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Research Article

Nanoprodrugs of NSAIDs Inhibit the Growth of U87-MG Glioma Cells

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Several recent reports have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the growth of various malignant cells suggesting their application as anticancer agents. In this study, we prepared six nanometer-sized prodrugs (nanoprodrugs) of NSAIDs, ibuprofen, indomethacin, and naproxen through the spontaneous emulsification mechanism using monomeric and dimeric derivatives of the NSAIDs. We evaluated their effect on the proliferation of U87-MG glioma cells by cell counting, WST-1 cell proliferation reagent, and propidium iodide incorporation. The two ibuprofen nanoprodrugs inhibited the cell growth more potently than the indomethacin nanoprodrugs, whereas the naproxen nanoprodrugs did not show any significant effect. Remarkably, ibuprofen did not show any effect at an equimolar concentration. Approximately, 4.4% of the ibuprofen nanoprodrugs was found in the cell, whereas no ibuprofen could be detected suggesting that the superior effect of the nanoprodrugs can be attributed to the efficient cellular uptake of the nanoprodrugs.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of pain, fever, and inflammation. The major mechanism by which NSAIDs exert their anti-inflammatory activity is the inhibition of cyclooxygenase (COX)-derived prostaglandin synthesis. COX is the first enzyme in the formation of prostaglandin (PG) and thromboxane (TX) from arachidonic acid at the site of inflammation or after infection [1]. There are two types of COX enzymes, namely COX-1 and COX-2. COX-1 is expressed constitutively in many tissues, whereas COX-2 is expressed only at the site of inflammation [2]. Recent studies have shown that high COX-2 expression has been detected in various cancers, including colorectal, lung, breast, liver, head and neck and brain tumors, whereas COX-1 expression was unaffected [3–5].

Human glioblastoma multiforme (GBM) is one of the most common tumors of the central nervous system with poor prognosis and high rate of recurrence. It is a highly aggressive and recalcitrant brain tumor, and despite intensive multimodal therapeutic interventions, only modest progress has been achieved over the last several decades in improving the treatment of patients with GBM [6]. Although the molecular mechanisms involved in the development of GBM are not yet fully understood, intensive studies have revealed some important molecular events correlated to the progress of malignant gliomas. The studies revealed that COX-2 have been expressed in brain tumors [7–9] and high COX-2 expression in gliomas is associated with poor prognosis [10].

A number of studies, clinical trials, and animal studies have demonstrated that NSAIDs may be effective in the prevention and treatment of certain types of cancers [11–14]. The molecular mechanisms by which NSAIDs exhibit antineoplastic effects are poorly understood and under intensive investigation. The chemopreventive and antitumorigenic effects of NSAIDs are partially attributed to the induction of apoptosis followed by inhibition of COX-2 [15–18]. Various studies have suggested that a COX-2-independent mechanism may also be involved because apoptosis induction by NSAIDs does not always correlate with their ability to inhibit COX-2 [19–22].

Indomethacin, ibuprofen and naproxen belong to the acidic NSAIDs which are widely used for the treatment

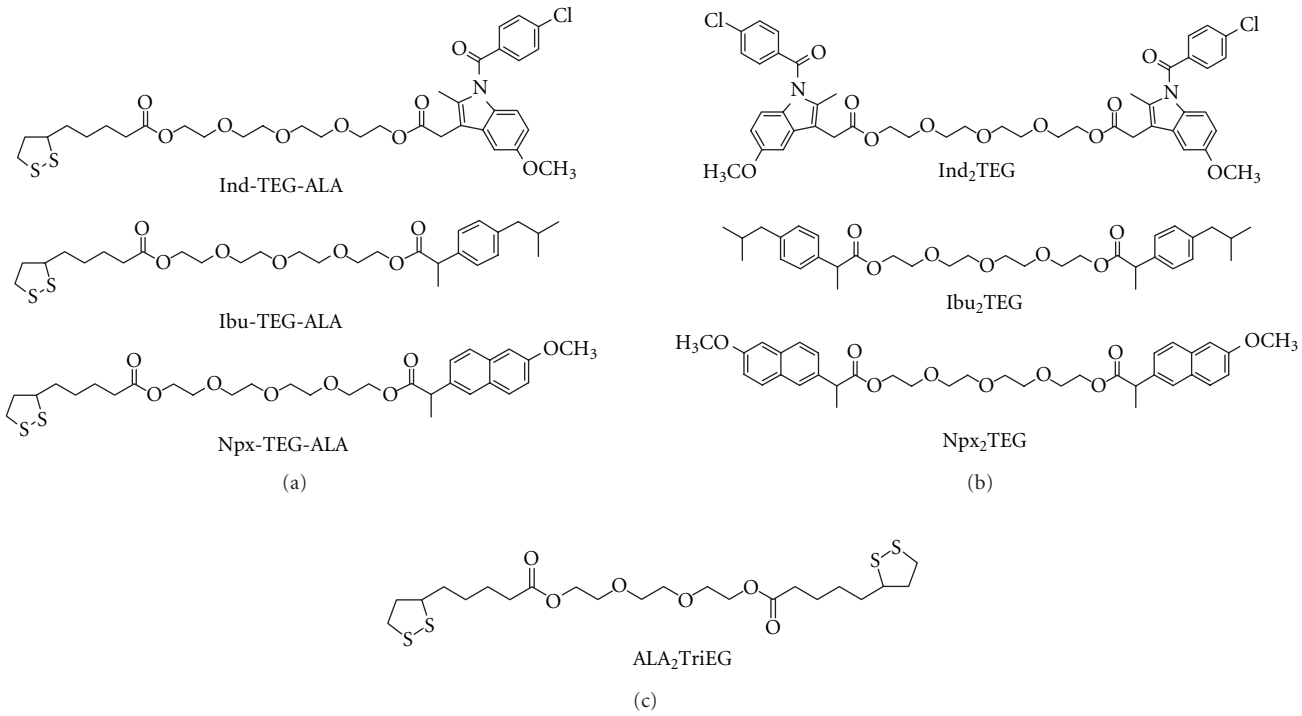

Figure 1: Derivatives of NSAIDs and ALA. ALA: α-lipoic acid; Ind: indomethacin; Ibu: ibuprofen; Npx: naproxen; TEG: tetraethylene glycol; TriEG: triethylene glycol.

of chronic inflammatory conditions. Ibuprofen is a potent COX-1 and COX-2 inhibitor. Besides its widespread use in the treatment of pain, fever, and inflammation, it has been shown that ibuprofen may be effective in the treatment of many cancers including prostate cancer [23], colon cancer [24, 25], and bladder cancer [26].

The inhibition of COX-derived prostaglandin synthesis, which is the major mechanism by which NSAIDs exert their anti-inflammatory activity, is also responsible for the adverse side effects, such as irritation and ulceration of the gastrointestinal (GI) mucosa [27]. These side effects are ascribed to the combined effect of the irritation caused by the free carboxylic groups in NSAIDs and blockage of prostaglandin biosynthesis in the GI tract [28]. In addition, the acidic moiety of these NSAIDs also contributes to the gastrointestinal side effect observed in response to these drugs [29]. Therefore, various prodrugs have been developed which attempt to alleviate the NSAID's adverse side effects as well as to improve their bioavailability by masking the carboxylic acid groups through the formation of bioreversible bonds [30–33].

In recent years, nanostructured biomaterials have received significant attention from the pharmaceutical industry, mainly because of their highly potential applicability as drug delivery vehicles. One of the most remarkable properties of nanostructured biomaterials is their improved bioavailability which can be ascribed to the generation of an enlarged surface area by transformation of bulk materials into the nanometer-sized structures [34, 35]. The surface-to-volume ratio increases with decreasing size of the nanostructures, which improves the bioavailability and enhances the biological efficacy of the materials [36]. The other advantage of nanostructures is that water-insoluble therapeutics can be transported more efficiently in the aqueous physiological environment when formed into stable nanostructures [37].

In an effort to combine the two concepts of nanomersized biomaterials and prodrugs we have developed nanomersized prodrugs (nanoprodrugs) of NSAIDs by spontaneous emulsification of hydrophobic derivatives of NSAIDs and demonstrated their antioxidant activity, oxidant responsiveness and enzymatic prodrug activation [38]. Despite the highly hydrophobic nature of the derivatives, NSAIDs were readily hydrolyzed enzymatically from the nanoprodrugs, which is a prerequisite condition for the nanoprodrugs to be used as a prodrug. Thus, the nanoprodrugs may have potential as an anti-inflammatory prodrug and also as a biodegradable anti-inflammatory drug delivery vehicle.

In this study, we demonstrated the anti-proliferative effect of NSAID nanoprodrugs on U87GM glioma cells.

2. Materials and Methods

2.1. Preparation of NSAID Nanoprodrugs. The synthesis and characterization of the monomeric NSAID derivatives (Figure 1(a)) and the dimeric NSAID derivatives (Figure 1(b)) were performed as described [38]. Nanoprodrugs were prepared according to the method using spontaneous emulsification as described [38] with modifications. Briefly, 25 mg of the NSAID derivatives and 5 mg of α-tocopherol were dissolved in acetone (5 mL) containing polysorbate 80 (0.1% w/v). The organic solution was poured under moderate stirring on a magnetic plate into an aqueous

phase prepared by dissolving 25 mg of Pluronic F68 in 10 mL distilled water (0.25% w/v). Following 15 minutes of magnetic stirring, the acetone was removed under reduced pressure at room temperature. The suspensions were filtered through 0.8 μm hydrophilic syringe filter (Corning, Part no. 431221, Fisher Scientific Co., Pittsburgh, PA, USA), dialyzed in cellulose membrane tube (Sigma, code D9777) overnight in distilled water and stored at 4°C. As control, nanospheres were prepared with 25 mg of α-tocopherol or 25 mg of ALA_2TriEG (Figure 1(a)) in the absence of NSAID derivatives using the same procedure as described above. The α-lipopic acid-containing compound ALA_2TriEG was synthesized and characterized as described previously [39].

2.2. Size Measurements. The hydrodynamic size measurement and size distribution of the nanoprodrugs were performed by the dynamic light scattering (DLS) using a Coulter N4-Plus Submicron Particle Sizer (Coulter Corporation, Miami, FL, USA) as described [38, 39]. For each preparation mean diameter and mean polydispersity index (P.I.) of three determinations were calculated. The error bar (S.D.) was calculated from triplicate determinations.

2.3. Stability of NSAID Nanoprodrugs during Long Term Storage. The stability of the nanoprodrugs was assessed by measuring the size and concentrations of prodrug molecules of NSAIDs after a 3-month storage at 4°C. The size of the nanoprodrugs was measured as described above (Section 2.2) and the changes were calculated as follows:

$$\text{Size \% of control} = \left(\frac{\text{Size}_{t=0}}{\text{Size}_{t=3\,\text{mo}}}\right) \times 100, \quad (1)$$

where $\text{Size}_{t=0}$ is the nanoprodrug size immediately after dialysis and $\text{Size}_{t=3\,\text{mo}}$ is the size after 3-month storage at 4°C. The amount of intact NSAIDs prodrugs was assessed by RP-HPLC as follows: the suspensions of nanoprodrugs (100 μL) were added to acetonitrile (400 μL) and analyzed using RP-HPLC as described [38]. The recovery yield was calculated as follows:

$$\text{Recovery yield (\%)} = \frac{\text{Amount of prodrugs after incubation}}{\text{Amount of prodrugs before incubation}} \times 100. \quad (2)$$

The error bar (S.D.) was calculated from triplicate determinations.

2.4. Enzymatic Hydrolysis of NSAID Nanoprodrugs. The nanoprodrugs were suspended in phosphate buffered saline (PBS, pH 7.4) to give the final concentration of 500 μM NSAID derivatives. Esterase (porcine liver, Sigma, code E3019) was added to the final concentration of 5 U/mL and the mixture was incubated for 1 hour in a water bath at 37°C.

To determine the amount of enzymatically hydrolyzed NSAIDs, samples were centrifuged for 10 minutes at 20,000 $\times$ g and the supernatants were analyzed by RP-HPLC using a C_{18} reversed phase column as described [38].

The error bar (S.D.) was calculated from triplicate determinations.

2.5. Maintenance of Cell Line. The U87-MG human glioma cell line was obtained from American Type Culture Collection (ATCC, Bethesda, MD, USA). The cells were grown and maintained in Minium Essential Medium (MEM, Invitrogen) containing antibiotics penicillin (100 U/mL) and streptomycin (100 μg/mL) and supplemented with 10% fetal bovine serum (FBS, Invitrogen). Cells were grown at 37°C at an atmosphere of 5% CO_2 in humidified air.

2.6. Cell Counting. The glioma cells were seeded at 10^5 cells per well in 6-well plates and grown for 24 hours. The cells were treated with NSAID nanoprodrugs for 3 days. After treatment, the culture medium was removed and cells were washed with PBS. 0.5 mL of 0.25% Trypsin/EDTA was added to each well and the detached cells were counted immediately in a hemocytometer. The antiproliferative effect of the nanoprodrugs was presented as a cell number % of control, which was calculated as follows:

$$\text{Cell number \% of control} = \left(\frac{\text{Cell number}_{\text{treated}}}{\text{Cell number}_{\text{control}}}\right) \times 100, \quad (3)$$

where $\text{Cell number}_{\text{treated}}$ is the number of cells after treatment with nanoprodrugs and $\text{Cell number}_{\text{control}}$ is the number of cells of control culture which was incubated with culture medium only. The cells were also treated with nanospheres prepared from α-tocopherol or ALA_2TriEG only. The error bar (S.D.) was calculated from triplicate determinations.

2.7. Assessment of Cell Viability Using Regent WST-1. The effect of the nanoprodrugs on the cell proliferation was quantified using the cell proliferation reagent WST-1(water-soluble tetrazolium salt) colorimetric assay (Boehringer Mannheim) according to the manufacturer's instructions. Nanoprodrugs were prepared from the monomeric derivative Ibu_2TEG or dimeric derivative Ibu-TEG-ALA (Figure 1). Ibuprofen was prepared as a 100 mM solution in DMSO. The human glioma cells were seeded on a 96-well microtiter plate at 2×10^3 cells/well for 24 hours. The cells were treated with drugs at a final concentration ranging from 10 to 100 μM for nanoprodrugs and 50 to 400 μM for ibuprofen. After 72 hours of treatment, culture medium containing the drugs was removed, cells were washed with 200 μL of PBS, and 90 μL of culture medium and 10 μL of WST-1 solution were added to each well. Cells were incubated at 37°C for 1–4 hours, and the absorbance was read by an ELISA plate reader at 450 nm. The cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \left(\frac{\text{Abs}_s}{\text{Abs}_c}\right) \times 100, \quad (4)$$

where Abs_s is the absorbance of cells treated with drugs and Abs_c is the absorbance of control cells incubated with

Table 1: Size and polydispersity index (P.I.) of the nanoprodrugs ($n = 3$, ±S.D.).

NSAIDs derivatives	Size (nm)	P.I.
ALA-TEG-Ind	149 ± 1 (253 ± 25)*	0.12 ± 0.02
ALA-TEG-Ibu	149 ± 15 (251 ± 13)*	0.09 ± 0.04
ALA-TEG-Npx	147 ± 6 (298 ± 6)*	0.11 ± 0.02
Ind_2TEG	140 ± 8 (159 ± 10)*	0.11 ± 0.04
Ibu_2TEG	141 ± 11 (186 ± 11)*	0.10 ± 0.02
Npx_2TEG	148 ± 1 (259 ± 9)*	0.06 ± 0.02

*Size of the nanoprodrugs in the absence of α-tocopherol [38]. ALA: α-lipoic acid; Ind: indomethacin; Ibu: ibuprofen; Npx: naproxen; TEG: tetraethylene glycol.

cell culture medium only. The cells were also treated with nanospheres prepared from α-tocopherol or ALA_2TriEG only. The error bar (S.D.) was calculated from triplicate determinations.

2.8. Propidium Iodide Assay. The glioma cells were treated with ibuprofen nanoprodrugs for 3 days. The cells were also treated with free ibuprofen and nanospheres prepared from α-tocopherol or ALA_2TriEG only. After treatment, the cells were incubated with 5 μM of propidium iodide (PI) (Sigma) for 1 hour. PI fluorescence was excited at 515–600 nm using an inverted microscope fitted with a standard rhodamine filter. Images were taken using a digital camera connected to the microscope.

2.9. Uptake of Ibu_2TEG Nanoprodrug and Ibuprofen by Glioma Cells. The glioma cells were plated in 75 cm^2 culture flasks containing 20 mL cell culture medium and grown up to approximate 70% confluent density. Cells were treated with 100 μM of Ibu_2TEG nanoprodrug suspension or ibuprofen dissolved in DMSO for 24 hours. Treated cells were washed three times with PBS to remove the drugs, and adherent cells were trypsinized. The cells were collected by centrifugation at 1,500 × g and the recovered pellets were washed three time with PBS by repeated resuspendeing and centrifugation. In order to determine the content of ibuprofen, cells were disrupted in 0.5 mL of lysis buffer (1% of Triton X-100, 10 mM Tris-HCl, pH 4.7) and cell debris was removed by centrifugation for 10 minutes at 10,000 × g and 25°C. The resulting supernatant was collected and frozen at −20°C. In order to determine the content of nanoprodrugs, 2 mL of acetonitrile was added to the cell lysates and the cell debris was removed by centrifugation for 10 minutes at 10,000 × g and 25°C. The supernatant was collected for analysis. The content of Ibu_2TEG nanoprodrug and ibuprofen was determined from the supernatants as described previously using RP-HPLC [38].

2.10. Statistical Analysis. The results were analyzed and expressed as mean ± standard deviation (S.D.). Statistical analysis of the results was carried out using Student's t-test. For all tests, differences with a $P < .05$ were considered to be significant.

3. Results and Discussion

3.1. Preparation of Nanoprodrugs of NSAIDs. In order to combine the concept of NSAIDs prodrug and nanostructured drug/drug delivery system, we have developed nanometer-sized prodrugs (nanoprodrugs) of NSAIDs [38]. Many favorable properties of nanostructured biomaterials have been characterized in respect to their applicability as a drug carrier. One of the most remarkable properties is their improved bioavailability which is attributed to an enlarged surface area by transformation of bulk materials into the nanometer-sized structures, leading to an enhanced biological efficacy of the materials [34, 40]. These properties of nanostructured biomaterials have been especially crucial for the development of nanoprodrugs based on the formation of nanostructures using the spontaneous emulsification method. This is because only water-insoluble hydrophobic prodrug molecules can be formed into nanometer-sized structures which is stable for a prolonged period of time in an aqueous biological environment, and in the other hand, the enzymatic activation of the hydrophobic prodrugs would be otherwise impossible due to the insolubility of the prodrugs in aqueous media.

Thus, the formation into the nanoprodrugs with an increased surface-to-volume ratio may improve the bioavailability and biological efficacy of the hydrophobic prodrug molecules by facilitating the interaction between hydrolytic enzymes and prodrugs [34, 40].

The hydrophobic derivatives of NSAIDs (Figure 1(a) and 1(b)) in organic solvents spontaneously formed into nanoprodrugs upon the addition into an aqueous solution containing hydrophilic surfactants by a spontaneous emulsification process [41–43].

The size and stability of nanoprodrugs depends on multiple factors, such as the nature and concentration of the compounds, the surfactants, and the ratio of organic solvent to water [42–44]. In this study, formulation parameters were kept the same as described [38] except for the addition of α-tocopherol (Section 2.1).

The hydrodynamic size was within the range of 140 and 150 nm and highly reproducible (Table 1). The size of the nanoprodrugs was significantly smaller when compared with the size of nanoprodrugs prepared without the addition of α-tocopherol [38].

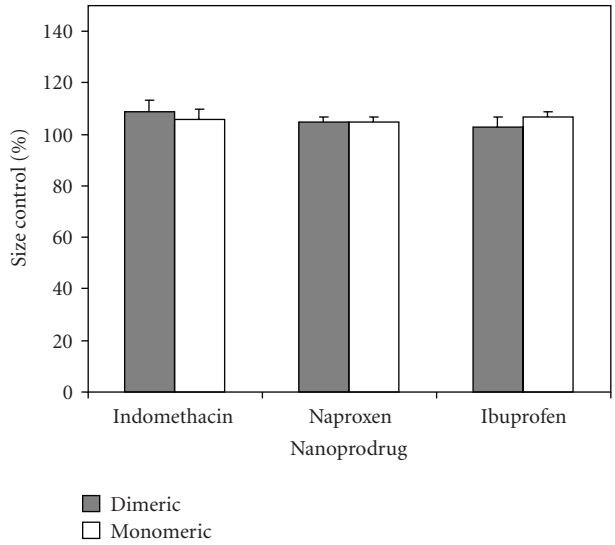

Figure 2: Long term stability of nanoprodrugs. Dimeric nanoprodrugs: Ind_2TEG, Npx_2TEG and Ibu_2TEG; Monomeric nanoprodrugs: Ind-TEG-ALA, Npx-TEG-ALA, and Ibu-TEG-ALA.

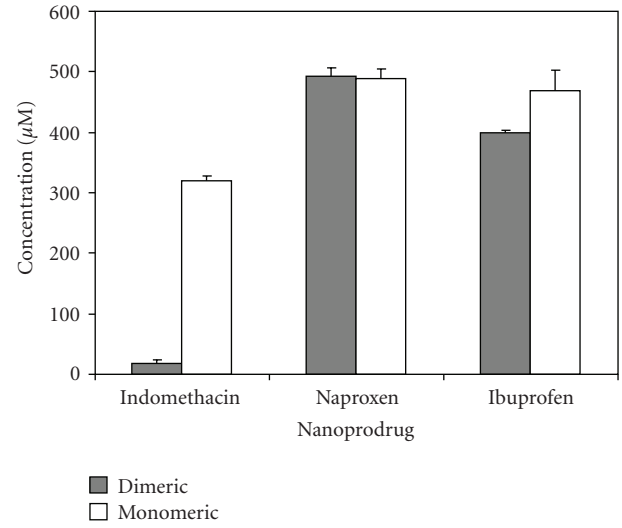

Figure 3: Enymatic hydrolysis of NSAID nanoprodrugs at 37°C.

Moreover, practically no differences in the size were observed between the nanoprodrugs of different NSAIDs when supplemented with α-tocopherol. This is especially important when the therapeutic efficiacy of different nanoprodrugs are compared. When the compositons of the nanoprodrugs are the same except for the active drug compounds and the size varies within a close range, the observed differences in the efficacy can be attributed directly to the different prodrug molecules involved.

The stability of the nanoprodrugs was assessed by measuring the size and contents of the intact prodrug molecules of NSAIDs after a 3-month storage at 4°C. In this study, the size of all the nanoprodrugs remained almost unchanged (Figure 2) and also no decreases were observed in the amount of the available prodrug molecules after the 3-month storage (data not shown). The observed chemical and physical stability of the nanoprodrugs may be ascribed to the strong assembly of the hydrophobic prodrug molecules and α-tocopherol which further reduces the interaction with water and increases the structural integrity of the nanoprodrugs.

3.2. Enzymatic Hydrolysis of Nanoprodrugs. In order to assess the differences in the rates of prodrug activation from the NSAID nanoprodrugs, the rate of enzymatic reconversion of the prodrugs into the parent drugs was investigated in vitro with porcine liver esterase. According to the molecular design based on ester bonds, the NSAID prodrug molecules were expected to be degraded by enzymatic ester hydrolysis. As shown in our previous investigation at room temperature [38], a different rate of enzymatic hydrolysis were observed, which was attributed to the different structures of the prodrug molecules. It has been shown that the indomethacin nanoprodrugs were more stable compared with the naproxen and ibuprofen nanoprodrugs, and the nanoprodrugs from dimeric Ind_2TEG, Npx_2TEG and Ibu_2TEG were more stable when compared with the nanoprodrugs from monomeric Ind-TEG-ALA, Npx-TEG-ALA and Ibu-TEG-ALA. As shown in Figure 3, at an elevated temperature of 37°C, the differences in the hydrolysis rates between the monomeric and dimeric nanoprodrugs were completely disappeared (naproxen), drastically diminished (ibuprofen) or retained (indomethacin). Notably, after a-1 hour incubation at 37°C, approximately 65% of drug was released from the nanoprodrug of Ind-TEG-ALA compared with 39% at room temperature [38], whereas only 6% was released from the nanoprodrug of Ind_2TEG, presumably due to the effect of the bulkier indomethacin and the replacement of one indomethacin with ALA on the enymatic hydrolysis rate [38].

3.3. Effect of NSAID Nanoprodrugs on Growth of Glioma Cells. In order to evaluate the effect of NSAID nanoprodrugs on tumor cell growth, we studied the effect of the NSAID nanoprodrugs on the cell growth of U87-MG glioma cells. Glioma cells were treated with six nanoprodrugs (10, 25, 50 and 100 μM) for three days (Section 2.6). Cells were also treated with nanospheres prepared from α-tocopherol or ALA_2TriEG only by exposing to an equimolar concentration of α-tocopherol or ALA unit.

As shown in Figure 4, the nanoprodrugs of ibuprofen were more potent at reducing the cell proliferation in comparison with the nanoprodrugs of indomethacin or naproxen. The concentration of 25 and 50 μM nanoprodrugs of Ibu_2TEG and Ibu-TEG-ALA, respectively, were sufficient to inhibit growth of the glioma cells. In addition, comparing the two ibuprofen nanoprodrugs, the nanoprodrug of dimeric Ibu_2TEG was more potent (Figure 4(a)). In Section 3.2, we showed that more than 80% of prodrugs were hydrolyzed from the nanoprodrugs of Ibu_2TEG and Ibu-TEG-ALA (Figure 3), suggesting that the observed difference may not be due to the slightly different rate of enzymatic prodrug activation. On the other hand, the indomethacin nanoprodrugs were able to cause a significant effect only in the concentration of 100 μM or higher (Figure 4(c)). The

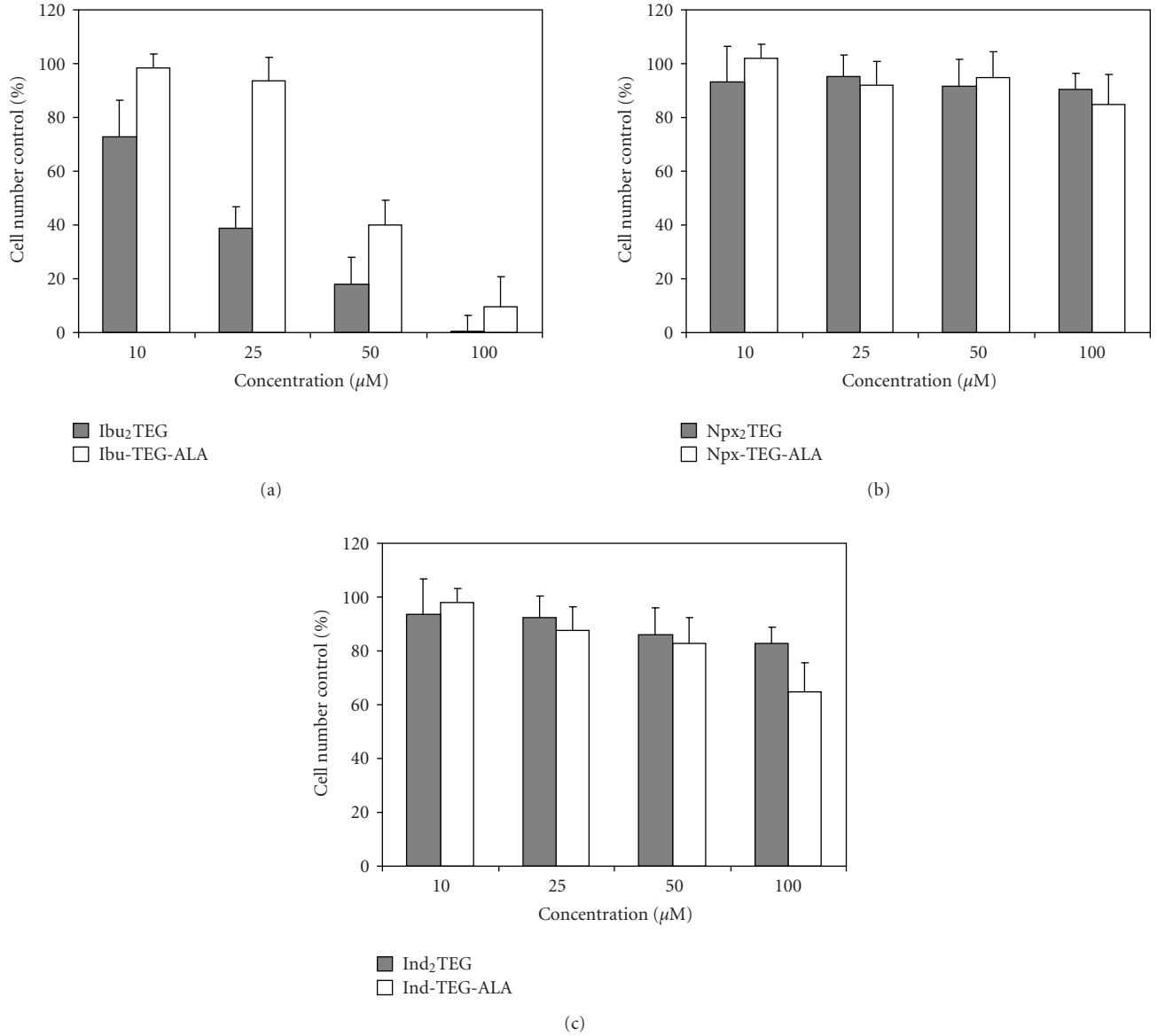

Figure 4: Effect of NSAID nanoprodrugs on glioma cell proliferation.

treatment with naproxen nanoprodrugs did not show any significant effect on cell proliferation (Figure 4(b)).

It is interesting to note that the nanoprodrug of the monomeric Ind-TEG-ALA was more potent than the nanoprodrug of dimeric Ind_2TEG ($P < .05$ at 100 μM each) (Figure 4(c)), which is in contrast to the ibuprofen nanoprodrugs. Considering the rate of the enzymatic hydrolysis of Ind-TEG-ALA and Ind_2TEG (Section 3.2), the more potent antiproliferative effect of the nanoprodrug Ind-TEG-ALA can be attributed to the more efficient prodrug activation. The treatment with control nanoprodrugs prepared from α-tocopherol or ALA_2TriEG only did not show any effect on the cell proliferation (data not shown).

3.4. Effect of Ibuprofen Nanoprodrug on Cell Viability. The WST-1 assay is based on the formation a water-soluble formazan crystal, which directly correlates to the number of viable cells with active mitochondrial dehydrogenases. In order to evaluate the effect of ibuprofen nanoprodrugs on tumor cell viability, U87-MG glioma cells were treated with the ibuprofen nanoprodrugs (10, 25, 50, and 100 μM) for three days (Section 2.7). Cells were also treated with nanospheres prepared from α-tocopherol or ALA_2TriEG only by exposing to an equimolar concentration of α-tocopherol or ALA unit. Similar to the results from Section 3.3, the nanoprodrug from the dimeric Ibu_2TEG were more potent than the nanoprodrug from Ibu-TEG-ALA (Figure 5(a)). The IC_{50} values were 25 and 47 μM for the Ibu_2TEG and Ibu-TEG-ALA nanoprodrugs, respectively. In Section 3.2, we showed that more than 80% of prodrugs were hydrolyzed from the nanoprodrugs of Ibu_2TEG and Ibu-TEG-ALA after 1 hour incubation at 37°C, suggesting that the observed difference may not be due to the slightly different rate of enzymatic prodrug activation.

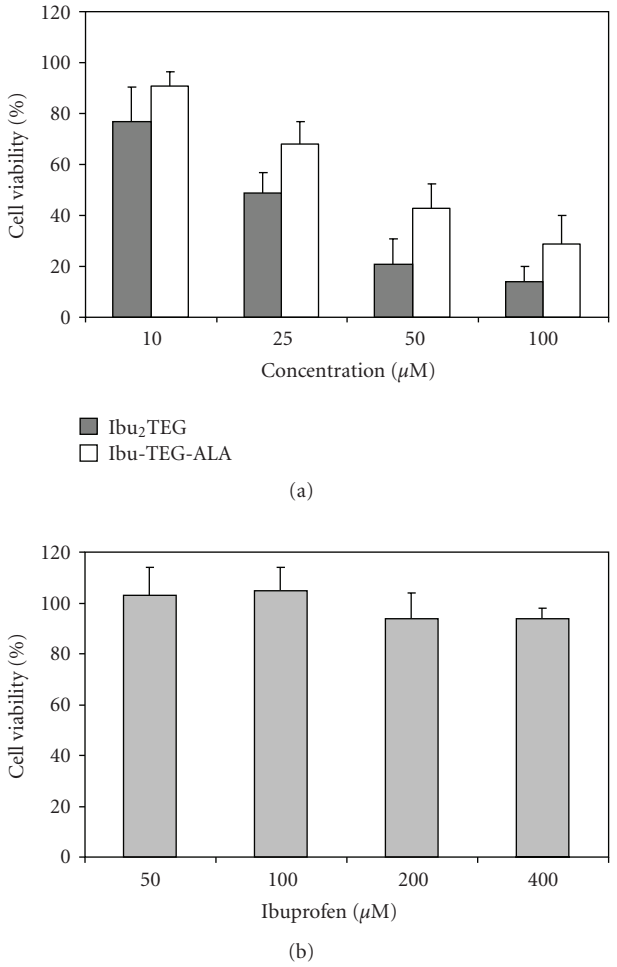

FIGURE 5: Effect of ibuprofen nanoprodrugs (a) and ibuprofen (b) on the viability of glioma cells.

Obviously, the more potent effect of the dimeric nanoprodug can be ascribed to the higher parent drug concentration wherein the total amount of ibuprofen available from the nanoprodrug of Ibu_2TEG is twice as much as that available from the Ibu-TEG-ALA nanoprodrug. Again, the nanoprodrugs prepared from α-tocopherol or ALA_2TriEG only did not show any effect on the cell viability (data not shown). More notably, the treatment with free ibuprofen did not show any significant effect on the cell proliferation even with a higher concentration of 400 μM (Figure 5(b)).

3.5. Cytotoxic Effect of Ibuprofen Nanoprodrug on Glioma Cells. In order to demonstrate that the NSAID nanoprodrugs induce cell death, glioma cells were treated for three days with ibuprofen nanoprodrugs which were found to have a potent effect on growth inhibition (Sections 3.3 and 3.4) and incubated with propidium iodide (PI). PI incorporated into the dead cells, binds to DNA and becomes fluorescent [45]. Figure 6 shows representative images of U87-MG glioma cells treated with 50 μM (c) and 100 μM (d) of Ibu_2TEG nanoprodrgs, 100 μM of Ibu-TEG-ALA nanoprodrugs and 200 μM of ibuprofen (f). The treatment of the glioma cells with the nanoprodrugs resulted in a

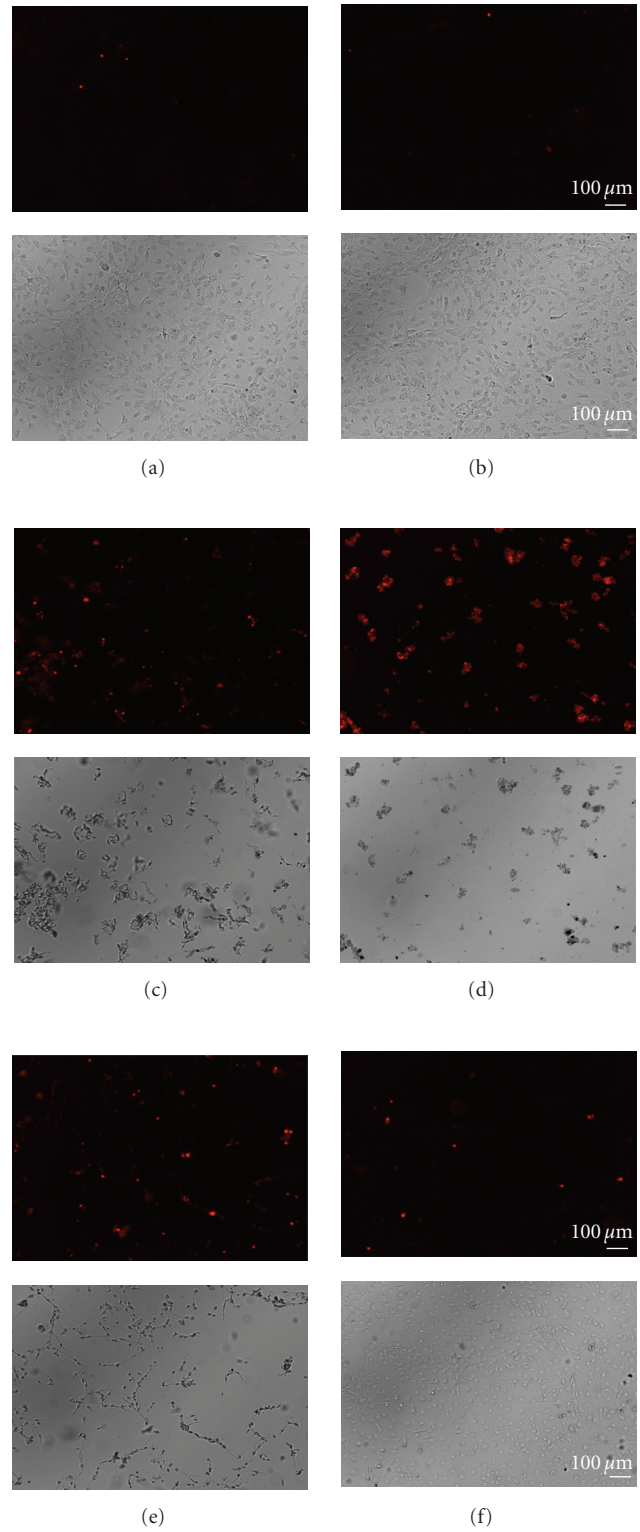

FIGURE 6: Effect of nanoprodrugs on cell death in U87-MG glioma cells by propium iodide incorporation. Representative pictures of: control cultures (a); cells treated with control nanoprodrug from α-tocopherol (b); cells treated with 50 μM (c) and 100 μM (d) nanoprodrug from Ibu2TEG; cells were treated with 100 μM nanoprodrug from Ibu-TEG-ALA (e); cells treated with 200 μM ibuprofen (f). Panels above (a), (b), (c), (d), (e), and (f) are correspondent contrast phase photomicrographs.

significant reduction in the cell number and induced cell death with significant PI incorporation, whereas ibuprofen did not show any effect.

3.6. Cellular Accumulation of Ibu_2TEG Nanoprodrug. In order to investigate the relationship between the cytotoxicity and the drug concentration in cell, the cellular uptake of Ibu_2TEG nanoprodrug and free ibuprofen were determined in U87-MG glioma cells after a 24-hour exposure to equimolar concentration of ibuprofen and Ibu_2TEG nanoprodrug (100 μM) corresponding to a total amount of 2 μmol of ibuprofen or Ibu_2TEG nanoprodrug per flask containing 20 mL of cell culture medium (Section 2.9). Cell lysate was prepared from the cells harvested from one 75 cm^2-flask which contained 5×10^6–6×10^6 cells/flask. The content of Ibu_2TEG found in the cell lysate was 88 nmol approximately corresponding to 4.4% of the initially added nanoprodrugs. No ibuprofen was detected in the cell lysates. These findings confirmed the previous assumption that the availability of parent drugs may be the crucial factor for the efficacy of the nanoprodrugs. The higher intracellular parent drug concentration can be achieved by a combination of efficient cellular uptake of the nanoprodrugs and prodrugs activation from the nanoprodrugs. The underlying mechanisms of the cellular uptake of the nanoprodrugs are under investigation.

As demonstrated in this study, water-insoluble drug compounds can be transformed into stable nanostructures obviating the need to dissolve the compounds in excessive amount of cosolvents and thus eliminating the interference of toxic side effects caused by cosolvents [46, 47]. The formation into the compact nanostructures confers an additional advantage of higher drug loading per volume, which is of crucial importance when high dosing is required. Probably, the most important advantage of the nanostructures in anticancer therapy is their increased accumulation within the tumor tissues, which is attributed to a phenomenon characterized as the enhanced permeability and retention (EPR) effect. The EPR effect was first described by Matsumura and Maeda [48] as a result of differences in tumor neovasculature compared to that of normal tissues. This includes leaky blood vessels and poor lymphatic drainage system. The leaky blood vessels allow the nanostructures penetrate more easily ino the tumor tissues than into the normal tissues. Because of the dysfunctional lymphatic drainage system, the penetrated nanostructures are retained and accumulated in tumors, which allows them to diffuse into the vicinity of the tumor cells. Studies have shown that particles with diameters <200 nm are more effective accumulated in the tumor tissues [49–52]. In this study, we showed that the NSAID nanoprodrugs could be prepared reproduciably in the size range of <150 nm and accumulated in the glioma cells to the amount sufficient to inhibit cell growth and elicit cell death.

4. Conclusion

In this study, nanoprodrugs of NSAIDs were prepared by spontaneous emulsification of hydrophobic prodrugs of NSAIDs and their antiproliferative effect was demonstrated using U87-MG glioma cells. Among the tested three NSAIDs, the nanoprodrugs of ibuprofen inhibited the cell growth most significantly and induced cell death. In contrast to the ibuprofen nanoprodrugs, free ibuprofen did not show any effect on cell growth and viability. In addition, no accumulated ibuprofen was found in the cells, whereas approximately 4.4% of ibuprofen nanoprodrug was recovered from the treated glioma cells suggesting that the superior antiproliferative effect of the nanoprodrugs can be attributed to the enhanced uptake by the cells. We are further investigating the mechanisms of the cellular uptake and the molecular events underlying the antiproliferative effect of the ibuprofen nanoprodrugs.

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