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Anti-tumor effect investigation of obtustatin and crude *Macrovipera lebetina obtusa* venom in S-180 sarcoma bearing mice

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ABSTRACT

Over the last few decades, research on snake venom toxins has provided not only new tools to decipher molecular details of various physiological processes, but also inspiration to design and develop a number of therapeutic agents. Isolated from the venom of Macrovipera lebetina obutusa (MLO), obtustatin represents the shortest known snake venom monomeric disintegrin specific inhibitor of α1β1 integrin. This low molecular weight peptide revealed a potent therapeutic effect on melanoma progression. Its oncostatic effect was related to the inhibition of angiogenesis. The aim of the proposed investigation was to study the influence of obtustatin and crude MLO venom on the S-180 sarcoma growth *in vitro* and *in vivo*. A S-180 sarcoma bearing mouse model, histological examination, DNA retardation assay were utilized to investigate the anti-tumor effects of MLO and obtustatin. In addition, some biochemical tests (chemiluminescence-ChL, TBA-test) were applied to elucidate the influence of obtustatin and crude MLO venom on the S-180 sarcoma. The size of tumor was significantly inhibited by MLO venom and obtustatin with the inhibitory rate of 50% and 33% at the doses of 10 μg/mouse and 1 mg/kg/day respectively. Both ChL and MDA decrease in the two treated groups. Both obtustatin and MLO venom have an anticancer activity and might be candidates for the treatment of malignant sarcoma. All our results have shown that both obtustatin and MLO venom have an anticancer activity and might be candidates for the treatment of malignant sarcoma.

1. Introduction

The medicinal value of snake venoms has been known from ancient times (Koh et al., 2006; Gawade, 2007). *Macrovipera lebetina obtusa* (*MLO*) is the most important poisonous snake in Armenia. A specific toxin was not identified in the venom of this snake and they have no real toxins in the venom (like three-finger toxins of *Elapidae*) but they form complexes with other non-enzymatic proteins to achieve higher efficiency through synergy (Sanz et al., 2008). According to these data the *MLO* venom consists of 38 protein components, venom proteins belong to only a few major protein families, including enzymes and proteins without enzymatic activity. Some proteins found in the venom of *MLO* are only characteristic of these venoms *e.g.* the obtustatin – the shortest known KTS-disintegrin (Calvete et al., 2003; Kisiel et al., 2004), a selective inhibitor of α1β1 integrin (Marcinkewicz, 2005). Structurally, obtustatin belongs to the monomeric short disintegrins, which resemble the previously reported short disintegrins such as echistatin or eristostatin (Marcinkiewicz et al., 1996). These disintegrins contain 8 cysteines in their polypeptide chain that are involved in the creation of 4 intramolecular disulfide bounds. The 3D structure of obtustatin was recently solved based on NMR coordinates (Moreno-Murciano et al., 2003). Therapeutic effectiveness of obtustatin was particularly remarkable in the mouse experiments, but only in the case of i.v. administration (Brown et al., 2008).

Malignant sarcomas are tissue tumors and are classified into bone and soft tissue tumors. It is known that bone sarcoma tumors arise from the skeleton while soft tissue sarcomas are tumors of mesenchymal tissue (blood, muscle, fat) (Fletcher et al., 2002). Although this soft tissue sarcomas account for less than 1% of malignant neoplasms, while bone sarcomas happen at a rate of one third of their soft tissue counterparts, a vast majority of these patients who have these tumors eventually die from metastatic diseases (Dorfman and Czerniak, 1995). Chemotherapy and

Abbreviations: MLO, Macrovipera lebetina obutusa; ChL, chemiluminescence; TBA, thio-barbituric acid

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biochemotherapy have been used to improve patients' condition who have sarcoma, but toxicity or significant side effects greatly restrict their clinical application (Dean and Whitwell, 2009).

There is considerable research supporting the role of oxidants in the development of cancer (Battisti et al., 2008; Chang et al., 2008; Wauquier et al., 2009). However, the function of oxidative stress in the above mentioned sarcomas remains to be explored further. That's why the aim of the proposed investigation was not only to study the influence of obtustatin and crude *MLO* venom on the proliferation of the S-180 sarcoma *in vitro* using the S-180 sarcoma bearing mouse model, histological examination, DNA retardation assay but also chemiluminescence-ChL, thio-barbituric acid test-TBA, were applied to elucidate the influence of obtustatin and crude *MLO* venom on the S-180 sarcoma which would provide a better understanding of the role of reactive oxygen species (ROS) in sarcomas that could lead to the development of new therapeutic strategies (Chavushyan et al., 2006).

2. Materials and methods

2.1. Chemicals

Obtustatin and *MLO* venom were purchased from the Tocris and Latoxan respectively.

2.2. S-180 sarcoma mouse model and administration

The S-180 sarcoma cells were transplanted subcutaneously into the right axilla of each mouse. When the tumor grew to 100–300 mm^3 we randomly divided mice into 3 groups: model (tumor control), and two S-180 sarcoma groups treated with *MLO* venom and obtustatin. Each group contained 5 mice. The mice were administered as follows: model group, PBS; one obtustatin treated group, 1 mg/kg body weight obtustatin; one MLO venom treated group, each animal received small doses of MLO (10 μg/mouse). Taking into consideration the content of obtustatin in the crude MLO venom (2.8% of total venom proteins) (Sanz et al., 2008), the doses of treatments were comparable. All solutions dissolved in PBS and administered daily by intra-tumor injection (50 μl) for 5 days. Twenty-four hours after the last drug administration, all animals were weighed and sacrificed by cervical dislocation. All procedures were done according to our institution's animal care rules and the IACUC's ethical guidelines for Decapitation of Unanesthetized Mice and Rats (http://www.utsouthwestern.edu/utsw/cda/dept238828/files/ 469088.html). The LD50 of MLO venom for mouse is: LD_{50} [μg] – 18.4 ± 1.4 (Kurtovic et al., 2014).

2.3. Antitumor activity assay in vivo

The antitumor activity was expressed as inhibitory rate (%) and was calculated as $[(A-B)/A]^*100\%$, where A and B was the average tumor weight of the model and that of treated groups, respectively.

2.4. Histological examination

Tumors from S-189 sarcoma mice were removed, weighed, fixed in 10% neutral formalin, embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin. Histological examination was carried out by microscope (EPI-Fluorescence Trinocular Microscope+5MP Camera, Model FM320-5M, Amscope, USA).

2.5. DNA retardation assay

A DNA retardation assay was employed to evaluate the DNA-binding ability of obtustatin and venom and was performed as previously reported (Wang et al., 2012). The genomic DNA was extracted from S-180 sarcoma cells by using a genomic DNA extraction kit (precellys tissue DNA kit, PeQlab, Germany). The DNA concentration was measured by using a spectrophotometric method (Eppendorf BioPhotometer plus, Germany) and then equal amounts of genomic DNA were mixed with different concentration of obtustatin and *MLO* venom at a 1:1 (vol:vol) ratio for 30 min. Next, 1% agarose gel electrophoresis (PerfectBlue™ Horizontal Mini Gel System, PeQlab, Germany) was used to detect the migration of DNA bands under a UV illuminator (E-BOX VX2-VILBER LOURMAT, PeQlab, Germany). DNA levels were quantified by density analysis with the use of Image J software, and DNA-binding rate (%) was calculated as $[1-(A/B)]^*100\%$, where A is the average density of the electrophoretic band and B is the total of the genomic DNA band (Wang et al., 2012).

2.6. Tissue processing for chemiluminescence analysis and lipid peroxidation

Non-purebred white rats were decapitated. Then the sarcoma was removed and homogenized for 5 min by the homogenizator of Potter–Elvehejm in Tris–HCl buffer (pH 7.4) with a final concentration of 20 mg/ml.

2.7. Chemiluminescence analysis

Reactive oxygen species (ROS) levels were measured by a ChL analyzing system: intensities of tissue homogenates were measured on a quantometric device equipped with a photomultiplier, which is an ultra fast single photon counter with spectral sensitivity range of 380–630 nm. All the experiments were performed by Junior LB 9509 portable tube luminometer (BERTHOLD Technologies, Germany).

2.8. Lipid peroxidation

Lipid peroxides are unstable and decomposed to a complex series of compounds. The most abundant compound is malonicdialdehyde (MDA). The MDA level of tissues was determined by spectrophotometric measurement (Stalnaja and Garishvili, 1985), using the TBA-test, based on the reaction of a chromogenic reagent, thio-barbituric acid (TBA) with MDA at 100 °C and two molecules of MDA reacting with one molecule of TBA to yield a stable threemethin complex dye. MDA concentration was measured at 532 nm with the CT-2600 spectrophotometer (CT-ChromTech, Taiwan).

2.9. Statistical analysis

For quantitative analysis of chemiluminescence intensity a Student's test was used to compare differences at each time point, considering $P<0.05$ as significant. All data were presented as mean ± S.E.M. (n=number of experiments).

3. Results

3.1. Obtustatin and crude Macrovipera lebetina obtusa venom inhibited the growth of S-180 sarcoma in mice

In the first stage of our experiment we modeled the S-180 sarcoma formation in the mouse. The cells transplanted

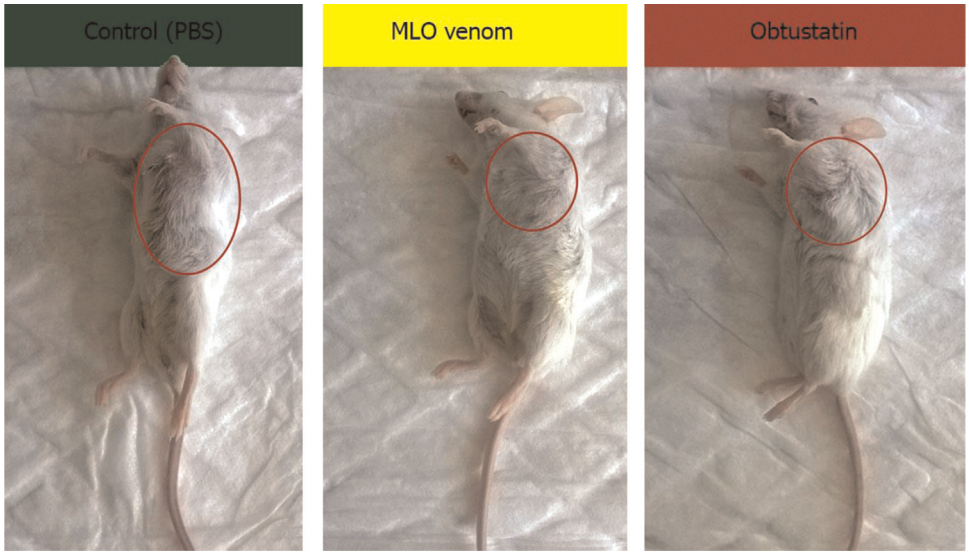

Fig. 1. Effective suppression of sarcoma growth in S-180 bearing mice was induced by *MLO* venom and obtustatin. After the sarcomas had reached 100–300 mm^3 in volume mice were randomly subdivided into three groups: model (tumor control), and two S-180 sarcoma groups treated with *MLO* venom and obtustatin. Each group contained 5 mice. The mice were administered as follows: model group, PBS; one obtustatin treated group, 1 mg/kg body weight obtustatin; one *MLO* venom treated group, each animal received small doses of *MLO* (10 μg/mouse). All solutions were dissolved in PBS and were administered daily by intra-tumor injection (50 μl) for 5 days. Twenty-four hours after the last drug administration, all animals were weighed and sacrificed by cervical dislocation. Tumor growth was significantly suppressed in mice treated with MLO venom compared to the control group and obtustatin. The red circle indicates the location and size of the S-180 sarcoma. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Body weight changes of S-180 sarcoma bearing mice with or without *MLO*, obtustatin treatment. The total body weights of the S-180 treated mice were similar to those of the model group, during treatment. Data are presented as the mean ± S.D., $n=5$.

Days	Samples	1	2	3	4	5	6
Body weight (g)	Control (PBS)	23.3 ± 0.6	22.9 ± 0.6	22.5 ± 0.6	22.3 ± 0.5	22.3 ± 0.4	22.7 ± 0.3
	MLO venom	25 ± 0.5	24.1 ± 0.8	23.3 ± 1.3	23.5 ± 1.6	23.7 ± 1.9	25.4 ± 2.3
	Obtustatin	25.3 ± 0.6	24.2 ± 0.6	23.2 ± 0.7	22.5 ± 0.8	22.3 ± 1.1	22.6 ± 1.1

subcutaneously into the right axilla of each mouse. When the tumor grew to 100–300 mm^3 we randomly divided mice into 3 groups: model (tumor control), and two S-180 sarcoma groups treated with *MLO* venom and obtustatin.

As shown in Fig. 1 *MLO* venom influence on S-180 sarcoma was the most powerful as compared with control and obtustatin groups. Furthermore, significant weight loss in the S-180 sarcoma-group mice was not observed indicating that there was no notable side effect on body weight during treatment (Table 1).

In vivo results have shown that S-180 sarcoma growth in mice was significantly inhibited by *MLO* venom and obtustatin with the inhibitory rate of 50% and 33% respectively (standard error ± 8 and ± 3 respectively).

3.2. Histological examination

For further evaluation of the antitumor effect of obtustatin and *MLO* venom in S-180 sarcoma bearing mice, tumor tissues from each group were removed and dissected for hematoxylin and eosin staining. Specimens from the obtustatin and *MLO* venom treated groups revealed that mean tumor value was substantially less than from that of the control group. More importantly, the configuration of tumor cells from the control group was compact, while that of the tumor cells in the S-180 sarcoma treated groups was slothful. Morphological changes in the S-180 sarcoma treated groups were also observed including focal necrosis, necrobiosis which is more abusive in the *MLO* treated group. In the place where tumors remained we noticed a nucleus polymorphism. The active part of the tumor volume is less than in the *MLO* venom

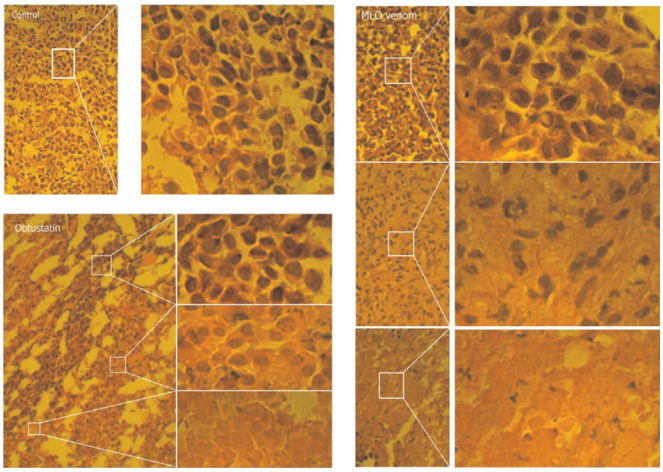

Fig. 2. Pathological changes in S-180 sarcoma tumor tissues from S-180 sarcoma bearing mice after administration of MLO venom and obtustatin. Each group contained 5 mice. The mice were administered as follows: model group, PBS; one obtustatin treated group, 1 mg/kg body weight obtustatin; one MLO venom treated group, each animal received small doses of MLO (10 μg/mouse). All the solutions were dissolved in PBS and were administered daily by an intra-tumor injection (50 μl) for 5 days. Twenty-four hours after the last drug administration, all the animals were weighed and sacrificed by cervical dislocation. Tumor tissues from mice treated with MLO venom contained large areas of necrosis and necrobiosis, compared with the obtustatin and control group. The configuration of tumor cells from the control group was compact, while that of the tumor cells in the S-180 sarcoma treated groups was slothful. Representative micrographs are shown 160 ×, 640 × respectively.

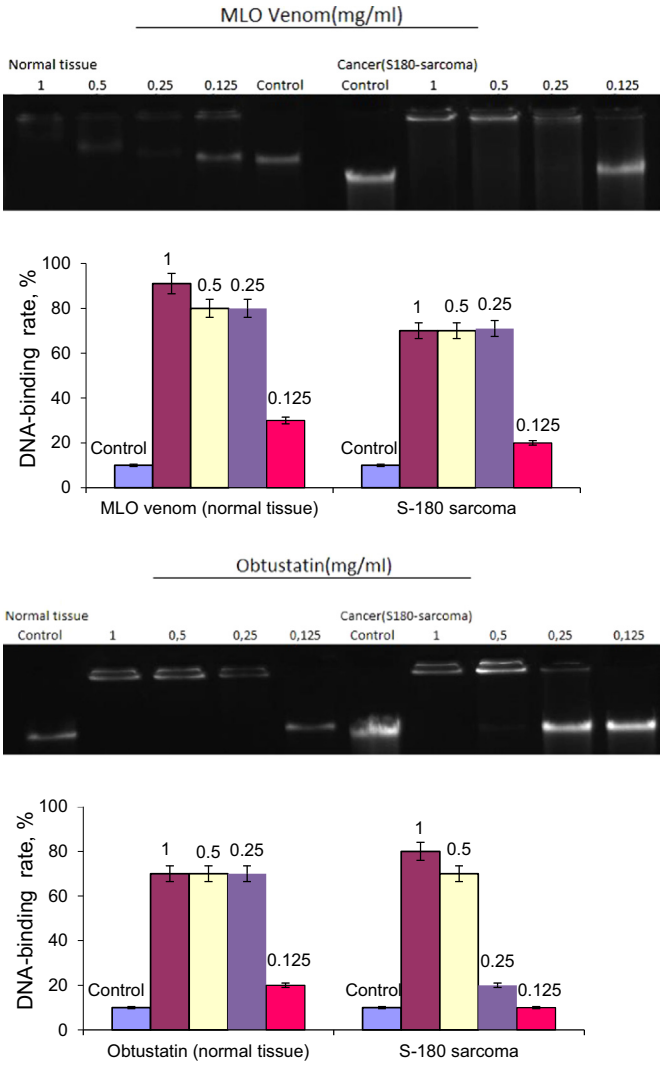

Fig. 3. Retarded electropheretic migration of *MLO* venom (above) and obtustatin (below) treated genomic DNA from S-180 sarcoma cells. Photograph of gel retardation assay of genomic DNA from S-180 sarcoma cells treated with *MLO* venom and obtustatin. Genomic DNA was extracted from S-180 sarcoma cells treated with *MLO* venom and obtustatin (0, 1, 0.5, 0.25, and 0.125 mg/ml) for 30 min and electrophoretically analyzed for 1 h. These results demonstrated that MLO venom and obtustatin were able to bind genomic DNA in S-180 sarcoma cells. Densitometric analysis of the relative ratios of genomic DNA levels in the gel retardation assay. The electrophoresis bands were photographed and analyzed using Image J software.

treated group compared with the obtustatin treated group and control group respectively (Fig. 2).

3.3. Obtustatin and crude Macrovipera lebetina obtusa venom binding to genomic DNA of the S-180 sarcoma cells

The results of the DNA retardation experiment (Fig. 3 above) demonstrated that both *MLO* venom and obtustatin were able to bind genomic DNA in S-180 sarcoma cells and suppress its electrophoretic mobility (genomic DNA was determined for a series of obtustatin and *MLO* venom concentrations in a SYBR green stained agarose gel). It is interesting that in case of *MLO* venom (at the same concentration) interaction with genomic DNA which was isolated from normal tissue interacts in a dose-dependent manner compared with the genomic DNA which was isolated from S-180 sarcoma cells (Fig. 3). In the second case 3 concentrations of the *MLO* venom (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml) have an analogous effect (DNA-binding rates were approximately 70%) which means that *MLO* venom interaction with genomic DNA from S-180 sarcoma cells was more potent.

In case of obtustatin (at the same concentration) interaction with genomic DNA isolated from normal tissue was very spectacular as 3 concentrations of the obtustatin (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml) have a similar effect and their DNA-binding rates were approximately 70%, the smallest concentration (0.125 mg/ml) barely interacts with DNA and DNA-binding rate was 20% (Fig. 3 below). Obtustatin interaction with DNA isolated from S-180 sarcoma cells was weaker. DNA-binding rate for 0.25 mg/ml and 0.125 mg/ml concentrations of obtustatin were approximately 20% and 10% respectively. These results indicated that obtustatin and *MLO* venom were able to bind to the genomic DNA of S-180 sarcoma cells and block the electrophoretic mobility of genomic DNA.

Summing up the above mentioned we can say that *MLO* venom might exert its inhibitory effect on S-180 sarcoma cells by binding genomic DNA and blocking gene expression.

3.4. Results of the chemiluminescence analysis and lipid peroxidation

The importance of lipid peroxidation processes in different cancers is already known: the imbalance between the pro-oxidants and antioxidants in favor of the former gives rise to oxidative stress that has been proven to lead to carcinogenesis (Battisti et al., 2008; Chang et al., 2008; Wauquier et al., 2009). *In vivo*, lipid peroxidation was measured in the tumor tissue of different experimental groups as an indicator of oxidative stress.

Our results have shown decreases in ChL intensity in all 2 treated group in course of *MLO* and obtustatin *in vivo* processing in comparison with control (Fig. 4). The data of ChL-analysis and TBA-test indicate antioxidant effect on all 2 groups which were

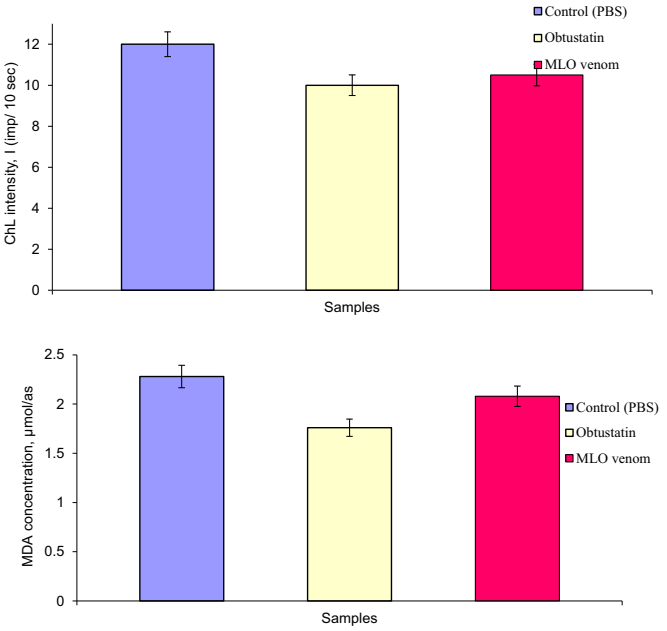

Fig. 4. Changes of spontaneous chemiluminescent levels and the concentration of malonic dialdehide of S-180 sarcoma tissues in course of *MLO* venom and obtustatin *in vivo* processing. Each group contained 5 mice. The mice were administered as follows: model group, PBS; one obtustatin treated group, 1 mg/kg body weight obtustatin; one *MLO* venom treated group, each animal received small doses of *MLO* (10 µg/mouse). All solutions were dissolved in PBS and were administered daily by an intra-tumor injection (50 µl) for 5 days. Twenty-four hours after the last drug administration, all the animals were weighed and sacrificed by cervical dislocation. Then the sarcoma was removed and homogenized for 5 min by homogenizator of Potter–Elvehejm in Tris –HCl buffer (pH 7.4) with a final concentration of 20 mg/ml. ChL intensity was significantly decreased in the mice treated with obtustatin and MLO venom compared to the control group (injected PBS).

treated with *MLO* venom and obtustatin. It is interesting that in healthy mice ChL intensity is high but MDA concentration was low compared with the mice which were a control S-180 sarcoma group (the date are not shown) (Fig. 4).

4. Discussion

Our previous results have shown the changes of native membrane condition and properties under *in vitro* and *in vivo* influence of the *MLO* venom (Ayvazyan et al., 2012; Zaqaryan et al., 2014). The addition of *MLO* venom to single mixed-lipid giant unilamellar vesicles from brain lipids of rats shows noticeable changes, both distortions in the vesicle membrane and shrinking of the vesicle size. These effects are likely to disturb the local packing structure of the lipids and fluidity of the bilayer (Ayvazyan and Ghazaryan, 2012; Ghazaryan et al., 2013, 2015). We decided to go further and develop these interesting results and to study how crude *MLO* venom and obtustatin influence was on the sarcoma model.

Sarcomas are defined as a type of cancerous connective tissue tumors (Dean and Whitwell, 2009). As results of different oxidative loads vary in tissues, the total antioxidant capacity varies. Individual tissues acquire their own antioxidant composition based on the oxidizing courses that it most likely would endeavor (Moller et al., 1996). Tumors arising in bone and soft tissue share common characteristic features (to not only common mesenchymal origin but also the anatomical surrounding). Soft tissue sarcomas are extracompartmental or found in an anatomical region that is not blocked off by anatomical barriers. The inhibitory effect of one of the *MLO* venom components obtustatin is already shown on the melanoma (Brown et al., 2008). However, whole crude *MLO* venom and obtustatin anti sarcoma effects have not been studied yet.

As our results have shown, *MLO* venom and obtustatin suppressed the S-180 sarcoma bearing mice tumor with 50% and 33% at the doses of 10 μg/mouse and 1 mg/kg/day respectively. This activity was confirmed by the hematoxyilin and eosin staining that showed morphological changes in the *MLO* and obtustatin treated groups including necrosis and necrobiosis and significant reduction of the number of tumor cells. Furthermore, significant weight loss in the treated groups was not observed which means that both obtustatin and *MLO* venom could be potential against sarcoma clinically with high efficiency and low toxicity. Besides, both obtustatin and *MLO* venom target additional anionic constituents of tumor cells, specifically genomic DNA. The results of the DNA retardation experiment demonstrated that obtustatin and *MLO* venom were able to bind to the genomic DNA of S-180 sarcoma cells and block the electrophoretic mobility of genomic DNA, which means they could be blocking gene expression. The results of our DNA retardation experiment demonstrated that obtustatincould bind to genomic DNA from the S-180 sarcoma cells and suppress its electrophoretic mobility in a dose-dependent manner while MLO venom binding rate to DNA increased to 70% as the concentration of venom increased to 0.25 μg/ml, which was less than the LD50 almost 75 times. These data indicated that, except of obtustatin, there is some other component of MLO venom, which significantly binds to genomic DNA.

Free radicals attack lipids mainly polyunsaturated fatty acids, giving rise to lipid peroxides that play an essential role in cell division regulation (Battisti et al., 2008). As a result MDA formed from lipid peroxidation functions as a tumor promoter and cocarcinogenetic agent and has the ability to hinder the role of antioxidant enzymes. The direct correlation between lipid peroxidation and cell proliferation with increased lipid damage in highly proliferated cells has been noted (Niedernhofer et al., 2003). In our study both ChL and MDA decrease in the two treated groups are compared with the model group which corroborate well with the oxidative stress of patients suffering from bone and soft tissue sarcomas (Nathan et al., 2011).

This is a new perspective direction for our research and our findings need to be confirmed by further experiments. We are planning to analyze the anti-angiogenic effect of MLO in complex with expression of the VEGF which is believed to be one of the most specific and pivotal regulators of the angiogenic signaling cascade (Carmeliet and Jain, 2000, 2011; Ferrara, 2002; Folkman, 2003; Naldini and Carraro, 2005; Lijnen, 2008), besides, we are going to study the antioxidant defense of the control and treated groups. In conclusion, as MLO venom is a complex of proteins we are planning to isolate other active, single components of this venom and to test those components on cancer cells and maybe we can find a more specific inhibitor for cancer cells but in this stage of our experiments our results suggested that obtustatin and *MLO* venom might be a potential candidate for the treatment of sarcoma, and might prevent tumor growth *in vivo* with low toxicity.

Acknowledgments

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