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A novel nitro-substituted benzothiadiazole as fluorescent probe for tumor cells under hypoxic condition

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ABSTRACT

Most of solid tumor cells are hypoxic and hard to trace and measure. A new compound, 4,7-bis(4-dodecylthiophen-2-yl)-5,6-dinitrobenzo[*c*][1,2,5]thiadiazole (**BTTD-NO_2**), was synthesized for labeling the hypoxic cells specially in this paper. **BTTD-NO_2** showed no cytotoxicity to MG63 cells by MTT method. When MG63 cells were cultured with **BTTD-NO_2** under hypoxic condition for 24 h, strong red fluorescence distribution in cytoplasm was observed. Flow cytometry results showed that 65% of MG63 cells were labeled with strong red fluorescence in hypoxic condition while only 2.4% in oxic condition. Furthermore, Real time RT-PCR proved that **BTTD-NO_2** could stimulate high gene expression of the nitroreductase in the cells which could improve the conversion rate of **BTTD-NO_2** to **BTTD-NH_2** in turn. It proved that the fluorescence of **BTTD-NO_2** was quenched by its two nitro groups, however, strong red fluorescence could emit in the cytoplasm after the reduction of its nitro groups to amino groups in the tumor cells under hypoxic condition. These results suggested that **BTTD-NO_2** had the potential as a superior fluorescent probe for tumor detection.

1. Introduction

Cancer is one of the diseases which are the most pressing public health now. The great challenges for future personalized oncology are to explore improved methodology for the early detection of localized and disseminated tumor cells in patients, which is very critical to the success of cancer therapy and improvement of patients' survival rates. Compared to other technologies of tumor detection, such as radioisotope labeling, magnetic resonance imaging (MRI), electron spin resonance (ESR) spectroscopy, and electrochemical detection, fluorescence imaging has many advantages, for example, it enables highly sensitive, less-invasive and safe detection using readily available instruments. Another advantage of fluorescence imaging is that the fluorescence signal of a molecule can be drastically modulated, so that probes relying on activation, not just accumulation, can be utilized.[1]

Many works had carried out on the fluorescence imaging by using luminescence probes for tumor cells in vitro.[2–6] Intravital microscopic imaging approaches, based on the use of fluorescence imaging, had been shown to allow the study of cell morphology and cell–cell interaction with high (single-cell) resolution in living organisms.[7] However, distinguishing of tumor cells compared with normal cells should be considered. With fast growth in the tumor of cancer, hypoxic condition would aroused in the center of the tumor. Hypoxic cells in some human solid tumors are thought to limit the effectiveness of radiotherapy and chemotherapy, and in some cases may contribute to failure to control the disease,[8] as most of solid tumor cells are hypoxic. Therefore, both before and during therapy, tracing and measurement of the hypoxic cell fraction in tumor would be considerable important in clinical treatment. Yin et al.[9] had designed a series of aliphatic N-oxide of naphthalimides and fluorescence image analysis showed that one of compounds had 17 times intensity of fluorescence in V79 cells under hypoxic condition compared with oxic environment. Many other researching works indicated that fluorescent detection for tumor cells could be effective by distinguishing those tumor cells under hypoxic condition.[10–12]

In the past few years, we had developed a few families of benzothiadiazole fluorophores which were optically stable and had easily tunable properties.[13,14] Due to the electron-withdrawing properties and high fluorescence quantum yields both in solution and in the solid state, benzo[*c*][1,2,5]thiadiazole (BTD) had been used for developing optoelectronic materials, liquid crystal displays (LCDs), organic semiconductors and light technology.[15] Previous researches showed that benzothiadiazole could selective label the mitochondria of the cells,[16,17] hence, we attempted to induce the stem of benzothiadiazole and synthesize a new derivative as a fluorescent probe for tumor cells.

After the new benzothiadiazole derivative was synthesized, we hypothesized that this compound could be transmitted into cells and emitted red fluorescence in the cells after bio-reduction under

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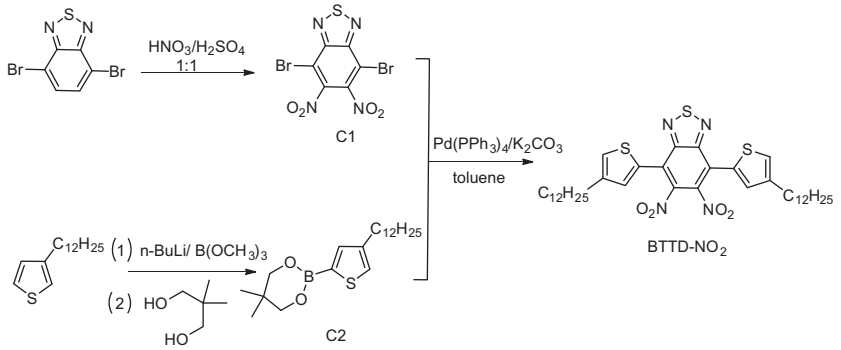

Figure 1. Synthesis of target compound, **BTTD-NO$_2$**.

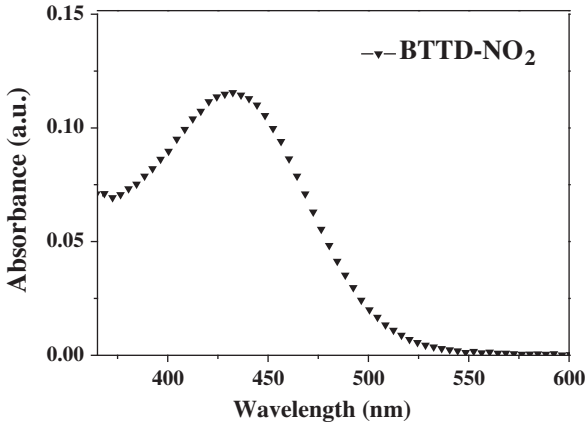

Figure 2. Absorption spectra of the target compound **BTTD-NO$_2$** (10^{-5} mol/L methanol).

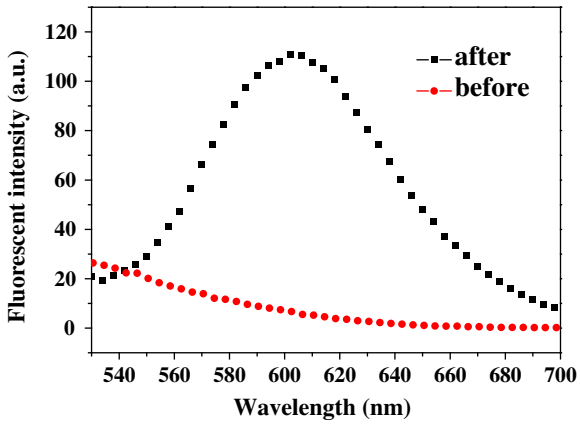

Figure 4. Fluorescence intensity of the target compound **BTTD-NO$_2$** before and after chemical reduction (10^{-6} mol/L methanol), excited at 480 nm.

hypoxic condition. To testify the hypothesis, the osteosarcoma cell line, MG63, was employed to the following biological experiments.

2. Results and discussion

2.1. Synthesis and spectra analysis

2.1.1. Synthesis

The new benzothiadiazole derivative, 4,7-bis(4-dodecylthiophen-2-yl)-5,6-dinitrobenzo[*c*][1,2,5]thiadiazole (**BTTD-NO$_2$**), was synthesized from 4,7-dibromobenzo[*c*][1,2,5]thiadiazole and 3-dodecylthiophene as shown in Figure 1. Their structures were confirmed by ^{1}H NMR, ^{13}C NMR.

2.1.2. Absorption spectra of BTTD-NO$_2$

The absorption spectra of **BTTD-NO$_2$** were shown in Figure 2. In methanol solution, its maximal absorption band lie at 432 nm, arising from π–π^* transitions ($\log\varepsilon = 4.06$).

2.1.3. Fluorescence spectra of BTTD-NO$_2$

To study the fluorescent features, the **BTTD-NO$_2$** was reduced firstly according to chemical reduction which was illustrated in Figure 3. The results of fluorescence intensity of the **BTTD-NO$_2$** before and after reduction were shown in Figure 4, no fluorescence was detectable before chemical reduction of **BTTD-NO$_2$**, whereas **BTTD-NH$_2$** (chemical reduction from **BTTD-NO$_2$**) had broad and bright red luminescence with the peak at 600 nm after it was excited at 480 nm. Previous reports had proved that emission wavelength at 600 nm could greatly reduce the background fluorescence and improve the resolution.[18] The fluorescent features of **BTTD-NO$_2$** had reached our initial expectation.

2.1.4. Biological reduction of BTTD-NO$_2$

To further testify whether **BTTD-NO$_2$** could be reduced by nitroreductase, liver microsomes provided as nitroreductase (P450 reductase) in this experiment, the possible reaction principle was shown in Figure 5. After different concentration of **BTTD-NO$_2$** was treated by liver microsomes for 4 h, the fluorescence spectra

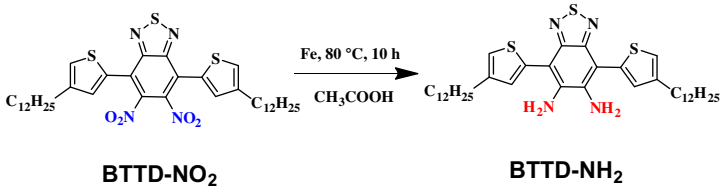

Figure 3. Chemical reduction of target compound **BTTD-NO$_2$**.

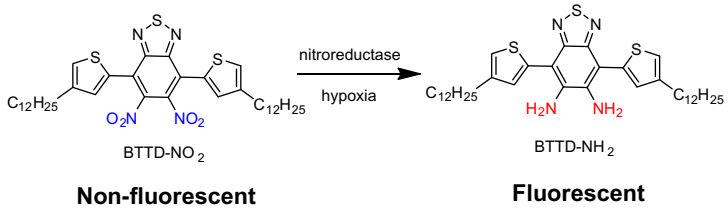

Figure 5. Proposed mechanism for fluorescent enhancement of **BTTD-NO$_2$**.

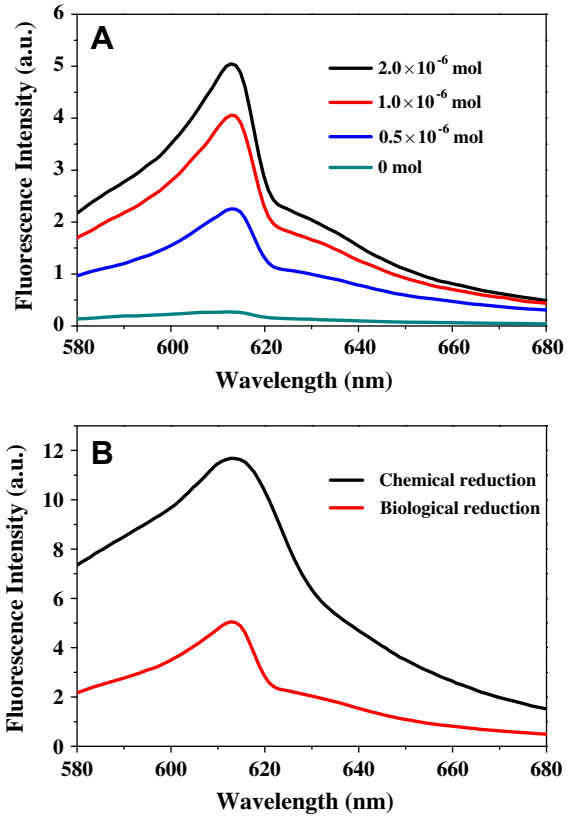

Figure 6. Fluorescence spectra excited at 500 nm. (A) The **BTTD-NO$_2$** with different concentration after reduction by nitroreductase for 4 h, the fluorescence intensity increased gradually with raised concentration of **BTTD-NO$_2$**; (B) fluorescence spectra of **BTTD-NO$_2$** according to chemical reduction or biological reduction (ultimate concentration: 2×10^{-6} mol/L). They had the same peak at 613 nm and the similar spectra shape.

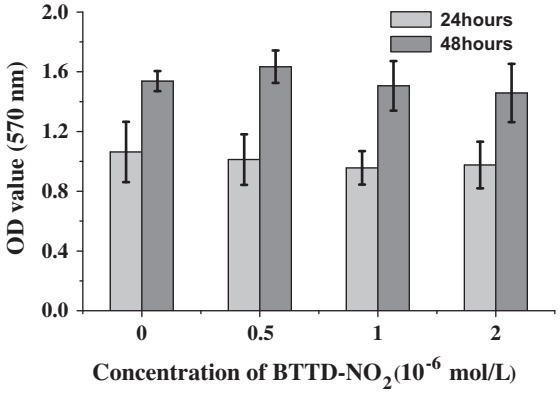

Figure 7. **BTTD-NO$_2$** had no cytotoxicity to MG63 cells according to MTT method.

were detected and the results were shown in Figure 6(A). Sharply red luminescence with the peak at 613 nm was observed after biological reduction of **BTTD-NO$_2$** and the fluorescence intensity increased gradually with raised concentration according to analysis by fluorescence spectrophotometer. In addition, to identify whether the results of the biological reduction of **BTTD-NO$_2$** were coincided with chemical reduction of **BTTD-NO$_2$** or not, fluorescence spectra of **BTTD-NO$_2$** by chemical reduction were compared with that of by biological reduction, the results were shown in Figure 6(B). They had the same peak at 613 nm and the shape of the spectra was almost unchanged, it indicated that the nitro group could be reduced to the amino group whatever the reduction method we adopted. However, the fluorescent intensity in the biological reduction group was considerable lower than that of chemical reduction group, which indicated **BTTD-NO$_2$** was not reduced to **BTTD-NH$_2$** totally according to biological reduction.

2.2. Tracing tumor cells with BTTD-NO$_2$ in vitro

2.2.1. Cytotoxicity of BTTD-NO$_2$

After the MG63 cells were co-cultured with **BTTD-NO$_2$** for 24 and 48 h, the MTT results showed that each concentration of **BTTD-NO$_2$** did not inhibit the growth of MG63 cells (Fig. 7), no significant differences (t-test, $P < 0.05$) were found in cellular proliferation between different concentration groups. The results indicated that the **BTTD-NO$_2$** compound had good biocompatibility as bio-imaging agents.

2.2.2. Fluorescent labeling of hypoxic cell by BTTD-NO$_2$

To assess the potential applications as fluorescent probe of the compound **BTTD-NO$_2$**, fluorescent imaging inside the MG63 cells treated by **BTTD-NO$_2$** under hypoxic or oxic condition was examined at 4 and 24 h, respectively. After exchanging medium, laser scanning confocal microscope (LSCM) observation was performed and the results were shown in Figure 8. Strong fluorescence in the MG63 cells was observed at 24 h under hypoxic condition compared with oxic condition. It proved that the fluorescence could be significant enhanced in the hypoxic cells according to LSCM observation.

2.2.3. Quantitative analysis of fluorescent cells by flow cytometry

In order to further determine the proportion of fluorescent cells labeled by **BTTD-NO$_2$**, cytometric analyses were performed by flow cytometry and the results were shown in Figure 9. The proportion of fluorescent labeling cells was reached 65% after cultured with **BTTD-NO$_2$** for 24 h under hypoxic condition while only 2.4% of cells were recognized as fluorescent cells in control group (without **BTTD-NO$_2$**).

Yin and Zhu, et al.[9,11] had reported that the nitro group could quench the fluorescence of the aromatic ring system, but when the nitro group was bio-reduced to amino group by nitroreductase in hypoxic cells, their naphthalimide and quinoxaline compounds

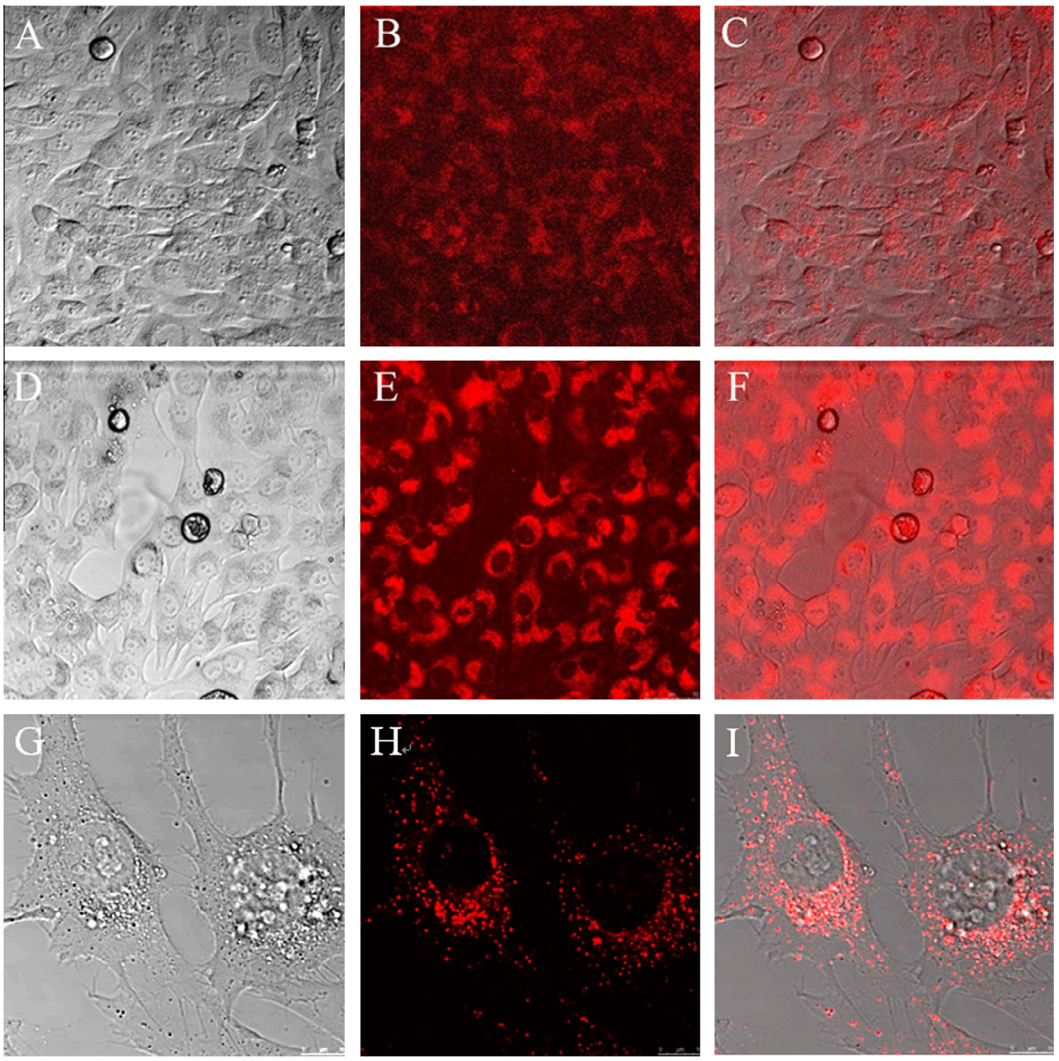

Figure 8. Fluorescence microphotograph of MG63 cells incubated with **BTTD-NO_2** for 24 h under oxic condition (A–C) and hypoxic condition (D–I). Left pictures: transmission channel; middle pictures: fluorescence channel; right pictures: composition. Strong fluorescence was observed in cytoplasm when these cells were cultured under hypoxic condition (E and H) compared with that of under oxic condition (B).

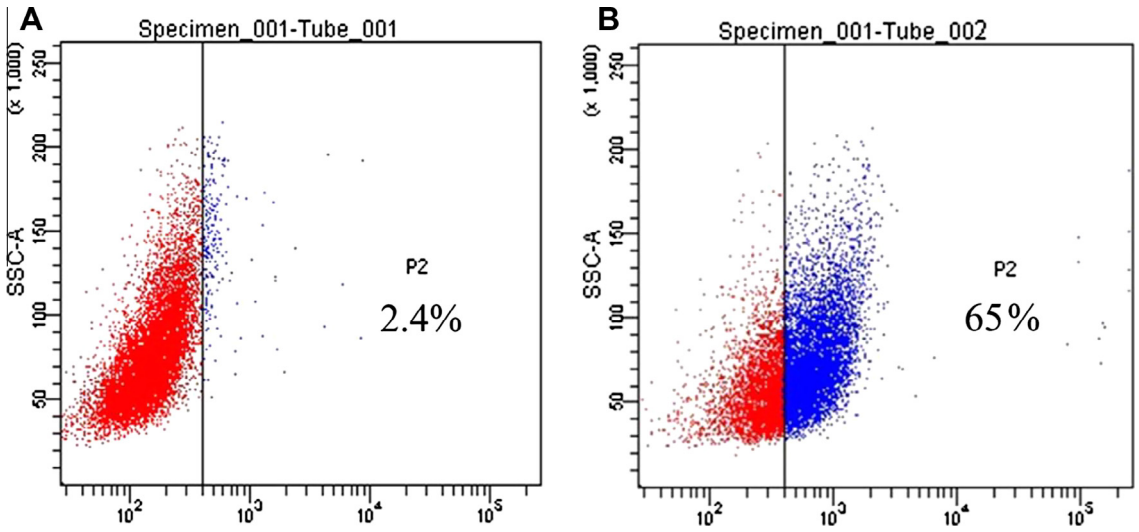

Figure 9. Flow cytometry results. Only 2.4% of cells were recognized as fluorescent cells after cultured with **BTTD-NO_2** for 24 h under oxic condition (A), while the proportion of fluorescent cells was reached 65% under hypoxic condition (B).

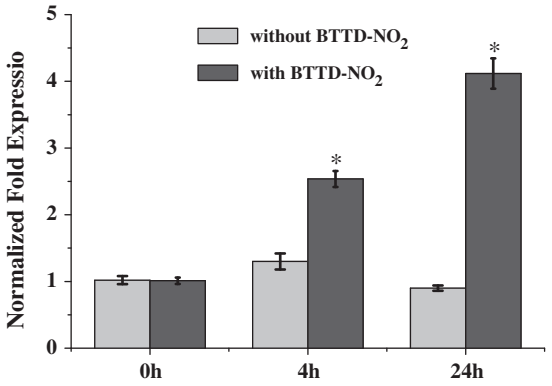

Figure 10. Gene expression of nitroreductase in MG63 cells cultured with or without **BTTD-NO$_2$**. *the mRNA expression of nitroreductase increased significantly after 4 and 24 h by adding **BTTD-NO$_2$** ($p < 0.05$).

could reach 11 and 17 times differential fluorescence between oxic and hypoxic cells. In this paper, based on the reduction of **BTTD-NO$_2$** to **BTTD-NH$_2$**, the proportion of fluorescent cells significantly enhanced under hypoxic condition compared with oxic condition.

2.2.4. Gene expression of nitroreductase in MG63 cells

To analyze the effect of **BTTD-NO$_2$** on the gene expression of the nitroreductase in MG63 cells, quantitative RT-PCR was carried out after 4 and 24 h. As shown in Figure 10. Real time RT-PCR analysis revealed that, after the MG63 cells treated with **BTTD-NO$_2$** for 4 and 24 h, the gene expression level of reductase reached 2.5 times and 4 times, respectively, compared with control group (without **BTTD-NO$_2$**).

These results indicated that **BTTD-NO$_2$** could transfer into MG63 cells and increase gene expression of nitroreductase, high expression of nitroreductase could accelerate reduction of **BTTD-NO$_2$** to **BTTD-NH$_2$** in turn, as a result, the MG63 cells showed significant fluorescent intensity which were confirmed by CLSM observation and Flow cytometry.

Previous reports had showed that thiophene fluorophores were capable of staining the cytoplasm[19] and some benzothiadiazole derivatives could selectively stain the mitochondria of the cells[16,17] or label the nucleus of the cells.[20–22] However, those kinds of compounds could only be as fluorescent probes for all kinds of cells and could not distinguish tumor cells specially. In this paper, **BTTD-NO$_2$** constituted of both thiophene group and the stem of benzothiadiazole, was also observed distribution in the cytoplasm of MG63 cells. Furthermore, with nitro group on the stem, the **BTTD-NO$_2$** could selectively trace the hypoxic tumor cells with strong red fluorescence. The possible mechanism was that **BTTD-NO$_2$** could be reduced by nitroreductase, and stimulated the high gene expression of the nitroreductase in MG63 cells, which improved conversion rate of **BTTD-NO$_2$** to **BTTD-NH$_2$** in turn.

Totally, the new benzothiadiazole derivative, **BTTD-NO$_2$**, had the considerably potential as fluorescent probe in cancer cells due to high specificity in hypoxic condition, negligible background fluorescence signal, ease of handling, and solution stability.

3. Conclusions

The new benzothiadiazole compound, **BTTD-NO$_2$**, had been successfully synthesized and showed no cytotoxicity to MG63 cells. It could significantly label the MG63 cells by emitting strong red fluorescence under hypoxic condition. The possible mechanism was that **BTTD-NO$_2$** could be reduced by nitroreductase, and could stimulate the high gene expression of the nitroreductase in MG63 cells, which improved conversion rate of **BTTD-NO$_2$** to **BTTD-NH$_2$** in turn. In conclusion, **BTTD-NO$_2$** had the potential as a superior fluorescent probe for tumor detection.

4. Experimental section

4.1. Synthesis of target compound (BTTD-NO$_2$)

All the chemicals commercially available were used directly without further purification unless otherwise stated. All the solvents were of analytical grade and freshly distilled prior to use, except spectrographically pure DMSO used for biological evaluation. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE-400 spectrometer in $CDCl_3$ using TMS as internal standard.

The detailed synthetic routes were shown in Figure 1. $Pd(PPh_3)_4$,[23] 4,7-dibromobenzo[*c*][1,2,5]thiadiazole,[24] 3-dodecylthiophene[25] were synthesized according to the literature procedures.

4.1.1. Synthesis of 4,7-dibromo-5,6-dinitrobenzo[*c*][1,2,5]thiadiazole (C1)

A mixture of concentrated H_2SO_4 (140 mL) and fuming HNO_3 (140 mL) was cooled to 0 °C. After stirring for 10 min at 0 °C, 4,7-dibromobenzo[*c*][1,2,5]thiadiazole (5.0 g, 17 mmol) was added in small portions. The reaction mixture was stirred for 6 h at 0 °C, then poured out into ice/water. The precipitate was filtrated, washed with water and purified by column chromatography on silica gel to afford beige solid (1.5 g, 40%). Melting point: 201–202 °C.

4.1.2. Synthesis of 2-(4-dodecylthiophen-2-yl)-5,5-dimethyl-1,3,2-dioxaborinane (C2)

3-Dodecylthiophene (5.04 g, 20 mmol) was dissolved in 40 mL of anhydrous THF under inert atmosphere, then the solution was cooled down to −78 °C, and 8.8 mL (22 mmol) of *n*-BuLi (2.5 M in *n*-hexane) was added dropwise. The mixture was allowed to be stirred at −78 °C for an additional hour, trimethyl borate (3.36 mL, 30 mmol) was added dropwise, followed by 1 h stirring at −78 °C, and 24 h stirring at room temperature. After that, the mixture was cooled to 0 °C and 2 mol/L HCl (50 mL) was added under stirring for 30 min, then extracted by ether. The organic layer was washed with brine for three times, finally anhydrous $MgSO_4$ (40 mmol, 4.8 g) and 2,2-dimethyl-1,3-propanediol (40 mmol, 4.72 g) were added. The reactant was stirred overnight. After evaporating off the solvent, the residue was purified by column chromatography on silica gel to afford white solid (4.8 g, 66%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.46 (s, 1H), 7.19 (s, 1H), 3.78 (s, 4H), 2.61 (t, J = 7.6 Hz, 2H), 1.60 (m, 2H), 1.30 (m, 18H), 0.99–0.87 (m, 9H). ^{13}CNMR (100 MHz, $CDCl_3$): δ (ppm) 144.6, 136.9, 126.5, 72.4, 32.0, 31.9, 30.1, 29.7, 29.6, 29.5, 29.4, 22.7, 21.9, 14.1.

4.1.3. Synthesis of 4,7-bis(4-dodecylthiophen-2-yl)-5,6-dinitrobenzo[*c*][1,2,5]thiadiazole (BTTD-NO$_2$)

To a 100 mL two-neck flask **C1** (0.38 g, 1.0 mmol), **C2** (1.09 g, 3.0 mmol), catalytic amount of $Pd(PPh_3)_4$ (0.5 mol %), aqueous potassium carbonate (2 mol/L, 20 mL) and deoxygenated toluene (20 mL) were added under the protection of argon. The mixture was stirred for 48 h at 90 °C. After cooling to room temperature, the reaction mixture extracted with toluene. The organic layer was washed with water before being dried over anhydrous magnesium sulfate. After evaporating off the solvent, the residue was purified by column chromatography on silica gel to afford light yellow solid (0.47 g, 65%). Melting point: 68–69 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.32 (s, 2H), 2.68 (t, J = 7.6 Hz, 2H), 1.68–1.61 (m, 2H), 1.33–1.26 (m, 18H), 0.89 (t, J = 6.4 Hz, 3H). ^{13}C

NMR (100 MHz, $CDCl_3$): δ (ppm) 152.3, 144.5, 141.8, 132.3, 129.3, 126.5, 121.5, 32.2, 32.1, 30.5, 30.4, 29.8, 29.8, 29.7, 29.6, 29.5, 29.4. IR (KBr), cm^{-1}: 2920, 2850, 1539, 1468, 1353, 827. ESI-MS: m/z 765.2946 ($M+K^+$); calcd for M_w+K^+: 765.2944.

4.2. Spectroscopic feature of BTTD-NO_2

4.2.1. Absorption spectrophotometry

The extinction co-efficient (ε) of **BTTD-NO_2** was calculated according to the Lambert–Beer law. Absorption spectra of the sample were measured by a Perkin Elmer Lambda 950 UV/VIS Spectrometer in CH_3OH.

4.2.2. Fluorescence spectrophotometry before and after chemical reduction

Reduction of **BTTD-NO_2** by iron dust in acetic acid and heating for 10 h at 80 °C, resulted in diamine **BTTD-NH_2**. The structure was confirmed by ^1H NMR and FTIR. ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.18 (s, 2H), 7.12 (s, 2H), 4.39 (br, 4H), 2.71 (t, J = 8.0 Hz, 4H), 1.72–1.65 (m, 4H), 1.43–1.26 (m, 36H), 0.90 (t, J = 8.0 Hz, 6H). IR (KBr), cm^{-1}: 3330, 2922, 2851, 1445. The detailed synthetic routes were shown in Figure 3. Fluorescence spectra of **BTTD-NO_2** before and after chemical reduction (10^{-6} mol/L methanol) were measured with excitation at 480 nm in CH_3OH. Fluorescence spectra were analyzed by a HITACHI F-7000 fluorescence spectrophotometer at 298 K.

4.2.3. Bioreductive activation of BTTD-NO_2 by nitroreductase

The liver microsomes were isolated from 10 rats (average weight: 150 g) according to the method reported previously,[26–28] the liver microsomes provided as nitroreductase (P450 reductase) in this experiment. After homogenization of the liver samples in 20 mL of 50 mM phosphate (with 0.25 M sucrose, pH 7.4), the microsomes were isolated by centrifugation at 10,000g to 100,000g, and resuspended in 5.0 mL of 0.1 M phosphate buffer (pH 7.4). The samples were prepared with microsomes (10 mg/mL), **BTTD-NO_2** (concentration: 0, 0.5×10^{-6}, 1.0×10^{-6}, 2.0×10^{-6} mol/L), NADPH (2×10^{-3} mol/L) and $MgCl_2$ (3×10^{-3} mol/L) in 0.1 M phosphate buffer pH 7.4. All these samples were incubated at 37 °C for 4 h. After incubation these samples were centrifuged at 10,000g and the fluorescence spectra of the supernatants were measured by the F-7000 FL Spectrophotometer (Hitachi). Furthermore, the fluorescence spectra of chemical reduced **BTTD-NO_2** was compared with that of biological reduced **BTTD-NO_2** at concentration of 2.0×10^{-6} mol/L.

4.3. Tracing tumor cells with BTTD-NO_2 in vitro

After the **BTTD-NO_2** was synthesized, it was initially dissolved at 1×10^{-4} mol/L in dimethyl sulfoxide (DMSO), followed by filtration with 0.22 μm filter membrane to eliminate contamination. Small volumes were added to cell suspensions to give the appropriate concentration in the following biological experiment (ultimate concentration of DMSO <2%).

4.3.1. Cytotoxicity of BTTD-NO_2

For cell cytotoxicity assay, MG63 cells were seeded in 96-well plates at a concentration of 1×10^4 cells/well for 24 h, then different concentration of **BTTD-NO_2** (0, 0.5×10^{-6}, 1×10^{-6} and 2×10^{-6} mol/L in ultimate concentration) were added in the well and cultured for another 24 and 48 h. Cytotoxicity was determined by MTT colorimetric method with 5 parallel wells at each time point. After cells were incubated with 5 mg/mL of MTT for 4 h at 37 °C, supernatant was discarded, 200 μL/well of DMSO was added to dissolve the formazen, and then the absorbance was measured at 570 nm with a microplate reader (BIO-RAD, model 550).

4.3.2. Fluorescence labeling of MG63 cells by using BTTD-NO_2

MG63 cells were cultured in DMEM medium with fetal bovine serum (10%, v/v) at 37 °C in 5% CO_2. The cells (5×10^4 cells) were seeded in culture flasks (25 cm^2, Corning co.). After cultured in a CO_2 incubator for 24 h, the culture medium was exchanged to the DMEM medium with **BTTD-NO_2** (ultimate concentration: 2×10^{-6} mol/L). The cells were then placed in a hypoxic chamber flushed with 5% CO_2 and 95% N_2 at 37 °C for hypoxic condition. For oxic condition, the flasks with cells directly placed in incubator at 37 °C. After treatment for 24 h, the cells were observed by Laser Scanning Confocal Microscopy.

4.3.3. Quantitative analysis of fluorescent cells by flow cytometry

In order to determine the proportion of fluorescent cells labeled by **BTTD-NO_2**, cytometric analyses were performed according to flow cytometry. The MG63 cells (2×10^4 cells) were seeded in culture flasks (25 cm^2, Corning co.), treated with **BTTD-NO_2** (ultimate concentration: 2×10^{-6} mol/L) for 24 h under oxic or hypoxic condition. Then the cell pellet was washed with phosphate buffered saline and centrifuged again. The pellet was resuspended in 1 mL PBS buffer and measured in a flow cytometer (Becton Dickinson). Data processing was carried out using the FACSDiva Version 6.1.1 Software (BD Biosciences).

4.3.4. Gene expression of nitroreductase in MG63

The **BTTD-NO_2** could be as substrate for the nitroreductase in the cells, to determine whether the **BTTD-NO_2** could promote the higher expression of the nitroreductase in cells. The gene expression of nitroreductase in MG63 cells was analyzed by RNA extraction followed by quantitative real-time polymerase chain reaction (QRT-PCR) with SYBR green.

After incubated for 4 and 24 h with **BTTD-NO_2** (2×10^{-6} mol/L), MG63 cells were collected for RNA extraction by Trizol (Invitrogen, USA) according to the instructions of the manufacturer. Briefly, cells were rinsed twice with PBS and lysed directly by adding 1 mL sample of Trizol reagent, and followed by passing with an 8# needle for several times. Then RNA samples was subsequently isolated and were dissolved in 30 μL of RNase- and DNase-free water, and the RNA concentration were measured using a NanoDrop (Thermo Scientific NanoDrop 2000, DE, USA).

Total RNA (1 μg) was reverse transcribed into complementary DNA (cDNA) using an iScrip cDNA Synthesis Kit (Bio-Rad, CA) in a 20 μL reaction by following the protocol of the manufacturer.

The PCR reactions were performed using SsoFast™ EvaGreen® Supermix (Bio-Rad, CA), in a CFX96 real-time thermo cycler (Bio-Rad, CA), and Triplicate PCR reactions were carried out. Relative mRNA abundance was analyzed according to Bio-Rad CFX manager software (version: 1.6.541) and reported as fold induction. GAPDH abundance was used for normalization. The primer sequences of the GAPDH were: (forward) GGAAG GTGAA GGTCG GAGTC and (reverse) TTAGGGTAGTGGTAGAAGGT; the primer sequences of nitroductase were: (forward) GTCCTGAAACTGGGAACTAACA and (reverse) TCCTCTTCTTCATCGGTGGTAA.

4.4. Statistical analysis

All data were expressed as means ± standard deviations (SD). An unpaired Student's t-test was adopted to test the significance of the observed differences between the study groups. A value of $p < 0.05$ was considered statistically significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.019.

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