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Original Article

Invasion of TC-1 cells to skeletal muscle fibers protect them from peroxisomicine A1 (T-514) treatment in a murine model of cancer

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Abstract: T-514 or Peroxisomicine A1 (PA1) is a toxin isolated from plants of genus *Karwinskia*. *In vitro* studies described selective toxicity of PA1 on malignant cell lines. A toxic effect of PA1 on TC-1 cells *in vivo* was reported. The objective was to evaluate the effect of PA1 over invasion of TC-1 cells to muscle fibers *in vivo*. TC-1 cells were implanted in 36 mice divided in two groups (n: 18): treated with PA1 or with vehicle, a control group was included. At 10 days, nine mice of each group were euthanized. TC-1 implant site was analyzed by light and electron microscopy, a morphometric study was also performed. Remaining mice were used to evaluate tumor growth and survival time. Results show tumor cells between muscle fibers, with diminution in diameter, change in the staining pattern, loss of continuity of external lamina, and sarcoplasm with tumor cells. Statistically difference was observed between treated group *vs* control group. PA1 decreased tumor growth and increased the survival time in treated mice. The degree of resistant activity, aggressiveness, and invasiveness of TC-1 cells described in present work; should be taken into account in studies that evaluate effectiveness of therapies using this cancer model.

Keywords: Cancer model, TC-1 cells, intrasarcoplasmic invasion, peroxisomicine A1

Introduction

T-514 or peroxisomicine A1 (PA1) is one of the dimeric anthracenones compounds isolated from fruit of plants of genus *Karwinskia*. Dreyer et al. (1975) named them according to their molecular weight as: T-496, T-514, T-516 and T-544 [1].

Piñeyro et al. (1994) described for the first time a selective toxicity of T-514 on human tumor cell lines derived from liver, lung and colon. Also in this study, *in vitro* effect of T-514 was compared with antineoplastics like vincristine, epidoxorubicin and 5-fluorouracil among others; and it was observed that normal cell lines were more sensitive to these agents than tumor cell lines [2]. This has prompted further studies to establish T-514 as a potential anticancer agent.

In studies in patients, Martínez de Villarreal et al. (1996) reported stable disease, and increased survival time of patients with recurrent cervical cancer who were treated with T-514 during a clinical phase I study [3]. Due to these results, Piñeyro-López in 1995 and 1996 obtained patents for the use of T-514 as a potential anticancer agent. These patents were granted by the European Economic Community, USA, Japan, Korea, Canada and Mexico [4, 5].

In studies *in vitro*, it was described the activation of apoptosis by PA1 in HL-60 and HL-60MX leukemia cells [6]; as well as in other transformed cell lines such as HeLa, breast adeno-

carcinoma, colon adenocarcinoma and hepatoma [7].

In studies *in vivo*, it was shown that administration of toxic doses of PA1 ($2DL_{50}$=28 mg/kg body weight) to normal mice, caused apoptosis in liver, kidney and lung [8]. Other *in vivo* study demonstrated that early administration of 1 mg of PA1/kg weight caused necrosis of tumor TC-1 cells implanted in C57BL/6 mice without affecting liver, kidney and lung [9].

Recently, in a study performed in our laboratory with the murine cancer model with TC-1 cells implanted in C57BL/6 mice, we observed that tumor cells induced histopathological alterations in adjacent skeletal muscle fibers [10]. This cancer model with TC-1 tumor cells has been extensively used in the evaluation of the effectiveness of experimental vaccines, to generate immune protection against tumors induced by HPV-16 [11-13]. The objective of this study was to evaluate the effect of early administration of 1 mg of PA1/kg weight, on the histopathological alterations, caused by invasion of implanted TC-1 cells to adjacent skeletal muscle fibers in this murine cancer model. Histological, histochemical, immunohistochemical, ultrastructural and morphometric methods were applied.

Materials and methods

TC-1 tumor cell line

TC-1 tumor cells (ATCC: CRL-2785) (Manassas, VA, USA) were cultivated, harvested and quantified according to procedures already described [9].

Experimental animals

Fifty four C57BL/6 mice were used with 5-6 weeks of age, and 25-30 gr of weight. Animals were supplied by Harlan Mexico, UNAM laboratory. Mice were maintained on a standard laboratory diet, *ad libitum* water access, and light and dark cycles of 12 hours in the animal facility of the Department of Histology, Faculty of Medicine, UANL. Experiments were carried out according with the International Guidelines on the Appropriate Use of Experimental Animals, and according to Mexican Norm NOM-062-ZOO-1999 on the Technical Specifications for Production, Care and Use of Laboratory Animals [14]. The protocol was approved by the Bioethical Committee of the Faculty of Medicine UANL in Monterrey, Nuevo León, México.

Animals were organized in three groups as follows: Absolute control group (AC) (n: 18): This group did not receive implantation of TC-1 cells nor any treatment.

Negative control group (NC) (n: 18): This group received implantation of tumor cells and treatment with carthamus oil as vehicle administered via intraperitoneal (i.p.).

PA1 treated group (n: 18): This group received implantation of tumor TC-1 cells, and treatment with 4 doses of 1 mg PA1/kg weight each. PA1 was dissolved in vehicle and was administered via i.p.

After examination of viability with trypan blue, tumor cells were implanted in a concentration of 5×10^4 cells/100 µL of PBS 1X subcutaneously in the right hind leg. The day of implantation of TC-1 cells was considered as day 0, at days 2, 4, 6 and 8 post-implant doses of PA1 or vehicle were administered.

T-514 or PA1

In this study, PA1 was extracted from the fruit of *Karwinskia parvifolia* according to the method described by Guerrero et al., 1987 [15]. Isolation, purification and evaluation of its biological activity were carried out in the Department of Analytical Chemistry, Faculty of Medicine, UANL.

Tissue samples collection

At 10 days after implantation of the TC-1 cells, nine mice of each study group were euthanized by cervical dislocation and perfused by left ventricular puncture, first with saline solution and then with 2.5% glutaraldehyde pH 7.2-7.4 in saline cacodylate buffer. After perfusion, site of implantation of tumor cells in right hind limb was collected, in AC group samples of the right limb were also collected.

Morphological analysis

A portion of the sample was fixed by immersion in a solution of 4% paraformaldehyde in PBS 1X, processed by conventional histology technique and embedded in paraffin blocks.

Histological sections of 4 μm were obtained and stained with hematoxylin and eosin (H&E), or Masson's trichrome staining for histological analysis.

For histochemical evaluation, sections of 5 μm were pretreated with diastase for 30 min, and then tested with method of Periodic Acid-Schiff (PAS), to distinguish between glycogen and polysaccharide complexes present in the external lamina of muscle fibers.

Immunohistochemical analysis was carried out in 4 μm sections. TC-1 cells were identified with antibodies anti-cytokeratin AE1/AE3 (1:100) for their epithelial origin, and skeletal muscle fibers were identified with anti-skeletal muscle specific actin (1:100). Invasiveness of TC-1 cells to muscle fibers was also determined with immunolabeling of matrix metalloproteinase-9 (MMP-9) (1:400). Envision® system was used as detection system, antibodies and detection system were purchased from Dako Cytomation Inc®. Positivity was identified with 3,3'diaminobenzidine (DAB) and nuclei were contrasted with Mayer's hematoxylin. Sections of palatine tonsil, tongue and jejunum respectively were used as technique positive controls; as negative control incubation with the monoclonal antibody was omitted. Samples were analyzed by light microscopy.

Morphometric analysis of diameter fibers

For morphometric analysis, sections stained with Masson's trichrome method were used to identify muscle fibers in cross-section. A random sampling to measure diameters of 200 muscle fibers was applied. Images were analyzed with the NIS elements software version 2.30. Results of all study groups were compared by paired mean test using the SPSS program v. 16, a value of $P \leq 0.05$ was considered as statistically significant.

Electron microscopy analysis

Simultaneously, other portion of the implant site of tumor cells was fixed by immersion in a solution of 4% paraformaldehyde, 5% glutaraldehyde and 0.05% picric acid in 0.1 M PBS [16]. After 24 hours the tissues were rinsed briefly in buffer and postfixed in 2% aqueous osmium tetroxide for one hour at room temperature. The tissues were then treated with a 1% aqueous solution of uranyl nitrate. After rapid dehydration with acetone, the tissues were embedded in epoxy resin using the technique of Luft [17]. Sectioning was done with a diamond knife on an RMC ultramicrotome. The sections were collected on 200-mesh copper grids without a supportive substrate, stained with uranyl acetate [18], and then by lead citrate [19]. Microscopy was done in a Carl Zeiss EM109 electron microscope. Semithin sections were mounted on glass slides and stained with 1% toluidine blue for study with the light microscope.

Analysis of tumor growth

The other nine mice of each group were left alive to determine the daily tumor growth, tumor measurements started when a tumor mass of 5 to 7 mm was detected. Since then, every third day measurements of tumor growth were performed checking major and minor diameters using a vernier caliper. These data were substituted into the formula: $A \times B^2 \times \pi/6$, where A is the maximal tumor diameter, and B is the perpendicular diameter to the maximal diameter to obtain the tumor volume/per observation day/per group of study [20]. Data were graphicated and a statistical student's *t* test was used with a degree of significance adjusted to $P \leq 0.025$ obtained by the Bonferroni method.

Analysis of survival

The total number of mice in each group (n:9) was taken as 100% of the population, and the percentage of death for each day was subtracted. The following characteristics were used to consider the death of animals: 1) Spontaneous death of mice, or 2) euthanizing the mice because major diameter of tumor growth was larger than 2.5 cm, and this was recorded as death. The data were analyzed with the SPSS 16.0 program to obtain a Kaplan-Meier graphic. Statistical analyses of the data with a significance value of $P<0.05$ was applied to determine differences among groups.

Results

Light microscopy evaluation

At 10 days post-implantation,in samples analyzed from NC and PA1 treated groups no tumor mass was observed. But in histopathological analysis of these samples the following alterations were observed: masses of TC-1 cells infil-

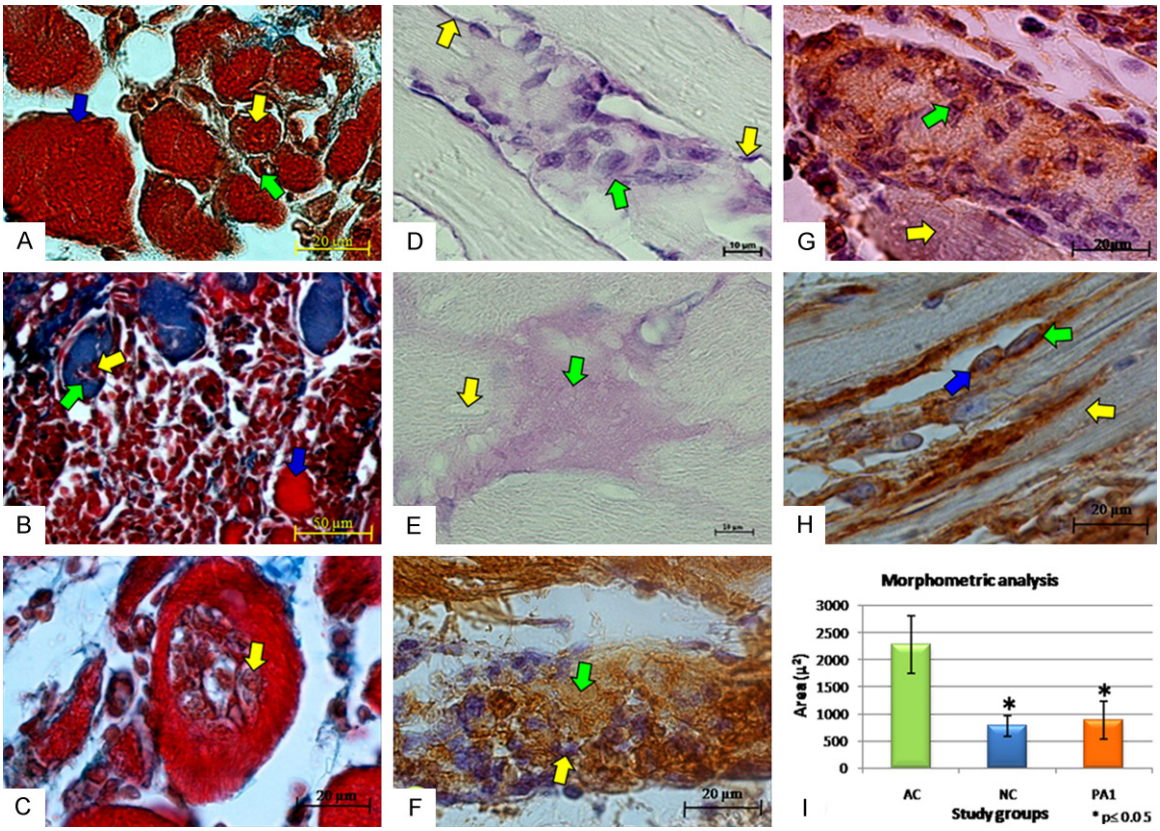

Figure 1. A: *Skeletal muscle fibers with diminution of the diameter*: Muscle fibers showing reduced diameter (yellow arrow), TC-1 cells (green arrow) and muscle fibers with normal diameter (blue arrow). B: *Changes in the staining pattern of skeletal muscle fiber*: blue muscle fibers instead of bright red for this technique (green arrow), TC-1 cells into the sarcoplasm of the fibers (yellow arrow), and normal red staining in muscle fibers (blue arrow). C: *TC-1 cells invading muscle fibers*: TC-1 cells in the sarcoplasm of skeletal muscle fibers (yellow arrow). D: *Loss of integrity of the external lamina*: skeletal muscle fibers that show loss of continuity of the external lamina (yellow arrows) on the site that were invaded by the TC-1 cells (green arrow). E: *PAS positive material between muscle fibers*: PAS positive material resistant to diastase without TC-1 cells (green arrow) between skeletal muscle fibers (yellow arrow). F: *Differentiation of skeletal muscle fibers of the TC-1 cells*: Muscle fiber (green arrow) with TC-1 cells into the sarcoplasm (yellow arrow). G: *Differentiation of TC- 1 cells of skeletal muscle fibers*: TC-1 cells (green arrow) into the sarcoplasm of muscle fibers (yellow arrow). H: *Positivity for synthesis and secretion of MMP-9*: TC-1 cells strongly positive for MMP-9 in the cytoplasm (blue arrow) and space (green arrow) between tumor and muscle fiber cells (yellow arrow). Light Microscopy, embedded in paraffin. A-C: Masson's trichrome. Bars: 20, 50 and 20 microns, respectively. D and E: Pre-treatment with diastase + PAS. Bar: 10 microns. F-H: Immunohistochemistry. Bar: 20 microns. I: *Statistical difference of muscle fibers diameters in the morphometric analysis*: Graph showing the decrease in the diameter of muscle fibers in groups with TC-1 cells implanted and treated with PA1 or vehicle. *P ≤ 0.05.

trating connective tissue between muscle fibers which showed reduced diameter (**Figure 1A**). Muscle fibers also showed variations and change in the staining pattern (**Figure 1B**). One important finding in this study was the observation of groups of TC-1 cells that invaded adjacent muscle fibers reaching the sarcoplasm (**Figure 1C**).

In histochemical analysis, loss of continuity of the external lamina surrounding the muscle fibers was observed in the site where fibers were invaded by TC-1 cells (**Figure 1D**). TC-1 cells in the extracellular space and into muscle fibers showed positivity to this method in the cytoplasm. Also PAS positive material resistant to pretreatment with diastase was observed between muscle fibers without presence of tumor cells (**Figure 1E**).

Immunohistochemical analysis confirmed the presence of TC-1 cells into the sarcoplasm of muscle fibers (**Figure 1F** and **1G**). Another finding was the positivity for MMP-9 both in the cytoplasm of tumor cells, and in the space between tumor cells and skeletal muscle (**Figure 1H**). Histological, histochemical and immunohistochemical alterations were similar

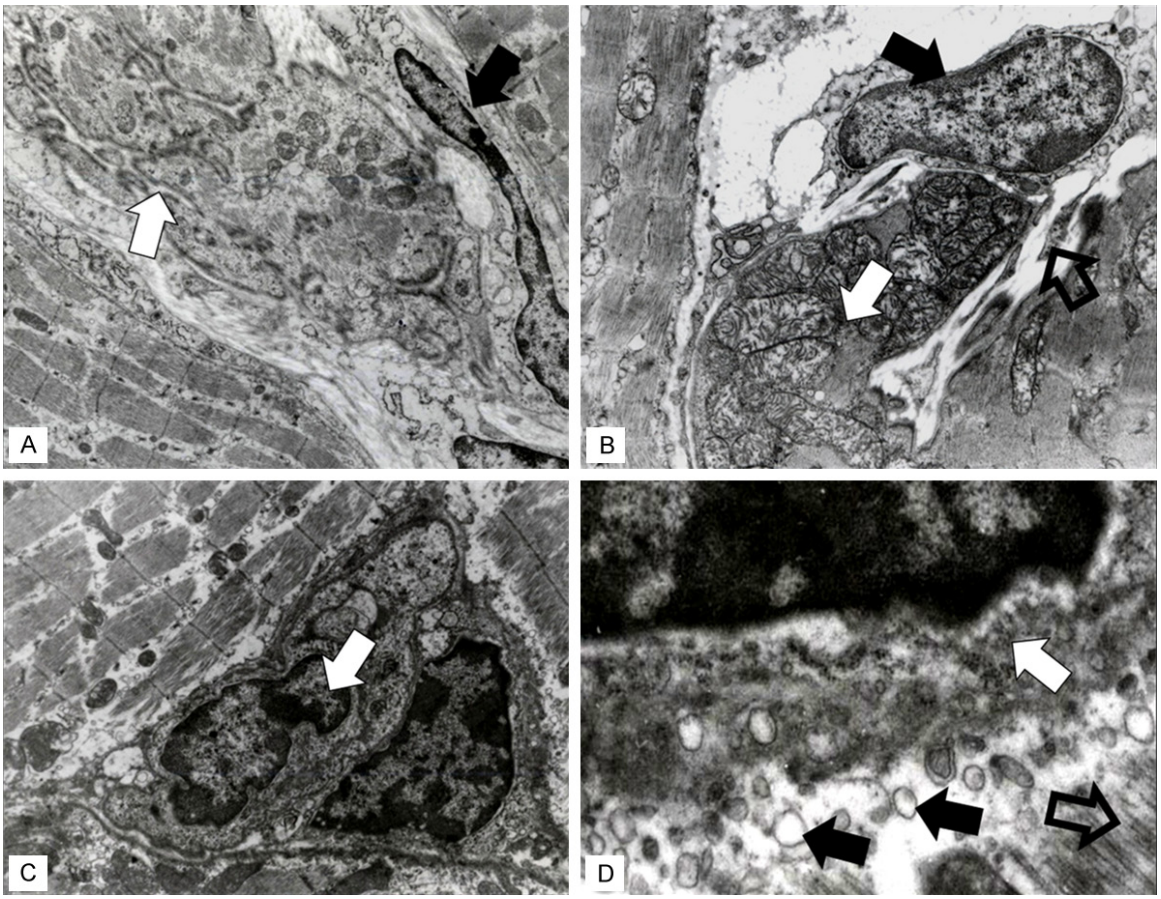

Figure 2. A: *Ultrastructural alterations in skeletal muscle fibers*: Tumor cells (black arrow) in the endomysium of muscle fibers that show spaces and disorganization of myofibrils (white arrow). B: *Invasion of TC-1 cells to adjacent skeletal muscle fibers*: TC-1 cell (black arrow) with shaped cytoplasmic pseudopodia (empty arrow), in contact with a muscle fiber that show alterations in myofibrils and organelles (white arrow). C: *TC-1 cells masses into the sarcoplasm of skeletal muscle fibers*: Tumor cells into the sarcoplasm (white arrow). D: *Rupture of external lamina and sarcolemma of muscle fibers:* Portion of a muscle fiber (empty arrow) showing subsarcolemmal vesicles (black arrows) resulted from sarcolemma rupture, TC-1 cell (white arrow). Embedded in epoxy resin, Contrasted with uranyl acetate and lead citrate. Transmission electron microscopy.

in NC and PA1 treated group. In AC a normal histology of skeletal muscle fibers was observed.

Morphometric analysis

In the morphometric analysis, the decrease in the diameter of muscle fibers observed in the histopathological analysis was corroborated. Contrasting the results of all study groups, a statistically significant difference was observed for a value of $P \leq 0.05$ (**Figure 1I**).

Ultrastructural evaluation

In the ultrastructural evaluation, muscle fibers infiltrated by TC-1 cells were observed,also muscle fibers showed ultrastructural signs of atrophy, destruction and separation of myofilaments, and variation in the electrodensity of myofibrils (**Figure 2A**). Tumor cells showed large euchromatic nucleus with prominent nucleoli, and scarce cytoplasm with few organelles. Another feature of TC-1 cells was the presence of ultrastructural signs of cell migration: elongated nuclei and constricted in the middle, as well as multiple membrane projections as pseudopodia, occurring in the areas where the external lamina of muscle fibers showed variations in thickness and fragmentation (**Figure 2B**). The presence of invading tumor cells within the sarcoplasm of muscle fibers was confirmed in the ultrastructural analysis (**Figure 2C**). Besides the destruction of the external lamina, small subsarcolemmal vesicles associ-

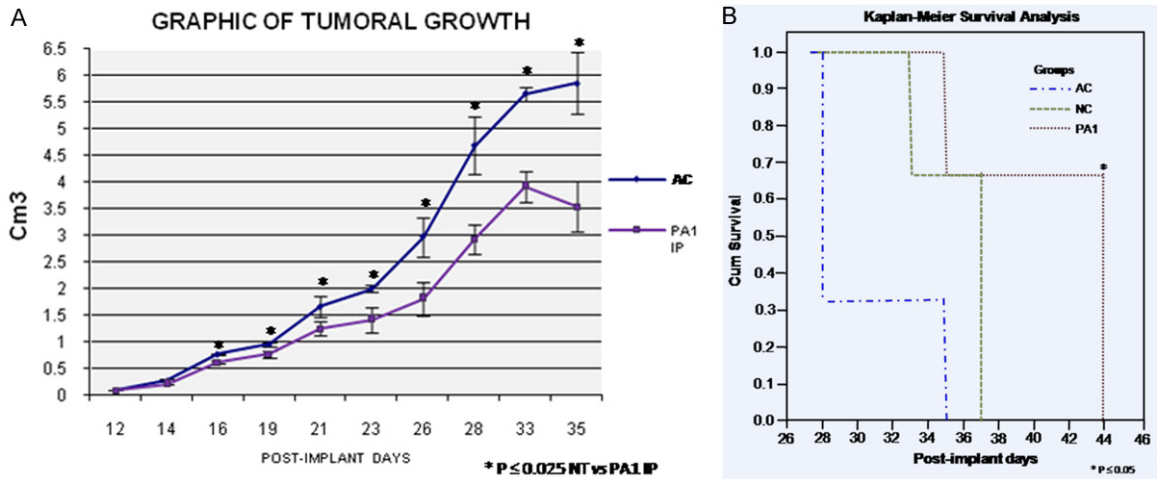

Figure 3. A: *Graphic of tumor growth:* Tumor mass was detected at day 12 after cell implantation and measurements were carried out until day 35 after cell implantation. Observe that in untreated animals tumors grew more rapidly than PA1 treated mice. At each daily measure point statistically significant differences were found. At the end of the observation period tumors from PA1 treated mice were smaller than those from untreated control. Bonferroni method and Student's *t*-test. *P ≤ 0.025. B: *Kaplan-Meier survival analysis*: observe that mice treated with PA1 showed an increased survival time. Statistic analysis shows that there is significant difference among mice of untreated control group *vs* mice of PA1 treated group. Student's *t*-test *P ≤ 0.05.

ated with sites of sarcolemmal rupture were observed in the site were tumor cells invade muscle fibers (**Figure 2D**).

Tumor growth analysis

In the tumor growth analysis, a tumor mass was detected at day 12 after cell implantation. Measurements of tumor diameters were made until day 35 after cell implantation and tumor growth was compared among groups. Despite all tumors grew in every group, we noted that in mice of AC and NC groups tumors grew faster compared to those treated with PA1. Values of the effect of vehicle were subtracted, and at the end of the observation period, tumors from the PA1 treated mice were smaller than those from control groups. At each daily measuring point, statistically significant differences were found (**Figure 3A**).

Survival analysis

We recorded that the tumor-bearing animals treated with PA1 had a longer survival time (33 to 44 days), than AC group (28 to 37 days) (**Figure 3B**).

Discussion

This study describes for the first time the resistance of tumor TC-1 cells adjacent to skeletal muscle fibers to treatment with PA1 in a murine model of cancer. At 10 days post-implant,no tumor was observed, but masses of TC-1 cells were observed infiltrating connective tissue between muscle fibers, these finding correlates with alterations described in an *in vivo* study with L1210 leukemia cells [21].

In this study, muscle fibers showed reduced diameter, variations and change in the pattern of staining. These histological alterations suggest that TC-1 cells release proteolytic factors, which can affect microenvironmental and structure of surrounding tissues, and their affinity to colorants. Proteolytic factors as TNF-α, IL-6 and IFN-γ have been reported in experimental animals with colon adenocarcinoma-26 cells [22, 23]. Also a proteolysis inducing factor has been demonstrated in mice with MAC16 cells, and in patients with cancer [24]. These factors cause an increase of 50% in protein catabolism, mainly of actin filaments and a decrease to 50% in protein synthesis [24-26]. Both mechanisms can occur simultaneously [27, 28], and result in severe muscle fibers atrophy that has been associated with the state of cachexia present in experimental animals, and in patients with cancer [29, 30]. Reduction in diameter of muscle fibers observed in groups with TC-1 implanted cells, were confirmed in the morphometric analysis that show a statisti-

cally difference of these groups, compared with group without tumor cells.

Furthermore, it was observed that in places were tumor cells invaded the muscle fibers; there is change in the staining pattern of the muscle fibers,which showed blue color instead of red bright characteristic with Masson's trichrome method. Changes in the pattern of staining may be related to intracellular physicochemical changes in muscle fibers apparently induced by the invasion of the TC-1 cells, as well as alterationsin the metabolism of proteins. These changes have also been associated with the invasion to nearby tissues, or may favor the generation of metastases by different type of cancer cells [31, 32]. It has been described that TC-1 cells caused metastasis to lung when they are injected by tail vein of syngeneic C57BL/6 mice [11].

Histochemical analysis showed loss of continuity of the external lamina surrounding muscle fibers, positive reaction at the cytoplasm of tumor cells invading muscle fibers, and in tumor cells infiltrating the connective tissue between the muscle fibers. PAS positive material resistant to pretreatment with diastase in the extracellular space without tumor cells was also observed. These results suggest that TC-1 cells synthesize and secrete components of the basement membrane, which promotes their migration. This mechanism has been described in cell types such as human lung carcinoma [33], and breast cancer [34], were the synthesis of basement membrane components enables them to migrate and invade distant tissues and organs to their site of origin, causing metastasis. In the literature we did not find reports describing this phenomenon regarding to TC-1 cells.

Immunohistochemistry studies with specific antibodies confirmed the presence of TC-1 tumor cell masses into the sarcoplasm of muscle fibers. This invasion of the muscle fibers has been associated with a worse prognosis in patients, and a higher degree of malignancy and invasiveness of tumor cells, as has been described in studies in humans and in experimental animals for tumor cell types different to TC-1 cells [35, 36].

In addition, positivity to MMP-9 was observed in cytoplasm of tumor cells and in the space between TC-1 cells and adjacent muscle fibers. This suggest that TC-1 cells synthesize and secrete this proteolytic enzyme to degrade extracellular matrix and external lamina surrounding muscle fibers, thereby facilitating the invasion of skeletal muscle. The synthesis and secretion of matrix metalloproteinases is related to resistance to antineoplastic agents, migration, and metastasis of tumor cells to distant organs and tissues at the site of origin [37].

When analyzing the ultrastructure of the TC-1 cells implanted in mice of NC and PA1 treated groups, tumor cells with cytoplasmic processes, few mitochondria and ribosomes, nuclei of variable size and heterogeneity in the arrangement of chromatin were observed. These ultrastructural signs correlate with those described for neoplastic cells that have few organelles by their high proliferation index without performing any secretory function [38].

Other findings in this study were the ultrastructural observation of disruption and changes in thickness of the external lamina surrounding the muscle fibers, as well as subsarcolemmal vesicles associated with the site of fragmentation of the sarcolemma were muscle fibers are invaded by tumor cells. These findings are consistent with those described by Brandes et al. (1967) in a murine model with L1210 leukemia cells [21].

Otherwise, in a previous work, we demonstrated that PA1 caused necrosis in TC-1 cells that are distant to the muscle fibers [9], but this did not occur in TC-1 cells that are adjacent or into the sarcoplasm of muscle fibers. This resistance may be due to the presence of different subpopulations of cells TC-1, as it has been reported in studies employing this murine cancer model testing other treatments [12, 39, 40].

The decreased volume size of tumors found at each measuring point in mice treated with PA1, compared to non treated controls, could be due to the necrotic process occurring at early times of tumor growth on the animals treated [9]. This fact could be the cause of the smaller size of tumors at the end of the observation period. At the same time the smaller growth observed in the tumors of treated animals may be related to

the longer survival time registered in this group. This could explain the findings of studies that reported stable disease, and increased survival time of patients with recurrent cervical cancer who were treated with T-514 during a clinical phase I study [3].

The degree of resistant activity to a potential antineoplastic agent as PA1, added to the aggressiveness, and invasiveness of TC-1 cellsas described in the present work; should be taken into account in studies that use this cancer model, to evaluate effectiveness of anticancer therapy. Currently in our laboratory, studies are conducted to evaluate the possible inhibitory effect of combination of antineoplastic agents on the invasion of the TC-1 cells to adjacent muscle fibers, as well as the effect of PA1 over other cancer cell lines in studies *in vitro* or *in vivo*.

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Disclosure of conflict of interest

None.

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