Enhanced in vitro antiproliferative effects of EpCAM antibody-functionalized paclitaxel-loaded PLGA nanoparticles in retinoblastoma cells

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Background: To specifically deliver paclitaxel (PTX) to retinoblastoma (RB) cells, the anionic surface-charged poly(lactic-co-glycolic acid) (PLGA) NPs loaded with paclitaxel were conjugated with epithelial cell adhesion molecule (EpCAM) antibody for enhancing site-specific intracellular delivery of paclitaxel against EpCAM overexpressing RB cells.

Methods: PTX-loaded PLGA NPs were prepared by the oil-in-water single emulsion solvent evaporation method, and the PTX content in NPs was estimated by the reverse phase isocratic mode of high performance liquid chromatography. Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride/N-hydroxysuccinimide chemistry was employed for the covalent attachment of monoclonal EpCAM antibody onto the NP surface. In vitro cytotoxicity of native PTX, unconjugated PTX-loaded NPs (PTX-NPs), and EpCAM antibody-conjugated PTX-loaded nanoparticles (PTX-NP-EpCAM) were evaluated on a Y79 RB cell line by a dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, while cellular apoptosis, cysteinyl-aspartic acid protease (caspase)-3 activation, Poly (adenosine diphosphate-ribose) polymerase (PARP) cleavage, and cell-cycle arrest were quantified by flow cytometry. By employing flow cytometry and fluorescence image analyses, the extent of cellular uptake was comparatively evaluated.

Results: PTX-NP-EpCAM had superior antiproliferation activity, increased arrested cell population at the G2-M phase, and increased activation of caspase-3, followed by PARP cleavage in parallel with the induction of apoptosis. Increased uptake of PTX-Np-EpCAM by the cells suggests that they were mainly taken up through EpCAM mediated endocytosis.

Conclusions: EpCAM antibody-functionalized biodegradable NPs for tumor-selective drug delivery and overcoming drug resistance could be an efficient therapeutic strategy for retinoblastoma treatment.

Advances in our knowledge of molecular biology of cancer and pathways involved in malignant transformation of cells are revolutionizing the approach to cancer treatment with a focus on targeted cancer therapy. The newer approaches to cancer treatment not only supplement conventional chemotherapy and radiotherapy but also aim to prevent damage to normal tissues and overcome drug resistance [1]. Nanoparticulate drug delivery systems using biodegradable polymeric carriers have attracted increasing attention in recent years. The major advantage of using these nanoparticles (NPs) is their sustained release property, and since the drug is encapsulated, it is unexposed to the cell membrane-associated efflux transporters [2-4]. In this way the efflux action of these transporters could be bypassed, resulting in greater cellular drug uptake than that with drug in solution. Polymeric NPs, primarily based on biodegradable poly (D,L-lactic-co-glycolic acid; PLGA) polymers, have been used for the administration of water insoluble anticancer agents, such as paclitaxel (PTX) [3,5-8]. Since PLGA NPs cannot be delivered to specific cells in a target-specific manner, using cell recognizable targeting ligands, such as monoclonal antibodies, endogenous targeting peptides, and low-molecular-weight compounds, such as folate, onto the surface of the NPs will enhance the intracellular delivery capacity of polymeric NPs to specific cells [9-13]. One possible approach of target-specific delivery could be using antibodies directed toward membrane protein overexpressed by cancer cells.

Earlier we showed that epithelial cell adhesion molecule (EpCAM), a transmembrane protein, is highly expressed in retinoblastoma (RB) primary tumors [14], and recently we demonstrated that EpCAM inhibition leads to decreased RB cell proliferation in vitro [15]. EpCAM is a 40,000 molecular weight, type I, transmembrane glycoprotein that consists of two epidermal growth factor-like extracellular domains, a cysteine-poor region, a transmembrane domain, and a short cytoplasmic tail. EpCAM is overexpressed in various epithelial cancers [16] and is an ideal therapeutic target because of the following reasons: (a) overexpression in cancer cells versus noncancerous cells, (b) apical expression in
cancer cells and basolateral expression in normal epithelial cells [17], and (c) not shed into the circulation [18].

In this context, we made use of EpCAM membrane protein for targeted delivery of the chemotherapy drug paclitaxel to retinoblastoma cells that express high EpCAM. We formulated paclitaxel-loaded PLGA NP surfaces functionalized with EpCAM monoclonal antibody and tested their efficacy in the retinoblastoma Y79 cell line in vitro.

**METHODS**

**Preparation of PTX-loaded nanoparticles:** PTX-loaded PLGA NPs were prepared by the oil-in-water, single emulsion, solvent evaporation method with little modifications. In this method, PTX (equivalent to 10% weight/weight [w/w] dry weight of polymer) was dissolved in 3 ml organic solvent (chloroform) containing 100 mg of polymer (PLGA) to form a primary emulsion. The emulsion was further emulsified in an aqueous poly vinyl alcohol (PVA) solution (12 ml, 2% volume/volume [v/v]) to form an oil-in-water emulsion. The emulsification was performed using a microtip probe sonicator (VC 505; Vibracell Sonics, Newtown, CT) set at 55 W of energy output for 2 min over an ice bath. The emulsion was stirred overnight on a magnetic stir plate at room temperature to evaporate the organic solvent. The excess amount of PVA was removed the next day by ultracentrifugation at 8,500 ×g, 4 °C for 20 min (Kendro/Sorvall UltraTurbine Centrifuge, Artisan Scientific Corporation, Champaign, IL), followed by three washes with double distilled water. The recovered nanoparticulate suspension was lyophilized for 2 days (−80 °C and <10 mm Hg; LYPHLOCK; Labconco, Kansas City, MO) to obtain lyophilized powder for further use.

**Particle size analysis and zeta potential measurement:** To determine the particle size and zeta potential, 1 mg/ml of NP solution was prepared in double distilled water. The sample (100 µl) was diluted to 1 ml, sonicated in an ice bath for 30 s, and subjected to particle size and zeta potential measurement using a zetasizer (Zetasizer nano-zs ZEN3600; Malvern Instrument, Worcestershire, UK).

**Transmission electron microscopic studies:** NPs were also evaluated for size by transmission electron microscopy (TEM; Philips/FEI Inc., Barliff Manor, NY). For this purpose, a sample of NPs (0.5 mg/ml) was suspended in water and sonicated for 30 s. One drop of this suspension was placed over a carbon-coated copper TEM grid (150 mesh; Ted PELLA Inc., Redding, CA) and negatively stained with 1% uranyl acetate for 10 min and then allowed to dry. Images were visualized at 120 kV under a TEM (Philips/FEI Inc., Barliff Manor, NY).

**Scanning electron microscopic studies:** The surface morphology of NPs was characterized by scanning electron microscopy (SEM; JEOL JSM-T220A; Tokyo, Japan) operating at an accelerating voltage of 10–30 kV. The NPs were sputtered with gold to make them conductive and placed on a copper stub before the acquisition of SEM images.

**Estimation of entrapment efficiency of Paclitaxel loaded nanoparticles:** The PTX content in NPs was estimated by the reverse phase isocratic mode of high performance liquid chromatography (HPLC) with slight modification, using an Agilent 1100 HPLC (Agilent Technologies, Boblingen, Germany), which consists of a Zorbax Eclipse XDB-C18, 150×4.6 mm internal diameter, with an internal standard of dimethylphthalate. Briefly, 1 mg of lyophilized PTX-loaded NPs was dissolved in 1 ml of acetonitrile and kept in a shaker at 37 °C and 150 rpm (Wadegati Labequip, Mumbai, India) for 48 h for proper dissolution of the particulate system and better release of the entrapped drug. After 48 h, samples were removed from the shaker and centrifuged at 1,000×g for 10 min at 25 °C (SIGMA 3K30; Life-Sciences, Bremen, Germany) to extract the drug present in the solution. Five hundred microliters of the supernatant was collected, and 20 µl of this supernatant was injected manually in the injection port and was analyzed using a mobile phase of methanol–acetonitrile–water (60:5:35; v/v/v). Separation was achieved by isocratic solvent elution at a flow rate of 1 ml/min with a quaternary pump (Model No-G1311A, Agilent Technologies) at 10 °C with a thermostat (Model No-G1316A, Agilent Technologies). The PTX level was quantified by ultraviolet
detection at 228 nm (with Diode Array Det [DAD], Model-G 1315A, Agilent Technologies). The amount of PTX in the NPs was determined from the peak area correlated with the standard curve. The standard curve of PTX was prepared under identical conditions. All analyses were performed in triplicate. Triplicate samples were analyzed, and the PTX encapsulation efficiency was calculated by dividing the amount of PTX entrapped by the total amount of PTX added, multiplied by 100.

In vitro release of paclitaxel from nanoparticles: In vitro release kinetics of PTX from NPs was determined in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4; pH adjusted to 7.4 containing 0.1% Tween-80) at 37 °C. Ten milligrams of NPs was dispersed in 3 ml of the PBS buffer. The NP suspension was equally divided (1 ml each) in three tubes. These tubes were kept on a shaker at 37 °C and 150 rpm (Wadegati Labequip, Mumbai, India). At particular time intervals these tubes were taken from the shaker and centrifuged at 1,000 × g, 4 °C for 10 min (Sigma 1–15K microfuge, Shropshire, UK). The supernatants were removed to estimate the amount of drug released at that particular time, using reverse phase (RP)-HPLC. The same amount of fresh PBS was added to the residue, which was placed back on the shaker for further in vitro release studies at different time points.

Conjugation of epithelial cell adhesion molecule antibody on the surface of nanoparticles: For covalent attachment of monoclonal EpCAM antibody onto the NP surface, EDC/NHS chemistry was employed. Briefly, 10 mg of PTX-loaded NPs was dissolved in 5 ml of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4; pH adjusted to 7.4), followed by drop-wise addition of 250 µl of EDC solution (1 mg/ml) and 250 µl of NHS solution (1 mg/ml) in 0.02 M PBS to the NP suspension. The sample was left at room temperature under agitation for 4 h on a magnetic stirrer. The sample was then ultracentrifuged at 8,500× g at 4 °C for 20 min (Sorvall Ultraspeed Centrifuge; Kendro) to remove unreacted EDC and NHS. The process was repeated three times, and the sediment was washed each time with 1 ml PBS (0.02 M, pH 7.4). Finally, to dissolve the pellet obtained after centrifugation, 2 ml of PBS (0.02 M, pH 7.4) was added. We used EpCAM-fluorescein isothiocyanate (FITC) for conjugation to identify the amount of EpCAM conjugated to NPs. For coupling EpCAM-FITC, the EDC-activated NPs were suspended in 2 ml of PBS (0.02 M) and 500 µl of EpCAM-FITC antibody (200 µg/ml in PBS, Santa Cruz Biotechnology, Santa Cruz, CA) was added drop wise and stirred for another 2 h at room temperature; this solution was incubated overnight at 4 °C. The next day unconjugated EpCAM-FITC was removed by ultracentrifugation and the supernatant was collected to calculate the conjugation efficiency. EpCAM-conjugated NP suspension was lyophilized to obtain the powder for further use. The collected supernatant was used to estimate the amount of EpCAM-FITC that was not conjugated to PTX-loaded NPs, using a fluorescence spectrophotometer (Synergy HT; BioTek® Instruments Inc., Winooski, VT), and the pellet was lyophilized for further studies. The above procedure was followed to conjugate EpCAM to NPs (EpCAM was used instead of EpCAM-FITC) for further experiments.
**Cell lines and cell culture:** The Y79 cell line (endogenously EpCAM-expressing cell line) was obtained from the cell bank, RIKEN BioResource Center (Ibaraki, Japan). Rosewell Park Memorial Institute (RPMI) 1640 media and fetal bovine serum (FBS) were purchased from Gibco-BRL (Rockville, MD). Y79 was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 0.1% ciprofloxacin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 4.5% dextrose and grown in suspension at 37 °C in a 5% CO₂-humidified incubator. The study adhered to the Declaration of Helsinki. This study was conducted at the Medical Research Foundation and Vision Research Foundation, Sankara Nethralaya, India, and was approved by the Vision Research Foundation ethics board.

**Y79 cells treatment with native Free Paclitaxel (PTX)/ Paclitaxel loaded Nanoparticles (PTX-NPs)/Epithelial cell adhesion molecule conjugated, paclitaxel loaded Nanoparticles (PTX-NP-EpCAM):** Y79 cells (1×10⁵ cells/ml) were seeded on tissue culture treated flat-bottomed cell culture plates (Axygen, Inc. Union city, CA) containing RPMI media and allowed to grow overnight. The next day, 0.5 µg/ml media containing native PTX and an equivalent concentration of freeze-dried PTX-NPs/PTX-NPs-EpCAM was added to the wells and the plates were incubated for 48 h in a CO₂ incubator (Hera Cell; Thermo Scientific, Waltham, MA). Void nanoparticle served as the control. After respective incubation periods, the cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄; pH adjusted to 7.4) and collected for mitogenic assay, apoptosis assay, cell-cycle analysis, cysteine-aspartic proteases (caspase)-3, and poly (ADP-ribose) polymerase (PARP) activity assay.

**Mitogenic assay:** In a dose–response study, cell viability was determined after 5 days following treatment. A standard (3-

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| Formulation       | Sizea | Zeta potentialb | PDFc | Encapsulation efficiencyd |
|-------------------|-------|----------------|------|---------------------------|
| PTX-NPs           | 272±1.6 | −14.8±2.2       | 0.17 | 83                        |
| PTX-NP-EpCAM      | 313±3.3 | −13.1±2.5       | 0.15 | 79                        |

*aSize in nm as measured by photon correlation spectroscopy. bZetapotential in mV measured by zetasizer. cPolydispersity index measured by photon correlation spectroscopy. dPercentage of encapsulation efficiency of NPs measured by RP-HPLC.*

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![Figure 4. Characterization of paclitaxel loaded nanoparticles by transmission electron microscopy (TEM). TEM was used to analyze the size and size distribution of the paclitaxel loaded nanoparticles.](http://www.molvis.org/molvis/v17/a295)
(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay was used to determine cell viability. Reagents were mixed and added to each well (10 µl/well), plates were incubated for 3 h at 37 °C in a cell culture incubator, and the color intensity was measured at 490 nm using a microplate reader (BioTek, Winooski, VT). The antiproliferative effect of different treatments was calculated as a percentage of cell growth with respect to the respective control.

Assessment of apoptosis by flow cytometry: The induction of apoptosis by PTX/PTX-NPs/PTX-NP-EpCAM was studied by flow cytometry, using the annexin FITC apoptosis detection kit (BD Biosciences, Mississauga, ON, Canada). For apoptosis study the cells were pelleted and then resuspended in 100 µl of 1× binding buffer (Clontech Laboratories, Inc., Palo Alto, CA). Thereafter, 5 µl annexin V-FITC (final concentration, 1 µg/ml; BD Biosciences) and 5 µl propidium iodide (10 µg/µl; MP Biomedicals Inc.) were added to the cells and incubated at room temperature in the dark for 20 min. Before flow cytometric analysis 400 µl of 1× binding buffer was added to the cells and the extent of apoptosis was determined by analyzing 15,000 ungated cells, using a FACSscan flow cytometer and Cell Quest software (FACS Calibur; Becton-Dickinson, San Jose, CA). All experiments were performed in triplicate.

Cell-cycle analysis: Cell-cycle analysis was studied by flow cytometry using the BD cycle test plus DNA Reagent kit (BD Biosciences) according to the manufacturer’s protocol. In brief, the treated cells were centrifuged, cells were centrifuged for 5 min at 300× g at room temperature and 1 ml buffer (DMSO [DMSO] in sucrose-sodium citrate) solution was added to the supernatant. Cells were resuspended by gentle vortexing at low speed (procedure repeated twice). Solution A 206 (250 µl) was added to the cells re-suspended in buffer solution, and the solution was mixed by tapping and then incubated for 10 min. Next, 200 µl of solution B was added and mixed by tapping, followed by incubation for 10 min. Then, 200 µl of cold solution C (propidium iodide [PI]) was added to each tube, mixed by tapping, and then incubated for 10 min in the dark on ice. The sample was filtered through a 35-µm cell strainer and analyzed by flow cytometry.

Measurement of intracellular uptake: One day before treatment, Y79 cells were plated in a 24-well format with 1×10^5 cells/well containing 1 ml of RPMI 1650 medium with 10% FBS. On the day of treatment, Y79 cells were incubated with coumarin-labeled NPs (NPs or NP-EpCAM) for 2 days. Untreated cells were kept as a control. Following incubation, the cells were harvested, washed twice with PBS, centrifuged for 5 min at 500× g, and resuspended in ice-cold PBS. Intracellular uptake was determined by flow cytometry [19].
Microscopic studies: To study the intracellular retention of the drug, cells were treated with either dye, coumarin in solution, or freeze-dried dye-loaded NPs (unconjugated or conjugated with EpCAM antibody; 10 μg/ml media). Untreated cells were used as a control to account for the autofluorescence, if any. The medium was changed on day 2 after treatment and then every alternate day, and no further dose of the dye was added. At different time points, the cells were washed thrice with PBS to remove any uninternalized dye and then visualized using an Axio Observer fluorescent microscope (Carl Zeiss, Berlin, Germany) equipped with an argon laser with an excitation wavelength set at 488 nm and emission at 525 nm [20]. Detection was with a band-pass emission barrier filter. The images were processed using Axio Vision 4.7 software (Carl Zeiss, Bangalore, India).

Caspase-3 and poly (Adenosine Diphosphate-ribose) polymerase activity assay by flow cytometer: For intracellular staining, cells were fixed and permeabilized with 2% paraformaldehyde and 0.05% Tween-20 to allow intracellular labeling with the respective cleaved PARP antibody (ab32064; Abcam, San Francisco, CA), and cleaved caspase-3 antibody (Cell Signaling, Danvers, MA; 1:200 dilution) was added and incubated for 1 h. Following incubation, cells were washed twice with ice-cold PBS and incubated with secondary FITC-antirabbit immunoglobulin (Sigma, 1:1,000 dilution) for 30 min at 4 °C. Cells were then washed twice with ice-cold PBS, resuspended in FACS buffer, and analyzed by FACS.

Statistical analysis: All experiments were repeated at least three times. ANOVA (ANOVA) was used for statistical analysis. The differences were considered significant for p values of <0.05.

RESULTS

Characterization of paclitaxel-loaded nanoparticles conjugated with epithelial cell adhesion molecule antibody: PTX-loaded NPs were prepared by the single emulsion method as described in the methodology. EpCAM was covalently conjugated to the carboxylic group of PLGA NPs by the EDC and NHS activation method (Figure 1). The amount of EpCAM-FITC antibody conjugated to the nanoparticle surface was determined by fluorescence spectrophotometry. Approximately 7.8 μg of EpCAM antibody was found to attach per milligram of the nanoparticle. Dynamic light scattering analysis revealed that the formulated nanoparticle had an average diameter of 272±1.6 nm (Figure 2) with a negative zeta potential of -14.8±2.2 mV (Figure 3). On measuring the size of EpCAM-conjugated NPs, a negligible increase in the size of NPs (272 to 313 nm) was observed after conjugation of EpCAM to the NP surface (Table 1). The zeta potential of NP-EpCAM was comparatively less than unconjugated NPs (-14.8±2.2 mV versus -13.1±2.5 mV; Table 1). TEM images showed a
discrete spherical outline and monodispersed size distribution (~100 nm) of PLGA NPs (Figure 4). The topology of the NPs as observed by SEM analysis confirmed the smooth and spherical nature of PTX-NPs (Figure 5). The encapsulation efficiency of the NPs was around 83% (i.e., 83% of the drug added in formulation was entrapped in NPs) as estimated by RP-HPLC. After conjugation the encapsulation efficiency slightly decreased to about 79%.

In vitro release of paclitaxel by nanoparticles: In the in vitro release study, NPs demonstrated a sustained release of the encapsulated drug, with approximately 38% cumulative drug release in 21 days (Figure 6). The NPs gave a comparatively faster release (~22% in 1 day) followed by a slow release (~38% in 21 days) of the drug from the polymeric matrix.

Cytotoxicity assay: The ability of the PTX-NPs (conjugated or unconjugated) to deliver PTX and induce cell death was examined on the retinoblastoma cell line (Y79) by the MTT assay. Results demonstrated that cell viability of the Y79 cell line was affected by different concentrations of PTX as well as PTX-NPs/PTX-NP-EpCAM. Greater antiproliferative activity was observed for almost all doses of PTX-NP-EpCAM compared to that of the native PTX and PTX-NP (Figure 7A). PTX-NP-EpCAM-treated Y79 cells exhibited an inhibitory concentration 50 (IC\textsubscript{50}) value as low as 0.005 µg/ml, while native PTX and PTX-NP had an IC\textsubscript{50} value as high as 0.05 and 3.5 µg/ml, respectively. Another important observation was the increase in the antiproliferative activity of the drug with incubation time (day 5) when cells were treated with low doses of the drug using PTX-NP-EpCAM (PTX dose=0.005 µg/ml; Figure 7B). Differences between the native PTX and PTX-NPs (conjugated or unconjugated) were significantly evident after 5 days of drug treatment as they showed significantly greater antiproliferative effect (42% inhibition by PTX-NP-EpCAM versus 71% and 60% inhibition by native PTX and PTX-NP, respectively). The Y79 cells were treated with native PTX and PTX-NP (conjugated and unconjugated) for 2 days in vitro. The cells were harvested on day 2 for an apoptotic assay using FACS. Following treatment with native PTX, Y79 cells showed 2.91% apoptosis (1.68% of early apoptosis, 1.23% of late apoptosis), and with PTX-NP, Y79 cells showed 8.06% of apoptosis (6.93% early apoptosis and 1.13% late apoptosis). In contrast, Y79 cells treated with PTX-NP-EpCAM showed increased late apoptosis (17.16% early apoptosis; 2.24% late apoptosis; Figure 8).

Figure 7. Dose and time-dependent cytotoxicity of paclitaxel (PTX) and PTX loaded nanoparticles (NPs) in Y79 cells. A and B: Different concentrations of PTX either as solution or PTX encapsulated in NPs or Epithelial cell adhesion molecule antibody-conjugated PTX-NPs were added to the wells with medium. The extent of growth inhibition was measured at 48 h and at day 5 by the (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Inhibition was calculated with respect to respective controls. Experiments were performed in triplicates and the data are represented as mean±standard error (*p<0.001).
Increased caspase-3 activation and Poly (adenosine diphosphate-ribose) polymerase cleavage in Y79 cells treated with epithelial cell adhesion molecule antibody conjugated paclitaxel loaded nanoparticles: To determine whether the apoptosis of Y79 cells induced by PTX is a specific caspase-dependent pathway, we analyzed the effect of native PTX, PTX-NPs, and PTX-NP conjugated with epithelial cell adhesion molecule antibody (PTX-NP-EpCAM) treatment on Y79 cells after 48 h incubation. The Y79 cells treated with PTX-NP-EpCAM showed significantly higher (p<0.001) early and intermediary apoptotic events compared to PTX-NP or native PTX. Abbreviation: PI represents propidium iodide.

Figure 8. Apoptotic effects of paclitaxel loaded nano-conjugates on Y79 cells. Flow cytometry analysis showing the effect of control (A), native paclitaxel (PTX; B), PTX-loaded nanoparticles (PTX-NP; C), and PTX-NP conjugated with epithelial cell adhesion molecule antibody (PTX-NP-EpCAM; D) treatment on Y79 cells after 48 h incubation. The Y79 cells treated with PTX-NP-EpCAM showed significantly higher (p<0.001) early and intermediary apoptotic events compared to PTX-NP or native PTX. Abbreviation: PI represents propidium iodide.

Figure 9. Analysis of caspase-3 expression in paclitaxel loaded nano-conjugates. Flow cytometry analysis showing caspase-3 expression in Y79 cells after the treatment with control (A); native paclitaxel (PTX; B); PTX-loaded nanoparticles (PTX-NP; C), and PTX-NP conjugated with epithelial cell adhesion molecule antibody (PTX-NP-EpCAM; D). Increased caspase expression was noted in Y79 cells treated with PTX-NP-EpCAM compared to PTX-NP or native PTX.
nanoparticles: We quantified the percentage of cells at various phases of the cell cycle and plotted them against the native PTX/PTX-NP/PTX-NP-EpCAM (Figure 11). In untreated Y79, 3.84% cells were at the G2-M phase. When exposed to native PTX and PTX-NP, the arrested population increased about 12.2% and 17.8%, and the increase continued progressively to 21.7% with PTX-NP-EpCAM.

Increased uptake of coumarin-6 labeled epithelial cell adhesion molecule antibody conjugated nanoparticles by Y79 cells: To explain the increased cytotoxicity of the PTX-NP-EpCAM formulation, the intracellular uptake of NP-EpCAM was quantitatively examined in an EpCAM-overexpressing Y79 cell line (Figure 12). In the case of NP-EpCAM, increased fluorescence intensity profiles to the right direction indicates that the cellular uptake was significantly enhanced due to EpCAM-mediated endocytosis.

The extent of cellular uptake for NP-EpCAM was about twofold greater than that of EpCAM antibody unconjugated NPs and tenfold greater than the free coumarin-6 dye, (Figure 12). The fluorescence intensity was twofold less in the HeLa cell line, which does not express EpCam compared to uptake in Y79 cells. This clearly shows that EpCAM on Y79 cells mediates the specific uptake of NP-EpCAM.

Prolonged retention of NP-EpCAM in Y79 cells: To further confirm the enhanced intracellular uptake of NP-EpCAM, fluorescence microscopic analysis was performed. Y79 cells incubated with free coumarin in solution demonstrated drug internalization within 4 h of incubation, but with increase in incubation time, the fluorescent intensity decreased slowly. EpCAM-conjugated coumarin-loaded NPs on the other hand were more strongly stained with clear visualization of the internalized TXNPs, and in addition they showed a significant increase in fluorescence intensity with incubation time, with strong fluorescence even after 5 days of treatment as compared to the unconjugated coumarin-loaded NPs and free coumarin (Figure 13). In this study we envisioned that the uptake of EpCAM-conjugated NPs via EpCAM-mediated endocytosis could have an intracellular disposition pathway different from that of unconjugated NPs. This could influence the intracellular retention of NPs and hence the therapeutic efficacy of the encapsulated drug in retinoblastoma. Our study shows that the EpCAM-conjugated PLGA NPs could be potentially applied to target-specific intracellular delivery of various hydrophobicanticancer agents.

DISCUSSION

Functionalization of NPs with tumor-targeting ligands, such as antibodies or peptides, directed against overexpressed tumor markers not only enhances localization of the particles to the solid tumor mass but also allows the NPs to target early stage tumors and metastatic tumor cells. Treatment with monoclonal antibody (mAbs) is a viable therapeutic option in cancer. Recently, these mAbs, such as cetuximab and herceptin, have been used as targeting agents to selectively deliver chemotherapeutics to cancerous cells [21]. For example, Reddy et al. has used folic acid-coated polymeric NPs and demonstrated enhanced localization and internalization of NPs for drug delivery to breast cancer cells [22], whereas Zhang et al. has shown that folic acid-coated magnetite NPs demonstrated improved localization and internalization intended for tumor imaging of breast cancer cells [23]. Similarly, tagging anti-HER2 to the nanoparticle surface greatly improved cell internalization of gelatin/albumin [24].

Our objective in the present study was to examine the target-specific intracellular delivery capacity of PLGA NPs by coating EpCAM antibody on their surface. Although the prepared PLGA NPs with a nanoscale size distribution accumulated at the solid tumor site in a passive targeting manner by an “enhanced permeation and retention” effect [22], cell-specific targeting ability by EpCAM antibody
Figure 11. Cell cycle analysis of Y79 cells treated with paclitaxel loaded nano-conjugates. Increased G2-M arrest was observed by flow cytometry analysis in Y79 cells treated with paclitaxel-loaded nanoparticles conjugated with epithelial cell adhesion molecule antibody (PTX-NP-EpCAM) at 48 h compared to paclitaxel-loaded nanoparticles (PTX-NP) or native paclitaxel (PTX). A: Untreated Y79 cells showing 3.8% of G2-M phase after doublet discrimination. B: Y79 cells treated with native paclitaxel showing increased G2-M cells (12.2%). C: Y79 cells treated with PTX-NP showing 17.8% of G2-M phase. D: Y79 cells treated with PTX-NP-EpCAM showing 21.7% G2-M phase.
conjugation is responsible for promoting their intracellular uptake within EpCAM-expressing cancer cells. PTX-loaded NPs were prepared by the single emulsion method, and EpCAM moieties were covalently conjugated to the carboxylic group of PLGA NPs by the EDC and NHS activation method for cell recognition. The enhancement of cellular uptake was gained via an EpCAM receptor-mediated intracellular delivery mechanism.

Fluorescent microscopy and flow cytometry analysis revealed increased uptake of NP-EpCAM by Y79 cells compared to unconjugated NPs or native coumarin-6 dye. Interestingly, PTX-NP-EpCAM showed an increase in the antiproliferative activity of the drug with incubation time when cells were treated with low doses of the drug using PTX-NP-EpCAM (PTX dose=0.005 µg/ml). PTX-NP-EpCAM showed higher apoptotic events in Y79 cells as evidenced by caspase-3 activation. The induction of apoptosis is considered to be one of the principle mechanisms by which PTX induces tumor regression in retinoblastoma [23,24]. Suarez et al. [23] has shown that paclitaxel administered through the subconjunctival route effectively inhibited the retinoblastoma tumor burden in the LH beta-Tag animal models. However, higher doses of native drug administration are associated with ocular tissue toxicities [23]. For improved sustain drug release, PLGA NPs were used to deliver drugs in diabetic rat models through subconjunctival administration [25]. This approach reduces the ocular tissue toxicity associated with native drugs and enhances sustained drug release for effective therapeutic response. Our present study has further modified the paclitaxel-containing PLGA NPs with EpCAM antibody for targeted and sustained drug release to RB cells.

One of the key initiation elements of the apoptotic pathway is the activation of caspases followed by cleavage of the caspase substrates [26]. The 113-kDa PARP-1, which is normally involved in DNA repair, DNA stability, and other cellular events, is cleaved by members of the caspase family during early apoptosis. PTX-NP-EpCAM showed increased G2-M phase arrest in Y79 cells compared to PTX-NP or native PTX. Previous studies have shown that PTX arrests cells at the G2-M phase of the cell cycle [27,28] and that defects of spindle assembly or the presence of detached chromosomes activates an internal signaling pathway that probably initiates the induction of PTX-induced apoptosis [29].

**Figure 12. Flow cytometry analysis of uptake of nanoparticles by Y79 cells**

Flow cytometry analysis showed significantly higher uptake of coumarin encapsulated nanoparticles conjugated with epithelial cell adhesion molecule antibody (COU-NP-EpCAM) by Y79 cells compared to that of unconjugated coumarin encapsulated nanoparticles. EpCAM negative cell line HeLa cells were used as negative control which showed relatively less uptake (mean intensity -1144.44) compared to EpCAM positive Y79 cells (mean intensity -2090.80).

| Key          | Name                     | Mean intensity |
|--------------|--------------------------|----------------|
| Unstained    |                          |                |
| Native coumarin |                        | (182.69)       |
| Coumarin-NP  |                          | (1175.74)      |
| Coumarin-NP-EpCAM in Y79 |                | (2090.80)      |
| Coumarin-NP-EpCAM in HeLa |               | (1144.44)      |
Figure 13. Microscopic analysis of uptake of nanoparticles by Y79 cells. Fluorescent microscopic analysis showing the uptake of native coumarin (A-D), coumarin encapsulated nanoparticles (COU-NP; E-H), and coumarin encapsulated nanoparticles conjugated with epithelial cell adhesion molecule antibody (COU-NP-EpCAM; K-N) at 4 h (A, E, K), day 1 (B, F, L), day 2 (C, G, M), and day 5 (D, H, N). The Y79 cells showed increased COU-NP-EpCAM uptake compared to unconjugated COU-NP at all time points. Increased coumarin-NP retention was observed in the Y79 cells even at day 5 when compared to free coumarin.
To conclude, this study demonstrates the proof of principle of using EpCAM antibody to specifically deliver the chemotherapy drugs to retinoblastoma cells. Further in vivo studies are warranted to use this formulation in clinical settings for retinoblastoma and other EpCAM-expressing cancer management.

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