International Journal of Biological Macromolecules 120 (2018) 566–577

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Anti-glioma properties of DVL, a lectin purified from *Dioclea violacea*

Ana Paula M. Nascimento [a,1], Jhônatas L. Knaut [b,1], Débora K. Rieger [c], Ingrid A.V. Wolin [a], Isabella A. Heinrich [b], Josiane Mann [b], Andrea V. Juarez [d,e], Liliana del V. Sosa [d,e], Ana Lucia De Paul [d,e], Cleane G. Moreira [f], Ivanice B. Silva [f], Clareane S. Nobre [f], Vinicius J.S. Osterne [f], Kyria S. Nascimento [f], Benildo S. Cavada [f], Rodrigo B. Leal [a,b,*]

[a] *Departamento de Bioquímica e Programa de Pós-graduação em Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário, 88040-900 Florianópolis, Santa Catarina, Brazil*
[b] *Programa de Pós-graduação em Neurociências, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário, 88040-900 Florianópolis, Santa Catarina, Brazil*
[c] *Departamento de Nutrição e Programa de Pós-graduação em Nutrição, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, Campus Universitário, 88040-900 Florianópolis, Santa Catarina, Brazil*
[d] *Universidad Nacional de Córdoba, Facultad de Ciencias Médicas, Centro de Microscopía Electrónica, Ciudad Universitaria, 5000 Córdoba, Argentina*
[e] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto de Investigaciones en Ciencias de la Salud (INICSA), Córdoba, Argentina*
[f] *Universidade Federal do Ceará, Departamento de Bioquímica e Biologia Molecular, BioMol-Lab, CEP 60440-970 Fortaleza, Ceará, Brazil*

ARTICLE INFO

Article history:
Received 30 May 2018
Received in revised form 17 July 2018
Accepted 21 August 2018
Available online 23 August 2018

Keywords:
Lectin
Autophagy
Glioma
Dioclea violacea
DVL
ConA

ABSTRACT

Plant lectins have been studied owing to their structural properties and biological effects that include agglutinating activity, antidepressant-like effect and antitumor property. The results from this work showed the effects of the lectin extracted from the *Dioclea violacea* plant (DVL) on the C6 rat glioma cell line. DVL treatment was able to induce caspase-3 activation, apoptotic cell death and cellular membrane damage. Furthermore, DVL decreased mitochondrial membrane potential and increased the number of acidic vesicles and cleavage of LC3, indicating activation of autophagic processes. DVL also significantly inhibited cell migration. Compared to ConA, a well-studied lectin extracted from *Canavalia ensiformes* seeds, some effects of DVL were more potent, including decreasing C6 glioma cell viability and migration ability. Taken together, the results suggest that DVL can induce glioma cell death, autophagy and inhibition of cell migration, displaying potential anti-glioma activity.

1. Introduction

Glioblastoma multiforme (GBM) is a grade IV astrocytoma, and it is the most common form of malignant brain tumor, accounting for 46.1% of all cases [1]. In addition, the prognosis for patients diagnosed with GBM is poor [2], resulting in an average survival rate of less than one year [3,4], and only 5.1% of patients survive five years or more after diagnosis [1]. This poor prognosis is associated with the infiltrative nature GMB, its rapid proliferation capacity and its resistance to multiple drugs. Hence, GBM is the most intractable of all malignant gliomas, and no therapy is currently available for the treatment of these tumors [4,5]. One of main reasons for lower rates of effective therapeutic response is the lack of tumor-specific drugs that can induce tumor cell death associated with low side effects [4,6]. In this context, lectins emerge as a potential pharmacological tool that recognizes cancer cells and possibly induces tumor regression as a result of specific binding to certain carbohydrates that may be present on the tumor cell-membrane surface or via internalization and targeting intracellular glycoproteins [7–10].

Plant lectins are a highly diversified class of proteins [11], widely distributed in plant species and associated with several important biological functions, such as antitumor, antifungal and antiviral activity [7,12]. Plant lectins draw attention because of their versatile antiproliferative abilities [7] and the induction of programmed cell death in cancer cells via apoptosis and autophagy [8,11,13,14].

A number of cellular processes, including development, differentiation, morphogenesis, autophagy and cell migration, are dependent on glycoconjugates and may be regulated by interactions between lectins and oligosaccharides present in glycoproteins [15,16].

It has been reported that some lectins, such as Concanavalin A (ConA), *Polygonatum cyrtonema* lectin (PCL) and Mistletoe lectins (MLs), can promote antitumor activity by inducing both apoptosis and autophagic cell death [11,17–19]. In addition to the cytotoxic effect, some plant lectins have been used as tools to differentiate malignant

* Corresponding author at: Departamento de Bioquímica, Centro de Ciências Biológicas (CCB), Universidade Federal de Santa Catarina (UFSC), Trindade, Florianópolis, Santa Catarina CEP 88.040-900, Brazil.
E-mail address: r.b.leal@ufsc.br (R.B. Leal).
[1] The first two authors contributed equally to this paper.

https://doi.org/10.1016/j.ijbiomac.2018.08.106
0141-8130/

tumors from benign ones and to mark the degree of glycosylation associated with tumor metastasis and identification of cancer stem cells [11,20].

The lectin extracted from the seed of *Dioclea violacea,* DVL, was purified for the first time in 1996 [21] by Moreira et al. DVL is a legume lectin with affinity for glucose/mannose, and it belongs to the subtribe Diocleinae, similar to ConA [22].

Structurally, DVL appears in tetrameric form in neutral pH and dimeric form in more acidic pH. In animal models, administration of DVL was associated with both anti-inflammatory [23] and proinflammatory action [24]. Moreover, DVL was capable of inducing relaxation in a rat aorta endothelium model [22]. Despite these activities, no studies have confirmed the antitumor activity of DVL. It is noteworthy that the antitumor potential of legume lectins has been largely indicated [25–28] and antitumor activity of ConA has been reported in HeLa and U87 human glioma cell line [17,19,29]. Additionally, four new purified legume lectins, including CaBo, ConV, DLL, and DlyL, have been shown to induce C6 glioma cell death [29–32]. Therefore, the present study aimed to investigate the antitumor activity of DVL against the rat C6 glioma cell line and compare this action with that provoked by the well-studied legume lectin ConA.

2. Materials and methods

2.1. Cell line culture and lectin treatment

The rat C6 glioma cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (*v*/v) of fetal bovine serum (FBS) (Gibco®), 100 units/mL penicillin and 100 mg/mL streptomycin (Gibco®). Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cells were seeded in 96- or 48-well plates at the density of 10^4 or 5×10^4 cells per well, respectively, and incubated for 24 h at 37 °C in an incubator (5% CO_2). Afterwards, the culture medium was exchanged, and the cells were incubated for 24 or 48 h in serum-free DMEM, but containing vehicle or lectin (ConA or DVL). Lectins were diluted with HEPES–saline buffer without glucose composed of NaCl 124 mM, KCl 4 mM, MgSO4 1.2 mM, HEPES 25 mM, and $CaCl_2$ 1 mM, pH 7.4. For all assays, the control cell cultures were incubated with the vehicle (HEPES–saline buffer without glucose).

2.2. Purified lectin

The purification of *Canavalia ensiformis* (ConA) and *Dioclea violacea* (DVL) lectins were performed by affinity chromatography in Sephadex®-G50, following protocol adapted from by Moreira et al. [33] and Mackler et al. [34]. Lectins purity was accessed by SDS-PAGE following protocol described by Laemmli [35] using a MiniProtean II apparatus (BioRad, Milan, Italy) in 0.75 mm vertical gel slabs with 15% acrylamide on separation gel and 4% on stacking gel. Lectins were solubilized in 0.88 M Tris-HCl (pH 6.8), 2% SDS, 1% bromophenol blue and 12.5% glycerol and heated at 100 °C by 10 min. Electrophoresis was conducted at 25 mA. Protein bands were visualized by Coomassie Brilliant blue G-250 staining as presented at Supplementary Fig. 1 (S1).

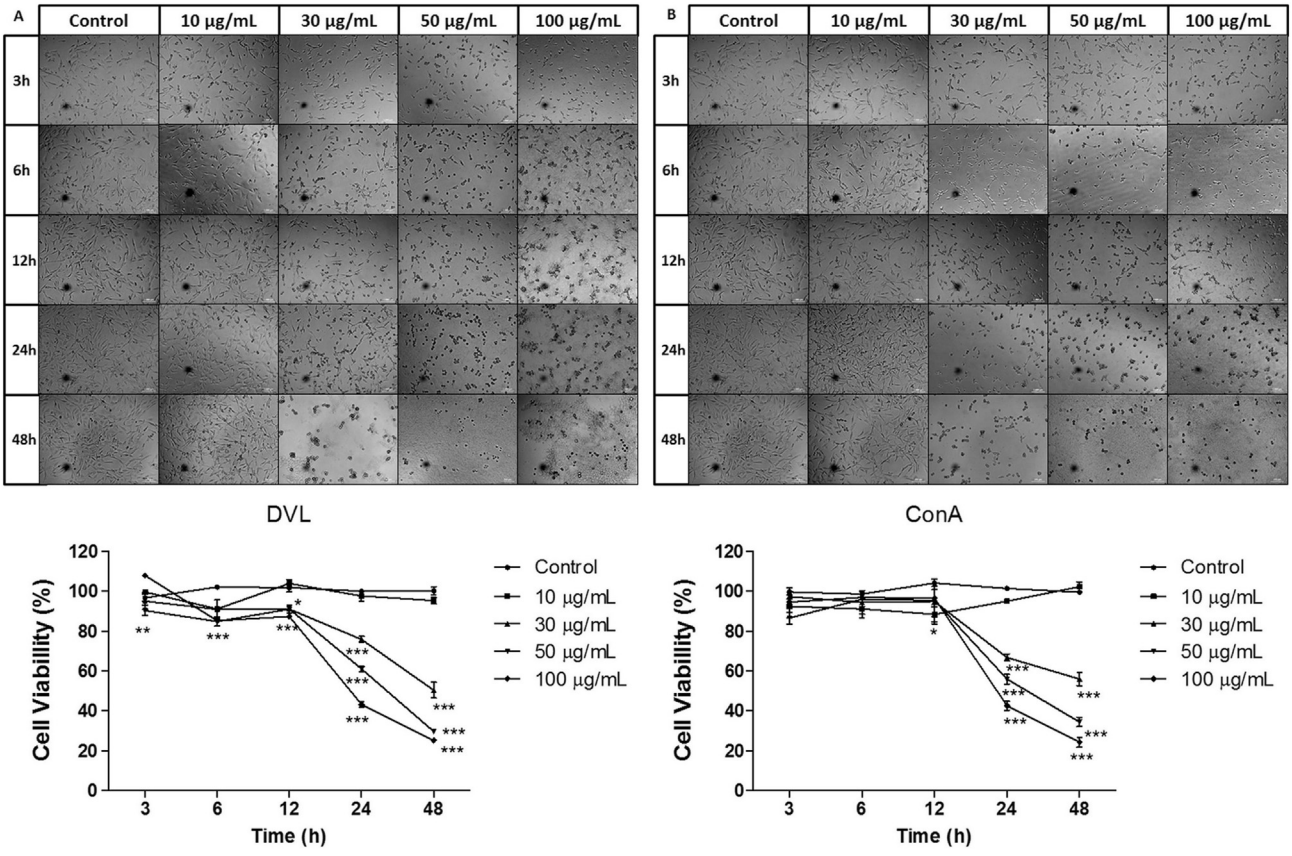

Fig. 1. ConA and DVL induce morphological alteration and decrease cell viability. C6 glioma cells were exposed for 3, 6, 12, 24 and 48 h to vehicle (HEPES-saline buffer; control) or lectins (DVL or ConA) at 10, 30, 50 and 100 µg/mL. Thereafter, cell morphology and viability were performed. (A) and (B) show representative images from four independent experiments performed in triplicate, showing cell morphology, as evaluated by optical microscopy, in response to DVL and ConA, respectively. Moreover, the graphics show cell viability measured by the MTT assay after treatment for 3, 6, 12, 24 and 48 h with DVL and ConA. The data are expressed as a percentage of control (considered 100%), and the values are presented as mean ± SEM of four independent experiments performed in triplicate. Cells were visualized by inverted NIKON® eclipse T2000-U microscope (20 X). *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, as compared to control.

Table 1
Cytotoxic concentration for 50% of cell viability (CC50).

Lectin	Leguminous	CC 50% (μg/mL)	Confidence interval
DVL	*Dioclea violacea*	58,84	51,14-67,70
ConA	*Canavalia ensiformis*	56,02	38,01-80,03

The table presents the lectin concentration that will reduced cell viability by 50% (CC50) and the confidence interval, both of which were determined through GraphPad® Prism software.

2.3. Light and fluorescence microscopy

In order to address morphological alterations in response to lectin treatment, cells were visualized in optical microscopy by inverted NIKON eclipse T2000-U microscope. Briefly, C6 cells were seeded in a 96-well plate and incubated for 24 or 48 h. Following treatment, the cells were visualized by optical microscopy.

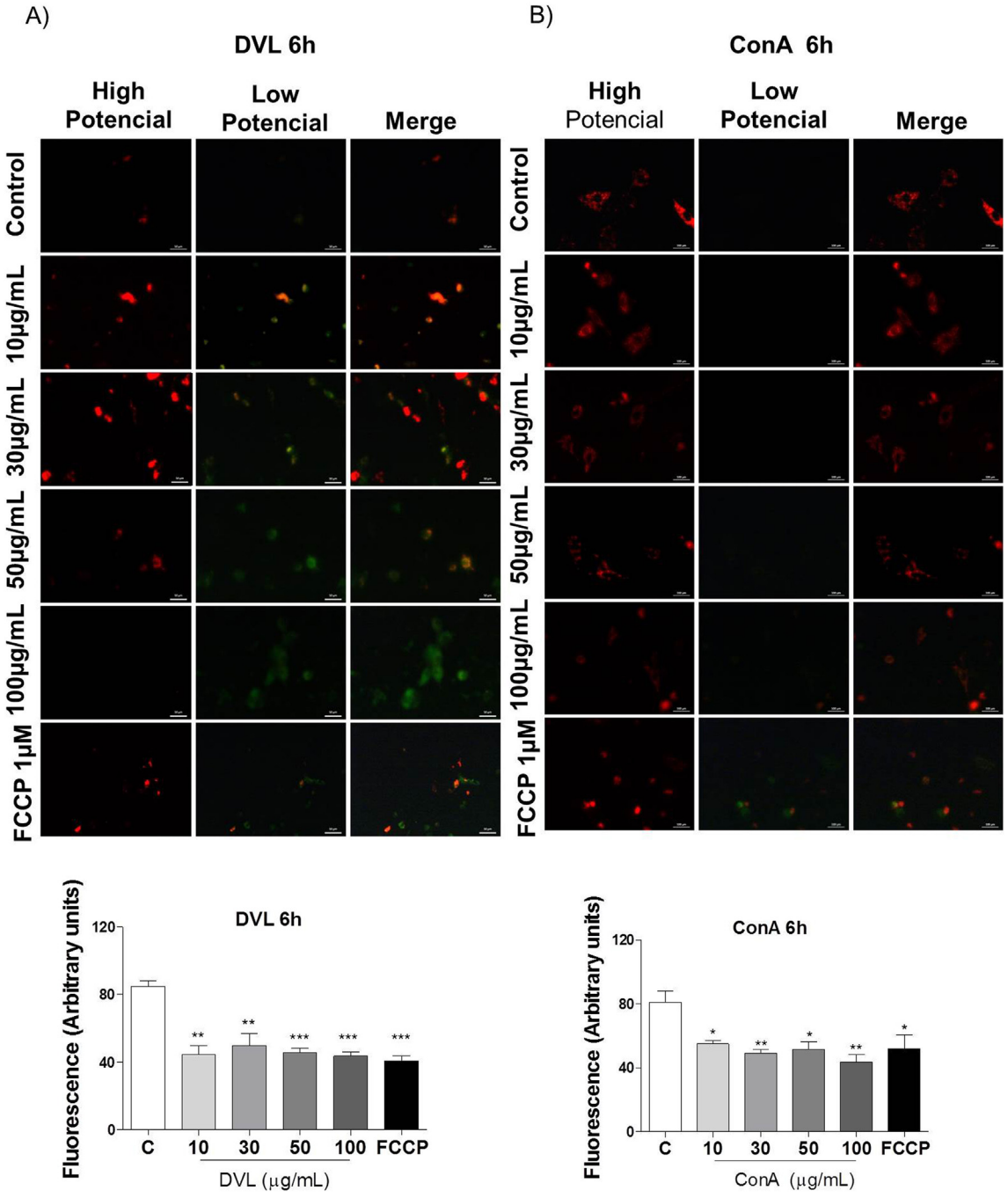

Fig. 2. ConA and DVL induce decrease of mitochondrial membrane potential in C6 cells. Mitochondrial membrane potential was evaluated by measuring JC-1 fluorescence in C6 cells exposed for 6 h to concentrations of 10, 30, 50 and 100 μg/mL of DVL (A) and ConA (B). FCCP treatment (1 μM; 6 h) was used as positive control. Cells were visualized by inverted NIKON® eclipse T2000-U microscope (20 X), and representative images are presented. Moreover, the fluorescence was quantified. For this purpose, the red fluorescence was measured using excitation wavelengths of 490 nm excitation and 525 nm emission, while green fluorescence was read using excitation wavelengths of 520 nm and 590 nm emission with the SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The fluorescence values were converted to percentages relative to the average of the cellular controls, considered as 100%, and they were presented in the graphics. All assays were made in 4 independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, as compared to control.

2.4. MTT assay

Cell viability was analyzed by the colorimetric MTT (Sigma®) [30-(4,5-dimethylthiazol-2yl) 2,5-diphenyltetrazolium bromide] reduction assay [36]. Cells were seeded in a 96-well plate and incubated for 24 or 48 h with vehicle (control) or ConA/DVL in the final concentration of 10, 30, 50 or 100 μg/mL. After treatment, medium was removed, and the cells were incubated for 1 h (37 °C) with 0.1 mL of 0.5 mg/mL MTT diluted in HEPES-saline buffer. The mitochondrial dehydrogenases in viable cells reduced the MTT to formazan crystals, which were dissolved in 0.1 mL of dimethylsulfoxide (DMSO) for 30 min at 37 °C. The absorbance was measured spectrophotometrically, using the Tecan Microplate Reader Infinite M200 (k = 540 nm). The results were expressed as a percentage of the control (vehicle), considered 100%.

2.5. Cell growth analysis

To evaluate the kinetics of cell growth in response to lectin treatment, cells were seeded in a 96-well plate and treated with vehicle or lectin. Thereafter, cells were trypsinized and then submitted to counting in a Neubauer chamber in the presence of the vital stain Trypan Blue (0.2%). Evaluations were performed at 0, 24 and 48 h after treatment with vehicle (control) or lectins (ConA or DVL) at 10, 30, 50 and 100 μg/mL.

2.6. JC-1 assay

In order to evaluate whether the lectins could cause disruption of mitochondrial electrochemical gradient, the JC-1 (Sigma®) assay was applied. Briefly, 10^4 cells per well were plated, and after 24 h, they were treated with DVL or ConA (10, 30, 50 and 100 μg/mL) for 6 h. As a control, cells were treated with vehicle (HEPES-Saline Buffer), and as positive control, cells were treated with FCCP (1 μM, Sigma®). After treatments, the medium was removed, and 100 μL of JC-1 (1 mg/mL) probe were added. Hence, plates were incubated for 20 min at 37 °C protected from light. After this time, the cells were washed once with PBS (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4), followed by the addition of 0.1 mL/well of PBS. The results were analyzed by SpectraMax (Molecular Devices®, Sunnyvale, CA, USA) through fluorescence reading performed using wavelengths of 490 nm of excitation and emission of 520 nm for detection of red fluorescence and excitation of 525 nm and 590 emission for green detection. The fluorescence values of red/green ratio of each sample were converted to percentages relative to the red/green ratio average of the cell control, considered as 100%. The assays were performed in 4 independent experiments and in triplicate.

2.7. Lactate dehydrogenase (LDH) assay

The activity of the cytosolic enzyme lactate dehydrogenase (LDH) was evaluated in the cell culture medium, as described by Rosa et al. [37]. To accomplish this, the cells were seeded in a 96-well plate and incubated for 12 or 24 h with vehicle or lectin at concentrations of 10, 30, 50 and 100 μg/mL. After the treatment, 50 μL of medium from each well were transferred to another 96-well plate, and 200 μL of 0.5 M potassium phosphate buffer plus pyruvate, $NaHCO_3$ and NADH were added. The kinetics of enzymatic activity was carried out by Infinite M200 (Tecan). Positive control consisted of incubating the well for 15 min at 37 °C with 0.02% Triton-X100 before withdrawing 50 μL of medium. The values of the absorbance variation of NADH, measured for each well, were converted to percentages relative to the average of the positive controls, considered as 100%.

2.8. Cell cycle analysis

To investigate if cell cycle impairment was part of the molecular mechanisms involved in lectin-induced cell death, cells were subjected to cell cycle analysis by flow cytometry, according to the method described by Riccardi and Nicoletti [38]. The cells were seeded in 12-well plates and treated with vehicle (control) or lectin at the concentrations of 10, 30, 50 and 100 μg/mL for 6 or 24 h. After the treatment, the cells were resuspended from the wells by chemical dissociation (300 μL/0.25% trypsin well) and washed twice with phosphate buffer solution (PBS), centrifuged at 500 rpm for 5 min, and fixed with 70% ethanol for 30 min at 4 °C. After fixation, the cells were incubated with 50 μg/mL RNase and 100 μg/mL Propidium Iodide for 30 min at room temperature. After this period, the cells were analyzed on a flow cytometer, recording 20,000 events for each sample.

2.9. Flow cytometry assay with propidium iodide staining

For propidium iodide (PI) staining of cells with membrane rupture, 12-well plates were seeded with 1 mL C6 cells at the density of 250,000 cells/mL and incubated at 37 °C in a 5% CO_2 greenhouse for 24 h until confluence. Afterwards, medium was replaced with new medium plus vehicle or lectin at concentrations of 10, 30, 50 and 100 μg/mL, and the plates were incubated in an oven at 37 °C with 5% CO_2 for 6 or 24 h. Following this, the cells were resuspended from the wells by chemical dissociation (300 μL/well of 0.25% trypsin), washed with PBS, incubated with binding buffer for 15 min, and then propidium iodide was added. The FACS Canto II flow cytometer (Becton Dickinson, USA) was used to analyze the cells, recording 20,000 events for each sample. The data obtained were analyzed using Flowing® software.

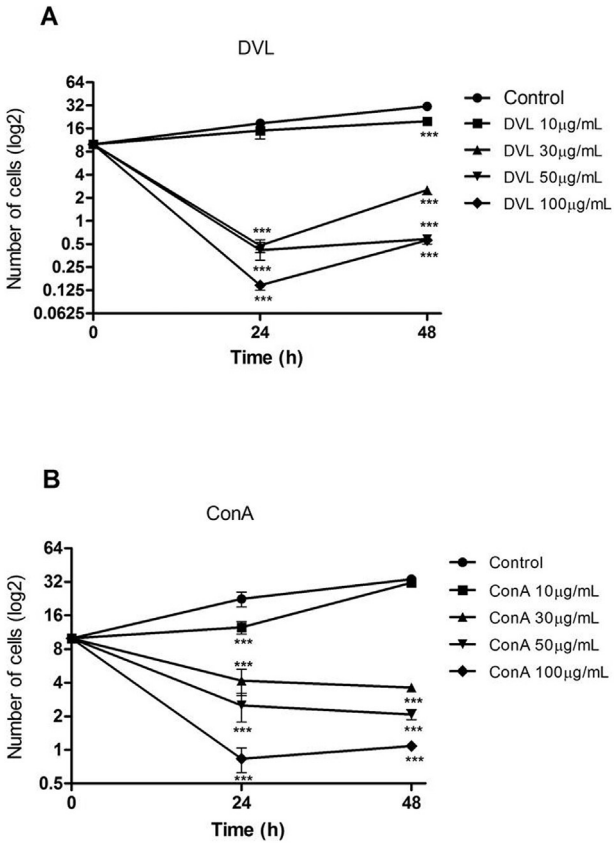

Fig. 3. DVL and ConA decrease of cell proliferation. The graphic shows cell number counting in a Neubauer Chamber after 0, 24 and 48 h treatment with vehicle (control; C), DVL (A) or ConA (B) at concentrations of 10, 30, 50 and 100 μg/mL.***$p < 0.001$) indicates statistical difference, as compared to control.

2.10. Flow cytometry assay with acridine orange staining

Quantification of acidic vacuoles stained with acridine orange was performed by flow cytometry. Hence, 12-well plates were seeded with 1 mL C6 cells at a density of 250,000 cells/mL and incubated at 37 °C in a 5% CO_2 greenhouse for 24 h until confluence. After this time, the medium was replaced with a new medium plus vehicle or lectin at concentrations of 10, 30, 50 and 100 μg/mL, and the plates were incubated in an oven at 37 °C with 5% CO_2 for 6 or 24 h. After treatment, the cells were resuspended from the wells by chemical dissociation (300 μL/0.25% trypsin well), stained with 0.1 μg/mL acridine orange, and incubated for 30 min in a 37 °C. After incubation, the cells were washed with PBS and analyzed by a FACS Canto II flow cytometer (Becton Dickinson, USA), recording 20,000 events for each sample. The data obtained were analyzed using Flowing® software.

2.11. Fluorescence microscopy assay with acridine orange

Staining of cells with acridine orange (AO) was performed to study cell death pattern and induction of acidic vesicular organelles by lectin

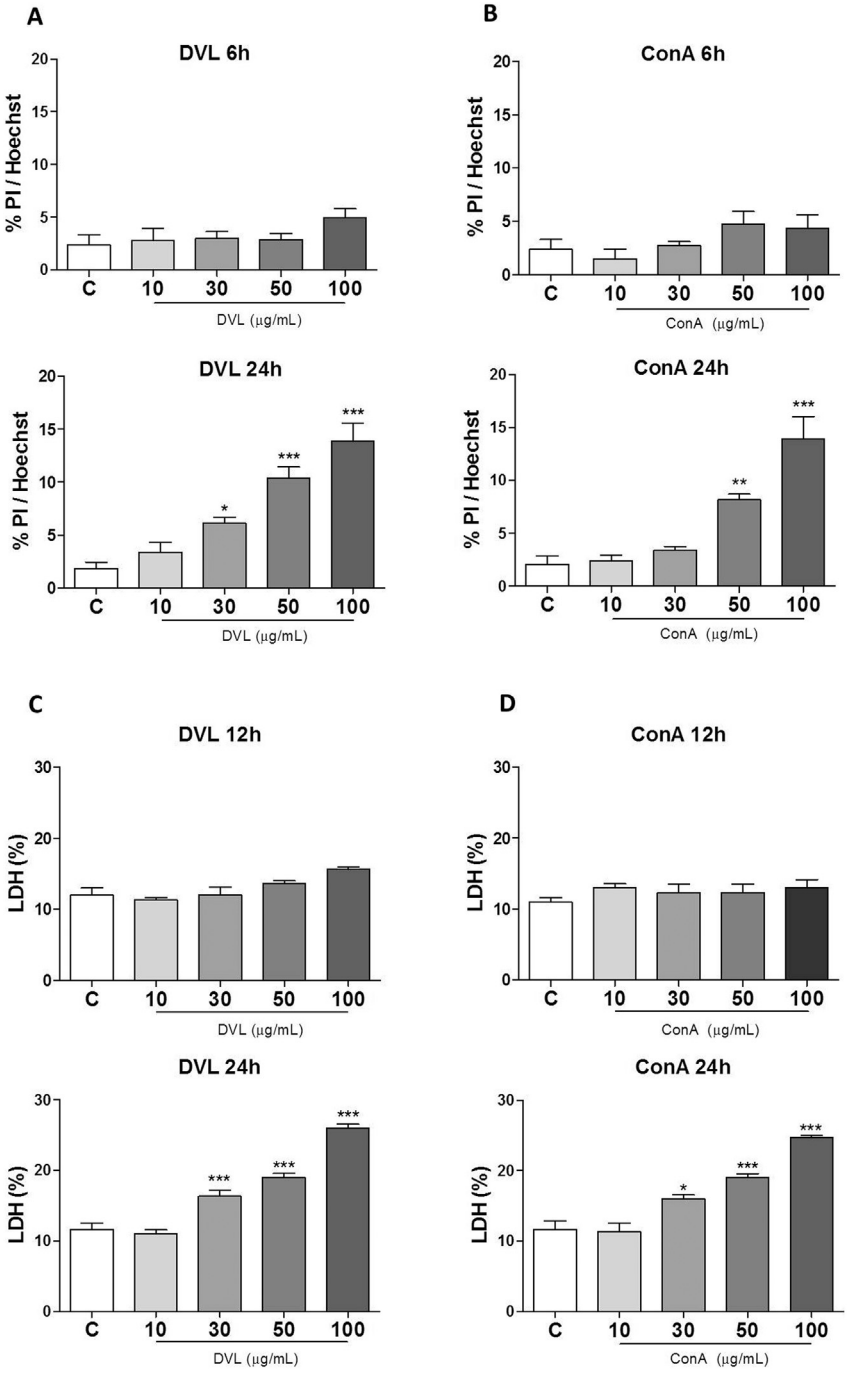

Fig. 4. ConA and DVL induce increment of propidium iodide staining and LDH activity in C6 glioma cells. Propidium iodide (PI) and Hoechst fluorescence of C6 cells exposed for 6 and 24 h to vehicle (control) or lectins (DVL and ConA) at concentrations of 10, 30, 50 and 100 μg/mL were analyzed by flow cytometry (FACS Canto BD). The ratio of PI/Hoechst fluorescence in response to DVL (A) and ConA (B) is presented as a percentage. The analysis of flow cytometry was performed by the open source Flowing® 2.5.1 software. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistical difference, as compared to control. Activity of lactate dehydrogenase (LDH) in the culture medium of C6 cells after treatment for 12 and 24 h with vehicle and the lectins DVL (C) and ConA (D) was also determined. The activity was expressed as a percentage of the positive control, considered as 100%. $^{***}p < 0.001$ indicates statistical difference, as compared to control.

after 24 and 48 h of incubation. C6 cells were seeded in a 48-well plate at the concentration of 5×10^4 cells per well for 24 h in DMEM supplemented with 10% SFB. Thereafter, the medium was exchanged by DMEM without serum, but with vehicle (control) or lectin (ConA or DVL) at 10, 30, 50 and 100 μg/mL. After 24 and 48 h, the cells were washed with PBS, fixed with 3.7% paraformaldehyde, and stained with AO (10 μg/mL; Sigma Chemicals®, USA) for 20 min in the dark. All procedures were performed at 37 °C. The cells were visualized by the inverted NIKON eclipse T2000-U microscope using filter sets, 470 nm excitation and 525 nm emission for chromatin detection (CR) and 350 nm excitation and 615 nm emission for detection of acidic vesicular organelles (AVO).

2.12. Fluorescence microscopy assay with propidium iodide

For propidium iodide (PI) assay, C6 cells were seeded in a 48-well plate under the same conditions as those described for the acridine orange assay (5×10^4 cells per well) and treated with vehicle or lectins at concentrations and time points as mentioned before. After incubation, the cells were washed with PBS and sequentially incubated in binding buffer (0.01 HEPES, pH 7.4, containing 140 mM NaCl and 25 mM CaCl2) containing Hoechst (1 μg/mL, Sigma Chemicals, USA) and Propidium Iodide (PI, 14 μg/mL, Sigma Chemicals®, USA) for 15 min in the dark [39]. Cells were visualized by an inverted NIKON eclipse T2000-U microscope using filter sets, 488 nm excitation and 560 nm emission for propidium iodide and 353 nm excitation and 483 nm emission for Hoechst.

2.13. Migration assay "Scratch"

C6 glioma cells were seeded in a 48-well plate at the concentration of 1.5×10^4 cells per well and incubated for 24 h. After this period, the culture medium was discarded, and a wound was introduced in each well by scraping confluent cell layers with a P200 pipette tip, followed by washing with PBS to remove any loosely adherent cells [40]. Thereafter, DMEM serum-free medium, containing vehicle (control) or the lectins (DVL or ConA) at the concentration of 10, 30, 50 and 100 μg/mL, was added. Images were captured at time points 0, 24 and 48 h after the treatments by inverted NIKON eclipse T2000-U microscope.

2.14. Migration/invasion assay "Transwell"

In the scratch assay, changes in cell proliferation ratio could interfere with measurement of the cytostatic/antimigration capacity of the lectins; therefore, the transwell migration assay was performed. This protocol is recognized as selective for cell migration analysis. Briefly, the transwells were placed in a 24-well plate, and then C6 cells suspended in DMEM media were plated inside of each transwell at density of 50×10^3 cells/well (volume 100 μL). Hence, cells were immediately submitted to treatment with vehicle (HEPES-saline buffer), ConA or DVL lectin at concentrations of 30 and 50 μg/mL. Ten minutes after addition of each treatment, the 24-well plate was filled with 600 μL/well of F12 DMEM medium supplemented with 20% FBS, as a chemotactic factor for cells placed inside the transwell. Treatments were performed for 24 h. After this incubation period, the transwells were removed from the 24-well plates, and with the aid of a swab, the cells from the transwell interior (i.e., those that did not migrate) were removed. Subsequently, each transwell was stained with Crystal Violet, and the cells were visualized under light microscopy and counted. The migration ratio for each treatment was calculated as a comparative to control (considered 100%).

2.15. Western blot assay

To analyze the expression of Beclin-1, Atg5 and cleavage of LC3, Western blotting assay was performed. Initially, C6 cells were plated at the density of 250×10^3 cells per well in a six-well plate, and after 24 h incubation, cells were treated for 6 and 12 h with the lectin DVL or ConA (10, 30, 50 and 100 μg/mL) and HEPES-Saline medium as a control. Subsequently, the cells were washed once with PBS and homogenized in 200 μL of Stop Solution (Tris 50 mM, EDTA 2 mM, SDS 4%, pH 6.8). Each of two wells was pooled, and the samples were placed in 1.5 mL tubes and immediately boiled at 100 °C for 5 min. Thereafter, the samples were vortexed, centrifuged (13,000 rpm for 5 min) and kept at −20 °C overnight. Then, the samples were thawed at 100 °C, vortexed, and centrifuged again under the same conditions.

The supernatant was then carefully collected, and to the sample were subsequently added dilution solution (40% glycerol, 100 mM Tris, bromophenol blue, pH 6.8) in the ratio 25:100 (v/v) and β-mercaptoethanol (final concentration 8%). The samples were stored at −80 °C until electrophoresis was performed. The same amount of

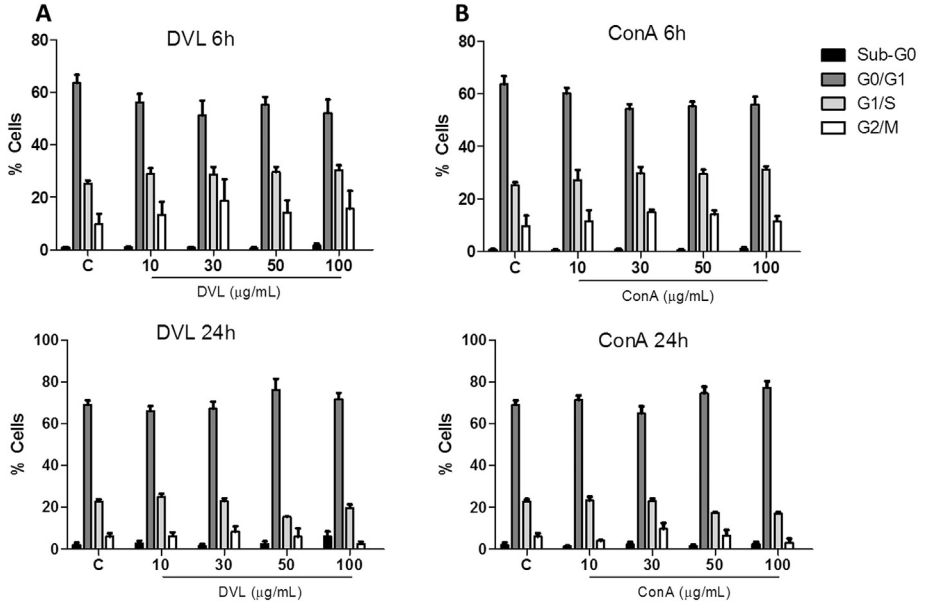

Fig. 5. DVL and ConA do not alter the cell cycle. Cell cycle of C6 cells exposed for 6 and 24 h to vehicle DVL (A) or ConA (B) at 10, 30, 50 and 100 μg/mL was analyzed by flow cytometry and using the open source Flowing® version 2.5.1 software.

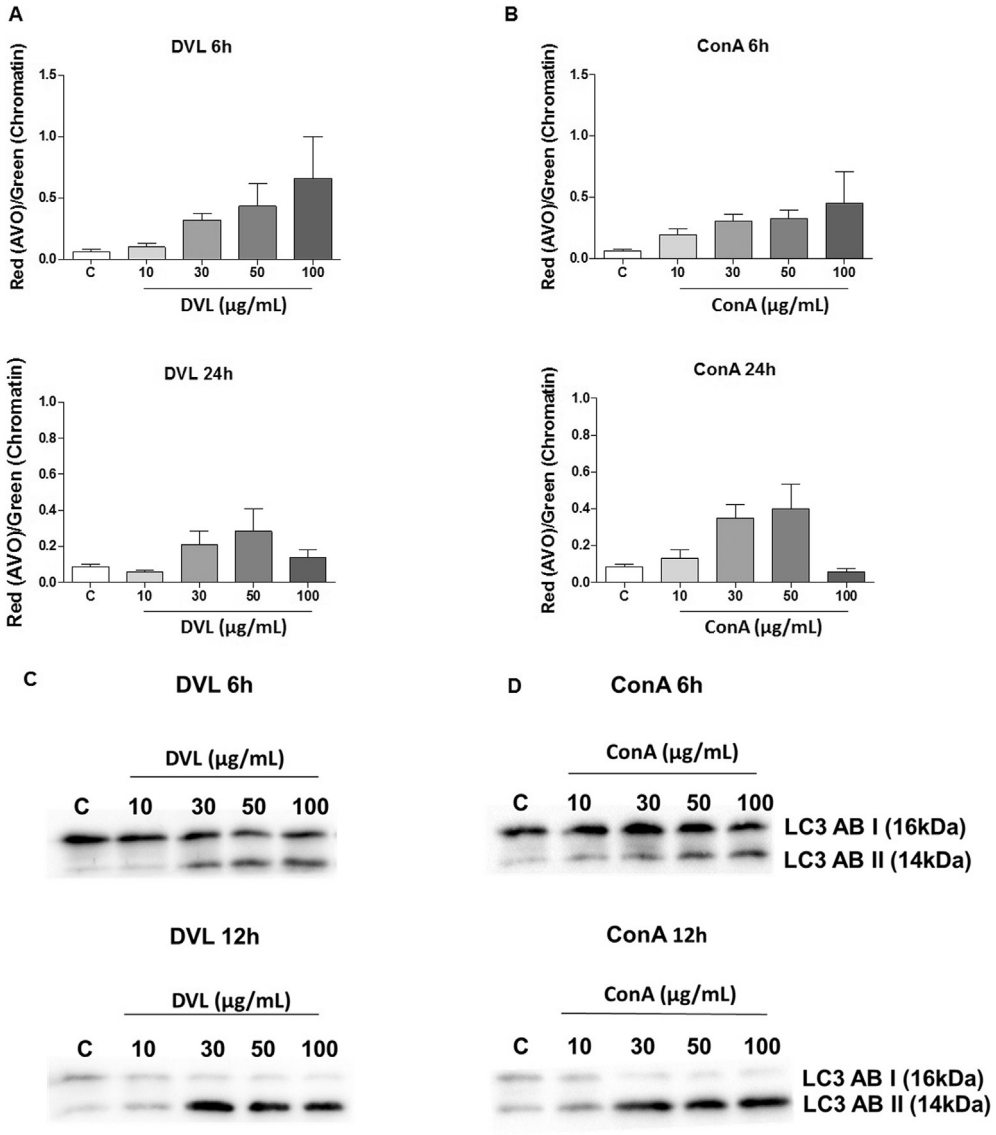

Fig. 6. Treatment with DVL and ConA induces the labeling of acidic vesicular organelles and LC3 cleavage. Cells were exposed for 6 or 24 h to vehicle (control; C) and DVL (A) or ConA (B) at concentrations of 10, 30, 50 and 100 μg/mL. The percentage of fluorescently labeled C6 cells for acidic vesicular organelles (AVO) was calculated through the red (AVO)/Green (chromatin) ratio performed by flow cytometry, using the open source Flowing® 2.5.1 software. C6 cells treated for 6 or 12 h with DVL (C) or ConA (D) were also analyzed by Western blot in order to determine the cleavage of LC3I to LC3II, a hallmark of autophagy. The images were captured and analyzed in the Image Lab Software, version 4.1.

protein for each sample (30 μg per lane) was electrophoresed in 12% SDS-PAGE minigels and transferred to nitrocellulose membranes using a semi-dry blotting apparatus (1.2 mA/cm^2; 1.5 h). To verify transfer efficiency, membranes were stained with Ponceau S.

The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) (Tris 10 mM, NaCl 150 mM, pH 7.5). The Beclin-1 and Atg5 levels, as well as LC3I cleavage to LC3II, were detected after overnight incubation with specific antibodies diluted in Tris-Buffered Saline and Tween 20 (TBST), containing 2% BSA in the dilutions of 1:1000 for Beclin-1 (Cell Signaling®), 1:1000 for Atg5 (Cell Signaling®), and 1:1000 for LC3I/II (Cell Signaling®). Moreover, the membranes were incubated for 1 h at room temperature with horse radish peroxidase (HRP)-conjugated anti-rabbit antibody for detection of Beclin-1, Atg5, and LC3I/II. The reactions were developed by chemiluminescence substrate (Super ECL, General Electric®). All blocking and incubation steps were followed by washing 3× (5 min) with TBS-T (Tris 10 mM, NaCl 150 mM, Tween-20 0.1%, pH 7.5). All membranes were incubated with mouse anti-β-actin (1:2000) antibody to verify that equal amounts of proteins were loaded on the gel. The expression levels of proteins associated with autophagic events were determined as a ratio of optic

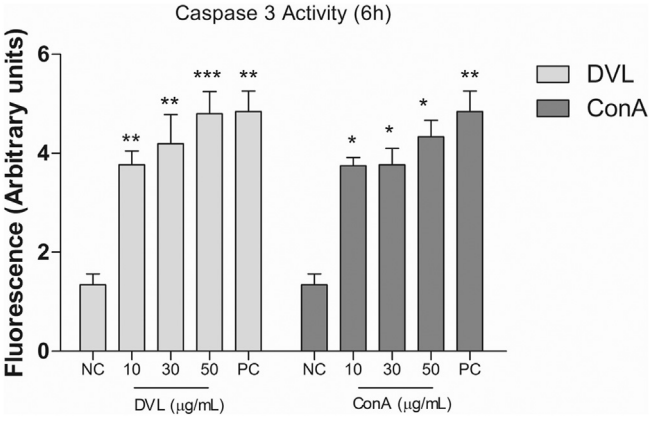

Fig. 7. DVL and ConA increased Caspase 3 activity. The graphic demonstrates the activity of Caspase 3 after 6 h treatment with vehicle, DVL and ConA at concentrations of 10, 30 and 50 μg/mL. Staurosporine (1 μg/mL) was used as positive control (PC). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ show statistical difference, as compared to control.

density (OD) of each protein tested by OD of β-actin and by the ratio of LC3II OD by LC3I OD. The bands were captured and quantified using the Image Lab® software. The antibody against Beclin-1 detected a single band at approximately 60 kDa. Anti-Atg5 detected a single band of approximately 55 kDa, and anti-LC3I/II detected two bands, one at approximately 16 kDa (LC3I) and the second at approximately 14 kDa (LC3II).

2.16. Caspase-3 activity assay

To detect whether ConA and DVL lectins could induce apoptosis, the detection of Caspase 3 activity was analyzed. For the test, EnzChek® Caspase-3 Assay Kit (Invitrogen) was used according to the specifications of the manufacturer.

2.17. Transmission electron microscopy (TEM)

With the objective of evaluating the morphological features of C6 cells at subcellular level, the transmission electronic microscopy was performed as previously described by De Paul et al. [41]. Bottles of 25 cm^2 were seeded with 5 mL of C6 cells at 600,000 cells/mL density and incubated at 37 °C in a 5% CO_2 oven for 24 h. After this period, the medium was replaced with a new medium plus vehicle or lectin at the concentration of 30 μg/mL, and the bottles were incubated in an oven at 37 °C with 5% CO_2 for 24 h. After treatment, cells were resuspended from the bottles by chemical dissociation (1 mL/bottle of 0.25% trypsin), washed with PBS, and fixed in Karnovsky mixture containing 4% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer plus 7%

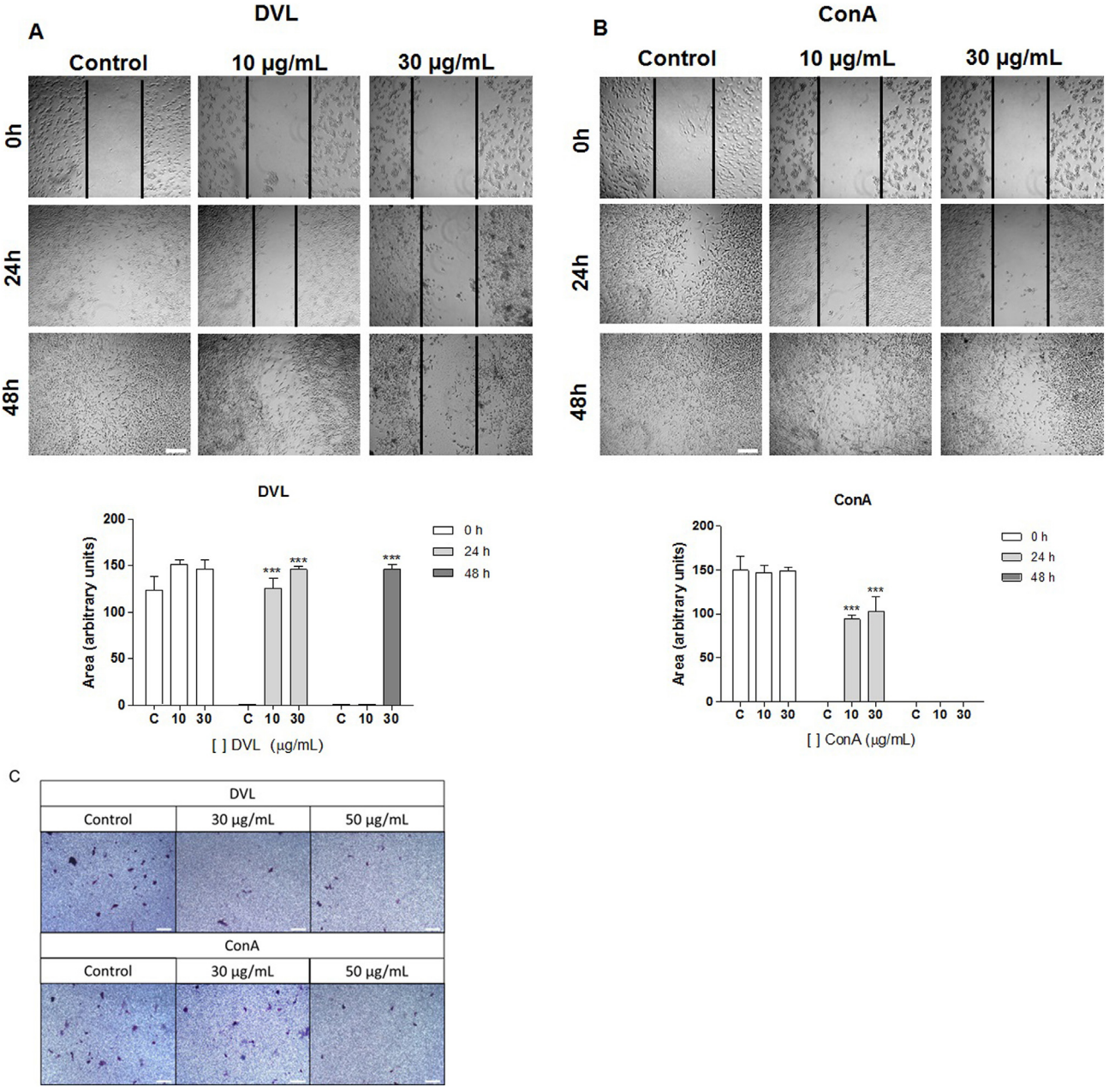

Fig. 8. Inhibitory effect of DVL and ConA on glioma C6 cell migration. C6 glioma cells were treated for 24 and 48 h with vehicle and DVL (A) or ConA (B) at 10 and 30 μg/mL. A linear area of attached cells was removed with a pipette tip after indicated concentrations of each lectin or vehicle had been added. Cells were photographed immediately after scratching the cells (time 0) and after 24 and 48 h. The line defined the area not covered with cells. The graphs show the magnitude of cell migration inhibition by the lectins. For quantification, the wound area at each time point was measured by the Image J® and the value expressed as arbitrary units. The bars represent 200 μm. The Transwell assay (C) was undertaken after 24 h treatment with DVL and ConA at 30 and 50 μg/mL. The cells were stained with Crystal Violet and visualized under light microscopy by inverted NIKON® eclipse T2000-U microscope (5 X) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ show significant difference in relation to the control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sucrose. These fixed C6 cells were centrifuged and the pellets treated with 1% OsO_4 for 1 h, before being stained in block with 1% uranyl acetate in 0.1 M acetate buffer pH 5.2 for 20 min. After dehydration with a series of graded cold acetones, the cells were embedded in Araldite resin (Electron Microscopy Sciences, Hatfield, PA). Thin sections were cut using a JEOL ultramicrotome (Nikon, Tokyo, Japan) with a diamond knife, which were then stained with uranyl acetate/lead citrate and examined using a Zeiss Leo 906-E electron microscope equipped with the digital camera Megaview III (Oberkochen, Germany).

2.18. Statistical analysis

The results were analyzed using GraphPad Prism® version 5.0 software (La Jolla, California, USA). The data presented had normal distribution, according to the Shapiro-Wilk test ($p < 0.05$). Thus, they were evaluated by analysis of variance (One-way, Two- way ANOVA), followed by Bonferroni the post-hoc test. p-Values < 0.05 ($p < 0.05$) were considered statistically significant.

3. Results

3.1. DVL and ConA induced morphological alteration and decreased cell viability in C6 cells

The purity degree of ConA and DVL was indicated by SDS-PAGE that showed the three bands corresponding to α, β, and γ chains, as expected from ConA-like lectins [42–44] presented at Supplementary Fig. 1. DVL and ConA in a concentration- and time-dependent manner provoked morphological alterations in C6 cells characterized by induction of spherical conformation and a possible impairment of cell adhesion (Fig. 1A). This effect was more pronounced in response to DVL exposure. Results from the MTT assay indicated that DVL treatment decreased cell viability in a time- and concentration-dependent manner from 6 up to 48 h at 100 μg/mL and from 12 up to 48 h exposure at 50 μg/mL concentration. DVL at 30 μg/mL decreased cell viability around 30% after 24 h. However, it caused a robust decrease (60%) after 48 h incubation. In contrast, the effect of ConA was not as pronounced since it started only after 24 h exposure; after 48 h, ConA at 30 μg/mL decreased C6 cell viability by only 30% (Fig. 1B). The CC_{50} for DVL and ConA were similar, while the confidence interval was considerably larger for ConA (Table 1).

3.2. DVL and ConA decrease mitochondrial membrane potential

To investigate if the effects of DVL and ConA were associated with mitochondrial damage, mitochondrial membrane potential (ΔΨm) was measured with JC1 probe. Cells treated with DVL or ConA (10–100 μg/mL) showed a significant reduction in ΔΨm after 6 h incubation (Fig. 2). It is noteworthy that DVL and ConA could generate more significant changes in this parameter compared to the positive control performed with the FCCP (1 μM), suggesting the existence of an important modulation of mitochondrial membrane potential by the lectins.

3.3. DVL and ConA decreased cell number after treatment

The results obtained with the growth curve show that DVL in the lower concentration (10 μg/mL) promoted a delay in cell multiplication at 24 and 48 h incubation as compared to control cells ($^{***}p < 0.001$) (Fig. 3A). ConA in the same concentration was effective at 24 h incubation, but it could not inhibit cell growth after 48 h incubation (Fig. 3B). At higher concentrations (30–100 μg/mL), both lectins displayed similar profile by their dramatic decrease in cell growth ($^{***}p < 0.001$) (Fig. 3A and B). The results in the proliferation assay reinforce the effects of DVL and ConA concerning the impairment on cell viability and mitochondrial membrane potential, as previously observed (Fig. 1 and Fig. 2).

3.4. DVL and ConA induced increased propidium iodide labeling and LDH activity

In order to determine membrane damage of C6 glioma cells, staining with propidium iodide (PI) was performed. Results showed that DVL (30–100 μg/mL) and ConA (50–100 μg/mL) induced a significant increment of PI labeling after 24 h treatment, as analyzed by flow cytometry (Fig. 4A and B) and fluorescence microscopy (S2). Notably, membrane damage was detected in <20% of C6 cells. Additionally, cell membrane disruption was also assessed through the lactate dehydrogenase **(LDH)** assay in the cell culture supernatant. Results showed that DVL and ConA (30–100 μg/mL) induced increment of LDH activity in the supernatant in a concentration-dependent manner after 24 h exposure (Fig. 4C and D). Taken together, these findings suggest delayed cell membrane damage in response to lectins.

3.5. ConA and DVL did not induce alterations in cell cycle in C6 cells

Based on the lectin capacity to impair cell growth, we asked if this cellular alteration was associated with cell cycle modifications, and we found that no cell cycle phase was altered in comparison with control (Figs. 5 and S3).

3.6. DVL and ConA induce autophagy in C6 cells

Acridine Orange is a cell-permeable green fluorophore that can be protonated and trapped in acidic vesicular organelles (AVOs). This assay has been applied as an accessible and reliable initial method to assess autophagy [29,45]. Using this assay, we evaluated the response of C6 glioma cells to lectin treatment from 6 to 24 h of exposure to detect autophagy. Our results obtained with flow cytometry assay with acridine orange (Figs. 6A, B and S4) and fluorescence microscopy (S5) reveal a tendency for autophagy stimulated by the lectins after 6 h incubation with 30–100 μg/mL of DVL or ConA. However, after 24 h, this effect was only observed in response to lectin concentrations of 30 and 50 μg/mL, but not in response to 100 μg/mL (Figs. 6A, B S4 and S5). In order to confirm these findings, the cleavage of LC3 was evaluated by Western blotting (Fig. 6C and D). The results showed that DVL and ConA stimulated LC3I cleavage to LC3II after 6 h and more intensely after 12 h treatment. The proautophagic proteins Beclin-1 and Atg5 were also analyzed at the same concentrations and times as LC3, but they did not show significant changes, except for Atg 5 after 6 h of treatment with DVL, $^{*}p < 0.05$ (data not shown). Therefore, the autophagic processes in response to DVL and ConA seem to be dynamic, starting early and reaching a robust effect after 12 h, as detected by different approaches, including: Western blotting, detection of acidic vesicular organelles (AVO) (Figs. 6; S4 and S5), and by transmission electron microscopy (TEM) supplementary fig. 6.

3.7. DVL and ConA increased caspase -3 activity

After 6 h incubation, both ConA and DVL (10–50 μg/mL) increased caspase-3 activity in a concentration-dependent manner (Fig. 7). It should be noted that caspase 3 activation was detected in response to the low concentration of 10 μg/mL for both lectins. These data suggest apoptosis as an important early event induced by DVL and ConA on the C6 glioma cell line.

3.8. DVL and ConA impair cell migration

Since lectin treatment could generate a cytostatic action (Fig. 4), we analyzed if it could also impair cell migration (Fig. 8). At concentrations of 10 and 30 μg/mL, results showed that DVL and ConA treatment decreased cell migration/proliferation for 24 h. However, when evaluated for 48 h, only DVL 30 μg/mL could maintain inhibitory effect on cell migration (Fig. 8A). ConA was no longer effective after 48 h since the cells

closed the open area on the cellular carpet (Fig. 8B). It is important to highlight, that both lectins were able to inhibit cell migration for 24 h in the low concentration (10 μg/mL) which did not alter cell viability (see Fig. 1). These data were reinforced by the transwell assay that showed the ability of lectins to prevent cell migration/invasion (Fig. 8C). Taken together, these findings suggest a lectin antimigration effect that was more evident in response to DVL.

4. Discussion

Plant lectins have been studied in recent years owing to their antitumor potential, particularly the leguminous lectin purified from the *Canavalia ensiformes* seeds (ConA), which has been thoroughly investigated in this respect [7,8,17,46]. Most legume lectins share monomeric structure similar to ConA [47]. DVL and ConA belong to the same subtribe (Diocleinaea) and share structural similarity and the same carbohydrate affinity (mannose/glucose). Despite the homology among ConA-like lectins, significant differences have been observed with respect to the effect of these proteins on various biological activities, such as anti-inflammatory, insecticidal, and antitumor [11,12,14,22,23,28,46,48,49].

In the present work, we performed a comparative study to test the anti-glioma activity of DVL and ConA. DVL and ConA displayed similar actions against C6 glioma cells. Mitochondrial membrane potential ($\Delta\Psi m$) was disrupted by both lectins after 6 h incubation, and both lectins could reduce cell viability and cell growth, analyzed by Neubauer chamber counting (Fig. 3) in a concentration- and time-dependent manner. However, in some parameters, DVL was apparently more potent with respect to antitumor potential, since DVL (50–100 μg/mL) could decrease cell viability after 6–12 h incubation, while ConA afforded this action only after 24 h. Moreover, DVL in the low concentration of 10 μg/mL impaired cell growth after 48 h incubation, while ConA did not alter cell growth at this concentration.

Previous studies have demonstrated that ConA can induce autophagy in U87 glioma cells [29,50], as well as hepatoma and melanoma cells [7,14,46,51]. In our study, it was evident that ConA elicited an increment in the autophagic process in rat C6 glioma cells. Applying three approaches, including acridine orange staining, Western blotting of LC3 and electron microscopy, we showed that DVL also promoted autophagy to a degree very similar to that observed in response to ConA exposure. This effect appears after 6 h and is very evident after 12 h incubation with DVL or ConA at concentration ranging from 30 to 100 μg/mL. Therefore, our study reinforces the capability of legume lectins to afford autophagic processes in early incubation periods. It is well known that autophagy is a complex and dynamic process associated with cell survival, as well as the recycling and reuse of certain proteins and even organelles [52]. However, autophagy is also associated with a type of cell death termed macroautophagy [52,53]. Interestingly, upon analyzing Beclin-1 and Atg5 (data not shown), which are essential proteins for autophagy, we did not observe statistical significance in their expression after lectin treatment. On the other hand, LC3, a conserved protein involved in autophagy [54–56], was markedly modulated by the treatments. These results, in association with the mitochondrial damage observed in our study, could be related to mitophagy. Reinforcing this possibility, previous studies have reported the induction of BNIP3 by ConA [29,50], which has been demonstrated to interact with LC3 and promote autophagy of both mitochondria and endoplasmic reticulum [52,53]. However, future studies with detection of the BNIP3 and p62, as well as the use of autophagy inhibitor, such as 3-MA and chloroquine, will be necessary to assess ConA and DVL mechanism concerning the modulation of autophagic event and determine its role for cell death induction.

Regarding the possible targets for the lectins in cell surface, some studies have indicated some possibilities, especially for ConA action. Membrane type-1 matrix metalloproteinase (MT1-MMP), for example, is a glycoprotein expressed in glioma cells already demonstrated to interact with ConA and to be involved in the apoptotic and autophagic response induced by the lectin [29] via signaling pathway dependent of JAK2/STAT3 [50]. Moreover, it has been reported in hepatoma cells that internalization of ConA, after binding unidentified glycoprotein on cell surface, promotes its accumulation onto the mitochondria and induction of autophagic pathway with LC3-II generation and BNIP3 induction [25]. Finally, other potential target is the glycoprotein CD73, which is a dimeric ecto-5′-nucleotidase expressed on the exterior side of the plasma membrane. Notably, lectins such as ConA showed specific binding to the CD73 protein [57]. This aspect is particularly important, since changes in the CD73 activity may modulate glioma progression [58,59]. Concerning DVL, there is no studies addressing possible targets, but we can expect similarity with that described for ConA, since DVL is a ConA like lectin with binding specificity for mannose/glucose. Though, all these caveats deserve future studies to determine the cell surface partners modulated by DVL and ConA, as well as the capacity of the lectins to be internalized in glioma C6 cell line and modulate direct or indirectly the BNIP3.

The present study provides evidence that DVL, similar to ConA, can induce early apoptosis (after 6 h incubation) as indicated by the strong increment in caspase 3 activity to a level similar that observed in the positive control (staurosporine). Several studies showed that ConA induces apoptotic cell death via the mitochondrial pathway [7,14,46,52,60]. Hence, the damage of mitochondrial membrane potential (Fig. 2) and the increment of caspase 3 activity (Fig. 7) in C6 cells treated with DVL or ConA may be consequences of direct or indirect modulation of mitochondria by lectins.

Finally, we demonstrated that DVL and ConA could both inhibit cell migration as analyzed by two different approaches: evaluation of wound closure, after scraping confluent cell layers, and by the transwell assay. It should be noted that this effect was more prominent with DVL treatment since the inhibition of migration was sustained for 48 h by DVL at 30 μg/mL, but not by ConA.

5. Conclusion

In conclusion, our results indicate, for the first time, that DVL is a legume lectin capable of promoting several cellular and molecular changes associated with induction of cell death, autophagy and inhibition of cell migration in the C6 tumor cell line. Noteworthy, further studies will be important to clarify the mechanisms and targets involved in DVL-induced cell death, especially which pathways are involved in these effects. However, the results from this work clearly indicate the antiglioma potential of DVL and reinforce the antitumor capability of legume lectins.

Acknowledgments

This study was supported by the National Council for Scientific and Technological Development (CNPq) Brazil (Projects #308449/2016-9; #481523/2013-8); CNPq/RENORBIO (Project #407945/2013-0); National Coordination for the Training and Improvement of Higher Education Personnel (CAPES/MINCyT Project #249/14); Santa Catarina State Research Foundation (FAPESC/PRONEX Program—NENASC Project; #1262/2012-9). KSN, BSC and RBL are recipient of Research Scholarship from CNPq. APN and JK received a fellowship from CAPES Foundation, Ministry of Education of Brazil. The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.08.106.

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