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Synthesis and Antitumor Properties of the Myelopeptide MP-1

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Abstract—The bone marrow-derived peptide Phe-Leu-Gly-Phe-Pro-Thr (MP-1) has been synthesized by the classical methods of peptide chemistry in solution, and its antitumor properties have been studied. It has been shown that MP-1 enhances the efficacy of the cytostatic therapy of lympholeukosis P388, increases the latent period of the growth of P388 tumors implanted in irradiated mice, and reduces the recurrence of the breast adenocarcinoma Ca-755 in mice after the surgery.

Keywords: myelopeptide MP-1, antitumor activity, immunodepression

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INTRODUCTION

The hexapeptide Phe-Leu-Gly-Phe-Pro-Thr belongs to the group of endogenous regulatory peptides (myelopeptides) produced by bone marrow cells and is called myelopeptide-1. The studies of the biological activity and the mechanisms of action of individual myelopeptides revealed that each of six peptides isolated to date produces a specific action on a particular type of immunocompetent cell, accomplishing the immunocorrection effects by natural mechanisms [1, 2].

It was shown earlier that MP-1 is an immunomodulator that normalizes the antibody formation in animals with immunodeficiencies of different etiology, caused in particular by radiation, cytostatics, and antibiotics [3–5].

The in vivo modeling of directed deficiencies in mice by selective elimination of T and B cells demonstrated that the immunocorrection effect of MP-1 is accomplished through a population of T cells [4]. A study of the binding of fluorescein-labeled MP-1 to the surface of lymphoid cells by the method of double fluorescence staining revealed that the target cell of this regulatory peptide is $CD4^+$ lymphocyte, i. e., T helper [6]. The mechanism of the immunocorrection effect is based on its ability to normalize the disturbed balance of CD4/CD8 cells (T helpers/T suppressors), which results in the restoration of the level of antibody formation [7].

Because it has been shown earlier that MP-1 normalizes the disturbed balance of the major regulatory subpopulations of CD4/CD8 cells, which is characteristic, among other things, for the tumor growth, it was of interest to study the ability of this peptide to produce the antitumor action: to inhibit the growth of various tumors under the conditions of immunodeficiency induced by irradiation or postsurgical stress.

The goal of the present work was to study the immunocorrection and antitumor properties of MP-1 under the conditions of immunodeficiency induced by both irradiation or surgical stress and the development of malignant tumors.

RESULTS AND DISCUSSION

MP-1 (Phe-Leu-Gly-Phe-Pro-Thr) was synthesized by the classical methods of peptide chemistry in solution by the scheme (3 + 3). N- and C-terminal tripeptides were obtained by successive elongation of peptide chains at the N-terminus by the method of activated *N*-oxysuccinimide esters. The tripeptides obtained were condensed by the carbodiimide method. The α-amino groups of amino acids were blocked by Boc- and Z-substituents, carboxylic groups were protected by salt formation, and the C-terminal carboxylic group of threonine was protected by amidation. The target hexapeptide MP-1 was isolated and purified by reversed-phase chroma-

Abbreviations: CP, cisplatin; DMF, *N*,*N*-dimethylformamide; HPLC, high-performance liquid chromatography; LTP, lifetime prolongation; MLT, mean lifetime; MP-1, myelopeptide-1; s.c., subcutaneously; ITG, inhibition of tumor growth.

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Table 1. Efficacy of MP-1 monotherapy and the application of MP-1 in combination with CP in mice with hypodermic lympholeukosis P388

Preparation	Dose, g/mouse	V_{mean} (mm^3)[&]	ITG, %[&]	MLT, day	LTP, %
Control	–	461; 2948; 6348	–	21.1	–
MP-1	1×10^{-8}	73; 1132; 2111	84*; 62*; 67*	21.5	
CP	8×10^{-8}	60; 362; 993	86*; 91*; 86*	24.7	28*
CP	1.6×10^{-7}	39; 175; 750	91*; 94*; 88*	27.8	31*
CP + MP-1	$8 \times 10^{-8} + 1 \times 10^{-8}$	9; 66; 367	98**; 97**; 94**	32.8	32*
CP + MP-1	$1.6 \times 10^{-7} + 1 \times 10^{-8}$	2; 79; 335	99**; 97**; 95**	33.7	39*

Note: [&] The three numbers correspond to the results obtained on day 11, 14, and 18 after the tumor inoculation, respectively. In a tenfold course of MP-1, the interval between injections was 24 h; difference from the control: * $p < 0.05$, ** $p < 0.01$; V_{mean} (mm^3), the mean size of the tumor in mm^3; MLT, mean lifetime; LTP, lifetime prolongation.

Table 2. Effect of MP-1 on the inoculability of lympholeukosis P388 under stress induced by irradiation (4.5 Gy)

Number of group	Dose of MP-1, g/mouse	Day after the implantation of P388 (death of animals, %)										
		14	15	16	17	18	19	20	21	22	23	24
1	– (control group)*	0	60	70	90	100						
2	1×10^{-5}	0	44	56	67	100						
3	1×10^{-6}	0	40	50	70	80	90	90	90	100		
4	1×10^{-7}	0	30	40	50	60	90	90	90	90	100	
5	1×10^{-8}	0	0	0	40	40	60	60	60	70	80	100

* Administration of the physiological solution.

tography; it was characterized by the data of analytical HPLC, mass spectrometry, and sequencing.

In the first series of experiments, the effect of MP-1 on the growth and development of P388 lympholeukosis and the efficacy of this peptide in the combined cytostatic antitumor therapy were studied. The antitumor effect of MP-1 was estimated using the cytostatic drug CP as positive control, which is applied in clinical practice.

The antitumor activity of MP-1 and CP was estimated in vivo on different types of implanted mouse tumors (lympholeukosis P388 and breast adenocarcinoma Ca755), obtained from the bank of tumor strains of the Blokhin Russian Oncological Research Center.

Experiments were performed on mice with s.c. implanted lympholeukosis P388. CP was injected singly intraperitoneally at doses of 8 and 4 mg/kg, which cause marked immunodepression. The treatment with CP was started 24 h after tumor inoculation. MP-1 was injected s.c. at a single dose of 1×10^{-8} g/mouse daily beginning from day 2 to day 11 after tumor implantation. The results of the experiments are presented in Table 1.

It was shown that the MP-1 monotherapy induced a 84% reduction of tumor growth by the last day of treatment and did not influence the survival of mice. CP in the monotherapy at doses of 8×10^{-8} and 1.6×10^{-7} g/mouse was nearly equal in efficiency to MP-1 and induced a maximum ITG, by 86–94%, which remained throughout the week at a level of 86–88%; in this case, the lifetime of mice increased by 28–31%. In groups with combined therapy, ITG after the injection of CP (at doses of 8×10^{-8} and 1.6×10^{-7} g/mouse) and MP-1 (at a single dose of 1×10^{-8} g/mouse for 10 days) increased to 94–95% by the end of the first week after the treatment. The lifetime of mice in these groups increased by 32 and 39%, i. e., was almost 1.5 times greater than in the case of the CP monotherapy.

Thus, it is evident that the combined therapy of lympholeukosis P388 by CP and MP-1 more strongly suppresses the tumor and increases the survival of animals than the monotherapy with each of these preparations.

Then the antitumor and prophylactic effects of MP-1 on the growth and development of lympholeukosis P388 under the conditions of immunosuppression induced by radiation was studied. Mice of the BDF_1 line were injected with MP-1 for 5 days (at an interval of 24 h) at doses of $1 \times 10^{-8} - 1 \times 10^{-5}$ g/mouse. Then mice were irradiated at a dose of 4.5 Gy, and after 24 h 10^3 lympholeukosis P388 cells were s.c. implanted; the injections of MP-1 were continued for another five days after implantation. The results of the experiments are presented in Table 2.

Table 3. Effect of MP-1 on spontaneous recidivation of breast carcinoma Ca-755 in mice under the conditions of immunodepression induced by surgical stress

Administration of MP-1, 1×10^{-8} g/mouse	Number of mice (total number)	Number of mice with tumor recurrence	Recidivation, %
– (control group)•	34	22	65
days 2–11 after the operation	28	13	46
for 5 days before and after the operation	32	14	44*

Note: •Administration of physiological solution daily for 10 days. * $p < 0.05$.

It is evident from the data in Table 2 that, in mice of the control group, tumors appeared on day 15 (the latent period). In experimental groups 2–4, tumors appeared also on day 15; however, they were found in a lesser number of animals. In group 4, tumors began to appear even later (on day 17). The injection of MP-1 for 10 days delayed the appearance of tumors to 22–24 days. The maximum delay in the inoculability of leukosis was 6 days (33–35%) at a single dose of MP-1 of 1×10^{-8} g/mouse, which is rather much, having regard to a short lifespan of mice with this tumor.

These results allow one to conclude that MP-1 produces a prophylactic effect: its injection distinctly slows down the appearance of tumors, prolongs the life of experimental animals, and decreases the level of radiation-induced immunodepression in mice.

In the second series of experiments, the antitumor and prophylactic effects of MP-1 on the spontaneous and artificial recidivation of tumors under the conditions of immunodepression induced by surgical stress, the removal of breast adenocarcinoma Ca-755 in mice. It is known that the postsurgical stress is accompanied by the stimulation of metastasizing and reappearance of tumors. The onset of the exponential phase in tumor growth, when tumors drastically increase in size within a short period of time, corresponds to a decrease in the immunological control of the organism, i. e., pronounced immunodepression [8].

Cells of the Ca-755 tumor (50 mg/mouse) were s.c. implanted into female mice of BDF1 hybrids. When the mean volume of the tumor reached 700–900 mm^3, they were removed under thiopental narcosis. Mice in which subcutaneous recurrences appeared on day 10 after the surgical removal of the tumor formed a group of "spontaneous recidivation." In a study of the effect of MP-1 on the spontaneous recurrence of breast adenocarcinoma Ca-755, mice of the first experimental group received MP-1 at a dose of 1×10^{-6} g/mouse beginning from day 2 to day 11 after operation (Scheme 1). The mice of the second experimental group were injected with MP-1 at a preventive dose of 1×10^{-8} g/mouse for five days prior to, and for 5 days after, the operation (Scheme 2). Thus, the animals of both experimental groups received MP-1 for 10 days, and the difference was that, according to Scheme 1, only the therapeutic effect of MP-1 was studied, whereas according to Scheme 2, the prophylactic effect of the peptide on the spontaneous recidivation of the breast carcinoma Ca-755 was also examined. The animals of the control group were injected with the physiological solution. The size of recurrent Ca-755 carcinoma was measured on day 10 after the removal of the tumor. The results are presented in Table 3.

It follows from the data in Table 3 that spontaneous recurrences in the control group after tumor removal were observed in 65% of cases. In the group of animals receiving MP-1 for 10 days after tumor removal the number of spontaneous recurrences decreased to 46%, which is 1.4 times less than in the control (Scheme 1). In the group of mice that received MP-1 during the maximum possible immunodepression, for 5 days prior to the operation (neoadjuvant therapy) and for 5 days after the operation (adjuvant therapy), the number of spontaneous recurrences decreased reliably to 44%, which is nearly 1.5 times less than in the control.

In a study of the effect of MP-1 on artificial recidivation, a suspension of Ca-755 tumor cells (50 mg/mouse) was repeatedly implanted into mice on days 10–11 after the operation. The mice of the control and experimental groups received MP-1 according to the same protocol as in the previous series of experiments. The size of the recurrent Ca-755 carcinoma was measured on day 7 after the implantation of the tumor. The results are presented in Table 4.

The experiments showed that the artificial recidivation in the control group occurred in 100% of cases after the removal of the tumor. The administration of MP-1 with a therapeutic purpose (on days 2–11 after the operation) did not lead to a decrease in the number of recurrences. At the same time, the peptide administered with the preventive purpose during the maximum possible immunodepression (5 days prior to, and 5 days after the operation) decreased the number of artificial recurrences of the breast carcinoma Ca-755 to 67% (by 33% less than in the control).

Thus, the study of the effect of MP-1 on the growth of implanted tumors under the conditions of radiation-induced immunodepression and after the occurrence of recidivation under the conditions of surgical

Table 4. Effect of MP-1 on artificial recidivation of breast carcinoma Ca-755 in mice under the conditions of immunodepression induced by surgical stress

Administration of MP-1, 1×10^{-8} g/mouse	Number of mice (total number)	Number of mice with tumor recurrence	Recidivation, %
– (control group)*	15	15	100
days 2–11 after the operation	17	17	100
for 5 days before and after the operation	18	12	67

* Administration of the physiological solution daily for 10 days.

stress showed that MP-1 exhibits the preventive immunocorrection effect. The peptide reliably prolonged the lifespan of experimental animals, decreased the level of radiation-induced immunodepression, increased the time of tumor inoculability in mice, and reduced the recidivation of the tumor after its surgical removal.

The preventive effect of MP-1, which manifested itself in the postsurgical period, is evidently related to its immunocorrection effect during immunodepression, which appears in animals in operation stress. The postoperative immunodepression promotes the resumption of tumor growth, and MP-1 provides the restoration of immunity, thereby reducing the probability of recurrence. The data obtained indicate that MP-1 holds promise in complex cancer therapy as an immunocorrection agent to prevent the recurrence in oncology patients who underwent surgery, intensive radiation or chemotherapy. MP-1 is an endogenous low-molecular-weight immunoregulatory peptide with the known primary structure and mechanism of action; the peptide holds promise as the basis for the design of new-generation drugs that target impaired immune functions and produce no side effects.

EXPERIMENTAL

Commercially available amino acids and their derivatives (Reanal, Hungary; Fluka, Switzerland) or derivatives obtained by standard methods were used. The purity of compounds obtained at the intermediate stages of the synthesis was controlled by TLC on silica gel plates (Merck, Germany) in the following solvent systems: chloroform–methanol–32% acetic acid 60 : 45 : 20 (A), chloroform–methanol–32% acetic acid 5 : 3 : 1 (B), chloroform–methanol–32% acetic acid 15 : 4 : 1 (C), *n*-butanol–acetic acid–water 3 : 1 : 1 (D), and chloroform–methanol–acetic acid 9 : 1 : 0.5 (E). Compounds were detected using ninhydrin or *o*-tolidine.

Analytical HPLC was carried out using an LC-10ADvp chromatographic system (Shimadzu, Japan) on an Ultrasphere C 18 column (4.6 × 250 mm) in a gradient of acetonitrile concentration in 0.1% trifluoroacetic acid (0–80%, 32 min). The elution rate was 1.6 mL/min, and the detection was at 214 and 280 nm.

Preparative HPLC of terminal and intermediate peptides was carried out on a column of Diasorb C 16-T (50 × 250 mm) in a gradient of buffer C in buffer A (10–50%, 120 min; buffer A, 0.01 M CH_3COONH_4; buffer C, 80% acetonitrile in buffer A). The flow rate was 50 mL/min, and the detection was at 226 nm. Fractions corresponding to the main peak were combined, acetonitrile was evaporated, and the resulting solution was diluted with water and lyophilized.

Synthesis of H-Phe-Leu-Gly-Phe-Pro-Thr-NH_2 (MP-1). H-Leu-Gly-OH × TFA. DMF (60 mL) and Boc-Leu-ONSu (6.58 g, 0.02 mol) were added to a solution of glycine (1.65 g, 0.022 mol) in 1 M NaOH (22 mL). The reaction mixture was stirred for 12 h at 20°C, DMF was evaporated, and the residue was dissolved in water (100 mL) and extracted with diethyl ether (3 × 30 mL). The water layer was acidified with 5% H_2SO_4, and the target compound was extracted with ethyl acetate (2 × 50 mL). The ethyl acetate solution was washed with water to pH 5 and evaporated. The oily residue was dissolved in TFA (70 mL) and kept for 1 h at room temperature, the acid was distilled in vacuo, and the residue was triturated with ether and filtered. The resulting precipitate was dried in a dessicator over KOH. Yield: 5.44 g (90%).

Z-Phe-Leu-Gly-OH (1). A solution of H-Leu-Gly-OH × TFA (5.44 g, 0.018 mol) in DMF (60 mL) was added to *N*-methylmorpholine (2 mL, 0.018 mol) and Z-Phe-ONSu (7.15 g, 0.018 mol). The reaction mixture was stirred for 12 h at 20°C, DMF was evaporated, and the residue was dissolved in ethyl acetate (100 mL) and washed with 5% H_2SO_4 (50 mL) and water (3 × 50 mL) to pH 5. The ethyl acetate solution was evaporated and crystallized from diethyl ether. Yield: 7.97 g (93%).

H-Pro-Thr(Bzl)-NH_2 × TFA. To a solution of H-Thr(Bzl)-NH_2 (4.6 g, 0.022 mol) in DMF (60 mL),

Boc-Pro-ONSu (6.26 g, 0.020 mol) was added, and the mixture was stirred for 12 h at 20°C. The reaction mixture was evaporated, and the residue was dissolved in ethyl acetate (100 mL) and extracted with a saturated $NaHCO_3$ solution (2 × 50 mL) and 5% H_2SO_4 (2 × 50 mL). The ethyl acetate solution was washed with water to pH 5, and ethyl acetate was evaporated. The oily residue was dissolved in TFA (70 mL), kept for 1 h at 20°C, evaporated, and triturated with ether. The precipitate was filtered and dried in a dessicator over KOH. Yield: 7.96 g (95%).

Boc-Phe-Pro-Thr(Bzl)-NH_2. To a solution of H-Pro-Thr(Bzl)-NH_2 (7.96 g, 0.019 mol) in DMF (60 mL), *N*-methylmorpholine (2.1 mL, 0.019 mol) and Boc-Phe-ONSu (6.9 g, 0.019 mol) were added, and the mixture was stirred for 12 h at room temperature. The reaction mixture was evaporated, and the residue was dissolved in ethyl acetate (100 mL) and washed with a saturated $NaHCO_3$ solution (2 × 50 mL and 5% H_2SO_4 (2 × 50 mL). The ethyl acetate solution was washed with water to pH 5, evaporated, and crystallized from diethyl ether. Yield: 9.38 g (89%).

H-Phe-Pro-Thr(Bzl)-NH_2 × TFA (2). A solution of Boc-Phe-Pro-Thr(Bzl)-NH_2 (9.38 g, 0.017 mol) in TFA (70 mL) was kept for 1 h at 20°C, evaporated, and the oily residue was triturated with ether and filtered. The filtrate was dried in a dessicator over KOH. Yield: 9.62 g (99.9%).

Z-Phe-Leu-Gly-Phe-Pro-Thr(Bzl)-NH_2. Peptide (**1**) (7.97 g, 0.017 mol), *N*-methylmorpholine (1.9 mL), and HONSu (1.04 g, 0.009 mol) were added to a solution of peptide (**2**) (9.62 g, 0.017 mol) in DMF (100 mL). The solution was cooled to −25°C, and dicyclohexylcarbodiimide (3.68 g, 0.178 mol) was added under stirring. The reaction mixture was kept for 12 h at 8°C. The resulting dicyclohexylurea was filtered. DMF was evaporated in vacuo, and the residue was dissolved in 100 mL of a mixture of ethyl acetate and butanol 1 : 1 and washed with a saturated $NaHCO_3$ solution (2 × 50 mL) and 5% H_2SO_4 (2 × 50 mL). The ethyl acetate–butanol solution was washed with water to pH 5, evaporated, and crystallized from ether. Yield: 13.06 g (85%).

H-Phe-Leu-Gly-Phe-Pro-Thr-NH_2. The peptide Z-Phe-Leu-Gly-Phe-Pro-Thr(Bzl)-NH_2 (13.06 g, 0.0145 mol) was dissolved in methyl alcohol (100 mL) and hydrated over 10% Pd/C until the starting compound disappeared, which was monitored by TLC. The catalyzer was filtered, and the solution was evaporated. The target peptide was purified by HPLC on a column (50 × 250 mm) of Diasorb C-16-T in a gradient of concentration (0–50%, 120 min) of buffer B in buffer A. The flow rate was 50 mL/min, and detection was at 226 nm. Acetonitrile was evaporated, and the resulting solution was diluted with water and lyophilized. The yield of the amide MP-1 was 6.16 g (62%).

Molecular weight of MP-1 was determined by mass spectrometry on a Thermo Bioanalysis Vision 2000 device (England) and was 681.

Antitumor efficacy of MP-1 was estimated on mice with s.c. implanted lympholeukosis P388 cells. MP-1 was injected s.c. at a single dose of 1×10^{-5} g/mouse daily beginning from day 2 to day 11 after tumor implantation. Experiments on the combined antitumor effect of MP-1 were performed using CP as a cytostatic agent. CP was injected intraperitoneally at doses of 8 and 4 mg/kg, which induce a marked immunodepression. The treatment with CP was started 24 h after the tumor implantation. The efficacy of treatment in both cases was estimated from standard parameters, ITG and LTP (both in percent). These parameters indicate the effect of preparations on tumor size and the life of mice.

$$\mathrm{ITG} = \frac{V_c - V_{exp}}{V_c} \times 100,$$

where V_c and V_{exp} are the volume of the tumor in the control and experiment;

$$\mathrm{LTP} = \frac{\mathrm{LT_c} - \mathrm{LT_{exp}}}{\mathrm{LT_c}} \times 100,$$

where LT_c and LT_{exp} are the lifetime of mice in the control and experiment.

Irradiation of mice (total dose of 4.5 Gy) was performed on a Stebel-3A device at a dose rate of 5 Gy/min.

Prophylactic effect of MP-1 on oncogenesis under the conditions of immunodeficiency induced by irradiation (4.5 Gy) was estimated from the delay of the 100% inoculability of leukosis compared with the control group (unirradiated animals). Lympholeukosis P388 cells capable of forming well palpable hypodermic tumors at the early stage of development were used as an implantable tumor. Studies were carried out on BDF_1 mice into which 10^3 lympholeukosis P388 cells in a volume of 0.2 mL were implanted 24 h after irradiation. Each group contained no less than eight animals. MP-1 was injected s.c. in a physiological solution, beginning from day 5 before tumor implantation and for the next five days after the implantation at single doses from 1×10^{-8} to 1×10^{-5} g/mouse.

Prophylactic and therapeutic effects of MP-1 on the recurrence of tumors under surgical stress were estimated on a model of solid nonmetastatic breast adenocarcinoma Ca-755. The scheme of the experiment involved the s.c. inoculation of a suspension of Ca-755 cells into BDF1 female mice (50 mg/mouse). Then, mice having tumors of a volume of 700–900 mm^3 were selected, and the tumors were removed under thiopental narcosis. Two schemes of the tumor recurrence experiment were used: spontaneous recidivation (formation of tumor nodes at the site of the surgical wound on day 10 after the removal of the primary tumor in mice) and artificial recidivation, the formation of

tumor nodes after the second inoculation (10–11 days after the removal of the primary node) of suspended Ca-755 cells (50 mg/mouse). With regard to these schemes, six groups of mice were formed, 15–34 animals in each.

Spontaneous recidivation. On day 10 after the removal of the primary tumor in mice, tumor nodes at the site of the surgical wound developed. MP-1 was injected s.c. at a single dose of 1×10^{-8} g/mouse daily beginning from day 2 to day 11 after the operation (Scheme 1) or for 5 days before and 5 days after the operation (Scheme 2); after 10 days, the development of new nodes was controlled by palpation. The mice of control groups received injections of the physiological solution from day 2 to day 11 after the removal of the primary tumor.

Artificial recidivation was caused by the repeated implantation of Ca-755 cells against the background of the injection of MP-1. MP-1 was injected s.c. at a single dose of 1×10^{-8} g/mouse according to Schemes 1 or 2 (see above). Seven days after the repeated implantation of Ca-755 cells, the development of tumor nodes was controlled.

Statistical processing of data was carried out using the STATGRAPH software package and a package of functions for the statistical processing of the Excel program.

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