products for further incubation of 72 hours. The cell numbers before and after treatment with test compounds were counted by a Coulter Counter model ZB1. The ID_{50} value, which is the concentration of each test compound required for 50% inhibition of growth, was obtained graphically by a plot of its log concentration versus the probit of inhibition rate.

Determination of inhibition of DNA synthesis

To 1 ml of the cell suspension of AH66 cells (1×10^6 cells/ml) in EAGLE's MEM medium supplemented with 10% calf serum was added 0.95 ml of the medium containing test compounds at various concentrations for the incubation of 30 minutes at 37°C. For incubation of further 30 minutes, [6-^{3}H]-thymidine (1 μCi/0.05 ml) was added to the mixture. The incorporation of [^{3}H]-thymidine was terminated by adding 2 ml of chilled 10% trichloroacetic acid. The precipitate obtained by centrifugation at 1,500 rpm for 5 minutes was washed twice with 5 ml of methanol by centrifugation at 3,000 rpm for 5 minutes. The precipitate thus obtained was solubilized by adding 0.7 ml of Hyamine-10X and the radioactivity in the solubilized sample was counted by a Packard model Tri-Carb 3380 liquid scintillation spectrometer.

Determination of DNA strand cleavage

The reaction mixture (0.5 ml) consisted of pH 7.4 (0.05 M) potassium phosphate buffer, 2 mM KCl, various concentrations of the Fe(II) complexes of PEP or its degradation products and 0.5 μg of [^{3}H]-DNA (24,000 dpm). The mixtures were incubated at 37°C for 5 minutes. To terminate the reaction, 0.1 ml of an aqueous solution of bovine serum albumine (10 mg/ml) and 0.4 ml of 25% trichloroacetic acid-12.5 mM sodium pyrophosphate solution were successively added at 0°C. After centrifugation at 3,000 rpm for 5 minutes, the radioactivity of acid-soluble DNA contained in 0.5 ml of the supernatant was counted by the liquid scintillation spectrometer with 10 ml of triton-toluene scintillation fluid.

Acute toxicity in mice

PEP or each of its degradation products were injected intraperitoneally in a dose of 100 mg/kg

to 6-week old male ICR-SLC mice (weighing 28~30 g), and their body weight changes, their general manifestations (*e.g.*, piloerection, contamination of fur, loss of nail, *etc.*), and their survival or death were observed during the following 14 days.

Pulmonary toxicity in mice

Fifteen-week old male ICR-SLC mice were used. Each group consisted of 5 or 10 mice to which 5 mg/kg of test compounds were injected intraperitoneally daily for 10 days. To the control group, physiological saline was injected. In order to examine the development of pulmonary toxicity 5 weeks after the last injection, all mice were sacrificed, the lungs were fixed with neutral formalin solution, and their histological specimens were prepared.

Pulmonary fibrosis was determined by the method reported by MATSUDA *et al.*[12] In this method, degrees of pulmonary fibrosis were divided into 5 classes by the following: grade (score);

− (0), indicating the absence of fibrosis;
± (1), the presence of areas with questionable fibrosis in alveolar septae;
+ (2), a few foci of fibrosis, often in subpleural area;
++ (4), scattered foci of fibrosis;
+++ (6), diffuse fibrosis.

The data were expressed as follows: incidence (%)=(number of mice with fibrosis in group/number of mice tested in group)×100; grade (mean score)=total score of specimens in group/number of specimens in group. For the evaluation of pulmonary toxicity of test compounds, the BLM mixture was taken as the control and the data were expressed as relative values to the latter.

Results

Activities *In Vitro* of PEP and its Degradation Products

PEP and its degradation products showed the following antimicrobial potencies against *Mycobacterium smegmatis* ATCC 607 (μg potency/mg); PEP (873), ISO (352), DC (242), DP (37) and RC

Fig. 2. Effects of PEP and its degradation products on the DNA synthesis of AH66 cells

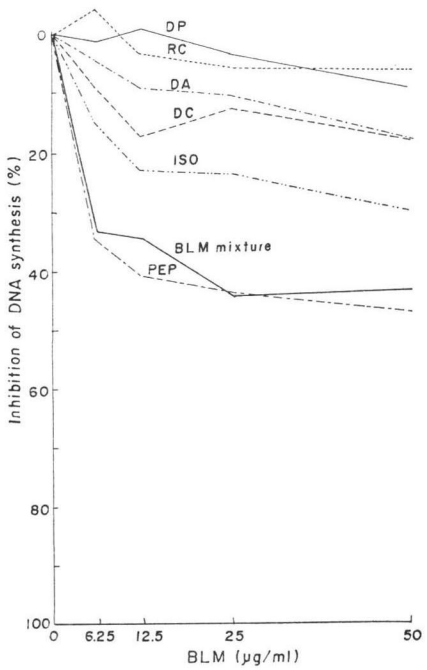
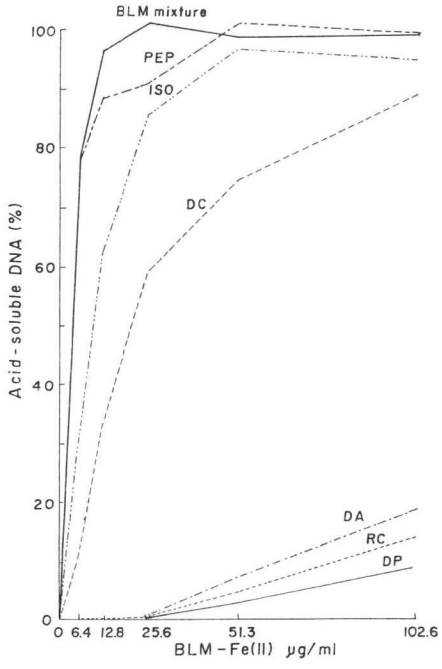


Fig. 3. *In vitro* DNA strand cleavage by various BLM-Fe(II) complexes



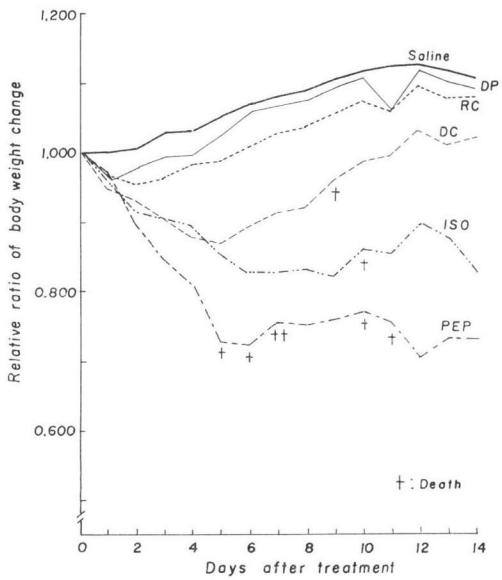
(28), and gave the following very correlative results on the growth inhibition of cultured HeLa S_3 cells (ID_{50}, μg/ml); PEP (0.84), ISO (3.2), DC (9.0), DP (above 100) and RC (above 100). The inhibitory effects on DNA synthesis in rat ascites hepatoma AH66 cells are shown in Fig. 2. PEP and the BLM mixture gave almost the same results, while the degradation products showed markedly reduced activities decreasing in the order of ISO, DC, DA, DP and RC. From the above results, all of the degradation products of PEP were found to have much less activity against the tested living cells in comparison to PEP.

Ferrous ions and oxygen are required for the action of BLM as described. Therefore, the activity on the isolated DNA was studied by using Fe(II) complexes of PEP and its degradation products. Fig. 3 shows the activity to break DNA strands. PEP and the BLM mixture gave almost the same amounts of acid-soluble fraction resulting from [^{3}H]-DNA cleavage, while all the degradation products were less active. Concentrations for PEP, ISO and DC enough to make DNA acid-soluble by 78%, were approximately 6.4, 20 and 60 μg/ml, respectively. Concentrations of DA, RC and DP required to make 11% of DNA acid-soluble were approximately 66, 84 and 115 μg/ml, respectively, while DC showed the same extent of solubilization at 6.4 μg/ml. Therefore, the activities of ISO, DC, DA, RC and DP were estimated at approximately 32%, 11%, 1%, 0.8% and 0.6%, respectively, to that of PEP.

Toxicity in Mice

The acute toxicity to mice of PEP and its degradation products is shown in Fig. 4 and Table 1. The changes of the mean values of body weights were observed to be very different, when each of the compounds was injected intraperitoneally in a dose of 100 mg/kg to the ICR-SLC mice. The rate of loss in body weight decreased in the order of PEP, ISO, DC, RC and DP. Table 1 shows the lethality of the treated groups. Lethality was 67% in the PEP treated group, while only one animal died in groups treated with ISO or DC, and no animal died in the other 2 groups treated with DP or RC.

Fig. 4. Change of body weights of mice treated with PEP or its degradation products



The observation of general manifestations and the autopsy in the above groups did not reveal more severe toxicological symptoms in the degradation products groups over those in the PEP treated group. Furthermore, no special toxic manifestations and no autopsical findings due to the degradation products were observed.

Since pulmonary toxicity is most important in the chemotherapy with BLM or PEP, it was tested in mice. The magnitude of PEP to cause pulmonary toxicity was 0.34 (grade) and 0.40 (incidence). ISO and DC gave the values 0.13 and 0.06 (grade). The other compounds, RC and DP gave no pulmonary toxic symptoms as shown in Table 2.

Table 1. Lethality of mice by the administration of PEP and its degradation products

Compounds (100 mg/kg, i.p.)	No. of mice	Cumulative No. of dead mice														Total No. of dead mice	Lethality (%)
		Days after administration															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14		
PEP	9	0	0	0	0	1	2	4	4	4	5	6	6	6	6	6	67
ISO	9	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	11
DC	10	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	10
DP	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RC	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Saline	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2. Pulmonary toxicities of PEP and its degradation products in mice

Compounds (5 mg/kg, i.p. × 10)	Body weight difference (g)		Fibrosis incidence		Fibrosis grade	
	at day 11	at day 45	No. of animals with fibrosis / No. of treated animals	Ratio	Total No. of fibrosis score / No. of specimens	Ratio
Saline	−0.4	0.0	0/4=0.00		0/12=0.000	
BLM mixture	−5.1	−1.1	4/4=1.00	1.00	7/12=0.583	1.00
PEP	−5.8	−0.5	2/5=0.40	0.40	3/15=0.200	0.34
DP	−0.6	+2.6	0/5=0.00	0.00	0/15=0.000	0.00
RC	+1.0	+4.4	0/5=0.00	0.00	0/15=0.000	0.00
Saline	+0.2	+3.0	0/5=0.00		0/15=0.000	
BLM mixture	−5.5	−1.4	7/9=0.78	1.00	15/27=0.556	1.00
ISO	−0.8	+1.9	2/9=0.22	0.28	2/27=0.074	0.13
DC	−0.7	+1.6	1/10=0.10	0.13	1/30=0.033	0.06

Discussion

In comparison with PEP, DP and RC showed the lowest antimicrobial activity, 1/24 and 1/31, respectively, against *Mycobacterium smegmatis* ATCC 607. Furthermore, against cultured HeLa S_3 cells, both compounds showed less than 1/119 of the activity of PEP. Compound DA, resulting from enzymatic inactivation, was 1/100 as potent as PEP in breaking isolated DNA strands. DP and RC were found to have still lower activity than DA as shown in Figs. 2 and 3. They produced very low acute and pulmonary toxicity in mice; no lethal effects, slight reduction of body weights and no evidence of pulmonary fibrosis.

ISO and DC showed somewhat higher activity than the above mentioned 3 degradation products. ISO and DC were 1/2.5 and 1/3.6 as potent as PEP against *Mycobacterium smegmatis* ATCC 607, and 1/3.8 and 1/11, respectively, against HeLa cells. The activity of ISO and DC to cause DNA strand cleavage was 1/3.1 and 1/9.1 of that of PEP. The activities against HeLa cells and isolated DNA are thought to be correlative. Acute toxicity in mice caused by these 2 compounds was low. The reduction in body weights showed good correlations to their *in vitro* activity; DNA strand cleavage activity and inhibition of DNA synthesis in AH66 cells.

The low potencies of DP, RC and DA, and the relatively high potencies of DC and ISO suggest the contribution of the ligands to the bioaction of BLM. The significance of the free α-amino group of V, the carbamoyl group and its location on the mannose molecule are evident. This study showed the relationships among the antimicrobial and anti-HeLa activities, the inhibition of DNA synthesis of AH66 cells, cell-free DNA strand cleavage, the acute toxicity and pulmonary toxicity of PEP and

its degradation products. These activities decreased in the order of PEP, ISO, DC, DA, RC and DP. From the viewpoint of drug-safety, the degradation products were shown to have less toxicity than PEP in all aspects.

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