Supplementary Materials

Chemo-biologic combinatorial drug delivery using folate receptor-targeted dendrimer nanoparticles for lung cancer treatment

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Materials

Dendrimer (G4-PAMAM, (Den), 14214 MW), polyethylene imine (PEI, 800 MW), O,O′-Bis[2-(N-Succinimidyl-succinylamino)ethyl]polyethylene glycol (NHS-PEG-NHS, 2000 MW), cis-diamminedichloridoplatinum(II) (CDDP)**,** O-Phenylenediamine (OPDA), N,N-dimethylformamide (DMF), DAPI (4′6-diamidino-2-phenylindole dihydrochloride) were purchased from Sigma Aldrich (St. Louis, MO, USA). RPMI-1640 and EMEM medium, trypsin-EDTA, and fetal bovine serum (FBS) were obtained from GIBCO BRL Life Technologies (New York, NY, USA). All siRNAs, control siRNA (5' UAA GGC UAU GAA GAG AUA C 3'), and HuR-siRNA (5' UCA AAG ACG CCA ACU UGU A 3') were purchased from Dharmacon (Lafayatte, CO, USA).

Characterization: Zeta Potential, Transmission Electron Microscopy.

The surface charge of each step of Den-PEI-CDDP-siRNA-FA synthesis starting from the G4 PAMAM platform was measured by zeta potential. All samples were dispersed in Tris-HCl (pH 7.4) buffer to measure the zeta potential using a Brookhaven ZetaPALS analyzer (Holtsville, NY, USA). The size and shape of the nanoparticles were characterized by Transmission Electron Microscopy (TEM) images (HITACHI, 80 kV, Schaumburg, IL, USA). Samples were dispersed in Tris-HCl, placed on copper grids, and air-dried.

Folic acid conjugation to Den-PEI-CDDP nanoparticles.

The Den-PEI-CDDP-FA molecule was synthesized by conjugating FA molecules to the free amine groups of Den-PEI polymer of the nanoparticles. The FA linker with an N-hydroxy succinamide (NHS) group (folic acid-PEG-NHS, Nanocs) can form a firm bond with amine

groups. 47 µg of Den in Den-PEI-CDDP was reacted with 20, 40, 80, or 160 µg of folic acid-PEG-NHS linker in Tris-HCl buffer for 3 h at room temperature, followed by centrifugation and removal of the unbound linker molecules. The conjugation was confirmed by measuring absorbance spectroscopy of FA at 280 nm.

OPDA assay.

To estimate the unknown CDDP concentration, the OPDA solution was incubated with known CDDP concentrations or unbound CDDP fractions in the presence of N,N-dimethylformamide (DMF) at 95 ^{o}C. After 30 min of incubation, a blue product indicated Pt-OPDA metal complex formation. The absorbance of this blue adduct was measured at 704 nm using a Denovix DS11 spectrophotometer (Wilmington, DE, USA) and the unknown CDDP concentrations were obtained from a standard graph[1]. The CDDP encapsulation efficiency was calculated using following equation:

% Encapsulation efficiency = (Initial amount of CDDP added - amount of CDDP left in the supernatant) /Initial amount of CDDP added X 100.

Agarose gel electrophoresis for siRNA encapsulation and protection assays.

Briefly, 40-µl aliquots of the Den-PEI-CDDP-siRNA and Den-PEI-CDDP-siRNA-FA nanoparticles were taken and subjected to gel electrophoresis in 1.2% agarose gel at 80V for 30 min in Tris-acetate-EDTA (TAE) buffer (pH 8.0). The siRNA complexation with Den-PEI nanoparticles was determined against the free siRNA used as a positive control. The electrophoresis gel stained with ethidium bromide (SIGMA St. Louis MO, USA) was visualized and imaged by Syngene gel documentation system.

Optimization of folic acid concentration.

To optimize the folic acid concentration of Den-PEI-CDDP-siGLO-FA nanoparticles to achieve the highest cell uptake efficiency, fluorescence measurements were conduct *in vitro* using fluorescently labeled siRNA (siGLO). Briefly, H1299 cells were treated with different amounts of folic acid-PEG-NHS linker (3.75, 7.5, 11, 18, 37, 56, or 75 µg per well) conjugated Den-PEI-CDDP-siGLO nanoparticles with a constant siGLO concentration of 50 nM per well. After 24 h of incubation with nanoparticles, cells were collected and the fluorescence intensity of each sample was recorded using an Envision® plate reader.

Optimization of CDDP concentration.

The CDDP concentration required to achieve the combination effect in H1299 cells was optimized by a cell viability assay. In brief, H1299 cells were treated with Den-PEI-CDDP nanoparticles or different concentrations of free CDDP (10, 25, 40, 50, 60, 80, or 100 µM). After 24 h of nanoparticle incubation, the cells were harvested and cell viability was measured using a trypan blue assay. Resulted were compared with those from the untreated control, as previously described[2, 3].

***In vitro* release profiles of CDDP and siRNA from Den-PEI-CDDP-siRNA and Den-PEI-CDDP-siRNA-FA nanoparticles.**

CDDP and siRNA release profiles from Den-PEI-CDDP-siRNA or Den-PEI-CDDP-siRNA-FA nanoparticle were analyzed *in vitro* in the presence and absence of 10% fetal bovine serum (FBS) in Tris-HCl buffer with pH 7.4. For CDDP release studies, the Den-PEI-CDDP-siRNA nanoparticle complex was collected in slide-A-lyzer mini dialysis tubes with 200-µl

capacity (3500 MW cutoff; Thermo Scientific, Rockford IL USA) and were inserted into 1.5-ml microfuge tubes containing Tris-HCl, with or without 10% FBS. Tubes were incubated at 37 °C with mild stirring. 200 µl of releasing medium was collected from each sample at predetermined intervals and was replaced with fresh buffer. The samples were collected until 7 h. The released CDDP for time point was estimated using a colorimetric OPDA assay, as mentioned earlier. The percent CDDP release was calculated by comparing it with stock CDDP concentrations. Analysis of the CDDP release profile from the Den-PEI-CDDP-siRNA-FA study followed the abovementioned protocol and was conducted in Tris-HCl (pH 7.4) buffer.

For siRNA release studies, the Den-PEI-CDDP-siRNA nanoparticle complex was incubated at 37 $^{\circ}$C with mild stirring with either 10% FBS or FBS-free Tris-HCl. At each time interval, samples were collected from the supernatants. The medium was replaced with fresh buffer solutions of respective composition. The siRNA concentration in each samples were obtained using PicoGreen assay following the manufacturers' protocol. Briefly, the collected supernatant was mixed with 100 µl (200 times dilution from stock) of Quant-iT PicoGreen reagent® (Thermo Fisher Scientific, USA). The reaction product was subjected to fluorescence measurement at 485 nm excitation and 535 nm emission wavelengths using an Envision multilabel reader (Perkin Elmer, Santa Clara, CA, USA). The amount of siRNA in the supernatant was quantified by comparing with the stock concentration of siRNA and is represented as the % of siRNA release from Den-PEI-CDDP-siRNA nanoparticles. The siRNA release kinetics from the Den-PEI-CDDP -siRNA-FA nanoparticles were also determined with a similar procedure.

Quantitative real-time PCR (qRT-PCR).

HuR mRNA expression was analyzed using quantitative real-time PCR **(**qRT-PCR) for cells treated with Den-PEI-CDDP, Den-PEI-HuR, or Den-PEI-CDDP-HuR nanoparticles compared with untreated cells, using the TRIZOL (Invitrogen, Grand Island NY, USA) method. Total RNA was isolated according to the manufacturer's protocol. First, strand cDNA was synthesized from 100 ng of total RNA with a Quant script cDNA synthesis kit (*Bio*-Rad, Richmond CA). The synthesized cDNA was diluted by 10 times. From the dilution, 5-µl aliquots of each cDNA sample were used for qRT-PCR analysis with the iQ SYBER Green Supermix kit (Bio-rad, Richmond CA, USA), with a total reaction volume of 20 µl. Each sample was run in triplicate. HuR-specific oligonucleotide primers (Forward- 5' ATGAAGACCACATGGCCGAAGACT 3'- Sense; Reverse - 5' TGTGGTCATGAGTCCTTCCACGAT 3'- Antisense) were used. The PCR conditions and calculated HuR gene expression were performed as described earlier [4].

Results

Optimization of folic acid concentration

The siGLO concentration in the nanoparticles was kept constant and folic acid concentration was varied. As illustrated in **Supplementary Figure 3A**, with increasing folic acid concentrations up to 3.75 µg the fluorescence intensity was increased. Fluorescence intensity gradually decreased with further increases in folic acid. The highest fluorescence intensity of ~19578 a.u. ($p < 0.01$) was observed with 3.75 µg of FA in Den-PEI-CDDP-siGLO. Hereafter, all of our studies were conducted with 3.75 µg of folic acid-PEG-NHS-conjugated dendrimer nanoparticles.

Optimization of CDDP concentration in H1299 cells

CDDP concentration was varied and tested for its *in vitro* cell killing efficiency in H1299 cells. The cells were treated with free CDDP and Den-PEI-CDDP nanoparticles with different concentrations of CDDP from 10 to 100 µM per well, as represented in **Supplementary Figure 3B.** Free CDDP group induced ~19% cell killing at a 10-µM concentration, which further increased to ~57% killing at a 50-µM concentration (**Supplementary Figure 3B**). The cell killing efficiency plateaued with higher concentrations of CDDP. In contrast, Den-PEI-CDDP nanoparticles showed ~22% killing efficiency at a 10-µM concentration and reached ~87% (p <0.01) killing efficiency at 100 µM. A gradual increase in cell killing efficiency was observed with Den-PEI-CDDP. This efficiency was significantly higher than that of the free-CDDP at equivalent concentrations. For our further therapeutic siRNA and CDDP combination studies, we chose a CDDP concentration of 10 µM, which is the IC_{20} value of CDDP in H1299 cells.

Validation of siRNA-based knockdown of HuR in H1299 and A549 cell lines

HuR-specific siRNA (HuR) transfection using Den-PEI nanoparticles was conducted in H1299 and A549 cell lines. In H1299 cells, HuR knockdown via Den-PEI-HuR nanoparticles showed ~30% (p <0.05) cell growth inhibition at 72 h of treatment, whereas the control scrambled siRNA (CSi) -carrying Den-PEI nanoparticles induced significantly lower toxicity at the respective time points (**Supplementary Figure 4A**). The trend was repeated in the A549 cell line, which showed ~29% (p <0.01) cell growth inhibition with Den-PEI-HuR nanoparticles, while the CSi-carrying Den-PEI nanoparticles showed significantly lower toxicity at 72 h (**Supplementary Figure 4A**).

Western blot analysis confirmed HuR knockdown as a result of treatment with Den-PEI nanoparticles carrying HuR siRNA in H1299 and A549 cell lines (**Figure 4B**). HuR knockdown downregulated the cyclin-dependent kinase protein cyclin E,[6] which was confirmed by reductions in cyclin E expression in both cell lines when treated with Den-PEI-HuR nanoparticles. The western blot analysis also confirmed that HuR and cyclin E proteins was significantly reduced by Den-PEI-HuR nanoparticles (p <0.01). The increased cell growth inhibition and significant reduction of HuR expression of Den-PEI-siHuR nanoparticles demonstrate that the HuR siRNA specifically knocked down HuR, resulting in significant cell growth inhibition compared with Den-PEI-CSi and untreated controls. The specific reduction of HuR and cyclin E expression correlates with the enhanced cell growth inhibition induced by Den-PEI-HuR nanoparticles in H1299 and A549 cells.

References

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Supplementary Figure Legends

Supplementary Figure 1: Schematic representation of synthesis of Den-PEI-CDDP-siRNA-FA nanoparticles. CDDP and siRNA were encapsulated in dendrimer nanoparticles through hydrolysis and electrostatic methods, respectively. Folic acid was conjugated to the surface amines of dendrimer nanoparticles.

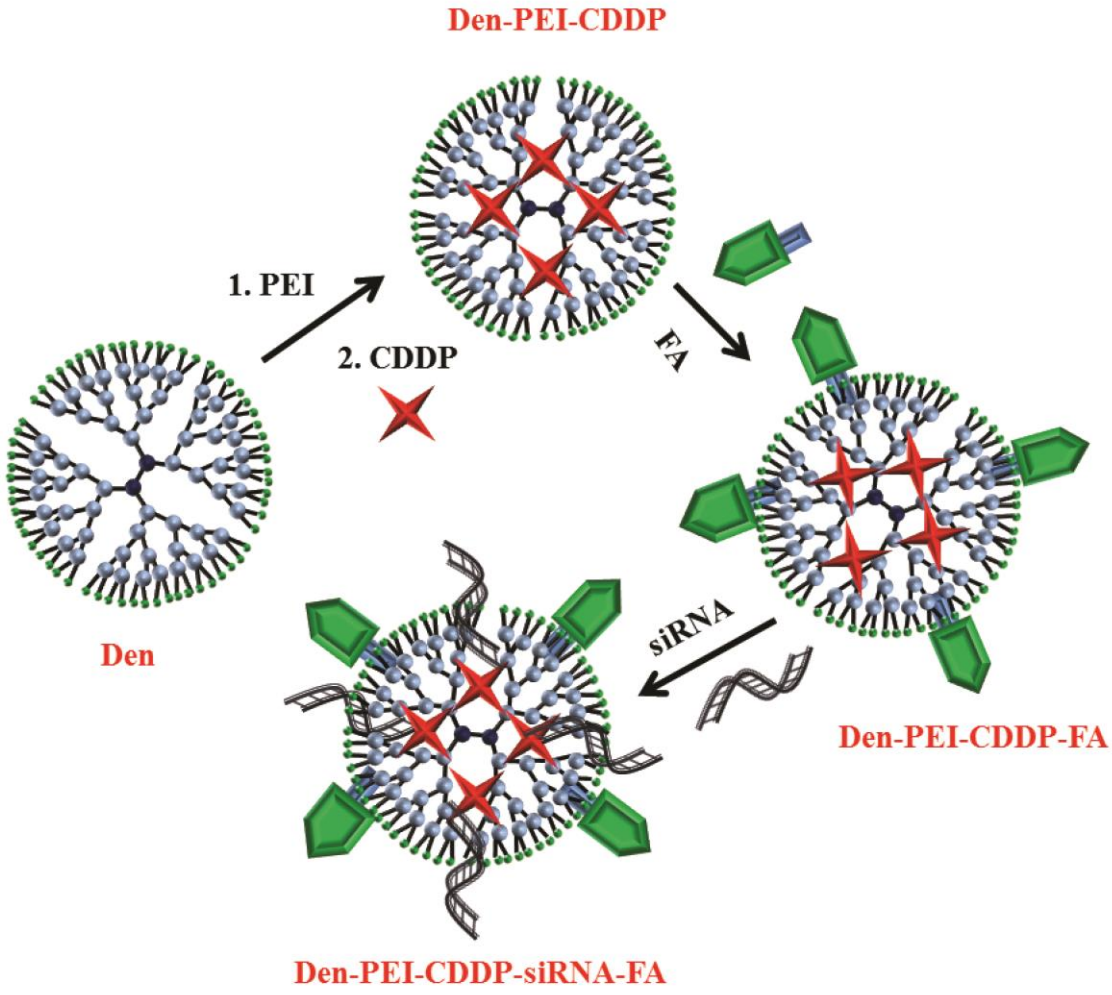
Supplementary Figure 2: Confirmation of folic acid conjugation to dendrimer nanoparticles by absorbance spectra of folic acid at 280 nm with Den-PEI-CDDP-FA nanoparticles using different Den-PEI-CDDP and FA ratios.

Supplementary Figure 3: Optimization of folic acid and CDDP concentration in H1299 cells by fluorescence uptake and cell viability, respectively. **(A)** Normalized fluorescence intensity of Den-PEI-CDDP-siGLO-FA nanoparticles with constant siGLO concentration and various folic acid concentrations. (n=3) **(B)** Cell viability of different concentrations of CDDP in free CDDP

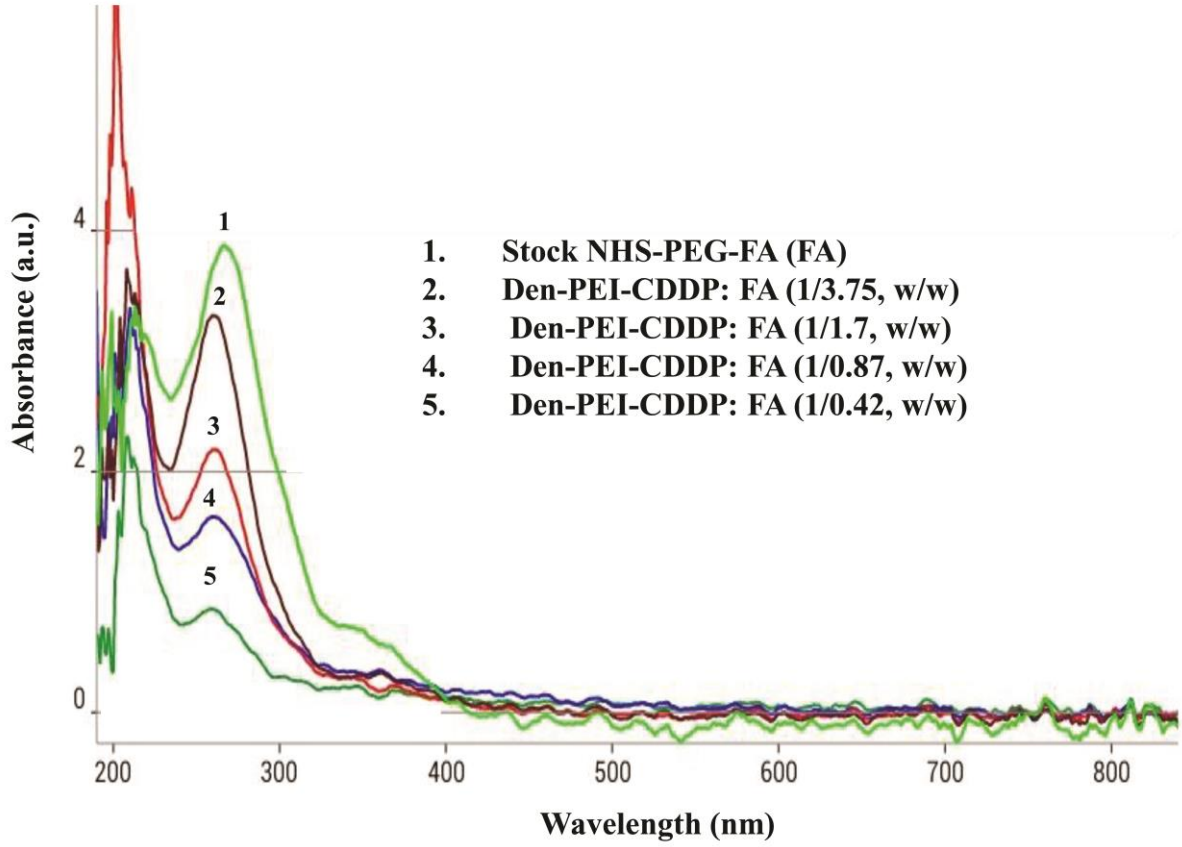
and Den-PEI-CDDP nanoparticles in H1299 cells at 24 h. (n=4) (p** <0.01, p NS = non significant).

Supplementary Figure 4: Cell growth inhibition and HuR protein knockdown study. HuR siRNA delivery into H1299 and A549 cells by Den-PEI-HuR nanoparticles, compared with Den-PEI-CSi and untreated controls study. **(A)** Cell growth inhibition of H1299 and A549 cells at 72 h. (n=4) **(B)** Western blot and **(C)** quantification studies for HuR and cyclin E protein knockdown in H1299 and A549 cells. (p** <0.01, p* < 0.05, p NS = non significant).

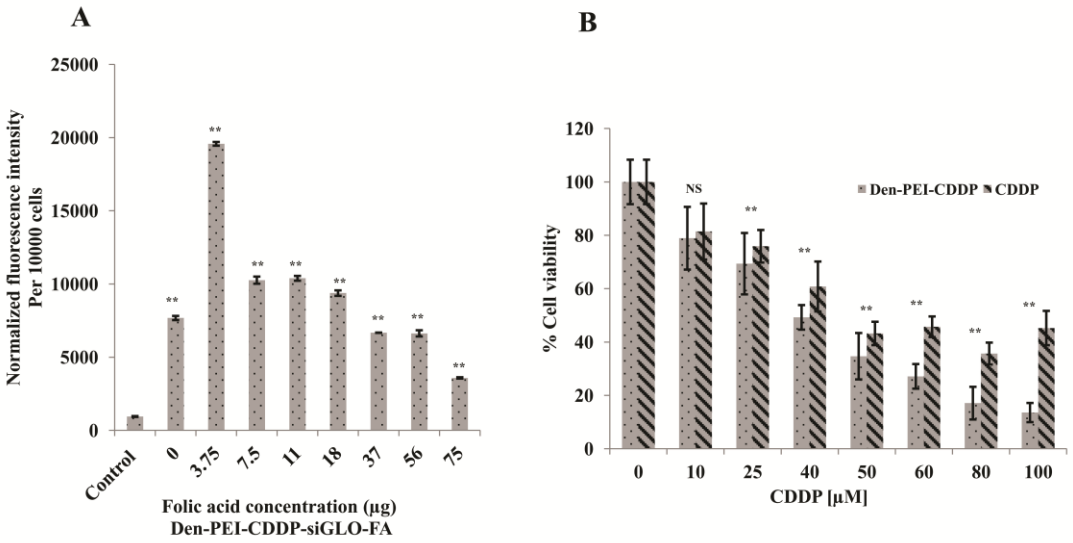
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4