Electronic Supplementary Material (ESI) for Biomaterials Science.
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A helical oncolytic polypeptide with potent membranolytic activity for cancer therapy

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Experimental Section

Materials. 1-Hexadecylamine, 3-buten-1-ol, glutaric dialdehyde and cysteamine hydrochloride were purchased from Tokyo Chemical Industry Co., Ltd (Shanghai). L-Glutamic acid was purchased from Gill biochemical Co., Ltd (Shanghai). Tris hydroxymethyl aminomethane, glycine, DTT, glycerol, bromophenol blue, TEMED and sodium dodecyl sulfate were purchased from Sangon Biotech Co., Ltd (Shanghai). Penicillin-Streptomycin Solution (100X), Trypsin-EDTA Solution with phenol red, LDH Cytotoxicity Assay Kit, Enhanced ATP Assay Kit, Fast Silver Stain Kit, Calcein/PI Live/Dead Viability/Cytotoxicity Assay Kit, Annexin V-FITC Apoptosis Detection Kit, Mycoplasma PCR Detection Kit and 30% Acr-Bis (29:1) were purchased from Beyotime Biotechnology Co., Ltd (China). 3-(4,5-dimethyl-2-Thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) and ammonium persulfate were purchased from Sigma-aldrich Trading Co., Ltd (Shanghai). DMEM medium and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. (USA). Wortmannin and methyl-β-cyclodextrin were purchased from Shanghai Macklin Biochemical Technology Co., Ltd (China). Z-VAD-FMK, VX-765, necrostatin-1, ferrostatin-1, cisplatin, gemcitabine and paclitaxel were purchased from MedChemExpress (USA). All other solvents and chemicals were of reagent grade, purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai) and used without further purification.

Characterization. ^{1}H NMR spectra were recorded on a Bruker AVANCE

NEO (600 MHz) or a Varian 400MHz spectrometer in deuterated solvent ($CDCl_3$). Chemical shifts were reported in ppm and referenced to the solvent proton impurities. The polydispersity (PDI) and number average molecular weight (Mn) of polypeptides were characterized by gel permeation chromatography (GPC), equipped with an isocratic pump (Model 1515, Waters, Milford, MA, USA), a refractive index detector (Model 2414, Waters), and size exclusion columns (HR0.5, HR1, HR2 and HR4 Styrene divinylbenzene columns, 5 μm, 300 × 7.8 mm, Waters, Milford, MA, USA) at 30 °C using DMF as the mobile phase. The PDI and Mn were calculated with poly (ethylene glycol) as the standard samples. Circular Dichroism (CD) measurements were carried out on a CS30320 CD spectrometer (Applied Photophysics Ltd, England). TECAN iControl (Version 2.0) was used to collect data of MTT assay, lactate dehydrogenase (LDH) leakage assay and adenosine-triphosphate (ATP) leakage assay. High-content screening-analysis was conducted by PerkinElmer Operetta CLS (PerkinElmer, USA). FACSCelesta was used for flow cytometry and Flowjo (Version 10) was used for data analysis. Confocal laser scanning microscope (CLSM) (Zeiss, LSM 880 with airyscan) was used to obtain confocal images. Cellsens software (Version 2.2) was used to obtain and analyse CLSM images. The cell morphology was examined by scanning electron microscopy (SEM) (Zeiss Marlin Compact, Germany). PerkinElmer IVIS Lumina III was used to evaluate the biodistribution of $OLPP_{10}$ (C16-$PButLG_{10}$-CA) and living Image (Version 4.4) was used for analysis.

CaseViewer (Version 2.1) was used for the analysis of images from H&E staining.

Cell lines. MDA-MB-231 human mammary carcinoma cells, CT26 murine colon carcinoma cells, Panc02 murine pancreatic cancer cells, 4T1 murine mammary carcinoma cells, MC38 murine colorectal cancer cells, EMT6 murine breast cancer cells, Caco-2 human colorectal cancer cells, E0771 murine mammary cancer cells, CAL27 human tongue squamous carcinoma cells were sourced from American Type Culture Collection (ATCC). Cisplatin (DDP) resistant human tongue squamous carcinoma cells (CAL27/DDP)[1] was a gift from Prof. Jin-song Li (from Sun Yat-sen Memorial Hospital, Sun Yat-sen University). Cells were maintained in DMEM medium or RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin solution (100X) and were cultured at 37 ℃ in a humidified atmosphere containing 5% CO_2. $Panc02_{mCherry/GFP}$ murine pancreatic cancer cells was constructed as previously reported.[2] To maintain resistance of CAL27/DDP, cisplatin (0.5 μM) was added in the medium. All cell lines were mycoplasma free as tested by Mycoplasma PCR Detection Kit.

Mouse and housing conditions. C57BL/6 mice (female, 6 weeks) were purchased from the Hunan SJA Laboratory Animal Co., Ltd (China). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. All mice were housed in pathogen-free conditions and kept in a room with controlled temperature (~22 ℃) and

humidity (45%-60%) under 12 h light/dark cycle.

LDH leakage assay. The LDH leakage assay was carried out according to the LDH Cytotoxicity Assay Kit. Specifically, Panc02 cancer cells were seeded in 96-well plates at the density of 1.5×10^4 per well in DMEM medium and cultured at 37 ℃ in a humidified atmosphere with 5% CO_2. After 12 h, the medium was replaced with fresh medium containing various concentrations of $OLPP_{10}$ (10, 20, 40, 80, 160 μg/mL) for 2 h. Then, the supernatant was collected and centrifuged at 400 g in a porous plate centrifuge for 5 min. LDH levels were detected by the kit mentioned above. LDH release (%) was calculated as following equation: $[(\Delta490_{sample} - \Delta490_{blank\ control}) / (\Delta490_{positive\ control} - \Delta490_{blank\ control})] \times 100\%)$. $\Delta490_{blank\ control}$ was the absorbance value of the cell-free wells at 490 nm. $\Delta490_{positive\ control}$ was the absorbance value of the wells treated with LDH release reagent (which provided by the kit) at 490 nm.

ATP leakage assay. The ATP leakage assay was carried out according to the ATP Assay Kit. Specifically, Panc02 cancer cells were seeded in 96-well plates at the density of 1.5×10^4 per well in DMEM medium and cultured at 37 ℃ in a humidified atmosphere with 5% CO_2. After 12 h, the medium was replaced with fresh medium containing various concentrations of $OLPP_{10}$ (10, 20, 40, 80, 160 μg/mL) for 2 h. The supernatant was collected and centrifuged at 12000 g at 4 ℃ in a porous plate centrifuge for 5 min. ATP levels were detected by the kit mentioned above. The actual absorbance value for each sample was calculated as following equation: $\Delta490 = \Delta490_{sample} - \Delta490_{blank}$

$_{control}$. $\Delta 490_{blank\ control}$ was the absorbance value of the untreated wells at 490 nm. The levels of ATP in the samples were calculated according to the standard curve.

Calcein AM/PI co-staining assay. The Calcein AM/PI co-staining assay was carried out according to the Calcein/PI Live/Dead Viability/Cytotoxicity Assay Kit. Specifically, Panc02 cancer cells were seeded in 24-well plates at the density of 1.5×10^5 per well in DMEM medium and cultured at 37 ℃ in a humidified atmosphere with 5% CO_2. After 12 h, the supernatant was replaced with fresh medium containing cisplatin, gemcitabine, paclitaxel or $OLPP_{10}$ separately. 2 h later, the cells were washed twice using PBS and then incubated with Calcein AM/PI detection solution in dark for 30 min. Finally, the cells were observed under fluorescence microscope.

The toxicity of $OLPP_{10}$ at 4 °C. Panc02 cancer cells were seeded in 96-well plates at the density of 1.5×10^4 per well in DMEM medium and cultured at 37 ℃ in a humidified atmosphere with 5% CO_2. After 12 h, the supernatant was replaced with cold DMEM medium containing different concentrations of $OLPP_{10}$ (10, 20, 40, 80, 160 μg/mL) at 4 °C for 2 h. And then the cytotoxicity of $OLPP_{10}$ was evaluated by MTT assay.

The toxicity of $OLPP_{10}$ in the presence of inhibitors. To determine whether the toxicity of $OLPP_{10}$ was depended on endocytosis, Panc02 cancer cells were treated with wortmannin (50 nM, an endocytosis inhibitor inhibiting phosphatidylinositol-3-kinase), methyl-β-cyclodextrin (50 μM, a caveolae-

dependent endocytosis inhibitor), sodium azide (200 mM, an endocytosis inhibitor blocking energy metabolism) for 0.5 h. Then the medium was replaced with fresh medium containing a series of $OLPP_{10}$ (10, 20, 40, 80, 160 μg/mL) and inhibitors. The cells were incubated for another 2 h before MTT assay.

To determine whether the toxicity of $OLPP_{10}$ was depended on classic death pathway, Panc02 cancer cells were treated with Z-VAD-FMK (40 μM, an apoptosis inhibitor by inhibiting pan-caspase), Necrostatin-1 (100 μM, a necroptosis inhibitor by inhibiting receptor-interacting protein kinase 1), Ferrostatin-1 (1 μM, a ferroptosis inhibitor) and VX-765 (40 μM, a pyroptosis inhibitor by inhibiting caspase 1) for 0.5 h. Then the medium was replaced with fresh medium containing a series of $OLPP_{10}$ (10, 20, 40, 80, 160 μg/mL) and inhibitors. The cells were incubated for another 2 h before MTT assay.

Silver staining. Panc02 cancer cells were seeded at the density of 1.5×10^5 per well in 24-well plates. After 12 h, cells were treated with FBS-free medium containing different concentrations of $OLPP_{10}$ (10, 20, 40, 80, 160 μg/mL). 2 h later, the supernatant was collected through centrifugation at 1000 rpm for 5 min and 12000 rpm for another 5 min. The supernatant was collected for SDS-PAGE and the gel was stained with a Fast Silver Stain Kit.

The biodistribution of $OLPP_{10}$. Panc02 tumor-bearing mice were used to evaluate the biodistribution of $OLPP_{10}$. 8 and 24 h post intra-tumoral injection of Cy5 labeled $OLPP_{10}$ (5mg/kg), fluorescence images were acquired by PerkinElmer IVIS Lumina III. Then the mice were sacrificed, and main

organs (heart, liver, spleen, lung and kidney) and tumors were collected for ex vivo fluorescence imaging. Living Image (Version 4.4) was used for analysis.

Results

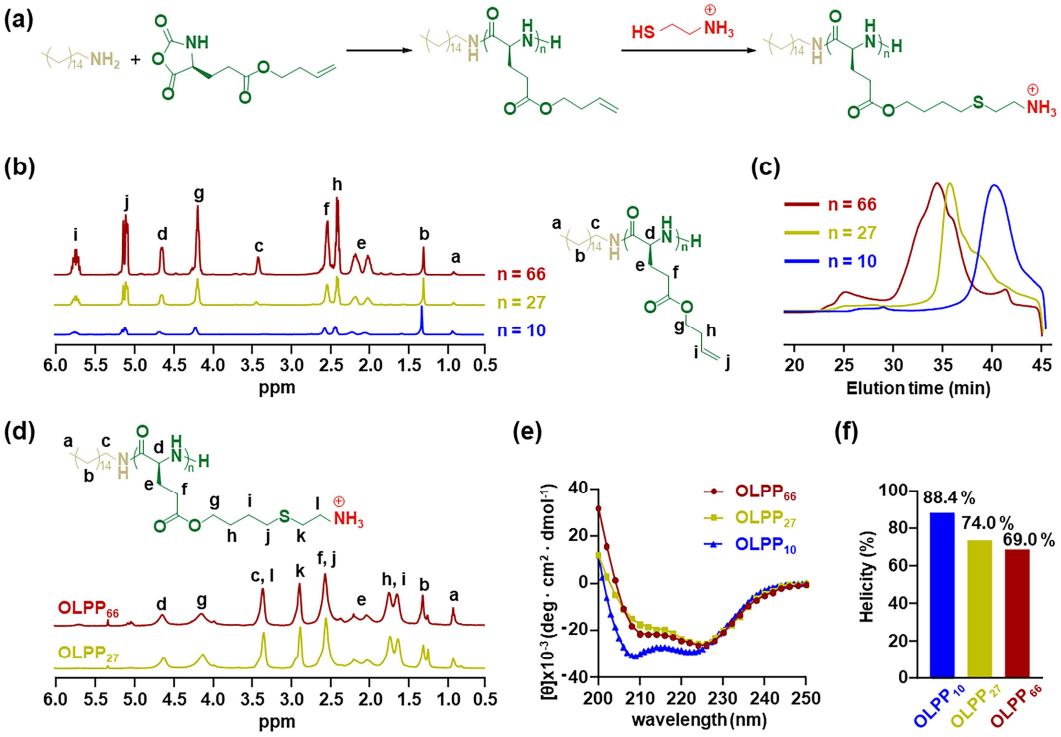

Fig. S1. (a) The synthesis route of $OLPP_n$ ($C16\text{-}PButLG_n\text{-}CA$). (b) The ^1H NMR spectra of $C16\text{-}PButLG_n$. (c) The GPC spectra of $C16\text{-}PButLG_n$. (d) The ^1H NMR spectra of $OLPP_{27}$ and $OLPP_{66}$. (e) Circular Dichroism spectra of $OLPP_{10}$, $OLPP_{27}$ and $OLPP_{66}$ at pH 7.4. (f) Helicity of $OLPP_{10}$, $OLPP_{27}$ and $OLPP_{66}$ at pH 7.4.

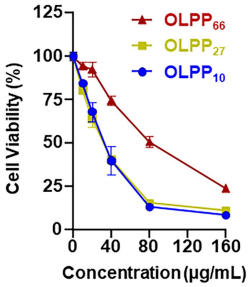

Fig. S2. The cytotoxicity of $OLPP_{10}$, $OLPP_{27}$ and $OLPP_{66}$ against Panc02 cancer cells.

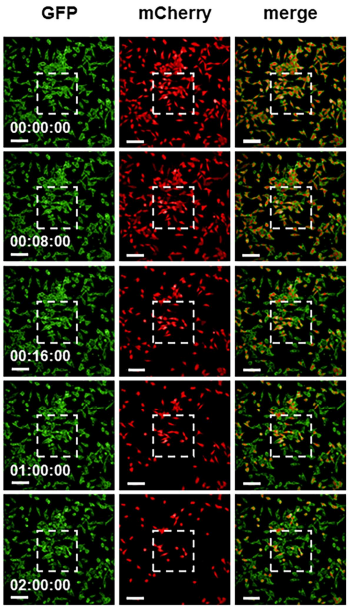

Fig. S3. Images of Panc02$_{mCherry/GFP}$ cancer cells treated with OLPP$_{10}$ (80 μg/ mL). Scale bar, 100 μm. The parts circled in white boxes were shown in **Fig. 3d.**

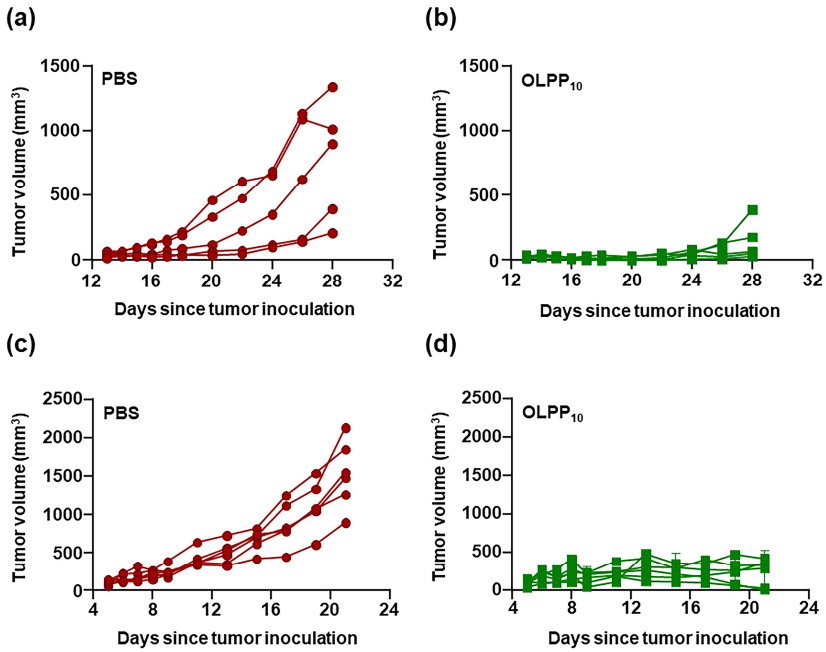

Fig. S4. Tumor volume of each Panc02 tumor-bearing C57BL/6 mouse received (a) PBS treatment or (b) OLPP$_{10}$ treatment (n = 5). Tumor volume of each MC38 tumor-bearing C57BL/6 mouse received (c) PBS treatment or (d) OLPP$_{10}$ treatment (n = 6). The dose of OLPP$_{10}$ was 5 mg/kg.

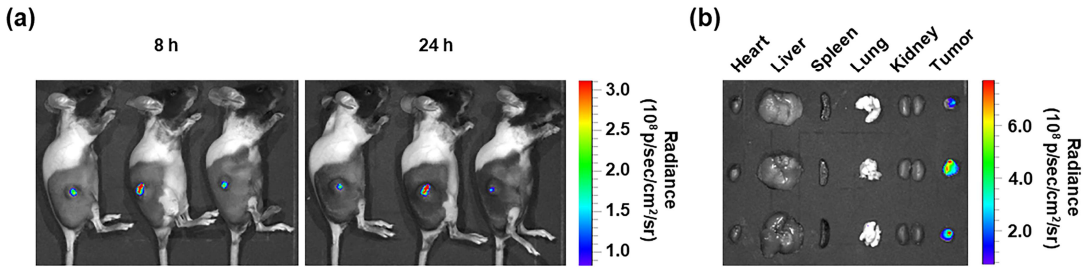

Fig. S5. (a) Fluorescence images of Panc02 tumor-bearing C57BL/6 mice after intra-tumoral injection of Cy5 labeled $OLPP_{10}$ (5 mg/kg). Images were acquired at 8 and 24 h post injection (n = 3). (b) Ex vivo fluorescence images of major organs (heart, liver, spleen, lung, kidney) and tumors at 24 h post intra-tumoral injection of Cy5 labeled $OLPP_{10}$ (n = 3).

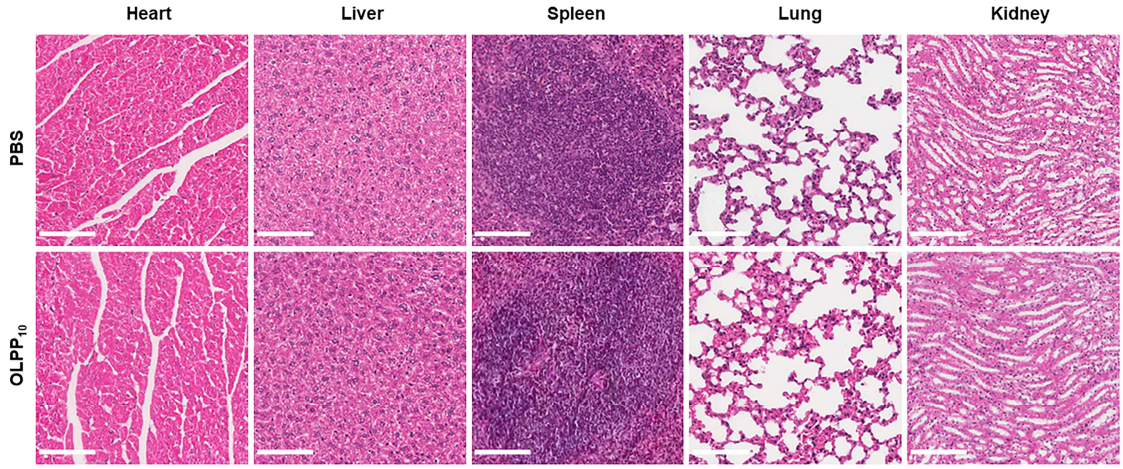

Fig. S6. The H&E staining of heart, liver, spleen, lung and kidney of Panc02 tumor-bearing C57BL/6 mice after intra-tumoral injection of $OLPP_{10}$. Scale bar, 100 μm.

References

1. L. Chen, Q. Zhu, L. Lu and Y. Liu, *Bioengineered*, 2020, **11**, 91-102.

2. M. Liu, L. Huang, W. Zhang, X. Wang, Y. Geng, Y. Zhang, L. Wang, W. Zhang, Y. J. Zhang, S. Xiao, Y. Bao, M. Xiong and J. Wang, *Nat. Nanotechnol.*, 2022, **17**, 541-551.