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Enhanced cytotoxicity of TATp-bearing paclitaxel-loaded micelles *in vitro* and *in vivo*

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ABSTRACT

Cell-penetrating peptide (TATp) was attached to the distal tips of polyethyleneglycol (PEG) moieties of polyethyleneglycol–phosphatidylethanolamine (PEG–PE) micelles loaded with paclitaxel (PCT). The TATp-modified micelles demonstrated an increased interaction with cancer cells compared to non-modified micelles resulting in a significant increase of the *in vitro* cytotoxicity to different cancer cells. TATp-modified PCT-loaded micelles were administered intratumorally in mice and the induction of apoptosis in tumor cells was studied after 48 h with the Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay using free PCT and TATp-free PCT-loaded PEG–PE micelles as controls. A significant apoptotic cell death was observed in tumors treated with PCT-loaded micelles modified with TATp, while the treatment with free PCT or with non-modified PCT-loaded micelles resulted in much smaller number of TUNEL-positive cells within tumors.

1. Introduction

One of the popular nanocarrier systems for the delivery of poorly soluble drugs, including anticancer drugs, is the polymeric micelles (Kataoka et al., 2001; Torchilin, 2007). Among different types of polymeric micelles reported, micelles composed of polyethyleneglycol–phosphatidylethanolamine (PEG–PE) conjugate draw a lot of attention because of their small size (10–100 nm), good solubilization efficiency, and high stability (Torchilin, 2001, 2007; Krishnadas et al., 2003; Lukyanov and Torchilin, 2004). The small size of micelles allows for their efficient accumulation in pathological tissues with leaky vasculature, such as tumors and infarcts, via the enhanced permeability and retention (EPR) effect (Maeda et al., 2000, 2008). Many efforts have been made to increase the delivery of the micellar nanocarriers to tumor cells by using monoclonal antibodies (mAb) (Torchilin et al., 2003b) or other tumor-specific ligands (Bae and Kataoka, 2006) attached to the micelle surface.

In order to facilitate the delivery of anticancer drug not only to the surface of cancer cells, but also inside those cells for an enhanced therapeutic effect, a promising approach for the intracellular delivery that is under development over the last decade—the use of cell-penetrating peptides (CPPs) (Schwarze et al., 1999; Snyder and Dowdy, 2004). In particular, the CPP derived from the human immunodeficiency virus-1 transactivator protein (TATp) has attracted much interest. TATp-mediated cytoplasmic uptake of polymers, plasmid DNA (Astriab-Fisher et al., 2002; Nguyen et al., 2008), nanoparticles (Lewin et al., 2000; Zhao et al., 2002; Rao et al., 2008), liposomes (Torchilin et al., 2001; Levchenko et al., 2003; Fretz et al., 2004) and micelles (Sethuraman and Bae, 2007; Sawant et al., 2008) has been reported. Various uptake mechanisms appear to be involved in different systems, and in some cases, the mechanism is cell-type or cargo-specific (Zorko and Langel, 2005). Smaller molecules attached to TATp seem to transduce directly into cells by the less energy-independent electrostatic interactions and hydrogen bonding (Vives et al., 2003; Rothbard et al., 2004), while the larger cargos seem to get into cells by the energy-dependent macropinocytosis pathway (Wadia et al., 2004). One of the major obstacles in using TATp-assisted intracellular delivery of pharmaceutical nanocarriers is the lack of selectivity of TATp. This non-selectivity provides concern about drug-induced toxic effects towards normal tissues. Thus intratumoral administration of TATp containing nanocarriers may serve as good solution to this problem for delivery of anticancer drugs at least in certain cases (Fujita et al., 2008).

Here, we prepared and studied paclitaxel (PCT)-loaded TATp-modified PEG–PE micelles. The potential of these micelles for the

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induction of apoptosis was further evaluated *in vivo* after the intratumoral injection in tumor-bearing mice.

2. Materials and methods

2.1. Materials

TAT-cysteine peptide (TATp-Cys) (12-mer: CysTyrGlyArgLysLysArgArgGlnArgArgArg) was synthesized at the Tufts University Core Facility (Boston, MA, USA). PCT was purchased from Sigma (St. Louis, MO, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (poly(ethylene glycol))-750] (PEG_{750}-PE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-PE), were from Avanti Polar Lipids (Alabaster, AL). 3-(4,5-Dimethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Promega (Madison, WI, USA). Cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Cell culture media, Dulbecco's modified eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), and concentrated solutions of sodium pyruvate and penicillin/streptomycin stock solutions were purchased from Cellgro (Herndon, VA, USA). All other chemicals were analytical grade preparations from Sigma (St. Louis, MO, USA).

2.2. Synthesis of TATp-PEG–PE conjugates

TATp-PEG_{1000}-PE was synthesized using a heterobifunctional NHS-PEG_{1000}-Mal derivative, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and TATp-Cys as in (Kale and Torchilin, 2007a,b).

2.3. Preparation of PCT-loaded micelles

PCT solubilized in micelles were prepared by adding PCT (1 mg/mL in methanol) to a PEG_{750}–PE solution in chloroform. The organic solvents were removed by rotary evaporation to form a thin film of drug/micelle material mixture. This film was further dried under high vacuum overnight to remove traces of remaining solvents. Drug-loaded micelles were formed by resuspending the film in 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-buffered saline (HBS), pH 7.4. The mixture was incubated in a water bath at 37 °C for 10 min. Excess non-incorporated drug was separated by centrifugation ($13{,}000 \times g$) before characterization. For preparation of TATp-containing micelles, 2.5 mol% of TATp-PEG_{1000}–PE in chloroform was added to the above preparation during micelle preparation. For cell interaction study, 0.5 mol% of the fluorescent probe, Rh-PE, was added to the micelle composition during micelle preparation.

2.4. Characterization of micelles

2.4.1. Size analysis and morphology

The micelle size (hydrodynamic diameter) was measured by the dynamic light scattering (DLS) using a N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL, USA). The micelle suspensions were diluted with the deionized, distilled water until the concentration providing a light scattering intensity of 5×10^4 to 1×10^6 counts/s was achieved. The particle size distribution of all samples was measured in triplicate.

2.4.2. Drug solubilization efficiency

The amount of drug in the micellar phase was measured by reversed phase-HPLC. The clear aqueous dispersion was diluted with the mobile phase prior to applying onto the HPLC column (since the mobile phase contains acetonitrile, micelles are disrupted and free drug is determined). The D-7000 HPLC system equipped with a diode array and fluorescence detector (Hitachi, Japan) and Spherisorb ODS2 column, 4.6 mm × 250 mm (Waters, Milford, MA, USA) was used. The column was eluted with acetonitrile/water (65:35, v/v) at 1.0 mL/min. PCT was detected at 227 nm. Injection volume was 50 μL; all samples were analyzed in triplicate.

2.4.3. Cell cultures

The MCF7 (human breast adenocarcinoma) and 4T1 (murine mammary carcinoma), cells were maintained in DMEM cell culture medium at 37 °C, 5% CO_2. DMEM media were supplemented with 10% FBS, 1 mM Na-pyruvate, 50 U/mL penicillin, and 50 μg/mL streptomycin.

2.4.4. Interaction of micelles with cells

The 4T1 cells were grown on cover slips placed in six-well tissue culture plates. After the cells reached a confluence of 60–70%, the plates were washed with DMEM and the cells were treated with various Rh-PE-labeled micelle (0.2 mg/mL of the micelle-forming material) samples (with and without TATp) in DMEM. After 1 h, the medium was removed and the plates were washed with sterile PBS, pH 7.4. Individual cover slips were mounted cell-side down onto fresh glass slides with PBS. Cells were viewed with a Nikon Eclipse E400 microscope under bright light or under epifluorescence with a rhodamine/TRITC filter.

2.4.5. Cytotoxicity assay

Cells were plated at a 5×10^3 cells per well density in 96-well plates (Corning Inc., Corning, NY, USA). After 24 h incubation at 37 °C, 5% CO_2, the medium was replaced with medium containing free drug dissolved in DMSO or drug-loaded micelles with or without TATp for 48 h. After the incubation, each well was washed twice with Hank's buffer and cell survival was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method. The absorbance of the degraded MTT was measured at 492 nm (the measurement of the cytotoxicity) using a Labsystems Multiskan MCC/340 microplate reader (Labsystems and Life Sciences International, UK).

2.4.6. Tumor growth and intratumoral administration of PCT-loaded micelles

Approximately 10^5 4T1 cells were inoculated in 6–8 weeks old female Balb/C mice by the subcutaneous injection into the left flank. Fourteen days after tumor inoculation, the mice were injected intratumorally with different PCT formulations equivalent to 5 mg/kg of PCT. After 48 h, the tumors were harvested, embedded in tissue freezing media and stored at −80 °C. Cryostat sections were made, stained with the Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay and examined under a microscope.

3. Results and discussion

For an efficient interaction of the micelle-attached TATp with the cell, the TATp moiety should be located "above" the carrier surface (Sawant et al., 2008) to allow for the unhindered interaction of TATp moieties with the cell surface (Torchilin et al., 2003a). With this in mind, micelles were prepared using PEG_{750}–PE as the main micelle-forming component with the addition 2.5 mol% TATp-PEG_{1000}–PE (see the schematics in Fig. 1). Such micelles were loaded with PCT. At 5 mM concentration of PEG_{750}–PE, with or without TATp, micelles were produced containing 151 ± 2.78 μg PCT/mL of micelle formulation. The average micelle size of all formulations was in the range of 8–25 nm.

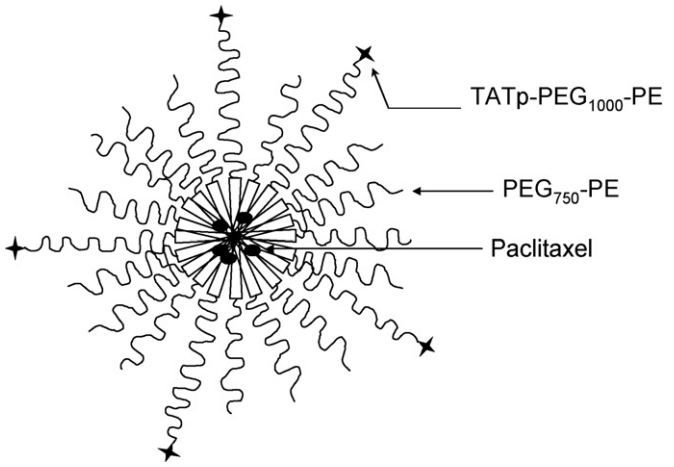

Fig. 1. Schematic representation of the micelles used in this study.

The *in vitro* cell interaction of the TATp-bearing PEG–PE micelles was confirmed by fluorescence microscopy with 4T1 cells. The interaction of different micelle preparations with 4T1 cells is shown in Fig. 2. Plain micelles composed of PEG_{750}–PE demonstrated a very limited interaction with the cells (Fig. 2A), however, the use of the TATp-bearing PEG–PE micelles resulted in the expected strong interaction with the cells (Fig. 2B).

The next question was, whether this strong interaction would help to increase the delivery of the drug (PCT in PCT-loaded TATp-bearing micelles) into cells compared to the free PCT or PCT-loaded micelles without TATp. The *in vitro* cytotoxicity of different formulations was investigated using MCF7 and 4T1 cells. The cells were incubated with free drug or with different drug-loaded micellar formulations for 2 days and analyzed for their survival using the MTT colorimetric assay for the dehydrogenase activity of viable cells. As seen in Fig. 3, there is a significantly higher cytotoxicity with PCT-loaded TATp-bearing micelles compared to PCT-loaded micelles without TATp or free PCT at both tested PCT concentrations (5 and 50 nM). This could be explained by the increased interaction

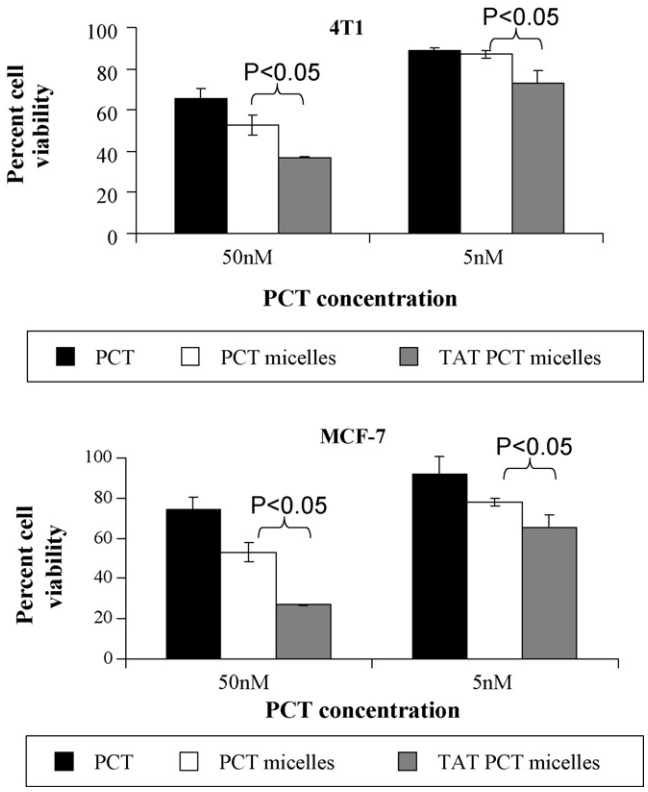

Fig. 3. *In vitro* cytotoxicity of various PCT formulations towards 4T1 and MCF-7 cells.

of TATp-bearing micelles with cells compared to micelles without TATp. Drug-free, plain or TATp-modified micelles, as well as the TATp itself were not toxic to cells at the concentrations used (data not shown).

In *in vivo* studies in tumor-bearing mice, to avoid any unwanted distribution of PCT-loaded TATp-micelles, those were injected intratumorally, and tumors were harvested after 48 h. The nuclear

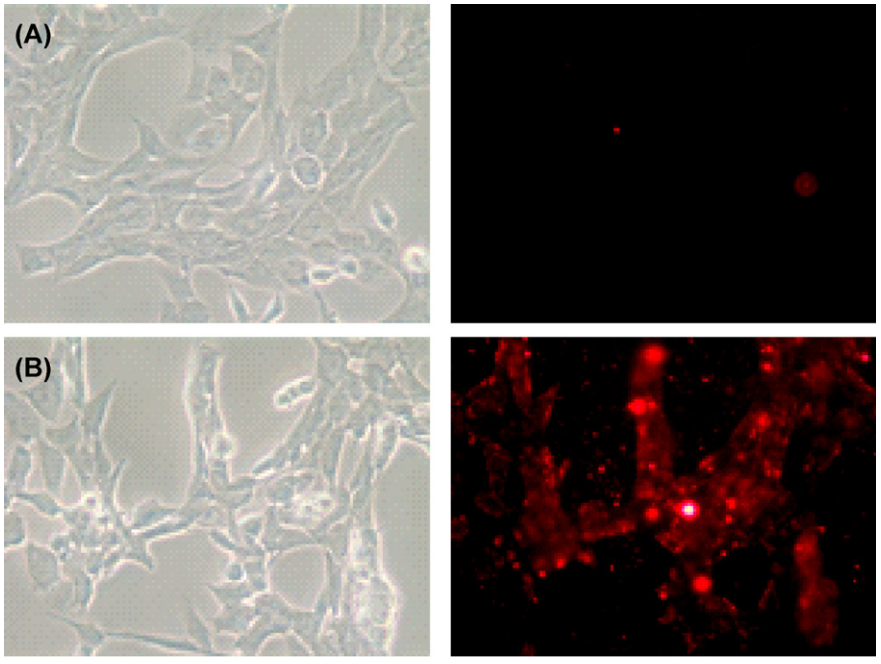

Fig. 2. Left panel shows the bright field and right panel shows the fluorescent microscopy of 4T1 cells treated with Rh-PE:PEG_{750}–PE micelles (A), Rh-PE:PEG_{750}–PE:TATp-PEG_{1000}–PE micelles (B). Magnification 40× objective.

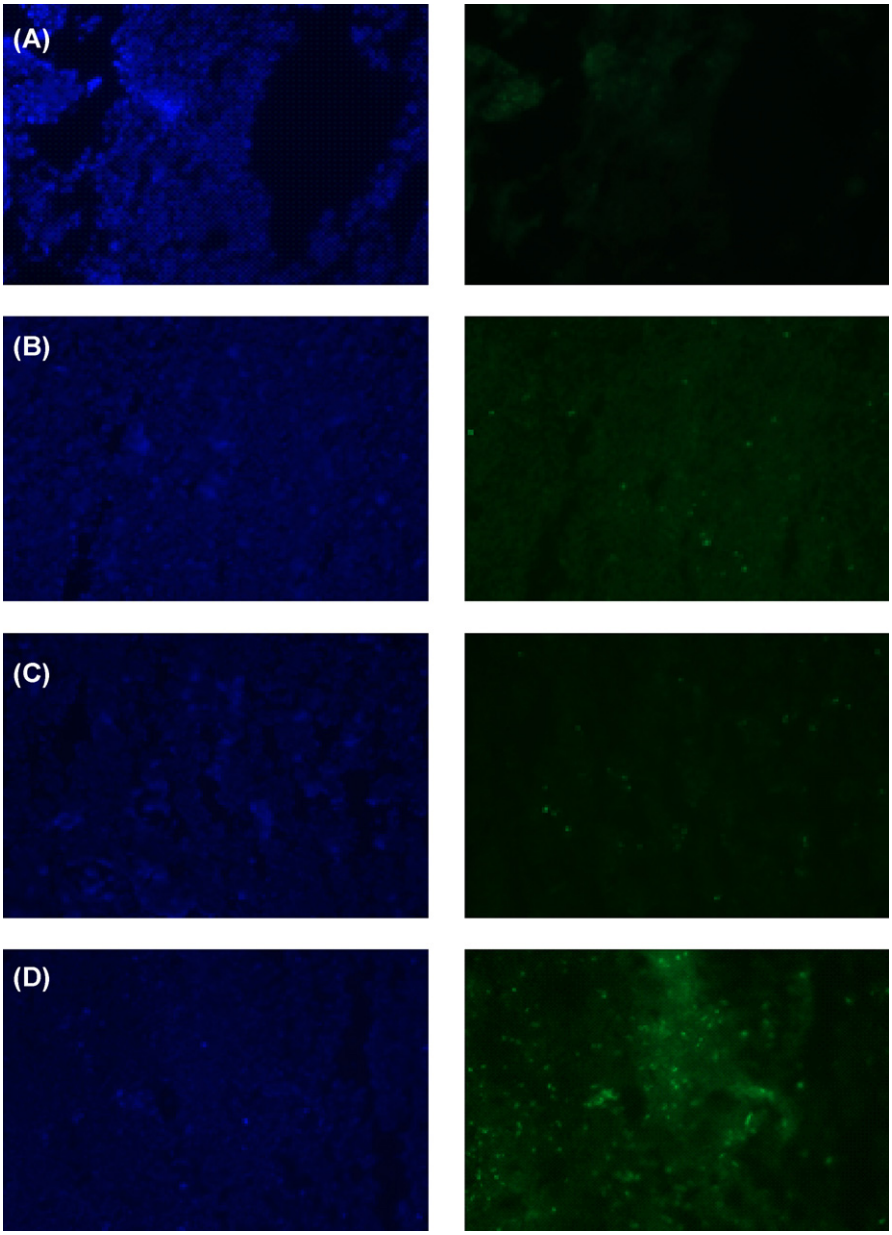

Fig. 4. Detection of the apoptotic cells by the fluorescence microscopy in frozen tumor sections. Apoptosis determined by TUNEL. The left panel shows the sections stained with DAPI and the right panel shows TUNEL. Negative control (A), free PCT (B), PCT-loaded micelles without TATp (C), PCT-loaded micelles with TATp (D). Magnification 20× objective.

DNA fragmentation in tumor sections (to confirm the presence of the apoptosis process) was followed by the TUNEL assay using the DNA fragmentation kit, Fluorescein FragEL™ according to the manufacturer's instructions. The TUNEL assay enables the differentiation of apoptosis from necrosis because DNA fragments produced by the apoptosis are exclusively labeled with the TUNEL method (Gold et al., 1994). The results of the TUNEL staining of tissue sections are shown in Fig. 4. In DAPI stained images, it is difficult to observe differences among groups since the cell nuclei of live cells represented bright blue fluorescence attributed to DAPI staining. However, the TUNEL assay images showed the very different results depending on the type of the treatment. The cell nuclei of non-apoptotic bodies did not exhibit the green fluorescence attributed to FITC-labeled TdT. Very few TUNEL-positive cells were observed in tumors injected with free PCT or with PCT-loaded micelles (Fig. 4). Significant apoptotic cell death was observed in tumors treated with PCT-loaded TATp-bearing micelles (Fig. 4). Thus, these *in vivo* results are in good agreement with our *in vitro* cytotoxicity data.

4. Conclusion

Micelles bearing a CPP function, TATp, demonstrated strong interaction with cells *in vitro*. This enhanced interaction translated into the increased cytotoxicity both *in vitro* and *in vivo* when such micelles were loaded with the anticancer drug PCT. Such CPP-mediated drug-loaded pharmaceutical nanocarriers represent promising candidates for intratumoral administration.

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