Biomedicine & Pharmacotherapy 66 (2012) 64–69

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Original article

Pro-apoptotic effects of Amblyomin-X in murine renal cell carcinoma "in vitro"

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ARTICLE INFO

Article history:
Received 3 August 2011
Accepted 1 November 2011

Keywords:
Renal cell carcinoma
Amblyomin-X
Apoptosis

ABSTRACT

Renal cell carcinoma (RCC) is one of the most lethal urologic cancers and is highly resistant to both radiotherapy and chemotherapy. The recombinant protein Amblyomin-X, characterized as a Kunitz-type protease inhibitor, was obtained from a cDNA library from the salivary glands of the *Amblyomma cajennense* tick. This paper reports the biological effect of Amblyomin-X on inducing cell death by apoptotic process *in vitro*. For this purpose, the changes in morphological aspects of cells, the phosphatidylserine exposition and DNA degradation were evaluated after treatment with Amblyomin-X. We found that Amblyomin-X was able to induce apoptosis in Renca cells in a dose-dependent manner. So, the results presented here open perspectives for new researches and developing for Amblyomin-X in the treatment of RCC.

1. Introduction

Renal cell carcinoma (RCC) is the most common kidney cancer, accounting for nine out of ten cases of kidney cancer. Its incidence has been increasing steadily over the past decades. According to the American Society of Cancer, kidney cancer is among the ten most common cancers in both men and women [1]. It is estimated that over 58,000 new cases occurred in 2010 and approximately 13,040 people died from this disease. RCC is one of the most difficult urological neoplasm to cure, as it is chemotherapy-resistant and nonradiosensitive. Patients with RCC already have metastasis at the time of diagnosis in 25–57% of the cases, and subsequently develop metastasis within 10 years in approximately 60% of all cases after curative nephrectomy [2,3].

Clinical trials have shown that cytokine therapy with interleukin 2 (IL-2) and/or interferon-alpha (IFN-α) can induce tumor regression in a subset of patients with metastatic RCC, but the therapy is associated with high toxicity. Novel targeted therapeutic approaches with higher response rates, especially inhibitors of tyrosine kinase receptors such as sunitinib or sorafenib, have been approved for treatment of advanced kidney cancer. Unfortunately, in the meantime, the clinical response to these agents is limited due to the development of tumor resistance by still unknown mechanisms. Therefore, there is a necessity to investigate clinical and pathological factors that can predict overall outcome of these treatments [4–6].

Apoptosis is a tightly controlled mechanism of cell death in which many catabolic enzymes, mainly proteases and nucleases, are activated leading to biochemical and morphological alterations that culminate in cellular collapse. This process of cell death plays an essential role in maintaining tissue homeostasis, and is important in certain pathological conditions, including cancer [7]. The first morphological characteristics of apoptosis include onset of blebbing, cell shrinkage, cytoskeletal rearrangement, chromatin condensation and nuclear fragmentation. Apoptosis occurs when cells prepare themselves to die in a cascade culminating in DNA degradation (via activation of endonucleases), nuclear disintegration and formation of apoptotic bodies. These apoptotic bodies are rapidly removed by tissue macrophages. Signaling for this is the translocation of phosphatidylserine from the inside to the outside of the membrane, marking the cells to be phagocytosed [8]. Many studies performed in recent years have shown that apoptosis is a constitutive way of death presented by almost all cells, if not all, and may be triggered by a large number of stimuli [9].

The recombinant protein Amblyomin-X, characterized as a Kunitz-type protease inhibitor (GenBank accession AAT68575), was obtained from a cDNA library from the salivary glands of the *Amblyomma cajennense* tick [10]. This protein is able to inhibit the coagulation factor Xa (FXa) and also promotes cytotoxic activity on several tumor cells, among them pancreatic and melanoma cells, with little or no activity on normal cells [11,12]. Furthermore, *in*

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doi:10.1016/j.biopha.2011.11.015

vivo treatment with Amblyomin-X induced a regression of tumor mass in a murine melanoma model (B16F10) and showed a decreased number of metastatic events. Previous studies have reported that recombinant proteins derived from ticks are capable of inducing cancer cell death [12,13]. Since RCC is highly resistant to cancer treatments, Amblyomin-X could be a possible and effective candidate to cancer therapy. Hence, in this work, the mode of cell death induced by Amblyomin-X treatment was evaluated in the RCC cell line.

2. Material and methods

2.1. Cell culture, reagents and recombinant protein

Murine renal adenocarcinoma cells (Renca) were kindly provided by PhD Maria Helena Bellini Marumo (Institute of Energy and Nuclear Research, IPEN, São Paulo, Brazil) and cultured according to ATCC recommendations. Briefly, the cells were cultured in RPMI supplemented with 10% fetal calf serum (v/v), 2 mM, L-glutamine, antibiotics (penicillin/streptomycin) in 75 cm^2 culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

RPMI 1640 medium (Gibco, Grand Island, NY - USA), fetal calf serum (Cultilab Ltda, São Paulo, Brazil), L-glutamine (Sigma-Aldrich Company, St Louis, USA), sodium pyruvate (Spectrum Chemical Mfg. Corp., New Jersey, USA), streptomycin and ampicillin (Cultilab Ltda, São Paulo, Brazil), Dulbecco's Modified Eagle's Medium (DMEM, Cultilab Ltda, São Paulo, Brazil). Dimethylsulfoxide (DMSO), propidium iodide (PI), fluorescein isothiocyanate (FITC) conjugated Annexin V, HEPES, proteinase K, RNase A, Hoechst: Bisbenzimide (Hoechst 33258), DAPI (blue) and acridine orange were purchased from Sigma-Aldrich Company (St Louis, MO, USA), sodium chloride and sodium monobasic phosphate. Vectashield mounting medium were from (Vector Laboratories, Burlingame, CA, USA). All other chemicals used were of the highest purity available in the market.

Amblyomin-X is a 13.5 kDa protein obtained in a recombinant form, as described by Batista et al. [11].

2.2. MTT assay

Renca cells were seeded in 96-well plates (10^4/well) and treated with 0.6 or 1.0 mM of Amblyomin-X during 24 or 48 hours followed by incubation with 20 μL MTT for 3 hours. Control using untreated cells was also performed. Afterwards, the supernatant was removed following centrifugation and 100 μL of DMSO was added. The absorbance at 540 nm was determined using an automatic microplate reader Spectra MAX 190 (Molecular Devices, USA).

2.3. Flow cytometry

To determine the rate of apoptosis that Amblyomin-X was able to induce, Renca cells were submitted to flow cytometry analysis using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (10^4 cells/well). Thus, cells were incubated with Amblyomin-X (0.6 or 1.0 mM) or saline for 24 or 48 hours. After that, the cells were centrifuged (1000 rpm, 10 min) and ressuspended in binding buffer (Sigma), pH 7.4, containing 10 mM HEPES; 140 mM NaCl; and 2.5 mM $CaCl_2$ at approximately 10^6 cells/mL. Fluorescein isothiocyanate (FITC)-Annexin V (5 μL) and PI (10 μL) in PBS were then added to cells, and then incubated for 10 minutes at room temperature in the dark. Cell fluorescence was immediately assessed in a flow cytometer (Guava Easy Cyte Mini, Guava Technologies). A total of 5000 events were analyzed using the software (CytoSoft, Hayward, USA). We considered cells to be in early apoptosis when marked by FITC/Annexin V only, and necrotic cells when stained by PI and FITC/Annexin V. The results were expressed as viability percentage.

2.4. DNA fragmentation analysis

Cells (10^5/well) were plated in 6-well plates and treated with 0.6 or 1 mM Amblyomin-X, or equal volume of culture medium (control). After 24-hour incubation, the cells were treated with a lysis buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0, 0.25% NP-40, 0.5 mg/mL proteinase K) at 50 °C for 2 hours. The DNA was precipitated with 2.5 mL of a solution containing ethanol 100% and NaCl 3 M overnight at room temperature. The samples were then centrifuged at 12,000 g for 30 minutes at 4 °C. The precipitate was washed with 70% ethanol and dried at room temperature. It was then dissolved in a buffer (10 mM Tris-HCl pH 8, 0, 0.1 mM EDTA) containing RNAse (600 mg/mL) and incubated at 37 °C for 1 hour. The concentration of extracted DNA was measured in a spectrophotometer (SPECTRAmax, Molecular Devices, USA) at 260 nm. DNA fragmentation was analyzed by electrophoresis on 1% agarose gel. Finally, the gel analysis was performed under ultraviolet light absorbance at 320 nm (Hoefer, Macrovue UV, 20) and photographed using the Doc-Print Photo Documentation system.

2.5. Cell death analysis by fluorescence staining

The Renca cells were cultured in 6-well plates (10^5 cells/well) and treated with 0.6 and 1 mM of Amblyomin-X at 37 °C in a humidified atmosphere with 5% CO_2 for 24 hours. Cell death was evaluated through *in situ* uptake of PI and Hoechst 33258 markers as described by Mckeague et al. [14]. Briefly, Amblyomin-X treated cells were washed with phosphate buffered saline (PBS) and incubated in PBS containing PI (1 ng/mL) and Hoechst 33258 (1 μM/mL) for 10 minutes. Cells were observed with a fluorescent microscope (Nikon TE 2000, USA). The apoptotic index was calculated comparing the number of apoptotic nuclei with the total number of nuclei on each visual field.

2.6. Morphological observation of dying cells

The Renca cells were cultured in 6-well plates (10^5 cells/well) and treated with 0.6 and 1 mM of Amblyomin-X at 37 °C in a humidified atmosphere with 5% CO_2 for 24 hours. The cells were stained with PI (50 mg/mL) and acridine orange (50 mg/mL) and incubated in the dark for 10 minutes. Afterwards, DAPI was added (to mark the nuclei of all cells) and they were subsequently analyzed through fluorescence microscopy (NIKON TE 2000).

We considered for analysis: the DAPI (blue) stains the cellular DNA content of all cells, the PI (red) stains the DNA of cells with damaged cell membrane, and acridine orange makes ionic interactions with acidic components in the cell (such as DNA, RNA, and cells phagocytized lysosome). Acidic pH appears in orange blush and neutral or basic pH stains appear green. Living cells are stained with DAPI and acridine orange, apoptotic cells are stained with DAPI and PI.

2.7. Statistical analysis

All the detection items in this study were performed using GraphPad Prism 5. Data was expressed as mean ± SD. Statistical significance (asterisks) of the difference between control cells and cells treated with Amblyomin-X was analyzed by ANOVA and Bonferroni's post-test ($^{***}P < 0.05$). All experiments were repeated at least three times.

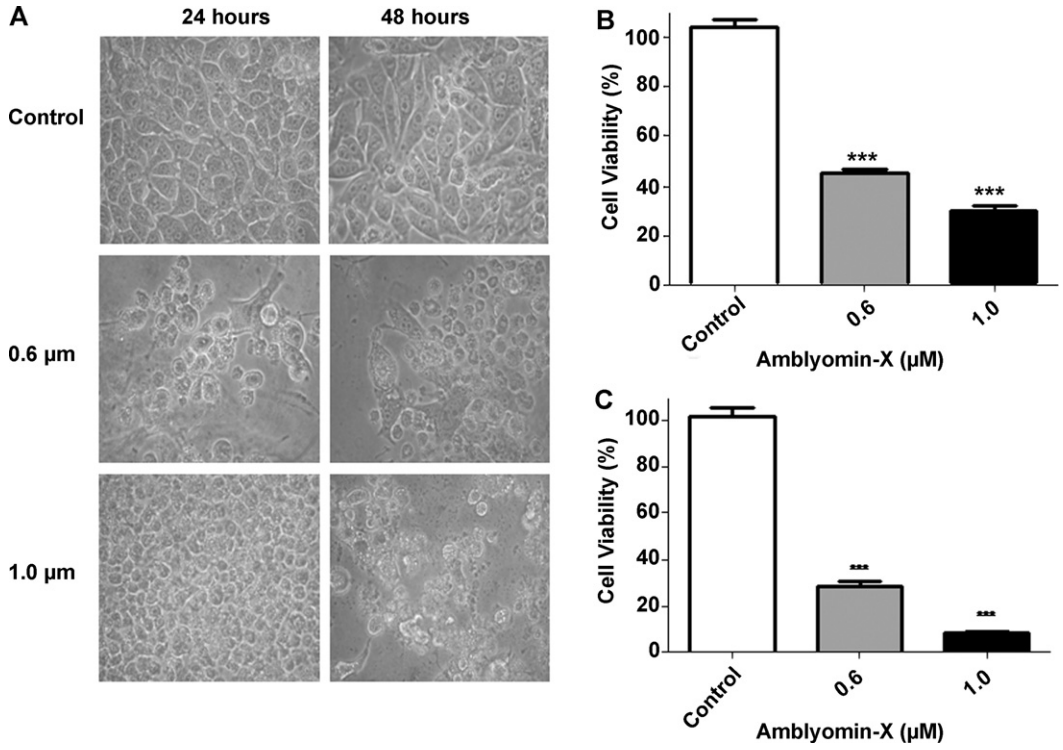

Fig. 1. Overview and MTT of Renca cells treated with Amblyomin-X. Cytological aspects of Renca cells treated with Amblyomin-X (0.6 or 1.0 μM) for 24 or 48 hours treatment. A. Changes on cell architecture were observed in both 24 and 48 hours treatment such as cell contraction, loss of inter-cell elongations and dispersed cell. After 48 hours there were formations of many cell aggregates. B and C. Viability assessed by the formation of formazan crystals in adhered cells. Mean ± SD determined from three independent experiments. $^{***}P < 0.05$ compared to control group, as determined by "one-way" ANOVA.

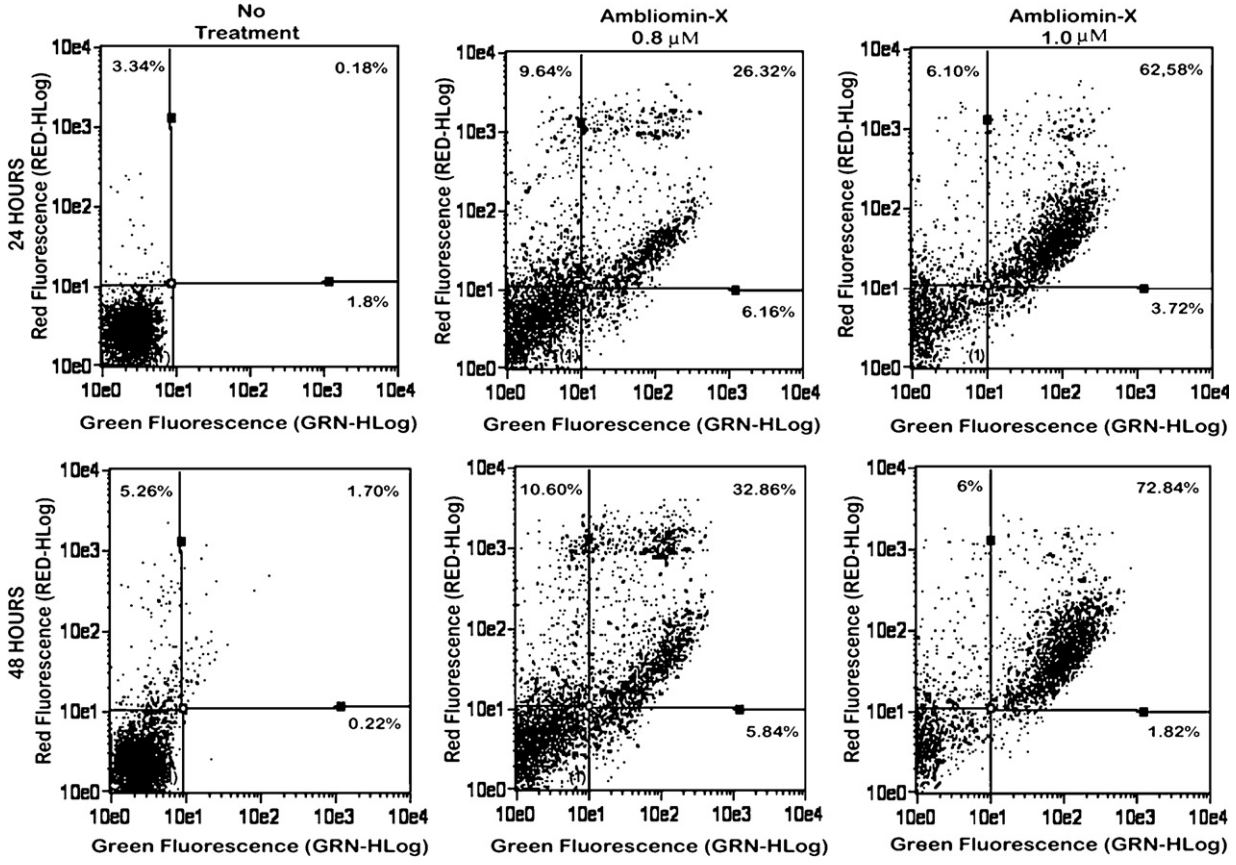

Fig. 2. Apoptosis inducing in Renca cells. The cells were incubated with 0.6 or 1.0 μM Amblyomin-X for 24 or 48 hours and analyzed through flow cytometry (Guava System) using propidium iodide and FITC-conjugated Annexin V. Quadrants: bottom left - viable cells; bottom right - early apoptotic cells; top right - late apoptotic; top left - necrotic cells.

3. Results

3.1. Overview of Renca cells treated with Amblyomin-X

To examine if Amblyomin-X inhibited Renca cell proliferation, the cells were treated with 0.6 or 1.0 μM of Amblyomin-X for 24 or 48 hours and the morphological changes were then observed by optical microscopy (Fig. 1). The general aspect of cells treated showed a dose-dependent effect that can be summarized as cell shrinkage, formation of cell aggregates and decreased cell adhesion.

Was examined the effects of Amblyomin-X on Renca cell viability by MTT assay, which showed that 24- or 48-hour treatment with Amblyomin-X (0.6 or 1.0 μM) was cytotoxic in a dose- and time-dependent manner (Fig. 1B and C). There was more than 50% decrease in viability of treated cells indicating a strong cytotoxic effect.

3.2. Flow cytometry

To determine the route of death induced by Amblyomin-X treated Renca cells (0.6 or 1.0 μM) after 24 or 48 hours, cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry (Fig. 2). To quantify the apoptotic phenotype, including early and late apoptosis, cells were labeled Annexin V positive (right quadrant in the dot density plot).

Cells treated with Amblyomin-X showed a higher percentage of apoptotic cells than the untreated control cells in both treatment periods (24 or 48 hours). This effect induces apoptosis presented as dose- and time-dependent (Fig. 2).

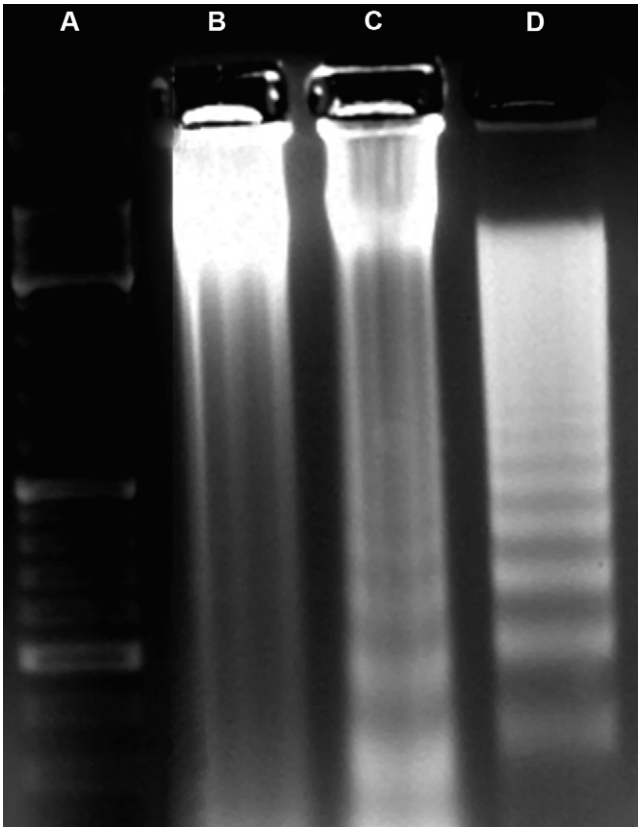

Fig. 3. DNA fragmentation in Renca cells treated with Amblyomin-X for 24 hours. A. Molecular weight marker (O'GeneRuler DNA Ladder Mix - Fermentas). B. Control cells without treatment; C and D. Renca cells treated with 0.6 or 1.0 μM Amblyomin-X, respectively.

3.3. DNA fragmentation of Renca cells treated with Amblyomin-X

Apoptosis induced by Amblyomin-X was also confirmed through analysis of DNA fragmentation (Fig. 3). The pattern of nuclear degradation can be observed in the gel, indicating apoptosis (staircase profile) of Renca cells treated for 24 hours with 0.6 or 1.0 μM Amblyomin-X (Lines C and D, respectively). These results showed that Amblyomin-X effectively induces a dose-dependent apoptosis in Renca cells.

3.4. Cell apoptosis analysis by fluorescence staining

To determine whether Amblyomin-X treated Renca cells undergo apoptosis, the cells were stained using Hoechst 33258/PI. The cells were stained blue (Hoechst 33258), marking the cellular DNA content; red (PI), marking the DNA of cells with damaged cell membrane; and pink (both markers) for apoptosis. Fig. 4 shows reductions in viable cells after treatment with Amblyomin-X (Fig. 4B and C) compared to the control cells (Fig. 4A). Our data also showed that the apoptotic index of Renca cells increased in a dose-dependent manner when treated by Amblyomin-X.

3.5. Morphology of apoptotic cells

The morphology of Renca cells treated or not with Amblyomin-X was evaluated by fluorescence microscopy. Cells were stained with DAPI, PI and acridine orange. Cells treated with 0.6 and 1.0 μM Amblyomin-X showed various morphological changes as shown in Fig. 5. These images show typical features of apoptosis, such as changes at the cell surface like bleb formation and apoptotic bodies (Fig. 5B arrow), cytoplasm retraction (Fig. 5C).

4. Discussion

The emergence of cells resistant to apoptosis is a challenge to the antitumor drug discovery and therapies. So, new drugs that induce cell death without toxicity to normal cells is a phenomenon that needs to be more deeply investigated. Many natural products extracted from plants, herbs and other living organisms are recognized to possess efficacy as potential anticancer drugs [15,16]. Ticks could be a source of such natural products due to bioactive molecules with pharmacologic activities presented on their salivary glands [17]. Our group has found that Amblyomin-X is able to inhibit coagulation factor Xa [11] and also promote cytotoxic activity in different tumor cell lines, without, however, inducing significant cytotoxic effects in normal cells, such as endothelial cells from human umbilical cord (HUVECs) and fibroblasts [12]. This inhibitory effect of Amblyomin-X in tumorigenesis through induction of apoptosis in several tumor cell lines indicates a possible therapeutic potential for cancer treatment.

The therapeutic options for RCC are limited and insufficient to control this disease, mainly due to strong resistance to conventional treatments (chemotherapy and radiotherapy). New anticancer drugs being launched do not bring great benefits to the patient and, in addition, they result in many side effects and toxicity [18]. Therefore, there remains a necessity to research new drugs that show an effective and definitive outcome on treatment of RCC, but without presenting side effects, and in this case Amblyomin-X is appointed as a possible candidate for this purpose.

In this work, we investigated the mechanisms underlying the anticancer activity of Amblyomin-X, a novel protein classified as a Kunitz-type serine protease inhibitor, encoded in the cDNA library of the tick *A. cajennense* [11]. Amblyomin-X was found to be similar

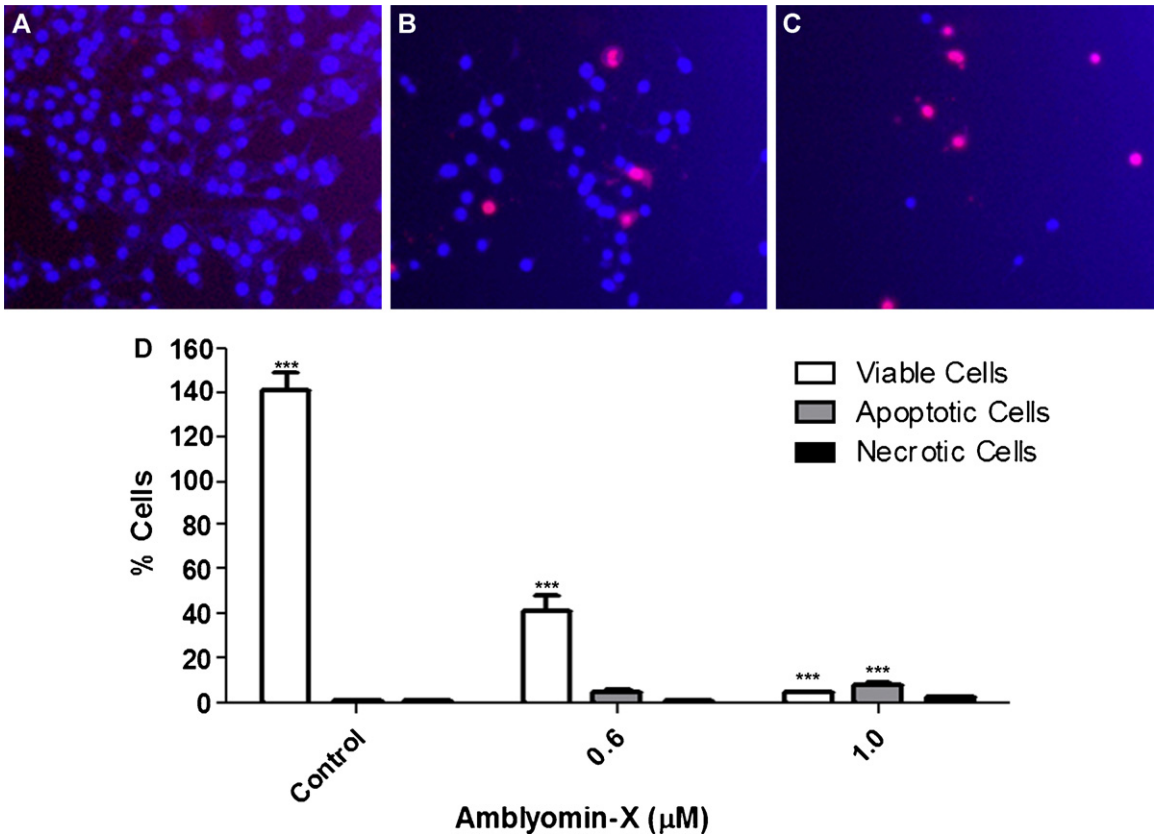

Fig. 4. Quantification of number of cells death by fluorescence microscopy. The were treated with 0.6 or 1.0 μM Amblyomin-X during 24 hours and stained with Hoechst/PI. A. Control (untreated). B and C. Treated with 0.6 or 1.0 μM of Amblyomin-X, respectively. Magnification 200 ×. D. The number of viable cells or in the process of death was assessed by direct counting, were blue fluorescent cells were considered viable cells and cells on different shades of red were considered dead or dying cells. Mean ± SD from three independent experiments. $^{***}P < 0.05$ compared to control group, determined by two-way ANOVA.

to another FXa inhibitor isolated from the *Ixodes scapularis* tick [19] and to share similarity with the Kunitz-type domain of the Tissue Factor Pathway Inhibitor (TFPI) [20].

RCC treated with Amblyomin-X showed visible morphological changes when compared to untreated cells. Moreover, increasing doses of Amblyomin-X suppresses the typical sprouting morphology and induces the cells to become rounded, culminating in a large number of detached cells. Besides this, Amblyomin-X also induced other morphological changes, such as plasma membrane blebbing, cell shrinkage and formation of apoptotic bodies in the final stages. In some cases, cytoplasmic vacuoles were also observed after incubation with Amblyomin-X (data not shown). These cytoplasmic findings were also described as autophagic vacuolization and these results could indicate that autophagy and apoptosis may be linked or may occur simultaneously when cells are incubated with Amblyomin-X.

The MTT assay, a method to access the metabolic activity of cells, demonstrated a significant dose-dependent loss of viability for cells treated with Amblyomin-X, indicating a metabolic impairment induced by incubation with Amblyomin-X. Although the phenomenon of induction of cytotoxicity in itself was an interesting finding, it is very important to know what type of cell death this molecule is able to induce. Thus, a flow cytometry of cells treated or not with Amblyomin-X was performed after staining with Annexin V/Propide Iodide. Apoptotic cells present phosphatidylserine on their surfaces as a hallmark of the apoptosis process while cells in other death processes do not show this phenomenon. Flow cytometry is a sensitive method to differenti-

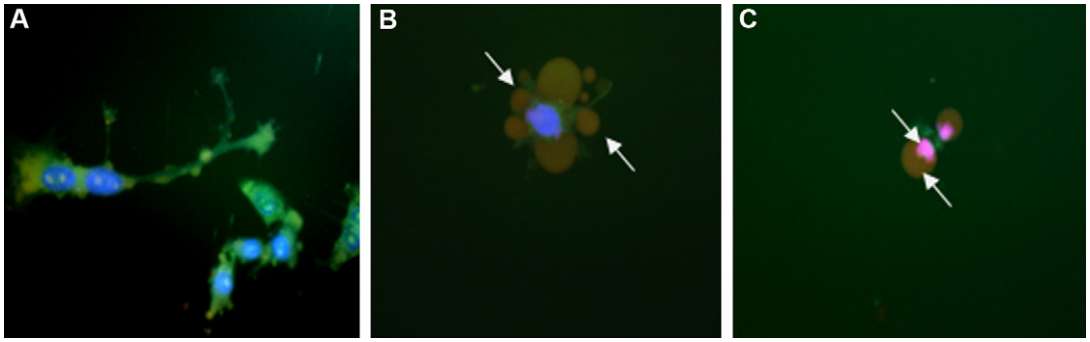

Fig. 5. Morphologic feature of cells treated with Amblyomin-X. The Renca cells were treated with Amblyomin-X (0.6 and 1.0 μM). A. Control (untreated). B and C. Depicts changes on cells consistent with apoptosis such as blebbing and formation of apoptotic bodies (B, arrow), cellular retraction (C, arrows). Magnification 400 ×.

ate between distinct processes of cell death, such as necrosis and apoptosis, and to allow characterization of early and late apoptosis stages. Our findings support the allegations that treatment with Amblyomin- X induced apoptosis and not necrosis in Renca cells.

Cells in apoptosis develop special morphological features, such as shrinkage, cytoskeletal rearrangements, nuclear fragmentation and others events. There are many ways to investigate these events. Staining cells with fluorescent dyes, including acridine orange and ethidium bromide, are frequently used to evaluate the nuclear and cytoplasmic morphology of apoptotic cells. Thus, to corroborate that apoptosis was induced by Amblyomin-X, Renca cells were analyzed in the presence of acridine orange and ethidium bromide staining. Several typical features of apoptosis, such as cell volume reduction, and formation of cytoplasmic blebs, were observed after treatment with Amblyomin-X corroborating other data presented here. This phenomenon was not observed in untreated cells. Besides this, when the cells were stained with Hoechst/PI, we found that the number of dead-red-cells was increased and the number of live-blue-cells was decreased. In this case, the total amount of cells also declined probably due to cells lysis as a consequence of apoptosis.

As the final stage on characterization of Amblyomin-X-induced cell death, we also analyzed if the treatment with this protein could induce DNA degradation. The fragmented DNA could appear in two patterns on agarose gel submitted to electrophoresis: in the first, as a necrotic pattern characterized by nonspecific DNA degradation resulting in a ''smear'' of randomly degradated DNA and a second pattern characterized by apoptotic DNA fragmentation resulting in the ''DNA ladder'' as a consequence of action of endonucleases [20]. As a biochemical hallmark of intrinsic apoptotic cell death, DNA fragmentation was used to determine whether the cytotoxic and antiproliferative effects of Amblyomin-X on cells were in fact due to apoptosis. We have found that the treatment of Renca cells with Amblyomin-X was able to induce DNA fragmentation consistent with apoptosis. The Renca cells were treated with two concentrations of Amblyomin-X (0.6 and 1 μM) and both dosages were able to induce typical DNA laddering characteristic of apoptosis. Untreated cells did not show DNA laddering.

In summary, these studies demonstrate that Amblyomin-X is a strong cytotoxic agent able to induce apoptosis on Renca cells. These results therefore are consistent with previous studies performed by our group where Amblyomin-X was demonstrated to induce apoptosis in human melanoma (Sk-Mel-28) and human pancreatic adenocarcinoma (Mia PaCa-2) without inducing apoptosis in normal cells (Fibroblasts). Thus, although many studies are yet to be done, Amblyomin-X emerges as a possible candidate for renal antitumor therapy.

5. Conclusion

The present results indicate that Amblyomin-X has cytotoxic activity on RCC cells *in vitro* and that its anticancer activity against RCC occurs by induction of apoptosis. These findings suggest a clinical possibility for Amblyomin-X in the treatment of drug-resistant and/or immunotherapeutic-resistant RCC, although additional studies are necessary to clarify what mechanism induces this kind of cell death.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgments

The authors thank Dr Alexandre Pereira (Laboratory of Genetic, Butantan Institute, São Paulo, SP, Brazil) for her collaboration on the Fluorescence Microscopy experiments. This work was supported by FAPESP, CNPq, INCTTOX and CAT-CEPID.

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