Bioorthogonal Turn-On BODIPY-Peptide Photosensitizers for Tailored Photodynamic Therapy

Greta Linden and Olalla Vázquez*[a]

Dedicated to Professor Jesús Jiménez–Barbero on the occasion of his 60th birthday

Abstract: Photodynamic therapy (PDT) leads to cancer remission via the production of cytotoxic species under photosensitizer (PS) irradiation. However, concomitant damage and dark toxicity can both hinder its use. With this in mind, we have implemented a versatile peptide-based platform of bioorthogonally activatable BODIPY-tetrazine PSs. Confocal microscopy and phototoxicity studies demonstrated that the incorporation of the PS, as a bifunctional module, into a peptide enabled spatial and conditional control of singlet oxygen (\(\text{O}_2^*\)) generation. Comparing subcellular distribution, PS confined in the cytoplasmic membrane achieved the highest toxicities (IC\(_{50} = 0.096 \pm 0.003 \text{ µm}\)) after activation and without apparent dark toxicity. Our tunable approach will inspire novel probes towards smart PDT.

Introduction

Recent years have witnessed an immense growth in interest in the photoregulation of biological events because light offers the possibility of exerting remote control of cellular functions and, consequently, cures for a variety of diseases too. A traditional clinical treatment based on light exposure is photodynamic therapy (PDT), which is nowadays the standard treatment for several illnesses, including some cancers. PDT requires three primary components to trigger cell death: an efficient photosensitizer (PS), light, and in situ production of cytotoxic species under photodynamic conditions. Notably, the efficiency of cell photodamage is strongly determined by the PS biodistribution. To investigate our hypothesis, we aimed for the synthesis and study of five bioorthogonally activatable BODIPY-tetrazine PSs. Confocal microscopy and phototoxicity studies demonstrated that the incorporation of the PS, as a bifunctional module, into a peptide enabled spatial and conditional control of singlet oxygen (\(\text{O}_2^*\)) generation. Comparing subcellular distribution, PS confined in the cytoplasmic membrane achieved the highest toxicities (IC\(_{50} = 0.096 \pm 0.003 \text{ µm}\)) after activation and without apparent dark toxicity. Our tunable approach will inspire novel probes towards smart PDT.

Now, we further expand this concept by exploring a modular platform, in which the halogenated BODIPY and the quencher tetrazine are separately integrated into a peptide scaffold via a cysteine residue. As yet, there are only few examples of BODIPY-peptide conjugates in PDT; they have been mostly used as fluorescent probes. However, if the new molecular arrangement maintains the precise control of the FRET process, peptide bioconjugation may not only improve the properties of our aPS in line with other hydrophobic PSs, but since peptide signalling sequences are powerful delivery vehicles, may also direct photodynamic action to specific subcellular locations. Notably, the efficiency of cell photodamage is strongly determined by the PS biodistribution. To investigate our hypothesis, we aimed for the synthesis and study of five bioorthogonally aPS peptide conjugates. We selected the conventional polyarginines (Rn) composed of five (R5) or eight (R8) PS confined in the cytoplasmic membrane achieved the highest toxicities (IC\(_{50} = 0.096 \pm 0.003 \text{ µm}\)) after activation and without apparent dark toxicity. Our tunable approach will inspire novel probes towards smart PDT.

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and Tz-[2I-BODIPY]-versus F10 than in the case of the Tz-C(2I-BODIPY)-PEPTIDE conjugate pair under irradiation. We planned to combine the hydrophilic tetra-arginine (R4) moiety with the palmitic acid (PA) to form the corresponding pair Tz-C(2I-BODIPY)-K(PA)R4 ([TCO:Tz]-C(2I-BODIPY)-PEPTIDE, 2-6ON) analogues from this project.

The synthetic route to these bioconjugates is shown in Scheme 1. Thus, the azide-functionalized BODIPY 7, which had previously been described as an accessible scaffold for the generation of various BODIPY dyads, was first iodinated with N-iodosuccinimide (NIS) in DCM to maximize the intersystem crossing process, and hence O2 production. The resulting 2I-BODIPY compound 8 was then converted to bromoacetamide 9 via a sequence of a 1,3-dipolar cycloaddition followed by substitution. For the modular attachment of our PS/quencher pair, all peptides included an N-terminal cysteine. The synthesis of these cysteinyl fragments (11–14) was performed using the classical Fmoc solid-phase methodology. After cleavage from the solid support and reversed-phase (RP) HPLC purification, the single cysteine residue enabled the efficient alkylation with the thiol-reactive 2I-BODIPY.

The remainder of the synthesis was then analogous to those described above. Lastly, all of the obtained Tz-C(2I-BODIPY)-PEPTIDE conjugates (2-6OFF) reacted successfully with the strained dienophile TCO to form the desired active peptide-based PSS (TCO:Tz)-C(2I-BODIPY)-PEPTIDE, 2-6ON).

Next, we explored the photodynamic activity and subcellular localization of all BODIPY peptide-based conjugates. First, we analysed their ability to produce O2 under irradiation. We used 1,3-diphenylisobenzofuran (DPBF) as a trap compound. In the presence of O2, DPBF decomposes in a 1:1 stoichiometry via a [4+2] cycloaddition to give 1,2-dibenzoylebenzene, resulting in reduced absorbance at 415 nm, and thereby, allowing the measurement of O2 production. Our kinetics (Figure 2, Figure S43–S45 and Table 1) together with the determined quantum yields (Table 1) showed that biocjugation, that is, conjugation to the peptide sequences, does not have any impact on the in vitro production of O2. The PS capacity of the turn-on [TCO:Tz]-C(2I-BODIPY)-PEPTIDE conjugates (2-6ON) is similar (kD = 0.60) to the one of standard PSS.

Under 525 nm irradiation, all pyridazine BODIPY [TCO:Tz]-C(2I-BODIPY)-PEPTIDE analogues (2-6ON) retained the same PS capacity as the halogenated azide-functionalized BODIPY 8 (k = 4.52 s⁻¹ 10⁻³ versus k = 4.97 s⁻¹ 10⁻³; kD = 0.59 versus kD = 0.61, respectively) while, gratifyingly, there was a slower decrease in the O2 generation of all Tz-C(2I-BODIPY)-PEPTIDE conjugates (2-6OFF) (k = 2.61 s⁻¹ 10⁻³; kD = 0.55). Despite the efficient O2 quenching observed, as expected from a FRET-quenching mechanism, this effect is higher when the tetrazine is directly conjugated with the 2I-BODIPY core, as in PSS 1 than in the case of the Tz-C(2I-BODIPY)-PEPTIDE compounds (2-6OFF).

Figure 1. Structures of our precedent work on the unimodular turn-off mTz-2I-BODIPY (1OFF) photosensitizer (PS) and the turn-on TCO:mTz-2I-BODIPY (1ON) PS, as well as the novel modular peptide-based turn-off Tz-C(2I-BODIPY)-PEPTIDE (2-6ON) PSS and turn-on [TCO:Tz]-C(2I-BODIPY)-PEPTIDE (TIDE) analogues from this project.

4ON; Tz-C(2I-BODIPY)-MPP2, 5OFF and [TCO:Tz]-C(2I-BODIPY)-MPP2, 5ON), as our targeting vectors. The latter peptides, MPP1[23] and MPP2,[24] are based on the combination of two unnatural amino acids: cyclohexylalanine (F) and d-arginine (r), the difference between them is only a single extra d-arginine residue in MPP2. We also pursued the preparation of a conjugate pair embedded in the cell membrane. To achieve the appropriate lipophilicity,[25] we planned to combine the hydrophilic tetra-arginine (R4) moiety with the palmitic acid (PA) to form the corresponding pair Tz:[C2I-BODIPY]-K(PA)R4 (6OFF) and [TCO:Tz]-C(2I-BODIPY)-K(PA)R4 (6ON).

Results and Discussion

For the assembly of the hybrids, we followed a convergent strategy that involves the synthesis of the 2-bromoacetamide BODIPY 9 and the corresponding cysteine-containing peptides (11–14) (Scheme 1). The tetrazine could successfully be introduced in solution via standard M-hydroxysuccinimide (NHS) conjugation[26] to yield the expected turn-off BODIPY-peptide PSS (Tz-C(2I-BODIPY)-PEPTIDE, 2-6OFF). These could be activated, in the presence of TCO, to afford the pyridazine analogues ([TCO:Tz]-C(2I-BODIPY)-PEPTIDE, 2-6ON) with the intention of generating the O2 upon irradiation.

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Afterwards, we evaluated the cellular uptake and subcellular localization of these derivatives in HeLa cells by flow cytometry and live-cell confocal microscopy. For this purpose, we prepared the fluorescent non-halogenated H$_2$N-C(BODIPY)-PEPTIDE analogues (2-6FL) lacking the tetrazine group. (Figure 3A). Flow cytometry enables cellular-uptake quantification by measuring fluorescence intensity. Thus, HeLa cells were incubated with the BODIPY-labelled peptides (H$_2$N-C(BODIPY)-PEPTIDE 2-6FL) at different concentrations for two hours, followed by a washing protocol including a trypsin treatment before the cytometry trials. At 2 µM concentration, all peptides displayed a pronounced fluorescence shift, which is clearly different to that of the untreated cells. This signal corresponds to quantitative labelling (Figure 3B–D). However, if the concentration were decreased 100 times (0.02 µM), cells were hardly labelled, except when using the lipopeptide H$_2$N-C(BODIPY)-K(PA)R$_4$ (6FL) (28 % labelled cells). Interestingly, this effect was

**Table 1. Photophysical properties and photodynamic activity of the 2I-BODIPY-peptide conjugates in HeLa cells.**

| Compound | k [10$^{-13}$ s$^{-1}$] | $\Phi_A$ | IC$_{50}$ [µM] |
|----------|----------------------|---------|----------------|
| 2OFF     | 2.62                 | 0.55    | 0.617 ± 0.024  |
| 3OFF     | 2.55                 | 0.55    | 0.716 ± 0.078  |
| 4OFF     | 2.82                 | 0.56    | 0.599 ± 0.043  |
| 5OFF     | 2.70                 | 0.56    | 0.598 ± 0.037  |
| 6OFF     | 2.40                 | 0.55    | 0.369 ± 0.030  |
| 2ON      | 4.50                 | 0.59    | 0.201 ± 0.012  |
| 3ON      | 4.96                 | 0.60    | 0.196 ± 0.017  |
| 4ON      | 4.14                 | 0.59    | 0.228 ± 0.008  |
| 5ON      | 4.32                 | 0.59    | 0.225 ± 0.011  |
| 6ON      | 4.68                 | 0.59    | 0.096 ± 0.003  |

[a] Rate constant of the DPBF consumption in MeCN; [b] $\Phi_A$ quantum yields calculated relative to: rose bengal ($\Phi_A = 0.76$) and erythrosine B ($\Phi_A = 0.60$) in MeOH; [c] half maximal inhibitory concentration obtained from resazurin cell viability assays (irradiation dose: 69.4 ± 0.6 W m$^{-2}$ for 160 s). Mean values derived from two independent experiments with errors ± 6% for $k$, ± 7% for $\Phi_A$. 

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more dramatic for the oligoarginine conjugates, where low labelling rates (~10%) were already detected at 0.2 μmol (Figure S67 and S68). The fluorescence intensity was directly proportional to the concentration and dependent on the type of peptide (Figure 3D). The lipopeptide H₂N-C(BODIPY)-K(PA)R₄ (6FL) displayed at least a three-fold signal increase compared to 2FL, 3FL, 4FL and 5FL at all concentrations (Table S4), with a maximum of 15-fold signal intensity increase for the 2 μmol concentration of H₂N-C(BODIPY)-K(PA)R₄ (6FL) compared to H₂N-C(BODIPY)-R₅ (2FL).

Since standard flow cytometry cannot discriminate between fluorescence from internalized fluorophores and that resulting from membrane-bound ones, we employed live-cell confocal microscopy (Figure 4). Images recording across consecutive Z-stacks and the use of commercial fluorescent markers specific for lysosomes (LysoTracker® Red DND-99), mitochondria (tetramethylrhodamine ethyl ester perchlorate: TMRE), and cytoplasmic membranes (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate: DiI) allowed us to decipher the intracellular distribution of our fluorescent conjugates. For comparison, we used the same parameters for all BODIPY-peptide derivatives (H₂N-C(BODIPY)-PEPTIDEs, 2-6FL), that is, 2 μmol concentration and two hours of incubation. The analysis of the results revealed that the lipopeptide H₂N-C(BODIPY)-K(PA)R₄ (6FL) was unable to penetrate the cells, despite having an oligoarginine sequence. Indeed, the incapacity of other R₄ peptide constructs to efficiently translocate the cell membrane has previously been reported.[25,35] In this case, we observed a very intense fluorescence signal around the cytoplasmatic membrane.

Co-localization experiments with H₂N-C(BODIPY)-K(PA)R₄ conjugate (6FL) and the membrane stain Dil demonstrated a close-to-perfect overlay with a Pearson’s correlation coefficient (Rr) of 0.907. However, the conjugates containing either at least five arginine residues (R₅ and R₈) or either the MPP sequences were efficiently taken up by the HeLa cells under the same conditions (Figure 4). For the polyarginine vectors, in general, it is assumed that a minimum of six arginine residues (R₆) are needed to induce intracellular uptake.[22a,36] However, we observed that five consecutive arginines (R₅) in our BODIPY-peptide H₂N-C(BODIPY)-R₅ (2FL) are enough to achieve almost the same cell-penetration as the R₈ derivatives (H₂N-C(BODIPY)-R₈, 3FL). Regarding the subcellular localization, significant differences between the Rₙ and MPP hybrids were observed. Thus, LysoTracker® colocalization experiments verified that the punctate BODIPY-Rₙ distribution—hybrids H₂N-C(BODIPY)-R₅ (2FL) and H₂N-C(BODIPY)-R₈ (3FL)—is predominantly within the lysosomes (Rr~0.7). Unexpectedly, the compounds designed specifically to target mitochondria (H₂N-C(BODIPY)-MPP₇, 4FL and H₂N-C(BODIPY)-MPP₂, 5FL) showed poor colocalization with TMRE (Rr~0.34) even when the incubation time was reduced to 30 min (Rr~
activation of our bioorthogonally activatable PS. To this end, the different OFF/ON BODIPY-peptide PSs were incubated at different concentrations, and irradiated at 525 nm with a custom-made 96-well plate LED array (69.4 ± 0.6 W m⁻² for 160 s) after two hours of incubation. Before the viability assays, the total incubation time was 20 h. The obtained IC₅₀ values (Table 1; Figure S47–S49) displayed a clear difference between the tetrazine Tz-(2I-BODIPY)-PEPTIDE derivatives (2-6OFF) and the corresponding pyridazine [TCO:Tz]-2I-BODIPY-PEPTIDE ones (2-6ON) in the intracellular environment. Importantly, without the PS motif, the peptide vectors for cellular-compartment targeting displayed a total absence of toxicity at the studied concentration range, up to 4 μM (Figure S51).

In concordance with the in vitro O₂ quenching experiments (Figure 2), the phototoxicity window increases when the tetrazine is directly conjugated with the 2I-BODIPY core, for example PS 1 (IC₅₀ = 1.92 ± 0.27 μM for the tetrazine derivative 1OFF versus IC₅₀ = 0.354 ± 0.017 μM for its pyridazine analogue 1ON after iEDDA with TCO).

As expected, there was almost no difference in the intracellular phototoxicity of the turn-off/tum-on pairs bearing oolithiogamines (Rn) or mitochondria penetrating peptides (MPPs), which were almost identical in both vitro reactivity (Figure 2 and Table 1) and cellular localization (Figure 4). It is noteworthy to highlight the BODIPY-lipopeptide conjugates 6 because, among our BODIPY-peptide PSs, the membrane-tagging turn-on [TCO:Tz]-2I-BODIPY-K(PA)R4 (6ON) was the most cytotoxic, and, simultaneously, this construct displayed the highest phototoxicity variation between turn-off Tz-(2I-BODIPY)-K(PA)R4 (6OFF) and turn-on [TCO:Tz]-2I-BODIPY-K(PA)R4 (6ON) (3.8-fold). Its cellular phototoxicity is higher than that of the other constructs (2-5ON) yet DPBF behaviour (Figure 2) is comparable. The superior phototoxicity of 6ON was verified in other cell types too (Figure S52–S54, Table S3). Importantly, in the highly metastatic PC-3 cells, the conjugates 6 displayed similarly the highest toxicity as well as the best OFF/ON ratio (3.3-fold; IC₅₀ = 0.392 ± 0.028 μM for 6OFF versus IC₅₀ = 0.119 ± 0.012 μM for 6ON) among the pairs. In addition, we demonstrated that the acidic pH of the lysosomes does not affect the O₂ generation by our PSs (Figure S44), unlike in other lysosome targeted BODIPYs. Consequently, the highest phototoxicity of [TCO:Tz]-2I-BODIPY-K(PA)R4 (6ON) must be due to the specific localisation within the cell. These factors jointly reveal the importance of photoinduced membrane damage. This observation is in agreement with the general theory that cell membranes are essential sites for the photosensitized cell damage, as reported for other PSs like rose bengal, or Kill-erRed. Of note, no dark toxicity was detected for any compound up to a maximum of 4 μM concentration (Figure S50).

Figure 4. Localization of the fluorescent BODIPY-peptides 2-6FL in live HeLa cells. Images displayed in green correspond to these peptides at 488 nm excitation/535 nm emission. Before confocal imaging, cells were incubated for 2 h (in FluoroBrite media, 2.5% FBS, 1.25% MeCN, 8.75% ultrapure H₂O). Concentrations of 2 μM BODIPY conjugates were used. The conditions were: A) BODIPY-oilogamine (Rn) peptides 2FL or 3FL: following a DPBS washing step, 50 nM of LysoTracker® Red DND-99 in FluoroBrite media (2.5% FBS, 0.5% DMSO) was added and left 2 h; cells were DPBS washed again before image acquisition; images displayed in red correspond to LysoTracker® Red DND-99 fluorescence (633 nm excitation/635–700 nm emission); or B) BODIPY-mitochondria penetrating peptides (MPPs) 4FL or 5FL: following a DPBS washing step, 25 nM of TMRE in FluoroBrite media (2.5% FBS, 0.5% DMSO) was added and left for 30 min; cells were DPBS washed again before image acquisition; images displayed in orange correspond to TMRE fluorescence (543 nm excitation/545–589 nm emission); or C) BODIPY-lipopeptide 6FL: following a DPBS washing step, 10 μM of DiI in FluoroBrite media (2.5% FBS, 0.5% DMSO) was added and left for 15 min; cells were DPBS washed again before image acquisition; images displayed in orange correspond to DiI fluorescence parameters (543 nm excitation/545–589 nm emission). All images collected were analysed by Zeiss ZEN; ImageJ software was used for the calculation of the Rr to determine the extent of overlay. Values reported were calculated by linear regression, using more than 10 cells analysed in two independent experiments. Scale bar: 20 μm; TMRE: tetramethylrhodamine ethyl ester perchorlate; DiI: 1,1'-dioctadecyl-3,3,3'-tetramethyl-indocarbocyanine perchlorate.
Similarly, conjugates of the well-validated PS tetraphenyl-porphyrin (TPP) with our peptides 12, 14 and 15 displayed an increased photocytotoxicity for the H$_2$N-C(2I-BODIPY)-K(PA)R4 conjugate ($IC_{50} = 2.310 \pm 0.301 \mu M$) compared to the H$_2$N-C(2I-BODIPY)-R8 ($IC_{50} = 3.894 \pm 0.453 \mu M$) and H$_2$N-C(2I-BODIPY)-MPP2 ($IC_{50} = 3.923 \pm 0.469 \mu M$) conjugates (Figure S49). These results suggest a general behaviour of PA-R4 conjugates, which is independent of the cargo, and therefore, applicable to other PSs.

Finally, we investigated whether we could achieve activation of our turn-off BODIPY-peptide PSs via an in situ bioorthogonal iEDDA, and whether there is an influence of PS localization. For this purpose, HeLa cells were treated with the corresponding turn-off BODIPY-peptide PSs (2-OFF) in varied concentrations from 0.13 $\mu M$ to 1 $\mu M$ for 90 min, followed by a washing step to remove any traces of extracellular derivatives. Afterwards, the dienophile TCO was added in excess (12.5 equiv). After 30 min, the plates were irradiated with 69.4 $\pm$ 0.6 W m$^{-2}$ for 160 s and incubated for another 18 h, before determining cell viability via a resazurin-based assay.25 We observed that the addition of TCO to the turn-off Tz-C(2I-BODIPY)-PEPTIDE probes (2-OFF) caused more cellular death than either compound alone, across the entire range of concentrations studied (Figure S56–S58). Importantly, such toxicity perfectly aligns to that measured for the synthesized turn-on [TCO:Tz]-C(2I-BODIPY)-PEPTIDE PSs, (2-OFF) (Figure S47–S49). Altogether, these results demonstrate that regardless of the cellular localization of the PSs, all conjugates are assembled inside the cells, and upon irradiation, the newly synthesized [TCO:Tz]-C(2I-BODIPY)-PEPTIDE cycloadducts (2-OFF) display enhanced phototoxicity. The Figure 5 compares the in situ activation process for the different peptide-based PSs at concentrations, where the cytotoxic difference between the turn-off and in situ activated analogues is the highest. Despite similar, satisfactory tendencies across conjugates in the in situ activation process, the membrane-embedded conjugates 6OFF/ON again surpassed the rest of the conjugates. Indeed, its effectiveness in triggering cellular mortality due to its specific membrane localization enabled the reduction of the concentration of the turn-off Tz-C(2I-BODIPY)-K(PA)R4 PS (6OFF) by half, which minimized background toxicity and improved its applicability as an aPS.

Conclusions

Most small-molecules fail to target cellular compartments specifically: instead, they distribute either between several areas simultaneously, or at random without reaching the desired localization. In this study, we successfully provided access to a modular peptide-based platform, in which the halogenated BODIPY photosensitizer (PS) and the quencher tetrazine are separated. Peptide conjugation not only represents a straightforward approach to improve the photochemical properties of PSs but also enables spatial activation due to the accumulation of the PS in specific organelles. We reported a range of dual-labelled peptides that only differ in their peptidic vectors, sharing the same PS/quencher pair. This type of bioorthogonally activatable PSs achieved control of the confined photodynamic effect. Thus, the bioorthogonal activation in specific subcellular organelles was successful. Importantly, PS distribution determined the cellular response to photodamage in multiple cell lines. Among our conjugates, the example capable of being activated specifically in the cytoplasmic membrane, Tz-C(2I-BODIPY)-K(PA)R4 (6OFF) via an bioorthogonal iEDDA surpassed the rest. Once active, the in situ formed, [TCO:Tz]-C(2I-BODIPY)-K(PA)R4 PS, (6ON) attained superior PDT performance, that is, enhancement of the photodynamic effect at lower concentrations without dark toxicity. Importantly, since our approach used a cysteine residue as workhorse, this methodology is compatible with the common bioconjugation strategies, making it highly versatile and tunable for the incorporation of other PS as well as other biopolymers. Thus, we believe that our modular approach will assist the exploration of further subcellular compartments and bioconjugation possibilities, as well as additional peptidic functionalities such as tumour-targeting peptides and antibodies to tackle the selectivity issues in PDT. Therefore, this modular peptide-based platform will further advance the applicability of the bioorthogonally activatable PSs.

Experimental Section

Materials

All commercially available compounds were used without further purification as delivered from the corresponding companies, 3-diphenylisobenzofuran from Alfa Aesar (USA); propargylamine from Acros Organics (Belgium); TMRE from Cayman Chemical (USA); DIL from MedChemExpress (USA); LysoTracker® Red DND-99 from Thermo Fisher (USA); bromoacetic anhydride, palmitic acid, trypsin-EDDA, and penicillin-streptomycin from Sigma Aldrich (USA); Oxyma, Fmoc-Lys(Alloc)-OH and 3-diphenylisobenzofuran from Alfa Aesar (USA); propargylamine from Acros Organics (Belgium); TMRE from Cayman Chemical (USA); DIL from MedChemExpress (USA); LysoTracker® Red DND-99 from Thermo Fisher (USA); bromoacetic anhydride, palmitic acid, trypsin-EDDA and penicillin-streptomycin from Sigma Aldrich (USA); Oxyma, Fmoc-Lys(Alloc)-OH and Fmoc-D-Arg(Pbf)-OH from Carbolution (Germany); Fmoc-F$_3$-OH from Fluorochem; DMF (peptide grade), Fmoc-protected amino acids and Tentagel S RAM resin from Iris Biotech (Germany); DPBS, DMEM and FluoroBrite
from Gibco Thermo Fisher Scientific (USA); FBS from PAN Biotech (Germany); MeCN (HPLC grade) from VWR (France). TEA, THF and DCM were dried using standard procedures. Ultrapure H₂O of type I was obtained with a MicroPura Water Purification System (TKA, Germany).

**Characterization**

NMR spectra were recorded at 300 K on Bruker AV III HD 300 and 600 MHz spectrometers, whereas HRMS (ESI) results were acquired with a LTQ-FT Finnigan Ultra mass spectrometer (Thermo Fischer Scientific), the resolution was set to 100,000. Peptide and conjugate characterization was performed via RP-HPLC-MS on an Agilent 1260 Infinity II HPLC-System (Agilent Technologies) with an UV detector (220 nm). The analytical column was an Agilent eclipse XDB-C18 column (5 μm, 4.6×150 mm) or Macherey-Nagel Nucleodur 100-C18 ec column (3 μm, 2×125 mm). The following two eluents were used: A) ultrapure H₂O with addition of 0.05% TFA, and B) MeCN with addition of 0.03% TFA.

**Singlet oxygen measurements**

Singlet oxygen production: This was determined in black μclear 96 well plates (Greiner Bio-One, Item: 655096) by recording endpoint absorbance at 415 nm with a Beckman 20 μl plate reader at 25 °C. Compound concentrations were determined from stocks using the molar extinction coefficients (Table S1). 50 μL of 800 μmol DPBF solution (200 μmol as final concentration per well) and 20 μL of 1 μmol PS (100 nm as final concentration per well) were added in MeCN (200 μL as final volume per well). As controls the background (MeCN), and a sample containing 200 μmol DPBF were used. Irradiation was performed in 20 s intervals with an irradiation of 69.4±0.6 Wm⁻² until 320 s by a custom-made LED-array bearing 96 LEDs (Broadcom Limited, 525 nm, 16,000–27,000 mcd, viewing angle: 23°, AVAGO HLM-P-2100D). For all measurements, before the first irradiation cycle, an initial measurement was performed as time zero without irradiation. Results were given as mean of the individual values obtained, in which the background was previously subtracted, displayed as percentage of DPBF present in each sample in triplicate. Measurements were performed at least in two independent experiments, that is, from two different stocks.

Singlet oxygen quantum yields (φₐ): They were determined as we described before. The procedure was alike the determination of ¹⁸O₂ described above, but using 1 μmol as final PS concentration. Erythrosine B (EB) and rose bengal (RB) in MeOH were used as reference (φₐ,EB = 0.76 for RB and 0.6 for EB). Irradiation was performed in 10 s intervals with irradiation of 69.4±0.6 Wm⁻² for 160 s.

**Peptide synthesis**

Peptides 11–14 were synthesized in a 20 μmol scale according to the standard SPPS methodology, using Fmoc-amino acids and Oxyma/DIC as coupling agent. Obtained peptides were purified by semipreparative RP-HPLC on a Varian (USA) ProStar Preparative HPLC system with a semi-preparative Phenomenex Jupiter 10 u C18 300 Å column. The eluants were ultrapure H₂O (A) and MeCN (B) with addition of 0.1% TFA. Analytical RP-HPLC chromatograms and used gradients are in Figure S1–54.

**Organic synthesis**

NMR spectra for compounds 19 and 20 are in Figure S1–27.

**Compound 9:** This was obtained in a two-step synthesis from 8, 8 (165 mg, 250 μmol, 1.00 equiv) and Cul (7.6 mg, 40.0 μmol, 0.16 equiv) were dissolved in dry THF (3.00 mL) under inert atmosphere. Propargylamine (240 μL, 3.75 μmol, 1.50 equiv) was added last, and the solution was stirred for three hours. The reaction mixture was concentrated under reduced pressure and purified by column flash chromatography (DCM to MeOH/TEA: 94:5, v/v/v). The intermediate 19 was obtained as red solid (82.9 mg, 116 μmol, 46%). TLC: Rₜ = 0.30 (DCM/MeOH/TEA, 94:5:1, v/v/v).

**H NMR (300 MHz, 300 K, CDCl₃): δ = 8.68 (s, 3H, NH₂), 8.09 (s, 1H, CH₃), 7.13 (d, J = 8.5 Hz, 2H, 2H), 7.01 (d, J = 8.6 Hz, 2H, 2H), 4.81 (t, J = 4.6 Hz, 2H, CH₂), 4.44–4.41 (m, 4H, 2H, CH₂), 2.61 (s, 6H, 2H), 1.38 ppm (s, 6H, 2H, CH₂).**

**HRMS-ESI+ (m/z):** calculated for C₂₇H₂₅BrF₂₂N₂O₂: [M+Na⁺]: 740.0216; found: 740.0212. The intermediate 19 (50.0 mg, 69.8 μmol, 1.00 equiv), and pyridine (5.6 μL, 69.8 μmol, 1.00 equiv) were dissolved in degassed DMF (2.50 mL) under inert atmosphere and placed in an ice bath. Bromoacetic anhydride (54.4 mg, 209 μmol, 3.00 equiv) was added dropwise. After 15 min the ice bath was removed, and the red solution was stirred for three hours at room temperature. The mixture was then concentrated under reduced pressure, diluted with EtOAc (10 mL), washed with distilled H₂O (2×2.5 mL) and brine (1×2.5 mL). The organic phase was dried over anhydrous MgSO₄, filtered, concentrated under reduced pressure and purified by column flash chromatography (EtOAc to obtain 9 as dark red solid (37.5 mg, 44.8 μmol, 64%). TLC: Rₜ = 0.36 (EtOAc). ¹H NMR (300 MHz, 300 K, CDCl₃): δ = 7.79 (s, 1H, CH₃), 7.15 (d, J = 8.7 Hz, 2H, 2H), 7.01 (d, J = 8.7 Hz, 2H, 2H), 4.81 (t, J = 5.1 Hz, 2H, CH₂), 4.59 (d, J = 5.9 Hz, 2H, CH₂), 4.44 (t, J = 5.1 Hz, 2H, CH₂), 3.88 (s, 2H, CH₂), 2.63 (s, 6H, 2H, CH₂), 1.41 ppm (s, 6H, 2H, CH₂).**

**HRMS-ESI+ (m/z):** calculated for C₂₇H₂₅BrF₂₂N₂O₂: [M+Na⁺]: 740.0216; found: 740.0212. The intermediate 19 (50.0 mg, 69.8 μmol, 1.00 equiv), and pyridine (5.6 μL, 69.8 μmol, 1.00 equiv) were dissolved in degassed DMF (1.50 mL) was added dropwise. After 15 min the ice bath was removed, and the red solution was stirred for three hours at room temperature. The mixture was then concentrated under reduced pressure, diluted with EtOAc (10 mL), washed with distilled H₂O (2×2.5 mL) and brine (1×2.5 mL). The organic phase was dried over anhydrous MgSO₄, filtered, concentrated under reduced pressure and purified by column flash chromatography (EtOAc to obtain 9 as dark red solid (37.5 mg, 44.8 μmol, 64%). TLC: Rₜ = 0.36 (EtOAc). ¹H NMR (300 MHz, 300 K, CDCl₃): δ = 7.79 (s, 1H, CH₃), 7.15 (d, J = 8.7 Hz, 2H, 2H), 7.01 (d, J = 8.7 Hz, 2H, 2H), 4.81 (t, J = 5.1 Hz, 2H, CH₂), 4.59 (d, J = 5.9 Hz, 2H, CH₂), 4.44 (t, J = 5.1 Hz, 2H, CH₂), 3.88 (s, 2H, CH₂), 2.63 (s, 6H, 2H, CH₂), 1.41 ppm (s, 6H, 2H, CH₂).**

**HRMS-ESI+ (m/z):** calculated for C₃₁H₃₄BrF₂₂N₂O₂: [M+Na⁺]: 754.0348; found: 754.0348. ¹H NMR (300 MHz, 300 K, CDCl₃): δ = 7.79 (s, 1H, CH₃), 7.15 (d, J = 8.7 Hz, 2H, 2H), 7.01 (d, J = 8.7 Hz, 2H, 2H), 4.81 (t, J = 5.1 Hz, 2H, CH₂), 4.59 (d, J = 5.9 Hz, 2H, CH₂), 4.44 (t, J = 5.1 Hz, 2H, CH₂), 3.88 (s, 2H, CH₂), 2.63 (s, 6H, 2H, CH₂), 1.41 ppm (s, 6H, 2H, CH₂).**

**HRMS-ESI+ (m/z):** calculated for C₃₁H₃₄BrF₂₂N₂O₂: [M+Na⁺]: 778.0480; found: 778.0480.
Conjugate synthesis

Compounds 2–5A: They were obtained in a two-step synthesis. Initially, in a microcentrifuge tube DIPEA (2.20 equiv) was added to a 0.12 m solution of the corresponding peptide (1.20 equiv) in degassed Tris buffer (0.10 M, pH 8.5). Afterwards, the 2-bromoaryl photosensitizer 9 (1.00 equiv, 0.10 M in MeCN) was added. The reaction was mixed at room temperature on an Eppendorf Thermoshaker, and followed by analytical RP-HPLC-MS until the complete conversion of the starting material 9 was observed, and the desired 2I-BODIPY-peptides 24–27 were observed (Figure S23–S26). These compounds were purified by semi-preparative RP-HPLC using a linear gradient over 30 minutes from 5% to 75% of solvent B. Detection of the signals was achieved with a dual wavelength UV detector at 220 nm and 260 nm. The 2I-BODIPY-peptides were characterized as explained before (characterization section above). Analytical RP-HPLC chromatograms and used gradients are in Figures S23–S26. Afterwards, to a 0.07 m solution of the corresponding 2I-BODIPY-peptide (1.00 equiv) dissolved in DMF, it was added first DIPEA (2.00 equiv), and subsequently the NHS-active ester 10 (2.00 equiv, 0.20 M in DMF). The reaction was mixed at room temperature on an Eppendorf Thermoshaker, and followed by analytical RP-HPLC-MS until complete conversion was observed. 2-5A conjugates were purified by semi-preparative RP-HPLC. A linear gradient over 30 minutes from 5% to 75% of solvent B was applied for compounds 2-5A. Detection of the signals was achieved with a dual wavelength UV detector at 220 nm and 260 nm. Analytical RP-HPLC chromatograms and used gradients are in Figures S52–S53.

Tz-C(2I-BODIPY)-RRRRR-CONH₂ (OFF): obtained from peptide 28 and compound 10; reaction time: 4 h; pink solid; yield: 42%.

[TCO:Tz]-C(2I-BODIPY)-K(PA)RRRR-CONH₂ (CON): obtained from peptide 25 and compound 10; reaction time: 3 h; pink solid; yield: 53%.

[tC(2I-BODIPY)-]F₇F₇F₁₇-r-CONH₂ (ON): obtained from peptide 26 and compound 10; reaction time: 3 h; pink solid; yield: 57%.

[Tz-C(2I-BODIPY)-]r-CONH₂ (CON): obtained from peptide 27 and compound 10; reaction time: 3 h; pink solid; yield: 57%.

Cell-bassed assays

Cell culture: Most of the studies were performed in HeLa cells (details on PC-3 cells see cell culture in SI). HeLa cells were grown in flat-bottomed culture flasks (75, Sarstedt, Germany) in a fully humidified cell-culture incubator (Galaxy CO-170 S incubator, New Brunswick Scientific, USA) at 37 °C under CO₂ (5% v/v). Growth medium was DMEM supplemented with FBS (10% v/v), penicillin (100 units/mL⁻¹), and streptomycin (100 μg/mL⁻¹). DMEM and FBS were stored at 6 °C, additives and trypsin at >20 °C, solutions were tempered to 37 °C prior usage by a water bath. Cells were grown to confluency and passaged every 2 to 4 days using trypsin-EDTA solution till passage 17. Cells were counted using a Neubauer Improved cell counter (Laboroptik Ltd., Germany) for the determination of seeding densities. All cell-based assays were performed under reduced media conditions (2.5% FBS). A detailed step-wise protocol of the cell-based assays is in the supporting information (Cell Culture).

IC₅₀ determinations: HeLa cells (2 x 10⁶) were seeded into black μclear 96-well microtiter plates (Greiner Bio-One, Item: 655090) in DMEM (200 μL, 2.5% FBS). After 21 h, 110 μL media were removed
from each well, and cells were treated with 10 µL of the conjugates 2-6OFF, 2-6ON and 29-31 in ultrapure H2O with 12.5% MeCN at different concentrations (4.00–0.03 µM as final conjugate concentrations), each concentration in triplicate. Compounds were incubated for 2 h. Then, the plate was aerated 69.4 ± 0.6 Wm−2 for 160 s at 525 nm and further aerated for 18 h. Resazurin fluorescence-based cell viability assay was performed to evaluate cell viability. IC50 values were determined using GraphPad Prism version 6 (GraphPad Software, USA) applying the log(inhibitor) vs. response—variable slope (four parameters) fit. Untreated cells and cells treated only with the vehicle solvent (12.5% MeCN in ultrapure H2O) were considered as negative controls. All conditions were assessed in triplicates per sample/ control. The IC50 derived from, at least, two independent experiments.

Intracellular IEDDA cell viability assays: HeLa cells were seeded as for the IC50 determination experiments. 21 h after seeding, 110 µL media per well were removed, and cells were treated with 10 µL of the conjugates 2-6OFF in ultrapure H2O with 12.5% MeCN at different concentrations (1.00–0.13 µM as final conjugate concentration), each concentration in triplicate. Compounds were incubated for 90 min. Then, the medium was carefully aspirated, and the cells were washed twice with DPBS, and once with media (DMEM, 2.5% FBS). Afterwards, fresh reduced serum culture media (100 µL DMEM, 2.5% FBS) was added to the wells as well as 12.5 equiv (according to the peptide concentration) of TCO. After 30 min of incubation, the plate was aerated 69.4 ± 0.6 Wm−2 for 160 s at 525 nm and incubated for 18 h. Untreated cells, cells treated only with the vehicle solvent (12.5% MeCN in ultrapure H2O) and only TCO were controls. All conditions were assessed in triplicates per sample/control. The results derived from, at least, two independent experiments.

Flow cytometry measurements

HeLa cells (1 × 10^5) were seeded in 3.0 mL DMEM (2.5% FBS) into 6-well plates (Sartetted, Item: 83.3920), and incubated for 18 h. The cells were treated with three different concentrations: 2.00 µM, 0.20 µM and 0.02 µM in 12.5% MeCN in ultrapure H2O of the fluorescent peptides 2-6FL for 2 h. Afterwards they were washed with DPBS (2×1.0 mL), and treated with a trypsin-EDTA solution (500 µL) for 3 min at 37 °C. After that time, DMEM (1000 µL, 2.5% FBS) was added, and the cell suspensions were transferred to microcentrifuge tubes and centrifuged (6000 rpm, 1 min) in a Biofuge pico (Heraeus, Germany), the supernatant was discarded. This was repeated one more time using DPBS. Finally, the cells were suspended in DPBS containing 0.5% FBS for measurement. Fluorescence analysis was performed immediately with a LSR Fortessa cell analyser (BD Biosciences, USA). A minimum of 50000 events per sample were analysed. The experiment was performed in two independent experiments.

Live-cell microscopy measurements

HeLa cells (2.5 × 10^5) were seeded in 8-well on cover glass cell culture chambers (Sarstedt, Item: 94.6190.802) in 500 µL FluoroBrite DMEM (2.5% FBS) and incubated for 18 h. Then, fluorescent conjugates 2-6FL were added from freshly prepared stocks (20 µM in 12.5% MeCN ultrapure H2O) resulting in a final 2 µM concentration and incubated for 2 h. After that, cells were rinsed with DPBS (3×500 µL). Cells were then treated with commercial dyes for labelling cell compartments. For lysosome labelling: cells were treated with 100 nM LysoTracker® Red DND-99 in FluoroBrite media (2.5% FBS, 0.5% DMSO) for 2 h. For mitochondria labelling, cells were treated with 25 nM TMRE in FluoroBrite media (2.5% FBS, 0.5% DMSO) for 30 min. For cytoplasmic membrane labelling, cells were treated with 10 µM Dil in FluoroBrite media (2.5% FBS, 0.5% DMSO) for 15 min. Afterwards, cells were rinsed with DPBS (2×500 µL), then, 500 µL FluoroBrite DMEM with 2.5% FBS was added to each well. Images were acquired on a LSM880 Laser Scanning Confocal Microscope (Zeiss, Germany) with a 63×1.4 oil immersion objective at 37 °C. Z-stack images (1 µm steps) corresponding to images in Figure 4 are depicted in Figures S59–S63.

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Conflict of interest

The authors declare no conflict of interest.

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