

A novel anti-tumor protein extracted from *Meretrix meretrix* Linnaeus induces cell death by increasing cell permeability and inhibiting tubulin polymerization

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Abstract. Discovery and development of new pharmaceuticals from marine organisms are attracting increasing interest. Several agents derived from marine organisms are under preclinical and clinical evaluation as potential anti-cancer drugs. We extracted and purified a novel anti-tumor protein from the coelomic fluid of *Meretrix meretrix* Linnaeus by ammonium sulphate fractionation, ion exchange and hydrophobic interaction chromatography. The molecular weight of the highly purified protein, designated MML, was 40 kDa as determined by SDS-PAGE analysis. MML exhibited significant cytotoxicity to several cancer cell types, including human hepatoma BEL-7402, human breast cancer MCF-7 and human colon cancer HCT116 cells. However, no inhibitory effect was found when treating murine normal fibroblasts NIH3T3 and benign human breast MCF-10A cells with MML. The cell death induced by MML was characterized by cell morphological changes. The induction of apoptosis of BEL-7402 cells by MML was weak by DNA ladder assay. The possible mechanisms of its anti-tumor effect might be

the changes in cell membrane permeability and inhibition of tubulin polymerization. MML may be developed as a novel, highly selective and effective anti-cancer drug.

Introduction

Marine organisms represent an essentially unexploited reservoir for discovery and development of potential products of genetic, biological and/or pharmacological interest, in particular for anti-cancer drugs (1-4). Some natural products derived from marine organisms are evaluated and developed in preclinical and/or clinical status as potential anti-cancer drugs and these kinds of products are dominated by low-molecular-weight compounds so far (4). However, a few macromolecular compounds with anti-tumor activity have also been reported. For example, Aplysianins (5-7), Julianins (8,9) and Dolabellans (10-13) are all glycoproteins isolated from different tissues of sea hare and they can inhibit the proliferation of multiple tumor cells and cause tumor lysis (5-13). Cyplasin is a 56 kDa cytotoxic protein isolated from the mucus of European sea hare *Aplysia punctata* and it shows preferential toxicity to autonomously growing transformed mammalian cells (4). Previous study from our laboratory confirmed that a novel polypeptide PG155 from shark cartilage showed potent anti-angiogenic activity by inhibiting vascular migration and tubulogenesis of human umbilical vein endothelial cells induced by vascular endothelial growth factor (VEGF). Further study revealed that PG155 could significantly inhibit angiogenesis of zebrafish embryos *in vivo* (14).

Meretrix meretrix Linnaeus has long been used as traditional Chinese medicine for the treatment of various cancers and other diseases. There is evidence to show that *M. meretrix* has anti-cancer efficacy, eliminating cysts and detoxification (15-18). Several peptides and proteins with anti-tumor activity have also been extracted from *M. meretrix*. Leng *et al* purified a peptide from *M. meretrix*, which exhibited strongly inhibitory effect on the growth of human stomach adenocarcinoma BGC-823 cells (18). In addition, two glycoproteins, MGP0405 (MW: 9,655 Da) and MGP0501 (MW: 15,878 Da), were reported to have anti-tumor effect *in vitro* (19,20).

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Abbreviations: VEGF, vascular endothelial growth factor; FCS, fetal calf serum; FPLC, fast protein liquid chromatography; BCA, bicinchoninic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; MTT, 3-(4,5-di-methylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; SEM, scanning electron microscopy; PI, propidium iodide; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PBS/FBS, PBS, pH 7.4, containing 10% fetal bovine serum (FBS)

Key words: *Meretrix meretrix* Linnaeus, anti-tumor protein, cytotoxicity, cell membrane permeability, cell cycle, tubulin polymerization, apoptosis

In the present studies, we extracted and purified a novel protein with potent anti-tumor activity from the coelomic fluid of *M. meretrix*. The effects of this protein on cell growth inhibition and morphological alterations were evaluated on several human cancer cell types. In order to find the possible mechanisms of the anti-tumor activity of MML, we investigated its effects on the changes of cell membrane permeability by SEM observation, Hoechst 33342/PI dual staining and LDH leakage assays. We also studied the effect of MML on the inhibition of tubulin polymerization by tubulin immunofluorescence staining. DNA ladder assay was employed to study apoptosis induced by MML in BEL-7402 cells.

Materials and methods

Materials, cell lines and cell culture. *M. meretrix* was purchased from the Fishery Company of Qingdao, China. The human hepatoma cell line BEL-7402 was provided by Cell Bank, Type Collection of Chinese Academy of Sci., China, while the other cells, including human breast cancer MCF-7, colon cancer HCT116 cells as well as murine fibroblasts NIH3T3 and human breast epithelial MCF-10A cells were obtained from American Type Culture Collection. Murine fibroblasts NIH3T3 cells were grown as monolayer in DMEM medium supplemented with 10% FCS, while other cell lines were cultured in RPMI-1640 medium. All cells were incubated at 37°C in a humidified air atmosphere containing 5% CO₂.

Procedures for extraction and purification. The fluids were collected from the cavity of *M. meretrix* by splitting the shells open and they were centrifuged at 8,000 x g for 30 min to remove the precipitates. Ammonium sulphate powder was slowly added to the supernatants to reach a saturation of 40%. After centrifugation at 12,000 x g for 30 min, the supernatants were collected and additional ammonium salt was added to reach 60% saturation. The precipitates were collected by centrifugation at 12,000 x g for 30 min, dialyzed against 0.05 M, pH 6.0 sodium acetate buffer, applied to a CM-cellulose-52 column and eluted with the same buffer containing 0.08 M, 0.3 M and 1 M sodium chloride.

All of the fractions were collected and analyzed their cytotoxicity by MTT assay. The active fractions were dialyzed against 0.05 M sodium acetate buffer, pH 6.0 containing 2.2 M sodium chloride. Then the supernatants were loaded to a Phenyl Sepharose FF column. The column was washed with the starting buffer and eluted with 1 M sodium chloride, H₂O and 5% ethylene glycol, respectively. The active fractions were pooled and dialyzed against 0.02 M Tris buffer, pH 7.8. The supernatants were applied to a Resource15Q column on FPLC (GE, USA). Elution was performed with the same buffer containing 0.1 M and 0.5 M sodium chloride. The protein concentrations of each fraction were determined by using the BCA protein assay kit (Pierce, USA).

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed to determine the purity of isolated proteins according to the method described by Laemmli (21). Purified protein (0.1 µg) was used in the analysis. Electrophoresis of protein was carried out on 12% polyacrylamide gel stained with Coomassie brilliant blue R250.

Assessment of cytotoxicity. The inhibitory effects of the anti-tumor protein on the growth of cancer cells as well as benign cells NIH3T3 and MCF-10A were assessed *in vitro* by MTT assay (22). Five thousand cells per well were seeded in a 96-well microplate. Cells were cultured in 180 µl media of RPMI-1640 or DMEM for 24 h. The purified protein with different concentrations was added to the medium. After 48 h, MTT solution (100 µl, 0.5 mg/ml) was added into each well and the cells were incubated for another 4 h. After adding 150 µl DMSO to each aspirated well, the plate was gently agitated until the color reaction was uniform. The OD₅₇₀ was determined by a microplate reader (Bio-Tek Instruments, USA) with subtraction of background absorbance.

Morphological alterations of BEL-7402 cells. Morphological alterations of BEL-7402 cells after MML treatment were investigated using phase contrast microscopy and SEM. The cells were seeded and cultured in 96-well plates, as described above. After incubation with 52.2 µg/ml MML, the morphology of cells was observed under the CKX41 phase contrast microscopy (Olympus, Japan) and photographed at 30, 60, 90 and 120 min, respectively. In the SEM experiment, BEL-7402 cells were grown onto poly-L-lysine-coated coverslips in 6-well plates for 24 h to allow firm attachment. Then they were treated with 52.2 µg/ml MML and incubated for 60 min. The medium containing MML was removed and subsequently the cells were fixed in glutaraldehyde. After fixation overnight at 4°C, the coverslips were dehydrated in ethanol and dried in a critical point dryer. Cells on coverslips were coated with gold and analyzed by the S-3400N SEM (Hitachi, Japan).

Hoechst 33342/PI dual staining. The BEL-7402 cells were seeded in 6-well plates at 5x10⁴ per well and treated with 52.2 µg/ml MML. After incubation for 24 h, the cells were collected into a 1.5 ml Eppendorf tube, centrifuged at 1,000 x g for 5 min to remove the medium and then stained with Hoechst 33342 and PI by using the dual staining Kit (Beyond, China). The cells were spread on slides and observed under the fluorescence microscopy (Zeiss, Germany).

LDH leakage assay. LDH leakage assay was performed to determine the membrane damage and loss of membrane integrity. The cells were seeded and cultured in 96-well plates, as described above. After incubation with various concentrations (26.1, 52.2, 104.4 µg/ml) of MML for 24 h, the activity of LDH in the medium supernatants (S) and remaining cells (C) was analyzed using the Colorimetric LDH cytotoxicity assay kit (Promega, USA). The percentage of LDH leakage was calculated as follows, leakage (%) = S/(S + C) x 100%.

Cell cycle analysis. BEL-7402 cells were seeded at 3x10⁴/cm² in 10-cm dishes and treated with 52.2 µg/ml MML. After incubation for 24 h, the cells were collected and washed with PBS for three times and then were resuspended in 5 µg/ml PI (1 ml) containing 100 U/ml RNase A. The DNAs of cells were stained by PI, while the RNAs were removed by digestion with RNase A at 4°C for 30 min. The DNA contents of the cells were determined by FACSCalibur™ cytometer (Becton-Dickinson, Germany).

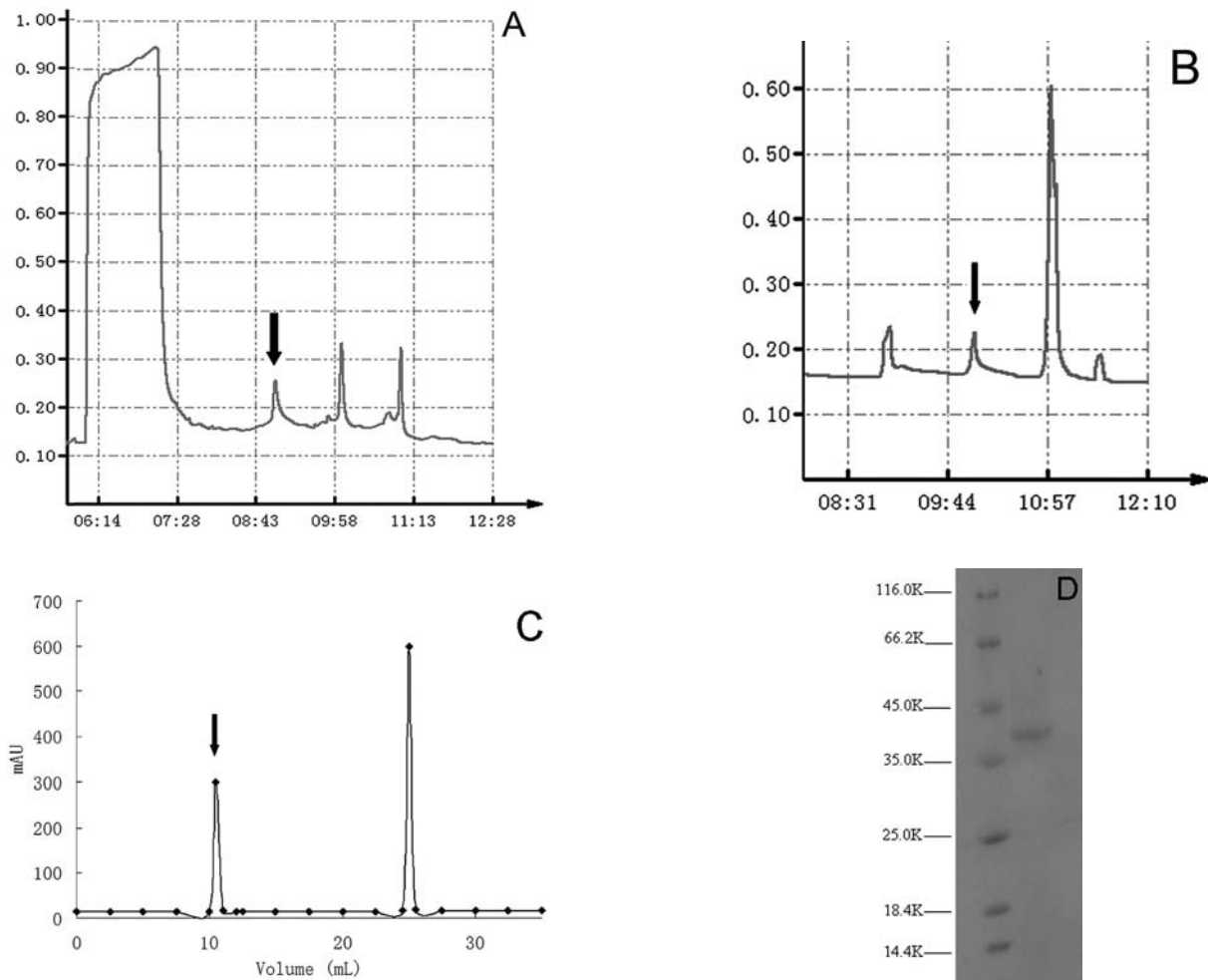


Figure 1. Elution patterns on column chromatography and SDS-PAGE analysis of MML. The arrow indicated the active component during the purification. (A) The dialyzed ammonium sulphate fraction was applied to a CM-cellulose-52 column equilibrated with 0.05 M, pH 6.0 sodium acetate buffer and was eluted with the same buffer containing 0.08, 0.3 and 1 M sodium chloride. The eluted active fraction was applied to Phenyl Sepharose FF column equilibrated with 0.05 M, pH 6.0 sodium acetate containing 2.2 M sodium chloride and was eluted with 1 M sodium chloride, water and 5% ethylene glycol (B). The active fraction from phenyl sepharose chromatography was applied to a RSource-15Q column equilibrated with 0.02 M Tris buffer, pH 7.8 and was eluted with the same buffer containing 0.1 and 0.5 M sodium chloride (C). SDS-PAGE analysis of the purified MML from the coelomic fluid of *M. meretrix* (D). The purified MML was resolved on 12% polyacrylamide gel and the gel was stained with Coomassie brilliant blue R250.

Indirect immunofluorescence detection of α -tubulin. BEL-7402 cells were grown onto glass coverslips in 6-well dishes. After incubation for 24 h, the cells were exposed to 52.2 μ g/ml MML for 30 min. Then the cells were fixed with 2% paraformaldehyde for 10 min at room temperature. After washing twice with PBS, the cells were blocked with PBS/FBS solution for 20 min and then incubated with mouse monoclonal anti- α -tubulin antibody (1:500, Calbiochem, USA) in 0.1% Triton/PBS/FBS solution for 60 min at room temperature in a humidified chamber. After washing twice with PBS/FBS solution, the cells were incubated with a second antibody (FITC-anti-mouse IgG, 1:500, Calbiochem) in the dark for 60 min at room temperature, washed again with PBS/FBS solution and covered with a glass slide. Cellular microtubules were observed under fluorescence microscopy (Zeiss, Germany).

DNA ladder assay. BEL-7402 cells were seeded at 3×10^4 /cm² in 10-cm dishes and incubated for 24 h. After treated with 52.2 μ g/ml MML for 48 h, the cells were collected and

total genomic DNA was extracted according to the protocol described in Universal Genomic DNA extraction kit (Takara, Japan). The DNA samples were resolved on 1.0% agarose gel stained with 0.5 μ g/ml of ethidium bromide and photographed by Quantity one system (Bio-Rad, USA).

Results

Extraction and purification of the protein from *Meretrix meretrix*. The protein was extracted and purified from the coelomic fluid by ammonium sulphate fractionation, ion exchange and hydrophobic interaction chromatography. The elution profiles on column chromatography are shown in Fig. 1. The precipitates formed at 40-60% ammonium sulphate saturation were applied to a CM-cellulose-52 column. The active fractions were eluted with 0.08 M sodium chloride solution (Fig. 1A) and further purified by Phenyl Sepharose FF (Fig. 1B). After hydrophobic chromatography, the active components were applied to a RSource-15Q pre-packed column eluted with 0.1 M sodium chloride solution, and

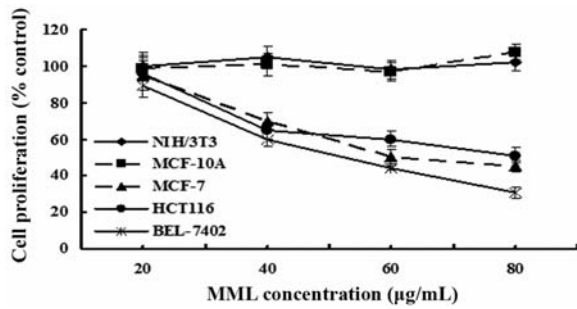


Figure 2. MML inhibited the growth of several types of cancer cells. Human hepatoma BEL-7402 (star), human colon cancer HCT116 (solid circle), human breast cancer MCF-7 (triangle), human breast epithelial cell MCF-10A (solid box) and murine fibroblast NIH3T3 cells (diamond) were incubated in the absence or presence of various concentrations of MML for 48 h at 37°C. MTT assay was performed to determine the growth inhibition of different cancer cells and benign NIH3T3 and MCF-10A cells by MML. The experiments were performed more than three times and the results were compared to untreated cells.

purified to homogeneity (Fig. 1C). The purified protein, designated MML, revealed as a single band of 40 kDa on SDS-polyacrylamide gel electrophoresis (Fig. 1D).

Cytotoxicity of MML against human cancer cells. During the purification process, MTT colorimetric method was employed to trace the active components for cell growth inhibition against human hepatoma BEL-7402 cells. The purified protein, MML, significantly inhibited the growth of BEL-7402 cells and the IC_{50} was determined to be 52.2 μ g/ml (Fig. 2). MML displayed cytotoxicity also to human breast cancer MCF-7 and human colon cancer HCT116 cells. However, it had no inhibitory effect on the growth of benign cells, including murine fibroblasts NIH3T3 as well as human breast epithelial cell line MCF-10A at a concentration of 80 μ g/ml (Fig. 2). Moreover, no growth inhibition of NIH3T3 and MCF-10A cells was found while increasing the concentration of MML to 300 μ g/ml (data not shown).

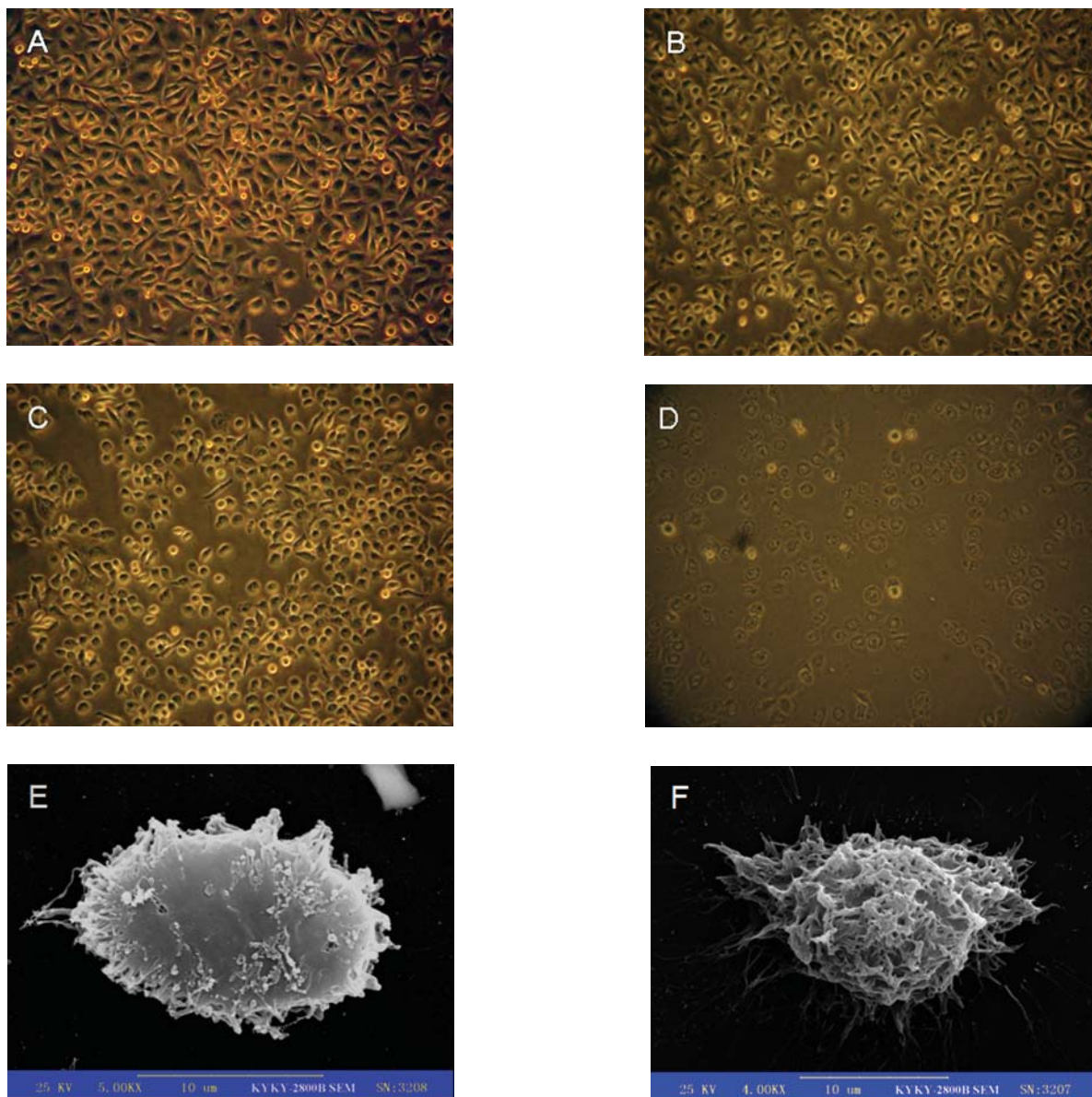


Figure 3. Morphological analysis of BEL-7402 cells induced by MML. The cells were untreated (Panel A) or treated with 52.2 μ g/ml MML for 30 min (Panel B), 60 min (Panel C), and 90 min (Panel D). In the SEM experiment, BEL-7402 cells were grown onto poly-L-lysine-coated coverslips for 24 h to allow firm attachment and treated with 52.2 μ g/ml MML for 60 min. Cells were fixed on coverslips coated with gold and analyzed by using the S-3400N SEM. Panel E represented the results of untreated cells and Panel F displayed the results of cells treated with MML (Scale bar = 10 μ m).

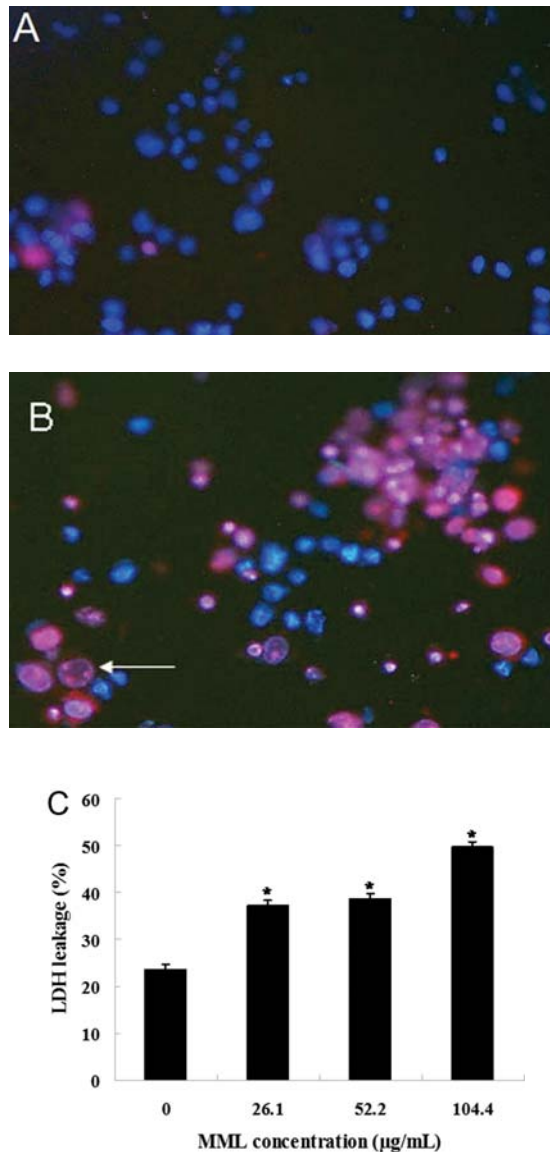


Figure 4. MML increased cell membrane permeability as determined by Hoechst 33342/PI staining and LDH leakage assay. After incubation for 24 h, the cells were collected stained with Hoechst 33342 and PI and then the cells were spread on glass slides and observed under the fluorescence microscopy. For the untreated cells, only Hoechst 33342 staining was found (A). In contrast, the cells treated with MML were stained by both Hoechst 33342 and PI (arrows, B). For LDH leakage assay, cells were incubated with various concentrations (26.1, 52.2 and 104.4 μg/ml) of MML for 24 h at 37°C. The percentage of LDH leakage (C) was analyzed, as described in Materials and methods. The asterisk indicates the significant difference between the group of untreated control and the groups of MML treatment ($P < 0.01$).

MML induces the morphological alterations of BEL-7402 cells. Remarkable alterations of cell membrane of BEL-7402 cells were observed after MML treatment. The architecture of untreated BEL-7402 cells displayed a typical spindle shape (Fig. 3A). However, the morphology of the cells started to change in a short time after incubation with MML. The cells detached from the substratum, became rounding (Fig. 3B) and separated from each other (Fig. 3C) after exposure to MML for 30-60 min. Membrane bulge and detachment from cytoplasmic inclusion were observed in 90 min after MML treatment (Fig. 3D). Obvious bulging of the membranes was found when treated with MML for 120 min (data not shown).

The morphologic changes of the cell membrane were also clearly visualized by SEM. The untreated BEL-7402 cells showed a normal smooth surface (Fig. 3E). In contrast, the cells treated with MML became rounded and the surface of the cell membrane was markedly disrupted (Fig. 3F).

MML increases the permeability of cell membrane of BEL-7402 cells. To demonstrate the effects of MML on cell permeability of BEL-7402 cells, Hoechst 33342/PI dual staining and LDH leakage assay were performed. The BEL-7402 cells displayed integrate cell membranes and did not stain by PI without MML treatment (Fig. 4A). In contrast, typical red fluorescence of PI in the BEL-7402 cells was visible after MML treatment (Fig. 4B). The results demonstrated that MML increased the membrane permeability of BEL-7402 cells. Furthermore, the percentage of LDH leakage was significantly increased ($P < 0.01$) in BEL-7402 cells treated with MML compared to that of untreated cells (Fig. 4C). The increase of LDH activity was in a dose-dependent manner and more LDH leakage was observed with higher MML concentrations (Fig. 4C).

MML induces cell cycle arrest in G2/M phase and inhibits tubulin polymerization. To further evaluate the mode of action of MML, the effects of MML on the location of BEL-7402 cells in the cell cycle were examined by using flow cytometry analysis. The result showed that MML blocked BEL-7402 cells in G2/M phase in a dose-dependent manner (Fig. 5). Since microtubule-targeted anti-cancer agents arrested cell cycle in G2/M phase, we examined the microtubule network of BEL-7402 cells treated with MML by indirect immuno-fluorescence approach. The result of MML affecting tubulin polymerization is shown in Fig. 6. The microtubule network in untreated cells showed normal organization (Fig. 6A) while tubulin polymerization was inhibited in treated cells (Fig. 6B).

DNA ladder assay. To further understand the molecular events underlining the MML on cancer cells, DNA ladder assay was performed to study MML inducing apoptosis. As shown in Fig. 7, BEL-7402 cells treated with VP16 (positive control) were characterized by fragmentation of DNA (DNA ladder) on the agarose gel. Electrophoresis of DNA from the cells treated with MML also displayed DNA ladders, but the fragmentation was much weaker than that of the VP16-treated cells.

Discussion

In the present study, we extracted and purified a novel anti-tumor protein from the coelomic fluid of *Meretrix meretrix* Linnaeus. MTT assay indicated that purified MML could selectively inhibit the proliferation of several human cancer cells. Treatment of human hepatoma BEL-7402 cells with MML resulted in increased permeability of cell membrane and inhibition of tubulin polymerization.

An interesting point in our study was that the novel protein, MML, displayed potent cytotoxicity to human hepatoma BEL-7402 cells. Also, MML could inhibit the growth of human breast cancer MCF-7 and human colon cancer HCT116

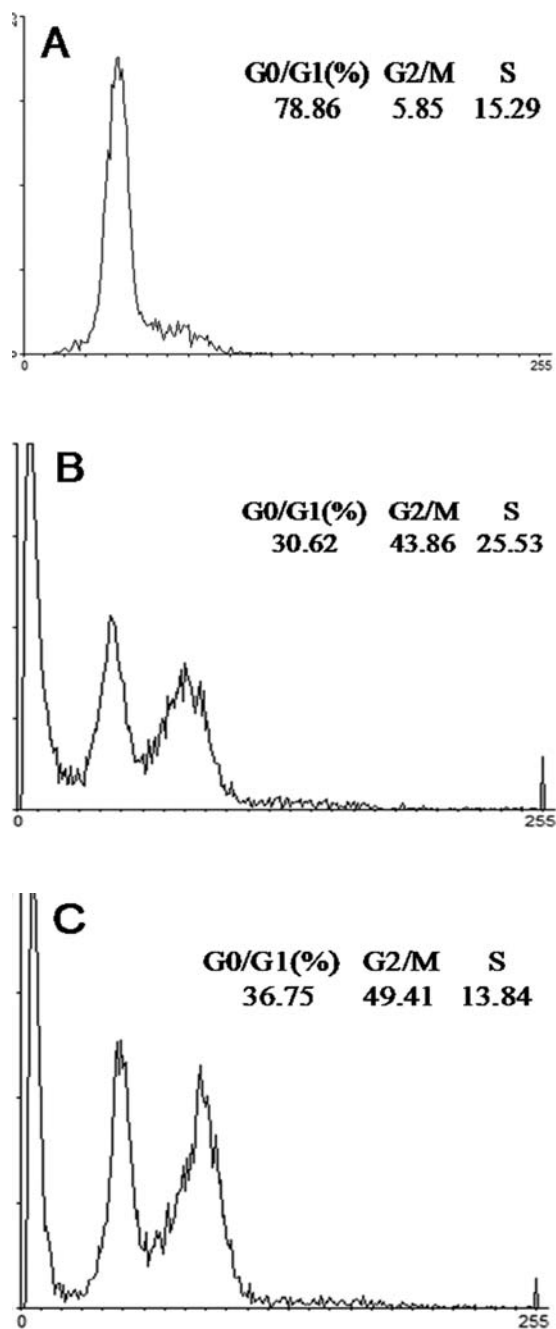


Figure 5. Effect of MML on cell cycle distribution. BEL-7402 cells were seeded at $3 \times 10^4/\text{cm}^2$ in 10-cm dishes with or without MML treatment. After 24 h incubation, the untreated cells (A) and the cells treated with MML at 52.2 $\mu\text{g}/\text{ml}$ (B), or 104.4 $\mu\text{g}/\text{ml}$ (C) were collected and the DNAs were stained by PI, while the RNAs were removed by digestion with RNase A. The DNA contents of the cells were determined with FACSCalibur™ cytometer.

cells significantly. However, no inhibition effect was found when treating murine fibroblast NIH3T3 cells and human benign breast MCF-10A cells with MML. These results demonstrated that MML could selectively inhibit tumor cells. Previous studies show that many anti-microbial peptides, such as insect cecropins (23) and magainins (24-26), exhibit anti-tumor effects *in vitro* and/or *in vivo*. Some of them are proved to have selective activity on different tumor cells. Magainin II displayed significant anti-tumor activities against various cancer cell lines *in vitro* and animal tumor models *in vivo*, but did not display cytotoxicity to the fibroblasts NIH3T3 cells

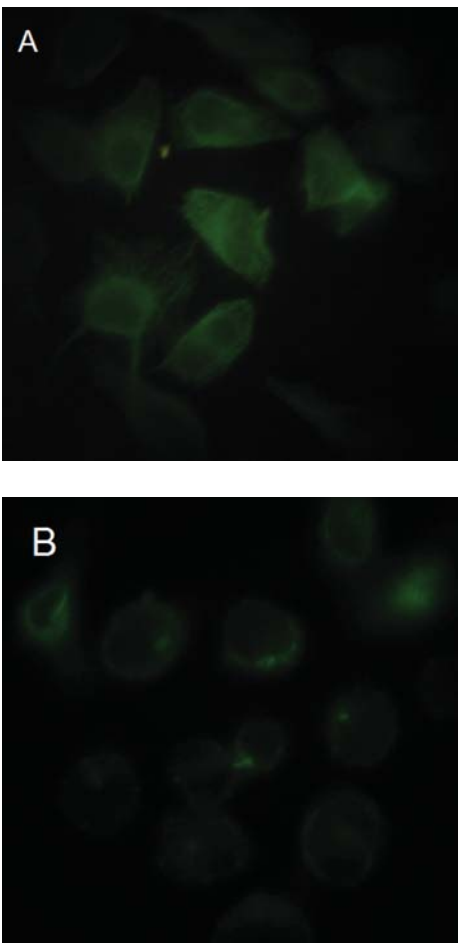


Figure 6. MML inhibits polymerization of cellular α -tubulin. BEL-7402 cells were grown onto glass coverslips placed in petri dishes. After incubation for 24 h, the untreated cells (A) and the cells treated with 52.2 $\mu\text{g}/\text{ml}$ MML (B) were fixed in 2% paraformaldehyde for 10 min at room temperature. After washing twice with PBS, the cells were incubated with mouse monoclonal anti- α -tubulin antibody (1:500) for 60 min. After washing twice with PBS/FBS solution, cells were incubated with a second antibody (FITC-anti-mouse IgG, 1:500) and covered with a glass slide. Cellular microtubules were observed under fluorescence microscopy.

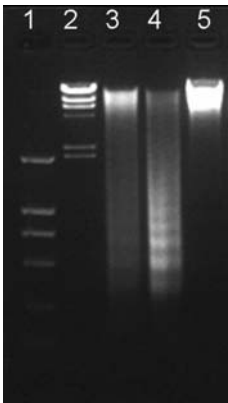


Figure 7. Effect of MML on apoptosis of BEL-7402 cells. The cells were seeded at $3 \times 10^4/\text{cm}^2$ in 10-cm dishes and incubated for 24 h. The untreated cells and the cells treated with 52.2 $\mu\text{g}/\text{ml}$ MML or 5 μM VP16 were incubated for another 48 h. The cells were collected and total DNA was extracted and analyzed by electrophoresis on 1.0% agarose gel. Lane 1 and lane 2 represents DNA marker. Lane 3 indicates the cells treated with MML. Lane 4 indicates the cells treated with VP16. The untreated cells are indicated in lane 5.

(27). It was found that magainin II could interact strongly with the anionic lipid of cell membranes (28). However, further study is needed to address if there are differences in the contents of anionic lipid between tumor and benign cells.

There is evidence to show that most of anti-microbial peptides cause cell death by increasing cell membrane permeability. Cecropin A and B, anti-microbial peptides isolated from the hemolymph of giant silk moth *Hyalophora cecropia* (29), possessed the ability to form specific amphipathic α -helices to target non-polar lipid cell membranes (30-32). The peptides targeted membrane by forming ion-permeable channels and subsequently resulted in cell depolarization, irreversible cytolysis and finally cell death (33,34). Previous studies have demonstrated that both Cecropin A and B family members showed cytolytic activity against various human cancer cell lines, including leukemia, lymphoma and colon carcinoma (23,35) as well as small cell lung cancer (36), gastric cancer (37) and bladder cancer (38). Intensive studies have proved that many anti-microbial peptides utilized a unique mechanism to lyse the membrane of cancer cells, either in a 'carpet' model (39-42) or in a 'barrel-stave' model (40,43,44). Compared with these anti-microbial peptides, MML displayed similar effects against human hepatoma BEL-7402 cells. However, since MML is a macromolecular protein, further studies are required to elucidate the action mode of MML on cancer cell membranes.

Microtubules are the major dynamic structural components in cells and used as the target by several classes of anti-cancer agents. Previous studies with mitotic inhibitors from marine organisms are dominated by the compounds of low-molecular-weight. Dolastatin 10, an anti-cancer peptide isolated from the sea hare, could block cells in G2/M phase by inhibiting tubulin polymerization (45-48). Our results suggested that MML, like Dolastatin 10, also induced cell arrest in G2/M phase and the effect might attribute to its inhibition effect on tubulin polymerization. A number of anti-cancer agents could inhibit the tubulin polymerization by direct interaction with tubulin. For example, cryptophycin, vinorelbine and colchicines could bind directly with different sites on tubulin molecule and affect the tubulin polymerization (49-51). Further studies are ongoing in our laboratory to investigate if MML interacts with tubulin directly or through receptor-mediated signal transduction events.

Most anti-cancer agents induce cell death by apoptosis and nuclear fragmentation was a typical indicator for cell apoptosis (52,53). In the present study, obvious DNA ladder was observed in the hepatoma cells treated with VP16, while only much weaker DNA fragmentation was presented in the MML-treated cells. Based on this finding, the induction of apoptosis might not be the main effect of MML for its cytotoxicity to human hepatoma cells.

In summary, MML is a novel protein isolated from a marine mollusk with remarkable *in vitro* anti-tumor activity against cancer cells. MML selectively inhibited the growth of several types of cancer cells. The inhibitory effect of MML on the proliferation of human hepatoma BEL-7402 cells appeared to involve increased cell membrane permeability and damage and inhibition of microtubules polymerization. However, the interaction of this protein with cell membrane and tubulin and the interacting sites on tubulin molecules still

require to be further investigated. MML may be a good candidate to be developed as a novel and effective anti-cancer drug.

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References

1. de Vries DJ and Beart PM: Fishing for drugs from the sea: status and strategies. *Trends Pharmacol Sci* 16: 275-279, 1995.
2. Wallace RW: Drugs from the sea: harvesting the results of aeons of chemical evolution. *Mol Med Today* 3: 291-295, 1997.
3. Fenical W: New pharmaceuticals from marine organisms. *Trends Biotechnol* 15: 339-341, 1997.
4. Petzelt C, Joswig G, Stammer H and Werner D: Cytotoxic Cytoplasm of the sea hare, *Aplysia punctata*, cDNA cloning, and expression of bioactive recombinants in insect cells. *Neoplasia* 4: 49-59, 2002.
5. Kisugi J, Kamiya H and Yamazaki M: Purification and characterization of aplysianin E, an antitumor factor from sea hare eggs. *Cancer Res* 47: 5649-5653, 1987.
6. Yamazaki M, Kimura K, Kisugi J, Muramoto K and Kamiya H: Isolation and characterization of a novel cytolytic factor in purple fluid of the sea hare, *Aplysia kurodai*. *Cancer Res* 49: 3834-3838, 1989.
7. Kamiya H, Muramoto K and Yamazaki M: Aplysianin A, an antibacterial and antineoplastic glycoprotein in the albumen gland of a sea hare, *Aplysia kurodai*. *Experientia* 42: 1065-1067, 1986.
8. Kamiya H, Muramoto K, Goto R, Sakai M, Endo Y and Yamazaki M: Purification and characterization of an antibacterial and antineoplastic protein secretion of a sea hare, *Aplysia Juliana*. *Toxicon* 27: 1269-1277, 1989.
9. Kamiya H, Muramoto K, Goto R and Yamazaki M: Characterization of an antibacterial and antineoplastic glycoprotein in a sea hare, *Aplysia Juliana*. *Nippon Suisan Gakkaishi* 54: 773-777, 1989.
10. Yamazaki M, Tansho S, Kisugi J, Muramoto K and Kamiya H: Purification and characterization of a cytolytic protein from purple fluid of the sea hare, *Dolabella auricularia*. *Chem Pharm Bull* 37: 2179-2182, 1989.
11. Kisugi J, Yamazaki M, Tansho S, Muramoto K and Kamiya H: Purification of a novel cytolytic protein from albumen gland of the sea hare, *Dolabella auricularia*. *Chem Pharm Bull* 37: 2773-2781, 1989.
12. Kisugi J, Kamiya H and Yamazaki M: Purification of dolabellin-C, an antineoplastic glycoprotein in the body fluid of a sea hare, *Dolabella auricularia*. *Dev Comp Immunol* 13: 3-15, 1989.
13. Yamazaki M: Antitumor and antimicrobial glycoproteins from sea hares. *Comp Biochem Physiol* 105C: 141-146, 1993.
14. Zheng LH, Ling PX, Wang Zh, Niu RL, Hu ChX, Zhang TM and Lin XK: A novel polypeptide from shark cartilage with potent anti-angiogenic activity. *Cancer Biol Ther* 6: 775-780, 2007.
15. Chen HY, Cong XQ, Zhang A and Zhu JM: The anticancer research of extraction from *Mercenaria*. *Cancer Res Prev Treat* (in Chinese) 4: 3-7, 1980.
16. Zhang JX and Xing YP: Studies on anti-cancer activity of *Meretrix Meretrix* nucleic acid. *Oceanol Et Limnol* (in Chinese) 21: 88-91, 1990.
17. Dou CH, Huang F, Huang LS, Li PZ and Xiao WD: An experimental study on the effects of *Meretrix* polysaccharide. *J Marine Drugs* (in Chinese) 2: 15-19, 1999.

18. Leng B, Liu XD and Chen QX: Inhibitory effects of anticancer peptide from *Mercenaria* on the BGC-823 cells and several enzymes. *FEBS Lett* 579: 1187-1190, 2005.
19. Zhang B and Wu JL: Isolation and characterization of glycopeptide MGP0405 from *Meretrix meretrix*. *Chin J Nat Med* (in Chinese) 4: 230-233, 2006.
20. Wu JL, Zhang B, Huang CH, Zhu XC and Wu WT: The antitumor activity of glycopeptide (MGP0501) from *Meretrix meretrix* *in vitro*. *Pharm Biotech* (in Chinese) 13: 260-264, 2006.
21. Laemmli UK: Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* 227: 680-688, 1970.
22. He QJ, Yang B, Lou YJ and Fang RY: Contragestazol (DL111-IT) inhibits proliferation of human androgen-independent prostate cancer cell line PC3 *in vitro* and *in vivo*. *Asian J Androl* 7: 389-393, 2005.
23. Chen HM, Wang W, Smith D and Chan SC: Effects of the anti-bacterial peptide cecropin B and its analogs, cecropins B-1 and B-2, on liposomes, bacteria and cancer cells. *Biochim Biophys Acta* 1336: 171-179, 1997.
24. Zasloff M: Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* 84: 5449-5453, 1987.
25. Baker MA, Maloy WL, Zasloff M and Jacob LS: Anticancer efficacy of Magainin2 and analogue peptides. *Cancer Res* 53: 3052-3057, 1993.
26. Cruciani RA, Barker JL, Zasloff M, Chen HC and Colamonici O: Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation. *Proc Natl Acad Sci USA* 88: 3792-3796, 1991.
27. Lehmann J, Retz M, Sidhu SS, Suttman H, Sell M, Paulsen F, Harder J, Unteregger G and Stockle M: Antitumor activity of the antimicrobial peptide Magainin II against bladder cancer cell lines. *Eur Urol* 50: 141-147, 2006.
28. Lad MD, Birembaut F, Clifton LA, Frazier RA, Webster JR and Green RJ: Antimicrobial peptide-lipid binding interactions and binding selectivity. *Biophys J* 92: 3575-3586, 2007.
29. Steiner H, Hultmark D, Engström A, Bennich H and Boman HG: Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 292: 246-248, 1981.
30. Steiner H, Andreu D and Merrifield RB: Binding and action of cecropin and cecropin analogues: antibacterial peptides from insects. *Biochim Biophys Acta* 939: 60-66, 1988.
31. Holak TA, Engström A, Kraulis PJ, Lindeberg G, Bennich H, Jones TA, Gronenborn AM and Clore GM: The solution conformation of the antibacterial peptide cecropin A: a nuclear magnetic resonance and dynamical simulated annealing study. *Biochemistry* 27: 7620-7629, 1988.
32. Christensen B, Fink J, Merrifield RB and Mauzerall D: Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. *Proc Natl Acad Sci USA* 85: 5072-5076, 1988.
33. Bechinger B: Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin. *J Membr Biol* 156: 197-211, 1997.
34. Boman HG: Antibacterial peptides: basic facts and emerging concepts. *J Intern Med* 254: 197-215, 2003.
35. Moore AJ, Devine DA and Bibby MC: Preliminary experimental anticancer activity of cecropins. *Pept Res* 7: 265-269, 1994.
36. Shin SY, Lee MK, Kim KL and Hahm KS: Structure-antitumor and hemolytic activity relationships of synthetic peptides derived from cecropin A-magainin 2 and cecropin A-melittin hybrid peptides. *J Pept Res* 50: 279-285, 1997.
37. Chan SC, Hui L and Chen HM: Enhancement of the cytolytic effect of anti-bacterial cecropin by the microvilli of cancer cells. *Anticancer Res* 18: 4467-4474, 1998.
38. Suttman H, Retz M, Paulsen F, Harder J, Zwergel U, Kamradt J, Wullich B, Unteregger G, Stöckle M and Lehmann J: Antimicrobial peptides of the Cecropin-family show potent antitumor activity against bladder cancer cells. *BMC Urol* 8: 5-11, 2008.
39. Shai Y: Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochim Biophys Acta* 1462: 55-70, 1999.
40. Papo N and Shai Y: Host defense peptides as new weapons in cancer treatment. *Cell Mol Life Sci* 62: 784-790, 2005.
41. Matsuzaki K, Murase O and Miyajima K: Kinetics of pore formation by an antimicrobial peptide, magainin 2, in phospholipid bilayers. *Biochemistry* 34: 12553-12559, 1995.
42. Ludtke SJ, He K, Heller WT, Harroun TA, Yang L and Huang HW: Membrane pores induced by magainin. *Biochemistry* 35: 13723-13728, 1996.
43. Ehrenstein G and Lecar H: Electrically gated ionic channels in lipid bilayers. *Q Rev Biophys* 10: 1-34, 1977.
44. Vogel H, Jahnig F, Hoffmann V and Stumpel J: The orientation of melittin in lipid membranes: a polarized infrared spectroscopy study. *Biochim Biophys Acta* 733: 201-209, 1983.
45. Jordan MA: Mechanism of action of antitumor drug that interact with microtubules and tubulin. *Curr Med Chem Anticancer Agents* 2: 1-17, 2002.
46. Li Y, Kobayashi H, Hashimoto Y, Shirai R, Hirata A, Hayashi K, Hamada Y, Shiori T and Iwasaki S: Interaction of the marine toxin dolastatin 10 with porcine brain tubulin: competitive inhibition of rhizoxin and phalloidin A binding. *Chem Biol Interact* 93: 175-183, 1994.
47. Bai R, Taylor GF, Schmidt JM, Williams MD, Kepler JA, Pettit GR and Hamel E: Interaction of dolastatin 10 with tubulin: induction of aggregation and binding and dissociation reactions. *Mol Pharmacol* 47: 965-976, 1995.
48. Turner T, Jackson WH, Pettit GR, Wells A and Kraft AS: Treatment of human prostate cancer cells with Dolastatin 10, a peptide isolated from a marine shell-less mollusk. *Prostate* 34: 175-181, 1998.
49. Smith CD and Zhang XQ: Mechanism of action of cryptophycin. *J Biol Chem* 271: 6192-6198, 1996.
50. Sorensen JB: Current position of vinorelbine in cancer chemotherapy. *Ann Oncol* 6: 105-107, 1995.
51. Bhattacharyya B, Panda D, Gupta S and Banerjee M: Anti-mitotic activity of colchicine and the structural basis for its interaction with tubulin. *Med Res Rev* 28: 155-183, 2008.
52. Arends MJ, Morris RG and Wyllie AH: Apoptosis: the role of the endonuclease. *Am J Pathol* 136: 593-608, 1990.
53. Compton MM: A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. *Cancer Metastasis Rev* 11: 105-119, 1992.