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Myelopeptide MP-5 and Fluorescent Derivatives: Synthesis and Biological Activity

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Abstract—The Val-Val-Tyr-Pro-Asp bone marrow peptide (MP-5) and its analogue (MP-5-Lys) were synthesized. Fluorescent derivatives, Ftc-MP-5 and MP-5-Lys(Ftc), were prepared. The iological activity of MP-5 and MP-5-Lys was studied in vitro and in vivo. The MP-5 peptide caused 60–84% inhibition of growth of the following mouse cancers: lymphatic leukemia P388, melanoma B-16, and cervical carcinoma CUC-5. These peptides also restored functional activity of T-lymphocytes that was inhibited by metabolic products of the HL-60 leukemic cell line. MP-5-Lys(Ftc) was shown to preserve the functional properties of MP-5 towards T-lymphocytes, but Ftc-MP-5 was practically inactive.

Key words: myelopeptide MP-5, VVYPD, fluorescent analogues, synthesis, T-lymphocytes, proliferative response, antitumor activity

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INTRODUCTION

The Val-Val-Tyr-Pro-Asp pentapeptide (MP-5) belongs to the group of endogenous regulatory peptides, myelopeptides, which are produced by cells of the bone marrow [1].[2] This peptide is homologous to the Leu-Val-Val-Tyr-Pro-Trp myelopeptide (MP-2) for which the mechanism of action and functional activity were studied in detail. MP-2 was shown to restore the proliferative response of T-lymphocytes to PHA that was suppressed by the products of the HL-60 tumor cells of human myeloleukemia [2]. This effect is accompanied by recovery of the $CD3^+$ and $CD4^+$ phenotypes of T-cells that were damaged by the cancer [3]. MP-2 were also found to inhibit the growth of a number of transplantable tumors of mice: lymphatic leukemia P-388, adenocarcinoma of the mammary gland Ca 755, sarcoma S-180, Lewis lung carcinoma, and melanoma B-16 [4]. We proposed on the basis of high homology of the MP-2 and MP-5 structures that MP-5 would exhibit the biological activity similar to that of MP-2.

The goal of this study is synthesis and investigation of biological activity of the MP-5 myelopeptide. Two fluorescent analogues of MP-5 were prepared for profound studies of the mechanism of its action and for detection of its target cell.

RESULTS AND DISCUSSION

MP-5 was synthesized by the solid phase method on the co-polymer of styrene and 1% divinyl benzene with the HMP acid-labile anchoring group according to the standard Fmoc-technique. The crude product of solid phase synthesis was purified by a preparative HPLC and characterized by ^{1}H NMR spectroscopy and mass spectrometry.

MP-5 peptide with the additional Lys residue on its *C*-terminus was prepared for the subsequent introduction of the fluorescent label. This peptide was synthesized by methods of conventional peptide synthesis in solution using Boc and Z protective groups and a combination of fragment condensation with a stepwise elongation of the amino acid chain by the method of

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[2] Abbreviations: CM, conditioned medium; DIC, diisopropylcarbodiimide; F-complex, adduct of pentafluorophenol with dicyclohexylcarbodiimide; FITC, fluorescein isothiocyanate; Ftc, fluoresceinthiocarbamoyl; HMP, hydroxymethylphenoxy; MP, myelopeptides, MP-5, Val-Val-Tyr-Pro-Asp pentapeptide of the MP family; NMP, *N*-methylpyrrolidone; HOBt, hydroxybenzotriazolyl; Pfp, pentafluorophenyl; PHA, phytohemagglutinin; TFA, trifluoroacetic acid.

pentafluorophenyl active esters. The *C*-terminal tetrapeptide was prepared by the condensation of the two dipeptides, and two valine residues were attached stepwise to this tetrapeptide. Benzyloxycarbonyl and *tert*-butyloxycarbonyl protections were used for blocking of the α-amino group and the ε-amino group of lysine residue, respectively. The β-carboxyl group of aspartic acid residue was temporary protected by benzyl ester on the stage of preparation of the dipeptide. The α amino group of the labeled hexapeptide was removed by the treatment with hydrogen bromide in trifluoroacetic acid.

The fluorescent label was introduced in MP-5 and MP-5-lys using fluorescein isothiocyanate (see Experimental). The labeled peptides were purified by the reversed phase HPLC.

Biological activity of the prepared peptides were determined according to a degree of restoration of the PHA-induced proliferation of human T-lymphocytes (in vitro) and according to the ability of peptides to inhibit growth of the mouse transplanted cancers (in vivo).

The cancer cells are known to release substances that inhibit functional activity of T-lymphocytes [5, 6]. Functional state of the lymphocytes subjected to the action of cancer toxins was determined according to their ability to blasttransformation in the presence of PHA. T-lymphocytes of peripheral blood of healthy donors were used in this study. Their functional activity was inhibited in CM of the cells of the HL-60 line of leukemic cells. Proliferative response of T-lymphocytes to PHA was evaluated according to [^{3}H]-thymidine incorporation in DNA. The proliferation level of the PHA-stimulated cells was taken to be 100%. The experimental results are presented in Table 1.

As one can see from Table 1, decrease in the proliferation level (to 46%) of the PHA-induced response of T-lymphocytes is observed in the presence of the CM of HL-60 cells. In this model, MP-5 and MP-5-Lys(Ftc) exhibit the equally high activity in a wide range of concentrations (from 1×10^{-6} to 1×10^{-9} g/ml) and restore the level of proliferation of T-lymphocytes to 76–93%. However, the MP-5 analogue with the *N*-terminal amino group labeled with fluorescein proved to be inactive. Preservation of the high functional activity of MP-5-Lys(Ftc) allows an application of this peptide as an adequate instrument for a search for a target cell for MP-5 peptide and studies of the mechanism of its action.

Cytotoxic activity of T-lymphocytes is known to be one of factors of the antitumor protection of an organism. Ability of MP-5 to restore the functional activity of T-lymphocytes impaired by the products of leukemic cells is a prerequisite for studies of the antitumor activity of this peptide on the in vivo models.

Three types of the transplanted mouse tumors were chosen for examination of the antitumor effect: cervical carcinoma CUC-5, lymphatic leukemia P-388, and melanoma B-16. Experiments demonstrated that administration of MP-5 to the animals with the transplanted tumors resulted in significant inhibition of growth of all the examined cancers (Table 2). The peptide was found to have no direct action on a tumor cell, but it supported active antitumor immunity in an organism of a tumor-bearer by the inhibition of action of tumor toxins on T-lymphocytes.

Table 1. Effects of MP-5, Ftc-MP-5, and MP-5-Lys(Ftc) on restoration of the PHA-induced proliferation of human T-lymphocytes that was decreased by the action of HL-60 CM

Added components		Peptide concentration, μg/ml	Proliferation level, %
PHA (control)		–	100
PHA + LH-60 CM		–	46 ± 4
PHA + HL-60 CM	MP-5	1	89 ± 10*
		0.1	93 ± 7*
		0.01	86 ± 9*
		0.001	82± 8*
	MP-5-Lys(Ftc)	1	86 ± 6*
		0.1	84 ± 11*
		0.01	90 ± 8*
		0.001	89 ± 16*
	Ftc-MP-5	1	44 ± 18
		0.1	48 ± 11
		0.01	48 ± 19
		0.001	47 ± 14

* $p < 0.01$.

Table 2. Effect of MP-5 (1 mg/kg, five times with 24-h intervals) on growth of the transplanted mouse tumors

Type of the tumor	Mouse line	Days after the tumor transplantation	ITG*, %
Cervical carcinoma CUC-5	CBA	7	84
		14	83
		21	81
Melanoma B-16	$C_{57}BL_b$	7	74
		14	66
		21	59
Lymphatic leukemia P-388	DBA_2	7	60
		14	63
		21	61

* ITG was calculated as a ratio of difference in the averaged volumes of tumors in the control and treated groups to the tumor volume in the control group.

Thus, the biological activity of MP-5 in the used tests in vitro and in vivo is practically the same as that of MP-2 described by us previously [7]. In the case of MP-2, the attachment of fluorescent label to its *N*-terminal amino group resulted in a complete loss of its functional properties. In this study, we demonstrated that elongation of MP-5 by an additional *C*-terminal Lys residue and the attachment of the fluorescent label to its ε-amino group resulted in the fluorescent analogue that preserved the activity in vitro. We can conclude on the basis of these facts that the common Val-Val-Tyr-Pro fragment of these peptides with unmodified *N*-terminal amino group was responsible for their functional activity, whereas the nature of *C*-terminal residue was unimportant. Therefore, various analogues with the specific functional activity could be prepared. It is interesting that the direction of a peptide chain is also unimportant, at least in the case of MP-2. For example, the Trp-Pro-Tyr-Val-Val *retro*-analogue, which was described by us previously, exhibits the activity similar to that of MP-2 in vitro and in vivo [7].

We propose on the basis of these results that the MP-5 endogenous immunoregulatory peptide, likely to MP-2, can participate in the processes of anti-carcinogenesis that occur in a healthy organism and prevent development of cancers resulting from mutations, carcinogenic actions and other factors.

EXPERIMENTAL

Commercially available amino acids and their derivatives (Merck, Germany and Fluka, Switzerland) were used for the peptide synthesis. The 10% palladium on activated charcoal (Merck, Germany) served as catalyst for hydrogenolysis. The fluorescent label was introduced by the treatment with FITC (AppliChem, Germany). Homogeneity of the compounds on the intermediate stages of synthesis was determined by TLC on a Kieselgel 60 plates (Merck, Germany) in the following chromatographic systems: (A) *n*-butanol–acetic acid–water (3 : 1 : 1), (B) chloroform–methanol–acetic acid (9 : 1 : 0.5), (C) chloroform–hexane–acetic acid (9 : 1 : 1), and (D) chloroform–methanol–acetic acid–water (60 : 45 : 6.4 : 13.6). The substances were detected with the use of the chlorine-benzidine reagent.

Analytical HPLC was performed on a LC-10ADvp chromatographic system (Shimadzu, Japan) equipped with an Ultrasphere C18 column (4.6 × 250 mm, Beckman, United States) in a concentration gradient of acetonitrile in 0.1% trifluoroacetic acid (from 0 to 80% within 32 min) at a flow rate of 1.6 ml/min with the detection at 214 and 280 nm.

Preparative HPLC was performed on a System Gold chromatographic system (Beckman, United States).

The ^{1}H NMR spectra were recorded on a Bruker WM500 spectrometer (Germany) with a working frequency of 500 MHz at 37°C in DMSO-d_6.

The peptides were characterized by mass spectrometry on a Thermo Bioanalysis Vision 2000 spectrometer (England).

Z-Asp(OBzl)-Lys(Boc)-OH·DCHA (I). The solution of Z-Asp(OBzl)-OPfp (3.14 g, 6 mmol) in dioxane (20 ml) was added to the solution of H-Lys(Boc)-OH (1.48 g, 6 mmol) in 1 N NaOH (6 ml) on stirring. The reaction mixture was kept for 24 h at 18°C, and dioxane was evaporated. The obtained aqueous solution was acidified with 0.5 N H_2SO_4 to pH 3. The oily precipitate was extracted with ethyl acetate (3 × 20 ml). The ethyl acetate solution was washed with water (2 × 20 ml) and saturated solution of NaCl (2 × 20 mm), dried over the anhydrous Na_2SO_4, filtered, and evaporated. The residue was dissolved in ether (20 ml), and dicyclohexylamine (1.2 ml, 6 mmol) was added. The precipitated crystals were filtered, washed with ether, and dried in vacuum. Recrystallization from ethyl acetate yielded 3.45 g (75%) of Z-Asp(OBzl)-Lys(Boc)-OH · DCHA; mp 144-145.5°C; R_f 0.88(A), 0.75 (B).

H-Asp-Lys(Boc)-OH (II). 0.5 N H_2SO_4 was added to the suspension of compound (**I**) (1.31 g, 1.71 mmol) to pH 3 on stirring. The ethyl acetate layer was washed with water (3 × 15 ml) and saturated solution of NaCl (2 × 15 ml), dried over Na_2SO_4, filtered, and evaporated. The oily residue was dissolved in methanol (30 ml), and 10% Pd/C (0.1 g) was added to the solution. The reaction mixture was subjected to the catalytic hydrogenolysis in the H_2 atmosphere to the disappearance of the starting compound according to TLC. The peptide precipitated in the course of hydrogenolysis was dissolved by the addition of 10% solution of aqueous ammonia. The catalyst was filtered off, and the filtrate was evaporated. The residue was dissolved in isopropanol (20 ml) and evaporated again. Acetone (50 ml) was added to the residue, and the formed precipitate was filtered, washed with petroleum-ether, and dried in vacuum. The yield of H-Asp-Lys(Boc)-OH was 0.61 g (98%); mp 165–166°C; R_f 0.61 (A).

Z-Tyr(Bzl)-Pro-OH (III). The solution of Z-Tyr(Bzl)-OPfp (4.61 g, 8.06 mmol) in dioxane (30 ml) was added to the solution of H-Pro-OH (1.11 g, 9.67 mmol) in 1 N NaOH (9.67 ml) on stirring. The reaction mixture was kept for 24 h at 18°C and evaporated. The residue was diluted with water (30 ml). The aqueous solution was washed with ether (2 × 15 ml) and acidified with 0.5 N H_2SO_4 to pH 3. The oily precipitate was extracted with ethyl acetate (3 × 15 ml). The ethyl acetate layer was washed with water (3 × 15 ml) and with saturated solution of NaCl (2 × 15 ml), dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue was crystallized in petroleum-ether, filtered, and dried in vacuum. The yield of compound (**III**) was 3.85 g (95%); mp 54–56°C. R_f 0.81 (A), 0.51 (C).

Z-Tyr(Bzl)-Pro-OPfp (IV). The solution of F-complex [8] (3.77 g, 4.97 mmol) in ethyl acetate (25 ml) was cooled to –5°C and added to the solution of compound (**III**) (2.5 g, 4.97 mmol) in ethyl acetate (20 ml).

The reaction mixture was kept at 0°C for 24 h. The precipitated crystalline dicyclohexylurea was filtered off and washed with ethyl acetate (2 × 20 ml). The filtrate was evaporated. The oily residue was dried in vacuum. They yield of compound (**IV**) was 3.2 g (96%); R_f 0.78 (C).

Z-Tyr(Bzl)-Pro-Asp-Lys(Boc)-OH (V). The solution of compound (**IV**) (3.6 g, 5.38 mmol) in dioxane (20 ml) was added to the solution of compound (**II**) (1.77 g, 4.89 mmol) in 1 N NaOH (9.78 ml, 9.78 mmol) on stirring. The reaction mixture was kept for 24 h at 18°C. Dioxane was evaporated. The residue was diluted with water (20 ml), washed with ether (2 × 15 ml), and acidified with 0.5 N H_2SO_4 to pH 3. The oily precipitate was extracted with ethyl acetate (3 × 20 ml), washed with water (2 × 20 ml) and with saturated solution of NaCl (2 × 20 ml), dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue was triturated with petroleum-ether (40 ml). The formed crystalline precipitate was filtered and dried in vacuum. Peptide (**V**) was prepared with the yield of 2.49 g (60%) as amorphous powder with R_f 0.81 (B).

Z-Val-Tyr-Pro-Asp-Lys(Boc)-OH (VI). The 10% Pd/C (0.15 g) was added to the solution of compound (**V**) (2.49 g, 2.94 mmol) in methanol (30 ml). The reaction mixture was subjected to the catalytic hydrogenolysis in current of hydrogen gas until the disappearance of the starting compound according to TLC. The catalyst was filtered off, and the filtrate was evaporated. The residue was dissolved in 1 N NaOH (5.88 ml, 5.88 mmol), and the solution of Z-Val-OPfp (1.23 g, 2.94 mmol) in dioxane (30 ml) was added on stirring. The reaction mixture was kept for 24 h at 18°C. Dioxane was evaporated, and the residue was acidified with 0.5 N H_2SO_4 to pH 2.5. The oily precipitate was extracted with ethyl acetate (3 × 20 ml). The ethyl acetate extract was washed with water (2 × 20 ml) and with the saturated solution of NaCl (2 × 20 ml), dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue was triturated with petroleum-ether (30 ml), and the formed crystals were filtered and dried in vacuum. The recrystallization from ethyl acetate yielded 1.52 g (61%) of peptide (**VI**); mp 124–126°C; R_f 0.9 (A), 0.42 (B).

Z-Val-Val-Tyr-Pro-Asp-Lys(Boc)-OH (VII). The solution of compound (**VI**) (1.52 g, 1.78 mmol) in methanol (20 ml) was subjected to catalytic hydrogenolysis in the presence of 10% Pd/C (0.1 g) in a current of hydrogen gas until the disappearance of the starting compound according to TLC. The catalyst was filtered off, and the filtrated was evaporated. The residue was dissolved in 1 N NaOH (3.56 ml, 3.56 mmol), and the solution of Z-Val-OPfp (0.74 g, 1.78 mmol) in dioxane (20 ml) was added on stirring. The reaction mixture was kept for 24 h at 18°C. Dioxane was evaporated, and the residue was acidified with 0.5 N H_2SO_4 to pH 3. The oily precipitate was extracted with ethyl acetate (3 × 20 ml). The extract was washed with water (2 × 20 ml) and with the saturated solution of NaCl (2 × 20 ml), dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue was triturated with ether (30 ml). The formed precipitate was filtered, washed with the mixture of ether and petroleum-ether (1 : 1) (2 × 20 ml), and dried in vacuum. The yield of peptide (**VII**) was 1.36 g (80%); mp 157–160°C. R_f 0.86 (A), 0.24 (B).

Z-Val-Val-Tyr-Pro-Asp-Lys-OH (VIII). Compound (**VII**) (0.22 g, 0.24 mmol) was treated with trifluoroacetic acid (10 ml) for 40 min at 18°C, evaporated, and mixed with ether. The precipitate was filtered, washed with ether (3 × 20 ml), and dried in vacuum. The product was purified by the preparative HPLC on a Diasorb-130-C16T column (25 × 250 ml) (BioKhimMak, Russian Federation) in a concentration gradient of isopropanol in 0.1 M acetic acid (from 20 to 50% within 30 min) at a flow rate of 40 ml/min. The yield of product (**VIII**) was 0.11 g (49%); mp 183–185°C.

Amino acid analysis: Asp 1.04 (1), Val 1.77 (2), Pro 0.95 (1), Lys 1.05 (1), Tyr 1.00 (1).

Z-Val-Val-Tyr-Pro-Asp-Lys(Ftc)-OH (IX). The solution of FITC (42.05 mg, 0.108 mmol) in dimethylformamide (2.27 ml) was added to the solution of compound (**VIII**) (73 mg, 0.086 mmol) in 0.1 M Na_2CO_3 (11.3 ml). The reaction mixture was kept for 2 h at 18°C in the dark and for 24 h at 0°C and evaporated. Isopropanol (10 ml) was added to the residue, and the solution was evaporated again. The residue was dissolved in water (5 ml) and acidified with 1 N HCl to pH 3. The oily precipitate was extracted with *n*-butanol (2 × 10 ml), washed with water (2 × 5 ml), and evaporated. The residue was dissolved in isopropanol (10 ml) and evaporated again. Triturating with ether yielded 92 mg of peptide (**IX**); R_t 20.1 (the analytic HPLC).

HBr·H-Val-Val-Tyr-Pro-Asp-Lys(Ftc)-OH (X). The solution of compound (**IX**) (60 mg) in trifluoroacetic acid (5 ml) was completely saturated with dry HBr gas. The reaction mixture was evaporated. The residue was dissolved in isopropanol (5 ml) and evaporated again. The residue was triturated with ether (10 ml). The formed precipitate was filtered washed with ether (10 ml), and dried in vacuum. The product was purified by HPLC on an Ultrasphere ODS C18 column (10 × 250 mm, 5 μm, Beckman, United States) in a concentration gradient of acetonitrile in 0.1% TFA (from 0 to 100% within 32 min) at a flow rate of 8 ml/min with the detection at 214 and 280 nm. The yield of peptide (**X**) was 25 mg (44%); R_t 14.6 min (the analytical HPLC); MALDI-MS (*m/z*): 1191.3 $[M + H]^+$ (calculated value was 1190.27).

TFA·H-Val-Val-Tyr-Pro-Asp-OH, **MP-5, (XI)** was synthesized on an Applied Biosystems 431A automatic peptide synthesizer (United States) starting from 375 mg (0.25 mmol) of the Wang polymer with the preliminary attached Fmoc-Asp(OBu^t) (0.67 mmol/g) purchased from Bachem (Switzerland).

The peptide was synthesized according to the standard protocol with a one-step condensation of Fmoc-

amino acids. The synthetic cycle involved deprotection of α-amino group by the treatment with 20% piperidine solution in NMP for 20 min, activation of 1 mmol of the attached amino acid in the presence of equivalent amounts of DIC and HOBt in NMP for 20 min, condensation with 1 mmol (fourfold excess) of the acylating agent in NMP for 37 min, and all the necessary intermediate washings of the peptidylpolymer.

For the final deprotection and cleavage of the peptide from the polymer, the peptidylpolymer was suspended in the deprotecting mixture of TFA and H_2O (10 : 1, 10 ml) and stirred for 2 h at 20°C. The resin was filtered off and washed with TFA (2 × 1 ml). The filtrate was evaporated to the volume of 3–5 ml and mixed with anhydrous ether (100 ml). The precipitate was filtered, washed with ether and ethyl acetate, and dried. The obtained precipitate was dissolved in 1% TFA (5 ml) and applied onto a Diasorb C-16T column (25 × 250 mm). The column was eluted with a gradient of acetonitrile in 0.1% TFA (0.5% per one min) at a flow rate of 12 ml/min with the detection at 226 nm. The fraction containing the target product (**XI**) were joined and evaporated. The residue was dissolved in water and lyophilized. The yield of MP-5 was 80%; the purity was 98% according to HPLC; R_t 9 min on the analytical HPLC column; MALDI-MS (*m/z*): 592.7 $[M + H]^+$ (the calculated molecular mass was 591.68).

Ftc-MP-5 (XII). The reaction was performed in the mixture of dimethylformamide and 0.1 M sodium carbonate (volume ratio of 1 : 3, pH 9.0) with 1.5-fold molar excess of FITC. The obtained Ftc-MP-5 was purified on a Diasorb C18 column (10 × 250 mm) in a concentration gradient of acetonitrile in 0.1% TFA (from 0 to 80% within 32 min) at a flow rate of 9 ml/min with the detection at 280 nm. R_t of Ftc-MP-5 was 15.9 min (the analytical HPLC); MALDI-MS (*m/z*): 981 $[M + H]^+$ (the calculated molecular mass was 980.05).

Proliferative response of T-lymphocytes on the PHA mitogen was determined according to the incorporation of $[^3H]$-thymidine in DNA as described in paper [2]. MP-5 and its labeled analogues were added to the culture of T-lymphocytes in the concentration range from 1×10^{-6} to 1×10^{-9} g/ml. The experimental results were processed using the Statgraphics software (Manugistics, United States).

Antitumor effect of MP-5 was evaluated in vivo on the various types of the transplanted mouse tumors (cervical carcinoma CUC-5, lymphatic leukemia P-388, and melanoma B-16). The tumor strains were obtained from the Bank of tumor strains of the Russian Cancer Research Center. For the experiments and support of the strains in vivo, the cancers were passed on the DBA_2, CBA, and $C_{57}BL_b$ male mice (18–20 g of the body weight) purchased from the Stolbovaya breeding nursery of the Russian Academy of Medical Sciences. Groups of 10–12 animals were formed. The cancers were subcutaneously transplanted in the amount of 1×10^6 cells per one mouse in 0.3 ml of the culture medium 199 [1]. MP-5 was dissolved ex tempore in 0.9% solution of NaCl and administered to the mice 48 h after the transplantation of carcinoma CUC-5 and melanoma B-16 or 24 h after the transplantation of lymphatic leukemia P-388. MP-5 was daily subcutaneously administered for 5 days at a dose of 1.0 μg/g. The 0.9% sterile solution of NaCl (0.1 ml) was administered to the control groups. The mice were observed until their death, and volumes of their cancers were measured three times. The antitumor effect of MP-5 was evaluated according to the dynamics of inhibition of growth of a tumor node (ITG) on the 7th, 14th, and 24th day after the tumor transplantation. ITG was calculated as a ratio of difference in the averaged volumes of tumors in the control and treated groups to the tumor volume in the control group.

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