Supplementary Information for

Inhibition of K-Ras4B-Plasma Membrane Association with a Membrane Microdomain-Targeting Peptide†

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Materials and Methods

Reagents

Reagents for solid phase peptide synthesis and HPLC purification were purchased from GL Biochem (Shanghai) Ltd, Bo Mai Jie Technology Co., Ltd and J&K Scientific. 1-Butanol was purchased from Alfa Aesar. 5(6)-Carboxyfluorescein was purchased from Acros Organics. Trans, trans-Farnesyl bromide, zinc acetate dihydrate, anti-c-K-Ras antibody (R3400), anti-β-actin antibody (A1978), anti-mouse IgG-peroxidase antibody (A9044), anti-rabbit IgG-peroxidase antibody (A6154) and X-tremeGENE HP DNA transfection reagent (06366244001) were purchased from Sigma-Aldrich. Lipofectamine 2000 transfection reagent (11668-027), LysoTracker Red DND-99 (L7528), halt protease and phosphatase inhibitor cocktail (78441), pierce BCA protein assay kit (23225) and SuperSignal west pico chemiluminescent substrate (34077) were purchased from ThermoFisher Scientific. Ampicillin, Kanamycin and G418 sulfate were purchased from Amercso LLC. Fetal bovine serum (FBS) was purchased from Biowest. RPMI-1640, DMEM (High glucose) and 0.25% trypsin-EDTA were purchased from Corning Life Sciences. Opti-MEM was purchased from Gibco Life Sciences. Anti-MEK1/2 (9122), anti-p-MEK1/2 (9121), anti-ERK1/2 (4695), anti-Akt (9272), anti-p-Akt (9271) antibodies and cell fractionation kit (9038) were purchased from Cell Signaling Technology. Anti-tubulin (ab6160), anti-Sodium potassium ATPase (ab76020) and anti-transferrin receptor (ab214039) antibody were purchased from Abcam. Anti-Ras antibody (60309-1-Ig) and anti-Rab35 antibody (11329-2-AP) were purchased from Proteintech. Anti-p-ERK antibody (sc-7383) was purchased from Santa Cruz Biotechnology. All lipids and liposome extruder equipment were purchased from Avanti Polar Lipids, Inc. EndoFree maxi plasmid kit was purchased from Tiangen biotech (Beijing) Co., Ltd. CellTiter-Glo luminescent cell viability assay kit was purchased from Promega Corporation. pCMV-myc-ERK2-L4A-MEK1_fusion was a gift from Melanie Cobb (Addgene plasmid # 39197; http://n2t.net/addgene:39197; RRID:Addgene_39197) and myrAkt delta4-129 was a gift from Richard Roth (Addgene plasmid # 10841 ; http://n2t.net/addgene:10841; RRID:Addgene_10841).

NMR, HPLC and MS

Nuclear Magnetic Resonance (NMR) spectra were recorded on a JEOL JNM-ECS400 spectrometer (Japan Electron Optics Laboratory Co., Ltd).

All peptides were purified by Shimadzu semi-preparative HPLC systems with LC-6AD as solvent pumps. YMC-Pack ODS-A column (250 x 20 mm, YMC Co., Ltd, Japan) was used for peptide purification at a flow rate of 10 mL/min. All peptides were analyzed by a Shimadzu LC-2010A HT HPLC system. YMC-Pack ODS-A column (150 x 4.6 mm, YMC Co., Ltd, Japan) was used for peptide analysis at a flow rate of 0.8 mL/min. UV absorption values at 215 nm and 254 nm were monitored and recorded. Double distilled water (with 0.6% TFA, phase A) and 80% acetonitrile (acetonitrile: water = 4:1, v/v, with 0.6% TFA, phase B) were chosen as mobile phase solvents for gradient elution. Analysis gradient was optimized according to each peptide. ESI-MS spectra were recorded on a SYNAPT G2-Si HDMS system (Waters Corporation) or Esquire-LC ion trap mass spectrometer system (Bruker Corporation).
Synthesis of Fmoc-Cys-OMe

To remove the trityl protection group of the thiol group, N-Fmoc-(S)-trityl-L-cysteine (4.8 g, 8.2 mmol) was treated with a mixed solution of TFA/CH$_2$Cl$_2$/H$_2$O (70 mL, 74:25:1, v) in the presence of triisopropylsilane (1.68 mL, 8.2 mmol) for 30 min at room temperature (1). Solvents were evaporated and the residue was suspended in MeOH (60 mL) followed by the addition of HCl (12N, 2 mL) (2). The suspension was stirred overnight at room temperature. After evaporation of MeOH under reduced pressure, the mixture was subsequently purified by column chromatography (cyclohexane: ethyl acetate 4:1) to obtain the desired product (2.52 g, 7.05 mmol, 86%).

R$_f$ = 0.55 (silica gel, CH:EE = 2:1, v/v); ESI-MS: for C$_{19}$H$_{19}$NO$_4$S [M+Na]$^+$: calcd 380.09, found 380.10;

$^1$HNMR (400MHz, CDCl$_3$): δ = 7.78 (d, 2H, 2xC$_{H_{arom Fmoc}}$); 7.61 (d, 2H, 2xC$_{H_{arom Fmoc}}$); 7.41(dd, 2H, 2xC$_{H_{arom Fmoc}}$); 7.33(dd, 2H, 2xC$_{H_{arom Fmoc}}$); 4.68-4.40 (m, 3H, C$_2$H$_2$Fmoc, α-CH); 4.24(dd, 1H, CH$_{Fmoc}$); 3.81(s, 3H, OCH$_3$); 3.01(d, 2H, β-C$_2$H$_2$);

Attachment of Fmoc-Cys-OMe onto 2-chlorotrityl chloride resin

The 2-chlorotrityl chloride resin (1.0 mmol/g, 1.0 g) was placed into a peptide synthesis vessel and swollen with DCM for 30 min. The resin was washed with DMF and DCM. Fmoc-Cys-OMe (125.1 mg, 0.35 mmol) was dissolved in DCM followed by the addition of DIPEA (244 μL, 1.4 mmol). The solution was added to the vessel. The mixture was left agitating on a shaking table for five hours. The solvent was removed by filtration and the resin was washed with DMF and DCM. Then a solution of DCM containing DIPEA (244 μL) and MeOH (400 μL) was added to cap unreacted functional groups on the resin (3). Finally the reagents were drained off the vessel and the resin was thoroughly washed with DMF and DCM. The loading of Fmoc-Cys-OCH$_3$ on the resin (0.2 mmol/g) was measured and calculated by UV-Fmoc assay (4).

Synthesis of Memrasin peptide series

| Name | Peptide Sequence |
|------|-----------------|
|      |                 |


The pre-farnesylated Memrasin precursor (aka C3) peptide was synthesized manually using 2-Cl Trt-Cl resin loaded with Fmoc-Cys-OMe via the standard Fmoc-based SPPS strategy. A solution of 20% piperidine in DMF was used for Fmoc deprotection while HATU (3.6 eq), HOAt (4.0 eq), protected amino acid (4.0 eq) and DIPEA (8.0 eq) in DMF were applied for amino acid coupling for 45 minutes in each cycle. Boc-protected amino acid was applied for the last cycle. After the peptide chain elongation was finished, the resin was washed thoroughly and dried under vacuum. The peptide was released and deprotected by treatment of Reagent K cleavage cocktail (TFA /thioanisole/phenol/water/ethanedithiol = 82.5:5:5:5:2.5) for three hours. The cleavage solution was evaporated and subsequently precipitated with cold diethyl ether. No further purification was needed before farnesylation in this case.

For Memrasin synthesis, the Memrasin precursor crude product (28.1 mg, 10 μmol) was dissolved in DMF/BuOH/H2O (4.0 mL, 2:1:1,v) followed by the addition of farnesyl bromide (13.6 μL, 50 μmol) and a solution of Zn(OAc)2·2H2O (11.0 mg, 50 μmol) in H2O separately. Then the pH of the reaction system was acidified to about 3 and the reaction system was left stirring for two hours (5). The solution was then filtered and purified by HPLC. The lyophilized product was obtained as white solid (7.9 mg, 2.62 μmol, 26.2%). The purity of Memrasin was analyzed at a gradient of 30 to 90% phase B in 30 minutes. \( t_R = 18.8 \) min.

ESI-MS for \( \text{C}_{145}\text{H}_{252}\text{N}_{34}\text{O}_{32}\text{S} \ [\text{M+5H}]^{5+} \); calcd 603.7772, found 603.7705.

C1 was synthesized and purified in a way similar to Memrasin. C1 precursor crude peptide (13.5 mg, 10 μmol) was used for undergoing farnesylation reaction and HPLC purification. The lyophilized product was obtained as white solid (6.3 mg, 4.06 μmol, 40.6%).

The purity of C1 was analyzed at a gradient of 30 to 70% phase B in 30 minutes. \( t_R = 15.4 \) min.
ESI-MS for C_{74}H_{141}N_{19}O_{14}S [M+3H]^3+; calcd 518.3542, found 518.3632.

C2 was synthesized using Rink Amide AM resin. The peptide chain was assembled via the standard Fmoc-based SPPS strategy. Peptide was released and deprotected with Reagent K cocktail and precipitated with cold diethyl ether. The crude product (20.0 mg, 13.5 μmol) was purified by HPLC. The lyophilized product was obtained as white solid (14.4 mg, 9.73 μmol, 72.0%).

The purity of C2 was analyzed at a gradient of 50 to 95% phase B in 30 minutes. t_R = 12.3 min.

ESI-MS for C_{71}H_{114}N_{16}O_{18} [M+2H]^{2+}; calcd 740.4249, found 740.4592.

C3 was purified using crude Memrasin precursor peptide synthesized above by HPLC. The crude product (10.9 mg, 3.88 μmol) was directly purified by HPLC. The lyophilized product was obtained as white solid (5.7 mg, 2.03 μmol, 52.3%).

The purity of C3 was analyzed at a gradient of 30 to 80% phase B in 30 minutes. t_R = 14.6 min.

ESI-MS for C_{130}H_{228}N_{34}O_{32}S [M+5H]^{5+}; calcd 562.9396, found 562.9527.

**Synthesis of fluorescent peptides**

| Name              | Peptide Sequence                                      |
|-------------------|-------------------------------------------------------|
| FAM-R7            | H-K(FAM)R7-NH2                                        |
| FAM-C1            | H-K(FAM)K5SKTKC(Far)-OMe                              |
| FAM-C3            | H-GLFDIIKKIAESF-K(FAM)K5SKTKC-OMe                     |
| FAM-Memrasin      | H-GLFDIIKKIAESF-K(FAM)K5SKTKC(Far)-OMe                |
FAM-R7 was synthesized using Rink Amide AM resin by standard Fmoc-based SPPS strategy. An additional Boc-Lys(Fmoc)-OH was coupled with the N-terminal Arginine. The Fmoc group was deprotected and 5(6)-carboxyfluorescein was coupled to the side chain of Lysine. The resin was dried in vacuo and the peptide was then cleaved and purified. The crude product (5.0 mg, 3.13 μmol) was directly purified by HPLC. The lyophilized product was obtained as yellow solid (2.3 mg, 1.44 μmol, 46.0%).

The purity of FAM-R7 was analyzed at a gradient of 10 to 50% phase B in 30 minutes. $t_R = 14.8 \text{ min.}$

ESI-MS for $C_{69}H_{109}N_{31}O_{14} [M+4H]^{4+} \text{;}$ calcd 399.9693, found 399.9824.

FAM-C1 was synthesized similarly to the synthetic strategy for C1. During the synthesis process of FAM-C1 precursor, the protected N-terminal lysine was chosen as Boc-Lys(Fmoc)-OH. 5(6)-carboxyfluorescein was introduced to the side chain of the N-terminal lysine. Precursor peptide was cleaved and precipitated as described above. FAM-C1 precursor crude peptide (8.5 mg, 5.0 μmol) was used for undergoing farnesylation and HPLC purification. The lyophilized product was obtained as yellow solid (4.6 mg, 2.41 μmol, 48.3%).

The purity of FAM-C1 was analyzed at a gradient of 35 to 80% phase B in 30 minutes. $t_R = 14.3 \text{ min.}$

ESI-MS for $C_{95}H_{151}N_{19}O_{20}S [M+3H]^{3+} \text{;}$ calcd 637.7035, found 637.7211.

FAM-C3 was synthesized similarly to C3. The protected lysine at the 14th position was chosen as Fmoc-Lys(ivDde)-OH and the N-terminal glycine was chosen as Boc-Gly-OH. After the peptide chain was assembled, 2 % $N_2H_4·H_2O$ in DMF was used to remove the ivDde group and 5(6)-carboxyfluorescein was coupled with the corresponding amino group. The resin was dried in vacuo and the peptide was then cleaved and purified. The crude product (129.5 mg, 40.85 μmol) was directly purified by HPLC. The purified and lyophilized product was obtained as yellow solid (10.1 mg, 3.19 μmol, 7.8%).

The purity of FAM-C3 was analyzed at a gradient of 30 to 90% phase B in 30 minutes. $t_R = 18.2 \text{ min.}$

ESI-MS for $C_{151}H_{238}N_{34}O_{38}S [M+5H]^{5+} \text{;}$ calcd 634.5491, found 634.5659.
FAM-Memrasin was achieved as the farnesylated product of purified FAM-C3. Purified FAM-Memrasin precursor peptide (6.0 mg, 1.89 μmol) was used for undergoing farnesylation and HPLC purification. The purified and lyophilized product was obtained as yellow solid (2.1 mg, 0.62 μmol, 32.9%).

The purity of FAM-Memrasin was analyzed at a gradient of 40 to 100% phase B in 30 minutes. \( t_R = 16.0 \text{ min} \).

ESI-MS for \( \text{C}_{166}\text{H}_{262}\text{N}_{34}\text{O}_{38}\text{S} \ [\text{M+5H}^+]^5; \) calcd 675.3867, found 675.4008.

**CD Spectra Analysis**

All L-Memrasin series peptide were synthesized and purified in small amounts similar to the synthesis of Memrasin as mentioned above.

All peptides were dissolved in 200 μL H2O to get a final concentration of 0.2 mg/mL. In addition 40 equivalents of SDS were dissolved together with the related peptide in amphipathic environment. The CD spectra were measured by a Chirascan-plus CD Spectrometer (Applied Photophysics Limited). The spectra were drawn by Graphpad software and the structural information were analyzed by Chirascan and CDNN.

**Serum Stability Assay**

L-C3 and C3 (30 μg) were diluted individually with 20% fetal bovine serum in a total volume of 100 μL and incubated at 37°C. The corresponding samples were treated with 200 μL 9% TCA on ice to precipitate serum proteins at indicated time points. After centrifugation the supernatants were analyzed with a C18 analytical column by RP-HPLC. The stabilities and peak areas were analyzed with Shimadzu analysis software.

**Cell Culture**

MDCK wt and Ras-overexpressed, A549, NCI-H1299, NCI-H358, NCI-H460, NCI-H441 cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂. Beas-2b, COS-7 cell lines were cultured in DMEM (high glucose) supplemented with 10% FBS at 37 °C with 5% CO₂.

**Flow Cytometry**

A549 cells were plated in 24-well plates and cultured overnight for adhesion and growth. The cells were treated individually with 2 μM DMSO, FAM-R7, FAM-C1, FAM-C3 and FAM-Memrasin in triplets at 37 °C for 10 minutes. Each sample was performed in triplicate. The concentrations of fluorescein-labeled peptides were
determined by Implen NanoPhotometer in protein-dye mode at pH 9.0 (50 mM Tris-HCl). Cells were gently washed with PBS twice and trypsinized. Cells were washed with PBS for another two times and resuspended in 200 μL PBS and then analyzed by Fluorescence Activating Cell Sorter (FACS, BD Caliber). 1x10^4 cells were collected and mean fluorescence intensity was analyzed in the form of histogram.

Construction of Ras-overexpressed MDCK Cell Lines

MDCK cells were plated in a 6-well plate and allowed to grow to 80-90% confluency. Cells were washed with Opti-MEM and serum starved for two hours. pmCitrine-C2-K-Ras4B and pmCitrine-C1-H-Ras plasmids were individually transfected with Lipofetamine 2000 in Opti-MEM for five hours. Opti-MEM was substituted with standard growth medium consisting of RPMI 1640 and 10% FBS for about 24 hours. The transfected cells were screened using growth medium containing G418 sulfate (800 μg/mL) for one week. The cells were then sorted into 96-well plates using BD FACS Aria III cell sorting system to generate monoclonal cells. After cultured for two weeks, monoclonal cells in good condition were picked manually via a fluorescence microscopy for amplification and further experiments.

Confocal Microscopy

A549 cells were plated in a 6-well plate with a sterile cover slip in each well and cultured overnight for cell adhesion. Cells were treated with FAM-C1 (5 μM), FAM-C3 (1 μM) and FAM-Memrasin (1 μM) individually for 10 minutes at 37°C. Cells were washed with PBS and stained with Lysotracker Red (2 μM) for 40 minutes. After lysosome staining cells were fixed and the cover slips were sealed with microscope slides prior to being observed. In further membrane labeling the cell membrane was stained with Wheat Germ Agglutinin (CF 405S conjugated, 5 μg/mL, Biotium,Inc.) for 10 minutes before fixation. Cells were imaged on Zeiss LSM 710 meta Confocal Microscope with 60X oil objective.

For Ras redistribution assay, mCitrine-K-Ras4B (or H-Ras)-overexpressed MDCK cells were plated in 6-well plates with sterile cover slips and cultured to over 90% confluency. Cells were treated with Memrasin or control peptides. After being washed the cells were fixed and the cover slips were sealed. The intracellular localization of fluorescent protein-tagged Ras protein was observed on Zeiss LSM 780 Confocal Microscope with 60X oil objective (6). The intensity-distance analysis was performed using Zeiss Zen2-3 software.

Competitive Fluorescence Polarization Assay

Artificial liposome composed of 1.94 mg DOPC/DOPG/DPPC/DPPG/Cholesterol (15:10:40:10:25, molar ratio) was prepared as model membrane via the extrusion method. Lipids (DOPC, DOPG, DPPC, DPPG, cholesterol) were dissolved in chloroform to generate stock solutions of 10 mg/mL. The organic stock solutions were mixed in accordance with the composition needed. The solvent was removed by argon stream followed by drying under vacuum. The lipid film was hydrated with 1 mL aqueous buffer (25 mM Heps, 100 mM NaCl, 3 mM MgCl₂, pH 7.4). After undergoing a freeze-thaw process for five times, lipid extrusion was carried out
by Avanti Mini Extruder through polycarbonate membrane with 0.1 μm pores to generate unilamellar vesicles of 100 nm (7).

The fluorescence polarization was performed with 96-well black plates. Pre-incubated liposome (60 μM) and FAM-C1 peptide (0.1 μM) was treated with Memrasin and control peptides at various concentration (0-8 μM) in polarization buffer (25 mM Hepes, 100 mM NaCl, 3mM MgCl2, pH 7.4). After a five-minute equilibrium, the fluorescence polarization values were measured using a Synergy 4 Multi Detection Microplate Reader (BioTek Instruments, Inc.). For the competitive fluorescence polarization experiments at higher concentrations, 0.6 mM liposome, 1 μM FAM-C1 and 0-60 μM Memrasin (or C1) were applied and tested.

**Atomic Force Microscopy (AFM)**

Liposome composed of 1.94 mg DOPC/DOPG/DPPC/DPPG/Cholesterol (20:5:40:5:25, molar ratio) was prepared by the extrusion approach as described above in 1 mL AFM analysis buffer (20 mM Tris, 5 mM MgCl2, pH 7.4) (8). Supported lipid bilayers (SLBs) were prepared by fusion of lipid vesicles. Lipid vesicle solution (30 μL) along with AFM analysis buffer (40 μL) was placed in contact with freshly cleaved mica (Φ=15 mm, Ted Pella, Inc.) and heated in a wet chamber at 70 °C for one hour (9). After cooling to room temperature, the sample was rinsed with buffer to remove unbound vesicles. During AFM analysis the SLBs should always be kept in buffer because they are not stable in air. For peptide-membrane interaction studies, the AFM analysis buffer was replaced by the corresponding peptide solution. Memrasin or C1 peptide solution (80 μL, 2 μM) was added on the SLBs and incubate for five minutes at room temperature. After incubation the peptide solution was removed by precision wipe and the SLBs were rinsed with AFM buffer. Measurements were carried out using MultiMode 8 Atomic Force Microscopy (Bruker Corporation). Images of the SLBs and the peptide-loaded SLBs were scanned in the ScanAsyst-Fluid mode with a fluidic cell. Scan frequencies were between 0.5 and 1.0 Hz. The acquired data were analyzed by Nanoscope Analysis software.

**Phosphosignaling Western Blot Analysis**

Cells (A549, NCI-H358 or NCI-H441) were plated in 6 cm dishes. After the confluency reached about 60-70%, the cells were washed with PBS and treated with peptide at the indicated concentrations (0 μM, 2 μM, 4 μM, 6 μM) in serum free RPMI 1640. After 10 minutes exposure to inhibitor peptide at 37°C, cells were washed with PBS and stimulated with hEGF (100 ng/mL) for 10 minutes. The cells were washed with cold PBS and collected into 1.5 mL pipe. The collected cells were lysed with 100 μL RIPA lysis buffer containing protease and phosphatase inhibitors for 30 minutes on ice. Each sample was centrifuged. The concentration of the supernatants were measured by BCA assay and samples were subjected to SDS-PAGE. Proteins in the gel were transferred to a PVDF membrane and detected with the corresponding primary antibodies. After incubation with second antibodies the signals were visualized by enhanced chemiluminescent kit.

**Cell Fractionation Assay**
mCitrine-K-Ras overexpressed MDCK cells were seeded in 10 cm cultural dishes at about 70% confluency. Cells were treated with 0/5/10 μM Memrasin in RPMI 1640 for 10 minutes at 37°C. Cells were washed, trypsinized, harvested and subsequently fractioned by 200 μL Cytoplasmic Isolation Buffer and Membrane Isolation Buffer containing protease inhibitors (Cell Fractionation Kit, Cell signaling Technology) as the instruction. One fold of the membrane fraction and two folds of the cytosolic fraction were loaded for SDS-PAGE for each concentration. Proteins were transferred to a PVDF membrane and detected with the anti-c-K-Ras antibody.

Cancer cells (A549 or NCI-H358) were plated in 10 cm dishes and allowed to grow to 60-70% confluency. Cells were treated with Memrasin peptide at 0 or 2 or 4 μM in serum-free RPMI 1640 for 10 minutes at 37°C. The cells were washed, collected and fractioned with the cell fractionation kit. The same volume of each fraction for each sample was loaded for SDS-PAGE. Proteins were transferred to a PVDF membrane and K-Ras, Na/K ATPase α1 subunit, transferrin receptor, Rab35 proteins were detected by corresponding antibodies. The C/M ratio was calculated for three samples respectively.

**Sensor Electroporation and Memrasin Treatment**

The K-Ras sensor was introduced into MDCK cells by electroporation using the Neon Transfection system (Invitrogen). For electroporation, a 10 cm cell culture dish was trypsinized and the cells were washed with 50 mL PBS buffer. The cells were counted and 1 x 10^6 cells suspended in 100 μL Buffer R of the Neon Transfection Kit. 20-30 μL of the protein solution (15-20 mg/mL) was premixed with Buffer R in a 1:1 ratio. 20 μL of the protein mixture were added to 100 μL of the cell suspension and electroporated with 3 pulses using the Neon pipette tip. Immediately after electroporation the cells were suspended in 15 ml PBS and excess protein removed by repeated washing and spinning (3 x 15 mL PBS, 4 min, 1500 rpm). Finally, the cells were incubated in imaging medium and transferred into an imaging dish previously coated with polylysine. The cells were ready for live cell imaging after incubation overnight at 37 °C and 5% CO₂.

For treatment with Memrasin, a stock solution (20 mM) of the inhibitor in DMSO was diluted to 120 μM in imaging medium. 1 mL of the diluted Memrasin solution was added to the cells for an overall concentration of 60 μM Memrasin in 2 mL cell culture medium. Uniform distribution was ensured by pipetting the imaging medium up and down using a 1 mL pipette. The effect of treatment was followed directly after addition of the inhibitor through confocal and fluorescence lifetime microscopy.

**Fluorescence Lifetime and Microscopy Confocal Laser Scanning**

Fluorescence lifetime imaging microscopy (FLIM) and confocal laser scanning microscopy (CLSM) were carried out using a FlouView FV1000 (Olympus) equipped with a time-correlated single-photon counting (TCSPC) LSM Upgrade Kit (PicoQuant). Cell images were collected through a 60x/1.35 UPlanSApo oil immersion objective (Olympus). All measurements were carried out in an incubation chamber at 37 °C and 5% CO₂. For FLIM measurements the samples were exited with a 470 nm pulsed diode laser (PicoQuant) at 36% and a repetation rate of 40 MHz. Photons were collected by a single-photon counting avalanche photodiode (PDM Series, MPD, PicoQuant) and timed using a time-correlated single-photon counting module.
(PicoHarp 300, PicoQuant) after being spectrally filtered using a narrow-band emission filter (HQ 525/15, Chroma) over a course of approximately 2 min with 1-2 x 10^5 photons.

**Cell Viability Assay**

The indicated cell lines were plated in 96 well plates at 5000 cells per well. After 12 hours the media was replaced by a solution of Memrasin, C1 or C2 respectively at indicated concentrations in RPMI-1640 containing 10% FBS. For Beas-2b and COS-7 cells, DMEM (high glucose) was used to take the place of RPMI-1640 for cell culture and Memrasin incubation. The cell viability was measured and calculated based on the ATP level at 24 hours using the CellTiter-Glo Luminescent Cell Viability Assay.

**Hemolysis Assay**

Memrasin solution was prepared using PBS (Hyclone) to avoid the possible hemolysis caused by osmotic pressure. Rabbit red blood cells (5% rRBCs, Guangzhou Ruite Biotechnology Co., Ltd) were mixed with Memrasin solutions of different concentrations and PBS (with or without BSA) in eppendorf pipes to reach a final concentration of 2% (v/v). For the test group containing 10 μM BSA, BSA was introduced to this system at a final concentration of 10 μM. For the positive control, triton X-100 was added to reach a final concentration of 1% (w/v) to represent 100% hemolysis. The setup of BSA-containing assay group was to validate the hemolytic capability of Memrasin in a serum-like condition considering serum albumin binding could change the pharmacological effects of drugs. Samples were incubated at 37 °C on an orbital shaker for 1h and each sample was replicated in triplets. After incubation the tubes were centrifuged at 500 g for 5 min and the supernatants were added to transparent 96-well plates. The values of absorbance at 540 nm were recorded using a microplate reader.

**Viability Recovery Assay**

The control plasmids were constructed from the corresponding plasmids purchased from Addgene. Primers were designed according to the individual plasmid maps for further PCR reactions, respectively. The primers for constructing ERK control plasmid were CMV-F: TAAGTGTGGAGCAACAAAGAGC and CMV-R:GGCAATTCTGACGGTTCACTAAACG and the primers for constructing Akt control plasmid were ECE-F: TGAAACGGTTCTCACCG and ECE-R: GAATTCCCCGGGTACC. Each plasmid (20 ng) with inserted gene was applied as template for each PCR reaction and the reactions were carried out with phusion hot start flex DNA polymerase (New England Biolabs Inc.) at annealing temperatures of 63 °C. After 30 cycles 1 μL of each PCR product was treated with KLD enzyme mix (New England Biolabs Inc.) and transformed into DH5α chemo-competent cells. Colonies were picked and sequenced for each constructed plasmid. The right colony for each control plasmid was cultured and the related plasmids were extracted with EndoFree plasmid kit.

For viability recovery assay, NCI-H441 cells (7.5×10^5/well) were seeded in 6-well plates. Each plasmid (2 μg) was transfected using 6 μL X-tremeGENE HP DNA transfection reagent in 200 μL Opti-MEM for 36 h after cell attachment. Then cells of each group were counted and seeded to 96-well plates at 5000 cells per well.
The remaining cells were collected and lysed in RIPA buffer with protease inhibitors. Memrasin was diluted in RPMI-1640 containing 10% FBS and added to the tested cells and the viabilities were measured with CellTiter-Glo assay. The expression of the transfected genes were identified by western blot.
Fig. S1. The MS (a) and $^1$H NMR spectra (b) of Fmoc-Cys-OMe.
Fig. S2. LC-MS analysis of Memrasin and the control peptides. (a) – (d) LC (left panel) and ESI-MS (right panel) analysis data of Memrasin, C1, C2 and C3. All peptides were analyzed by C18 column using the condition mentioned in the experimental procedures.
Fig. S3. LC-MS analysis of fluorescent peptides. (a) – (d) LC (left panel) and ESI-MS (right panel) analysis data of FAM-R7, FAM-C1, FAM-C3 and FAM-Memrasin. All peptides were analyzed by C18 column using the condition mentioned in the experimental procedures.
Fig. S4. CD spectra of Memrasin & L-Memrasin peptide series. a) Peptide sequence of L-Memrasin and L-Memrasin series peptides. b) CD spectra of L-Memrasin, and Memrasin and C2 peptide series in aqueous environment and amphipathic environment. c) CD spectra of L-C1 and C1. All peptides together with 40 eq. SDS were dissolved in water to reach a peptide concentration of 0.2 mg/mL. The CD spectra were recorded with a Chirascan-plus CD spectrometer. The analysis of the structure was performed with Graphpad software.
Fig. S5. In vitro stability analysis of L-Memrasin and Memrasin peptides. a) – d): HPLC analysis of a) serum protein precipitation, b) L-Memrasin as well as Memrasin, c) stability of L-C3 and d) C3. Peptides (30 μg) were incubated with 20% FBS in 100 μL, followed by 200 μL 9% TCA treatment. Samples were centrifuged and the supernatant were analyzed using RP-HPLC with a C18 analytical column. Treatment of each sample was carried as mentioned above. All sampled were analyzed at a linear gradient from 0 to 100% phase B (80% acetonitrile with 0.6‰ TFA) of 30 minutes with C18 analytical column by RP-HPLC. Related curves and peak areas were analyzed with Shimadzu software. FBS: fetal bovine serum, TCA: trichloroacetic acid, TFA: trifluoroacetic acid.
Fig. S6. Intensity-distance graph of Memrasin redistribution assays. The intensity-distance information was acquired using Zen2-3 software provided by Zeiss. The images were exported and the intensity-distance graphs were drawn with Graphpad software.
Fig. S7. Cell-based mCitrine-K-Ras redistribution experiment with or without Memrasin treatment for different time. mCitrine-K-Ras overexpressed MDCK cells were seeded on coverslips plated in 6-well plates. Memrasin was solved in RPMI-1640 containing 10% FBS. Cells were treated with DMSO or Memrasin (80 μM) for 10 minutes or 12 hours and monitored by confocal microscopy.
Fig. S8. Cellular uptake of fluorescent peptides (2 μM) into A549 cells. 2 μM FAM-R7, FAM-C1, FAM-C3 and FAM-Memrasin were incubated individually with A549 cells in triplets at 37 °C for 10 minutes. Cells were then analyzed by flow cytometry. The cell penetrating abilities for all peptides were evaluated by comparing the mean fluorescence intensity values.
Fig. S9. Cell permeability and intracellular localization of fluorescent Memrasin peptides. (a) Confocal microscopy images of A549 cells treated with 5(6)-carboxyfluorescein labeled C1 (5 μM, 10 minutes) or C3 (1 μM, 10 minutes) or Memrasin (1 μM, 10 minutes) followed by Lysotracker Red staining (2 μM, 40 minutes) at 37°C. 5(6)-FAM, green; Lysotracker Red (lysosome), red. (b) 5(6)-FAM labeled Memrasin peptide displays a plasma membrane and cytosol localization in A549 cells. Additional Wheat Germ Agglutinin (WGA, CF405 conjugated) labeling (5 μg/mL, 10 minutes) was performed after peptide incubation and lysosome staining. 5(6)-FAM, green; Lysotracker Red (lysosome), red; WGA (plasma membrane), blue.
Fig. S10. Interaction analysis between Memrasin as well as C1 and model membrane. (a) Competitive fluorescence polarization assay performed with liposome (DOPC/DOPG/DPPC/DPPG/Cholesterol =15:10:40:10:25, molar ratio, 100 nm, 0.6 mM), FAM-C1 (1 μM) and Memrasin or C1 (1-60 μM, as indicated). The experiment was carried out in black 96-well plates and fluorescence polarization was measured by multiplate reader. A brief pre-incubation of liposome and FAM-C1 at room temperature was applied before the addition of Memrasin or C1 peptide. All groups were performed in triplets. (b) Surface morphology of empty (left panel) and C1 treated (right panel) supported lipid bilayers (SLBs) detected by AFM microscopy. The SLBs were generated by heat fusion of 30 μL liposome (DOPC/DOPG/DPPC/DPPG/Cholesterol =20:5:45:5:25, molar ratio, 100 nm, 0.6 mM) on a freshly cleaved mica at 70 °C for an hour. 2 μM C1 was incubated with the supported lipid bilayers for 5 minutes at room temperature. Dark brown, liquid disordered (l_d) region; light brown, liquid ordered (l_o) region; scale bar, 1 μm.
Fig. S11. Height analysis of the supported lipid bilayers before (a) or after (b) Memrasin treatment. Data acquired was analyzed by Nanoscope Analysis software. A horizontal line was drawn and three individual pairs of differences in vertical height were chosen and measured in the height diagram in the section analysis mode. The vertical height differences between the crosses were listed below in the corresponding color (blue, red and green). The vertical color scale in the AFM image corresponds to the height range in the height diagram, respectively.
Fig. S12. Height analysis of the supported lipid bilayers before (a) or after (b) C1 treatment. Analysis was performed as described in Fig. S11.
Fig. S13. Inhibition of ERK and Akt phosphorylation in NCI-H441 by Memrasin. NCI-H441 cells were treated with the indicated doses of Memrasin at 37 °C for 10 minutes and stimulated with human EGF (100 ng/mL, 10 minutes) prior to cell collection and cell lysis.
Fig. S14. Phospho-signaling activation level of Raf/MEK/ERK and PI3K/PDK1/Akt pathways in A549 cells after C1 or C2 treatment. A549 cells were treated with the indicated doses of C1 (a) or C2 (b) at 37 °C for 10 minutes and stimulated with human EGF (100 ng/mL, 10 minutes) prior to cell collection and cell lysis.
Fig. S15. Fractionation assay of two non-raft marker proteins (Na⁺/K⁺ ATPase α1 subunit and transferrin receptor) and Rab35 GTPase in HCl-H358 and A549 cell lines treated with Memrasin. Cells were seeded in 10 cm plates and incubated with Memrasin at indicated concentrations, after which cells were trypsinized. The cytosolic fraction (C) and the membrane fraction of the cells were extracted with cell fractionation kit as described in the experimental procedures. The localizations of Na⁺/K⁺ ATPase α1 subunit, transferrin receptor and Rab35 were tested by western blot.
Fig. S16. Anti-proliferative activity of Memrasin and related control peptides in K-Ras-expressed lung tumor cells. K-Ras dependent (a), K-Ras independent (b) (10) and other cancer cells (c) were treated with Memrasin, C1 or C2 at the indicated concentrations. Cell viability was measured at 24 hours by CellTiter-Glo cell viability assay.
Fig. S17. Expression level identifications of the transfected genes in H441 cell lines. After transfected with related plasmids for 36 h, a part of cells from each group were seeded into 96-well plates while the remaining cells were lysed with RIPA buffer containing protease inhibitors and the protein concentration of each lysis sample was measured. Then 30μg protein from each sample was loaded and detected by western blot with anti-ERK, Akt and Ras antibodies, respectively. The related exogenous and the endogenous proteins were discriminated according to their different molecular weights.
Fig. S18. Toxicity of Memrasin in normal cell lines. MDCK (Madin-Darby Canine Kidney) cells were epithelial cells from dog’s kidney. Beas-2b cells were epithelial cells from human’s lung. COS-7 cells were fibroblast-like cells from African green monkey’s kidney. 5000 cells/well were seeded into 96-well plates followed by Memrasin working solution incubation for 24 h. The viabilities of the normal cell lines were measured by CellTiter-Glo assay. The dash line represented the viability of K-Ras sensitive NCI-H441 cells against Memrasin treatment.
**Fig. S19.** Hemolytic activities of Memrasin with rabbit red blood cells (rRBCs) without or with bovine serum albumin (10 μM). Peptide was diluted and gently mixed with rRBCs (2%, v/v) and incubated at 37 °C for 1h followed by centrifugation. The supernatants were added into 96-well microplates and read at 540 nm. 1% (final concentration) triton X-100 was applied as positive control. The hemolytic percentage were calculated with the following formula Hemolysis (%) = (A_x-A_0)/(A_{positive}-A_0).
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