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

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ABSTRACT

The aim of this study was to evaluate the anti-tumor activity of Amblyomin-X, a serine protease Kunitz-type inhibitor. Amblyomin-X induced tumor mass regression and decreased number of metastatic events in a B16F10 murine melanoma model. Alterations on expression of several genes related to cell cycle were observed when two tumor cell lines were treated with Amblyomin-X. PSMB2, which encodes a proteasome subunit, was differentially expressed, in agreement to inhibition of proteasomal activity in both cell lines. In conclusion, our results indicate that Amblyomin-X selectively acts on tumor cells by inducing apoptotic cell death, possibly by targeting the ubiquitin-proteasome system.

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1. Introduction

Some natural products extracted from plants, herbs, and other living organisms are recognized to possess efficacy as potential anticancer drugs (Shiah et al., 2007). Ticks could be a source of such natural products. Tick saliva contains a wide range of physiologically active molecules that participate in several physiological processes, such as coagulation and fibrinolysis, immunity and inflammation, and angiogenesis [(Hovius et al., 2008) and (Francischetti et al., 2009)]. The Amblyomma cajennense tick is classified as a member of the hard ticks (Ixodidae) family and is considered to be one of the most important and widespread tick species because of its low host-specificity and ability to feed on all classes of vertebrates, including man (Aragão, 1936).

A cDNA library of the A. cajennense salivary glands was constructed (Batista et al., 2008) and used to identify a gene encoding a Kunitz-type protease inhibitor (GenBank accession AAT68575). A recombinant protein, named Amblyomin-X (from A. cajennense inhibitor of Factor Xa) was overexpressed in E. coli (Batista et al., 2010). The expressed protein is able to inhibit Factor Xa (FXa) from coagulation and also promotes cytotoxic activity on several tumor cells, among them pancreatic and melanoma cells.

Coagulation inhibitors acting on tumor cells are described in the literature and also an association between cancer and thrombosis has been recognized for more than a century. However, the manner by which tumor growth is regulated by coagulation in vivo remains unclear. Coagulation factor inhibitors from haematophagous organisms have been studied in tumor lines cultures [(Hembrough
et al., 2003) and (Tuszynski et al., 1987)]. Cutaneous melanoma is one of the fastest rising malignancies in the last several decades. In contrast to many other cancer types, melanoma affects a relatively younger population and is notorious for its propensity to metastasize and poor response to current therapeutic regimens (Li et al., 2002). It is also recognized that 95% of melanoma cases are curable if diagnosed early and surgically excised. The treatment options for metastatic melanoma are limited, as the tumors are radio and chemo-resistant, and hence resistant to the anti-tumor activity of Amblyomin-X (Satyamoorthy and Herlyn, 2002). Pancreatic adenocarcinoma also stands out as a highly lethal disease that is usually diagnosed at an advanced state and is one of the leading causes of cancer-related death. The natural resistance of pancreatic adenocarcinoma to chemotherapy and radiotherapy makes the survival rates of affected individuals very low (less than 5%). Both adjuvant chemotherapy and radiation therapy are not very effective, extending survival time by an average of 3–6 months (Nawrocki et al., 2004).

We have shown that Amblyomin-X elicits cell death in several tumor cell lines especially in those derived from solid tumors (PI 0406057–1). In the present work we show that Amblyomin-X has an anti-tumor activity in vivo by decreasing tumor mass and reducing metastasis in a murine melanoma model. In addition, we show that Amblyomin-X has a pro-apoptotic activity in other SK-Mel-28 (melanoma) or Mia-PaCa-2 (pancreas adenocarcinoma) cell lines. The increased expression of the proteasome β2 catalytic subunit gene (PSBM2), decreased proteasomal activity and increased pool of poly-ubiquitinated proteins in both cell lines suggest that the proteasome is targeted by Amblyomin-X, leading to cell death and providing a probable mechanistic explanation for the pro-apoptotic effect elicited by Amblyomin-X.

2. Material and methods

2.1. Amblyomin-X

The Amblyomin-X is a 15 kDa protein obtained in a recombinant form, as described before (Batista et al., 2010).

2.2. Tumor model

A murine B16F10 (H2b) variant of the B16 melanoma cell line originated from C57BL/6J mice was used in the experiments. Murine melanoma B16F10 cells (American Type Culture Collection ATCC – CRL 6475) were cultured according to ATCC recommendations. Cell viability was determined by the Trypan Blue exclusion test, which showed that more than 95% of the cells were viable.

Male C57BL6 mice (25 g) were housed at 22 ± 2 °C under a 12/12 h light/dark cycle with free access to food and water. Animal handling and experimental procedures were conducted according to institutional approved guidelines (Butantan Institute Ethics Committee). B16F10 cells (5 × 10^5) suspended in saline were injected subcutaneously into the flank regions of mice (n = 10 per group) to induce tumor formation. Ten days after injection (3–5 mm diameter of the tumor mass), treatment with Amblyomin-X (1.0 mg/kg/day) or vehicle (saline) by intra-peritoneal injection was initiated. The treatment was continued for 14 days. Tumor size was measured daily using a caliper-like instrument and converted to tumor volume by the equation: volume = length × width^2/2. The anti-tumor activity was assessed according to the guidelines established by the National Cancer Institute. Data are expressed as means ± SD.

2.3. Metastasis analysis

C57BL6 mice were intravenously injected with 5 × 10^4 tumor cells. After ten days, treatment with Amblyomin-X (1.0 mg/kg/day) or vehicle (saline) by intra-peritoneal injection was initiated and continued for 14 days (n = 10 per group). Metastasis quantification was performed by macroscopic analysis using digital photographic documentation and by histopathological analysis of all internal organs and primary dorsal tumors. Animals underwent autopsy at day 26 after cell implantation and metastatic foci were assessed. Statistic analyses were carried out using ANOVA followed by the multiple comparative Tukey–Kramer test. A significance threshold of p < 0.05 was used. The values are expressed as means ± SD.

2.4. Cell culture

Human melanoma (SK-Mel-28) and primary pancreatic adenocarcinoma (Mia-PaCa-2) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to ATCC recommendations. Human fibroblasts were obtained from Centro de Estudos do Genoma Humano (Instituto de Biociência da Universidade de São Paulo – IB-USP). Cells were treated with 0.5 and 1.0 μM of Amblyomin-X for 24 and 48 h and the cell viability was evaluated by the MTT assay (Mosmann, 1983). After treatment, cells were pelleted and resuspended in 50 mM Tris–HCl, pH 7.5 containing 150 mM NaCl, 1 mM EGTA, 10% (v/v) glycerol, 0.5% (v/v) Nonidet P-40, and 1 mM MgCl2. The cells were lysed by passing the suspension through insulin-syringes (10 times) followed by 30-min incubation on ice. The samples were centrifuged at 15,000 × g for 30 min, and the supernatant was used for the biochemical determinations.

2.5. Gene expression analysis

Total RNA was isolated from Amblyomin-X-treated cells and untreated control cells using TRIzol reagent (Invitrogen, Carlsbad, CA), as recommended by the manufacturer. The integrity of RNA samples was assessed by analysis of 28S/18S rRNA ratios following capillary electrophoresis (2100 Bioanalyser, Agilent Technologies Inc). Ratios equal to or greater than 1.5 were considered satisfactory. Gene expression measurements were performed using human Whole Genome Code Link Bioarrays (GE Healthcare, Chandler, AZ) containing approximately 55,000 30-mer probes, using reagents and protocols recommended by the manufacturer.

For each cell line, six hybridizations were performed for each condition (Amblyomin-X-treated or untreated control
cells) using cRNA targets obtained from independent preparations. Cy3-labeled hybridized targets were detected with an arrayWoRx scanner (Applied Precision Inc., WA, USA).

A set of 11,606 (Mia-PaCa-2) or 7854 (SK-Mel-28) probes that showed valid measurements (i.e., above the average array background) in at least five out of six (Mia-PaCa-2) or four out of five (SK-Mel-28) independent replicate hybridizations with Amblyomin-X-treated or untreated control samples were further analyzed. For each cell line, the intensity data obtained from different hybridizations were normalized using the quantile method (Bolstad et al., 2003).

A Signal-to-Noise Ratio (SNR) metric with statistical significance ascertained by bootstrap resampling following 10,000 random permutations was used to identify gene expression changes induced by Amblyomin-X treatment (Golub et al., 1999). Functional analysis of gene expression changes in the context of known biological responses and regulatory networks was performed by Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Mountain View, CA). A p ≤ 0.05 (Fisher’s exact test) and used to select significantly enriched biological function/pathways. Raw and normalized microarray intensities were deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE18124.

2.6. Apoptosis assay

The sensitivity of tumor cells to apoptosis was determined by two-color fluorescence-activated cell sorting (FACS) analysis using propidium iodide and FITC–conjugated Annexin V (Sigma). The cells were incubated with Amblyomin-X (0.5 and 1 μM) or saline (24 and 48 h). Aliquots of freshly collected cells suspended in PBS were centrifuged (1000 rpm, 10 min) and resuspended in binding buffer, pH 7.4 containing 10 mM HEPES (Sigma), 140 mM NaCl, and 2.5 mM CaCl2 at approximately 1 × 106 cells/mL. Fluorescein isothiocyanate (FITC)-annexin V (5 μL) and PI (10 μL, 50 μg/mL in PBS) were then added to the cells, and then incubated for 10 min at room temperature in the dark. The fluorescence of the cells was immediately assessed in a flow cytometer (Guava Easy Cyte Mini, Guava Technologies).

2.7. Cell cycle analysis

Exponentially growing cells (1 × 106) were incubated with 0.5 and 1 μM Amblyomin-X for 24 and 48 h. Cells kept without the drug were used as a control. Amblyomin-X–treated and control cells were harvested by trypsin, washed in cold PBS, and resuspended in 70% ethanol for about 16 h at 4 °C, and washed again three times with cold PBS. The cells were stained (staining solution: 9.5 mL of 0.1% Triton X-100, 0.2 mL of 10 mg/mL RNase A, and 0.25 mL of 1 mg/mL propidium iodide). The fractions of cells in G0/G1, S, and G2/M phase were analyzed by the cell cycle software Guava Express Plus (Guava Technologies).

2.8. Proteasome activity

Fluorogenic peptides (AMC, 7-amido-4-methylcoumarin as the fluorescent probe; Calbiochem, San Diego, CA, USA) were utilized to determine proteasomal activity. Suc-LVY-AMC was utilized as a standard peptide to assess the chymotrypsin-like activity and z-ARR-AMC to determine the trypsin-like activity. Cell extracts (25–100 μg protein) obtained as described above were incubated at 37 °C with 125–500 μM of the fluoropeptides. Fluorescence emission at 440 nm (excitation at 365 nm) was recorded for 1 h. Results are expressed as the percentage of hydrolysis measured in total extracts from untreated control cells and were calculated based on the difference between the total hydrolytic activity of the cell extracts and the activity determined in samples previously incubated with standard proteasome inhibitors (lactacystin or NLV). Experiments were performed in triplicate.

2.9. Immunoassays

Immunoblotting was performed as described by the protocol supplied with the ECL Western Blotting System (GE Biosciences, Piscataway NJ-USA). Membranes containing aliquots of total soluble protein of cellular samples were hybridized with anti-ubiquitin (Santa Cruz Biotechnologies California USA) and anti-actin (ICN Biomedicals, Inc., Costa Mesa, CA, USA) antibodies. Bound primary antibodies were detected following incubation with horseradish-peroxidase-conjugated secondary antibodies using enhanced chemiluminescence Western Blotting Detection Reagents (GE Biosciences).

3. Results

3.1. Effect of Amblyomin-X on tumor growth and metastasis in a murine melanoma model

Ten days after tumor implantation, the animals were daily treated with Amblyomin-X, as described in Materials and Methods. The tumor mass progression was significantly decreased when compared to the control group (Fig. 1a).

A considerable reduction in the percentage of internal metastasis was observed in Amblyomin-X-treated animals. The presence of metastatic nodules in the lung, kidney, and lymph nodes as well as splenic metastasis decreased 60% in treated animals compared to the metastatic lesions distributed in the internal organs of the control group (Fig. 1b). Macroscopic analysis of the pulmonary parenchyma showed that the diameter of the nodules in the control group varied between 0.1 and 0.3 cm. In the Amblyomin-X-treated group, besides presenting a smaller number of tumors, the nodule diameter varied between 0.1 and 0.2 cm and lacked the characteristics of invasion and dissemination. Reduction in tumor volume and mass was also observed in most individuals (Fig. 1c). Furthermore, tumor regression was detected (Fig. 1d and e).

3.2. Cell cycle and cytotoxicity analyses

Our next step was to evaluate Amblyomin-X effects in vitro, by examining its effect on the cycle progression and viability of two tumor cell lines, derived from melanoma...
(SK-Mel-28) or pancreatic adenocarcinoma (Mia-PaCa-2), and in non-transformed fibroblasts.

Both SK-Mel-28 and Mia-PaCa-2 cells presented an apparently time and Amblyomin-X concentration-dependent death. Analysis of cell morphology after 4 h of treatment revealed slight changes and loss of cell adhesion. After 24 h of treatment, loss of inter-cell elongations and dispersed cell aggregates were observed. Forty-eight hours after treatment, cell contraction, loss of adhesion, and the formation of many cell aggregates were verified (Fig. 2a). Conversely, when non-transformed fibroblasts were assayed under the same conditions no cytotoxic effect was observed. Moreover, the morphology of these cells did not change even after 48 h of treatment (Fig. 2b).

We also evaluated the presence of apoptotic cells after Amblyomin-X treatment, as measured by Annexin-V/PI binding. Apoptosis was verified 24 h after treatment in both tumor cells (Fig. 3a and b).

To elucidate the mechanism of cell damage in both SK-Mel-28 and Mia-PaCa-2 cells, we investigated whether the effects of Amblyomin-X were associated with specific changes in cell cycle progression. The DNA content assessed by PI staining revealed increased percentage of cells in G0/G1 and decreased percentage in S or G2/M phases after Amblyomin-X treatment, as compared to untreated control cells (Table 1).

3.3. Gene expression profiling of Mia-PaCa-2 and SK-Mel-28 cells exposed to Amblyomin-X

To highlight the molecular pathways that are involved in the cytotoxic effects of Amblyomin-X, we investigated the changes in global gene expression in Mia-PaCa-2 and SK-Mel-28 cells treated for 4 h with 1 μM Amblyomin-X. After data extraction and normalization, 1091 Mia-PaCa-2 genes and 465 SK-Mel-28 were differentially expressed, compared to their respective controls ($p < 0.05$). Genes modulated by Amblyomin-X exposure in both cell lines were enriched in networks affecting cell death, cell growth, proliferation and cell cycle. Among the genes differentially expressed in Mia-PaCa-2 cells, over expression of Histone deacetylase 4 (HDAC4) and anti Müllerian hormone (AMH) has been associated with increased apoptosis [(Hoshiya et al., 2003) and (Liu et al., 2004)]. Over expression of NGF inducible anti proliferative protein PC3 or BTG family member 2 (BTG2) was shown to have an anti proliferative effect on prostate cancer cells (Tsui et al., 2008) and to cause cell cycle arrest in breast cancer cells (Kawakubo et al., 2004). Among the genes differentially expressed in SK-Mel-28 cells is Oxidative stress included growth inhibitor 1 (OSGIN1) whose over expression has been shown to increase cell death (Ong et al., 2004) and to inhibit cell proliferation (Huynh et al., 2001).
Fig. 2. (a) Cytological aspects of SK-Mel-28 (up) and Mia-PaCa-2 (down) treated with Amblyomin-X (0.5 and 1.0 μM) for 4 h, 24 h and 48 h. 4 h treatment: slight changes and a loss of cell adhesion; 24 h treatment: loss of inter-cell elongations and dispersed cell aggregates formation; 48 h treatment, cell contraction, loss of cell adhesion and formation of many cell aggregates. (b) Cytological aspects fibroblasts treated with Amblyomin-X (0.5 and 1.0 μM) 48 h. No cytotoxic effects were observed, the morphology of the normal cells did not change even after 48 h.
Fig. 3. Induction of apoptosis by Amblyomin-X in (a) SK-Mel-28 and (b) Mia-PaCa-2 cells. The cells were incubated with 0.5 and 1.0 μM Amblyomin-X for 24 and 48 h and analyzed by flow cytometry using propidium iodide and FITC-conjugated Annexin-V. Quadrants 1–4: viable cells; early apoptotic cells; late apoptotic or necrotic cells; dead cells, respectively.
Interestingly, we observed that the expression of the gene encoding the proteasome catalytic subunit (PSMB2) was significantly increased in Mia-PaCa-2 as compared to SK-Mel-28 cells. Amblyomin-X treatment and displayed inverted patterns of expression changes (Bandyopadhyay et al., 2002), responded differently to Receptor III (Betaglycan, 300 kDa) \((\text{TGFBR3})\) and Transforming Growth Factor, Beta some genes associated with metastatic potential, such as \(\text{SHOX2}\) and \(\text{SCD5}\), responded differently to Amblyomin-X \(0.5 \text{M}\) Amblyomin-X (according to statistical analysis; Fig. 4a), both concentrations used \(0.5 \text{M}\) and \(1 \text{M}\) significantly increased the pool of poly-ubiquitinylated proteins (Fig. 4b and c). Although the trypsin-like activity decreased only after cell incubation with \(1 \mu \text{M}\) Amblyomin-X (Fig. 4a), in agreement with an increased pool of poly-ubiquitinated proteins (Fig. 4b and c). However, 24 h later both trypsin- and chymotrypsin-like proteasomal activities decreased (Fig. 4d) along with an increased pool of poly-ubiquitinated proteins (Fig. 4e and f) after treatment with both Amblyomin-X concentrations tested. Similar results were observed in both cell lines after Amblyomin-X treatment (Table 2). While the trypsin-like activity decreased only after 4 h of incubation of the cells with \(1 \mu \text{M}\) Amblyomin-X (Fig. 4a), in agreement with an increased pool of poly-ubiquitinylated proteins (Fig. 4b and c).

### Table 1
Cytotoxic and Cell Cycle effects induced by Amblyomin-X treatment in SK-Mel-28 and Mia-PaCa-2 cells.

<table>
<thead>
<tr>
<th></th>
<th>SK-Mel-28</th>
<th>4h</th>
<th>Mia-PaCa-2</th>
<th>4h</th>
</tr>
</thead>
<tbody>
<tr>
<td>%G0/G1</td>
<td>100</td>
<td>53.5</td>
<td>100</td>
<td>51.5</td>
</tr>
<tr>
<td>%S</td>
<td>16.5</td>
<td>29.0</td>
<td>18.0</td>
<td>20.5</td>
</tr>
<tr>
<td>%G2/M</td>
<td>27.0</td>
<td>100</td>
<td>18.0</td>
<td>27.0</td>
</tr>
</tbody>
</table>

Cell lines were incubated with the indicated concentrations of Amblyomin-X for 4, 24 and 48 h and the viability was measured by MTT reduction as described in Materials and Methods and cell cycle distribution of Amblyomin-X-treated cells and non-treated cells are expressed as percentage of three independent experiments. Results are expressed as percentage of total cells.

Remarkably, only 24 genes were altered in both cell lines after Amblyomin-X treatment (Table 2). While the expression changes observed for these 24 transcripts in both cell lines were predominantly in the same direction, some genes associated with metastatic potential, such as Antigen CD49C, Alpha 3 Subunit of VLA-3 Receptor \((\text{ITGA3})\) (Wang et al., 2004) and Transforming Growth Factor, Beta Receptor III \((\text{TGFBR3})\) (Bandopadhyay et al., 2002), responded differently to Amblyomin-X treatment and displayed inverted patterns of expression in Mia-PaCa-2 as compared to SK-Mel-28 cells. Interestingly, we observed that the expression of the gene encoding the proteasome beta2 catalytic subunit \((\text{PSMB2})\) was upregulated in both cell lines following Amblyomin-X treatment. This observation suggests that apoptosis induced by Amblyomin-X may result from perturbation of proteasome complex, thus prompting us to further investigate the effects of Amblyomin-X on proteasome function, as described below.

### Table 2
Twenty four genes differentially expressed in MIA PaCa-2 and SK-Mel-28 cells after treatment with Amblyomin-X \((\text{SNR } p < 0.05)\).

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene name</th>
<th>Log2 treated/control (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL273703</td>
<td>Spliced EST; nearby phospholipase C, beta 2 (PLCB2) locus</td>
<td>SK-Mel-28 -0.4</td>
</tr>
<tr>
<td>AK024243</td>
<td>Hypothetical gene supported by AK024243</td>
<td>SK-Mel-28 -0.5</td>
</tr>
<tr>
<td>AX747345</td>
<td>Homo sapiens cDNA FLJ43579 bss, clone NT2NE2001874</td>
<td>SK-Mel-28 -0.3</td>
</tr>
<tr>
<td>BC022082</td>
<td>Homo sapiens cDNA clone IMAGE: 4824304</td>
<td>SK-Mel-28 -0.5</td>
</tr>
<tr>
<td>BIN2</td>
<td>Bridging integrator 2</td>
<td>SK-Mel-28 0.4</td>
</tr>
<tr>
<td>CAPRIN1</td>
<td>GPI-anchored Membrane Protein 1</td>
<td>SK-Mel-28 0.4</td>
</tr>
<tr>
<td>CRYZL1</td>
<td>Crystallin, Zeta (Quinone Reductase)-Like 1</td>
<td>SK-Mel-28 -0.4</td>
</tr>
<tr>
<td>DAAM1</td>
<td>Dishevelled Associated Activator of Morphogenesis 1</td>
<td>SK-Mel-28 -0.4</td>
</tr>
<tr>
<td>DTD1</td>
<td>Histidyl-tRNA Synthetase 2</td>
<td>SK-Mel-28 0.9</td>
</tr>
<tr>
<td>DYNC1L2</td>
<td>Dynactin, Cytoplasmic 1, Light Intermediate Chain 2</td>
<td>SK-Mel-28 2.5</td>
</tr>
<tr>
<td>FAM134B</td>
<td>Hypothetical Protein FLJ20152</td>
<td>SK-Mel-28 -0.9</td>
</tr>
<tr>
<td>ITGA3</td>
<td>Integrin, Alpha 3 (Antigen CD49C, Alpha 3 Subunit of VLA-3 Receptor)</td>
<td>SK-Mel-28 0.7</td>
</tr>
<tr>
<td>LOC441178</td>
<td>Hypothetical LOC441178</td>
<td>SK-Mel-28 -0.4</td>
</tr>
<tr>
<td>MAP1A</td>
<td>Microtubule-Associated Protein 1A</td>
<td>SK-Mel-28 -0.7</td>
</tr>
<tr>
<td>MYO1D</td>
<td>Myosin ID</td>
<td>SK-Mel-28 -0.3</td>
</tr>
<tr>
<td>PLCG2</td>
<td>Phospholipase C, Gamma 2 (Phosphatidylinositol-Specific)</td>
<td>SK-Mel-28 -0.4</td>
</tr>
<tr>
<td>PRKACB</td>
<td>Protein kinase, cAMP-dependent, catalytic, beta</td>
<td>SK-Mel-28 -0.4</td>
</tr>
<tr>
<td>PSMB2</td>
<td>Proteasome (Prosome, Macropain) Subunit, Beta Type, 2</td>
<td>SK-Mel-28 1.2</td>
</tr>
<tr>
<td>SCD5</td>
<td>Stearoyl-CoA Desaturase 5</td>
<td>SK-Mel-28 0.5</td>
</tr>
<tr>
<td>SHOX2</td>
<td>Short Stature Homeobox 2</td>
<td>SK-Mel-28 -1.4</td>
</tr>
<tr>
<td>TGFBR3</td>
<td>Transforming Growth Factor, Beta Receptor III (Betaglycan, 300 kDa)</td>
<td>SK-Mel-28 0.5</td>
</tr>
<tr>
<td>TLD1</td>
<td>TLD Domain Containing 1</td>
<td>SK-Mel-28 -0.4</td>
</tr>
<tr>
<td>TXNDC9</td>
<td>Thioredoxin Domain Containing 9</td>
<td>SK-Mel-28 -0.7</td>
</tr>
<tr>
<td>VPS3</td>
<td>Vacuolar Protein Sorting 53 Homolog ((S). cerevisiae)</td>
<td>SK-Mel-28 -0.5</td>
</tr>
</tbody>
</table>

### 3.4. Proteasome function

The ubiquitin-proteasome system was examined in the cell extracts of SK-Mel-28 and Mia-PaCa-2 cells after 4 h and 24 h of Amblyomin-X treatment. Results obtained from representative experiments with the SK-Mel-28 cell line are depicted in Fig. 4. As observed, only the proteasomal trypsin-like activity decreased after 4 h of incubation of the cells with \(1 \mu \text{M}\) Amblyomin-X (Fig. 4a), in agreement with an increased pool of poly-ubiquitinated proteins (Fig. 4b and c). Although the trypsin-like activity decreased only after cell incubation with \(1 \mu \text{M}\) Amblyomin-X (according to statistical analysis; Fig. 4a), both concentrations used \(0.5 \text{M}\) and \(1 \mu \text{M}\) significantly increased the pool of poly-ubiquitinylated proteins (Fig. 4b and c). However, 24 h later both trypsin- and chymotrypsin-like proteasomal activities decreased (Fig. 4d) along with an increased pool of poly-ubiquitinated proteins (Fig. 4e and f) after treatment with both Amblyomin-X concentrations tested. Similar results were observed.
were obtained using the Mia-PaCa-2 cell line (Supplementary Figure 1). Notably, no proteasome inhibition was observed in the extracts of fibroblast cells incubated with same Amblyomin-X concentrations (not shown). Taken together, these findings suggest an important alteration of the ubiquitin-proteasome system of both tumor cell lines following exposure to Amblyomin-X, in accordance with the described cellular death.

One important question raised from the results described above was whether Amblyomin-X interacts directly with proteasome components, which would support the notion that the proteasome is an Amblyomin-X cellular target. To test this hypothesis, Amblyomin-X (20 μg) was in vitro incubated for 2 h in the presence of purified proteasome preparations (10 μg). After incubation, Amblyomin-X was washed out through filtration (Millipore) and re-dilution. Finally, these purified proteasome samples incubated with Amblyomin-X in vitro were assayed for the chymotrypsin-, trypsin- and caspase-like activities. In agreement with results obtained with cellular extracts, only the trypsin-like activity was 70% inhibited when compared to control samples, i.e., purified proteasome preparations incubated for 2 h in the absence of Amblyomin-X followed by filtration and re-dilution (data not shown). Similar experiments were performed to follow Amblyomin-X degradation by the proteasome (Supplementary Figure 2). Taken together, these results strongly suggest that the proteasome might be one Amblyomin-X cellular target.

4. Discussion

Many drugs are used to treat melanoma and pancreatic adenocarcinoma, but new therapies must be developed, as the existing ones often provide unsatisfactory results (Bold et al., 2001). Since we had already observed a pro-apoptotic role of Amblyomin-X upon several tumor cell lines, herein we examined its effect in a cutaneous melanoma model. In vivo experiments demonstrated that a 14-day Amblyomin-X treatment promoted a decrease in tumor mass progression without visible changes in the normal tissues (Fig. 1).

Here we investigated the mechanisms underlying the anti-tumor activity of this novel protein, a Kunitz-type serine protease inhibitor encoded in the transcriptome of the tick *A. cajennense* (Batista et al., 2008 and Batista et al., 2010). The recombinant protein obtained was found to be similar to another FXa inhibitor isolated from the *Ixodes scapularis* tick (Francischetti et al., 2002) and to share similarity with the Kunitz-type domain of the Tissue Factor Pathway Inhibitor (TFPI) (Salemink et al., 1999). Tissue factor pathway inhibitor (TFPI) is a multivalent Kunitz-type protein that inhibits FXa via its second domain and factor Vlla/TF complex via its first domain. TFPI is able to inhibit the extrinsic pathway and affects both PT and APTT tests (Han et al., 1999). Amblyomin-X was able to inhibit the Factor Xa activity and also extended global coagulation tests such as APTT, PT and PCA of normal human plasma (Batista et al., 2010 and Supplementary Figure 3). This capability together with the structural
features of the molecule, suggests that this protein can be classified as a member of the TFPI family. Inhibitors of the TF/FVIIIa complex, including TFPI, have been shown to possess a potent anti-tumor and anti-angiogenesis activity independent of their anti-thrombotic effect, leading to an additional non-hemostatic mechanism. Thus, because of the anti-tumor activity and similarity with TFPI, the effect of Amblyomin-X on cell death was analyzed.

The physiological role of the Amblyomin-X in the tick is difficult to discuss at this moment, because the native protein that corresponds to our molecule was not yet characterized, but, as Amblyomin-X is an anticoagulant protein probably it is enrolled in the feeding process.

To evaluate the mechanism of action of Amblyomin-X on cells, we tested its effect in two tumor cell lines: the melanoma SK-Mel-28 and the pancreas adenocarcinoma Mia-PaCa-2. Amblyomin-X was able to reduce cell viability in a time and dose-dependent manner. Interestingly, the protein did not reduce the viability of normal cells under the same experimental conditions (Fig. 1b). Moreover, the MTT assay and flow cytometry (Annexin-V/PI) confirmed cell death through an apoptotic mechanism, respectively. Because apoptosis is a way to eliminate abnormal cells without activating the immune system, agents that selectively promote apoptosis in tumor cells are especially advantageous when dealing with aggressive tumors.

Global gene expression changes in both cell lines after exposure to Amblyomin-X were observed. Although both cell lines presented transcriptional changes in genes related to biological processes that affect growth and survival, only a few of these genes were common to both cell lines. The overlap observed between genes differentially expressed in both cell lines was quite small (Table 2). Notably, PSMB2, which encodes the proteasomal catalytic subunit β2, was consistently upregulated upon Amblyomin-X exposure in both cell lines studied here. Inhibition of the proteasome is a pro-apoptotic event, as attested by countless reports in the literature ([Adams, 2003] and [Moore et al., 2008]). Although still not well understood, inhibition of proteasomal activity is known to result in the differential killing of many tumor cells (Moore et al., 2008). Furthermore, according to the data in the literature (Satyamoorthy and Herlyn, 2002) and to the results of experiments performed in this work, conventional proteasome inhibitors fail to induce apoptosis at least in the SK-Mel-28 cell line (results not shown). Nevertheless, our results showed that Amblyomin-X treatment modifies the ubiquitin-proteasome system in both cell lines, causing cell death via apoptosis (Fig. 4 and Supplementary Figure 1).

The 20S catalytic particle contains six active sites that present chymotrypsin-like, trypsin-like, and caspase-like activities and a threonine as the active site. According to the present results, Amblyomin-X treatment promoted partial loss of the trypsin-like proteasomal activity just after 4 h of incubation (Fig. 4a and Supplementary Figure 1a) and of both chymotrypsin- and trypsin-like activities after 24 h (Fig. 4d and Supplementary Figure 1d). In agreement with the partial loss of catalytic activity, an increased pool of poly-ubiquitinated proteins was observed in response to all treatments (Fig. 4b and Supplementary Figure 1b and c).

It is noteworthy that all conventional proteasome inhibitors, which are largely utilized to promote apoptosis, promptly inhibit the chymotrypsin-like proteasomal activity differing from Amblyomin-X, which preferentially inhibited the proteasomal trypsin-like activity in both cell lines here described (Fig. 4 and Supplementary Figure 1). Trypsin-like activity is most likely associated with a specific regulation of the cell cycle, including the apoptotic response, in the tumor cell lines studied here. It has been demonstrated that proteasome inhibition may lead to transcriptional activation of proteasome genes in an auto-regulated feedback loop (Meiners et al., 2003). In fact, we observed that the decreased 20S proteasome function correlates with an increased expression of the β2 subunit gene in Amblyomin-X-treated cells.

Our in vitro experiment showing 70% inhibition of the trypsin-like proteasomal activity after incubation of Amblyomin-X with purified preparations of the proteasome catalytic unit suggests that the 20S proteasome is probably the one intracellular target of Amblyomin-X, resulting in partial proteasomal catalytic inhibition. Proteasome inhibition may in turn induce de novo formation of the catalytic 20S proteasome by transcriptional activation, as supported by our data showing increased expression of genes related to the 20S proteasome subunit (Table 2). Interestingly, Amblyomin-X is degraded in vitro by purified preparations of the 20S catalytic core particle (Supplementary Figure 2). Moreover, preliminary results showed that a peptide sequence of Amblyomin-X, which is not related to the Kunitz domain, inhibited the trypsin-like activity of purified proteasome preparations. We hypothesize that Amblyomin-X intracellular hydrolysis by the proteasome generates a peptide fragment that is resistant to further degradation and would therefore promote site-specific proteasome inhibition. This possibility is currently being investigated in our laboratory.

Our findings document the profound cytotoxic effect of Amblyomin-X against tumor cells specially demonstrated in melanoma cells. Although further investigation is warranted, we propose that Amblyomin-X targets the ubiquitin-proteasome system perturbing its catalytic activity and ultimately leading to the apoptosis of tumor cells.

5. Conclusion

Amblyomin-X is a recombinant protein with cytotoxic activity on tumor cells and little or no activity on normal cells. Global gene expression as well as cytotoxicity, cell cycle modulation and ubiquitin-proteasome system function were evaluated in pancreas adenocarcinoma and melanoma human cell lines, and, after treatment with Amblyomin-X, the expression of several genes related to the cell cycle were altered, consistent with the G0/G1 phase alteration observed. Furthermore, in vivo treatment with Amblyomin-X induced a regression of tumoral mass in a mouse murine melanoma model (B16F10) and decreased the number of metastatic events. In conclusion, our results indicate that Amblyomin-X acts selectively on tumoral cells, focusing the ubiquitin-proteasome system as target.
Authors' contributions

AM Chudzinski-Tavassi conceived all experiments performed in this study and was responsible, together with D Maria, for the in vivo assays. AM Chudzinski-Tavassi and D Maria also contributed to the writing of the manuscript together with M Demasi, PL De-Sá-Júnior, and EM Reis. E Durães and EM Reis were responsible for the gene expression analysis, M Demasi worked on the proteasome analysis, PL De-Sá-Júnior and JS Ventura were responsible for the cell death experiments, and SM Simons, IFC Batista, and F Faria produced the recombinant Amblyomin-X used on the experiments. EM Reis and M Demasi contributed equally to the study.

Conflict of interest statement

None declared.

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Appendix. Supplementary material

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.toxicon.2010.04.019.

References