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Research Paper

Farnesylthiosalicylic acid-loaded lipid–polyethylene glycol–polymer hybrid nanoparticles for treatment of glioblastoma

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Keywords
brain tumour; farnesylthiosalicylic acid; glioblastoma; hybrid nanoparticles; intratumoral injection

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Abstract

Objectives We aimed to develop lipid–polyethylene glycol (PEG)–polymer hybrid nanoparticles, which have high affinity to tumour tissue with active ingredient, a new generation antineoplastic drug, farnesylthiosalicylic acid (FTA) for treatment of glioblastoma.

Method Farnesylthiosalicylic acid-loaded poly(lactic-co-glycolic acid)-1,2 distearoyl-glycerol-3-phospho-ethanolamine-N [methoxy (PEG)-2000] ammonium salt (PLGA-DSPE-PEG) with or without 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) hybrid nanoparticles has been prepared and evaluated for in-vitro characterization. Cytotoxicity of FTA-loaded nanoparticles along with its efficacy on rat glioma-2 (RG2) cells was also evaluated both in vitro (in comparison with non-malignant cell line, L929) and in vivo.

Key findings Scanning electron microscopy studies showed that all formulations prepared had smooth surface and spherical in shape. FTA and FTA-loaded nanoparticles have cytotoxic activity against RG2 glioma cell lines in cell culture studies, which further increases with addition of DOTAP. Magnetic resonance imaging and histopathologic evaluation on RG2 tumour cells in rat glioma model (49 female Wistar rats, 250–300 g) comparing intravenous and intratumoral injections of the drug have been performed and FTA-loaded nanoparticles reduced tumour size significantly in in-vivo studies, with higher efficiency of intratumoral administration than intravenous route.

Conclusion Farnesylthiosalicylic acid-loaded PLGA-DSPE-PEG-DOTAP hybrid nanoparticles are proven to be effective against glioblastoma in both in-vitro and in-vivo experiments.

Introduction

Glioblastoma is the most malignant and the most aggressive primary brain tumour and comprises more than 50% of glial tumours and 16% of all primary brain tumours.[1] Current treatment approaches for glioblastoma commonly include surgery, radiotherapy and chemotherapy.[2,3] Even after complete surgical resection, infiltrative tumour cells around surgical resection cavity inevitably cause the recurrence of glioblastoma.[4] Radiotherapy and chemotherapy are usually applied after surgical treatment.[5]

Blood–brain barrier is the main reason for the failure of antineoplastic drugs in treating the brain tumours. To obtain effective results from chemotherapy, high doses of antineoplastic drugs are needed, causing damage in healthy tissue along with tumour tissue and lead to unacceptable, fatal toxicity.[6,7] Therefore, despite recent advances in

multiple therapeutic approaches, the median survival of patients with glioblastoma is around 14 months.[8] For more effective treatment of glioblastoma, drug delivery systems that bring high/desired concentration of antineoplastic drugs to tumour tissue without causing any systematic damage are in great need.

Nanoparticles have particle sizes of 10–1000 nm, and they provide good penetration of the therapeutic and diagnostic agents to target tissues, and extended and controlled release of drugs.[9–12] Several studies have shown that polymer-based nanoparticles are successful in delivering the chemotherapeutic agents to the brain tumours.[13–16] Furthermore, enhancing the surface properties of nanoparticles by targeting or coating materials or combining different properties of nanostructures in one nanoparticulate system such as hybrid nanoparticles may be able to deliver a higher amount of drugs selectively to tumour sites and improve the efficacy of existing treatment of brain tumour.[17–21] Hybrid nanoparticles are formed by a hydrophobic polymeric core and a hydrophilic polymeric shell separated by a lipid monolayer. They integrate the advantages of both liposomes and polymeric nanoparticles, which are the most popular drug delivery vehicles approved for clinical use.[22]

In this study, poly(lactic-co-glycolic acid) (PLGA) and 1,2-distearoyl-glycerol-3-phosphoethanolamine-N [methoxy (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG) were used to form the hybrid nanoparticles. PLGA is a Food and Drug Administration-approved polymer, and its biocompatibility and biodegradability have been demonstrated in brain.[23] DSPE-PEG, a PEG-modified lipid, increases glioma-specific drug accumulation providing longer blood circulation time of micelle formulation.[24,25] 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) has been shown to exhibit a high affinity to negatively charged cell membrane compounds and increases the internalization and presentation of the drug by molecular, passive or electrostatic interactions.[26] We used DOTAP in some formulations to evaluate the influence of cationic charge on efficacy of nanoparticles. By collecting these functional materials in same formulation, we developed a hybrid nanoparticle which can be used for both intratumoral and intravenous applications. Thus, in this study, two routes of administration can also be compared for developed formulation to determine the most efficient approach for the treatment of brain tumours.

Farnesylthiosalicylic acid (Salirasib, FTA), a new generation antineoplastic agent, is a specific inhibitor of Ras protein, which presents in high rate in most of malignant tumours.[27] Although it was shown to be effective against brain tumours in in-vitro cell culture studies,[27] there is limited number of in-vivo studies.[28] FTA-loaded nanoparticles have not been studied so far. As FTA cannot cross blood–brain barrier,[29] development of FTA-loaded lipid–PEG–polymer hybrid nanoparticles may be an alternative and promising treatment approach for glioblastomas.

In the light of available information, we aimed to develop PLGA-DSPE-PEG hybrid nanoparticles with or without DOTAP, which have high affinity to brain tumour tissue and provide extended and controlled release of drug, FTA. Additionally, we aimed to determine in-vitro characterization and cytotoxicity of FTA-loaded nanoparticles along with their efficacy on rat glioma-2 (RG2) tumour model in Wistar rats comparing intratumoral and intravenous routes of administration.

Materials and Methods

Materials

Farnesylthiosalicylic acid was purchased from Concordia Pharmaceuticals Inc. (Ft. Lauderdale, FL, USA). Resomer RG503 (PLGA 50 : 50; MW 24 000–38 000 Da) and Resomer RG 753 S (PLGA 75 : 25; MW 36 610 Da) were purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany). DSPE-PEG (2000), lecithin and DOTAP were provided by Avanti Polar Lipids Inc. (Alabaster, AL, USA). Foetal bovine serum (FBS) and penicillin G sodium–streptomycin sulphate solution were provided by Biochrom (Berlin, Germany). RG2 cell lines and L929 (mouse fibroblast) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). All other chemicals were of high-performance liquid chromatography (HPLC) grade or research grade.

Assay of farnesylthiosalicylic acid

Level of FTA was determined by HPLC according to the modified method of Shimek *et al.*[30] FTA was assayed by reversed-phase liquid chromatography using an Agilent HPLC system (Agilent 1100, Santa Clara, CA, USA) equipped with ultraviolet (UV) detector (322 nm) and a column (Waters Spherisorb S10 ODS2 C18 200 × 4.6 mm Milford, MA, USA). The mobile phase consisted of acetonitrile : water : acetic acid solution (90 : 10 : 0.1, v/v) and was run through the HPLC system at a rate of 1 ml/min at room temperature. The peak area used throughout this study and the chromatographic method were validated by linearity, sensitivity, precision, accuracy and specificity.

Solubility of farnesylthiosalicylic acid

As FTA is insoluble in water, but soluble in organic solvents such as ethanol, dimethyl sulfoxide (DMSO) and dimethylformamide, the solubility of FTA in in-vitro release and cell culture media was studied. Three different media were used

for solubility studies: phosphate-buffered saline (PBS; pH 7.4), PBS containing Tween-20 (0.1%) (TPBS), PBS containing 10% FBS supplemented with 0.1% DMSO (Biochrom). The solutions ($n = 6$) were mixed with 1 mg FTA at 37°C for 12 h (equilibrium time point), filtered through 0.22-μm membrane filters and then analysed with HPLC.

Formulation of nanoparticles

PLGA-DSPE-PEG hybrid nanoparticles were prepared using emulsion sonication method.[31] PLGA (copolymer ratio 50 : 50 or 85 : 15) was dissolved in acetonitrile (2.5 μg/ml) to obtain organic phase. In FTA-loaded formulations, FTA (5%, 10% and 20% (w/w) of polymer amount) was added into organic phase. To obtain water phase, lecithin and DSPE-PEG (and DOTAP for DOTAP-containing nanoparticles) were separately dissolved in 4% (v/v) ethanol and then mixed with deionized water. Two different DSPE-PEG/DOTAP ratios (50 : 50 μM and 75 : 25 μM) were used in DOTAP-containing formulations. This water phase was added into organic phase (ratio, 10 : 1) and sonicated in a capped glass vial for 5 min using a Branson B 220 Smith Kline (Pittsburgh, PA, USA) bath sonicator at a frequency of 42 kHz and power of 100 W. The nanoparticles were centrifuged in filter centrifuge tubes containing a 10-kDa molecular size permeable membrane (Millipore, 10 kDa Billerica, Massachusetts, USA), and was washed three times using PBS (pH 7.4). Nanoparticle dispersions obtained were frozen at −80°C and lyophilized for 24 h.

Studies to characterize nanoparticles

To measure particle size and zeta potentials of nanoparticle formulations ($n = 6$), Malvern Zetasizer Nano Series ZS device (Malvern Instruments Ltd., Worcestershire, UK) was used.

To calculate the amount of FTA in nanoparticle formulations, acetonitrile was added into precisely weighed nanoparticles to extract FTA by breaking down the nanoparticle structure of the organic solvent. The solutions were ($n = 6$) filtered through 0.2-μm membrane filters and was injected into HPLC column.

The surface characteristics of nanoparticles were determined by scanning electron microscopy (SEM) and atomic force microscopy (AFM). For both evaluations, samples were fixed on metal plates by two-sided adhesive tape. For SEM, samples were coated with 100-A°-thick gold in brand coating device (Bio-Rad Laboratories Inc., Hercules, CA, USA). Following the coating process, samples were evaluated by SEM (Nova™ NanoSEM 430; FEI, Muntinlupa City, Philippines, USA). For AFM, nanoparticles were visualized with Q-Scope TM 350 Multimode AFM (Ambios Technology, Santa Cruz, CA, USA).

In-vitro FTA release test was performed with dialysis bag method.[32] Nanoparticles ($n = 6$) were dispersed in TPBS, medium with highest FTA solubility, and placed inside the dialysis membrane with pore diameter of 2000 Da. The dialysis bag was fitted into the glass tube containing 3 ml release media. The apparatus was fully immersed in a water bath which was stirred magnetically at 100 rpm (Heidolph, Schwabach, Germany) and kept at 37°C. The tubes were prepared individually for each sample collection. At predetermined time intervals, the whole release medium was withdrawn, and the drug content of the samples was determined by the previously described HPLC method and experiments continued until FTA release ended.

Cell culture studies

To evaluate in-vitro bioactivity of the nanoparticles, cell culture studies were performed using RG2 cell line, which is one of the most similar cell groups to human glioma cells. Also, L929 (mouse fibroblast) cells were used as non-malignant control cells.

The medium for RG2 cells and L929 cells was prepared by adding FBS, penicillin–streptomycin and L-glutamine to the Dulbecco's modified Eagle's medium (DMEM) to have the final concentration of 10% v/v, 50 U/ml–50 μg/ml and 0.002 mM/ml, respectively.

A 100-μl cell suspension (RG2 or L929 cells) containing 5×10^3 cells was introduced into each of the 96-well, flat-bottomed plates. Cells were kept overnight in the incubator to allow them to attach to the wells. In the following day, cells were treated with formulations ($n = 6$) (Table 1) and kept in the incubator for 72–120 h. WST-1 [sodium 5-[2,4-disulfophenyl]-2-[4-iodophenyl]-3-[4-nitrophenyl]-2H-tetrazolium inner salt) (Clontech Laboratories Inc., Mountain View, CA, USA) test was used for cell viability measurements of nanoparticle formulations.

As this is the first study evaluating the cytotoxic effects of FTA on RG2 cells, lethal concentration (LD50) value was also determined using different concentrations of FTA vs 0.1% DMSO in complete medium as control.

Poly(lactic-co-glycolic acid) 50 : 50 formulations with DOTAP were chosen for cell culture experiments due to their higher drug loading values comparing to those for PLGA 85 : 15 formulations (see Table 2). Also to investigate the absence of DOTAP in formulations on efficacy of nanoparticles, PLGA 50 : 50 formulations without DOTAP were included the experiments with RG2 cells. To evaluate the cellular uptake of nanoparticle formulations, fluorescent marker was loaded on the nanoparticles and treated with RG2 cells.[33] The samples were then visualized under fluorescent microscopy in Hacettepe University Brain Research Laboratory.

Table 1 Particle size and zeta potential of hybrid nanoparticle formulations ($n = 6$)

Nanoparticle formulation	Particle size (nm)[a]	Zeta potential (mV)[b]
1. Without FTA		
1a. 50 : 50 PLGA/DSPE-PEG	94.0 ± 2.3	−28.4 ± 2.0
1b. 85 : 15 PLGA/DSPE-PEG	102.0 ± 2.0	−30.1 ± 1.4
1c. 50 : 50 PLGA/DSPE-PEG with DOTAP*	120.0 ± 3.4	−11.1 ± 1.1
1d. 85 : 15 PLGA/DSPE-PEG with DOTAP*	145.0 ± 2.5	−13.0 ± 1.2
2. 5% FTA-loaded		
2a. 50 : 50 PLGA/DSPE-PEG	121.0 ± 3.2	−30.2 ± 2.0
2b. 85 : 15 PLGA/DSPE-PEG	147.0 ± 0.7	−32.4 ± 2.3
2c. 50 : 50 PLGA/DSPE-PEG with DOTAP*	127.0 ± 2.0	−11.2 ± 1.2
2d. 85 : 15 PLGA/DSPE-PEG with DOTAP*	155.0 ± 2.7	−13.0 ± 1.5

FTA, farnesylthiosalicylic acid; PLGA, poly(lactic-co-glycolic acid); DSPE-PEG, 1,2 distearoyl-glycerol-3-phospho-ethanolamine-N [methoxy (PEG)-2000] ammonium salt; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane. Data are given as mean ± standard deviation. The polydispersity index values were below 0.25 for all the formulations. [a]There was no significant statistical difference for 1a vs 1b, 1c vs 1d, 2a vs 2b, 2c vs 2d ($P > 0.01$), but significant difference for 1a vs 2a, 1b vs 2b, 1c vs 2c, 1d vs 2d ($P < 0.01$). [b]There was no significant statistical difference for 1a vs 2a, 1b vs 2b, 1c vs 2c, 1d vs 2d ($P > 0.01$), but *significant difference between 1a vs 1c, 1b vs 1d, 2a vs 2c, 2b vs 2d ($P < 0.01$). (DSPE-PEG: DOTAP (75 : 25 μM)).

In-vivo studies

Animals

The in-vivo study protocol was approved by Hacettepe University Animal Experiments Local Ethics Committee (No. 2011/68-4). Forty-nine adult female Wistar rats (250–300 g) were housed in a standard animal facility under controlled temperature, humidity with a regular lighting schedule of 12-h light : 12-h dark and fasted overnight, and however were permitted free access to water before the experiment. All experiments were carried out in accordance with institutional guidelines.

Rats were anesthetized with intraperitoneal 0.75–1.5 ml/kg ketamin (100 mg/ml) and xylazin (100 mg/ml) mixture (with 2 : 1 ratio). Body temperature was continuously monitored by a rectal probe and maintained at 37.0 ± 1°C with a thermal pat.

Tumour cell implantation

For induction of brain tumour in rats, the modified method of Yemisci *et al.*[34] and Geletneky *et al.*[35] was used. RG2 cell suspension was added into DMEM, and centrifuged at 1000*g* for 5 min, and cells were counted under microscopy. Samples were inoculated to obtain 50×10^4 RG2 cells/5 μl. Rats were placed in stereotaxic instrument (Lab Standard Stereotaxic; Stoelting, Chicago, IL, USA). The scalp was shaved, and 5 μl cell suspension containing 50×10^4 RG2 cells was injected with Hamilton glass syringe (Hamilton 32G, Bonaduz, GR, Switzerland) unilaterally into the right striatum with coordinates of anteroposterior −0.5 mm, lateral +3.0 mm and ventral −6 mm.

After RG2 cell implantation, rats were followed up for daily activity and general well-being, and those who did not show interest to their environment, not respond to external stimuli, not self-fed and with red eye were sacrificed.

Magnetic resonance imaging

To determine the presence and size of tumour, magnetic resonance (MR) imaging which is commonly used technique for in-vivo studies[36,37] was performed with 3 Tesla MR unit (Magnetom Trio; Siemens Healthcare, Erlangen, Germany; slew rate, 200 mT/m per ms; maximum amplitude, 45 mT/m) in National Magnetic Resonance Research Center (UMRAM).

For imaging of the rats, a handmade 8-channel receive-only phase array surface coil (with circular loops elements

Table 2 Drug loading (%) and entrapment efficiency (%) values of hybrid nanoparticle formulations ($n = 6$)

Nanoparticle formulation	The amount of FTA loaded (μg)	Loading percentage (%)	Loading efficiency (%)	*P* values for loading efficiency
1. Without DOTAP				
1a. 50 : 50 PLGA/DSPE-PEG loaded with 5% FTA	7.0 ± 1.9	1.52 ± 0.01	21.0 ± 2.0	<0.01
1b. 85 : 15 PLGA/DSPE-PEG loaded with 5% FTA	9.0 ± 2.0	1.76 ± 0.01	28.0 ± 1.2	
2. With DOTAP				
2a. 50 : 50 PLGA/DSPE-PEG/DOTAP (50 : 50 μM)	12.0 ± 1.1	2.60 ± 0.01	24.0 ± 1.3	>0.01
2b. 85 : 15 PLGA/DSPE-PEG/DOTAP (50 : 50 μM)	9.3 ± 2.0	2.10 ± 0.01	19.0 ± 1.6	
2c. 50 : 50 PLGA/DSPE-PEG/DOTAP (75 : 25 μM)	25.0 ± 1.6	5.10 ± 0.03	60.0 ± 1.2	<0.01
2d. 85 : 15 PLGA/DSPE-PEG/DOTAP (75 : 25 μM)	14.6 ± 1.4	2.80 ± 0.02	29.0 ± 1.1	

FTA, farnesylthiosalicylic acid; PLGA, poly(lactic-co-glycolic acid); DSPE-PEG, 1,2 distearoyl-glycerol-3-phospho-ethanolamine-N [methoxy (PEG)-2000] ammonium salt; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane. Data are given as mean ± standard deviation.

of 3.5 cm diameter) was developed and placed on a semi-cylinder body to support the rat body. Each element of the coil was tuned with four nonmagnetic capacitors and was decoupled by both capacitive and overlapping decoupling techniques. The gain and noise figures of the used low-noise amplifiers are 20 dB and <1 dB. Preamplifier decoupling was also implemented.

The first MR examination was performed 10 days after tumour cell implantation with the rats anesthetized. T2-weighted turbo spin-echo imaging was obtained parallel to the long axis of the rat in prone position (coronal plane) using the following parameters: repetition time/echo time: 4420/94; field of view: 78 × 78 mm; slice thickness: 2 mm; interslice gap: 0; and number of excitations: 5. The images were evaluated for the presence and size of tumour by an experienced neuroradiologist (K.K.O).

Treatment groups

After measuring of tumour sizes by MR, rats with brain tumour between 15 and 30 mm^2 were included in further treatment study.

Based on the chemical characterization and in-vitro studies, PLGA 50 : 50 DSPE-PEG-DOTAP loaded with 100 μM FTA, which has the highest drug loading value, longest drug release and highest cytotoxicity against RG2 cells, was selected for in-vivo studies.

The volume of drug solution or nanoparticle dispersion (prepared in PBS containing Tween-20 (0.1%, w/v) (TPBS)) for both intratumoral and intravenous applications was 20 μl, which was the intratumoral tolerable amount by rats. The formulations were sterilized under UV light for 30 min before injection.[35]

The rats were randomly divided into the groups as follows: (1) untreated (control group), (2) treated with blank nanoparticles (without FTA), (3) treated with FTA-loaded nanoparticles and (4) treated with drug solution. On the same day of MR imaging, the drug solution or formulation dispersion was injected into the tail vein of rats in intravenous injection group while they were injected to the brain with the same coordinates of cell implantation for intratumoral treatment group. Although six rats had been planned to be included in each injection groups and control group, only three could be involved per group due to the animal losses during experiments. Five days after initial MR imaging and injection of formulations, MR imaging was repeated to evaluate the interval change in tumour size.

Evaluation of tumour size by haematoxylin and eosin staining

To confirm tumour size measurements obtained by MR study, histopathological analysis which is more sensitive technique[38] was carried out. For this purpose, rats were sacrificed after last MR imaging study, and their brains were removed and postfixed in 10% formalin. Brains then were cut into 2-mm-thick slices in the coronal plane and then each slice embedded in paraffin. Serial 6-μm-thick sections were obtained from each paraffin-embedded tissue block, and the sections were stained with haematoxylin and eosin. Evaluations and measurements were performed under light microscopy. Largest transverse and longitudinal diameters of tumour were measured on the slice in which tumour was seen largest at ×10 magnification. Then product of two measurements was recorded as area of the tumour in mm^2. The histopathology was evaluated by a neuropathologist (F.S.).

Statistical analysis

Statistical analysis was performed using commercially available software (Statistical Package for Social Sciences, version 18.0; SPSS Inc., Chicago, IL, USA). One-way ANOVA was used for the statistical evaluation of the results. Tukey HSD test was performed to investigate the difference between the groups. Statistical level of significance was defined as $P < 0.01$. Results showed mean ± standard deviation (SD) with '*n*' representing the number of observations.

Results

Solubility of farnesylthiosalicylic acid

The percentages of soluble FTA were 2.12 ± 1.8%, 18.4 ± 2.1% and 40.1 ± 3.1%, in PBS, TPBS, PBS containing 10% FBS supplemented with 0.1% DMSO, respectively, showing that TPBS is more appropriate for release medium.

In-vitro characterization of hybrid nanoparticles

The particle size of all nanoparticle formulations ranged between 94 ± 2.3 and 155 ± 2.7 nm, and nearly homogenous distribution was observed. The particle size of PLGA 50 : 50 and PLGA 85 : 15 DSPE-PEG formulations with or without DOTAP loaded with FTA was significantly greater than those not containing drug ($P < 0.01$, Table 1).

The zeta potential values were between −30.2 ± 2.0 mV and −11.1 ± 1.1 mV for all formulations. The zeta potential was not significantly different between nanoparticles with and without drug, for both DOTAP-containing and lacking formulations ($P > 0.01$). As DOTAP is cationic, nanoparticles containing DOTAP showed significant

positive shift of *z* potential values, which were still negative ($P < 0.01$) (Table 1).

The loading efficiencies of FTA-loaded nanoparticles with or without DOTAP were summarized in Table 2. Considering all results of drug loading, highest efficiency of FTA loading was obtained with formulation of PLGA 50 : 50 copolimer ratio and DSPE-PEG/DOTAP ratio of 75 : 25.

The SEM and AFM photographs revealed that the nanoparticles were almost spherical in shape and did not contain any drug crystals on their surface (Figure 1).

The cumulative release of FTA was faster and higher in DOTAP-free nanoparticles in comparison with DOTAP-containing nanoparticles. The FTA release lasted in 150 h for DOTAP-containing formulations, while it was completed at 72 h for nanoparticles not containing DOTAP. The formulations with PLGA 85 : 15 copolymer showed faster FTA release profile than those with PLGA 50 : 50 copolymer (Figure 2).

Cell culture studies

In cytotoxicity studies, 30% of RG2 cells died at the end of third day and 90% at the end of fifth day at 200 μM of FTA concentration. On the other hand, 25 μM FTA caused viability in 98% and 80% at the end of third and fifth days, respectively. The LD50 value of FTA was calculated as 100 μM, and this concentration was used for cell culture cytotoxicity experiments (with RG2 cells or L929 cells) of other formulations and for in-vivo studies.

For RG2 cells, the in-vitro bioactivity experiments of PLGA 50 : 50 nanoparticles with or without DOTAP loaded with 100 μM FTA revealed that DOTAP-containing nanoparticles had the highest anticancer activity at the end of fifth day (Figure 3A). Thus, DOTAP-containing nanoparticles were used to evaluate the in-vitro bioactivity of formulations on L929 cells.

For L929 cells, higher cell viability values were obtained with FTA-loaded and blank nanoparticles (DOTAP containing) compared to RG2 cells (Figure 3B). Cell viability values with L929 cells were found as 82.5% and 110.4% for FTA-loaded and blank nanoparticles, respectively (Figure 3B), while these values were measured as 24.8% and 96.7% for drug-loaded and blank nanoparticles, respectively, with RG cells (Figure 3A). The results indicated that developed formulations had more cytotoxic effects on cancerous RG2 cells compared to non-cancerous L929 cells and specific drug targeting of the cancerous cell lines over non-cancerous cell lines can be provided by nanoparticle formulations.

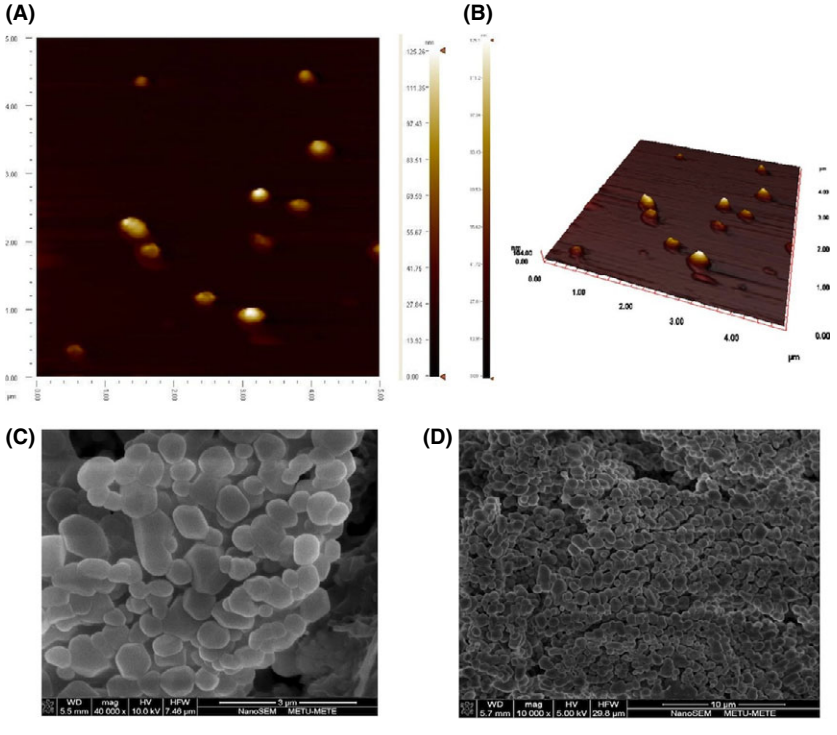

Figure 1 Atomic force microscopy photographs of farnesylthiosalicylic acid-loaded PLGA 85 : 15 DOTAP nanoparticles (A, B). Scanning electron microscopy photographs of farnesylthiosalicylic acid-loaded PLGA 85 : 15 DOTAP nanoparticles (×4000) (C) and of farnesylthiosalicylic acid-loaded PTGA 50 : 50 DOTAP nanoparticles (×1000) (D). PLGA, poly(lactic-co-glycolic acid); DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane. [Colour figure can be viewed at wileyonlinelibrary.com]

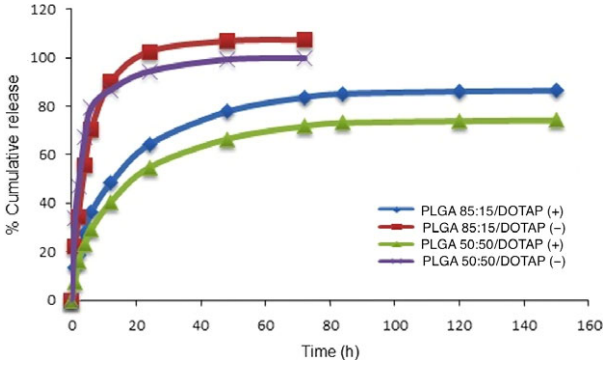

Figure 2 In-vitro release profiles of farnesylthiosalicylic acid-loaded PLGA/DSPE-polyethylene glycol without or with DOTAP (DSPE-polyethylene glycol: DOTAP (75 : 25 μM)) hybrid nanoparticles (n = 6). PLGA, poly(lactic-co-glycolic acid); DSPE-PEG, 1,2 distearoyl-glycerol-3-phospho-ethanolamine-N [methoxy (PEG)-2000] ammonium salt; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane. [Colour figure can be viewed at wileyonlinelibrary.com]

Fluorescent microscopy, which assessed cellular uptake qualitatively, also indicated that uptake of nanoparticles containing DOTAP into RG2 cells was higher than those not containing DOTAP (Figure 3C).

In-vivo studies

In-vivo studies were performed only on the Wistar rats that developed adequate size of brain tumour after 10 days of RG2 injection. On MR imaging, tumour was observed as a rough oval-shaped, T2 hyperintense mass with compression and herniation effects depending on its size. As coronal plane was the best imaging plane to show the lesion, cranio-caudal (height) and right-to-left (transverse) length were measured from the slice section with the largest tumour (Figure 4A). Tumour area was calculated as multiplication of these two measurements.

On macroscopical examination of the brains, grey-coloured space occupying lesion was observed at the injection site of RG2 cells. Microscopically, the main tumoral mass was observed generally in the periventricular deep grey matter and rarely in the cortical inoculation zone with an extension to the underlying white matter (Figure 4B).

The large tumours showed a mushroom-like growth pattern. Histologically, the neoplasm was composed of oval-to-spindle cells with vesicular nuclei and eosinophilic cytoplasm, forming interlacing fascicles. There were scattered multinucleated or uninucleated giant cells. Necrosis was not observed. High mitotic activity was detected. The neoplasm was rich in vessels, but did not show microvascular proliferation.

According to post-treatment measurements performed with both MR imaging and pathology (Figure 4), tumour size – determined by only MR imaging before treatment –

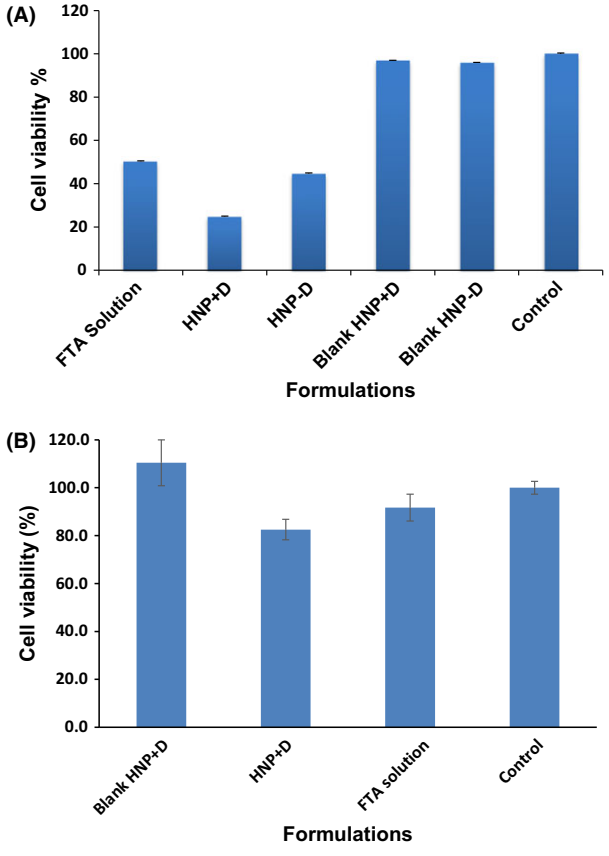

Figure 3 (A) Effect of the farnesylthiosalicylic acid solution (100 μM) or formulations on the viability of rat glioma-2 cells at the end of fifth day (n = 6, bars indicated standard deviation). Blank HNP-D: Blank (no drug) PLGA 50 : 50 hybrid nanoparticles without DOTAP; blank HNP+D: Blank (no drug) PLGA 50 : 50 hybrid nanoparticles with DOTAP; HNP-D: 100 μM farnesylthiosalicylic acid-loaded PLGA 50 : 50 hybrid nanoparticles without DOTAP; HNP+D: 100 μM farnesylthiosalicylic acid-loaded PLGA 50 : 50 hybrid nanoparticles with DOTAP. (B) Effect of the farnesylthiosalicylic acid solution (100 μM) or formulations on the viability of L929 cells at the end of fifth day (n = 6, bars indicated standard deviation). Blank HNP+D: Blank (no drug) PLGA 50 : 50 hybrid nanoparticles with DOTAP; HNP+D: 100 μM farnesylthiosalicylic acid-loaded PLGA 50 : 50 hybrid nanoparticles with DOTAP. (C) Fluorescent microscopy images of cellular uptake into rat glioma-2 cells of nanoparticles containing DOTAP (×40 in A, ×100 in B) and not containing DOTAP (×40 in C). PLGA, poly(lactic-co-glycolic acid); DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane. [Colour figure can be viewed at wileyonlinelibrary.com]

decreased significantly after 5 days of intravenous or intratumoral injection of nanoparticle loaded with FTA, while it significantly increased in all other groups ($P < 0.01$ for all) (Table 3). Tumour sizes measured with MR imaging and pathology after treatment were similar ($P > 0.01$). While tumour size in control group has increased by 106% from initial size (from 23.99 to 49.56 mm^2 on MR imaging), this increase was in rate of 63.47% (from 23.49 to 38.04 mm^2)

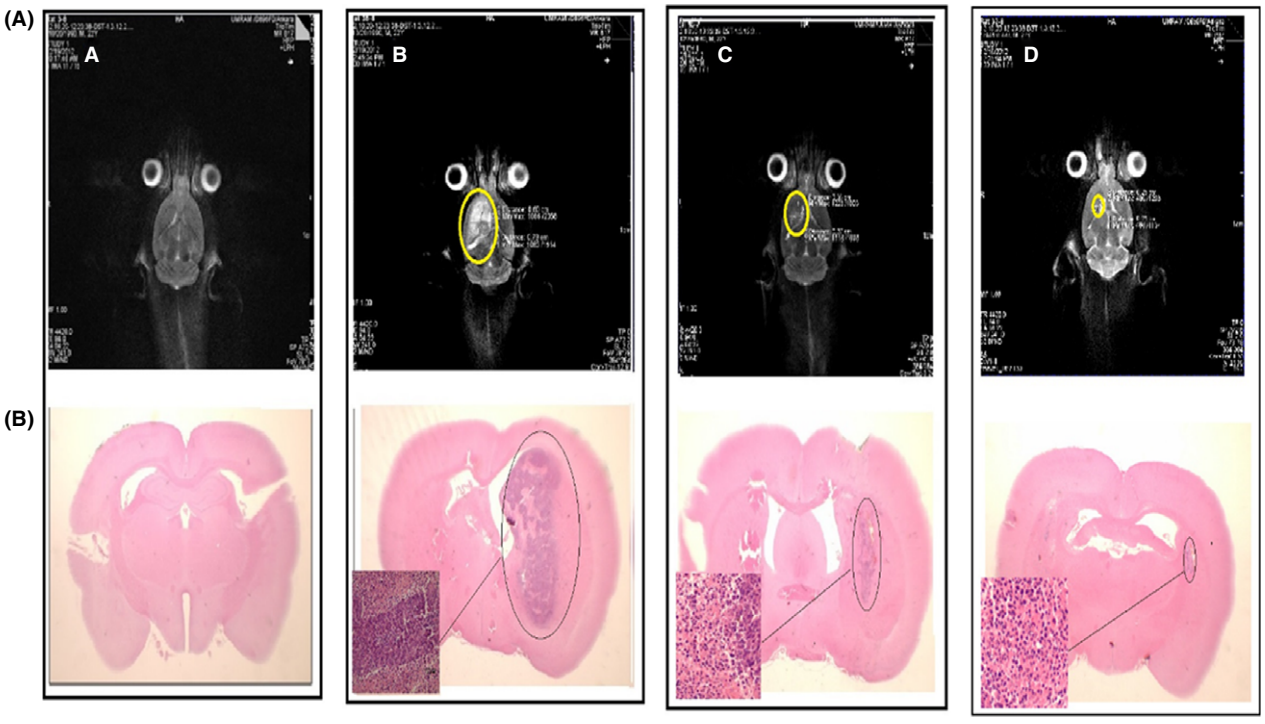

Figure 4 Samples of magnetic resonance images (A) and light microscopy images (B) (H&E, ×10) of pathology specimens, obtained from different group of rats without brain tumour (a) with brain tumour (b) and after 5 days of intravenous injection (c) or intratumoral injection (d) of PLGA 50 : 50-DSPE-polyethylene glycol/DOTAP (75 : 25) nanoparticles loaded with 100 μM farnesylthiosalicylic acid. In light microscopy images, tumour area was hyperchromatic (darker, purple colour) and pleomorphic, containing chromatin-rich cells (inner frame image in a [H&E, ×100]). Increased mitosis and endovascular proliferation of intratumoral vessels were also noted in specimens of rats with brain tumour (a). Please note that magnetic resonance images were obtained in the prone position, and thus, tumour is seen in the opposite hemisphere on slices obtained from paraffin-embedded tissue. PLGA, poly(lactic-co-glycolic acid); DSPE-PEG, 1,2 distearoyl-glycerol-3-phospho-ethanolamine-N [methoxy (PEG)-2000] ammonium salt; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane. [Colour figure can be viewed at wileyonlinelibrary.com]

and 130.02% (from 21.18 to 48.72 mm^2) in groups injected with drug solution and those with blank nanoparticles, respectively. On the other hand, tumour size decreased by 76.20% (from 22.65 to 5.39 mm^2) after intravenous application of FTA-loaded nanoparticles, which shows that only FTA-loaded nanoparticles have the capacity to decrease the tumour size (Figure 5A).

After intratumoral injection, tumour size increased by 106% (from 23.99 to 49.56 mm^2) in control group, 59% (from 19.71 to 31.04 mm^2) in drug solution group and 74.72% (from 24.45 to 42.72 mm^2) in blank nanoparticle group. On the other hand, tumour size decreased by 88.62% (from 22.15 to 2.52 mm^2) after intratumoral injection of FTA-loaded nanoparticles (Table 3, Figure 5B). Therefore, among intratumoral injection groups, only FTA-loaded nanoparticles decreased tumour size.

Discussion

Glioblastoma is among the most devastating cancers, with poor prognosis and short survival rates. Glioblastoma presents unique challenges to therapy due to its location, aggressive biological behaviour and diffuse infiltrative growth. Glioblastoma is one of the most resistant tumours to the chemotherapeutic approaches due to several reasons including presence of the blood–brain barrier, lack of effective targeted therapies and activation of multiple signal transduction pathways. Novel methods of intracranial drug delivery are needed to deliver higher concentrations of chemotherapeutic agents to the tumour cells while avoiding the adverse systemic effects of these medications.[13–16,22–26]

The nanoparticles demonstrate superiority over conventional therapies in the treatment of brain tumours. If they are designed by means of incorporating more than one type of nanostructure in a nanoassembly as hybrid nanoparticles, they could provide higher and permanent drug doses in target tissue. As a result of application of such system to the brain tumours, increased drugs' intake and crossing through blood–brain barrier[39] could be achieved. Thus, intensive research in recent years has been focused on hybrid nanoparticles particularly for treatment of gliomas.[17–20,40–42]

Poly(lactic-co-glycolic acid) was chosen in our nanoparticle formulation because of its long-acting controlled

Table 3 MR imaging- or pathology-defined pre- and post-treatment tumour sizes of in-vivo study groups that received intravenous or intratumoral injection (n = 3 for each group)

	MR imaging			Pathology	
	Pretreatment tumour size (mm^2)	Post-treatment tumour size (mm^2)	P values[a]	Post-treatment tumour size (mm^2)	P values[b]
Intravenous application					
Drug solution	23.49 ± 5.07	38.04 ± 3.02	<0.01	37.21 ± 3.02	<0.01
Nanoparticle without FTA	21.18 ± 7.17	48.72 ± 7.03	<0.01	46.63 ± 6.29	<0.01
Nanoparticle loaded with FTA	22.65 ± 6.43	5.39 ± 5.08	<0.01	5.93 ± 5.23	<0.01
Control (no injection)	23.99 ± 5.03	49.56 ± 6.33	<0.01	49.01 ± 7.01	<0.01
Intratumoral application					
Drug solution	19.71 ± 2.04	31.04 ± 1.35	<0.01	18.01 ± 2.44	<0.01
Nanoparticle without FTA	24.45 ± 1.76	42.72 ± 2.03	<0.01	22.45 ± 5.76	<0.01
Nanoparticle loaded with FTA	22.15 ± 2.23	2.52 ± 1.33	<0.01	3.15 ± 1.39	<0.01
Control (no injection)	23.99 ± 5.03	49.56 ± 6.33	<0.01	49.01 ± 7.01	<0.01

FTA, farnesylthiosalicylic acid. Data are given as mean ± standard deviation. [a]Pre- vs post-treatment tumour sizes by MR imaging. [b]Pretreatmnet tumour size by MR imaging vs post-treatment tumour size by pathology.

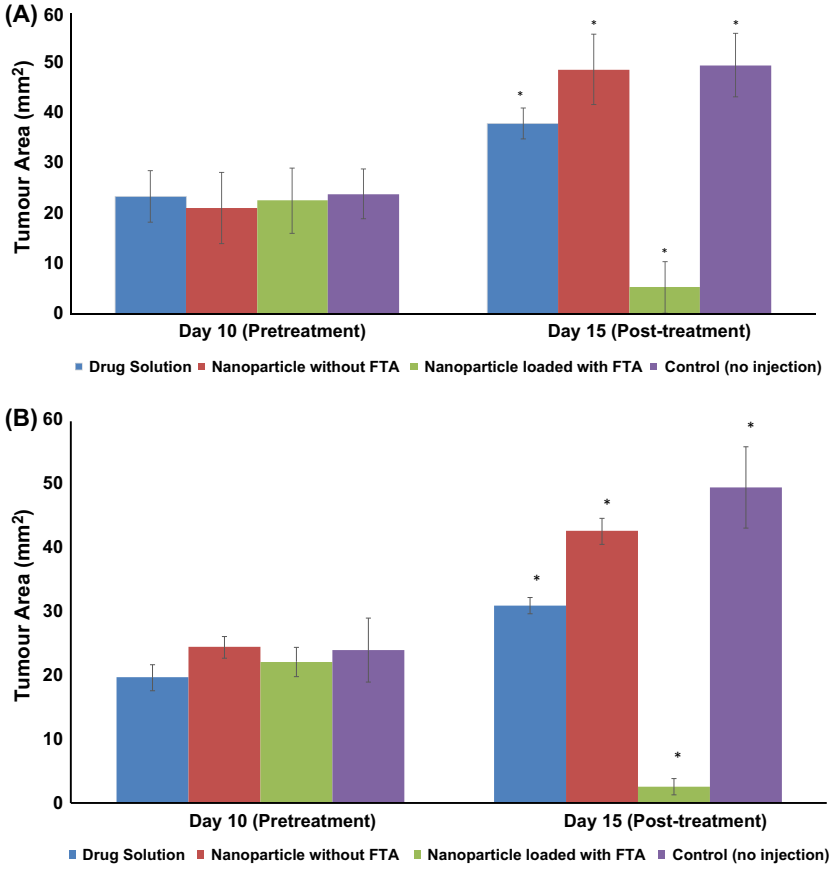

Figure 5 Magnetic resonance imaging defined pre- and post-treatment tumour sizes of in-vivo study groups that received intravenous (A) or intratumoral (B) injection (bars indicated standard deviation, n = 3 for each group). [Colour figure can be viewed at wileyonlinelibrary.com]

release, DOTAP was employed in this study because of its ability to increase cellular uptake in the brain. Furthermore, PEG is a convenient hydrophilic polymer to coat nanoparticles or to co-polymerize (PEGylation) the used polymer in order to obtain a longer retention time of nanoparticles in the bloodstream.[39–42]

In the previous studies, the particle size was below 200 nm at the systems prepared for the treatment of brain

tumours by nanoparticular systems.[43,44] For this reason, in our preformulation studies, it was strived to catch this particle size range, and the particle size of all the prepared nanoparticle formulations were found to be between 94 ± 2.3 and 111 ± 2.7 nm. There was a significant difference between the particle sizes of the formulations with and without drug ($P < 0.01$). Similarly in the mPEG-PLGA particles prepared by Wang *et al.*,[45] the particle size of the drug-loaded formulations was bigger in comparison with the nanoparticles without drug. In a study on drug loading to the lipid-based cationic nanoparticles, it was necessary to provide the optimum formulation rates to obtain high drug loading values.[46] In our study, the highest drug loading value was obtained by the formulation in which PLGA 50 : 50 copolymer ratio was used, and DSPE-PEG/DOTAP ratio was 75 : 25. Therefore, these formulations were used in in-vivo studies.

When the in-vitro release findings were evaluated, the formulations prepared using PLGA 85 : 15 copolymer showed a faster release compared to the nanoparticles prepared using 50 : 50 copolymer. Contrary to our findings, some previous studies reported that the drug release profiles obtained from the formulations prepared by PLGA 85 : 15 copolymer were slower than those prepared by PLGA 50 : 50.[47,48] This was explained in literature in the way that increase in the lactic acid ratio (from 50 to 85) causes the slowing down of the degradation of polymer and possibility of hydrophobic interaction between polymer and drug, which result in slower drug release. In this study, formulations have PEG chains besides PLGA copolymer making the surface of the nanoparticle hydrophilic. The PEG chains may lead to the diffusion of water to into the nanoparticle by forming a hydrophilic region in the nanoparticle surface, which eliminates the impact of polymer hydrophobicity on the release properties of the formulations and provides release of drug with diffusion.

Farnesylthiosalicylic acid is a specific inhibitor of Ras, a major component of mitogen-activated protein kinase (MAPK) pathway. This pathway is located downstream of epidermal growth factor receptor (EGFR), which is overexpressed in glioblastoma. EGFR and MAPK pathway activation has a substantial role in proliferation and survival of glioblastoma cells. In in-vitro studies on cell culture, FTA, which is effective against gliomas,[27,49] cannot cross through blood–brain barrier in active concentration.[29] Goldberg *et al.*[28] applied intratumoral FTA to the rats having brain tumours using convection-enhanced delivery technique and reported more decreased tumour size than control group. However, there is no study with nanoparticle system containing FTA.

This is the first study that has evaluated the cytotoxicity of FTA on RG2 cells. From this perspective, the results are important. The formulations with PLGA 50 : 50 copolymer ratio (with or without DOTAP) were used based on in-vitro characterization findings. The findings revealed that 100 μM value of FTA showed cytotoxic effect on the cells. The formulations with active substance and DOTAP showed the highest level of anticancer activity. In fluorescent microscopy images through which cellular uptakes of the nanoparticle formulations are assessed qualitatively, RG2 cells held more amount of the formulations containing DOTAP compared to the formulations without DOTAP (Figure 3B). The reason for this is that DOTAP enables the formulation to be carried into the cell efficiently by means of its positive loading.[50,51]

For in-vivo studies, PLGA 50 : 50 DSPE-PEG/DOTAP (75 : 25) was selected as the nanoparticle formulation because of its the highest level of drug loading, the longest release time of FTA and the most cytotoxic characteristic against RG2 cells in cell culture studies. FTA dose was identified as 100 μM considering the result obtained from cell culture experiments, and the same volume 20 μl was used for both intratumoral and intravenous applications. In the literature, the studies in which nanoparticle systems are applied in intravenous or intratumoral ways are available separately. However, there is no study comparing both application ways. In our study, with the formulations containing FTA, meaningful decrease in tumour size was measured with MR imaging after both intravenous and intratumoral treatment, and it was seen that tumour decrease ratio obtained with intratumoral application was significantly higher than that obtained with intravenous application (88.62% vs 76.20%). These results can be explained as follows: although chemotherapy drugs reach tumour area by crossing through blood–brain barrier, their concentration in tumour cannot reach to desired level due to avascular structure of many solid brain tumours. In many solid tumours, cell population can be 100 μm or more away from the vessels, which restricts the molecules such as nutrition, oxygen and drug to reach the remote cells. In addition, high cell density, pressure among the tissues, hypoxia and acidic pH lead to insufficient penetration, distribution and accumulation of the drugs into tumour.[52] By the implication of chemotherapy drugs on the core of tumour with intratumoral application, drug concentration in tumour can be increased more than with intravenous application, and its distribution can be enhanced.[52,53] In our histological evaluation, the existence and size of tumour were confirmed, and no statistically significant difference was found between the radiological and pathological tumour sizes.

This is the first study that developed an FTA-loaded hybrid nanoparticle formulation for treatment of glioblastoma and showed its both in-vitro activity and in-vivo activity. In future studies, we plan to increase the number of animals in each group which was limited by the animal

loses during the experiments and to test the formulations on larger animal species.

Conclusion

In summary, FTA can be effectively loaded into PLGA-DSPE-PEG nanoparticles, and addition of DOTAP into the formulation has positive effects on in-vitro characterization, cytotoxicity and cellular uptake. FTA-loaded PLGA-DSPE-PEG-DOTAP nanoparticles have significant cytotoxicity on RG2 cells. Intravenous and intratumoral applications of FTA-loaded hybrid nanoparticles are both effective in in-vivo tests, but intratumoral application was more effective in reduction of tumour size than intravenous application. On the basis of these findings, further animal studies on same and larger animal species (including RG2 or human-based glioma cell line) and normal tissue reaction and clinical studies should be implemented for common use of FTA-loaded PLGA-DSPE-PEG-DOTAP nanoparticles in the treatment of glioblastoma patients in clinical practice.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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