Original Paper

A Tick Cysteine Protease Inhibitor RHcyst-1 Exhibits Antitumor Potential

Nana Wei^{a,b} Zhibing Lin^c Zhengmao Xu^b Jie Cao^b Yongzhi Zhou^b
Houshuang Zhang^b Haiyan Gong^b Jinlin Zhou^b Guoqing Li^a

^a Guangdong Provincial Zoonosis Prevention and Control Key Laboratory, College of Veterinary Medicine, South China Agricultural University, Guangzhou, ^b Key Laboratory of Animal Parasitology of Ministry of Agriculture, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, ^c State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, Shanghai, China

Key Words

RHcyst-1 • Cathepsins • Antitumor • Proliferation • Migration • Invasion

Abstract

Background/Aims: We previously identified a potent and tight-binding inhibitor of cysteine proteases from *Rhipicephalus haemaphysaloides*, RHcyst-1, which belongs to the cystatin type 1 family. Cathepsins, which are members of the cysteine protease family, participate in various pathological processes, including the initiation and development of cancers. The present study aimed to investigate the antitumor effects of RHcyst-1 and to explore the underlying mechanism of these effects. **Methods:** Different tumor cells were treated with RHcyst-1 *in vitro*. Proliferation activity was evaluated using Cell Counting Kit-8, and migration and invasion were determined by wound healing and Transwell® invasion assays. In addition, a mouse tumor therapy model was established by inoculating the left forelimb of mice with B16-F10 cells, and tumor progression was evaluated by assessing tumor volume and survival. Flow cytometry was conducted to evaluate myeloid-derived suppressor cells (MDSCs), CD4⁺, and CD8⁺ T cell levels in PBMCs and spleens. Immunohistochemistry was performed to analyze immune cell infiltration and angiogenesis in the tumors. **Results:** RHcyst-1 significantly inhibited the proliferation, migration, and invasion of all four different tumor cells *in vitro*. Additionally, it inhibited tumor growth and improved survival *in vivo*. A decrease and an increase in MDSCs levels were observed in PBMCs and in the spleen, respectively, after RHcyst-1 application. **Conclusions:** Tick RHcyst-1 has potential antitumor efficacy, and the observed antitumor activities may be partly attributable to changes in cathepsin expression and MDSCs levels in the PBMCs and spleens. The findings of the present study suggest that RHcyst-1 may have the potential to be utilized in cancer treatment.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Guoqing Li
and Jinlin Zhou

Guangdong Provincial Zoonosis Prevention and Control Key Laboratory, College of Veterinary Medicine, South China Agricultural University, 483 Wushan Rd, Tianhe Qu, 510640 Guangzhou (China)
Tel. +8620 8528-0241 +8621 3429-3138 E-Mail gqli@scau.edu.cn, jinlinzhou@shvri.ac.cn

Introduction

Cancer is a major disease with a high mortality rate. Although numerous drugs and treatments have been reported, anticancer therapy remains insufficient. Therefore, there is a need to identify novel treatment regimens to circumvent certain negative features of current anticancer drugs, including poor efficacy and side effects [1]. Proteinases play a major role in immune regulation, inflammation, angiogenesis, apoptosis, and cell-cell communication. Proteinases have been reported to promote cancer progression and metastasis [2, 3]. Cathepsins, such as cathepsins B, H, K, and L, belong to the family of cysteine proteases that have been associated with cancer progression, tumor burden, overall survival, tumor cell proliferation, angiogenesis, and metastasis [4-7]. Cathepsins are involved in different cancers and exhibit various characteristics and functions [8-17]. Serum cathepsin levels can be used as prognostic markers for melanoma [18]. Cathepsins are thus considered as promising targets for cancer therapy.

Inhibitors targeting cathepsin activity or their expression have been developed and used in cancer therapy [19-21]. Cancer cells could secrete cathepsins into their environment [22]. Inhibitors that block cathepsin activity or decrease the cathepsin level in the peripheral environment can prevent tumor progression and metastasis. The combination of cathepsin inhibitors with chemotherapy and radiation for cancer treatment has been reported [9, 10]. However, cathepsins comprise several subclasses; thus, the use of one cathepsin may not be effective for another cancer type [23]. The development of more effective inhibitors is therefore warranted.

Cystatins are inhibitors that are reversibly tight-binding with cathepsins and legumains. Cystatins are classified into four subgroups, and they are present in almost all organisms including protozoa [24]. As modulators of cathepsins, cystatins are involved in various physiological and pathological processes such as antigen processing and presentation, cytokines expression, tumor cells proliferation, tumor cells apoptosis, and other tumor genesis and process [25]. A novel natural bioactive molecule, named RHcyst-1, identified from *Rhipicephalus haemaphysaloides* belongs to type 1 cystatins.

Various bioactive molecules such as natural peptides or proteins isolated from honey, snakes, leeches, ticks, and other blood-sucking arthropods have been identified and studied in relation to the treatment and prevention of disease [26]. Hirudin isolated from leeches possesses strong anticoagulation ability and has been used in antithrombotic therapy and in combination with tumor chemotherapy and radiotherapy. Ticks are blood-feeding ectoparasitic arthropods that produce various bioactive molecules that display both pluripotency and redundancy, particularly proteins present in the saliva and saliva gland [27]. Other studies have described different native and recombinant proteins with antitumor effects. Ixolaris, a protein isolated from *Ixodes scapularis* showed potential in decreasing primary tumor growth, metastasis, and angiogenesis [28]. The antitumor effect of Amblyomin-X, a serine protease inhibitor derived from *Amblyomma cajennense*, has also been reported [29]. HLTnI from *Haemaphysalis longicornis* is a potent angiogenesis inhibitor that may be potentially utilized as a multi-functional agent for the control of cancer and tick-borne diseases [30]. Additionally, these bioactive molecules have low toxicity and immunogenicity in humans and animals and are thus regarded as ideal and potential antitumor drugs [31]. All these studies indicate that tick-derived bioactive molecules have the potential to be employed in cancer therapy.

We previously cloned and identified RHcyst-1 in our laboratory. It has a strong and broad spectrum of cathepsin inhibitory ability [32]. In the present study, we assessed the antitumor ability of RHcyst-1 in several cancer cell lines *in vitro* and B16-F10 *in vivo*. Here, we found that tick-derived RHcyst-1 could inhibit the proliferation, migration, and invasion of different tumor cells *in vitro* as well as suppress tumor growth and improve overall survival *in vivo*.

Materials and Methods

Cell culture

The human cervical (HeLa), lung (A549), and ovarian (SKOV-3) cancer cell lines and murine melanoma B16-F10 cell line were purchased from the American Type Culture Collection (ATCC). The HeLa, A549, and B16-F10 cells were maintained in high glucose DMEM medium (Gibco, Grand Island, NY, USA), and the SKOV-3 cells were maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA); both media were supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 100 U/mL penicillin (Gibco, Grand Island, NY, USA) and 100 µg/mL streptomycin (Gibco, Grand Island, NY, USA) in an incubator at 37°C in 5% CO₂ and saturated humidity.

Animals and experimental design

Female C57BL/6 mice were purchased from Slac Laboratory Animal, Inc. (Shanghai, China) and housed in the Laboratory Animal Center. All experiments involving mice were conducted following the US National Institutes of Health Guide for the Care and Use of Animals. Female C57BL/6 mice were randomly divided into two groups and eight each group. B16-F10 cells (2×10^5 cells/mouse) were inoculated into the left forelimb of C57BL/6 mice. After four days, purified RHcyst-1 protein (50 µg/mouse) was administered by intratumoral injection and the control group were received a vehicle of PBS buffer, once a week for three weeks. The length and width of the tumors were measured each day with a caliper, and the volume of the tumors was calculated using the formula: Tumor volume = (Width² × Length)/2 [33]. For animal ethics reasons, mice bearing tumors of 2 cm³ in size were sacrificed and recorded as dead. All animal experiments were repeated twice.

Expression and purification of RHcyst-1

The recombinant protein with a GST tag was expressed in *Escherichia coli* BL-21 cells by induction using 1 mM IPTG for 12 h at 37°C and was purified with an AKTA Pure system (GE) using GST resin according to the manufacturer's protocol. The GST tag was removed by PreScission Protease (GE) treatment. LPS was removed from the RHcyst-1 using Detoxi-Gel™ endotoxin-removing columns (Thermo Scientific™, USA). Endotoxin levels were monitored using a *Limulus* amoebocyte lysate test (LAL, GenScript, China). The proteins were stored in PBS at -80 °C for later use.

Recombinant plasmids construction and cell transfection

The ORF (Open Reading Frame) of tick RHcyst-1 gene was obtained by PCR amplification and inserted into the eukaryotic expression vector pcDNA3.1-GFP digested by *NheI* and *EcoRI* restriction enzymes (NEB, USA) at 37°C for 30 min. Subsequently, the recombinant pcDNA3.1-GFP-RHcyst-1 plasmid and pcDNA3.1-GFP control vector were obtained by EndoFree Maxi Plasmid Kit (Tiangen, China). Cells in the logarithmic growth phase were plated and cultured at 37°C overnight. When the cell grew to 80%-90% confluence, plasmids (4 µg) were transfected into B16-F10 cells by using Lipofectamine™ 2000 transfection reagent with Opti-MEM according to the manufacturer's instructions (Invitrogen, USA). After culture in medium for 48 h, the cells were collected. The transfection efficiency was evaluated by using Western blotting.

Proteinase inhibitor assays

To calculate the inhibitory activity of RHcyst-1 protein, the IC₅₀ of RHcyst-1 was measured. Purified protein was preincubated with each enzyme (0.15 µM) in a reaction buffer for 30 min. Then, 0.25 mM of the protease-specific substrates was added to each well, and residual enzyme activity was measured [32, 34]. The following enzymes were used: cathepsin B (from bovine spleen), cathepsin C (from bovine spleen), cathepsin L (from human liver) and cathepsin S (from human spleen). These enzymes were all purchased from Sigma Co. (Sigma, USA). The reaction buffer consisted of 100 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, 1 mg/mL cysteine, and 0.005% Triton X-100 [32, 34]. The substrates purchased (Sigma, USA) were as follows: Z-Phe-Arg-AMC·HCl for cathepsin B and cathepsin L; Pro-Arg-4-methoxy-β-naphthylamide acetate salt for cathepsin C and Ac-Lys-Gln-Lys-Leu-Arg-AMC for cathepsin S, and the final concentration of different substrates is 0.25 mM.

CCK-8 assay

Cells (5×10^4 cells/well) were seeded into each well of 96-well culture plates. After overnight incubation, the medium was removed and replaced with 100 μ L fresh culture medium with or without RHcyst-1 protein. At the indicated time points, 100 μ L of the fresh medium along with 10 μ L of a cell counting kit-8 solution (CCK-8, Dojindo) were added to each well, and the cells were incubated for 2 h at 37°C. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Tek, USA). The IC₅₀ values were calculated based on the log values using GraphPad Prism 6 software.

Cell migration and invasion assays

Cell migration was assessed using wound healing assays, with cultured cells resuspended and seeded into six-well plates. Cells at 90% confluency in six-well plates were manually scratched with a sterile 200- μ L plastic pipette tip. After 0 h and 24 h, images of the wound areas under each condition were photographed. The width of the wound area was quantitated using Image-Pro Plus software (IPP).

Cell invasion assays were performed using 24-well Transwell® chambers with 8- μ m pore size polycarbonate membrane filters as previously described [35]. Briefly, the upper surface of the filter was pre-coated with Matrigel® (BD Biosciences, USA). The Matrigel® was dried at 37°C for 1 h and placed at room temperature overnight. Cells (5×10^4) in 200 μ L of serum-free medium were added to the upper chamber, whereas 500 μ L of medium containing 10% FBS were added to the lower chamber. Cells were then allowed to migrate for 24 h, and invading cells were stained with 0.1% crystal violet, counted, and photographed under an inverted microscope.

Western blotting

The cells were harvested and lysed in IP buffer for 20 min on ice and centrifuged for 10 min (12,000 rpm, 4°C). Protein concentrations were quantified using a BCA protein assay kit (Thermo Scientific™, USA). Then, 50 μ g of proteins from each sample were electrophoresed in a 12% SDS-PAGE gel and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% nonfat milk for 1 h at 37°C and probed with a mouse polyclonal anti-RHcyst-1 antibody prepared in-house (1:500) and a mouse monoclonal actin antibody (Protech, USA, 1:3,000) overnight at 4°C, followed by secondary antibody goat anti-mouse IgG (CST, USA, 1:6,000) for 1 h at 37°C. Band signals were visualized with fresh diaminobenzidine (DAB, Tiangen, China), which was used as a chromogenic substrate for 5 min.

Indirect immunofluorescent assay (IFA)

At various time points after different treatments, the cells were washed three times with PBS, fixed in 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, and rewashed with PBS. The fixed cells were then permeabilized with 0.1% Triton X-100 for 10 min (room temperature) and then blocked with 3% BSA for 1 h (37°C). Then, the cells were incubated overnight at 4°C with anti-GST antibody (Protech, USA, 1:2,000), followed by secondary antibody Alexa Fluor-488 goat anti-mouse IgG (Thermo Scientific™, USA, 1:5,000) for 1 h (37°C). The cells were washed with PBST, stained with 10 mg/mL DAPI (Sigma-Aldrich, USA) and imaged under a fluorescence microscope.

Immunohistochemistry

Tumors and organs were fixed in formalin and embedded in paraffin. The 5 μ m sections were cut and stained with hematoxylin and eosin (H&E). For immunohistochemical detection of antigens, the sections were incubated with mouse monoclonal antibodies of anti-CD8⁺ at 4°C overnight. After washing with PBS three times, the slides were incubated with biotinylated secondary antibodies, and then it was detected using DAB detection kit (DAB, Tiangen, China). The observations and photography of the sections were performed using Nikon microscope (Nikon, ECLIPSE, JAPAN).

Table 1. Primer sequences used in qRT-PCR analysis. Note: F means forward primer; R indicates reverse primer

Gene name	Primer sequence (5'→3')	Length (bp)
Cathepsin B	F: CAGGCTGGACGCAACTTCTAC	104
	R: TCACCGAACGCAACCCTTC	
Cathepsin H	F: ACCGTGAACGCCATAGAAAAG	186
	R: TGAGCAATTCTGAGGCTCTGA	
Cathepsin S	F: CCATTGGGATCTCTGGAAGAAA	155
	R: TCATGCCCACTTGGTAGGTAT	
Cathepsin L	F: ATCAAACCTTTAGTGCAGAGTGG	136
	R: CTGTATTCCCGTTGTGTAGC	
Mus-actin	F: AGAGGGAATCGTGCGTGAC	195
	R: CCATACCCAAGAAGGAAGGCT	

RNA extraction and quantitative real-time PCR (qRT-PCR) experiments

Total RNA was extracted from cells or tissues using TRIzol® (Life Technologies, USA). cDNA was synthesized using Prime Script™ RT Reagent with genomic DNA removal (Takara, Japan) by following the manufacturer's recommendations. RT-PCR was performed using a SYBR Green mix (Takara, Japan) for 40 amplification cycles of 95°C for 5 min, 95°C for 15 s, and 60°C for 1 min. The relative expression levels of the target genes in each sample were evaluated using the $2^{-\Delta\Delta Ct}$ method. Mouse β -actin, a housekeeping gene, was used as internal control. The qRT-PCR primers used in this study are listed in Table 1.

Flow cytometry

To characterize and quantify CD4⁺ cells, CD8⁺ T cells, and MDSCs by FACS, the mice were sacrificed and spleen cell suspensions and PBMCs were prepared, washed with PBS, and then resuspended in a staining buffer. The cell suspensions were then incubated with their respective primary antibodies (anti-CD3-FITC, anti-CD4-PerCP-cy5.5, and anti-CD8-PerCP-cy5.5 for the detection of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells, and anti-CD45-FITC, anti-CD11b-PerCP-cy5.5, and anti-Ly6G-PE for the detection of MDSCs, isotype-matched mAbs for control staining) bought from BD Biosciences for 30 min in the dark at 4°C and then washed with staining buffer twice. Finally, the cells were resuspended in 100 μ L of staining buffer for flow cytometry analysis (Beckman CytoFLEX, USA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

To test the possible toxicity, the apoptosis assay was performed. After 48 h of 72 μ M RHcyst-1 treatment, cells were washed twice with cold 1 $\times$ PBS, resuspended in 100 μ L 1 $\times$ binding buffer at a concentration of 1×10^6 cells/ml, and incubated with 5 μ L Alexa Fluor® 488 annexin V and 1 μ L 100 μ g/mL PI working solution. Samples were incubated for 15 min at room temperature in the dark. After the incubation period, samples were added 400 μ L 1 $\times$ binding buffer and kept on ice. Flow cytometry was performed within 1 hour by flow cytometer. The scatter diagram was distributed as follows: healthy cells (FITC⁺/PI⁻); apoptotic cells at an early stage (FITC⁺/PI⁻); apoptotic cells at an advanced stage (FITC⁺/PI⁺).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 software. The IC50 values were calculated based on the log values using GraphPad Prism 6 software. Numerical data were expressed as the mean $\pm$ SD, and most of the data were analyzed by the Student *t* test. Cumulative survival time was calculated using the Kaplan–Meier method and analyzed with the log-rank test. In all statistical analysis, * indicates *p* < 0.05, ** indicates *p* < 0.01, and *** indicates *p* < 0.001.

Results

Expression and purification of RHcyst-1

The open reading frame of the RHcyst-1 gene was successfully subcloned into the vector pGEX-6p-1. The structure of the recombinant RHcyst-1 (rRHcyst-1) is shown in Fig. 1A. The recombinant protein incorporating a GST tag was successfully expressed with an expected size of 37 kDa. After removal of the GST tag, SDS-PAGE analyses demonstrated that about 2 mg RHcyst-1 (11 kDa) was obtained, with a purity of >95%, and LPS was removed using Detoxi-Gel™ endotoxin-removing columns (Fig. 1B). Western blot analyses demonstrated that the bioactive recombinant RHcyst-1 and purified RHcyst-1 had molecular weights of about 37 kDa and 11kDa, respectively (Fig. 1C).

RHcyst-1 exhibits high cysteine protease inhibitory activity

To further confirm whether the RHcyst-1 has the same target protease-inhibiting efficiency as rRHcyst-1, purified RHcyst-1 was used to test its inhibitory activity against cysteine cathepsins. The results showed that RHcyst-1 can also effectively inhibit the enzyme activities of cathepsins B, L, and S but not C (Fig. 2). The IC50s of RHcyst-1 targeting cathepsins B, L, and S were 524.4 nM, 37.03 nM, and 28.04 nM, respectively.

RHcyst-1 inhibits tumor cells proliferation but not Normal Cells

CCK-8 assays were used to evaluate the proliferation of different cancer cell lines *in vitro*. Cell viability in these four tumor cells treated with different concentrations of RHcyst-1

Fig. 1. Clone construction, expression, and purification of RHcyst-1. (A) The structure of recombinant RHcyst-1. The protein was purified using the AKTA Pure system. (B) SDS-PAGE analyses of rRHcyst-1 (with GST tag) and RHcyst-1 (GST tag removed). (C) Western blot analyses after purification show a single band with a molecular mass of 37 kDa (rRHcyst-1) and 11 kDa (RHcyst-1), respectively. Lane M: protein standard markers.

was determined by using CCK-8 assays. RHcyst-1 inhibited cell viability in a dose-dependent manner, with an IC₅₀ value of ranging from 40.93 to 60.55 μ M for A549, from 30.65 to 57.26 μ M for HeLa, from 40.15 to 54.78 μ M for SKOV-3 and from 26.14 to 38.66 μ M for B16-F10 cell, respectively (Fig. 3A and Fig. 3B). To explore the safety of RHcyst-1, the normal cell lines 293T, Vero and CHO were treated with RHcyst-1 with a concentration of 18 μ M and 72 μ M. We found there was no significant effect between RHcyst-1 treatment and control group even at the maximum concentration (Fig. 3C). In addition, we evaluated the toxicity of RHcyst-1 on tumor and normal cells by flow cytometry. According to the data, RHcyst-1 treatment didn't induce apoptosis on tumor and normal cell lines compared with PBS control groups (Fig. 3D and Fig. 3E).

Next, we calculated the proliferation of four different tumor cells at the minimum effective concentration of 18 μ M (under IC₅₀). The results showed that twenty-four hours after RHcyst-1 treatment with a concentration of 18 μ M, a significant decrease in proliferation was observed compared with the control in HeLa and B16-F10 cells, and 48 h after treatment with a concentration of 18 μ M, cell proliferation was also suppressed in the A549 and SKOV-3 cells ($p < 0.05$, Fig. 4). These results indicate that RHcyst-1 significantly suppressed the proliferation of the four cancer cell lines *in vitro*.

RHcyst-1 restrained tumor cell migration and invasion in vitro

Cell migration and invasion are critical steps in tumor progression, and we evaluated the effect of RHcyst-1 on tumor migration and invasive ability by wound healing and Transwell® invasion assays. In the wound healing assay, a significant inhibition in migration and invasion was observed in the four cancer cell lines that were treated with RHcyst-1 compared with the negative control. The mean reductions in the migration of A549, HeLa, SKOV-3, and B16-F10 cells were 73.14%, 36.02%, 25.76%, and 51.10%, respectively ($p < 0.05$, Fig. 5). In addition, the mean invasive suppression rates of the A549, HeLa, SKOV-3 and B16-F10 cells were 45.5%, 67.3%, 58.6%, and 49.7%, respectively ($p < 0.05$, Fig. 6). These findings indicate that RHcyst-1 possesses anti-migratory and anti-invasive activities.

Fig. 2. Inhibition of protease activities by purified RHcyst-1. Cathepsins B, C, L, and S were incubated with each of the substrates in the presence of different concentrations of RHcyst-1 after incubation for 1 h. Incubation of cathepsins without RHcyst-1 leads to 100% enzyme activity.

Fig. 3. Cell viability was evaluated by using CCK-8 and apoptosis assay. (A) Different tumor cells were treated with different concentrations of RHcyst-1 for different time points, and relative cell viability was detected. (B) The dose-response curves after RHcyst-1 treatment were fitted with nonlinear regression, and dotted lines indicate the IC₅₀ value for each curve. (C) Cell viability was detected in normal cell lines 293T, Vero and CHO treated with different concentrations of RHcyst-1 for different time points. The apoptosis of different tumor cells (D) and different normal cells (E) were incubated with RHcyst-1 and PBS control for 48 h and analyzed by flow cytometry following staining with PI and Annexin V. Bars mean SD obtained from three independent experiments. * means $p < 0.05$, ** represents $p < 0.01$, and *** indicates $p < 0.001$.

RHcyst-1 acts on the extra-cellular environment

To determine specific binding, active uptake, or direct action of RHcyst-1 on the extracellular environment, B16-F10 cells were treated with 18 μ M RHcyst-1 for 12 h, 24 h, and 48 h. The cell supernatants and cell lysis solution were obtained at different times, and Western blotting was performed to assess the level of RHcyst-1 expression in the B16-F10 cell supernatants and cell lysis solution. The purified protein RHcyst-1 which is loaded into the gel was used for the positive control. The results showed that RHcyst-1 could be detected only in the cell supernatants but not in the cell lysis solution (Fig. 7A and 7B). In addition, IFA was used to determine the expression patterns of RHcyst-1 at different time points using anti-GST McAb, and pcDNA3.1-GST overexpression transfected in B16-F10 cell was used for the

Fig. 4. The proliferation of A549, HeLa, SKOV-3, and B16-F10 cells was evaluated using a CCK-8 assay between the control and RHcyst-1 treatment groups. Bars mean SD obtained from three independent experiments. * means $p < 0.05$, ** represents $p < 0.01$, and *** indicates $p < 0.001$.

Fig. 5. Wound healing assay showing the effect of RHcyst-1 treatment with a concentration of 18 μ M on the migration of A549, HeLa, SKOV-3, and B16-F10 cells, compared to the control group. Bars mean SD obtained from three independent experiments. * represents $p < 0.05$, ** indicates $p < 0.01$, and *** signifies $p < 0.001$.

Fig. 6. The invasion ability of A549, HeLa, SKOV-3, and B16-F10 cells was evaluated using Transwell® assays between the control and RHcyst-1 treatment groups with a concentration of 18 μ M. Bars mean SD obtained from three independent experiments. * indicates $p < 0.05$, ** shows $p < 0.01$, and *** represents $p < 0.001$.

Fig. 7. The location and expression of RHcyst-1 at different time points were evaluated using western blotting and IFA analyses. Different tumor cells were treated with 18 μ M RHcyst-1 for 12 h, 24 h, and 48 h. The cell supernatant (A, D, G, J) and the cell lysis solution (B, E, H, K) showing the western blotting results of RHcyst-1 at different time points. Lane M: protein standard markers. The IFA analyses results (C, F, I, L) using anti-GST tag McAb (FITC-labeled).

positive control. As shown in Fig. 7C, we did not detect the expression of RHcyst-1 at any time point. And we also found the same results in other three tumor cell lines (Fig. 7D-L). These results suggested that RHcyst-1 exerted antitumor effects that may act on the extracellular environment and not on active uptake or specific binding.

RHcyst-1 inhibits tumor progression in vivo

The B16-F10 cell line that was derived from murine melanoma and the B16-F10 mouse melanoma model system have been widely used in numerous studies [36-39]. To investigate whether the antitumor effect would also occur *in vivo*, B16-F10 cells were inoculated into the left forelimbs of C57BL/6 mice to establish a tumor therapy model. Approximately 7 days after B16-F10 cell inoculation, the emerging tumors were measured and recorded. A significant decrease in tumor size was observed in the RHcyst-1 treatment group compared with that in the negative control group ($p < 0.05$, Fig. 8A), and the survival of the RHcyst-1 intratumor injection group was significantly prolonged relative to the negative control group ($p < 0.05$, Fig. 8B). The mean life span of the RHcyst-1 treatment group increased by about 11.76% relative to the negative control group. These data indicate that RHcyst-1 inhibited tumor progression *in vivo*.

To explore the safety of RHcyst-1 *in vivo*, the sections of collected organs from different treatment groups including liver, lung and kidney were stained with H&E [40-42]. The results of H&E showed no differences between RHcyst-1 and vehicle treatment (Fig. 8C), indicating RHcyst-1 intratumor injection is safe.

RHcyst-1 mediates specific immune response

MDSCs, as well as T cell levels and balance, play important roles in controlling tumor metastasis and progression [43]. In the present study, we investigated whether RHcyst-1 influenced MDSCs and T cells, which are related to antitumor responses and cancer immunotherapy. We focused on the level and balance of MDSCs, CD8⁺ T cells, and CD4⁺ T cells in the spleen and PBMCs. Helper T cells (CD3⁺CD4⁺), Effector T cells (CD3⁺CD8⁺), and MDSCs (CD45⁺CD11b⁺Ly6G1⁺) were stained and analyzed by flow cytometry (Gating strategies

Fig. 8. The effect of RHcyst-1 on B16-F10 tumor model *in vivo*. The B16-F10 cells were inoculated into the left forelimbs of C57BL/6 mice. RHcyst-1 at 50 $\mu\text{g}/\text{mouse}$ and control (intratumor injection) were started 4 days after inoculation with B16-F10 cells. (A) The mean tumor volumes $\pm$ SD were recorded 7 days after B16-F10 cell inoculation. (B) A survival curve was constructed when tumor volume reached 2,000 mm^3 . Each value ($n = 5/\text{group}$) represents the mean. (C) Sections of the Heart, Liver, Lung and Kidney were stained with H&E ($\times 200$), respectively. Scale bar 100 μm . Bars mean SD. * indicates $p < 0.05$, ** represents $p < 0.01$, and *** signifies $p < 0.001$.

for frequency analysis are shown in Fig. 9A). As shown in Fig. 9B, MDSCs in the PBMCs significantly decreased after RHcyst-1 treatment (RHcyst-1-22.20% vs. Control-63.53%, $p < 0.05$), but MDSCs in the spleens increased after RHcyst-1 treatment (RHcyst-1-6.16% vs. Control-4.20%, $p < 0.05$). In addition, no significant difference in the levels of CD3⁺CD8⁺ T cells was observed between the RHcyst-1 treatment and the control, both in the PBMCs (RHcyst-1-20.32% vs. Control-19.38%) and spleens (RHcyst-1-9.56% vs. Control-9.77%). Further analysis showed no significant difference in the levels of CD3⁺CD4⁺ cells between the RHcyst-1 treatment group and the control, also both in the PBMCs (RHcyst-1-12.70% vs. Control-14.69%) and spleens (RHcyst-1-14.51% vs. Control-16.31%). MDSCs are strongly

Fig. 9. The level of MDSCs was changed and T cells infiltration was enhanced after RHcyst-1 treatment. (A) Gating strategies for frequency analysis of MDSCs, CD3⁺CD4⁺ Help T cell and CD3⁺CD8⁺ Effector T cell. (B) The level of MDSCs and T cells was analyzed by flow cytometry. (C) CD8⁺-positive cells were detected by immunohistochemical staining ($\times 200$). Scale bar 100 μ m. Bars mean SD obtained from three independent experiments. * represents $p < 0.05$, ** indicates $p < 0.01$, and *** signifies $p < 0.001$.

Fig. 10. The mRNA expression levels of four different cathepsins were affected after RHcyst-1 treatment in vitro and in vivo. (A) Western blot analyses after RHcyst-1 and vector control transfection on B16-F10 cells. (B) The apoptosis analyses after RHcyst-1 and vector control transfection on B16-F10 cells. (C) The mRNA expression levels of cathepsins in B16-F10 cells after transfected with pcDNA3.1-GFP-RHcyst-1 and pcDNA3.1-GFP vector control were analyzed. (D) The mRNA expression of the tumor tissues of RHcyst-1 intratumor injection and Control group was determined. Bars mean SD obtained from three independent experiments. * indicates $p < 0.05$, ** represents $p < 0.01$, and *** signifies $p < 0.001$.

associated with immune suppression, and these cells infiltrate and accumulate in the peripheral blood of tumor-bearing mice [44]. These results suggest that RHcyst-1 mediated specific antitumor immunity partly by influencing MDSC from both PBMCs and spleens of tumor-bearing mice.

To evaluate whether RHcyst-1 could promote immune cells infiltration to tumor tissue, we analyzed the amount of CD8⁺ T cells in the tumor tissue from RHcyst-1 and PBS intratumor injected mice, respectively. The number of CD8⁺-positive cells in the tumor from RHcyst-1 treatment mice was higher than the control group (Fig. 9C). These results suggest that the antitumor immunity of RHcyst-1 was at least in part caused by increasing T cell infiltration.

RHcyst-1 affects mRNA expression of cathepsins

The expression and activity of cathepsins is regulated by their natural endogenous inhibitors that belong to the cystatin family. Previous research studies have reported that the imbalance between cathepsins and cystatins play an important role in tumorigenesis [10, 45, 46]. We analyzed the expression of different cathepsins using qRT-PCR *in vitro* and *in vivo*. We construct the overexpression plasmid of RHcyst-1 named pcDNA3.1-GFP-RHcyst-1 and pcDNA3.1-GFP for the control. To evaluate the transfection efficiency and the toxicity after transfection, the Western blotting and the apoptosis assay were performed. RHcyst-1 can be tested and highly expressed in RHcyst-1 transfection group by Western blotting (Fig. 10A). The apoptosis assay showed that overexpression of RHcyst-1 by transfection cannot induce the apoptosis (Fig. 10B). The cells were harvested after transfection for 48 h and the expression of different cathepsins *in vitro* was analyzed using qRT-PCR. We found that the relative expression of all four different cathepsins was downregulated in the high RHcyst-1 expression group (pcDNA3.1-GFP-RHcyst-1 transfection) compared with the negative control (pcDNA3.1-GFP transfection) on B16-F10 cell (Fig. 10C). The tumor tissues of RHcyst-1 intratumor injection and control were collected and the expression of different cathepsins

in vivo was analyzed using qRT-PCR. The results showed that RHcyst-1 intratumor injection (RHcyst-1) regulated the expression of cathepsins B, L, and S but not H (Fig. 10D). Therefore, RHcyst-1 affects mRNA expression of different cathepsins *in vitro* and *in vivo*. We speculate the imbalance between cathepsins and RHcyst-1 may partly contribute to the antitumor of RHcyst-1, but it need to be further studied in the following research.

Discussion

Studies have shown that cysteine proteases such as cathepsins are related to tumor growth and progression, which are often upregulated during early- and late-stage tumor cell progression. However, cysteine protease inhibitors such as cystatins regulate the expression and activity of cathepsins. The imbalance between cysteine proteases and cystatins may be clinically useful in combatting cancer [11, 47]. Therefore, cystatins could be considered a potential therapeutic strategy for cancer [8]. In the present study, the small bioactive molecule RHcyst-1 from tick belonging to the type 1 cystatin family has been proven to have effective antitumor activity *in vitro* and *in vivo*.

To date, approximately 20 tick cystatins, all belonging to family types 1 and 2, have been reported [48]. Their functions include immunomodulatory (host or itself), *Borrelia* and *Anaplasma phagocytophilum* infections, and tick feeding, attachment, and hatching. RHcyst-1 is the member of cystatin family isolated in our lab. To the best of our knowledge, this is the first report of a cystatin isolated from *Rhipicephalus haemaphysaloides* tick that exhibits antitumor activity *in vitro* and *in vivo*. This antitumor effect is similar to various other reported native or recombinant cystatins from other species [45, 49-51]. However, the molecular mechanism underlying the antitumor effect of RHcyst-1 remains unclear. MDSCs are negatively correlated with tumor prognosis and vasculogenesis [52]. Similarly, in the present study, the number of MDSCs from the PBMCs decreased with RHcyst-1 treatment, indicating that MDSCs may have regulated the antitumor mechanism of RHcyst-1. MDSCs accumulate in the spleens of tumor-bearing mice, where they contribute to immunosuppression [53]. However, we also observed that the MDSCs from the spleens were augmented after RHcyst-1 treatment. It is thus possible that a cross-connection exists between MDSCs and tumor immune regulation [54]. How RHcyst-1 mediates and influences antitumor immune responses by regulating MDSCs from the spleens and PBMCs requires further investigation.

In the present study, no significant binding or uptake in tumor cells was detectable by Western blotting and IFA (Fig. 7). These results revealed that RHcyst-1 exerted an antitumor effect that possibly involves acting on its extracellular environment. Inhibition of the expression and activities of cathepsins B, L, and S that could be released from cells and function in the degradation of components of the extracellular matrix may influence the tumor progression. The intracellular events induced by cystatins are complex. The present study observed alternations in the relative expression of different cathepsins after RHcyst-1 treatment *in vitro* and *in vivo* (Fig. 10). It is thus possible that the antitumor mechanism of RHcyst-1 is associated at least partly with the level of cathepsins; however, additional investigations confirming this activity are warranted. Using mRNA microarray analysis, we recently showed the RHcyst-1 overexpression by transfecting pcDNA3.1-GFP-RHcyst-1 on HeLa cell lines could induce the upregulation or downregulation of many genes that are primarily involved in cell growth, cell adhesion/migration, and apoptosis, such as Rap1, PI3K-Akt, ErbB, and the calcium signaling pathway (data not shown). Further studies are required to identify the molecular targets of RHcyst-1 that results in the inhibition of tumor cells.

Oncolytic virus therapy is currently considered to be a potential anticancer biological treatment regimen because these viruses can specifically replicate in cancer cells and kill them without damaging normal tissues [55-57]. Our research team is presently conducting oncolytic virus construction.

In conclusion, tick-derived RHcyst-1 exhibits potential antitumor efficacy. Our research direction will concentrate on evaluating the safety and efficacy of this bioactive molecule and identify its targets in tumor cells using oncolytic virus therapy.

Acknowledgements

The National Natural Science Foundation of China (Grant no. 31711540022, 31572512 and 31672541) supported this study.

Disclosure Statement

The authors declare to have no conflict of interests.

References

- 1 Sousa ACP, Szabo MPJ, Oliveira CJF, Silva MJB: Exploring the anti-tumoral effects of tick saliva and derived components. *Toxicon* 2015;102:69-73.
- 2 Sevenich L, Bowman RL, Mason SD, Quail DF, Rapaport F, Elie BT, Brogi E, Brastianos PK, Hahn WC, Holsinger LJ, Massague J, Leslie CS, Joyce JA: Analysis of tumour- and stroma-supplied proteolytic networks reveals a brain-metastasis-promoting role for cathepsin S. *Nat Cell Biol* 2014;16:876-888.
- 3 Aggarwal N, Sloane BF: Cathepsin B: multiple roles in cancer. *Proteomics Clin Appl* 2014;8:427-437.
- 4 Bian B, Mongrain S, Cagnol S, Langlois MJ, Boulanger J, Bernatchez G, Carrier JC, Boudreau F, Rivard N: Cathepsin B promotes colorectal tumorigenesis, cell invasion, and metastasis. *Mol Carcinog* 2016;55:671-687.
- 5 Sudhan DR, Pampo C, Rice L, Siemann DW: Cathepsin L inactivation leads to multimodal inhibition of prostate cancer cell dissemination in a preclinical bone metastasis model. *Int J Cancer* 2016;138:2665-2677.
- 6 Wilkinson RD, Young A, Burden RE, Williams R, Scott CJ: A bioavailable cathepsin S nitrile inhibitor abrogates tumor development. *Mol Cancer* 2016;15:29.
- 7 Andrade SS, Gouveia IE, Silva MCC, Castro ED, de Paula CA, Okamoto D, Oliveira L, Peres GB, Ottaiano T, Facina G: Cathepsin K induces platelet dysfunction and affects cell signaling in breast cancer-molecularly distinct behavior of cathepsin K in breast cancer. *BMC cancer* 2016;16:1.
- 8 Eatemadi A, Aiyelabegan HT, Negahdari B, Mazlomi MA, Daraee H, Daraee N, Eatemadi R, Sadroddiny E: Role of protease and protease inhibitors in cancer pathogenesis and treatment. *Biomed Pharmacother* 2017;86:221-231.
- 9 Sudhan DR, Siemann DW: Cathepsin L targeting in cancer treatment. *Pharmacol Ther* 2015;155:105-116.
- 10 Nalla AK, Gorantla B, Gondi CS, Lakka SS, Rao JS: Targeting MMP-9, uPAR, and cathepsin B inhibits invasion, migration and activates apoptosis in prostate cancer cells. *Cancer Gene Ther* 2010;17:599-613.
- 11 Olson OC, Joyce JA: Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nat Rev Cancer* 2015;15:712-729.
- 12 Loser R, Pietzsch J: Cysteine cathepsins: their role in tumor progression and recent trends in the development of imaging probes. *Front Chem* 2015;3:37.
- 13 Tan GJ, Peng ZK, Lu JP, Tang FQ: Cathepsins mediate tumor metastasis. *World J Biol Chem* 2013;4:91-101.
- 14 Reiser J, Adair B, Reinheckel T: Specialized roles for cysteine cathepsins in health and disease. *J Clin Invest* 2010;120:3421-3431.
- 15 Palermo C, Joyce JA: Cysteine cathepsin proteases as pharmacological targets in cancer. *Trends Pharmacol Sci* 2008;29:22-28.
- 16 Gocheva V, Joyce JA: Cysteine cathepsins and the cutting edge of cancer invasion. *Cell Cycle* 2007;6:60-64.
- 17 Mohamed MM, Sloane BF: Cysteine cathepsins: multifunctional enzymes in cancer. *Nat Rev Cancer* 2006;6:764-775.

- 18 Kos J, Stabuc B, Schweiger A, Krasovec M, Cimerman N, KopitarJerala N, Vrhovec I: Cathepsins B, H, and L and their inhibitors stefin A and cystatin C in sera of melanoma patients. *Clin Cancer Res* 1997;3:1815-1822.
- 19 Duong LT, Wesolowski GA, Leung P, Oballa R, Pickarski M: Efficacy of a cathepsin K inhibitor in a preclinical model for prevention and treatment of breast cancer bone metastasis. *Mol Cancer Ther* 2014;13:2898-2909.
- 20 Jensen AB, Wynne C, Ramirez G, He W, Song Y, Berd Y, Wang H, Mehta A, Lombardi A: The cathepsin K inhibitor odanacatib suppresses bone resorption in women with breast cancer and established bone metastases: results of a 4-week, double-blind, randomized, controlled trial. *Clin Breast Cancer* 2010;10:452-458.
- 21 Le Gall C, Bellahcene A, Bonnelye E, Gasser JA, Castronovo V, Green J, Zimmermann J, Clezardin P: A cathepsin K inhibitor reduces breast cancer induced osteolysis and skeletal tumor burden. *Cancer Res* 2007;67:9894-9902.
- 22 Mikhaylov G, Klimpel D, Schaschke N, Mikac U, Vizovisek M, Fonovic M, Turk V, Turk B, Vasiljeva O: Selective targeting of tumor and stromal cells by a nanocarrier system displaying lipidated cathepsin B inhibitor. *Angew Chem Int Ed Engl* 2014;53:10077-10081.
- 23 Akkari L, Gocheva V, Quick ML, Kester JC, Spencer AK, Garfall AL, Bowman RL, Joyce JA: Combined deletion of cathepsin protease family members reveals compensatory mechanisms in cancer. *Genes Dev* 2016;30:220-232.
- 24 Abrahamson M, Alvarez-Fernandez M, Nathanson CM: Cystatins. *Biochem Soc Symp* 2003;179-199.
- 25 Zavasnik-Bergant T: Cystatin protease inhibitors and immune functions. *Front Biosci* 2008;13:4625-4637.
- 26 Dossey AT: Insects and their chemical weaponry: new potential for drug discovery. *Nat Prod Rep* 2010;27:1737-1757.
- 27 Chmelar J, Kotal J, Kopecky J, Pedra JHF, Kotsyfakis M: All For One and One For All on the Tick-Host Battlefield. *Trends Parasitol* 2016;32:368-377.
- 28 de Oliveira Ada S, Lima LG, Mariano-Oliveira A, Machado DE, Nasciutti LE, Andersen JF, Petersen LC, Francischetti IM, Monteiro RQ: Inhibition of tissue factor by ixolaris reduces primary tumor growth and experimental metastasis in a murine model of melanoma. *Thromb Res* 2012;130:e163-170.
- 29 Chudzinski-Tavassi AM, De-Sa-Junior PL, Simons SM, Maria DA, de Souza Ventura J, Batista IF, Faria F, Duraes E, Reis EM, Demasi M: A new tick Kunitz type inhibitor, Amblyomin-X, induces tumor cell death by modulating genes related to the cell cycle and targeting the ubiquitin-proteasome system. *Toxicon* 2010;56:1145-1154.
- 30 Fukumoto S, Sakaguchi T, You M, Xuan X, Fujisaki K: Tick troponin I-like molecule is a potent inhibitor for angiogenesis. *Microvasc Res* 2006;71:218-221.
- 31 Simons SM, Junior PL, Faria F, Batista IF, Barros-Battesti DM, Labruna MB, Chudzinski-Tavassi AM: The action of Amblyomma cajennense tick saliva in compounds of the hemostatic system and cytotoxicity in tumor cell lines. *Biomed Pharmacother* 2011;65:443-450.
- 32 Wang YJ, Zhou YZ, Gong HY, Cao J, Zhang HS, Li XR, Zhou JL: Functional characterization of a cystatin from the tick Rhipicephalus haemaphysaloides. *Parasite Vector* 2015;8:140.
- 33 Faustino-Rocha A, Oliveira PA, Pinho-Oliveira J, Teixeira-Guedes C, Soares-Maia R, da Costa RG, Colaco B, Pires MJ, Colaco J, Ferreira R, Ginja M: Estimation of rat mammary tumor volume using caliper and ultrasonography measurements. *Lab Animal* 2013;42:217-224.
- 34 Yamaji K, Tsuji N, Miyoshi T, Islam MK, Hatta T, Alim MA, Anisuzzaman M, Kushibiki S, Fujisaki K: A salivary cystatin, HISC-1, from the ixodid tick Haemaphysalis longicornis play roles in the blood-feeding processes. *Parasitol Res* 2009;106:61-68.
- 35 Pulkuri SM, Gorantla B, Knost JA, Rao JS: Frequent loss of cystatin E/M expression implicated in the progression of prostate cancer. *Oncogene* 2009;28:2829-2838.
- 36 Baird JR, Byrne KT, Lizotte PH, Toraya-Brown S, Scarlett UK, Alexander MP, Sheen MR, Fox BA, Bzik DJ, Bosenberg M, Mullins DW, Turk MJ, Fiering S: Immune-mediated regression of established B16F10 melanoma by intratumoral injection of attenuated Toxoplasma gondii protects against rechallenge. *J Immunol* 2013;190:469-478.
- 37 Kim DY, Kwon DY, Kwon JS, Park JH, Park SH, Oh HJ, Kim JH, Min BH, Park K, Kim MS: Synergistic anti-tumor activity through combinational intratumoral injection of an in-situ injectable drug depot. *Biomaterials* 2016;85:232-245.

- 38 Ma Q, Zhou D, DeLyria ES, Wen X, Lu W, Thapa P, Liu C, Li D, Bassett RL, Overwijk WW, Hwu P, Li C: Synthetic Poly(L-Glutamic Acid)-conjugated CpG Exhibits Antitumor Efficacy With Increased Retention in Tumor and Draining Lymph Nodes After Intratumoral Injection in a Mouse Model of Melanoma. *J Immunother* 2017;40:11-20.
- 39 Ohkuri T, Kosaka A, Ishibashi K, Kumai T, Hirata Y, Ohara K, Nagato T, Oikawa K, Aoki N, Harabuchi Y, Celis E, Kobayashi H: Intratumoral administration of cGAMP transiently accumulates potent macrophages for anti-tumor immunity at a mouse tumor site. *Cancer Immunol Immunother* 2017;66:705-716.
- 40 Sun Z, Song X, Li X, Su T, Qi S, Qiao R, Wang F, Huan Y, Yang W, Wang J, Nie Y, Wu K, Gao M, Cao F: In vivo multimodality imaging of miRNA-16 iron nanoparticle reversing drug resistance to chemotherapy in a mouse gastric cancer model. *Nanoscale* 2014;6:14343-14353.
- 41 Zeng L, Pan Y, Tian Y, Wang X, Ren W, Wang S, Lu G, Wu A: Doxorubicin-loaded NaYF₄:Yb/Tm-TiO₂ inorganic photosensitizers for NIR-triggered photodynamic therapy and enhanced chemotherapy in drug-resistant breast cancers. *Biomaterials* 2015;57:93-106.
- 42 Xu A, Zhang L, Chen Y, Lin Z, Li R: Immunogenicity and efficacy of a rationally designed vaccine against vascular endothelial growth factor in mouse solid tumor models. *Cancer Immunol Immunother* 2017;66:181-192.
- 43 Welte T, Kim IS, Tian L, Gao X, Wang H, Li J, Holdman XB, Herschkowitz JI, Pond A, Xie GR, Kurley S, Nguyen T, Liao L, Dobrolecki LE, Pang L, Mo QX, Edwards DP, Huang SX, Xin L, Xu JM, Li Y, Lewis MT, Wang T, Westbrook TF, Rosen JM, Zhang XHF: Oncogenic mTOR signalling recruits myeloid-derived suppressor cells to promote tumour initiation (vol 18, pg 632, 2016). *Nature cell biology* 2016;18:822-822.
- 44 Kodumudi KN, Woan K, Gilvary DL, Sahakian E, Wei S, Djeu JY: A Novel Chemoimmunomodulating Property of Docetaxel: Suppression of Myeloid-Derived Suppressor Cells in Tumor Bearers. *Clinical Cancer Research* 2010;16:4583-4594.
- 45 Kos J, Lah TT: Cysteine proteinases and their endogenous inhibitors: target proteins for prognosis, diagnosis and therapy in cancer (review). *Oncol Rep* 1998;5:1349-1361.
- 46 Duivenvoorden HM, Rautela J, Edgington-Mitchell LE, Spurling A, Greening DW, Nowell CJ, Molloy TJ, Robbins E, Brockwell NK, Lee CS, Chen M, Holliday A, Selinger CI, Hu M, Britt KL, Stroud DA, Bogoy M, Moller A, Polyak K, Sloane BF, O'Toole SA, Parker BS: Myoepithelial cell-specific expression of stefin A as a suppressor of early breast cancer invasion. *J Pathol* 2017;243:496-509.
- 47 Cox JL: Cystatins and cancer. *Front Biosci* 2009;14:463-474.
- 48 Chmelar J, Kotal J, Langhansova H, Kotsyfakis M: Protease Inhibitors in Tick Saliva: The Role of Serpins and Cystatins in Tick-host-Pathogen Interaction. *Front Cell Infect Microbiol* 2017;7:216.
- 49 Magister S, Kos J: Cystatins in immune system. *J Cancer* 2013;4:45-56.
- 50 Xie Q, Tang N, Wan R, Qi Y, Lin X, Lin J: Recombinant snake venom cystatin inhibits the growth, invasion and metastasis of B16F10 cells and MHCC97H cells *in vitro* and *in vivo*. *Toxicon* 2011;57:704-711.
- 51 Keppler D: Towards novel anti-cancer strategies based on cystatin function. *Cancer Lett* 2006;235:159-176.
- 52 Talmadge JE, Gabrilovich DI: History of myeloid-derived suppressor cells. *Nat Rev Cancer* 2013;13:739-752.
- 53 Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM: Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res* 2005;11:6713-6721.
- 54 Nausch N, Galani IE, Schlecker E, Cerwenka A: Mononuclear myeloid-derived "suppressor" cells express RAE-1 and activate natural killer cells. *Blood* 2008;112:4080-4089.
- 55 Cai J, Lin Y, Zhang HP, Liang JK, Tan YQ, Cavenee WK, Yan GM: Selective replication of oncolytic virus M1 results in a bystander killing effect that is potentiated by Smac mimetics. *P Natl Acad Sci USA* 2017;114:6812-6817.
- 56 Fukuhara H, Ino Y, Todo T: Oncolytic virus therapy: A new era of cancer treatment at dawn. *Cancer Sci* 2016;107:1373-1379.
- 57 Miest TS, Cattaneo R: New viruses for cancer therapy: meeting clinical needs. *Nat Rev Microbiol* 2014;12:23-34.