Preclinical evaluation of Amblyomin-X, a Kunitz-type protease inhibitor with antitumor activity

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ABSTRACT

Amblyomin-X, a Kunitz-type protease inhibitor, is a recombinant protein that selectively induces apoptosis in tumor cells and promotes tumor reduction in vivo in melanoma animal models. Furthermore, Amblyomin-X was able to drastically reduce lung metastasis in a mice orthotopic kidney tumor model. Due to its antitumor activity, Amblyomin-X potential to become a new drug is currently under investigation, therefore the aim of the present study was to perform preclinical assays to evaluate Amblyomin-X toxicity in healthy mice. Exploratory toxicity assays have shown that treatment with 512 mg/kg of Amblyomin-X lead to animal mortality, therefore two groups of treatment were evaluated in the present work: in the acute toxicity assay, animals were injected once with doses ranging from 4 to 256 mg/kg of Amblyomin-X, while in the subacute toxicity assay, animals were injected with 0.25, 0.57 and 1 mg/kg of Amblyomin-X daily, during 28 days. Following this treatment regimens, Amblyomin-X did not cause any mortality; moreover, toxicity signs were discrete, reversible and observed only at the higher doses, thus establishing a safety profile for administration in mice, which can be further used to determine the dose translation of this novel drug candidate for treatment in other species.

1. Introduction

According to the American Cancer Society, more than 1,7 million cancer cases and more than 609 thousand cancer associated deaths are projected in 2018, in the United States alone [1]. Worldwide, data from the GLOBOCAN 2018 predicts 18.1 million cancer cases and 9.6 million cancer deaths in 2018, a projection for countries with different human development indexes, with the most prevalent types being lung cancer, breast cancer on females and colorectal cancers [2].

Based on this data, novel cancer treatments are under continuous investigation and a recombinant protein, Amblyomin-X, is currently under investigation by our research group as a potential novel drug for tumor treatment. Amblyomin-X protein sequence was originally identified in the transcriptome of the salivary glands of the Amblyomma sculpturn tick (GenBank accession nº. AAT68575) [3–5]. Among the molecules present in tick saliva, some directly affect hemostasis, inflammation and immunity [6–8]. Amblyomin-X is a serine protease Kunitz-type inhibitor with a molecular mass of 12.295 Daltons and a structure that contains three disulfide bonds. Previous studies have shown that this recombinant protein inhibits factor Xa in the blood coagulation cascade and extends global blood clotting time, as was demonstrated in aPTT assays [6].

Amblyomin-X also induced cell cycle arrest and apoptosis in several tumor cell lines, such as SK-MEL-28 (human melanoma) and Mia PaCa-2 (human pancreatic carcinoma), without causing any effect on normal cells [9]. It induced apoptosis in murine renal adenocarcinoma (RENCA) cells by causing an imbalance between pro- and anti-apoptotic Bcl-2 family proteins, and induced dysfunction/mitochondrial damage and the production of reactive oxygen species (ROS) [10]. Apoptosis seems to be caused by endoplasmic reticulum-associated stress and the inhibition of the ubiquitin - proteasome system [11]. It was also shown that Amblyomin-X treatment lead to a decrease in cell migration and actin cytoskeleton disruption in tumoral cell models [12] In addition, tumor regression and the reduction of lung metastasis have been observed in animal models [13,14]. Recently, as a part of toxicity studies, the biodistribution and pharmacokinetics of Amblyomin-X administered to healthy mice was evaluated, indicating its rapid elimination from plasma, short term accumulation of the protein in the liver, following by elimination through urine [15].

In the present study, Amblyomin-X toxicity in healthy mice was evaluated under acute and subacute treatments. General animal behavior, body weight variation, water and food consumption, mortality as...
well as biochemical, hematological and histopathological parameters were evaluated after Amblyomin-X treatment, establishing a safety profile for this novel drug candidate.

2. Material and methods

2.1. Experimental conduct and design

The study was conducted at the Molecular Biology Laboratory at the Butantan Institute in accordance with Good Laboratory Practice (GLP) guidelines. Experiments were designed following the guidelines from the Brazilian Regulatory Agency [16] which determines the guidelines for performing preclinical studies in the country. Briefly, for single-dose acute toxicity studies the limit dose is 1000 mg/kg/day and animals should be observed for a minimal of 14 days after treatment. For repeated toxicity assays, the same dose limits must be observed, with a minimal duration of 2 weeks and LD50 (lethal dose) are not considered necessary [16]. All animal experiments comply with the ARRIVE
Table 1
Hematological profile of male and female mice in the Acute Toxicity Group.

| Acute Dose | Male 24 h | Vehicle | 4 mg/kg | 16 mg/kg | 32 mg/kg | 64 mg/kg | 256 mg/kg | 4 mg/kg | 16 mg/kg | 32 mg/kg | 64 mg/kg | 256 mg/kg |
|------------|-----------|---------|---------|----------|----------|----------|-----------|---------|---------|----------|----------|-----------|
| RBC (10^6/mm³) | 8.78 ± 0.41 | 9.32 ± 0.14 | 10.00 ± 0.24 | 7.25 ± 0.61 | 12.00 ± 0.63 | 13.00 ± 0.44 | 9.00 ± 0.24 | 9.00 ± 0.14 | 12.00 ± 0.68 | 12.00 ± 0.18 |
| HGB (g/dL) | 12.85 ± 0.44 | 13.53 ± 0.31 | 14.00 ± 0.55 | 14.00 ± 0.29 | 12.25 ± 0.52 | 13.00 ± 0.63 | 13.00 ± 0.44 | 12.00 ± 0.68 | 12.00 ± 0.18 |
| HCT (%) | 42.56 ± 1.01 | 45.23 ± 0.88 | 45.00 ± 1.03 | 46.00 ± 1.00 | 41.18 ± 1.85 | 40.00 ± 2.05 | 43.00 ± 1.47 | 42.00 ± 2.42 | 41.00 ± 2.64 |
| PCT (10^3/mm³) | 24.00 ± 0.19 | 22.00 ± 0.15 | 17.50 ± 0.34 | 16.00 ± 0.15 | 16.00 ± 0.34 | 8.00 ± 0.58 | 8.00 ± 0.58 | 10.00 ± 0.58 | 14.00 ± 1.06 |
| WBC (10^3/mm³) | 3.10 ± 1.00 | 2.70 ± 0.51 | 2.80 ± 0.57 | 3.60 ± 1.15 | 2.40 ± 0.50 | 2.80 ± 0.50 | 2.80 ± 0.58 | 5.00 ± 0.58 | 10.00 ± 0.58 |
| Neutrophils (%) | 16.90 ± 5.95 | 12.00 ± 2.83 | 12.00 ± 1.73 | 12.00 ± 2.52 | 19.00 ± 2.06 | 23.00 ± 2.65 | 18.00 ± 1.83 | 18.00 ± 2.45 | 0.75 ± 0.50 |

| Acute Dose | Female 24 h | Vehicle | 4 mg/kg | 16 mg/kg | 32 mg/kg | 64 mg/kg | 256 mg/kg | 4 mg/kg | 16 mg/kg | 32 mg/kg | 64 mg/kg | 256 mg/kg |
|------------|-------------|---------|---------|----------|----------|----------|-----------|---------|---------|----------|----------|-----------|
| RBC (10^6/mm³) | 9.04 ± 0.31 | 9.23 ± 0.27 | 9.00 ± 0.22 | 8.99 ± 0.96 | 8.00 ± 0.81 | 9.00 ± 0.77 | 8.89 ± 0.19 | 9.00 ± 0.39 | 9.14 ± 0.46 | 9.00 ± 0.48 | 9.00 ± 0.23 |
| HGB (g/dL) | 13.88 ± 0.73 | 13.93 ± 0.49 | 13.00 ± 0.55 | 13.20 ± 1.21 | 13.00 ± 1.22 | 14.00 ± 1.18 | 14.00 ± 0.46 | 14.23 ± 0.30 | 14.50 ± 0.16 | 14.50 ± 0.15 | 14.50 ± 0.15 |
| HCT (%) | 39.14 ± 1.03 | 45.90 ± 1.17 | 41.00 ± 1.21 | 43.00 ± 1.34 | 44.00 ± 0.74 | 44.00 ± 1.35 | 45.00 ± 2.27 | 47.00 ± 0.87 | 47.00 ± 0.87 | 47.00 ± 0.87 | 47.00 ± 0.87 |
| PCT (10^3/mm³) | 24.00 ± 0.19 | 22.00 ± 0.15 | 17.50 ± 0.34 | 16.00 ± 0.15 | 16.00 ± 0.34 | 8.00 ± 0.58 | 8.00 ± 0.58 | 10.00 ± 0.58 | 14.00 ± 1.06 |
| WBC (10^3/mm³) | 3.10 ± 1.00 | 2.70 ± 0.51 | 2.80 ± 0.57 | 3.60 ± 1.15 | 2.40 ± 0.50 | 2.80 ± 0.50 | 2.80 ± 0.58 | 5.00 ± 0.58 | 10.00 ± 0.58 |
| Neutrophils (%) | 16.90 ± 5.95 | 12.00 ± 2.83 | 12.00 ± 1.73 | 12.00 ± 2.52 | 19.00 ± 2.06 | 23.00 ± 2.65 | 18.00 ± 1.83 | 18.00 ± 2.45 | 0.75 ± 0.50 |

* RBC: Red blood cells - Erythrocytes; HGB: hemoglobin; HCT: Hematocrit; PCT: platelet; WBC: White Blood Cells - Leukocytes.

Amblyomin-X was administered in a single dose and parameters were evaluated 24 h and 14 days after treatment. (Values are presented as means with standard deviation).
|               | Control | 0.25 mg/kg | 0.57 mg/kg | 1 mg/kg | 0.25 mg/kg | 0.57 mg/kg | 1 mg/kg | 0.25 mg/kg | 0.57 mg/kg | 1 mg/kg | 0.25 mg/kg | 0.57 mg/kg | 1 mg/kg |
|---------------|---------|------------|------------|---------|------------|------------|---------|------------|------------|---------|------------|------------|---------|
| RBC (10^6/mm³) | 9.67 ± 0.1 | 9.12 ± 0.45 | 9.44 ± 0.32 | 9.04 ± 0.82 | 9.49 ± 0.30 | 9.00 ± 0.30 | 9.57 ± 0.46 |
| HGB (g/dL)    | 13.51 ± 0.3 | 12.64 ± 0.66 | 12.72 ± 0.48 | 12.41 ± 1.02 | 13.55 ± 0.42 | 12.70 ± 0.35 | 13.14 ± 0.69 |
| HCT (%)       | 45.32 ± 0.5 | 42.32 ± 2.06 | 42.90 ± 1.57 | 41.99 ± 3.36 | 45.48 ± 1.47 | 42.76 ± 1.03 | 44.76 ± 3.46 |
| PCT (10³/mm³) | 739.7 ± 15.7 | 753.33 ± 118.72 | 916.56 ± 46.23 | 886.60 ± 138.59 | 713.50 ± 90.00 | 682.60 ± 22.66 | 776.00 ± 141.18 |
| WBC (10³/mm³) | 2.9 ± 0.5 | 3.40 ± 1.17 | 3.00 ± 0.64 | 2.56 ± 0.80 | 1.55 ± 0.44 | 3.46 ± 0.39 | 4.36 ± 1.25 |
| Neutrophils (%) | 9.9 ± 3.0 | 6.58 ± 2.17 | 11.25 ± 2.97 | 9.90 ± 2.83 | 9.58 ± 3.07 | 7.82 ± 1.33 | 4.97 ± 1.55 |
| Lymphocytes (%) | 88.63 ± 3.0 | 92.34 ± 2.44 | 87.39 ± 3.44 | 88.40 ± 3.11 | 89.00 ± 3.68 | 91.20 ± 1.54 | 88.40 ± 9.57 |
| Monocytes (%) | 1.16 ± 0.1 | 0.78 ± 0.28 | 0.96 ± 0.47 | 1.17 ± 0.52 | 0.98 ± 0.64 | 0.76 ± 0.33 | 0.38 ± 0.19 |
| Eosinophils (%) | 0.06 ± 0.1 | 0.11 ± 0.26 | 0.11 ± 0.25 | 0.10 ± 0.15 | 0.23 ± 0.19 | 0.04 ± 0.09 | 0.02 ± 0.04 |
| Basophils (%) | 0.25 ± 0.1 | 0.18 ± 0.09 | 0.26 ± 0.22 | 0.43 ± 0.46 | 0.23 ± 0.15 | 0.18 ± 0.08 | 1.08 ± 2.03 |

RBC: Red blood cells; Erythrocytes; HGB: Hemoglobin; HCT: Hematocrit; PCT: Platelet; WBC: White Blood Cells. Values are presented as means ± standard deviation.
| Acute Dose | Male 24 h | Female 24 h | Male 14 days | Female 14 days |
|------------|-----------|-------------|-------------|---------------|
|            | Vehicle   | 4 mg/kg     | 16 mg/kg    | 32 mg/kg      |
| Creatinine (mg/dL) | 0.39 ± 0.10 | 0.38 ± 0.11 | 0.39 ± 0.07 | 0.52 ± 0.18 |
| Urea (mg/dL)    | 51.59 ± 11.30 | 27.0 ± 13.11 | 69.58 ± 9.32 | 70.66 ± 4.46 |
| Albumin (g/dL)  | 2.57 ± 0.22  | 2.95 ± 0.16  | 2.51 ± 0.06  | 7.63 ± 0.48  |
| Globulin (g/dL) | 1.52 ± 0.34  | 1.87 ± 0.28  | 1.65 ± 0.09  | 5.26 ± 0.16  |
|               |            | 1.60 ± 0.16  | 1.53 ± 0.11  |               |
|               | 4 mg/kg    | 16 mg/kg    | 32 mg/kg    |               |
| Creatinine (mg/dL) | 0.63 ± 0.20 | 0.61 ± 0.19 | 0.59 ± 0.18 | 0.52 ± 0.21 |
| Urea (mg/dL)    | 90.25 ± 35.09 | 118.0 ± 54.5 | 131.5 ± 38.52 | 115.3 ± 17.01 |
| Albumin (g/dL)  | 2.37 ± 0.24  | 1.87 ± 0.28  | 1.65 ± 0.09  | 5.52 ± 0.34  |
| Globulin (g/dL) | 1.52 ± 0.34  | 1.87 ± 0.28  | 1.65 ± 0.09  | 5.26 ± 0.16  |
|               | 16 mg/kg   | 32 mg/kg    | 64 mg/kg    |               |
| Creatinine (mg/dL) | 0.49 ± 0.10 | 0.49 ± 0.10 | 0.59 ± 0.18 | 0.52 ± 0.21 |
| Urea (mg/dL)    | 90.25 ± 35.09 | 118.0 ± 54.5 | 131.5 ± 38.52 | 115.3 ± 17.01 |
| Albumin (g/dL)  | 2.37 ± 0.24  | 1.87 ± 0.28  | 1.65 ± 0.09  | 5.52 ± 0.34  |
| Globulin (g/dL) | 1.52 ± 0.34  | 1.87 ± 0.28  | 1.65 ± 0.09  | 5.26 ± 0.16  |

Amblyomin-X was administered in a single dose and parameters were evaluated 24 h and 14 days after treatment. (Values are presented as means ± standard deviation).
|                |            | Repeated Dose |              |              | Satellite |              |              |              |
|----------------|------------|---------------|--------------|--------------|----------|--------------|--------------|--------------|
|                |            | Vehicle       | 0.25 mg/kg   | 0.57 mg/kg   | 1 mg/kg  | 0.25 mg/kg   | 0.57 mg/kg   | 1 mg/kg      |
|                |            |               |              |              |          |              |              |              |
| Creatinine (mg/dL) |            | 0.02 ± 0.0    | 0.02 ± 0.04  | 0.08 ± 0.06  | 0.03 ± 0.05 | 0.02 ± 0.04  | 0.00 ± 0.00  | 0.00 ± 0.00  |
| Urea (mg/dL)     |            | 22.9 ± 1.3    | 23.78 ± 2.54 | 20.50 ± 1.27 | 23.00 ± 2.79 | 24.20 ± 2.59 | 23.00 ± 1.22 | 23.80 ± 4.38 |
| AST (U/L)        | 59.2 ± 20.1| 53.97 ± 18.32 | 55.10 ± 25.45| 51.20 ± 27.28| 85.20 ± 14.75| 81.00 ± 14.65| 93.90 ± 16.41|
| ALT (U/L)        | 43.2 ± 14.4| 44.67 ± 10.71 | 42.40 ± 24.82| 38.10 ± 7.99 | 55.60 ± 13.50| 59.60 ± 15.13| 51.80 ± 6.14 |
| Total Protein (g/dL) |        | 4.61 ± 0.0    | 4.64 ± 0.13  | 4.93 ± 0.19  | 4.85 ± 0.22 | 4.62 ± 0.20  | 4.62 ± 0.22  | 4.60 ± 0.26  |
| Calcium (mg/dL)  | 8.24 ± 0.1 | 8.29 ± 0.34   | 8.46 ± 0.28  | 8.14 ± 0.34  | 8.24 ± 0.05 | 8.66 ± 0.29  | 8.24 ± 0.27  |
| Albumin (g/dL)   | 1.95 ± 0.0 | 1.90 ± 0.14   | 1.91 ± 0.14  | 1.95 ± 0.19  | 2.10 ± 0.12 | 2.06 ± 0.09  | 2.02 ± 0.13  |
| Globulin (g/dL)  | 2.76 ± 0.2 | 2.90 ± 0.28   | 2.99 ± 0.21  | 2.88 ± 0.24  | 2.52 ± 0.13 | 2.58 ± 0.11  | 2.58 ± 0.33  |
| Alk. Phosphatase (U/L) | 108.6 ± 32.2 | 89.33 ± 24.93 | 87.30 ± 14.14| 74.30 ± 20.11| 110.20 ± 41.23| 104.40 ± 33.15| 106.40 ± 14.57|

|                |            | Satellite     | 0.25 mg/kg   | 0.57 mg/kg   | 1 mg/kg  | 0.25 mg/kg   | 0.57 mg/kg   | 1 mg/kg      |
|----------------|------------|---------------|--------------|--------------|----------|--------------|--------------|--------------|
|                |            |               |              |              |          |              |              |              |
| Creatinine (mg/dL) |            | 0.01 ± 0.0    | 0.04 ± 0.05  | 0.08 ± 0.04  | 0.00 ± 0.00 | 0.00 ± 0.00  | 0.00 ± 0.00  |              |
| Urea (mg/dL)     |            | 20.90 ± 3.35  | 19.78 ± 4.55 | 21.00 ± 2.55 | 23.40 ± 2.41 | 28.00 ± 4.25 | 19.40 ± 3.05 |
| AST (U/L)        | 79.8 ± 34.5| 64.40 ± 24.50 | 62.33 ± 18.00| 60.00 ± 27.70| 94.60 ± 17.49| 126.00 ± 52.71| 95.40 ± 30.88|
| ALT (U/L)        | 63.4 ± 26.9| 41.50 ± 7.63  | 40.89 ± 7.56 | 45.67 ± 12.40| 63.40 ± 17.27| 59.50 ± 10.75| 45.40 ± 12.66|
| Total Protein (g/dL) |        | 4.71 ± 0.1    | 4.99 ± 0.11  | 4.72 ± 0.25  | 4.79 ± 0.17 | 4.64 ± 0.30  | 4.40 ± 0.41  | 4.52 ± 0.26  |
| Calcium (mg/dL)  | 8.23 ± 0.3 | 8.47 ± 0.41   | 8.49 ± 0.26  | 8.42 ± 0.19  | 8.70 ± 0.38 | 8.63 ± 0.41  | 8.66 ± 0.31  |
| Albumin (g/dL)   | 1.98 ± 0.1 | 1.99 ± 0.13   | 2.03 ± 0.13  | 2.02 ± 0.08  | 2.14 ± 0.17 | 2.03 ± 0.21  | 2.06 ± 0.17  |
| Globulin (g/dL)  | 2.72 ± 0.0 | 2.77 ± 0.14   | 2.70 ± 0.13  | 2.76 ± 0.15  | 2.52 ± 0.13 | 2.37 ± 0.21  | 2.46 ± 0.11  |
| Alk. Phosphatase (U/L) | 107.5 ± 39.7 | 91.00 ± 14.06 | 104.44 ± 25.82| 98.22 ± 18.86| 123.00 ± 38.33| 111.25 ± 52.71| 87.60 ± 17.74|

Amblyomin-X was administered daily for 28 days and parameters were evaluated at the 28th and 42nd days. (Values are presented as means with standard deviation).
Fig. 2. Histopathological analysis 24 h after acute treatment with Amblyomin-X. Each line represents a group and a row represents an organ. A, D, G, J, N, R = Spleen; B, E, H, L, O, S = kidney and C, F, I, M, Q, T = Liver.
Fig. 3. Histopathological analysis 14 days after acute treatment with Amblyomin-X. Each line represents a group and a row represents an organ. A, D, G, J, N and R = Spleen, B, E, H, L, O and S = kidney; C, F, I, M, Q and T = liver.
guidelines and were carried in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. All animal procedures performed in this work were approved by the CEUAIB/BUTANTAN Ethics Committee under protocol n. 2588090517.

2.2. Amblyomin-X production

Recombinant Amblyomin-X was produced using an *E. coli* expression system as described previously [14] and representative batch-to-batch quality controls and cytotoxic activities can be found in a recent work from our group [15].

2.3. Animals care

Male and female Balb-c mice (*Mus musculus*), weighing 20 to 30 g, were obtained from the IPEN (São Paulo, Brazil). Animals were kept in ventilated shelving units under a controlled temperature and humidity and with a 12-hour photoperiod. Each cage was provided with CR1-Nuvital Nuvilab® commercial food and water *ad libitum*.

2.4. Animal groups and Amblyomin-X administration

Amblyomin-X was injected via an intravenous route into the retro orbital plexus. Animals received local anesthetic (0.5% proxymetacaine chloridrate) before administration and no eye changes or secondary infections were observed. Each randomized animal groups consisted of 10 males and 10 females mice, which received acute or subacute doses as described below. Each group had its respective 10 animal control group, which were injected with only the vehicle (vehicle group). An additional group did not receive any injection (control group). In the acute toxicity group, animals were injected once with the acute dose of 4, 16, 32, 64 and 256 mg/kg and analyzed 24 h or 14 days after the injection. The animals from the subacute group were injected with 0.25, 0.57 and 1 mg/kg daily, and were evaluated at the 28th day. In a satellite group, animals were injected daily for 28 days, observed for 14 days and analyzed at the 42nd day. The choice of concentrations for the experimental development of the toxicity tests was based on a manuscript published by our group, where the following therapeutic effects of tumor burden reduction and inhibition of the formation of metas- 

tases in organs at a distance were observed in mice treated with 1 mg/kg of animal weight through the intravenously into the orbital plexus.

Fig. 4. Histopathological analysis after subacute treatment with Amblyomin-X. Each line represents a group and a row represents an organ.
2.5. Clinical evaluation of mice

Body mass and water consumption were evaluated throughout the experiment. Hippocratic screening was performed by means of individual observations of the animals in periods of 15 min, 30 min, 1, 2, 4 h after the injection, and subsequently at each 24 h for 14 days. The state of consciousness and general disposition, motor coordination, muscle tone, reflexes and autonomic nervous system activity were evaluated.

2.6. Kaplan–Meier survival curves

Previous results from exploratory toxicity tests were compiled and the evaluation of toxicity effects and the survival probability of Amblyomin-X treated animals at doses of 0.25 to 512 mg/kg were assessed by the Kaplan-Meier method. Also, the nonparametric log-rank test was applied, for comparison between the different treated and control groups.

2.7. Blood collection for hematological profiles and biochemical analysis

Animals were deeply anesthetized (xylazine hydrochloride 10 mg/kg and ketamine hydrochloride 100 mg/kg) [17]. Blood was collected by cardiac puncture. Hematological and biochemical parameters were evaluated using specific kits according to the manufacturer's instructions (Randox Laboratories). All measurements were performed using an automated Randox RX Daytona Chemistry Analyzer (Crumlin, UK). Prothrombin time was analyzed on venous blood samples using a portable coagulometer (CoaguChek XS, Roche Diagnostics, Mannheim, Germany).

2.8. Histopathological analysis and Relative mass of organs

Animals under deep anesthesia were euthanized, following a detailed external (color and integrity of the skin and hair, changes of all the tissues and the shape of abdomen) and internal examination. Organs (kidney, spleen, liver, heart, lung, lymph nodes, testis and ovaries) from each animal were removed and weighted. The relative mass of the organs of each animal was calculated by dividing the weight of each organ (g) by the body weight of each animal on the day of collection and multiplying the result by 100.
All tissues were fixed in medium of 10% solution of buffered formalin (pH 7.4) followed by dehydration and then enclosed in paraffin. Thin sections were stained with hematoxylin/eosin (H&E), and slides were scanned (Olympus VS110 system) at 20x/N.A. 0.75 and 40x/N.A. 0.95.

2.9. Statistical analysis

Instat v3.01 and Prism 5 software programs (GraphPad Software Inc., USA) were used to calculate statistical significance using one-way analysis of variance (ANOVA) and multiple-comparison Tukey-Kramer tests. The results are presented as the mean ± standard deviation. Significant differences in results between groups were considered when p < 0.05.

3. Results

3.1. Protein analysis

Recombinant Amblyomin-X production process and representative quality controls and cytotoxic activity towards tumoral cells can be found in a recently published work from our group [15].

3.2. Clinical evaluation of mice

Exploratory toxicity assays in mice were conducted previously to this work, where the survival probability of Amblyomin-X treatment at doses from 0.25 to 512 mg/kg were assessed by the Kaplan-Meier method to estimate the probability of survival at the various time intervals after 24 h of administration of the compound up to 90 days (Suppl. Fig. 1).

Data showed that treatment of healthy mice with concentrations higher than 256 mg/kg lead to acute toxicity, with mortality higher than 50% of animals 24 h after treatment For humanized issues and standards established internationally by the Canadian Council Animal Care (CCAC), LD50% was not determined, since the concentration of 256 mg/kg was toxic after 24 h of administration. Therefore, the toxicity range utilized in this work was in the range of 0.25 mg/kg to 256 mg/kg. Within this range, no mortality was observed upon Amblyomin-X treatment in any acute dose treated group throughout the study. In the hippocratic screening, animals which received doses above 64 mg/kg showed alterations (moderate to intense) in the motor and / or sensorial signals, including decreased motor activity, piloerection and tail tightening. These signals appeared after 15 min of injection and at different times up to 6 days and then disappeared (Suppl. Table B1). Subacute administration of repeated doses of Amblyomin-X did not significantly alter the investigated parameters of the treated groups at concentrations less than 1 mg/kg. Animals treated with 1 mg/kg of Amblyomin-X presented piloerection, which was observed after the 5th day of application, intensifying until the 29th day, and disappearing upon treatment termination (Suppl. Tables B1, B2).

3.3. Determination of body weight

A reduction in body mass gain (ponderal weight gain percentage) was observed on animals treated with acute doses above 25 mg/kg, and on animals treated with multiple doses above 0.25 mg/kg, but this effect was reversible, since no body weight loss was observed on the satellite group (Fig. 1A–C). Additionally, the average weight curves during the subacute treatment showed continuous increase on the animal body weight throughout the treatment (Suppl. Fig. 2).

3.4. Hematological analysis

No morphological alterations were observed in blood cells (Suppl. Fig. 3), and no changes were observed in the total number of erythrocytes or hemoglobin levels after the acute or subacute administration of Amblyomin-X (Tables 1 and 2). A decrease in platelet numbers was detected 24 h after the acute treatment (Table 1, 24 h). Nevertheless, this effect was reversible, as it was not observed 14 days after treatment (Table 1, 14 days). A small decrease in the leukocytes count was observed 24 h after acute treatment with doses above 32 mg/kg, which was reverted 14 days after administration (Table 1).

No statistical differences were observed in the other hematological parameters evaluated. The coagulation time of the blood samples obtained from the groups of animals treated with different concentrations of Amblyomin-X, acute and subacute groups, did not present significant variations in mean prothrombin time (Suppl. Tables B3, B4).

3.5. Biochemical analysis

Biochemical analysis showed no alterations on the levels of urea and creatinine (Tables 3 and 4), and histological analysis of the kidneys did not show any alterations (Figs. 2–4) indicating no toxicity upon Amblyomin-X treatment. Biochemical analysis showed no statistically significant alterations on AST and ALT levels in the acute and subacute dose treatment group (Tables 3 and 4).

3.6. Histopathological analysis

Histological analysis of spleen (Fig. 2, left column) and kidney (Fig. 2, middle column) showed no alterations with any treatment, with renal corpuscle with the glomerulus and the Bowman’s space well defined. Changes were observed only in the liver of animals treated with the acute dose of 256 mg/kg of Amblyomin-X, after 24 h of treatment (Fig. 2T, arrows), such as focal alteration, with the well delimited Glisson’s capsule consisting of relatively coarse cords of hyperesinophilic hepatocytes with smaller nuclei of condensed chromatin, karyolysis, necrosis and moderate diffuse neutrophil infiltrates. Hepatic histological alterations were reverted after 14 days (Fig. 2T). The injection of subacute doses of Amblyomin-X did not cause any alteration on spleen, kidneys and liver, as shown in the Fig. 4.

No morphological alterations were observed in the ovaries and testis of any animals (Fig. 5). Section of ovaries from control animals showed normal small follicles and large follicles. Ovaries from Amblyomin-X treated groups had a normal aspect, with small follicles and large follicles. The testis from control and Amblyomin-X treated animals (all doses) had well-layered seminiferous tubules with germ cells with normal seminiferous epithelium with spermatooza in the lumen and interstitial tissue (Fig. 5).

4. Discussion

Recombinant Amblyomin-X induces tumor cell death and does not affect the viability of normal cells. On model mice bearing tumors, treatment with Amblyomin-X caused a significant reduction in tumor mass sizes and in the number of metastasis [10,13,14]. Previous studies from our group demonstrated that Amblyomin-X antitumor activity involves induction of tumor cells apoptosis and inhibition of proteasome [13]. Due to its central role in the control of proteolysis, the ubiquitin-proteasome system (UPS) is an important target for diseases and Bortezomib, the first proteasome inhibitor drug approved by the FDA in 2003, is currently used for the treatment of multiple myeloma and mantle cell lymphoma [18], presenting several adverse effects, such as thrombocytopenia [19] and liver toxicity [20].

Herein, we present the first toxicity studies for Amblyomin-X administration in healthy mice, in order to establish a safety profile. In general, Amblyomin-X presented a profile of low toxicity, since most toxicity effects were reversible, as could be observed by monitoring the satellite experimental group.

Amblyomin-X treatment resulted in decreased body weight gain during the acute treatment with doses greater than 25 mg/kg and in the...
The subacute of 0.57 mg/kg, which was reverted after treatment termination, as observed in the satellite group. There were no deaths of the mice during the administration of Amblyomin-X, and no abnormality was observed in behavioral performance at concentrations below 32 mg/kg in the acute and sub-chronic tests at the concentrations tested.

No morphological alterations were observed in blood cells, but a decrease in platelets and leukocytes count was detected 24 h after the acute treatment, which returned to normal levels 14 days after treatment. Transient thrombocytopenia and leucopenia were also observed as adverse effects upon Bortezomib treatment [21,22]. No cytological changes were detected, such as large or giant platelets in the blood smear of animals from acute or subacute toxicity studies, suggesting the absence of toxicity effects of Amblyomin-X as a component of peripheral destruction leading to megakaryocyte hyperplasia in bone marrow or a structural defect of platelets.

In our study, no acute changes were observed in PT values, 14 days after the acute treatment and in the subacute toxicity group, which is in agreement with previous results from our group, where aPTT and PT prolongation was observed after Amblyomin-X administration in rabbits, but were either partially (PT) or fully (aPTT) normalized 24 h after injection [23].

Biochemical analysis showed no alterations on the levels of urea and creatinine, and histological analysis of the kidneys did not show any alterations indicating no toxicity to this organ upon Amblyomin-X treatment. The level of the enzymes AST and ALT are important parameters for evaluation of drug metabolism, since alterations on liver function are usually observed, as is the case of Bortezomib treatment which was reported to result in an increase in hepatic enzymes levels [20]. AST and ALT levels alterations upon Amblyomin-X treatment were not statistically significant, and these levels remained in the normal range previously reported for mice. These results are in agreement with histological analysis of the liver of the animals, where alterations were observed only on the acute group treated with 256 mg/kg of Amblyomin-X. Reversible effects of toxicity are commonly found in the liver because of its ability to regenerate and its adaptive capacities following contact with toxic compounds, even when the parenchyma is necrotic. [24]. Keles et al. [24], demonstrated extensive areas of liver degeneration induced by treatment with 0.2 mg/kg Bortezomib, which contrary to the data obtained with Amblyomin-X, were not reversible, with an indication of damaged hepatocytes as well as the vacuolization in the hepatocytes.

5. Conclusions

In this study, the maximum tolerable dose (MTD) and the no observed adverse effect level (NOAEL) was determined, based on the acute and subacute treatments. Based on the results, doses above 16 mg/kg represent the MTD and NOAEL was observed at concentrations of 0.57 mg/kg of Amblyomin-X. At these doses, no important histopathological changes were observed, and low toxicity effects were shown to be tolerable and reversible in the satellite group, thus establishing a safety profile for treatment with this novel drug candidate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxrep.2018.11.014.

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Conflict of interest statement

Recombinant Amblyomin-X is patent granted (WO/2006/029492). The authors declare no other conflicts of interest.
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