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ORIGINAL RESEARCH

2-(2-Methoxyphenyl)-3-((Piperidin-1-yl)ethyl) thiazolidin-4-One-Loaded Polymeric Nanocapsules: In Vitro Antiglioma Activity and In Vivo Toxicity Evaluation

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Abstract

Among gliomas types, glioblastoma is considered the most malignant and the worst form of primary brain tumor. It is characterized by high infiltration rate and great angiogenic capacity. The presence of an inflammatory microenvironment contributes to chemo/radioresistance, resulting in poor prognosis for patients. Recent data show that thiazolidinones have a wide range of pharmacological properties, including anti-inflammatory and antiglioma activities. Nanocapsules of biodegradable polymers become an alternative to cancer treatment since they provide targeted drug delivery and could overcome blood–brain barrier. Therefore, here we investigated the in vitro antiglioma activity and the potential in vivo toxicity of 2- (2-methoxyphenyl) -3- ((piperidin-1-yl) ethyl) thiazolidin-4-one-loaded polymeric nanocapsules (**4L-N**). Nanocapsules were prepared and characterized in terms of particle size, polydispersity index, zeta potential, pH, molecule content and encapsulation efficiency. Treatment with **4L-N** selectively decreased human U138MG and rat C6 cell lines viability and proliferation, being even more efficient than the free-form molecule (**4L**). In addition, **4L-N** did not promote toxicity to primary astrocytes. We further demonstrated that the treatment with sub-therapeutic dose of **4L-N** did not alter weight, neither resulted in mortality, toxicity or peripheral damage to Wistar rats. Finally, **4L** as well as **4L-N** did not alter makers of oxidative damage, such as TBARS levels and total sulfhydryl content, and did not change antioxidant enzymes SOD

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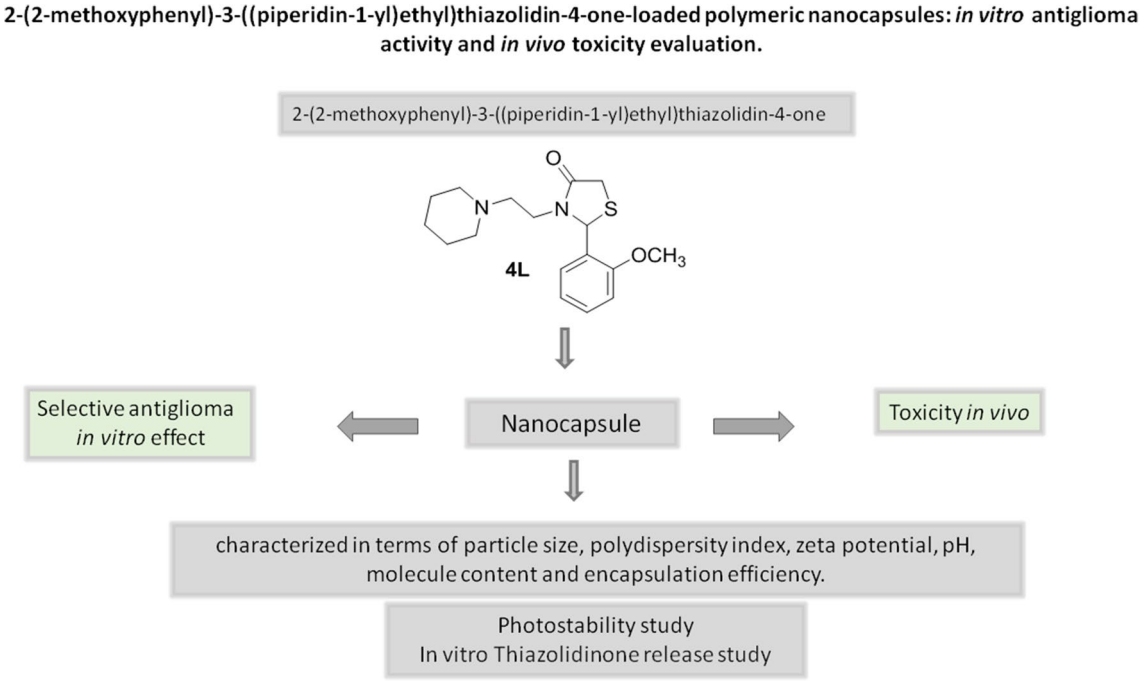
and CAT activity in liver and brain of treated rats. Taken together, these data indicate that the nanoencapsulation of **4L** has potentiated its antiglioma effect and does not cause in vivo toxicity.

Graphical Abstract



Keywords Thiazolidinones · Nanocapsules · C6 glioma · U138MG glioma · Astrocytes · Toxicity

Introduction

Glioblastoma (GB) is the most common and lethal brain tumor, accounting for about 40% of all primary tumors and 78% of the malignant tumors from central nervous system (CNS) (Dunn et al. 2007). GB exhibits morphological and gene expression similarities with glial cells, such as astrocytes, oligodendrocytes, and their precursors (Holland 2001). Currently, the protocol adopted for the treatment of patients diagnosed with glioblastoma is surgical resection whenever possible (Louis et al. 2007), followed by radio/chemotherapy with temozolomide (TMZ), which is considered the chemotherapy of first choice. The adjuvant therapy is maintained for at least 6 months (Mrugala 2013; Stupp et al. 2007). These tumors exhibit significant resistance to treatment because they have a high rate of proliferation and angiogenesis and ability to infiltrate adjacent tissues making neurosurgery difficult. Thus, therapeutic strategies are limited by chemoresistance development and high recurrence rates (Stupp et al. 2007; Soffietti et al. 2007). Another characteristic that limits the treatment of these tumors is the difficulty of chemotherapy to cross the blood–brain barrier (BBB), being a limiting factor for an efficient treatment. In the research for new and effective therapeutic targets, heterocyclic compounds, such as 4-thiazolidinones, stand out to have a wide range of pharmacological properties, including antimicrobial activity (Patel et al. 2012), anti-inflammatory (Deep et al. 2012), antifungal (Lesyk and Zimenkovsky 2004) antioxidants (Saundane et al. 2012), anticancer, among others (Tripathi et al. 2014; Havrylyuk et al. 2009). Recent studies have shown that these compounds may possibly control cell proliferation through induction of apoptosis (Moorkoth 2015; Wan et al. 2015) and inhibition of

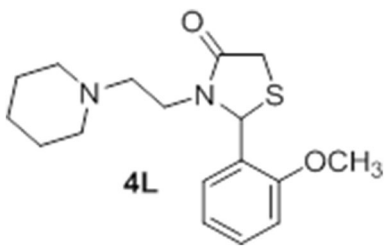


Fig. 1 Structure of 2-(2-methoxyphenyl)-3-((piperidin-1-yl)ethyl) thiazolidin-4-one **4L** (Kunzler et al. 2013)

cyclooxygenases (Abdelazeem et al. 2014), there by controlling cancer-related inflammation (Abdelazeem et al. 2014).

In the search for new medicines carriers, nanocapsules have been considered as a promising tool to improve the passage of bioactive molecules through BBB, contributing to increased therapeutic efficacy and reduced side effects of conventional chemotherapy. These particles are capable of becoming invisible to the phagocytic system, which favors the targeting of the formulation to the tumor microenvironment promoting its uptake (Obermeier et al. 2013). Moreover, nanocarriers overcome multiresistance drug problems, exhibit low in vivo toxicity and enables decreased frequency of administration (Dong et al. 2016; Maji et al. 2015). In a recent work, we have demonstrated the synthesis (Kunzler et al. 2013), and the in vitro and in vivo antiglioma potential of a series of thiazolidinones, wherein the 2-(2-methoxyphenyl)-3-((piperidin-1-yl)ethyl)thiazolidin-4-one 4L exhibited prominent potential for cancer therapy (da Silveira et al. 2017) (Fig. 1). However, 4L low solubility in water and its susceptibility to photodegradation may result in poor bioavailability and pharmacological effect, which make difficult the further use of this thiazolidinone in therapy. By the other hand, these chemical characteristics make 4L a good candidate for nanoencapsulation, which is a strategy to overcome these issues and to potentiate its biological activity. Indeed, previous studies from our group have shown that the nanoencapsulation of ketoprofen, an anti-inflammatory drug, improved its anticancer activity, probably by promoting cancer-target delivery (Silveira et al. 2013).

In an attempt to circumvent the possible limitations of 4L molecule and those clinical problems for cancer treatment, including incidence of side effects, low selectivity, and inability to achieve CNS, the aim of the present work was to develop nanocapsules loading 2-(2-methoxyphenyl)-3((piperidin-1-yl)ethyl)thiazolidin-4-one (4L) in order to evaluate its in vitro antiglioma potential comparing to the 4L-free formulation as well as its in vivo toxicity.

Experimental Procedures

Reagents

Dulbecco's modified Eagle's medium (DMEM), Fungizone, penicillin/streptomycin, 0.25% trypsin/EDTA solution, and fetal bovine serum (FBS) were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA). Poly (Ɛ-caprolactone) (PCL) (MW: 80 kDa) and Span 80® (sorbitan monooleate) were acquired from Sigma Aldrich (São Paulo, Brazil). Tween 80® (polysorbate 80) and MCT (medium chain triglycerides) were furnished by Delaware (Brazil). All other chemicals and solvents used were of analytical or pharmaceutical grade.

Preparation of Nanocapsule Suspensions

Nanocapsule suspensions were prepared by interfacial deposition of preformed polymer. An organic phase consisting of polymer [PCL] (0.100 g), acetone (27 mL), Span 80® (0.077 g), compound **4L** (0.01 g), and MCT (330 µL) were maintained for 60 min under moderate magnetic stirring at 40 °C. After all components were solubilized, the organic phase was injected into 53 mL of an aqueous dispersion of Tween 80® (0.077 g) and kept under magnetic stirring for 10 min. Subsequently, organic solvent and part of water were removed by evaporation under reduced pressure to achieve a final volume of 10 mL and **4L** at a concentration of 1.0 mg/mL (**4L-N**). For comparison purposes, formulations without drug were also made (**NC**). All formulations were prepared in triplicate.

Characterization of Nanocapsule Suspensions

Granulometric distribution was performed by laser diffraction (Mastersizer® 3000E, Malvern Instruments, UK) after diluting the samples in distilled water. A laser obscuration of 15% and refractive index of 1.59 was used to perform the measurements. Particle sizes and polydispersity indexes were determined by photon correlation spectroscopy (Zetasizer Nanoseries, Malvern Instruments, UK) after dilution of samples with ultrapure water (1:500). The zeta potentials were measured using the same instrument after the dilution of the samples in 10 mM NaCl (1:500) by microeletrophoresis. The pH of the nanocapsule suspensions was verified by direct immersion of electrode from a calibrated potentiometer (Model PH 21, Hanna Instruments, São Paulo, Brazil) in the formulations. Measurements were taken at room temperature (25 ± 2 °C).

The total **4L** content in nanocapsule suspensions was determined by the HPLC method. For this, 150 µL of the nanocapsule suspension were diluted in 10 mL of methanol, sonicated for 30 min, filtered through a 0.45 µm membrane and injected into HPLC system. The **4L** quantification was performed on a LC-10A HPLC system (Shimadzu, Japan) equipped with a LC-20AT pump, an UV–VIS SPD-M20A detector, a CBM-20A system controller and a SIL-20A HT valve sample automatic injector. Separation was achieved at room temperature using a Gemini C_{18} Phenomenex column (150 mm × 4.60 mm, 5 µm; 110 Å) coupled to a C_{18} guard column. The isocratic mobile phase consisted of methanol and water (90:10, v/v) at 1 mL/min of flow rate. The **4L** was detected at 279 nm. The method was linear ($r = 0.995$) in the concentration range of 5.0–25.0 µg/mL.

For the encapsulation efficiency determination, an aliquot of formulation was placed in a 10,000 MW centrifugal device (Amicon® Ultra, Millipore) and free molecules were separated from the nanocapsules by the ultrafiltration/centrifugation technique for 10 min at 2200×*g*. The ultrafiltrate was analyzed by HPLC. The difference between **4L** total and free concentrations, and the ultrafiltrate, was respectively calculated as encapsulation efficiency (EE %) of the NCs following equation: EE = [(total content − free content)/total content] × 100.

Photostability Study

For the purposes of photostability study, 700 µL of **4L** methanolic solution (1 mg/mL) and **4L-N** (1 mg/mL) were placed individually in cuvettes with lids arranged at a fixed distance and subsequently exposed to ultraviolet radiation (Phillips TUV Long Life Lamp-UVC, 30 W) for 120 min in a mirrored chamber (1 m × 25 cm × 25 cm). At time intervals (0, 15, 45, 60, 90, and 120 min), aliquots of 150 µL were withdraw and diluted in methanol to determine the concentration of thiazolidinone in each sample by HPLC. To discard the influence of other factors, the cuvettes containing the samples, wrapped in aluminum foil (dark controls), were also evaluated.

In Vitro Thiazolidinone Release Study

The thiazolidinone in vitro release from nanocapsules was evaluated by the dialysis bag diffusion technique. For this, 1.0 mL of **4L** methanolic solution or nanocapsules (**4L-N**) were placed in the dialysis bag (MWCO 10,000, Spectra Por 7) and immersed in 150 mL of phosphate buffer (pH 7,4): ethanol (70:30, v/v), maintained at 37 °C and under continuous magnetic stirring at 50 rpm. At predetermined times, 1 mL aliquots were withdrawn and replaced with the same volume of fresh medium to maintain *sink* conditions. The amount of released thiazolidinone **4L** was quantified by HPLC as previously described.

General Cell Culture Procedures

Rat C6 and Human U138MG Glioma Cell Lines

C6 and U138MG glioma cells were purchased from American Type Culture Collection (Rockville, MD, USA) and were cultured in DMEM containing 0.1% Fungizone and 100 U/L penicillin/streptomycin and supplemented with 5% or 10% (v/v) fetal bovine serum (FBS, Gibco), respectively. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Rat Cortical Astrocyte Primary Cultures

Astrocyte primary culture was prepared as previously described (da Silveira et al. 2017). Briefly, the cerebral cortex of newborn (1–2 day old) Wistar rats (CEEA protocol number 9219) was removed and mechanically dissociated in Ca^{+2}/Mg^{+2}-free buffer (pH 7.4). After centrifugation at 1000×*g* for 10 min, pellet was suspended in DMEM supplemented with 10% FBS and cells (3×10^4) were seeded in 96-well plates previously coated with poly-L-lysine for better adherence of cells. Following 4 h of cell seed, culture medium was changed. Cultures were allowed to grow to confluence, around 20–25 days. Culture medium was replaced every 4 days.

In Vitro Treatment of Cell Cultures

Synthetic 4L was first dissolved in sterile 0.01% DMSO at 100 mM concentration (stock solution) and further diluted in DMEM/5% FBS (C6 glioma) or DMEM/10% FBS (U138MG or astrocytes) to obtain the desired concentrations; 4L-N was prepared as described above. C6, U138MG glioma cells or astrocyte cultures were exposed for 48 or 72 h to 4L or 4L-N at the following concentrations 6.25, 12.5, 25, and 50 µM. Control cells were treated with equivalent volume of vehicle (DMSO) or unloaded drug-nanocapsule (NC).

Cell Viability Assay

Cell mitochondrial viability was assessed by MTT assay. This experiment is based on the ability of cells, which remain viable, in reducing MTT and forming a blue formazan product. Following treatment, medium was withdrawn and cells were exposed to MTT solution (0.5 mg/mL) prepared in DMEM/5% or 10% FBS for 90 min at 37 °C in a 5% CO_2 humidified atmosphere. The solution was then removed and the plates were shaken with DMSO for 30 min. The optical density of each well was measured at 492 nm. The results were expressed as absorbance.

Determination of Cell Proliferation by Sulforodamine B Assay

Following 48 or 72 h treatment, cells were gently washed and 50% trichloroacetic acid was added for 45 min for cell attachment. After this period, acid was removed and five washes were performed with distilled water until the reagent was completely removed, followed by 30 min incubation with 0.4% sulfarodamine. The solution was then removed and 5 washes were made with 1% acetic acid for the complete removal of non-complexed dye with proteins. Finally, SRB was eluded with 10 mM Tris solution. The

optical density of each well was measured at 530 nm in a spectrophotometer.

Propidium Iodide Assay

Cell damage was assessed by fluorescent imaging of propidium iodide (PI) uptake. At the end of the treatments, C6 glioma cells were incubated with PI (7.5 µM) for 1 h. PI fluorescence was excited at 515–560 nm using an inverted microscope (Olympus IX71, Tokyo, Japan) equipped with a standard rhodamine filter. The images were captured using a digital camera connected to microscope.

In Vivo Treatment

Animals

Male Wistar rats (60 days old) were obtained from Central Animal House of Federal University of Pelotas (Pelotas, RS, Brazil). Animals were maintained under conventional conditions on a 12/12 h light/dark cycle at an air-conditioned constant temperature (22 ± 1 °C) colony room. Rats had free access to 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with Federation of Brazilian Societies for Experimental Biology and was approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas (CEEA protocol number 9219).

Treatment Protocol

Animals were randomly divided into five groups as follows: (1) Control (rats water treated); (2) Control canola oil; (3) **4L** (rats treated with 5 mg/Kg/day of thiazolidin-4-ones dissolved in oil); (4) **4L-N** (rats treated with 5 mg/Kg/day of thiazolidin-4-ones-loaded nanocapsules); and (5) Blank nanocapsules (rats treated with drug-unloaded nanocapsules) (**NC**). Animals received the treatments by gavage for 5 consecutive days according to (Villalba et al. 2016) adapted from (Khachane and Nagarsenker 2011a; b). During the treatment period, animals were weighed daily. After treatment time, animals were anesthetized and euthanatized. Immediately blood, brain, and liver were removed for further analysis.

Serum Preparation and Biochemical Analysis

Blood was collected without anticoagulant and immediately centrifuged at 2500×*g* for 15 min at RT. The clot was removed and the resulting serum was stored at − 80 °C for further biochemical determination. Hepatic and renal functions were evaluated using the enzymatic assays alanine aminotranspherase (ALT), aspartate aminotranspherase (AST), creatinine, and urea. These analyses were performed using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

Tissue Preparation

For oxidative stress parameter determination, tissues from brains and livers were homogenized in 10 volumes (1:10 w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 2500×*g* for 10 min at 4 °C. Pellet was discarded and supernatant was separated and used for biochemical determination.

Oxidative Stress Parameters in Liver and Kidney

Thiobarbituric Acid Reactive Substances (TBARS) Quantification

TBARS levels were determined as previously described by (Esterbauer and Cheeseman 1990). For the assay, the already homogenized tissues were mixed with trichloroacetic acid (10%) and thiobarbituric acid (0.67%) and incubated in a dry block at 100 °C for 30 min. TBARS levels were determined by absorbance at 535 nm. Results were reported as nmol of TBARS per mg of protein.

Total Quantification of Sulfhydryl (SH) Content

Total sulfhydryl content was quantified as previously described by (Aksenov and Markesbery 2001), which is based on the reduction of DTNB by thiols, which in turn becomes oxidized (disulfide) generating a yellow derivative (TNB) whose absorption is measured at 412 nm in a spectrophotometer. First, homogenates were added to a PBS buffer at pH 7.4 containing EDTA. Reaction was started by the addition of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB). Results were reported as nmol TNB per mg protein.

Superoxide Dismutase (SOD) Activity

Total SOD activity was measured according to described by (Misra and Fridovich 1972). This assay is based on inhibition of self-oxidation of superoxide dependent adrenaline to adenocarbon in a spectrophotometer set at 480 nm. The intermediate in this reaction is superoxide, which is eliminated by SOD. A unit of SOD was defined as the amount of enzyme to cause 50% inhibition of adrenaline self-oxidation. The specific activity of SOD was reported as units per mg protein.

Catalase (CAT) Activity

CAT activity was assayed by the method of (Aebi 1984). The decomposition of 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) was continuously monitored with a spectrophotometer at 240 nm for 180 s in a thermostat (37 °C). One unit of the enzyme is defined as the number of hydrogen peroxide consumed per minute and the specific activity reported for units per mg protein.

Protein Determination

Protein was determined by the method of s(Lowry et al. 1951) using bovine serum albumin as standard.

Statistical Analysis

Data sets were analyzed using one-way or two-way ANOVA followed by a Tukey test for multiple comparisons. Significance was considered when $p < 0.05$ in all analyses. Data are expressed as mean ± SD ot mean ± SEM when indicated.

Results

Preparation and Characterization of Thiazolidinone 4L Nanocapsules

After preparation, **4L**, **4L-N,** and **NC**, respectively, exhibited homogeneous appearance without visible precipitates. Table 1 shows physicochemical characterization of formulations. In order to discard the presence of particles in the micrometer size range, laser diffraction analysis was performed. Any microparticle population was observed for both formulations. Besides that, 50% of particle presented size around 554 nm and the *Span* was less than 2.0, indicating a narrow distribution. By photon correlation spectroscopy particles with size between 276–243 nm and polydispersity index around 0.2 were observed. Zeta potential values were negative and pH was in the neutral range. The thiazolidinone did not significantly alter the physicochemical parameters. The molecule content was close to the theoretical value (1 mg/mL) and the encapsulation efficiency was higher than 98%.

Table 1 Physicochemical characterization of **4L-N** and **NC**

	4L-N	NC
Mean diameter (nm)	243 ± 3	276 ± 2
PDI	0.215 ± 0.010	0.234 ± 0.012
Zeta potential (mV)	−9.0 ± 0.4	−7.9 ± 0.2
pH	6.7 ± 0.2	8.0 ± 0.1
Drug content (%)	97.74 ± 4.71	–
EE (%)	98.85	–
Granulometric distribution		
$D_{4,3}$ (μm)	0.671	0.597
$D_{0,9}$ (μm)	0.986	0.688
$D_{0,5}$ (μm)	0.554	0.450
$D_{0,1}$ (μm)	0.367	0.306
Span	1.120	0.844

Physicochemical characterization of formulations: *PDI* polydispersity index; EE%: encapsulation efficiency

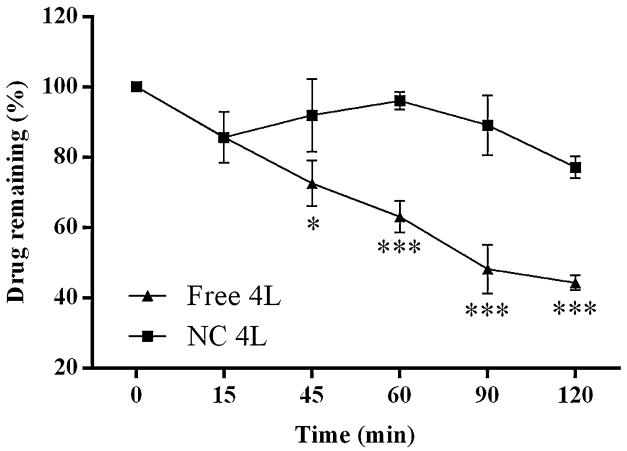


Fig. 2 Photostability of 4L and 4L-N. Evaluation of the ability of nanostructures to protect 4L thiazolidinone against photodegradation. Values represent mean ± SD of at least three independent experiments. Data were analyzed by ANOVA two-way followed by post hoc comparisons (Tukey–Kramer test). *, **, ***Significantly different from control ($p < 0.05$; $p < 0.01$; $p < 0.001$, respectively)

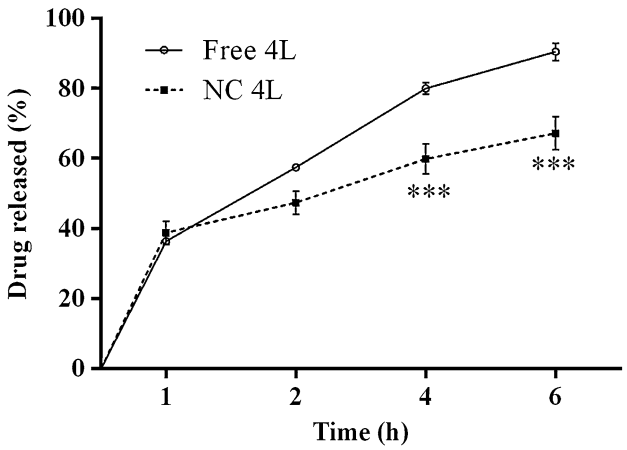


Fig. 3 Thiazolidinone release. The values represent the mean ± SD of at least three independent experiments. Data were analyzed by ANOVA two-way followed by post hoc comparisons (Tukey–Kramer test). *,**,***Significantly different from control ($p < 0.05$; $p < 0.01$; $p < 0.001$, respectively)

In Vitro Photostability Study

In order to evaluate the nanostructures ability to protect **4L** thiazolidinone against photodegradation, **4L-N** and **4L** methanolic solution (**4L-**free molecule) samples were exposed to UVC radiation (Fig. 2). After 120 min of exposure, approximately 78% of thiazolidinone **4L-N** associated to nanocapsules kept unchanged. On the other hand, only 44% of free molecule **4L** was remained under the same conditions. Statistical analysis shows that the degradation of thiazolidinone methanolic solution starts in 45 min of exposure. For dark control, **4L** concentration was close to 100%, which discards the influence of chamber temperature on drug degradation *(data not shown)*.

In Vitro Thiazolidinone Release Study

Concerning in vitro drug release experiments, Fig. 3 shows that **4L-N** could control thiazolidinone release in comparison to **4L**. In a 6 h period, almost 95% of free **4L** was released while 67% of thiazolidinone **4L** was released from **4L-N** in the same period, showing that the nanocarrier was able to prolong thiazolidinone release. Two-way ANOVA analyses shows that nanocapsules began to prolong the release from 4 h incubation.

2- (2-Methoxyphenyl) -3 - ((Piperidin-1-yl) ethyl) thiazolidin-4-one) (4L) Selectively Decrease the Viability of Rat C6 and Human U138MG Glioma Cells

As shown previously, the class of 2-aryl-3-((piperidin-1-yl) ethyl)thiazolidin-4-ones shows a promising antiglioma activity and compound 2-(2-methoxyphenyl)-3–(piperidin-1-yl)ethyl)thiazolidine-4-one (4L) showed the best in vitro antiglioma potential and did not induce cytotoxicity to astrocytes. Also, this compound reduced 73% in vivo rat glioma growth (da Silveira et al. 2017). Here, we nanoencapsulated 4L compound in order to potentiate its activity. Firstly, we evaluated if 4L and 4L-N differentially reduce C6 glioma cell viability (Fig. 4). For this, glioma cells were exposed to 6.25, 12.5, 25, and 50 µM of 4L or 4L-N and cell viability was assessed by MTT after 48 and 72 h of treatment. Cells exposed to DMEM/5% FBS, DMSO (0.01%), or NC were considered controls.

As shown in Fig. 4 (panels a and b), 4L was able to reduce 27% of C6 cell viability at 50 µM following 48 h of treatment when compared to control, which reproduces data already published by our group (da Silveira et al. 2017). In the same way, 4L-N reduced cell viability by 32.44, 27.62, 33.44, and 25.12% at 6.25, 12.5, 25, and 50 µM, respectively, after 48 h of treatment. When the same experiment was carried out for 72 h, 4L reduced cell viability in ~ 30% at 25 and 50 µM, while 4L-N induces viability reduction at all concentrations, starting from 21.81% at 6.25 µM until 60.51% at the highest concentration tested (50 µM). We also notice that the nanoencapsulated molecule showed an increase of 34.15% of cell toxicity in relation to 4L-free molecule at highest concentration tested (50 µM), showing the efficiency of nanoencapsulation to improve antiglioma activity. In general, treatment with 4L or 4L-N decreased cell viability when compared to controls, exhibiting concentration and time dependence. Of note, NC (drug-unloaded nanocapsule) caused significant decrease in glioma cell viability only in at longest time (72 h) and in the highest concentrations tested. We suggest that this effect can be related to interpolation of polymer and Tween 80® present in the composition of the nanocapsule and, furthermore, by the prolonged cell incubation time. In addition, experiments were performed in U138MG glioma cells, in order to verify if the 4L-N induced citotoxicity remains significant and mimicking the efficacy in human tumor cells (Fig. 4, panel c and d). Cell viability tests were also performed after 48 and 72 h of treatment. Following 48 h of treatment, 25 and 50 µM of 4L-N reduced 18.5% and 23.5% U138MG cell viability, respectively, when compared to control. Similarly, 72 h of 4L-N exposition reduced cell viability in all concentrations tested, reaching 48.7% of decrease in the highest concentration tested (50 µM).

In parallel, primary astrocyte cultures were used as an untransformed cell to evaluate 4L-N citotoxicity selectivity (Fig. 4, panel e). Notably, neither 4L free molecule (da Silveira et al. 2017) nor 4L-N promoted significant astrocyte cytotoxicity after 72 h of exposure. Therefore, data indicate that 4L-N exhibit selective citotoxic effect in rat and in human glioma cell lines, indicating its potential for translational purposes.

Synthetic 2-(2-Methoxyphenyl)-3-((Piperidin-1-yl) ethyl)thiazolidin-4-one (4L) Decreases C6 Glioma Cell Proliferation

Through the sulfarodamine B assay, we evaluated whether **4L** and **4L-N** were able to reduce C6 glioma cell proliferation (Fig. 5). Glioma cells were exposed to **4L** or **4L-N** and after 48 or 72 h of treatment, proliferation was evaluated. Controls containing 0.01% DMSO, DMEM/5% FBS, or blank nanocapsule (NC) were used. As shown in Fig. 5, **4L** decreased glioma cell proliferation by 35 to 65% following treatment with increasing concentrations of thiazolidinones (6.25–50 µM) for 72 h, while after 48 h of treatment no changes were observed. On the other hand, **4L-N** reduced C6 cell proliferation in all times (48 or 72 h) and concentrations tested (6.25-50 µM). Indeed, the decrease of cell proliferation ranged from 25–56% and 60–70% following 48 or 72 h of exposure, respectively. We note that while

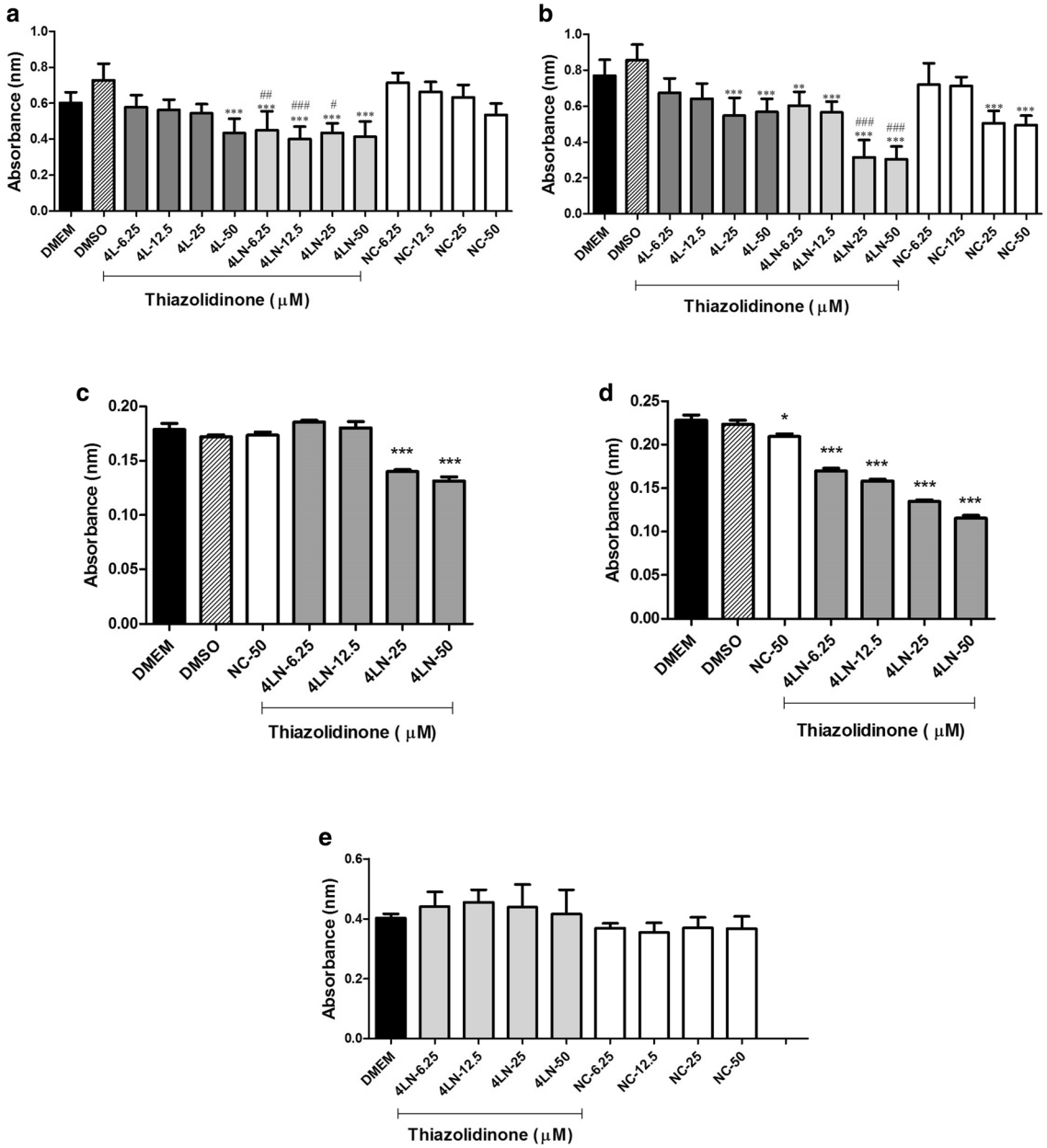
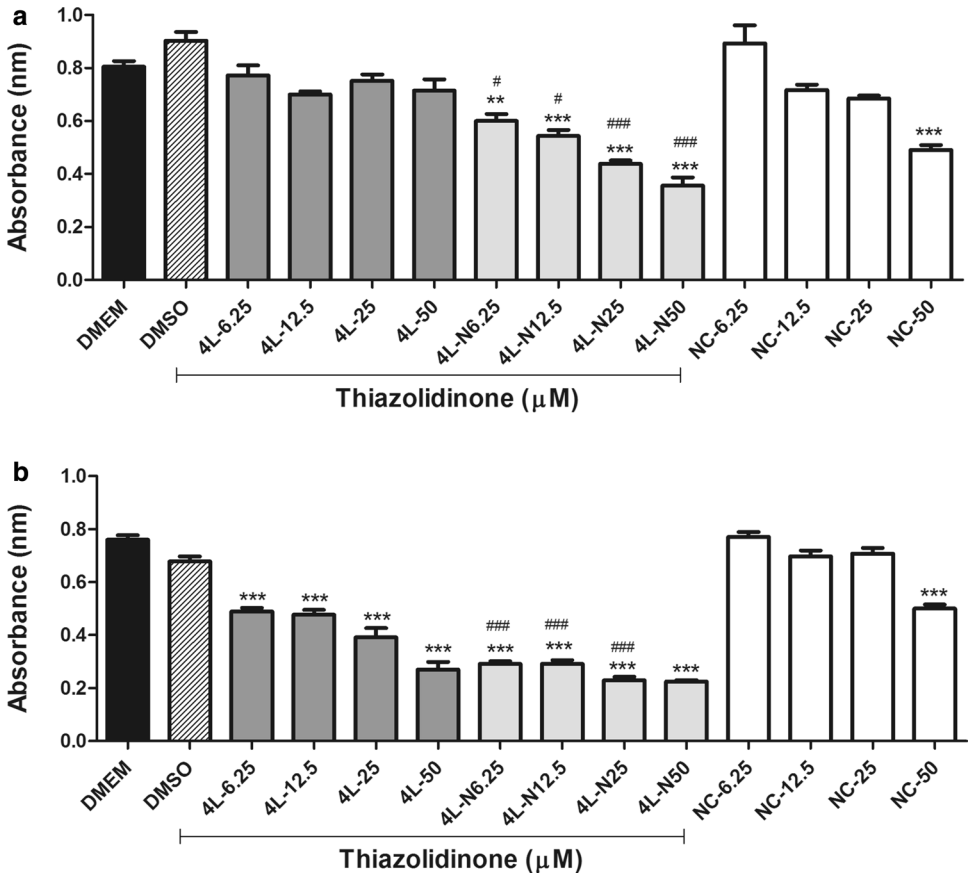


Fig. 4 Evaluation of 4L thiazolidinone effect on glioma and astrocyte cell viability. Rat C6 glioma (**a**, **b**), human U138MG glioma (**c**, **d**) and rat primary astrocyte cultures (**e**) were treated for 48 h (panel **a**, **c**) or 72 h (panel **b**, **d** and **e**) with increasing concentrations of 4L or 4L-N as indicated and MTT test was performed. Appropriate controls were performed for each group. Values represent the mean ± SD of at least three independent experiments. Data were analyzed by ANOVA, followed by post hoc comparisons (Tukey–Kramer test). Significantly different between the free (4L) or nanocapsulated (4L-N) molecule ($p < 0.05$, $p < 0.01$), $p < 0.001$), respectively)

in its free form the **4L** thiazolidinone did not prove to be efficient in decreasing glioma cell proliferation at 48 h, the **4L-N** nanoencapsulated molecule was efficient in all tested concentrations, including in the smaller ones.

Experiments with molecule-unloaded nanocapsules (NC) have also been performed and the result is close to that shown in MTT, where NC alone is able to reduce glioma viability at higher concentrations. In general, both **4L** or **4L-N** treatments decreased C6 glioma cell proliferation compared to controls, and we conclude that 4L nanoencapsulation may further enhance its promising antiglioma effect. Notably, results show that even at lowest concentration tested, **4L-N** is 25% more effective than **4L** in reducing glioma proliferation.



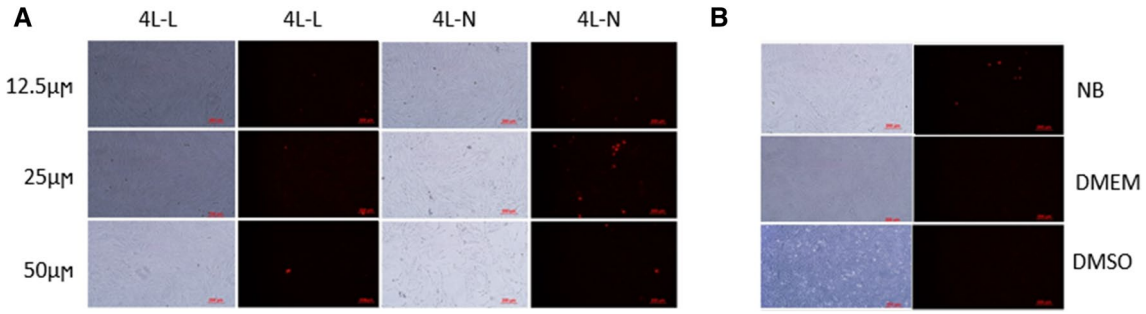


Fig. 5 Effect of **4L** or **4L-N** on C6 glioma cell proliferation. C6 glioma cell line was treated for 48 h (panel **a**) or 72 h (panel **b**) at different concentrations (6.25–50 μM) of **4L** synthetic thiazolidinone (free or in nanocapsules). The sulfarodamine B assay was performed. Appropriate controls were performed for each group. Values represent mean ± SD of at least three independent experiments. Data were analyzed by ANOVA followed by post hoc comparisons (Tukey–Kramer test). *, **, ***Significantly different from control cells, #, ##, ###Significant difference between free or nanocapsule molecule) ($p < 0.05$; $p < 0.01$; $p < 0.001$, respectively)

Fig. 6 Incorporation of propidium iodide (PI) into C6 glioma cells after treatment with 4L or 4L-N. Cells were exposed to increasing concentrations of 4L or 4L-N and after 48 h of treatment the glioma cells (panel **a**) were incubated with diluted PI in culture medium. Appropriate controls (panel **b**) containing DMEM, 0.01% DMSO or blank nanocapsules were performed. Fluorescence (right panel) and phase contrast (left panel) micrographs were performed using an Olympus inverted microscope

2- (2-Methoxyphenyl)-3-((Piperidin-1-yl)ethyl) thiazolidin-4-one (4L) Promotes C6 Glioma Cell Death

Permeability of cell membrane was assessed by incorporation of propidium iodide (PI). Concentrations of 12.5, 25, or 50 μM were chosen and treatment with **4L** or **4L-N** were performed for 48 h (Fig. 6). Cells exposed to DMEM/5% FBS, DMSO (0.01%) or drug-unloaded nanocapsules were used as controls. **4L** in its free form was able to decrease viability of cancer cells, whereas **4L-N** promoted cell morphological alterations and PI incorporation when compared to controls. These results suggest that the antiproliferative effect of **4L** is potentiated when the molecule is nanoencapsulated and its effect can be mediated by necrosis. However, the involvement of other cell death pathways could not be excluded.

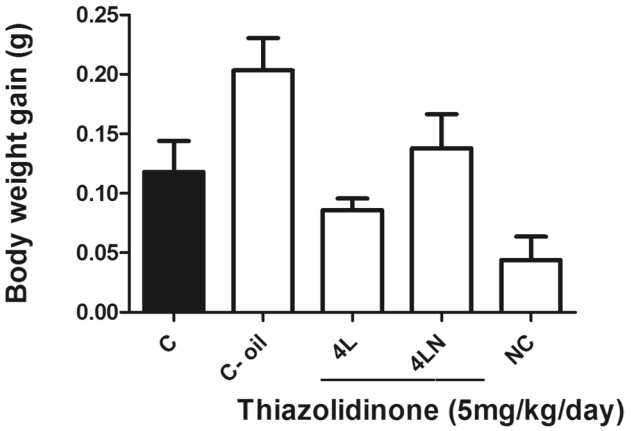


Fig. 7 Analysis of weight gain in controlled or treated rats ($n=8$). Animals were treated with free **4L** thiazolidinone (5 mg/kg/day) or in nanocapsules (**4L-N**) or controls (canola oil or molecule-free nanocapsule). The values represent the mean ± SD. Data were analyzed by ANOVA

4L or 4L-N Does Not Cause Weight Loss, Metabolic Toxicity and Mortality in Rats

We also evaluated the toxicity of **4L** and **4L-N** in vivo. Animals (60 days old Wistar rats) were orally treated with 4L or 4L-N (5 mg/kg/day) for 5 days. Control groups received an equivalent volume of canola oil (vehicle) or drug-unloaded nanocapsule (NC). Treatment with both **4L** and **4L-N** did not cause weight loss or mortality (Fig. 7). Cellular toxicity was assessed by ALT and AST activity (liver damage markers) and creatinine and serum urea levels (markers of kidney damage), and no alterations were observed in these parameters (Table 2). Taken together, these results support the idea that both **4L** and **4L-N** did not promote peripheral toxicity.

Evaluation of Oxidative Stress Parameters in Brain and Liver of Rats

First, no changes were observed in both brain and liver of the **4L** or **4L-N** group compared to the control group. Regarding the profile of oxidative stress parameters in the brain, that can be seen in Fig. 8, there was no change in the levels of TBARS ($F_{(4-13)}=0.37$, $p>0.05$) and total sulfhydryl content ($F_{(4-18)}=0.64$, $p>0.05$) as well as in the activity of the antioxidant enzymes SOD ($F_{(4-17)}=1.15$, $p>0.05$) and CAT ($F_{(4-18)}=1.57$, $p>0.05$) in the **4L**, **4L-N**, and **NC** groups when compared to the control group.

Similar results were found in the liver as shown in Fig. 9. No change was observed in TBARS ($F_{(4-18)}=3.12$, $p>0.05$) levels, total sulfhydryl content ($F_{(4-17)}=3.87$, $p>0.05$) and activity of antioxidant enzymes SOD ($F_{(4-18)}=1.91$, $p>0.05$) and CAT ($F_{(4-20)}=0.62$, $p>0.05$) liver of rats treated with **4L**, **4LN** as well as **NC**.

Discussion

Glioblastoma is an extremely aggressive, uncured tumor with a high rate of proliferation and infiltration, as well as a large angiogenic capacity (Borisov and Sakaeva 2015). Despite intense efforts to develop new therapies for patients diagnosed with this type of tumor, GBM eradication is still not available making it a challenge for most antineoplastic agents (Chumak et al. 2014). Thiazolidinones have a large number of different biological applications (Tripathi et al. 2014). In a previous study published by our group, some 2-aryl-3-((piperidin-1-yl)ethyl)thiazolidin-4-ones demonstrated in vitro and in vivo antiglioma activities (da Silveira et al. 2017). Among all compounds tested, the best result was found for 2-(2-methoxyphenyl)-3-((piperidin-1-yl) ethyl)thiazolidin-4-one **4L**. In this work, **4L** was nanoencapsulated with the aim of further improving its antitumor ability. Nanocapsules are promising alternatives in therapeutics and are able to become invisible to phagocytic system, which favors the targeting of the formulation to the microenvironment of the tumor and facilitate the passage of bioactive molecules through BBB, thereby increasing therapeutic efficacy and reducing side effects (Obermeier et al. 2013). In addition, nanocarriers increase the drug stability, protect against photo- or chemical degradation, resulting in increased molecule stability and reduced drug toxicity

Table 2 Serum markers of tissue damage in controls, 4L or 4L-N-treated rats

	Control	C-oil	**4L**	**4LN**	NC
AST (U/L)	96.60 ± 33.31	152.80 ± 20.14	157.40 ± 45.19	144.00 ± 23.70	141.60 ± 36.71
ALT (U/L)	42.00 ± 3.93	45.00 ± 6.20	47.60 ± 5.94	44.80 ± 6.30	48.20 ± 3.42
Urea (mg/dL)	43.20 ± 3.19	45.80 ± 5.11	43.20 ± 3.03	47.00 ± 3.53	43.20 ± 3.19
Creatinine (mg/dL)	0.46 ± 0.07	0.53 ± 0.08	0.55 ± 0.04	0.52 ± 0.02	0.50 ± 0.05

Animals were treated via gavage with **4L** or **4L-N** (5 mg/kg/day) for 5 days ($n=8$). Control rats were treated with equivalent volume of canola oil (vehicle) or NC. Values represent mean ± SD. Data were analyzed by ANOVA

Serum markers of tissue damage: *AST* aspartate aminotransferase, *ALT* alanine aminotransferase

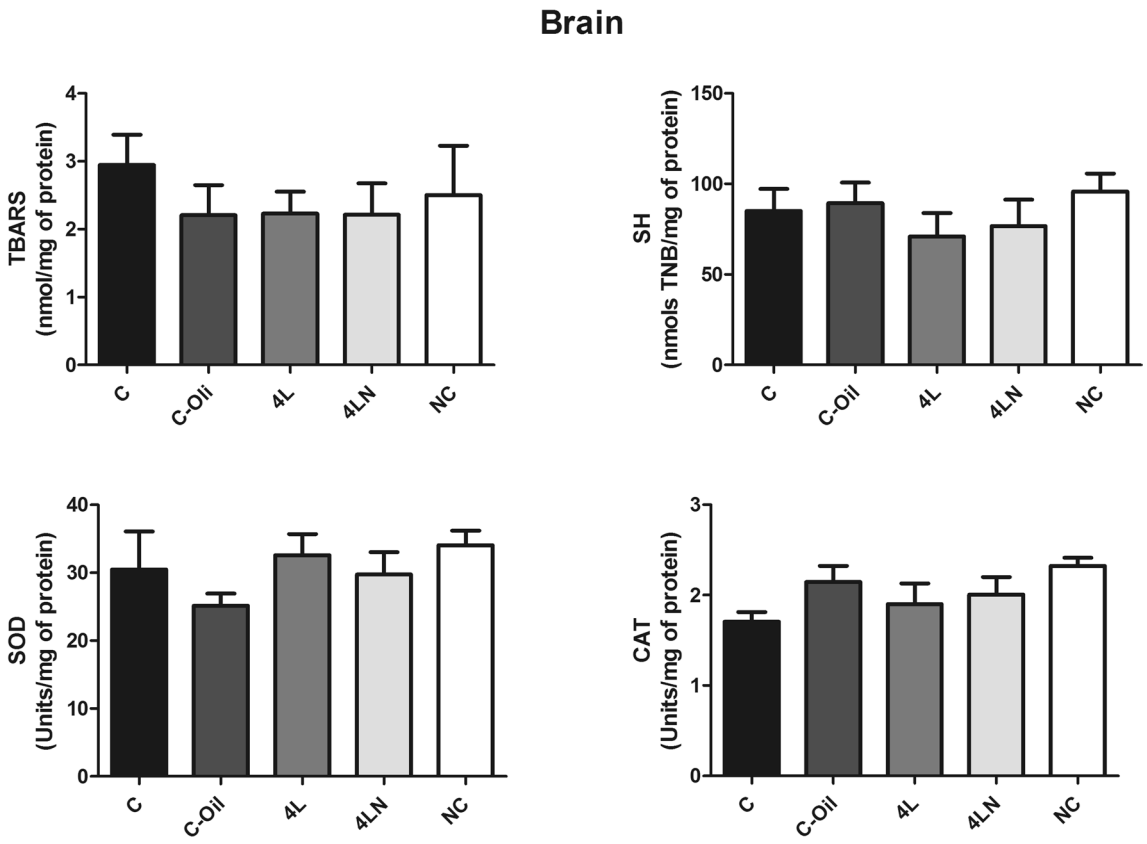


Fig. 8 Analysis of oxidative stress parameters TBARS (**a**), total sulfhydryl content (**b**) and activities of antioxidant enzymes superoxide dismutase (SOD) (**c**) and catalase (CAT) (**d**) in brain of rats 5 days after treatment with **4L**, **4LN** and **NC** compounds. Values are expressed as mean ± SD ($n = 4$–5). TBARS levels were reported as nmol TBARS per mg protein, total sulfhydryl content as nmol TNB per mg protein and SOD and CAT are express as unit per mg pf protein. Data were analyzed by ANOVA followed by Tukey–Kramer test

(Santos et al. 2014; Chassot et al. 2015). Features like these are usually found into nanocapsules prepared by the interfacial deposition of preformed polymer employing PCL as polymer and MCT as oil core (Ourique et al. 2008; Friedrich et al. 2008). The zeta potential values were negative which is probably due to the PCL, which is a polyester (Chassot et al. 2015) and to polysorbate 80 that confers a steric stabilization and presents a negative charge surface density (Fontana et al. 2009). The pH values are in the neutral range and also are in accordance to the nanocapsules prepared by the method employed in this study and are suitable for parenteral administration.

Aiming to determine **4L** photostability and nanocapsules ability to protect the molecule against degradation, the formulations were exposed to UVC radiation. As a first point, we can suggest that **4L** is a photolabile molecule, since after 120 min of exposure the content decrease significantly. On the other hand, when this molecule is nanoencapsulated, **4L** content kept unchanged. This result can be explained by PCL presence in nanocapsule vesicular structure because it is a semi-crystalline polymer and is able to reflect and/or scatter ultraviolet light (Pohlmann et al. 2013; Almeida et al. 2010). Additionally, nanometric size of polymeric nanocapsules can contribute to photostability improvement and scattering radiation.

Concerning in vitro drug release study, it is possible to observe a molecule controlled release in comparison to **4L** methanolic solution. After 6 h of experiment, 100% of free **4L** had been released, while around 70% were released from nanocapsules. Controlled release is one advantage attributed to nanostructured systems and the presence of the polymer can be the main barrier to drug delivery. The drug release can be dependent on the polymer erosion and/or drug diffusion to the dissolution medium and the lipophilic molecules have high affinity to oil core and consequently present a slow drug delivery (Fontana et al. 2009; Chassot et al. 2015).

We analyzed the effect of 4L and 4L-N in in vitro cell viability and proliferation of glioma cells. Treatment with 4L selectively decreased cell viability and proliferation of C6 glioma line, while the molecule 4L-N at both treatment times (48 and 72 h) exhibits a greater efficiency to reduce cell viability and proliferation when compared to 4L-free formulation. In addition, **4L-N** was efficient to reduce human U138MG glioma cell viability. We also verified that **4L-N**

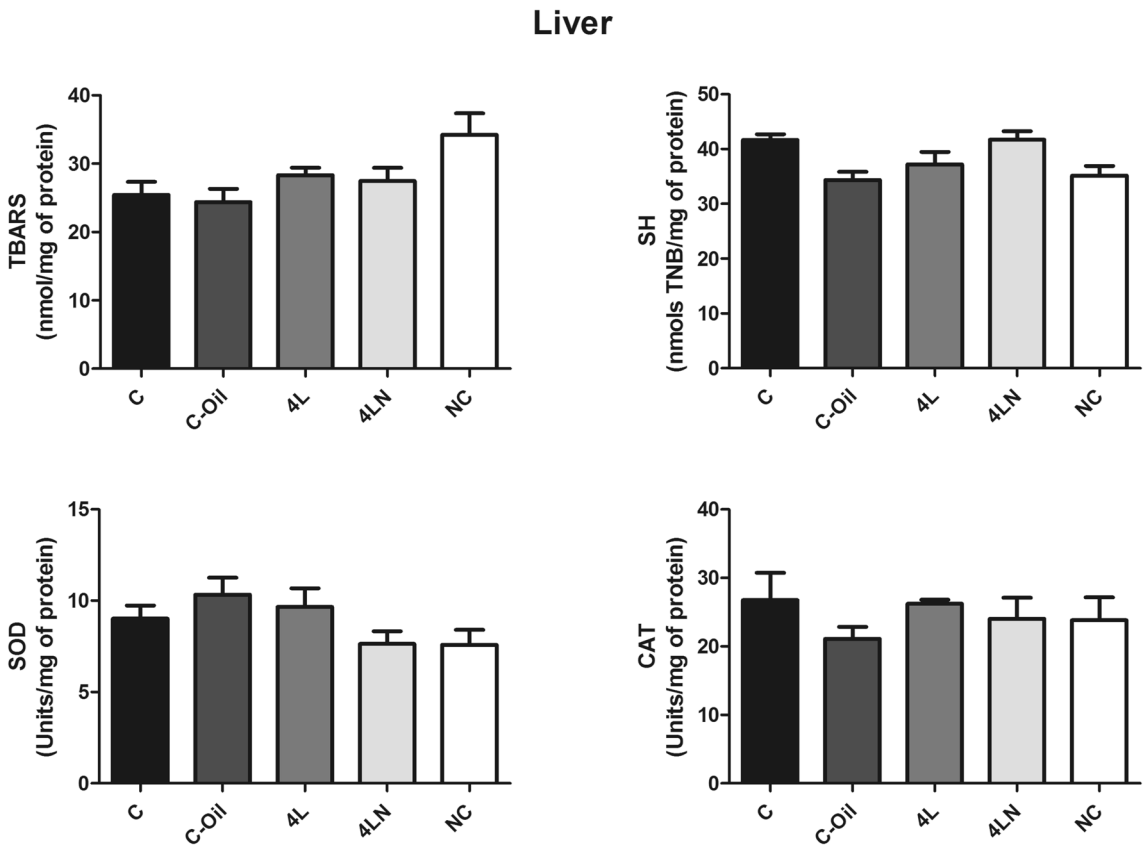


Fig. 9 Analysis of oxidative stress parameters TBARS (**a**), total sulfhydryl content (**b**) and activities of antioxidant enzymes superoxide dismutase (SOD) (**c**) and catalase (CAT) (**d**) in liver of rats 5 days after treatment with **4L**, **4L-N** and **NC** compounds. Values are expressed as mean ± SD ($n = 4$–6). TBARS levels were reported as nmol TBARS per mg protein, total sulfhydryl content as nmol TNB per mg protein and SOD and CAT are express as unit per mg pf protein. Data were analyzed by ANOVA followed by Tukey–Kramer test

induced C6 glioma morphological changes and incorporation of PI when compared to control. These results suggest that the antiproliferative effect of compound is enhanced by nanoencapsulation and its effect can be mediated by necrosis. However, the involvement of other cell death pathways can not be excluded since changes in cell viability and survival, as well as the occurrence of cell damage, is secondary to cell cycle dysregulation and development of apoptotic cell characteristics (Degterev et al. 2001). Recent studies show that the class of 5-ene-4-thiazolidinones have anticancer effects through reversible blockade of the cell cycle progression at the G2/M phase boundary leading to the induction of apoptosis (Senkiv et al. 2016). In addition, thiazolidinones groups still act as inhibitors of anti-apoptotic protein–protein interactions—occurring between Bcl-2 and Bax family (Lugovskoy et al. 2002), as selective inhibitors of extracellular signal-regulated kinases-1 and 2 (ERK1/2) (Jung et al. 2013) and still as inhibitors of CDK1 (Vassilev et al. 2006; Chen et al. 2007) and CDK1/cyclin B (Chen et al. 2007). Thiazolidinones have affinity for different molecular targets, however, apoptosis induced by 4-thiazolidinones and their derivatives has been demonstrated in different types of cancer cells (Chandrappa et al. 2009; Onen-Bayram et al. 2012; Li et al. 2007).

The research for new molecules with important antitumor activity and low toxicity for normal cells is strongly prioritized (Chumak et al. 2014). In previous studies, we have already shown that the thiazolidinone **4L** was not toxic to primary astrocytes (da Silveira et al. 2017). Here we demonstrate that this thiazolidinone in its nanoencapsulated form (**4L-N**) also shows no toxicity to normal cells, being selective to tumoral astrocytes. Current chemotherapy presents many options with little specificity and high toxicity and this represents a limitation to cancer treatment (Chari 2007). Thus, considering the therapeutic potential of the **4L** or **4L-N** thiazolidinone demonstrated in in vitro tests, investigation of the potential toxicity of these molecules becomes relevant. It is important to emphasize that in this study **4L-N** did not promote either mortality or systemic toxicity to the animals, with normal, ALT, AST, creatinine, and urea levels. In addition, animals treated with **4L-N** did not exhibit weight loss throughout the treatment. Similar data to these

have already shown that these polymer nanostructures did not promote systemic toxicity (Chassot et al. 2015). However, additional studies of pharmacokinetics are necessaries to evaluate whether 4L-N is better than 4L in in vivo drug delivery.

Oxidative stress reflects an imbalance between pro-oxidants and antioxidant compounds that can cause irreversible damage to biomolecules that lead to cell dysfunction (Gonsette 2008; Ljubisavljevic 2016). However, oxidative stress can be defined as homeostatic, non-specific defensive response of the organism to the challenges, in addition to contributing to pathogenesis of various diseases. Therefore, this is an important point to be investigated in the search for new therapeutic potentials. Recent studies with molecules derived from 4-thiazolidinones did not alter the level of ROS, whereas doxorubicin elevated the indices significantly; in addition, doxorubicin decreased the activity of antioxidant enzymes such as, SOD, GPO, and Cat in rats blood, whereas thiazolidinones had less proeminetes results. These data show that 4-thiazolidinone derivatives have a greater safety regarding alterations in the antioxidant defense system when compared to doxorubicin, a chemotherapeutic already used in the clinic (Kobylinska et al. 2016). Our results indicated that the thiazolidinone **4L** as well as **4L-N** do not alter the parameters of oxidative stress in liver and brain. As shown above, they did not alter markers of oxidative damage, such as TBARS levels, a marker for lipid peroxidation and total sulfhydryl content. In addition, no change in the activity of antioxidant enzymes such as SOD and CAT was observed in the liver and brain of rats. Taken together, these data suggest that exposure to the **4L** or **4L-N** thiazolidinone did not induce oxidative stress in rats.

Conclusion

In conclusion, our data show for the first time **4L** thiazolidinone-loaded nanocapsules as an alternative for the treatment of glioma. In addition, nanocapsule formulation (**4L-N**) has in vitro antitumor activity even more efficient than molecule in its free form (**4L**), it does not induce toxicity to normal astrocytes in culture or to Wistar rats, as indicated in in vivo experiments, which suggests a possible the safety of formulation. Data shown here support additional studies to test 2-(2-methoxyphenyl)-3-((piperidin-1-yl)ethyl)thiazolidin-4-one-loaded polymeric nanocapsules in a preclinical glioma model as a novel therapeutic modality for the treatment of brain tumors in the future.

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Authors Contribution EFS: Experimental design, culture preparation and treatment, discussion of results and manuscript preparation. LMF: Experimental design and preparation and characterization of nanocapsules. MG: Experimental design, preparation and characterization of nanocapsules and manuscript preparation. LC: Experimental design and preparation and characterization of nanocapsules. NSP: Experimental design, culture preparation and treatment, viability and cell proliferation assays. PTR: Culture preparation and treatment, viability and cell proliferation assays. NPB: Preparation of astrocyte cultures, treatment of cultures with nanocapsules, analysis of viability and cell proliferation data, treatment of animals in vivo and oxidative stress tests. MSPS: Preparation of astrocyte cultures, treatment of cultures with nanocapsules, analysis of viability and cell proliferation data, treatment of animals in vivo and oxidative stress tests. RR: Biochemical toxicity and data analysis. RMS: Experimental design, statistical analysis, interpretation and discussion of results and manuscript preparation. WC: Synthesis and design of thiazolidinones. FMS: Experimental design and interpretation and discussion of the oxidative stress results. JHA: Preparation of cell cultures, cell treatments, propidium iodide assay and analysis of the results. APH: Experimental design, statistical analysis, interpretation and discussion of results and manuscript preparation. EB: Experimental design, statistical analysis, interpretation and discussion of results and manuscript preparation.

Compliance with Ethical Standards

Conflict of interest All the authors declares that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures used in the present study followed the Principles of Laboratory Animal Care from NIH and the Brazilian laws and were approved by the Ethical Committee of the Federal University of Pelotas (Protocol Number 9219).

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