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Marine lysozyme from a marine bacterium that inhibits angiogenesis and tumor growth

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Abstract Recent studies suggest that lysozyme, rich in hen egg, has an antitumor function. In the present study, we investigated the antitumor and antiangiogenesis effects of a newly isolated marine lysozyme both in vitro and in vivo. First, we showed that this marine-derived lysozyme specifically inhibits the proliferation of endothelial cells (ECV304) in a dose-dependent manner with no cytotoxicity (IC_{50}=3.64 μM). Second, we showed that this marine lysozyme directly suppresses neovascularization in chicken embryos using chorioallantoic membrane assay. Third, we demonstrated that this marine lysozyme markedly inhibits tumor growth in mice bearing either sarcoma 180 or hepatoma 22. Unexpectedly, hen egg lysozyme has no effects on the proliferation of endothelial cells in vitro or neovascularization in chicken embryos or tumor growth in nude mice at the same dosage range. Taken together, our studies clearly show that the newly identified marine lysozyme is a potent antitumor molecule, which may inhibit tumor growth and inhibit angiogenesis. We believe that this marine lysozyme may have a therapeutic value in antitumor drug development.

Keywords Lysozyme · Antiangiogenesis · Endothelial cells · CAM assay · Antitumor · Marine lysozyme

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Introduction

Lysozyme (1,4-β-*N*-acetylmurmidase) is known as a powerful antibacterial protein widely distributed in various biological fluids and tissues (Blake and Koening 1965). Basic experiments and clinical trials have also shown that lysozyme could function as a nonspecific immunoregulator to enhance phagocytic activity of polymorphonuclear leukocytes and macrophages, stimulate proliferation of these cells, and inhibit the growth of malignant tumor (Cappuccino et al. 1962; LeMarbre et al. 1981; Warren et al. 1981; Sava et al. 1989).

Marine-derived lysozyme is a new class recently extracted from marine invertebrates (Nilsen et al. 1999; Nilsen and Myrnes 2001). As marine invertebrates need to retain their activity at low temperature, hypoxia, and high-pressure environment, marine-derived lysozyme exhibits special activity compared to vertebrate lysozyme. Crude extractions of shark cartilage, with high homology in molecule structures to lysozyme, have been demonstrated as a strong angiogenesis inhibitor (Brem and Folkman 1975; Moses et al. 1990; Sheu et al. 1998; Shen et al. 2000; Chen et al. 2000). Our previous studies found that cold-adapted lysozymes extracted from marine bacterium possessed some different features from chicken-type lysozyme such as broad optimum pH range, broad range of optimum temperatures, and more broad-spectrum antibiotic reaction (Wang et al. 2000; Zou et al. 2005). In the present study, we studied the antiangiogenesis and antitumor bioactivities of a lysozyme from a marine bacterium initially. We investigated the effects of marine lysozyme and hen egg lysozyme on angiogenesis in vitro and in vivo. Furthermore, the antitumor growth effects of two lysozymes on mouse sarcoid S180 and hepatoma 22 models were studied.

Materials and methods

Extraction and purification of marine lysozyme

The marine lysozyme was isolated and purified from a marine bacillus at Open Laboratory of Marine Enzyme and Enzyme Engineering, Yellow Sea Fisheries Research Institute as described previously (Wang et al. 2000). Briefly, the marine bacillus strain was cultured at 20°C for 40 h on a shaker in the medium consisting of 1% glucose, 0.5% yeast extract, 3.5% tryptone, and artificial seawater. The supernatant was acquired after centrifugation at $1.5\times10^4\times g$ for 1 h at 4°C, and the lysozyme was purified by the procedures of ultrafiltration technology using a hollow fiber (molecular weight cut off, 50 kD), CM-Sepharose-FF positive ion column chromatography and perfusion chromatography. Lysozyme was analyzed by reversed-phase high-performance liquid chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12%). The amino acid sequence analysis was performed by automatic Edman degradation assays. A suspension of lysozyme was dissolved in phosphate-buffered saline (PBS) sterilized at a stock concentration of 2.8%.

Cell-proliferation assay in vitro

3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used as described previously to evaluate the effect of lysozyme on the proliferation of vascular endothelial cells (Carmichael et al. 1987). The endothelial cell (ECV304) and the non-endothelial cell lines: human hepatoma 22 cells, murine sarcoma S180 cells, and fibroblast cells (NIH3T3; type culture collection of Chinese Academy of Science, TCCCAS) were grown in RPMI-1640 (Sigma) containing 10% fetal calf serum (Gibco) supplemented with penicillin (100 U/ml, Sigma) and streptomycin (100 μg/ml, Sigma) at 37°C in a 5% CO_2 atmosphere. Four cell lines were plated in triplicate onto 96-well culture plate (Falcon, Franklin Lakes, NJ, USA) at a density of 2×10^3 cells/100 μl starvation medium containing 1% new calf serum (0.1 ml/well). After 72 h, different concentrations of marine lysozyme or hen egg lysozyme (Sigma), ranging from 6 to 36 μM was added to each well. Cells grown in the presence or absence of basic fibroblast growth factor (bFGF; 10 ng/ml) were divided as positive controls and negative controls. About 3 days later, 20 μl MTT solution (0.25%; Sigma) was added to the cells and incubated for 4 h at 37°C. The medium was pipetted out from each well, and 150 μl dimethyl sulphoxide (Sigma) was added. The absorbance was determined at 570 nm with a microplate reader (Model 3550, Bio-Rad). Each independent dosage and experiments were repeated three times.

Chorioallantoic membrane assay in vivo

Inhibition of angiogenesis in vivo was measured using a modified chorioallantoic membrane (CAM) assay (Sharma et al. 2001). All procedures were carried out in a laminar-flow hood under sterile conditions. Approximately 80 fertilized 6-day eggs were incubated at 90% of humidity and 37°C. A window was made on the top of each egg on day 7. Then, the eggs were divided into ten groups randomly, and sterile 6 mm diameter circular filter paper discs soaked with total 10 μl different concentration medium were applied onto the surfaces of the growing CAMs treated with 0.1 M PBS or 100 nM suramin as the negative or positive groups, respectively, and four different concentrations of marine lysozyme or hen egg lysozyme as treatment groups. About 72 h later, vascularization degree was quantified by counting the number of branches of blood vessels around the filter papers within 100 mm^2 under a dissecting microscope (Nikon USA) and a charge-coupled device imaging system (Scion Corporation, Frederick, MD, USA) and photographed at a magnification of ×40. The inhibition rate of angiogenesis was calculated as following: Inhibition rate of angiogenesis = [1 − (vessel branch points of test group)/(vessel branch points of negative control] × 100%. The assay was performed twice to ensure reproducibility.

In vivo tumor growth inhibition assay

Female 19–21 g Kunming mice (Animal Center of Shanghai Institute of Pharmaceutical Industry) were used to study inhibition of tumor growth. The experimental protocol was approved by the China Institutional Ethics Review Committee for Animal Experimentation and was in accordance with guidelines of the Experimental Animal Association of China. The sterilely harvested murine sarcoma 180 cells or hepatoma 22 cells with ascites, diluted with sterilized physiological saline at a ratio of 1:4 (*v*/*v*, cell concentration was adjusted to 1×10^7/ml), were implanted subcutaneously into the axillary fossa of 80 mice as described by others (0.2 ml per mouse; Lu et al. 2003). The mice were divided into eight groups randomly (ten mice per group) on next day. Different doses of marine lysozyme 1.25, 2.5, and 5 mg/kg, hen egg lysozyme 2.5, 5, and 10 mg/kg dissolved in normal saline once a day were given intraperitoneally as treatment groups. The equal volume of saline and 100 mg/kg cytoxan (CTX) intraperitoneally once a day were given as negative or positive controls. The mice were killed 10 days later, and the tumors were cut and weighed. The rate of antitumor activity was calculated as: tumor growth inhibitory rate(%)=(1−tumor weight of test group/tumor weight of negative control group)×100.

Table 1 Purification of marine lysozyme

Procedure	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	4.04×10^6	2.63×10^4	154.6	100	
Ultrafiltration	3.88×10^6	0.54×10^4	718.5	96.3	4.9
CM-Sepharose	2.89×10^6	1,436.7	2,011.6	71.5	18.3
Sephadex G-100	1.96×10^6	884.2	2,216.7	48.5	29.7

Statistical analysis

Data were expressed as mean±S.D., *n* representing the number of experiments or the animals per group. Statistical analysis was performed by one-way analysis of variance (ANOVA) analysis. Qualitative data was performed by χ^2 with SPSS version 10.0. $P<0.05$ was considered to be statistically significant.

Results

Extraction and purification of marine lysozyme

The crude lysozyme was electrophoreticly purified 29.7-fold with a recovery of 48.5% and specific activity 2,216.7 U/mg by purification procedures (Table 1). The molecular weight of this marine lysozyme was finally determined as 15,800 Da by SDS-PAGE (Fig. 1) and 16,464 Da by Matrix-assisted laser desorption ionization time-of-flight mass spectrometry assay. The automatic Edman degradation assays showed the protein containing 143 amino acids including asprines (21.68%), serines (8.39%), and histidines (7.69%). N-terminal amino acid sequence is Ala-Asn-Leu-Thr-Glu-Asp-Ala-Phe-Ile-Glu. Its isoelectric point is 9.28, and Km value is 72 μg/ml. The optimum pH of marine lysozyme reacting is 6.5, and the temperature ranges from 5 to 50°C (the optimum is 35°C; data not shown).

Inhibitory effects of lysozyme on the proliferation of vascular endothelial cell in vitro

As angiogenesis involves local proliferation of endothelial cells, we first invested the effects of lysozyme on proliferation of endothelial cells (ECV304) by MTT assays. The optical density (OD) value showed that the proliferation of ECV304 and non-endothelial cells (2×10^3/well) grown in the absence of 10 ng/ml bFGF for 3 days was very slow and almost kept in resting state, which is almost same as the day after cells seeded (data not shown). bFGF (10 ng/ml) markedly enhanced the proliferation of ECV304 and non-endothelial cells, which is showed by the increased OD value. As shown in Fig. 2a, in the concentration ranging from 6 to 24 μM marine lysozyme inhibited the proliferation of ECV304 induced by bFGF significantly in a concentration-dependent manner. 12 μM marine lysozyme could inhibit cell proliferation by 79%. And at dosage more than 24 μM, the inhibition rate is almost unchanged (IC_{50} value is 3.64 μM). To confirm the specificity of marine lysozyme on angiogenesis, we determined the cytotoxicity of the marine lysozyme on some non-endothelial cell lines: two tumor cell lines (murine sarcoma S180 cells and hepatoma 22 cells) and murine fibroblast cell line (NIH3T3). The results showed that no significant detectable stimulating or inhibitory effects were observed on the growth of all examined non-endothelial cells (Fig. 2b–d), which indicated that the inhibitory effect of this concentration range of marine lysozyme were specific rather than the results of general cytotoxicity. Meanwhile, hen egg lysozyme showed no significant inhibitory effect on ECV304, and non-

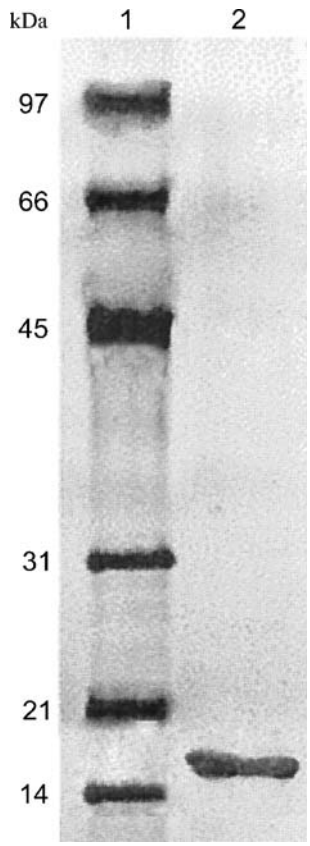

Fig. 1 SDS-PAGE assay of purified marine lysozyme. *Lane 1* Marker; *lane 2* purified marine lysozyme

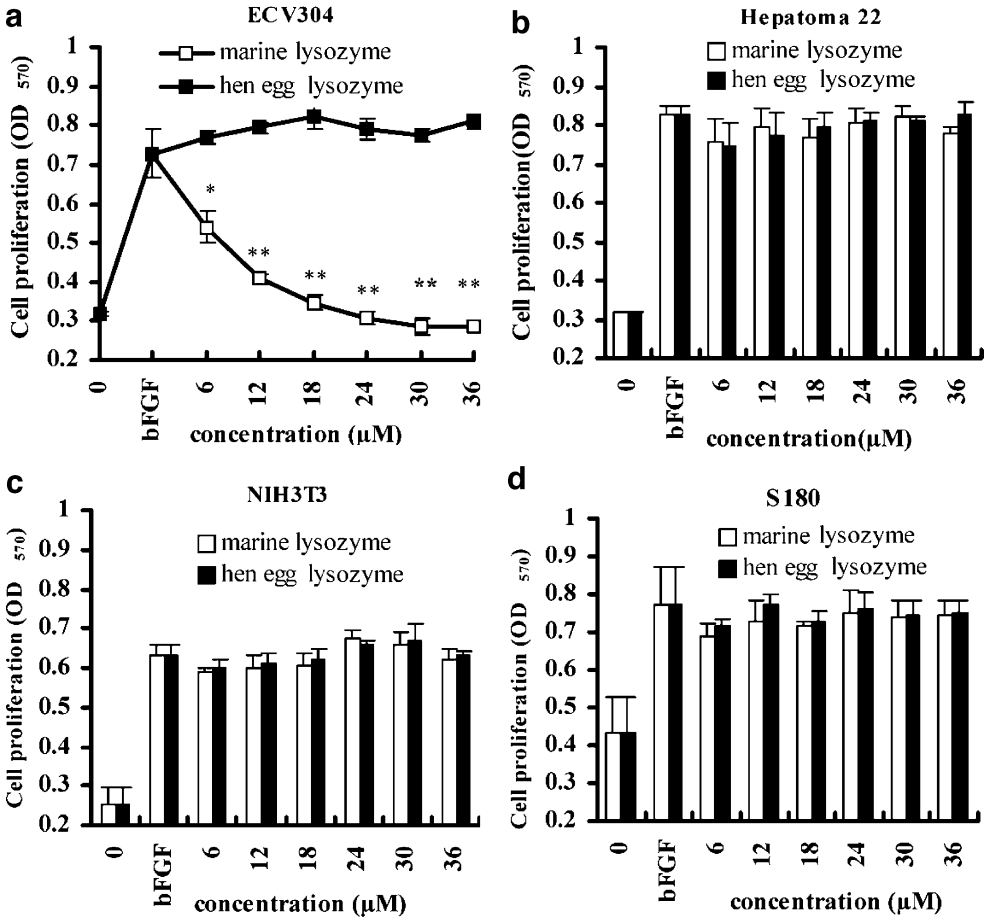

Fig. 2 Effects of lysozyme on the proliferation of bFGF-treated endothelial and non-endothelial cells by MTT assay. Treated with two lysozymes at various dosages for 72 h, **a** the proliferation of ECV304 was significantly inhibited in a concentration-dependent manner by marine lysozyme (6–24 μM), and no significant proliferatory effect was observed in hen egg lysozyme-treated groups. No significant effect of marine lysozyme and hen egg lysozyme on examined non-endothelial cells: hepatoma 22 (**b**), NIH3T3 (**c**), and S180 (**d**). One-way ANOVA test followed by LSD test, $N=3$, values represent mean OD absorbance±SEM. *Single asterisk* $P<0.05$ or *double asterisk* $P<0.01$ vs bFGF-treated positive control group

endothelial cell growth in the tested concentration ranged from 6 to 36 μM (Fig. 2a–d). These results indicated that hen egg lysozyme had no obvious antiproliferative effect on endothelial cells, while marine lysozyme may specifically inhibit the proliferation of endothelial cells.

Antiangiogenesis of lysozyme in chicken chorioallantoic membrane in vivo

The antiangiogenic activity of marine lysozyme was further observed by in vivo CAM assay. In the PBS controls, blood vessels in CAM formed densely branching vascular networks (Fig. 3a). Marine lysozyme strongly inhibited CAM angiogenesis, clearly producing an avascular zone in a dosage-dependent manner (Fig. 3b), with concentrations of 0.6, 1.2, and 2.4 nM/egg yielding inhibition rates of 53, 70, and 85.2%, respectively (Fig. 3e). At the dosage of 1.2 nM/egg, the inhibition rate of marine lysozyme almost resembled that of 100 nM/egg of suramin, a well-known angiogenesis inhibitor (71% inhibition, Fig. 3d,e). In contrast, there are no significant differences in the number of blood branch points between the PBS control and different concentration of hen egg lysozyme groups. No avascular zones were observed in any embryos of these five groups (Fig. 3c,e).

Inhibition of tumor growth of tumor-bearing mice in vivo

The broad-spectrum antitumor activity of marine lysozyme in vivo was shown in two xenograft mouse models: harboring tumors induced by implantation of sarcoma 180 cells, or hepatoma 22 cells. In this experiment, marine lysozyme significantly inhibited the growth of subcutaneous xenograft of S180 sarcoma and hepatoma 22 in dose-dependent manner (Tables 2 and 3). Administration of 1.25, 2.5, and 5 mg/kg marine lysozyme for 10 days following implantation of sarcoma 180 cells hindered tumor growth by 29.7, 49.8, and 83.1%, respectively (Table 2). At dosage of 5.0 mg kg^{-1} day^{-1}, the tumor inhibitory rate is 83.1%, which is higher than CTX group. While administration of 1.25, 2.5, and 5 mg/kg marine lysozyme for 10 days following implantation of hepatoma 22 cells could suppress tumor growth by 26.4, 55.6, and 73.1%, respectively (Table 3), the inhibitory effects of different dosages of hen egg lysozyme on the growth of two tumor models were not significant. These results indicate that lysozyme

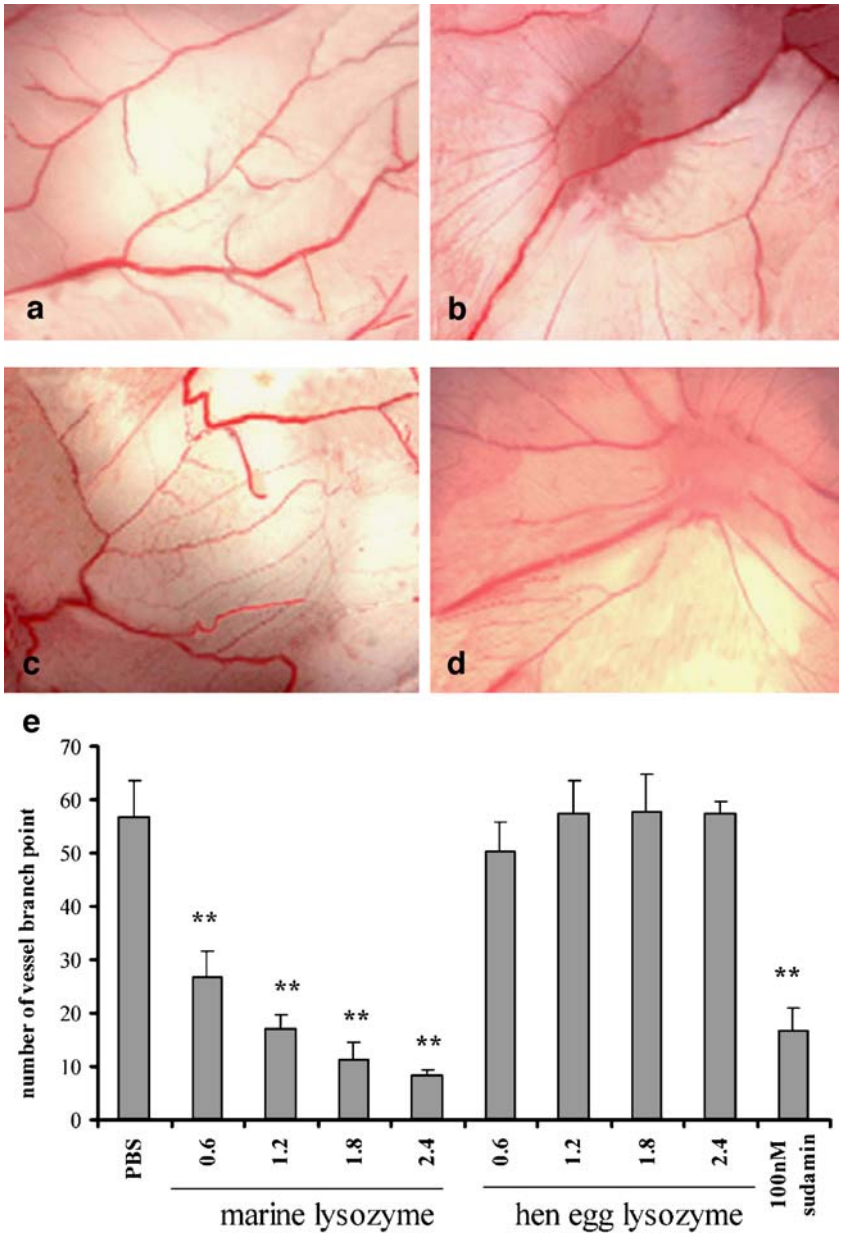

Fig. 3 Effect of marine lysozyme on CAM. Fertilized eggs were incubated continuously for 6 days, and then a window was opened to expose the CAM, and lysozyme at different concentrations was added on sterilized filter paper discs on day 7. The eggs were incubated for another 72 h, and then the treated CAMs were harvested and photographed. **a** 0 nM/egg (PBS negative control), **b** 1.2 nM/egg (marine lysozyme), **c** 1.2 nM/egg (hen egg lysozyme), **d** 100 nM/egg (suramin). **e** Macroscopic assessment of vascular density conducted by counting the number of branch points within a 100-mm^2 area surrounding filter paper in each photograph. The number of branch points was markedly decreased with marine lysozyme in a dose-dependent manner compared to PBS control; there are no obvious differences between hen egg lysozyme-treated groups and PBS control (n=8; mean±SEM); *double asterisk* P<0.001 compared to PBS control

has antitumor growth activity in vivo, and the effects of marine-derived lysozyme are more potential than hen egg lysozyme.

Discussion

The growth and metastasis of a solid neoplasm is always accompanied by neovasculrization or new capillary development (Carmeliet and Jain 2000; Robert et al. 2003; Carmeliet 2005). To achieve new blood vessel formation, endothelial cells must proliferate to provide the necessary number of cells for making a new vessel. In the present study, we found that marine lysozyme exhibited an inhibitory effect on the proliferation of endothelial cells induced by bFGF in a concentration-dependent manner, while hen egg lysozyme showed no significant inhibitory effect on endothelial cells. As bFGF is a well-known potential endothelial cell mitogen (Millauer et al. 1993), our data suggested that the inhibitory effect of marine lysozyme on endothelial cells may be related with bFGF pathway. To confirm the specificity of marine lysozyme on antiangiogenic activity, we determined whether marine lysozyme had cytotoxicity on non-endothelial cell lines: Hepatoma 22, NIH3T3, and S180. We found that marine lysozyme had no stimulating or inhibitory effects on tested cell lines, which

Table 2 Effect of marine lysozyme on the growth of sarcoma 180 tumors

Treatment group	Dosage (mg kg^{-1} day^{-1})×day	Mice number (initial/end)	Body weight (g) (initial/end)	Body weight gain vs controls (%)	Tumor weight (g)	Inhibition rate (%)
Saline	–	10/10	18.9/25.4	–	2.96±0.79	–
Marine lysozyme	1.25×**7**	10/10	18.8/25.2	98.5	2.08±0.55	29.7
Marine lysozyme	2.5×**7**	10/10	19.0/24.9	90.8	1.54±0.38*	49.8
Marine lysozyme	5×**7**	10/10	19.1/25.1	92.3	0.50±0.32**	83.1
Hen egg lysozyme	2.5×**7**	10/10	18.7/24.4	87.7	2.81±0.99	5.1
Hen egg lysozyme	5×**7**	10/10	19.0/23.3	66.2	2.65±1.08	10.4
Hen egg lysozyme	10×**7**	10/10	19.2/26.8	117	2.40±0.77	18.9
CTX	100×**2**	10/10	19.1/22.4	50.8	0.51±0.26**	82.7

Sarcoma 180 cells were implanted in mouse models, and the indicated drugs were administered once daily for 10 days. On day 11 after implantation of cells, mouse tumor tissues were harvested and weighed, and the tumor growth inhibition rates were calculated. The data were expressed as mean±SEM from three independent experiments. One-way ANOVA test followed by least significant difference (LSD) test.
*$P<0.05$ compared with saline-treated groups
**$P<0.01$ compared with saline-treated groups

indicated that the tested concentrations of lysozyme had no cytotoxicity on cells, and the inhibitory effects of marine lysozyme on endothelial cells were specific.

Further in vivo CAM assay showed that marine lysozyme significantly inhibited the growth of vessels in a dosage-dependent manner, while no avascular zones were observed in hen egg lysozyme-treated chick embryos. These results strongly suggested the antiangiogenesis activity of marine lysozyme. We also found that marine lysozyme could significantly inhibit tumor growth of subcutaneous xenograft of S180 sarcoma and hepatoma 22 in dose-dependent manner in vivo, whereas the inhibitory effects of hen egg lysozyme on tumor growth were not significant. Angiogenesis is a critical process required by primary solid tumor to support their growth. As the in vitro study showed no inhibition of marine lysozyme on the proliferation of the two tumor cell lines, we proposed that the inhibition of marine lysozyme on tumor growth may target to the angiogenesis of solid tumor. This may be related with the special molecular structure and activities of marine invertebrate's lysozyme (Wang et al. 2000). However, tumor angiogenesis is a complex, multistep process, and the genome structure of marine lysozyme genes is not clear; future work will be focused on the structure–activity relationship, the cellular mechanisms of marine lysozyme on the different processes of angiogenesis, and tumor growth.

Taken together, marine lysozyme was a potent antitumor molecule which may inhibit tumor growth and angiogenesis. Being of higher activities, easier extraction, lower optimum temperature, and more cold-adapted than hen egg lysozyme and abundant in natural resources, marine lysozyme might have a therapeutic value in antitumor drug development.

Table 3 Effect of marine lysozyme on the growth of hepatoma 22 tumors

Treatment group	Dosage (mg kg^{-1} day^{-1})×day	Mice number (initial/end)	Body weight (g; initial/end)	Body weight gain vs controls (%)	Tumor weight (g)	Inhibition rate (%)
Saline	–	10/10	19.2/24.6	–	2.90±0.94	–
Marine lysozyme	1.25×**7**	10/10	18.9/ 24.1	96.3	2.13±0.87	26.4
Marine lysozyme	2.5×**7**	10/10	19.0/25.1	113	1.29±0.22*	55.6
Marine lysozyme	5×**7**	10/10	19.1/24.8	105.6	0.78±0.13**	73.1
Hen egg lysozyme	2.5×**7**	10/10	19.2/ 25.2	111.1	2.75±0.96	5.2
Hen egg lysozyme	5×**7**	10/10	19.1/24.3	96.3	2.62±0.73	9.7
Hen egg lysozyme	10×**7**	10/10	19.0/24.1	94.4	2.50±0.51	13.8
CTX	100×**2**	10/10	19.2/22.5	61.1	0.16±0.04**	94.5

Hepatoma 22 cells were implanted in mouse models, and the indicated drugs were administered once daily for 10 days. On day 11 after implantation of cells, mouse tumor tissues were harvested and weighed, and the tumor growth inhibition rates were calculated. The data were expressed as mean±SEM from three independent experiments. One-way ANOVA test followed by LSD test.
*$P<0.05$ compared with saline-treated groups
**$P<0.01$ compared with saline-treated groups

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