Original Article

Targeting CXCR4-expressing Cancer Cells with Avidin-poly (lactic-co-glycolic acid) Nanoparticle Surface Modified with Biotinylated DV1 Peptide

Abstract

Background: Chemokine receptor CXCR4 is frequently present in cells of various cancers. Hence, targeted therapy using CXCR4 ligands, such as DV1 peptide, on drug-loaded nanoparticles, has the potential to enhance the efficiency of cancer treatment. **Aim:** The present study created a CXCR4-targeting drug delivery system using avidin-poly (lactic-co-glycolic acid) (PLGA) nanoparticle surface tagged with biotinylated DV1 peptide ligand. **Materials and Methods:** A double-emulsion solvent evaporation technique was employed to prepare avidin-PLGA nanoparticles and characterized by transmission electron microscopy (TEM) and dynamic light scattering. Uptake was studied by confocal microscopy after incorporating fluorescein isothiocyanate (FITC)-labeled albumin inside the nanoparticles during their synthesis. Peptide-biotin-avidin-PLGA nanoparticles were tested *in vitro* on CXCR4-expressing U87MG cells. Photomicroscopy was done by a Nikon A1 Confocal Microscope, and pictures were analyzed by Nikon NIS-Elements BR software. **Results:** Experimental results confirmed the specificity of DV1 peptide-tagged avidin-PLGA nanoparticles for cells expressing CXCR4 receptors. The avidin-PLGA nanoparticles were successfully synthesized and the same was confirmed by tagging them with FITC-labeled biotin. **Conclusion:** Avidin-PLGA nanoparticle surface tagged with biotinylated DV1 peptide ligand has potential clinical application in the treatment of various cancers as targeted therapy for CXCR4-expressing cancer cells.

Keywords: *Cancer therapeutics, CXCR4 receptors, DV1 peptide, poly(lactic-co-glycolic acid) nanoparticles*

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Introduction

The field of cancer therapeutics is rapidly evolving. Chemotherapy, a mainstay of cancer treatment, is limited by its poor localization to tumor sites, especially in the core of solid tumors and the risk of systemic side effects.[1] The concept of targeted cancer therapy has widely evolved recently to get over these limitations. Many such targets such as transferrin receptors, folate receptors, and epidermal growth factor receptor (EGFR) have been identified in context of various cancers. The ligands for these molecular targets can be proteins (antibodies), nucleic acids (aptamers), peptides, carbohydrates, etc.[1] Chemokine receptor CXCR4 is one such potential target for anticancer drug delivery systems for therapeutic purposes.[2] Chemokine ligand, CXCL12, binds to CXCR4 receptors and activates various regulatory pathways that influence the migration and proliferation of cells.[3] This specific chemokine receptor has shown to be overexpressed in many types of cancers and cancer stem-like cells and is involved in the proliferation, migration, and distant metastasis of these cells. CXCR4 is overexpressed in different types of human cancers including kidney, lung, brain, breast, prostate, ovarian, pancreas, colon, and melanoma.[4] Multiple studies have reported the use of CXCR4 ligands for developing targeted drug delivery systems to CXCR4-expressing cancer cells. Various peptides, small organic molecules, and antibodies have been explored in this regard.[5,6] ALX-40C, AMD3100, T22 and its downsized analog T140, and N-terminal of viral macrophage-inflammatory protein-II (vMIP-II) have been reported to bind and inhibit CXCR4.[6] DV1 peptide is a modified derivative of vMIP-II comprising only 1–21 residues from the N-terminus and modified with D-amino acid analogs. It

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shows high affinity and antagonistic activity for chemokine receptor CXCR4 and thereby disrupts CXCR4-mediated signaling.[6] The use of DV1 peptide as a CXCR4 ligand for cancer drug delivery systems has not been investigated so far.[7]

Nanoparticles are the preferred medium for targeted drug delivery. Poly(lactic-co-glycolic acid) (PLGA)-based nanoparticles present many advantages for the specific delivery of drugs, proteins, peptides, or nucleic acids to their target tissue.[8] Treatment with this delivery vehicle increases bioavailability, reduces the frequency of administration, and promotes the targeting of drugs to specific sites. PLGA is biocompatible and the United States Food and Drug Administration approved. Efficient cellular uptake, rapid lysosomal escape, and sustained intracellular drug release characteristics make PLGA nanoparticles a promising option as a drug delivery vehicle.[9] Further, the surface of nanoparticles can be modified with specific ligands for active targeting to specific target sites. We aimed to target CXCR4-expressing cancer cells by PLGA nanoparticle surface tagged with DV1 peptide.

Materials and Methods

All ethical practices have been followed in carrying out this work after due permission from the institute's Ethics Committee (IESC/T-326/August 02, 2013).

Preparation of avidin-palmitic acid conjugate

Avidin (Sigma) at 5 mg/mL was reacted with a 10-fold molar excess of NHS-palmitic acid (Sigma) in phosphate-buffered saline (PBS) containing 2% deoxycholate buffer and the solution was gently mixed at 37°C for 2 h. To remove the excess fatty acid and hydrolyzed ester, the solution was purified by centrifuging at 18,000 g in Centrifugal Filter Units (Amicon Ultra-0.5 mL Centrifugal Filters, Merck Millipore).

Preparation of avidin-poly(lactic-co-glycolic acid) nanoparticles

The PLGA nanoparticles were prepared by the double-emulsion solvent evaporation method. 100 µL aqueous solution of bovine serum albumin (2 mg/mL) tagged with fluorescein isothiocyanate (FITC) (Sigma) was added dropwise to 10 mg PLGA (lactide: glycolide 50:50 MW 30–60 kDa, Sigma) dissolved in 330 µL chloroform. FITC served as a fluorescent marker for nanoparticle uptake by cells. The mixture was sonicated for 30 s in an ice bath (ultrasonic processor UP100H) at maximum amplitude to form a water-in-oil emulsion. This solution was then added to a 400 µL aqueous solution consisting of 200 µL (5 mg/mL) avidin-palmitic acid conjugate and 200 µL 5% polyvinyl alcohol (PVA) (MW 30–70 kDa, Sigma-Aldrich) followed by sonication (24W for 3 min) over an ice bath to form a water-in-oil-in-water emulsion.[10,11] PVA is used as an emulsifier that facilitates the formation of relatively small-sized particles with uniform size distribution. The solution was stirred for 18 h at ambient conditions to remove the residual chloroform. Nanoparticles were recovered by ultracentrifugation (Beckman Coulter) at 20,000 g for 30 min at 4°C and were washed two times with PBS to remove PVA and unincorporated bovine serum albumin (BSA). The final pellet was resuspended in 1 ml of PBS for working suspension or lyophilized for long-term storage at −20°C.

Preparation of avidin-poly(lactic-co-glycolic acid)-polyethylenimine nanoparticles

These PLGA nanoparticles were prepared by the double-emulsion solvent evaporation method. 10 mg PLGA was dissolved in 330 µL chloroform containing 35 µg polyethylenimine (PEI) (MW 25 kDa, Sigma-Aldrich) followed by dropwise addition of 100 µL aqueous solution of BSA (2 mg/mL). The rest of the steps were repeated the same as for avidin-PLGA nanoparticles.

Preparation of poly(lactic-co-glycolic acid)-polyethylenimine nanoparticles

These nanoparticles were prepared following the same protocol with some modifications. During the preparation of the first emulsion, PEI was added to chloroform to impart a positive charge to the nanoparticles to enhance their uptake by cells. During the preparation of the second emulsion, PVA (6 mL of 2.5% solution) was used. Positively charged nanoparticles allow a higher extent of internalization, apparently as a result of the ionic interactions established between positively charged particles and negatively charged cell membranes. These nanoparticles were used as a positive control with respect to nonspecific internalization by cells.

Tagging of nanoparticles by biotin-fluorescein isothiocyanate and biotinylated DV1 peptide

5X molar excess of biotin-FITC (Sigma) with respect to avidin was added to avidin-PLGA-PEI nanoparticle suspension and rotated for 1 h at room temperature for tagging. Subsequently, nanoparticles were washed with PBS to remove the unbound ligand and resuspended in PBS for further use. For tagging with DV1 peptide, 5X molar excess of biotinylated DV1 peptide (custom synthesized) was added to avidin-PLGA nanoparticle suspension and rotated for 1 h at room temperature for tagging. Subsequently, nanoparticles were washed with PBS to remove the unbound ligand and resuspended in PBS for further use.

The biotinylated DV1 peptide used in this study was synthesized by GenScript with the sequence (D)L(D)G(D)A(D)S(D)W(D)H(D)R(D)P(D)D(D)K-CCLGYQKRPLP-GGSGK (7), MW 3079.60 and purity 95.7%. The first ten aminoterminal residues were D-amino acids and a lysine was added at the carboxyl-terminal

that was biotinylated at its epsilon amino group and was preceded by a GGSG linker. A working solution of this peptide was prepared in a 10 mM DTT solution.

Electron microscopy of nanoparticles

The nanoparticles were resuspended in PBS, mounted on carbon-coated copper grids, and stained with 2% phosphotungstic acid. Then, the grids were visualized under a transmission electron microscope at 80 kV for size and shape determination.

Dynamic light scattering

The hydrodynamic size distribution and zeta potential of the nanoparticles were measured by the dynamic light scattering method using a Zetasizer (Malvern, Model: ZEN3600). The parameters were set by taking water as a solvent for all samples during testing.

Cell culture

The cell lines used in the experiments were human glioblastoma cell line U87MG (ATCC name: HTB-14) that is known to express CXCR4 and murine neuroblastoma cell line Neuro-2a (ATCC name: CCL-131) which does not express CXCR4. These cell lines were authenticated by Lifecode Technologies Pvt. Ltd., India. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Germany) with 10% (v/v) Fetal Calf Serum from BioWest, USA, 3.7 g/L sodium bicarbonate, and 10 µg/mL ciprofloxacin at 37°C with 5% CO_2.

Treatment of cells with nanoparticles

Cells were seeded at 1×10^4 per well in a 96-well plate. 24 h later, cells were washed and fresh working DMEM media was added. Specific nanoparticle suspension was added as 10 µL per well in a 96-well plate and incubated for 24 h. The cells were then washed with PBS followed by fixation, staining, and microscopy.

Confocal microscopy

The washed cells were fixed with 4% paraformaldehyde for 15–20 min and washed twice with 1X PBS. 4', 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) at 1 µg/mL working concentration was added to each well and kept for 2–5 min. Then, cells were again washed with 1X PBS twice and examined under a Confocal Microscope, A1 (Nikon, Japan). Fluorescence quantification was done using Nikon NIS-Elements BR software.

Results

Characterization of poly(lactic-co-glycolic acid) and avidin-poly(lactic-co-glycolic acid) nanoparticles

Scanning electron microscope and transmission electron microscopy (TEM) analyses revealed that particles were spherical and the size of nanoparticles ranged from 50 to 200 nm [Figure 1]. Physicochemical characterization studies showed that PLGA-Nanoparticles (NPs) were negatively charged with a zeta potential of −23 mV and particle size of 225 nm.

Confirmation of avidin on the surface of avidin-poly(lactic-co-glycolic acid)-polyethylenimine nanoparticles

For confirmation of functional avidin groups on the surface of nanoparticles, a batch of avidin-PLGA-PEI nanoparticles was prepared without FITC. These nanoparticles were tagged with biotin-FITC (test NPs) while untagged nanoparticles were taken as control (control NPs). U87MG cells were treated with these test and control NPs for 24 h and FITC fluorescence quantification was done using confocal microscopy [Figure 2]. The mean fluorescence intensity of cells treated with biotin-FITC-tagged avidin-PLGA-PEI nanoparticles was highly significant as compared to cells treated with untagged nanoparticles alone (Mean Fluorescence Intensity (MFI) 107.79 vs. 11.86, $P < 0.01$).

Specific delivery of targeted avidin-poly(lactic-co-glycolic acid) nanoparticles to CXCR4-expressing cells

Creation of targeted avidin-poly(lactic-co-glycolic acid) nanoparticles

Avidin-PLGA NPs were tagged with 5X molar excess of biotinylated DV1 peptide-forming peptide-avidin-PLGA NPs. The peptide-tagged nanoparticles thus formed could target the CXCR4 receptors due to the DV1 peptide on their surface. Untagged nanoparticles were used as control nanoparticles (avidin-PLGA NPs). FITC-albumin was included in NP preparation as a fluorescent marker to track the uptake of particles by cells.

Cell culture and treatment with nanoparticles

U87MG and Neuro-2A cells were selected for the present study based on the evidence from the literature, Neuro-2a cells were used as the control cell line as it does not express CXCR4 receptor while U87MG cells were used as the test cell line since it expresses CXCR4 receptors.[12,13] The cells were successfully cultured under standard conditions as detailed in the methodology section. To treat the cells with

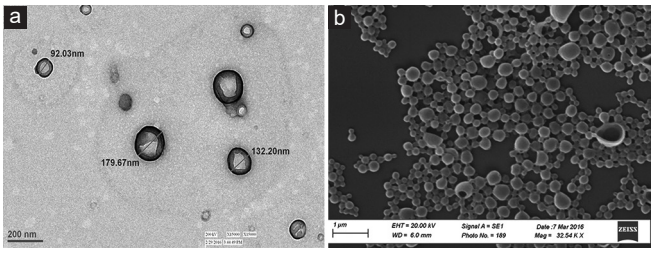

Figure 1: (a) Transmission electron microscopy image of the poly (lactic-co-glycolic acid)-polyethylenimine nanoparticles showing sizes displayed the varied sizes ranging from 50 nm to 200 nm. (b) Scanning electron microscopy of nanoparticles revealed that the nanoparticles were spherical

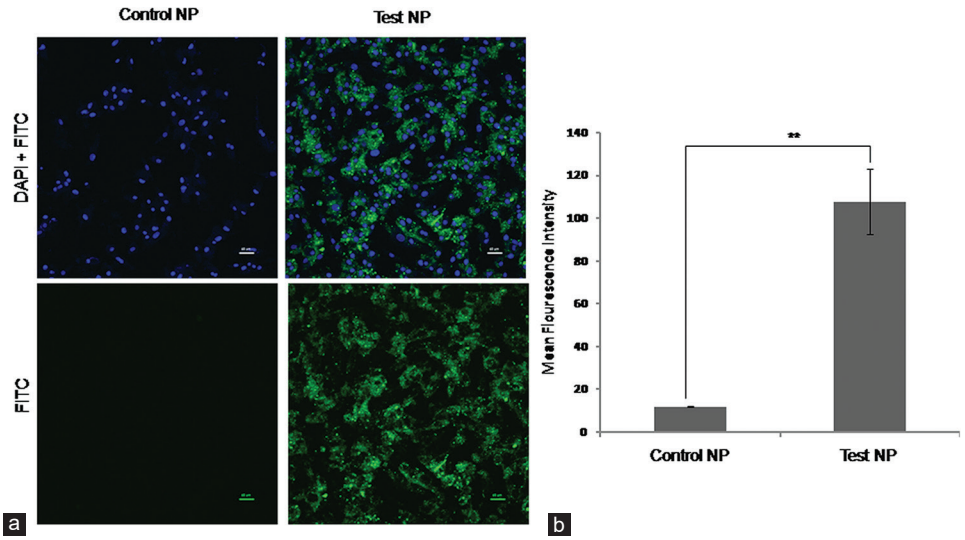

Figure 2: (a) Confocal microscopy images of U87MG cells after 24 hours of treatment with control NPs, avidin-poly (lactic-co-glycolic acid)-polyethylenimine (Avidin-PLGA-PEI) nanoparticles and test NPs, avidin-PLGA-PEI nanoparticles tagged with biotin-FITC. (b) Fluorescence quantification in U87MG cells after treatment with control and test NPs ($P < 0.01$). FITC: Fluorescein isothiocyanate, NP: Nanoparticle, DAPI: 4', 6-diamidino-2-phenylindole

nanoparticles, initially, the cells were counted and seeded in a 96-well plate at a density of 1×10^4 per well and grown in working DMEM media. After 24 h of growth, the cells exhibited their ideal morphology and were treated with specific nanoparticle suspensions. The cells were allowed to grow under standard conditions for another 24 h. Thus, U87MG and Neuro-2a cells were exposed to both peptide-avidin-PLGA NP and avidin PLGA NP uptake in different wells for 24 h.

Analysis of peptide-avidin-poly(lactic-co-glycolic acid) NPs delivery to U87MG and Neuro-2a cells

After 24 h of treatment, cells were fixed and stained with DAPI. Fluorescence quantification for FITC was done using confocal microscopy [Figure 3]. U87MG cells treated with peptide-avidin-PLGA nanoparticles showed increased mean fluorescence intensity than cells treated with avidin-PLGA nanoparticles alone (MFI – 45.5 and 25.36, respectively, $P < 0.01$). Furthermore, nanoparticle uptake by U87MG cells with peptide-avidin-PLGA nanoparticles was significantly more as compared to Neuro-2a cells (MFI - 45.5 and 15.86, respectively, $P < 0.01$).

Discussion

A targeted drug delivery system has the potential to enhance the therapeutic efficacy of drugs in cancer treatment and effectively minimize the bystander effects. Given its critical role in cancer metastasis, CXCR4 is an emerging druggable target with great potential for tumor sensitization to anticancer therapies. This is affirmed by reports proving the role of the CXCL12-CXCR4 axis in tumor angiogenesis and metastasis.[3,14] CXCR4 activates intracellular signaling pathways such as PI3K, MAPK, and ERK 1/2 and plays a critical role in cancer cell survival, proliferation, and migration.[15,16] There is high expression of CXCR4 on cancer stem cells that are mainly responsible for tumor resistance to anticancer therapies and relapse.[17-19] Targeting cancer stem cells through CXCR4 receptors is a promising approach to combat various malignancies effectively. Although many studies reporting various targeted delivery systems exist,[20] only a few revolve around CXCR4 receptor targeting. Most of the CXCR4-targeting studies show convincing results for hepatic, lung, and breast cancers, but the nanocarrier used is mostly lipid based or polymer based with lipid coating.[21-24] Since the biocompatibility of delivery systems is a preferred requisite to evolve translational therapeutics, PLGA-based systems have been studied extensively in this regard. The advantages of PLGA-based delivery systems include high stability, efficient cellular uptake by endocytosis, ability to target specific cells or tissues by ligand binding, biodegradability, low toxicity, and sustained release characteristics. Likewise, many researchers have reported PLGA-based drug delivery systems for various cancers. Jin *et al*.[25] designed PLGA-polyethylene glycol (PEG) nanoparticles targeting EGFR as a curcumin delivery system for breast cancer and Pereira *et al*.[26] devised carcinoembryonic antigen-targeting PLGA nanoparticle system for colorectal cancer therapy. Both these studies report the successful targeting of cancer cells using PLGA-PEG nanoparticles as delivery vehicles. Although PEG is widely used and offers several advantages such as particle stability and increased circulation time along with enhanced permeability and retention effect, it also has some undesirable effects[27,28] such as inhibition of cellular uptake and endosomal escape (that significantly decreases the activity of the delivery system), unexpected pharmacokinetic behavior, nonbiodegradability, and rare complement activation and hypersensitivity reactions.

Considering these aspects, we designed a PLGA delivery system without using PEG. Chittasupho *et al*.[29] reported

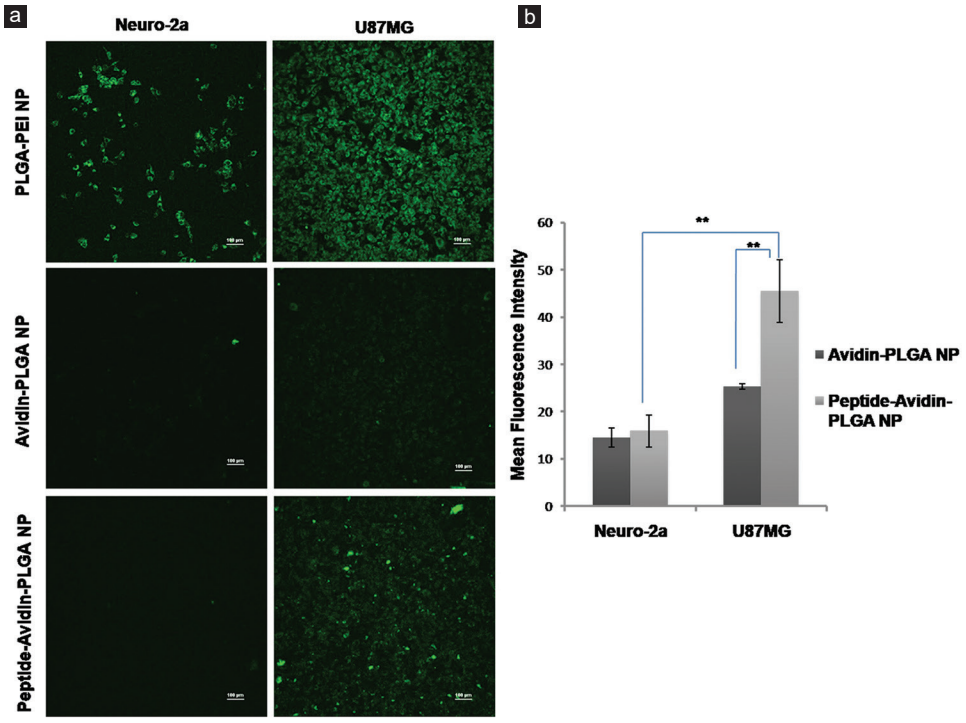

Figure 3: Confocal microscope images of (a) Neuro-2a and U87MG cells after treatment with PLGA-PEI NPs, avidin-PLGA NPs, and peptide-avidin-PLGA NPs for 24 h. (b) Fluorescence quantification in U87MG cells as compared to Neuro-2a cells after uptake of avidin-PLGA and peptide-avidin-PLGA NPs ($P < 0.01$). PLGA: Poly(lactic-co-glycolic acid), PEI: Polyethylenimine, NP: Nanoparticle

CXCR4-targeting LFC131-Dox-NP for lung cancer where the authors used PLGA nanoparticles as a delivery vehicle and T140 derivative peptide-LFC131 as a CXCR4 inhibitor and reported convincing results. However, short peptide molecules containing only L-amino acids are prone to proteolytic degradation. Synthetic D-peptides can be advantageous over natural L-peptides, as they are highly stable and resistant to proteolytic degradation.[5] One such CXCR4-ligand containing D-amino acid analogs is the DV1 peptide. This peptide was initially used by various study groups for inhibition of HIV1 entry into its reservoir cells. DV1 peptide is a modified derivative of vMIP-II comprising only 1–21 residues from the N-terminus and made up of D-amino acid analogs. The N-terminal of vMIP-II has been reported to inhibit CXCR4.[6] We have used this DV1 peptide analog as the targeting ligand for CXCR4 receptors in our study. We designed an avidin-PLGA nanoparticle surface conjugated with biotinylated DV1 peptide and exploited the avidin-biotin system to ensure efficient and strong tagging of DV1 peptide to PLGA nanoparticle surface.

To assess the presence of avidin groups on the surface of nanoparticles formed following the discussed protocol, a batch of avidin-PLGA-PEI nanoparticles was prepared without FITC. Surface functionality of nanoparticles for avidin groups was confirmed by tagging those using biotin-FITC conjugate and subsequently treating U87MG cells with biotin-FITC-tagged avidin PLGA-PEI NP and untagged avidin PLGA-PEI NP. Any green fluorescence observed in cells would be due to biotin-FITC which in turn attributes to its binding to functional avidin groups on the surface of nanoparticles. PEI was used to ensure maximal internalization of nanoparticles by the cells by giving a less negative charge. Significantly high fluorescence intensity in cells treated with biotin-FITC-tagged avidin PLGA-PEI NPs as compared to cells treated with untagged avidin PLGA-PEI NPs confirmed the functionality and specificity of incorporated avidin to biotinylated ligands [Figure 2].

Neuro-2a and U87MG cells were treated with PLGA-PEI nanoparticles and nonselective uptake of NPs was observed in both the cell lines. To specifically target CXCR4 receptors, avidin-PLGA nanoparticles (without PEI) were prepared and a part of them was tagged with biotinylated DV1 peptide. PEI was eliminated to overcome the nonspecific uptake of nanoparticles by cells. On the treatment of U87MG and Neuro-2a cells with peptide-tagged and untagged avidin-PLGA nanoparticles, we observed significantly enhanced uptake of peptide-avidin-PLGA nanoparticles by U87MG cells as compared to the uptake of avidin-PLGA NP. Furthermore, the uptake of peptide-avidin-PLGA nanoparticles was significantly more in U87MG cells as compared to Neuro-2a cells [Figure 3]. These results suggest the specificity of our designed drug delivery system for CXCR4 receptors expressed on U87MG cells.

The CXCR4-targeting drug delivery system demonstrated in this study can be used to effectively deliver

chemotherapeutic drugs or other small molecules specifically to cancer cells and cancer stem cells. Nanoparticles targeted to target cells are predominantly uptaken by the cells and are released intracellularly into the cytoplasm following lysosomal escape due to localized destabilization of the endolysosomal membrane. Thus, such targeted nanosystems offer the advantage of intracellular drug delivery. Since PLGA nanoparticles have the potential to cross the blood–brain barrier, our CXCR4-targeting drug delivery system can also be used as therapy for CNS neoplasm. However, *in vivo* studies are needed to be done for the translation of this work to clinical therapeutic practice.

Conclusion

CXCR4-targeting drug delivery system surface modified with DV1 peptide successfully targets CXCR4-expressing cancer cells. DV1 peptide being resistant to proteolytic degradation would be effective for a longer period on the nanoparticles. The application of this targeted therapy will significantly enhance the maximum tolerable doses of chemotherapeutic drugs and reduce the systemic side effects. This drug delivery system can act as an effective tool against the cancer stem cells that attribute to chemoresistance in various cancers. Further studies are needed to establish the promising role of this drug delivery system in clinical therapeutics.

Ethical statement

The study was approved by the Institute Ethics Committee of All India Institute of Medical Sciences, New Delhi, vide Reference number IESC/T-326/August 02, 2013.

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Conflicts of interest

There are no conflicts of interest.

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