Supplementary Information

Supramolecular Nanoparticle-Mediated Targeting of Phosphatidylinositol 3 Kinase Enhances Anti-tumor Efficacy and Overcomes Insulin Resistance.

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Materials and Methods:

All chemical reagents were of analytical grade, used as supplied without further purification unless indicated. All reactions were performed under inert conditions unless otherwise indicated. Dichloromethane (DCM), anhydrous DCM, Methanol, Cholesterol, Dimethylamino Pyridine (DMAP), Succinic Anhydride, Sodium Sulfate, Pyridine, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), L-α-phosphatidylcholine and Sephadex G-25 were purchased from Sigma-Aldrich. PI-103 was purchased from Selleckchem and PI828 was purchased from Tocris Biosciences. 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Amino(Polythylene Glycol)2000] and the mini handheld Extruder kit (including 0.2 µm Whatman Nucleopore Track-Etch Membrane, Whatman filter supports and 1.0 mL Hamiltonian syringes) were bought from Avanti Polar Lipids Inc. Analytical thin-layer chromatography (TLC) was performed using precoated silica gel Aluminium sheets 60 F_{254} bought from EMD Laboratories. Spots on the TLC plates were visualized under UV light, and/or by treatment with alkaline permanganate solution followed by heating. MTS reagent was supplied by Promega. Column chromatography was conducted using silica gel (230-400 mesh) from Qualigens. ^{1}H spectra were recorded on Bruker DPX 400MHz spectrometer. Chemical shifts are reported in δ (ppm) units using residual ^{1}H signals from deuterated solvents as references. Spectra were analyzed with Mest-Re-C Lite (Mestrelab

Research) and/or XWinPlot (Bruker Biospin). Electrospray ionization mass spectra were recorded on a Micromass Q Tof 2 (Waters) and data were analyzed with MassLynx 4.0 (Waters) software.

Synthesis of PI103-cholesterol conjugate:

Cholesterol (500 mg, 1.29 mmol) was dissolved in 5 ml of anhydrous pyridine. Succinic anhydride (645 mg, 6.45 mmol) and catalytic amount of DMAP was added to the reaction mixture to form clear solution. The reaction mixture was flushed with argon and allowed to stir under argon atmosphere for 12h. Then, pyridine was removed under vacuum and the crude residue was diluted in 30 ml DCM. It was washed with 1N HCl (30 ml) and water (30 ml). The organic layer was separated and dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. Completion of the reaction was confirmed by performing a TLC in 1:99 Methnol:DCM solvent mixture. The product was used for next step without further purification. PI-103 (25 mg, 0.072 mmol) was dissolved in 3 ml anhydrous DCM followed by addition of cholesterol-succinic acid (0.216 mmol, 105 mg), EDC (0.216 mmol, 41.4 mg) and DMAP (0.216, 26 mg). The reaction mixture was stirred at rt for 12h under argon. Upon completion of reaction as monitored by TLC, the reaction mixture was diluted with 10 ml DCM and washed with dilute HCl and water. The organic layers were separated, combined and dried over anhydrous sodium sulfate. The solvent was evaporated under vacuum and the crude product was purified by using column chromatography, eluting with methanol:methylene chloride gradient, to give the PI-103 cholesterol conjugate as a light yellow solid (52 mg, 90 %). ^{1}H NMR ($CDCl_3$, 400MHz): δ 8.65 – 8.53 (m, 1H), 8.36 (d, *J* = 8.3 Hz, 1H), 8.19 (d, *J* = 1.7 Hz, 1H), 7.56 – 7.41 (m, 1H), 5.29 (s, 1H), 4.28 – 4.15 (m, 2H), 3.97 – 3.86 (m, 2H), 3.64 (s, 1H), 2.93 (d, *J* = 7.0 Hz, 1H), 2.76 (d, *J* = 7.0 Hz, 1H), 2.35 (s, 1H), 2.17 (s, 1H), 1.59 (s, 4H), 1.29 (d, *J* = 34.2 Hz, 3H), 1.25-1.23 (m, 6H), 1.13 – 0.80 (m, 13H), 0.66 (s, 2H), 0.03 (m, 12H). HRMS Calculated for $[C_{50}H_{64}N_4O_6+H]^+$:817.4899 Found: 817.4883 .

Synthesis of PI828-cholesterol conjugate:

20.0 mg (0.044 mmol) of cholesteryl chloroformate was dissolved in 2.0 mL dry DCM. To it 28 mg (0.088 mmol) of PI-828 dissolved in 2.0 mL of dry DCM was added. Finally 15.5 µL (0.088 mmol) of dry DIPEA was added to it dropwise at room temperature in an inert condition. Progress of the reaction was monitored by thin layer chromatography. After 24h, it was quenched with 100 mL 0.1(N) HCl and the compound was extracted in DCM. The desired product was separated by column chromatography using a solvent gradient of (0-5)% MeOH in DCM. ^{1}HNMR(300 MHz) δ(ppm) = 8.165-8.13(m); 7.59-7.40(m, aromatic); 6.72(s); 5.98-5.93(m); 5.42-5.40(m); 4.67-4.59(m); 3.75-3.74(m); 3.44-3.40(m); 2.43-2.34(m); 2.04-1.93(m); 1.86-1.77(m); 1.65-1.43(m); 1.35-1.43(m); 1.32-0.85(m).

Preparation of PI3K-inhibiting NPs:

PI103-SNP: 3.5 mg (50 mol%) of L-α-phosphatidylcholine, 2.5 mg (20 mol%) PI103-cholesterol conjugate and 7.5 mg (30 mol%) of 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Amino(Polythylene Glycol)2000](DSPE-PEG) were dissolved in 1.0 mL DCM. Solvent was evaporated into a thin and uniform lipid-drug film using a rotary evaporator. The lipid-drug film was then hydrated with 1.0 mL H_2O for 1 h at 55°C. The hydrated nanoparticles looked light yellow to white with little viscous texture. It was passed though Sephadex G-25 column and extruded at 55°C to obtain sub 200 nm particles. A standard curve of PI103-Choletserol conjugate in DMF was generated by measuring absorbance at 285 nm using UV-Vis spectrophotometry (Shimadzu 2450). A known concentration of nanoparticle was dissolved in DMF and the absorbance value at 285 nm was used to calculate the loading from standard curve. This was validated using HPLC method. The iRGD tagged PI103 nanoparticles were synthesized by the same procedure but iRGD peptide was conjugated to DSPE-PEG-maleimide using thiol conjugation as described by Prof. Ruoslahti.

PI828-SNP: PI-828 conjugated cholesteryl chloroformate, phosphatidylcholine and DSPE-PEG (in 5:10:1 weight ratio) were dissolved in DCM. The resulting solution evaporated in a round-bottomed flask with the help of a rotary evaporator and thoroughly dried. The resulting thin film was hydrated with PBS with constant rotation at 70°C for 2h. Nanoparticles were eluted through a Sephadex column followed by extrusion by a hand-held mini extruder (filter size 200 nm). The size was checked by DLS and drug loading was determined by UV-VIS spectroscopy.

PI103-encapsulated NP: Phosphatidylcholine, Cholesterol, DSPE-PEG and PI103 were taken in 10:5:1:1 weight ratio. PI103 was soluble in Methanol and Cholesterol, DSPE-PEG and Phosphatidylcholine were soluble in dry DCM. The solution was taken in a round-bottomed flask and evaporated using a rotary evaporator. The thin film was dried thoroughly and was hydrated by 1.0 mL of dd H_2O. It was stirred at the highest possible speed in rotary evaporator @ 55˚C. The white colored suspension was passed through Sephadex G-25 column. The eluent was extruded with the help of a hand-held mini extruder (Avanti) using 200nm polycarbonate filter membranes. Drug loading was performed by UV spectrophotometer.

Release kinetics studies:

Drug loaded nanoparticles (1mg drug/ml, 5ml) were suspended in PBS buffer (pH 7.4), 4T1 cell lysate and 4306 cell lysate (additionally for PI828-SNPs) and sealed in a dialysis tube (MWCO= 3500 Dalton, Spectrum Lab). The dialysis tube was suspended in 1L PBS ph7.4 pH with gentle stirring to simulate the infinite sink tank condition. A 100 µL portion of the aliquot was collected from the incubation medium at predetermined time

intervals and replaced by equal volume of PBS buffer, and the released drug was quantified by UV-VIS Spectrophotometer and plotted as cumulative drug release.

Nanoparticle characterization and stability studies:

The mean particle size of the nanoparticles was measured by Dynamic Light Scattering method using Zetasizer Nano ZS90 (Malvern, UK). 10µL of nanoparticles solution was diluted to 1ml using DI water and 3 sets of 10 measurements each were performed at 90 degree scattering angle to get the average particle size. The zeta potential was measured using a Zetasizer ZS90 with the nanoparticles diluted in water for measurement according to the manufacturer's manual. The physical stability of nanoparticles was evaluated by measuring changes in mean particle size and zeta potential during storage condition at 4^0C.

Cryo-Transmission Electron Microscopy for PI103-SNPs:

The sample was preserved in vitrified ice supported by holey carbon films on 400 mesh copper grids. The sample was prepared by applying 3µL of sample suspension to a cleaned grid, blotting away with filter paper and immediately proceeding with vitrification in liquid ethane. Grids were stored under liquid nitrogen until transferred to the electron microscope for imaging. Electron microscopy was performed using an FEI Tecnai Cryo-Bio 200KV FEG TEM, operating at 120KeV equipped with 2 Gatan Sirius CCD cameras one 2K*2K and one 4K*4K pixel. Vitreous ice grids were transferred into the electron microscope using a cryostage that maintains the grids at a temperature below -170°C. Images of the grid were acquired at multiple scales to assess the overall distribution of the specimen. After identifying potentially suitable target areas for imaging at lower magnification, high magnification images were acquired at nominal magnification of 52,000x (0.21 nm/pixel), and 21,000x (0.50 nm/pixel). Images were acquired at a nominal underfocus of -5µm (21,000x) and -4µm (52,000x) at electron doses of ~10-15 e/A^{o2}.

Cell Viability Assay:

4T1 and MDA MB 468 breast cancer cells were cultured in RPMI, 4306 ovarian cancer cells were cultured in DMEM, supplemented with 10% FBS and 1% of Antibiotic-Antimycotic 100x solution (Invitrogen, 15240-062). 4×10^3 Cells were seeded into 96-well flat-bottomed plates. Free drug or drug loaded nanoparticles (normalized to equivalent amounts of free drug) was added in triplicate in each 96-well plate at appropriate concentrations (1, 10, 100 nM and 1, 10, and 50 µM) and then plates were incubated in 5% CO_2 atmosphere at 37° C. After desired time period of incubation, cells were washed and incubated with 100 µl phenol-red free medium (without FBS) containing 20 µl of the CellTiter 96 Aqueous One Solution reagents (Promega, WI).

After 2 hours incubation in a 5% CO_2 atmosphere at 37° C, the absorbance in each well was recorded at 490 nm using an Epoch plate reader (Biotek instruments, VT). The absorbance reflects the number of surviving cells. Blanks were subtracted from all data and results analyzed using GraphPad Prism software (GraphPad, San Diego, CA). Each experiment was independently repeated thrice and data shown is mean ± SE of n=3.

PI103-SNPs Internalization Assay:

4T1 breast cancer cells (1 x 10^6 cells) were seeded in 10 ml petri dish and incubated with serum free media for 6 hours after it reached 70% confluency. Then, free PI103 or equivalent amount of PI103-SNPs was added in 20 μM concentration in serum deprived media (1% FBS). After 4 hours incubation in 5% CO_2 atmosphere at 37^0C, cells were washed thrice with PBS and replenished with fresh media. After desired time of incubation, 2 x 10^6 cells were lysed from each sample, centrifuges and supernatant was collected. Amount of drug in the samples were measured by UV-Vis spectroscopy using drug free cells as control.

Western Blot Assay:

For western blot 5×10^4 Cells were seeded in each well of a 6 well plate. When cells were 70% confluent, they were incubated in serum free media for 6 hours. Then, free drug or equivalent amount of drug loaded nanoparticles were added in appropriate concentration in serum deprived media (1% FBS). After 24 hours of incubation in 5% CO_2 atmosphere at 37° C, cells were washed twice with ice cold PBS and protein was collected by scraping using RIPA buffer supplemented with protease inhibitor (Roche diagnostic). Amount of protein was measured by BCA assay and equal amount of protein lysates were electrophoresed on a 4-20% polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and blocked in TBST-T with 5% dry milk. Then membranes were incubated in TBST with Phospho AKT (S473) (1:500 dilution), total AKT (1:1000 dilution), and actin (1:2000 dilution) antibodies (all antibodies from Cell Signalling Technology) overnight at 4° C. After appropriate amount of washing with TBST, membranes were incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour. Detection was done using G-box from Syngene and densitometric quantification was done by image J software.

Efficacy study of PI828-SNPs and PI103-SNPs in murine 4T1 breast cancer model:

4T1 breast cancer cells (1 X 10^5) were implanted subcutaneously in the flanks of 4-week-old BALB/c mice (weighing 20g, Charles River Laboratories). The drug therapy was started on day 9. For PI828-SNPs, the drug therapy consisted of administration of PBS (for Control group), free PI-828 (5mg/kg) and PI828-SNPs (5mg/kg). For PI103-SNPs, the drug therapy consisted of administration of PBS (for control group), free PI-103

(5mg/kg) and PI103-SNPs (5mg/kg) and iRGD-PI103-SNPs (5mg/kg) (administered by tail-vain injection). The tumor volumes and body weights were monitored on every alternate day for 11 days. The tumor volume was calculated by using the formula, L X $B^2/2$, where the longest diameter was considered as L and the shortest diameter as measured using a vernier caliper as B. Tumor volume increments were calculated as V_t/V_0 (V_0 was tumor volume at the time of first injection). The animals were sacrificed when the average tumor volume of the control exceeded 2000 mm^3 in the control group. The tumors were harvested immediately following sacrifice and stored in 10% formalin for further analysis. All animal procedures were approved by the Harvard Institutional Use and Care of Animals Committee.

Western Blot Assay of *In Vivo* Tumor Samples:

For animal tissue, tumor stored in -80° C were pulverized in a morter and pestle using liquid nitrogen, then treated with RIPA buffer to extract the protein. Amount of protein was measured by BCA assay and equal amount of protein lysates were electrophoresed on a polyacrylamide gel, then transferred to polyvinylidene difluoride membrane, (Bio-Rad) and blocked in with 5% milk soultion. Then membranes were incubated with appropriate concentration of primary antibody (all antibodies from Cell Signalling Technology) overnight at 4° C, followed by horseradish peroxidase–conjugated secondary antibody for 1 hour. Detection was done using G-box from Syngene and densitometric quantification was done by image J software.

Efficacy study of PI103-SNPs in Murine Ovarian Cancer Tumor Model:

Ovarian adenocarcinomas were induced in genetically engineered K-ras $^{LSL/+}$/Pten$^{fl/fl}$ mice via intrabursal delivery of adenovirus-carrying Cre recombinase. Tumor cells were also engineered to express luciferase once activated by Adeno-Cre to make tumor imaging possible before and after drug treatment. Once mice developed medium to large tumors, they were placed into one of four treatment groups (vehicle, Free-PI-103 5 mg/kg, PI-103-SNP 5 mg/kg, and iRGD-PI103-SNP 5 mg/kg) and all drugs were administered via tail vein injection. Treatment was administered five times over a 10 d period with a 1 d period between treatments for Free-PI-103 and PI103-SNP, while iRGD-PI103-SNP was administered three times over a 7 d period with 1 d break between treatments. Tumor imaging in vivo was performed using an IVIS Lumina II Imaging System. Quantification of bioluminescence was achieved by using Living Image Software 3.1 (Caliper Life Sciences). Prior to imaging, mice received 150 mg/kg of D-luciferin firefly potassium salt via intraperitoneal injection. Five minutes post-luciferin injection, mice were anesthetized in a 2.5% isoflurane induction chamber where they were kept under anesthesia by a manifold supplying isoflurane and their body temperature was maintained steady by a 37 ^{o}C temperature stage. Bioluminescent signal was collected 15 min after luciferin administration for an exposure

time of 30 s. Images were taken a day prior to initial treatment (day 0, baseline image), after 3 treatments, and 1 d after 5 treatments.

Western Blot Analysis of *In vivo* Tumor Samples:

Treatment efficacy was quantified by examining expression of PI3K/mTOR proteins following treatment cycle via western blot analysis. Tissue lysates were examined on a 4-20% Tris Glycine gel (Invitrogen) and transferred to a 0.4µM PVDF membrane (Perkins Elmer). Non-specific proteins were blocked with 5% nonfat dry milk. Primary antibodies (Cell Signaling Technologies, 1:1000 dilution) used with pAkt S473, pS6, pmTOR, p4E-BP1, Akt, S6, mTOR, and 4E-BP1 were incubated overnight. B-Actin was used as a loading control. Anti-rabbit IgG secondary antibody (Cell Signaling Technologies, 1:2000) was then incubated at room temperature for 1 h. Expressed proteins were detected using Supersignal West Pico Chemiluminescence substrate (Thermo Scientific).

Tumor slice staining and Imaging:

For IHC and TUNEL study, tumor slices (5 Am) were cut after frozen in OCT medium at Harvard Medical School Core facility. For iRGD targeting images these sections were directly imaged under the green filter of Nikon TE2000 microscope. For TUNEL imaging studies, tumor sections were stained with standard TMR red fluorescent terminal deoxynucleotidyl tranferase-mediated dUTP nick end labeling (TUNEL) kit following the manufacturer’s protocol (In Situ Cell Death Detection Kit, TMR-Red, Roche). Images were obtained using a Nikon Eclipse TE2000 fluorescence microscope equipped with red filter. Significant internalization of FAM-iRGD tagged PI103 –cholesterol was imaged. Blood vessels were stained with vWF staining kit.