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Quercetin promotes glioma growth in a rat model

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ABSTRACT

We have previously demonstrated that quercetin (Quer), a polyphenol widely found in vegetables, decreased glioma cell growth *in vitro*. Here, we asked whether this compound could affect glioma growth in an *in vivo* rat glioma model. We found that daily intraperitoneal Quer (50 mg/kg) injections lead to a concentration of 0.15 μg of Quer per gram of brain tissue, which increased the tumor volume in a time dependent manner. We observed a small reduction in lymphocytic infiltration, a marker of good prognosis in gliomas that was accompanied by a small reduction in cell viability of peripheral T-cells. Moreover, after Quer treatment neither body weight alteration nor liver pathology markers were detected. Although *in vitro* studies and massive literature reports point to the antitumoral properties of Quer, the present results indicate that great caution has to be taken in the design of clinical trials and the indiscriminate use of this polyphenol as dietary supplement.

1. Introduction

Gliomas are the most common primary brain tumors, with a worldwide annual incidence of around 20 cases per 100,000 individuals (Furnari et al., 2007). The most frequent and most malignant glioma is glioblastoma (GBM) that occurs typically in older adults. Grossly, GBMs are heterogeneous intraparenchymal masses that show evidence of necrosis and hemorrhage. Microscopically, they consist of several cell types: the glioma cells proper, hyperproliferative endothelial cells, macrophages and trapped cells of the normal brain structures that are overrun by the invading glioma (Kleihues and Cavenee, 2000; Russel and Rubenstein, 1989). Peritumoral brain tissue shows various types of inflammatory responses, including activated macrophages and microglia, hypertrophic reactive astrocytes, vascular invasion and edema formation (Schiffer, 1997). Despite the multimodal therapy approach, that basically consist in surgery followed by radio and/or chemotherapy, the prognosis remains very poor, with the majority of patients succumbing to the disease within a year (Sathornsumetee et al., 2007).

Quercetin (3,3′,4′,5,7-pentahydroxylflavone) (Quer) is a typical flavonoid present in the plant kingdom as a secondary metabolite found in red onions, grapes, apples, berries, cherries, broccoli, citrus fruits, tea (*Camellia sinensis*) and, at particularly high concentrations (180 mg per 100 g), in capers and lovages (Bischoff, 2008; Hertog et al., 1993). Abroad spectrum of beneficial properties have been described for Quer, including anti-inflammatory, anti-oxidant, anti-carcinogenic, cardioprotective, neuroprotective effects, among others (reviews available on these fields include: Bischoff, 2008; Boots et al., 2008; Dajas, 2012; Russo et al., 2012).

The anti-carcinogenic properties of Quer have been widely studied (Middleton et al., 2000) and include inhibition of the growth of cells derived from human cancers such as those of stomach (Yoshida et al., 1990), colon (Hosokawa et al., 1990; Pawlikowska-Pawlega et al., 2001), prostate (Kampa et al., 2000), breast (Damianaki et al., 2000), uterine cervical cancer (Vidya Priyadarsini et al., 2010), melanomas (Rosner et al., 2006), brain (Braganhol et al., 2006) and intestinal tumors in *in vivo* model of mice (Mahmoud et al., 2000). At the molecular level, Quer is suggested to acts as anticancer agent by down-regulating the expression of oncogenes (H-ras, c-myc and K-ras) (Ranelletti

Abbreviations: Quer, quercetin; GBM, glioblastoma; DMEM, Dulbecco's modified Eagle's medium; C, control; γ-GT, γ-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PHA, phytohaemagglutinin.

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et al., 2000) and proliferative pathways such as PI3k/PKB pathway (Gulati et al., 2006); or up-regulating tumor suppressor genes p53 (Chan et al., 2013) and cell cycle control proteins ($p21^{WAF1}$ and $p27^{KIP1}$) (Casagrande and Darbon, 2001). Furthermore, Quer emerges as a potential drug to reduce multidrug cancer resistance, acting as a chemosensitizer compound (Chen et al., 2010), as well as modulating the activity of MEK/ERK, Nrf2/keap1 (reviewed by Murakami et al., 2008) and activating Sirt1 pathways (reviewed by Chung et al., 2010). In addition, in primary cultures of mouse cortical neurons, Quer inhibits different tyrosine and serine–threonine kinases, whose activities are linked to survival pathways such as MAPK and AKT/PKB (Spencer et al., 2003).

We have previously demonstrated that Quer decreased cell growth in glioma cell lines by inducing apoptosis and senescence (Zamin et al., 2009). In this work we asked whether this drug could inhibit and/or decrease tumor growth in an *in vivo* rat model of glioma extensively used to test antitumoral interventions (Bernardi et al., 2009; Chekhonin et al., 2007; Takano et al., 2001).

2. Material and methods

2.1. Cell culture

All culture materials were obtained from GIBCO Laboratories (Grand Island, NY). C6 rat glioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, pH 7.4) plus 0.1 % amphoterecin B, 1% penicillin/streptomycin and 5 % fetal bovine serum. Cells were kept at 37 °C, humidity of 95 %, and an atmosphere of 5 % CO_2.

2.2. Glioma implantation

Rats (male *Wistar*, 8-weeks old) anesthetized with 90 mg/kg ketamine plus 12 mg/kg xylazine (Agener, PousoAlegre, MG, Brazil) i.p. (intraperitoneal) were placed in a stereotactic frame and 5×10^5 C6 cells suspended in 3 μL DMEM medium (pH 7.4) were injected at a depth of 6 mm in the right *striatum* with a Hamilton syringe (Bernardi et al., 2009; Braganhol et al., 2009; Li et al., 2012; Morrone et al., 2006; Takano et al., 2001;Wilson et al., 2012). Injection coordinates with regard to bregma were 0.5 mm posterior and 3.0 mm lateral. Animals from in-house breeding colonies at the Departamento de Biofísica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil were maintained under standard conditions (12-h light/dark, 22 ± 2 °C) with food and water *ad libitum*. All procedures used in the present study followed the "Principles of Laboratory Animal Care" of the National Institutes of Health and were approved by the local Ethical Committee (protocol number 2008229).

2.3. Treatments

Quer suspension was prepared in 0.2 % Tween-80 saline solution (10 mg/mL) followed by sonication (Moreira et al., 2004). Five days after glioma implantation, the rats were divided in the following groups: control group (C) which received the vehicle where drug was dissolved (0.2 % Tween-80 saline solution) i.p. and Quer group which received the drug i.p. daily at 50 mg/kg (Sigma Chemical, St. Louis, MO). The concentration of Quer was selected based on published tested dose for cancer prevention and those used in prior animals studies (Cho et al., 2006; Indap et al., 2006; Joshi et al., 2005; Molina et al., 2003; Moreira et al., 2004; Suzuki et al., 1998).

Ten or fifteen days after treatment, rats were sacrificed by decapitation and the isolated brain was immersed in 10% paraformaldehyde in phosphate saline. After fixation for 24 h, the brains were sectioned into 4 μm and stained with Hematoxylin/Eosin (HE). Blood samples were collected for posterior enzymatic assays (γ-glutamyltransferase – γ–GT-, alanine aminotransferase – ALT-, aspartate aminotransferase – AST-) (Labtest, Lagoa Santa, MG, Brazil).

The liver was also removed, sectioned and fixed with 10 % paraformaldehyde for pathological analysis. For body weight analysis, Quer group was compared to the control group. The spleen was removed and placed in DMEM medium for posterior T-cell proliferation test.

2.4. Pathological analysis and tumor volume quantification

At least five HE sections from each animal were analyzed by a pathologist, blinded for the experimental treatment regimen, for mitotic index, coagulative necrosis and lymphocytic infiltration determination. For tumor size quantification, the slices were imaged using a digital camera and tumor volume was calculated using Image J software (NIH Image, Rockville, MD).

2.5. Quantification of Quer in cerebral tissues

After 15 days of Quer treatment, the brain was removed and minced with scissors and placed in a homogenizer vessel; acetonitrile was added (5 mL) and tissues were subsequently homogenized. The homogenized samples were transferred to 50 mL conical glass tubes and vortexed for 5 min prior to centrifugation at 2800*g* for 30 min at 4 °C. The supernatant was placed into a clean tube, filtered (Millipore 0.45 μm) and placed in a sealed amber vial for HPLC analysis. The injection volume used was 20 μL for all samples. The quantity of Quer was calculated by comparing the peak area ratio from tissue samples of treated animals with those of the corresponding concentration standards of Quer in acetonitrile injected directly into the HPLC system (Frozza et al., 2010).

2.6. Colony formation assay

C6 cells were seeded in a density of 10^3 cells in 24-wells plate and treated with Quer. Each well received 500 μL of medium (DMEM plus 0.1 % amphoterecin B, 1 % penicillin/streptomycin and 5% fetal bovine serum) plus Quer in a final concentration of 5, 10, 25, 50 and 100 μM. During 10 days, the medium and treatment was changed every day to better simulate the *in vivo* rat treatment. After 10 days of treatment, cells were trypsinized, counted, and plated at a concentration of 100 cells/well into six-well plates, one well for each treatment. These cells were maintained only in DMEM plus 0.1 % amphoterecin B, 1 % penicillin/streptomycin and 5 % fetal bovine serum for 7 days. After 7 days without treatments, colonies were stained with crystal violet 0.5 % and manual quantification was performed to define the numbers of colonies.

2.7. Cell proliferation/viability assay

T-cell proliferative responses were determined by a modified colorimetric assay (Mosmann, 1983) as described (Motta et al., 2007). A single cell suspension of splenocytes from rats was obtained and cells were incubated at 8×10^5 cells/mL with 1 % phytohaemagglutinin (PHA) (Sigma Chemical). In the last 4 days of culture, 100 μL of the supernatant was gently discarded and 30 μL freshly prepared MTT [3-(4,5-diamethyl 2-thiazolyl) 2,5 diphenyl-2H-tetrazolium] (Sigma) solution (5 mg/mL in RPMI-1640) was added to each well. The cell cultures were incubated for 4 h at 37 °C in a 5 % CO_2 atmosphere. After complete removal of the supernatant, 100 μL of dimethyl sulphoxide (Sigma) was added to each well. The optical density was determined using a Biorad ELISA plate reader at wavelengths of 570 and 630 nm.

2.8. Statistical analysis

Data are expressed as mean ± S.E.M (standard error of the mean) and analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by post-hoc for multiple comparisons (Tukey's test) using Biostat 5.0 software (Belem, PA, Brazil). Differences between mean values were considered significant when $p < 0.05$. For the pathological parameters, the Chi-Square test was used.

3. Results

In order to investigate whether the *in vitro* glioma cell death induction by Quer (Zamin et al., 2009) could also occur *in vivo* we implanted C6 glioma cells in the *striatum* of adult *Wistar* rats. After 5 days of glioma implantation treatment was started with daily i.p. injections of Quer (50 mg/kg). There was no reduction in the tumor volume after 10 days of treatment with Quer (Fig. 1a and c).

Gliomas from the untreated group presented a high mitotic index, nuclear pleomorphism, *foci* of tumor necrosis and lymphocytic infiltration, which are characteristics of GBMs in humans (Fig. 1b) (Chekhonin et al., 2007). Although no statistical significant difference was observed among groups in histopathological analysis, a slight reduction in coagulative necrosis and mitotic index in Quer treated group was observed (Table 1).

These findings led us to hypothesize that the time of treatment was insufficient to observe the effects of Quer on tumor size (Zamin et al., 2009), therefore we treated the animals for 15 days. However, this new approach only increased the growth-inducing effect produced by Quer on tumors (Fig. 1a and c), highly increasing its volume when compared to vehicle treated animals.

To investigate if the absence of growth reducing effect of Quer on tumor volume was due to the concentration that reached the

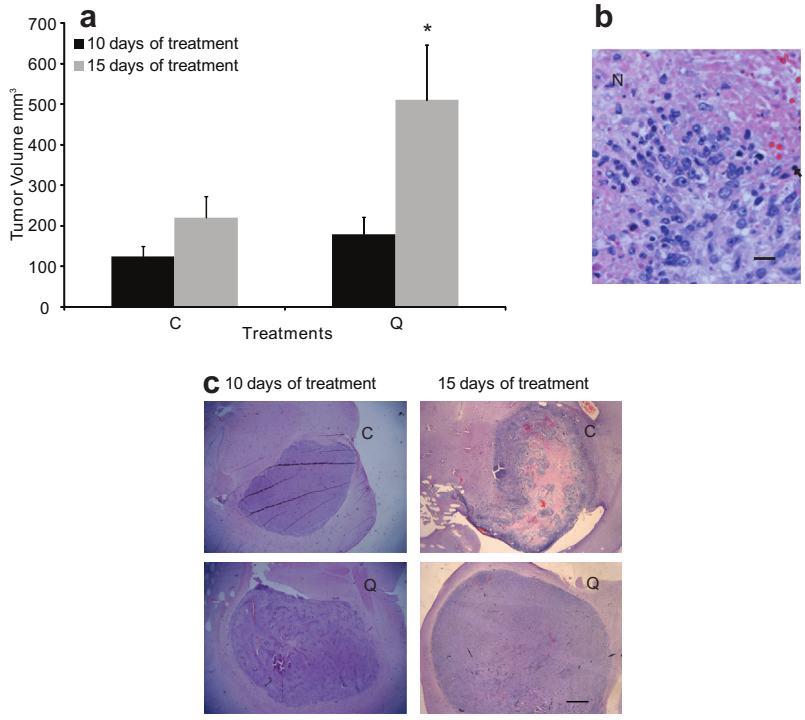

Fig. 1. Effect of Quer on tumor volume of implanted glioma. (a) Tumor volume was measured 15 days after implantation of C6 cells, corresponding to 10 days of Quer treatment (black bar) or 20 days after implantation of C6 cells, corresponding to 15 days of Quer treatment (grey bar). The values are presented as means ± S.E.M of 8 animals per group. Data were analyzed by ANOVA followed by Tukey's post-test. *Significantly different from control with $p < 0.05$. Scale bars 1 mm. (b) Histological analysis of implanted gliomas. The sections of implanted rat glioma were stained with HE, as described in Section 2. Representative pictures of histological characteristics that define GBMs, as seen in rats implanted with gliomas (control group). Necrosis (N), and lymphocytic infiltration (arrow) were observed. Scale bars 10 μm. (c) Representative tumor images stained with HE are shown.

Table 1
Histological characteristics of implanted gliomas. The histological variables (coagulative necrosis and lymphocytic infiltration) were regarded as present or absent. Mitosis was counted in ten high power fields (HPF) of the tumor, and the average of this counting was used as mitotic index (means ± S.E.M.). Each variable in the left column indicates the characteristics after 15 days of glioma implantation (10 days of Quer treatment) and the right column represent the characteristics after 20 days of glioma implantation (15 days of Quer treatment)

Treatments	Lymphocytic	Infiltration	Coagulative	Necrosis	Mitotic index (mitosis/HPF)	
C	5/10 (50%)	6/7 (85%)	6/10 (60%)	7/7 (100%)	15.36 ± 2.29	9.97 ± 3.21
Q	4/8 (50%)	2/3 (66%)	4/8 (50%)	3/3 (100%)	11.83 ± 0.57	6.93 ± 2.33

brain we measured the Quer concentration on brain after Quer treatment. We found a concentration of 0.16 ± 0.06 (mean ± SEM) μg of Quer per gram of tissue. Considering the density of the brain tissue as 1 g/mL, the Quer concentration on brain was 530 nM, which is lower than the concentration used in the previous *in vitro* work (Zamin et al., 2009). For this reason we performed another *in vitro* analysis using lower Quer concentration. The results showed in Fig. 3 indicate that concentrations below 10 μM *in vitro* had no effect in decreasing cell colony formation.

Since we found a slight decrease in lymphocytic infiltration in tumor area after Quer treatment we wondered whether this effect was mediated by decreasing systemic lymphocytic proliferation or just the tumor lymphocytic recruitment. Therefore, we analyzed the T-cell proliferation stimulated with PHA. We found a slight reduction in T-cell proliferation (Fig. 2a).

Finally, we investigated whether this drug was inducing hepatotoxicity by measuring the hepatic enzymes γ-GT, ALT and AST in the rat blood serum. None of the treated animals presented significant alterations in the investigated enzymes, discarding hepatic alterations (Fig. 2b–d). Microscopic investigation of the liver by HE analysis demonstrated absence of signs of toxicity (data not shown). We did not find any difference in the body weight of rats with implanted gliomas (Fig. 2e and f).

4. Discussion

We have previously demonstrated that Quer treatment caused cytotoxic effects on glioma cell lines (Zamin et al., 2009). It is well known that *in vitro* assays for growth and invasion only represent isolated aspects of the multiples process of the *in vivo* tumor growth (Kaczarek et al., 1999). For this reason, in the present work, we examined the effect of Quer treatment using an *in vivo* glioma model that, despite its limitations, is useful for the study of growth, angiogenesis, immunology and invasion of gliomas (Chekhonin et al., 2007). These advantages come mainly from the fact that this model is established in the brain of immunocompetent rats and presents several features observed in human GBMs. The main disadvantage of this model is that it uses only one rat tumor type, which does not fully represent the complexity and variability of human gliomas.

Numerous reports on the chemopreventive and anti-genotoxic effects of Quer have been published (reviewed in Murakami et al., 2008). There are, however, studies that show an increased risk of tumors development with Quer administration, despite not being mutagenic or teratogenic (reviewed by Verschoyle et al., 2007). In a study conducted by Pamukcu et al. (1980), albino Norwegian rats were fed a diet supplemented with 0.1 % Quer for

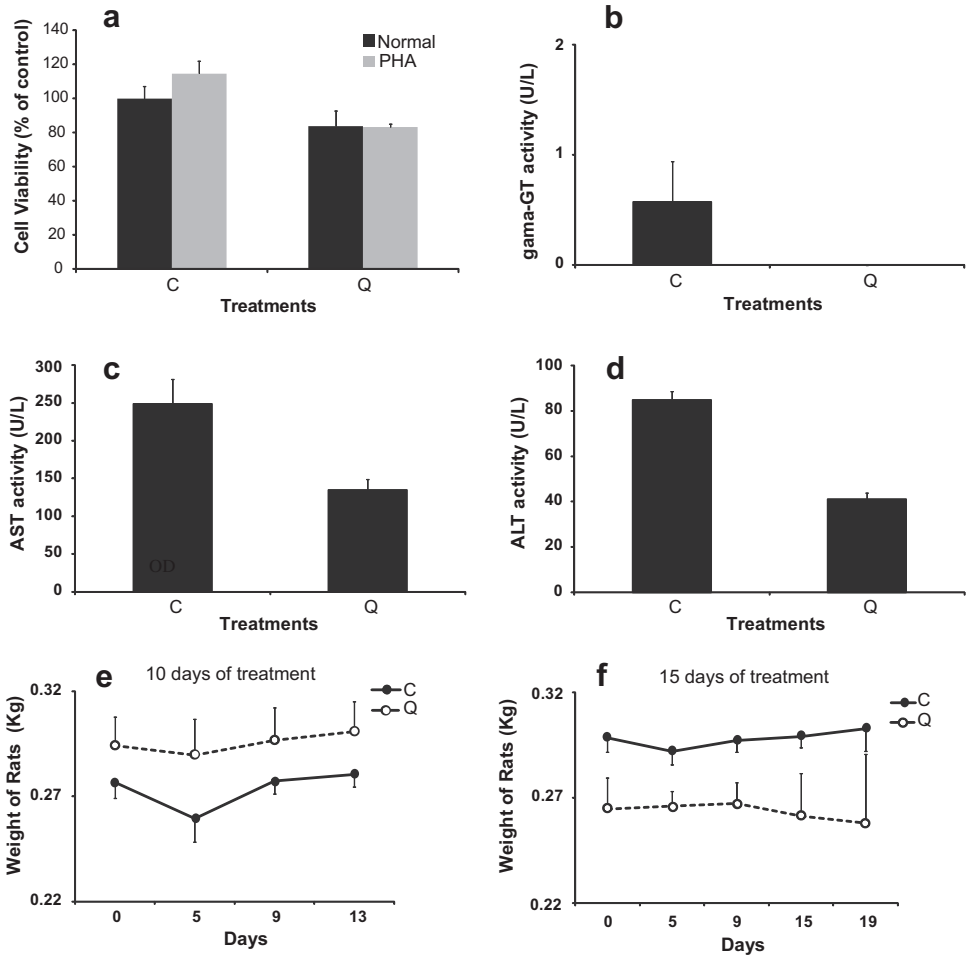

Fig. 2. T-cell proliferation *in vitro*. (a) Splenocytes from 4 rats after 20 days of glioma implantation were incubated with PHA or only DMEM (normal) for 3 days. After, MTT assay was performed. Data are expressed as percentage of control. Bars indicate mean ± S.E.M, ANOVA, followed by Tukeýs test, $n = 4$ in triplicates. (b, c and d) Effect of Quer on plasma transaminases activity after 20 days of glioma implantation. After 15 days of treatment, plasma was acquired followed by measurement of the activity of γ-glutamyltranspherase (b), AST (aspartate aminotransferase) (c) and ALT (alanine aminotransferase) (d). The values are represented as means ± S.E.M for 4 animals per group. Data were analyzed by ANOVA followed by Tukeýs test. (e and f) Body weight of animals with implanted gliomas. Animals were treated for 10 (e) or 15 days (f) as described in methods. Weights were observed daily. The values are represented as means ± S.E.M of the animals used in this study ($n = 8$ animals per group). Data were analyzed by ANOVA followed by Tukeýs test.

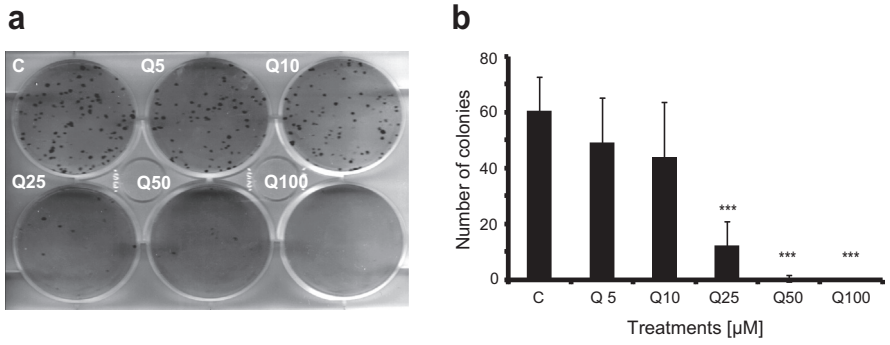

Fig. 3. Colony formation efficiency of Quer-treated C6 cells *in vitro*. C6 cells were exposed to different Quer (Q) treatments followed by 7 days in drug-free medium. (a) Representative dishes showing clonogenic assay. (b) Colony-forming quantification. Results shown are representative of three independent experiments. Data were analyzed by ANOVA followed by SNK post test. $^{***}p < 0.001$.

406 days. By the conclusion of the experiment, 80 % of treated rats had developed intestinal tumors and 20 % had bladder tumors. No tumors of either type were seen in control animals. Mean survival time was similar in treated and untreated animals (Pamukcu et al., 1980). Singh et al. (2010) examined the effects of Quer on estrogen-induced breast cancer *in vivo*. The ACI rat model of estrogen-induced breast cancer was used. Female ACI rats were given Quer via the diet (2.5 g/kg food) for a period of 240 days. At the end of the experiments, Quer did not prevent oxidative changes in the breast and did not confer protection against breast cancer.

In another study Quer did not show significant inhibitory effect on the growth of established mammary tumor inoculated in C3H/J mice (Indap et al., 2006). To our knowledge this is the first study showing tumor-growth promoting effects of Quer in gliomas.

What makes treatment regimen that reduces cell number due to apoptosis and senescence *in vitro* increases the tumor size *in vivo*, mainly considering the use of the same cell line? We considered three hypotheses: 1. modulation of the immune system, 2. angiogenesis and 3. stress from cell culture.

The immunosuppressive state associated with gliomas was described more than twenty years ago. Initially, it was noted that the cellular immune response of patients and animals with gliomas was impaired (Wood and Morantz, 1982, 1983; Yamasaki et al., 1983). The first reports described a lack of proliferation of the peripheral T cells of these patients after exposure to PHA (Wood and Morantz, 1983; Yamasaki et al., 1983). The aggressive nature of gliomas is closely related to their capacity to evade the anti-tumoral immune response. Palma et al. (1978) investigated two hundred selected patients who were operated for GBM for the incidence of the lymphocytic infiltration in the histological slides and its possible relevance to a better clinical course. The group that exhibited a defined lymphocytic infiltration had a significantly longer preoperative history and postoperative survival than the groups that presented slight or no infiltration. The mechanisms implicated in this phenomenon are only partially understood. Here, we found that lymphocytic infiltration was slightly reduced by Quer. We tested if peripheral T cells of these rats were also affected. After exposure T cells to PHA we observed that T-cells proliferation was slightly reduced in rats treated with Quer, suggesting that this drug had a small effect on peripheral T-cells proliferation, which may be responsible for part of the lymphocytic infiltration reduction in the tumor. Therefore, we conclude that probably this is not the main mechanism by which Quer induces glioma growth.

Regions of necrosis and microvascular proliferation characterize high-grade malignant gliomas and are associated with bad prognosis (Kleihues et al., 2000; Louis, 2006). Evidence suggests that the necrotic regions may initially represent a migratory response to local hypoxia (perhaps brought about by coagulopathic events), followed by frank necrosis. The cells that palisade around the necrosis liberate angiogenic (e.g., VEGF – vascular endothelial growth factor) and other growth factors, contributing to the marked angiogenesis in these tumors (including the so-called glomeruloid blood vessels) (Kleihues et al., 2000). In a number of studies, Quer was found to inhibit several steps of angiogenesis, including proliferation, migration and differentiation of endothelial cells (Fan et al., 2003; Igura et al., 2001; Tan et al., 2003). Following *in vivo* administration of Quer, the glucuronide (3G) and sulfate (3S) derivatives of Quer predominate (Day et al., 2001). Quer conjugates exhibit divergent effects on the endothelium and on angiogenesis (Donnini et al., 2006). On the one hand, Quer3G and Quer itself have no effect, at least at the concentration of 1 μM, on quiescent endothelium, while they inhibit endothelial functions and *in vivo* angiogenesis elicited by VEGF. On the other hand, Quer3S increases significantly the growth of quiescent endothelial cells reaching maximal activity at 1 μM, while it has no effect on cell proliferation stimulated by VEGF.

Another important feature to mention is the concentration achieved in the brain after the treatments. We found 0.53 μM of Quer in the brain tissue after 15 days of 50 mg/kg/day Quer treatment. This dosage is very low when compared with the *in vitro* concentrations that we have previously used (Zamin et al., 2009). When we tested the effect of Quer lower concentrations in colony formation assay we found that concentrations below 10 μM did not inhibited colony formation indicating that low concentrations of Quer may be ineffective in decreasing tumor cell growth. Other *in vitro* studies have indicated that toxic concentrations of Quer is between 20 and 100 μM range for several cancer cells (Cos et al., 2001; Jakubowicz-Gil et al., 2010; Kuntz et al., 1999; Seibert et al., 2011; Zhang et al., 2009). More importantly, when less than 5 μM of Quer was used, no effect on C6 glioma or MOGGCCM astrocytoma cells (Jakubowicz-Gil et al., 2010; Seibert et al., 2011) was observed and 0.5 μM Quer even promoted growth of the MDA-MB-231 human breast cancer cell line (Schlachterman et al., 2008).

The high concentrations required may be explained by the low stability of the Quer in culture medium. In a recent work, Sun et al., 2013 tested Quer stability on DMEM medium, and found that after 4-h incubation of breast cancer cell line with 200 μM of Quer, 50 % was degraded. After 24 h, the Quer was completely undetectable in the medium. Furthermore, the experimental conditions used can have a major impact on the nominal concentrations determined to cause a specific toxic effect. For example, Quer binds to albumin which reduces their available free fraction (Bolli et al., 2010; Rawel et al., 2005). Thus, the presence of serum in the culture medium should affect the nominal effective concentrations. In the experiments presented here the cells were exposed under serum medium conditions which may explain, at least in part, its low nominal sensitivity. However, caution must be used with the interpretation of *in vitro* data because the compounds that were added to the tissue culture cells are relatively stable in their aglycone forms compared to the dietary polyphenols that accumulate in the blood following oral administration (Murakami et al., 2008; Murota and Terao, 2003).

The lack of liver toxicity in animals opens the possibility for higher concentrations of Quer to be tested. But the glioma tumor increasing induced by Quer, despite being a key finding considering the clinical use of Quer, strongly discourage the dose escalation strategy.

The opposite effect between *in vitro* and *in vivo* results may lie in some aspects of cell culture, such as heterogeneity of tumor cell lines, the animal model, the time lapse between cell implantation and the beginning of treatments, the amount of cell implanted, and others. When non-transformed cells are cultured, they normally enter senescence (Hayflick, 1965) after a time in culture. Cell lines somehow are able to circumvent this senescence, but which, as we have shown, can be re-activated (Zamin et al., 2009). It is reasonable to think that this reactivation with Quer requires the stressful conditions of the cell culture, since we did not observe tumors volume reduction of rats treated with Quer and also no clear reduction in the mitotic index was observed in these tumors.

Several clinical trials in humans are being made to test the efficiency of Quer to treat several diseases, including cancer, cardiac diseases and aging-related pathologies (Egert et al., 2009; Ferry et al., 1996; Morrow et al., 2001). Moreover, Quer has been commercialized as "miracle" phytotherapic drug and people are taking it as if the chance of unwanted side effects was null. Thus, considering the results found here it is very important to better elucidate the effects of this compound in animal models before jumping to widespread human use.

Ethical standard

All procedures used in the present study followed the "Principles of Laboratory Animal Care" of the National Institutes of Health and were approved by the local Ethical Committee (protocol number 2008229).

Conflict of Interest

The authors declare that there are no conflicts of interest.

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