The anti-tumor activities of cerebrosides derived from sea cucumber *Acaudina molpadioides* and starfish *Asterias amurensis* *in vitro* and *in vivo*

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Abstract: The present study was undertaken to examine the effect of cerebrosides derived from the sea cucumber *Acaudina molpadioides* and the starfish *Asterias amurensis* on the anti-tumor activity *in vitro* and *in vivo*. The results indicated that both *Acaudina molpadioides* cerebrosides (AMC) and *Asterias amurensis* cerebrosides (AAC) exhibited an inhibitory effect on cell proliferation through induction of apoptosis in S180 cells. Moreover, administration of AMC and AAC (50 mg/kg BW) on S180 tumor bearing mice reduced the tumor weight by 45.24 % and 35.71 %, respectively. In S180 ascites tumor model, AMC and AAC (50 mg/kg BW) treatment exhibited a significant ascites fluid growth inhibition of 31.23 % and 22.72 %. Furthermore, the ascites tumor cell viability ratio in AMC and AAC groups reduced to 50.89 % and 51.69 %, respectively. The life span of AMC and AAC administrated groups increased by 55.28 % and 35.77 % compared to control. Quantitative real-time PCR analysis demonstrated that the administration of AMC and AAC down-regulated the expression of Bcl-2, Bcl-xL, while on the other hand, up-regulated Bax, Cytochrome c, caspase-9 and caspase-3 mRNA level of the S180 ascites tumor cells. It was concluded that AMC and AAC should have potential anti-tumor activity both *in vitro* and *in vivo* by inducing apoptosis through the mitochondria-mediated apoptosis pathway. AAC seemed to be more effective than AMC *in vitro* but less potent *in vivo*. It may depend on the structural differences in their fatty acid groups and sphingoid bases.

Key words: *Acaudina molpadioides*, *Asterias amurensis*, cerebrosides, anti-tumor, S180 cells, mitochondria-mediated apoptosis pathway

1 INTRODUCTION

The marine echinoderms mainly include sea cucumber (Holothuroidea), starfish (Asteroidea) and sea urchin (Echinoidea). They occur in various habitats from intertidal zone down to the bottom of the deep sea trenches and from sand to rubble coral reefs through cold and tropical seas. Sea cucumbers have been used as a traditional tonic food in China and other Asian countries for thousands of years^{1,2)}. The major edible part of sea cucumber is the body wall, which contains numerous bioactive substances, including collagen, acidic polysaccharides, triterpenoid saponins, gangliosides and cerebrosides³⁾. Except a few species that inhabit in brackish water, starfish are basically benthic organisms found in all oceans. There are over 1,500 known

species, and many are still undiscovered. Various secondary metabolites including steroids, steroidal glycosides, anthraquinones, alkaloids, phospholipids, peptides, and fatty acids were reported from starfish. These chemical constituents exhibit cytotoxic, hemolytic, antiviral, antifungal, and antimicrobial activities⁴⁾. *Acaudina molpadioides* is an abundant low valued sea cucumber widely distributed in sandy coastal regions of China. However it has not been fully exploited nor utilized. *Asterias amurensis* is a widely distributed starfish in the Northern Pacific and causes severe damage to the fishery and aquacultural grounds for benthic shellfish⁵⁾. Therefore, sufficient exploiting and utilization of *Acaudina molpadioides* and *Asterias amurensis* is aspired.

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Cerebrosides are one of the simplest lipid classes of glycosphingolipids and consist of monosaccharides (such as glucose and galactose), an amide-linked fatty acid and sphingoid base which is also called long-chain base⁶⁾. Cerebrosides have been shown to display variety of biological activities such as anti-tumor, immunomodulatory, and antimicrobial activities⁷⁻¹⁰⁾. The studies of prominent anti-tumor activities of marine-derived cerebrosides and their sphingoid bases during the past two decades have been attracted our attention. The glycosphingolipids of marine invertebrates, including sea cucumber and starfish, have unique sphingoid bases with a conjugated diene such as 2-amino-1, 3-dihydroxy-4-heptadecene (d17:1), 2-amino-1, 3-dihydroxy-4, 8, 10-octadecatriene (d18:3) or 2-amino-1, 3-dihydroxy-9-methyl-4, 8, 10-octadecatriene (d19:3)^{11, 12)}. Recent significant progress in biochemical and molecular studies of cerebrosides metabolism and function revealed that these cerebroside metabolites regulate many cellular processes including apoptosis¹³⁻¹⁵⁾. Sugawara *et al* reported that sea cucumber (*Stichopus variegates*) sphingoid bases *e.g.* d17:1, 4, 8-sphingadienine (d18:2), d18:3 and d19:3 have strong cytotoxicity against some colon cancer cell lines *via* induction of apoptosis¹⁶⁾. A novel identified glycosphingolipid, ophidiacerebroside C was isolated from *Ophidiaster ophidiamus*. The major component of that cerebrosides was (2*S*, 3*R*, 4*E*, 8*E*, 10*E*)-1-(β -D-glucopyranosyloxy)-2-hydroxy-2-[(*R*)-2-hydroxydocosanoyl]amino]-9-methyl-4, 8, 10-octadecatriene. And this showed strong cytotoxicity on L1210 leukemia cells *in vitro*¹⁷⁾. It is demonstrated that the marine-derived cerebrosides and their sphingoid bases have significant anti-tumor activity. And it is considered that the antitumor activity rests on the sphingoid base structure.

Apoptosis is the process of programmed cell death that occurs in multicellular organisms. It is physiologically and pathologically involved in cellular homeostasis¹⁸⁾. In the recent years, apoptosis has become an important area for cancer research because most anti-cancer strategies, including radiation therapy, immunotherapy, or targeted therapies, activate apoptosis signal pathways¹⁹⁻²¹⁾. Apoptosis occurs through two main pathways. The first, referred to as the extrinsic or cytoplasmic pathway, is triggered through the Fas death receptor²²⁾. The second pathway is the intrinsic or mitochondrial pathway that when stimulated leads to the release of cytochrome c from the mitochondria and activation of the death signal²³⁾. Bcl-2 family genes comprise one of the most important regulators of this pathway. Both pathways converge to a final common pathway involving the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell²⁴⁾.

The aim of this work is to assess the anti-tumor activities, in particular, the structure-activity relationships of AMC and AAC both *in vitro* and *in vivo*. Furthermore, to

investigate the anti-tumor molecule mechanism of AMC and AAC, we assayed the gene expression of Bcl-2, Bcl-xL, Bax, cytochrome c, caspase-9 and caspase-3, which are strongly associated with the mitochondrial apoptotic pathway.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

Sea cucumber (*Acaudina molpadioides*) was purchased from the Zhou-Shan Fishery Company (Zhejiang Province, China) in September, 2008. Starfish (*Asterias amurens*) material was collected in southern Hokkaido in April, 2007. The sample of AAC was provided by Professor Takahashi from the Hokkaido University. Roswell Park Memorial Institute-1640 Medium (RPMI-1640), newborn calf serum (NCS) were all obtained from Gibco (Grand Island, NY). Trypsin, 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and Trypan blue were purchased from Sigma (St. Louis, MO, USA). Tissue culture plates were obtained from Corning (NY, USA). Acridine orange (AO), ethidium bromide (EB) and Moloney murine leukemia virus reverse transcriptase (MMLV) were obtained from Promega (Madison, WI). TRIzol reagent was obtained from Invitrogen (Carlsbad, CA, USA). Maxima SYBR Green qPCR Master mix was purchased from Fermentas (Glen Burnie, Maryland).

2.2 Preparation and analysis of AMC and AAC

AMC were prepared and analyzed using the procedure described by Xu *et al*⁶⁾. The preparation of AMC and AAC solution was followed by the liposome preparation done by Z. Hossain *et al*²⁵⁾, and diluted to the desired concentrations by medium before use.

2.3 Cell lines and cell culture

Murine sarcoma cells (S180) were obtained from Shanghai Cell Bank (Shanghai, China). S180 cells were maintained in RPMI-1640 medium, supplemented with 10% heat-inactivated NCS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

2.4 Animals

BALB/c male mice (18-22 g, 8-10 weeks) were purchased from Vital River Laboratory Animal Laboratory Animal Center (Beijing, China). The animals were provided with standard laboratory food and water, and were kept at 24°C, relative humidity at 65 ± 15% and under 12 h light-dark cycle. All experiments were carried out according to the P.R. China legislation regarding the use and care of laboratory animals and were approved by the Bioethics Committee of Ocean University of China.

2.5 Cell viability assay

Cell viability was determined by the MTT assay²⁶⁾. S180 cells (2×10^4 cells/well) were seeded in 96-well plates. After 24 h, the cells were treated with different concentrations of AMC and AAC liposomes for 24 h, 48 h and 72 h respectively. At the end of the treatment, MTT solution (0.5 mg/mL in RPMI-1640 medium) was added and further incubated for 4 h. The absorbance was subsequently measured at 570 nm in each well by using microplate reader (Model 680, Bio-Rad, Tokyo, Japan). Triplicate experiments were performed in a parallel manner for each concentration. Cell viability ratio was calculated by the following formula: cell viability ratio (%) = $(A_t/A_c) \times 100\%$, where A_t and A_c are the average absorbance of three parallel experiments from the treated and control groups, respectively.

2.6 Cellular morphological analysis of apoptosis

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by fluorescence microscopy using AO and EB staining²⁷⁾. S180 cells (2×10^5 cells/well) were treated with various concentrations of AMC and AAC liposomes (0, 62.5 and 250 μ M) for 48 h. The cells were then trypsinized, harvested, centrifuged and washed with phosphate buffered saline (PBS). The 20 μ L of cell suspension was stained with 100 μ g/mL AO and 100 μ g/mL EB for 10 min at room temperature. Cells were then observed through fluorescent microscope (IX-51, Olympus, Tokyo, Japan) equipped with an automatic photomicrograph system.

2.7 Anti-tumor activity in S180 tumor bearing mice

Tumors were established by subcutaneous injection of S180 cells. Namely, 5×10^6 cells were injected into the right axilla of the BALB/c mice²⁸⁾. The mice were then randomly divided into three groups (10 mice in each group): control group (0.85% normal saline) and AMC-treated or AAC-treated groups (AMC or AAC, 50 mg/kg BW). After 24 h, the animals in each group were administered intragastrically (i.g.) of normal saline or the test cerebrosidases once per day for totally 10 consecutive days. Mice conditions were carefully monitored, and their body weight was measured every other day. Animals were sacrificed and weighed immediately 24 h after the final administration. Tumor was dissected and weighed, Tumor inhibitory ratio was calculated as follows: tumor inhibitory ratio (%) = $[1 - (W_t/W_c)] \times 100\%$, where W_c is the average tumor weight of the control group and W_t is the average tumor weight of test groups.

2.8 Anti-tumor activity in S180 ascites tumor mice

A total of 10^6 S180 cells suspended in 0.2 mL PBS were injected into the BALB/c mice via the abdominal cavity²⁹⁾. The mice were then randomly divided into three groups (20 mice in each group): model control group (0.85% normal

saline) and AMC-treated or AAC-treated groups (AMC or AAC, 50 mg/kg BW). After 24 h, the animals in each group were given intragastrically (i.g.) of normal saline or the test cerebrosidases once per day for totally 10 consecutive days. Half mice of each group were sacrificed 24 h after the final medication and the rest mice were kept alive for the assessment of increase in life span. The ascites tumor cells were collected into nuclease-free tubes and dissolved in TRIzol reagent to extract total RNA. The volume of the ascites fluid and the viable tumor cells counts were measured. Trypan blue was used for the assessment of viable ascites tumor cells. Ascites fluid inhibitory ratio (AFIR) was calculated by the formula $AFIR(\%) = [1 - (V_t/V_c)] \times 100\%$, where V_c is the average volume of the ascites fluid of the control group and V_t is the average volume of the ascites fluid of test groups. Ascites tumor cell viability ratio (ATCVR) was calculated by the formula $ATCVR(\%) = (N_v/N_t) \times 100\%$, where N_v and N_t were the average numbers of the viable tumor cells and total number of cells, respectively. The anti-tumor effect was evaluated by increase in life span using the formula $ILS(\%) = [(D_t/D_c) - 1] \times 100\%$, where D_t is the average number of survival days in the test groups, and D_c is the average number of survival days in the control group.

2.9 RNA isolation and quantitative real-time PCR

Total RNA was extracted from S180 ascites tumor cells using TRIzol reagent. RNA concentrations were determined spectrophotometrically, and 1 μ g of RNA was converted to first strand cDNA using M-MLV reverse transcriptase and random primers. Real-time PCR was performed in the Bio-Rad iCycler iQ5 system³⁰⁾. Approximately 25 μ L of reaction volume was used for the quantitative real-time PCR assay that consisted of 12.5 μ L Maxima SYBR Green qPCR Master mix, 10 μ M of primers (0.3 μ L each of forward and reverse primer), 5.9 μ L nuclease-free water, and 6 μ L of template. The thermal conditions consisted of an initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s and extension at 72°C for 30 s. Data normalization was accomplished using the endogenous reference β -actin. The gene expression level was analyzed by relative quantification using the standard curve method. The sequence of the primers used was described in Table 2.

2.10 Statistical analysis

All the values in tables and figures were expressed as mean $\pm$ standard error of the mean. Statistical comparisons of the results were performed using one-way analysis of variance (ANOVA). *P* value less than 0.05 was considered statistically significant.

3 RESULTS

3.1 Chemical structure of AMC and AAC

The major structure of AMC and AAC were analyzed by GC-MS. As shown in Table 1, the glycosyl group of both cerebrosides was glucose. The amide-linked fatty acid units of AMC were composed by C18:0h, C22:1h, C23:1h and C24:1h. Its sphingoid base only contained d17:1, which is a typical predominant sphingoid base in sea cucumber. However, the composition of fatty acids and long-chain bases of AAC was more complicated than AMC due to the length of carbon chains and the number of the double

bonds. The main amide-linked fatty acid units and long-chain base components in AAC were C24:1h, d18:3 and d18:2, respectively.

3.2 Effects of AMC and AAC on proliferation of S180 cells

The inhibitory effects of AMC and AAC on the proliferation of S180 cells were measured by MTT assay. As shown in Fig. 1A, AMC exhibited a dose-dependent inhibitory effect on the viability of S180 cells, showing a 36.6% reduction ($P < 0.01$) when the cells were treated with 250 μM

Table.1 Fatty acids and sphingoid bases Composition of cerebrosides from *Acaudina mol-padioides* and *Asterias amurensis* by GC-MS.

	Fatty acids	Ratio of peak areas (%)	Sphingoid bases	Ratio of peak areas (%)
AMC	C18:0h	43.32	d17:1	100.00
	C22:1h	20.79		
	C23:1h	18.53		
	C24:1h	17.36		
AAC	C16:0h	9.49	d18:2	39.92
	C18:0h	6.66		
	C22:0h	12.45		
	C24:0h	11.96		
	C24:1h	52.16		
	C25:1h	7.28		

Table.2 Sequences of the primers used in the quantitative real-time PCR.

Gene name	Accession No.	Forward primer	Reverse primer
Bcl-2	NM_177410	GCTACCGTCGTGACTTCGC	ACCCAGCCTCCGTTATCC
Bcl-xL	X83574	AACCCATCCTGGCACCTG	GCATCTCCTTGTCTACGCTTT
Bax	NM_007527	GGTTGCCCTCTTCTACTTTGC	CCACCATCCCAACCCCTC
Cytochrome-c	XM_975140	ATCTCCACGGTCTGTTCGG	GCCCTTTCTCCCTTCTTCTTA
Caspase-9	NM_015733	ACAATAAATCTTCGGCAATAGG	TGGGGACCAGGCTCACTT
Caspase-3	NM_009810	TGACTGGAAAGCCGAAACT	CTGGATGAACCACGACCC
β -actin	NM_007393	CAGGCATTGCTGACAGGATG	TGCTGATCCACATCTGCTGG

Fig. 1 Effects of AMC and AAC on proliferation of S180 cells. S180 cells were treated with various concentrations of AMC and AAC for 24, 48 and 72 h. AMC and AAC inhibited S180 cell proliferation in a dose- and time-dependent manner. Data represent the mean $\pm$ SD of three independent experiments.

for 72 h. AAC also exerted dose-dependent suppressive effects on cell proliferation (Fig. 1B). The IC_{50} value of AAC at 72 h was 216.36 μ M. These results indicate that AMC and AAC inhibited S180 cell proliferation effectively and AAC showed a stronger effect than AMC.

3.3 Effects of AMC and AAC on the induction of S180 cell apoptosis

To ascertain whether the AMC and AAC inhibited S180 cell proliferation by inducing apoptosis, S180 cellular morphologic characteristics were evaluated by staining with AO and EB under fluorescence microscopy. These included viable cells with apoptotic nuclei, which contain bright green chromatin with a highly condensed or fragmented structure as well as nonviable cells with apoptotic nuclei, which contain orange chromatin with a highly condensed or fragmented structure. Results revealed that control cells exhibited uniform bright-green nuclei with organized structures (Fig. 2A and D). Compared to the control, S180 cells treated with low amount of AMC or AAC (62.5 μ M) showed slightly increased percentages of early apoptotic and late apoptotic cells (Fig. 2B and E). High doses of AMC

or AAC (250 μ M) significantly increased apoptosis in S180 cells, with most cells showing condensation of chromatin as dense orange areas (Fig. 2C and F). These results suggested that the AMC and AAC induced apoptosis in S180 cells.

3.4 Effect of AMC and AAC on S180 tumor bearing mice

To confirm the *in vivo* anti-tumor effects of AMC and AAC, we employed S180 tumor bearing mice model and S180 ascites tumor model. In S180 tumor bearing mice model, after the ten-day given intragastrically (i.g.) treatments of AMC and AAC, the average tumor weights significantly decreased to 45.24% and 35.71% compared to the untreated control mice (Table 3). Changes in body weight of the AMC and AAC administered groups were not significantly different compared to the control group. These results suggested that the AMC and AAC treatment had a high anti-tumor activity in mice.

3.5 Effect of AMC and AAC on S180 ascites tumor mice

In S180 ascites tumor model, ten days AMC or AAC administered (i.g. 50 mg/kg BW) groups exhibited a significant

Fig. 2 Effects of AMC and AAC on the apoptosis of S180 cells. The changes in cell morphology were examined by AO/EB staining after various concentrations of AMC and AAC exposure. (A) and (D) control S180 cells at 48 h; (B) S180 cells treated with 62.5 μ M AMC at 48 h; (C) S180 cells treated with 250 μ M AMC at 48 h; (E) S180 cells treated with 62.5 μ M AAC at 48 h; (F) S180 cells treated with 250 μ M AAC at 48 h. Compare to control cells, S180 cells treated with 62.5 μ M AMC or AAC at 48 h showed mixed population of viable, and early apoptotic (arrow 1 and arrow 3) cells. Cells treated with 250 μ M AMC or AAC at 48 h exhibited appearance of late-apoptotic cells seen as orange stained cells (arrow 2 and arrow 4).

Table 3 Effect of AMC and AAC on S180 tumor bearing mice by i.g. route. S180 cells (including 5×10^6 cells) were injected into the right axilla of BALB/c mice, and mice were administered AMC and AAC at a frequency of one time per day for a total of 10 consecutive days by intragastrically (i.g.). The tumor weight and body weight were measured in each group. Each value represents a mean $\pm$ S.D. (n = 10). * $P < 0.05$ versus control, ** $P < 0.01$ versus control.

Groups	Dose (mg/kg BW)	Weight of mice (g)		Weight of tumor (g)	Inhibitory rate (%)
		Pre-medication	Post-medication		
Control		18.29 $\pm$ 0.65	20.29 $\pm$ 1.37	0.42 $\pm$ 0.06	
AMC	50	18.43 $\pm$ 0.73	20.63 $\pm$ 0.86	0.23 $\pm$ 0.02**	45.24
AAC	50	18.44 $\pm$ 0.84	20.74 $\pm$ 0.78	0.27 $\pm$ 0.03*	35.71

Table 4 Effect of AMC and AAC on S180 ascites tumor mice by i.g. route. S180 cells (10^6 cells/mouse) were injected intraperitoneally (i.p.) into BALB/c mice, and mice were administered AMC and AAC at one time per day for a total of 10 consecutive days by intragastrically (i.g.). The volume of the ascites fluid, ascites fluid inhibitory ratio (AFIR), ascites tumor cells count, ascites tumor cell viability ratio (ATCVR), survival days and ILS values were estimated in each group. Each value represents a mean $\pm$ S.D. (n = 10). * $P < 0.05$ versus control, ** $P < 0.01$ versus control.

Groups	Dose (mg/kg BW)	Volume of ascites fluid (mL)	AFIR (%)	Ascites tumor cells count (10 ⁸ /mL)	ATCVR (%)	Survival days	ILS (%)
Control		19.72 $\pm$ 1.23		12.96 $\pm$ 1.49	85.06 $\pm$ 4.68	12.30 $\pm$ 1.49	
AMC	50	13.56 $\pm$ 1.39**	31.23	7.28 $\pm$ 1.36**	50.89 $\pm$ 3.05**	19.10 $\pm$ 1.97**	55.28
AAC	50	15.24 $\pm$ 1.48**	22.72	7.89 $\pm$ 1.71**	51.69 $\pm$ 4.51**	16.70 $\pm$ 1.16**	35.77

ascites fluid growth inhibition by 31.23% and 22.72%, respectively (Table 4). A count of 12.96×10^8 cells/mL ascites tumor cells were counted in peritoneal fluid of control mice, whereas, in AMC or AAC groups showed only 7.28×10^8 cells/mL ($P < 0.01$) or 7.89×10^8 cells/mL ($P < 0.01$) counts. Furthermore, the ascites tumor cell viability ratio in AMC and AAC groups were reduced by 50.89% and 51.69%, respectively (Table 4). Control mice died within 12.30 ± 1.49 days, while the AMC and AAC administered mice prolonged the survival time to 19.10 ± 1.97 days ($P < 0.01$) and 16.70 ± 1.16 days ($P < 0.01$). The ILS of AMC and AAC treatment increased by 55.28% and 35.77% compared to control (Table 4, Fig. 3). These results indicated that AMC and AAC would strongly inhibit tumor growth in S180 ascites fluid and increase the life span of the mice significantly.

3.6 AMC and AAC induce apoptosis through the mitochondrial pathway

To investigate the mitochondrial events involved in AMC- and AAC-induced apoptosis in S180 ascites tumor cells, expression differences in genes involved in the mitochondrial apoptosis pathway were analyzed by quantitative real-time PCR. As shown in Fig. 4A, Cytochrome-c mRNA level was significantly up-regulated by AMC and AAC treatment (1.23 and 1.38 fold, respectively). AMC treatment decreased the mRNA levels of Bcl-2 and Bcl-xL by 63.32%

Fig. 3 Kaplan-Meier survival curve of AMC and AAC in S180 ascites tumor mice. S180 cells (10^6 cells/mouse) were injected intraperitoneally (i.p.) into mice, then the mice were administered intragastrically (i.g.) with AMC and AAC at once per day for totally 10 consecutive days. The survival rate was followed up to 21 days after inoculation.

and 19.84% ($P < 0.01$ and $P < 0.05$, respectively). Meanwhile, the expression levels of Bcl-2 and Bcl-xL mRNA also decreased by 51.12% and 14.06%, by the treatment with AAC (Fig. 4B and C). In contrast, AMC and AAC dramatically up-regulated Bax mRNA level which increased by 1.84 and 1.42 fold, respectively, compared to the control (Fig. 4D). In addition, as shown in Fig. 4E and F, AMC and AAC

Fig. 4 Effects of AMC and AAC on the mRNA expression of mitochondrial apoptotic pathway. The mRNA expression of Cytochrome-c (A), Bcl-2 (B), Bcl-xL (C), Bax (D), caspase-9 (E), caspase-3 (F) and β -actin in S180 ascites tumor cells were measured by real time PCR after treatment with AMC and AAC (50 mg/kg BW by i.g.). Data normalization was accomplished using the endogenous reference β -actin. Value are a mean $\pm$ SD (n = 10), * P < 0.05 versus control, ** P < 0.01 versus control.

treatment increased caspase-9 and caspase-3 mRNA expression. These results indicate that AMC and AAC induce apoptosis through the mitochondrial pathway.

4 DISCUSSION

In recent years, the bioactivity of marine-derived natural products has witnessed an upsurge. The marine environment is a source of extremely potent natural products that have confirmed significant activity as anti-tumor and tumor cells cytotoxic drugs³¹. Carcinogenesis is a multistep process in which an accumulation of genetic alterations within a single cell line leads to a progressively dysplastic cellular appearance deregulated cell growth, and finally carcinoma³². Apoptosis is now recognized as the major pathway by which cytotoxic agents induce cancer cell death and a critical process that contributes to both drug sensitivity and resistance in cancer cells^{33,34}. In the present study, the results showed that AMC and AAC, two novel marine natural cerebroside, had significant inhibition on

murine sarcoma S180 both *in vitro* and *in vivo*. AMC and AAC strongly inhibited the proliferation of S180 cells and induced apoptosis. Moreover, we also certified that treatment with AMC or AAC resulted in remarkable growth inhibition of HepG2 cells (hepatocellular carcinoma cells), 95D cells (lung carcinoma cells), Caco-2 cells (human epithelial colorectal adenocarcinoma cells) and HGC-27 (gastric carcinoma cells) *in vitro* (data not shown). Our findings also indicated that the anti-tumor activity of AAC was more potent than AMC *in vitro*.

To better understand the anticancer effects of AMC and AAC, we employed S180 tumor bearing mice model and S180 ascites tumor model. These results indicated that AMC and AAC inhibited prominently S180 ascites fluid volume and solid tumor growth, and increased the life span of the S180 ascites tumor mice significantly. At present, most of the anti-tumor medicines have serious side effects. In our animal experiments, AMC and AAC showed strong anti-tumor activity without any serious weight loss on S180 tumor bearing mice. To the best of our knowledge, this was the first to establish that the dietary marine-derived cere-

brosides increased the life span *in vivo*.

It is now established that Bcl-2 family genes regulate apoptosis mainly via the mitochondrial pathway. Bcl-2 family genes involved in the process of apoptosis are highlighted not only in the clinical treatments of malignancy, but also in cancer chemoprevention. Bcl-2 family genes can regulate caspase activation through the regulation of cytochrome c release, which is inhibited by the death antagonists (Bcl-2 or Bcl-xL), and promoted by the death agonists Bax³⁵. Cytochrome c is released then binds with apoptotic protease activating factor-1 (Apaf-1) and ATP, which then bind to pro-caspase-9 to make up a protein complex known as an apoptosome which cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector caspase-3 that contribute to cell apoptosis³⁶. In this study, the down-regulation of the death antagonists Bcl-2 and Bcl-xL genes and up-regulation of the death agonists Bax which promoted the cytosolic release of Cytochrome c and leads to the activation of caspase-9, followed by caspase-3, thus paving the way for AMC- and AAC-induced apoptosis.

In the present study, we also found that in contrast to the experiments results *in vitro*, AMC treatment was more effective than AAC in both of the S180 ascites tumor and S180 tumor bearing mice model. Several studies had been confirmed the protective role of cerebroside against carcinogenesis. And this was considered to be the result of conversion of cerebroside to bioactive metabolites including the variation types of carbon-chain structure of sphingoid bases and compositions of amide-linked fatty acid^{27,37}. Oku *et al* reported that the sugar group in cerebroside was not responsible for the selective toxicity. In fact, unglycosylated ceramides of short acyl carbon-chain also selectively inhibited the growth of cancer cells⁸. Obeid *et al* indicated that only ceramides with a Δ^4 double bond induced apoptosis *in vitro*¹⁵. In contrast, sphingoid bases of a wide range of structural types also are cytotoxic³⁸. In animal experiments, the uptakes of AMC and AAC in S180 tumor mice were administered through i.g. route. Digestion of cerebroside releases the backbone ceramides and sphingoid bases^{39,40}; therefore, it is possible that the sphingoid bases that are produced by digestion of AMC or AAC were responsible for the suppression of tumorigenesis *in vivo*. The evident differences in anti-tumor effects between AMC and AAC may be correlated with the difference in structure of sphingoid bases and/or divers compositions of amide-linked fatty acids.

5 CONCLUSION

In conclusion, the present study provides evidence that AMC and AAC have prominent anti-tumor activity both *in vitro* and *in vivo*, by inducing apoptosis through the mito-

chondrial pathway. This study also described the structure-activity relationships of AMC and AAC. Abundant marine-derived natural products with high activity such as AMC and AAC might provide novel drugs for tumor therapeutics.

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