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# A highly selective near-infrared fluorescent probe for imaging H<sub>2</sub>Se in living cells and *in vivo*†

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Hydrogen selenide (H<sub>2</sub>Se), a highly reactive Se species, is an important selenium metabolism intermediate involved in many physiological and pathological processes. This compound is of scientific interest with regard to the real-time monitoring of H<sub>2</sub>Se in living cells and *in vivo* to understand the anti-cancer mechanism of selenium. However, monitoring H<sub>2</sub>Se in living cells is still challenging due to the lack of straight forward, highly selective and rapid methods. Here, we developed a novel small-molecule fluorescent probe, **NIR-H<sub>2</sub>Se**, for imaging endogenous H<sub>2</sub>Se. **NIR-H<sub>2</sub>Se** exhibited high selectivity toward H<sub>2</sub>Se over selenocysteine (Sec), H<sub>2</sub>S and small molecule thiols and was successfully used to image the H<sub>2</sub>Se content in HepG2 cells during Na<sub>2</sub>SeO<sub>3</sub>-induced apoptosis. Increased H<sub>2</sub>Se content and reduced ROS levels were observed under hypoxic conditions compared to normoxic conditions, which indicated that the cell apoptosis induced by Na<sub>2</sub>SeO<sub>3</sub> under a hypoxic environment is *via* a non-oxidative stress mechanism. Thus, this probe should serve as a powerful tool for exploring the physiological function of H<sub>2</sub>Se and Se anticancer mechanisms in a variety of physiological and pathological contexts.

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## Introduction

Selenium (Se) is an essential trace element for various physiological functions in the human body. Insufficient or excessive Se intake has been associated with a number of diseases.<sup>1,2</sup> Importantly, selenium plays a role in cancer prevention and treatment.<sup>3</sup> However, the anticancer mechanism for Se is still not fully understood. The effectiveness of Se compounds as anticancer agents is correlated to their chemical form and dose.<sup>4</sup> H<sub>2</sub>Se, a reduced form of selenium, is an important metabolite of dietary Se compounds.<sup>5</sup> Endogenous H<sub>2</sub>Se is generated by reducing selenite *via* GSH and other reduction systems<sup>6</sup> and is involved in many physiological and pathological processes.<sup>7</sup> A reliable and rapid method to determine H<sub>2</sub>Se *in vivo* must be developed to investigate the physiological function of H<sub>2</sub>Se and the anticancer mechanism of Se.

Fluorescent probes with high sensitivity, good selectivity, and short response times *via* direct observation are powerful tools for the *in vivo* detection of biomolecules.<sup>8</sup> Several fluorescent probes for detecting Se species were recently reported. Maeda *et al.* developed the first fluorescent probe, BESThio, to

discriminate selenols from their Cys counterparts based on their different nucleophilicity at pH = 5.8.<sup>9</sup> Using the recognition group Maeda reported, Fang and Lin exploited probes by adjusting the fluorophore to detect selenols with high selectivity under physiological conditions.<sup>10</sup> In addition, Fang *et al.* developed another novel fluorescent probe (TRFS-green) using a 1,2-dithiolane reporter group to selectively image thioredoxin reductase (TrxR) at pH = 7.4.<sup>11</sup> Wu reported a cadmium sulfide (CdS) quantum dot (QD) probe for HSe<sup>−</sup> ions in aqueous solution.<sup>12</sup> However, a small-molecule fluorescent probe for imaging the highly reactive H<sub>2</sub>Se in living cells and *in vivo* has not been reported.

Previously, our group discovered that the Se–N bond can be cleaved using sulfhydryl groups and we developed a series of fluorescence methods to monitor thiols and their redox state in living cells.<sup>13–15</sup> We subsequently found that the Se–N bond in 2,1,3-benzoselenadiazole (BS) can be specifically broken by selenol *via* direct nucleophilic addition and we designed a new fluorescent probe (HB) to image endogenous Sec in living cells and *in vivo*.<sup>16</sup> These results inspired us to develop a H<sub>2</sub>Se fluorescent probe based on the BS group by modifying the probe molecule structure.

Here, we integrated the BS moiety into a near infrared (NIR) merocyanine dye<sup>17</sup> to develop a novel small-molecule fluorescent probe for detecting H<sub>2</sub>Se (**NIR-H<sub>2</sub>Se**). **NIR-H<sub>2</sub>Se** responds to H<sub>2</sub>Se rapidly, with a high selectivity over H<sub>2</sub>S, Sec and biological thiols and was used to successfully image endogenous H<sub>2</sub>Se in living cells and *in vivo*. Moreover, we used a radical oxygen species (ROS) probe and found that the H<sub>2</sub>Se contents increased during HepG2 cell apoptosis induced by Na<sub>2</sub>SeO<sub>3</sub> in a time- and

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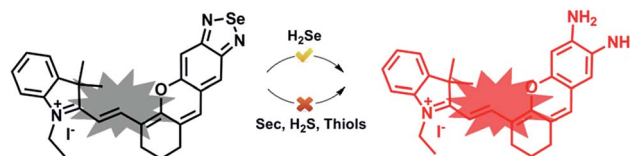
dose-dependent manner, while the ROS level did not change under a hypoxic environment. This probe will provide a powerful tool for investigating the metabolism and antitumor mechanism of Se compounds.

## Results and discussion

The syntheses are illustrated in Scheme 1, and the probe and intermediate structures were fully characterized by  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, and HRMS. We first examined the spectroscopic properties of this probe and optimized the fluorescence measurement conditions (Fig. S1 and S2, ESI†). **NIR-H<sub>2</sub>Se** exhibits an emission maximum at 735 nm with weak fluorescence ( $\Phi = 0.019$ ) in aqueous solutions buffered at physiological pH (10 mM phosphate buffered saline, PBS, pH = 7.4) due to effective fluorescence quenching of the BS *via* the heavy atom effect of Se. As predicted, the BS moiety in **NIR-H<sub>2</sub>Se** reacts selectively with H<sub>2</sub>Se over Sec, H<sub>2</sub>S, and small molecule thiols. The HPLC analysis confirmed that the fluorescent product was a diamino product (Scheme 2 and Fig. S3 and S4, ESI†).

We then tested the fluorescence response to H<sub>2</sub>Se. Adding H<sub>2</sub>Se increased the fluorescence intensity of **NIR-H<sub>2</sub>Se** *ca.* 10-fold ( $\Phi = 0.13$ , Fig. 1a). There was good linearity between the fluorescence intensities and H<sub>2</sub>Se concentrations for the range 0–12  $\mu\text{M}$  (Fig. 1b). The regression equation was  $F = 2096.7 + 1772.3[\text{H}_2\text{Se}] \mu\text{M}$  with a linear coefficient of 0.9941. The limit of detection was 7.0 nM (standard deviation 3.5%,  $n = 11$ ).

To evaluate the selectivity of **NIR-H<sub>2</sub>Se** for H<sub>2</sub>Se, we measured the fluorescence spectra for the probe with H<sub>2</sub>Se, H<sub>2</sub>S and thiols. High concentrations of thiols such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) (1 mM for each) produced low fluorescence responses, and **NIR-H<sub>2</sub>Se** also gave a negative response for H<sub>2</sub>S, Sec, Na<sub>2</sub>SeO<sub>3</sub>, *N*-acetyl-L-cysteine (NAC), thioredoxin reductase (TrxR) and vitamin C (Vc). The reactivity of **NIR-H<sub>2</sub>Se** towards ROS was then tested. Fig. 2a shows that biologically relevant ROS, including H<sub>2</sub>O<sub>2</sub>,  $^-\text{OCl}$ , O<sub>2</sub> $^{2-}$ , and ROO $^{\cdot}$ , did not trigger any fluorescence changes in the probe. Moreover, considering that nitric oxide (NO) may be a potential interfering species,<sup>18</sup> the fluorescence response of **NIR-H<sub>2</sub>Se** to differing amounts of NO were studied both with and without H<sub>2</sub>Se. The results indicated that even high NO concentrations (10 equiv.) did not induce an obvious fluorescence change in the detection system. The interference from



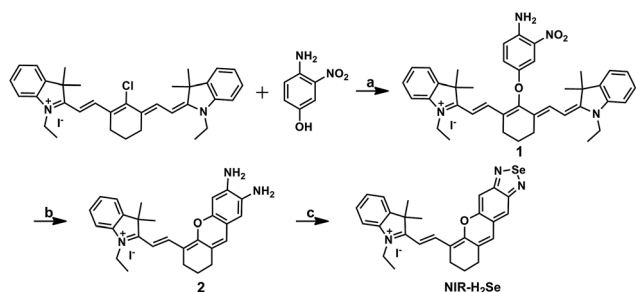
Scheme 2 Proposed mechanism for the selective reaction of H<sub>2</sub>Se.

metal ions and other amino acids were also tested as shown in Fig. S5 and S6.† Collectively, **NIR-H<sub>2</sub>Se** selectively recognized H<sub>2</sub>Se under physiological conditions.

Considering the variable nature and quick metabolism of endogenous H<sub>2</sub>Se in biological systems, a fast-responding method for H<sub>2</sub>Se detection is necessary. The response of **NIR-H<sub>2</sub>Se** to H<sub>2</sub>Se was evaluated *via* a kinetics experiment. The results indicated that the fluorescence intensity immediately increased to its maximum after adding H<sub>2</sub>Se to the probe solution, which indicated that the probe instantly responds to H<sub>2</sub>Se (Fig. 2b). In addition, the cytotoxicity of the probe in HepG2 cells was determined *via* a conventional MTT assay (Fig. S7, ESI†), which indicated that the **NIR-H<sub>2</sub>Se** probe exhibited low biotoxicity and could be used as a viable probe for detecting H<sub>2</sub>Se in biological samples.

After confirming the high sensitivity, selectivity and rapid response of this probe for H<sub>2</sub>Se, we explored its applications to image endogenous H<sub>2</sub>Se in living cells. During these cell imaging experiments, Na<sub>2</sub>SeO<sub>3</sub> was used as the H<sub>2</sub>Se precursor because sodium selenite can be metabolized to hydrogen selenide (H<sub>2</sub>Se) *via* selenodiglutathione (GSSeSG) and glutathione selenenylsulfide (GSSeH).<sup>19</sup> HepG2 cells were treated with 2–10  $\mu\text{M}$  Na<sub>2</sub>SeO<sub>3</sub> for 12 h or incubated with 5  $\mu\text{M}$  Na<sub>2</sub>SeO<sub>3</sub> for 2–12 h, as our previous study found that these action concentrations and times of Na<sub>2</sub>SeO<sub>3</sub> can induce HepG2 cell apoptosis.<sup>16</sup> After treatment, the cells were loaded with 10  $\mu\text{M}$  of **NIR-H<sub>2</sub>Se**. The experimental results are shown in Fig. 3. Higher H<sub>2</sub>Se contents were observed in hypoxic environments than under normoxic conditions during these parallel experiments.

We found that the apparent discrepancies above were related to the aerobic metabolism of H<sub>2</sub>Se. Under normoxic conditions (20% pO<sub>2</sub>), H<sub>2</sub>Se rapidly oxidized to generate a mass of superoxide anion radicals (O<sub>2</sub> $^{\cdot-}$ ) and an accumulation of reactive



Scheme 1 Synthesis of **NIR-H<sub>2</sub>Se**. Reaction conditions: (a) NaH, DMF, rt, 73%; (b) SnCl<sub>2</sub>, HCl, CH<sub>3</sub>OH, 70 °C, 45%; (c) SeO<sub>2</sub>, grind, 71%.

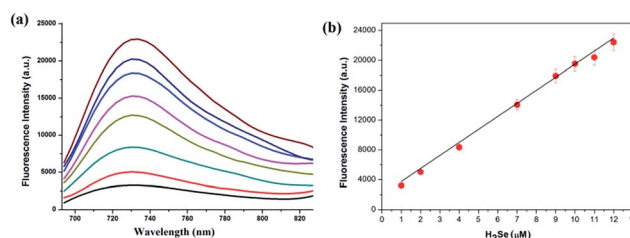


Fig. 1 (a) Fluorescence response of 10  $\mu\text{M}$  **NIR-H<sub>2</sub>Se** to differing amounts of H<sub>2</sub>Se. (b) Linear correlation between the emission intensity and H<sub>2</sub>Se concentration. All spectra were acquired in 10 mM PBS with a pH of 7.4 ( $\lambda_{\text{ex}}/\lambda_{\text{em}} = 688/735 \text{ nm}$ ). The data were expressed as the mean  $\pm$  standard deviation (SD) across three experiments.



**(a)** Selenite 12 h

	Control	2 $\mu\text{M}$	5 $\mu\text{M}$	10 $\mu\text{M}$
20% $\text{pO}_2$				
1% $\text{pO}_2$				

**(b)** Selenite 5  $\mu\text{M}$

	Control	2 h	6 h	12 h
20% $\text{pO}_2$				
1% $\text{pO}_2$				

**(c)** Na<sub>2</sub>SeO<sub>3</sub> Concentration ( $\mu\text{M}$ )

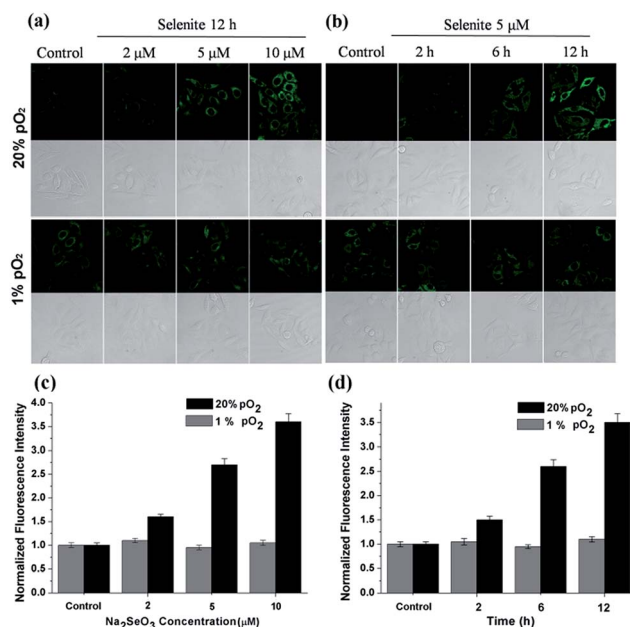
Na <sub>2</sub> SeO <sub>3</sub> Concentration ( $\mu\text{M}$ )	20% $\text{pO}_2$	1% $\text{pO}_2$
Control	1.0	1.0
2	1.5	1.6
5	1.8	2.7
10	2.3	3.4

**(d)** Time (h)

Time (h)	20% $\text{pO}_2$	1% $\text{pO}_2$
Control	1.0	1.0
2	1.5	1.6
6	1.8	2.5
12	2.4	3.5

oxygen species during the tumor apoptosis process induced by  $\text{Na}_2\text{SeO}_3$ .<sup>20</sup> However, under a hypoxic environment (1%  $\text{pO}_2$ ),  $\text{H}_2\text{Se}$  cannot quickly metabolize due to the lack of oxygen, which allows  $\text{H}_2\text{Se}$  to effectively accumulate. To further confirm our

The above results indicate that **NIR-H<sub>2</sub>Se** has potential for detecting endogenous H<sub>2</sub>Se *in vivo*. To evaluate this proposal, mice bearing subcutaneously implanted tumors grown from murine hepatoma cell line H22 were subcutaneously injected with buffer solutions containing **NIR-H<sub>2</sub>Se** (10 μM) and sodium selenite (10 μM), and fluorescence images were then obtained at



Chem. Sci., 2016, 7, 1051–1056 | 1053



different times using an *in vivo* imaging system (IVIS). Fig. 5 shows that the fluorescence signal of the probe was exclusively observed in the tumor region without a background signal, and that the fluorescence intensity increased from 3 h to 12 h post injection. The results indicated that the hypoxic solid tumor generated  $\text{H}_2\text{Se}$  from sodium selenite and that the  $\text{H}_2\text{Se}$  gradually accumulated.

## Conclusions

In summary, we developed a novel small-molecule fluorescent probe, **NIR- $\text{H}_2\text{Se}$** , to detect  $\text{H}_2\text{Se}$ . The probe rapidly responded to  $\text{H}_2\text{Se}$ , exhibited high sensitivity and good selectivity for  $\text{H}_2\text{Se}$  over  $\text{Sec}$ ,  $\text{H}_2\text{S}$ , ROS and other sulfur-containing species and was successfully used to image endogenous  $\text{H}_2\text{Se}$  in living cells and *in vivo*. Furthermore, we found that normoxic conditions increased the  $\text{H}_2\text{O}_2$  content, while the  $\text{H}_2\text{Se}$  level rose only slightly during HepG2 cell apoptosis induced by  $\text{Na}_2\text{SeO}_3$ . However,  $\text{H}_2\text{Se}$  accumulated gradually and the ROS remained low in a hypoxic environment. These findings indicate that the anticancer mechanism of Se for hypoxic solid tumors is *via* non-oxidative stress. We anticipate that the current probe will provide an ideal tool for further studies into the biological functions of  $\text{H}_2\text{Se}$  and Se anticancer mechanisms.

## Experimental

### Materials and instruments

All chemicals were available commercially and the solvents were purified by conventional methods before use. Cysteine (Cys), homocysteine (Hcy), glutathione (GSH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company.  $\text{Na}_2\text{SeO}_3$ , *N*-acetyl-L-cysteine (NAC), thioredoxin reductase (TrxR) and vitamin C (Vc) were purchased from Sigma-Aldrich Co. Ltd. The silica gel (100–200 mesh) was used for the flash chromatography. Cy.7.Cl was synthesized in our laboratory. Sartorius ultrapure water (18.2 M $\Omega$  cm) was used throughout the analytical experiments.  $\text{H}_2\text{Se}$  was prepared by the reaction of  $\text{Al}_2\text{Se}_3$  with  $\text{H}_2\text{O}$  in an  $\text{N}_2$  atmosphere for 30 min at room temperature before use every time.<sup>24</sup>  $\text{H}_2\text{O}_2$ , *tert*-butylhydroperoxide (TBHP), and hypochlorite (NaOCl) were delivered from 30%, 70%, and

10% aqueous solutions respectively. Nitric oxide (NO) was used from a stock solution prepared by sodium nitroprusside. Superoxide ( $\text{O}_2^{\cdot-}$ ) was delivered from  $\text{KO}_2$  in DMSO solution or from xanthine oxidase.

$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were determined using 300 MHz and 400 MHz Bruker NMR spectrometers. The mass spectra were obtained using a Bruker maXis ultra-high resolution-TOF MS system. The melting points were measured using a SGW X-4 Melting Point Tester. The fluorescence spectra measurements were performed using an FLS-920 Fluorescence Spectrometer (Edinburgh Instruments, UK). All pH measurements were performed with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass/calomel electrode. The fluorescence images of cells were taken using a TCS SP5 confocal laser scanning microscope with an objective lens ( $\times 40$ ). The absorbance was measured using a TRITURUS microplate reader in the MTT assay.

### Synthesis and characterization of NIR- $\text{H}_2\text{Se}$

**Synthesis of compound 1.** 4-Amino-3-nitrophenol (39 mg, 0.25 mmol) and NaH (60% in mineral oil) (10 mg, 0.25 mmol) were dissolved in anhydrous *N,N*-dimethylformamide (DMF) (7 mL). The mixture was stirred at room temperature for 10 min under an argon atmosphere. Then a solution of heptamethine cyanine chlorine (Cy.7.Cl) (64 mg, 0.10 mmol) in anhydrous DMF (2 mL) was added to the mixture *via* a syringe. The reaction mixture was further stirred for 4 h at room temperature. The solvent was removed under reduced pressure, then the crude product was purified by silica gel chromatography with 6% MeOH in dichloromethane (DCM) to afford the desired product as a dark green solid (55 mg, 73%). mp = 149–150 °C.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.26–1.40 (m, 18H), 2.03 (s, 4H), 2.17 (s, 2H), 2.68 (s, 4H), 4.08 (s, 4H), 5.97 (d,  $J$  = 12 Hz, 2H), 7.09 (d,  $J$  = 8 Hz, 2H), 7.21–7.29 (m, 5H), 7.54 (s, 3H), 7.80 (d,  $J$  = 8 Hz, 1H), 7.92 (d,  $J$  = 12 Hz, 2H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  12.2, 21.0, 24.3, 27.8, 39.4, 49.1, 99.3, 108.3, 110.2, 122.1, 122.4, 125.3, 128.7, 141.1, 141.5, 142.2, 143.4, 164.4, 171.7. HR MS [ $\text{M} - \text{I}$ ] $^+$ :  $m/z$  calcd 629.3486, found 629.3455.

**Synthesis of compound 2.**  $\text{SnCl}_2$  (900 mg, 4 mmol) and concentrated HCl (0.8 mL) were added to a solution of compound 1 (151 mg, 0.20 mmol) in MeOH (6 mL), stirred, and maintained at 70 °C overnight under an argon atmosphere. The mixture was neutralized with 2 N NaOH, and the precipitate was removed by filtration and washed with DCM. The filtrate and washings were washed with water. The organic layer was dried over  $\text{Na}_2\text{SO}_4$ , and the solvent was evaporated to yield a dark green solid (49 mg, 45%). mp = 225–228 °C.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.31 (s, 3H), 1.69 (s, 6H), 1.84 (s, 2H), 2.57 (s, 2H), 2.70 (s, 2H), 3.89 (s, 2H), 4.63 (s, 1H), 5.68 (s, 1H), 6.75 (s, 1H), 6.86–6.92 (m, 2H), 7.03 (s, 1H), 7.28 (s, 2H), 7.39 (s, 2H), 8.10 (d,  $J$  = 12 Hz, 1H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  11.6, 21.0, 24.5, 28.3, 28.7, 48.0, 97.5, 107.2, 108.4, 115.7, 121.5, 128.2, 137.1, 139.3, 148.5, 152.6, 160.8, 166.8. HR MS [ $\text{M} - \text{I}$ ] $^+$ :  $m/z$  calcd 412.2383, found 412.2378.

**Synthesis of NIR- $\text{H}_2\text{Se}$ .** To a porcelain mortar was added compound 2 (108 mg, 0.20 mmol) and  $\text{SeO}_2$  (22 mg, 0.2 mmol).

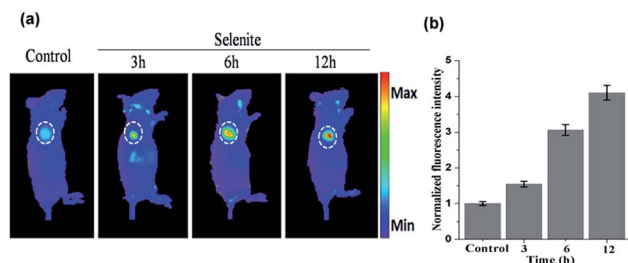


Fig. 5 (a) *In vivo* fluorescence imaging of H22 tumor-bearing mice injected with the probe NIR- $\text{H}_2\text{Se}$  (10  $\mu\text{M}$ ) in response to sodium selenite (10  $\mu\text{M}$ ) at different times. (b) The fluorescence intensity for (a). The data were normalized to the control.



The mixture was fully grinded for 30 min, and TLC showed full conversion of compound 2 to the probe. The resulting mixture was extracted by MeOH (20 mL) and the solvent was removed under reduced pressure, then the crude product was purified by silica gel chromatography with 6% MeOH in DCM to afford the desired product as a dark purple solid (87 mg, 71%). mp = 252–253 °C.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.61 (t,  $J$  = 6 Hz, 3H), 1.86 (s, 6H), 2.01 (t,  $J$  = 3 Hz, 2H), 2.41 (s, 2H), 2.75 (t,  $J$  = 3 Hz, 2H), 3.00 (t,  $J$  = 3 Hz, 2H), 5.00 (q,  $J$  = 6 Hz, 2H), 7.38 (d,  $J$  = 16 Hz, 1H), 7.49 (s, 1H), 7.54–7.59 (m, 4H), 7.68 (s, 1H), 7.74 (d,  $J$  = 16 Hz, 1H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  13.7, 14.1, 20.6, 24.5, 29.3, 30.9, 42.9, 51.7, 104.7, 110.7, 114.3, 119.0, 119.6, 122.5, 124.7, 126.5, 128.0, 128.8, 129.7, 132.6, 140.7, 142.9, 146.6, 152.7, 155.5, 179.6. HR MS:  $[\text{M} - \text{I}]^+$ :  $m/z$  calcd 488.1218, found 488.1237.

### Fluorescence analysis

Fluorescence spectra were obtained with an FLS-920 Fluorescence Spectrometer (Edinburgh Instruments, UK). After dilution to 10  $\mu\text{M}$  of the probe with 10 mM PBS, various amounts of  $\text{H}_2\text{Se}$  were added. The fluorescence intensity was measured at  $\lambda_{\text{ex}}/\lambda_{\text{em}}$  = 688/735 nm.

### Cell culture

HepG2 cells were maintained following the protocols provided by the American Type Tissue Culture Collection. Cells were first grown in a circular Petri dish (60 mm) using high glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g of glucose per L) supplemented with 10% fetal bovine serum (FBS),  $\text{NaHCO}_3$  (2 g  $\text{L}^{-1}$ ) and 1% antibiotics (penicillin/streptomycin, 100 U  $\text{mL}^{-1}$ ). Cultures were maintained in a humidified incubator at 37 °C, in 5%  $\text{CO}_2$ /95% air. One day before imaging, cells were passed and plated on 18 mm glass coverslips in a culture dish. The culture medium was refreshed every 24 h. All cells used were in the exponential growth phase.

### Confocal imaging

Fluorescence imaging studies were performed with a TCS SP5 confocal laser scanning microscope (Germany Leica Co., Ltd) with an objective lens ( $\times 40$ ). Excitation of the  $\text{H}_2\text{Se}$  probe-loaded cells at 633 nm was carried out with an argon laser, and emission was collected using a META detector between 650 and 750 nm. Excitation of the  $\text{H}_2\text{O}_2$  probe-loaded cells at 532 nm was carried out with an argon laser, and emission was collected using a META detector between 650 and 750 nm. Prior to imaging, the medium was removed. Cell imaging was carried out after washing cells with PBS (pH = 7.4, 10 mM) three times.

### Animal and tumor models

All animal experiments were carried out according to the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, and approved by the local Animal Care and Use Committee. Six- to eight-week-old female Kunmin male mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. During

procedures, the mice were anesthetized with inhaled isoflurane.  $1 \times 10^6$  H22 cells were injected into the enterocoelia of each Kunmin mouse, ascites were formed after 5 or 7 days, which were further used after three passages. Four- to six-week-old nude mice received a subcutaneous injection of  $1 \times 10^6$  H22 ascite tumor cells into the axillary lateral subcutaneous of their right forelimbs. Tumors were then allowed to grow over a period of 15 to 20 days until reaching 0.5–1.5 cm in diameter.

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