Co-delivery of drug nanoparticles and siRNA mediated by a modified cell penetrating peptide for inhibiting cancer cell proliferation

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Experimental sections

Materials and reagents
Ellipticine (EPT) (MW, 246.31; >98% purity) was purchased from EMD-Merck (Mississauga, Canada). STR-H16R8 was synthesized by Pepscan (Lelystad, The Netherlands). R8 was synthesized by CanPeptide Inc. (Pointe-Claire, Canada). siRNA targeting Bcl-2 oncogene was purchased from Sigma, (Oakville, Canada) with a sense sequence of GUGAAGUCAACAUGCCUGCdTdT and antisense sequence of GCAGGCAUGUGACUUCACdTdT. The scrambled siRNA from Life Technologies (Carlsbad, USA) was employed as negative control in the experiments.

Cell culture
The non-small lung carcinoma A549 cells (ATCC, Manassas, USA) were cultured in Dulbecco’s Modified Eagle Media-high glucose (DMEM) (Invitrogen, Burlington, Canada) with 10% heat-inactivated FBS. All of the cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Preparation of nanoparticles
2 mL of 2 mM EPT dichloromethane solution was added into 10 mL water at 50 °C under sonication. 2 min later, 10 mL of water or the peptide solution was added into the EPT suspension, which was stirred at 200 rpm overnight to remove the organic solvent, the final volume of which was adjusted to 50 mL by adding water. The EPT NP suspension was then mixed with water or 2 µM siRNA solution, respectively (1:1, v/v). The complexes of peptide/siRNA was prepared by mixing 2 µM siRNA solution and peptide solutions (1:1, v/v) before characterization and cell culture assays.

Transmission electron microscopy (TEM)
6 µL of NP suspension was placed on a perforated copper grid (R 3.5/1, Quantifoil Micro Tools GmbH, Jena, Germany), and then the sample was frozen at −180 °C in a cryo box (Carl Zeiss NTS GmbH, Germany). The sample was then transferred with a liquid nitrogen cooled holder (626, Gatan Inc., USA) into the pre-cooled cryo-electron microscope (CM 120, Philips, Netherlands) operated at 120 kV and viewed under low dose conditions.

Particle size and zeta potential
The hydrodynamic diameter of the complexes were measured on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a 4 mW He-Ne laser operating at 633 nm. A quartz microcell (45 µL) with a 3 mm light path was used and the scattered light intensities were collected at an angle of 173°. Zeta potential measurements were also performed on the same machine using clear disposable zeta cells. The zeta potential values were obtained with the multimodal algorithm CONTIN, Dispersion Technology Software 5.0. Three measurements were performed to generate the intensity-based size and zeta potential plot reported herein.

Fluorescence-activated cell sorting (FACS)
Cellular uptake of EPT was studied using BD FACSCalibur Flow Cytometry (BD Biosciences, Mississauga, Canada). The nanosuspension was diluted to 1/10 of the original concentration with Opti-MEM medium. A549 cells (80,000 cells/well) were plated in a 24-well cell culture plate.
h later, the medium was removed and washed with PBS. Afterwards, A549 cells were treated with 300 μL of the sample solutions. Nontreated cells served as a negative control. After 3 h incubation, the culture medium was discarded and cells were washed with PBS, Trypsin-EDTA was then added to detach the cells from the plate; cells were suspended in 4% paraformaldehyde solution and collected.

**Gene silencing**

A549 cells (40,000 cells/well) were plated in a 24-well cell culture plate. 24 h later, the medium was removed and washed with PBS. 1.28 μL of Lipofectamine 2000 (Lipo) (Life Technologies, Carlsbad, USA), a commercial transfection reagent, was diluted in 30.72 μL of RNase free water and mixed with 32 μL of BCL-2 siRNA solution to form complexes with the final concentration of siRNA at 1 μM, which was then diluted in 596 μL of OPTI-MEM medium (Life Technologies, Carlsbad, USA). The nanosuspension was also diluted to 1/10 of the original concentration with Opti-MEM medium with a final concentration of siRNA at 100 nM. 300 μL of the sample solutions containing the lipoplexes of Lipo/siRNA or the complexes of STR-H16R8/siRNA were added to the cells.3 h later, 300 μL of DMEM with 20% FBS was added. Afterwards, the cells were incubated for 48 hours at 37 °C in a 5% CO₂ atmosphere. The cultures were then washed with PBS. Total RNA was extracted from the cells with TRIzol reagent (Life Technologies, Carlsbad, USA), then treated with chloroform (Sigma, Oakville, Canada) and 2-propanol (Sigma-Aldrich, Oakville, Canada) as recommended by the manufacturer. RNA concentrations were measured by Nanodrop spectrophotometer ND-1000 (Thermo scientific, Ottawa, Canada). All RNAs were reverse transcribed with Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Mississauga, Canada). The cDNA synthesis was primed with a unique blend of oligo (dT) and random primers. The following pairs of primers were used for PCR: 5'-GGTGAGGTCATGTGTGTGG-3' and 5'-CGGTTTCAGGTACTCAGTCATCC-3' (Sigma, Oakville, Canada). Here, the housekeeping gene cyclophilin was chosen as an internal control to normalize the Bcl-2 gene. The normalization was performed by the amplification of human cyclophilin with the following primers: 5'-GGTGATCTTTGGTCTCTTCCGCG GTG-3' and 5'-TAGATGCTCTTTCCTCCTGTG-3' (Sigma, Oakville, Canada). PCR reaction was performed with Brilliant II Fast SYBR Green QPCR Master Mix (Agilent Technologies, Wilmington, USA) on an Mx3005P™ Real-Time PCR System (Agilent Technologies, Wilmington, USA).

**Cytotoxicity assay**

A549 cells were plated in to 96-well plates (8,000 cells/well). 24 h later, the medium was removed and washed with PBS. The nanosuspension was diluted to 1/10 of the original concentration with Opti-MEM. 60 μL of the sample solution was added to the cells. 3 h later, 60 μL DMEM medium with 20% FBS was added. After incubation for 48 hours, the cultures were washed with PBS. 100 μL of Opti-MEM medium with CCK-8 reagent was then added to each well. Cell viability was assessed by measuring the absorbance at 570 nm with a FLUOstar OPTIMA microplate reader and expressed as the ratio of the cells treated over the nontreated cells (negative control).