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Selective cytotoxicity of indomethacin and indomethacin ethyl ester-loaded nanocapsules against glioma cell lines: An *in vitro* study

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Abstract

Gliomas are the most common and devastating tumors of the central nervous system. Several studies have suggested that nonsteroidal anti-inflammatory drugs (NSAIDs) are promising anticancer agents. Biodegradable nanoparticulate systems have received considerable attention as potential drug delivery vehicles. The aim of this study was to evaluate the effects of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules on glioma cell lines. In addition, the effect of these formulations on normal neural tissue was also evaluated. In order to investigate this, glioma cell lines (U138-MG and C6) and hippocampal organotypic cultures were used. The main finding of the present study is that indomethacin-loaded nanocapsules formulation was more potent than a solution of indomethacin in decreasing the viability and cell proliferation of glioma lines. Indomethacin and indomethacin ethyl ester associated together in the same nanocapsule formulation caused a synergic effect decreasing glioma cell proliferation. In addition, when the glioma cells were exposed to 25 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules, a necrotic cell death was observed. Interestingly, 5 μM of indomethacin-loaded nanocapsules was able to cause an antiproliferative effect without promoting necrosis in glioma cells. Another important finding was that the cytotoxic effect induced by 25 μM or 50 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules, in glioma cells was not observed in the organotypic cultures, indicating selective cytotoxicity of those formulations for tumoral cells. Further investigations using *in vivo* glioma model should be helpful to confirm the distinct effects of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules, in normal versus tumoral cells.

Keywords: Glioma cell; Hippocampal organotypic culture; Cell death; Indomethacin; Indomethacin ethyl ester; Polymeric nanocapsule

1. Introduction

Malignant gliomas are the most common primary brain tumors, representing 50–60% of this type of tumor (Bigner et al., 1988; Holland, 2001). Glioblastomas, malignant glioma of grade IV, are poorly responsive to multimodaly therapeutic interventions, including surgery, radiotherapy, and chemotherapy (Deen et al., 1993). Despite treatment, malignant gliomas recur early, leading to a mean survival of less than 12 months (Holland, 2001). The highly lethal nature of this tumor results from the acquisition of an invasive phenotype that allows the glioblastoma cells to infiltrate surrounding brain tissue (Greemberg et al., 1993). Anticancer drugs are toxic to both tumoral and normal cells and the efficacy of chemotherapy is often limited by important side effects (Brigger et al., 2002a).

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Besides, the presence of the blood-brain barrier limits the penetration of antineoplasic drugs into brain tumors (Gelperina et al., 2002). An alternative approach for the treatment of gliomas is the employment of a drug delivery system, as nanocarriers, which are able to improve or even target delivery of the anti-tumoral agents to the brain (Gelperina et al., 2002).

Nanoparticles are submicronic (<1 μm) systems generally, but not necessarily, made of polymers, which have been widely studied as drug delivery systems (Soppimath et al., 2001; Brigger et al., 2002a, Schaffazick et al., 2003; Garcia-Garcia et al., 2005; Wong et al., 2007). Generally, nanoparticles have a size around 200 nm and the drugs or other molecules may be dissolved, entrapped, encapsulated, adsorbed and/or attached to the nanoparticles (Brigger et al., 2002a). Applying the nanoprecipitation and the interfacial deposition techniques (Fessi et al., 1989), nanostructured systems such as nanospheres or nanocapsules can be obtained, according to the formulation composition. Nanocapsules are vesicular systems in which the drug can be confined to an aqueous or oily cavity surrounded by a single polymeric wall (Jäger et al., 2007). Nanocapsules may, thus, be considered as a 'reservoir' system (Brigger et al., 2002a). The most promising application of polymeric nanoparticles is their use as carriers for anticancer drugs (Kim and Lee , 2001). It has been recently reported that novel nanoparticles could be used as potential drug carriers across the blood-brain barrier (Lockman et al., 2003; Koziara et al., 2003). Additionally, it has been found that the polymer-anticancer drug conjugates in comparison with low-molecular-weight anticancer drugs were accumulated more in the tumor tissues than in the normal tissues due to the enhanced permeability and retention effect (Kim and Lee , 2001). The literature also reported that polymer-anticancer drug conjugates could prolong the antitumoral activity by releasing the drug at a controlled rate (Alonso, 1996; Seijo et al., 1990; Vinogradov et al., 2002). The approach of treating glioma cells by liposomal carriers in order to obtain an increased efficiency or reduced side effects has been reported (Hu et al., 1995; Koukourakis et al., 2000). Moreover, nanoparticles of biodegradable polymers coated with polysorbate 80 (Tween 80®) were reported in the literature for cerebral delivery of several substances (McCarthy et al., 2005; Zhang and Feng , 2006; Brioschi et al., 2007).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used drugs for the treatment of autoimmune and chronic inflammatory diseases (Baek et al., 2002). Numerous experimental, epidemiological, and clinical studies suggest that NSAIDs are promising anticancer agents (Thun et al., 2002). Recent studies have provided evidence of an additional role for NSAIDs in preventing growth of cancer cells by inhibiting cyclooxygenase (COX) enzymes (Grubbs et al., 2000; Williams et al., 2000). Other studies revealed that the antiproliferative effect of NSAIDs may be, in part, independent of cyclooxygenase inhibition (Baek et al., 2002). The exact mechanisms by which NSAIDs contribute to the antitumor activity remain controversial and it is peculiar for each tumor (Thun et al., 2002). The data from the literature suggest the involvement of these drugs on the induction of apoptosis, on the control of cell proliferation and invasion and/or on the inhibition of angiogenesis (Tegeder et al., 2001).

We have recently shown that indomethacin, a powerful NSAID derived from indolacetic acid, causes antiproliferative effects in glioma cell lines due to an arrest of cell cycle progression (Bernardi et al., 2006). In addition, we demonstrated that these antiproliferative effects of indomethacin on glioma cells are mediated, at least in part, by increasing the catabolism of extracellular purines (Bernardi et al., 2007). Considering these effects recently demonstrated by our group (Bernardi et al., 2006; Bernardi et al., 2007), the aim of the present study was to investigate the effect of indomethacin-loaded nanocapsules on the glioma cell lines. Additionally, the effects of nanoencapsulated indomethacin ethyl ester, a selective inhibitor of cyclooxygenase-2 (COX-2) (Kalgutkar et al., 2000), were also evaluated.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), HBSS, horse serum, Fungizone®, penicillin/streptomycin, 0.25% trypsin/EDTA solution were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA). Fetal bovine serum was obtained from Cultilab (Cultilab, Campinas, SP, Brazil). Gentamicin was obtained from Schering do Brazil (Rio de Janeiro, RJ, Brazil). Indomethacin, dimethylsulphoxide, propidium iodide (PI) and MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) were obtained from Sigma (St. Louis, USA). Poly(ε-caprolactone) (PCL) (M_w=65,000) was supplied by Aldrich (Strasbourg, France). Caprilic/capric triglyceride mixture was delivered from Brasquim (Porto Alegre, Brazil). Span 60® (sorbitan monostearate) and Tween 80® (polysorbate 80) were obtained from Delaware (Porto Alegre, Brazil). Indomethacin ethyl ester was synthesized by our group (Cruz et al., 2006) after adapting the methodology described by Kalgutkar et al. (2000). All other chemicals and solvents used were of analytical or pharmaceutical grade.

2.2. Preparation of nanocapsules

Nanocapsule suspensions were prepared by interfacial deposition (Fessi et al., 1989). At 40 °C, indomethacin or indomethacin ethyl ester (0.010 g), poly(ε-caprolactone) (0.100 g), capric/caprylic triglyceride (0.33 ml) and sorbitan monostearate (0.077 g) were dissolved in acetone (27 ml). In a separate flask, polysorbate 80 (0.077 g) was added into 53 ml of water. The organic solution was poured into the aqueous phase under magnetic stirring at room temperature. After 10 min, the acetone was eliminated and the suspensions concentrated under reduced pressure. The final volume was adjusted to 10 ml. Control formulations (drug-unloaded nanocapsules) were prepared omitting the drug (indomethacin or indomethacin ethyl ester). Additionally, a suspension containing both indomethacin (0.0054 g) and indomethacin ethyl ester (0.0046 g) was also prepared.

2.3. Characterization of nanocapsules

After preparation, the pH values of nanocapsule suspensions were determined using a potentiometer (Micronal B-474). The particle size, polydispersity and zeta potential of the systems were determined using a Zetasizer®nano-ZS ZEN 3600 model (Malvern, UK). The samples were diluted with water (MilliQ®) (particle size) or in 10 mM NaCl aqueous solution (zeta potential). The measurements were made in triplicate. The total concentrations of indomethacin or indomethacin ethyl ester in the formulations were measured by HPLC (Perkin-Elmer S-200, with injector S-200, detector UV–vis, a guard-column and a column Lichrospher 100 RP-18, 250 mm, 4 mm, 5 μm, Merck). The mobile phase (1.2 ml/min) consisted of acetonitrile/water (70:30, v/v) adjusted to apparent pH 5.0±0.5 with 10% (v/v) acetic acid. Each suspension (100 μl) was treated with acetonitrile (10 ml), the solution was filtered (Millipore 0.45 μm) and injected (20 μl). The HPLC method was validated following the ICH (1996). Linear calibration curves for indomethacin ester and for indomethacin were obtained in the range of 1.00–25.00 μg/ml presenting correlation coefficients higher than 0.9994 (indomethacin ethyl ester) and 0.9992 (indomethacin).

2.4. Maintenance of cell lines

The human glioblastoma cell line U138-MG (derived from spontaneously occurring human malignant gliomas) and the rat glioma cell line C6 (derived from *N*-nitrosomethylurea-induced glioma in rat) were obtained from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics penicillin/streptomycin 0.5 U/ml, and supplemented with 5% (C6) or 15% (U138-MG) (v/v) fetal bovine serum (FBS). Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air. All the experiments throughout this study were conducted in serum supplemented DMEM.

2.5. Drug exposure

Indomethacin or indomethacin ethyl ester in solution was dissolved in cell culture-grade dimethylsulphoxide (DMSO; Sigma). The indomethacin-loaded nanocapsules, the indomethacin ethyl ester-loaded nanocapsules and drug-unloaded nanocapsules were prepared as described above. The glioma cells were seeded according each experiment and after reaching subconfluence the cultures were exposed for 1, 3, 24 or 48 h to formulations: indomethacin, indomethacin ethyl ester, indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules (5, 10, 25, 50 or 100 μM). Control cells were treated with vehicle, i.e., 1.0% of DMSO or with drug-unloaded nanocapsules. In experiments made in hippocampal organotypic cultures, the cultures received 25 or 50 μM of indomethacin-loaded nanocapsules, indomethacin ethyl ester-loaded nanocapsules or drug-unloaded nanocapsules for 24 h. Control cultures were performed without nanocapsules.

2.6. Assessment of glioma cell viability

The method MTT provides a quantitative measure of the number cells with metabolically active mitochondria and it is based on the mitochondrial reduction of a tetrazolium bromide salt, MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]), to a chromophore, formazan product, whose absorbance can be determined by spectrophotometric measurement. Glioma cells were plated in a 96-well plate at 10^3 per well and, after reaching semi-confluence, the cultures were treated with 5, 10, 25, 50 or 100 μM of indomethacin, indomethacin-loaded nanocapsules, indomethacin ethyl ester or indomethacin ethyl ester-loaded nanocapsules for 48 h. Control cultures were also treated with DMSO or drug-unloaded nanocapsules. After 48 h of treatment, each culture medium containing the drug was removed and the cells were washed twice with 100 μl of PBS. After removing the PBS, 90 μl of culture medium and 10 μl of MTT were added to each of the wells. The cells were incubated for 3 h and the solution was then removed from the precipitate. A total of 100 μl of DMSO were added to the wells and the level of absorbance was read by an ELISA plate reader at 490 nm. This absorbance was linearly proportional to the number of live cells with active mitochondria. The cell viability was calculated using Eq. (1):

$$\text{Cell viability}\,(\%) = (\text{Abs}_s/\text{Abs}_{\text{control}})100 \quad (1)$$

where Abs_s is the absorbance of cells treated with different formulations and $\text{Abs}_{\text{control}}$ is the absorbance of control cells (incubated with cell culture medium only).

2.7. Cell counting

The human glioma cells (U138-MG) were seeded at 1×10^4 cells per well in DMEM/15% FBS in 24-well plates, and allowed to grown for 24 h. After reaching semi-confluence, glioma cells were treated with 5, 10, 25, 50 or 100 μM of indomethacin, indomethacin-loaded nanocapsules, indomethacin ethyl ester or indomethacin ethyl ester-loaded nanocapsules for 1, 3, 24 or 48 h. Control cultures were treated with DMSO or drug-unloaded nanocapsules. At the end of the treatment, the medium was removed. Cells were washed with phosphate buffered saline (PBS) and 200 μl of 0.25% trypsin/EDTA solution was added to detach the cells, which were counted immediately in a hemocytometer. The procedure was the same for the rat glioma cells (C6) except that they were seeded at 5×10^3 cells per well in DMEM/5% FBS.

2.8. Propidium iodide assay

Glioma cell lines were treated with 5, 10, 25, 50 or 100 μM of indomethacin, indomethacin-loaded nanocapsules, indomethacin ethyl ester or indomethacin ethyl ester-loaded nanocapsules for 24 h. Control cultures were also treated with DMSO or drug-unloaded nanocapsules. After the end of treatment, glioma cells were incubated with 5 μM of propidium iodide (PI) (Sigma Chemical) for 1 h. PI is excluded from healthy cells, but following loss of membrane integrity this molecule enters cells,

binds to DNA and becomes highly fluorescent (Macklis and Madison, 1990). PI fluorescence was excited at 515–560 nm using an inverted microscope (Nikon Eclipse TE300) fitted with a standard rhodamine filter. Images were captured using a digital camera connected to the microscope.

2.9. Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al. (1991). Briefly, 400-μm-thick hippocampal slices were prepared from 6- to 8-day-old male *Wistar* rats using a Mcllwain tissue chopper (all animal use procedures were approved by local Animal Care Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals) and separated in ice-cold Hank's balanced salt solution (HBSS) composed of (mM): glucose 36, $CaCl_2$ 1.26, KCl 5.36, NaCl 136.89, KH_2PO_4 0.44, Na_2HPO_4 0.34, $MgCl_2$ 0.49, $MgSO_4$ 0.44, HEPES 25; Fungizone® 1% (Gibco, Grand Island, NY,USA) and gentamicine 36 μl/100 ml (Schering do Brasil, Rio de Janeiro, RJ, Brazil); pH 7.2. The slices were placed on Millicell culture insert (Millicell®-CM, 0.4 μm, Millipore®, Bedford, MA, USA) and the inserts were transferred to a 6-well culture plate (Cell Culture Cluster, Costar®, New York, NY, USA). Each well contained 1 ml of tissue culture medium consisting of 50% minimum essential medium (MEM) (Gibco), 25% HBSS (Gibco), 25% heat inactivated horse serum (Gibco) supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; Fungizone® 1% and gentamicine 36 μl/100 ml; pH 7.3. Organotypic cultures were maintained in a humidified incubator gasified with 5% CO_2 atmosphere at 37°C. The medium was changed every 3 days and experiments were carried out after 14 days *in vitro* when the cells received 25 or 50 μM of indomethacin-loaded nanocapsules, indomethacin ethyl ester-loaded nanocapsules or drug-unloaded nanocapsules for 24 h. Control cultures were performed without nanocapsule formulations.

2.10. Quantification of cellular death in organotypic hippocampal cultures

Cell death was assessed by fluorescent image analysis of propidium iodide (IP) uptake (Noraberg et al., 1999). After a period of 22 h in the presence of the different formulations, 5 μM PI was added to the cultures and incubated for 2 h. Cultures were observed with an inverted microscope (Nikon Eclipse TE300) using a standard rhodamine filter set. Images were captured and then analyzed using Scion Image software (http://www.scioncorp.com). The area where PI fluorescence was determined using the "density slice" option of Scion Image software and compared to the total hippocampus area to obtain the percentage of damage (Valentin et al., 2003).

2.11. Statistical analysis

Data are expressed as mean±S.E.M. and analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by *post-hoc* for multiple comparisons (Tukey test) using an Instat software package (GraphPad Software, San Diego, CA, USA). Differences between mean values were considered significant when $P<0.05$.

IC_{50} values were calculated by linear regression analysis of $\log_{10}$ cell viability versus $\log_{10}$ concentration. The values were compared using one-way analysis and Tukey test for simultaneous comparisons between groups.

3. Results

3.1. Physico-chemical characterization of nanocapsule formulations

All nanocapsule formulations were prepared by interfacial deposition of polymer without the need of any subsequent step

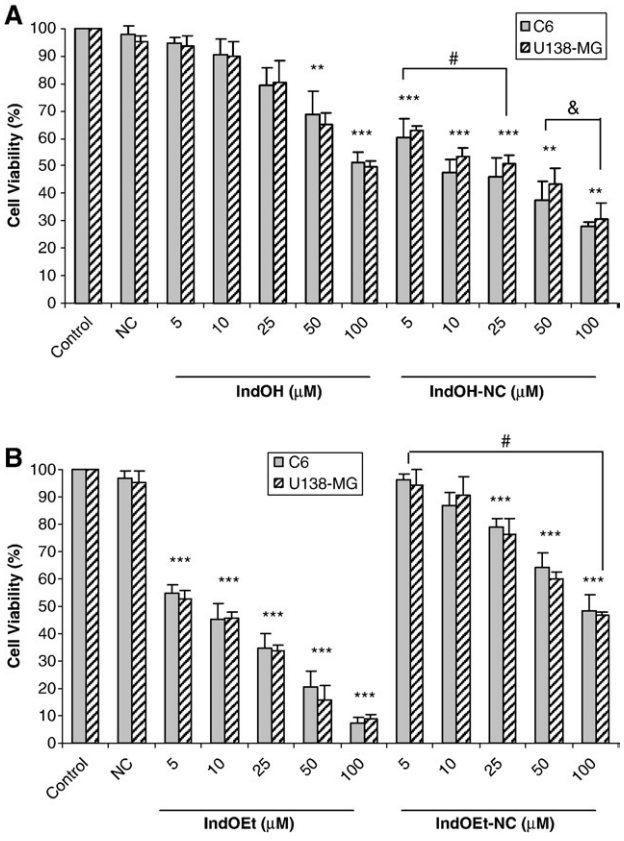

Fig. 1. Effect of indomethacin and indomethacin ethyl ester in solution or in nanocapsule formulations on cell viability of glioma cell lines. U138-MG and C6 glioma cell lines were grown on a 96-well plate and, after reaching approx. 80% confluence, the cultures were treated with 5, 10, 25, 50 or 100 μM of (A) indomethacin (IndOH) or indomethacin-loaded nanocapsules (IndOH-NC) and (B) indomethacin ethyl ester (IndOEt) or indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC) for 48 h. Cultures were also treated with drug-unloaded nanocapsules (NC). After 48 h of treatment, the cell viability was evaluated by MTT assay, as described in Materials and methods. The cell viability was represented in relation of control cells (100% of cell viability). The values are represented as means±S.E.M. of six independent experiments made in quadruplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). **Significantly different from the control group ($P<0.01$); ***Significantly different from the control group ($P<0.001$); #Significantly different from the respective group treated with drug in solution ($P<0.001$); &Significantly different from the respective group treated with drug in solution ($P<0.01$).

of purification. Indomethacin-loaded nanocapsules, indomethacin ethyl ester-loaded nanocapsules and drug-unloaded nanocapsules presented macroscopic homogeneous aspect like milky white bluish opalescent liquids. After preparation, the particle sizes were 240 nm (Indomethacin-loaded nanocapsules), 234 nm (indomethacin ethyl ester-loaded nanocapsules) and 226 nm (drug-unloaded nanocapsules). The suspensions showed monomodal size distributions and polydispersity indexes lower than 0.19, indicating narrow distributions. The pH values were 5.95 (Indomethacin-loaded nanocapsules), 6.14 (indomethacin ethyl ester-loaded nanocapsules) and 6.05 (drug-unloaded nanocapsules). The zeta potential values were −6.9, −7.4 and −7.3 mV, respectively. The drug contents were 0.991 ± 0.012 mg/ml (Indomethacin-loaded nanocapsules) and 1.003 ± 0.017 (indomethacin ethyl ester-loaded nanocapsules) and the encapsulation efficiencies were close to 100% for both formulations.

3.2. Indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules decrease cell viability in human and rat glioma cells

To investigate whether indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules affect the cell viability, we used an MTT assay that measures the mitochondrial activity and, indirectly, the cell viability of cells treated with these formulations. Cultured cells were treated with several concentrations (5, 10, 25, 50 or 100 μM) of indomethacin or indomethacin ethyl ester in solution or in nanocapsule formulations for 48 h. Analysis of MTT assay showed that, in solution, the effect of indomethacin ethyl ester was more pronounced than that of indomethacin. All the concentrations of indomethacin-loaded nanocapsules caused a significant reduction in cell viability compared to the control culture cells (Fig. 1A). An important finding of the present study is that the effect of indomethacin-loaded nanocapsules was more pronounced than indomethacin in solution at the same tested concentrations (Fig. 1A). The IC_{50} values showed that indomethacin-loaded nanocapsules increases 2.5 fold the cytotoxicity for C6 and 2.0 fold for U138-MG, when compared to indomethacin in solution for same cell lines (Table 1). A significant reduction on cell viability was also observed after the treatment with indomethacin ethyl ester-loaded nanocapsules at the concentrations of 25, 50 or 100 μM (Fig. 1B). However, on the contrary to indomethacin, the indomethacin ethyl ester in solution was more efficient than its nanocapsule formulations, in all tested concentrations (Fig. 1B). These effects were similar in human and rat glioma cell lines and the treatment of drug-unloaded nanocapsules did not show significant alterations in cell viability (Fig. 1).

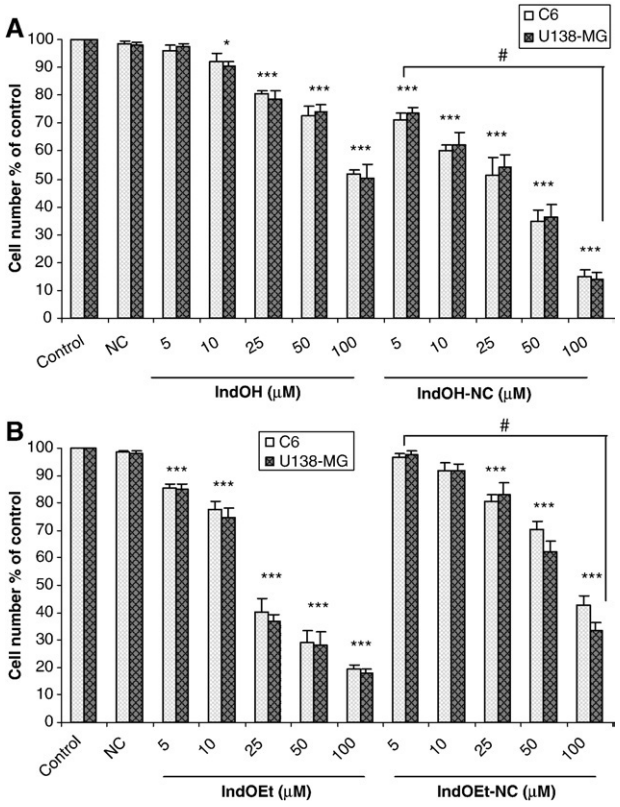

Fig. 2. Effect of indomethacin and indomethacin ethyl ester in drug solution or nanocapsule formulations on glioma cell proliferation. Semi-confluent cultures of glioma cells were treated with 5, 10, 25, 50 or 100 μM of (A) indomethacin (IndOH) or indomethacin-loaded nanocapsules (IndOH-NC) and (B) indomethacin ethyl ester (IndOEt) or indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC) for 48 h. Cultures were also treated with drug-unloaded nanocapsules (NC). After 48 h of treatment, the cells were detached with 0.25% trypsin-EDTA and counted in hemocytometer. The values are represented as means ± S.E.M. of four independent experiments made in triplicate. The average number of cells in untreated controls (100%) was 110,000 ± 5600 for U138-MG and 232,000 ± 9800 for C6 glioma cells. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). *Significantly different from the control group ($P<0.05$); ***Significantly different from the control group ($P<0.001$); #Significantly different from the respective group treated with drug in solution ($P<0.001$).

Table 1
IC_{50} values of indomethacin and indomethacin ethyl ester in solution or in nanocapsules formulations in glioma cell lines

	C6	U138-MG
IndOH	97.08 ± 2.379	92.15 ± 2.269
IndOH-NC	38.44 ± 4.723 [a]	45.9 ± 4.376 [a]
IndOEt	22.60 ± 5.346	20.88 ± 5.642
IndOEt-NC	89.99 ± 2.125 [a]	84.43 ± 1.975 [a]

Mean (S.E.M.) IC_{50} values (μM) for 48 h of treatment with Indomethacin (IndOH), indomethacin-loaded nanocapsules (IndOH-NC), indomethacin ethyl ester (IndOEt) or indomethacin ethyl ester-loaded nanocapsules (IndOEt) by MTT assay, as described in Materials and methods. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test).

[a] Significantly different from the group treated with respective drug in solution for same cell line ($P<0.001$).

3.3. Indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules inhibit growth of the glioma cell lines

In order to investigate the effect of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules

on cell growth, we studied the effect of these formulations in the proliferation of glioma cell lines. Treatment of glioma cells with indomethacin-loaded nanocapsules (5, 10, 25, 50 or 100 μM) for 48 h resulted in significant decrease in cell number when compared to the control cultures (Fig. 2A). Again, indomethacin-loaded nanocapsules were more potent to reduce the cell proliferation in comparison with indomethacin in solution (Fig. 2A). It is important to note that the concentration of 5 μM indomethacin-loaded nanocapsules is already sufficient to cause a significative antiproliferative effect in glioma cells (Fig. 2A). On the order hand, indomethacin ethyl ester in nanocapsule formulations was able to significantly decrease the cell proliferation only in the concentrations of 25, 50 or 100 μM, being more efficient in solution (Fig. 2B). The treatment of drug-unloaded nanocapsules did not show significant alterations in cell proliferation (Fig. 2). These effects were observed in human and rat glioma cell lines and are in agreement with the results observed in MTT assay (Fig. 1).

To address the effect of short exposure to nanocapsule formulations, we treated U138-MG glioma cells with 25 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules for 1 or 3 h. After 1 or 3 h of exposure with these formulations, the medium (DMEM) was changed to DMEM formulations-free. After 48 h in culture, the assay of cell proliferation was performed. Our results showed that the short exposure of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules is enough to cause a significant decrease in cell proliferation of U138-MG glioma cells (Fig. 3). In 3 h and 48 h of exposure, the effects were significantly similar (Fig. 3). Similar effects were observed in C6 glioma cell line (data not shown).

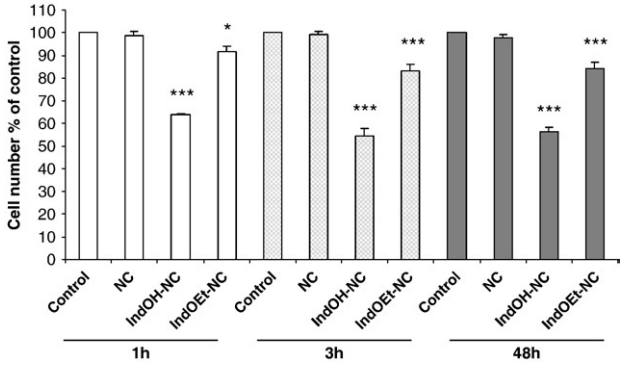

Fig. 3. Effect of short exposure to indomethacin and indomethacin ethyl ester in nanocapsule formulations on glioma cell proliferation. U138-MG glioma cells were exposed to 25 μM of indomethacin-loaded nanocapsules (IndOH-NC) or 25 μM of indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC) for 1, 3 or 48 h. Cultures also were treated with drug-unloaded nanocapsules (NC). After 1 or 3 h of exposure with formulations, the medium (DMEM) was changed to DMEM formulations-free. After 48 h in culture, the cells were detached with 0.25% trypsin-EDTA and counted in hemocytometer. The values are represented as means±S.E.M. of four independent experiments made in triplicate. The average number of cells in untreated controls (100%) was 116,000±4200. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). *Significantly different from the respective control group ($P<0.05$); ***Significantly different from the respective control group ($P<0.001$).

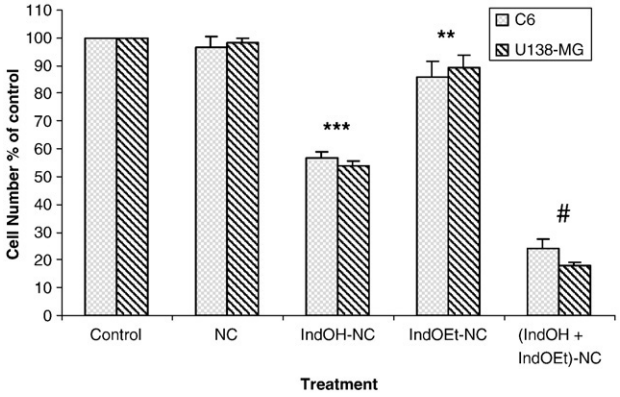

Fig. 4. Synergic effect of indomethacin and indomethacin ethyl ester in nanocapsule formulations on glioma cell proliferation. Semi-confluent cultures of glioma cells were treated for 48 h with 25 μM of nanocapsule formulations containing simultaneously indomethacin and indomethacin ethyl ester [(IndOH+IndOEt) −NC]. Cultures also were treated with drug-unloaded nanocapsules (NC). After 48 h of treatment, the cells were detached with 0.25% trypsin-EDTA and counted in hemocytometer. The values were represented as means±S.E.M. of four independent experiments made in triplicate. The average number of cells in untreated controls (100%) is 108,000±3800 for U138-MG and 225,000±7200 for C6 glioma cells. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). ***Significantly different from the control group ($P<0.001$); **Significantly different from the control group ($P<0.01$); #Significantly different from the control, indomethacin-loaded nanocapsules (IndOH-NC) and indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC) groups ($P<0.001$).

3.4. Indomethacin and indomethacin ethyl ester in the same nanocapsule formulation cause synergic effect in decreasing cell growth of glioma cells

Considering the antiproliferative effect observed when the glioma cell line was exposed to indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules, we studied the effect on cell proliferation after the treatment with nanocapsule formulation containing simultaneously 25 μM of both indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules. Treatment for 48 h of culture cells with indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules caused a significant inhibition on cell proliferation when compared to the control cultures (Fig. 4). Interestingly, the antiproliferative effects of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules treatment (76% for C6 and 82% for U138-MG) were higher than the effects observed for each nanocapsule formulation containing indomethacin (44% for C6 and 47% for U138-MG) or indomethacin ethyl ester (15% for C6 and 17% for U138-MG) (Fig. 4). These results suggest a synergic effect between indomethacin and indomethacin ethyl ester in decreasing cell growth of C6 and U138-MG glioma cell lines.

3.5. Cell death induced by Indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules in glioma cell lines

To verify whether indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules could induce

necrosis cell death, glioma cells were treated with several concentrations (5, 10, 25, 50 or 100 μM) of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules for 24 h and then analyzed for cell death. Fig. 5 shows representative pictures of U138-MG glioma cells treated with 5 or 25 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules. As observed, the glioma cells treated with 5 μM of indomethacin-loaded nanocapsules presented a reduction in the cell number with no significant PI incorporation (Fig. 5D). Similar results were obtained with 10 μM (data not shown). When the cells were treated with 25 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules it was observed a significant PI incorporation, indicating an intense cell necrosis (Fig. 5E and F). Similar results were obtained with 50 and 100 μM (data not shown). The treatment of drug-unloaded nanocapsules did not show significant alterations in PI incorporation (Fig. 5B).

3.6. Indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules did not cause cytotoxic effect on organotypic hippocampal slice cultures

To evaluate the effect of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules on normal neural cells, organotypic hippocampal slice cultures were used as model. These cultures provide an excellent *in vitro* model system to study physiological factors, cellular and molecular mechanisms of neural death, and pharmacological compounds to neural survival (Holopainen, 2005). After 14 days in culture, the organotypic cultures were treated with 25 or 50 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules for 24 h and the cell death was analyzed by PI uptake (Fig. 6). As a positive control of cell damage we used organotypic hippocampal slice cultures exposed to oxygen and glucose deprivation (OGD), which showed significant cell damage (approximately 50%) (Fig. 6). It is important to note that either indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules did not promote organotypic hippocampal culture damage (25 μM: 1.9% for indomethacin ethyl ester-loaded nanocapsules and 3.1% for indomethacin-loaded nanocapsules with 4.8% of PI incorporation in control cultures; 50 μM: 2.7% for indomethacin ethyl ester-loaded nanocapsules and 2.9% for indomethacin-loaded nanocapsules with 3.3% of PI incorporation in control cultures) (Fig. 6). Taken together, these results showed that the cell death induced by 25 μM indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules in glioma

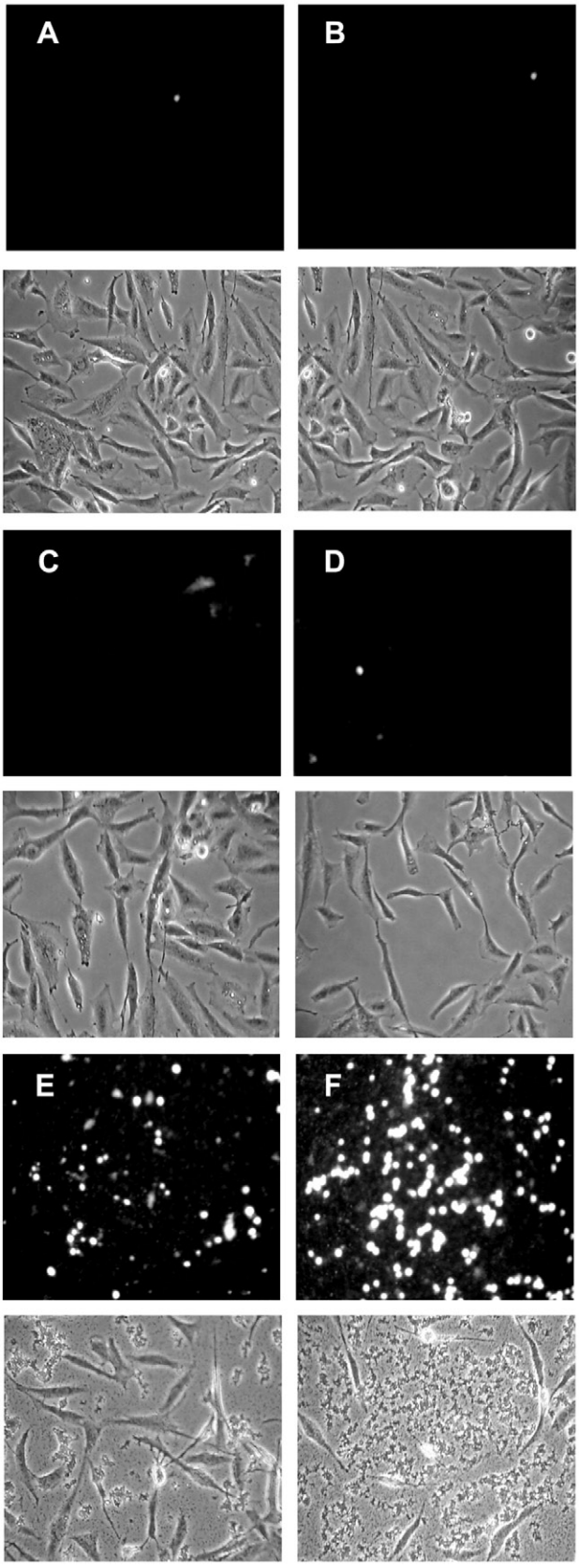

Fig. 5. Effect of indomethacin and indomethacin ethyl ester in nanocapsule formulations on cell death in U138-MG glioma cells by propidium iodide incorporation. Representative pictures of: control cultures (A); cells treated with drug-unloaded nanocapsules (NC) (B); cells treated with 5 μM (C) or 25 μM (E) of indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC), 5 μM (D) or 25 μM (F) of indomethacin-loaded nanocapsules (IndOH-NC). After 24 h of treatment, glioma cells were incubated with 5 μM of propidium iodide (PI). Cellular death was analyzed by PI incorporation that was visualized using a Nikon inverted microscope. Panels below A, B, C and D are correspondent contrast phase photomicrographs. The data are representative of three different experiments.

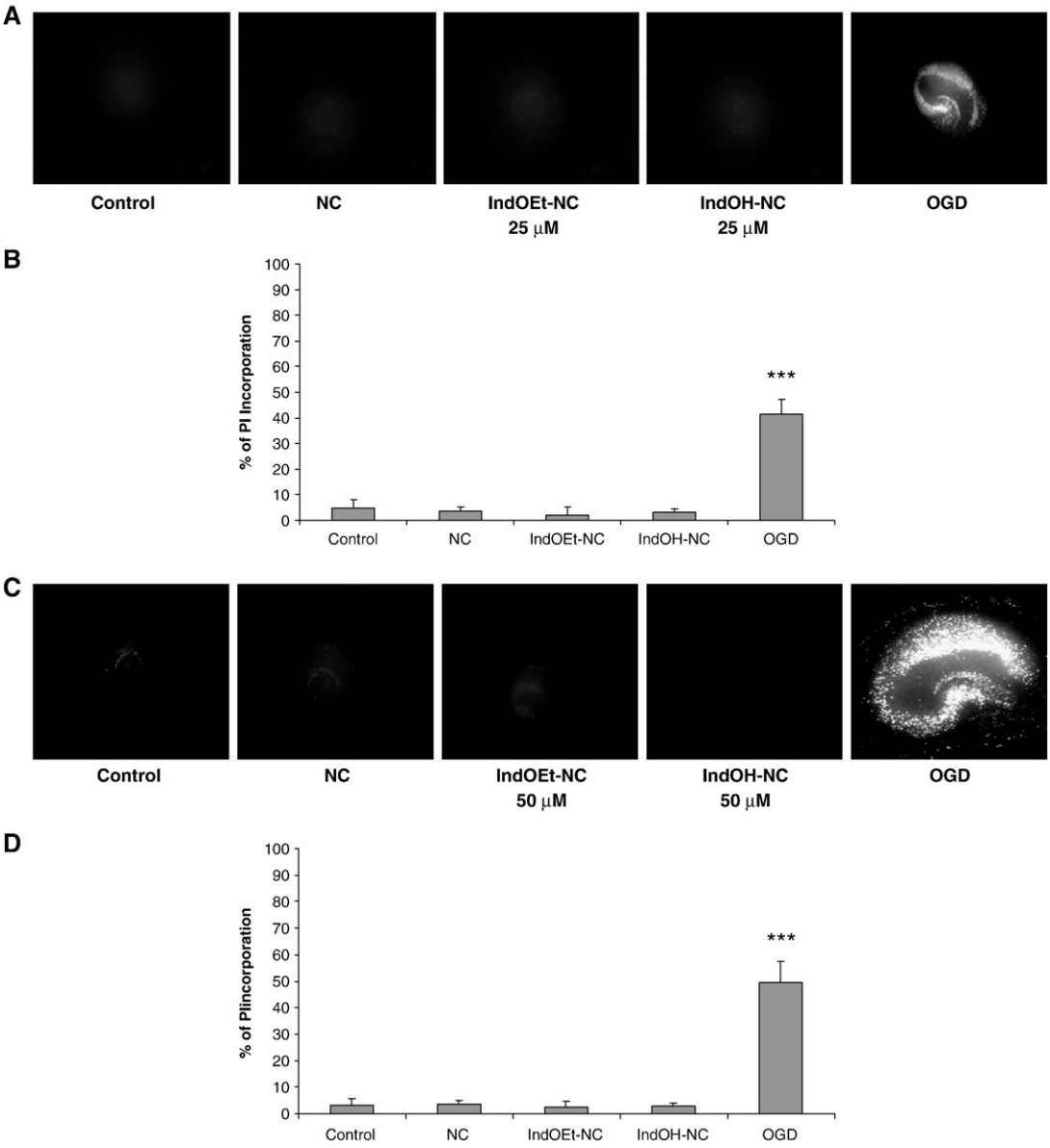

Fig. 6. Effect of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules on organotypic hippocampal slice cultures. (A) and (C) Representative pictures of organotypic hippocampal cultures. (B) and (D) Quantitative analysis of hippocampus damage after treatment of formulations. Organotypic hippocampal slices at 14 days were treated with drug-unloaded nanocapsules (NC), 25 or 50 μM of indomethacin-loaded nanocapsules (IndOH-NC), 25 or 50 μM of indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC) for 24 h. (A) and (B): treatment with 25 μM of formulations, (C) and (D): treatment with 50 μM of formulations; Control: organotypic cultures not exposed to formulations; OGD: neural cultures had been exposed to oxygen glucose deprivation and then used as a positive control of cell damage. Cellular death was analyzed by propidium iodide (PI) incorporation, which was visualized using a Nikon inverted microscope (at 40× magnification). Data represent the means ± S.E.M. of nine independent experiments made in duplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). ***Significantly different from the control group ($P<0.001$).

cultures was not observed in the organotypic cultures treated with 25 or even 50 μM.

4. Discussion

Gliomas present a particular therapeutic challenge because of their poor response to chemotherapy. Standard chemotherapy for brain tumors includes highly lipophilic drugs (nitrosurea, temozolomide) able to cross the blood-brain barrier (Brigger et al., 2004). The blood-brain barrier represents an insurmountable obstacle for a large number of drugs, including antibiotics, antineoplasic agents and a variety of central nervous system (CNS)-active drugs (Aktas et al., 2005). One of the possibilities to overcome this barrier is the use of nanoparticles in order to target the drug to the brain (Calvo et al., 2001; Brigger et al., 2002b). Several authors showed very encouraging results obtained at the cellular level with nanoparticles: efficient drug protection (Chavany et al., 1994; Raffin et al., 2006), cell internalization (Couvreur et al., 1977), drug release and drug transport (Beck et al., 2007), controlled release or reversion of the multidrug resistance (MDR) (Bennis et al., 1994; Colin de Verdiere et al., 1997; Koziara et al., 2004).

The mechanisms of encapsulation and release for several drugs in nanoparticulated systems have been extensively studied by our group (Guterres et al., 2000; Schaffazick et al., 2003; Pohlmann et al., 2004; Beck et al., 2007; Schaffazick et al., 2007; Poletto et al., 2007). In the present study, we investigated the cytotoxic effects of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules in U138-MG and C6 glioma cell lines. Previously we have showed that indomethacin caused antiproliferative effects in glioma cell lines due to an inhibition of cell cycle progression and by increasing the catabolism of extracellular purines (Bernardi et al., 2006; Bernardi et al., 2007). Within this context, we hypothesize that NSAIDs in nanocapsule formulations could be more cytotoxic for glioma cells than the respective drugs in solution. Our results show that indomethacin-loaded nanocapsules were at least 2 folds more cytotoxic than indomethacin in solution for both glioma cell lines (Table 1). On the order hand, indomethacin ethyl ester-loaded nanocapsules formulation was less efficient in reducing the cell viability in comparison with indomethacin ethyl ester in solution (Fig. 1, Table 1; Fig. 2). One plausible explanation for these effects is related to the mechanism of encapsulation of each drug in the nanocapsules. Indomethacin is adsorbed at the particle/water interface, while its ethyl ester is predominantly entrapped within the nanocapsules (Pohlmann et al., 2004; Cruz et al., 2006). Thus, different magnitudes of time are required for the total release of indomethacin or its ester from the nanocapsules (Cruz et al., 2006). Furthermore, these differences in the mechanism of drug encapsulation could explain, at least in part, the synergic effect observed in cell proliferation when the glioma cells were treated with nanocapsule formulations containing simultaneously indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules (Fig. 4). Probably the indomethacin causes an initial antiproliferative effect, which is maintained and sustained for indomethacin ethyl ester released from nanocapsules.

Another interesting finding of the present study was that the short exposures (1 and 3 h) of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules were enough to cause significant decreases in cell proliferation after 48 h of culture (Fig. 3). We suggest that the antiproliferative effect of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules is not a temporal response. According to literature, the higher cytotoxic effect of nanocapsules in glioma cells may be triggered by a higher potential cell division activity or an increased endocytotic activity resulting in enhanced nanocarrier uptake (Lamprecht and Benoit, 2006). Thus, in our experiment the cell uptake of drug-loaded nanocapsules was probably sufficient to achieve a high intracellular concentration of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules, and the subsequent intracellular release of drug led to the glioma cell death. Further studies, however, are necessary to give support to this hypothesis.

Necrosis is the pathological process, which occurs when cells are exposed to a serious physical or chemical insult. The literature reports that compounds that can induce antiproliferative effects in tumor cells without promoting a necrotic cell death are considered as being good candidate as antitumor drugs. In the present study, we observed that even though 5 μM of indomethacin-loaded nanocapsules is sufficient to cause antiproliferative effects in glioma cell lines, this drug concentration in nanocapsules did not cause necrotic cell death, which was observed with concentrations above 25 μM (Fig. 5). Considering that 400 μM of indomethacin in solution is necessary to cause a necrotic death in glioma cell lines (Bernardi et al., 2006), the results presented here, confirm that nanocapsule formulations are more efficient for glioma cell lines compared to the respective drugs in solution despite a cytotoxic effect was observed.

A major problem of drugs used in cancer chemotherapy is nonspecific toxicity against normal cells and tumoral cells. Such toxic action to normal cells limits the dose of the anticancer drugs to be administered to patients (Kim and Lee, 2001). Thus, to evaluate the selective cytotoxic effect of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules on glioma cell cultures, hippocampal organotypic cultures were used as model of normal neural cells. Thus, an important outcome of the present study is that, even though 5 μM of indomethacin-loaded nanocapsules is sufficient to cause cell viability and cell proliferation inhibition (Figs. 1A and 2A) without necrosis in glioma cell lines (Fig. 6), over 10-fold concentration of this formulation (50 μM) did not cause cell death in the organotypic cultures. These results indicate a selective cytotoxicity of these formulations for the tumor cells, which could be explained, at least in part, by the high metabolism of glioma cells that have a potential higher cell division activity and an increased endocytotic activity, enhancing the uptake of nanocapsules.

Although indomethacin is not an agent used in the treatment of brain tumors, our results imply that indomethacin in nanocapsule formulations may be considered a potential candidate for glioma treatment, being more potent than this drug in solution, without the undesirable side effects of conventional chemotherapy. Moreover, it is important to note that *in vitro* studies are limited and that further investigations using *in vivo* glioma model could be helpful to confirm the distinct effects of indomethacin-loaded nanocapsules in normal versus tumoral cells, as well as to determine the appropriate doses for therapy of gliomas.

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