Supplementary Information

Effective delivery of a rationally designed intracellular peptide drug with gold nanoparticle-peptide hybrid

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**Methods**

**Preparation of GNP-peptide hybrids**

GNP-peptide hybrids were fabricated by mixing a pre-mixed 50 μM peptide stock solution with an aqueous solution of 20 nm spherical GNPs (Ted Pella; concentration of ~1 nM) at 10:1 volume ratio. The mixture was gently shaken overnight at 25°C in the dark. Different centrifugation speed was tested for optimization by measuring surface plasma absorption of supernatant GNP. GNP-peptide hybrids were centrifuged at 12,000 revolutions per minute (rpm) for 30 minutes to eliminate excess non-conjugated peptides.

To prepare the pre-mixed peptide solution, P2 (CAAAAE) and P4 (CAAAAW) peptides (CanPeptide; >95% pure, C-terminal protected by amidation) were first mixed at 95:5 molar ratio in deionized water. This 95P2P4 solution was subsequently mixed with a stock solution of δV1-1 (CSFNSYELGSL, named as PKCi) (Mimotopes; >95% pure, C-terminal protected by amidation). Due to its high hydrophobicity, 50% acetonitrile (Sigma-Aldrich, Saint Louis, USA; 99.8% pure) was used as the solvent for PKCi peptide, and peptide purity was confirmed using LC-MS/MS. <20% of the peptides in the mixture were shown to be dimerized. Five different GNP-peptide hybrids were prepared based on the peptide mixture composition (Fig. 1c).

**Stability of GNP-peptide hybrids at physiologic ionic condition**

Stability of GNP (dispersed vs. aggregated) in water and in 150 mM saline solution was tested based on changes in the surface plasma absorption. The nanoformulations were first centrifuged at 12,000 rpm for 30 minutes and the supernatant was replaced with either double distilled H₂O (ddH₂O) or 150 mM phospho-buffered saline (PBS), and incubated for 2 hours at room temperature. A Model Cary 50 Varian UV-Vis spectrophotometer (Palo Alto) was employed to
obtain the absorption spectra of unmodified GNP and GNP-peptide hybrids. A µQuant microplate reader (Bio-Tek Instrument) was also used to confirm the spectra results and for high-throughput screening of GNP-peptide hybrids.

The change in surface plasma absorption, ΔOD, was calculated to represent the differences between the absorption at the peak and at 440 nm as background (ΔOD = A_{peak} - A_{440}). When GNP aggregates, the absorption peak shifts to higher wavelengths and the peak absorption intensity decreases; therefore a decrease in ΔOD represents a decrease in stability. Solutions were also photographed using a digital camera to capture the colour change that occurs due to this absorption peak shift (from red to blue/purple).

**Hydrodynamic size and serum protein adsorption**

Hydrodynamic size of the GNP-peptide hybrids was measured by dynamic light scattering (DLS) assay using Zetasizer Nano ZS90 (Malvern Instruments). The samples were run at 25°C with the refractive index setting as gold and solvent viscosity setting as pure water. GNP formulations were prepared using the protocol previously described and incubated overnight at room temperature. After incubation, GNP formulations were centrifuged at 12,000 rpm for 30 minutes and the supernatant was replaced with either ddH$_2$O, 150 mM PBS + 10 µM bovine serum albumin (BSA) (Sigma-Aldrich, ~66kDa), or pure fetal bovine serum (FBS) (Invitrogen) and incubated for 2 hours at room temperature. For each sample, 1 ml of solution was used and the experiment was repeated three times. The intensity-based size distribution of the nanoparticles was generated by a computer software package (Dispersion Technology Software, v 5.03).
Zeta potential

The surface charge of the nanoparticles after peptide coating was determined by the zeta potential measurements using Zetasizer Nano ZS90 at 25°C. The GNP-peptide hybrid solutions were centrifuged at 12,000 rpm for 30 minutes, and the supernatant was replaced with the same volume of PBS (~pH 7.0). One ml of samples were injected into a disposable cell (folded capillary DTS-1060 from Malvern Technologies) and analyzed at constant voltage. The zeta potential distribution (in mV) was automatically calculated by the software package (Dispersion Technology Software, v 5.03) based on the electrophoretic mobility distribution. At least three measurements were carried out per sample. The zeta potentials reported corresponds to the average peak values of the distribution profiles from the three measurements.

Loading capacity of 20 nm GNP

The number of PKCi peptide molecules attached per 20 nm gold nanoparticle was quantified using the UV-Vis spectrophotometer. In the first experiment, overall concentration of the peptide mixture was varied and the stability of GNPs in 150 mM PBS was studied by monitoring the change in their surface plasma absorption. In the second experiment, concentration of the peptide mixture remained the same but the GNP concentration was varied. The solutions were then centrifuged at 13,200 rpm for 20 minutes and the concentration of the unconjugated peptide was measured using UV-Vis spectrophotometer. Since only Tyrosine or Tryptophan residues absorb light at 280 nm, and PKCi contains one Tyrosine while P2 doesn’t (and P4 has a Tryptophan), only GNP-100PKCi was used to quantify GNP peptide loading using the second experiment.
Cellular uptake using confocal microscopy

Cellular uptake of GNP-peptide hybrids was studied using human BEAS2B lung epithelial cells. Cells were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% FBS (Invitrogen) at 37°C with 5% CO₂. When cells were >80% confluent, they were collected and seeded to an 8-well cover glass-bottom chamber (Sigma-Aldrich) at a concentration of 2x10⁴ cell/well. The cells were then grown at 37°C for two days. After incubation, the cells were treated with unmodified GNP or one of the GNP-peptide hybrids at 2 nM GNP concentration for 2 hours. Cells were then washed three times with warm PBS and fixed with 4% paraformaldehyde for 30 minutes. The cell membrane was permeabilized with 0.1% Triton-X for 10 minutes and washed two times with PBS. WGA-Alex fluor 488 (Invitrogen) at the concentration of 5 μM was then used to stain cell membrane for 15 minutes at room temperature, followed by PBS washing three times in the dark. Hoechst 33342 dye (Invitrogen) at the concentration of 5 μg/ml was used to stain the cell nucleus for 20 minutes and again washed with PBS three times in the dark. Cells were washed with H₂O and mounted with Dako fluorescent mounting medium (Dako Canada).

The florescent signals were captured using an Olympus FV1000 laser confocal scanning microscopy with the excitation wavelengths of 405, 488 and 633 nm for Hoechst 33342, Alex fluor 488 and GNP aggregates, respectively. A lambda scan of unmodified GNP aggregates (pH ~3) was performed with the 633 nm laser to generate an emission spectrum, with step size and bandwidth set to 2 nm. GNP uptake was quantified by the average florescence signals per volume inside the cells. For each cell, three sections, z = ¼, ½ and ¾ were selected from the bottom of the cell. Volume of each section was calculated as the focal depth (0.2 μm) x the
section area that was determined by drawing a line along the cell membrane. Average florescence intensity was calculated for each cell.

**Evaluation of therapeutic efficacy using cell culture**

Therapeutic efficacy of GNP-peptide hybrids was evaluated by treating BEAS2B epithelial cells with GNP-0PKCi as a control and GNP-20/50/80/100PKCi for testing. We followed the original procedure reported in 2000. Tat-PKCi and Tat peptides (Mimotopes; >95% pure) were also included for comparison. The testing formula was pre-incubated with cells for 1 hour in DMEM supplemented with 10% FBS, and also at 4°C in the Perfadex® (Vitrolife), a lung preservation solution, during simulated lung preservation. The cold Perfadex® solution was replaced with therapeutic-free DMEM plus 10% FBS at 37°C to simulate reperfusion. Cell viability and cytokine production were used as endpoints to compare the efficacy between GNP-delivered and Tat-delivered PKCi.

For the cell viability experiment, cells were first seeded in a 96-well plate (Sarstedt) at 13,000 cells/well and grown overnight at 37°C with 5% CO₂. DMEM with 10% FBS was then replaced with DMEM + 10% FBS containing one of the nanoformulations, Tat-PKCi or Tat-alone, and incubated for 1 hour at 37°C with 5% CO₂. For comparison, the nano-drug concentrations were calculated in terms of PKCi molar concentration. Following 1 hour incubation, DMEM was replaced with Perfadex® lung preservation solution, and cells were subjected to 18 hours of cold ischemic time (CIT) at 4°C with oxygen. Cells were switched back to serum-containing DMEM for 4 hours to simulate reperfusion. MTS was added at the end of the second hours, and a μQuant microplate reader (Bio-Tek Instrument) was used to obtain the absorbance at 630 nm, minus the absorbance at 490 nm as background. Wells without cells that underwent treatment and CIT
were used to ensure that the absorbance data was not influenced by gold nanoparticles. The experiment was repeated at least three times with 5 treatment wells per group.

For the cytokine production experiment, cells were first seeded in a 6-well plate (Nest Biotech) at 300,000 cells/well and grown overnight at 37°C with 5% CO₂. The cells were subjected to the same simulated ischemia-reperfusion environment as described above, except the cold ischemic time was reduced to 6 hours. The cell culture medium was collected, centrifuged at 13,000 RPM for 10 minutes at 4°C and stored in -80°C. The experiment was repeated at least three times with 3 wells per group. In a separate experiment, pictures of cells were taken after reperfusion using an Olympus light microscope to examine any changes in cell morphology. ELISA kits (R&D Systems) were used to measure the levels of IL-6, IL-8 and GROα, in the collected medium. Results are shown in pg/ml concentration.

**Hemolytic activity assay**

To ensure the safety of the GNP-50PKCi nano-formulation for intravenous administration, hemolytic activity was examined as described previously⁵. Fresh Yorkshire pig blood was collected in a glass flask without any anti-coagulants. The blood sample was immediately stirred vigorously with a glass rod for 10 minutes to remove fibrinogen. The blood sample was then washed with 0.9% saline solution at least three times until the supernatant became clear. Red blood cell (RBC) suspensions were prepared in 0.9% saline with a final concentration of 2%. The test samples were then mixed with the RBC solution (2.5 ml RBC, 2.0 ml saline, 0.5 ml GNP sample). RBC suspension in water was used as the positive control. After mixing gently, the samples were incubated in a 37°C water bath for 3 hours. After incubation, pictures of the samples were taken and absorbance was read at 415 nm using a μQuant microplate reader (Bio-Tek Instrument). Percentage hemolysis was calculated using the following equation:
\[
\text{Hemolysis(\%)} = \frac{\text{Abs(sample)} - \text{Abs(-)}}{\text{Abs(+)} - \text{Abs(-)}} \times 100\%
\]

**In situ pulmonary ischemia-reperfusion model**

Male Sprague-Dawley (SD) rats were purchased from Charles River. All animals were cared for according to Principles of Laboratory Animal Care regulated by the National Society for Medical Research (NIH publication No. 85-23, Revised 1985, U.S. Government Printing Office, Washington DC, US) and Canadian Council on Animal Care (Guide to the Care and Use of Experimental Animals, Ottawa, Canada).

We used the same ischemia-reperfusion model described in Gao, et al. (2014). Briefly, rats were anesthetized in an isoflurane chamber, tracheotomised, intubated with a 12-gauge intravenous catheter and ventilated with a Model 683 Small Animal Ventilator (Harvard Apparatus. Ventilation was set to 70 breaths/min with 2 cm H\(_2\)O positive end-expiratory pressure (PEEP). Fraction of inspired oxygen (FiO\(_2\)) was kept at 0.6 with a tidal volume of 10 ml/kg. A 24-gauge intravenous catheter was inserted into the left jugular vein for drug administration. Throughout the experiment, rats were given ketamine (100 mg/kg) and xylazine (10 mg/kg) continuously via the let jugular vein at 1 ml/hour to maintain anesthesia. Atropine (0.2 mg) was administered intramuscularly to reduce airway secretions. A heating pad was placed underneath the animals to keep a constant body temperature at around 38°C. A pulse oximeter (Nonin PalmSAT® 2500A) was clamped to the right hind limb to monitor heart rate and oxygen saturation (SpO\(_2\)). An intravenous catheter was also inserted into the right carotid artery and connected to a blood pressure monitor to monitor systolic, diastolic, and mean blood pressures and pulse waveforms during the experiment.
Half ml of nano-drug dissolved in 0.9% saline (equivalent to 150 mM NaCl) was administered via the left jugular vein 30 minutes before inducing left lung ischemia. The GNP-peptide hybrids were centrifuged at 12,000 rpm for 30 minutes and the supernatant was replaced with 0.9% saline. Animals were placed on their right side, and a lateral thoracotomy in the fifth intercostal space was performed. The left pulmonary artery was dissected and 500 µL of saline with heparin (50 IU/animal) was injected intravenously. Five minutes after heparin administration, the tidal volume was adjusted to 6 ml/kg and the left pulmonary hilum was clamped immediately with two mini Sugita® aneurysm clips (Mizuho Medical) (one for the pulmonary artery and the other for pulmonary vein and left bronchus) to induce tissue ischemia in the left lung.

The clips were removed after 90 minutes of ischemia to reperfuse the left lung and the tidal volume was adjusted to the original setting of 10 ml/kg. The GNP-peptide hybrids (0.5 ml) were administered again immediately after unclamping. The thoracic cavity was sutured and placed to a supine position for the 120 minutes reperfusion period. At the end of the reperfusion stage, the rats were laparotomized and the right main bronchus and pulmonary vessels were clamped. After 5 minutes, 0.5 ml of blood from the aorta was drawn with a 23-gauge needle attached to a 1 ml syringe for arterial blood gas analysis using a RAPIDPoint® 400/405 Systems analyzer (Siemens).

For comparison, Tat-PKCi and Tat was also tested. Tat-PKCi or Tat alone were dissolved in 0.9% saline and administered 1 minute before ischemia was induced instead of 30 minutes, to account for the short half life in vivo. The Tat-PKCi and Tat administered rats were subjected to the same experimental procedure as described above. We did not include a sham surgery group or a saline control group, since these groups have been previously tested in our lab using the same animal model.
At the end of every procedure, animals were sacrificed to collect the left and right lungs. Plasma samples were collected by centrifuging blood drawn with a heparinised syringe immediately after sacrifice, at 3,000 rpm at 4°C for 10 min. These samples were used for further analysis.

**Lung function measurement**

After 2 hours of reperfusion, a laparotomy was performed to expose the thoracic cavity. The right hilum was clamped with two mini Sugita® aneurysm clips, and the left lung was ventilated for 5 minutes with a tidal volume of 4 ml/kg and positive end-expiratory pressure (PEEP) of 70 breaths/min using the small animal ventilator. Ventilation was then switched to flexiVent™ (Scireq) to measure lung function mechanics of the left lung. Dynamic resistance (Rrs), compliance (Crs), elastance (Ers), and peak airway pressure (Pmax) were measured in triplicates using the flexiVent Snapshot function⁶.

**Lung wet/dry ratio**

Lung wet/dry ratio was measured to assess the extent of pulmonary edema⁶. The cranial lobe of the right lung and 1/3 of the left lung was cut and placed on a Kimwipe® for 10 minutes with the cut surface either facing up or to the side. The lung sections were weighed before and after dried in an 80°C oven for 72 hours. Wet weight was divided by the dry weight to calculate the wet/dry ratio.

**Lung injury score**

Sections of the left and right lungs were washed by injecting cold 10% buffered formalin through the trachea at a constant pressure of 20 cm H₂O. Tissues were fixed in 10% buffered formalin for 24 hours and stored in 70% ethanol at 4°C. Tissue samples were then embedded using paraffin wax, cut into 4 μm sections and stained with hematoxylin and eosin (H&E). A Nanozoomer 2.0
RS slide scanner (Hamamatsu Photonics) was used to scan the tissue sections at x40 magnification. Six representative images were taken at x20 magnification for each tissue section. Lung injury was semi-quantitatively scored in a blinded manner by giving a score based on a 4-point scale (0 = minimal, 1 = mild, 2 = moderate, 3 = severe damage) for each of the following; alveolar wall thickening, intra-alveolar edema, neutrophil infiltration and intra-alveolar hemorrhage. Scores for each image was combined (0 – 12) and averaged among the six fields for each tissue sample.

**TUNEL staining for apoptosis quantification**

Apoptosis in the lung tissue samples were assessed using *in situ* terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining (TMR red *In Situ* Cell Death Detection Kit). Briefly, 4 µm fixed tissue sections were deparaffinized using xylene (2 x 5 min), 100% ethanol (2 x 3 min), 95% ethanol (1 minute) and 70% ethanol (1 minute). Sections were washed with distilled water and incubated at 37°C for 15 minutes with proteinase K solution (15 µg/ml in 10 mM Tris/HCl, pH 7.4). Tissue samples were rinsed two times with PBS and 50 µl of TUNEL reaction mixture (combination of red and blue vials provided with the TUNEL assay kit) was added and slides were incubated for 60 minutes at 37°C in the dark. The slides were washed with PBS three times and mounted with DAPI-containing mounting medium.

The stained slides were examined with an Axiovert 200M fluorescent microscope (Zeiss) and images were taken using a CoolSnap HQ 12 bit CCD camera (Roper) and Axiovision 4.8 software. The whole tissue section was first examined at a magnification of x100. At x200 magnification, 8 random DAPI stained (blue) fields of alveolar tissue area without major airways and blood vessels were taken. TUNEL-stained images were also taken at the same 8 fields. Only
cells with both TUNEL and DAPI staining were considered apoptotic. The total number of apoptotic cells was averaged among the 8 images and the results were expressed in terms of average number of apoptotic cells per x200 field.

**Statistical analysis**

All experiments were repeated a minimum of 3 times. All statistical analyses were carried out using either Microsoft Excel 2010 or GraphPad Prism 5. Unpaired two-tailed Student’s t-test was performed when comparing two groups. One-way ANOVA analysis was used when comparing multiple samples with each other. Differences were considered significant when the P-value was less than 0.05. All results are expressed as the Mean ± Standard Error of Mean (SEM).

**References**

Supplementary Figure 1. UV-Vis Spectroscopic graphs of GNP and GNP-peptide hybrids in water and in 150 mM saline.
Supplementary Figure 2. Loading capacity, Zeta potential and hydrodynamic size measurements. a. Total number of peptide molecules attached per GNP for GNP-100PKCi formulation using UV-Vis spectrophotometry. b. Zeta potential values of GNP-peptide hybrids in PBS. GNP-peptide hybrids were prepared by mixing 50 µM peptide and 1 nM GNP in 1:10 volumetric ratio. c. Pictures of GNP and GNP-peptide hybrids in FBS before and after centrifugation at 12,000 rpm for 30 minutes. Black arrow indicates a big pellet size under GNP alone condition. d. DLS size distribution graphs of GNP and GNP-peptide hybrids in water (blue) and in serum (red).
Supplementary Figure 3. Estimation of GNP-peptide hybrid uptake using confocal microscopy. 

**a.** Aggregated 20 nm GNP was excited by the 633 nm laser, with a fluorescence emission at 642 – 655 nm. 

**b.** For each cell, three z-stack sections were used to quantify fluorescence. 

**c.** Estimation of GNP uptake by lung epithelial cells using the average fluorescence signals per volume of three cellular sections. Uptake of GNP decreased as the percentage of 95P2P4 peptides reduced (n = 3).
Supplementary Figure 4. No changes in BEAS2B cell density or morphology were observed after treatment for 24 hours at 1 nM GNP concentration (2.5 µM for Tat and Tat-PKCi).

Supplementary Figure 5. GNP-50PKCi rescued cells from ischemia-reperfusion-induced cell death in a dose-dependent fashion. mean ± SEM, n = 3, *p < 0.05, **p < 0.01, vs. GNP-50PKCi=0 group.
Supplementary Figure 6. Safety tests of GNP-50PKCi. a. Light microscope images of porcine red blood cells. Positive control: cells were lysed with water. Negative control: normal cells. Neither GNP-50PKCi nor GNP-0PKCi induced cell lysis or aggregation. b. Solution colour of red blood cells. c. Quantified hemolytic percentage and evaluation criteria. No red blood cell aggregation or significantly cell lysis was observation at concentrations tested. Tat-PKCi, for comparison, also passed these tests. d. Complement activation assay at the doses tested.
Supplementary Figure 7. Left lung mechanics after 90 min ischemia and 2 h reperfusion. A trend towards improved lung mechanics with GNP-50PKCi treatment was observed, as shown by a decrease in peak airway pressure (Pmax), increase in compliance (Crs), and a decrease in resistance (Rrs) and elastance (Ers). p-values are shown above each graph.
**PaO$_2$/FiO$_2$ at the end of 2 hours of reperfusion**

<table>
<thead>
<tr>
<th>Tat</th>
<th>Tat-PKCi</th>
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<tbody>
<tr>
<td>259.1</td>
<td>501.4</td>
</tr>
<tr>
<td>Died after 1 h R</td>
<td>242.5</td>
</tr>
<tr>
<td>Died after 0.5 h R</td>
<td>303.5</td>
</tr>
<tr>
<td>91.7*</td>
<td>475.5</td>
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<tr>
<td>472.3</td>
<td>59.6*</td>
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* indicates that the rat could not tolerate right hilum clamping due to severity of acute injury on the left lung.

Supplementary Figure 8. PaO$_2$/FiO$_2$ values of rats treated with Tat alone or Tat-PKCi at the end of 2 hours of reperfusion following 90 minutes of pulmonary ischemia. Pictures of the lungs for the best and worst survived cases are shown. * indicates that the rat could not tolerate right hilum clamping due to severity of acute injury on the left lung.