Enzyme-Assisted Self-Delivery System of Lonidamine-Peptide Conjugates for Selectively Killing of Cancer Cells

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subcutaneous injections of rhodamine-loaded **LND-GFFpY** (22 mM) with 3 unit/mL ALP (D, 0.5 h; E, 3 h; F, 12 h); (G, H, I), Peritumoral injections of rhodamine-loaded **LND-GFFpY** (22 mM) with 3 unit/mL ALP (G, 0.5 h; H, 3 h; I, 12 h); (J, K, L), Peritumoral injections of rhodamine-loaded (22 mM) **LND-GFFpY** without of ALP (J, 0.5 h; K, 3 h; L, 12 h). Dotted circles indicate presence of dye in tissues. “T” indicates the tumor tissues.

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**Figure S16.** Histological images of H & E stained tumor tissues of mice after treatment of PBS (A), free LND (B), **LND-GFFpY** (C). Bar, 100 μm.

**Table S1.** IC\textsubscript{50} values of LND, **LND-GFFpY**, **LND-GFFY**, and mixture of LND and **GFFpY** (LND+**GFFpY**) against cancer cells (HepG2, HeLa) and normal cells (LO2, NIH3T3) for 48 h.

3. References
1. Materials and methods

1.1 Materials
Lonidamine was purchased from Meilun Reagent Company (Dalian, China). 2-Chlorotrityl chloride resin, Fmoc-protected amino acids and reagents used in solid peptide synthesis were gained from GL Biochem Ltd (Shanghai, China) and Aladdin Reagent Company (Shanghai, China). Fetal bovine serum (FBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide (MTT), newborn Calf Serum (NBCS), Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin and RIPA Lysis Buffer were the products of Gibco (US). Alkaline phosphatase was obtained from Sigma-Aldrich. Human Hepatocyte LO2 cells were purchased from Procell Life Science & Technology Corporation. Human Liver Cancer HepG2 cells, Human Cervical Carcinoma HeLa cells and Murine fibroblast NIH3T3 cells were obtained from America Type Culture Collection. All cells were cultured at 37 °C and 5% CO2.

1.2 Syntheses of peptide derivatives
The peptide derivatives LND-GFFpY, LND-GFFY and GFFpY were synthesized by solid phase synthesis using 2-chlorotriyl chloride resins. Briefly, the first N-Fmoc-protected amino acid was linked to the activated resin for 3 h. Then the unreacted active sites on the resin were protected by using the capping reagent (DCM: methanol: DIEA=80:15:5 V/V/V) and the Fmoc group of the first amino acid was removed for coupling with the next amino acid. The peptide derivatives were extended according to this procedure. Finally, the products were cleaved from the resin using trifluoroacetic acid and precipitated by cold ether. The products were characterized by MS, 1H NMR and purified by preparative high-performance liquid chromatography (HPLC) equipped with a Shim-Pack RRC-ODS column utilizing 75% methanol and 25% water (0.1% TFA).

1.3 Preparation of the hydrogel with peptide derivatives
LND-GFFpY, LND-GFFY or GFFpY of varying weight was dissolved in 200 μL PBS (pH 7.4), and Na2CO3 was used to adjust the pH of the solution. Alkaline phosphatase (ALP, 3 unit/mL) was added to trigger the gelation of LND-GFFpY or mixture of GFFpY and LND. A heating and cooling process was used for the gelation of LND-GFFY. The minimum gelation concentration (MGC) were determined by a tube inversion method. And the critical micelle concentration (CMC) of LND-GFFpY was tested by utilizing a Malvern Zetasizer (NANO ZS90) with a laser light scattering spectrometer at 632.8 nm at 25 °C.

1.4 Conversion efficiency of LND-GFFpY
Briefly, alkaline phosphatase (ALP, 0.3 unit/mL) was added into 1 mg/mL LND-GFFpY in PBS and incubated at 37 °C. At fixed time points, 150 μL solution was take out and mixed with 150 μL methanol. The samples were then analyzed by HPLC (Shimadzu, SPEAX GP-C18 column) utilizing 75% methanol and 25% water (0.1% TFA) and compared with elution curves of pure LND-GFFpY and LND-GFFY.

1.5 Characterization of hydrogel
Transmission Electron Microscopy (TEM)
Hydrogel was formed with EISA at 1.0 wt%, diluted to 0.5 mg/mL with PBS and stained with phosphotungstic acid. The samples were deposited on a copper grid for TEM observations under an accelerating voltage of 100 kV (JEOL JEM-2100f TEM). For the observation of intracellular fiber formation, HepG2 cells were incubated with LND-GFFpY (150 μM) for 12 h. After being washed with PBS to remove precursors outside cells, the cells were lysed with RIPA buffer followed by sample preparation for TEM.

Circular dichroism (CD) spectra
CD spectra were employed to study the secondary structure of peptide derivatives in the hydrogel. 1.0 wt% hydrogel of LND-GFFpY formed by EISA was diluted to 0.5 mg/mL and then CD spectra ranging from 245 to 190 nm were recorded on a CD spectropolarimeter (Jasco J-810, Tokyo, Japan).
Rheology study
The viscoelastic properties of LND-GFFpY/ALP hydrogel was measured on a rheometer (Discovery HR-2) with a 40 mm-diameter parallel plates and 0.3 mm gap distance. Dynamic time sweep test was tested at a frequency of 6.28 rad/s with the strain of 0.2%. Dynamic strain sweep was performed at a frequency of 6.28 rad/s with strain ranging from 0.1% to 100%. Dynamic frequency sweep was carried out at 0.2% strain and frequency varied from 0.1 to 100 rad/s. For cyclic strain sweep, G' and G'' were recorded at fixed frequency and repeated stain cycle (2% and 50% strain alternatively).

1.6 In vivo gelation experiments
To make xenografted tumor model, suspension of A549 cells were mixed with Matrigel (1:1 v/v, Corning, USA) and injected subcutaneously to the right flank of Balb/c nude mice (male, 6-8 weeks, Zhejiang Academy of Medical Sciences, China) for tumor inoculation and mice bearing tumors (~200 mm³) were selected for further experiment. For in situ gelation in mice, LND-GFFpY (22 mM) was first dissolved in PBS and mixed with Rhodamine B (2.0 mg/mL). Then 200 μL of above solution was mixed with or without 3 unit/mL of ALP and immediately injected subcutaneously or peritumorally in nude mice, and gel residues were observed after 0.5 h, 3 h and 12 h, respectively. LND solution (200 μL, 22 mM) loaded with Rhodamine B (2.0 mg/mL) served as control. All animal procedures were conducted according to the Guidelines for Care and Use of Laboratory Animals of China Pharmaceutical University and approved by the Animal Ethics Committee of China Pharmaceutical University in Nanjing, China.

1.7 Drug release behaviour of LND-GFFpY in vitro
LND-GFFpY hydrogels (500 μL, 1.0 wt%) were prepared in glass bottles and then 300 μL of PBS solution (pH 7.4) with varying concentrations of protein kinase K (0, 10, and 30 unit/mL) were added on top of hydrogels for incubations in an oscillator (40 rpm, 37 °C). At fixed time points (1-72 h), 250 μL PBS was taken out and fresh PBS solution of equal volume was added. The release amount of intact LND moieties was determined by HPLC with isocratic elution of 75% methanol and 25% water (0.1% TFA). The cumulative release rate was calculated according to pre-established standard curve of LND. The release of free LND from LND solution served as a control, and was examined using dialysis bags (MWCO = 3000).

1.8 Cell viability test
For cell viability test, HepG2, HeLa, LO2, or NIH3T3 cells were seeded in 96-well plates at a density of 3000 cells/well. After culturing for 24 h, the cells were treated with varying concentrations (600 to 37.5 μM) of LND, LND-GFFpY, LND-GFFY or mixture of GFFpY and LND, respectively. After 48 h of incubation, 20 μL MTT solution (5 mg/mL) was added to each well. Four hours later, the medium was removed and 200 μL DMSO was added to dissolve the formazan formed. The absorbance at 490 nm of each well was then recorded with a microplate reader (Tecan Sunrise, Austria).

1.9 Alkaline Phosphatase Activity
NBD-GFFpY was synthesized according to previously reported protocols 1-2 and used to compare the expression levels of alkaline phosphatase (ALP) in varying cell types (cancer cells, HepG2 and HeLa; normal cells, NIH3T3 and LO2). For staining with NBD-GFFpY, cells were first plated on 35 mm dishes. Then 300 μM of NBD-GFFpY were added and incubated for 4 h. After removing the medium, the cells were washed with PBS and fixed with 3.7 % formaldehyde for DAPI staining (1 μg/mL). The fluorescence of cells was examined with a confocal laser scanning microscope (Carl Zeiss, LSM700) and the fluorescence intensity of each cell was quantified by an ImageJ software. Relative fluorescence intensity of various cells was then normalized with that of NIH3T3 cells.

1.10 Cellular uptake experiments
To measure the cellular uptake of LND-GFFpY or LND in cancer cells (HepG2, HeLa) or normal cells (NIH3T3 and LO2), 350 μM of LND-GFFpY or LND were incubated with each cell type for 4 h. Then cells were washed with PBS and lysed with a mixture solution of 500 μL RIPA buffer and 500 μL methanol with ultra-sonification. After centrifugation (10000 rpm, 5 min), the amounts of compounds in supernatants were quantified by HPLC with pre-established standard curves. The intracellular uptake of LND-GFFpY was then calculated by combination of intracellular LND-GFFpY and LND-GFFY, and was expressed in unit of nmol that was normalized with total cell number.

1.11 Actin Staining

To examine effect of LND-GFFpY to the formation of cytoskeleton network (actin and microtubules), HepG2 cells were first plated on 35 mm dish and incubated with 150 μM LND or LND-GFFpY. After culturing for 24 h, the cells were washed, fixed and permeabilized with 0.1% Triton X-100. Then, 500 μL of Alexa Fluor 633 Phalloidin (33 nM) was added and incubated with cells for 20 min. After washing with PBS, these cells were stained with DAPI (1 μg/mL) for 10 min. Finally, cells were observed under a confocal laser scanning microscope (Carl Zeiss, LSM700).

1.12 In vivo efficacy of LND-GFFpY hydrogel to HeLa xenografted tumor in mouse

HeLa cell suspension (5.0 × 10⁶ cells) with a volume of 75 μL were mixed with 75 μL Matrigel (Corning, USA) and then subcutaneously injected into the right flank of male BALB/C nude mice (Zhejiang Academy of Medical Sciences). When the tumor volume reached about 80 mm³, the nude mice were randomly divided into three groups: (1) PBS group (control); (2) free LND solution group; (3) LND-GFFpY group. Various drug formulations were injected in peritumoral regions once a week for two weeks. The dose of LND were fixed at 40 μmol/kg body weight. The tumor sizes (L × W²/2, where L and W are length and width of tumor respectively) and body weights were measured every 3 days for 21 days. Finally, the entire tumor tissues of all mice were collected for histological study by hematoxylin-eosin (H&E) staining and slides were examined under a microscope (NIKON Eclipse ci). All animal procedures were conducted according to the Guidelines for Care and Use of Laboratory Animals of China Pharmaceutical University and approved by the Animal Ethics Committee of China Pharmaceutical University in Nanjing, China.
2. Results

Figure S1. Structural characterizations of LND-GFFpY. (A) TOF-MS: C_{44}H_{41}Cl_{2}N_{6}O_{10}P, calc. MW = 915, obsvd. [M] = 915.3, [M-2H] = 913.3; (B) 'HNMR spectrum. (1HNMR, 500 MHz, ([D6] DMSO) d = 9.76 (s, 1 H), 8.25-8.21 (d, 3 H, J = 20 Hz), 8.18-8.14 (m, 1 H), 7.80-7.78 (d, 1 H, J = 10 Hz), 7.70 (s, 1 H), 7.53-7.50 (m, 1 H), 7.38-7.34 (m, 2 H), 7.27-7.25 (d, 4 H, J = 10 Hz), 7.21-7.20 (d, 3 H, J = 5 Hz), 7.15-7.12 (m, 5 H), 7.09-7.07 (d, 3 H, J = 10 Hz), 6.94-6.93 (d, 1 H, J = 5 Hz), 5.84 (s, 2 H), 4.62-4.58 (m, 1 H), 4.56-4.53 (m, 1 H), 4.46-4.43 (m, 1 H), 3.94-3.91 (d, 2 H, J = 15 Hz), 3.84-3.82 (d, 2 H, J = 10 Hz), 3.01-3.00 (d, 2 H, J = 5 Hz), 2.86-2.84 (d, 2 H, J = 10 Hz), 1.66 (s, 1 H), 1.55 (s, 1 H)).
Figure S2. Structural characterizations of LND-GFFY. (A) TOF-MS: C$_{44}$H$_{40}$Cl$_{2}$N$_{6}$O$_{7}$, calc.MW = 835, obsvd. [M]$^-$ = 835.4, [M-2H]$^-$ = 833.4; (B) $^1$HNMR spectrum ($^1$HNMR, 300 MHz, ([D$_6$] DMSO) d = 12.70 (s, 1 H), 8.50-8.47 (d, 1 H, J = 9 HZ), 8.37-8.34 (d, 1 H, J = 9 HZ), 8.26-8.23 (m, 1 H), 8.09-8.06 (d, 1 H, J = 9 HZ), 7.68 (s, 1 H), 7.51-7.49 (m, 1 H), 7.23-7.22 (d, 7 H, J = 3 HZ), 7.14-7.12 (d, 4 H, J = 6 HZ), 7.02-6.97 (m, 4 H), 6.88-6.86 (d, 1 H, J = 6 HZ), 6.67-6.63 (m, 3 H), 5.82 (s, 1 H), 4.61-4.51 (m, 3 H), 4.36 (s, 2 H), 3.88-3.82 (d, 2 H, J = 18 HZ), 3.04-3.01 (d, 2 H, J = 9 HZ), 2.95-2.92 (d, 2 H, J = 9 HZ), 2.83-2.80 (d, 2 H, J = 9 HZ)).
Figure S3. Structural characterizations of GFFpY. (A) TOF-MS: C_{29}H_{33}N_{4}O_{9}P, calc. MW = 612, obsvd. [M+H]^+ = 613.3; (B) $^1$HNMR spectrum of GFFpY ($^1$HNMR, 300 MHz, ([D6] DMSO) δ = 9.02 (s, 1 H), 8.39-8.37 (d, 2 H, J = 6 HZ), 8.06-8.05 (d, 1 H, J = 3 HZ), 7.27-7.23 (m, 6 H), 7.18-7.15 (d, 5 H, J = 9 HZ), 7.10-7.07 (d, 3 H, J = 9 HZ), 4.54-4.48 (m, 1 H), 4.44-4.39 (m, 2 H), 3.42-3.35 (d, 2 H, J = 21 HZ), 3.04-2.98 (d, 4 H, J = 18 HZ), 2.88-2.81 (m, 2 H), 2.27 (s, 1 H), 1.64-1.55 (m, 2 H), 1.24 (s, 1 H)).

Figure S4. TEM images of 2.0 wt% LND-GFFY after a heat and cooling process.
Figure S5. HPLC elution curves of LND-GFFpY (A, 1 mg/mL), LND-GFFpY/ALP (1 mg/mL, 0.3 unit/mL ALP) at 6 h (B) and LND-GFFY (C, 1 mg/mL).

Figure S6. Conversion ratio of 1 mg/mL LND-GFFpY to LND-GFFY with 0.3 unit/mL ALP in 12 h.
Figure S7. Structural characterization of NBD-GFFpY. (A) TOF-MS: C_{38}H_{39}N_{8}O_{13}P, calc. MW = 846, obsvd. [M]^- = 846.3, [M-H]^-= 845.3; (B) HPLC elution curve; (C) $^1$H NMR spectrum ($^1$H NMR, 300 MHz, ([D6] DMSO) d = 9.43 (s, 1 H), 8.51-8.49 (d, 1 H, J = 6 Hz), 8.27-8.25 (d, 1 H, J = 6 Hz), 8.19-8.13 (m, 1 H), 8.05-8.02 (d, 1 H, J = 9 Hz), 7.25-7.20 (m, 6 H), 7.17-7.12 (d, 7 H, J = 15 Hz), 7.08-7.06 (d, 3 H, J = 6 Hz), 4.58-4.55 (m, 1 H), 4.47-4.43 (m, 2 H), 3.68-3.66 (m, 1 H), 3.59-3.57 (d, 2 H, J = 6 Hz), 3.39-3.37 (d, 2 H, J = 6 Hz), 3.04-3.00 (m, 2 H), 2.95-2.91 (d, 2 H, J = 12 Hz), 2.82-2.79 (d, 2 H, J = 9 Hz), 2.61-2.56 (m, 3 H), 1.24 (s, 1 H)).
Figure S8. Confocal images of HepG2 (A), HeLa (B), LO2 (C) and NIH3T3 (D) cells after treatment with 300 μM NBD-GFFpY for 4 h.

Figure S9. Relative mean fluorescence intensity of HepG2, HeLa, LO2 and NIH3T3 cells after treatment with 300 μM NBD-GFFpY for 4 h. Fluorescence intensities of various cells were normalized with that of NIH3T3 to calculate the relative fluorescence intensity.
**Figure S10.** *In situ* examinations of hydrogel/solution residues with rhodamine. (A, B, C), Subcutaneous injections of aqueous solution of 22 mM LND mixed with 2 mg/mL rhodamine (A, 0.5 h; B, 3 h; C, 12 h); (D, E, F) subcutaneous injections of rhodamine-loaded LND-GFFpY (22 mM) with 3 unit/mL ALP (D, 0.5 h; E, 3 h; F, 12 h); (G, H, I), Peritumoral injections of rhodamine-loaded LND-GFFpY (22 mM) with 3 unit/mL ALP (G, 0.5 h; H, 3 h; I, 12 h); (J, K, L), Peritumoral injections of rhodamine-loaded (22 mM) LND-GFFpY without of ALP (J, 0.5 h; K, 3 h; L, 12 h). Dotted circles indicate presence of dye in tissues. “T” indicates the tumor tissues.

**Figure S11.** Viscoelastic characterizations of LND-GFFpY/ALP hydrogel (1.0 wt%, 3 unit/mL ALP). (A) Dynamic time sweep; (B) Dynamic frequency sweep; and (C) Cyclic strain sweeps.
Figure S12. (A) Viabilities of HepG2 and LO2 cells after 48 h treatment of LND-GFFY with IC\textsubscript{50} value of LND-GFFY listed (B). (C) Viabilities of HeLa and NIH3T3 cells after 48 h treatment of LND-GFFY with IC\textsubscript{50} value of LND-GFFY listed (D).

Figure S13. (A) Viabilities of HepG2 and LO2 cells after 48 h treatment of the mixture of LND and GFFpY with IC\textsubscript{50} value of the mixture of LND and GFFpY listed (B). (C) Viabilities of HeLa and NIH3T3 cells after 48 h treatment of the mixture of LND and GFFpY with IC\textsubscript{50} value of the mixture of LND and GFFpY listed (D).
Figure S14. Standard curves of (A) LND; (B) LND-GFPpY; (C) LND-GFpY.

Figure S15. CLSM images of HepG2 cells after treatment with 150 μM LND (D-F) or LND-GFPpY (G-I) for 24 h. Blank cells without any treatment served as control (A-C). (A, D, G), Alexa Fluor 633 phalloidin conjugate staining for actin/microtubules; (B, E, H), DAPI for nucleus staining; (C, F, I), merged images of blue and red channels.

Figure S16. Histological images of H & E stained tumor tissues of mice after treatment of PBS (A), free LND (B), LND-GFPpY (C). Bar, 100 μm.
Table S1. IC$_{50}$ values of LND, LND-GFFpY, LND-GFFY, and mixture of LND and GFFpY (LND+GFFpY) against cancer cells (HepG2, HeLa) and normal cells (LO2, NIH3T3) for 48 h.

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<th>HeLa</th>
<th>LO2</th>
<th>NIH3T3</th>
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3. References