Electronic Supplementary Material (ESI) for Chemical Science.

Ratiometric Fluorescence Imaging of Golgi H_2O_2 Reveals a Correlation between Golgi Oxidative Stress and Hypertension

Hui Wang‡, Zixu He‡, Yuyun Yang, Jiao Zhang, Wei Zhang, Wen Zhang, Ping Li*, Bo Tang*

Abstract: Golgi oxidative stress is significantly associated with the occurrence and progression of hypertension. Notably, the concentration of hydrogen peroxide (H_2O_2) is directly proportional to the degree of Golgi oxidative stress. Therefore, based on a novel Golgi-targeting phenylsulfonamide group, we developed a two-photon (TP) fluorescent probe, Np-Golgi, for in situ H_2O_2 ratiometric imaging in living systems. The phenylsulfonamide moiety effectively assists Np-Golgi in precise location by binding to cyclooxygenase-2 (COX-2). In addition, the raw material of phenylsulfonamide is easily available, and chemical modification is easily implemented. By application of Np-Golgi, we explored the generation of endogenous H_2O_2 during Golgi oxidative stress, and also successfully revealed increase on the levels of Golgi H_2O_2 in the kidneys of mice with hypertension. This work provides an ideal tool to monitor Golgi oxidative stress for the first time and novel drug targets for the future treatment of hypertension.

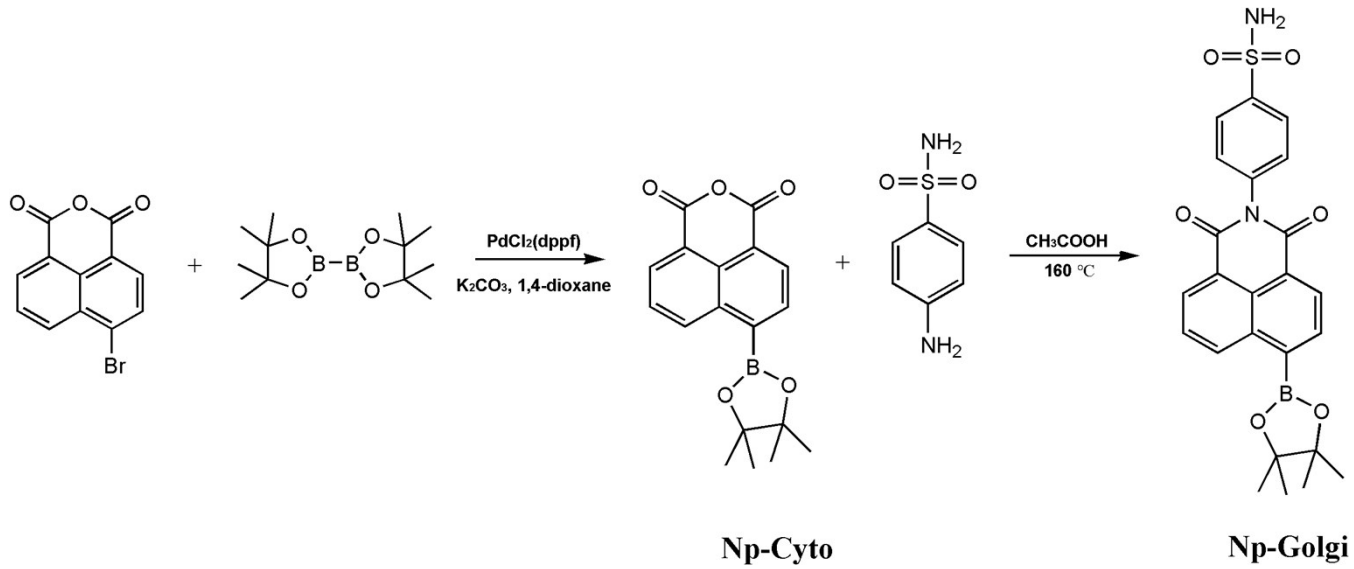
Table of Contents

Experimental Procedures

Materials and Instruments

All reagents used were purchased from commercial companies. Sulfanilamide was purchased from Aladdin. 1,1'-Bis (diphenylphosphino) ferrocene palladium (Ⅱ) was purchased from Strongerscience Company of Beijing. 4-Bromo-1,8-naphthalic Anhydride was purchased from Energy Chemical. Bis (pinacolato) diboron was purchased from nine Ding Chemistry. 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) were purchased from Sangon Biotech. SiRNA COX-2 was purchased from Genepharma. The commercial Tracker was purchased from Beyotime.

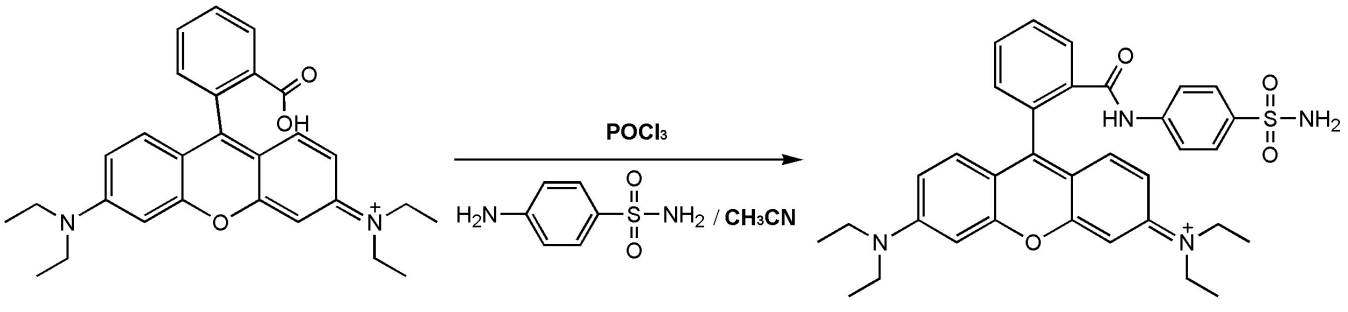
^{1}H NMR and ^{13}C NMR spectra were measured at 400 and 100 MHz on Bruker NMR spectrometers. The high-resolution mass spectra (HRMS) were measured by BrukerMaxis ultrahigh-resolution TOF MS system. The fluorescence spectra were detected by An FLS-920 Edinburgh fluorescence spectrometer. The cellular fluorescence imaging was performed by a Zeiss LSM 880 NLO confocal laser scanning microscope. *In vivo* fluorescence imaging was performed by a two-photon confocal microscope.



Scheme S1. Synthetic route of Np-Golgi.

Synthesis of Np-Golgi

Np-Cyto was synthesized according to the previously reported literature.[1] Np-Cyto (324 mg, 1 mmol) and sulfanilamide (1.7220 g, 10 mmol) was dissolved in 20 mL acetic acid and stirred at 160 °C over-nignt. The solution was removed under reduced pressure and the residue was purified by column chromatography with CH_2Cl_2/CH_3OH (v/v, 20:1) as the eluent.The compound Np-Golgi was obtained as a yellow solid (46 mg, yield 10 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.20 (d, J = 8.5 Hz, 1H), 8.64 – 8.57 (m, 2H), 8.34 (d, J = 6.6 Hz, 1H), 8.09 (d, J = 7.9 Hz, 2H), 7.82 (t, J = 7.8 Hz, 1H), 7.50 (d, J = 7.9 Hz, 2H), 4.99 (s, 2H), 1.47 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 129.92, 127.70, 84.78, 29.71, 25.02. HRMS (ESI) m/z: [M+Na] calculated for $C_{24}H_{23}BN_2O_6S$, 501.1268 found 501.1197.



Scheme S2. Synthetic route of Rd-Golgi.

Synthesis of Rd-Golgi

Rhodamine B (480 mg, 1 mmol) was dissolve in $POCl_3$ (2.5 mL, 27 mmol) return at 80 °C for 12 h. The solvent was removed at reduced pressure and the residue dissolved in 10 mL CH_3CN, added sulfanilamide (430 mg, 2.5 mmol) to the mixture and stirring at room temperature over-nignt.[2] The solution was filtered to remove insoluble solids and then gasified to remove the solvent. The residue was purified by column chromatography with CH_2Cl_2/CH_3OH (v/v, 20: 1) as the eluent. The compound Rd-Golgi was obtained as a pink solid (125 mg, 21 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.91 (dd, J=6.2, 1.7, 1H), 7.60 – 7.56 (m, 2H), 7.43 (t, J=5.9, 2H), 7.10 – 7.02 (m, 3H), 6.52 (d, J=8.2, 2H), 6.23 (s, 4H), 4.88 (s, 2H), 3.24 (dd, J=13.7, 6.7, 8H), 1.58 (s, 1H), 1.08 (t, J=7.1, 12H). ^{13}C NMR (101 MHz, CDCl3) δ = 167.27, 151.76, 147.94, 140.26, 137.54, 132.53, 128.56, 127.37, 125.94, 124.83, 122.92, 122.51, 107.27, 104.20, 96.85, 66.66, 43.30, 28.68, 11.55. HRMS (ESI) m/z: [M^+] calculated for $C_{34}H_{37}N_4O_4S$, 597.2517 found 597.2452; [M+Na] calculated for $C_{34}H_{37}N_4O_4S$, 619.2355 found 619.2266.

Fluorescence Measurement

We measured the fluorescence intensity of Np-Golgi in One-photon with 405nm excitation. And we measured the fluorescence intensity in Two-photon fluorescence imaging with 810nm and collected at 430-480nm and 530-580nm.

Selectivity of Np-Golgi for H_2O_2

For this experiment, Np-Golgi (2 μM) and several tested species (100 μM) were mixed together to reach a final volume of 1 mL and incubated at 37 °C for 30 min in 10 mM PBS (pH 7.4). The tested species included NaCl, $ZnCl_2$, KCl, $Na_3C_6H_5O_7$, $Na_2S_2O_3$, Cys, GSH, vitamin C, NO, $^1O_2$, ·OH, $O_2^{\cdot-}$, ROO·, t-BuOOH, ClO^- and H_2O_2. After co-incubation, the fluorescence intensity of Np-Golgi was measured at 470 nm and 560 nm with 405 nm excitation. Each experiment was repeated at least three times.

Effect of pH on Np-Golgi

The experiment was performed on two groups. Np-Golgi (2 μM) with or without H_2O_2 (100 μM) was added to PBS solution with different pH values (100 mM, pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0) and the mixtures were incubated at 37 °C for 30 min. The Fluorescence Intensity at 470 nm and 560 nm was collected under 405 nm excitation. Each experiment was repeated at least three times.

Measurement of Two-photon Absorption Cross Section

We used the reported method[3] to measure the two-photon absorption cross section (δ). Two-photon-induced fluorescence intensity was measured with 810nm laser and using fluorescein (10^{-4} M, pH 11) as a reference. Np-Golgi (10^{-3} M) were dissolved in DMSO and then Two-photon-induced fluorescence intensity was measured at 810 nm. Used the fluorescence intensity at the same emitted to calculated the two-photon absorption cross section (δ) by using Eq (1) from the reference.[4]

$$\delta_s = \delta_r \frac{\Phi_r}{\Phi_s}\frac{C_r}{C_s}\frac{n_r}{n_s}\frac{F_s}{F_r} \quad \text{Eq (1)}$$

The subscripts s and r means sample and reference material, respectively. δ is the two-photon absorption cross section, C is the concentration of solution, n is their refractive index of the solution, F is two-photon excited fluorescence integral intensity, Φ is their quantum yield.

HPLC Analysis

HPLC analyses were performed on an Agilent 1260 system equipped with a G1311B pump, UV detector and an Agilent Zorbax SB-C18 RP (9.4 × 250 mm) column, with CH_3OH (0.1 % of TFA) and water (0.1 % of TFA) as the eluent. The HPLC condition was shown in Table S1.

Table S1. HPLC condition for Np-Golgi with or without H_2O_2.

Time(min)	Flow(mL/min)	CH_3OH%	H_2O%
0	0.5	50	50
20	0.5	50	50
45	0.5	100	0
60	0.5	100	0
61	0.5	50	50

75	0.5	50	50

Cell Culture

The Hela cells, Human Hepatoma cells SMCC-7721 and Human liver cells HL-7702 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in DMEM medium with 10 % FBS (Fetal Bovine Serum) at 37 °C in humidified air containing 5 % CO_2.

Cell Viability

The cytotoxicity of Np-Golgi in HeLa, SMCC-7721 and HL-7702 cells was assessed.[5] An MTT cell proliferation kit was purchased from Samgon Biotech (Shanghai) Co. The cells were seeded at a density of 2 × 10^3 cells/well on a 96-well cell culture plate. The volume of medium in each well was 100 μL. The plate was incubated at 37 °C for 24 h in a humidified incubator with 5% CO_2. Different concentrations of NP-Golgi (0, 0.1, 1, 5, 10, 20 μM) were added to the wells. After incubating the plate for 12 h, 10 μL of MTT reagent (5 mg/mL) was added at a final concentration of 0.5 mg/mL to each well, and the plate was incubated at 37 °C for 4 h. Thereafter, the solvent was removed, and 100uL of DMSO was added to dissolve the blue crystals. The plate was placed on a shaker to mix gently for 10 minutes and the optical density (OD_{570}) value (Abs.) was measured by a TRITURUS microplate reader. The experiment was conducted according to the product instructions. Each experiment was repeated at least three times.

Golgi Co-localization Cell Imaging Experiment

A volume of 1 mL DMEM including Np-Golgi (10 μM) and Commercial Tracker Golgi-Red (0.5 μM, Ex=561 nm, Em=610-630 nm), ER-Red (0.5 μM, Ex=561 nm, Em=600-630 nm), Lyso-Red (0.5 μM, Ex=633 nm, Em=650-700 nm) and Mito-Red (0.5 μM, Ex=633 nm, Em= 650-700 nm) were added respectively into cells for 30 min. And then cell imaging was performed by using proper wavelength. Each group was repeated at least three times. Image Pro Plus 6.0 was used to calculate Pearson's co-location coefficients. For Rd-Golgi and Golgi-Red Golgi co-localization experiment, in order to avoid the influence of optical spectrum, we used different laser (Rd-Golgi: 514nm laser; Golgi-Red: 561nm laser). We collected 565nm-580nm for Rd-Golgi at 514nm excitation and 610nm-630nm for Golgi-Red at 561nm excitation.

Endogenously Stimulated H_2O_2 Cell Imaging Experiment

SMCC-7721 cells were incubated with different stimulators and then stained with Np-Golgi (10 μM) for 30 min before imaging. (a-c) Control cells: cells without any stimulation. (d-f) PMA cells: cells were incubated with PMA (1 μg/mL, 30 min). (g-i) PMA+NAC cells: cells were incubated with PMA (1 μg/mL, 30 min) and then NAC (20 mM, 1 h) was added. (j-l) NAC cells: cells were incubated with NAC (20 mM, 1h). And then cell imaging was performed by using proper wavelength. Each group was repeated at least three times. Ex=405 nm, Blue Chanel: 430-480 nm, Green Chanel: 530-580 nm.

Golgi H_2O_2 Cell Imaging Experiment

All the SMCC-7721 cells were pretreated with NAC (20 mM) for 1 h as control. After that, Monensin (10 μM) was add into the cell to induce Golgi Oxidative Stress. And then cells were incubated with different stimulators to change the concentration of $O_2^{\bullet -}$ at Golgi apptantus. (b, f, j) Monensin cells: The cells were incubated with NAC and then monensin (10 μM) was added for 6 h. (c, g, k) Tiron cells: The cells were incubated with NAC, and then monensin and Tiron (10 μM) were added for 6 h. (d, h, l) 2-ME cells: The cells were incubated with NAC, and then monensin were added for 6 h. After that, 2-ME (1 μg/mL) were added for 30 min. Cell imaging was performed by using proper wavelength. Each Group was repeated at least three times. Ex=405 nm, Blue Chanel: 430-480 nm, Green Chanel: 530-580 nm.

LPS-induced Abdominal Inflammatory model

Saline or LPS were injected into the two sides of the abdominal cavity of mice, respectively. (a) Control: left abdominal cavity of the mice were pretreated with Saline (400 μL) for 4 h and the injected Np-Golgi (10 μM, 100μL) for 30 min. (b) LPS-induced mice: right abdominal cavity of the mice were pretreated with LPS (2mg/mL, 400 μL) for 4 h and the injected Np-Golgi (10 μM, 100μL) for 30 min. Mice were removed their hair on the ventral surface of the abdomen. Two-photon fluorescence imaging were acquired by LSM 880 NLO confocal laser scanning microscope with 20× water objective lens. Ex=810 nm, Green Chanel: 430-480 nm, Red Chanel: 530-580 nm. (n=3)

High-Blood Pressure (HBP) Animal Model

The animal experiments were performed in compliance with the relevant laws and guidelines issued by the Etheical Committee of Shandong University and were in agreement with the guidelines of the Institutional Animal Care and Use Committee. The establishment of the High-Blood Pressure (HBP) animal model was based on previously reported methods.[6] Female Kunming mice (average weight about 20 g) were purchased from the Shandong Laboratory Animal Center. The mice were divided into a control group and an HBP group. The mice in the HBP group were injected with 100 μL of ouabain/saline solution (50 μg/mL) by intraperitoneal injection every day, while the mice in the control group were injected with saline instead. The blood pressure of the mice was monitored and recorded daily with a Metlab tail artery blood pressure meter at least three times per day.

Two-photon Fluorescence Imaging *in Vivo*

Two-photon fluorescence imaging was performed on the kidneys of the mouse after dropping the probe for 30 min. At 810 nm excitation, the proper wavelength was collected. Blue channel: 430-480 nm, Green channel: 530-580 nm. Each experiment was repeated at least three times.

Results and Discussion

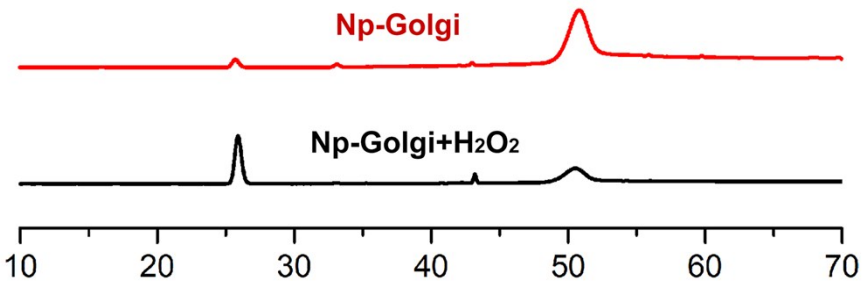


Figure S1. HPLC analysis of Np-Golgi with or without 200 eq H_2O_2.

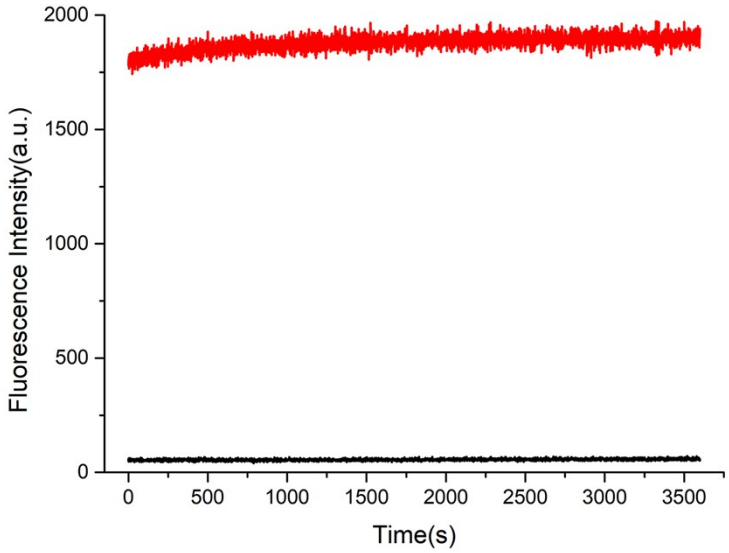


Figure S2. Photostablility of Np-Golgi (2 μM) reacted with H_2O_2 (100 μM) after 2 h. Ex=405; Em=560 nm.

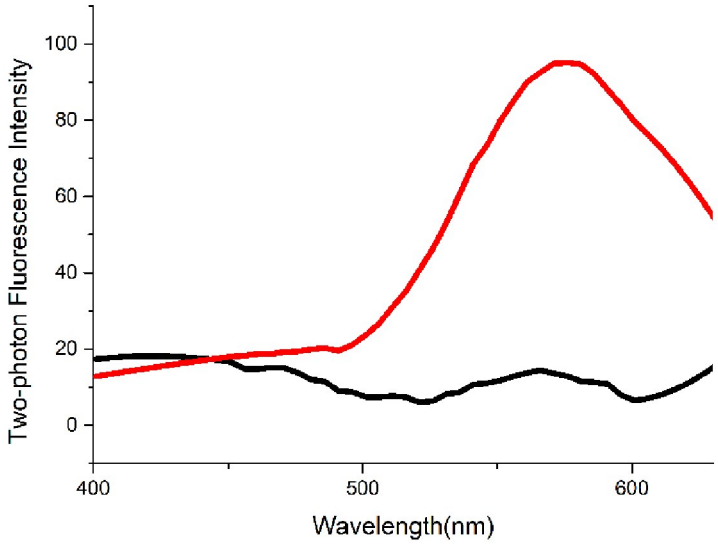


Figure S3. Two-photon fluorescence spectra of 10 μM Np-Golgi (black line) alone and after the addition of 200eq H_2O_2 (red line) in PBS (pH 7.4) at TP Ex= 810 nm.

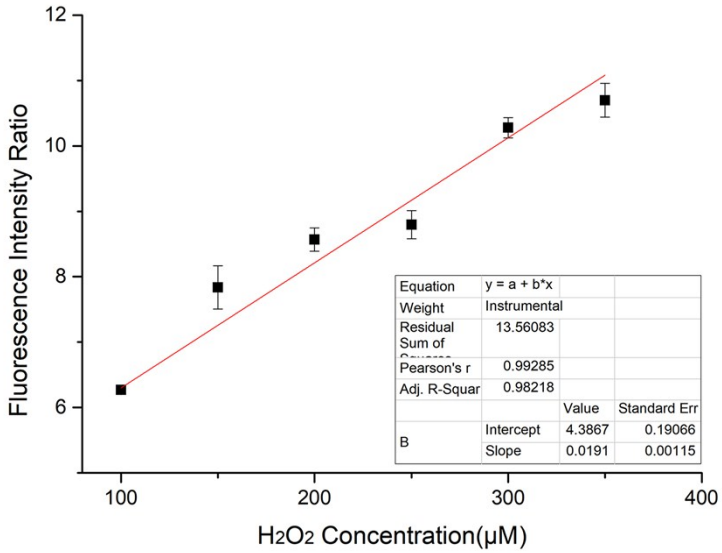


Figure S4. The linear relationship between FI of Np-Golgi and H_2O_2 level. The linear relationship between fluorescence intensity of Np-Golgi (2 μM) and H_2O_2 (100-350 μM) concentration.

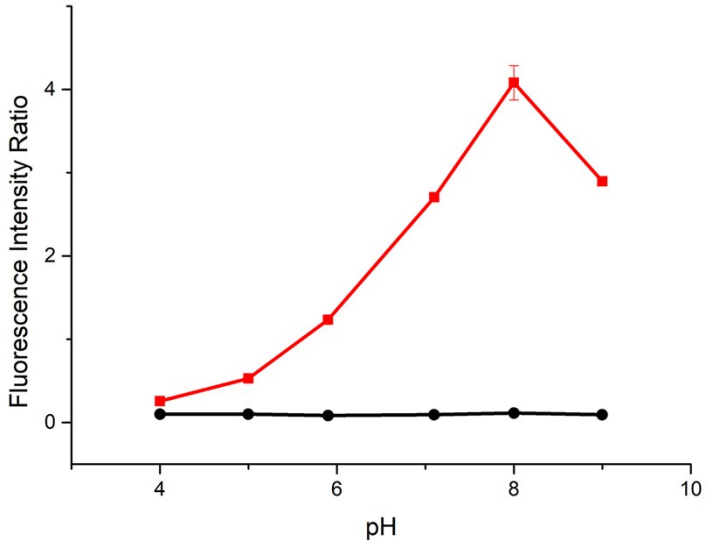


Figure S5. Effect of pH value on the fluorescence intensity of Np-Golgi reacted with H_2O_2. Fluorescence intensity ratio of Np-Golgi (2 μM) towards H_2O_2 (100 μM) at different pH (4.0 - 9.0). Np-Golgi reacted with H_2O_2 was shown in red curve, and Np-Golgi was shown in black.

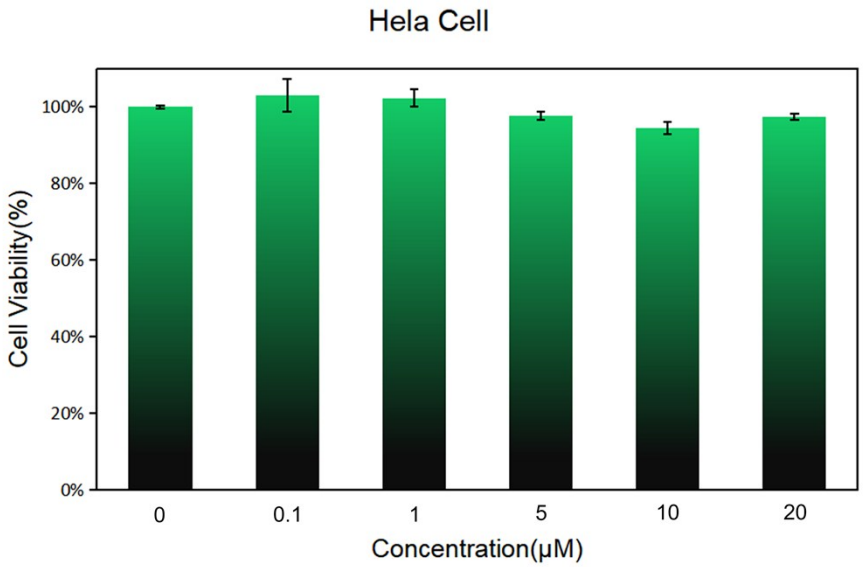


Figure S6. Cell viability of Hela cells after incubation with Np-Golgi. Hela Cells were incubated with different concentration of Np-Golgi (0, 0.1, 1, 5, 10, 20 μM).

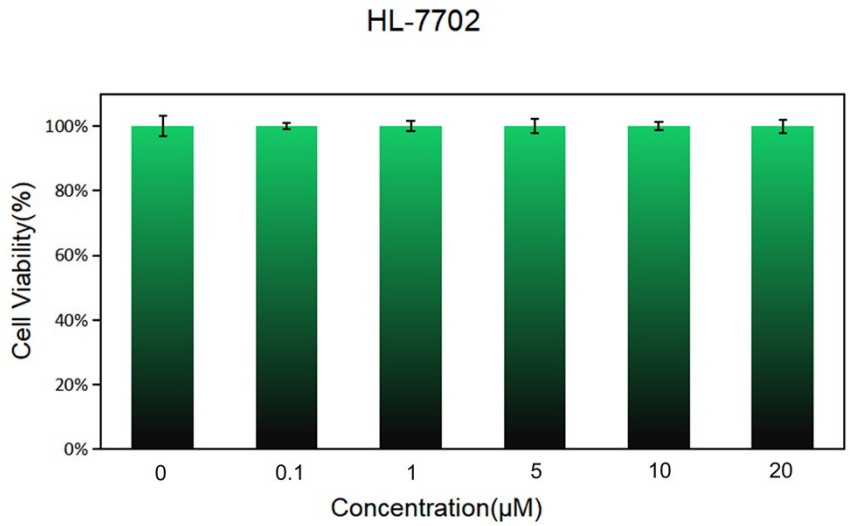


Figure S7. Cell viability of HL-7702 after incubation with Np-Golgi. HL-7702 were incubated with different concentration of Np-Golgi (0, 0.1, 1, 5, 10, 20 μM).

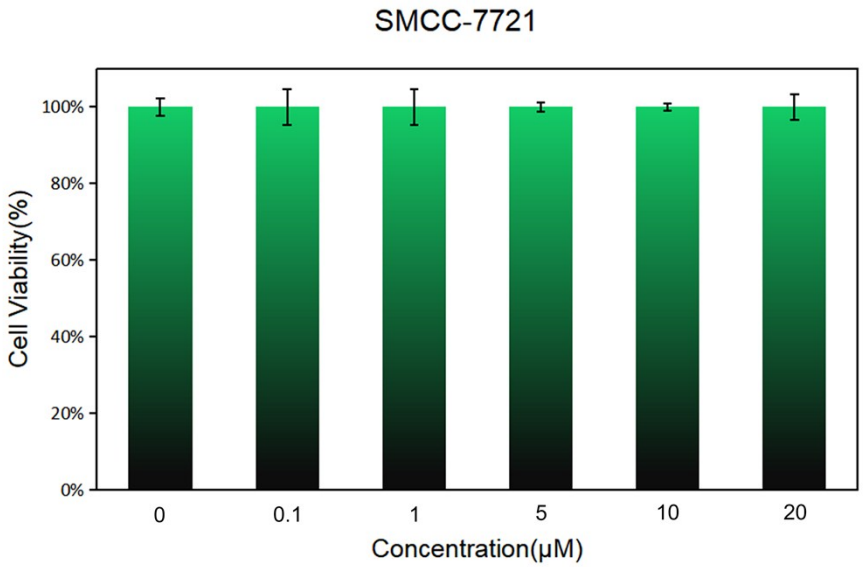


Figure S8. Cell viability of SMCC-7721 after incubation with Np-Golgi. SMCC-7721 were incubated with different concentration of Np-Golgi (0, 0.1, 1, 5, 10, 20 μM).

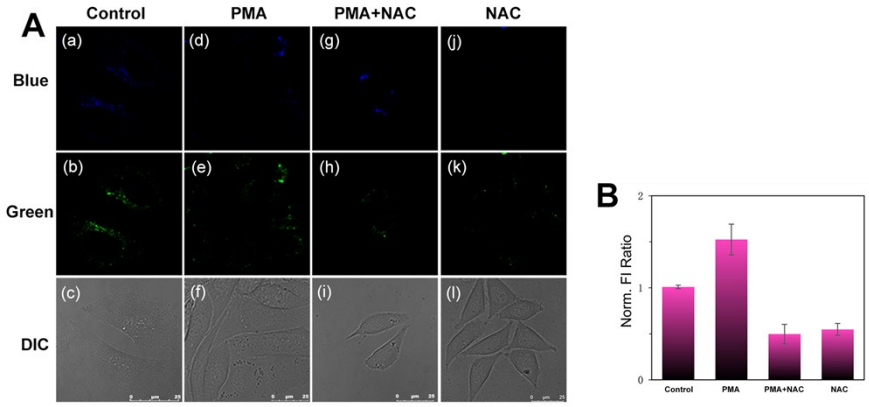


Figure S9. Fluorescence imaging of H_2O_2 in cells. (A) Fluorescence images of endogenously stimulated H_2O_2 in SMCC-7721 cells. Cell were incubated with different stimulators and then stained with Np-Golgi (10 μM, 30 min) before imaging. (a-c) Control cells: cells without any stimulation. (d-f) PMA cells: cells were incubated with PMA (1 μg/mL, 30 min). (g-i) PMA+NAC cells: cells were incubated with PMA (1 μg/mL, 30 min) and then NAC (20 mM, 1 h) was added. (j-l) NAC cells: cells were incubated with NAC (20 mM, 1h). Scale bar: 25 μm. (B) Relative fluorescence intensity of Np-Golgi-labeled cells from images. Error bars represent standard deviation (n=3).

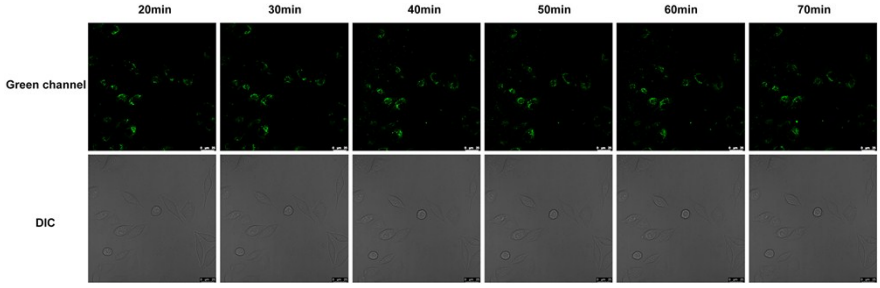


Figure S10. Photostability of Np-Golgi in cells. Cellular fluoresecence imaging of Np-Golgi (10 μM, 10 min) for 1 h.

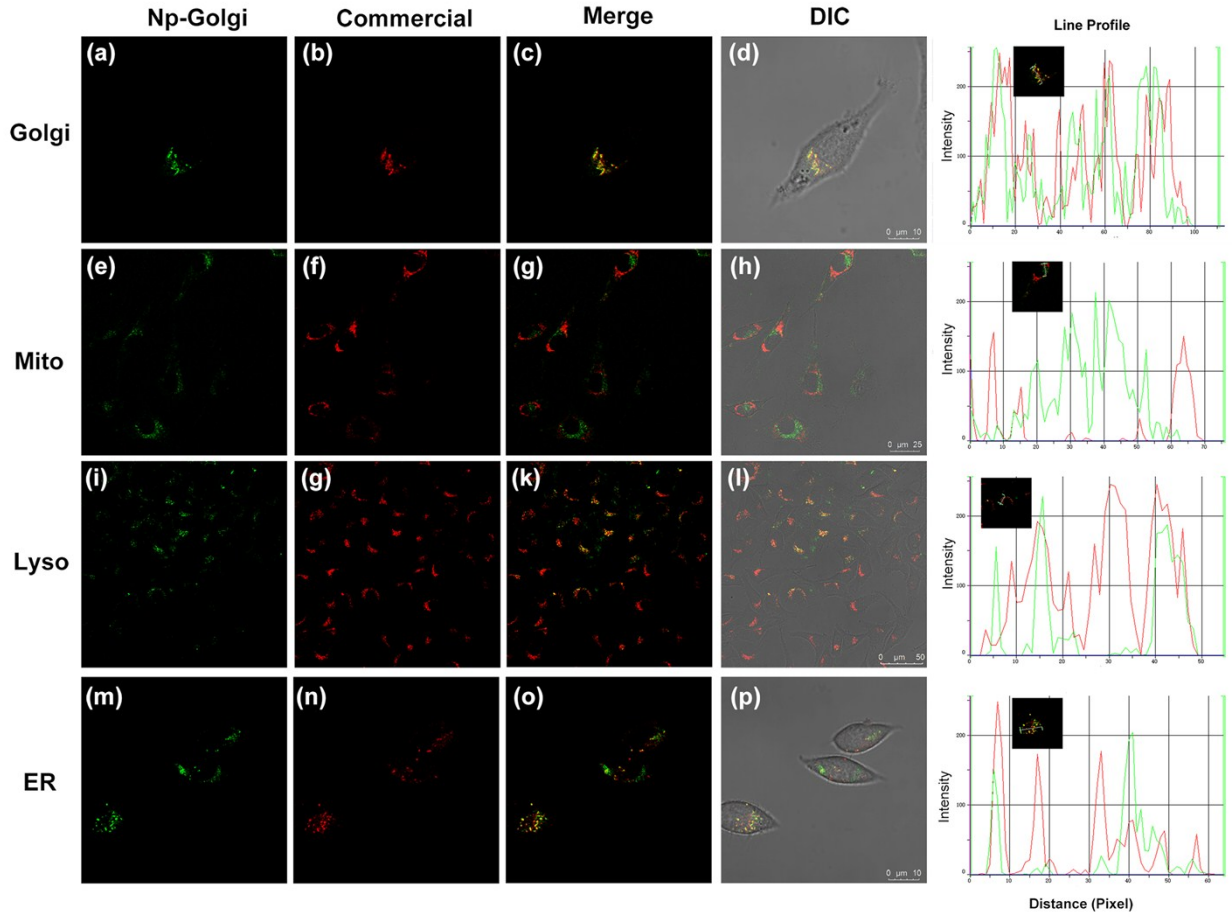


Figure S11. Co-localization cell imaging of Np-Golgi and commercial dyes in SMCC-7721. Golgi (a, b, c, d), mitochondria (e, f, g, h), lysosomes (i, j, k, l), endoplasmic reticulum (m, n, o, p).

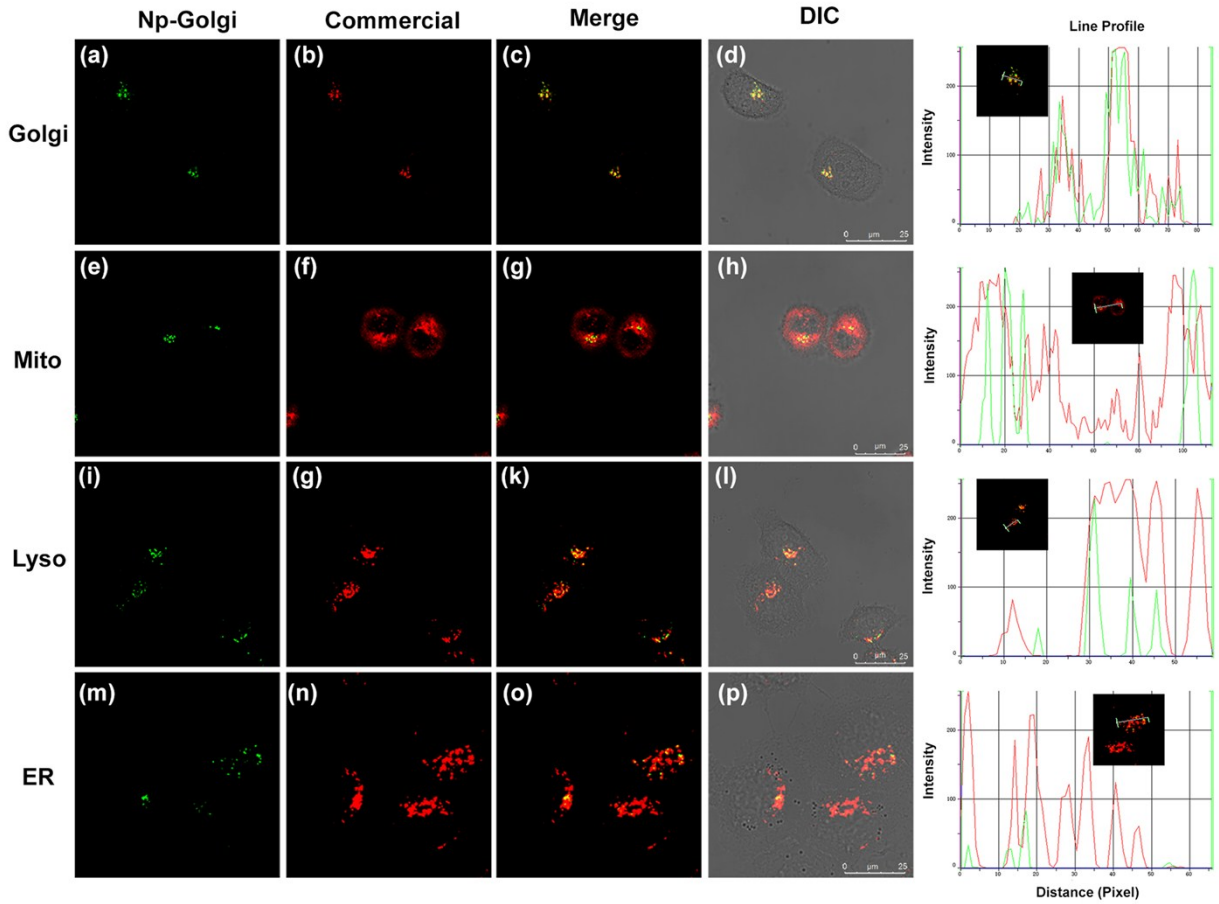


Figure S12. Co-localization cell imaging of Np-Golgi and commercial dyes in 7702 cells. Golgi (a, b, c, d), mitochondria (e, f, g, h), lysosomes (i, j, k, l), endoplasmic reticulum (m, n, o, p).

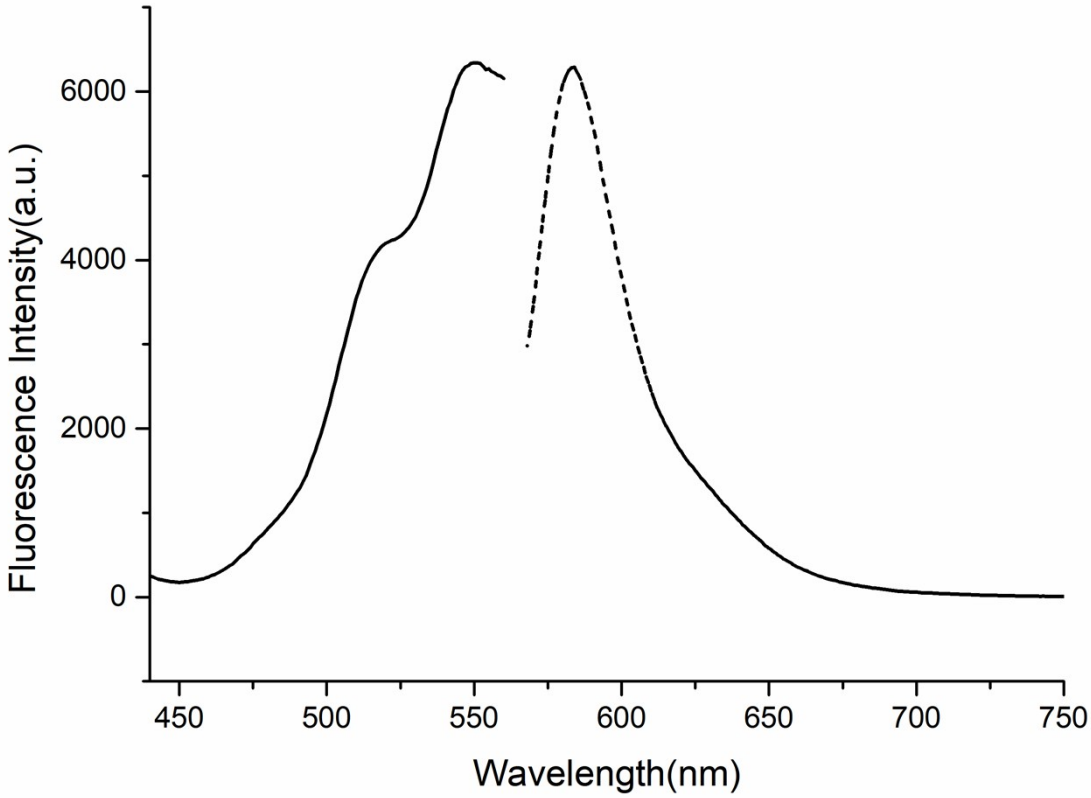


Figure S13. The absorption and emission spectra of Rd-Golgi (5 μM) in CH_2Cl_2.

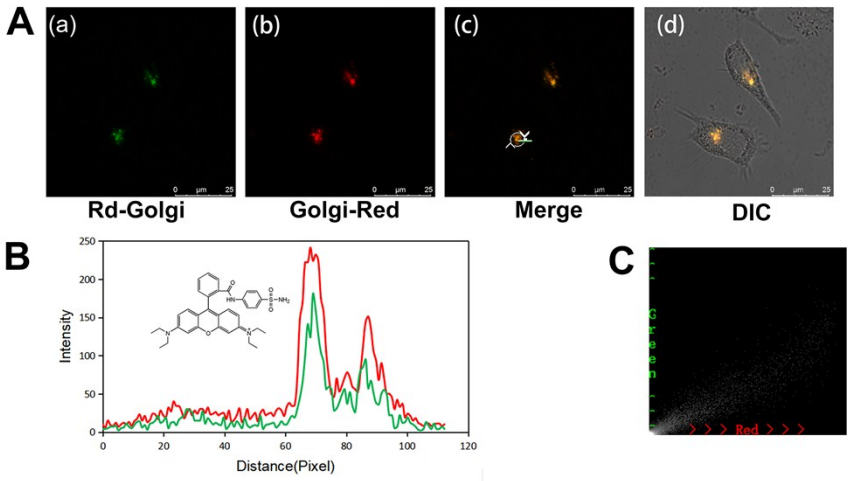


Figure S14. Cell location of Rd-Golgi in SMCC-7721. (A) Co-localization cell imaging of Rd-Golgi and commerial Golgi dyes Golgi-Red in SMCC-7721 cells. Golgi-Red: Ex=561nm, Em=610-630; Rd-Golgi: Ex: 514nm, Em:565-580. (B) Intensity profile along the white circle in the image A (c). (C) Scatter Plot of the white circle of the image A (c).

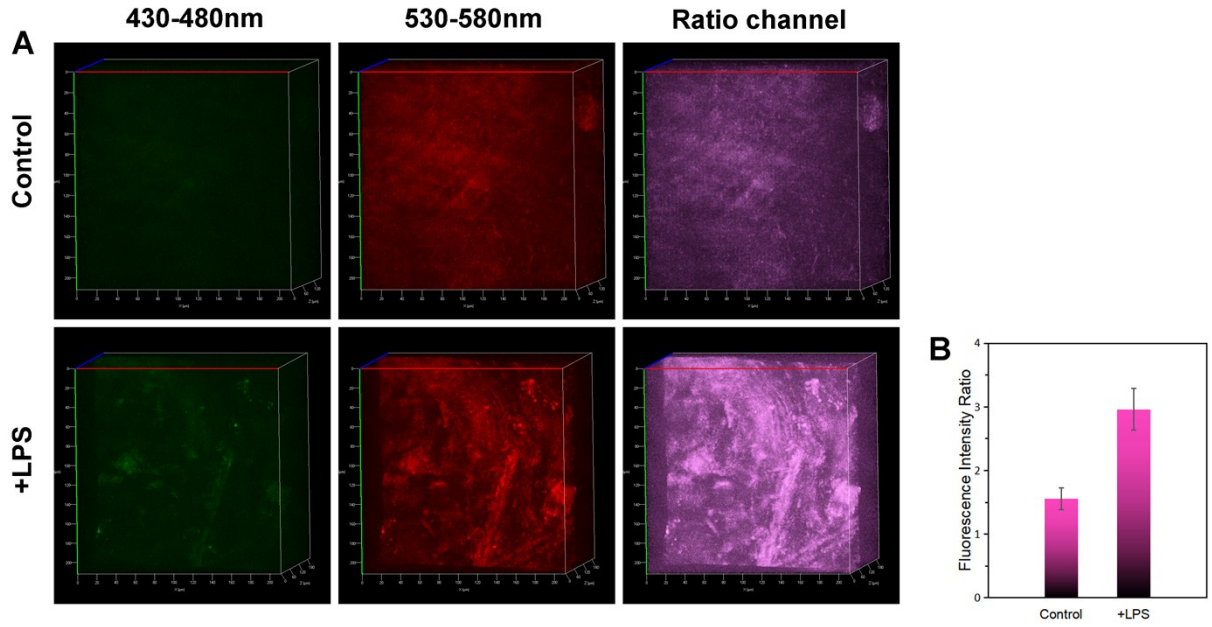


Figure S15. TP fluorescence imaging of LPS-induced mice. (A) Two-photon fluorescence imaging on the left side (control: without stimulated) or on the right side (LPS-induced) of abdominal cavity of mice. (B) The Fluorescence intensity ratio of control and LPS-induced site. TP Excited: 810 nm.

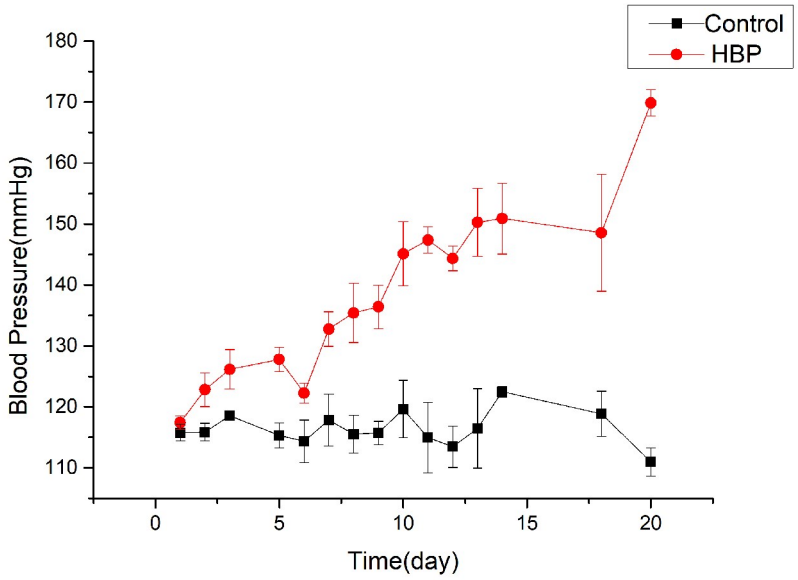


Figure S16. The blood pressure of mice intraperitoneally injected with ouabain (red) or saline (black) was measured in 20 days. (n=8)

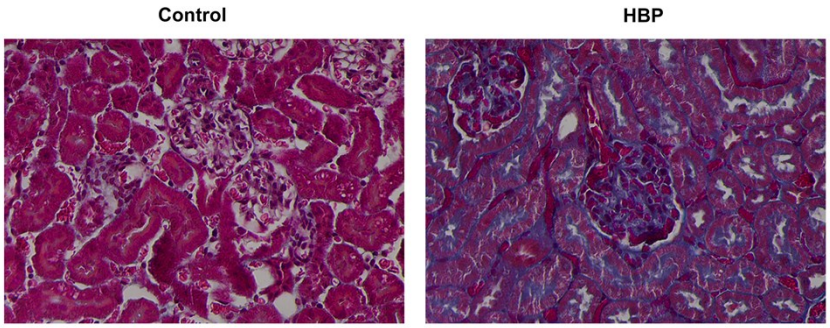


Figure S17. The Masson staining analysis of kidneys in normal mice and HBP mice. As shown in the figure, the blue staining are fibrotic tissue aeras. The kidneys of HBP mice showed obvious fibrosis compared with control mice.

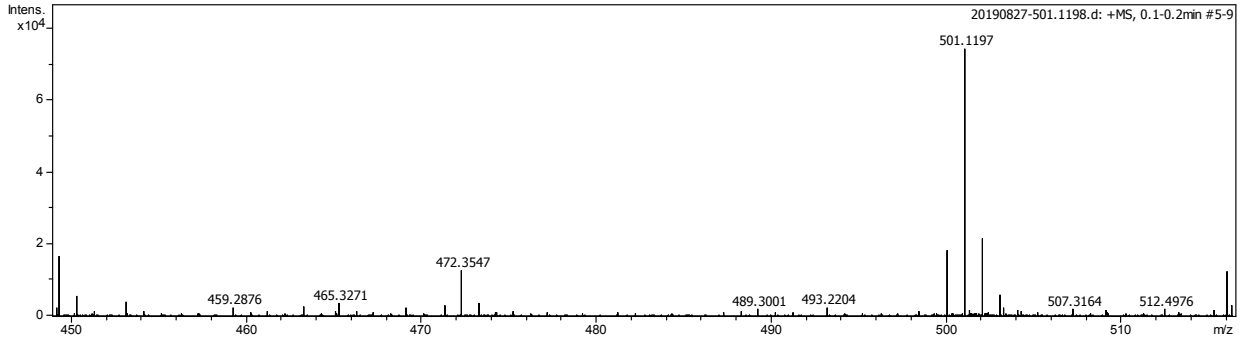


Figure S18. Mass spectrum of Np-Golgi.

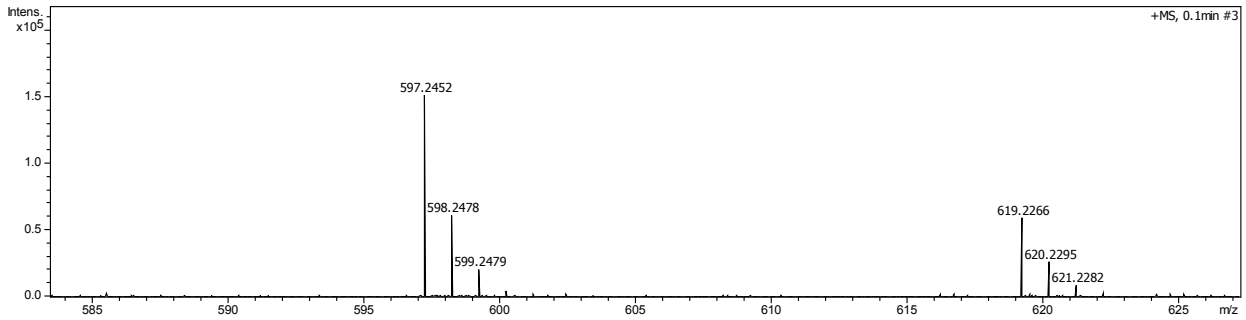


Figure S19. Mass spectrum of Rd-Golgi.

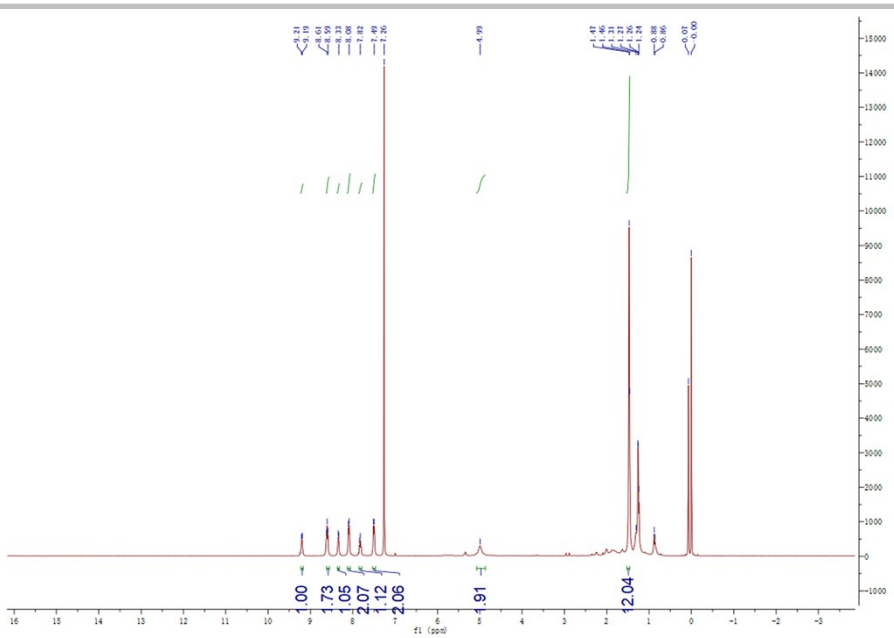


Figure S20. ^{1}H NMR spectrum of Np-Golgi.

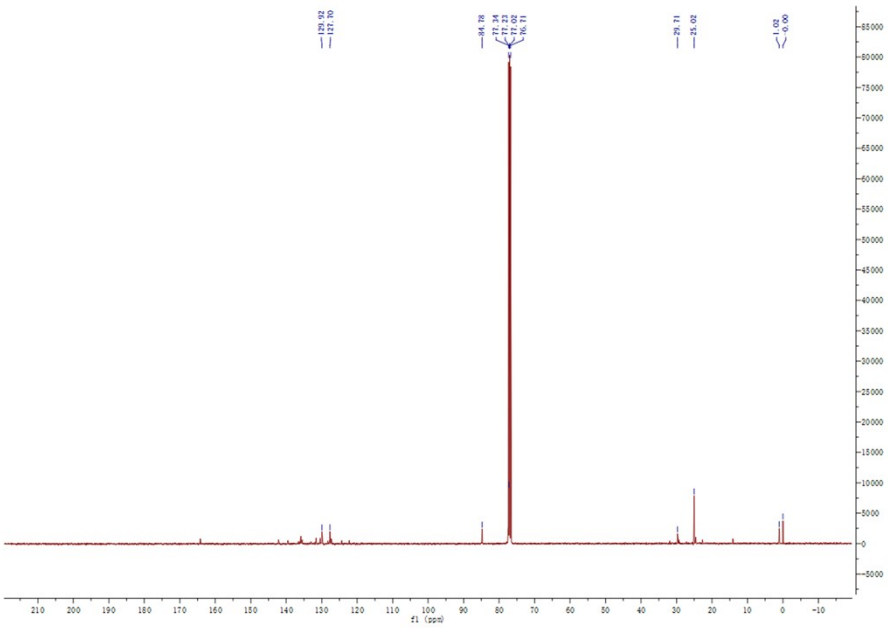


Figure S21. ^{13}C NMR spectrum of Np-Golgi.

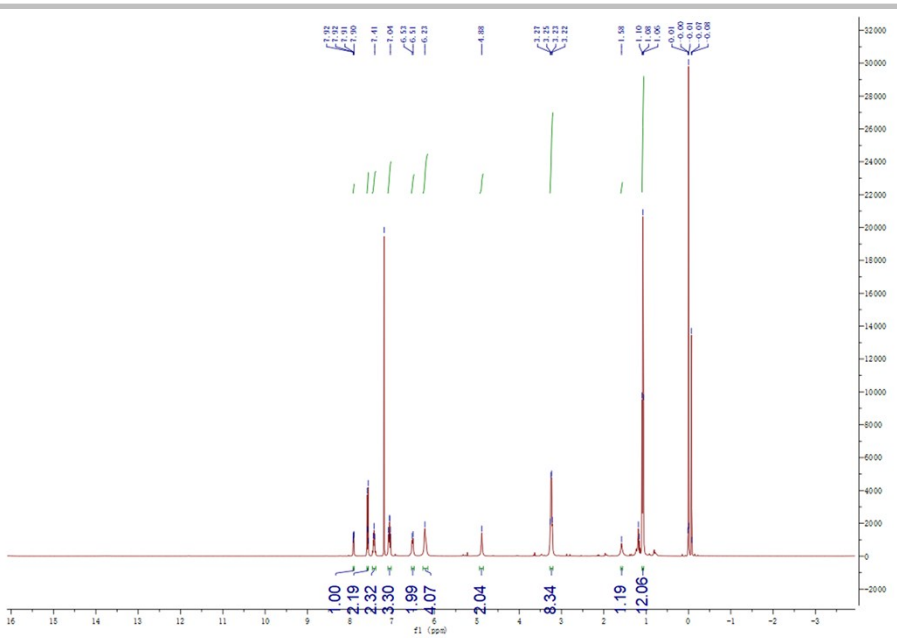


Figure S22. ^{1}H NMR spectrum of Rd-Golgi.

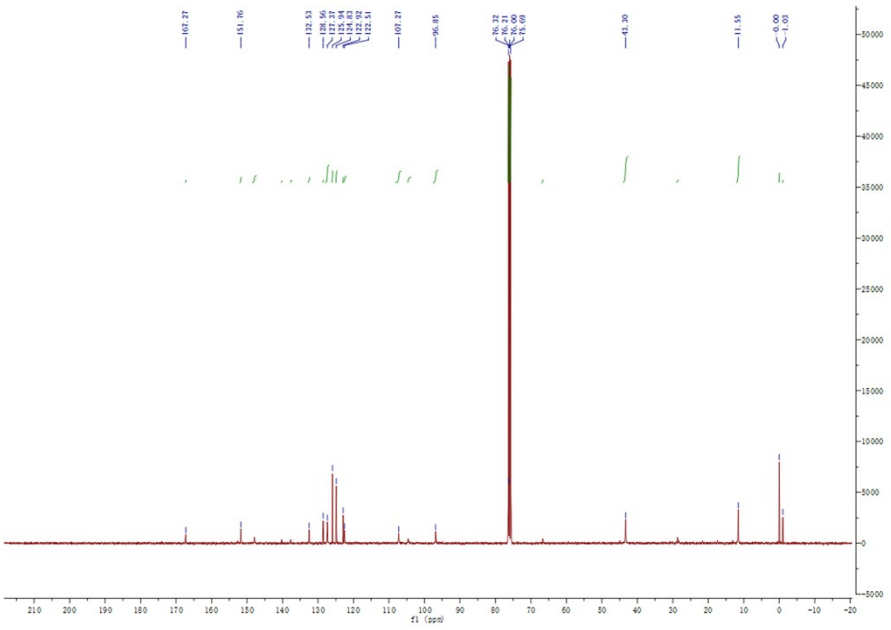


Figure S23. ^{13}C NMR spectrum of Rd-Golgi.

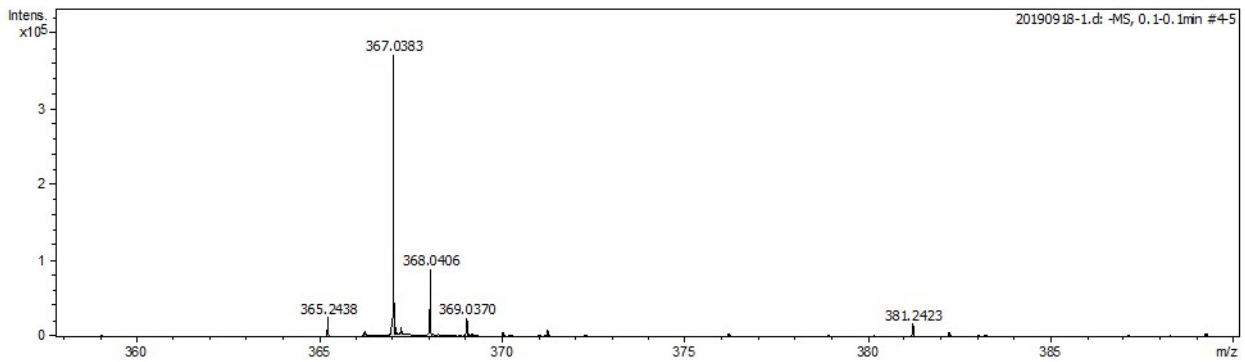


Figure S24. Mass spectrum of Np-OH. HRMS (ESI) m/z: [M-H] calculated for $C_{18}H_{12}N_2O_5S$, 367.0407 found 367.0383.

References

[1] X. Liang, X. Xu, D. Qiao, Z. Yin, L. Shang, *Chem Asian J* **2017**, *12*, 3187-3194.
[2] M. Rami, A. Innocenti, J. L. Montero, A. Scozzafava, J. Y. Winum, C. T. Supuran, *Bioorg Med Chem Lett* **2011**, *21*, 5210-5213.
[3] M. A. Albota, C. Xu, W. W. Webb, *Appl Opt* **1998**, *37*, 7352-7356.
[4] W. Zhang, J. Zhang, P. Li, J. Liu, D. Su, B. Tang, *Chem Sci* **2019**, *10*, 879-883.
[5] P. Kumar, A. Nagarajan, P. D. Uchil, *Cold Spring Harb Protoc* **2018**, *2018*, pdb prot095505.
[6] aW. J. Cheung, M. A. Kent, E. El-Shahat, H. Wang, J. Tan, R. White, F. H. Leenen, *Am J Physiol Heart Circ Physiol* **2006**, *291*, H624-630; bI. Dostanic, R. J. Paul, J. N. Lorenz, S. Theriault, J. W. Van Huysse, J. B. Lingrel, *Am J Physiol Heart Circ Physiol* **2005**, *288*, H477-485.

Author Contributions

H. W., P. L. and B. T. designed the experiments. Z. H. and H. W. performed the experiments and analyzed the data. J. Z., Y. Y., W. Z. and W. Z. assisted with the experiments. H. W., P. L., Z. H. and B. T. wrote the manuscript.