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meso-Acetoxymethyl BODIPY dyes for photodynamic therapy: improved photostability of singlet oxygen photosensitizers

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We report two BODIPY based photosensitizers (**Br_2BOAc** and **I_2BOAc**) featuring an acetoxymethyl substituent at the *meso*-position. These photosensitizers show improved photostability against singlet oxygen, when compared to a BODIPY photosensitizer lacking the acetoxymethyl group. Both compounds were evaluated for photodynamic therapy against HeLa cells and photodynamic inactivation against *E. coli* bacteria. We show that the compounds readily embed in the lipid membranes of HeLa cervical cancer cells and efficiently induced light-dependent apoptosis at nanomolar concentration. Also, both compounds showed a substantial degree of photoinactivation of *E. coli* bacteria when used at low micromolar concentrations.

Introduction

Photodynamic therapy (PDT) is an established approach for the targeted destruction of pathologic cells and tissue.[1–5] It provides a potent platform for the treatment of cancers[2,5] and drug-resistant microbes.[3] PDT relies on the interaction of a photosensitizer, light, and molecular oxygen. Following light excitation of a photosensitizer and subsequent intersystem crossing (ISC), an excited triplet state is formed which acts as an energy donor to ground state molecular oxygen ($^3O_2$) yielding a highly-reactive singlet oxygen ($^1O_2$) molecule. The sensitized $^1O_2$ may next oxidatively damage nearby lipids, proteins, and nucleic acids inducing cell death.

The search for optimal PDT photosensitizers seeks, from a photophysical–photochemical perspective, a high quantum yield of singlet oxygen generation (Φ_Δ), photostability (in particular assorted to $^1O_2$ mediated oxidation), and absorption with a high extinction coefficient in a region of the spectrum where excitation of biomolecules is minimal. The search additionally pursues, from a synthetic perspective, ease of preparation and functionalization of core chromophores. Modifications may then be envisioned that (a) involve the addition of heavy atoms such as Br or I to a chromophore to enhance its rate of ISC. Modifications may also seek (b) the incorporation of electron-withdrawing groups to prevent the addition of $^1O_2$ (an electrophile) to the chromophore, thus enhancing the chemical stability of parent dyes and PDT sensitizers.[6–10] Modifications may additionally explore (c) the extension of conjugation to satisfy the requirement for large extinction coefficients and suitable absorption wavelengths.

Over the past decade, bromo- or iodo-substituted boron dipyrromethene (BODIPY[11,12]) dyes have emerged as a new class of photosensitizers that fulfil many of the above requirements.[13] Enhanced photostability however has as of yet to be explored in the context of resilience towards $^1O_2$ mediated photodegradation. We reasoned that heavy atom substituted BODIPY dyes bearing an electron-withdrawing acetoxymethyl moiety[14] in the *meso*-position would be promising candidates for PDT given their potential for enhanced photostability. Here we report the photostability and efficiency as photosensitizers for photodynamic therapy of two such compounds bearing either bromine (**Br_2BOAc**) or iodine (**I_2BOAc**) at the equatorial (2 and 6) positions of the BODIPY scaffold (Fig. 1).[15] Acetoxymethyl substitution at the *meso* position was found to substantially improve the photostability of the sensitizers against $^1O_2$ without significantly affecting their Φ_Δ, when compared to a related compound bearing H at the *meso* position (**2I-BDP**,[16] Fig. 1). The new compounds readily embedded in the lipid membranes of HeLa cervical cancer cells and efficiently induced light-dependent apoptosis at nanomolar concentration. Additionally, both compounds showed a substantial degree of photoinactivation of *Escherichia coli* bacteria at low micromolar concentrations.

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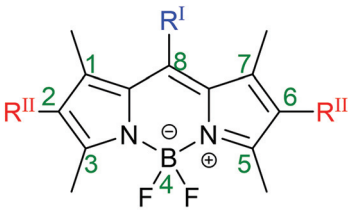

	R^I	R^{II}	Φ_Δ
H_2BOAc	CH_2OAc	H	n.a.
Br_2BOAc	CH_2OAc	Br	0.84
I_2BOAc	CH_2OAc	I	0.98
2I-BDP	H	I	0.98

Fig. 1 Structure of the BODIPY dyes used in this work. Numbering of the BODIPY scaffold shown in green.

Experimental

Materials

HPLC grade solvents, growth media and other reagents for cell culture and bacterial assays were purchased from ThermoFisher Scientific. The BODIPY derivatives were synthesized according to previously reported procedures.[14–16] Molecular biology grade DMSO for photocytotoxicity studies and all other chemicals were supplied by Sigma-Aldrich, Co. and used without further purification.

Cell culture

HeLa cells (ATCC CCL-2) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose with L-glutamine, phenol red, and sodium pyruvate (Gibco), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (growth media). Cells were maintained at 37 °C (5% CO_2) in a humidified atmosphere. Cells were trypsinized and split 1/15 twice a week when confluence of cells was reached. Cells were passaged a maximum of 20 times.

Bacterial strains and culture conditions

Escherichia coli ATCC 25922 stock cultures were preserved at −70 °C using glycerol 10% (v/v) as the cryoprotectant, and the strain was grown in LB broth (Lennox). Bacterial culture was prepared by inoculating a single colony from a pure culture. Microorganism growth determination was done using a Biotek Synergy 2 multimode microplate reader, recording the absorbance at 600 nm. After overnight incubation at 37 °C for 18 h, the culture reached a density of approximately 1×10^9 colony forming units (CFU) mL^{-1} and it was diluted to 10^6 CFU mL^{-1}.

Microscopy

Fluorescence and differential interference contrast (DIC) images of HeLa cells were acquired using a wide-field objective-based total internal reflection fluorescence (TIRF) microscopy setup consisting of an inverted microscope (Nikon Eclipse Ti) equipped with a Perfect Focus System (PFS) and stage-top incubator (Tokai Hit) to maintain the cells at 37 °C (5% CO_2).

For fluorescence imaging of dye localization, samples were excited with the evanescent wave of a 488 nm diode laser output of a laser combiner (Agilent Technologies, MLC-400B), obtained by focusing the collimated laser beam at the back focal plane of a high numerical aperture oil-immersion objective (Nikon CFI SR Apochromat TIRF 100×, NA = 1.49) and launching the beam past the critical angle. A dual band dichroic mirror (ZT488/640rpc-UF2, Chroma) directed the excitation beam to the sample while the emission was cleaned by a dual band pass filter (ZET488/640m, Chroma). The fluorescence emitted from the BODIPY dyes was collected through the same objective and captured on a back illuminated electron multiplying charge coupled device (EM-CCD) camera (Andor iXon Ultra DU-897).

For photocytotoxicity studies, a lower magnification objective (Nikon CFI Plan Apo VC 20× objective, NA = 0.75) was used and the fluorescence of ethidium homodimer was imaged in widefield configuration. A 561 nm diode laser output of the laser combiner was passed through a multiband clean-up filter (ZET405/488/561/647x, Chroma Technology) and coupled into the microscope objective using a multiband beam splitter (ZT405/488r/561/640rpc, Chroma Technology). The fluorescence light was spectrally filtered with an emission filter (ZET405/488/561/647M, Chroma Technology).

The excitation laser powers measured out of the objective with the beam projecting normal to the objective lens surface was 50 μW for all experiments. DIC images were collected using the same objective used for fluorescence imaging.

Image processing

All images were processed using the FIJI imaging processing package.[17]

Photobleaching studies

Photosensitizer samples were dissolved in acetonitrile and placed in 10 × 10 mm quartz cuvettes. The initial absorbance of the sample was 0.3 at 532 nm. Samples were bubbled with oxygen for a minimum of 30 minutes. Samples were next photobleached utilizing the second harmonic output (532 nm) of an Nd:Yag laser (Continuum model Surelite I-10). The excitation rate was 10 Hz, the pulse width was 6 ns, and the laser power was adjusted to 10 mJ per pulse. Absorption spectra were recorded using a Hitachi U-2800 UV-Vis-NIR spectrophotometer at regular time intervals.

Cellular uptake and localization

HeLa cells were plated on a 35 mm glass imaging dish (World Precision Instruments, Inc.) pre-coated with fibronectin (1 μg cm^{-2}), and cultured in DMEM containing growth factors 1 day prior to use. The growth media was removed and the cells were washed with phosphate buffer saline (PBS) before exchanging with imaging media (1 mL of DMEM containing high glucose, L-glutamine, and 10% FBS, without phenol red, or sodium pyruvate). A total of 500 μL of imaging media containing 3 μM

photosensitizer and 1% DMSO or vehicle alone was then added, with final solutions containing 1 μM photosensitizer and 0.33% DMSO. The cells were then returned to the incubator for 30 minutes.

Following incubation with the photosensitizer, the solution was removed and the cells were washed with PBS before exchanging with fresh imaging media. The cells were then imaged by fluorescence and DIC. DIC images were corrected by normalizing with a "flat-field" reference image of a blank region collected with the same illumination intensity and scaling the result to the mean intensity of the reference image.

Photocytotoxicity studies

Cells were plated on clear, tissue-culture treated 96-well plates (Corning) 1 day prior to use such that each well contained 10 000 cells in 100 μL of DMEM containing growth factors, without phenol red. Two plates were prepared for each photosensitizer, one to be irradiated and one to be maintained as a dark control (see below). Additionally, a plate was prepared for the non-sensitizing **H_2BOAc**.

Each plate was prepared as follows. Solutions of the photosensitizer were prepared in DMEM by serial dilution to afford 9 concentrations ranging from 20 nM to 10 μM. A solution of DMEM containing only DMSO (0.66%) was also prepared.

100 μL of the photosensitizer solutions were next added to the respective well for a total volume of 200 μL containing between 10 nM and 5 μM photosensitizer. Each concentration was done six times. Six wells were also treated with the DMSO vehicle (0.33%) alone. The plates were then returned to the incubator for 30 minutes.

The cells were next removed from the incubator and irradiated for 30 minutes with an LED panel (EXPO-LED, Luzchem Research) containing five 4 watt tube-style lamps with a peak wavelength of 520 nm and a fwhm of 31 nm. The output power was 2.6 mW cm^{-2}. The dark control plates were wrapped in aluminium foil prior to being removed from the incubator for 30 minutes. Following irradiation, the plates were returned to the incubator for 24 hours. The plates were then stained with ethidium homodimer to assess the viability of the cells; to three of the six wells per dye concentration, 20 μL of 44 μM ethidium homodimer solution was added for a final concentration of 4 μM ethidium homodimer and returned to the incubator for 70 minutes. Cell viability was assessed by measuring the fluorescence emission of the ethidium homodimer using a Biotek Synergy 2 multimode microplate reader and subtracting any fluorescence from the photosensitizer by measuring the fluorescence of the ethidium-free wells. The concentration that gave half maximal response (EC_{50}) was calculated by fitting a dose–response curve in the OriginPro software package.

To image the morphology of the HeLa cells following PDT treatment, cells were plated on tissue culture treated 8 well plates (μ-Slide, Ibidi) 1 day prior to use such that each well contained 15 000 cells in 300 μL of DMEM containing growth factors and phenol red. The media was exchanged with DMEM with growth factors without phenol red and 500 nM photosensitizer and 0.33% DMSO. The cells were returned to the incubator for 30 minutes, before being irradiated with the LED panel as above. A dark control plate was prepared in the same manner and covered in aluminium foil before being removed from the incubator for 30 minutes. Following irradiation, the plates were returned to the incubator for 24 hours.

To each well, 50 μL of 20 μM ethidium homodimer solution was added (4 μM final concentration) and the cells incubated for 70 minutes before imaging.

Bacterial photoinactivation studies

Stock solutions of **Br_2BOAc** and **I_2BOAc** (400 μM) were prepared in acetonitrile. To determine the photodynamic minimal inhibitory concentration (PD-MIC), samples were prepared in sterile glass vials by diluting the stock solution with PBS and LB broth (Lennox) in 70 : 30 (v/v) ratio to obtain concentrations between 0.078 μM and 40 μM. The final concentration of solvent was less than 10% for the experiments. Control samples containing only acetonitrile were also prepared. The samples were then inoculated with bacterial suspensions and incubated at 37 °C for 30 minutes. The samples were next irradiated for 30 minutes and incubated at 37 °C for 18 hours. Control samples were wrapped in aluminium foil prior to irradiation. The PD-MIC was determined as the lowest photosensitizer concentration at which growth was completely inhibited after incubation.

To determine the minimal bactericidal concentration (PD-MCB), samples were prepared as above with final photosensitizer concentrations of 5, 7.5, and 10 μM. The samples were irradiated for 8, 15, 23 or 30 minutes. Viability counts were performed in triplicate by preparing 10-fold serial dilutions of each sample and plating 0.1 mL of the dilution onto LB agar (Lennox L agar) plates and incubating the plate at 37 °C for 18 hours. The experiments were repeated three times on different days. The time-kill curves were constructed for each photosensitizer concentration by plotting the CFU mL^{-1} surviving for each sample as a function of irradiation time. The PD-MCB was defined as the lowest concentration that kills 99.9% of the initial inoculum.

Results and discussion

Photostability of *meso*-acetoxymethyl BODIPY dyes

To test the photostability of the *meso*-acetoxymethyl compounds **Br_2BOAc** and **I_2BOAc**, oxygen saturated solutions of the photosensitizers were subjected to pulsed irradiation at 532 nm, and the absorbance was monitored at regular intervals (Fig. 2 and 3). For comparison, we also measured the photostability of Rose Bengal (**RB**),[18] a commonly used photosensitizer as well as compound **2I-BDP**, a highly-photostable photosensitizer featuring iodine atoms at the 2 and 6 positions of the BODIPY scaffold but lacking the electron-withdrawing ester at the *meso* position.[16]

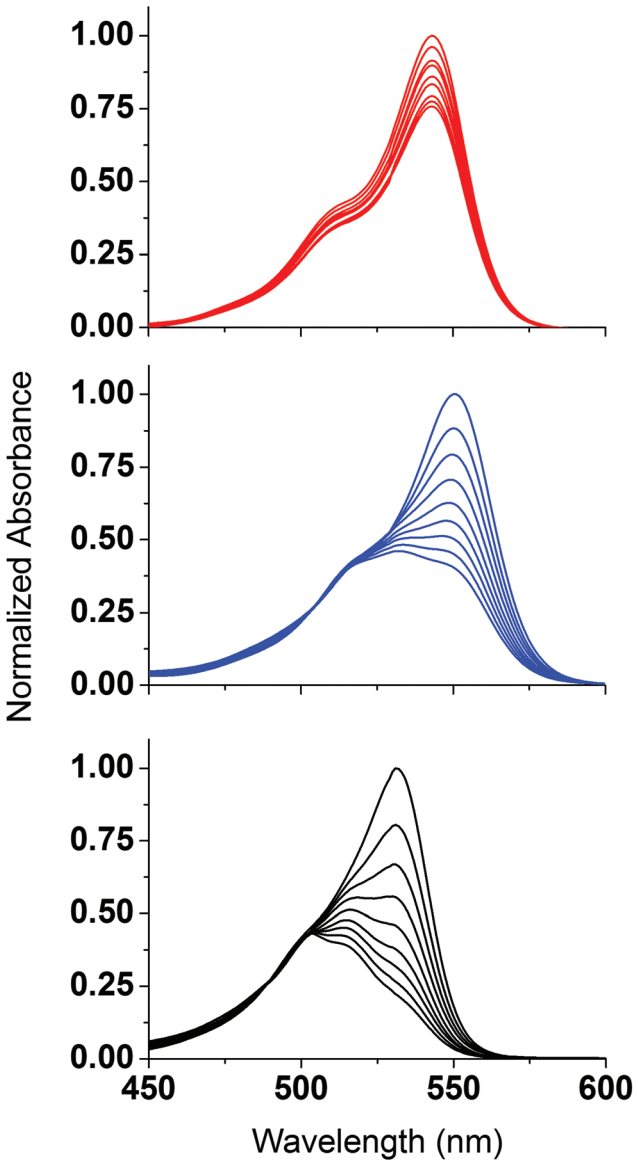

Fig. 2 UV-vis absorption spectra of **Br_2BOAc** (panel A, red), **I_2BOAc** (panel B, blue), and **2I-BDP** (panel C, black) measured in oxygen-saturated acetonitrile solutions recorded at regular intervals (5×10^3 shots) following photoexcitation at 532 nm, 10 mJ per shot.

A significant increase in photostability is observed when comparing **I_2BOAc** to the unsubstituted **2I-BDP**. Exchanging the iodine atoms for bromine further improved the stability of the PDT sensitizer. Bromine is more electron-withdrawing than iodine, further stabilizing the BODIPY scaffold against unwanted oxidation. It is important to note that compound **Br_2BOAc** has a lower quantum yield of singlet oxygen generation (0.84 *vs.* 0.98 for **I_2BOAc**), and thus additional improvement in stability may be attributed to the lesser amount of oxidant being generated. To better compare the results, we reported the relative efficiency of the photosensitizer (E_{rel}, see eqn (1)) given by the product of quantum yield of singlet oxygen (Φ_Δ) and the fitted survival time (τ) (Table 1), whereby

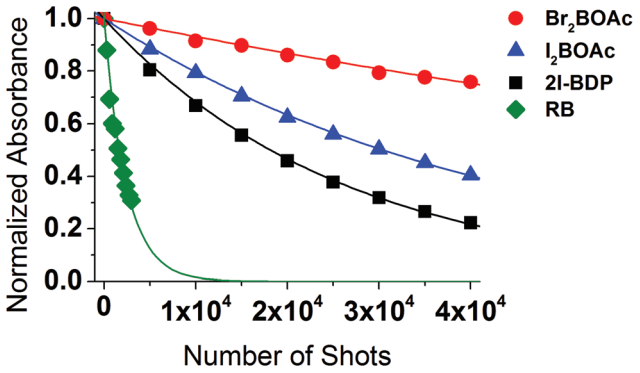

Fig. 3 Photobleaching curves of **Br_2BOAc** (red circles), **I_2BOAc** (blue triangles), **2I-BDP** (black squares) and **RB** (green diamonds) measured in oxygen-saturated acetonitrile solutions recorded at regular intervals (5×10^3 shots) following photoexcitation at 532 nm, 10 mJ per shot. Solutions were optically matched at 532 nm. The absorbance was measured at 543 nm, 550 nm, 531 nm and 518 nm for of **Br_2BOAc**, **I_2BOAc**, **2I-BDP** and **RB** respectively. The solid lines correspond to the result of fitting the data to an exponential decay function to obtain the survival time (τ).

Table 1 Efficiencies (Φ_Δ) and relative efficiencies (E_{rel}) of the photosensitizers tested

Compound	Φ_Δ	τ (per shots)	E_{rel}
Br_2BOAc	0.84[a]	$(1.41 \pm 0.04) \times 10^5$	91
I_2BOAc	0.98[a]	$(4.39 \pm 0.03) \times 10^4$	33
2I-BDP	0.98[b]	$(2.62 \pm 0.03) \times 10^4$	20
RB	0.54[c]	$(2.4 \pm 0.1) \times 10^3$	1

[a] Taken from ref. 15. [b] Taken from ref. 16. [c] Taken from ref. 18.

E_{rel} represents the relative number of singlet oxygen molecules generated before destruction of the photosensitizer.

$$E_{rel} = \Phi_\Delta \times \tau \quad (1)$$

Compound **Br_2BOAc** is 91 times more efficient than **RB** and 4.5 times more efficient than **2I-BDP** towards sensitizing singlet oxygen before photobleaching of the sensitizer. Similarly, **I_2BOAc** was found to be 1.6 fold more efficient than **2I-BDP** toward singlet oxygen sensitization before photobleaching of the sensitizer.

Cellular uptake and localization

We next investigated the cell-permeability and localization of **Br_2BOAc** and **I_2BOAc** compounds by fluorescence microscopy. We utilized the highly fluorescent hydrogen substituted analogue **H_2BOAc** as well as the innate fluorescence of the Br substituted compound (**Br_2BOAc**, $\Phi_f = 0.14$), to investigate the localization of the compounds in HeLa cervical cancer cells (Fig. 4). **I_2BOAc** could not be visualized in this manner due to its low fluorescence intensity. Consistent with the lipophilic nature of the BODIPY scaffold,[19–21] the compounds were seen in the various lipid membranes of the cell.

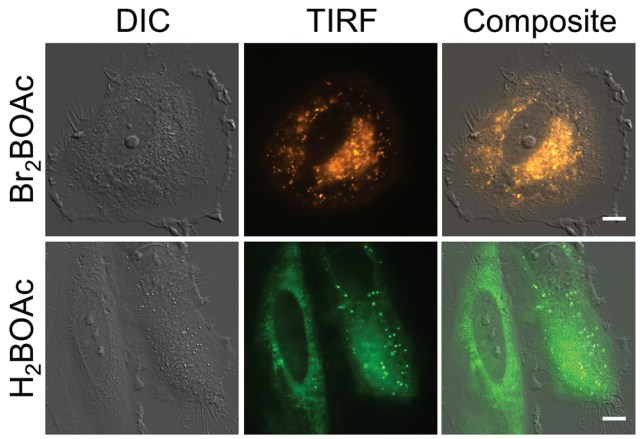

Fig. 4 HeLa cells stained with **Br_2BOAc** (top row) and **H_2BOAc** (bottom row) imaged in DIC mode (left column) and TIRF mode (middle column). The fluorescence of **Br_2BOAc** and **H_2BOAc** is shown in orange and green respectively. Scale bar is 10 μm.

Photocytotoxicity studies

We next tested the potency of **Br_2BOAc** and **I_2BOAc** compounds towards PDT against cultured cancer cells. HeLa cervical cancer cells were treated with solutions of **Br_2BOAc**, **I_2BOAc** or the non-singlet oxygen generating control **H_2BOAc** and irradiated for 30 minutes under the 520 nm LED panel. The cell viability was then assessed 24 hours following irradiation by ethidium homodimer fluorescence, see Fig. 5A. Compounds **Br_2BOAc** and **I_2BOAc** showed significant light-dependent toxicity. The EC_{50} values obtained were 140 nM and 180 nM respectively. Interestingly, while **Br_2BOAc** is less absorbing at the excitation wavelength used than **I_2BOAc**, this compound was more effective toward PDT than the iodine-substituted analogue. We speculate that the greater efficacy of **Br_2BOAc** may be due to the higher photostability conferred on the photosensitizer by the bromine substitution over the iodine substitution. The non-sensitizing dye **H_2BOAc** had an EC_{50} of 2.6 μM.

To further assess the mechanism of cell death, we conducted DIC and fluorescence microscopy of HeLa cells following treatment with the BODIPY photosensitizers. Cells were treated with 500 nM solutions of the photosensitizer in 0.33% DMSO and irradiated for 30 minutes under the 520 nm LED panel and incubated for an additional 24 hours before staining with ethidium homodimer. Dark and vehicle only controls were also conducted (Fig. 5B).

DIC images of the cells irradiated with compounds **Br_2BOAc** and **I_2BOAc** were consistent with cell death *via* apoptosis, which is the favourable mechanism of cell death for PDT treatment to reduce undesired inflammation. Taken together, these results highlight the efficiency of these compounds toward their application in PDT.

We next evaluated these compounds toward bacterial photodynamic inactivation against Gram negative strain *E. coli*. Concentrations of the photosensitizer in the range of 0.078–40 μM were administrated to the inoculum in order to identify an effective dose. The toxicity of the compounds in the absence of photoexcitation was found to be 40 μM. When the samples were irradiated with the 520 nm LED panel for 30 minutes the photodynamic minimal inhibitory concentration (PD-MIC)[22] observed was 5 μM for both **Br_2BOAc** and **I_2BOAc**. The light dose applied was harmless to the strain in the absence of the photosensitizer.

Time-kill curves (Fig. 6) showed no bactericidal activity at 5 μM during the irradiation period. At a slightly higher concentration (7.5 μM) no significant increase of cell lethality was observed. The photodynamic minimal bactericidal concentration (PD-MBC)[22] was reached at 10 μM in a short irradiation

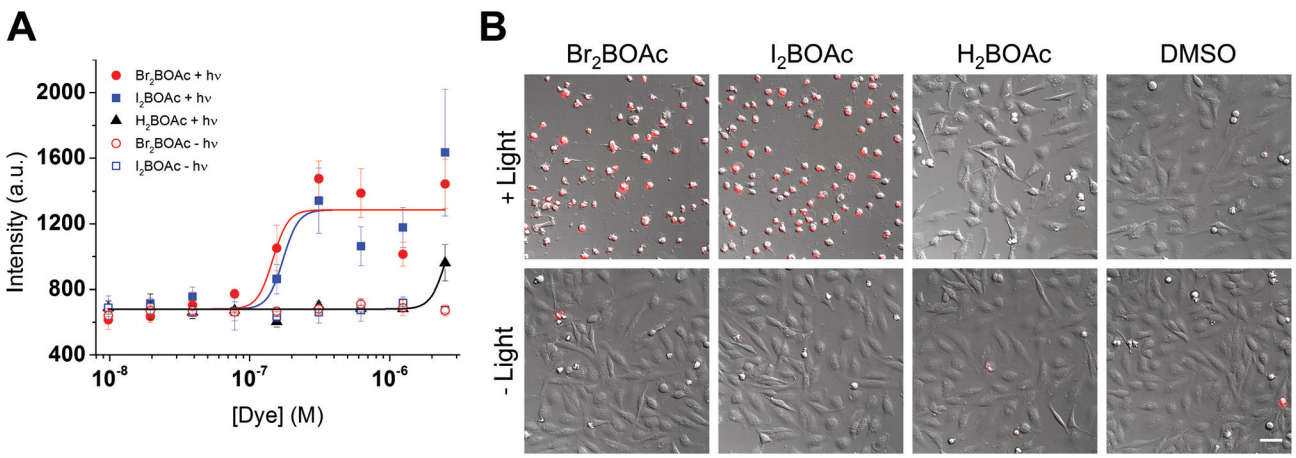

Fig. 5 Panel A: *In vitro* dose–response curves for the BODIPY photosensitizers **Br_2BOAc**, **I_2BOAc**, and the non-sensitizing control **H_2BOAc** in 0.33% DMSO in HeLa cells following with and without 30 minute irradiation. Cell viability was assessed by ethidium homodimer fluorescence 24 hours after light treatment. Panel B: Composite images of DIC microscopy and ethidium homodimer fluorescence (red) of HeLa cells treated with 500 nm solutions of the BODIPY photosensitizers **Br_2BOAc**, **I_2BOAc**, and the non-sensitizing control **H_2BOAc** in 0.33% DMSO as well as control cells treated with DMSO alone. Top panel: Cells were irradiated for 30 minutes. Bottom panel: Dark controls. Images were recorded 24 hours after light treatment. Scale bar is 50 μm.

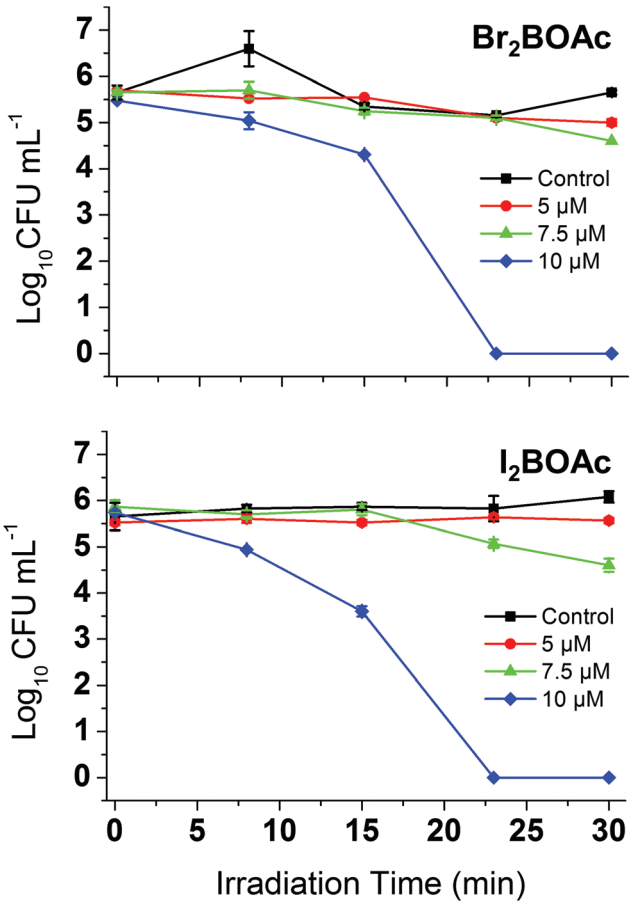

Fig. 6 Time-kill curves of *E. coli* ATCC 25922 treated with Br_2BOAc and I_2BOAc at different concentrations of photosensitizer.

time (~23 minutes). These results demonstrate that both compounds **Br_2BOAc** and **I_2BOAc** are very promising photosensitizers to be used against *E. coli* and possibly other Gram negative strains.

Conclusions

In conclusion the bromo- and iodo-fuctionalized *meso*-acetoxymethyl BODIPY dyes **Br_2BOAc** and **I_2BOAc** are promising photosensitizers for photodynamic therapy against cancer cells and bacteria. The electron-withdrawing acetoxymethyl moiety improved the photostability of the sensitizers to singlet oxygen rendering photosensitizers characterized by their high relative efficiencies, E_{rel}, of photosensitization. Fluorescence microscopy experiments showed that **Br_2BOAc** and **H_2BOAc** compounds readily embedded in the lipid membranes of HeLa cervical cancer cells. Presumably the analogous compound **I_2BOAc** also effectively embedded in the same manner. Light-dependent apoptosis of HeLa cells was observed at nanomolar concentration after only 30 minute irradiation times. Both **Br_2BOAc** and **I_2BOAc** compounds were effective at photoinactivation of *E. coli* bacteria with only 5 μM photosensitizer and applying a short irradiation period. We observed that **Br_2BOAc** was more potent than **I_2BOAc** even if working under conditions that favoured excitation of **I_2BOAc** over **Br_2BOAc**. We may speculate that this enhanced PDT action in HeLa cells arises from the higher photostability of the Br-substituted BODIPY over iodine. In fact, under conditions of low PDT agent dosage and high irradiation power/periods the PDT agent photodegradation may come into play, however such an effect would not be observable under high compound dosage and low irradiation conditions. We do note as well that no distinction was observed between both compounds with *E. coli* photoinactivation studies.

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