Attachment of \( -tBu \) groups to aza-BODIPY core at 3,5-sites with ultra-large Stokes shift to enhance photothermal therapy through apoptosis mechanism

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**ABSTRACT**

By the introduction of the \(-tBu\) groups into aza-BODIPY core, di-tert-butyl-substituted aza-BODIPYs at 3,5-sites (tBuazaBDPs) were prepared for the first time. Based on the X-ray analysis of CN-tBuazaBDP, this molecular structure is twisted. Near-infrared dye SMe-tBuazaBDP has the ultra-large Stokes shift (152 nm) in aza-BODIPY system, combining with the twisted intramolecular charge transfer and the free rotation of the \(-tBu\) groups at 3,5-sites. Although the barrier-free rotors of the distal \(-tBu\) groups in SMe-tBuazaBDP result in low fluorescence quantum yield, the photothermal conversion efficiency is markedly enhanced. SMe-tBuazaBDP nanoparticles with low power laser irradiation were proven to block cancer cell cycle, inhibit cancer cell proliferation, and induce cancer cell apoptosis in photothermal therapy (PTT). The strategy of “direct attachment of \(-tBu\) groups to aza-BODIPY core” gives a new design platform for a photothermal therapy agent.

1. Introduction

Cancers seriously threaten human life and health. So, drug therapy has become a widely used treatment for cancers since 1942 [1-5]. However, the intractable problems of high toxicity, side effects and drug resistance are still the main obstacles in clinical drug treatment of cancer. Recently, phototherapeutic, as a burgeoning tumor therapy method, has been developed rapidly with advantages of high spatial-temporal resolution, non-invasive property and low side effects [6-8]. Phototherapeutic mainly involves two species of treatments, photodynamic therapy (PDT) and photothermal therapy (PTT) [9-12]. PDT depends upon the efficient intersystem crossing (ISC) of the triplet photosensitizers (PSs) to sensitize the oxygen to produce reactive oxygen species (ROS) [13-15]. PTT converts light energy into heat by photothermal agents to increase the temperature of the surrounding environment, resulting in localized thermal damage [16-18]. Compared with PDT, the advantage of PTT is not restricted by the tumor hypoxic environment [19]. However, the high temperature (\(\geq 47\) °C) could lead to a damage to the normal tissues and may be also prone to inflammation and tumor metastasis [20,21]. Therefore, based on American National Standard for Safe Use of Lasers Outdoors, the maximum permissible exposure (MPE) for skin exposure is 0.2 W cm\(^{-2}\) at the 635 nm laser [22,23]. So, the temperature for the safe PTT under low laser power density should be slightly above 42 °C (usually 42–46 °C) [20,21]. A relaxed molecule in the lowest vibrational level of the excited state is known to return to the ground state mainly via the radiative transition (fluorescent emission), the non-radiative transition (heat release) and ISC to the triplet, sensitizing oxygen to produce ROS, that is, three channels to release energy [24,25]. Therefore, according to the Jablonski energy level diagram [26], by restricting the release of fluorescence and strengthening the other energy release process, the combination of PTT/PTD could maximize the effect of tumor treatment.

Aza-borondipyrromethene (aza-BODIPY) as a classic dye is known to widely applied in fluorescence imaging, phototherapy, photocatalysis and optoelectronic materials and so forth, owing to its near-infrared (NIR) absorption characteristic, high molar extinction...
Different intensity could usually trigger cell death through either necrosis or apoptosis [47,48], however PTT through apoptosis mechanism is rarely reported, comparing to necrosis mechanism [17]. In this work, SMe-tBuazaBDP under low laser radiation of 0.2 W cm⁻², was proven to inhibit cell viability, block cell cycle, inhibit cancer cell proliferation, induce cancer cell apoptosis, and lead to the death of cancer cells.

2. Experimental section

2.1. General

All chemical reagents and organic solvents were analytical grade, purchased from Energy Chemical & Technology (Shanghai) Co. Ltd without further purification unless otherwise specified. ¹H NMR spectra were recorded on a VARIAN Mercury 500 MHz spectrometer. ¹H NMR chemical shifts (δ) are given in ppm downfield from Me₄Si, determined by residual chloroform (δ = 7.26 ppm). ¹³C NMR spectra were recorded on a VARIAN Mercury 125 MHz spectrometer in CDCl₃, reporting with internal chloroform signal at δ = 77.0 ppm as standard. A high resolution mass spectrometer is used to give the exact molecular weight of the product. Fluorescence spectra were recorded on an F-128 spectrophotometer and are reported as cm⁻¹. Absorption spectrum were recorded on a UV-2550 spectrophotometer at 298 K. The absolute fluorescence quantum yield was measured by a F-98 spectrophotometer. Laser particle size analyzer purchased from Malvern. The temperature of the sample is monitored by a temperature measuring camera. A 635 nm laser was applied as the light source for light irradiation, controlling by a fiber coupled laser system for the laser output power and purchased from Changchun New Industries Optoelectronics Technology. Optical power density was measured by a CEL-NP 2000 power meter, purchased from Beijing Zhong Jiao Jin Yuan Technology Co, Ltd. BioTek Synergy H1 microplate reader was used in CCK8 assay. Confocal laser fluorescence microscope FV1200 (Olympus, Japan) was applied to estimate fluorescence imaging.

2.2. X-ray analysis

Crystals suitable for the X-ray structural determination were mounted on a Mac Science DIP2030 imaging plate diffractometer and irradiated with graphite monochromated Mo-Kα radiation (λ = 0.71073 Å) for the data collection. The unit cell parameters were determined by separately

Fig. 1. Design strategies for tBuazaBDPs with the –Bu groups at 3,5-sites.
auto-indexing several images in each data set using the DENZO program (MAC Science). For each data set, the rotation images were collected in 3° increments with a total rotation of 180° about the φ axis. The data were processed using SCALEPACK. The structures were solved by a direct method with the SHELX-97 program. Refinement on F² was carried out using the full-matrix least-squares by the SHELX-97 program. All non-hydrogen atoms were refined using the anisotropic thermal parameters. The hydrogen atoms were included in the refinement along with the isotropic thermal parameters.

2.3. Theoretical details

All theoretical calculations were performed based on density functional theory (DFT) and time-dependent density functional theory (TDDFT) methods via Gaussian 16 program suite. Becke’s three-parameter hybrid exchange function with Lee-Yang-Parr gradient-corrected correlation functional (B3LYP) and 6-31G(D) basis set were selected to serve the calculations. And the solvation effects of dichloromethane were also taken into consideration with the solvation model based on density (SMD).

2.4. Preparation of SMe-tBuazaBDP nanoparticles (SMe-tBuazaBDP NPs)

1 ml THF solution containing 1 mg SMe-tBuazaBDP and 5 mg DSPE-PEG2000 is slowly and gradually added to the 10 ml aqueous solution. Subsequently, THF was volatilized by vigorous stirring for 24 h with the purpose of dye molecules dispersed into the solution equally. The desired SMe-tBuazaBDP NPs could be obtained by centrifugation at 6000 rpm for 5 min.

2.5. Evaluation of singlet oxygen (1O₂) production efficiency

As the capture agent of 1O₂, 1,3-diphenylisobenzofuran (DPBF) can indicate the production capacity of 1O₂ in the solution. When the photosensitized agent produces 1O₂ under light radiation, DPBF will react with 1O₂ to open the loop, decreasing the absorption value at 416 nm. The absorption decline slope of DPBF can preliminarily indicate the capacity of 1O₂ production. Meanwhile, the 1O₂ production efficiency of the dyes can be calculated by the following formula under the reference 2,6-diiododibipyr with known 1O₂ production rate efficiency (φ₅ = 0.85 in toluene).

\[
\varphi_{\text{exp}} = \varphi_{\text{SDBE}} \left( \frac{m_{\text{exp}}}{m_{\text{DPBF}}} \right) \left( \frac{F_{\text{SDBE}}}{F_{\text{exp}}} \right)
\]

where “exp” represents the unknown dye molecule and “BDP” represents the reference 2,6-diiododibipy, “m” is the slope of DPBF absorption peak decline, “F” is the absorption correction factor, \( F = 1 - 10^{-0.01D} \). O.D. represents the absorption value of the sample at the wavelength of light radiation.

2.6. Measurement of photothermal capacity

The temperature change of dye nanoparticles in aqueous solution was evaluated by using 635 nm laser with different power density and different concentration of nanoparticles solution. The cooling process is studied via natural cooling. The temperature of the solution was measured by a temperature camera. The photothermal conversion efficiency was calculated using the reported formula [16–18]. For instance, SMe-tBuazaBDP NPs aqueous solution (80 μM) was irradiated by a 635 nm laser (0.3 W cm⁻²) for 10 min. Then, the solution was cooled to room temperature naturally, and the temperature was monitored every 1 min.

The calculation of photothermal conversion efficiency was based on the previously reported studies, and the specific calculation method was described by the following formula.

\[
\eta = \frac{\left( \Delta T_{\text{max}} - T_{\text{sur}} \right) - Q_{\text{he}}}{I \times (1 - 10^{-4})}
\]

Among them, \( \eta \) represents photothermal conversion efficiency, \( h \) is the heat transfer coefficient, \( s \) is the surface area of the container, \( Q_{\text{he}} \) represents heat dissipated from the laser mediated by the solvent and container, \( I \) is the laser power and \( A \) is the absorbance at 635 nm.

\[ m = \frac{m_{\text{CH}_2}}{\theta_c} \]

\( m \) is the mass of the solution containing the PTAs, \( C \) is the specific heat capacity of the solution, and \( \tau_c \) is the associated time constant.

\[ T = -\ln\left(\theta \right) \]

\( \theta \) is a dimensionless parameter, known as the driving force temperature.

\[ \theta = \frac{(T - T_{\text{sur}})}{(T_{\text{max}} - T_{\text{sur}})} \]

\( T \) is the current temperature. \( T_{\text{max}} \) and \( T_{\text{sur}} \) represent the maximum steady state temperature and the environmental temperature, respectively.

2.7. Evaluation of cytotoxicity by CCK8 assay

Human colon cancer (SW-620) cells or gastric cancer (SGC-7901) cells were cultured in 96-well plates for 24 h. The cells were treated with different treatments including no treatment (control group), SMe-tBuazaBDP NPs treatment, light treatment and SMe-tBuazaBDP NPs with light treatment, and different illumination time gradients (0-40 min) were added to light group and NPs with light group to evaluate the photothermal effects of light. Meanwhile, the 1O₂ production efficiency of the cells was measured by confocal microscopy. The absorbance value at 450 nm was measured by a microplate reader.

2.8. AM/PI staining of live-dead cells

Human colon cancer (SW-620) cells or gastric cancer (SGC-7901) cells were cultured in a 24-well plate for imaging experiments. The cells were treated in different groups to evaluate the dark-toxicity and phototoxicity of SMe-tBuazaBDP NPs. Group 1: natural growth (control group); Group 2: 30 μM dye NPs treatment (dye group); Group 3: 635 nm irradiation (0.2 W cm⁻² for 20 min) as light group; Group 4: After the dye NPs entered the cell (30 μM), 635 nm irradiation (0.2 W cm⁻² for 20 min) was applied as dye NPs + light group. After continuing culture for 30 min, Calcein-AM/PI double stain kit was added into each group of cells, continuing culture for 30 min for confocal fluorescence imaging. Among them, Calcein AM emits green fluorescence to indicate living cells with excitation wavelength of 488 nm and fluorescence collection channel of 500–529 nm, while PI emits red fluorescence to indicate dead cells with excitation wavelength of 559 nm and fluorescence collection channel of 570–619 nm.

2.9. Cell cycle analysis by flow cytometry

Human gastric cancer (SGC-7901) cells were cultured in 6-well plates for 24 h. The cells were treated in different groups to evaluate the dark-toxicity and photo-toxicity of SMe-tBuazaBDP NPs. Group 1: natural growth (control group); Group 2: 30 μM dye NPs treatment (dye group); Group 3: 635 nm irradiation (0.2 W cm⁻² for 20 min) as light group; Group 4: After the dye NPs entered the cell (30 μM), 635 nm irradiation (0.2 W cm⁻² for 20 min) was applied as dye NPs + light group. After continuing culture for 2 h, adherent cells were harvested by trypsinization and washed with cold PBS. Subsequently, the cells were fixed with ice-cold 70% ethanol at 4 °C overnight. Before analysis, ethanol was discarded.
and propidium iodide was used to stain the cells for 1 h in the dark. Then, the treated cells were subjected to flow cytometric analysis using the BD FACSVersé™ Flow Cytometer. A total of 10,000 events were acquired for each sample.

2.10. Cell apoptosis analysis by annexin-v/propidium iodide

Human gastric cancer (SGC-7901) cells were cultured in 6-well plates and treated in different groups. Group 1: natural growth (control group); Group 2: 30 μM dye NPs treatment (dye group); Group 3: 635 nm radiation (0.2 W cm⁻² for 20 min) as light group; Group 4: After the dye NPs entered the cell (30 μM), 635 nm radiation (0.2 W cm⁻² for 20 min) was applied as dye NPs + light group. Cells after treatment were collected by trypsinization, and then the extent of apoptosis was evaluated by using the Annexin V/PI apoptosis detection kit (Beyotime Biotechnology). Cells were analyzed immediately by using the BD LSRSFortessa (BD Biosciences) flow cytometer.

2.11. mRNA levels related to cell growth by RT-qPCR

Human gastric cancer (SGC-7901) cells were cultured in 6-well plates and treated in different groups. Group 1: natural growth (control group); Group 2: 30 μM dye NPs treatment (dye group); Group 3: 635 nm radiation (0.2 W cm⁻² for 20 min) as light group; Group 4: After the dye NPs entered the cell (30 μM), 635 nm radiation (0.2 W cm⁻² for 20 min) was applied as dye NPs + light group. Total RNA was isolated by the TRIzol (0.2 W cm⁻² for 20 min) was washed with brine (2 × 50 ml), and the organic layer was washed with anhydrous CH₂Cl₂. The mixture was extracted with CH₂Cl₂ (2 × 50 ml), and the organic phase was separated by column chromatography (CH₂Cl₂/CH₃CN: 2:1) to afford dark brown liquid as a crude mixture. After cooling to room temperature, the solvents were removed by evaporation, and the resulting crude mixture was separated by column chromatography (CH₂Cl₂/CH₃CN: 2:1) to afford compound 1-CN (570 mg, 2.7 mmol, 71%).

2.13. Synthesis of 3-(5,5-dimethyl-1-nitro-4-oxohexan-2-yl)benzonitrile (2-CN)

Triethylamine (2 ml) and nitromethane (1.5 ml) were added to 1-CN (0.57 g, 2.7 mmol) in anhydrous methanol (20 ml). The mixture was refluxed for 8 h. After cooling to room temperature, the solvent was removed under reduced pressure, and resulting crude mixture was separated by column chromatography (n-hexane: CH₂Cl₂: 1:1) to afford 2-CN (0.44 g, 1.6 mmol, 59%) as dark brown liquid. H NMR (500 MHz, CDCl₃): δ (ppm) 7.83 (d, J = 8.0 Hz, 2H), 7.67 (d, J = 16 Hz, 1H), 7.65 (d, J = 8.0 Hz, 2H), 7.18 (d, J = 16 Hz, 1H), 1.24 (s, 9H). 13C NMR (125 MHz, CDCl₃): δ (ppm) 204.1, 168.5, 141.4, 138.6, 134.8, 128.5, 128.1, 122.8, 43.5, 26.3.

2.13.2. Synthesis of 4-(5,5-dimethyl-1-nitro-4-oxohexan-2-yl)benzonitrile (2-CN)

2.13.3. Synthesis of CN-tBuazaBDP

HN₃OAc (10 g, 129.7 mmol) was added to a suspension of compound 2-CN (0.44 g, 1.6 mmol) in anhydrous methanol (20 ml). The mixture was refluxed for 8 h. After cooling to room temperature, the mixture was extracted with CH₂Cl₂ (2 × 50 ml), and the organic layer was washed with brine (2 × 50 ml) and dried over anhydrous Na₂SO₄. After solvent removal by evaporation, the residue 3-CN was dissolved in 10 ml anhydrous CH₂Cl₂/CH₃Cl. Triethylamine (0.5 ml, 3.6 mmol) was added and stirred at room temperature for 2 h, followed by dropwise addition of BF₃-Et₂O (1 ml, 7.9 mmol) with stirring at room temperature for 2 h. The mixture was then heated in 70 °C for 2 h. After cooling to room temperature, the mixture was extracted with CH₂Cl₂ (2 × 50 ml), and the organic layer was washed with brine (2 × 50 ml) and dried over anhydrous Na₂SO₄. After solvent removal by evaporation, and the resulting crude product was separated by column chromatography (CH₂Cl₂/n-hexane = 2:1) to afford CN-tBuazaBDP (142 mg, 0.28 mmol, 35%) as a dark red solid. H NMR (500 MHz, CDCl₃): δ (ppm) 7.98 (d, J = 8.5 Hz, 4H), 7.63 (d, J = 8.5 Hz, 4H), 6.79 (s, 2H), 1.56 (s, 18H). 13C NMR (125 MHz, CDCl₃): δ (ppm) 145.1, 136.9, 133.2, 132.6, 130.1, 130.9, 119.0, 113.0, 112.4, 36, 30.5. HRMS (ESI) m/z calc for C₂₀H₂₂BF₃N₃Na⁺ (M + Na)⁺ 530.22980, found 530.23041.

2.13.4. Synthesis of 1-CF₃

4-(Trifluoromethyl)benzaldehyde (0.44 ml, 2.9 mmol) was used as the starting material, and 1-CF₃ was obtained as reddish-brown solids (515.1 mg, 2.0 mmol, 69%). H NMR (500 MHz, CDCl₃): δ (ppm) 7.59 (d, J = 15.5 Hz, 1H), 7.58 (d, J = 8.0 Hz, 2H), 7.52 (d, J = 8.0 Hz, 2H), 7.15 (d, J = 15.5 Hz, 1H), 1.15 (s, 9H).

2.13.5. Synthesis of 2-CF₃

1-CF₃ (515.1 mg, 2.0 mmol) was used as the starting material, and 2-CF₃ was obtained as reddish-brown solids (350.9 mg, 1.1 mmol, 55%). H NMR (500 MHz, CDCl₃): δ (ppm) 7.15 (d, J = 8.0 Hz, 2H), 7.11 (d, J = 8.0 Hz, 2H), 4.68 (dd, J = 12.5 Hz, J = 6.0 Hz, 1H), 4.59 (dd, J = 12.5 Hz, J = 6.0 Hz, 1H), 4.07 (quint, J = 6.0 Hz, 1H), 2.97 (dd, J = 12.5 Hz, J = 6.0 Hz, 1H), 2.87 (dd, J = 12.5 Hz, J = 6.0 Hz, 0.98 (s, 9H).

2.13.6. Synthesis of CF₃-tBuazaBDP

2-CF₃ (350.9 mg, 1.1 mmol) was used as the starting material, and CF₃-tBuazaBDP was obtained as reddish-brown solids (137.8 mg, 0.23 mmol, 42%). H NMR (500 MHz, CDCl₃): δ (ppm) 8.01 (d, J = 8.0 Hz, 4H), 7.66 (d, J = 8.0 Hz, 4H), 6.88 (s, 2H), 1.57 (s, 18H). 13C NMR (125 MHz, CDCl₃): δ (ppm) 173.7, 144.6, 142.1, 135.6, 130.8, 129.3, 125.3, 120.3, 118.1, 35.3, 30.0. HRMS (ESI) m/z calc for C₂₀H₂₂BF₃N₃Na⁺ (M + Na)⁺ 616.21408, found 616.21423.
2.13.7. Synthesis of 1-OMe

4-Methoxybenzaldehyde (0.45 g, 3.7 mmol) was used as the starting material, and 1-OMe was obtained as reddish-brown solids (545.1 mg, 2.5 mol, 67%). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ (ppm) 7.55 (d, $^3J = 15$ Hz, 1H), 7.41 (d, $^3J = 8.5$ Hz, 2H), 6.94 (d, $^3J = 15$ Hz, 1H), 6.77 (d, $^3J = 8.5$ Hz, 2H), 3.67 (s, 3H), 1.12 (s, 9H).

2.13.8. Synthesis of 2-OMe

1-OMe (545.1 mg, 2.5 mmol) was used as the starting material, and 2-OMe was obtained as reddish-brown solids (390.6 mg, 1.4 mmol, 56%).

2.13.9. Synthesis of OMe-tBuazaBDP

2-OMe (390.6 mg, 1.4 mmol) was used as the starting material, and OMe-tBuazaBDP was obtained as reddish-brown solids (79.6 mg, 0.15 mmol, 22%). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ (ppm) 7.95 (d, $^3J = 9.0$ Hz, 4H), 6.95 (d, $^3J = 9.0$ Hz, 4H), 6.71 (s, 2H), 3.88 (s, 6H), 1.55 (s, 18H). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ (ppm) 160.5, 146.9, 143.1, 130.7, 125.4, 124.3, 123.8, 118.9, 55.2, 34.9, 31.2. HRMS (ESI) $m/z$ calcld for C$_{30}$H$_{34}$BF$_2$N$_3$O$_2$Na$^+$ (M + Na)$^+$ 540.26044, found 540.26074.

2.13.10. Synthesis of 1-SMe

4-(Methylthio)benzaldehyde (0.44 ml, 3.3 mmol) was used as the starting material, and 1-SMe was obtained as reddish-brown solids (461.9 mg, 1.97 mmol, 60%). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ (ppm) 7.63 (d, $^3J = 15.2$ Hz, 1H), 7.48 (d, $^3J = 8.0$ Hz, 2H), 7.33 (d, $^3J = 8.0$ Hz, 2H), 7.07 (d, $^3J = 15.2$ Hz, 1H), 2.51 (s, 3H), 1.22 (s, 9H).

2.13.11. Synthesis of 2-SMe

1-SMe (461.9 mg, 1.97 mmol) was used as the starting material, and

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Scheme 1. a) Synthesis of dyes R-tBuazaBDPs (R = CF$_3$, CN, OMe, SMe) with 3,5-di-tert-butyl groups at 3,5-sites. b) Molecular structures of dyes. c) ORTEP views of CN-tBuazaBDP (CCDC: 2,150,981) (displacement ellipsoids at the 30% probability level). (I) Front, (II) side of molecular structure.
2-SMe was obtained as reddish-brown solids (291.1 mg, 0.98 mmol, 50%). \[^1\text{H}\] NMR (500 MHz, CDCl₃): δ (ppm) 7.15 (d, \[^3\text{J} = 8.5\text{ Hz}, 2\text{H}\]), 7.11 (d, \[^3\text{J} = 8.5\text{ Hz}, 2\text{H}\]), 4.64 (dd, \[^3\text{J} = 12.5\text{ Hz}, 2\text{J} = 6.5\text{ Hz}, 1\text{H}\]), 4.55 (dd, \[^3\text{J} = 12.5\text{ Hz}, 2\text{J} = 6.5\text{ Hz}, 1\text{H}\]), 3.96 (quint, \[^3\text{J} = 6.5\text{ Hz}, 1\text{H}\]), 2.94 (dd, \[^3\text{J} = 12.5\text{ Hz}, 2\text{J} = 6.5\text{ Hz}, 1\text{H}\]), 2.84 (dd, \[^3\text{J} = 12.5\text{ Hz}, 2\text{J} = 6.5\text{ Hz}, 1\text{H}\]), 2.51 (s, 3H), 1.05 (s, 9H).

2.13.12. Synthesis of SMe-tBuazaBDP

2-SMe (291.1 mg, 1 mmol) was used as the starting material, and SMe-tBuazaBDP was obtained as reddish-brown solids (135.4 mg, 0.246 mmol, 49%). \[^1\text{H}\] NMR (500 MHz, CDCl₃): δ (ppm) 7.92 (d, \[^3\text{J} = 8.5\text{ Hz}, 4\text{H}\]), 7.27 (d, \[^3\text{J} = 8.5\text{ Hz}, 4\text{H}\]), 6.75 (s, 2H), 2.54 (s, 6H), 1.55 (s, 18H).

\[^13\text{C}\] NMR (125 MHz, CDCl₃): δ (ppm) 140.5, 129.4, 129.1, 127.3, 127.1, 126.6, 126.3, 125.7, 35.1, 29.9, 15.2. HRMS (ESI) m/z calcld for C₃₀H₃₄BF₂N₃S₂Na⁺ (M + Na)⁺ 572.21475, found 572.21466.

3. Results and discussion

3.1. Synthesis and structure of tBuazaBDPs

Synthetic routes of 3,5-diptert-butyl substituted aza-BODIPYs were outlined in Scheme 1a. 3,3-Dimethylbutan-2-one and aryl-containing aldehyde were ingeniously employed as the starting materials for the aldol condensation reaction. Nitromethane was added to produce nitro derivative 2, which was dealt with ammonium acetate to provide the precursor 3 under the reflux condition. By the complexation with BF₃·
EtO, the final dye tBuazaBPs with 3,5-di-tert-butyl groups are obtained for the first time. tBuazaBPs were clearly characterized by NMR spectroscopy and high resolution mass spectrometry. For example, by the identification of the $^1$H NMR spectrum, two sets of the singlet distinct hydrogen signals for the –tBu groups ($\delta = 1.55$ (s, 18H) ppm) and the –SMe groups ($\delta = 2.54$ (s, 6H) ppm) were observed respectively [49], which convincingly confirmed this structure of SMe-tBuazaBPA. Moreover, the molecular structure of CN-tBuazaBPA was indisputably demonstrated by the X-ray analysis of the prepared single crystal (Scheme 1c). The sp$^3$ hybridized boron center in CN-tBuazaBPA appeared in a distorted tetrahedron geometry with angles N2–B1–N3 of 106.7 (2)$^\circ$, N2–B1–F2 of 106.5 (2)$^\circ$ and F1–B1–F2 of 111.4 (2)$^\circ$, which seriously deviated from the ideal value of 119.5$. Consequently, the boron atom was found to be severely upwarped from the core plane of CN-BuazaBPA. Moreover, the C17–C16–C1–C2 dihedral angles were 22.9 (5)$^\circ$ and C14–C9–C6–C7 were –29.0 (4)$^\circ$ respectively, therefore two –C$_2$H$_4$(p-CN) groups at 1,7-sites in CN-tBuazaBPA located at both sides of the core and are non-coplanar (Scheme 1c–B), whereas two corresponding –Ph groups in PhazaBPA (Scheme 1b) nearly lie on the coplane of the aza-BODIPY core (Fig. S1) [39]. In short, the molecular structure of tBuazaBPA is more twisted than PhazaBPA [39].

3.2. Photophysical properties

Since 3,5-di-tert-butyl substituted tBuazaBPs were synthesized for the first time, we are very curious to gain insight into their spectral properties. In stark comparison to SMe-tBuazaBPA, 3,5-diphenyl substituted aza-BODIPY SMe-PhazaBPA (Scheme 1b) is also prepared [50]. The tert-butyl substitution at 3,5-sites in SMe-tBuazaBPA ($\lambda_{abs}/\lambda_{em}$ = 602/708 nm) indeed undergoes the hypochromic shift of the maxima absorption and emission by 70 and 27 nm respectively (Fig. 2a and b), comparing to those ($\lambda_{abs}/\lambda_{em}$ = 672/735 nm) of SMe-PhazaBPA. However, SMe-tBuazaBPA possesses ultra-large Stokes shift (106 nm) and still has the NIR emission wavelength (Fig. 2a and b). Furthermore, the introduction of the push-pull electron groups at 1,7-sites in tBuazaBPs (Scheme 1a) results in the bathochromic shift (4–14 nm) of absorption in CH$_2$Cl$_2$ solution (Fig. 2a and Table 1), compared with that ($\lambda_{abs}$ = 588 nm) of H-tBuazaBPA ([R = H, Scheme 1a]) [51]. It is noteworthy that the Stokes shift slightly decreased (22 nm for CF$_3$-tBuazaB, 24 nm for CN-tBuazaB) by the introduction of the electron-withdrawing groups, comparing to that (25 nm) of H-tBuazaB, although the absorption and emission spectra showed bathochromic shift simultaneously. Nevertheless, wide full width at half maximum (FWHM: 85 nm) of SMe-tBuazaBPA is broader than that (33 nm) of CF$_3$-tBuazaB. Moreover, half width of the fluorescence band of SMe-tBuazaBPA is even broader and about three times than that of CF$_3$-tBuazaB. The molar extinction coefficients increase slightly, along with the change of the pull or push electron groups. Especially, we were surprised to discover that the introduction of the –SMe group led to low fluorescence quantum yield ($\Phi_f = 0.05$), compared with other tBuazaBPs. In sharp contrast to Rev-SMe-tBuazaB [52] with the group exchange at 1,7 and 3,5-sites (Scheme 1b) from SMe-tBuazaBPA, although the maxima emission between Rev-SMe-tBuazaB (H-tBuazaB (l$_{em}$ = 713 nm) and SMe-tBuazaB (l$_{em}$ = 708 nm) could indeed be comparable, the maximum absorption of SMe-tBuazaB ($\lambda_{abs}$ = 602 nm) is obviously hypochromic shift relative to that ($\lambda_{abs}$ = 666 nm) of Rev-SMe-tBuazaB, so SMe-tBuazaB shows a larger Stokes shift (106 nm) than that (47 nm) of Rev-SMe-tBuazaB. Therefore, a breakthrough of ultra-large Stokes shift was found in the spectral properties of 3,5-di-tert-butyl-substituted aza-BODIPYs in this work.

In order to further deeply gain insight into the influence of the electron donating/withdrawing modifications on the spectral properties, we investigated the solvent effects of H-tBuazaBPA, CF$_3$-tBuazaB and SMe-tBuazaB. As shown in Fig. 2c and d and Table S1, the solvent effect between H-tBuazaBPA and CF$_3$-tBuazaB is not significantly different. For instance, the absorption and emission maxima of CF$_3$-tBuazaB in nonpolar solvent n-hexane locate at 588 and 610 nm, respectively. In the large polar solvent DMSO, a slight red-shift was monitored at 590 and 619 nm for absorption and emission maxima, while molar extinction coefficients and Stokes shifts were similar in different solvents. Different from CF$_3$-tBuazaB, the solvent effect on SMe-tBuazaB was comparatively evident. As shown in Fig. 2e and f, although there is a slight difference in absorption spectra in different solvents, the emission spectra of SMe-tBuazaB show dramatic discrepancy. In nonpolar solvent n-hexane, the absorption and emission maxima of SMe-tBuazaB are 598 and 670 nm, respectively. However, in the large polar solution DMSO, although the absorption only red-shifted by 6 nm–604 nm, the maximum emission was unexpectedly red-shifted to 756 nm and Stokes shift spectacularly reached 152 nm. This is probably due to the synergic action of TICT effect and the release energy by free rotation of the –tBu group at 3,5-sites in SMe-tBuazaB (see the following Fig. 3a). Since the large Stokes shift can improve the sensitivity of photochemical application and avoid the self-quenching of the excited and scattered light affiliated measurement error, Peng et al. synthesized the heptamethine cyanine dyes by introducing alkyalamino groups with large Stokes shift (140 nm), which is facilitated for optical applications such as fluorescent labeling [53]. Hence, SMe-tBuazaB with the ultra-large Stokes shift is highly desirable for a photothermal therapy. FWHM of SMe-tBuazaB becomes wider with the increase of solvent polarity, but the light-harvesting ability has not changed significantly in different solvents. It is worth mentioning that in DMF and DMSO solution, the fluorescence quantum yield of SMe-tBuazaB is significantly reduced ($\Phi_f = 0.02$) (Table S1).

3.3. Theoretical calculations

To further rationalize the special optical properties of SMe-tBuazaB, theoretical simulations are conducted based on DFT and TDDFT methods, and SMe-PhazaB is selected to serve as a scrupulously comparative molecule (Scheme 1b) [50]. The calculated absorption spectra of SMe-tBuazaB and SMe-PhazaB are displayed in Fig. S3. The agreement between theoretical and experimental data confirms the current calculation level and the reliability of the theoretical results. To figure out the causality of the large Stokes shift of SMe-tBuazaB, the frontier molecular orbital analysis is carried out. The primary absorption and emission peaks of SMe-tBuazaB both correspond to the $\pi\rightarrow\pi^*$ transition (Table S4–6), which is composed of the transition between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO). As shown in Fig. S4, the electron density of HOMO mainly distributes on the non-tBu groups, whereas the LUMO mainly concentrates on the BODIPY core. The different location of HOMO and LUMO results in the transition between them, undergoing a TICT process, which usually leads to a weak fluorescence and an ultra-large Stokes shift [54,55]. Moreover, the rotational process between SMe-tBuazaB and SMe-PhazaB are compared, as shown in Fig. 3a. The scan process covers 180$^\circ$ rotation divided into 6 steps. SMe-tBuazaB has smaller energy barriers (2.8 kcal/mol) along the rotation of the –tBu group, comparing to that (3.2 kcal/mol) of the –Ph.

| Aza-BODIPY | $\lambda_{abs}$/$\lambda_{em}$ (nm) | Stokes shift (nm) | FWHM (nm) | $r$ [M$^{-1}$cm$^{-1}$] | $\Phi_f$ |
|------------|-------------------------------|-------------------|-----------|-----------------|--------|
| H-tBuazaBPA | 588/613 | 25 | 31 | 74,000 | 0.12 |
| CF$_3$-tBuazaB | 592/614 | 22 | 29 | 73,500 | 0.12 |
| CN-tBuazaB | 600/624 | 24 | 33 | 73,000 | 0.11 |
| OMe-tBuazaB | 596/672 | 76 | 71 | 75,500 | 0.13 |
| SMe-tBuazaB | 602/708 | 106 | 85 | 76,000 | 0.05 |
| SMe-PhazaB | 672/735 | 63 | 75 | 80,000 | 0.07 |
| Rev-SMe-tBuazaB | 666/713 | 47 | 73 | 85,000 | 0.04 |
group in SMe-PhazaBDP. Such small energy barriers promise their free rotation in solution [56,57], which may cause more system energy released through non-radiative decay. Hence, the ultra-large Stokes shift of SMe-tBuazaBDP may attribute to the TICT and rotational energy release of the -tBu groups.

3.4. Singlet oxygen generation ability

To further investigate the effect of the electron donating/withdrawing group modification on the excited state release energy of CF₃-tBuazaBDP and SMe-tBuazaBDP, 1,3-diphenlisobenzofuran (DPBF), the singlet oxygen (¹O₂) capture agent, is applied to indicate ¹O₂ production in toluene solution. Once the solution is irradiated by the light, the absorption of DPBF at 416 nm gradually decreases with the increase of light radiation time, while the absorption maximum of SMe-tBuazaBDP did not change, indicating that SMe-tBuazaBDP has a favorable photostability (Fig. 3b). Then we also investigated the DPBF decline extent of CF₃-tBuazaBDP under the same conditions. SMe-tBuazaBDP was found to possess a relatively faster reduction efficiency of DPBF ($S = -0.0226$), compared with that ($S = -0.0124$) of CF₃-tBuazaBDP (Fig. 3c). Their singlet oxygen yields are low and calculated to be 8.1% for SMe-tBuazaBDP and 4.4% for CF₃-tBuazaBDP, respectively [58]. In terms of the calculation formula, one can find that ISC ($k_{ISC}$) rate is closely related to singlet-triplet energy differences ($ΔE_{st}$) [59–61]. So, to estimate the difficulty of the ISC, the first ten lowest-lying singlet and triplet transitions of SMe-tBuazaBDP have been calculated, as listed in Table S4. Obviously, the energy gap $ΔE_{st}$ between S₁ and T₁ state is about 1 eV, which is not a favorable energy difference for ISC [59–61]. In short, SMe-tBuazaBDP exhibits a weak ISC effect.

3.5. Synthesis and characterization of SMe-tBuazaBDP NPs

The low conversion efficiency for two above described pathways from the excited state to the ground state urges us to investigate the third pathway, that is, non-radiative decay. Since the rotation of the bulk groups (such as -CF₃, -tBu) for non-radiative decay can promote the heat energy conversion [39,45], we next investigated the photothermal conversion effect of SMe-tBuazaBDP. First, to improve the water solubility and biocompatibility of SMe-tBuazaBDP for better application in photo-imaging and therapy in biological systems, SMe-tBuazaBDP and

![Fig. 3. (a) Potential energy curves of SMe-tBuazaBDP and SMe-PhazaBDP along the rotation of the substituted group. (b) Absorption curve of SMe-tBuazaBDP and DPBF in toluene with time under light irradiation; (c) Comparison of absorption decline rate of DPBF in SMe-tBuazaBDP ($S = -0.0226$) and CF₃-tBuazaBDP ($S = -0.0124$) with time. T = 298 K. (d) DLS and (e) TEM of SMe-tBuazaBDP NPs in aqueous solution; (f) Photo of pure water and (g) Photo of SMe-tBuazaBDP NPs in water. (h) Absorption spectra of SMe-tBuazaBDP (black curve) in CH₂Cl₂ and SMe-tBuazaBDP NPs (red) in aqueous solution.](image)
polymer materials DSPE-PEG$_{2000}$ were self-assembled into dye nanoparticles (SMe-tBuazaBDP NPs) [62,63]. Transmission electron microscopy (TEM) photograph demonstrated the spherical morphology of the nanoparticles, and their sizes were less than 110 nm (Fig. 3d and e). Dynamic light scattering (DLS) of SMe-tBuazaBDP NPs showed a suitable hydrodynamic diameter (30–110 nm) in Fig. 3d, and the average hydrodynamic diameter and the polydispersity index (PDI) of SMe-tBuazaBDP NPs were about 85.5 nm and 0.31, respectively [64,65]. After being placed in aqueous solution for two weeks, no precipitate was observed and the particle size did not change significantly, suggesting that the prepared nanoparticles are stable (Fig. 3f and g). Due to the bulky tert-butyl substituents as spacers in the aggregation (Fig. S5), the absorption of SMe-tBuazaBDP NPs in aqueous solution is red-shifted ($\lambda_{\text{abs}} = 624$ nm) and the peak becomes wider (Table S7), covering from 450 to 750 nm, comparing to that ($\lambda_{\text{abs}} = 602$ nm) of SMe-tBuazaBDP in organic solvent CH$_2$Cl$_2$ (Fig. 3h).

### 3.6. Photothermal conversion performance

As depicted in Fig. 4, the temperature of the solution increased significantly under the light irradiation of SMe-tBuazaBDP NPs in the aqueous solution. For instance, after 1 min light irradiation, the solution temperature rapidly increased by 11.3 °C and its temperature even reached 56.0 °C after only 4 min light irradiation (Fig. 4a), achieving strong light absorption ability and thermal energy release properties. Since hyperthermia (above 42 °C) generated by nanomaterials under laser irradiation could effectively destruct cancer cells [20,21,66], SMe-tBuazaBDP NPs are consequently suitable for hyperthermia. Next, we further monitored the temperature enhancement at different concentrations (20, 40 and 80 μM) of SMe-tBuazaBDP NPs and different optical power densities (0.1, 0.2 and 0.3 W cm$^{-2}$) (Fig. 4b and c). As shown in Fig. 4b and c, temperature promotion is intimately dependent on concentration and power density. Higher concentration and stronger power density contribute to better thermal conversion effect. The temperature change under natural cooling phase after 10 min light radiation was investigated. In Fig. 4d, the temperature change under natural cooling phase after 10 min light radiation was investigated. On the 5th, the temperature of the solution cooled rapidly to near room temperature after 5 min, and the cooling effect was not significant for the next 5 min. Hence, light radiation for 10 min and natural cooling for 10 min were selected as one cycle to inspect three heating-cooling cycles testing. As can be seen from Fig. 4d, the temperature changes during the heating-cooling cycle of each group are almost the same, suggesting that SMe-tBuazaBDP NPs have excellent photothermal stability and can be applied for the photothermal therapy for multiple cycles. Furthermore, to investigate the photothermal conversion efficiency, the associated time constant obtained by mapping the cooling time and driving force temperature was brought into the reported formula for calculating the photothermal conversion efficiency. The photothermal conversion efficiency of SMe-tBuazaBDP NPs was calculated to be 49.3% (Fig. 4e and f), which is near to the highest one (50.5%) in aza-BODIPY-based system [67,68]. Moreover, this is obviously higher than that (45%) of the contrastive dye SMe-PhazaBDP [50] or those of the classical photothermal agents, such as ICG (27.9%) [17], indicating that this strategy of the 3,5-tert-butyl substitution in aza-BODIPY system is an effective way to enhance the photothermal conversion.

### 3.7. Cytotoxicity assay and PTT performance in vitro

To determine the suitable time of light radiation to SMe-tBuazaBDP NPs, we performed CCK8 assay with human colon cancer (SW-620) cells and gastric cancer (SGC-7901) cells. As is shown in Fig. 5a, the inhibition of cell viability was significant when the laser radiation time exceeded...
15 min, indicating that SMetBuazaBDP NPs possess low dark cytotoxicity and high phototoxicity [17]. Therefore, the laser radiation time of 20 min was selected as the condition of the subsequent experiment to continue to investigate the phototherapeutic performance of SMetBuazaBDP NPs. Then, to further verify the inhibitory effect of SMetBuazaBDP NPs with laser radiation time of 20 min on various cancer cell viability, we performed live-dead cell staining by using calcine AM (green) and propidium iodide (red) dyes. Human lung cancer (NCI-H2030) cells also were used for the experiment. As expected, SMetBuazaBDP NPs induced death of NCI-H2030 cells and SGC-7901 cells after laser irradiation (0.2 W cm$^{-2}$) for 20 min, respectively. Scale bar: 100 μm. For clarity, SMetBuazaBDP NPs is abbreviated as Sme in Fig. 5.

3.8. Determination on cell death mechanism

Since apoptosis and necrosis are well-known to be two important modes during cell death in PTT [47,48], therefore the cell death mechanism induced by SMetBuazaBDP NPs was herein investigated. To determine whether SMetBuazaBDP NPs with laser radiation has effect on the cycle and apoptosis of cancer cells, we first performed flow cytometry with SGC-7901 cells. As shown in Fig. 6a, compared with other groups, cells treated with SMetBuazaBDP NPs and laser irradiation group showed an increase in G2 phase and a decrease in S phase, which caused G0/G1 arrest, suggesting that SMetBuazaBDP NPs blocked cell cycle progression and inhibited proliferation of cancer cells via laser radiation (Fig. 6a). Moreover, Fig. 6b showed that the percentage of apoptotic cells increased from 3.9% (blank control) to 30.5% after treatment with SMetBuazaBDP NPs and laser radiation, while SGC-7901 cells treated with SMetBuazaBDP NPs or light irradiation displayed lower apoptosis rate (18.4%, 4.6%), indicating the efficient apoptosis-inducing capacity of SMetBuazaBDP NPs to cancer cells. Next, we further performed RT-qPCR and western blot to verify the effects of SMetBuazaBDP NPs with laser radiation on apoptosis and cell cycle at mRNA and protein levels with various factors related to the regulation of cell cycle and apoptosis (Fig. 6c and d). Herein, Bcl2 is a negative regulator of apoptosis, while Bax is a positive regulator of apoptosis (Fig. 6c). In the group treated with SMetBuazaBDP NPs and light irradiation, the mRNA level and protein level of Bcl2 decreased significantly compared with other groups, while the mRNA level of Bax increased significantly, which further proved that the photothermal effect of the SMetBuazaBDP NPs could induce cancer cell apoptosis (Fig. 6c). Meanwhile, as shown in Fig. 6d, the decrease of expression level of C-myc and cyclin-D1 showed that treatment with SMetBuazaBDP NPs and light irradiation induced cell cycle arrest, blocked cancer cell cycle and inhibited cell proliferation. These results demonstrated strong consistency with the Calcein AM/PI results, suggesting that
Fig. 6. a) Cell cycle analysis using flow cytometry in SGC-7901 cells after treatment with SME-tBuazaBDP NPs (30 μM) alone, light alone or their combination. Light irradiation (0.2 W cm⁻², 20 min) was conducted after cells were incubated with SME-tBuazaBDP NPs. b) Apoptosis analysis using flow cytometry toward SGC-7901 cells after treatment with SME-tBuazaBDP NPs (30 μM) alone, light alone or their combination. Light irradiation (0.2 W cm⁻², 20 min) was conducted after cells were incubated with SME-tBuazaBDP. SME-tBuazaBDP NPs treatment with laser irradiation was shown to induce more apoptosis. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001. c) mRNA expression level related to regulation of cell cycle (cyclin-D1) and apoptosis (Bcl-2, Bax) were evaluated using RT-qPCR in SGC-7901 cells after treatment with SME-tBuazaBDP NPs (30 μM) alone, light (0.2 W cm⁻², 20 min) alone or their combination. d) The expression level of protein related to regulation of cell cycle (cyclin-D1), cell proliferation (C-myc) and apoptosis (Bcl-2) were evaluated by western blot in NCI-H2030 cells after treatment with SME-tBuazaBDP NPs (30 μM) alone, light (0.2 W cm⁻², 20 min) alone or their combination. For clarity, SME-tBuazaBDP NPs is abbreviated as Sme in Fig. 6.

SMe-tBuazaBDP NPs with low laser radiation effectively blocked cancer cell cycle, inhibited cancer cell proliferation and induced cancer cell apoptosis. Therefore, SMe-tBuazaBDP NPs provide great possibilities as a cancer photothermal agent.

4. Conclusion

In summary, we successfully synthesized a series of innovative dyes, 3,5-di-tert-butyl-substituted (BuazaBDP) for the first time. Based on the X-ray analysis of the prepared single crystal CN-tBuazaBDP, the molecular structure of tBuazaBDP is twisted. SMe-tBuazaBDP has the ultra-large Stokes shift in az-aBODIPY system, which may attribute to the TICT and the rotational energy release of the –Bu groups based on the experiment and theoretical calculation. Although the barrier-free rotors of the distal –Bu groups in SMe-tBuazaBDP results in low fluorescence quantum yield, its photothermal conversion efficiency (η = 49.3%) is obviously enhanced. SMe-tBuazaBDP NPs realized the photo-toxicity to effectively block cancer cell cycle, inhibit cancer cell proliferation and induce cancer cell apoptosis by the optical radiation driven for photothermal therapy. The strategy of “direct attachment of –Bu groups to az-aBODIPY core at 3,5-sites” provides a meaning idea for opening a new platform for the future design of PTAs.

Credit author statement

R. L, X. J. carried out the synthetic work and analytical characterization, including the crystallographic studies. J. R, Z. W, H. W, S. Z performed the experiments on cell apoptosis and fluorescence imaging. M. L. carried out the computational studies. D. Z. assisted with data analysis. D. Z, J. D, X. J, G. W. wrote the manuscript. X. J. supervised the research. All authors reviewed and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mibio.2022.100446.

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