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Antitumor effect of lysine-isopeptides

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Abstract

Isopeptides (ε-peptides) of lysine, with a given Mw and low polydispersity (10–400 units), were synthesized to study the relationship between their chemical structure and biological effect. The designed compounds were of high purity, low polydispersity and high stereochemical purity. The effect of the compounds was tested on a human erythroleukemia cell line (K-562) and on four transplantable mouse tumors (L1210 lymphoid leukemia, P38 macrophage derived tumor, Ehrlich ascites carcinoma, Lewis lung tumor /LLT/). In case of the L1210 and P388 tumors and the Ehrlich carcinoma, survival of the animals was used as an indicator of the effect. In case of the Lewis lung tumor, the number and size of metastases in the lung and/or liver of treated and untreated mice were used as indicators. The polymers of polymerisation degree 80–120 (Mw 10.2–15.4 KD) showed the strongest antiproliferative effect both on K562 cells and the tumors growing *in vivo*. This effect was manifest with a significantly higher survival rate as compared to the control (L1210, P38, Ehrlich ascites), furthermore, by a decrease in the number and size of liver and lung metastases (LLT).

Introduction

The biological activity of poly-cations includes the immunoglobulin production stimulatory activity of epsilon poly-L-lysine. This compound also enhances interferon beta production, without stimulating cell proliferation [1]. On the other hand, alpha-poly-L-lysine enhances the proliferation of astrocytes in culture [2].

Clavicepamines are lysine-rich basic proteins isolated from cultures of ergot (*Claviceps purpurea*). These proteins could be fractionated to yield compounds of Mw 2–17 kD with a high lysine content (between 30–95 mole%). Biological investigation of these compounds showed a cell-proliferation retarding effect in animal tumors, without toxic side effects [3]. Structure determinations indicate that ε-lysine (poly) peptides are the fundamental structural units of clavicepamines.

In the present study, active oligomers for polycondensation have been prepared by a process which protected the

carboxyl groups with active ester groups, and the peptide coupling was carried out at a considerably higher rate than the aminolysis of the protecting active ester group. This coupling method provides a higher degree of activation than the active ester method [4].

We found that the "backing off" activation method is highly applicable for preparing isopeptides and salts consisting of diamino carboxylic acids. For temporary protection and simultaneous activation of the COOH groups, p-nitrophenylester is the most suitable.

The biological activity of the polyisolysines prepared in our laboratory was studied *in vitro* and *in vivo*.

Materials and methods

Enzymatic cleavage of lysine isopeptides

Enzymes: trypsin (2× cryst. lyophil.), chymotrypsin (N: 14.52%), papain (cryst.), pronase-P (protease from *Streptomyces griseus*), subtilisin (cryst.), carboxypeptidase-B (N: 15.4%) were obtained from SERVA Entwicklungslabor (Heidelberg, Germany); hog kidney leucineaminopeptidase from Koch Light Lab. Ltd. (USA).

Reference substrates

human serum albumin (lyophil.), benzyloxy-carbonyl-glycyl-L-phenylalanine, leucineamide hydrochloride (SERVA Entwicklungslabor, Heidelberg, Germany).

Compounds labelling of SZTP:Polyiso-L-lysines produced by our method (see Table 1)

SZTP-14: Polyiso-L-lysine HBr salt, molecular weight calculated on free bases: 12700± 200, polydispersity: 3.7 (number average/weight average).

Table 1: Characterization of isopolylysines (*å-poly-lysines-HBr*)

Charge No	*Preparation***method**	**Mw** (± 300)	**Polym. Degree** (± 2)
K-17	Kushawa (6)	9400	73
H-17	Hull (5)	900–3200	7–23
SZTP-14	„backing off" ONp	12700	99
SZTP-15	Dialyzed (H_2O)	11600	90
SZTP-16	Dialyzed (NaCl)	13400	105
SZTP-17	Ion exchanged	14500	113
SZTP-18	Kushawa (6) OPep	9500	74

Further characterisation of SZTP-14.

The free polymer was available as HBr salt.

Br content: 37.7% (calc.), 36.8% (found).

A_{254} (0 (i.e. no UV absorbance).

It was purified by precipitation from alcohol with ether.

$(\alpha)^{20}$ D (32.4° (C = 2; water)

Mw = 12700 D ± 200

SZTP-15: Polyiso-L-lysine HBr salt, molecular weight calculated on free bases: 11600 ± 200.

SZTP-16: Polyiso-L-lysine HBr salt, molecular weight calculated on free bases: 13400 ± 200.

SZTP-l7: Polyiso-L-lysine HBr salt, molecular weight calculated on free bases: 14500 ± 200.

SZTO-18: Polyiso-L-ornithine HBr salt, molecular weight calculated on free bases: 8900 ± 200.

H-17: Polyisolysine produced according to Hull *et al*. [5].

K-17: Polyisolysine produced according to Kushawa *et al*. [6]

A-3: α-Polylysine. Mw = 300 kD (SIGMA Ltd., USA)

Method of digestion and analysis

Digestion of the oligomers, polymers and the reference substrates by different enzymes was followed by paper electrophoresis. Incubation mixtures containing 0.1–2% of substrates in suitable buffers (pH 7 for trypsin and pH 8.5 for the others) were kept at 37°C in small test tubes. The reaction was initiated by the addition of the enzyme. 1–10 µl aliquots of the digests were taken at 0.2 and 24 h respectively, and were applied directly to Whatman No. 1 paper. Electrophoretic patterns were obtained in a horizontal electrophoretic assembly at 1500 V. Digestion was indicated by the appearance of new lysine ninhydrin-positive spots, by the change in intensity of ninhydrin color with time and finally, and by the disappearance of the starting materials.

In vitro studies on cell proliferation

Cells: K-562 human erythroleukemia cell line (Karolinska Institute, Stockholm) was used.

Cell number: 50×10^3/ml, in 5–6 ml medium containing glass test tubes.

Number of probes: 3 test tubes/dose.

Table 2: Effect of various polylysines on K-562 cell cultures Cell number: × 10^3 /ml culture medium (average ± SD)

Compound		**SZTP-14**				**SZTP-15**		
dose (μg/ml)	Control	1	10	100	Control	1	10	100
Hours after treatment 24	120 ± 10	93.3 ± 11	90 ± 20	0	120 ± 15	100 ± 11	60 ± 15	50 ± 15
48	223.3 ±	156.6 ± 5	83.3 ± 5	0	190 ± 11	140 ± 15	120 ± 19	20 ± 5
72	240 ± 17	173.3 ±	136.6 ± 15	0	250 ± 15	190 ± 10	110 ± 25	10 ± 5
Compound		**SZTP-16**				**SZTP-17**		
dose (μg/ml)	Control	1	10	100	Control	1	10	100
Hours after treatment 24	130 ± 10	120 ± 13	120 ± 14	120 ± 15	150 ± 15	130 ± 12	120 ± 10	120 ± 10
48	200 ± 15	210 ± 20	160 ± 17	120 ± 10	220 ± 18	210 ± 16	200 ± 17	190 ± 14
72	260 ± 20	280 ± 22	240 ± 23	140 ± 20	280 ± 20	280 ± 18	260 ± 20	210 ± 20
Compound		**SZTO-18**				**H-17**		
dose (μg/ml)	Control	1	10	100	Control	1	10	100
Hours after treatment 24	150 ± 14	140 ± 12	120 ± 10	100 ± 11	150 ± 14	120 ± 10	130 ± 11	110 ± 10
48	230 ± 18	210 ± 17	160 ± 15	130 ± 15	230 ± 18	214 ± 14	240 ± 12	210 ± 16
72	280 ± 20	260 ± 22	230 ± 20	180 ± 20	260 ± 22	290 ± 22	280 ± 21	270 ± 20
Compound		**K-17**				**A-3**		
dose (μg/ml)	Control	1	10	100	Control	1	10	100
Hours after treatment 24	130 ± 12	120 ± 11	135 ± 11	110 ± 5	120 ± 11	120 ± 10	120 ± 10	12" ± 11
48	200 ± 15	220 ± 15	240 ± 10	210 ± 10	240 ± 14	110 ± 5	110 ± 10	110 ± 5
72	280 ± 17	280 ± 20	290 ± 19	290 ± 19	290 ± 20	110 ± 7	110 ± 5	100 ± 10

Medium: Parker's M-199 supplemented with 10 % fetal bovine serum (Flow Laboratories, Irvine, Scotland).

Temperature, atmosphere: 37°C, 5% CO_2 + 95% air.

Treatment: 24 h after the dilution of cell cultures.

Doses: 1–10–100 ug/ml

Evaluation: Cell counts 24, 48, 72 and 96 h after the dilution of cultures, using Buerker's chamber.

Cells were counted in 3 test tubes per dose and in 3 test tubes as control at each time-point.

Table 3: The effect of 50 mg/kg daily treatment on the survival of DBA/2 mice inoculated with L1210

Days after inoculation	Living controls	Living treated mice
9	3	5
10	-	5
11	-	5
12	-	5
13	-	4
14	-	3
15	-	-

In vivo studies on the effect of SZTP 14

a) L1210 tumor

Origin of tumor: Chester Beatty Institute, London.

Animal strain: DBA/2 inbred mice, own breed.

Weight and sex of animals: 20–23 g females, 5 animals/group.

Tumor cell number: 10^5/animal injected intraperitoneally.

Treatment: 50 mg/kg, injected intraperitoneally 24 h after tumor inoculation, for 8 days, once a day.

Control group: physiological saline solution, 0.2 ml intraperitoneally.

Evaluation: survival of animals.

b) P-388 ascites tumor

Origin of tumor: National Institute of Oncology, Budapest, Hungary.

Animal strain. BDF/1 hybrid mice, own breed.

Weight and sex of animals: 20–23 g, males 5 mice/group.

Cell number: 2.8×10^6/animal, injected intraperitoneally.

Treatment: 10 mg/kg injected intraperitoneally daily, started 24 h after tumor inoculation.

Control: 0.2 ml of physiological saline solution daily, intraperitoneally.

Evaluation: survival of animals.

c) Ehrlich ascites tumor

Table 4: Number of surviving animals, untreated and treated with SZTP-14 on subsequent days after inoculation of Ehrlich ascites carcinoma

Control			**50 mg/kg**		**75 mg/kg**	
Days after inoculation	**?**	**?**	**?**	**?**	**?**	**?**
16	5	5	4*	5	5	5
17	5	4	4	5	5	5
18	5	4	4	5	5	5
19	3	2	4	5	5	5
20	2	2	4	5	5	5
21	2	2	4	5	5	5
22	0	1	4	5	5	5
23	0	0	4	5	5	5
24	0	0	4	5	5	5
| ?						
50	0	0	4	4	5	5
51	0	0	4	4	5	5
52	0	0	4	4	5	5
53	0	0	4	4	5	5
54	0	0	4	4	5	5
55	0	0	4	4	4	4

* = died of unrelated causes

Table 5: Ascites and solid tumor in the peritoneal cavity of Swiss mice inoculated with Ehrlich ascites tumor, treated with 50 mg/kg and 75 mg/kg of SZTP-14 and sacrificed 55 days after tumor inoculation

Treatment				
	75 mg/kg ?	**75 mg/kg ?**	**50 mg/kg ?**	**50 mg/kg ?**
Ascites ml	- - 0 - 4	1 6 - 0 -	- 5 2 4 0	- 1 - 4 0
Solid tumor in the abdominal cavity	+ + 0 + +	+ + + 0 +	+ + + + 0	+ + + + 0

0: the animal died earlier, +: solid tumor in the abdominal cavity, - : no ascites

Origin of tumor: National Insitute of Oncology, Budapest, Hungary.

Animal strain: Swiss mice, not inbred, own breed.

Weight and sex of animals: 20 g, 5 males and 5 females/group.

Tumor cell number: 10^6/animal

Treatment: 50 mg/kg and 75 mg/kg resp., injected intraperitoneally daily, started 24 h after tumor inoculation, for 20 days.

Control: 0.2 ml of physiological saline solution, daily, intraperitoneally.

Evaluation: measurement of body weight, daily follow up on survival. Animals alive 55 days after tumor inoculation were sacrified, autopsied, amount of ascites measured, occasional solid tumor formation in the peritoneal cavity registered.

Inhibition of tumor metastases

Lewis lung tumor (LLT) inoculation into the spleen.

Origin of the tumor: NCI, Bethesda, MD, USA.

Animal strain: inbred C57Bl mice, LATI, Gödöllõ, Hungary.

Weight and sex of animals: 20–23 g females (6 controls and 4 treated).

Tumor cell number: 5×10^6 LLT cells inoculated into the spleen.

Treatment: 75 mg/kg for 8 days, started 25 h after tumor inoculation.

Evaluation: animals were killed and liver metastases counted on the 9th day after tumor inoculation.

Lewis lung tumor (LLT) inoculation into thigh muscle.

Animal strain: C57Bl inbred, LATI, Gödöllõ, Hungary.

Weight and sex of animals: 20–22 g females.

Number of animals: 5/group.

Tumor cells

5×10^5 cells/animal, injected intramuscularly into the thigh muscles. Ten days after tumor inoculation the tumor-bearing extremity was removed. After the operation, between the 11th and 17th days subsequent to tumor inoculation, a daily intraperitoneal dose of 50 mg/kg SZTP-14 was given. Controls intraperitoneally received 0.2 ml of physiological saline solution daily.

Evaluation

Animals were sacrified on the 18th day after tumor inoculation. The number and average volume of the lung metastases were determined under a stereomicroscope.

Results

In vitro studies

Table 2 shows the effect of polyisolysines produced by various processes and of α-polylysine on the proliferation of K-562 cells. SZTP-14, SZTP-15, SZTP-16, SZTP-17 and SZTP-18 – but especially SZTP-14 – treatment resulted in a significant, dose-dependent inhibition of cell proliferation. It was noteworthy that even 1 μg/ml treatment caused a well-defined antiproliferative effect.

However, H-17 and K-17 did not influence the proliferation of K-562 cells. á-polylysine caused the stagnation of cell number when compared to the control, but did not

cause increase or decrease of cell number even in a high dose (100 μg/ml).

In vivo experiments: the effect of SZTP-14 on tumor growth and metastasis L1210 tumor

Table 3 shows that the survival of treated animals exceeded that of the controls. Ten days after the tumor inoculation not a single control mouse was alive. At the same time all treated animals were alive. The first treated animal died 13 days, the last one 15 days after tumor inoculation.

Ehrlich ascites tumor

Table 4 shows the survival of mice from 16 days after tumor inoculation. The control animals died between 17 and 22 days. In each treated group the animals were alive (except one/group) 55 days after tumor inoculation, when the animals were sacrificed. Table 5 shows the amount of ascites and the presence of solid tumor in the peritoneal cavity. This means that no tumor-free animal was found, but out of 20 treated mice 16 were alive 33 days after the death of the last control mouse. No difference in response to SZTP-14 treatment regarding gender was observed.

P 388 tumor

All control mice died by day 10 following tumor inoculation. The treated mice all survived until day 15, but all died by day 18 (Table 6).

Lewis lung tumor

Spleen-liver model

Table 7 shows the number of liver metastases. Not a single liver metastasis was found after SZTP-14 treatment, whereas 20–63 metastatic nodules were found in control animals.

Muscle-lung model

The number and average volume of lung metastases are shown in Table 8. SZTP-14 treatment significantly decreased the number and average volume of lung metastases.

Table 6: The effect of 10 mg/kg daily SZTP-14 treatment on the survival of BDF/1 mice inoculated with P-388 ascites tumor

Days after inoculation	Living controls	Living treated mice
9	4	5
10	-	5
11	-	5
12	-	5
13	-	5
14	-	5
15	-	4
16	-	2
17	-	1
18	-	-

Discussion

Our studies revealed that among the poly-L-isolysines (polymerisation degree:10–400) prepared by our method, the polymers of polymerisation degree 40–200 (Mw 5.5–20.3 KD) retarded cell proliferation; i.e. they showed antitumor activity *in vitro* and *in vivo*, and inhibited the formation of tumor metastases. The antitumor effect was dependent on the degree of polymerisation.

In vitro, SZTP-14 and some other peptides decreased the number of tumor cells dose-dependently when compared to the control. This effect appeared as stagnation of cell number in case of small doses, whereas high doses killed cells. Isopolylysines K 17 and H 17 were ineffective, α-polylysine only led to static cell numbers.

In vivo treatment with SZTP-14 increased the survival of the treated animals inoculated with L1210, P 388 and Ehrlich ascites tumor. In the case of Ehrlich tumor, the treatment caused a very long tumor-free period and tumor growth was observed only after the cessation of treatment.

Table 7: Effect of SZTP-14 treatment (75 mg/kg intraperitoneally for 8 days) on liver metastases in female C57 Bl mice inoculated with LLT into the spleen

Number of liver metastases						
Control	31	20	63	36	38	56
	(ξ = 40.66; s = 16.02; n = 6)					
SZTP-14	0	0	0	0	(n = 4)	

Table 8: The effect of SZTP-14 treatment (50 mg/kg daily, intraperitoneally, between the 11th and 17th day after tumor inoculation) on lung metastases and average metastasis volume of C57 Bl mice intramuscularly inoculated with Lewis lung tumor

Treatment	Number of metastases in average	Volume of metastases (mm^3) in average
Control	63.13 ± 27.53	49.45 ± 29.07
SZTP-14	26.29 ± 18.87	20.10 ± 35.38

After SZTP treatment no metastasis formation was seen in the liver when the tumor was inoculated into the spleen, and there was a decrease in the number and size of lung metastases when the tumor was inoculated into the muscle.

The compounds with Mw 10.2–15.4 kD (polymerisation degree 80–120) showed the highest activity, surpassing that of clavicepamines [3]. The other substances tested were ineffective, presumably because of high polydispersity, low Mw and racemization. The results of this study indicate that peptides with promising biological effects can be synthesized with high reproducibility.

Although alpha-poly-L-lysine is known as a cell proliferation enhancing agent [2], L-lysine has been reported to have inhibitory effect on the growth of several transplantable animal tumors [7]. D-lysine, however, and methylated derivatives of L-lysine enhance cell proliferation under various experimental conditions [8]. The antitumor activity of TNF-alpha is likely to depend on the lysine amino groups of this compound [9]. Our studies indicate that epsilon-poly-L-lysine inhibits proliferation of several tumor lines, *in vitro* and *in vivo*. The *in vivo* effectiveness of this peptide indicates a direct effect on tumor cells, but in the case of *in vivo* experiments the immunostimulatory activity cannot be excluded as at least a contributor to the tumor growth inhibiting activity. The presence of other amino acids (Glu, Phe, Gly, Asp, Met) seems unnecessary and the change of Lys to Orn did not to improve the effects.

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