A Visible Light-triggered Ferroptosis Probe: Visualization, Endoplasmic Reticulum Localization and Photo-induced Lipid Peroxidation

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Abstract:

Accumulation of lethal lipid peroxidation plays the central role in ferroptosis initiation and execution. However, it remains unclear whether the location of lipid peroxidation accumulation in subcellular organelles contributes to ferroptosis initiation due to the lack of specific small-molecule probe. Here we report a quinazolinone-pyridine difluoroboron complex (**BODIQPy**s) with vinyltriphenylamine modification, **BODIQPy-TPA**, as a near-infrared (NIR) bioimaging and photo-induced ferroptosis bifunctional probe. **BODIQPy-TPA** with D- π -A structure exhibits a good endoplasmic reticulum (ER) localization and photo-activated lipid reactive oxygen species (ROS) generation. The photo-induced ferroptosis is confirmed by morphological changes of live cell fluorescence imaging, suppression of cell death by a ferroptosis inhibitor (Fer-1) and down-regulation of ferritin heavy polypeptide 1 (FTH1). This study is the first example of visual photo-induced ferroptosis by small organic molecular probe, which provides a promising tool for further investigation of the direct relationship between lipid peroxidation accumulation and ferroptosis. Moreover, ferroptosis induced by photodynamic therapy (PDT) process in ER will be a good strategy to improve the efficiency of PDT for apoptosis-resistant tumor cells.

INTRODUCTION

Ferroptosis is a recently recognized form of programmed cell death that is different from other forms of cell death in morphological, genetic and biochemical levels[1-4]. Caused by iron-dependent accumulation of lethal lipid peroxidation, ferroptosis can generate mild immunogenicity and cannot be suppressed by drug resistance related mutations in tumor cells[5,6]. Thus, the development of ferroptosis inducers becomes a promising strategy for cancer treatment, especially to drug-resistant tumors[7-9]. More than that, ferroptosis inducers as chemical probes also provide effective tools to explore and understand the mechanism of ferroptosis initiation and execution[4]. For instance, the glutathione peroxidase 4 (GPX4) inhibition, GSH depletion and cystine starvation (the GPX4-GSH-cysteine axis) have been identified as an important pathway for inducing ferroptosis by small-molecule reagents such as erastin and RSL3[1, 10, 11]. Depleting lipophilic antioxidant ubiquinol/coenzyme Q₁₀ (CoQ₁₀) has been reported as another pathway for

inducing ferroptosis by compounds FIN56 and iFSP1[12,13]. On the other side, ferroptosis is also achieved by affecting iron metabolism[14]. Excess redox-active iron in the process of iron metabolism is able to catalyze the Fenton reaction to accelerate the lipid reactive oxygen species (ROS) production and lead to ferroptosis[1,8]. Accordingly, some iron containing nanomaterials as ferroptosis inducers were then developed for ferroptosis-enhanced tumor therapy[15-17]. Therefore, the development of new types of ferroptosis inducers not only provides a powerful tool for exploring the mechanism of ferroptosis, but also provides benefits to the treatment of tumors. Despite the excess of lipid-based ROS that has been proved to be the central role for initiation of ferroptosis[18], it is still unclear whether the enrichment of lipid peroxide in specific organelles leads to cell death[3,4,6]. Mitochondria, as the very important organelle in lipid metabolism and cellular ROS generation, have been proved to be a crucial player in cysteine deprivation-induced ferroptosis[19]. Nevertheless, there are some results which indicate that the role of mitochondria in ferroptosis caused by other forms of cellular ROS aggregation and GPX4 inhibition is not significant[4,20]. In addition, ferrostatins, as ferroptosis inhibitors, can still inhibit ferroptosis in mitochondria deficient cells[20]. These results indicated that mitochondria may not be the primary site of lipid peroxidation for ferroptosis initiation except for cysteine deprivation pathway. Endoplasmic reticulum, as a source of lipids for most membranes in other organelles, is another important organelle in the lipid metabolism regulation. Recently, NADPH-cytochrome P450 reductase (POR) in ER has been reported to play a substantial role in membrane phospholipids peroxidation and ferroptosis execution[21,22]. In addition, it is worth noting that ferrostatins located in the ER may be the key factor for prevention of lipid peroxidation and ferroptosis suppression[23]. Therefore, the development of novel chemical probes with good ROS generation ability and ER localization would help to further understand the ferroptosis initiation and execution.

Photo-triggered ROS production by photosensitizers such as organic dyes, metal complexes and nanoparticles is a very efficient way for photodynamic therapy (PDT) and chemical transformation[24-27]. However, most of the reported photosensitizers caused apoptosis rather than ferroptosis through light-induced ROS production, which led to the fact that tumor cells could enhance the resistance to this common cell death mode of PDT by upregulating the expression of antioxidants[28,29]. Although some photosensitizers as PDT agents have been developed to enhance tumor therapy by the synergism of ferroptosis and apoptosis recently[29], those PDT agents are not suitable as chemical probe for investigation of the process of ferroptosis initiation due to the complicated roles of ROS produced by such photosensitizers in cell death. To the best of our knowledge, the small organic molecule PDT agents that located in endoplasmic reticulum as ferroptosis inducers were rare[30]. In this work, we designed a novel quinazolinone-based difluoroboron complex (**BODIQPy-TPA**). This probe showed good ability of ER localization and photoinduced ROS generation, which could effectively realize the photo-activated ferroptosis in B16 cells. In addition, **BODIQPy-TPA** showed good near infrared (NIR) fluorescence emission in live cells, which can be used as a NIR bioimaging probe to observe the process of ferroptosis.

RESULTS AND DISCUSSION

Synthesis and optical properties of **BODIQPy-TPA**

Based on our previous results[31,32], quinazolinone-pyridine difluoroboron complex (**BODIQPys**)

exhibited more electron deficiency compared with boron dipyrromethene (**BODIPY**), which can be a better electron acceptor. Therefore, we aim to construct a D- π -A structure of **BODIQPy**s with vinyltriphenylamine group modification (Figure 1). The introduction of triphenylamine (TPA) group can improve the interaction between **BODIQPy**s and lipids [33,34].

Figure 1. Synthesis and single crystal structure of **QPy-TPA** and **BODIQPy-TPA**

BODIQPy-TPA was prepared via two simple steps and characterized by ^1H NMR and HRMS. The single crystal of **BODIQPy-TPA** (CCDC No. 2071259) was obtained from mixed solution of acetonitrile and water (Figure 1C, S3 and S4). Compared with the single crystal of quinazolinone-pyridine ligand (**QPy-TPA**) (Figure 1B), the π - π interaction between **BODIQPy-TPA** molecules in crystal is weaker due to the hydrogen bonding between **BODIQPy-TPA** and water molecules, which leads to the change of molecular packing mode in the crystal. The absorption and emission spectra of **BODIQPy-TPA** and **QPy-TPA** in different solvents were then examined. The maximum absorption wavelength of **BODIQPy-TPA** (≈ 488 nm) and **QPy-TPA** (≈ 400 nm) did not change much in the selected solvents (Figure S5 and S7). Because of the better conjugation of BF_2 complex, compared with **QPy-TPA**, the absorption and emission wavelengths of **BODIQPy-TPA** red shifted (Table 1, S2 and S3). The maximum emission wavelength of **BODIQPy-TPA** in different solvents is above 640 nm except in toluene (Figure 2A, Table 1), which is located in the red/NIR region. Due to the D- π -A structure, both **BODIQPy-TPA** and **QPy-TPA** exhibited good solvochromic fluorescence properties (Figure 2A, 2B and Figure S6, S8). And **BODIQPy-TPA** also showed response to polarity (Figure 2F and Figure S8). The fluorescence quantum yield of **BODIQPy-TPA** and **QPy-TPA** was subsequently analyzed in different solvents (Table 1 and S2). **BODIQPy-TPA** showed very good emission efficiency in low polar solvent such as toluene and dichloromethane (Table 1). Although the emission efficiency of **BODIQPy-TPA** decreases in polar solvents such as DMSO, **BODIQPy-TPA** also showed good fluorescence in the DMSO/water (DMSO: H_2O = 4:6) or THF/water (THF: H_2O = 1:9) mixed solvent (Figure 2b, S9 and Table S4), due to the aggregation

induced emission (AIE) characteristics of TPA group. In addition, the emission intensity of **BODIQPy-TPA** significantly increased in the DMSO/glycerol system (Figure 2E-2G), which further indicated the AIE property of **BODIQPy-TPA** due to the suppression of the rotation of TPA group. It is worth noting that **BODIQPy-TPA** also showed good photostability (Figure S10) and considerable two-photon fluorescence (Figure S11), which can be used for two-photon fluorescence bioimaging.

Figure 2. The luminescent properties of **BODIQPy-TPA**. (A) The emission spectra of **BODIQPy-TPA** in different organic solvents. (B) The pictures of **BODIQPy-TPA** in organic solutions excited at 365 nm using a UV lamp. (C) The pictures of **BODIQPy-TPA** in the DMSO/water mixed solvent. (D) The emission spectra of **BODIQPy-TPA** in the DMSO/water mixed solvent. (E) The emission spectra of **BODIQPy-TPA** in the DMSO/glycerol system. (F) The relationship of **BODIQPy-TPA** between maximum emission wavelength and viscosity. (G) The relationship of **BODIQPy-TPA** between emission intensity and viscosity.

Table 1. The optical properties of **BODIQPy-TPA** in solution and solid state.

Solvent	$\lambda_{\text{abs}}^{\text{max[a]}}(\text{nm})$	$\varepsilon (\text{M}^{-1}\text{cm}^{-1})$	$\lambda_{\text{ex}}^{\text{F [c]}}(\text{nm})$	$\lambda_{\text{em}}^{\text{F}}(\text{nm})$	Stokes Shift (nm)	$\Phi_{\text{F}}^{(\text{c})}$
Toluene	474	31500	476	566	92	0.99
DCM	486	33600	485	673	187	0.57
THF	467	38300	465	650	183	0.55
EA	462	43000	460	643	181	0.41
DMSO	473	19600	478	724	251	<0.01
DMSO:H ₂ O(4:6)	487	18200	473	677	190	0.10
Solid	430		539	650	220	0.23

Cell imaging and ER localization of BODIQPy-TPA

On the basis of the good emission of **BODIQPy-TPA** in low polar solvent and aggregation state, the fluorescence cell imaging experiments with **BODIQPy-TPA** were conducted in live B16 cells. As showed in Figure 3, **BODIQPy-TPA** exhibited good cell membrane permeability with bright green and red/NIR fluorescence in B16 cells excited by both one and two-photon (Figure 3 and Figure S12). The **QPy-TPA** also showed blue and green fluorescence in B16 cells. The colocalization imaging experiments were further performed using commercial dyes (ER Tracker® Blue for ER imaging and Nile Red for lipid droplets imaging). Interestingly, **BODIQPy-TPA** and **QPy-TPA** showed different subcellular localization abilities. As shown in Figure 3, **BODIQPy-TPA** was mainly distributed in ER with Pearson's coefficient of 0.87, whereas **QPy-TPA** was mainly distributed in lipid droplets (LDs) with Pearson's coefficient of 0.86. Based on the recent literature[35], although both LDs and ER contain the lipid substances, the water content is significantly different. Based on the crystal structure analysis of **BODIQPy-TPA** and **QPy-TPA**, (Figure 1B and 1C), some water molecules was observed to form hydrogen bonds with **BODIQPy-TPA**, and water molecules were not found in the crystal structure of **QPy-TPA**, which indicated that **BODIQPy-TPA** showed better interaction with water than **QPy-TPA**. Therefore, the different hydrophilic ability of **BODIQPy-TPA** and **QPy-TPA** may be the reason for their differences in organelle localization.

Figure 3. One-photon confocal fluorescent images of **BODIQPy-TPA** in B16 cells and the colocalization with ER traker. (A) Merged image. (B) ER Tracker®Blue (200 nM, 30 min). (C) **BODIQPy-TPA** signal channel collected at 500-550nm ($\lambda_{\text{ex}} = 488$ nm), (D) **BODIQPy-TPA** signal channel collected at 663-738nm ($\lambda_{\text{ex}} = 488$ nm). (E) Bright-field image. (F) Colocalization scatter plot with a Pearson's correlation coefficient of 0.87. (G) Intensity profile of the white line in image.

Figure 4. One-photon confocal fluorescent images of **QPy-TPA** in B16 cells and the colocalization with Nile Red. (A) Merge image. (B) Fluorescence image of **QPy-TPA** (5 μ M, Blue channel, λ_{ex} = 405 nm, collected 425-475 nm) (C) Green channel, λ_{ex} = 488 nm, collected 500-570 nm. (D) Fluorescence image of Nile Red (1 μ M, 10 min, Red channel, λ_{ex} = 561 nm, collected 570-620 nm) (E) Bright-field image. (F) Colocalization scatter plot with a Pearson's correlation coefficient of 0.86. (G) Intensity profile of the white line in image.

Photo-induced ferroptosis by **BODIQPy-TPA**

According to the results of cell imaging, the morphology of the cells changed obviously under continuous light irradiation (Figure 5, Movie 1). The cell membrane was broken and bubbled. The nuclei of cells remained structural integrity. Interestingly, some red fluorescent circles gradually appeared in the cells at the later stage of cell death process (Figure 5 and S13). On consideration of these characteristics of cell morphological changes, the ferroptosis may occur. To determine the effect of light irradiation on the cytotoxicity of B16 cells, cell viabilities were tested with blue LED light irradiation. As shown in Figure 6, cell viability was almost unaffected by blue LED irradiation. Furthermore, the toxicity of **BODIQPy-TPA** (without irradiation) to B16 cells was very low (IC_{50} = 42.11 μ M) (Figure S14A and Table S5). By comparison, **BODIQPy-TPA** exhibited very high cytotoxicity (IC_{50} = 0.51 μ M) under blue LED irradiation (Figure S14B and Table S5). The photocytotoxicity index (PI), ratio of $(IC_{50})_{dark}/(IC_{50})_{irrad}$, was about 82.57, confirming good PDT effect of **BODIQPy-TPA**[29]. In order to further verify the process of photo-induced ferroptosis, Fer-1 as ferroptosis inhibitor was used[36,37]. As showed in Figure 6, Fer-1 prevents cells from photo-induced cell death in the presence of **BODIQPy-TPA**. In addition, higher concentration of Fer-1 gave better cell viability. The cell survival rate was above 80 % in the presence of **BODIQPy-TPA** (2.5 μ M, with irradiation) when the concentration of Fer-1 increased to 50 μ M. And the toxicity of **BODIQPy-TPA** to B16 cells was decreased (IC_{50} = 8.87 μ M) by adding Fer-1 (Figure S14C and Table S5). More than that, western blotting results indicated that the expression of ferritin heavy polypeptide 1 (FTH1), which plays a crucial antioxidant role in the regulation of ferroptosis[38,39], was down regulated after light irradiation in the presence of **BODIQPy-TPA** (Figure 6B and 6C). In turn, after adding Fer-1, FTH1 expression could not be affected by light irradiation with **BODIQPy-TPA**. These results

demonstrated that **BODIPY-TPA** could induce ferroptosis under light irradiation. It is noteworthy that **QPy-TPA** also showed good photo-cytotoxicity (Figure 6D and S14B). However, Fer-1 cannot rescue cells from **QPy-TPA** mediated photo-induced cell death (Figure 6D and S14D), which suggested that **QPy-TPA** cannot cause ferroptosis under light irradiation.

Figure 5. The morphology of the cells changed under continuous light irradiation. (A) Bright-field (B) fluorescence images change with irradiation (0-10 min)

Figure 6. Photo-induced ferroptosis by **BODIQPy-TPA**. (A) Cell viability was measured using MTT assay with **BODIQPy-TPA** (2.5 μM) and different doses of Fer-1. (B) Expression of FTH1 in B16 cells incubated with **BODIQPy-TPA** and Fer-1 after light irradiation. (C) Quantification of the immunoblot analysis in panel A. All quantitative data are presented as mean ± SD from three independent experiments, and p values were calculated with unpaired t test; **p < 0.005. (D) Cell viability was measured using MTT assay with **QPy-TPA** (5 μM) and different doses of Fer-1.

Photo-induced ROS production and lipid peroxidation *in vitro* by **BODIQPy-TPA**

Given the close relationship between ferroptosis and ROS accumulation as well as the good photophysical properties of **BODIQPy-TPA**, we further explored the photo-triggered ROS generation of **BODIQPy-TPA**. Firstly, the emission spectra of **BODIQPy-TPA** in nitrogen and oxygen atmosphere were acquired. The fluorescence intensity of **BODIQPy-TPA** was enhanced in nitrogen (Figure S15), which indicated a possible energy transfer process between **BODIQPy-TPA** and molecular oxygen. And 1, 3-diphenylisobenzofuran (DPBF) was used as singlet oxygen scavenger to explore the possibility of $^1\text{O}_2$ generation. The absorption band of DPBF decreased gradually with continuous light irradiation, further indicating the possible energy transfer process between **BODIQPy-TPA** and molecular oxygen (Figure S16). Subsequently, the electron paramagnetic resonance (EPR) spectroscopy was performed to detected the generation of ROS by using 2,2,6,6-tetramethylpiperidine (TEMP) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as trapping agents. As shown in Figure 7A, the three characteristic peaks of 2,2,6,6-tetramethyl-4-piperidinol-N-oxyl (TEMPO) in EPR spectra, which are derived from oxidation of TEMP by ROS[27,40], were observed in dichloromethane solution of **BODIQPy-TPA** under visible light irradiation. And the signal intensity increased with the increase of visible light irradiation time (Figure S17). In the same vein, obvious signals were detected in mixed solution (DMSO/H₂O=6:4) of **BODIQPy-TPA** under visible light irradiation by using DMPO as trapping agent (Figure 7B and S18). The typical spin-trapping adduct DMPO-•OOH can be identified through simulation ($g = 2.0057$, $\alpha^N = 14.07$ G, $\alpha^H = 10.99$ G), which may be attributed to peroxy radical or superoxide radical[41,42]. Accordingly, **BODIQPy-TPA** exhibited good photo-incudced ROS generation in organic solvent and aqueous solution probably through a combination of energy transfer and electron transfer processes. In

consideration of the photo-toxicity of **QPy-TPA**, the photo-induced ROS production by **QPy-TPA** was also investigated through EPR spectroscopy using DMPO and TEMP as trapping agents. Similar results to **BODIQPy-TPA** were obtained (Figure S19), suggesting that **QPy-TPA** also has the ability to generate ROS.

Figure 7. EPR spectra of **BODIQPy-TPA** under visible light irradiation (A) TEMP as trapping agent in CH_2Cl_2 ; (B) DMPO as trapping agent in $\text{DMSO}/\text{H}_2\text{O} = 6:4$; (C) DMPO as trapping agent with oleic acid in CH_2Cl_2

In addition, oleic acid was chosen to explore the possible formation of lipid peroxide under light irradiation by **BODIQPy-TPA**. As showed in Figure 7C, two major spin trapped adducts of DMPO- $\bullet\text{OR}$ (hyperfine splitting constants, $\alpha^{\text{N}} = 13.45 \text{ G}$, $\alpha^{\text{H}} = 8.95 \text{ G}$) and DMPO- $\bullet\text{R}$ (hyperfine splitting constants, $\alpha^{\text{N}} = 14.60 \text{ G}$, $\alpha^{\text{H}} = 21.51 \text{ G}$) was well resolved after simulation[43,44]. Furthermore, the conversion of oleic acid by visible light irradiation in the presence of **BODIQPy-TPA** was investigated via HPLC analysis (Figure S20-S26) and ESI-HRMS. A new peak appeared at retention time of 4.59 min (Figure 8A and S23). And the intensity of this new peak increased gradually with the the increase of illumination time (Figure S21). This new peak was then collected and was further characterized by ESI-HRMS. As shown in Figure 8B and 8C, three major peaks with m/z 281.2489, 295.2280 and 313.2384 were detected under negative ion detection, which could be attributed to oleic acid and two possible lipid peroxidation products (carbonylation products of oleic acid and peroxide of oleic acid) respectively (Figure 8C and S27). More than this, the characteristic peaks of two peroxidation products were also detected in the reaction mixture by ESI-HRMS under positive ion detection mode (Figure S27). To further investigate whether the electron transfer process is energetically favorable, Gibbs energy calculated by the Rehm-Weller equation (Eq. S1)[45]. The E^{ox} (Oleic acid = -0.725 V) and E^{red} (**BODIQPy-TPA** = 0.709 V) were measured by cyclic voltammetry (Figure S28). E_{00} (2.20 eV) is

the zero-zero transition energy, which can be calculated from the cross-point of the absorption and luminescence spectra at 563 nm ($E_{00} = 1240/563$ nm in eV, Figure S29). The calculated Gibbs energy (ΔG) is -3.634 eV, which suggested that the electron transfer process between **BODIQPy-TPA** and oleic acid is favorable. These results imply that **BODIQPy-TPA** could effectively promote the formation of ROS and lipid peroxidation process *in vitro*, which may be the main factor of photo-induced ferroptosis.

Figure 8. HPLC analysis and ESI-HRMS for the conversion of oleic acid by visible light irradiation in the presence of **BODIQPy-TPA**

Conclusion

In summary, vinyltriphenylamine modified quinazolinone-pyridine difluoroboron complex (**BODIQPy-TPA**) was developed as a photo-induced ferroptosis and NIR bioimaging bifunctional probe. The D- π -A structure of **BODIQPy-TPA** not only showed good red/NIR fluorescence and large Stokes shift (> 90 nm) in low polar organic solvents (green fluorescence in toluene), but also showed aggregation induced fluorescence enhancement (AIEE) properties in aqueous solutions. **BODIQPy-TPA** exhibited bright green and red/NIR emission in live cells by one or two photon excitation. Benefiting from the good interaction between quinazolinone-pyridine difluoroboron moiety and H_2O via hydrogen bond formation as well as the good lipophilic affinity of triphenylamine, **BODIQPy-TPA** mainly located in endoplasmic reticulum. More importantly, **BODIQPy-TPA** could effectively induce ROS and lipid peroxide generation under visible light irradiation, which might be the cause of ferroptosis. Therefore, **BODIQPy-TPA** would be a good candidate of visual probe for further investigation of the direct relationship between lipid peroxidation accumulation in ER and ferroptosis. Considering that ferroptosis could improve the efficiency of PDT for apoptosis-resistant tumor cells, we anticipate that these efforts also provide a promising strategy for the design of novel PDT agents.

Accession Codes

CCDC 2014059 and 2071259 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

Supporting Information

Supporting Information is available.

Conflict of Interest

There is no conflict of interest to report.

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