Supplementary Materials

Ammonium Tetrathiomolybdate as a Water-Soluble and Slow-Release Hydrogen Sulfide Donor

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Materials and Methods

Ammonium tetrathiomolybdate (TTM, 99.95%) and sodium sulfide nonahydrate (Na_2S $9H_2O$, 98%+) and hexadecyltrimethylammonium bromide (CTAB, 99%+) were purchased from Acros (Swedesboro, NJ). WSP-5 was synthesized as previously reported procedures.[1] All buffers were prepared using standard methods with nano-pure DI water. pH 5, 6, 7.4, 8 were standard 1× PBS buffers. All UV-vis absorbance measurements were performed using a Thermo Scientific Evolution 300 UV-vis spectrometer. All fluorescence measurements were performed on an Agilent Technologies Cary Eclipse Fluorescence Spectrophotometer.

H_2S Measurement by Zinc-Sulfide Precipitation based Methylene Blue Method

The measurements of H_2S release were run in triplicate. In each test, 500 μM TTM in buffer at certain pH were freshly prepared and distributed into 1.5 ml Eppendorf vials. The caps on the vials were then closed. At different time interval, one vial was open and 1.0 mL solution was transferred to another Eppendorf vial containing zinc acetate (100 μL, 10% w/v in H_2O), sodium hydroxide (12.5 μL, 1.5 M). The mixture was left for precipitation for 15 minutes before centrifuging at 10200 rcf for 20 minutes. The resulting supernatant was removed. To the solid precipitate was added $FeCl_3$ (200 μL, 30 mM in 1.2 M HCl), and N,N-dimethyl-1,4-phenylenediamine sulfate (200 μL, 20 mM in 7.2 M HCl). After 20 min, the solution was then transferred into a UV–Vis cuvette and the absorbance at 670 nm was measured. H_2S concentrations were calculated according to a standard calibration curve obtained with a series of Na_2S solutions.

Trapping H_2S Gas from TTM Solutions and Fluorescence Measurements

The stock solutions of both TTM and Na_2S (30 mM) were freshly prepared in nano-pure DI water. The stock solution of WSP-5 (3.5 mM) was prepared in DMSO. The stock solution of CTAB (3.5 mM) was prepared in ethanol. 100 μL of TTM or Na_2S stock solution was diluted to 2 mL (to make 1.5 mM solution) in pH 5, 6, 7.4 and 8 buffers. These solutions were put into 20 mL glass vials. To each vial, an Eppendorf tube containing 12 μL WSP-5 stock solution and 30 μL CTAB stock solution and 500 μL pH 7.4 buffer and a filter paper (1 cm × 2 cm) was then placed. The vials were then sealed with parafilm, and incubated at 37 °C for 3 hours. The solution in the Eppendorf vial was then diluted to 3 mL with pH 7.4 buffer. These were incubated for 30 minutes at 37 °C, then the fluorescence emission was measured (excitation= 502 nm, 800 V, emission= 525 nm).

Cell Culture

HaCaT cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose medium supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2.

Cell Viability Assay

The cell counting kit (CCK)-8 (Dojindo Laboratory, Kumamoto, Japan) was applied to measure HaCaT cell viability cultured in 96-well plates. After the indicated treatments, 100 μL of CCK-8 solution at 1:10 dilution with FBS-free DMEM was added to each well, and cells were incubated for a further 3 h at 37 °C. The absorbance was measured at 450 nm with a microplate reader (Tecan Infinite F200, Switzerland). The mean optical density (OD) of 4 wells in each group was used to calculate cell viability as follows:

$$\% \text{ Cell viability} = \frac{\text{OD treatment group} - \text{OD blank}}{\text{OD control group} - \text{OD blank}} \times 100$$

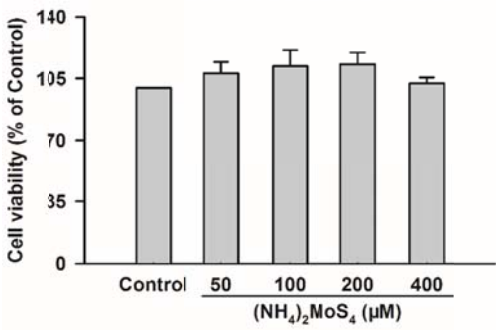

Figure S1 Cell viability of TTM in various concentrations.

Protective effects of TTM on H_2O_2-Induced Cellular Damage

HaCaT cells were exposed to 400 μM H_2O_2 for 5 h to set up an *in vitro* model of oxidative damage. Before the incubation with H_2O_2, the cells were treated with TTM at varied concentrations (50−400 μM) for 1 h. The protective activity of TTM was investigated with the CCK-8 assay.

LDH Measurement

The rate of LDH release was determined with a commercial LDH kit supplied by Thermo Fisher Scientific Inc. (Pittsburgh PA, US). Briefly, HaCaT cells were inoculated in a 96-well plate and grew to about 80% confluence. After the indicated treatments, 50 μL of supernatant per well was carefully removed and transferred into corresponding wells for the determination of extracellular LDH levels. Then, 100 μL of DMEM with 2% Triton X-100 was added to the adherent cells to lyse the cells. Fifty microliters of cell lysate was transferred to a 96-well plate to determine intracellular LDH levels. The same volume of prepared reaction mixture was added to the supernatant or homogenate, separately, and reacted for 10 min at room temperature by gentle shaking. Stopping solution was added to stop the reaction. Absorbance was measured at 490 and 600 nm with a microplate reader (Tecan Infinite F200, Switzerland). LDH release rate was calculated as follows:

$$\% \text{ LDH release} = \text{extracellular LDH}/(\text{extracellular LDH} + \text{intracellular LDH}) \times 100$$

MMP Measurement

Mitochondrial membrane potential (MMP) was observed using a fluorescent dye, rhodamine

123 (Rh123) followed by photofluorography. Rh123 is a cell permeable cationic dye that preferentially enters the mitochondria based on the highly negative MMP. Depolarization of MMP leads to a decreased intake of Rh123 and an intracellular weak fluorescence. After the indicated treatments, 10 mg/L Rh123 was added to cell cultures for 30 min at 37 °C in the dark, and fluorescent photos were taken using a fluorescent microscope (Advanced Microscopy Group, Seattle, US). The mean fluorescent intensity (MFI) of Rh123 from 6 random fields was analyzed using Image J software.

Statistical Analysis

All the data were expressed as Mean ± SE. The significance of inter-group differences was evaluated by one-way analysis of variance followed by SNK-q test with SPSS software. A probability of $P < 0.05$ was accepted as the level of statistical significance. Experiments were performed for 6 times.

References

[1] Peng, B.; Chen, W.; Liu, C.; Rosser, E. W.; Pacheco, A.; Zhao, Y.; Aguilar, H.; Xian, M. *Chem. Eur. J.* **2014**, *20*, 1010.