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A hydrogen sulfide probe activates Nrf2, inhibits cancer cell growth and induces cell apoptosis

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It has been demonstrated that a relatively high level of endogenous hydrogen sulfide (H$_2$S) promotes cancer cell growth. Thus, reducing endogenous H$_2$S levels with a small molecule that selectively targets H$_2$S in cancer cells is an attractive strategy to treat cancer. In this study, we synthesized a 3-hydroxyflavone-based dual probe (**HF-NBD**) targeting H$_2$S with favourable photostability, and found that it could also activate the nuclear factor erythroid-2-related factor 2 (Nrf2) and upregulate heme oxygenase-1 (HO-1) expression. **HF-NBD** inhibited the growth of A549 lung cancer cells, U87 malignant glioma cells and HeLa cervical cancer cells, and induced apoptosis in HeLa cells. Herein we provide **HF-NBD**, a novel Nrf2 activator that promotes HO-1 expression downstream of Nrf2. The data suggested that **HF-NBD** might be a potential lead compound for the development of new anti-cancer drugs.

Introduction

Cancer has become an increasingly serious problem in recent years. Especially in developing countries, cervical cancer is one of the most frequently occurring malignancies causing death in women, making it a pressing issue.[1] Likewise, lung cancer is a significant public health concern worldwide,[2] and treating malignant glioma in the central nervous system is one of the most formidable challenges, with high rates of recurrence and mortality.[3] Although there are some effective ways to prevent and reduce the occurrence of cancer, much remains to be done, such as finding more effective therapies to treat cancer or finding new effective drugs that can inhibit the growth of cancer cells without causing damage to healthy cells.

Hydrogen sulfide (H$_2$S), known for being a highly toxic gas with an unpleasant smell, is actually an endogenous signalling gas molecule[4] that regulates a range of physiological processes *via* the sulfhydration of target proteins.[5] Thus, H$_2$S plays an important role in cell activation. Nowadays, owing to advances in chemistry, biology research has progressed dramatically, and fluorescent probes can help us understand the cellular-level and subcellular-level better.[6] Thus, developing reaction-based biocompatible probes for the sensitive and selective fluorescence detection of H$_2$S can help to elucidate the mechanism of

the modulation of its signalling pathway, cellular function and involvement in diseases.[7] It has been reported that H$_2$S at low levels can protect mitochondria from oxidative stress damage.[8] In addition, a study showed that the H$_2$S-mediated formation of Cys151-persulfide (SSH) in the Kelch-like ECH-associated protein 1 (Keap 1) can cause Keap 1 to dissociate from Nrf2, leading to Nrf2 translocation into the nucleus, where it binds to antioxidant response elements and triggers the transcription of protective genes.[9,10] Therefore, low levels of endogenous H$_2$S can exert cytoprotective effects in healthy cells. However, the evidence shows that cystathionine b-synthase (CBS), which participates in the synthesis of endogenous H$_2$S, is overexpressed in cancer cells, suggesting that relatively high levels of endogenous H$_2$S can promote cancer cell growth.[11–13] Thus, reducing endogenous H$_2$S levels with small molecules that selectively target H$_2$S in cancer cells is an attractive approach for cancer treatment.

It is known that Nrf2, which is encoded by the nuclear factor erythroid 2-like 2 (NFE2L2) gene and is expressed in several tissues, belongs to the basic-leucine zipper (bZIP) family of transcription factors.[14] Cisplatin, an anticancer drug, leads to oxidative damage and death in HEp-2 cells by increasing Nrf2 and HO-1 expression, suggesting an anticancer role for Nrf2. Connecting H$_2$S with Nrf2, H$_2$S probes that may enhance the level of ROS and reduce endogenous H$_2$S could act as an Nrf2 activator and contribute to cancer treatment.

In this research, we investigated the effects of a 3-hydroxyflavone-based fluorescent probe, **HF-NBD**, which can detect and consume endogenous H$_2$S,[15] on the activity of Nrf2. We found that **HF-NBD** could increase the level of ROS in a time-dependent manner, and elevate the activity of Nrf2 and HO-1 mRNA expression. **HF-NBD** also inhibited the growth of A549 lung cancer cells, U87 malignant glioma cells and HeLa cervical

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cancer cells, and promoted apoptosis of HeLa cervical cancer cells.

Results and discussions

The HF-NBD probe can detect both endogenous and exogenous H₂S with favorable photostability

Although many drugs have been used in the clinical practice to treat cancer, it is still crucial to explore new efficient drugs that have specific targets. Endogenous H_2S plays an important role in cancer cell proliferation and therefore, a H_2S probe able to reduce endogenous H_2S levels may represent a strategy to treat cancer.[13] **HF-NBD** was synthesized and proven to be a H_2S probe,[15] and we further investigated it in HeLa cervical cells to detect its effects. We detected the levels of fluorescence in HeLa cervical cells after treatment with 1‰ DMSO or **HF-NBD** at 1, 5, and 10 μM for 0.5, 1, and 3 h, respectively (Fig. 1B). The data showed that upon treatment with **HF-NBD**, fluorescence increased in a dose-dependent and time-dependent manner. Based on these results, we decided to treat the cells with **HF-NBD** at 5 μM for 1 h for the subsequent experiments. It is well-known that cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) can catalyse the degradation of cysteine in living cells for H_2S production.[16] Similarly, the inhibition assay was also conducted by inhibiting CBS and CSE.[17,18] We utilized **HF-NBD** to detect the levels of fluorescence in HeLa cervical cells. Compared with fluorescence in the green channel without the addition of cysteine (control), the level of fluorescence with the addition of 200 μM cysteine was significantly higher. When cells were incubated with 1 mM propargylglycine (PAG) or 1 mM aminooxyacetic acid (AOAA) or PAG/AOAA, followed by incubation with the **HF-NBD** probe, almost no fluorescence change was observed compared to the control group (Fig. 1C). We also detected the level of fluorescence in HeLa cervical cells after treatment with 0, 0.1, 0.5, and 1 mM NaHS, followed by incubation with the **HF-NBD** probe and found that the relative fluorescence increased in a dose-dependent manner (Fig. 1D). The results confirmed that **HF-NBD** can target both endogenous and exogenous H_2S in HeLa cervical cells. We further investigated 5 μM **HF-NBD** and incubated it with HeLa cervical cells for 1 h to detect the photo stability during 0, 60, 120, 180, and 300 s, and the data suggested that **HF-NBD** does exhibit favourable photostability (Fig. 1E).

HF-NBD up regulated Nrf-2 activity as well as the expression of HO-1 in HeLa cells significantly

Because Nrf2 activators have shown strong potential in disease prevention,[19] we firstly investigated the effect of **HF-NBD** on Nrf2 activity. HeLa cells stably transfected with a luciferase-based Nrf2 reporter plasmid were treated with 10 μM **HF-NBD** for 12 h and 24 h (Fig. 2A). The luciferase assay showed that **HF-NBD** markedly increased Nrf2 activity compared with the control. Nrf2, a redox sensitive transcription factor, plays an important role as an antioxidant, protecting cells from oxidative stress.[20] It has been reported that oxidative stress can induce HO-1 gene transcription in tumorous cells through activation of
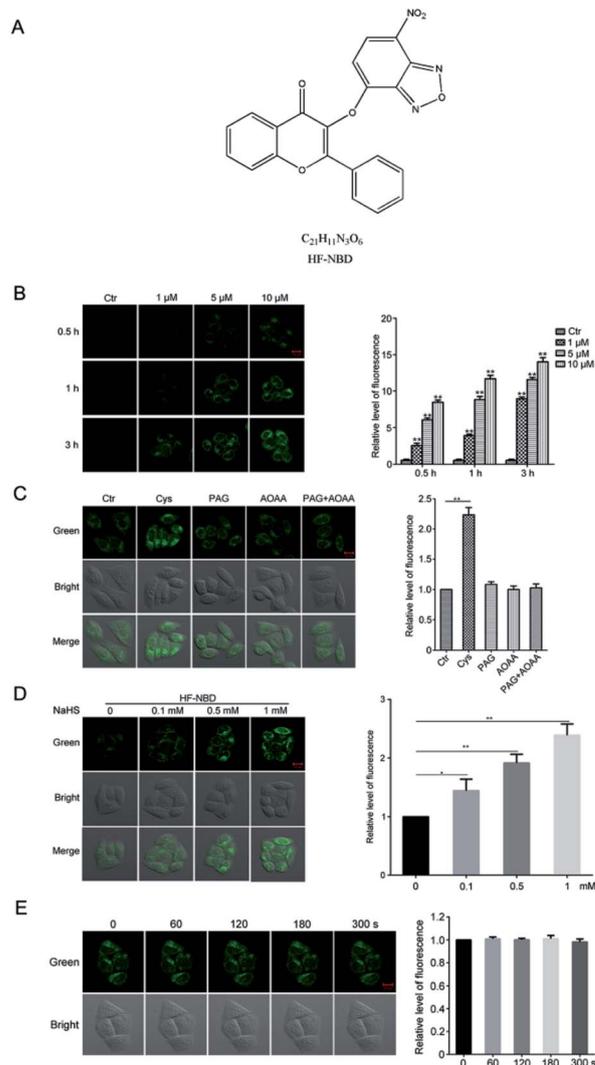

Fig. 1 HF–NBD can detect both endogenous and exogenous H_2S with favourable photostability. (A) Structure of **HF-NBD**. (B) Fluorescence in HeLa cells after treatment with 1‰ DMSO or **HF-NBD** at 1, 5, and 10 μM for 0.5, 1, and 3 h. (C) HeLa cells respectively treated with 200 μM cysteine, 1 mM propargylglycine (PAG), 1 mM aminooxyacetic acid (AOAA) or PAG/AOAA, fluorescence was measured after incubation with 5 μM **HF-NBD** for 1 h. (D) HeLa cells treated with 0, 0.1 and 0.5, and 1 mM NaHS, fluorescence was measured after incubation with **HF-NB**. (E) HeLa cells incubated with 5 μM **HF-NBD** for 1 h to evaluate photostability during 0, 60, 120, 180, 300 s. Data are presented as the mean ± SEM, *$p < 0.05$, **$p < 0.01$, $n = 3$.

Nrf-2,[21] where HO-1 is the downstream gene that is regulated by Nrf2 and responds to the stress. Thus, we detected the mRNA levels of HO-1 after treatment with 10 μM **HF-NBD** for 0.5, 1, 3, 6, 12, and 24 h (Fig. 2B). The results showed that **HF-NBD** could markedly upregulate the expression of HO-1 after treating with it for 12 h.

HF-NBD increases oxidative stress in a time-dependent manner

It has been reported that oxidative stress can alter the redox balance in tumorous microenvironments and impact metabolic
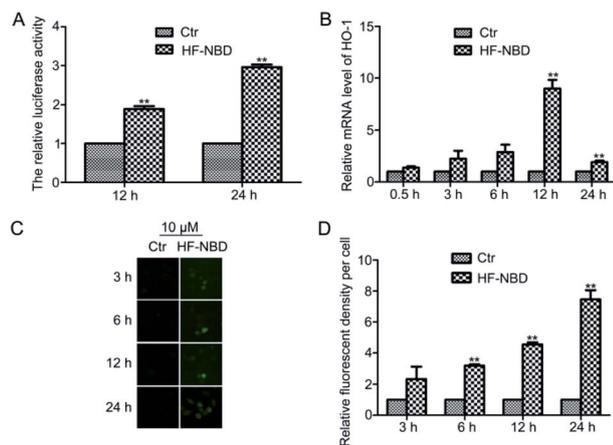
Fig. 2 HF-NBD upregulates Nrf2 activity, HO-1 expression, and induces oxidative stress in HeLa cells. (A) HeLa cells stably transfected with a luciferase-based Nrf2 reporter plasmid were treated with 10 μM HF-NBD for 12 h and 24 h. (B) HO-1 mRNA levels measured after treatment with 10 μM HF-NBD for 0.5, 1, 3, 6, 12, and 24 h. (C) ROS levels measured after treatment with 10 μM HF-NBD for 3, 6, 12, and 24 h. (D) Relative ROS levels normalized to the ROS levels in the control and expressed as percent of control. Data are presented as the mean ± SEM, *$p < 0.05$, **$p < 0.01$, $n = 3$.

Fig. 3 HF-NBD inhibits the growth of A549 lung cancer cells, U87 malignant glioma cells and HeLa cells with low IC$_{50}$ values. Cell viability of A549 cells (A), U87 cells (B) and HeLa cells (C), determined by the SRB assay after treatment with 1‰ DMSO (control) and HF-NBD at 1, 5, and 10 μM for 12 h and 24 h. (D) Morphological changes in HeLa cells after treatment with HF-NBD at 0.5, 1, 5, 10, and 20 μM for 6, 12, 24 and 48 h, as observed with a phase contrast microscope. Data are presented as the mean ± SEM, *$p < 0.05$, **$p < 0.01$, $n = 3$.

pathways in cancer cells.[22] Cancer cells are vulnerable to high levels of reactive oxygen species (ROS).[23] The evidence indicates that ROS influences proliferation and apoptosis in various types of cancer cells.[24] The overproduction of ROS results in oxidative stress and induces cell apoptosis.[25] We supposed that the increased Nrf2 activity and HO-1 expression may be a response to oxidative stress caused by HF-NBD, which reduces H$_2$S levels in cells. Thus, we detected the level of ROS after treatment with HF-NBD at 10 μM for 3, 6, 12, and 24 h (Fig. 2C). The data showed that HF-NBD increased ROS levels in a time-dependent manner (Fig. 2D), which conformed to our hypothesis that HF-NBD triggered the oxidative stress by reducing H$_2$S levels in cells.

Table 1 IC$_{50}$ values (μM, 24 h) for HF-NBD in A549 lung cancer cells, U87 malignant glioma cells and HeLa cells

Cell type	IC$_{50}$ values
A549 lung cancer cell	1.77
U87 malignant glioma cell	2.09
HeLa cervical cancer cell	6.73

HF-NBD inhibits the growth of A549 lung cancer cells, U87 malignant glioma cells and HeLa cervical cancer cells with low IC$_{50}$ values

We investigated the cell viability of A549 lung cancer cells (Fig. 3A), U87 malignant glioma cells (Fig. 3B) and HeLa cervical cancer cells (Fig. 3C) using sulforhodamine B (SRB) assay after treatment with HF-NBD at different concentrations for 12 h and 24 h to know if HF-NBD could affect the growth of cancer cells. We found that HF-NBD significantly inhibited the growth of these three types of cancer cells at low IC$_{50}$ values (Table 1).

In order to find out how HF-NBD influenced the growth of cancer cells, we selected HeLa cells for the subsequent experiment, since the HeLa cervical cancer cell line is the most widely used cell line in biomedical research.[26] We also observed the morphological changes in HeLa cells after treatment with 0.5, 1, 5, 10, and 20 μM HF-NBD for 6, 12, 24 and 48 h using a phase contrast microscope (Fig. 3D). The living cell density decreased and the cells shrank, with apoptotic bodies being released in
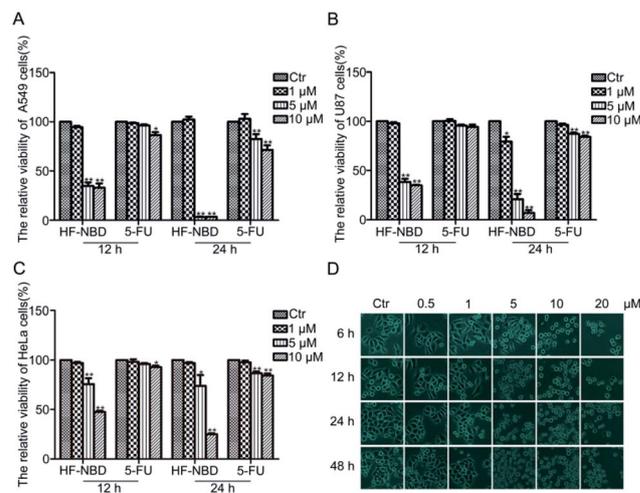
response to treatment with HF-NBD compared with the control group.

HF-NBD did not lead HeLa cervical cancer cells to necrosis

In natural conditions, for elucidating the mechanism of toxicity of a xenobiotic, several cell death pathways need to be taken into account.[27] According to a recent research, necrosis is a kind of cell programmed death.[28] Thus, we tested whether HF-NBD would lead to necrosis of HeLa cervical cancer cells using an LDH assay kit, and no significant differences were found between HF-NBD-treated cells and control cells. The result showed that HF-NBD did not induce necrosis of HeLa cells at doses lower than 10 μM for 24 h (Fig. 4).

HF-NBD induces apoptosis in HeLa cells

Apoptosis is one of the important programmed cellular processes that allows cells to cope with the damage induced by various stressors.[29] To detect whether HF-NBD could induce apoptosis in HeLa cells, we performed a western blot experiment to measure the levels of cleaved-PARP protein. The data showed that the protein level of cleaved-PARP (89 kDa)
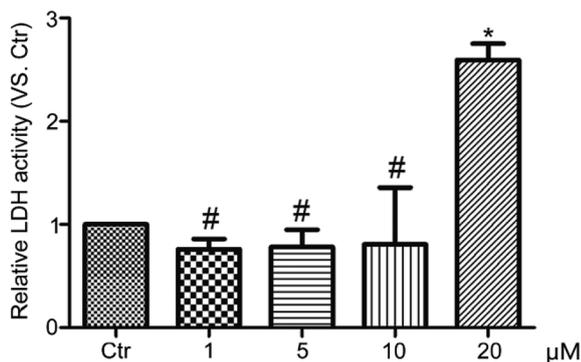
Fig. 4 HF-NBD did not lead HeLa cervical cancer cells to necrosis. HeLa cells treated with 1‰ DMSO (control) or HF-NBD at 1, 5, 10, and 20 μM for 24 h. Data are presented as the mean ± SEM, *$p < 0.05$, **$p < 0.01$, $n = 3$.

increased after treatment with 1‰ DMSO (as control group) and **HF-NBD** at 1, 5, 10 μM for 24 h (Fig. 5A) and with 1‰ DMSO (as control group) for 12 h and **HF-NBD** at 5 μM for 6, 12, 24 h (Fig. 5B). These results demonstrated that **HF-NBD** could induce apoptosis in HeLa cells.

It has been reported that ROS may induce cell apoptosis,[30] which is consistent with our data. However, some reports claim that activating Nrf2 may induce cancer cell apoptosis.[31] Reducing the endogenous H_2S levels in cells may affect sulfhydration or sulfidation,[9] impair the balance between H_2S production and clearance in cells,[10] and modulate the levels of some protein kinases or proteins, as well as the level of gene expression associated with apoptosis, including Nrf2 expression.[32–35] However, specific issues need specific analysis. Our next step is to establish which proteins are affected by reduced H_2S levels, triggering ROS production and activating Nrf2, but here, we propose a potential pathway and mainly focus on the
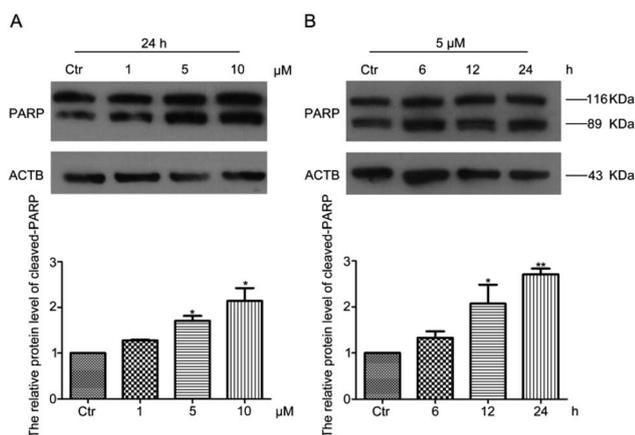

Fig. 5 HF-NBD induces apoptosis in HeLa cells. HeLa cells were treated with 1‰ DMSO or **HF-NBD** at 1, 5, and 10 μM for 24 h (A), and with 1‰ DMSO for 12 h or **HF-NBD** at 5 μM for 6, 12, and 24 h (B); cleaved-PARP (89 kDa) protein levels were determined by western blot. Relative cleaved-PARP levels were normalized to ACTB levels and results were expressed as percent of control. Data are presented as the mean ± SEM, *$p < 0.05$, **$p < 0.01$, $n = 3$.

applications of **HF-NBD**. Thus, we reached the conclusion that **HF-NBD** can inhibit cancer cell growth by reducing endogenous H_2S levels and increasing ROS levels, activating the Nrf2/HO-1 pathway and inducing cell apoptosis.

Experimental

Materials and methods

Cell culture. The human cervical cancer cell line HeLa was cultured in Dulbecco Modified Eagle Medium (DMEM, Gibco, 12800-058) with 10% (v/v) new born calf serum. The human malignant glioma cell line U87 was cultured in Dulbecco Modified Eagle Medium (DMEM, Gibco, 12800-058) with 10% (v/v) fetal bovine serum. The human lung cancer cell line A549 was cultured in RPMI-1640 medium (Gibco, 3180-022) with 10% (v/v) new born calf serum. HeLa cells stably transfected with a luciferase-based Nrf2 reporter plasmid were cultured in Dulbecco Modified Eagle Medium (DMEM, Gibco, 12800-058) with 10% new born calf serum. All cell lines were allowed to grow in air with 5% CO_2 and appropriate humidity levels at 37 °C. Cells were seeded in 96-well plates or other types of dishes at a density of 40 000 cells per ml. HeLa cells and A549 cells were purchased from the ATCC (American Type Culture Collection), U87 cells were provided by Bing Yan (Institute of Analytic Chemistry, School of Chemistry and Chemical Engineering, Shandong University, Jinan, China), and HeLa cells stably transfected with a luciferase-based Nrf2 reporter plasmid were provided by Qing Ye and Fan Jiang (The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Health, Qilu Hospital, Shandong University, Jinan, China).

Fluorescence imaging. HeLa cells were seeded in appropriate dishes or plates and subsequently treated with 1‰ DMSO or 1, 5, 10 μM **HF-NBD** for 0.5, 1, and 3 h. HeLa cells were seeded and 200 μM Cys, 1 mM PAG, 1 mM AOAA, or 1 mM PAG/AOAA were separately added followed by incubation with 5 μM **HF-NBD**. HeLa cells were seeded and 0, 0.1, 0.5, 1 mM NaHS was added followed by incubation with 5 μM **HF-NBD**. HeLa cells were seeded and treated with 5 μM **HF-NBD** for 1 h. After treatment, cells were washed twice with PBS and imaged under a laser scanning confocal microscopy (LSM 700) instrument at an excitation wavelength of 405 nm.

Luciferase assay. HeLa cells stably transfected with a luciferase-based Nrf2 reporter plasmid were seeded in 96-well plates at a density of 40 000 cells per ml and cultured for at least 12 h, then incubated with **HF-NBD** at 10 μM for 12 h or 24 h. Luciferase activity in cells was determined with the Luciferase Reporter Gene Assay Kit (Beyotime, China) and results were normalized to cell viability as measured by the SRB assay.

Quantitative real-time PCR. Total RNA was extracted using the Trizol reagent (Invitrogen, USA). The PrimeScript RT reagent kit with gDNA Eraser (DRR047, TAKARA) was used for the reverse transcription. Relative HO-1 mRNA levels were quantified by RT-PCR with the SYBR Premix Ex Taq kit (Tli RNaseH Plus). Results were normalized to β-actin expression with a melting curve for each reaction. Primers for HO-1 were: sense TGCACATCCGTGCAGAGAAT; antisense CTGGGTTCT

GCTTGCTTGTTTCGC. Primers for β-actin were: sense GAAGTGTGACGTGGACATCC; antisense CCGATCCACACGGA GTACTT. Primers for Nrf2 were: sense ACACGGTCCACAGC TCATC; antisense GTCCTGTACCTAAACTAACTGT.

Measurement of intracellular ROS levels. HeLa cells were seeded in 24-well plates and intracellular ROS levels were measured with 2′,7′-dichlorodihydrofluorescein (DCHF, Sigma-Aldrich). Cells were incubated with 10 μM DCHF at 37 °C for 30 min after treatment with 10 μM **HF-NBD** for 3, 6, 12, and 24 h. Then, cells were washed 3 times with PBS and photographed using an Olympus BH-2 (Japan) fluorescence microscope.

Cell viability assay (SRB). HeLa cells were cultured in 96-well plates at a density of 40 000 cells per ml, with each well containing 100 μl of cell suspension. Cells were treated with 1‰ DMSO or **HF-NBD** and 5-FU at 1, 2, 5, and 10 μM for 12 h or 24 h. Cell viability was measured by the SRB assay following the manufacturer's instructions. U87 cells and A549 cells were treated under the same conditions.

LDH assay. Cell culture medium was collected after treatment with 1‰ DMSO (control) and **HF-NBD** at 1, 5, 10, and 20 μM for 24 h. The LDH assay was performed with the lactate dehydrogenase (LDH) kit (Nanjing Jiancheng Co, China) following the manufacturer's instructions.

Western blot. HeLa cells were treated with 1‰ DMSO or **HF-NBD** at 1, 5, and 10 μM for 24 h, and with 1‰ DMSO for 12 h or **HF-NBD** at 5 μM for 6, 12, and 24 h. Subsequently, cells were washed twice with PBS and lysed in 80 μl of protein lysis buffer (Shanghai beyotime Co., China). All cell lysates were centrifuged at 12 000 × g for 15 min at 4 °C. Then, protein concentrations were analyzed with the bicinchoninic acid (BCA) protein assay kit (Beyotime Co, China). The SDS-PAGE assay was performed at 4 °C for 2 h, then proteins were transferred to PVDF membranes (Millipore, USA) at 4 °C for 2 h. The PVDF membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 1‰ Tween 20 (TBST) at room temperature for 1 h. Membranes were incubated with anti-PARP (Cell Signaling, Beverly, MA, USA) and anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies overnight at 4 °C. Then, membranes were washed 3 times with TBST for 5 min and incubated with HRP-conjugated secondary antibodies in 5% non-fat milk in TBST for 1 h at room temperature. Subsequently, membranes were washed 3 times with TBST and incubated with HRP substrate for 3 min. Fluorescence was measured using X-ray films and relative protein levels were quantified with the Image J software.

Statistical analysis. All data were presented as the mean ± SEM of the results from at least three independent repeated experiments and analyzed using the Statistical Package for the Social Sciences or the GraphPad Prism software. When the p value was <0.05, differences were considered statistically significant.

Conclusions

HF-NBD, which targets H_2S with good photostability, can activate Nrf2 and induce cell apoptosis. We suggested that **HF-NBD** could reduce endogenous H_2S levels in cells, increase intracellular ROS levels and activate the Nrf2/HO-1 pathway, ultimately inducing apoptosis in HeLa cells. **HF-NBD**, which consumes endogenous H_2S and reducing the its levels in cancer cells, functions as an Nrf2 activator, and may have potential as an anticancer drug with specific targeting to H_2S.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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