Supporting information for

GPCR Activation and Endocytosis Induced by a 2D Material Agonist

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Content list

S1. Additional figures S1-S8

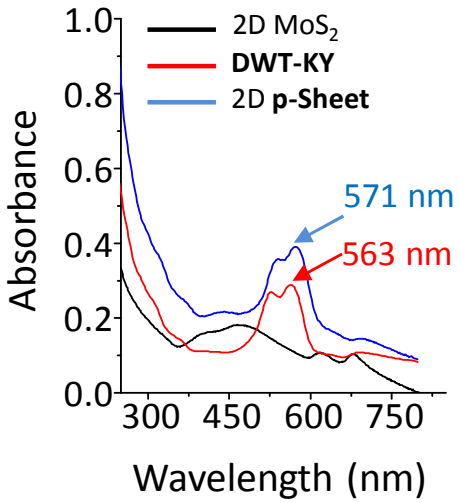

Figure S1. UV-vis absorbance spectra of 2D MoS_2 (35 µg mL^{-1}), **DWT-KY** (1 µM) and 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 1 µM/35 µg mL^{-1}) measured in phosphate buffered saline (0.01 M, pH 7.4).

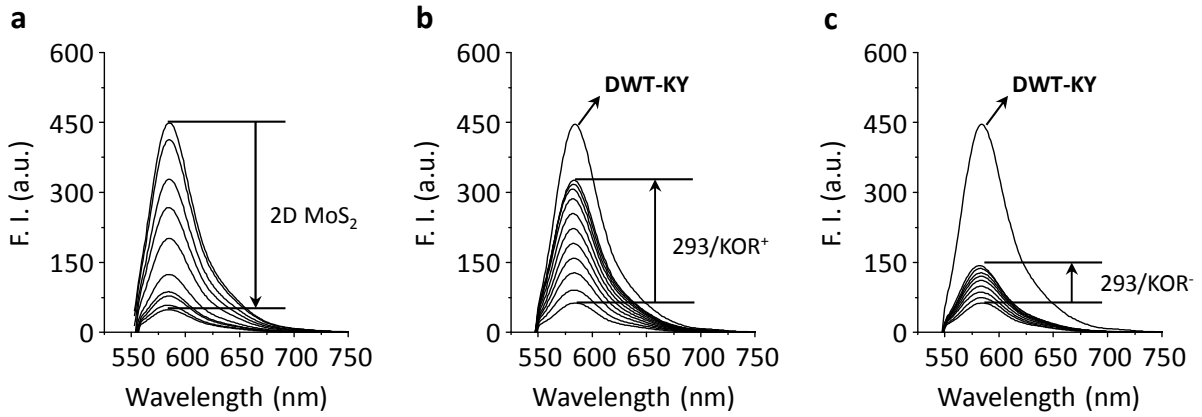

Figure S2. Fluorescence spectra of (a) **DWT-KY** (1 µM) with increasing 2D MoS_2 (from top to bottom curve: 0-35 µg mL^{-1}), (b) 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 1 µM/35 µg mL^{-1}) with increasing HEK293 (human embryonic kidney 293) cells stably expressing the κ-opioid receptor ($293/KOR^+$, from top to bottom curve: 0-300 000 cells mL^{-1}) and (c) 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 1 µM/35 µg mL^{-1}) with increasing HEK293 cells without κ-opioid receptor expression ($293/KOR^-$, from top to bottom curve: 0-300 000 cells mL^{-1}). All fluorescence measurements were carried out in phosphate buffered saline (0.01 M, pH 7.4) with an excitation wavelength of 530 nm.

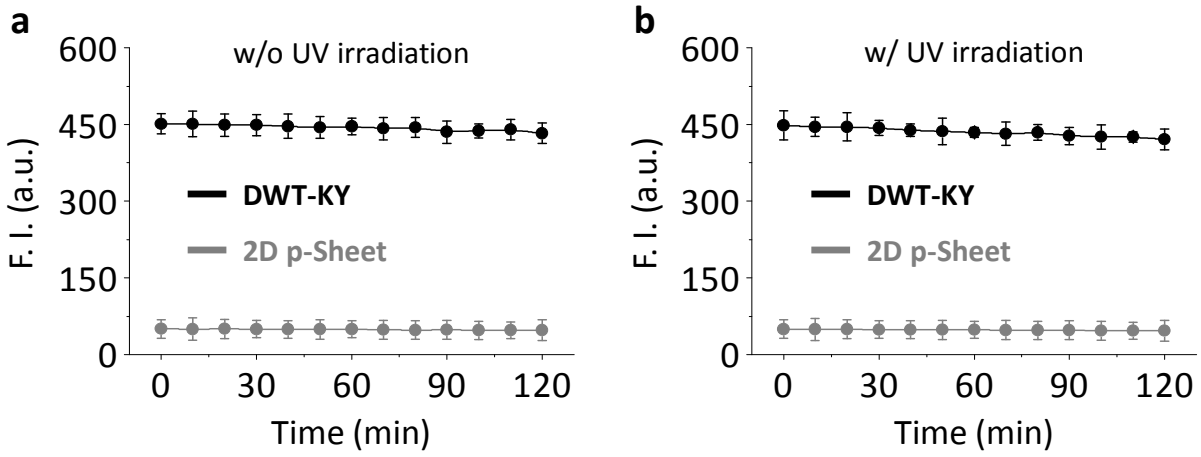

Figure S3. (a) Plotting the fluorescence intensity (F. I.) of **DWT-KY** (1 μM) and 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 1 μM/35 μg mL^{-1}) as a function of time (w/o means without). (b) Plotting the fluorescence intensity (F. I.) of **DWT-KY** (1 μM) and 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 1 μM/35 μg mL^{-1}) as a function of time; the material solutions were irradiated with UV light (365 nm) every 10 min. All fluorescence measurements were carried out in phosphate buffered saline (0.01 M, pH 7.4) with an excitation wavelength of 530 nm.

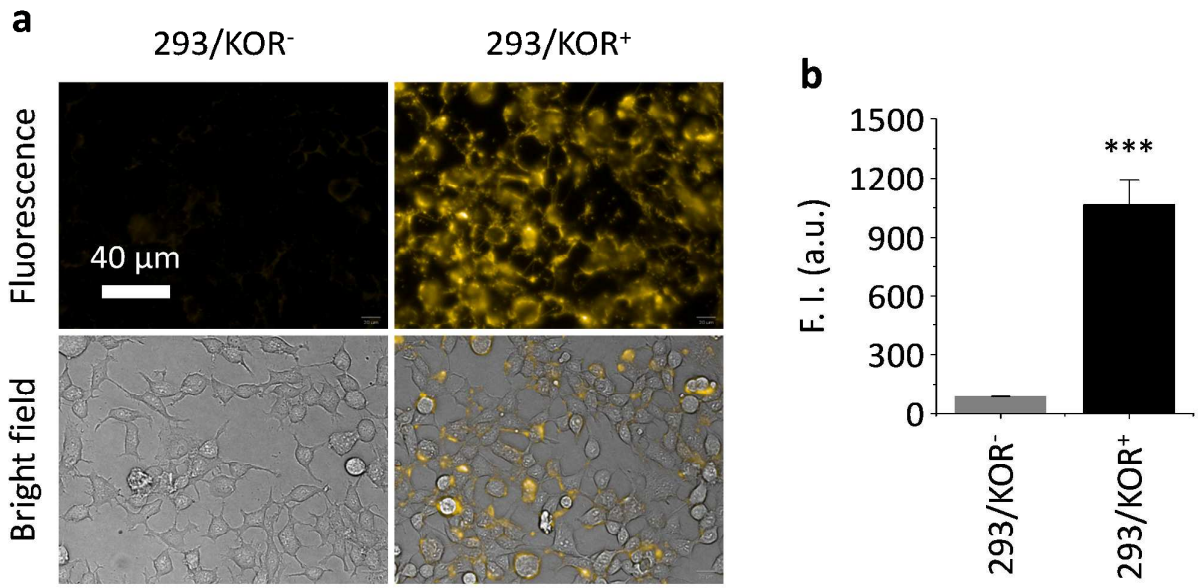

Figure S4. Fluorescence imaging (a) and quantification (b) of HEK293 cells stably expressing the κ-opioid receptor (293/KOR$^+$) and HEK293 cells without KOR expression (293/KOR$^+$) with 1 µM **DWT-KY** (***P < 0.001). The images were taken by an Operetta high content imaging system (the excitation channel for **DWT-KY** is 520-550 nm and the emission channel is 560-630 nm) and quantified by the Columbus image data analysis system (Perkinelmer, US).

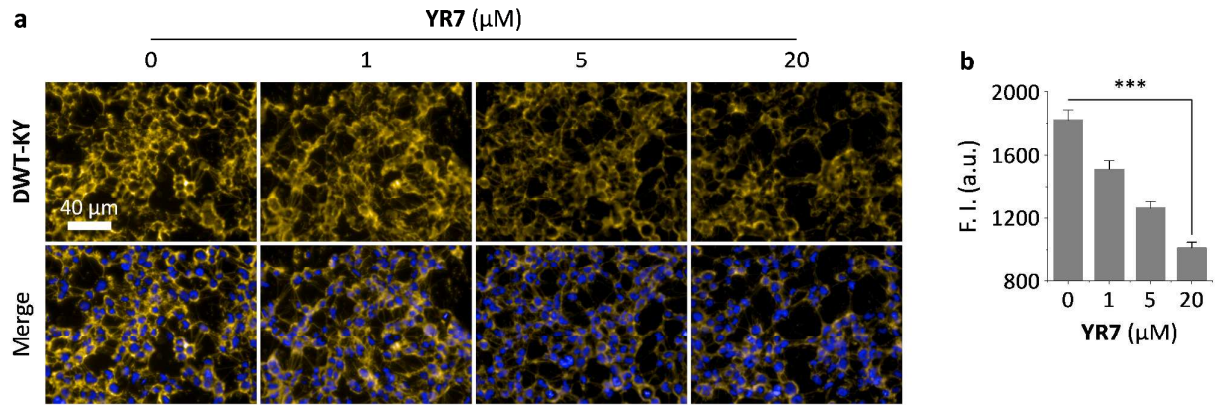

Figure S5. Fluorescence imaging (a) and quantification (b) of HEK293 cells stably expressing the κ-opioid receptor (293/KOR^{+}) with 1 μM **DWT-KY** (the cells were pretreated with increasing free **YR7** (YGGFLRR), which can selectively binds to KOR; the scale bar is applicable to all images) (***P < 0.001; **YR7** 20 μM with respect to the absence of **YR7**). The images were taken by an Operetta high content imaging system (the excitation channel for **DWT-KY** and nucleus (stained by Hoechst) is 520-550 nm and 360-400 nm, and the emission channel is 560-630 nm and 410-480 nm, respectively) and quantified by the Columbus image data analysis system (Perkinelmer, US).

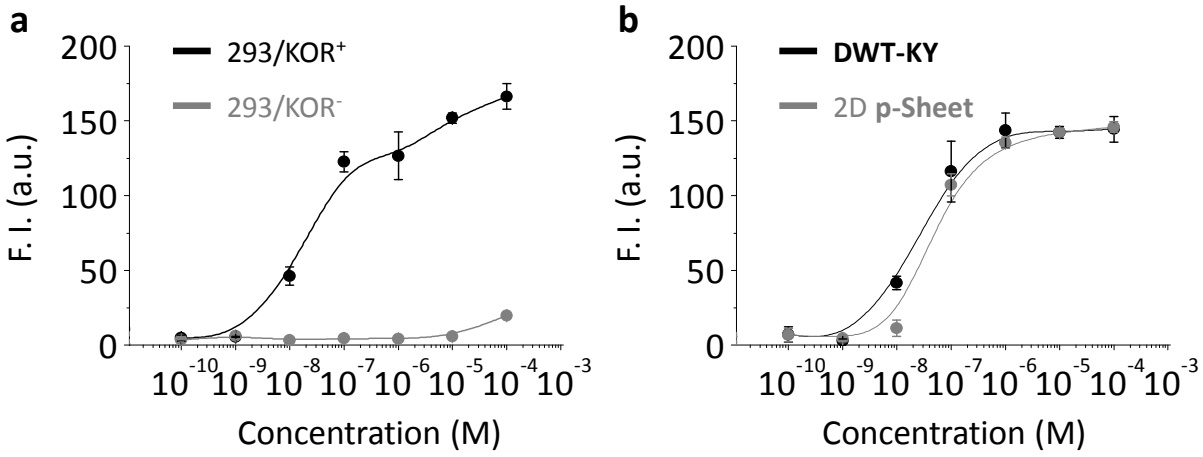

Figure S6. Calcium change of (a) HEK293 cells stably expressing the κ-opioid receptor (293/KOR$^+$) and HEK293 cells without κ-opioid receptor expression (293/KOR$^-$) stimulated with various concentrations of **DWT-KY**, and of (b) 293/KOR$^+$ cells stimulated with various concentrations of **DWT-KY** and 2D **p-Sheet** (where 2D MoS_2 = 5 μg mL^{-1}). The dose-dependent calcium responses were recorded with an FDSS 7000EX microplate reader (Hamamatsu, Bridgewater, NJ) with an excitation wavelength of 485 nm and emission wavelength of 525 nm.

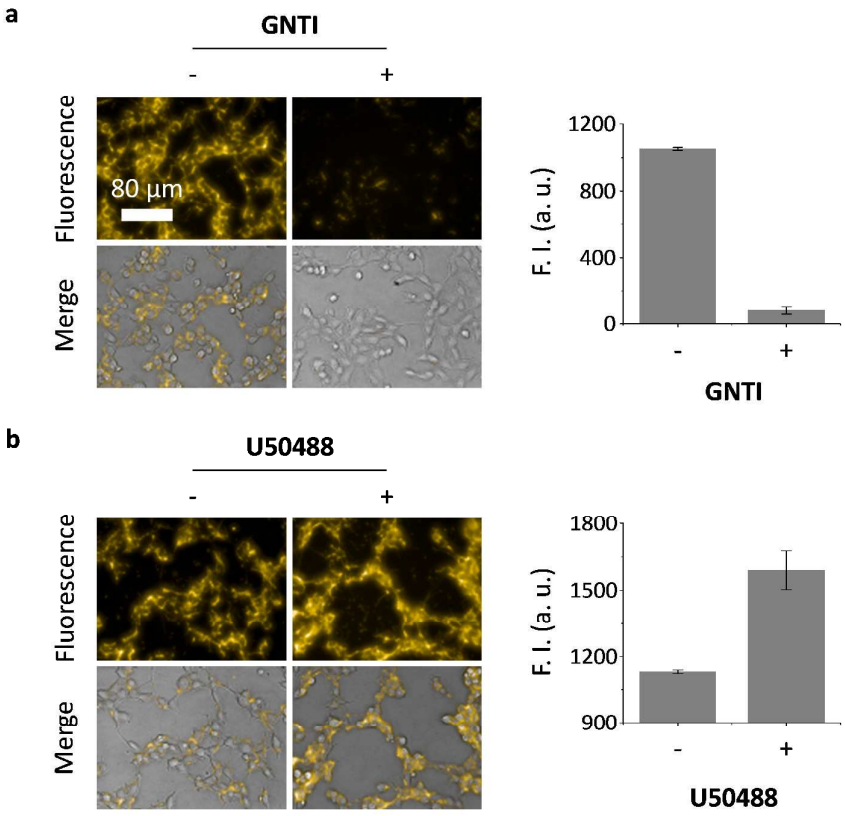

Figure S7. Fluorescence imaging (a) and quantification (b) of HEK293 cells stably expressing the κ-opioid receptor (293/KOR^{+}) with 2D **p-Sheet** (**DWT-KY** = 1 μM with 5 μg mL^{-1} 2D MoS_2. The cells were pretreated with increasing **GTNI** (an arrestin recruitment inhibitor, 20 μM) or **U50488** (a selective KOR agonist, 20 μM) for 120 min (the scale bar is applicable to all images). The images were taken by an Operetta high content imaging system (excitation channel: 520-550 nm; emission channel: 560-630 nm) and quantified by the Columbus image data analysis system (Perkinelmer, US).

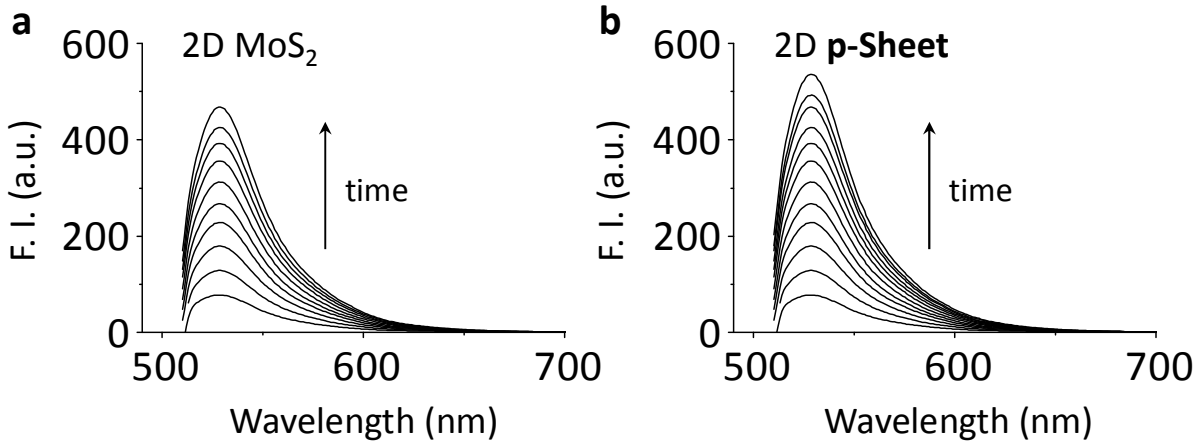

Figure S8. Fluorescence analysis of the ROS-releasing capacity of (a) 2D MoS_2 (35 μg mL^{-1}) and (b) 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 1 μM/35 μg mL^{-1}) irradiated by white light for 0-60 min (from bottom to top curve) with dihydrorhodamine 123 as a fluorescent ROS probe. The fluorescence measurement was carried out in phosphate buffered saline (0.01 M, pH 7.4) with an excitation wavelength of 485 nm.

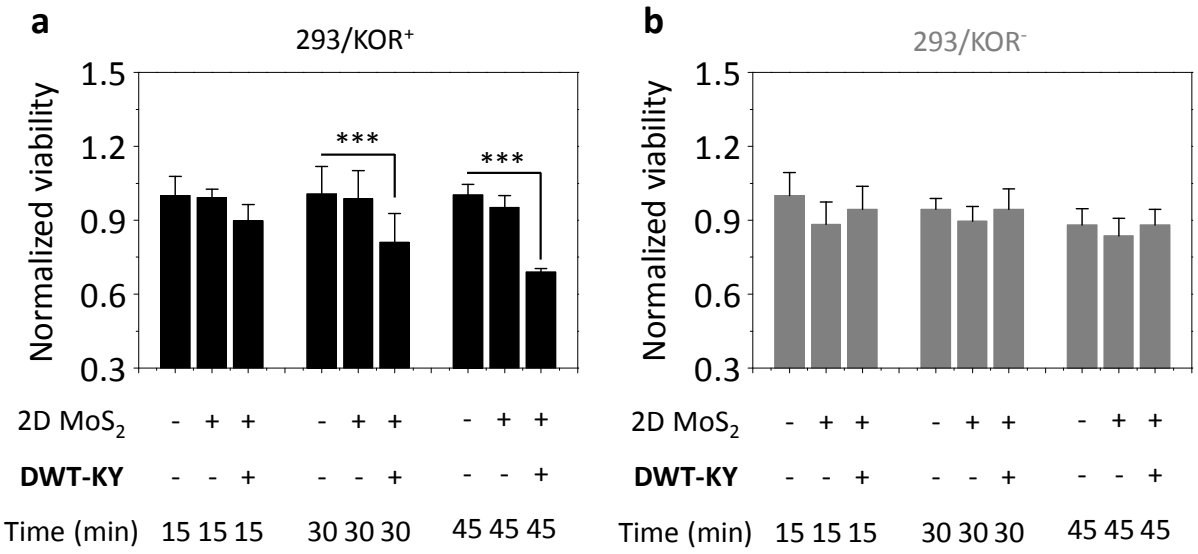

Figure S9. Normalized viability of (a) HEK293 cells stably expressing the κ-opioid receptor (293/KOR^{+}) and (b) HEK293 cells without κ-opioid receptor expression (293/KOR^{-}) treated with 2D MoS_2 (5 μg mL^{-1}) or 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 6 μM/5 μg mL^{-1}) with irradiation of white light for indicated times (15, 30 and 45 min) (***P < 0.001; the double "+" group with respect to the double "-" group). The cell viability was measured by the cell counting method.

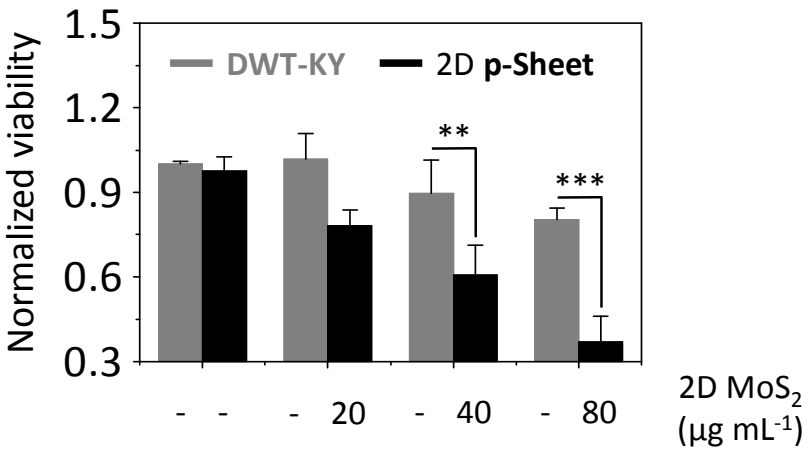

Figure S10. Normalized viability of HEK293 cells stably expressing the κ-opioid receptor (293/KOR^{+}) treated with 2D **p-Sheet** (**DWT-KY** = 6 μM with increasing 2D MoS_2 concentrations as indicated) with irradiation of white light for 70 min (**P < 0.05; ***P < 0.001). The cell viability was measured by cell counting method.

S2. Experimental section

General remarks. All chemicals and reagents used are of the highest commercial grade available. DHR123 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored in a brown plastic bottle. Proteins were purchased from Sigma-Aldrich. Molybdenum sulfide (MoS_2 crystalline powder, < 2 µm, 99%) was purchased from Sigma-Aldrich Co. (USA). Ultrapure water was obtained from a Milli-Q integral Pure/Ultrapure Water Production unit. Transmission electron microscopy (TEM) images were obtained on a JEOL 100CX transmission electron microscope operating at an accelerating bias voltage of 100 kV. High-resolution transmission electron microscope (HRTEM) images were obtained with JEOL 2100 equipped with a Gatan Orius charged-coupled device camera and Tridiem energy filter operating at 200 kV. Dynamic light scattering and zeta potential were carried out on a Horiba LB-550 DLS Nano-Analyzer and zeta potential Analyzer, respectively. Raman spectra were obtained using a Renishaw InVia Reflex Raman system (Renishaw plc, Wotton-under-Edge, UK) employing a grating spectrometer with a Peltier-cooled charge-coupled device (CCD) detector coupled to a confocal microscope, which were then processed with Renishaw WiRE 3.2 software. The Raman scattering was excited by an argon ion laser (I = 514.5 nm).

Synthesis of DWT-KY. The agonist probe was synthesized by the solid-phase method using reagent systems and methodologies of standard Fmoc-chemistry. Purified probe was analyzed by RP-HPLC (C-18) using the following gradient system with solvent A (0.1% TFA in acetonitrile) and solvent B (0.1% TFA in water): 0-0.01 min 20% A/80% B, 0.01-25 min linear gradient to 45% A/55% B (t_R = 9.1 min over 25 min, purity 99.6%). MS (MALDI-TOF): 761.9 and 1521.8; calcd for $[M+2H]^{2+}$ and $[M+H]^{+}$ 761.70 and 1522.8, respectively.

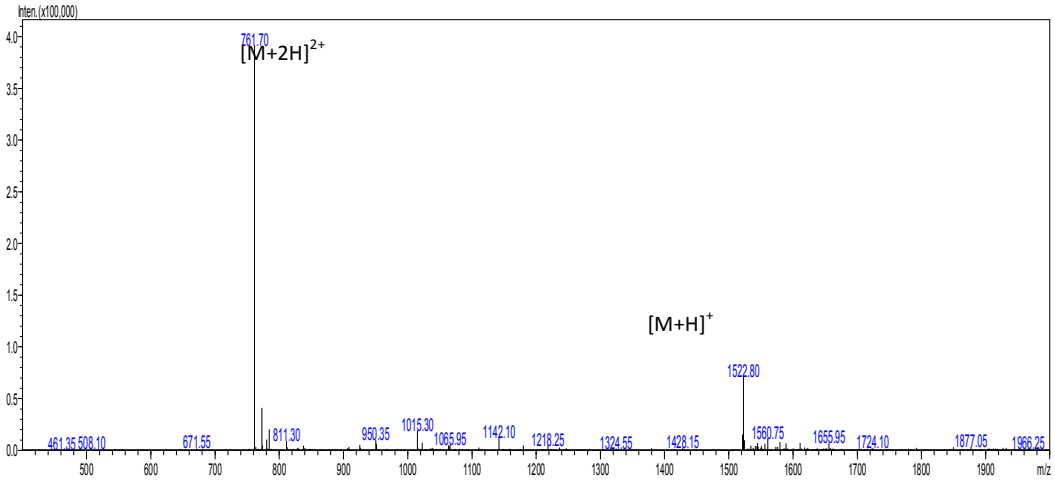

Figure S11. Mass spectrum of **DWT-KY**.

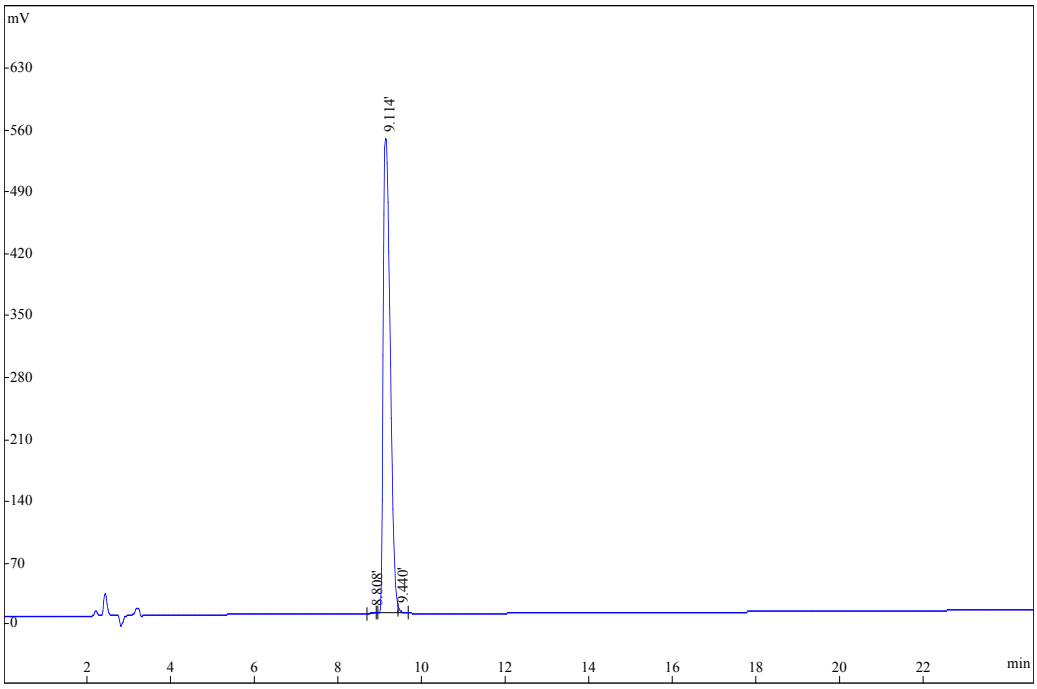

Figure S12. HPLC trace of **DWT-KY**.

Preparation of 2D MoS_2. Typically, 100 mg of bulk MoS_2 crystals precursor was added into a 100 mL glass vial. Then, 50 mL of ultrapure water and ethanol mixture (1:1, v/v) was added as the exfoliation and dispersion solvent. The mixture was bath-sonicated for 48 h using a KQ-100DE at a frequency of 28 kHz. The resulting suspensions were first centrifuged using a H1560 medical centrifuge at 3000 rpm for 30min, and then the top 2/3 portions of the supernatants were carefully collected by pipet. After collecting the supernatant and drying it under 60 ^{o}C in a drying oven, an army green powder was obtained. The obtained powder was dissolved in Milli-Q ultrapure water and sonicated for 30 min to provide a homogeneous stock solution.

Construction of 2D p-Sheet. 2D MoS_2 was suspended in ultrapure water at an initial concentration of 2 mg mL^{-1}. **DWT-KY** was dissolved in a DMSO and phosphate buffered saline mixture (1:4, v/v) at a concentration of 1 mM. Then, aliquots of **DWT-KY** and 2D MoS_2 were mixed in phosphate buffered saline (0.01 M, pH 7.4), followed by the addition of polyethylene glycol 400 at 1% volume ratio. The mixture was stirred overnight in a brown glass bottle to produce the 2D **p-Sheet**.

Fluorescence spectroscopy. In a typical fluorescence quenching assay, **DWT-KY** (with a final concentration of 1 μM) was incubated with 2D MoS_2 solution of different concentrations (0-35 μg

mL^{-1}) in phosphate buffered saline (0.01 M, pH 7.4) for 2 min, and then the fluorescence was measured on a Varian Cary Eclipse fluorescence spectrophotometer with an excitation wavelength of 530 nm. In a typical fluorescence recovery assay, **DWT-KY** (with a final concentration of 1 μM) was first incubated with 2D MoS_2 solution (35 μg mL^{-1}) in PBS phosphate buffered saline (0.01 M, pH 7.4) for 2min. Then living embryonic kidney 293 cells stably expressing the κ-opioid receptor (293/KOR^+) and those without KOR expression (293/KOR^-) at a series of concentrations were added to the mixture. The resulting mixture was incubated at 37.5 °C for 5 min. The fluorescence measurements were carried out at room temperature with an excitation wavelength of 530 nm.

Determination of reactive oxygen species (ROS) in solution. Dihydrorhodamine-123 (DHR123) was used to probe ROS. Oxidation of DHR123 by ROS produces the fluorescent Rhodamine 123. In a typical assay, 2D MoS_2 (35 μg/mL) or 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 1 μM/35 μg mL^{-1}) was added to the phosphate buffered saline (0.01 M, pH 7.4) solution of DHR123 (2 μM). Then, the mixture was irradiated under white light (40 mW cm^{-2}) for 0-60 min, and the emission intensity was recorded on a Varian Cary Eclipse fluorescence spectrophotometer with an excitation of 485 nm.

Plasmid design and construction. To fuse the genes encoding κ-opioid receptor (KOR) and green fluorescence protein (GFP), the cDNA encoding full-length KOR (OPRK1, NCBI reference sequence: NM_000912.4) was used as a template for amplification. The forward primer 5'-CCGCTC GAGGCCACCATGGA CTCCCCGATCCA-3', containing an XhoI restriction site and reverse primer 5'-ACGCG TCGACGATACTGGTTTATTCATC-3', containing a SalI restriction site. After digestion with XhoI and SalI (Takara), the fragments were ligated into pd2EGFP-N1.

Cell culture. HEK293 (human embryonic kidney 293)/KOR^+ cells stably co-expressing the κ-opioid receptor and Gα16 (a G-protein α subunit which plays an important role in GPCR signal-transduction pathways) was a kind gift from Prof. Xin Xie at SIMM. HEK293/KOR^+, HEK293 and HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Gland Island, NY, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Fluorescence imaging of cells. Cells were seeded on a black 96-well microplate with optically clear bottom (Greiner bio-one, Germany) overnight. Then the cells were incubated with 2D **p-Sheet** (**DWT-KY** = 1 μM with 0-5 μg mL^{-1} 2D MoS_2) for 15 min, and then were gently washed with phosphate buffered saline. The fluorescence images were recorded using an Operetta high content imaging system and quantified by the Columbus image data analysis system (Perkinelmer, US).

Receptor Internalization Assay. HEK293T cells transfected with plasmids encoding KOR–green fluorescent protein (GFP) were seeded onto a black 96-well microplate. After 24 h, cells were transferred into serum-free HG-DMEM medium containing 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 1 μM/5 μg mL^{-1}) and incubated for different durations. Cells were then washed with phosphate buffered saline and fixed in 4% paraformaldehyde. After counterstaining of nuclei with Hoechst, fluorescent images were obtained with Operetta (PerkinElmer, US).

Confocal laser-scanning microscopy (CLSM). HEK293T cells transfected with plasmids encoding KOR–green fluorescent protein (GFP) were seeded onto poly-L-ornithine-coated 12 mm glass coverslips. After 24 h, cells were transferred into serum-free HG-DMEM medium containing 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 1 μM/5 μg mL^{-1}) and incubated for 45 min. Cells were then washed with phosphate buffered saline and fixed in 4% paraformaldehyde. After counterstaining of nuclei with Hoechst, fluorescent images were obtained with a Leica TCS SP8 STED laser scanning confocal microscopy (Leica DMi8, Germany) with a 100x 1.32 NA oil-immersion objective.

Super-resolution live cell imaging. HEK293T cells transfected with plasmids encoding KOR–green fluorescent protein (GFP) were seeded onto poly-L-ornithine-coated 3.5 cm dish with a glass bottom. After 24 h, cells were transferred into serum-free HG-DMEM medium containing 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 1 μM/5 μg mL^{-1}). Live cell fluorescent images were obtained with Leica TCS SP8 STED (stimulated emission depletion) laser scanning confocal microscopy (Leica DMi8, Germany) with a 63x 1.32 NA oil-immersion objective.

Determination of ROS with cells. 293/KOR^+ and 293/KOR^- cells were cultured in DMEM supplemented with 10% FBS. Then, cells were seeded on a black 96-well microplate with optically clear bottom (Greiner bio-one, Germany) overnight. The cells were preincubated with 2D MoS_2 (5 μg mL^{-1}) or 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 3.5 μM/5 μg mL^{-1}) for 15 min. Then, cells were gently washed with phosphate buffered saline once. DHR123 (4 μM) was then added to the cells under dark. Samples (n = 3) with a total volume of 100 μL in 96-well plates were irradiated by white light (40 mW cm^{-2}). The fluorescence was recorded using an EnSpire Multimode Plate Reader (Perkinelmer, US) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Cell viability assay. Cells were seeded in 96-well plates and treated on the following day with indicated concentrations of 2D MoS_2 or 2D **p-Sheet** for indicated time (see Fig. S6 and Fig. S7). Then, cells were irradiated by white light (40 mW cm^{-2}) for 70 min. After 48 h, cells were washed with phosphate buffered saline and fixed in 4% paraformaldehyde. Cell viability was obtained by

counting Hoechst-stained-nucleus, recorded with the Operetta high content imaging system and quantified with the Columbus image data analysis system (Perkinelmer, US).

Analysis of apoptosis using FCM of AV/PI dual staining. Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) staining for FCM was used to detect apoptosis quantitatively and qualitatively. After treatment with phosphate buffered saline (negative control), 2D MoS_2 (5 μg mL^{-1}) or 2D **p-Sheet** (**DWT-KY**/2D MoS_2 = 6 μM/5 μg mL^{-1}) for 45 min, cells were irradiated by white light (40 mW cm^{-2}) for 70 min. Then, the cells were processed with an Annexin V-FITC kit (Keygene, Nanjing, China) according to the manufacturer's instructions. Next, the samples were analyzed using the NovoCyte D2060R (ACEA) to quantify the apoptotic rate. Different subpopulations were distinguished using the following criteria: Q1, Annexin V-negative, but PI positive (i.e., necrotic cells); Q2, Annexin V/PI-double positive (i.e., late apoptotic cells); Q3, Annexin V/PI-double negative (i.e., live cells); Q4, Annexin V-positive, but PI- negative (i.e., early apoptotic cells).

Real-time quantitative polymerase chain reaction (RT q-PCR). Total RNA was isolated from cells using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Complementary DNA generated using a PrimeScript® RT reagent kit (TaKaRa, Dalian, China) was analyzed by quantitative PCR using SYBR® Premix Ex TaqTM. RT q-PCR was performed carried out by Stratagene Mx3005P (Agilent Technologies). GAPDH was detected as the housekeeping gene. Primers for qPCR were as follows:

GAPDH forward, 5'- TCTCCTGCGACTTCAACA-3'
and reverse, 5'- TGGTCCAGGGTTTCTTACT-3'
KOR forward, 5'- ACATTGCCGTGTGCCACCCC-3'
and reverse, 5'- TGCCACCACCACCAGGACCA-3'

Competition assay. Cells were seeded on a black 96-well microplate with optically clear bottom (Greiner bio-one, Germany) overnight. Then, the cells were incubated with different concentrations of **YR7** (YGGFLRR, a KOR binder, 0, 1, 5 and 20 μM) for 45 min, gently washed with phosphate buffered saline, and then incubated with 1 μM **DWT-KY**. Then, the cells were again gently washed with phosphate buffered saline and fixed in 4% paraformaldehyde. After counterstaining of nuclei with Hoechst, fluorescence images were recorded using an Operetta high content imaging system and quantified by the Columbus image data analysis system (Perkinelmer, US).

Calcium Mobilization Assay. HEK293 cells stably expressing the κ-opioid receptor (293/KOR^+) and HEK293 cells without κ-opioid receptor expression (293/KOR^-) were seeded onto 96-well plates and

cultured overnight. The culture medium was then changed to Hanks' balanced salt solution (HBSS) buffer containing 2 mM Fluo-4 AM and 250 mM sulfinpyrazone, and then incubated at 37°C for 45 min. After removal of the excess dye, the cells were rinsed with HBSS once. Then, 50 ml of HBSS was added to the dye-loaded cells, and 25 mL of HBSS containing various concentrations of **DWT-KY** or 2D **p-Sheet** (where 2D MoS_2 = 5 μg mL^{-1}) were added. The dose-dependent calcium responses were recorded with an FDSS 7000EX microplate reader (Hamamatsu, Bridgewater, NJ) with an excitation wavelength of 485 nm and emission wavelength of 525 nm.