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In vitro and in vivo evaluation of nanoliposomes loading quercetin and 3-bromopyruvate against glioma

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Abstract

Background Diffuse astrocytoma (a type of glioma) and its prevalence are matters of concern worldwide. Patients with this type of tumour have a poor prognosis because after surgical treatment, radiotherapy and/or chemotherapy, these tumours eventually regrow or progress. To date, there is no effective treatment that can cure affected patients. Quercetin and 3-bromopyruvate are chemical compounds that have been proven to have antitumour effects alone or in combination with other compounds. Nevertheless, combination treatments including these agents are not used for treating diffuse astrocytoma.

Methods The use of nanoliposomes loaded with quercetin and 3-bromopyruvate as combination therapy was evaluated by treating C6 cells in vitro and in vivo (in Sprague–Dawley rat brain).

Results The 0.5 mg/mL quercetin + 0.75 mg/mL 3-bromopyruvate combination treatment decreased the expression of the biomarkers Annexin V and Caspase-3 and inhibited tumour growth; this was consistent with the in vivo results that revealed the administration of this treatment resulted in improved animal survival.

Conclusions The observations in the present study support the further exploration of this combination of active agents in the treatment of high-grade diffuse astrocytoma, especially in cases for which wide resection is possible.

Keywords High-grade diffuse glioma, Loaded liposomes, Apoptosis, Tumour volume

Background

The incidence rates of all primary malignant brain tumours range from 6.10 to 8.65 per 100,000 person-years; among these tumours, 80% are diffuse gliomas and 76% are high-grade astrocytomas and glioblastomas [1]. These tumours have become issues of concern worldwide since the population will increase to 2 billion people in the next 30 years [2]. The reason for the appearance of diffuse gliomas is unclear, but the process seems to be multifactorial. Factors that contribute to the development of diffuse gliomas include population ageing, overdiagnosis, ionizing radiation, air pollution, virus infection, etc. [3, 4]. The degree of malignancy depends on location, patient age, growth rate, infiltration of healthy tissues, and the presence of established and specific molecular

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markers [5]. Nevertheless, in most patients with grades 3 and 4 gliomas, aggressive evolution results in poor prognosis, and risk of mortality increases one year after diagnosis [6, 7]. Despite increasing technological advances to achieve more significant tumour surgical resection, effective treatment is lacking [5, 8, 9]. Consequently, the recurrence of these tumours is frequent [10, 11]. Therefore, searching for new treatments and using more effective drugs to target tumour progression or regrowth is a significant issue in neuro-oncology [12, 13].

In vitro and in vivo studies make it possible to evaluate the efficacy of new cancer treatments. The direct administration of chemical compounds and drugs to C6 cell cultures and the intrathecal or intraperitoneal administration of these agents to tumour cell transplantation rat models are both widely used to study gliomas [14]. These models have histopathological and molecular features that are similar to those of developing adult-type diffuse gliomas in humans [15]. The pharmaceutical preparations that have been proposed for treating high-grade gliomas include formulations with nanoparticles, particularly formulations with nanoliposomes (liposomes with a radius smaller than 100 nm), which have more attractive characteristics such as enhanced bioavailability of carried substances and increased efficacy due to the active substances and liposome components [16–18].

Moreover, nanoliposome formulations containing quercetin (Quer, a flavonoid with anti-inflammatory, antioxidant, and antineoplastic properties) have been shown to exert an antitumour effect against high-grade glioma [19, 20]. In recent reports, Ersoz et al. found that quercetin-loaded nanoparticles improve cytotoxic effects and antioxidant activity in C6 glioma cells [21]. Wang et al. also showed that PEGylated Quer-containing nanoparticles exert similar effects [22]. Zang et al. reviewed several Quer-containing formulations that are characterized by high encapsulation efficiency, stability, sustained release, prolonged circulation time, improved accumulation at tumour sites, and therapeutic efficiency. In addition, the authors suggested that combining quercetin with specific agents enhances the ability to detect or treat tumours [23].

One active compound that been used to treat glioblastoma is 3-bromopyruvate (3BP), which is a pyruvate-like alkylating compound that inhibits hexokinase II and glyceraldehyde-3-phosphate dehydrogenase [24]. 3BP exerts effects against several tumour cells, and its cytotoxicity is associated with the induction of autophagy; however, at the doses required for 3BP to be effective against glioblastoma cells, 3BP exerts toxic effects against healthy cells, which indicates a need to administer lower doses to limit systemic adverse effects [25]. In this sense, combinations of valproate, antimycin, menadione, and other

antineoplastic agents with low doses of 3BP have also been suggested as effective and safe combinations for targeting some neoplastic processes [26, 27].

Hence, in this work, a nanoliposome formulation loaded with Quer and 3BP was evaluated as a combination therapy for treating C6 glioblastoma cells in vitro and in vivo. The current observations support further exploration of this combination of active agents for the treatment of diffuse astrocytoma.

Methods

Cellular culturing and selection of cells

The rat cell-line C6 (ATCC, USA) was cultured in culture recipients of 75 cm² with D-MEM FK12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, ATCC, USA) media, supplemented with 20% horse foetal serum (HSF, ATCC, USA); 10% bovine foetal serum (BFS, ATCC, USA) and 2% antibiotic/antimycotic media (A.A., GIBCO, USA). The culture plates were kept in an incubator (Water Jacketed, Nuair, USA) at stable condition, 5% CO₂ at 35 °C for growth and propagation.

The sub-cell-cluster (Sub-C6) was obtained with serial dilutions 1:10 from the starting C6-cells. The last dilution was seeded in a 96 wells-plate/100 µL with supplemented D-MEM FK12 (20% HSF, 10% BSF, 10% A.A.). The wells with one cell were selected, then, growth to confluence. After that, sub-clusters were recovered with trypsin (Trypsin- EDTA 0.05%, cat 25,300,054, ThermoFisher) and spread into 6 well-plates and bottles of 75 cm³. The cluster with highest FOXM1 (K19 clone SC500, SantaCruz biotechnology, USA) and VEGF (C20 clone-SC152, SantaCruz biotechnology, USA) protein expression was selected.

Apoptosis-markers detection

Cells, each for treatment from C6 cluster and sub-clusters, were seeded in 24 well-plates with 5 × 10⁴ cells each well in supplemented D-MEM FK12 (20% HSF, 10% BSF, 10% A.A.) and treated with 5.7 ng, 17.1 ng y 34.2 ng of 3BP (376,817-M, Millipore) and/or 3.8 ng, 11.4 ng and 22.8 ng of Quer (Q4951, Sigma-Aldrich) during 3, 6, 12, 24, 48 and 72 h. Next, cells were washed with PBS and fixed by using paraformaldehyde 2%. Treated cells were incubated with the primary antibody against Annexin V (1:500, Santa Cruz Biotechnology, mouse sc-74438) by 48 h at 4 °C and Caspase 3 (1:500, Santa Cruz Biotechnology, mouse sc-56053), then with the secondary antibody Alexa 546 (anti-mouse 1:1000, cat A-11030, ThermoFisher) during 48 h at 4 °C, finally contrasted with Hoechst 33,342 (cat H3570, Invitrogen) by 20 min.

Samples from cultures were analysed with an inverted confocal microscope (Nikon Ti Eclipse with A1 through the NIS Elements v.4.5.0 software). Three photography

were acquired to different fields at 20× in each treatment. The quantification and analysis were determined by a binary mask (black and white) to discard noise or artefacts by using the Image-Fiji software (London SW7 2AZ, UK). Values considered as Caspase 3 activation and translocation by Annexin V were calculated as mean density by each cell.

Liposomal formulations preparation and characterization

10 mL of each unilamellar liposome preparation was made with a mixture of Quer and 3BP. Three different classes of liposomes were prepared: (1) control liposomes or empty liposomes, (2) low-dose liposomes (0.5 mg/mL Quer + 0.75 mg/mL 3BP) and (3) high-dose liposomes (0.75 mg/mL of Quer + 1.125 mg/mL of 3BP), based on the reverse phase evaporation method [28]. Briefly, for the formation of the lipid bilayer, 10 mg of cholesterol were dissolved in 2–3 mL of chloroform and added, together with 440 µL of phosphatidylcholine, in a conical flask with two necks (one with vacuum connection and the other with ability to place a removable filter) from 13 to 100 mm in diameter, and dried under vacuum so that the lipids remain uniformly distributed at the bottom of the flask.

Simultaneously the active ingredient was dissolved in milli-Q® water. As far as the Quer is concerned, it was dissolved in chloroform and a (1:1) proportional amount of water was added to them. Subsequently, the lipid bilayer was dissolved with 3 mL of diethyl ether and the previously dissolved active ingredient was added to this solution. The flask was then vortexed for 1 min and through immersion in a sonicator for an additional 1 min (5 s pulses). To carry out the control of liposomes, water was added instead of the active principle. Returning the flask to the vortex, a vacuum was applied for approximately 1 min, during which the ether was evaporated [29]. Maintaining erasure, 6 mL of saline solution containing 0.13% spermine was added. At this point, the preparation was evaluated by visualization (a cloudy suspension is considered adequate; the formation of lumps, inadequate). The liposome preparations obtained were filtered by extrusion, with Millipore Swinnex® membranes with 0.22 µm pore diameter (pressure 100–100 psi), to homogenize the size of the vesicles and to preserve them under sterile conditions. This suspension of liposomes was titrated with saline to a volume of 10 mL, pH 7.0.

Morphological characterization was done by means of atomic force microscopy, using a Nanos-Senterra (Bruker Optiks, Ettlingen, Germany) in the non-contact / tapping-mode; analysis was done on a plate with a maximum xy scan range of 40×40 µm and a z range of 8 µm. Cantilevers were standard microfabricated

(POINTPROBE-PLUS® Silicon-SPM-Sensor, Nanosensors® Wetzlar-Blankenfeld, Germany). The length of the AFM tip was 200 mm, and the resonance frequency was 165 kHz. The average height and roughness of the cell surface were analysed, and images were processed by using SPIP® software (Image Metrology, Hørsholm, Denmark) as previously [30].

Animal model

Animals

Forty-two male *Sprague–Dawley* rats, ageing 10 weeks, and weighting 210–255 g at the start of assays, were used. They were contained in acrylic boxes (50×40×40 cm), maintained under 12:12 h light/dark cycles, with Formu-Lab Diet #5008 food and water ad libitum.

This protocol was evaluated by the local committee for research and ethics in health research 3601 (Registering Number: R-2012–3601-106). The surgical procedures were done in the vivarium by using all the aseptic and antiseptic protocols and materials. The project followed the local laws for animal care and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (available at <https://arriveguidelines.org/arrive-guidelines>) for avoiding suffering to involved animals.

Stereotaxic approach was done in anaesthetized animals (90 mg/kg ketamine + 10 mg/kg xylazine). Once the head was fixed, the skull was exposed a trephine was done at anteroposterior 2 mm, lateral 2 mm and deep 2 mm from bregma [31]; at this site, 2.5×10^5 or 1×10^6 cells suspended in D-MEM FK12 medium (ATCC, USA) were injected and the skin was sutured. Then, a 5-day recovery period was permitted, being at the first three days treated with antibiotics (Gentamicin 80 mg + Benzathine penicillin 1,200,000 IU) and analgesic (Tramadol dose, 10 U).

Individual and combined treatments

The animals were divided into four groups (N=24, n=6 per group): Control) Treated with empty liposomes; Experimental 1) Treated with liposomes + Quer (1 mg/kg of Quer); Experimental 2) Treated with liposomes + 3BP (1.5 mg/kg of 3BP) and Experimental 3) Treated with liposomes + Quer + 3BP (1 mg/kg of Quer + 1.5 mg/kg of 3BP).

Low and high combined treatments

The animals were divided into three groups (N=18, n=6 per group): Control) Treated with saline solution; Experimental 1) Treated with low-dose liposomes (1 mg/kg of Quer + 1.5 mg/kg of 3BP) and Experimental 2) Treated with high-dose liposomes (1.5 mg/kg of Quer + 2.25 mg/kg of 3BP).

Administration of treatments and obtaining samples

The liposomal formulations were administered 3 times, at 72 h intervals, via i.p. being the first administration on the sixth day of the implant. Finally, the animals were sacrificed three weeks after implantation, due to an overdose of pentobarbital (100 mg/kg), to obtain and dissect the brains.

Samples processing

The brains were perfused and fixed in 4% paraformaldehyde for posterior paraffin embedding. After that, 5 μm slices were obtained every 250 microns for tumour identification. Briefly, the slices were mounted on slides covered with poly-L-lysine (10%, P4832, Sigma), the excess paraffin was removed (55°C, oven), and they were rehydrated (xylol, 100% alcohol, 96% and 70%, and water). Subsequently, the sections were stained with Harris haematoxylin for 5 min, rinsed with water, treated with lithium bicarbonate, and counterstained with eosin. Before mounting the slides with Entellan resin (107,960, Merck-Millipore), they were dehydrated (70%, alcohol, 96% and 100%, and xylol) [32].

The images were photographed with a NI5_Elements D (5.110064 bit, Nikon, Japan) microscope 40 \times and the calculation of the tumour areas were calculated with the Image Pro7 (Media Cybernetics, Rockville, MD 20852 USA) software. Tumour volumes were calculated with the formula:

$$v(\mu\text{m}^3) = \sum_{V_n}^{V_{n+1}} \left(\frac{1}{3}\right) * (250) * (A_n + A_{n+1} + \sqrt{A_n * A_{n+1}})$$

Subsequently, the values were converted to mm^3 and 75% of the volume of water was added.

Statistic analysis

The differences in the medians of Caspase 3 activation and translocation detected by Annexin V in C6-cells culture by exposure to Quer, 3BP or the combination were analysed using the nonparametric Kruskal–Wallis test. Differences in tumour size were analysed using the Mann–Whitney U nonparametric median comparison statistical test. All performed by using the Prima STAT v 12.0 software, a p -value < 0.05 was considered significant.

Results

Liposomal formulations were obtained as described for similar liposome formulations that contain nanoparticles (with radii ranging from 20 to 200 nm, Fig. 1). The Quer and 3BP encapsulation efficiency was not determined, but it was estimated to be $> 90\%$, as described in multiple previous reports of similar systems [28, 33, 34]. When

in solution, the rest of the described compounds were administered as original formulations and were used in our *in vivo* evaluations.

Effects of Quer and/or 3BP on C6 cell culture

Annexin V detection.

Decreased cell viability in the initial stage of apoptosis induction was reflected by Annexin V-stained Alexa 546-positive cells [35]. The administration of Quer to cell cultures induced significant differences in proportion of apoptotic cells, which differed with both Quer concentration and treatment time ($p < 0.0001$). The highest proportions of Annexin V-positive cells were observed at 12 and 72 h, and the lowest proportion of Annexin V-positive cells was observed at 6 h after treatment with all Quer concentrations (Fig. 2A).

The administration of 3BP to cell cultures induced significant differences in the proportion of Annexin 5-positive cells at all the concentrations that were tested and at all the time points that were studied ($p < 0.0001$). The highest proportion of Annexin V-positive cells was observed at 72 h, and the lowest proportions of Annexin V-positive cells were observed at 3, 6 and 24 h (Fig. 2B).

When both compounds were added to cell cultures, significant differences were observed in the proportions of Annexin V-positive cells at all the concentrations that were tested and at all the time intervals that were studied ($p < 0.0001$), except for 6 and 48 h. The highest proportions of Annexin V-positive cells were observed at 12 and 72 h, and the lowest proportion of Annexin V-positive cells was observed at 6 h (Fig. 2C).

Finally, when individual treatments were compared with the combination treatment, a higher proportion of Annexin V-positive cells was observed in the group that received the combination treatment.

Caspase 3 activation.

The apoptosis execution pathway was studied by measuring Caspase 3 activation in cultured cells as described elsewhere [36]. The administration of Quer to cell cultures induced significant differences in Caspase 3 activation, which differed with both Quer concentration and treatment time ($p < 0.0001$). In fact, the highest degree of Caspase 3 activation was observed at 12 h, followed by 48 and 72 h. In contrast, the lowest degree of Caspase 3 activation was observed at 6 h (Fig. 3A).

Additionally, 3BP administration to cell cultures induced significant differences in Caspase 3 activation, which differed with both Quer concentration and treatment time ($p < 0.0001$); these results similar to those after Quer treatment (Fig. 3B).

When both compounds were administered, significant differences were observed in the activation of Caspase 3,

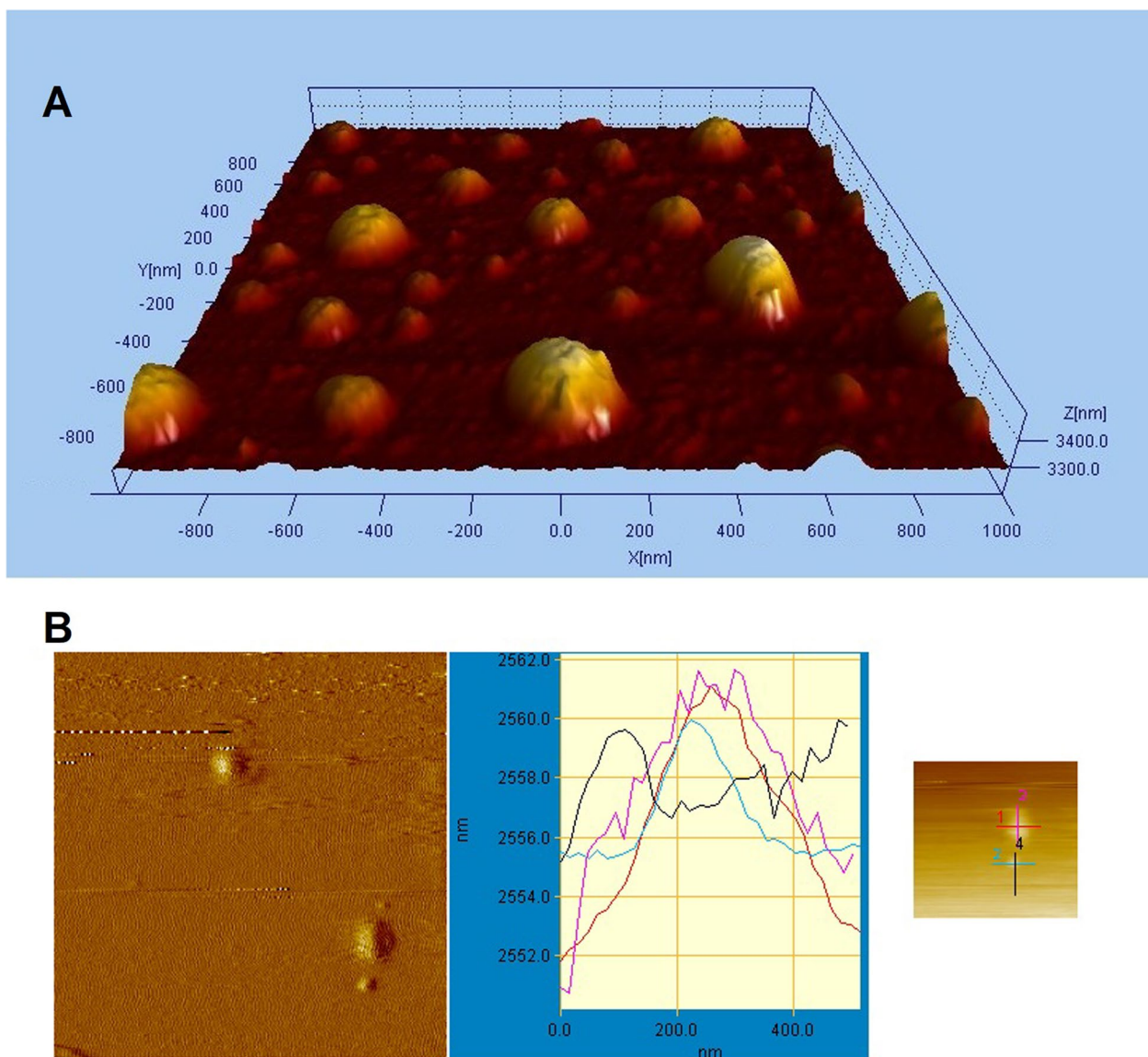


Fig. 1 Morphological approach of nanoliposome formulation containing Quer and 3BP. **A** Three-dimensional topographic image was obtained by atomic force microscopy and shows liposomal nanoparticles. **B** Topographic mode view on the left. Length values are presented in the centre and are marked in cross-sectional lines of amplitude view on the right

which differed with both concentration and treatment time ($p < 0.0001$), except for the 3-h timepoint. In this case, the highest degree of Caspase 3 activation was observed at 3 and 12 h, and the lowest degree of Caspase 3 activation was observed at 6 h and 12 h (Fig. 3C).

Finally, when the effects of the single treatments were compared with those of the combination treatment, the highest degree of Caspase 3 activation was observed after treatment with 3BP.

In vivo effects of liposomal formulations with combined treatment on tumour growth

A subclone of the C6 cell line was used, and the number of implanted cells that allowed the longest model survival was determined; this number of cells was used for subsequent experiments. For this experiment, intracranial tumour growth was measured in rats ($n = 20$) that received 1×10^5 cells ($n = 10$) or 2×10^5 cells ($n = 10$). A placebo treatment of empty liposomes ($n = 5$) or saline

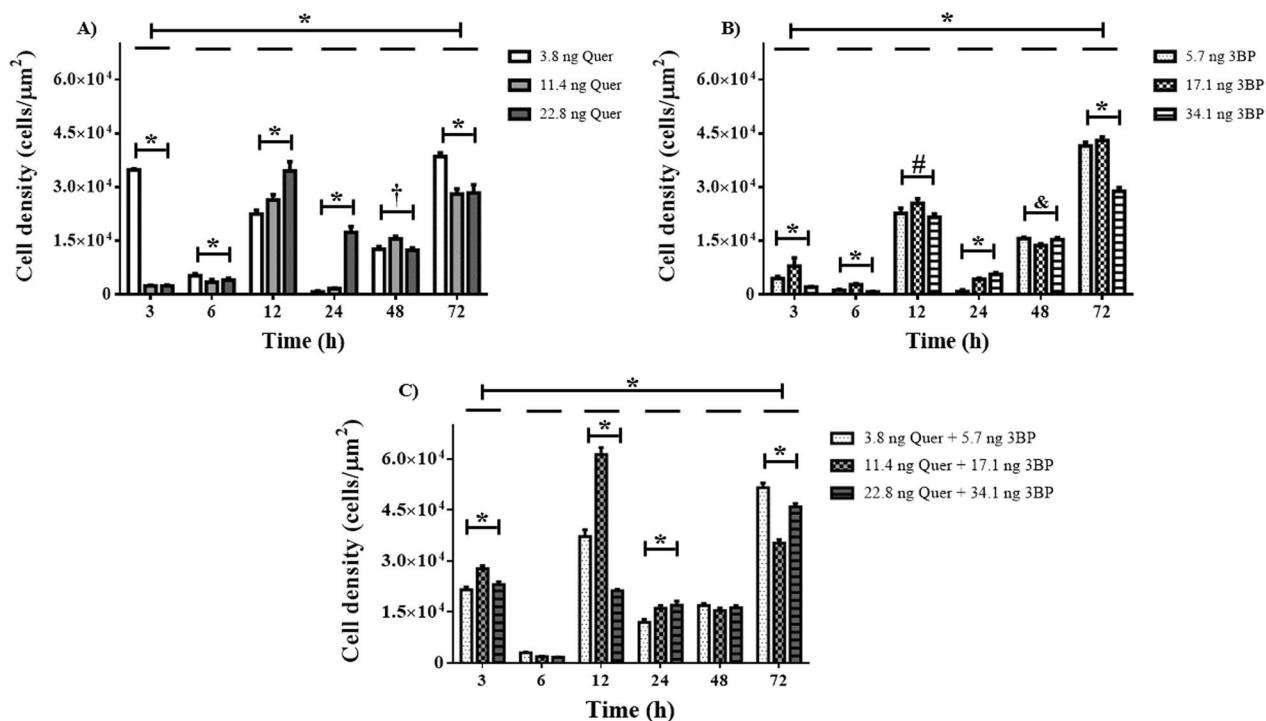


Fig. 2 Annexin V (as a marker of initial phase of cellular death involving cell membrane dysfunction) staining of C6-cells treated with Quer and/or 3BP. C6 cells were treated with different concentrations of Quer or 3BP, and then, the mean cell density was measured at 3, 6, 12, 24, 48 and 72 h. **A** Cells treated with 3.8, 11.4 or 22.8 ng Quer. **B** Cells treated with 5.7, 17.1 and 34.1 ng 3BP. **C** Cells treated with 3.8 ng Quer + 5.7 ng 3BP, 11.4 ng Quer + 17.1 ng 3BP and 22.8 ng Quer + 34.1 ng 3BP. Significant differences were determined by the Kruskal–Wallis test and are indicated by &p < 0.01, †p < 0.001, #p < 0.005 and *p < 0.0001

solution (n = 5) was administered, and the experiment was continued for three weeks. One hundred per cent of the animals in the 1×10^5 cell implant group survived at 21 days; however, only 80% of the animals in the 2×10^5 cell implant group survived at 21 days (data not shown).

Subsequently, the Quer and/or 3BP individual and combination treatments that had been tested in cell cultures and had elicited the best apoptotic pathway response (11.4 ng Quer, 17.1 ng 3BP and 11.4 ng Quer + 17.1 ng 3BP) were administered.

Figure 4 shows representative tumour growth in the section with the largest area that was identified in each case. A tumour region was observed mainly in the control groups, and similar regions were observed in the groups treated with either Quer or 3BP alone; in contrast, no areas of necrosis and less angiogenesis were observed in the combination treatment group (liposomes with Quer + 3BP), and only the inoculated cells, without obvious tumour formation, was observed.

Consequently, it was decided to test the combination treatment at a higher dose and to increase the cellular inoculum to further test the efficacy of the treatments.

After the administration of low and high doses of the liposomal formulations of the combination treatment, the animals were reactive, and obvious tumour formation was observed. Figure 5 shows representative tumour growth after the administration of low and high concentrations of liposomes. Smaller tumours were observed in the low-dose treatment group, but an apparent reversal of the therapeutic effect was observed in the high-dose treatment group.

Figure 6 shows a plot of the mean tumour volumes in the three groups of liposome-treated animals; there was a significant decrease between the control group and the group treated with low-dose combination treatment of Quer + 3BP ($p < 0.05$).

Discussion

The increasing incidence of glioblastoma and the regrowth of tumours after gross total resection followed by adjuvant treatment with temozolomide (gold standard in chemotherapy) and radiation therapy require the development of pharmacological tools for glioblastoma treatment [37, 38].

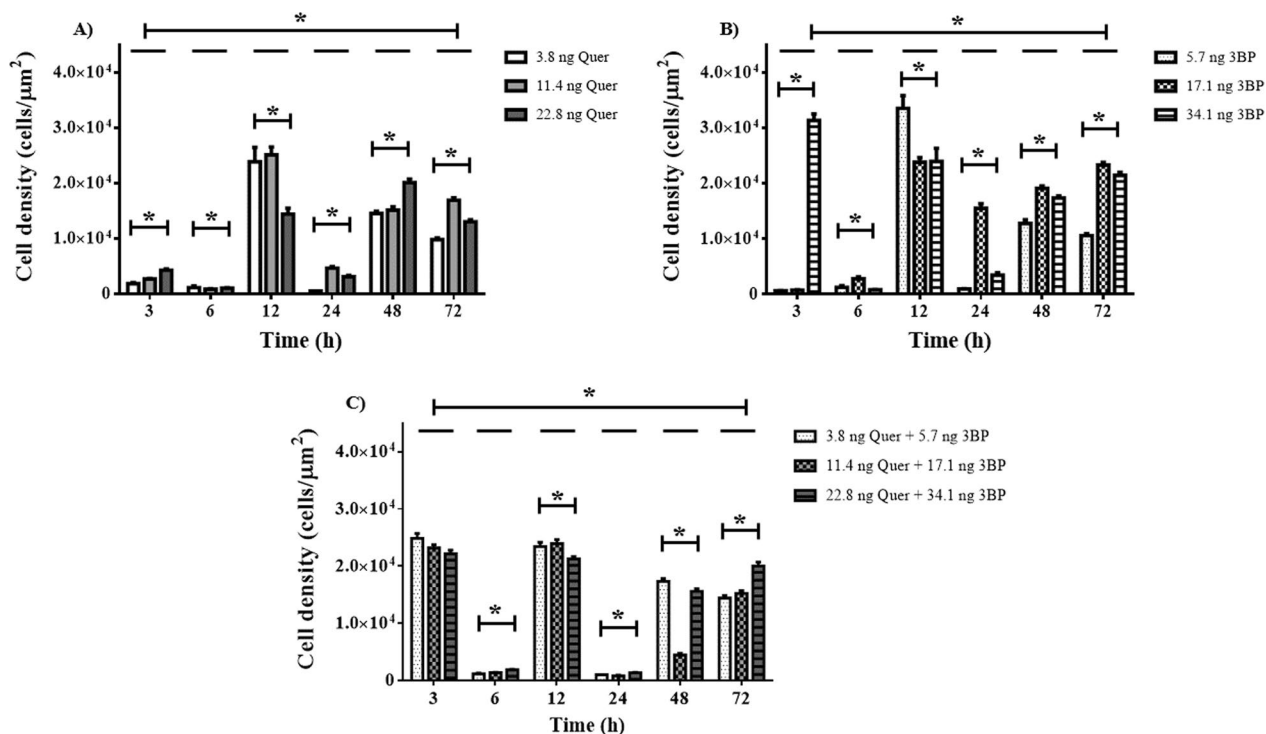


Fig. 3 Caspase 3 (marker of apoptosis pathway) expression in C6-cells treated with Quer and/or 3BP. C6 cells were treated with different concentrations of Quer or 3BP, and then, the mean cell density was measured at 3, 6, 12, 24, 48 and 72 h. **A** Cells treated with 3.8, 11.4 or 22.8 ng Quer. **B** Cells treated with 5.7, 17.1 and 34.1 ng 3BP. **C** Cells treated with 3.8 ng Quer + 5.7 ng 3BP, 11.4 ng Quer + 17.1 ng 3BP and 22.8 ng Quer + 34.1 ng 3BP. Significant differences were determined by the Kruskal–Wallis test and are indicated by an asterisk, * $p < 0.0001$

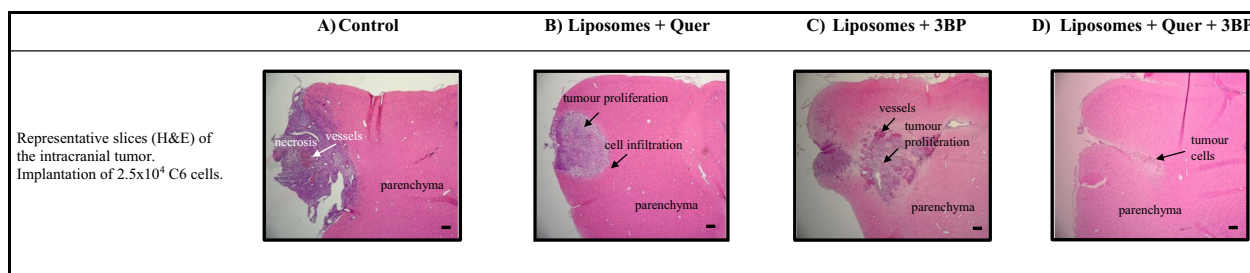


Fig. 4 Sections of the brain from rats implanted with 2.5×10^4 C6 cells and treated with liposomes containing Quer or 3BP were stained with H&E. **A** Treated with empty liposomes, **B** treated with liposomes loaded with 0.5 mg/mL Quer, **C** treated with liposomes loaded with 0.75 mg/mL 3BP and **D** treated with liposomes loaded with 0.5 mg/mL Quer + 0.75 mg/mL 3BP. These sections were obtained from rats 3 weeks after C6 cell implantation. The arrows indicate histopathological alterations due to the tumour. Scale bar 100 μm

The implantation of glial cells into rat brains has been used to model human glioma for more than four decades; effective models have been used to test different treatments that control the growth and development of these cells [39]. Auer et al. (1981) determined the number of cells necessary to obtain a reliable model by studying an implant concentration gradient; they determined that the implantation of 1×10^4 cells results in a 100% glioma formation rate [40]. In this work, we implanted 2.5 and

10 times more cells than the number originally recommended by Auer et al. because preliminary experiments in our laboratory (not shown) revealed no tumour development after implantation of the original cell number.

The aim of this study was to test a liposomal formulation of Quer and 3BP combination treatment. These two components have been separately tested as neuro-protectors and glioblastoma cell regulators, although not in a combined formulation, and they have been

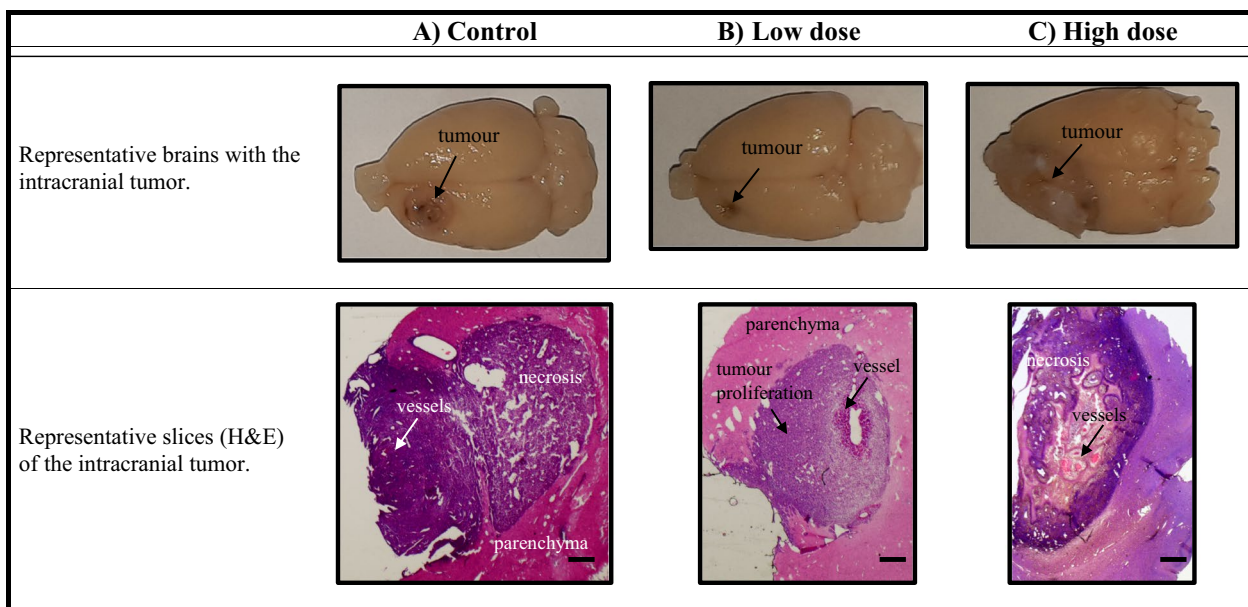


Fig. 5 Comparison of brain sections with free development of implanted C6 cells and brain sections from rats implanted with 1×10^5 C6 cells and treated with liposomes containing Quer or 3BP. Above are the entire brains, below are brain sections. All sections were stained with Haematoxylin & Eosin. **A** Treated with saline solution, **B** treated with liposomes loaded with 0.5 mg/mL Quer + 0.75 mg/mL 3BP and **C** treated with liposomes loaded with 0.75 mg/mL Quer + 1.125 mg/mL 3BP. These sections were obtained from rats 3 weeks after C6 cell implantation. The arrows indicate histopathological alterations due to the tumour. Scale bar 100 μ m

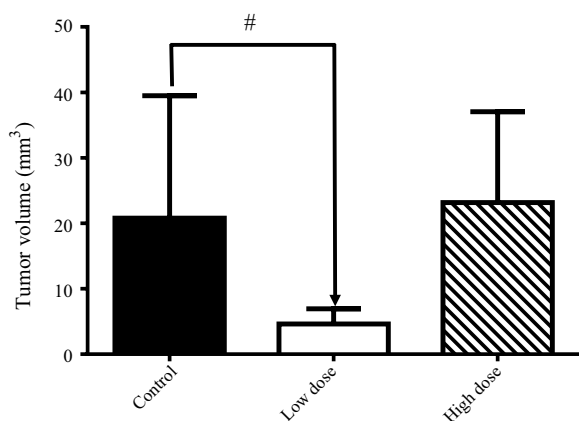


Fig. 6 Tumour volumes and effect of liposomal formulation treatment. Tumour volume from rats 3 weeks after implantation of 1×10^5 C6-cells treated with **A** empty liposomes, **B** liposomes loaded with 0.5 mg/mL Quer + 0.75 mg/mL 3BP and **C** liposomes loaded with 0.75 mg/mL Quer + 1.125 mg/mL 3BP. Significant differences were determined by the Kruskal–Wallis test and are indicated by # $p < 0.05$

demonstrated to exert effects in animal glioma models [21, 26, 41–44]. Notably, the material in the liposomal formulation used to improve drug availability to neoplastic cells is a third component of this formulation. In this work, the methodology used in the formulation of liposomes assumes a high encapsulation efficiency as

has been reported [28]. Originally, the encapsulation efficiency was reported > 65%, but currently is near 90% ($84.7 \pm 5\%$) [45].

Quercetin is insoluble in water due to its lipophilic property, so it has poor absorption, low bioavailability, and a limited ability to cross the BBB, therefore could not be used for the treatment of gliomas. The use of this liposome formulation increased the solubility of quercetin and guaranteed the probability of acting in the brain as well as limiting peripheral effects and reduction of drug-related toxicity [20, 33, 34, 46, 47]. In fact, several concentrations were evaluated for treating rats acting as a murine model of grade 4 astrocytoma, with the purpose of evaluating the possible therapeutic effects of adjuvant treatment after wide (implantation of 2.5×10^4 cells) or partial resection (implantation of 1.0×10^5 cells).

Anticancer properties of Quer are a consequence of different mechanisms that favour the progression of cancer cells. The antioxidant property is reflected in being an effective reactive oxygen species (ROS) scavenger and inhibiting lipid peroxidation; also, regulating signal transduction pathways, such as NrfB, MAPK and AMPK, as demonstrated by in vitro studies [48–50]. The anti-inflammatory properties are related to the inhibition of pro-inflammatory cytokines (TLR4 pathway) and a decrease in the production of cyclooxygenase (COX) and lipoxygenase (LOX) [51, 52]. Cell cycle progression

of different cancer cells is affected by the arrest of the G0/G1 and G2/M phases, because of the inhibition of cyclins, release of p53 and caspase activation [53–55]. The synergistic effect of Quer with different chemotherapeutic agents and with radiotherapy has been reported; these studies are complemented by the cytotoxicity of Quer in glioma cells when the late stage of autophagy is inhibited [56, 57].

The suggested mechanisms of 3BP action include decreasing ATP by disrupting the function of several cysteine-rich proteins [58, 59]. Additionally, 3BP-mediated inhibition of hexokinase II, which is involved in the survival of glioblastoma cells, has been demonstrated, probably providing additional sources of ATP in neoplastic cells [60, 61]. In addition, several reports showed increased intracellular ROS production in diverse malignancies after 3BP exposure as well as the specific pyruvilation of glyceraldehyde 3-phosphate dehydrogenase, which is a major intracellular biochemical mechanism, resulting in the metabolic disruption of cells and inducing apoptosis [62–64].

Thus, considering that tumour growth and progression are favoured by acidic microenvironments and the reactive oxygen and nitrogen species that are produced during anaerobic glycolysis (Warburg effect), in which hexokinase II (HK II) degrades glucose into pyruvate, producing two molecules of ATP and various glycolytic intermediates that are fed into multiple biosynthetic pathways, it is suggested that the combined effect of this formulation is additive, as differences were observed with formulations that included only one of these compounds. 3BP can also act directly because pyruvate is converted into lactate in the cytoplasm by the enzyme lactate dehydrogenase (LDH). In contrast, normal astrocytes use the combination of acute aerobic glycolysis (Crabtree effect) and slow aerobic glycolysis, favouring high glucose contents and ATP generation without affecting the integrity of the mitochondrial membrane and maintaining a balance between glycolysis and respiration [65, 66].

Annexin V, which is specifically related to the processes that are associated with the initial phases of cell death, is considered a marker of membrane dysfunction [35]. Quer administration modulated the changes in the cell cycle that are associated with the initial phase of apoptosis, as shown by the increased proportion of Annexin V-positive cells described in prostate, colorectal and other neoplastic cells [67–69]. 3BP administration also induced high expression of Annexin V or high proportions of Annexin V-positive cancer cells, such as was observed in melanoma and lung neoplastic cells [70, 71].

Complementarily, in this study, caspases (cysteine proteases) are considered key proteins in apoptotic processes, specifically in the execution phase. Caspase 3

expression and activation have been shown to be modulated by Quer administration in different cells [72–74]. Similarly, 3BP induced an increase in the Caspase 3 staining intensity in potentially different types of neoplastic (liver, lung, colorectal) cells, including glioma cells [64, 71, 75].

Regarding the formulation used (simple bilayer liposomes with charges neutralized by spermidine addition), it should be noted that the main advantages of the use of liposomes is efficient transport to the target site and evasion of natural barriers in the organism; however, other functional properties have been described in the treatment of gliomas; among these the facilitation of drug transport across the blood–brain barrier, the improvement of cellular uptake and the reduction of P-glycoprotein (P-gp) excretion of drugs, reversing of multidrug resistance, regulation of autophagocytosis and induction of apoptosis [34].

Additionally, formulations could include active protein members of the ATP-binding cassette (ABC) transporter superfamily. In the brain, these proteins are found in the blood–brain barrier, the blood-cerebrospinal fluid barrier, and the blood-arachnoid barrier, while in tumour cells, the overexpression of these ABC transporters is associated with drug resistance and regulated by metabolites that are generated during aerobic glycolysis; thus, 3BP could act indirectly by inhibiting the expression of these transporters [76–78]. On the other hand, there are reports that Quer favours the overexpression of P-gp, which is considered a substrate of these transporters [79, 80].

It should be noted that *in vitro* (Annexin V and Caspase 3 expression) and *in vivo* (limitation of growth) results are congruent and in line with the involvement of the mechanism(s) of action linked to regulation of death cells, mainly by apoptosis. However, other mechanisms such as metabolism disorders, avoiding of drugs expulsion of neoplastic cells and limitation of vascular development could be key in the observed effect. In this sense, an interesting finding of our *in vivo* experiments is that the combination treatment strategy seems to inhibit vascular formation. An antiangiogenic effect of Quer (at different doses and in different formulations) has been reported in multiple *in vitro* and *in vivo* studies, including those using liposomes as carriers [41, 81–85]. In contrast, scarce data suggest an effect of 3BP on vascularization in tumours; truly, its effects are linked to metabolism disruption, as briefly described above. In this work, the inhibition of neovascularization by the combination treatment could be an effect of both disrupted metabolism (particularly aerobic glycolysis) and direct inhibition of the production and actions of stimulating factors, such as VEGF (Vascular endothelial growth factor) [86, 87].

Further studies are required to support or refute these hypotheses. The determination of efficient doses of each component, the exploration of additional nanoliposome formulations as well as the comparison of effects from diverse administration pathways are desirable for testing this formulation in human cells, since this would increase the potential outcomes of this study and the possible clinical application. Also, an intentioned toxicity evaluation in higher or prolonged administration (studies of posology) compared with those used in this study should be done.

Conclusions

The tested combination liposomal formulation (Quer+3BP) inhibited the expression of the biomarkers caspase-3 and Annexin V and tumour growth, but in vivo, its administration resulted in higher animal survival and lower volumes of developed tumours than in control groups. These observations support the further exploration of these active agents in the treatment of high-grade diffuse astrocytoma. Specifically, this exploratory study suggests that postsurgical treatment with liposomes loaded with Quer 1 mg/kg+3BP 1.5 mg/kg allows glioma inhibition in cases where wide resection is possible or slows tumour growth when partial resection is possible. Further studies are required to support these findings.

Abbreviations

AA	Antibiotic/antimycotic solution
BFS	Bovine foetal serum
D-MEM FK12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
FOXM1	Forkhead Box M1
H&E	Haematoxylin–eosin
P-gp	P-glycoprotein
Quer	Quercetin
3BP	3-Bromopyruvate
Sub-C6	Sub-cell-cluster from rat cell-line C6
VEGF	Vascular endothelial growth factor

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Author contributions

The author's contributions are follows: Conceptualization was done by MASU and IAFR; methodology was done by AVG, VBA, ALON and JMM; formal analysis was done by MASU and IAFR; investigation was done by MASU and IAFR; resources were done by IAFR; writing—original draft preparation was done by MASU and IAFR; supervision was done by IAFR; funding acquisition was done by IAFR. All authors have read and agreed to the published version of the manuscript.

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Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The local ethics committee evaluated this protocol (Registering Number: R-2012-3601-106). The project followed the local laws for animal care NOM-062-ZOO-1999 and the ARRIVE guidelines for avoiding suffering to involved animals.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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