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Bombesin conjugated solid lipid nanoparticles for improved delivery of epigallocatechin gallate for breast cancer treatment

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ABSTRACT

Epigallocatechin-gallate (EGCG) is a potent anti-cancer therapeutic which effectively controls the growth of cancerous cells through a variety of different pathways. However, its molecular structure is susceptible to modifications due to cellular enzymes affecting its stability, bioavailability and hence, overall efficiency. In this study, we have initially encapsulated EGCG in the matrix of solid lipid nanoparticles to provide a stable drug carrier. To confer additional specificity towards gastrin releasing peptide receptors (GRPR) overexpressed in breast cancer, EGCG loaded nanoparticles were conjugated with a GRPR-specific peptide. *In-vitro* cytotoxicity studies showed that the peptide-conjugated formulations possessed greater cytotoxicity to cancer cell lines compared to the non-conjugated formulations. Further, *in-vivo* studies performed on C57/BL6 mice showed greater survivability and reduction in tumour volume in mice treated with peptide-conjugated formulation as compared to the mice treated with non-conjugated formulation or with plain EGCG. These results warrant the potential of the system designed in this study as a novel and effective drug delivery system in breast cancer therapy.

1. Introduction

Nanoparticle-based delivery systems have shown highly significant potential in the delivery of a wide range of therapeutic agents (Goethals et al., 2013; Pecorelli et al., 2010; Zhang et al., 2008). These nanosystems are not only able to deliver the therapeutic moieties effectively in the biological systems but also improve their physicochemical properties. Furthermore, they can be modified with one or more targeting ligands which direct them to the desired target-site. Therefore, these targeted nanosystems can increase the drug concentration in the diseased cells or tissues, which improves the efficacy of the therapeutic agent while simultaneously restricting unwanted circulation and accumulation of the therapeutic agent in healthy cells or tissues.

Catechins, one of the constituents found abundantly in green tea, have been known for their anti-oxidative, anti-bacterial, anti-inflammatory, anti-cancer and anti-viral activities (Cabrera et al., 2006). Epigallocatechin gallate (EGCG) is one of the most studied catechins owing to its extensive pharmacological activities (Lee et al., 2009; Tachibana, 2009; Tedeschi et al., 2002). It shows anti-cancer activity against many cancer cell lines by inhibiting tumorigenesis, proliferation and angiogenesis (Braicu et al., 2013). Besides, it has been seen that using EGCG in combination with traditional chemotherapy drugs leads to an increase in cytotoxic potential when compared to that of the drug alone (Pons-Fuster López et al., 2019). The structure of EGCG consists of 4 heterocyclic rings with 8 free hydroxyl groups. These hydroxyl groups are found to be susceptible to easy glucuronidation, sulphation and *o*-methylation (Dufresne and Farnworth, 2001) in physiological systems. These modifications interfere with the therapeutic efficiency of EGCG, as the presence and action of the free hydroxyl groups on the molecule are commonly linked with its efficacy (Tachibana, 2009). In our previous study we have addressed this issue of stability by encapsulating EGCG within solid lipid nanoparticles (SLNs) and studied the increase in cytotoxicity against various cancer cell lines (Rasika Radhakrishnan et al., 2016). The encapsulation enhanced the cytotoxicity of EGCG in cancer cell lines by up to 8 times. Solid lipid nanoparticles possess advantages including an improvement in drug stability, drug entrapment and biocompatibility, as they are composed of easily biodegradable excipients (Schwarz et al., 1994). SLNs can be

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used to incorporate both hydrophilic and hydrophobic drugs alike, and also can be administered through a variety of routes (Ramteke et al., 2012). These nanoparticles, therefore, worked as a protective as well as biodegradable vehicle for delivery.

One of the major advantages that EGCG holds is its pH-dependent degradation, with the molecule degrading as the pH moves from acidic pH towards physiological pH, with complete degradation at basic pH (Radhakrishnan et al., 2016). As tumour microenvironments have been shown to be slightly acidic in nature, only EGCG that is delivered in the cancer tissue will be active, while that delivered in normal cells will be degraded due to pH-dependent instability.

In the present study, we have developed a system that would act against cells marked with the targeted tumour marker. Non-specificity has to be circumvented to reduce non-specific organ damage which gives rise to serious side-effects (Monsuez et al., 2010). The dissipation of the drug in other areas also results in the concomitant increase in drug dosages, as the intended organ may not get the total therapeutic dose required (Coates et al., 1983).

To provide this additional specificity we have grafted a ligand onto the nanoparticle vehicle specific for breast cancer. Throughout the years, targeting has been mediated via monoclonal antibodies (Kulhari et al., 2015), biomolecules such as sugars, peptides and proteins (Pooja et al., 2015), and chemicals such as folates (Ruoslahti et al., 2010; Shohdy and Alfaar, 2013). Peptides provide an increasing advantage as they are small in size, thus allowing the conjugation of more peptide molecules per nanoparticle. They are also intrinsically biocompatible and non-toxic which leaves them undetected by the macrophages of the RES. GRPR is a glycoprotein receptor with 7 transmembrane domains consisting of 384 amino acids. It has been shown to overexpress in a number of cancers including breast cancer (Halmos et al., 1995; Zhou et al., 2019). In a recent study of 1432 primary breast tumours, GRPR overexpression was found in 75.8% of the cases (Morgat et al., 2017). Thus, the lower expression of GRPR in normal tissues and comparatively higher expression levels in tumour tissue, gives a greater molecular basis for choosing this receptor for targeting in chemotherapy approaches. These findings validate the use of GRPR as a promising indication for cancerous tissue, consequently aiding the meticulous delivery of nanoparticle cargo. Bombesin (BBN), a tetradecapeptide, is an amphibian analogue to the mammalian gastrin releasing peptide which is the natural ligand to GRPR (Begum et al., 2016). GRP and bombesin have a highly conserved 7 amino-acid terminal sequence that is essential for its high affinity binding and immunogenicity towards GRPR (Sunday et al., 1988). It has shown a very strong affinity towards GRPR and hence was used a ligand towards the tumour marker (Reubi et al., 2002).

The aim of this study was the construction of a breast-cancer targeted, biocompatible nanoparticle vehicle with minimum toxicity to non-specific tissues. Herein, bombesin was conjugated to the EGCG-encapsulated SLN system and bombesin-conjugated solid lipid nanoparticles were then explored for their anti-cancer efficacy by *in-vitro* cytotoxicity and uptake studies. *In-vivo* studies in C57BL/6 mice were performed to evaluate changes in tumour volumes and survivability.

2. Materials

Glycerol mono-stearate (GMS) was purchased from Alfa Aesar (Johnson Matthey Chemicals, Hyderabad, India). EGCG, bombesin acetate salt hydrate (Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2), stearic acid, Pluronic F68 (P-F68), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), 2-(n-morpho-lino)ethanesulfonic acid (MES), Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), trypsin–EDTA, phosphate buffered saline (Ca^{2+} and Mg^{2+} free), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). Lecithin soy was purchased from HiMedia (Mumbai, India). MDA-MB-231 human breast cancer cell lines and B16F10 mouse melanoma cells were obtained from American Type Culture Collection (ATCC, Manassas, USA). Dichloromethane was obtained from Merck Chemicals (Mumbai, India). Tween 80 was purchased from sd Fine Chemicals Limited (Hyderabad, India).

3. Methods

3.1. Preparation of nanoparticles

SLNs were prepared by the double emulsification-evaporation method as optimized in our previous work (Pooja et al., 2015). Briefly, GMS, stearic acid and lecithin soy were dissolved in the organic phase containing dichloromethane. The inner aqueous phase (1% w/v P-F68) containing dissolved EGCG was added to this organic phase under sonication to form a primary water-in-oil (w/o) emulsion. This emulsion was added to 2% w/v P-F68 solution to give a water-in-oil-in-water (w/o/w) emulsion. The nanoparticle suspension was stirred for 2 h for evaporation of the solvent and further centrifuged for 30 min at 11,200 g to obtain nanoparticle pellets. Nanoparticles were washed thrice with MilliQ water to remove the free drug present on the nanoparticle surface. The supernatant was used for determination of the entrapment efficiency.

3.2. Entrapment efficiency

The drug entrapment efficiency (EE) was calculated by an indirect method. Briefly, the supernatant obtained after centrifugation of the nanoparticle suspension was used for estimation of the unloaded drug content by UV–vis spectroscopy on a Jasco UV650 spectrophotometer at the absorption maxima of EGCG at 274.5 nm. The percent EE was calculated by the following formula:

$$\%EE = \frac{\text{Total amopunt of EGCC added} - \text{Amount of EGCG insupernatant}}{\text{Total amount of EGCG added}} \times 100$$

3.3. Bioconjugation of bombesin to nanoparticles

For the synthesis of targeted nanoparticles, drug-loaded nanoparticles (EGCG-SLN) were dispersed in MES buffer (pH 6.2) and incubated with NHS and EDC as reported earlier ((Bartczak and Kanaras, 2011). Bombesin was added to this suspension and allowed to incubate. The nanoparticle dispersion was collected, centrifuged and washed with distilled water. The supernatant was used to estimate the conjugation efficiency using a standard Bradford protein assay. The supernatant was diluted appropriately, and the absorbance was measured at 595 nm to determine the concentration of free or unconjugated bombesin using a calibration curve of bovine serum albumin.

The percent bombesin conjugation efficiency (% CE) was determined as follows:

$$\%\,CE = \frac{BA - Bs}{Bs} \times 100$$

B_A: Amount of bombesin added, B_S: Amount of bombesin present in supernatant

3.4. Physico-chemical characterization of nanoparticles

Particle dimensions and surface charge of nanoparticles were determined by photon correlation spectroscopy. Briefly, mean particle size, polydispersity index and surface potential of EGCG-loaded SLNs (EGCG-SLN) and bombesin-conjugated SLNs (EB-SLN) were measured using a Malvern Zetasizer Nano ZS (Malvern Instrument Ltd., Malvern, UK). Bioconjugation of bombesin on the surface of the nanoparticles was analysed by Fourier transform infrared analysis by scanning EGCG-

SLN and EB-SLN in the range of 4000–400 cm^{-1} using an IR spectrophotometer (Perkin Elmer, USA).

3.5. In-vitro cytotoxicity studies

MDA-MB-231 and B16F10 cells were grown in DMEM and RPMI medium, respectively and supplemented with 10% fetal bovine serum, 100 g/mL penicillin, 200 g/mL streptomycin and 2 mM L-glutamine. The cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

The cytotoxicity of formulations was determined by MTT assay against MDA-MB-231 human breast cancer cells based on mitochondrial reduction of yellow MTT tetrazolium dye to a highly coloured blue formazan product which shows absorption at 570 nm (Edmondson et al., 1988). About 1×10^4 cells were seeded (counted by the trypan blue exclusion dye method) in 96-well plates in 100 µL of medium. Cells were allowed to adhere overnight and then incubated with EGCG, EGCG-SLN or EB-SLN with a series of concentrations for 48 h at 37 °C. The above media was then replaced with fresh serum free media and 10 µL of MTT reagent (5 mg/mL), then plates were incubated at 37 °C for 4 h. After removing the media, DMSO was added to dissolve formazan crystals. The absorbance was measured at 570 nm using a spectrophotometer (Spectramax Plus, Molecular Devices, USA). The half maximal inhibitory concentration (IC_{50}) was calculated by using Probit software.

3.6. Cellular uptake studies

Rhodamine-B loaded nanoparticles (R-SLN) were prepared by replacing EGCG with Rhodamine B and conjugated with BBN (RB-SLN) for comparative cellular uptake studies (Xu et al., 2009). For quantitative studies, 1×10^5 cells per well were seeded in 12-well plates and allowed to adhere for 24 h. Cells were incubated with R-SLN or RB-SLN formulations for time intervals of 12 and 24 h. After removal of the culture media, the cells were washed twice with cold PBS and observed under a fluorescence microscope.

3.7. Migration assay

The effect of EGCG formulations on migration of the cells was determined by wound healing assay. MDA-MB-231 (5×10^5 cells/well) were seeded in petri dishes and allowed to grow to 80% confluent monolayer. Wounds were created through sterile 250 µL pipette tip by scratching the monolayer. Cells debris were removed by washing the cells twice with PBS. Cells were incubated with EGCG, EGCG-SLN or EB-SLN, equivalent to 100 µg/mL EGCG. The zone of wound healing was observed at 0 and 24 h using a bright field microscope. The percentage of wound closure was determined by measuring the wound area using Image J analysis software

3.8. Animal studies

The animal experimental protocol for this study was approved by the Institutional Animal Ethics Committee of the CSIR-Indian Institute of Chemical Technology, Hyderabad (approval no. IICT/PHARM/SRK/FEB/2013/8). All the animal studies were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Forty female C57/BL6 mice (6–8 weeks), weighing between 20–25 g, were used for the survival studies. Animals were kept in polypropylene cages under standard laboratory conditions (12:12 h light/dark cycles) at 24 °C. The animals were housed five per cage and had free access to food and water.

3.8.1. Induction of melanoma tumour

Mice were injected with B16F10 cells (3×10^5 /mice in 100 µL PBS) subcutaneously into the right flank via a 30 G needle. After induction of tumour, mice were randomly divided into four groups: control group, EGCG group, EGCG-SLN group and EB-SLN. (The control group was administered sterile PBS, EGCG group received pure drug at a dose of 50 mg/Kg body weight while EGCG-SLN or EB-SLN group received nanoparticle formulation containing EGCG equivalent to 50 mg/Kg body weight. PBS, EGCG, EGCG-SLN and EB-SLN were administrated independently via intra-peritoneal injection every third day from day 14 after tumour implantation.

3.8.2. Measurement of body weights and tumour volumes

The tumour size and body mass were measured regularly following the inoculation of tumour cells. Callipers were used to assess tumour growth by measuring two bisecting diameters in each tumour. The following equation was used to calculate the tumour volume:

$$\text{Tumour volume } (mm^3) = \frac{(\text{width} \times \text{length}^2)}{2}$$

3.8.3. Survivability studies

The administration of formulations to the groups of mice was continued until the animals died.

The body weight, tumour volume and overall survival of the mice were recorded, and the mean survival time was determined using a Kaplan-Meier survival plot.

4. Results and discussion

4.1. Particle size and surface charge

EGCG was encapsulated in the lipid matrix of GMS and stearic acid by a double emulsification method. Bombesin was conjugated on the surface of EGCG-SLN by EDC/NHS reaction. The bombesin-conjugated nanoparticles (EB-SLN) had a hydrodynamic diameter of 163.4 ± 3.2 nm, PD index of 0.341 ± 0.15 and a negative surface charge of -25.2 ± 2.8 mV (Table 1). There was a decrease in negative zeta potential in comparison to EGCG-SLN (−37.2 mV, owing to the free carboxylic group in the nanoparticle matrix), indicating the conjugation of bombesin on the surface of nanoparticles. The EE of EGCG within SLNs was approximately 67.2 ± 3.5%, which shows an efficient loading of EGCG within the lipid core of nanoparticles.

BBN peptide conjugation efficiency calculated by the standard Bradford assay showed a conjugation efficiency of about 92.4 ± 2.4%. The conjugation of bombesin was further confirmed by FTIR studies. EGCG-SLN showed peaks at 3358 cm^{-1} for O—H stretching in GMS, peaks at 2917 cm^{-1} for the OH stretch and 1731 cm^{-1} for the C–O stretch in stearic acid and at 1468 cm^{-1} for the C–H bend in alkanes. EB-SLN did not show the peaks at 1730 cm^{-1} for carboxylic acid groups, suggesting the conjugation of the carboxyl groups due to the formation of an amide bond with the amine group of bombesin. Furthermore, a prominent peak at 1640 cm^{-1}, characteristic of C–N stretching of an amide bond, was observed in the spectrum for EB-SLN. These changes point to the formation of amide bonds between the constituent lipids of the nanoparticle matrix and the peptide ligand.

Table 1
Physicochemical parameters of bombesin conjugated, EGCG-loaded solid lipid nanoparticles (EB-SLN).

Properties	Values
Hydrodynamic diameter	163.4 ± 3.2 nm
Polydispersity index	0.341 ± 0.15
Zeta potential	−25.2 ± 2.8 mV
Entrapment efficiency (%)	67.2 ± 3.5 %
Conjugation efficiency (%)	92.4 ± 2.4 %

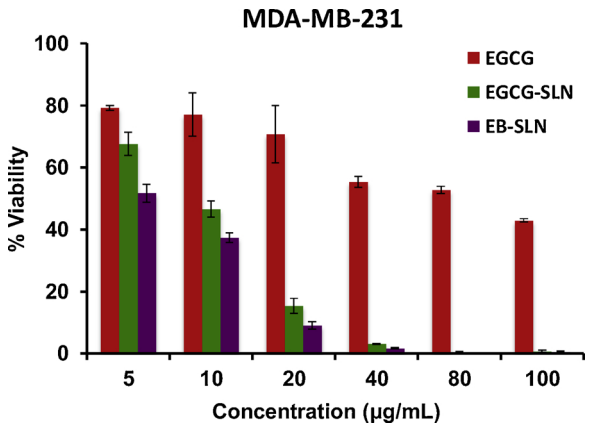

Fig. 1. Percent cell viability of MDA-MB-231 human breast cancer cells after 48 h of treatment with pure EGCG, EGCG-loaded SLN (EGCG-SLN) and bombesin-conjugated EGCG-SLN (EB-SLN).

4.2. In-vitro cytotoxicity

The *in-vitro* cytotoxicity of EGCG, EGCG-SLN or EB-SLN against MDA-MB-231 human breast cancer cells and B16F10 mouse melanoma cells was evaluated by MTT assay (Figs. 1 and 2). The cell viability of EGCG, EGCG-SLN and EB-SLN-treated cells after 48 h of treatment at a concentration range of 5–100 µg/mL was determined. The observed IC_{50} values for EGCG, EGCG-SLN and EB-SLN in MDA-MB-231 were 65.4 ± 4.9 µg/mL, 6.9 ± 1.1 µg/mL and 3.2 ± 1.7 µg/mL, respectively (Table 2). The cell viability of MDA-MB-231 following treatment with EGCG was 93.9% at the lowest tested concentration of 5 µg/mL. At the same concentration, EGCG-SLN and EB-SLN showed approximately 62% and 54.5% viability, respectively, demonstrating the comparative effectiveness of the nanoformulations. Similarly, these formulations were also more effective against B16F10 cells with IC_{50} values for EGCG, EGCG-SLN and EB-SLN found to be 59.3 ± 6.4 µg/mL, 28.2 ± 1.9 µg/mL and 15.6 ± 1.3 µg/mL, respectively. The results indicate that the activity of EGCG increased more than 9 times after encapsulation in SLNs and more than 20 times post conjugation with bombesin against MDA-MB-231. Similarly, against B16F10, the cytotoxicity increased more than 2 times for EGCG-SLN and almost 4 times for EB-SLN. We hypothesize that the increased cytotoxicity of EGCG in the nanoparticle formulations to the decreased degradation, and hence prolonged efficiency of EGCG because of the protection provided by the lipid core. Comparing targeted and non-targeted formulations, EB-SLN showed more cytotoxicity may be due to the interaction between BBN and GRPR receptors present in these cells, which enhance the internalization through receptor-mediated endocytosis.

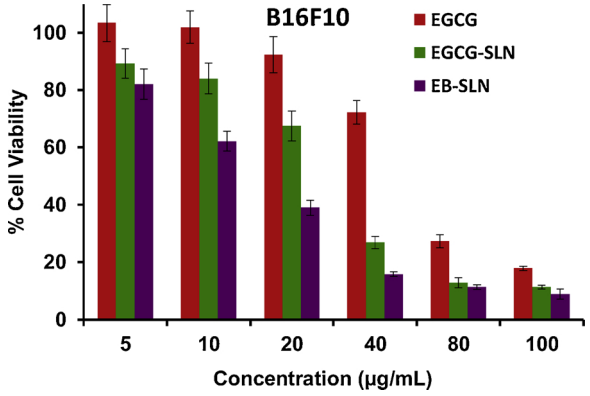

Fig. 2. Percent cell viability of B16F10 mouse melanoma cells after 48 h of treatment with pure EGCG, EGCG-loaded SLN (EGCG-SLN) and bombesin-conjugated EGCG-SLN (EB-SLN).

Table 2
IC_{50} values (µg/mL) for epigallocatechin gallate (EGCG), EGCG-loaded solid lipid nanoparticles (EGCG-SLN) and bombesin conjugated SLN (EB-SLN) against MDA-MB-231 human breast cancer cells and B16F10 mouse melanoma cells after 48 h of treatment.

Formulation	IC_{50} values (µg/mL)	
	MDA-MB-231	**B16F10**
EGCG	65.4 ± 4.9	59.3 ± 6.4
EGCG-SLN	6.9 ± 1.1	28.2 ± 1.9
EB-SLN	3.2 ± 1.7	15.6 ± 1.3

4.3. Phase contrast microscopy studies

Phase contrast microscopy was used to observe changes in the morphology of MDA-MB-231 cells after incubation with different EGCG formulations. Fig. 3 shows MDA-MB-231 cells treated with blank SLNs, EGCG, EGCG-SLN and EB-SLN at 24 h and 48 h post treatment with the respective formulations. Control cells were used as negative controls in the experiment. When cells are under stress and undergoing apoptosis, a few distinct morphological changes can be observed. MDA-MB-231 cells are characteristically elongated and tapered. During cell death, the cell becomes spherical and apoptotic bodies can be seen with the help of a nuclear staining dye (Elmore, 2007).

Blank SLNs do not show any observable cytotoxicity, as is evident by the elongated healthy cells at 24 and 48 h (Fig. 3). The EGCG treated cells showed apoptotic cells, demonstrated by the rounding of the cells when compared to the control cells at both time points. Cells incubated with EGCG-SLN showed more spherical cells compared to EGCG-incubated cells which suggests greater toxicity of EGCG-SLN. This could be attributed to the increased stability of the drug within the SLNs as well as the sustained release due to encapsulation (Radhakrishnan et al., 2016). EB-SLN treated cells showed more apoptotic cells, with the number of dead or dying cells increasing at 48 h compared to 24 h. This could again be attributed to the sustained release of the drug within SLNs and more importantly greater uptake due to targeting of bombesin to GRPR receptors (Kulhari et al., 2014). This enhanced uptake would automatically lead to increased concentrations of the drug within the cells and hence greater cytotoxicity.

4.4. Apoptosis studies

Apoptosis is a process of programmed cell death that occurs after a cell is no longer needed in the body. In cancer cells, the factors affecting the extrinsic and intrinsic pathways of apoptosis are downregulated, causing the cells to continue to proliferate uncontrollably. EGCG has shown the ability to induce apoptosis in cancer cells by triggering both the extrinsic and intrinsic apoptotic pathways (Wang and Bachrach, 2002).

To study apoptosis caused by the different formulations, MDA-MB-231 cells were incubated with blank-SLN, pure EGCG, EGCG-SLN and EB-SLN for 48 h followed by staining with Hoechst 33,342. Nuclear condensation is an important process during apoptosis, forming the principle of the nucleus-staining apoptosis assay. Hoechst 33,342 is a cell-permeable, blue fluorescence dye that emits blue fluorescence at 495 nm on binding with DNA (Allen et al., 2001). Fluorescence images in Fig. 4 clearly showed marked differences in nuclear staining in each of the treated cells. Hoechst 33,342 stains all nuclei, hence blue fluorescence is visible throughout the samples. However, the intensity of fluorescence is more in cells undergoing apoptosis, owing to nuclear condensation. Control cells and cells treated with blank-SLN show no intense fluorescence. However, cells treated with EGCG show several intense fluorescence specks. These specks increase in the EGCG-SLN; however, the highest intensity is visible in the EB-SLN treated cells. It can be explained by an increased uptake of these nanoparticles in

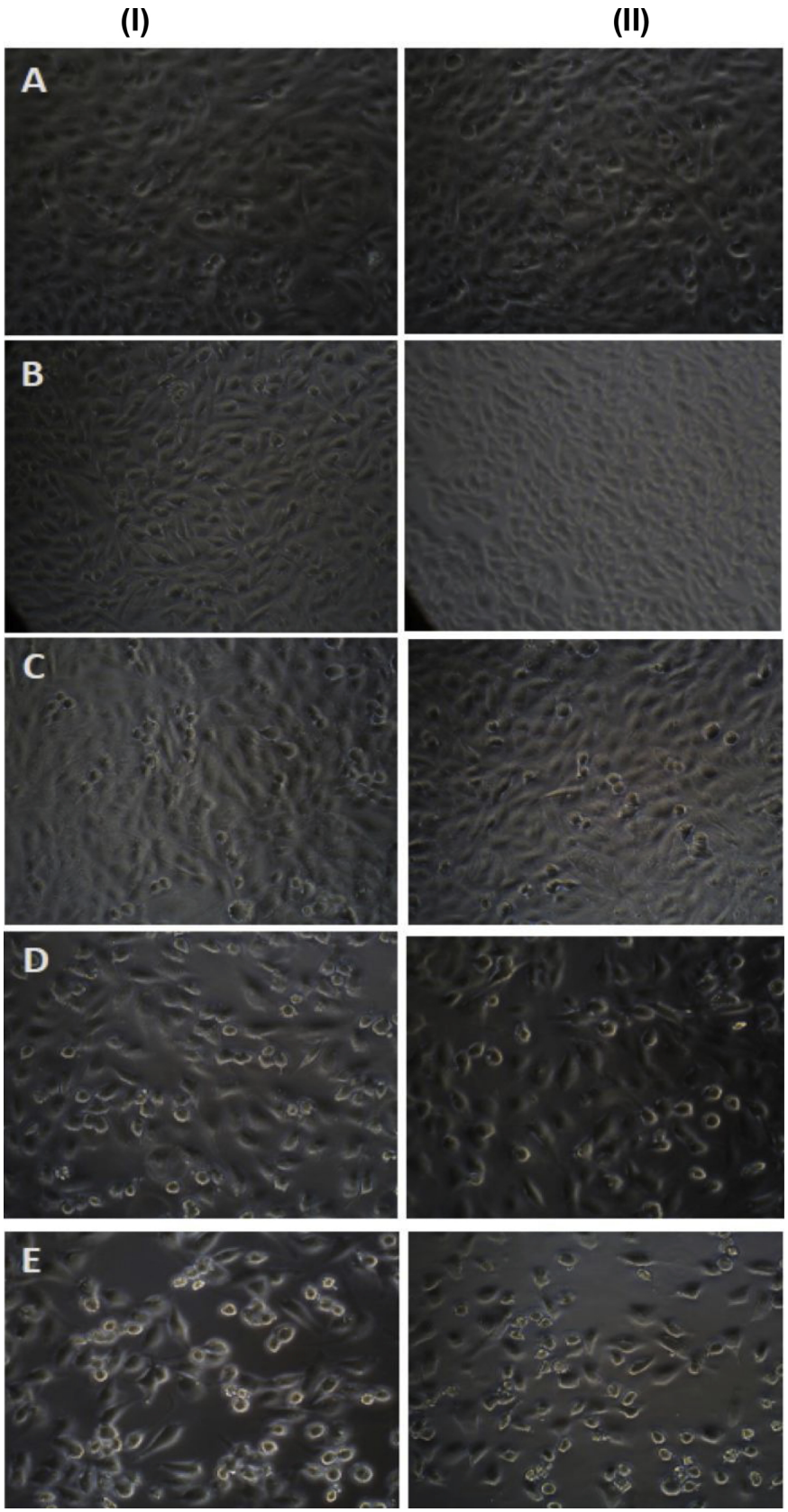

Fig. 3. Phase contrast microscopy images of MDA-MB-231 human breast cancer cells after 24 h (I) and 48 h (II) of treatment with A) control (without treatment), B) pure epigallocatechin gallate (EGCG), C) blank solid lipid nanoparticles (blank SLN), D) EGCG-loaded SLN (EGCG-SLN) and E) bombesin conjugated SLN (EB-SLN) equivalent to 20 μg/mL EGCG.

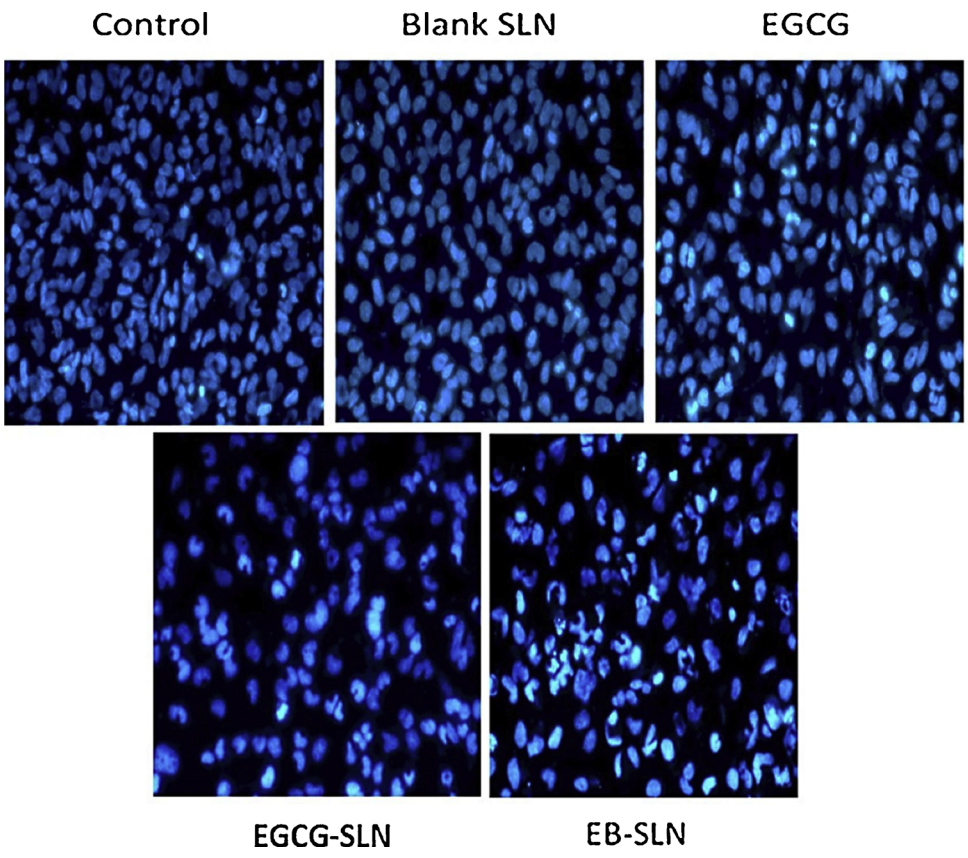

Fig. 4. Nuclear fragmentation studies: Fluorescent microscopic images of MDA-MB-231 human breast cancer cells after 48 h of treatment with blank solid lipid nanoparticles (blank SLN), pure epigallocatechin gallate (EGCG), EGCG-loaded SLN (EGCG-SLN) and bombesin conjugated SLN (EB-SLN) equivalent to 20 μg/mL EGCG followed by staining with Hoechst 33,342.

comparison to the EGCG-SLN due to the presence of the targeting ligand. These results indicate the successful and increased delivery of the cytotoxic EGCG inside the cells, thus causing a greater extent of apoptosis.

4.5. Cellular uptake studies

Uptake of nanoparticles by cancer cells was performed by using a fluorescent dye (i.e. Rhodamine-B) loaded in the lipid nanoparticles and observing fluorescence through microscopic detection as followed in previous works (Yuan et al., 2008). In this study, Rhodamine-B was chosen due to its hydrophilic nature, and therefore it can be loaded in the same phase as that of the hydrophilic drug. Uptake was studied in the MDA-MB-231 breast cancer cell line (Fig. 5). Rhodamine-B loaded SLNs (R-SLN) were prepared by the same method as EGCG-SLN, by replacing the drug with Rhodamine-B dye. R-SLN were further conjugated with bombesin to get BBN conjugated, Rhodamine-B loaded nanoparticles (RB-SLN). Cells were observed at three-time points (0 h, 1 h and 2 h) following incubation of MDA-MB-231 cells with the dye-loaded formulations (Fig. 5). At 1 h, visibly more intense red fluorescence was observed in cells treated with RB-SLN compared with the unconjugated nanoparticles (R-SLN). At 2 h, this difference is more pronounced with not only an increase in the number of RB-SLN treated cells showing fluorescence, but there is also visible intensification in the fluorescence of individual cells. These findings point to continual and increased uptake of nanoparticles in the cancerous cells conjugated with the targeting ligand bombesin as opposed to the unconjugated formulation.

4.6. Migration studies

Angiogenesis is the process by which a tumour develops new blood vessels from existing ones in order to maintain continual growth and nutrition (Folkman, 2002). Therefore, inhibition of angiogenesis aids in preventing the growth and metastasis of tumours, by essentially cutting off its nutrition. EGCG has shown anti-angiogenic effects in a number of cell lines (Jung and Ellis, 2002). In the present study, this effect was found to be increased when it was encapsulated as EGCG-SLN and further conjugated with bombesin (Fig. 6). Wound closure is found to occur in a greater extent in control cells as compared to EGCG-treated cells. Wound closure is almost negligible in EGCG-SLN and EB-SLN. Enhanced activity in EGCG-SLN and EB-SLN could be attributed to the increased stability of the drug within the solid lipid nanoparticles and hence greater activity. Sustained release of EGCG from the nanoparticles also aids in the continued anti-migratory effect of the drug, leading to better wound closure in EGCG-loaded nanoparticles than pure drug (Andreani et al., 2016).

4.7. In-vivo studies

The *in-vivo* anti-cancer efficacy of a therapeutic agent is assessed in terms of prolonging the quality of life and increasing the survival time. The anti-tumour efficacy of EGCG, EGCG-SLN or EB-SLN was evaluated on tumour-bearing mice upon administration of multiple doses equivalent to 50 mg/kg EGCG. *In-vivo* anti-tumour efficacy was evaluated in a syngeneic C57BL/6 mouse model.

The B16F10 cancer cell line was used to develop melanoma in this study. This mouse melanoma cell line shows expression of GRPR receptors and therefore was used for evaluating the efficacy of the bombesin conjugated formulation (Fang et al., 2009). Mice were first injected subcutaneously with 3×10^5 B16F10 cells on the flank to grow melanomas *in-situ*. Forty tumour-bearing C57BL/6 mice were randomly divided into 4 groups (n = 10) and intraperitoneally administered EGCG, EGCG-SLN or EB-SLN at a dose equivalent to 50 mg/kg body weight of EGCG every third day. The control group was administered an equivalent volume of saline every third day.

The body weight, tumour volume and overall survival of the mice were recorded. The mean survival time was determined using a Kaplan-

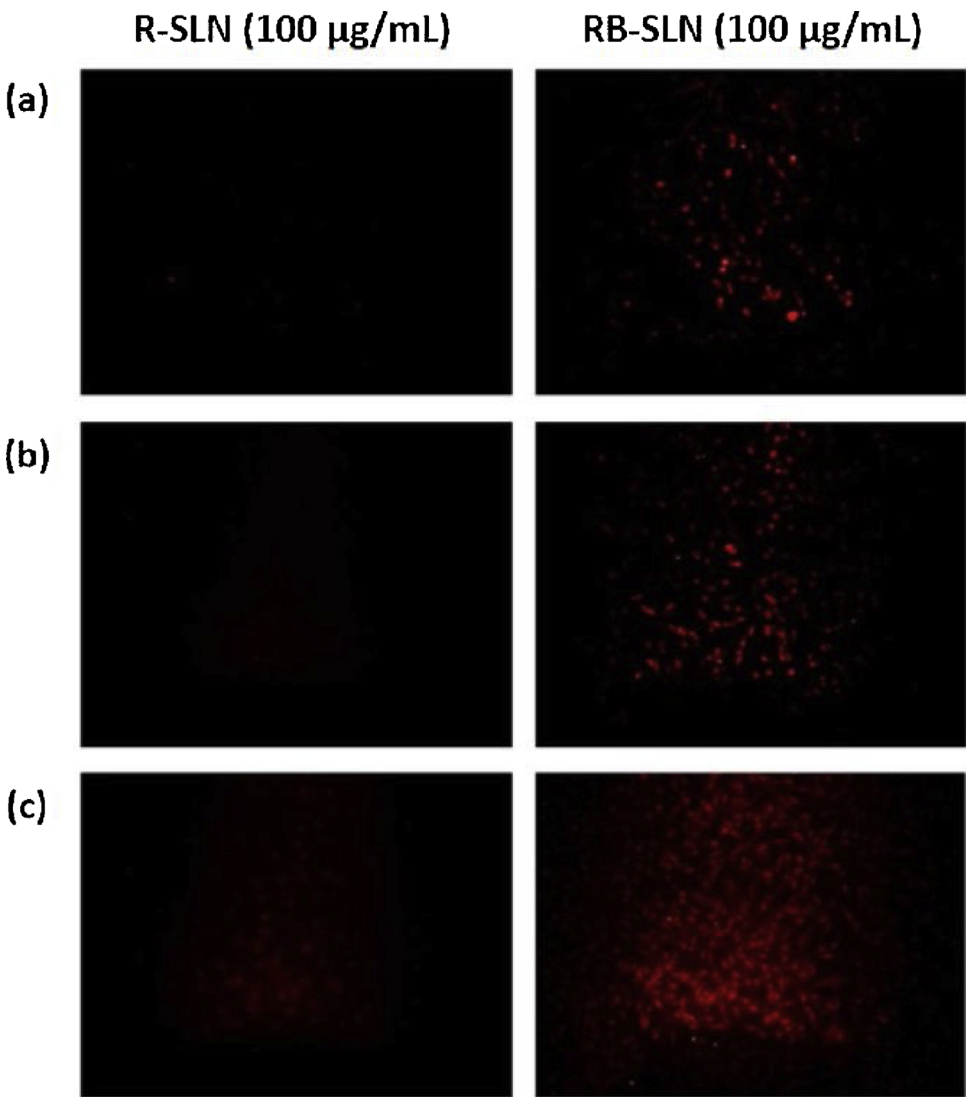

Fig. 5. Cellular uptake studies: Fluorescent microscopic images of MDA-MB-231 human breast cancer cells after 0 h (a), 1 h (b) and 2 h (c) of treatment with Rhodamine-loaded SLN (R-SLN) and bombesin conjugated SLN (RB-SLN).

Meier survival plot (Fig. 7). A median survival of 41, 48 and 55 days were observed for EGCG, EGCG-SLN and EB-SLN treated mice, respectively, compared to control mice (37 days) which were administered with normal saline. Thus, there was a significant extension in the life span of mice treated with EB-SLN.

The change in body weight of animals is an indicator of systemic toxicity caused by the formulation (Criscitiello et al., 2012). There was a rapid loss of body weight in the saline treated control group in comparison to EGCG formulation treated animals (Fig. 8). The control group receiving only saline recorded a loss of 7.01% in total body weight in 30 days whereas both EGCG-SLN groups did not show any significant change in their body weights.

The change in tumour volume was also observed and results revealed that the tumour growth was inhibited in the mice receiving the EGCG formulations (Fig. 9). The percent increase in tumour volume in mice treated with EGCG-SLN and EB-SLN was 3.78% and 0.78%, respectively, as compared to mice receiving pure EGCG and the control group which were 5.45% and 7.97%, respectively.

5. Conclusions

In this work, we prepared bombesin conjugated, EGCG loaded solid lipid nanoparticles by the double-emulsification solvent evaporation method followed by conjugation using EDC–NHS reaction. FTIR analysis validated the formation of the amide bond between the carboxylic C–O group of the lipid with the free amine group of the peptide. Antitumor effects of EB-SLN are shown by MTT assay, cell morphology and apoptosis induction. Targeted formulations show enhanced cytotoxicity, higher cellular uptake, increased apoptosis and better control over cell migration compared to non-targeted formulations. *In-vivo* tumour activity of nanoparticles are shown in syngeneic mouse model. Targeted nanoparticles show better control over tumour growth and enhanced survivability. These findings suggest that conjugation of BBN as a targeting ligand to the EGCG-encapsulated SLN system, further corroborating the potential of EGCG-encapsulated systems in cancer therapeutics.

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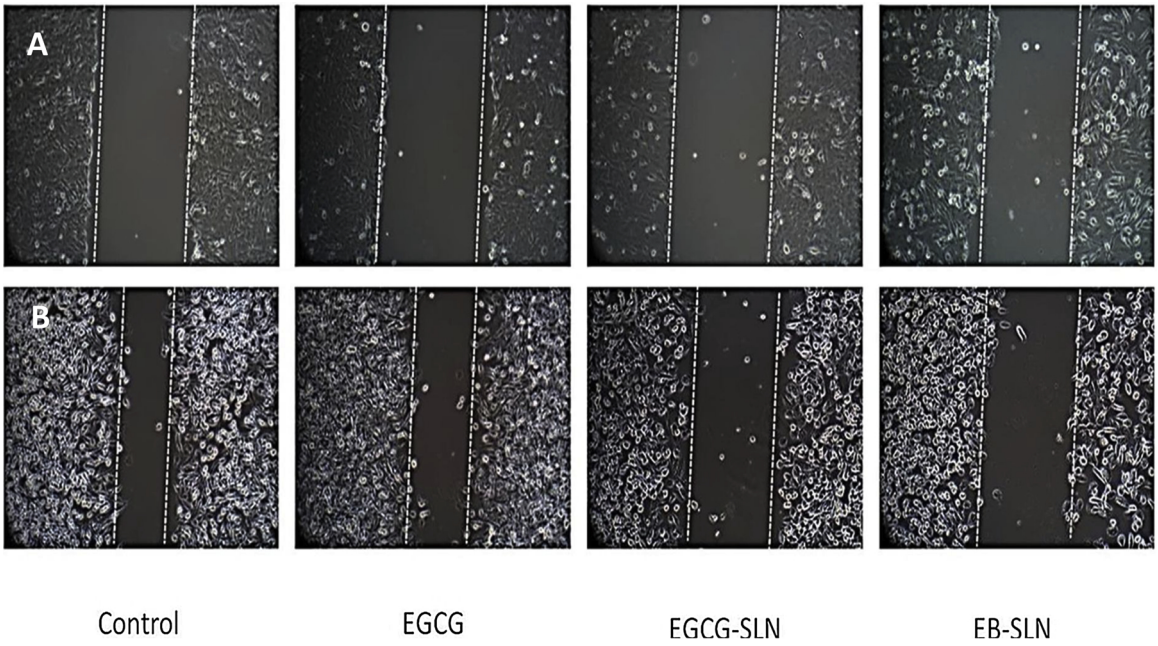

Fig. 6. Scratch assay images for MDA-MB-231 human breast cancer cells after 0 h (A) and 24 h (B) of treatment with blank solid lipid nanoparticles (blank SLN), pure epigallocatechin gallate (EGCG), EGCG-loaded SLN (EGCG-SLN) and bombesin conjugated SLN (EB-SLN) equivalent to 20 μg/mL EGCG, along with control cells for reference.

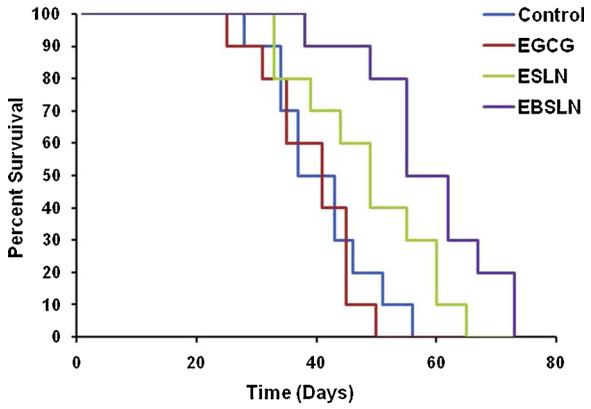

Fig. 7. Kaplan-Meier plot for survival following administration of pure epigallocatechin gallate (EGCG), EGCG-loaded SLN (EGCG-SLN) and bombesin conjugated SLN (EB-SLN) formulations, with control mice for reference.

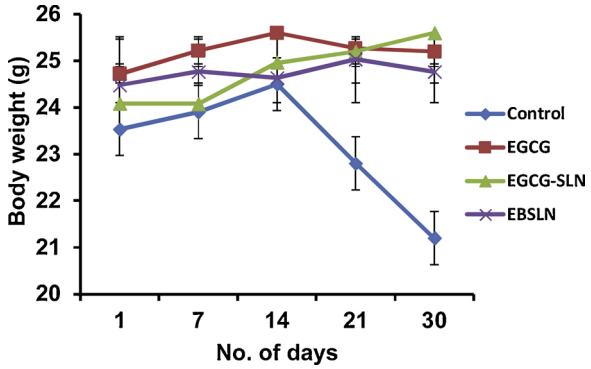

Fig. 8. Change in body weight in mice with the number of days following administration of pure epigallocatechin gallate (EGCG), EGCG-loaded SLN (EGCG-SLN) and bombesin conjugated SLN (EB-SLN) formulations with control mice for reference.

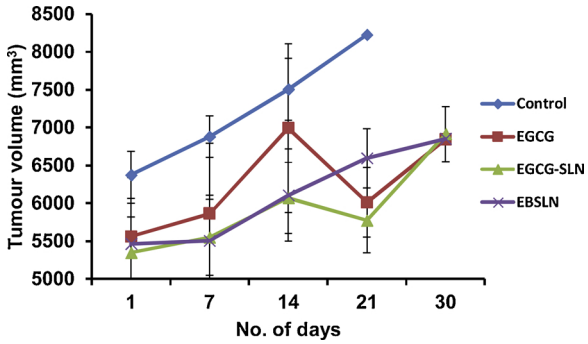

Fig. 9. Change in tumour volume in mice with the number of days following administration of pure epigallocatechin gallate (EGCG), EGCG-loaded SLN (EGCG-SLN) and bombesin conjugated SLN (EB-SLN) formulations with control mice for reference.

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