Supporting Information

Dual Functional Liposome Specifically Target Melanoma Cells Through Integrin and Ephrin Receptors

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Experimental Section

Materials:

Chemicals: Wang resin and all required fmoc protected amino acids were purchased from Novabiochem (Merck). N,N\textsuperscript{-}dimethylformamide (DMF), dichloromethane (DCM), trifluoroacetic acid (TFA), pipyridine, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU), diisopropylethylamine (DIPEA), methanol and di-tert-butyl dicarbonate were purchased from Spectrochem. 1,4-dioxan was purchased from RANKEIM. Ethanedithiol (EDT) and phenol were purchased from Merck. 5,6 carboxy fluorescence, 5-diphenyltetrazolium bromide (MTT), dulbecco\textsuperscript{'}s modified eagle\textsuperscript{'}s medium (DMEM), kanamycin sulfate, trypsin-EDTA solution, dimethyl sulfoxide for cell culture and formaldehyde solution for molecular biology were purchased from Sigma Aldrich. 1-Bromohexadecane and ethelene diamine were purchased from sigma. Penicillin-Streptomycin and fetal bovine serum (FBS) were purchased from Invitrogen. Annexin V and propidium iodide apoptosis detection kit were purchased from Santa Cruz Biotechnology. Bisbenzimide H 33258 (hoechst) was purchased from Calbiochem. Cholesterol and DOPC were purchased from Avanti polar lipid. Ethanol was purchased from Merck. Cover glass bottom dishes were purchased from SPL. Agarose was purchased from Fisher scientific. Goat polyclonal anti-Rabbit IgG was purchased from Abcam. These compounds were used without further purification. We used Shimadzu HPLC equipped with C-18 semi preparative reverse phase column for peptide purification. Experimental water was used of milli Q grade. HPLC grade acetonitrile was purchased from J.T. Baker. Olympus IX83 inverted microscope was used for cell imaging. Synthesized lipopeptide masses were characterized by MALDI-TOF.

Preparation of LDV and YSA tripeptide: About 300 mg wang resin was taken in a peptide vessel and swelled for overnight in DMF-DCM (1:1) solvent. Five equivalent of excess fmoc protected valine, aspartic acid and leucine were coupled successively followed by fmoc deprotection by CEM microwave peptide synthesizer equipped with Liberty1. Coupling and deprotection steps were maintained for eight and five minutes respectively and microwave power was 35 watt. Five equivalent excess of N,N\textsuperscript{-}Diisopropylethylamine (DIPEA) and
HBTU were used as an activator base and activator respectively. DMF was used as solvent. Resin attached YSA tripeptide was also synthesized following same procedure.

**Preparation of Lipopeptides:**

**Synthesis of P1:** Two equivalent of monobromo hexadecane was reacted with one equivalent of monoboc ethylene diamine in presence of anhydrous potassium carbonate (4 equivalent), ethyl acetate for 48h under reflux condition. It was purified by column chromatography (5% ethyl acetate in n-hexane). Thus, Monoboc protected N,N’-dihexadecyl ethylamine was synthesized. After that, Boc group was cleaved by the TFA-DCM (1:1 ratio). Next, TFA was removed by nitrogen gas flow and product was worked up. Finally, purified N,N’-dihexadecyl ethylamine (P1) was obtained.

**Synthesis of L1:** Synthesized resin attached tripeptides (LDV) were reacted with succinic anhydride (3 equivalent) in presence of triethyl amine (3 equivalent) for 48h at room temperature under nitrogen gas. DMF was used as solvent. After completion of the reaction, synthesized product (L1) was washed with DMF and DCM.

**Synthesis of L2:** Both the Synthesized L1 and P1 were reacted in presence of DIPEA and HBTU for 24h. DMF:DCM (1:1) was used as solvent. After completion of the reaction, synthesized product (L2) was washed thoroughly by DMF and DCM.

**Synthesis of L3:** Next, resin was cleaved by standard resin cleavage cocktail solution containing 92.5% TFA, 2.5% milli Q water, 2.5% EDT and 2.5% phenol. Lipopeptide was precipitated in cold diethyl ether. Finally, C₁₆-LDV (L3) lipopeptide was purified by reverse phase C-18 column and characterised by MALDI-TOF.

C₁₆-YSA (Y3) lipopeptide was synthesized following the above procedure.

**Preparation of targeted liposome:** Chloroform solution of DOPC, cholesterol, lipopeptide (C₁₆-LDV or C₁₆-YSA or 1:1 mixture of C₁₆-LDV and C₁₆-YSA) were mixed for preparation of liposome targeted with LDV lipopeptide (Lip.LCLDV), YSA lipopeptide (Lip.LCYSA), LDV-YSA lipopeptide [Lip.LCLDV:LCYSA (1:1) or TL] and control liposome (without lipopeptide). For preparation of TRITC labelled liposome we have used TRITC-DHPE lipid into the lipid mixture. The concentration of DOPC, cholesterol, lipopeptide in the liposome formulation was 1 mM, 0.25 mM and 1 mM respectively. For docetaxel (DX) encapsulation we have used chloroform solution of docetaxel in the liposome formulation. Chloroform solution of all the components were taken in a glass vial, it was evaporated by nitrogen flash, dried under vacuum and hydrated over night at 4 °C. Then, it was vortexed for 2-3 min to form multi-lamellar vesicles (MV). MVs were then sonicated for 5 min followed by probe sonication under ice-cold condition. Next, it was centrifuged for 30 min at 5000 rpm to remove free DX. Concentration of DX was measured in TL-DX by UV-Spectrophotometer (Cary 60 UV-Vis of Agilent technologies). Components of different liposomes are mentioned in tabular form below-
Analysis of drug loading efficacy: DX was labelled with alexa 561 dye covalently and was purified through centrifugation. Fluorescence of alexa 561 labelled DX (LDX) in chloroform was measured by fluorimeter (PTI, Quanta Master Spectrofluorometer, QM-40) and concentration measured by spectrophotometer. After that, liposomes encapsulated with labelled DX (LDV-LDX, YSA-LDX and TL-DX) were prepared following same procedure. To get the loading capacity of liposome, all liposomes were incubated for 24 h. Next, emission spectrum (Excitation: 561 nm and Emission range: 570-700 nm) of LDX containing different liposomes were measured. Drug loading capacity of liposome was determined by comparing the fluorescence intensity of drug loaded liposome with free labelled drug used for liposome preparation.

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\text{Drug loaded efficacy (DLE)} = \frac{\text{FI}_{\text{lip}}}{\text{FL}_{\text{free}}} \times 100.
\]

Here, \( \text{FI}_{\text{lip}} \) indicates fluorescence intensity of liposomal drug and \( \text{FL}_{\text{free}} \) indicates fluorescence intensity of free labelled drug.

Transmission electron microscopy (TEM): A 5 \( \mu \)L normal, LDV-DX, YSA-DX and LDV-YSA-DX or TL-DX used for liposome size determination by transmission electron microscope. Imaging was performed using TECNAI G2 POLARA, 300KV, equipped with (4KX4K) FEI eagle camera.

Dynamic light scattering (DLS) study for liposome size determination: To characterize the size and zeta potential of liposomes i.e normal, LDV-DX, YSA-DX and TL-DX, DLS study has been performed. Experiment was performed by Malvern particle size analyser (Model no. ZEN 3690 ZETASIZER NANO ZS 90).

Cell Culture: A375 (human melanoma) and WI38 (normal lung fibroblast) cells were purchased from National Centre for Cell Science (NCCS) Pune, India. Cells were cultured in 5% CO\(_2\) incubator at 37 \(^\circ\)C using dulbecco’s modified eagle’s medium (DMEM) having fetal bovine serum (10%), kanamycin sulfate (110 mg/L), penicillin (50 units/mL) and streptomycin (50 \( \mu \)g/mL). Trypsin-EDTA (1X) solution was used for cell detachment.
Imaging of cellular uptake using fluorescence microscope: A375 and WI38 cells were harvested in DMEM medium containing 10% fetal bovine serum on cover glass bottom disc for overnight prior to treatment. Cells were treated with TRITC labeled liposome solutions [Lip.LCLDV, Lip.LCYSA, Lip.LCLDV:LCYSA (1:1) and control (non-targeted)] for 120 min. After 120 min, proper washing by PBS, 4% formaldehyde (in PBS buffer) was added for half an hour to fix the cell in each cover glass. Next, formaldehyde solution was removed and washed with PBS buffer. Nucleus was stained with hoechst 33258 (1 µg/mL) for 1h. Hoechest 33258 solution was removed and washed by PBS buffer for three times. Finally, each cover slip was ready for the microscopic imaging. Cell imaging was performed by Olympus IX83 fluorescence microscope under 40X objective (Olympus) equipped with EMCCD camera in bright field, 561 and 405 nm wave lengths.

Cellular uptake using FACS: A375 and WI38 cells were seeded in 6-well plate containing 10% fetal bovine serum on cover glass bottom disc for overnight prior to treatment. Cells were treated with TRITC labelled liposome solutions [Lip.LCLDV, Lip.LCYSA, Lip.LCLDV:LCYSA (1:1) and control (non-targeted)] for 120 min. Uptake analysis was performed by BD LSRFortessa™ flow cytometer.

Cell viability study: A375 and WI38 cells were seeded in 96-well plate before 24h of treatment. Cell were treated with bared docetaxel (DX) and liposomal docetaxel targeted with LDV lipopeptide (LDV-DX), YSA lipopeptide (YSA-DX), LDV-YSA lipopeptide [LDV-YSA-DX or TL-DX] for 4h. Docetaxel concentration in the treatment solution was 1000 nM. Media was replaced with fresh media and incubated for 44h. Cell viability has been performed by MTT assay as mentioned before.1 Then, purple coloured formazan was dissolved in 1:1 (v/v) DMSO/MeOH and absorbance of each well were measured at 550 nm by micro-plate ELISA reader. Percent of viability was measured from this absorbance values. Percentage viability has been calculated as [(A_550 Treated Cells - A_550 Backgrounds)/ (A_550 Untreated Cells - A_550 Backgrounds) X 100.

Apoptosis study: Flow cytometric detection of cell death pathway has been performed using previously described method.2 In brief, A375 cells (~5 X 10^5 Cells/mL) were harvested overnight in a 6-well plate and treated with docetaxel (DX) and LDV-YSA dual targeted liposomal docetaxel (TL-DX) for 48h. Cells were trypsinized and washed with assay buffer. Cells were taken in suspension having 100 µL solution of assay buffer contained with propidium iodide (PI) and annexin V and incubated at 37 °C for 15 min. Emission of annexin V and PI has been detected using FITC and PI channels of BD LSRFortessa™ flow cytometer using emission filters at 530 and 610 nm respectively. In the represented data, cells in the Q1, Q2 and Q4 quadrants are regarded as necrotic, late apoptotic and early apoptotic cells respectively and Q3 quadrant cells are considered as normal or healthy cell population. FACS DIVA software was used for quantification.

Cell cycle analysis: A375 cells (~5 X 10^5 Cells/mL) were seeded overnight in a 6-well plate and treated with docetaxel (DX) and LDV-YSA dual targeted liposomal docetaxel (TL-DX) for 48h. Cells were trypsinized and washed with PBS. Cells were fixed with 70% ethanol and kept at -20 °C before analysis. Then cells were incubated with PI (100 µg/mL) and RNase (10
μg/mL) for 45 min at 37 °C. The experimental analysis was performed using PI channels of BD LSRFortessa™ flow cytometer at 610 nm emission filter.

**Effect of TLDX and DX on microtubule network:** A375 cells were seeded in a cover glass bottom disc prior to 24h treatment. A375 cells were treated with 1000 nM solution of DX and TL-DX for 24h. Next, cells were washed by PBS and treated with 4% paraformaldehyde solution for 30 minutes for cell fixing. We have treated the cells with cell permeable solution (0.1% Triton X-100 in PBS) for 25 minutes. Cells were washed with PBS and incubated with 5% BSA in PBS for 30 minutes to block nonspecific binding sites. Antibody solutions were prepared in PBS. After washing with PBS, cells were incubated with monoclonal anti-α-tubulin IgG [EP1332Y] antibody with dilution of 1:300 for 2 hours. After that cells were washed with PBS and incubated with secondary antibody (Cy3.5 pre-absorbed goat anti-rabbit IgG) with dilution 1:600 for 2 hours. Cells were washed with PBS and incubated with Hoechst 33258 (1 μg/mL) for 30 minutes before imaging. Cell imaging was performed by Olympus iX83 fluorescence microscope under 40X objective (Olympus) equipped with EMCCD camera in bright field, 561 and 405 nm wave lengths.

**Growth inhibition study of multicellular tumor spheroidal (MCTS) culture:** Multicellular tumor spheroidal generation of A375 cells was performed using non adhesive cell culture system. Briefly, the parental monolayer of A375 cells were collected and seeded in 35 mm cover disk coated with 1% agarose (w/v). The cell concentration was maintained 5×10³ cells/well. A375 cells were cultured in DMEM containing media and incubated in a humidified atmosphere with 5% CO₂ for the days till size of the spheroid reached upto the 100 μm. Then spheroid morphologies were captured in DIC mode using inverted Olympus iX83 microscope equipped with EMCCD camera under 40X objective (Olympus) and it was mentioned as day zero. Then, the spheroids were divided in three groups; without treatment (control), treated with DX and treated with TL-DX. The docetaxel concentration was 1000 nM. Size of the spheroids checked by above mentioned microscope for five days successively. Volume of the sphere was calculated and imaged taken by microscope. Formula used for volume calculation was mentioned below-

\[ V = 0.5 \times \text{Length} \times \text{Width}^2 \]

**LDH assay:** In order to demonstrate the membrane integrity affected due to the LDV-YSA-DX or TL-DX treatment, surfactant behavior of the complex was analyzed using LDH assay as performed earlier. In the present experiment, LDH activity was not observed in the media collected from control cells or TL-DX treated cells till 4h. Again longer time incubation, A375 cells will die due to apoptosis mediated by TL-DX interaction with cellular microtubule. Hence, to avoid this condition we have chosen 4h of incubation. The media collected from 10% Triton-X (TX) (non-ionic surfactant) treated cells taken as positive control showed elevated LDH activity after 4h. In our earlier apoptotic study, we have shown that TL-DX induces apoptosis, microtubule shrinkage in A375 cells and it is well documented that apoptosis induces cell damage or lysis releasing LDH into the medium. So from the above results, conclusions can be drawn that TL-DX does not damage the membrane integrity immediately as observed after 4 h.
Briefly, A375 cells were harvested in 96 wells tissue culture plate for 24 h. After that cells were treated with 1000 nM [DX] of TL-DX for 4h in serum free condition. Untreated cells were cultured as control and 10% Triton X (TX) (non-ionic surfactant) treated cells were considered as positive control. Then 96 well plates have been centrifuged at 400 ×g for 10 min and supernatant media has been transferred to a new 96 well plate. Then, enzyme assay reaction mixture containing NAD+, lactic acid, tetrazolium salt (INT) and diaphorase in assay buffer has been added to the supernatant media followed by incubation for 30 min at room temperature with continuous shaking. The overall method follows two step reactions. Initially, LDH (released from cytosol) catalyses the reduction of NAD$^+$ to NADH and H$^+$ by oxidation of lactate to pyruvate. Then, diaphorase uses NADH and H$^+$ to catalyse INT to highly coloured formazan which absorb light at 490 nm. Thus the amount of formazan represents amount of LDH released into the culture media. To quantify the LDH activity we have performed above experiment with known concentration of LDH and prepared a standard curve. The LDH activity thus measured using following formulae:

**LDH activity** = \( \frac{A_{490} - c}{m} \)

Where, \( A_{490} \) represents absorbance of formazan produced in the sample, c and m represents y-intercept and slope of the LDH activity standard curve.

**References:**

Figure S1: Mass spectroscopy of P1.
Figure S2: $^1$H NMR (300 MHz, CDCl$_3$) of P1, δ 2.7 (t, 2H, -C$_1$H$_2$), 2.5-2.3 (t, 6H, -C$_2$H$_2$ & -C$_3$H$_2$), 1.5-1.2 (m, 56H, -C$_4,4'$H$_2$ to -C$_17,17'$H$_2$), 0.88 (t, 6H, -C$_{18,18'}$H$_3$).

Figure S3: $^{13}$C NMR of P1 (75 MHz, CDCl$_3$) δ 14.06, 22.65, 25.87, 26.01, 26.79, 27.20, 27.53, 28.57, 29.22, 29.33, 29.67, 31.89, 39.85, 53.34, 54.37, 57.26 64.62, 65.32.
Figure S4: HPLC chromatogram of long chain LDV lipopeptide (C$_{16}$-LDV).

Figure S5: MALDI-TOF mass of long chain LDV lipopeptide (C$_{16}$-LDV).

Figure S6: HPLC chromatogram of long chain YSA lipopeptide (C$_{16}$-YSA).
**Figure S7:** MALDI-TOF mass of long chain YSA lipopeptide (C$_{16}$-YSA).

**Figure S8:** Transmission electron microscope (TEM) images of (a) normal liposome (b) LDV-DX (c) YSA-DX and (d) TL-DX respectively. Scale bar corresponds to 100 nm.
Figure S9: Dynamic light scattering (DLS) study reveals sizes of (a) Normal liposome (44.14 nm) (b) LDV-DX (46.56 nm) (c) YSA-DX (48.87 nm) (d) TL-DX (70.63 nm) and zeta potential of (e) normal liposome (-5.45 mv) (f) LDV-DX (-17.5 mv) (g) YSA-DX (-2.36 mv) (h) TL-DX (-12.0 mv).

Figure S10: Drug loading capacity of different liposomes (LDV-DX, YSA-DX and TL-DX) after 24 h incubation.
**Figure S11:** Cellular uptake study of TRITC labelled liposome in A375 cells.

**Figure S12:** Cellular uptake study of TRITC labelled liposome in WI38 cells.
Figure S13: Lactate dehydrogenase (LDH) assay study performed after TL-DX treatment for 4h.