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STRUCTURE–ANTITUMOR ACTIVITY RELATIONSHIP OF A D-MANNO-D-GLUCAN FROM *Microellobosporia grisea*: EFFECT OF PERIODATE MODIFICATION ON ANTITUMOR ACTIVITY

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ABSTRACT

An antitumor D-manno-D-glucan from *Microellobosporia grisea*, an actinomycete, has a tetrasaccharide repeating-unit structure, a single α-D-mannosyl group being located at both O-3 and O-6 of every other β-D-(1→4)-glucosyl residue. The D-mannosyl groups and D-glucosyl residues of the mannoglucan were polyhydroxylated to various extents by controlled periodate oxidation followed by borohydride reduction. The derivatives (PA mannoglucans) were further subjected to mild hydrolysis with acid, to give partially debranched mannoglucans (PA-H mannoglucans). These derivatives were tested for antitumor activity against Ehrlich carcinoma solid tumor in mice. The PA mannoglucans having degrees of polyhydroxylation of less than ~50 and 2% of the D-mannosyl groups and D-glucosyl residues, respectively, showed high antitumor activities, similar to that of the original mannoglucan, whereas further polyhydroxylation resulted in a marked decrease in, or complete loss of, the activity. The PA-H mannoglucans, lacking 5–40% of the D-mannosyl branches, still had potent antitumor activities, comparable to that of the original mannoglucan. On the basis of these results, the relationship of the structure of the mannoglucan to the antitumor activity is discussed.

INTRODUCTION

In previous studies, a polysaccharide exhibiting potent antitumor activity was isolated[1] from the culture filtrate of *Microellobosporia grisea*, and it was shown[2] to be a D-manno-D-glucan (MG) having a tetrasaccharide, repeating-unit structure as follows.

It was suggested that the D-mannosyl groups are responsible for the antitumor activity and water solubility of MG; partial introduction of some ester groups into MG resulted[3] in a decrease in, or complete loss of, the activity. The previous information prompted us to investigate the role of the D-mannosyl branches in the antitumor activity, and the interrelation between the activity and the structure of MG.

We now report a comparison of the antitumor effects of periodate-modified

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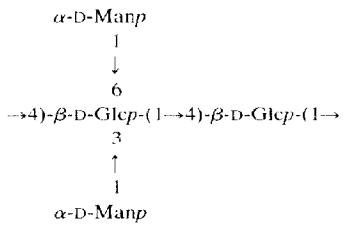


mannoglucans (obtained by controlled periodate oxidation followed by borohydride reduction) having various degrees of polyhydroxylation, and the effects of partial debranching on the antitumor activity.

RESULTS AND DISCUSSION

Preparation and characterization of MG derivatives. — MG was subjected to periodate oxidation, using a limited ratio of sodium periodate to mannoglucan (0.05–3.0 mol of periodate per sugar residue) for 14 days at 5°. The resulting, partially oxidized mannoglucan was then reduced with sodium borohydride, to give a mannoglucan polyalcohol (PA mannoglucan) with a certain degree of polyhydroxylation. The yield of the PA mannoglucan decreased with increased molar ratio of periodate to sugar residue (see Table I), as accounted for by release of formic acid during oxidation (see Table III).

The D-mannosyl groups and (1→4)-linked D-glucosyl residue of the tetrasaccharide repeating-unit were expected to be susceptible to periodate oxidation, whereas the doubly branched, (1→4)-linked D-glucosyl residue would be resistant.

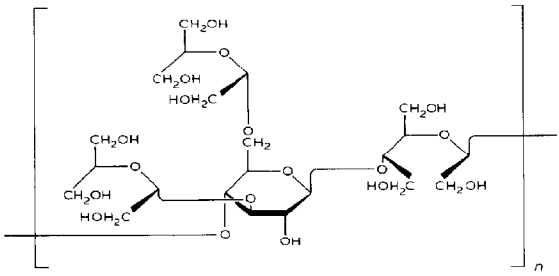
TABLE I

PHYSICOCHEMICAL PROPERTIES OF PA MANNOGLUCANS

PA mannoglucan	*Oxidation conditions*[a]	*Yield (%)*	*Molecular weight*[b] *(× 10⁻⁴)*	$[\alpha]_D$[c] *(degrees)*
MG			100	+65
PA-5	0.05	96	92	+60
PA-10	0.10	95	105	+51
PA-25	0.25	94	120	+30
PA-35	0.35	94	85	+15
PA-50	0.50	88	120	−5
PA-100	1.00	84	50	−19
PA-300	3.00	82	18	−32

[a]Molar ratio of periodate to sugar residue. [b]Estimated by gel-permeation chromatography with dextran as the standard. [c]0.5; water

Of the PA mannoglucans, PA-300, obtained by oxidation of MG with a large excess of periodate followed by borohydride reduction, corresponds to MG polyalcohol (see formula **1**), which has been used[2] for Smith degradation of MG; complete hydrolysis of PA-300 with acid yielded, in fact, glycerol, erythritol, and glucose in the molar ratios of 2.0:1.1:1.0.



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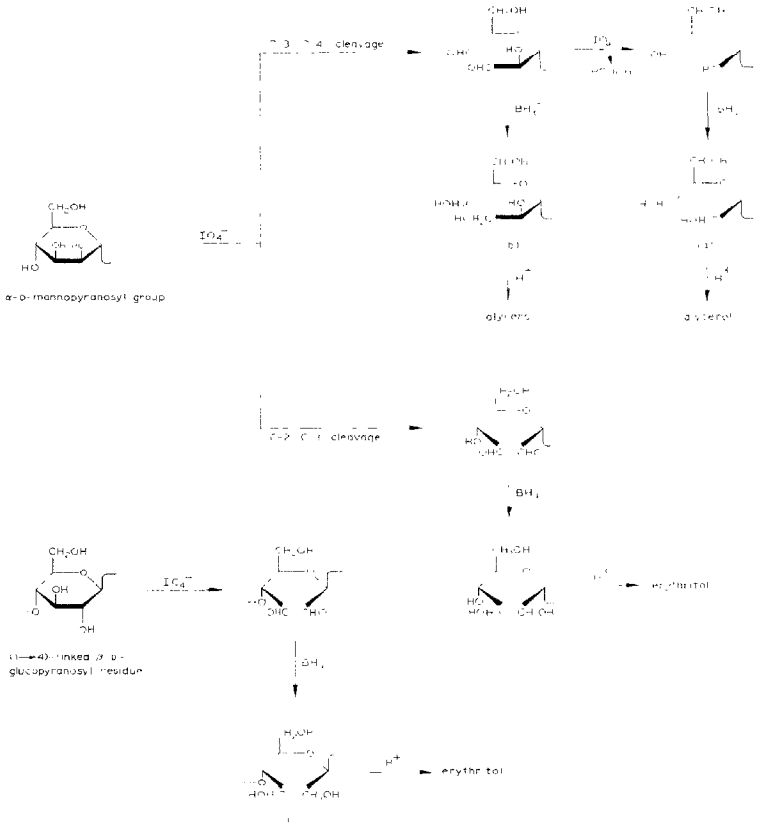
In the other PA mannoglucans, all of the C–C bonds of glycol groups in MG should not be split by periodate oxidation, because of a shortage of periodate for complete oxidation, such as in the case of PA-300. In this instance, the D-mannosyl groups of MG may undergo periodate cleavage of either the C-2–C-3 or the C-3–C-4 bond, in addition to cleavage of both bonds. Information on the mode of cleavage can be obtained by complete hydrolysis of the PA mannoglucans with acid; glycerol in the hydrolysis products arises both from single cleavage of C-3–C-4 bonds and cleavage of both the C-2–C-3 and C-3–C-4 bonds in the D-mannosyl groups, and erythritol from both single cleavage of C-2–C-3 bonds in its groups and of that in the (1→4)-linked D-glucosyl residues (see Scheme 1).

In order to elucidate the mode of cleavage, the PA mannoglucans were subjected to complete hydrolysis with acid, and the resulting sugars were analyzed as alditols by g.l.c. The analytical data are summarized in Table II. The production of erythritol from PA-5, PA-10, and PA-25 was negligible, and slight from PA-35 and PA-50. This indicates that, under oxidation conditions employing a molar ratio of periodate to sugar residue of <0.5:1, there occurs essentially neither single cleavage of C-2–C-3 bonds in the D-mannosyl groups nor cleavage of C-2–C-3 bonds in the (1→4)-linked D-glucosyl residues. The slight production of erythritol appears to proceed by the latter cleavage.

On the other hand, the production of glycerol from the PA mannoglucans increased with the ratio of periodate to sugar residue employed for their preparation, and was accompanied by degradation of the D-mannosyl groups (see Table II). The production of formic acid during periodate oxidation (see Table III) was significantly less than the degree of degradation of the D-mannosyl groups in PA-25, PA-



Scheme 1. Modes of cleavage of the C–C bonds by periodate oxidation.

50, and PA-100, indicating the occurrence of a certain single cleavage of C–C bonds (C-3–C-4 bonds) in the D-mannosyl groups, besides the cleavage of both bonds.

From these results, it may be concluded that periodate oxidation of the D-mannosyl groups proceeds by primary cleavage of the C-3–C-4 bonds, followed by secondary cleavage of the C-2–C-3 bonds, and that the glycerol is a product of the

TABLE II

ANALYSES OF PA MANNOGLUCANS[a]

PA mannoglucan	*Molar ratios of acid hydrolysis products*[b]				*Degrees of polyhydroxylation (%)*	
	Glucose	*Mannose*	*Glycerol*	*Erythritol*	*α-D-Mannosyl group*[c]	*(1→4)-Linked β-D-glucosyl residue*[d]
PA-5	1.00	0.86	0.06	<0.01	6 (2)[e]	<2
PA-10	1.00	0.80	0.15	<0.01	16 (9)	<2
PA-25	1.00	0.61	0.33	<0.01	35 (31)	<2
PA-35	1.00	0.43	0.45	0.01	51 (51)	2
PA-50	1.00	0 16	0.76	0 02	83 (82)	4
PA-100	1.00	0.00	1.1	0.20	100 (100)	32
PA-300	1.00	0.00	2.0	1.1	100 (100)	100
MG	1.00	0.88				

[a]Mannoglucan (MG) was modified by controlled periodate oxidation and borohydride reduction. [b]PA mannoglucans (PA-50 and PA-100) were first hydrolyzed with 18:7 sulfuric acid–water at room temperature, and then with 0.5M sulfuric acid for 5 h at 100°, and the others with 0.5M sulfuric acid for 5 h at 100°. [c]Calculated, based on the formation of glycerol; [glycerol/(mannose + glycerol)] × 100. [d]Calculated, based on the formation of erythritol; [erythritol/(glucose + erythritol)] × 2.1/1.1 × 100. [e]Calculated, based on the degradation of mannosyl groups in MG; [1 − mannose/(glucose + erythritol) × 0.88^{-1}] × 100.

TABLE III

RELEASE OF FORMIC ACID DURING PERIODATE OXIDATION[a]

PA mannoglucan	*Release of formic acid per sugar residue (mol)*	*Cleavage of C–C bond (%)*	
		Type (a)[b]	*Type (b)*[c]
PA-25	0.05 (0.11)[d]	11	20
PA-50	0.10 (0.21)	21	61
PA-100	0.35 (0.75)	75	25
PA-300	0.45 (0.96)	96	4

[a]Mannoglucan (MG) was oxidized with periodate, to give the PA mannoglucan. [b]Cleavage of both C-2–C-3 and C-3–C-4 bonds in α-D-mannosyl group. [c]Single cleavage of C-3–C-4 bond in α-D-mannosyl group. [d]Per α-D-mannosyl group.

acid hydrolysis of the PA mannoglucans other than PA-300, derived both from the single (C-3–C-4) and the double cleavage of the D-mannosyl groups in MG.

Thus, such polyhydroxy groups as groups a and b, and c (see Scheme 1), respectively originating from the D-mannosyl groups and the (1→4)-linked D-glucosyl residues, are present in the PA mannoglucans in the percentages given in Table II. The percentages of groups a and b present in some PA mannoglucans are shown in Table III, where the ratios of group a were obtained from the production of formic acid per D-mannosyl group. The degrees of polyhydroxylation of D-mannosyl groups, calculated on the basis of the production of glycerol, were in good agreement with those of degradation of D-mannosyl groups, except in PA-5 and PA-10

(see Table II); this supports the conclusion as to the modes of periodate cleavage just described.

The favored periodate-cleavage of the C-3–C-4 bonds in the D-mannosyl groups of MG is consistent with such an observation on some monosaccharides that was reported by Honda *et al.*[4]. Also, the resistance of the (1→4)-linked D-glucosyl residues to periodate oxidation is presumably attributable to steric hindrance due to the complicated structure of MG. In this connection, Yamaguchi *et al.*[5] reported that an internal residue, even of cellotriose, is not susceptible to periodate oxidation.

The PA mannoglucans showed specific rotations that were lessened in proportion to their degrees of polyhydroxylation (see Table I), and the removal of their polyhydroxy groups resulted in an increase in their specific rotations (see Table V), as described later. These results indicate that the polyhydroxy groups derived from the D-mannosyl groups have a levorotatory property.

As shown in Table I, differences in molecular weight between MG and the PA mannoglucans (PA-5–PA-50) were relatively small, but PA-100 and PA-300 showed molecular weights much lower than that of MG. The former observation may be explained by conformational changes occurring almost exclusively in side chains, and the latter, by those in both the side and the main chains, the ring fission of the D-glucosyl residues comprising the main chain should cause a remarkable change in conformation, to give a compact structure.

The PA mannoglucans thus obtained and characterized were subjected to mild hydrolysis with acid to remove the polyhydroxy groups derived from the D-mannosyl groups. On this treatment, the PA mannoglucans (PA-5–PA-35) gave their respective PA-H mannoglucans (PA-5H–PA-35H), which were water-soluble derivatives. On the other hand, PA-50 and PA-100 yielded water-insoluble materials during the treatment with acid; the products appear to be cellulose-like polymers or oligomers, produced by cleavage of acetal linkages of the polyhydroxy groups derived from the D-mannosyl groups and from the (1→4)-linked D-glucosyl residues, as expected from the structure of MG. The mild, acid hydrolysis of PA-300 corresponds to the controlled Smith degradation of MG.

These four PA-H mannoglucans were subjected to complete hydrolysis with acid, and the products were analyzed by g.l.c. Table IV shows that every one of the PA-H mannoglucans so far prepared lacks ~80% of the polyhydroxy groups present as branches in the respective PA mannoglucans. Thus, partially debranched derivatives, lacking 5–40% of the D-mannosyl side-chains of MG, were obtained by chemical modification.

As shown in Table V, their yields reasonably decreased with elimination of the polyhydroxy groups from their starting PA mannoglucans. PA-25H and PA-35H showed molecular weights significantly lower than that of MG, suggesting the occurrence of cleavage in the main chains of PA-25 and PA-35 with slight degrees of polyhydroxylation during the mild hydrolysis. There were positive differences in specific rotation between the PA-H mannoglucans and the respective PA manno-

glucans, increasing with the proportion of the polyhydroxy groups eliminated (see Tables I and V); the meaning of this observation has already been discussed.

Antitumor activities of MG derivatives. — These derivatives of MG were tested for antitumor activity against Ehrlich carcinoma in mice. As shown in Table VI, PA-50 and PA-100 showed little effect on the tumor by i.p. administration at a dose of 100 mg/kg, and PA-300 had lost the activity, whereas PA-25 had a high antitumor effect. Other PA mannoglucans (PA-5–PA-35) exhibited potent antitumor activities at a dose of 10 mg/kg, comparable to that of MG (see Table VII).

These results indicate that the polyhydroxylation of at least ~50% of the D-mannosyl branches keeps MG from loss of antitumor activity, so long as polyhydroxylation of the main chain is slight. In addition, a marked decrease in the activity of PA-50, polyhydroxylated to the extent of only 4% of the (1→4)-linked D-glucosyl residues in the main chain and to ~80% of the side chains, suggests that the main chain (cellulose), besides the presence of a certain proportion of the D-mannosyl branches, plays an important role in the antitumor activity. It is not clear whether the polyhydroxylation of the side chains or that of the main chain resulted

TABLE IV

ANALYSES OF PA-H MANNOGLUCANS[a]

PA-H mannoglucan	*Molar ratios of acid hydrolysis products*[b]			*Polyhydroxy group (%)*		*Loss of α-D-mannosyl group*[e] *(%)*
	Glucose	*Mannose*	*Glycerol*	*Residual*[f]	*Eliminated*[d]	
PA-5H	1.00	0.87	0.01	17	83	5 (1)
PA-10H	1.00	0.81	0.03	20	80	13 (3)
PA-25H	1.00	0.59	0.07	21	79	28 (7)
PA-35H	1.00	0.42	0.10	22	78	40 (11)

[a]PA mannoglucans were treated with 0.25M sulfuric acid for 24 h at room temperature. [b]PA-H mannoglucans were hydrolyzed with 0.5M sulfuric acid for 5 h at 100°. [c]Polyhydroxy groups non-eliminated from PA mannoglucans. [d]Polyhydroxy groups eliminated from PA mannoglucans. [e]Loss of polyhydroxy groups originating from α-D-mannosyl groups of mannoglucan (MG). [f]Residual polyhydroxy groups in PA-H mannoglucans.

TABLE V

PHYSICOCHEMICAL PROPERTIES OF PA-H MANNOGLUCANS

PA-H mannoglucan	*Yield (%)*	*Molecular weight*[a] *($\times 10^{-4}$)*	$[\alpha]_D$[b] *(degrees)*
PA-5H	94	100	+63
PA-10H	90	92	+59
PA-25H	80	79	+44
PA-35H	76	62	+32

[a]Estimated by gel-permeation chromatography with dextran as the standard. [b]*c* 0.5, water.

in such a decrease for PA-50. The antitumor activity of MG may, however, be more sensitive to the polyhydroxylation of the main chain than of the side chains.

As shown in Table VII, every one of the PA-H mannoglucans exhibited potent antitumor activity by i.p. administration at a dose of 10 mg/kg, comparable to that of MG. This demonstrates that a certain proportion, at least 40%, of the D-mannosyl groups is not essential for the antitumor activity of MG.

Thus, the presence of a certain proportion, not more than 60%, of the D-mannosyl groups in the cellulose chain should be important for antitumor activity and water-solubility. It is probable that the cellulose chain plays an important role (as a back-bone) in the antitumor activity.

An effect of polyhydroxylation of a highly branched, (1→3)-β-D-glucan on

TABLE VI

ANTITUMOR EFFECT OF PA MANNOGLUCANS ON EHRLICH CARCINOMA[a]

Sample[b]	*Dose (mg/kg)*	*Tumor weight*[c] *(mean ± SE) (g)*	*T/C*[d] *(%)*	*Complete regression*[e]
PA-25	100	0.15 ±0.09[f]	4.8	1/6
PA-50	100	1.95 ±0.49	62.3	0/5
PA-100	100	2.01 ±0.58	64.2	0/6
PA-300	100	3.12 +1.14	99.7	0/6
Control		3.13 ±0.53	100	0/10

[a]ICR mice were inoculated s.c. with tumor cells (3×10^6) on day 0. [b]Administered i.p. twice on days 12 and 17. [c]Weighed on day 30. [d]Average tumor-weight of treated group/that of control group. [e]No. of tumor-free mice/no. of mice tested. [f]Significant difference from control group (*$p < 0.001$)

TABLE VII

ANTITUMOR EFFECT OF MODIFIED MANNOGLUCANS ON EHRLICH CARCINOMA[a]

Sample[b]	*Dose (mg/kg)*	*Tumor weight*[c] *(mean ± SE) (g)*	*T/C*[d] *(%)*	*Complete regression*[e]
PA-5	10	0.40 ±0.25[f,g]	14.4	2/6
PA-10	10	0.74 ±0.61[f]	26.7	1/6
PA-25	10	0.84 ±0.38[f]	30.3	2/6
PA-35	10	0.86 ±0.24**	31.0	1/5
PA-5H	10	0.20 ±0.08***	7.2	2/6
PA-10H	10	0.36 ±0.26[f,g]	13.0	2/6
PA-25H	10	0.04 ±0.02[f,g]	1.4	3/6
PA-35H	10	0.61 ±0.36[f]	22.0	2/5
MG	10	0.34 ±0.22[f,g]	12.3	2/6
Control		2.77 ±0.40	100	0/14

[a]ICR mice were inoculated s.c. with tumor cells (3×10^6) on day 0. [b]Administered i.p. twice on days 12 and 17. [c]Weighed on day 30. [d]Average tumor-weight of treated group/that of control group. [e]No. of tumor-free mice/no. of mice tested. [f]Significant difference from control group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

antitumor activity has been reported by Misaki *et al.*[6], and it was suggested that the polyhydroxy groups derived from the side chains are responsible for the antitumor activity of the periodate-modified glucan. In the case of MG, a highly branched D-manno-D-glucan, the partial polyhydroxylation of the D-mannosyl side chains did not affect the antitumor activity, so long as the polyhydroxylation of the main chain was slight. These results indicate the great dependence of the antitumor activity of polysaccharides on their higher structures.

EXPERIMENTAL

Materials. — The D-manno-D-glucan (MG) was prepared as described previously[1].

Preparation of PA mannoglucans. — MG (5.0 g) in water (1.0 L) was oxidized with a 1.65% aqueous solution of sodium periodate (20 mL) for 14 days at 5° in the dark. The partially oxidized mannoglucan was then reduced with sodium borohydride (0.5 g) for 1 day at room temperature. After decomposition of the excess of borohydride by addition of acetic acid to a final pH of 5, the mixture was dialyzed against de-ionized water at 5°. The non-dialyzable solution was concentrated to ~350 mL, centrifuged to remove a trace of insoluble materials, and the supernatant liquor poured into ethanol (1.0 L). The resulting precipitate was collected by filtration, washed successively with ethanol and acetone, and dried over P_2O_5 *in vacuo*, to give PA-5 (4.78 g).

Other PA mannoglucans, PA-10 (4.75 g), PA-25 (4.72 g), PA-35 (4.68 g), PA-50 (4.38 g), PA-100 (4.21 g), and PA-300 (4.10 g) were prepared from MG (5.0 g) as just described, by oxidation with 40, 100, 140, 200, 400, and 1200 mL of the periodate solution, followed by reduction with 1.0, 2.0, 3.0, 4.0, 5.0, and 7.0 g of sodium borohydride, respectively; in the preparation of PA-300, the excess of periodate was decomposed with ethylene glycol before addition of borohydride.

Preparation of PA-II mannoglucans. — The samples (2.0 g in 150 mL of water) of PA-5, PA-10, PA-25, and PA-35 were treated with 0.5M sulfuric acid (150 mL) for 24 h at room temperature. After neutralization of the acid with 3M sodium hydroxide, each mixture was dialyzed against de-ionized water. The non-dialyzable solution was concentrated to ~200 mL, and the concentrate was poured into ethanol (600 mL). The respective precipitates resulting were collected by filtration, washed successively with ethanol and acetone, and dried over P_2O_5 *in vacuo*, to give PA-5H (1.87 g), PA-10H (1.79 g), PA-25H (1.60 g), and PA-35H (1.52 g).

Analyses of MG derivatives. — The samples (10 mg) of PA-50 and PA-100 were hydrolyzed sequentially in 18:7 sulfuric acid–water for 0.5 h at room temperature, and then in 0.5M sulfuric acid for 5 h at 100°; this procedure prevented the formation of insoluble, cellulose-like polymers or oligomers during the acid hydrolysis. Other derivatives (10 mg) of MG were hydrolyzed in 0.5M sulfuric acid (2 mL) for 5 h at 100°. The resulting sugars and alditols were analyzed as the alditol acetates by g.l.c.[2]; the column temperature was programmed from 80 to 190° at the rate of 5°/min.

Molecular weight was estimated by gel-permeation chromatography[1] with dextran T-500, T-70, and T-40 as standards, and optical rotation was measured with a Perkin–Elmer model 141 polarimeter.

Production of formic acid on periodate oxidation. — MG (100 mg) was oxidized with periodate under the respective conditions used for the preparations of PA-25, PA-50, PA-100, and PA-300, and the formic acid production was determined by titration with 10mM sodium hydroxide.

Assay of antitumor activity. — Antitumor activities of MG and its derivatives against Ehrlich carcinoma solid tumor in mice were assayed as previously described[1]. The tumor was implanted s.c. into the right groin of mice, and the test samples, dissolved in physiological saline, were administered i.p., twice on days 12 and 17, starting 24 h after tumor implantation.

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