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Uncaria tomentosa extract alters the catabolism of adenine nucleotides and expression of ecto-5′-nucleotidase/CD73 and P2X7 and A1 receptors in the MDA-MB-231 cell line

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ABSTRACT

Ethopharmacological relevance: *Uncaria tomentosa* (Willd.) DC. (Rubiaceae) (Ut), also known as cat's claw, is a woody liana widely spread throughout the Amazon rainforest of Central and South America, containing many chemical constituents such as oxindole alkaloids, which are responsible for various biological activities. Since ancient times, the indigenous people of Peru have used it as a bark infusion for the treatment of a wide range of health problems gastric ulcers, arthritis and rheumatism. Recently, Ut is distributed worldwide and used as an immunomodulatory and anti-inflammatory herbal remedy. Additionally, *U. tomentosa* also has antitumural activity. However, little is known about the action of *U. tomentosa* on the purinergic system mechanisms, which is involved in tumor progression.

Aim of the study: Considering the pharmacological properties of *U. tomentosa*, we sought to evaluate the hydroalcoholic extract *U tomentosa* is able to influence the purinergic system in breast cancer cells, MDA-MB-231. Through the activity and expression of ectonucleotidases (NTPDase – CD39; Ecto-5′-nucleotidase – CD73) and purinergic repceptores (P2X7 and A1).

Materials and methods: A hydroalcoholic extract was prepared in two concentrations, 250 and 500 μg/mL. (Ut250; Ut500). The effect of these concentrations on the activity and expression of ectonucleotidases, as well as on the density of purinergic receptors were investigated in MDA-MB-231 breast cancer cells. Cells were treated with the hydroalcoholic extract of *Uncaria tomentosa* and/or doxorubicin (Doxo 1 μM; Ut250+Doxo; Ut500+Doxo) for 24 h.

Results: Although the results were not significant for the hydrolysis of the ATP, they presented an increase in the ADP hydrolysis in the Ut500+Doxo group when compared to the control group. Additionally, the activity of 5′-nucleotidase was inhibited in all groups when compared with the untreated group of cells. Inhibition of the enzyme was more evident in groups with *U. tomentosa* per se. The expression of CD39 was increased in the Ut250 and Ut250+Doxo groups when compared to the control group. No changes were found in the CD73 expression. Furthermore, a reduction in the density of the P2X7 receptor in all treated groups was detected. On the other hand, the density of the A1 receptor increased in all groups compared to the control group, with the exception of the Ut500+Doxo group.

Conclusion: Therefore, we conclude that hydroalcoholic extract of *U. tomentosa* may be responsible for the reduction of adenosine levels in the extracellular medium, which accelerates tumor progression. Interestingly, the dysregulation of A1 and P2X7 receptors in the MDA-MB-231 cells exacerbate the

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proliferation of this cells and *U. tomentosa* treatment may be stimulate the antitumor activity of adenosine A1 receptor and control the P2X7 effects. Our study demonstrates the significant participation of purinergic pathway in the regulation of MDA-MB-231 progression; additionally, *U. tomentosa* treatment alone or combined with chemotherapy may favor the action of doxorubicin.

1. Introduction

Uncaria tomentosa (Willd.) DC. (Rubiaceae), also known as cat's claw, is a woody liana widely spread throughout the Amazon rainforest of Central and South America (Rizzi et al., 1993). Since ancient times, the indigenous people of Peru used it as a bark infusion for the treatment of a wide range of health problems such as cancer, gastric ulcers, arthritis and other inflammatory process (Keplinger et al., 1999; Heitzman et al., 2005). A wide spectrum of biological activities of this plant results from the presence of many secondary metabolites, and several phytochemical studies have shown that more than 50 chemical constituents exist in cat's claw, including tetracyclic and pentacyclic oxindole alkaloids (Keplinger et al., 1999; Heitzman et al., 2005). *U. tomentosa* is a medicinal herb that possesses important antioxidant properties (Pilarski et al., 2006) used for various therapeutic purposes including arthritis, rheumatism, inflammatory diseases, and cancer (Laus et al., 1997).

Currently, natural products play a relevant role in cancer therapy, in which a substantial number of anticancer agents used in clinical treatments originate from various natural sources such as plants, animals and microorganisms (Nobili et al., 2009). Various alkaloids, many secondary metabolites isolated from plants are also well known anticancer alkaloids, which act selectively on cancer cells affecting cell cycle progression (Urra et al., 2013). *U. tomentosa* presents additional activities, including immune stimulation (Sheng et al., 2005), induction of apoptosis in different cell lines (Sheng et al., 2000; De Martino et al., 2006), as well as antioxidant activity (Pilarski et al., 2006, 2010) and antitumoral activity in the Walker-256 cancer model (Dreifuss et al., 2010).

Breast cancer is the most common cancer in women. It is routinely classified by stage, pathology, grade and expression of estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor (Her2/neu) (Fernandez et al., 2010; Beamont and Leadbeater, 2011). The absence of expression in all these receptors is known as triple-negative breast cancer (TNBC) (Foulkes et al., 2010; Elias, 2010). Notably, approximately 15–20% of globally diagnosed breast cancer is characterized as TNBC (Foulkes et al., 2010). The MDA-MB-231 cell, which is an invasive ductal carcinoma line, is an example of TNBC (Cailleau et al., 1978; Engel and Young, 1978; Tokunaga, 2006).

Some authors have proven that there is a positive relationship between the presence of hormone receptors and a more favorable prognosis for breast cancer (Osborne, 1990; Blows et al., 2010). Thus, patients with negative tumors for both receptors (ER, PR and HER2) presented worse prognosis than those negative for only one receptor (Reiner et al., 1990; Cianfrocca and Goldstein, 2004; Blows et al., 2010).

One of the treatment options for breast cancer may be chemotherapy, which can be a single or combined therapy utilizing multiple drugs (Ozer et al., 2000). Anti-carcinogenic drugs may act through different mechanisms preventing cell proliferative capacity (Nobili et al., 2009; Rosales-Hernandez et al., 2009). However, conventional chemotherapy causes severe side effects, with limited success rates due to insufficient drug concentrations, high systemic toxicity, lack of selectivity, and increased tumor resistance to drug therapy after prolonged treatment (Xu and Mcleod, 2001).

Solid tumors, such as those seen in breast cancer, often cause chronic hypoxia and necrosis that lead to the release of adenine nucleotides. Hypoxic tumors release purines into their immediate environment (Spychala, 2000); in this context, the metabolism of such purines is proposed to be associated with tumor progression in breast cancer (Spychala et al., 2004).

Extracellular purines and pyrimidines are signaling molecules that have many different effects on biological processes (Burnstock and Knight, 2004). The hydrolysis of ATP to AMP is catalyzed either-by ecto-ATPDases or NTPDase (Zimmermann, 2001) and the AMP formed by this enzyme is hydrolyzed to adenosine by the action of an ecto-5′-nucleotidase (Zimmermann, 1992).

Ecto-5′-nucleotidase/CD73 is expressed in many different tissues and this enzyme has been described as an important molecule in cancer progression, involved in the control of cell growth, maturation, and differentiation, as well as in drug resistance and tumor-promotion (Spychala, 2000; Wang et al., 2008).

The extracellular ATP is capable of activating ionotropic receptors such as P2X7, and the activation of this receptor can lead to inhibition or proliferation of cancerous cells according to the type of tumor. (Roger et al., 2014). Additionally, the metabotropic receptor A1, which is adenosine sensitive, possesses pro-tumor or anti-tumor activity, depending on the type of cancer cell (Gessi et al., 2011).

Considering the pharmacological properties of *U. tomentosa* is still necessary to trace their possible mechanisms of action or properly which will be their target molecular pathway. Our primary aim in this study is try to supply evidence if the hydroalcoholic extract of *U. tomentosa* may be regulate the alterations in purinergic system in the MDA-MB-231 breast cancer cells.

2. Materials and methods

2.1. Uncaria tomentosa extract

Uncaria tomentosa extract (5%) was prepared by ultra-turrax extraction (Biotron-Kinematica AG) of ground bark of plants from Peru (Naturex) with 70% ethanol (Dipalcool). The fluid was centrifuged (Centrifuge Suzuki), concentrated in a heating tank (MCA-ALW) to remove the alcohol, and spray-dried (Kohls) using silicon dioxide (Evonik) and microcrystalline cellulose 102 (Blanver) as excipients. The *Uncaria tomentosa* extract was gently donated by Herbarium Botanic Laboratory, Curitiba, Brazil.

2.2. Analysis of dry Uncaria tomentosa extract

The following reagents were used: acetonitrile (JTBaker), triethylamine (Fluka), acetic acid (JTBaker), polyamide (Fluka), ethanol (Vetec), and ultrapure water. Sample extraction was performed using a Unique ultrasound, model USC 5000 A, at 40 kHz. Chromatographic analyses were performed on the Agilent 1100 HPLC system and a Zorbax XDB C-18 column (150 mm × 4.6 mm, 3.5 μm Agilent) at 15 °C. Samples (80 mg) were diluted in 60% ethanol (10 mL) and subjected to sonication (20 min at 30 °C). Next, 2 mL of sample was passed through a column containing 200 mg of

polyamide, and the eluate was injected into an HPLC system. Separation was achieved using gradient elution of water (0.2% acetic acid) adjusted to pH 6.9 with triethylamine (A) and acetonitrile (B) at a flow rate of 0.8 mL/min, detection was performed at 245 nm, and the concentration of oxindole alkaloids (OA) was calculated as previously described in Bertol et al. (2012).

2.3. Cell lines and cell culture

MDA-MB-231 cells were obtained from Banco de Células do Rio de Janeiro – BCRJ (Code BCRJ: 0164). Cells were cultured and frozen in liquid nitrogen for storage. Subsequently they were grown in culture in Eagle's medium, modified according to Dulbecco: Nutrient Mixture (DMEM-F12) (Sigma Chemical Co., Italy) supplemented with 100 UI/mL of penicillin, 100 μg/mL of streptomycin, 0,25 μg/mL of amphotericin and 10% fetal bovine serum (FBS, Cultilab). The cells were cultured in bottles and stored at 5% CO_2 at the temperature of 37 °C until the formation of cell monolayer, after, the cell bottles were subjected to trypsinization.

2.4. Cell treatments

Cells were seeded in 24-multiwell plates at densities of 1×10^4 cells/well in a final volume of 1 mL of culture medium. And in cell culture flasks, surface area 25 cm^2, at densities of 1×10^6 cells/well in a final volume of 5 mL of culture medium, for flow cytometry. Either was allowed to adhere overnight at 37 °C. The cells were treated with *Uncaria tomentosa* (250 e 500 μg/mL) and doxorubicin (1 μM) (Eurofarma, SP), a commonly used anthracycline chemotherapy. for 24 h as described below:

i. Control group: cells with culture medium only
ii. Ut250 group: cells with *Uncaria tomentosa* extract at 250 μg/mL
iii. Ut500 group: cells with *Uncaria tomentosa* extract at 500 μg/mL
iv. Doxo group: cells with doxorubicin at 1 μM
v. Ut250+Doxo: cells with *Uncaria tomentosa* extract at 250 μg/mL and doxorubicin at 1 μM
vi. Ut500+Doxo: cells with *Uncaria tomentosa* extract at 500 μg/mL and doxorubicin at 1 μM

2.5. Cell counting

The cells harvested by incubation for 5 min with 0,05% (w/v) trypsin (Sigma) in Hanks buffered solution (Gibco/Invitrogen) conataining 0.3 mmol/L EDTA followed by the addition of 10% FBS (Cultilab) to inactivate the trypsin. Cells were quantified using a Neubauer chamber, and cellular viability was assessed using the trypan blue exclusion method.

2.6. Cell viability by MTT assay

Cell viability was evaluated using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma) reduction assay. In brief, MDA-MB-231 cells were seeded at a density of 1×10^4 cells/well in 200 μL culture medium, in 96-well plates and incubated overnight at 5% CO_2 at the temperature of 37 °C to adhere. After, cells were treated as described in the previous section and incubated for 24 h. The cells were washed with culture medium and MTT (0.5 mg/mL) was added to each well. The cells were grown for a further period of 2.5 h and then carefully washed with PBS. DMSO (200 μL) was added to each well and absorbance was read at 595 nm.

2.7. Enzymatic assay

The MDA-MB-231 cells were washed 3 times with phosphate-free incubation medium in the absence of substrate. The reaction was started by the addition of 200 μL of the incubation medium containing 120 mM NaCl, 5 mM KCl, 10 mm glucose, 20 mM HEPES, pH 7.4, 2 mM $CaCl_2$ for ecto-NTPDase assay and 2 mM $MgCl_2$ for determining ecto-5′-nucleotidase. The reaction was started by adding ATP, ADP or AMP to the reaction medium to a final concentration of 2.0 mM and the incubation proceeded for 30 min at 37 °C. The reaction was stopped by removing an aliquot of the incubation medium and transferring to a tube containing TCA 10% previously placed on ice. The release of inorganic phosphate (Pi) was measured by malachite green method (Chan et al., 1986), using KH_2PO_4 as a Pi standard. Controls to correct non-enzymatic hydrolysis of nucleotides were performed by measuring the Pi released into the same reaction medium incubated without cells. All assays were performed in triplicate. Activity was expressed as nmol Pi released/min/mg of protein (nmol Pi/min/mg).

2.8. Protein determination

Cells in the 24 multiwell plates were dried and solubilized with 100 μL of NaOH 1N and frozen overnight. An aliquot was then removed and the protein was measured by the Coomassie blue method, using bovine serum albumin as standard.

2.9. Flow citometry

For flow citometry analysis, MDA-MB-231 cell line was maintained in culture flasks until reach confluence. Cells were then trypsinized, dissociated and counted immediately in a hemocytometer. Then 1×10^6 cells were centrifuged for 5 min at 400x*g* and washed twice with phosphate buffered saline (PBS) plus 3% fetal bovine serum (SFB). The sediments were suspended and incubated for 30 min with purified mouse anti-human CD39 and anti-human CD73 antibody (BD Pharmingen TM) (1:10). The same number of cells was incubated without antibodies. All samples were washed with PBS and immediately analyzed by flow cytometry (BD ACCURI C6) and FlowJo software.

2.10. Western blot analysis

The determination of the density of P2X7 and A1 receptors were carried out by Western blot analysis, as previously described (Duarte et al., 2009). Briefly, each sample was diluted with five volumes of SDS-PAGE buffer containing 30% (v/v) glycerol, 0.6 M dithiothreitol,10% (w/v) sodium dodecyl sulfate and 375 mM Tris–HCl pH6.8, and boiled at 95 °C for 5 min. These diluted samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% separation gel with a 4% concentrate in gel in the top) under reducing conditions, in two or three different protein concentrations, together with pre-stained molecular weight markers (Biorad, USA), and then electro transferred to polyvinylidene difluoride membranes (0.45 μm, from Amersham Biosciences, UK). After blocking for 1 h at room temperature with5% milk in Tris-buffered saline (Tris 20 mM, NaCl 140 mM, pH7.6), containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight at 4 °C with the primary antibodies against P2X7R (1:1000, Santa Cruz Biotecnology, Brazil) and A1R (1:800, Santa Cruz Biotecnology, Brazil). The selectivity of the tested antibodies P2X7 and A1 receptors has previously been validated. After three 15 min washing periods with TBS-T containing 0.5% milk, the membranes were incubated with the alkaline phosphatase–conjugated anti-rabbit IgG or anti-goat IgG secondary antibody (dilution 1:10,000) in TBS-T containing 1% milk during 90 min at room temperature. After three 20 min was TBS-T with 0.5% milk the membranes were incubated with enhanced chemi-fluorescent substrate (Amersham Biosciences) and then

analyzed with a Amershan Imager 600 (GE Healthcare life Sciences). The membranes were then reprobed and tested for β-actin immunoreactivity to confirm that similar amounts of protein were applied to the gels. Briefly, the membranes were incubated at room temperature for 30 min with 40% (v/v)methanol and 1 h with 0.1 M glycine buffer pH 2.3, and then blocked as previously described before incubation with an -anti-β-actin antibody (dilution 1:10,000) for 2 h at room temperature. The membranes were then washed, incubated with an anti-mouse IgG alkaline phosphatase-conjugated secondary antibody and analyzed as described above.

2.11. Statistical analysis

All results are presented as mean ± SD. Data were analyzed by one-way ANOVA, followed by Tukey post-hoc test. Differences between mean values were considered significant at $p < 0.05$.

3. Results

3.1. Analysis of extract and Cell viability

Fig. 1A shows the HPLC analysis of dry *Uncaria tomentosa* extract has a content of 4.20% oxindole alkaloids (OA) quantified as the sum of speciophylline, uncarine F, mitraphylline, rhynchophylline, isomitraphylline, uncarine C, isorhynchophylline, and uncarine E.

Cell viability was assessed by MTT assay, which indicates the degree of cell damage by quantifying mitochondrial dehydrogenase activity. No significant difference was observed in cell viability while cells were incubated for 24 h with Ut at concentrations of 250 and 500 μg/mL alone (Ut 250 and Ut 500 groups, respectively) or in combination with 1 μM Doxo (Ut250+Doxo and Ut500+Doxo groups, respectively), compared with Doxo group. The groups treated with different concentrations of Ut, alone or in combination with Doxo, showed a significant decrease in cell viability ($p < 0.05$) when compared with the control (Fig. 1B).

A viable cell count was also performed using trypan blue (data not shown), where the control group showed a viability of 93% and the *U. tomentosa* groups and/or doxorubicin treatment obtained 78–84% cell viability, respectively.

3.2. NTPdase and 5′-nucleotidase activities

Fig. 2A shows the ATP hydrolysis in MDA-MB-231 breast cancer cell line. The statistical analysis has not shown significant difference in the ATP hydrolyses in the Ut250 and Ut500 groups compared with the control and Doxo group. *U. tomentosa* extract 500 μg/mL associated with doxorubicin 1 μM (Ut500+Doxo group) showed an increase in ATP hydrolysis when compared to the Ut500 group.

In the Fig. 2B, the Ut500+Doxo group showed an increase in ADP hydrolysis, therefore the combination of Ut500 with doxorubicin showed an increase in the NTPDase activity in MDA-MB-231 breast cancer cells. However, when we observed the ADP hydrolysis by the other groups was not found significant differences.

Fig. 2C represent the 5′-nucleotidase activity in the MDA-MB-231 breast cancer cells. The 5′-nucleotidase inhibition was found in all groups compared to the control group. The Ut250 (0.632 ± 0.210 nmol Pi/min/mg) and Ut500 groups (0.328 ± 0442 nmol Pi/min/mg) significantly reduced enzyme activity compared to the control group (1.592 ± 0.318 nmol Pi/min/mg).

On the other hand, when *U. tomentosa* (Ut250 and Ut500) was associated with doxorubicin, which is the most widely used chemotherapy in breast cancer treatment, was observed a reduction in AMP hydrolysis in the Ut250+Doxo (0.279 ± 0.120 nmol Pi/min/mg) and Ut500+Doxo (0.330 ± 0.095 nmol Pi/min/mg) (Fig. 2C).

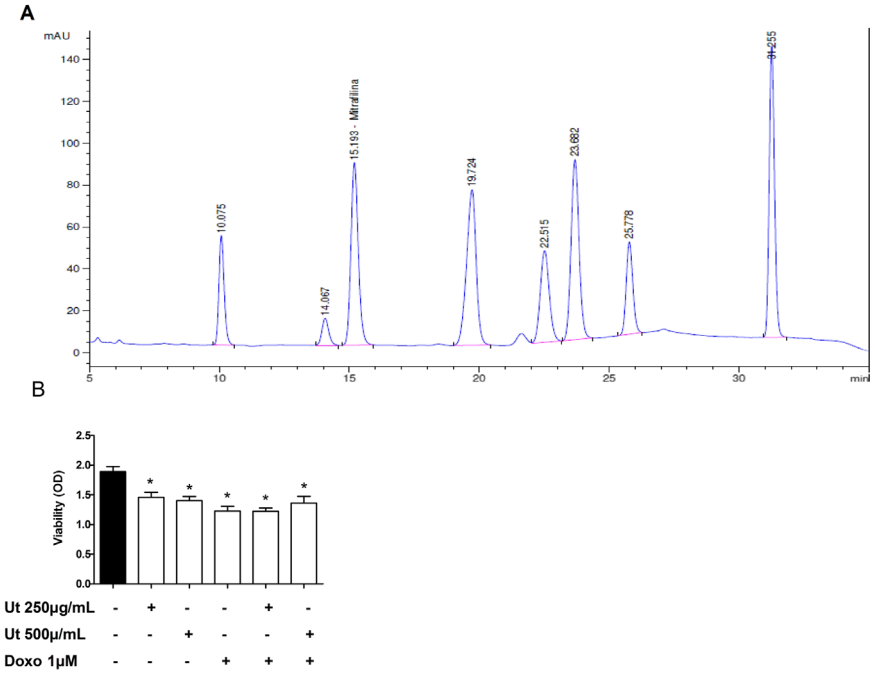

Fig. 1. HPLC-Fingerprint analysis of dry extract from *Uncaria tomentosa* and cellular viability. (A) 1- Speciophylline (10.075 min), 2- Uncarine F (14.067 min), 3- Mitraphylline (15.193 min), 4- Rhynchophylline (19.724 min), 5- Isomitraphylline (22.515 min), 6- Uncarine C (23.682 min), 7- Isorhynchopylline (25.778 min), and 8- Uncarine E (31.255 min); (B) Viability of the MDA-MB-231 cells. Cell cultures were treated with different concentrations of the hydroalcoholic extract *Uncaria tomentosa* (Ut250 and Ut500) and/or Doxorubicin 1 μM (Doxo) for 24 h. Experiments were performed in triplicate. Results are presented as mean ± SD. (*) Indicates a significant difference at $P < 0.05$.

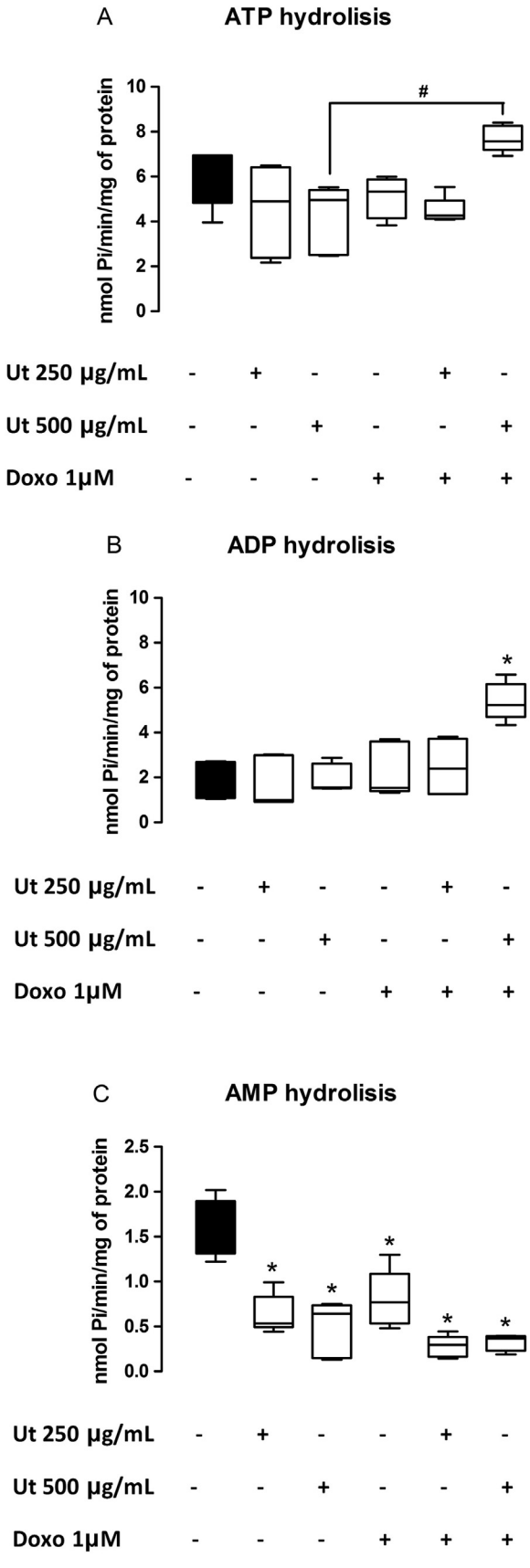

Fig. 2. NTPDase activity in the MDA-MB-231 cells using ATP (A), ADP (B) as substrate and 5′-nucleotidase activity in the MDA-MB-231 cells using AMP (C) as substrate at a final concentration of 2 mM. Cell cultures were treated with different concentrations of the hydroalcoholic extract *Uncaria tomentosa* (Ut250 and Ut500) and/or Doxorubicin 1 µM (Doxo) for 24 h. Experiments were performed in triplicate. Results are presented as mean ± SD. (*) Indicates a significant difference at $P < 0.05$ between untreated cells and treated cells. (#) Indicates a significant difference at $P < 0.05$ between treated groups of cells.

3.3. Ectonucleotidases expression by flow citometry

In the Fig. 3A, NTPDase1/CD39 expression was increased in the Ut250 and Ut250+Doxo groups, which shows the percentage of CD39-positive cells, which depicts histograms showing the CD39 expression (Fig. 3C and E).

A significant reduction was observed in the CD73 expression on the Ut500 group when compared with the Doxo group (Fig. 3B) (93.35 ± 4.031 frequency Doxo group). However, there no differences were founded in the expression of ecto-5′-nucleotidase/CD73 in the MDA-MB-231 cells treated with *U. tomentosa* and/or doxorubicin, although the Ut500 (59.30% ± 1.414) and Ut500+Doxo (66.05% ± 2,192) groups revealing a tendency to decrease the expression of CD73 in relation to control group (90.95% ± 6,293) (Fig. 3B), which shows the percentage of CD73-positive cells, which depicts histograms showing the CD73 expression (Fig. 3D and F).

3.4. P2X7 and A1 receptors by Western blot analysis

Fig. 4 shows the purinergic receptor expression, as adenosine A1 and P2X7 receptors. There was a significant increase in the adenosine A1 receptor expression in Ut250, Ut500, Doxo and Ut250+Doxo groups, compared with the control. The immunoreactivity was 305.9 ± 5.0% for the Doxo group, while it was 218.2 ± 10%, 331.9 ± 25.17%, 253 ± 45.09% for Ut250, Ut500, and Ut250+Doxo groups, respectively (Fig. 4A).

Ut500+Doxo group presented no difference when compared to the control group. When this group was compared with the Ut500 and Doxo groups, a significant reduction in the expression of the A1 receptor was observed. Similarly, the Ut250 group showed a decrease in the expression of this receptor compared to the Doxo.

For the P2X7 receptor, all treatment groups exhibited a significant reduction in the expression of this receptor when compared with the control group (Fig. 4B). Besides the all the groups treated, the Ut500+Doxo group showed a pronounced reduction in the P2X7 expression. Futhermore, we observed that Ut500+Doxo group had a significant reduction in the P2X7 receptor expression compared to the Ut500 and Doxo groups (Fig. 4B).

4. Discussion

In nature, Cat's claw occurs in two different chemotypes characterized by pentacyclic (POA) or tetracyclic (TOA) patterns of indole and oxindole alkaloids (Keplinger et al., 1999). Most of the pharmacological activity has been attributed to the one containing more pentacyclic (rather than tetracyclic) oxindole alkaloids (Philp, 2004). The *U. tomentosa* extract used in our study had an oxindole alkaloids concentration of 4.20%. Currently, there is a considerable amount of attention towards studies of anticancer properties of *Uncaria tomentosa*.

A study fulfilled by Oliveira et al. (2014) showed an increase in apoptosis in colorectal adenocarcinoma cells (HT29) treated with *Uncaria tomentosa* 750 µg/mL and oxaliplatin. As previously reported, there was antiproliferative activity in the promyelocytic leukemia cells (HL-60) (Sheng et al., 1998;. Pilarski et al., 2007) as well as in the breast cancer cells, MCF-7 (Riva et al., 2001).

Recently, our research group conducted studies with *Uncaria tomentosa* and concluded that this plant stimulates proliferation of myeloid precursor cells (Farias et al., 2011). The other study was a randomized clinical trial with patients diagnosed with breast cancer. In this study the patients received tablets of Ut (250–350 mg) between the chemotherapy cycles. The use of *U. tomentosa* in patients with breast cancer reduced neutropenia, one of the side effects of chemotherapy (Araújo et al., 2012).

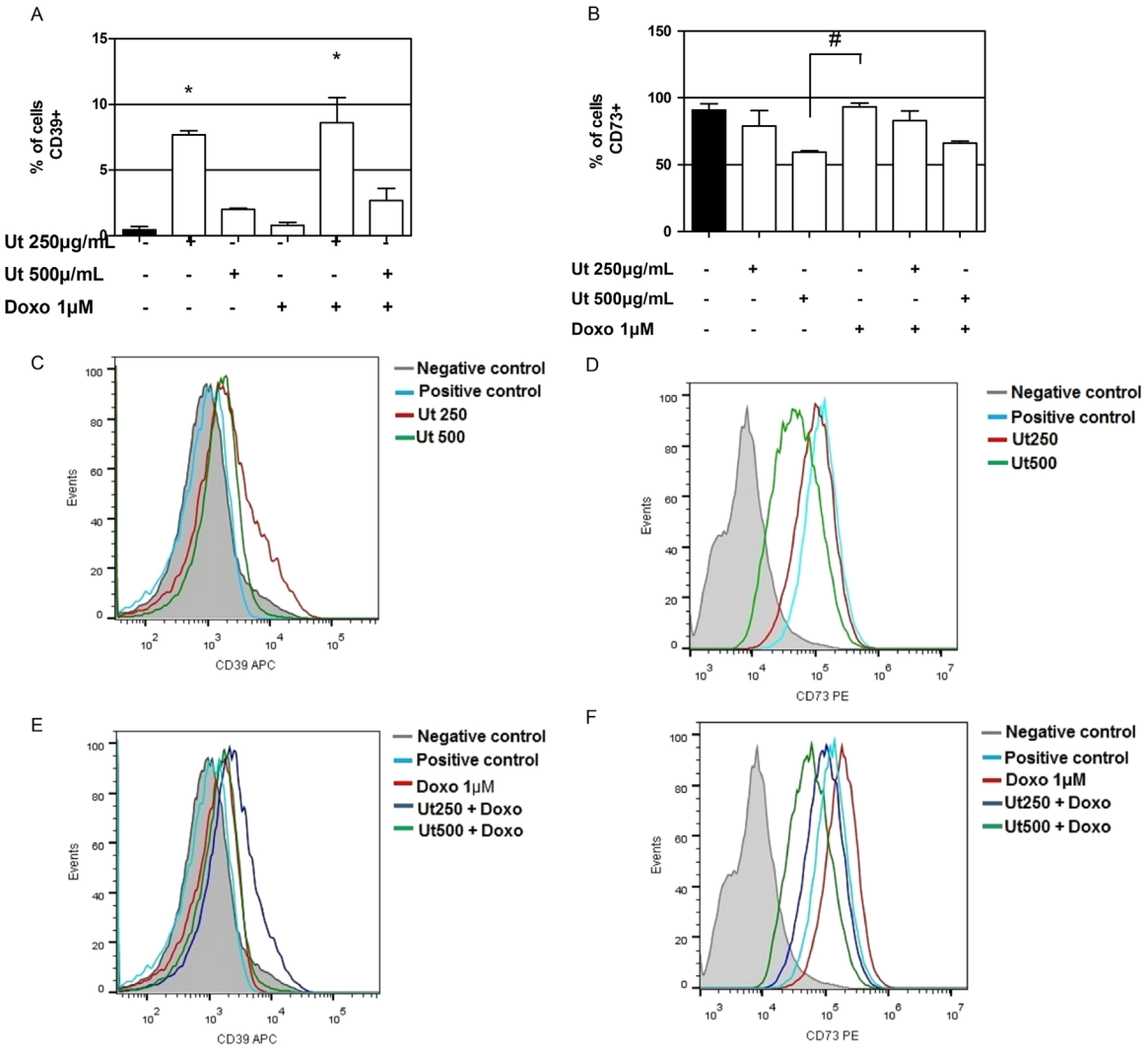

Fig. 3. Expression of C39 and CD73 in MDA-MB-231 cells. Cell cultures were treated with different concentrations of hydroalcoholic extract *Uncaria tomentosa* and/or Doxorubicin 1 µM (Doxo) for 24 h. (A) Percentage frequency (%) of the CD39 cells positive (NTPDase), (B) and CD73 cells positive (ecto-5′-nucleotidase), (C) and (E) expression of CD39 (NTPDase), (D) and (F) Expression of CD73 (ecto-5′nucleotidase). Experiments were performed in duplicate. Results are presented as mean ± SD. (*) Indicates a significant difference at $P < 0.05$ between untreated cells and treated cells. (#) Indicates a significant difference at $P < 0.05$ between treated groups of cells.

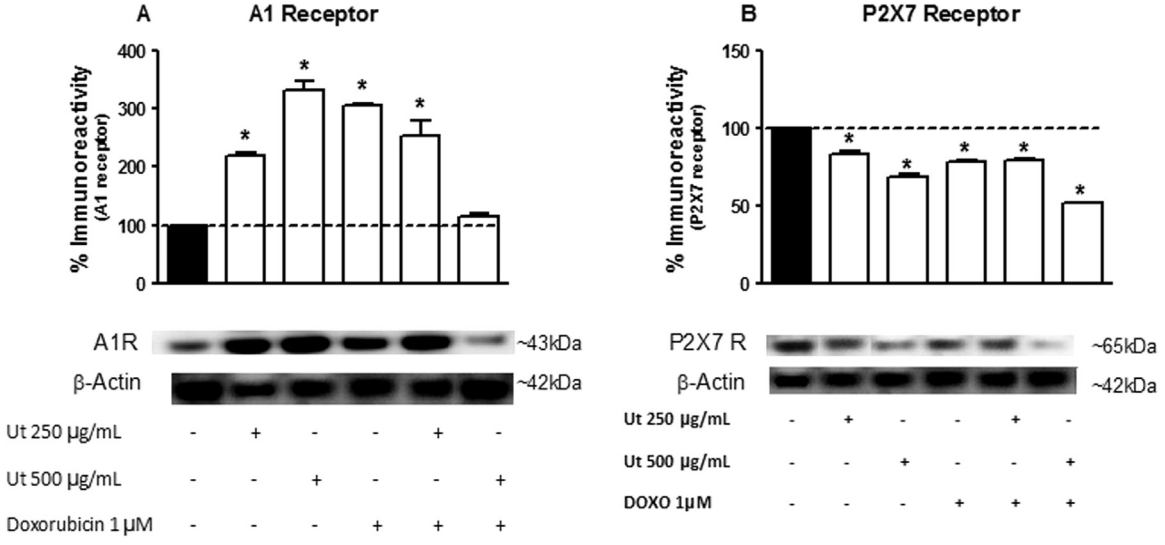

Fig. 4. Expression profiles of the A1 receptor (A) and P2X7 (B) receptor in the MDA-MB-231 breast cancer cells detected by western blotting (WB). Cell cultures were treated with different concentrations of the hydroalcoholic extract *Uncaria tomentosa* (Ut250 and Ut500) and/or Doxorubicin 1 µM (Doxo) for 24 h. Experiments were performed in triplicate. β-actin was used as an internal control. (*) Indicates a significant difference at $P < 0.05$.

Based on the promising results found in the in vivo study carried out by our research group and the results in vitro performed by other researchers with *Uncaria tomentosa*, in a triple-negative breast cancer cell line (MDA-MB-231), which it represents a subtype of breast cancer with worse prognosis (Marméa and Schneeweissa, 2015). However, it is important to note that cancer is a vast group of diseases that may vary substantially amongst themselves and present different outcomes among tumor lineages (Pilarski et al., 2010).

Adenosine, which is generated from AMP, has been shown as an important metabolite released by cancer cells that elicits physiological responses and promotes tumor progression (Spychala et al., 2004). Furthermore, adenosine has immunosuppressive effects, which can increase the migration of tumor cells in vitro and metastasis in vivo through the activation of the adenosine A2B receptor (Stagg et al., 2010). AMP is generated by the stepwise catabolism of ATP into AMP via the intermediate ADP and is subsequently converted to adenosine. The conversion of ATP into AMP is predominantly catalyzed by NTPDase1/CD39 with only trace amounts of ADP being released whereas ecto-5′-nucleotidase/CD73 catalysis the conversion. The NTPDase 1/CD39 is an enzyme responsible for the hydrolysis of ATP to ADP and ADP to AMP (Zimmermann, 2001). Our results showed that the *U. tomentosa* extract does not influence the activity of NTPDase, and in the same way doxorubicin, an anthracycline antibiotic that is widely used for solid tumors such as breast cancer (Gewirtz, 1999), also does not alter the enzymatic activity of NTPDase.

ATP is a multifunctional molecule that acts as the primary source of energy in living cells, as well as a signaling molecule that regulates diverse extracellular processes, including ion transport, apoptosis, secretion and tumor growth (White and Burnstock, 2006). Depending on the dose and the purinergic P2 receptor subtype engaged, ATP can trigger many different cellular responses, ranging from cell death to proliferation (Jin et al., 2014). The balance between adenosine and ATP is crucial in immune homeostasis because ATP is a danger signal released by damaged and dying cells that acts to prime immune responses through the ligation of P2X and P2Y purinoreceptors. ATP, via the activation of the P2X7 of dendritic cells (DCs) and the secretion of interleukin (IL)-1β and IL-18 where these chemical mediators can stimulate T cells $CD8^+$ that when activated to produce IFN-α triggering anti-tumor immune response. By contrast, adenosine suppresses immune responses through the activation of G-protein-coupled receptors (Stagg and Smyth, 2010).

An increase in ATP hydrolysis in the Ut500+Doxo group was observed when compared with the Ut500 group, thereby reducing the concentration of this nucleotide in the target area and generating more ADP (Fig. 2A). In this same group, an increase in the ADP hydrolysis was observed in comparison with the control group (Fig. 2B), generating more AMP. In contrast to adenosine, AMP, which is a precursor of adenosine, has an inhibitory effect on breast cancer cell growth (Mazurek et al., 1997). Furthermore, it was possible to observe an increase of NTPDase activity in the Ut500+Doxo group, and although there is no significant difference, this same group presented a tendency for increased expression of CD39 in relation to the untreated cells group (Fig. 3A and E). Consequently, we can infer that an increase in activity and expression of CD39 may be release AMP amounts in the medium, which is essential to inhibit tumor progression.

All treatments reduced the activity of the ecto-5′-nucleotidase enzyme when compared with the untreated MDA-MB-231 breast cancer cells. This reduction represents an increase in AMP levels in the cell line MDA-MB-231. The reduction in ecto-5′-nucleotidase activity was more prevalent in the groups in which the treatment was associated with Ut at both concentrations with doxorubicin (Fig. 2C).

According to Canbolat et al. (1996), the increased activity of ecto-5′-nucleotidase, which converts AMP to adenosine, appears to promote the growth of breast cancer cells. In our study, there was a reduction in the activity of 5′-nucleotidase in breast cancer cells treated with hydroalcoholic *U. tomentosa*. This decrease in the enzymatic activity reduces the amount of adenosine generated, maintaining a larger amount AMP levels. It is known that adenosine promotes tumor proliferation and AMP is an inhibitor of tumor growth, as can be concluded that the treatments conducted with *U. tomentosa* appear to inhibit tumor proliferation by reduction in 5′-nucleotidase activity.

In this manner, we suggest that the association of *U. tomentosa* 500 μg/mL with the doxorubicin has shown promise regarding the activities of ectoenzymes. The increased activity of NTPDase can generate a larger amount of AMP in the extracellular medium, which exerts an opposite effect of adenosine by inhibiting tumor growth. The activity of ecto-5′-nucleotidase was shown to be inhibited in cells that received this combined treatment. Thus, we suggest that by inhibiting the activity of ecto-5′-nucleotidase in the treatment, Doxo+Ut500 may inhibit tumor growth.

Additionally, the increased expression of ecto-5′-nucleotidase has been observed in breast cancer, as compared with the non-malignant surrounding tissue. In the present study, we observed a high expression level of CD73 (Fig. 3B), consistent with Spychala et al. (2004) who reported a higher expression level of CD73 in ER-negative than in ER-positive breast cancer cells.

Although we found a significant reduction in CD73 activity, the expression of this enzyme exhibited no significant difference between the study groups. Only the Ut500 and Ut500+Doxo groups, without any difference, displayed a tendency to reduce this expression when compared to the control group, and as a result it could be associated with a inhibition of ecto-5′-nucleotidase by U. tomentosa in these groups.

The activity of ectonucleotidases varies between different tumor types. According to the study performed by Stella et al. (2010), different bladder tumor cell lines were able to metabolize nucleotides with a distinct pattern of extracellular hydrolysis. The less malignant RT4 cell line revealed high levels of ATP and ADP hydrolysis, while AMP hydrolysis remained low. In contrast, the T24 line that represents an invasive tumor exhibited a markedly decreased capacity in hydrolyzing both ATP and ADP, along with a high capacity to hydrolyze AMP. These reports indicate that a possible relationship between malignancy and the altered profile of ectonucleotidases activities may also exist in bladder tumor cells, as it is seen in the brain (Canbolat et al., 1996) and other tumor cells (Durak et al., 1993, 1994).

Loi et al. (2013) showed that the high CD73 expression that is associated with the resistance to doxorubicin therapy in TNBC, and the TNBC patients with high CD73 expression have a higher risk of distant metastasis. In addition, their data revealed that CD73-mediated immunosuppression suppress the therapeutic activity of doxorubicin in vivo. Furthermore, CD73 inhibition potentiated the CD8-dependent anti-tumor immune response following doxorubicin treatment. We can thus suggest that somehow *U. tomentosa* inhibits the activity of CD73 of which could contribute to a successful treatment with doxorubicin.

Studies have shown that doxorubicin is the greatest up-regulation of CD73 and CD39 expression in human melanoma (A2058), leukemia (RPMI 8226) and breast cancer cells (MDA-MB-231) (Loi et al., 2013). This suggests that some chemotherapy drugs have the role of changing the enzyme cascade that converts the ATP to immunosuppressive adenosine. In our study, doxorubicin did not alter the expression of CD39 and CD73 in the MDA-MB-231 cells in 24 treatments. The expression of CD39 increased in the Ut250 and Ut250+Doxo groups compared to the control group, since the CD73 expression was significantly lower in the Ut500

group when compared to the cells treated group only with doxorubicin.

Our results revealed the expression of the A1 adenosine receptor in MDA-MB231 cells, but when these cells received different types of treatment there was a significant increase in the expression of the A1 receptor, except in the Ut 500+Doxo group in which A1 the receptor expression levels remained similar to those of the control group.

The A1 receptor adenosine has been associated to carcinogenesis in previous investigations where the expression of this receptor has been demonstrated in colorectal adenocarcinoma (Khoo et al., 1996). The antitumor activity of the adenosine A1 receptor has been reported as reducing tumor proliferation in glioblastoma cancer cells and MCF-7 breast cancer. Additionally, evidence of pro-tumor action has been observed increasing cell proliferation in breast cancer cells MDA-MB-468 (TNBC) (Mirza et al., 2005; Gessi et al., 2011). Nevertheless, little is known about the expression and activity of this receptor in the MDA-MB-231 breast cancer cells.

The role of hypoxia in regulating tumor progression is controversial (Burnstock and Di Virgilio, 2013). However, in the MCF-7 and the MDA-MB-231 breast cancer cell lines, the expression of the P2X7 receptor is increased by hypoxia (Tafani et al., 2011). In addition, there is increased expression of P2X7 receptors in breast tissue undergoing malignant change (Slater et al., 2004; Tafani et al., 2011). The increased expression of P2X7 receptors by hypoxia enhances invasion and migration of tumor cells (Burnstock and Di Virgilio, 2013).

Studies have shown that emodin is a potent P2X7 antagonist, which suppressed the invasiveness of breast and lung cancer ((Burnstock and Di Virgilio, 2013; Jelassi et al., 2013). This evidence indicates an important role for the P2X7 receptors in mediating cancer cell metastasis, the major cause for high mortality (Roger et al., 2014).

In our study, the untreated MDA-MB-231 cell group showed high density of the P2X7 receptor. This receptor density presented a significant reduction when cells were only treated with *U. tomentosa* and when this was associated with doxorubicin (Fig. 4B). Notably, the Ut500+Doxo group had performed better once again.

Finally, the present study suggests that *U. tomentosa* 500 μg/mL alone or associated with doxorrubicin can reduce tumor proliferation by inhibiting the ecto-5′-nucleotidase, reducing the amount of adenosine which is generated by hydrolysis of nucleotides through ectoenzymes. The combination of treatments (Ut500+Doxo) expressed greater reduction in density of the P2X7 receptor, demonstrating that the effect of the hydroalcoholic extract *U. tomentosa* can be effective when combined with chemotherapy.

5. Conclusions

Therefore, we conclude that hydroalcoholic extract of *Uncaria tomentosa* to inhibit the activity of ecto-5′-nucleotidase may be contributing to prevent tumor progression, because reduces conversion AMP in adenosine, and adenosine is a tumor proliferative agent. In addition, *U. tomentosa* extract also altered the expression of purinergic receptors. The role of the A1 receptor expression in MDA-MB-231 cells is unclear. The reduction in P2X7 receptor expression can reduce the chance of tumor invasion and metastasis. Our results showed a significant influence of the *Uncaria tomentosa* extract on enzymes and receptors purinergic system, which could contribute to the reduction of tumor progression. And also indicate that the combined *Uncaria tomentosa* and doxorrubicin may be promising effects on the purinergic system.

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