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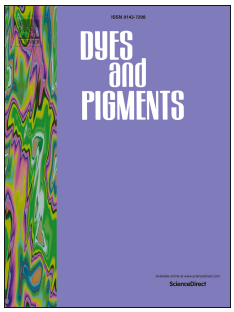

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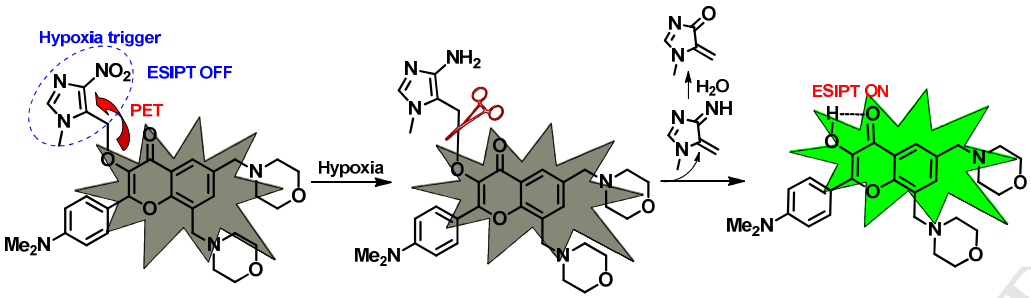

4-Nitroimidazole-3-hydroxyflavone conjugate as a fluorescent probe for hypoxic cells

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ABSTRCT

A novel off-on fluorescent probe for selective detection hypoxia or nitroreductase (NTR), 2-(4-(dimethylamino)phenyl)-3-((1-methyl-4-nitro-1H-imidazol-5-yl)methoxy)-6,8-bis(morpholino methyl)-4H-chromen-4-one (3-HF-NO_2), was developed using 4-nitroimidazole moiety as a hypoxic trigger and introducing two morpholine groups into 3-HF scaffold at 6,8-position. The design was based on a NTR-catalyzed reduction of the 4-nitroimidazole moiety in the presence of reduced nicotinamide adenine dinucleotide (NADH) as an electron donor and followed by the 1,6-rearrangement-elimination and the release of free 3-HF dye. The detection limit (S/N = 3) for NTR was 63 ng/mL. This probe displayed desired properties such as high selectivity, "Turn-On" fluorescence response with suitable sensitivity, no cytotoxicity, large Stokes' shift, and dual emission. This probe was successfully applied for imaging the hypoxic status of tumor cells (e.g. HeLa cells). We hope to apply this novel probe in the biomedical research fields for the imaging of disease-relevant hypoxia.

Key words: 4-nitroimidazole, hypoxic trigger, 3-HF-NO_2, off-on fluorescent probe, monitor hypoxic level

1. Introduction

Hypoxia is an important character of many solid tumors and influences malignant disease progression, development of metastases, clinical behavior, and response to conventional treatments [1,2]. The Hypoxic status has been considered an indicator of an adverse prognosis for solid tumors because it indicates tumor progression toward a more malignant phenotype with increased metastatic potential and resistance to treatment. Therefore, there has been increasing interest in developing clinical method which can rapidly, selectively and sensitively detect characterize the hypoxic tumor cells [3]. To date, many approaches have been developed to detect hypoxia, including immunostaining [4,5], positron emission tomography [6,7], single-photon emission computed tomography [8,9], magnetic resonance imaging [10,11], doppler optical coherence tomography [12], phosphorescence imaging [13,14], and fluorescence imaging [15~19]. Among these approaches, fluorescence imaging method offers various advantages, including high sensitivity, high resolution, non-invasiveness, and simple operation.

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In solid tumor, nitroreductase (NTR) is over expressed, and the NTR level is directly correlated with the Hypoxic status [20~22]. Thus, the detection of the NTR level has been used to investigate the hypoxic status of a tumor, and some fluorescent probes were developed to detect hypoxia [23~26]. These fluorescent probes usually utilize nitro aromatics (e.g. pimonidazole, nitrofuran, nitrobenzene, and 2-nitroimidazole) as exogenous trigger for detection hypoxia [27~29]. The trigger is a key factor when design an optical probe to detect hypoxia. However, so far, the hypoxic trigger for the detection of hypoxia is still lacking. Therefore, it is necessary to develop a hypoxic trigger and fluorescent probe for the detection of hypoxia.

3-Hydroxyflavone (3-HF) derivatives, based on excited-state intramolecular photon-transfer (ESIPT) process, exhibited excellent optical properties such as a large Stokes shift, dual emission, good photostability, and reasonable fluorescent quantum yield, which has been used in the study of molecular interactions in solution or biological systems [30~35]. Modification of the hydroxyl group of 3-HF can block the ESIPT process and results in quenching of the emission. Conversely, the regeneration of the free hydroxyl group leads to restore the emission of 3-HF. In view of their optical properties, some 3-HF derivatives have been applied for the detection of fluoride anion and cysteine anion [36~38].

In this study, we developed an off-on fluorescent probe 2-(4-(dimethylamino)phenyl)-3-((1-methyl-4-nitro-1H-imidazol-5-yl)methoxy)-6,8-bis(morpholino methyl)-4H-chromen-4-one (3-HF-NO_2) using 4-nitroimidazole moiety as a hypoxic trigger for detecting hypoxic status or NTR level of tumor cells. Under hypoxic conditions, 4-nitroimidazole moiety is reduced, 3-HF is released by means of rearrangement-elimination, and fluorescence is restored concomitantly. As we expected, 3-HF-NO_2 displayed a high selectivity, sensitivity, quickly response, no cytotoxicity for the detection of hypoxic status, and could be applied to monitor hypoxic status in living cells.

2. Experimental section

2.1. Instrumentation and materials

NMR spectra were measured on a Bruker AVIII 600 NMR spectrometer in DMSO-d_6. Electrospray ionization mass spectra (ESI-MS) were recorded in negative mode with a HRMS apex ultra 7.0T US+. The IR spectra were recorded on a Perkin-Elmer Model-683 spectrophotometer using KBr pellets. Fluorescence measurements were performed on a Hitachi F-7000 luminescence spectrophotometer in 10×10 mm quartz cells. A liquid chromatography system from Agilent Technologies (Agilent, American) was applied to all chromatography tests. The optical density (OD) was measured on a microplate spectrophotometer (Bio-Rad Model 680, USA). The absorbance for MTT analysis was recorded on a microplate spectrophotometer (MolecularDevices, VersaMax, USA). Fluorescence imaging experiments were performed on confocal microscope (Olympus, IX81, JPN) at λ_{ex} = 405 nm.

NTR (≥100 units/mg) and reduced nicotinamide adenine dinucleotide (NADH) were purchased from Sigma-Aldrich. The lyophilized powder of NTR was dissolved in pure water, and the solution was divided into 20 parts as suitable amounts for daily experiments. The enzyme solutions were frozen immediately at −20 °C for storage and allowed to thaw before use, which results in no change of the enzyme activity [29]. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin and phosphate-buffer saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3

mmol/L Na_2HPO_4, 1.4 mmol/L KH_2PO_4, pH 7.4) were obtained from Gibco (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), benzyl penicillin and streptomycin were obtained from Sigma. All other chemicals used were local products of analytical grade. A stock solution of 3-HF-NO_2 (1 mmol/L) was prepared by dissolving an appropriate amount of 3-HF-NO_2 in DMSO. Ultrapure water (over 18 MΩ/cm) was used.

The reagents and solvents were purchased as reagent grade and used without purification. All reactions were performed under nitrogen or argon and monitored by TLC. The products were purified by column chromatography on silica gel (200~300 mesh).

2.2. Synthesis and characterization

*2.2.1. 3,5-bis(chloromethyl)-2-hydroxyl acetophenone (**1**)*

To a 50 mL round-bottom flask equipped with a magnetic stirrer, 1.24 g paraformaldehyde, 1.16 g 2-hydroxyacetophenone (8.5 mmol) and concentrated hydrochloric acid (10 mL) were added and heated at 50 ℃ for 10 h. After cooling to room temperature, the mixture was extracted with dichloromethane. The organic phase was washed with saturated sodium bicarbonate solution and dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under reduced pressure. The precipitate was purified by recrystallization in mixed solvent (carbon tetrachloride: *n*-hexane = 1:1) to afford **1**. Light yellow solid, yield 65%; mp: 83.2-84.6 ^{o}C.

*2.2.2. 2-(4-(dimethylamino)phenyl)-6,8-bis(ethoxymethyl)-3-hydroxyflavone (**2**)*

To a 50 mL round-bottom flask equipped with a magnetic stirrer, 15 mL solution of 703 mg 4-(dimethylamino)benzaldehyde (6 mmol) in EtOH, 1.399 g compound **1** (6 mmol) and 30% NaOH (6 mL) were added and stirred at room temperature for 24 h. Then, 7 mL of 30% H_2O_2 solution was added drop wise to the reaction mixture in an ice-water bath, and the resulting mixture was stirred at 45 ℃ for 3 h. After cooling to room temperature, the reaction mixture was neutralized with 2 M HCl solution to pH 7 and extracted with dichloromethane. The organic phase was dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel to give **2**. Brown red solid, yield 41%; mp: 170.5- 171.3 ℃; ^{1}H NMR (DMSO-d_6, 600 MHz) 9.25 (s, 1H), 8.12 (d, 2H, *J* = 7.8 Hz), 7.96 (s, 1H), 7.70 (s, 1H), 6.87 (d, 2H, *J* = 7.8 Hz), 4.87 (s, 2H), 4.59 (s, 2H), 3.64 (q, 2H, *J* = 6.6 Hz), 3.54 (q, 2H, *J* = 6.6 Hz), 3.03 (s, 6H), 1.24~1.18 (m, 6H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ (ppm): 171.86, 151.20, 150.99, 146.42, 137.24, 134.40, 131.13, 128.78, 127.88, 122.11, 121.05, 118.07, 111.42, 70.79, 66.23, 65.65, 65.11, 15.10; HRMS (ESI) *m/z* calcd. for $C_{23}H_{28}NO_5$: 398.19620 $[M+H]^+$, found 398.19586.

*2.2.3. 2-(4-(dimethylamino)phenyl)-6,8-bis(morpholinomethyl)-3-hydroxyflavone (**3**)*

A mixture of 557 mg compound **2** (1.4 mmol), and 18 mL hydrobromic acid were heated at 100 ℃ for 4 h. After cooling, the reaction mixture was treated with saturated sodium bicarbonate solution until pH was 7 with the gradual formation of precipitation (6,8-bis(bromomethyl)-2-(4-(dimethylamino)phenyl)-3-hydroxyflavone). The collected precipitation was dried and the crude product was used for the next step without further purification.

To a round-bottom flask (50 mL) equipped with a magnetic stirrer, the crude product of 6,8-bis(bromomethyl)-2-(4-(dimethylamino)phenyl)-3-hydroxyflavone dissolved in 30 mL of THF, and 870 mg morpholine (10 mmol) were added and stirred at room temperature for 10 h. The

solvent was removed under reduced pressure. Then the crude product was purified by column chromatography on silica gel to give **3** as a pure compound. Yellow solid, yield 51%; mp: 207.5-208.6 ^{o}C; ^{1}H NMR (DMSO-d_6, 600 MHz) 9.22 (s, 1H), 8.14 (d, 2H, *J* = 9.0 Hz), 7.89 (s, 1H), 7.67 (s, 1H), 6.86 (d, 2H, *J* = 9.0 Hz), 3.84 (s, 2H), 3.65~3.54 (m, 10H), 3.01 (s, 6H), 2.56~2.31 (m, 8H); ^{13}C NMR (DMSO-d_6, 150 MHz) δ (ppm): 172.46, 151.49, 140.18, 137.67, 134.96, 133.93, 130.52, 130.12, 129.36, 127.29, 123.91, 118.70, 111.97, 111.13, 66.68, 62.08, 59.51, 56.56, 53.83, 53.56; HRMS (ESI) *m/z* calcd for $C_{27}H_{34}N_3O_5$: 480.24930 $[M+H]^+$, found: 480.24869.

2.2.4. 2-(4-(dimethylamino)phenyl)-3-((1-methyl-4-nitro-1H-imidazol-5-yl)methoxy)-6,8-bis (morpholinomethyl)-4H-chromen-4-one *(3-HF-NO_2)*

To a round-bottom flask (50 mL) equipped with a magnetic stirrer, 240 mg compound **3** (0.5 mmol) dissolved in 3 mL of dry DMF and 138 mg potassium carbonate (1 mmol) were added and stirred at room temperature for 30 min, followed by addition of 110 mg 5-(bromomethyl)-1-methyl-4-nitro-1H-imidazole (0.5 mmol). The mixture was stirred room temperature for 12 h. Then the reaction mixture was extracted with ethyl acetate (15 mL×3). The organic phase was washed with water twice, dried over Na_2SO_4. After filtration and concentration in vacuum, the crude product was purified by column chromatography on silica gel to give probe 3-HF-NO_2. Orange solid, yield 38%; mp: 182-184 ^{o}C; ^{1}H NMR (DMSO-d_6, 600 MHz,) δ (ppm): 8.17 (d, *J* = 9.0 Hz, 2H), 8.09 (s, 1H); 8.04 (d, *J* = 9.0 Hz, 2H), 7.71 (s, 1H), 7.63 (s, *J* = 8.4 Hz, 2H), 6.73 (d, *J* = 8.4 Hz, 2H), 5.22 (s, 2H), 3.85 (s, 2H), 3.75 (t, *J* = 4.8 Hz, 4H), 3.72 (t, *J* = 4.8 Hz, 4H), 3.60 (s, 2H), 3.09 (s, 6H), 2.57 (s, 4H), 2.47 (s, 4H); ^{13}C NMR (DMSO-d_6, 150 MHz,) δ (ppm): 173.70, 156.93, 152.60, 152.19, 145.36, 137.92, 137.48, 135.49, 134.58, 129.99, 128.62, 127.41, 124.11, 123.75, 116.66, 66.68, 61.98, 60.68, 56.51, 53.75, 53.56, 33.05; HRMS (ESI) *m/z* calcd for $C_{32}H_{39}N_6O_7$: 619.28747 $[M+H]^+$, found 619.28759.

2.3. NTR assay

All the fluorescence measurements were carried out according to the following procedure. In a 5 mL tube, 2 mL of PBS (10 mmol/L, pH = 7.4), and 2.5 mL 10 μmol/L 3-HF-NO_2 were mixed, followed by addition of NADH (final concentration, 1 mmol/L), and an appropriate volume of NTR solution. The final volume was adjusted to 5 mL with PBS; the reaction solution was mixed rapidly and degassed with nitrogen for 30 min to remove oxygen. After incubation at 37 ^{o}C for 15 min, the spectra were measured ($\lambda_{ex/em}$ = 412/560 nm) with 10/10 nm slit widths. In the meantime, a blank solution containing no NTR (control) was prepared and measured under the same conditions for comparison.

2.4 HPLC for the determination of reduction assay of NM-3-HF

A liquid chromatography system from Agilent Technologies (Agilent, American) was applied to all chromatography tests. The HPLC system was performed with a quaternary G1311C pump, a G1314F UV-vis detector. The data acquisition and processing was performed throughout a LC solution chromatographic workstation (Agilent, Japan). The analytes column was a Agilent HC-C18 column (250 mm × 4.6 mm, 5 um) from Bonna-Agela Technologies. The optimized mobile phase consisted of acetonitrile-0.01M K_2HPO_4 solution (20:80, *V/V*) and the flow rate was maintained at 0.5 mL/min. The UV detector was set at 254 nm and injected aliquots of 10 μL sample into the column.

The solution of NM-3-HF (0.5 mM) in DMSO and H_2O (1:1, *V/V*) was added $Na_2S_2O_4$ (10 mg)

and then incubated for 4 min in 25 ^{o}C. The mixture solution was filtered and then detected through HPLC.

2.5. MTT Assays

HeLa cells were cultured under normoxic condition (5% CO_2 at 37 ^{o}C) in a humidified incubator (SANYO, MCO-20AIC, JPN) or under hypoxic condition (2% O_2, 5% CO_2, and 93% N_2 at 37 ^{o}C) in a humidified incubator (Heal Force, HF 100, CHN). The effect of the compounds on cells viability was determined by MTT method [39]. In brief, the HeLa cells harvested from the flask at subconfluent stage were seeded in 96 well culture plates at density of 2×10^4 cells per well and incubated overnight. Then 3-HF-NO_2 was added to the wells to achieve final concentrations. Control wells were prepared by addition of culture medium. Wells containing culture medium without cells were used as blanks. At the end of incubation, 10 μL of MTT (5.0 mg/mL) was added into each well and incubated for 4 h. Then, the supernatant was removed and 100 μL DMSO was added to each well for dissolve the MTT formazan. The *OD* of formazan solutions were recorded on a microplate spectrophotometer (Molecular Devices, Versa Max, USA) at 570 nm. The cell viability was presented as the fold over the control group and was calculated according to formula: cell viability (%) = (OD_{sample}-OD_{blank})/($OD_{control}$-OD_{blank}×100).

2.6. Hypoxic conditions in cell imaging

HeLa cells were cultured under normoxic condition (5% CO_2 at 37 ^{o}C) in a humidified incubator (SANYO, MCO-20AIC, JPN) or under hypoxic condition (2% O_2, 5% CO_2, and 93% N_2 at 37 ^{o}C) in a humidified incubator (Heal Force, HF 100, CHN). After treatment of 3-HF-NO_2 at certain concentration (5 μmol/L) for different time, then the cells were washed with PBS twice. The fluorescence imaging experiments were performed on a confocal laser scanning microscope with FV5-LAMAR for excitation at 405 nm and variable band pass emission filter set to 545-645 nm through a 60×1.42 NA objective. Optical section was acquired at 0.8 um.

3. Results and discussion

3.1. Synthesis and structural characterization

The synthetic route of 3-HF-NO_2 is depicted in **Scheme 1**. 5-(Bromomethyl)-1-methyl-4-nitro-1*H*-imidazole was prepared *via* the reported method [40]. 3-HF-NO_2 was synthesized from *o*-hydroxyl acetophenone, 4-(dimethylamino)cinnamaldehyde and 5-(bromomethyl)-1-methyl-4-nitro-1*H*-imidazole by Blanc chloromethylation, Aldol condensation, Algar-Flynn-Oyamada (AFO) reaction, bromination reaction, and substitution reaction. Their structures were characterized by NMR and HRMS (**Fig. S3-S11**, ESI†). By modifying the structure of flavonoids with two morpholine groups at 6,8-positions, the water-solubility and cell permeability of 3-HF-NO_2 were improved. Thus, we selected a pH 7.4 PBS buffer solution containing 0.5% (v/v) DMSO for further experiments. In contrast, compound **4** (unmodified by morpholine group, **Fig. 1**) and **5** (using a *p*-nitrobenzyl in place of 4-nitroimidazole moiety, **Fig. 1**) were precipitated out in MTT assays at reasonable concentration.

Scheme 1

Fig. 1

*3.2. Photophysical properties of 3-HF-NO_2 and compound **3***

UV-Vis absorption and emission spectra of 3-HF-NO_2 (5 μmol/L) and compound **3** (5 μmol/L) in in 10 mmol/L PBS were presented in **Fig. 2**. The UV spectra of compound **3** exhibited absorption at λ_{max} 412 nm, 3-HF-NO_2 exhibited two bands at λ_{max} 314 (attributed to 4-nitroimidazole moiety) and 402 nm (**Fig. 2a**). The blue shift (10 nm) is due to the modification of the hydroxyl group of **3** by 4-nitroimidazole moiety. The fluorescence spectra of **3** and 3-HF-NO_2 were shown in **Fig. 2b**. Compound **3** exhibited a relatively strong fluorescence emission at 560 nm (excitation at 412 nm), whereas 3-HF-NO_2 displayed no fluorescence due to the combined effects of the photo-induced electron-transfer (PET) process and the blocking ESIPT process by 4-nitroimidazole moiety. However, upon incubation of 3-HF-NO_2 with NTR and NADH in PBS buffer (pH 7.4, 0.01 mol/L, 0.5% DMSO) at 37 ^{o}C, the reaction mixture produces a remarkable fluorescence change from almost non-fluoresce to strong green fluorescence ($\lambda_{ex/em}$= 412/560 nm, **Fig. 2b**). The absorption at 412 nm and obvious fluorescence enhancement at 560 nm suggested that the 4-nitroimidazole moiety of 3-HF-NO_2 was reduced by NTR, thus eliminating the possibility of PET-induced quenching, and concomitantly allowing fluorescence to occur. In addition, the above data suggested that the hypoxic probe has a larger stokes shift (148 nm).

Fig. 2

3.3 Response towards NTR or hypoxia

Next, the fluorescence response of 3-HF-NO_2 to NTR at varied concentrations in the presence of NADH was also tested. As shown in **Fig. 3**, the fluorescence intensity of the reaction solution increased gradually with the increase of NTR concentration, and a good linearity is obtained in the concentration range of 1-4 μg/mL NTR, with the regression equation y = 51.7952x+12.7415 (R^2 = 0.997). The detection limit is 63 ng/mL NTR. Thus, 3-HF-NO_2 exhibits high sensitivity to NTR and hypoxia.

Fig. 3

3.4. Selective response of probe 3-HF-NO_2 to NTR

To evaluate the effect of 3-HF-NO_2 selective response to NTR, considering the complexity of the intracellular environment, we investigated the influence of various analytes, including reductants (V_C, glutathione), biothiols (cysteine, homocysteine, and dithiothreitol), inorganic salts ($CaCl_2$, $MgCl_2$), saccharide (glucose), amino acid (arginine), and reactive oxygen species (H_2O_2, NaOCl) on the fluorescence behaviour of 3-HF-NO_2 in PBS buffer. As shown in **Fig. 4**, 3-HF-NO_2 uniquely responds to NTR to produce a prominent fluorescence enhancement at 560 nm, whereas has nearly no fluorescence responses to other reductants, biothreitol, saccharide, amino acids, metal salts, and reactive oxygen species under same conditions. The above results demonstrated that the fluorescent change of 3-HF-NO_2 was induced by NTR in presence of NADH. Therefore, it can be concluded that 3-HF-NO_2 displays extremely high selectivity for sensing NTR.

Fig. 4

3.5. The effect of pH on the reaction between 3-HF-NO_2 and NTR

The fluorescent response of 3-HF-NO_2 toward NTR in PBS buffer (10 mmol/L, 0.5% DMSO) at different pH conditions were further investigated in biological systems. As shown in **Fig. S2** (ESI†), the probe 3-HF-NO_2 response to NTR displayed pH-dependent. The fluorescence intensity of the reaction mixture increased obviously when pH is increased from 5.0 to 9.0. This trend is

consistent with the fluorescence intensity change of compound **3** along with pH increasing from 5.0 to 9.0 (**Fig. S1**, ESI†). The stable optical property of this probe reveals that it functions well under physiological conditions.

3.6 The cytotoxicity of 3-HF-NO_2

In order to evaluate the cytotoxicity of 3-HF-NO_2, MTT assay was performed in HeLa cells under normoxic (20% O_2) and hypoxic conditions (2% O_2) at 37 °C for 24 h. As shown in **Fig. 5**, the HeLa cell viability is found to be upon 90% when the concentration of 3-HF-NO_2 is lesser than 10 μmol/L under normoxic/hypoxic condition. The results showed that 3-HF-NO_2 exhibited low cytotoxicity and good biocompatibility under experimental conditions.

Fig. 5

3.7 *The mechanism of 3-HF-NO_2 to specifically recognize NTR*

4-Nitroimidazole moiety is not only a receptor for hypoxia/NTR but also an excellent fluorescence-quench moiety. 3-HF-NO_2 displays an extremely low background signal due to the PET process based on electron withdrawing property of nitro group, combined with the effects of blocking ESIPT process. Under hypoxic condition, the 4-nitroimidazole moiety is reduced *via* a series of one-electron reduction processes to form an unstable 4-aminomidazol derivative, which leads to rearrangement-elimination. Thus, it is expected that 3-HF which exhibits distinct photophysical property is released, which concomitantly cause the fluorescence to be restored (as shown in **Scheme 2**). The reduction of 3-HF-NO_2 was suppressed by molecular oxygen under aerobic conditions. Hence, the fluorescent response to hypoxia/NTR with high selectivity is expected using this probe.

Scheme 2

To examine this plausible mechanism of 3-HF-NO_2 to specifically recognize NTR or hypoxia, we determined the reduction product by HPLC and MS. First, $Na_2S_2O_4$ was used as the reductant to convert 3-HF-NO_2 to fluorophore **3** as expected. The formation of **3** was confirmed by HPLC analysis (**Fig. 6**). Then, the reaction of 3-HF-NO_2 with NTR in the presence of NADH under hypoxia was investigated and determined by MS analysis. After being separated and purified, the HRMS of the product showed a peak at 480.24945 attributing to compound **3** (same to calcd for $C_{27}H_{34}N_3O_5$ 480.24869 $[M+H]^+$, **Fig. S12**, ESI†). This is responsible for the observed enhancement of fluorescence emission. A signal of 149.1 (**Fig. S13**, ESI†) is responsible for **7** (calcd for $C_5H_6N_2O$ 149.0 $[M+K]^+$) obtained from the hydrolysis reaction from the immediate **6** in PBS buffer. Under hypoxia (or in the presence of NTR and NADH), the 4-nitroimidazole moiety was reduced *via* a series of one-electron reduction processes to form the amino intermediates that leads to the rearrangement-elimination.

Fig. 6

3.8 Cell imaging

To further evaluate the potential of 3-HF-NO_2 for practical application, HeLa cell was selected as cell model for cellular fluorescence imaging. HeLa cells were first incubated under normoxic (20% O_2) and hypoxic conditions (10%, 5%, and 2% O_2) at 37 °C for 16 h, and then incubated with 5 μmol/L 3-HF-NO_2. In order to determine the reduction rates of 3-HF-NO_2 under different

hypoxic conditions, HeLa cells were incubated with 3-HF-NO_2 for 20 and 40 min under the respective conditions. As expected, incubated with 3-HF-NO_2 under normoxic conditions for different times (20 and 40 min), no considerable fluorescence was observed (**Fig. 7a**, **g**). A significant green fluorescence was observed under hypoxic conditions with the aid of a confocal fluorescence microscope (**Fig. 7d**, **j**). The reduction of 4-nitroimidazole moiety of 3-HF-NO_2 under hypoxic conditions leads to the establishment of a sensitive and selective method for imaging the hypoxic cells. The results showed that cells under normoxic and hypoxic conditions could be differentiated successfully by this method.

In addition, HeLa cells rapidly respond to 3-HF-NO_2 under hypoxic conditions (less than 20 min). More importantly, the fluorescence intensity increases in the cells was observed with the decrease of the O_2 concentration from 10% to 2% (**Fig. 7m~u**). This implied that 3-HF-NO_2 can indicate the hypoxic status in the cells rapidly.

Fig. 7

4. Conclusions

In summary, by taking advantage of an efficient hypoxic marker 4-nitroimidazole moiety, a novel fluorescent probe 3-HF-NO_2 for hypoxia detection and imaging hypoxic status in tumor cells was synthesized. 3-HF-NO_2 is quenched by 4-nitroimidazole moiety. Under hypoxia or in the presence of NTR with NADH, 3-HF-NO_2 generated highly fluorescent 3-HF *via* the selective reduction and rearrangement-elimination. The probe displayed excellent properties such as high selectivity, sensitivity, no cytotoxicity, large Stokes' shift, and low detection limit. This probe was successfully applied in imaging of hypoxic HeLa cells. Thus, the conjugation of a 4-nitroimidazole moiety to a fluorophore is a useful strategy for designing hypoxia-sensitive probe. The simple chemical structures, large Stokes' shift and dual emission makes 3-HF-NO_2 a practically useful tool to investigate a variety of hypoxia-related biological phenomena.

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Legends to Tables and Figures

Scheme 1 Synthetic route for 3-HF-NO_2

Scheme 2 Proposed mechanism of 3-HF-NO_2 detection NTR or hypoxia

Fig. 1 The structure of compounds **4** and **5**

Fig. 2 UV-Vis absorption spectra and fluorescence spectra of **3** and 3-HF-NO_2
a: UV-Vis absorption spectra of **3** (5 μmol/L) and 3-HF-NO_2 (5 μmol/L)
b: Fluorescence spectra of **3** (5 μmol/L), 3-HF-NO_2 (5 μmol/L), and 3-HF-NO_2 (5 μmol/L) treated with NTR (3 μg/mL) in presence of NADH (1 mmol/L) for 0.5 h at λ_{ex}=412 nm

Fig. 3 Fluorescence response of 3-HF-NO_2 (5 μmol/L) to different concentrations of NTR at $\lambda_{ex/em}$ = 412/560 nm

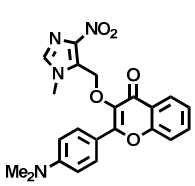
Fig. 4 Fluorescence responses of 3-HF-NO_2 (5 μmol/L) to various species ($\lambda_{ex/em}$=412/560 nm)
1: $CaCl_2$ (2.5 mmol/L); **2**: $MgCl_2$ (2.5 mmol/L); **3**: H_2O_2 (10 μmol/L); **4**: NaOCl (10 μmol/L); **5**: Vc (1 mmol/L); **6**: glutathione (DTT, 1 mmol/L); **7**: cysteine (GSH, 2 mmol/L); **8**: homocysteine (1 mmol/L); **9**: dithiothreitol (1 mmol/L); **10**: glucose (10 mmol/L); **11**: arginine (Arg, 1 mmol/L); **12**: NTR (3 μg/mL) with NADH (1 mmol/L)

Fig. 5 Cell viability assays of 3-HF-NO_2 under normoxic and hypoxic conditions

Fig. 6 HPLC profiles of a) **3**; b) 3-HF-NO_2; c) mixture of **3** and 3-HF-NO_2; d) solution of 3-HF-NO_2 incubated with $Na_2S_2O_4$

Fig. 7 Confocal fluorescence imaging of HeLa cells incubated with 3-HF-NO_2
(a-l) Incubated with 3-HF-NO_2 for 20 min (**a**, **b**, and **c**: 20%O_2; **d**, **e**, and **f**: 2%O_2) and 40 min (**g**, **h**, and **i**: 20%O_2, **j**, **k**, and **l**: 2%O_2); **(m-u)** Incubated with 3-HF-NO_2 under different hypoxic conditions: 10% O_2 (**m**, **n**, and **o**); 5% O_2 (**p**, **q**, and **r**); 2% O_2 (**s**, **t**, and **u**)

Fig 1



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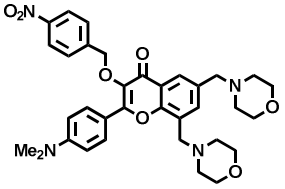


5

Fig. 2

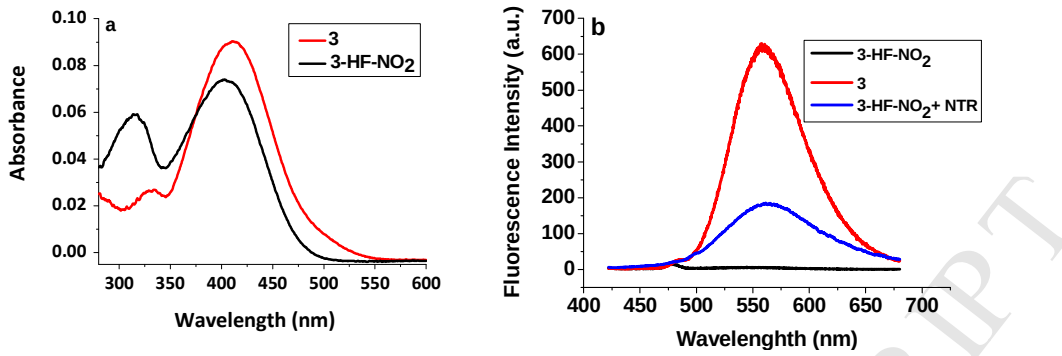


Fig. 3

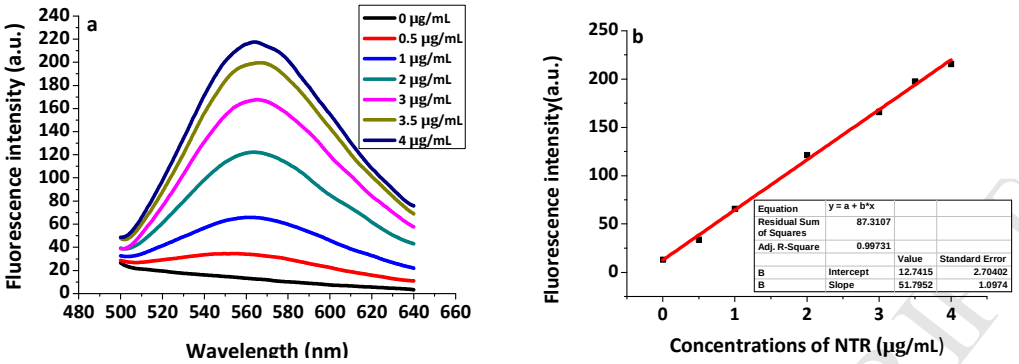
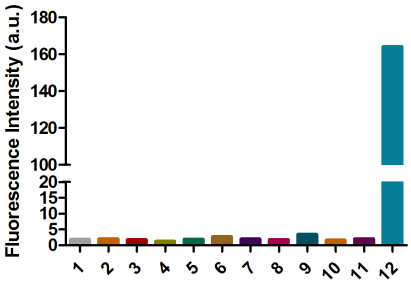
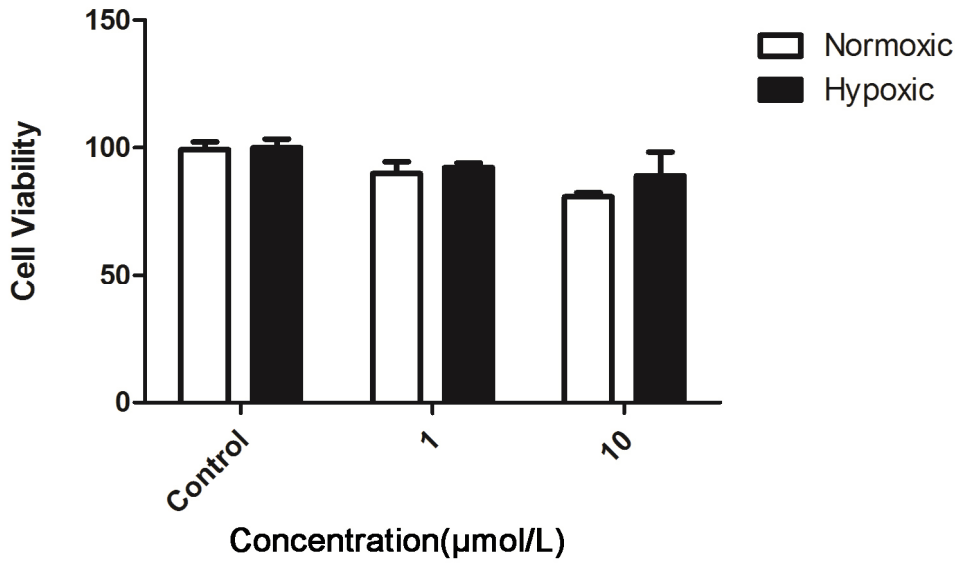
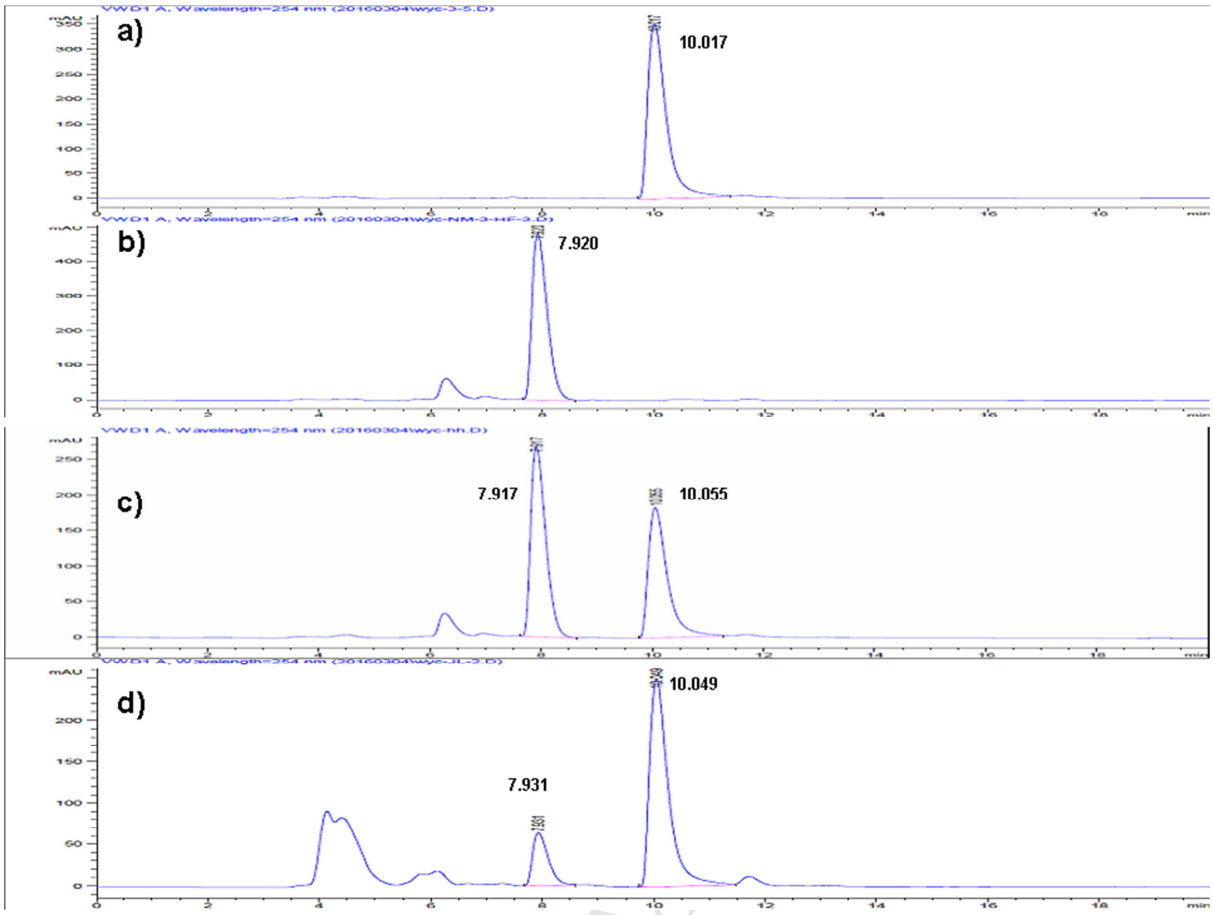
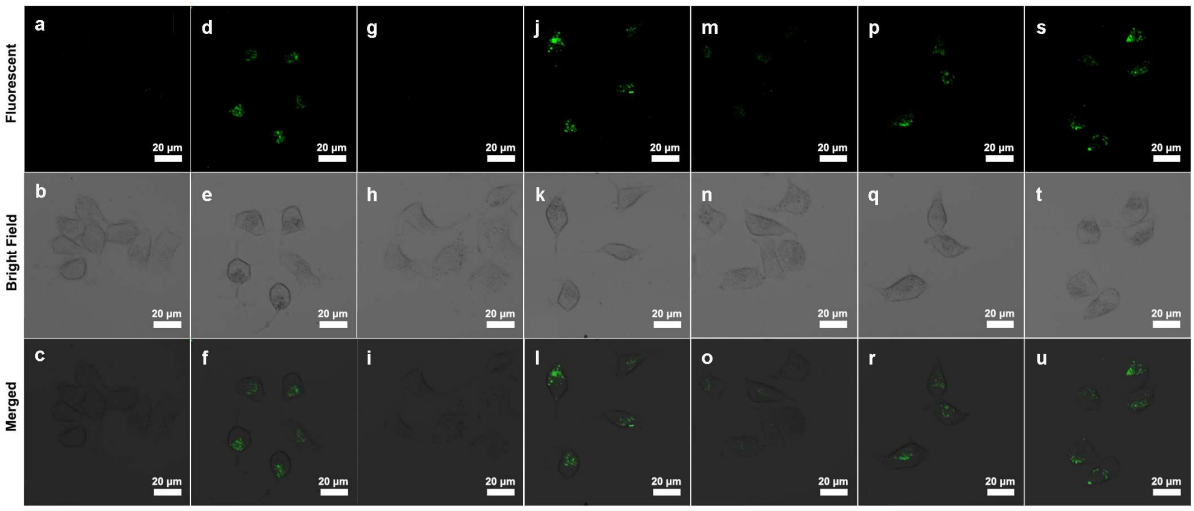


Fig 4

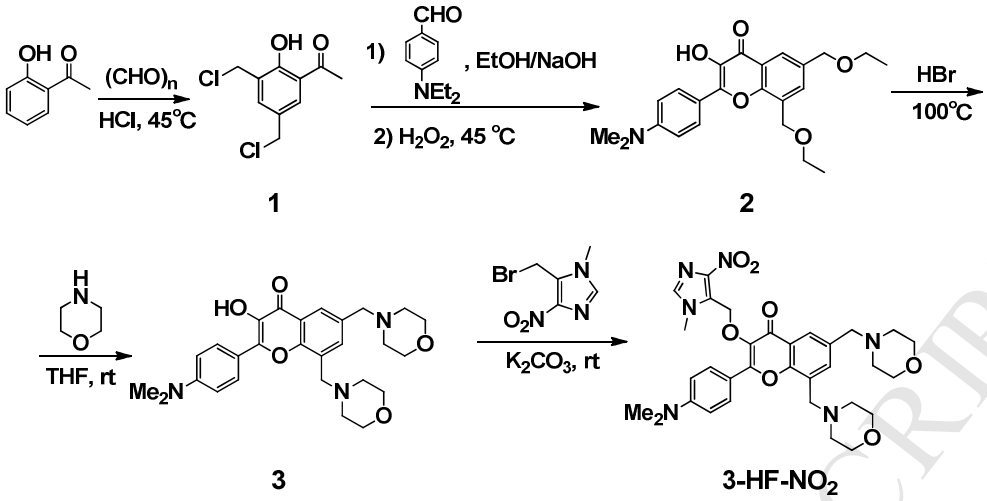




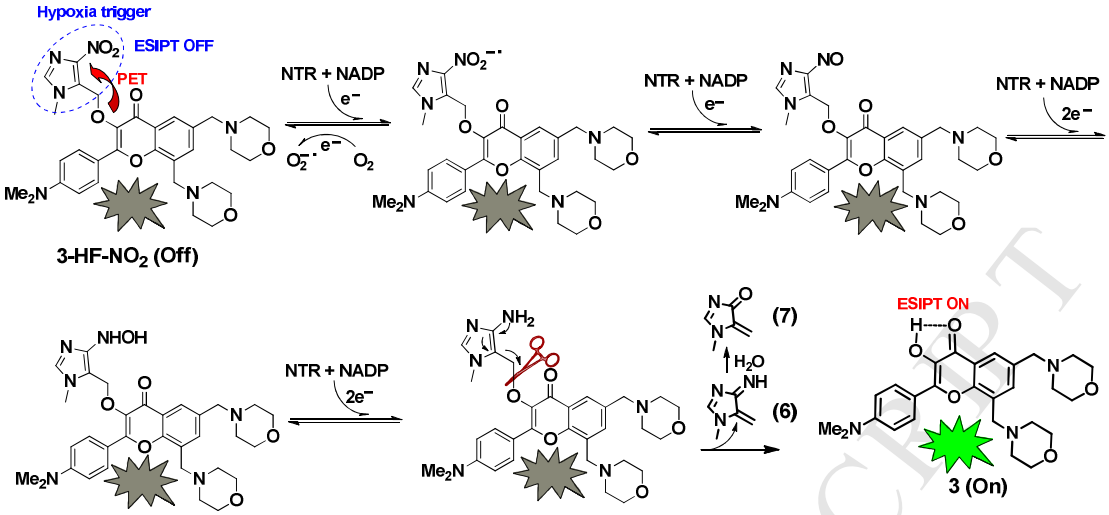




Scheme 1



Scheme 2



Highlights

- 4-Nitroimidazole moiety is an efficient hypoxic trigger.
- A novel probe 3-HF-NO_2 for the selective detection hypoxia or NTR was developed.
- 3-HF-NO_2 showed excellent selectivity, sensitivity, and low detection limit.
- 3-HF-NO_2 was successfully used for monitoring hypoxic status in HeLa cells.