Electronic Supplementary Material (ESI) for Molecular BioSystems.

Supporting Information for

Membrane Activity of a Supramolecular Peptide-based Chemotherapeutic Enhancer

Dawei Xu,[a] Linhai Jiang,[a] Louis DeRidder,[a] Brendan Elmore,[a] Maurish Bukhari,[b] Qiang Wei,[c] Damien S. K. Samways,*[b] He Dong*[a]

[a] Department of Chemistry and Biomolecular Science, Clarkson University, Potsdam, NY
[b] Department of Biology, Clarkson University, Potsdam, NY
[c] Department of Chemistry, State University of New York at Binghamton, Binghamton, NY

*Corresponding author: hdong@clarkson.edu, dsamways@clarkson.edu

General method

MBHA rink amide resin, Fmoc-protected amino acids, 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) were purchased from Novabiochem. Piperidine was purchased from Sigma-Aldrich. All other reagents and solvents for peptide synthesis and purification were purchased from Fisher Scientific and used as received. Desalting column VariPure IPE was ordered Agilent Technologies (Apple Valley, MN). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Dulbecco's modified Eagle medium (DMEM) culture medium, hoechst 33342 and LysoTracker Red DND-99 was purchased from Life Technologies. Fetal Bovine Serum (FBS) was ordered from VWR. CCK8 assay kit was obtained from Dojindo Molecular Technologies (Rockville, MD). Large unilamellar vesicles was prepared using Avanti Polar Lipids Mini-Extruder with Heating Block through polycarbonate filter with 0.1μm pores. Fluorescence measurements were performed on Varian Cary Eclipse fluorescence spectrophotometer. Reversed-phase HPLC was carried out using HITACHI L-7100 pump, Waters 717 plus auto-sampler and Higgins analytical column (proto 300 C4 10 μm, 250*10 mm). UV absorbance were measured on a micro-plate reader (Vitor^2 1420 Multilabel Counter, PerkinElmer) for cell toxicity quantification.

Synthesis and purification of peptides

The syntheses of MDPs followed standard Fmoc-solid phase peptide synthesis method. Briefly, Fmoc group was deprotected in 20 % (V/V) piperidine/DMF for 5 min and repeated once. Fmoc-protected amino acids were added in five equivalents of the resin for the coupling reaction. Five molar equivalents of HBTU were used in the coupling reaction with ten molar equivalents of diisopropylethylamine (DIPEA) with respect to the scale of the synthesis. The coupling reaction was carried out for 45 mins. Upon completion of the synthesis, the N-terminus of the peptide was acetylated in the presence of acetic anhydride and DIPEA at a molar ratio of 1:50:10 (peptide/acetic anhydride/DIPEA) in

DMF for 1 hr. Completion of the reaction was confirmed by the Kaiser test. Peptide was cleaved from the resin with a mixture of TFA / triisopropylsilane (TIS) / H_2O (95/2.5/2.5 by volume). After 3 hrs, cleavage solution was collected and the resin was rinsed twice with neat TFA. TFA was evaporated by using air blow and the residual peptide solution was triturated with cold diethyl ether. Peptide precipitate was centrifuged at 6500 rpm for 5 min, washed with cold diethyl ether for three times and dried under vacuum overnight before HPLC purification. Preparative reverse phase C18 column was used in peptide purification with a linear gradient of binary water/acetonitrile mixed solvent containing 0.05% TFA at a flow rate of 6 ml/min. The elution HPLC program starts with an initial 5-minute equilibration using 95% water/5% acetonitrile followed by a 3% linear gradient increase to reach 95% acetonitrile/5% water. HPLC elution was monitored at 280 nm. The HPLC fraction was collected, combined and using desalting column to remove the TFA residue. The desalted HPLC fraction was frozen in liquid nitrogen and placed in the lyophilizer for 3 days. The molecular weights of the peptides were confirmed by ESI-MS. For $K_{10}(QW)_6$, expected $[M+H]^+$: 3225.80, observed MW:3226.10; For $K_5(QW)_6K_5$, expected $[M+H]^+$: 3225.80, observed MW: 3226.87.

Preparation of lipid vesicles

Large unilamellar vesicles (LUV) were prepared by following the conventional extrusion method. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was dissolved in a chloroform/ methanol (10/1) mixture and placed into a vacuum desiccator overnight to form a lipid film. Calcein was dissolved in 20 mM HEPES buffer at pH 6.8 to achieve a desired concentration at 51 mM and used for lipid rehydration. 5 cycles of freezing (liquid nitrogen)-thawing (40 °C water bath) were performed to ensure maximum calcein encapsulation. The suspension was extruded through a 0.1 μm polycarbonate filter for 10 times. Non-encapsulated calcein was removed through gel filtration using a Sephadex G-50 column equilibrated with 20 mM HEPES buffer at pH 7.4.

Giant unilamellar vesicles (GUV) were prepared by following the fast evaporation

method.[1] POPC was dissolved in 1 ml of chloroform/ methanol (10/1) mixture and added to a 50 ml round-bottom flask. 7 ml of HEPES buffer (20 mM, pH 7.4) was added slowly along the flask wall. Organic solvent was removed in a rotary evaporator under reduced pressure of 10 mmHg and 40 rpm rotation speed at 40 °C. GUVs suspension was collected and stored at 4 ^{o}C for further use. Rhodamine labeled GUV was prepared by following the same method of fast evaporation with the addition of 0.5 mol% rhodamine-labeled phosphatidylethanolamine in POPC chloroform/ methanol solution.

Circular Dichroism (CD) Spectroscopy

MDPs were diluted in HEPES buffer (20 mM, pH 7.4) to reach a final concentration of 200 μM. Prior to the measurement, 100 μL of 200 μM of MDPs was mixed with equal volume of HEPES buffer or 20 mM GUV. The spectrum was acquired at 10 sec and 2 hr time point. The data were collected from 240 nm to 200 nm at room temperature with a scan rate of 100 nm/min, a response time of 2 secs and a bandwidth of 1 nm. The final spectra were an average of 10 scans. The mDeg of rotation was converted to molar residual ellipticity via the formula $\theta=(mDeg*1000)/(c*n*l)$, where c is the concentration of the peptide solution expressed in mM, n is the number of amino acids in the peptide sequence and l is the cell path length in mm.

Calcein release assay

Concentration of calcein loaded LUVs was adjusted to 1.6 mM in HEPES buffer (20 mM, pH 7.4). Prior to the measurement, 180 μl of calcein loaded LUV was mixed with 20 μl of 160 μM MDP solution and the mixture was transferred into a cuvette. The sample was exited at 495 nm and the emission was monitored at 515 nm. The change of emission intensity was measured over 2 hrs.

Fluorescence spectroscopic assay of membrane activity

Fluorescence measurements were performed on a Varian Cary Eclipse fluorimeter. 1.6 mM GUV was mixed with 20 µl of 160 µM MDP solution and the mixture was transferred into a cuvette. Fluorescence of tryptophan was monitored with an excitation wavelength at 280 nm and emission wavelengths between 295 nm and 495 nm. The emission spectrum was acquired at 10 sec and 2 hr time point. The kinetics of fluorescence intensity change was measured for 2 hrs with an excitation wavelength at 280 nm and emission wavelengths 350 nm.

Membrane localization

2 ul of 160 µM FAM-labeled MDP was mixed with 40 µl of 160 µM non-labeled MDP and incubated at room temperature for overnight. 40 µl of the above mixture was added into 360 µl of 1.6 mM rhodamine-labeled GUV solution, incubated for 1 hr and transferred into a confocal dish (MatTek Corporation). Images were captured using a laser scanning confocal microscope (Leica TCS SP2, Germany) and processed with ImageJ software.

Patch clamp electrophysiology

For patch clamp electrophysiology experiments, HEK293 cells were seeded onto glass coverslips, and then transferred to a bath positioned on the stage of an inverted Olympus IX51 microscope. Cells were continuously perfused with a divalent-free extracellular solution containing: 140 mM NaCl, 10 mM glucose, 10 mM HEPES, 4 mM NaOH (pH 7.4). Cells were patched the broken patch whole cell configuration according to conventional methods[2] using low resistance (0.5 3 MΩ), lightly fire polished, borosilicate glass electrodes. Membrane potential was held at -40 mV for the duration of the experiment. Current recordings were sampled at 20 KHz and filtered at 5 KHz using an AxoPatch 200B amplifier (Molecular Devices) and digitized via a 1440 Digidata (Molecular Devices). The MDPs were applied using a Perfusion Fast-Step System SF-77 (Warner Instruments). Data was analyzed offline using IGOR Pro (Wavemetrics, Inc) software.

Cytotoxicity test

HeLa cells were seeded into a 96-well plate with a density of 10000 cells/well. 10 μl of peptide solution was added into cell culture to reach a final concentration at 16 μM for MDPs and 20 μM for TAT. After 24 hrs of incubation, CCK-8 assay was performed to quantify the cell viability by monitorning the UV absorbance at 450 nm. All the experiments were performed in six replicates.

To evaluate the effect of peptide on cytotoxicity of doxorubicin toward HeLa cells, doxorubicin was first added into each well plate to reach a final concentration at 4 μM. Peptides were added to the cell culture to reach a final concentration of 16 μM for MDPs and 20 μM for TAT. After 24 hrs incubation, cell viability cytotoxicity was determined through CCK-8 assay according to the manufacturer's protocol. The optical absorbance of each well plate was determined on a microplate reader (Vitor^2 1420 Multilabel Counter, PerkinElmer, USA) at the wavelength of 450 nm. All the experiments were performed in six replicates.

Cell uptake of doxorubicin

Doxorubicin was dissolved in DMSO and diluted in HEPES buffer (20 mM, pH 7.4) as the stock solution. HeLa cells were seeded in a confocal dish at a density 5×10^4 cells/well and incubated for 24 hrs. The medium was replaced with 90 μl of fresh DMEM containing 10% FBS. Doxorubicin was added in the cell culture media to reach a final concentration of 4 μM. Then, 10 μl of 160 μM MDP was added into the cell culture to reach a final concentration of 16 μM and incubated for 1, 4 and 8 hrs. Cells were washed with PBS buffer for three times. Images were captured using a laser scanning confocal microscope (Leica DMi8, Germany) and processed with ImageJ software.

Cell uptake of MDPs

HeLa cells were used to investigate the cell uptake of MDPs. Cells were seeded onto a confocal dish at density of 1 x 10^5 cells/well. 2 μl of 160 μM FAM-labeled MDP was mixed with 40 μl of 160 μM non-labeled MDP and incubated at room temperature for overnight. 20 μL of the above solution was added to the cell culture to reach a final peptide concentration at 16 μM. After 1 hr and 24 hrs of incubation with MDP-1, cells were washed with PBS buffer for 3 times. After 1 hr of incubation with 50 nM LysoTracker Red DND-99, cells were incubated with Hoechst 33342 (1μg/ml) for 5 mins. Images were captured using a laser scanning confocal microscope (Leica DMi8, Germany) and processed with ImageJ software.

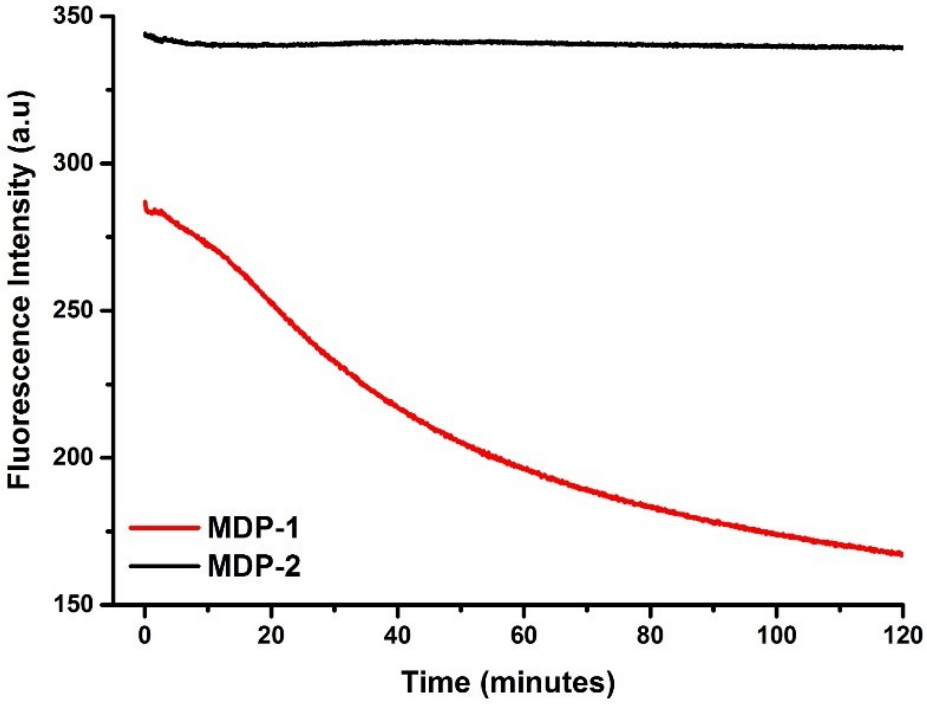

Figure S1. The kinetics plot of fluorescence intensity changes of MDPs upon incubation with GUVs.

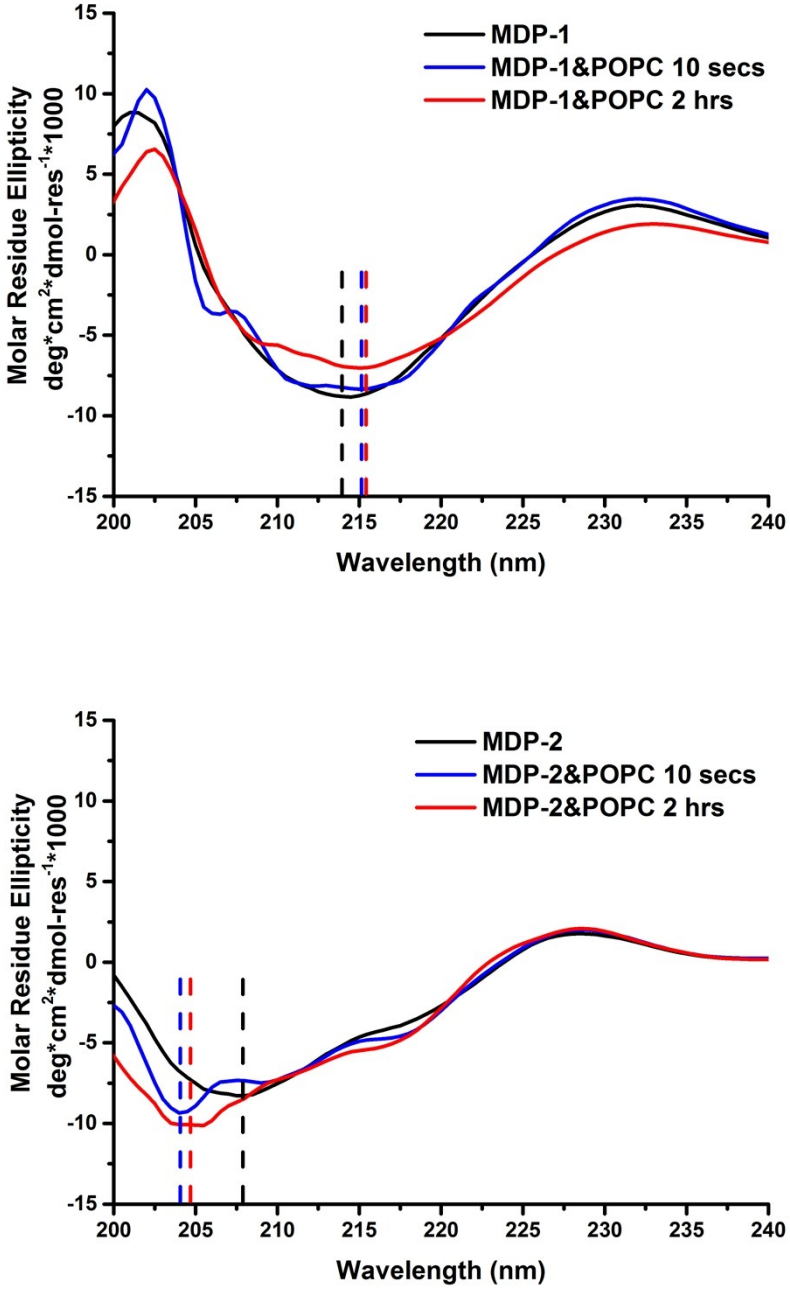

Figure S2. CD spectra of MDP-1 and MDP-2 in the presence of lipid membranes. The CD intensity was normalized to the mean residual ellipticity. Peptide concentration: 0.1 mM, lipid concentration: 10 mM.

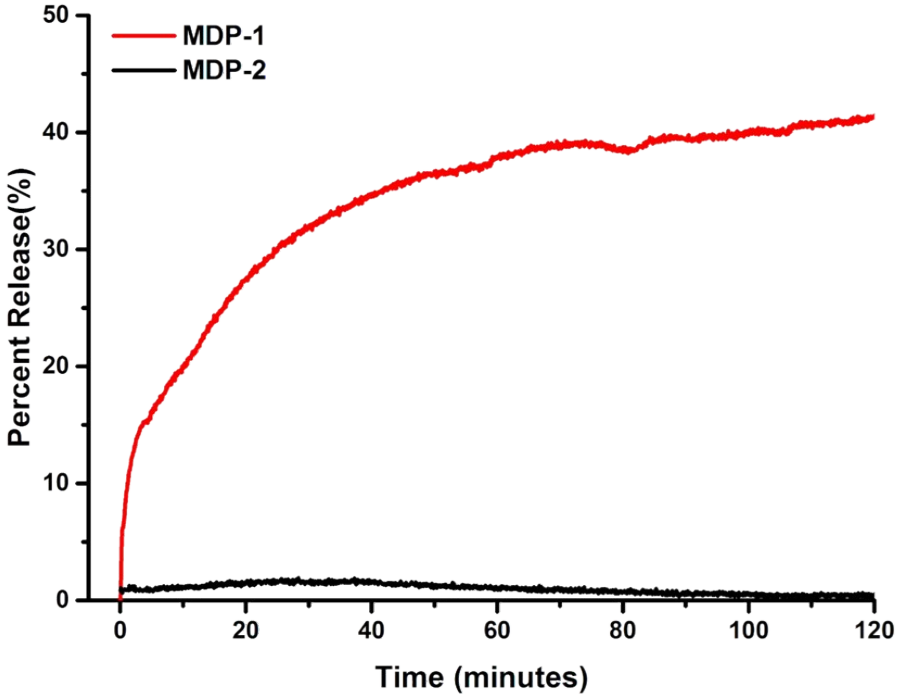

Figure S3. Percentage of calcein release upon treatment with MDPs. MDPs were mixed with LUVs at a lipid/peptide ratio of 100:1 in HEPES buffer (20 mM, pH 7.4).

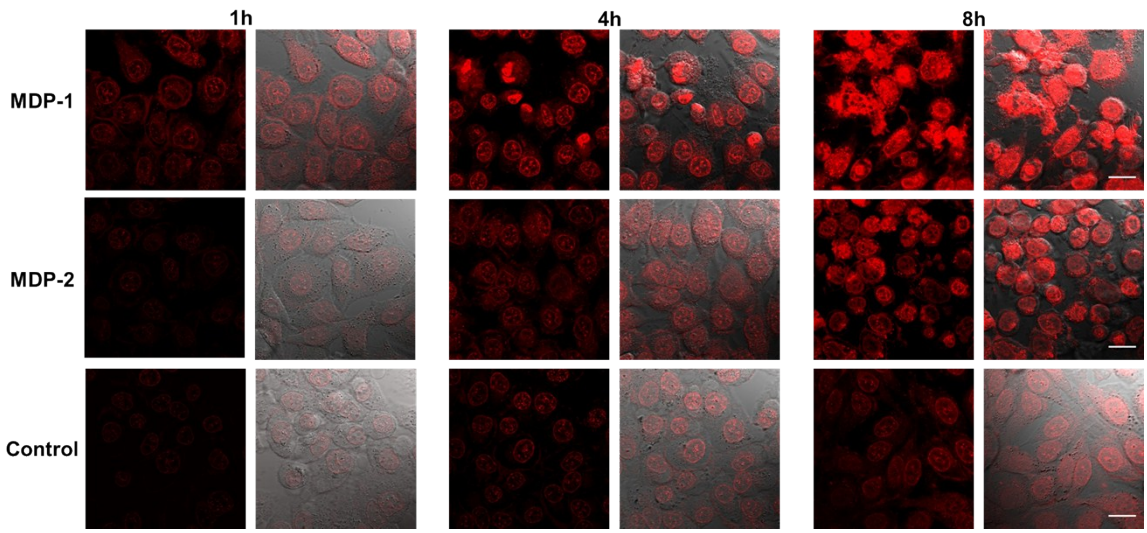

Figure S4. Time-dependent DOX uptake at 1 hr, 4 hrs and 8 hrs. For each image group taken at different time point, the left panel is comprised of fluorescence images and the right panel are the overlay of fluorescence and bright field images. Scale bar: 20 μm

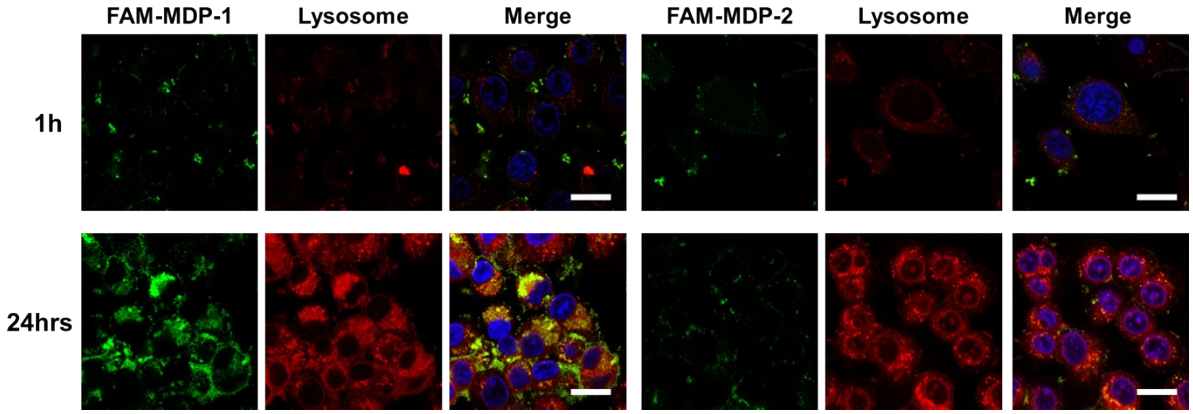

Figure S5. HeLa cell membrane location of MDP-1 and MDP-2 at different time points. Top panel: MDP-1 and MDP-2 localization after 1 hr of incubation with HeLa cells, Bottom panel: MDP-1 and MDP-2 localization after 24 hrs of incubation with HeLa cells. Scale bar: 20 μm.

1 A. Moscho, O. Orwar, D. T. Chiu, B. P. Modi and R. N. Zare, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 11443-11447.

2 M. Cahalan and E. Neher, *Methods Enzymol.*, 1992, **207**, 3-14.