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Sodium orthovanadate associated with pharmacological doses of ascorbate causes an increased generation of ROS in tumor cells that inhibits proliferation and triggers apoptosis

Tânia Mara Fischer Günther [a], Maicon Roberto Kviecinski [a], Carla Cristine Baron [a], Karina Bettega Felipe [a], Mirelle Sifroni Farias [a], Fabiana Ourique da Silva [a], Nádia Cristina Falcão Bücker [a], Claus Tröger Pich [b], Eduardo Antonio Ferreira [c], Danilo Wilhelm Filho [d], Julien Verrax [e], Pedro Buc Calderon [e], Rozangela Curi Pedrosa [a,*]

[a] Departamento de Bioquímica, Universidade Federal de Santa Catarina, Florianópolis, Brazil
[b] Campus de Araranguá, Universidade Federal de Santa Catarina, Araranguá, Brazil
[c] Universidade de Brasília, Faculdade de Ceilândia, DF, Brazil
[d] Departamento de Ecologia e Zoologia, Universidade Federal de Santa Catarina, Florianópolis, Brazil
[e] Toxicology and Cancer Biology Research Group, Louvain Drug Research Institute, Université Catholique de Louvain, Brussels, Belgium

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ABSTRACT

Pharmacological doses of ascorbate were evaluated for its ability to potentiate the toxicity of sodium orthovanadate (Na_3VO_4) in tumor cells. Cytotoxicity, inhibition of cell proliferation, generation of ROS and DNA fragmentation were assessed in T24 cells. Na_3VO_4 was cytotoxic against T24 cells (EC_{50} = 5.8 μM at 24 h), but in the presence of ascorbate (100 μM) the EC_{50} fell to 3.3 μM. Na_3VO_4 plus ascorbate caused a strong inhibition of cell proliferation (up to 20%) and increased the generation of ROS (4-fold). Na_3VO_4 did not directly cleave plasmid DNA, at this aspect no synergism was found occurring between Na_3VO_4 and ascorbate once the resulting action of the combination was no greater than that of both substances administered separately. Cells from Ehrlich ascites carcinoma-bearing mice were used to determine the activity of antioxidant enzymes, the extent of the oxidative damage and the type of cell death. Na_3VO_4 alone, or combined with ascorbate, increased catalase activity, but only Na_3VO_4 plus ascorbate increased superoxide dismutase activity (up to 4-fold). Oxidative damage on proteins and lipids was higher due to the treatment done with Na_3VO_4 plus ascorbate (2–3-fold). Ascorbate potentiated apoptosis in tumor cells from mice treated with Na_3VO_4. The results indicate that pharmacological doses of ascorbate enhance the generation of ROS induced by Na_3VO_4 in tumor cells causing inhibition of proliferation and apoptosis. Apoptosis induced by orthovanadate and ascorbate is closer related to inhibition on Bcl-xL and activation of Bax. Our data apparently rule out a mechanism of cell demise p53-dependent or related to Cdk2 impairment.

1. Introduction

Vanadium salts may be promising for anticancer therapy. They present cytotoxicity against several types of human tumor cell lines. Earlier reports have demonstrated that subcutaneous injections of orthovanadate into mice bearing a highly metastatic murine lymphosarcoma MDAY-D2 triggered significant reduction of tumor growth [1–3]. Multiple biochemical and molecular actions of vanadium have been associated with its antitumor properties [4]. It is well-known that it can act on the apoptotic process by modulating the extent and duration of phosphorylation on a great variety of key-proteins from cell signaling mediated by reactive oxygen species (ROS) and DNA damage [5]. Evidence demonstrates the possible involvement of vanadium in redox reactions associated with ROS generation that can culminate in cell cycle arrest at the G2/M phase and cell death [6,7].

Exogenously added compounds containing orthovanadate or vanadium (5^+ or V) can permeate the cancer cells, where they are reduced to vanadyl (4^+ or IV) consuming reducing agents common to cells such as ascorbate and glutathione [8]. The reaction

* Corresponding author. Address: Laboratório de Bioquímica Experimental – LABIOEX, Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, CEP 88040-900 Florianópolis, SC, Brazil. Fax: +55 483721 9672.
E-mail addresses: rozangelapedrosa@gmail.com, roza@ccb.ufsc.br (R.C. Pedrosa).

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with ascorbate involves rapid NADH oxidation and some studies suggest that vanadyl can also react with O_2 yielding H_2O_2, hydroxyl radicals and related oxygen species [9,10].

Cancer cells often exhibit high levels of ROS and low antioxidant defense activity [11]. Due to an overexpression of GLUT transporters, cancer cells may increase the uptake and accumulate ascorbate [12,13]. Thus, in this study, we explored this vulnerability (high ROS and low antioxidant defenses) by evaluating the administration of sodium orthovanadate, alone or combined with pharmacological doses of ascorbate. We hypothesized that ascorbate, by impairing the cancer cells redox status, can potentiate orthovanadate antitumor activities, leading them to a loss of cell growth capability and/or to cell death. Thus, we evaluated the type of cell death and whether orthovanadate combined with ascorbate provokes increased cytotoxicity, ROS generation, inhibition of proliferation and antitumor effects in mice.

2. Materials and methods

2.1. Chemicals and antibodies

Sodium orthovanadate, sodium ascorbate, N-acetylcystein (NAC), catalase (CAT) and dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics and other cell culture material were obtained from Cultilab (São Paulo, Brazil). The kit for apoptosis detection containing annexin V-FITC and propidium iodide was from BD Pharmingen (San Diego, CA, USA). Rabbit polyclonal antibody against P53 and cyclin-dependent kinase 2 (Cdk2) and mouse polyclonal antibody against Bcl-xL and Bax were from Santa Cruz Biotechnology Inc. (USA). Mouse antibody against β-actin, the secondary antibodies and the kit for chemiluminescence detection of HRP-coupled antibodies were from Millipore (USA). All other products were from ACS analytical grade reagents.

2.2. In vitro assays

Human bladder carcinoma-derived T24 cells were obtained from the Rio de Janeiro cell bank, Brazil. Cells were grown in DMEM supplemented with FBS 10%, penicillin 100 U/mL and streptomycin 100 μg/mL. Cytotoxicity was assessed by the MTT assay as described by Mosmann [14]. Adhered cells (10^4) in 96-well plates were exposed for 24 h to medium containing ascorbate up to 100 μM or orthovanadate (0.5–10 μM) separately or combined. Fresh medium was utilized for the negative controls. Three independent experiments were done and the results were presented in the form of EC_{50}.

Cell proliferation was evaluated according to Franken et al. [15]. Cells were seeded at single cell density (500) in 6-well plates, and after adherence they were treated respectively. They were then washed twice and fresh medium was added. After 10 days, cells were fixed and stained by crystal violet. Colonies with more than 50 cells were counted.

Intracellular ROS were evaluated as reported by Kviecinski et al. [16]. Cells (15.000) were loaded with 10 μM DCFH-DA in HBSS and incubated for 30 min. Excess DCFH-DA was removed by washing with fresh HBSS. Cells were then incubated for 2 h with the test compounds, washed twice more with HBSS, and then 100 μL of HBSS/well was added. Fluorescence was measured with a microplate reader (Victor™ ×2, PerkinElmer) at 485 nm for excitation and at 530 nm for emission. Changes in ROS levels were determined by calculating $\Delta F = (F - F0)/F0$, where F represents the fluorescence read at each time point and $F0$ the control fluorescence.

DNA fragmentation was evaluated as reported by Rey et al. [17]. Plasmid DNA (600 ng) in HEPES buffer 20 mM (pH 7.4) was exposed for 16 h at room temperature to orthovanadate (10 μM) or ascorbate (100 μM) separately or combined. EcoRI 50kU (1 μL) was utilized for the positive controls. Samples were then submitted to agarose gel electrophoresis and stained with ethidium bromide, from which we obtained bands that represented the profile of a breach in DNA. Bands were quantified by densitometry analysis using Image J 1.30 software (NIH, USA).

Western blots were performed as described by Dejeans et al. [18], using whole cells protein homogenates from treated with orthovanadate (5 μM) or ascorbate (1 mM) separately or combined for 6 h. Control conditions represented protein homogenates from non-treated cells. Equal amounts of protein (20 μg) were subjected to SDS–PAGE followed by electroblot to PVDF membranes. Membranes were incubated with the primary antibodies and further incubated with the secondary ones. Immune detection was done using a chemiluminescence kit for HRP-coupled antibodies. β-Actin served as a loading control.

2.3. In vivo antitumor activity

Male BALB/c inbred mice (20–22 g) were kept, receiving water and food *ad libitum*. Procedures were done in accordance with legal requirements (NIH publication #80-23, revised in 1978) and with the approval of the local ethics committee. Previous tests were done to select the maximal safe doses of orthovanadate, afterwards ascorbate was administered at 10-fold higher the dose of orthovanadate. On Day zero, Ehrlich carcinoma cells (5×10^6) were inoculated i.p. into mice from four groups ($n = 12$). i.p. treatments were done every 24 h for 9 days. Control animals receiving saline i.p. injections. Test groups received orthovanadate (18.75 mg/kg) or ascorbate (187.5 mg/kg). The last group received both orthovanadate and ascorbate in combination. On the tenth day, six mice from each group were euthanized. The inhibition on tumor growth was found taking into consideration the change in the abdominal circumference measured on days 0 and 10 [19]. Peritoneal cell suspensions were collected to determine oxidative stress markers and type of cell death. The remaining animals were kept alive to evaluate parameters of survival. The percentage of increased life span was calculated by recording the mortality on a daily basis for 30 days according to Mazumdar et al. [20] as follows:

$$\text{Mean survival time (MST)} = \text{day of the first death} + \text{day of the last death}/2 \quad (1)$$

$$\text{ILS (\%)} = [(\text{MST of treated group}/\text{MST of control group}) - 1] \times 100 \quad (2)$$

2.4. Oxidative stress in tumor tissue

Catalase activity was determined kinetically through the method described by Aebi [21] based on the decomposition of H_2O_2 and the decrease in absorbance at 240 nm. Superoxide dismutase (SOD) activity was measured monitoring the oxidation of adrenaline to adrenochrome as described by Misra and Fridovich [22]. Oxidative damage to proteins was quantified as carbonyl proteins as originally described by Levine et al. [23]. Carbonyl groups from proteins in the sample react covalently with 2,4-dinitrophenylhydrazine in acid, which leads to the formation of a 2,4-dinitrophenylhydrazone product. The reaction was followed by spectrophotometric quantification of the acid hydrazones at 370 nm. Lipid peroxidation was estimated by measurement of malondialdehyde formation using the thiobarbituric acid method, as described by Okawa et al. [24].

The results were normalized to the protein content using the Bradford method [25].

2.5. Type of tumor cell death in vivo

Tumor cells obtained from the peritoneal cavity of mice were washed (10^6) and treated with a binding buffer 100 μL (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and Annexin V-FITC (2 μL) and propidium iodide (2 μL) were added using a kit from BD Pharmingen (San Diego, CA, USA) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after cell labeling. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software. Another 5×10^6 cells washed in PBS (1 mL) were treated with a dye solution (1 μl) containing ethidium bromide 100 μg/mL and acridine orange 100 μg/mL (1:1). These cells were observed on slides under a fluorescence microscope. For each sample 300 cells were counted in triplicate. They were photographed for a visual record. The results were expressed as a percentage of viable, apoptotic and necrotic cells [26].

2.6. Statistical analysis

In vitro assays were repeated three times independently. Biochemical assays were done in triplicate. Results expressed as mean ± standard deviation. Data were analyzed with the ANOVA test followed by the Bonferroni test. Comparisons and the EC_{50} values were done using GraphPad Prism software (San Diego, USA). Values of $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

There is special attention on vanadium salts due to their cytotoxicity. Vanadium is truly bioactive by modifying xenobiotic enzymes, inhibiting tyrosine phosphatases, and at the same time, activating phosphorylases triggering a number of cellular metabolic pathways, which can lead to cell death mainly through apoptosis [4,27]. The results of the MTT assay reveal that orthovanadate increased T24 cells death in a concentration-dependent manner, whereas ascorbate up to 100 μM did not induce cytotoxicity. Consequently, the EC_{50} value estimated at 24 h for orthovanadate was 5.8 μM. Orthovanadate, when administrated in combination with 100 μM ascorbate presented decreased EC_{50} equivalent to 3.3 μM.

Evidence from literature also indicates that vanadium compounds can exert antiproliferative effects mainly through cell cycle arrest [6,7]. Fig. 1(A) shows that orthovanadate reduced in a concentration-dependent manner the proliferation of T24 cells. The treatments done only with ascorbate up to 100 μM did not affect cell proliferation. Contrarily, the addition of ascorbate to orthovanadate-treated cells resulted in a strong inhibition of cell growth as shown in Fig. 1(A).

As explained in the introduction section, some evidence indicates that within cells, vanadium V may be reduced by cellular electron donors such as ascorbate, thereby inducing ROS formation [8–10]. Data showed in Fig. 1(B) demonstrates the formation of ROS suggesting a role of free radicals in orthovanadate–ascorbate toxicity. Here it is possible to verify that the addition of ascorbate to orthovanadate-treated cells in culture increased by about 4-fold the content of intracellular ROS, whereas the antioxidants NAC or CAT decreased in a substantial way the formation of ROS.

A previous study indicated that vanadium compounds induce DNA cleavage, not directly reacting with DNA but acting through the production of ROS [7]. Fig. 1(C) shows the integrity of plasmid DNA which was estimated from its electrophoretic mobility under the different experimental conditions. FI represented the predominant intact form of DNA as observed in control conditions, whereas DNA treated by EcoRI was cleaved presenting altered

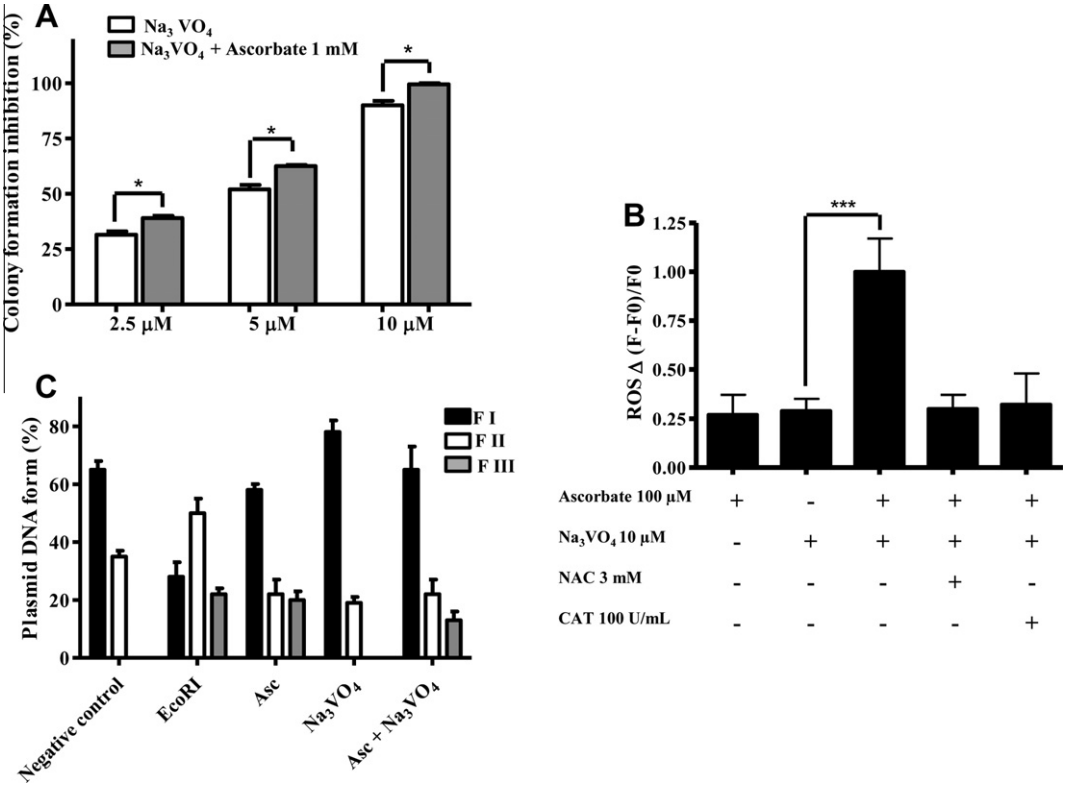

Fig. 1. Na_3VO_4 plus ascorbate (Asc) caused increased inhibition of cell proliferation (A) and generation of intracellular ROS (B) but did not directly cleave plasmid DNA (C). N-acetylcystein (NAC) or catalase (CAT) can restrict ROS formation. (*) and (***) denote difference at $p < 0.05$ and $p < 0.001$ between indicated treatments.

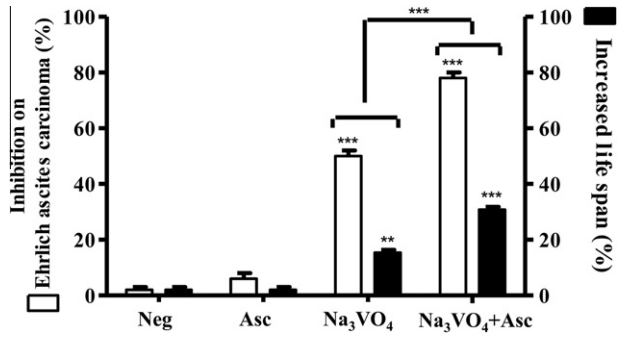

Fig. 2. Na_3VO_4 plus ascorbate (Asc) caused increased inhibition on tumor growth. The animals under this treatment lived more. (**) and (***) denote difference at $p < 0.01$ and $p < 0.001$ compared to the negative control (NEG) or between indicated treatments.

motility with most of DNA under the FII and FIII forms. We observed no differences between control and orthovanadate treated-DNA plasmids. In addition, these data indicated that no synergism is occurring between orthovanadate and ascorbate. Indeed, the DNA cleaving effect of both products administered together was not greater than the effects of the products administered separately (Fig. 1C).

Years ago, vanadium tested along with other metals was inactive against spontaneous mice tumors [28]. However, experiments carried out during the last two decades have demonstrated that orthovanadate and various peroxovanadates actually exert significant anticancer effects on tumor bearing mice [3,29,30]. Fig. 2 shows that Na_3VO_4 inhibited Ehrlich ascites carcinoma proliferation and enhanced the survival of mice. Very interestingly, this effect was more than 2-fold higher when we combined with ascorbate at a pharmacological dose. The inoculation (i.p.) of Ehrlich carcinoma in saline-treated mice resulted in regular development of ascites fluid. As illustrated in Fig. 2, the treatment done only with ascorbate was unable to produce a considerable difference neither in terms of tumor growth inhibition nor ILS. Animals treated with orthovanadate showed approximately 50% reduction in the tumor growth on average in comparison to saline-treated animals (open bars). Interestingly, the inhibition was superior by up to 80% when the animals' treatment combined orthovanadate–ascorbate. Animals treated with orthovanadate lived more than saline-treated mice presenting ILS of about 20% (closed bars). However, the animals that received orthovanadate combined with ascorbate lived about 35% more than animals from the control group (Fig. 2).

Previous studies have already shown that administration of vanadium salts to mouse epidermal cells induced apoptosis through the production of H_2O_2 and other ROS, which in turn, activated p53 [31,32]. Once we confirmed that the administration of ascorbate to orthovanadate-treated tumor bearing mice resulted in an increased survival, we decided to monitor ROS generation and some oxidative markers *in vivo,* also evaluating the dying tumor cells trying to differentiate them according to the type of death. Data shown in Fig. 3 relate to the effects of orthovanadate alone or with ascorbate on some oxidative markers here measured in cellular suspensions from mice peritoneal cavity. By looking Fig. 3, a similar profile for all the markers was observed. Increased activity of catalase and superoxide dismutase (Fig. 3A/B) plus

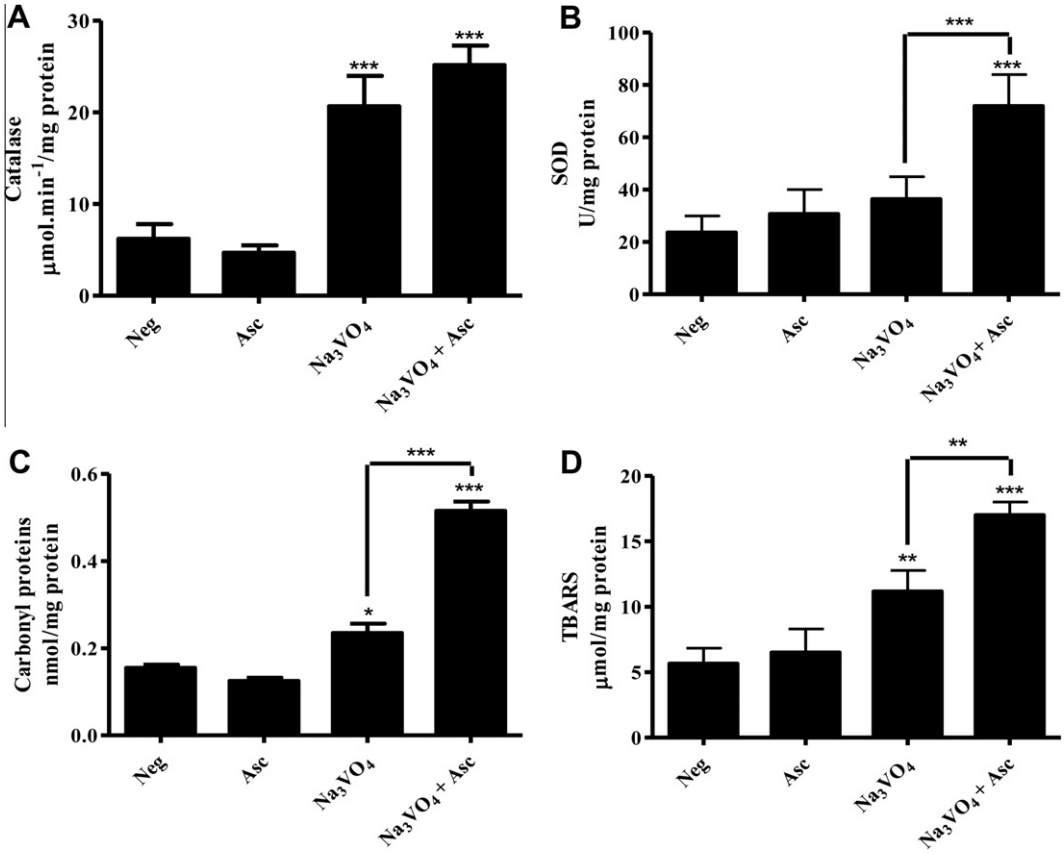

Fig. 3. Na_3VO_4 alone and combined with ascorbate (Asc) increased catalase activity (A) but only Na_3VO_4 plus ascorbate increased superoxide dismutase (SOD) activity (B). Oxidative damage on proteins (C) and lipids (D) was higher due to the treatment done with Na_3VO_4 plus ascorbate. (*), (**) and (***) denote difference at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively compared to the negative control (NEG) or between indicated treatments.

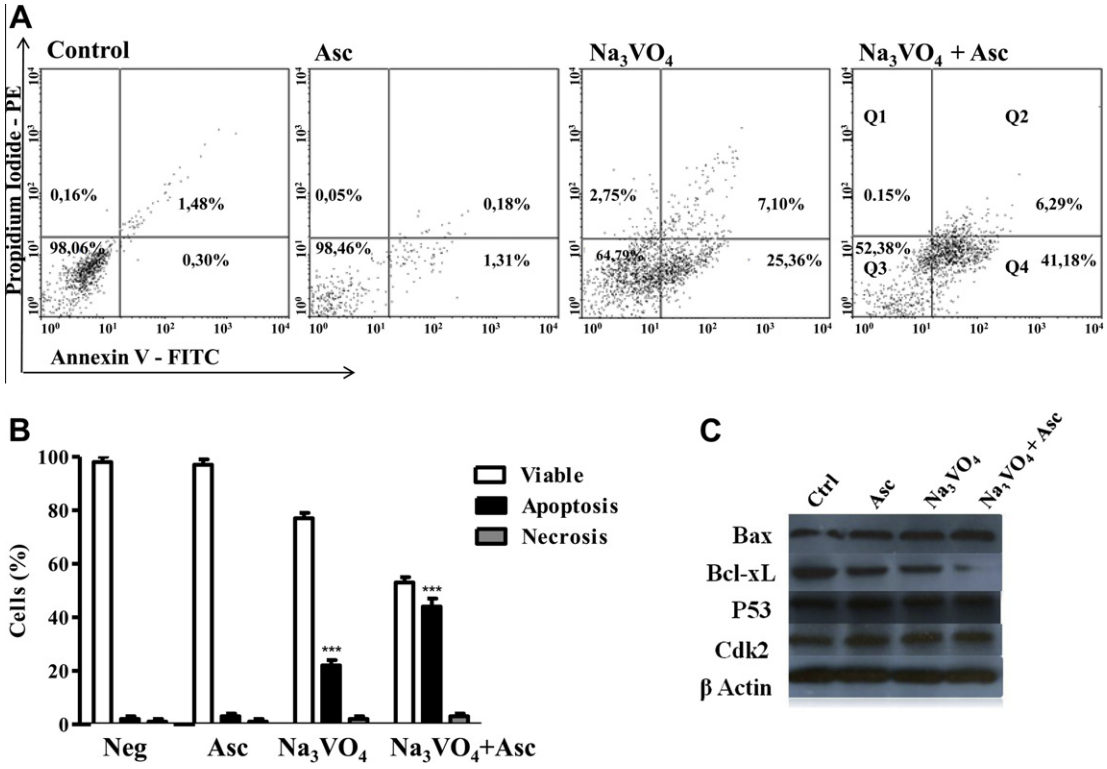

Fig. 4. Ascorbate (Asc) potentiated apoptosis in tumor cells from mice treated with Na_3VO_4 as indicated by the increase in annexin V-FITC positive cells (A) and cells with bright green nucleus and condensed or fragmented chromatin double stained with acridine orange and ethidium bromide (B). Orthovanadate plus ascorbate inhibits Bcl-xL and apparently activates Bax, whereas P53 and Cdk2 are not modified by the treatments (C).

increased levels of carbonyl proteins and lipid peroxidation (Fig. 3C/D) we detected in samples of tumors in remission from mice treated with orthovanadate. This reinforced that an oxidative stress does occur *in vivo*. Once again the addition of ascorbate to orthovanadate resulted in a 2–3-fold increase on average in oxidative markers compared to samples from only orthovanadate-treated mice.

In the ideal anticancer therapy, the drugs would be able to specifically target the cancer cells. Identifying cell-specific inducers of apoptosis is a great challenge. In order to assess the demise of Ehrlich cells triggered by orthovanadate–ascorbate *in vivo*, we performed two assays reflecting the cell membrane integrity and phosphatidylserine translocation by a double Annexin-V and propidium iodide staining (Fig. 4). Both results confirm that orthovanadate can actually lead cells to apoptosis, although the combination orthovanadate–ascorbate can kill more cells than orthovanadate alone. The increased occurrence of dying cells in Quadrant 4 suggests the presence of cells mainly in early and mid-stage apoptosis (Fig. 4A). Apoptosis occurred in cancer cells from mice treated with orthovanadate very possibly because orthovanadate triggered ROS over production. We found impairment to cell proliferation as well. Nevertheless, considering the data in Fig. 4, ascorbate potentiates apoptosis caused by orthovanadate.

The step of decision of apoptosis is controlled by the genes Bcl-2 and p53. Bcl-2 proteins regulate apoptosis on mitochondria membranes and endoplasmic reticulum; they can act on the calcium channels. Proteins such as Bcl-2 and Bcl-xL prevent apoptosis, whereas X protein-associated Bcl-2 such as Bax, Bak and Bcl-xS promote it [33]. Data illustrated in Fig. 4(C) show that the treatments with orthovanadate and ascorbate had little effect on Bax, but Bcl-xL was clearly inhibited. Recently, Morita et al. [34] demonstrated that apoptosis mediated by p53 can be inhibited by orthovanadate. However, our data show that vanadate induces apoptosis potentiated by ascorbate, probably due to a mechanism p53-independent. Indeed, data in Fig. 4(C) confirm that p53 was not affected either by vanadate alone or by its association with ascorbate. Moreover, according to data in Fig. 4(C), it seems that the mechanism of apoptosis induced by vanadate and ascorbate does not include actions on Cdks. Actually the bands corresponding to Cdk2 from cells treated with vanadate and/or ascorbate were not different from that of the controls either.

Assuming that cancer cells are taking up more ascorbate than normal cells [12,13], this study showed evidence that encourages the development of a novel way to explore vanadium antitumor properties by favoring its selectivity.

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