Photoactivatable metabolic warheads enable precise and safe ablation of target cells in vivo

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Photoactivatable molecules enable ablation of malignant cells under the control of light, yet current agents can be ineffective at early stages of disease when target cells are similar to healthy surrounding tissues. In this work, we describe a chemical platform based on amino-substituted benzoselenadiazoles to build photoactivatable probes that mimic native metabolites as indicators of disease onset and progression. Through a series of synthetic derivatives, we have identified the key chemical groups in the benzoselenadiazole scaffold responsible for its photodynamic activity, and subsequently designed photosensitive metabolic warheads to target cells associated with various diseases, including bacterial infections and cancer. We demonstrate that versatile benzoselenadiazole metabolites can selectively kill pathogenic cells - but not healthy cells - with high precision after exposure to non-toxic visible light, reducing any potential side effects in vivo. This chemical platform provides powerful tools to exploit cellular metabolic signatures for safer therapeutic and surgical approaches.
Since the early 1990s, photodynamic therapy (PDT) has been clinically used as a therapeutic approach to ablate pathogenic and malignant cells in various diseases, from bacterial infections to cancer. PDT combines light, oxygen, and photosensitizers to produce toxic reactive oxygen species locally, and cause cell death. A broad range of chemical architectures have been reported as effective PDT agents, including organic and inorganic molecules, superoxide radical generators, core-shell metal nanoparticles, metal-organic frameworks, and mesoporous organosilica nanoparticles. Recently, the groups of Gasser, Chao, and Sadler described the use of highly oxidative iridium photocatalysts to induce intracellular redox imbalance and kill cancer cells even in the presence of low concentrations of oxygen. In addition to exhibiting high phototoxic potential, a rare-earth iridium photocatalyst has been found to directly deliver photosensitizers to individual pathogenic and malignant cells under in vivo physiological conditions. Not only does this platform demonstrate how small photosensitizers can be conjugated to antibodies, which favor accumulation in tissues that express specific cell-surface markers. Whereas these approaches can be effective in the presence of abundant biomarkers, they show limitations at early stages of disease when the biochemical and proteomic signatures of target cells are not dissimilar to those of surrounding healthy tissues (e.g., microtumors). Cellular metabolism is a reliable indicator of disease onset and frequently used in the clinic (e.g., metabolomics can assist in the detection and therapy stratification of infection and cancer patients). The metabolic patterns of cells belonging to different taxa (e.g., prokaryotic vs eukaryotic cells) or with altered metabolic patterns of infection and cancer can be achieved with tolerable light doses and minimal side effects in healthy tissues.

Different strategies have been described to fine-tune the structures of photosensitizers and enhance their cell selectivity. Activatable agents exploit some of the biochemical features of tumors (e.g., low pH, glutathione, proteases) to release photosensitizers in cancerous tissues, showing promise in reducing bystander toxicity. Alternatively, photosensitizers can be conjugated to antibodies, which favor accumulation in tissues that express specific cell-surface markers. Whereas these approaches can be effective in the presence of abundant biomarkers, they show limitations at early stages of disease when the biochemical and proteomic signatures of target cells are not dissimilar to those of surrounding healthy tissues (e.g., microtumors). Cellular metabolism is a reliable indicator of disease onset and frequently used in the clinic (e.g., metabolomics can assist in the detection and therapy stratification of infection and cancer patients). The metabolic patterns of cells belonging to different taxa (e.g., prokaryotic vs eukaryotic cells) or with altered metabolic demands (e.g., cancer vs healthy cells, due to the Warburg effect) are unique; however, light-controlled ablation of cells on the basis of differential or altered metabolism has been negated by the lack of photosensitive chemical structures that retain the properties of native metabolites.

Most photosensitizers are derived from bulky fluorescent scaffolds, which impair their active uptake as photosensitive metabolites. Smaller fluorophores, such as pyrroloindolizines, nitrobenzodiazoles, and naphthylamines are more appropriate to retain metabolite function. In this work, we have designed a platform based on amino-substituted benzosenalenazoles as the smallest photosensitizers reported to date and used them to build photodynamic agents that recapitulate the uptake of native metabolites, unlike some commercial photosensitizers. Benzosenalenazole probes can selectively kill cells exhibiting altered metabolism with high spatial resolution and only after exposure to nontoxic light. Importantly, we demonstrate that benzosenalenazoles are compatible with different metabolite structures (e.g., amino acids, saccharides) and can be used to ablate a variety of disease-causing cells, from bacterial pathogens in vitro to glioblastoma microtumors in vivo. This chemical platform demonstrates how small photosensitizers can harness early metabolic signatures to safely eliminate harmful cells in vivo without damaging healthy tissues.

Results
Amino-substituted nitrobenzeneselenadiazoles are small photosensitizers. In order to generate photosensitizers that would not impair the biomolecular properties of native metabolites, we focused on the nitrobenzodiazole core as an example of a small and uncharged chromophoric scaffold. With the exception of nitrobenzeneselenadiazoles (e.g., NBD-CI, Fig. 1a), which are commercially available, we employed the diamine 1 to prepare nitrobenzeneselenadiazoles including different bridging atoms, namely sulfur, selenium, or carbon (Fig. 1a, full synthetic and characterization details in Supplementary Information). Thioxo- and selenoderivatives (3, 4) were prepared by condensation of the diamine 1 with N-sulfinylalanine or SeO₂ respectively, followed by nitration of the position 7 under acidic conditions. The synthetic route for the carbon derivative 5 was modified due to the lability of aminocetals under strong acidic conditions, which would deter the nitration step. Therefore, we treated nitrobenzeneselenadiazole 4F with HF and HCl to remove the selenium atom and performed a Cu-catalyzed condensation with acetone to obtain the corresponding carbon-bridged nitrobenzeneselenadiazoles (5). All nitrobenzeneselenadiazoles were treated with diethylamine and isolated as nitrobenzoselenadiazole 4F with HF and HCl to remove the selenium atom and performed a Cu-catalyzed condensation with acetone to obtain the corresponding carbon-bridged nitrobenzeneselenadiazoles (5). All nitrobenzeneselenadiazoles were treated with diethylamine and isolated as nitrobenzoselenadiazole 4F with HF and HCl to remove the selenium atom and performed a Cu-catalyzed condensation with acetone to obtain the corresponding carbon-bridged nitrobenzeneselenadiazoles (5). All nitrobenzeneselenadiazoles were treated with diethylamine and isolated as nitrobenzoselenadiazole 4F with HF and HCl to remove the selenium atom and performed a Cu-catalyzed condensation with acetone to obtain the corresponding carbon-bridged nitrobenzeneselenadiazoles (5).
improve their phototoxic potential. We employed the commercially available mono-iodinated diamine 10 to synthesize 9-diethylamino-6-iodo-7-nitrobenzoselenadiazole (11a) as described above (Fig. 1c; full synthetic details in Supplementary Information) and compared its behavior to the parent non-iodinated compound (4a). Both compounds 4a and 11a presented similar excitation wavelengths around 500 nm, with the latter exhibiting dark cytotoxicity and the non-iodinated derivative showing higher ratios of phototoxicity, making it more suitable for the preparation of photosensitive metabolites. Finally, we examined the mechanism by which amino-substituted nitrobenzoselenadiazoles induce oxidative death and confirmed that, upon light irradiation, they predominantly produce singlet oxygen over radical anions (approximately in a 75:25 ratio, Supplementary Fig. 2). Amino-substituted nitrobenzoselenadiazoles are therefore one of the smallest photosensitizer structures reported to date.

**Fig. 1 Synthetic routes for the preparation of small photosensitizers.** a Condensation of 1 with PhNSO or SeO2 followed by nitration led to compounds 3 and 4F, respectively. Reduction of 4F followed by Cu-catalyzed addition of acetone rendered compound 5. Nucleophilic substitutions of 2–5 with diethylanilinium yielded 2a-5a. b Human U87 cells were incubated with 2a-5a (100 μM) in Krebs-Ringer buffer and illuminated (red bars) or not (gray bars) with a ThorLabs M530L3 LED (10 mW, 37 J cm⁻², red). Cell viability was assessed 16 h post illumination using a TACSR MTT cell proliferation assay with values normalized to the viability of untreated cells. Data presented as mean values ± SEM (n = 3 independent experiments). c Nucleophilic substitution of 4F with piperidine, 4-methoxyphenol, and 4-methoxythiophenol led to compounds 6, 7, and 8, respectively. Suzuki coupling of 4Cl with methoxybenzene-4-boronic acid rendered compound 9. Condensation of compound 10 with SeO2 followed by nitration and nucleophilic substitution resulted in compound 11a. d Maximum excitation wavelengths and phototoxicity of compounds 2a–11a and 4F in human cells as determined in (b). Values indicate the decrease in viability between irradiated and non-irradiated cells after incubation with compounds 2a–11a (100 μM). P values were obtained from two-tailed unpaired t tests. Source data are available.

Benzosenadiazole-conjugated amino acids retain specific recognition of bacterial cells. In view of the suitable properties of benzosenadiazoles as small and uncharged photosensitizers, we examined their impact on the molecular recognition properties of metabolites, such as amino acids. VanNieuwenhze, Brun and co-workers have demonstrated that derivatives of d-amino acids (e.g., d-alanine) but not L-amino acids can be recognized by the metabolic cell wall machinery of bacterial cells and incorporated into
their peptidoglycan cell walls (Fig. 2a)\textsuperscript{40, 41}. Therefore, we prepared benzoselenadiazole derivatives of D- and L-alanine to examine whether the stereospecific incorporation of the D-enantiomer would be retained after conjugation to the photosensitizer. Two amino acids (12, the L-alanine surrogate and 13, the D-alanine surrogate, Fig. 2b) were synthesized by nucleophilic substitution of the fluorinated benzoselenadiazole 4F with the two enantiomers of 2,3-diaminopropionic acid (Dapa) (Fig. 2b, full synthetic details in Supplementary Information). Both amino acids exhibited similar optical properties and singlet oxygen quantum yields, indicating comparable phototoxicity potential (Supplementary Table 1).

During the synthesis of peptidoglycans in the bacterial cell wall, the nascent peptide chains are specifically capped with D-alanines, which can be selectively exchanged by exogenously provided unnatural D-amino acids (Fig. 2a)\textsuperscript{42}. To monitor the effect of the amino acids in bacterial cells, we grew Gram-negative \textit{Escherichia coli} in the presence of compounds 12 or 13 and imaged the cells shortly after by fluorescence microscopy. We observed that \textit{E. coli}
cells incubated with the d-amino acid 13-but not the l-amino acid 12—showed uniform labeling of the bacterial septa, indicating that the benzoselenadiazole scaffold retained the selectivity of d-alanine (Fig. 2c). We confirmed by mass spectrometry that the d-amino acid 13 was covalently incorporated into the peptidoglycan cell wall (Supplementary Fig. 3) and that the incorporation was independent of the bacterial strain, as shown in experiments with Gram-positive bacteria, such as the model species Bacillus subtilis and the pathogen Staphylococcus aureus (Supplementary Fig. 3). We also examined whether compound 13 could kill E. coli after light irradiation. E. coli bacterial colonies were treated with compounds 12 (as a negative control) or 13 and kept in the dark or irradiated with light, followed by proliferation measurements under both conditions. As shown in Fig. 2c, high levels of cell death were observed in 13-treated and light-irradiated E. coli cells (<10% cell viability) whereas minimal toxicity was detected in non-irradiated cells or cells that had been incubated with the control compound 12. Finally, we performed time-lapse imaging of E. coli cocultures that had been separately pre-labeled with compound 13 or NADA (Fig. 2b). NADA is a reported nitrobenzoxadiazole d-alanine analog lacking the selenium atom and able to fluorescently stain bacterial cell walls43. The amino acids NADA and 13 fluorescently labeled E. coli cells (λem (NADA): 530 nm, λem (13): 590 nm), and their growth was monitored for 30 min while irradiating the cells at regular intervals. Remarkably, most NADA-labeled cells showed clear signs of continued growth and proliferation over time, whereas cells that had been pretreated with compound 13 appeared as lysed or showed arrested cell growth (Fig. 2d). These results support that the local production of singlet oxygen after the irradiation of peptidoglycan-incorporated benzoselenadiazoles can selectively target and kill bacterial cells with high spatial resolution and without affecting neighboring cells in the close vicinity. Given that compound 13 can be effectively incorporated into Gram-negative and Gram-positive bacteria and cause cell death upon light irradiation, these molecules could open avenues to develop antibiotic regimes overcoming complications associated to antimicrobial resistance in pathogenic bacteria.

A 2-deoxyglucose benzoselenadiazole photosensitizer enables light-controlled ablation of human glioblastoma cells in vitro and in 3D spheroids. Glioblastomas are the most common and one of the most aggressive types of brain cancer, where surgical treatments require highly precise and safe strategies to remove malignant cells without affecting healthy tissues. Brain tumor cells are very glycolytic and show increased levels of glucose consumption when compared to healthy tissue44, hence we decided to assess whether glucose-derivatized benzoselenadiazoles could kill human glioblastoma cells after exposure to nontoxic light.

Two d-glucose analogs (compounds 14 and 15, Fig. 3a) were synthesized by condensation of the benzoselenadiazole scaffold 4F with two 2-d-deoxyglucose analogs. Both compounds were isolated as the mixture of α- and β- anomers of d-glucose (characterization data in Supplementary Information). Compound 15 showed better water solubility and photoactivity than compound 14, thus we assessed whether it could be actively taken up by GLUT1/3 transporters in glioblastoma cells, the most abundant glucose transporters in human U87 cells45. As controls, we also prepared d-glucoseamine derivatives of previously reported photosensitizers, such as Nile Blue reported by Fowley and co-workers46 and tetraphenylethylene (TPE) reported by the Bin Liu et al. for metabolic PDT47 (compounds 16 and 17, respectively, Fig. 3b). First, we compared the uptake for all d-glucose derivatives in U87 cells. As shown in Fig. 3c, compound 15—but not Nile Blue (16) or TPE (17) derivatives—exhibited reduced uptake in cells that had been pre-incubated with the GLUT inhibitor cytochalasin B, suggesting that compound 15—unlike other photosensitizers—can enter glioblastoma cancer cells through active uptake via GLUT transporters. We also analyzed whether the phototoxicity of compound 15 in U87 cells was dependent on GLUT-mediated transport and observed that its activity was significantly blocked with cytochalasin B (Fig. 3c). Furthermore, compound 15 showed the highest singlet oxygen generation quantum yield among all aminobenzoxadiazoles (Fig. 3d), with values around 25%—comparable to TPE—and produced effective amounts of singlet oxygen by single-photon (520 nm) in vitro and in cells as well as by two-photon (970 nm) excitation (Supplementary Fig. 4). Given the technical complexity of two-photon in vivo experiments and the limited availability of multi-photon illumination in clinical settings, we decided to perform our biological characterization using single-photon excitation.

We cultured human U87 glioblastoma cells and incubated them with compound 15 to directly image the viability of the cells in the dark as well as after light irradiation. Because photosensitizers producing singlet oxygen induce oxidative cell death via apoptosis48 we used the apoptotic marker Annexin V as an indicator of the toxicity caused by compound 15. Confocal fluorescence microscopy confirmed that U87 glioblastoma cells that had taken up compound 15 (as shown by intracellular red staining, Fig. 4a) were apoptotic (blue cell membrane staining by Annexin V-Pacific Blue, Fig. 4a) only after light irradiation but not in the dark. On the other hand, single treatments (i.e., compound 15 only or light irradiation only) did not cause cell death as confirmed by fluorescence microscopy and quantitative flow cytometry (Fig. 4b). We also confirmed that compound 15 would compete with unlabeled d-glucose, which drastically reduced the uptake and phototoxicity of compound 15 (Fig. 4a). Furthermore, as a control we used 2-NBDG, the non-photosensitive nitrobenzoxadiazole glucose analog (Fig. 3a) and confirmed that the selenium atom in the benzoselenadiazole core is essential to cause light-induced oxidative death (Fig. 4b). Finally, we examined the toxicity of compound 15 in 3D cultures from U87-nlsCrimson glioblastoma cells, which express the fluorescent protein E2Crimson in their nuclei as a cell viability marker (λexc/em: 610/645 nm), U87-nlsCrimson cells were cultured in ultra-low adherence plates at 37°C, forming spheroids of around 250 μm in diameter after 3 days. We compared the size of the spheroids in the absence and presence of compound 15 as well as before and after irradiation (Fig. 4c, d). Compound 15-treated and irradiated spheroids showed a significant decrease in the size of the cell viable core when compared to any of the other incubation conditions, indicating that compound 15 can effectively kill cells in multiple layers of 3D glioblastoma spheroids.

Compound 15 shows high potency and selectivity for glioblastoma cells in cocultures of human brain cells. In view of the potential of compound 15 to act as a PDT agent for glioblastoma, we examined its ability to selectively kill glioblastoma cells when they were cultured in the presence of non-cancerous brain cells. We cocultured the glioblastoma cell line U87-nlsCrimson with human HCN-2 cells as representative non-cancerous neuronal precursor cells49. HCN-2 cells were pre-stained with CellMask Green and cocultured with U87-nlsCrimson cells (Fig. 5a). Under normal coculture conditions, both HCN-2 and U87 cells were viable, as indicated by fluorescence emission of their respective markers (i.e., CellMask Green for HCN-2, E2Crimson for U87, Fig. 5b). Cocultures were then incubated with compound 15, irradiated with visible light at 37°C/cm², and monitored by fluorescence confocal microscopy. Notably, the combination...
treatment of compound 15 and light-induced cell death in most U87 cells—as shown by the lack of fluorescence in the E2Crimson channel—but not in HCN-2 cells, which are less glycolytic and metabolically-active (Fig. 5b). Plot profile analysis confirmed that HCN-2 cells remained intact after the treatment whereas U87 cells were devoid of E2Crimson fluorescence (Fig. 5c). Furthermore, we confirmed that cocultures which had not been treated with compound 15 or not exposed to light showed no signs of death in any cell type (Supplementary Fig. 6). Finally, we analyzed the solubility and phototoxicity profiles of compound 15 and 5-aminolevulinic acid (5-ALA) (Fig. 5d), the current gold standard in image-guided surgery for brain tumor resection. 5-ALA stimulates the production of protoporphyrin IX in tumor cells and is currently evaluated in clinical trials for postoperative and interstitial PDT. Both compound 15 and 5-ALA showed full water solubility in the high micromolar range, and we did not detect the formation of any aggregates with diameter size >10 nm. We also compared the in vitro toxicity of different concentrations of compound 15 and 5-ALA in U87 glioblastoma cells and observed that compound 15 was able to induce significant levels of cell death at similar doses as 5-ALA and with complete lack of toxicity in HCN-2 cells (Fig. 5e). Taken together, these results highlight the biocompatibility and potential utility of compound 15 for light-controlled ablation of cancer cells with comparable phototoxicity to PDT agents under clinical evaluation.

Compound 15 enables safe removal of microtumors in vivo. Encouraged by the selective photoreactivity of compound 15 in metabolically-active cancer cells, we evaluated whether it could be used to eliminate small tumors derived from human glioblastoma cells in vivo. We used a zebrafish model where low numbers of glioblastoma cells (around 50 ± 10 cells per organism) were injected into the yolk sac of 2-day-old embryo and allowed to form microtumors. In order to quantify the viability of tumor cells by fluorescence confocal microscopy, we used the above described U87-nlsCrimson cells since they express a live-cell fluorescent reporter that can be detected in the optically-transparent zebrafish. As expected, the injection of U87-nlsCrimson cells into zebrafish...
embryo at 48 hpf (hours post-fertilization) led to the formation of fluorescent tumor mass of several microns in diameter (Fig. 6a). Tumor-bearing zebrafish were then treated with compound 15 (microinjection of 6 pg into the yolk sac) and illuminated with visible light at a dose of 37 J cm$^{-2}$. Confocal microscopy images were taken from each group, and we quantified the number of remaining fluorescent U87 cells in each larva (Fig. 6a, b). The sequential treatment of compound 15 followed by light irradiation led to a dramatic reduction of the size of the microtumors, with some zebrafish being entirely free of glioblastoma cells after the treatment (Fig. 6b). The injection of compound 15 or the light irradiation of zebrafish on their own did not cause any significant reduction in the total number of cancer cells (Fig. 6b). Finally, we examined whether the in vivo treatment with compound 15 caused side effects in zebrafish. First, we compared the morphology of treated and untreated zebrafish, with no evident abnormalities between the two groups (Supplementary Fig. 7). Next, we examined their behavior and vital signs after the PDT treatment. Larvae from control and treated groups showed similar swimming behavior, as shown by the tracks of individual larva ($n \geq 10$ per group, Fig. 6c) with no significant differences in distance covered or velocity (Fig. 6d and Supplementary Movies 1, 2). Heartbeat monitoring in both groups also revealed that treatment with compound 15 and light was safe and did not cause any evident deleterious side effects (Supplementary Movies 3 and 4). In summary, our data indicate that photosensitive metabolites represent a viable chemical strategy to harness the aberrant early metabolic features of aggressive cancer cells and to open avenues for minimally-invasive therapeutic strategies to safely eliminate microtumors.

In summary, this study demonstrates the utility of amino-substituted benzoselenadiazoles as small photosensitizers to kill metabolically-active cells in vitro and in vivo under full chemical and light control. Unlike in other current photosensitizers, the reduced size of benzoselenadiazoles retains key recognition properties of native metabolites and enables cell-selective uptake via various routes (e.g., peptidoglycan incorporation in bacteria, glucose transport in glioblastoma cells). This chemical approach opens therapeutic avenues for the selective ablation of cells that do not present discriminatory cell-surface markers, such as malignant cancer cells at early stages of disease, or that may become resistant to existing treatments, such as pathogenic bacteria. We also show that the dosing regime and light exposure
required for photosensitive metabolites meet high safety standards and are compatible with current procedures used in clinical PDT. The possibility to ablate cells with high precision and purely because of differential or altered metabolism will enable mechanistic studies to identify unknown subsets of disease-causing cells and to develop safer therapeutic approaches with enhanced spatiotemporal resolution.

Discussion

The possibility to directly harness aberrant metabolism represents a strategy to improve the selectivity of PDT agents\(^5\). Photosensitizers that can enter target cells via endogenous metabolic transporters, especially those that are expressed differentially between target cells and their surrounding tissue, would increase selective cell ablation and minimize potential side effects. However, among the many chemical structures reported to date, very few photosensitizers can utilize endogenous metabolic transporters. Herein we describe benzoselenadiazoles as a generic photosensitizer scaffold to produce phototoxic metabolites. The synthesis of aminobenzoselenadiazoles from small metabolites is straightforward, with high-yielding and scalable reactions (Fig. 1). Given that many metabolites contain reactive amino groups (e.g., amino acids, aminoglycans, peptides), this chemical strategy is translatable to a broad range of biological transporters and cell types.

The lack of charged chemical groups and the small size of benzoselenadiazoles are essential to retain recognition and uptake through cell-surface transporters. Aminobenzoselenadiazoles mimic the stereospecificity of chiral amino acids (as shown in L-Ala vs D-Ala derivatives, Fig. 2) and replicate the uptake of natural biomolecules, as demonstrated with the glucose analog 15 in GLUT transporters (Fig. 3). Of note, GLUT-mediated transport was not observed for other glucose-based photosensitizers, such as Nile Blue or TPE. We have demonstrated that the active uptake of aminobenzoselenadiazoles is essential to 1) minimize off-target toxicity in healthy cells, as shown in human cocultures and in vivo (Figs. 5 and 6), and 2) accumulate concentrations of photosensitizer inside target cells that can generate cytotoxic levels of singlet oxygen under clinical PDT exposure (Figs. 4, 5, and 6). Furthermore, given that aminobenzoselenadiazoles can be excited by single and two-photon
sources (Fig. 3 and Supplementary Fig. 4), near-infrared multi-photon endoscopes—increasingly used for in vivo applications—could be considered as excitation sources. Recent in human studies combining local administration of benzodiazepines with endoscope-based imaging suggest that this approach might be translatable to the clinic.

Importantly, aminobenzoselenadiazoles show PDT efficacy at a power of 37 J cm\(^{-2}\), which is compatible with clinical use. Under these conditions, we have demonstrated that benzodiazepine 9-amino acids can selectively kill bacterial cells upon irradiation. In mammalian cells, we observed similar cytotoxicity between compound 15 and 5-ALA, the only FDA-approved photosensitizer able to enter cancer cells through endogenous transporters (i.e., PepT1 and PAT1 transporters). Unlike 5-ALA, compound 15 enters cancer cells within minutes and does not need to be metabolized to be phototoxic (5-ALA is converted to PPIX after several hours). This feature allows local administration of the photosensitizer to minimize systemic side effects, something that cannot be done with 5-ALA. In summary, this chemical platform offers a safe and rapid therapeutic avenue for the metabolic ablation of pathogenic cells, such as early-stage cancers and drug-resistant bacteria, which prove difficult to target with existing PDT approaches.

**Methods**

**Density Functional Theory (DFT) calculations.** B3LYP, PBE0, M06-2X, and wB97XD functionals along with 6–31 G\(^*\) and def2tzvp basis sets were tested for the closest match between the experimental absorption maxima in ethanol and the energy calculated for the S\(^0\) → S\(^1\) transitions of compounds 2a–5a. TD-B3LYP/def2tzvp achieved the closest match and was used for all subsequent computations.

**Bacterial cell growth.** E. coli, B. subtilis, and S. aureus cells were grown to exponential phase in LB medium at 37 °C. Cells were incubated with compounds 12, 13, or NADA at the indicated concentrations. Saccell from labeled cells were isolated by adding cell suspensions to boiling sodium dodecyl sulfate (SDS, 5% w/v) and incubation for 30 min. The SDS-insoluble material was collected by ultracentrifugation at 39,000 x g for 10 min at 30 °C, resuspended in water and boiled again in SDS (4% w/v) with stirring for 30 min. Samples were then washed, pelleted, and resuspended in the minimal amount of water. Growth curves were measured via absorbance at 600 nm over 18 h post-incubation with probes and/or illumination. Time-lapse microscopy experiments were performed with pre-labeled E. coli cells spotted on LB-agarose pads with images acquired every 1 min using a Nikon Ti2 Eclipse inverted microscope equipped with a Lumenor SPECTRA light engine and consecutively irradiating cells under optimized conditions for NADA (λ\(_{exc/em}\): 470/530 nm, 196 mW) and compound 13 (λ\(_{exc/em}\): 508/590 nm, 62 mW).

**Cell culture.** U87 cells (ATCC), U87-nlsCrimson cells (provided by Dr Dirk Sieger (University of Edinburgh)), and HCN-2 cells (ATCC) were grown in DMEM medium supplemented with 10–20% FBS, antibiotics (100 U mL\(^{-1}\) penicillin, 100 mg mL\(^{-1}\) streptomycin), and 2 mM l-glutamine at 37 °C in a humidified atmosphere with 5% CO\(_2\). Cells were passaged in T-75 cell culture flasks using trypsin-EDTA.

**Photoviability experiments.** U87 cells were incubated in Krebs–Ringer bicarbonate buffer (KRB) for 2 h, incubated with or without compounds (2a–17) at the indicated concentrations and illuminated or not with either a ThorLabs M530L3/ M625L4 LED or a white light source (10 mW, 37 J cm\(^{-2}\)). Medium was replaced with fresh DMEM, and cell viability was measured after 24 h using an MTT Cell...
Proliferation assay following the manufacturer’s instructions. Values were normalized to the viability of untreated cells and analyzed by GraphPad Prism.

**Determination of singlet oxygen quantum yields.** Solutions of DPBF (300 µM) and compounds 2a–16 (50 µM, unless indicated otherwise) in ETOH were illuminated with visible light (0.3 mW) for 0.5–4 min, followed by absorbance measurements at 410 nm using a Spectro flow photometer. Rates of singlet oxygen production (k) were calculated by plotting change in absorption of DPBF at 410 nm against time using the equation below:

\[
k = \frac{\ln(DPBF_{abs})}{\ln(DPBF_{abs})} - \frac{\ln(PS_{abs})}{\ln(PS_{abs})}
\]

Relative singlet oxygen quantum yields were determined using the equation below with Rose Bengal as the reference. k: rates of singlet oxygen production, Φ_S: singlet oxygen quantum yields.

**Flow cytometry.** U87 cells (300,000 cells/well) were incubated in glucose-free KRB buffer including or not compound 15 (100 µM) for 1 h. Cells were illuminated or not with visible light (10 mW). Following incubation in fresh DMEM for 16 h, cells were detached, resuspended in KRB buffer, and analyzed by flow cytometry using Annexin V-Pacific BlueTM (4 µg/mL, 405/450 nm). Flow cytometry data were analyzed using FlowJo.

**Live-cell fluorescence confocal microscopy.** U87 cells were plated in glass chamber slides (Nunc™ Lab-Tek™ II) in supplemented DMEM medium (20,000 cells/well). After 4 h, cells were incubated with or without compounds 15 or 2-NBDG (100 µM) in KRB buffer for 1 h and illuminated or not with visible light (10 mW, 37 °C). Prior to imaging experiments, cells were incubated with Annexin V-Pacific BlueTM (4 µg/mL) and imaged in a Leica SP8 confocal microscope (λ_ex/em: 405/450 nm for Annexin V, λ_ex/em: 488/530 nm for 2-NBDG, and λ_ex/em: 488/590 nm for compound 15). All images were processed and analyzed using ImageJ.

**Glioblastoma spheroids.** U87-nlsCrimson cells were plated in Corning ultra-low attachment 96-well plates (500 cells/well) in supplemented DMEM medium and incubated for 72 h at 37 °C in a humidified atmosphere with 5% CO2. Spheroids were incubated with or without compound 15 (100 µM) for 1 h and illuminated or not with visible light (10 mW, 37 °C) before being washed and imaged on a widefield fluorescence EVOS microscope (Olympus) (λ_ex/em: 610/645 nm). The spheroids diameter was measured after 48 h incubation at 37 °C and size decrease measurements were normalized to untreated spheroids. All images were processed and analyzed using ImageJ.

**Human brain cell cocultures.** HCN2 cells (15,000 cells/well) were plated in 6-well glass chamber slides (Nunc™ Lab-Tek™ II) and incubated with CellMask Green for 30 min. U87-nlsCrimson cells (10,000 cells/well) were then added to the wells and co-incubated for 4 h at 37 °C in a humidified atmosphere with 5% CO2. Cocultures in glucose-free KRB buffer were incubated with or without compound 15 for 1 h and illuminated or not with visible light, followed by imaging in Leica SP8 confocal microscope (λ_ex/cm: 488/520 nm for CellMask Green and λ_ex/cm: 561/645 nm for E2Crimson). All images were processed and analyzed using ImageJ.

**In vivo zebrafish experiments.** Experiments were carried out in accordance with Committee-approved ethical procedures. 48 hpf larvae were anesthetized using MS222 and mounted in 3% methylcellulose. U87-nlsCrimson (~50 cells/embryo) and injected with compound 15 (6 pg/embryo) and illuminated or not with visible light (150 µM) for 1 h and illuminated or not with visible light, followed by imaging in Leica SP8 confocal microscope (λ_ex/cm: 488/520 nm for CellMask Green and λ_ex/cm: 561/645 nm). All images were processed and analyzed using ImageJ.

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Author contributions
S.B., F.d.M., and A.F performed chemical synthesis and characterization of the compounds. J-.S.L. carried out DFT calculations. E.K., N.L.A., and J-.S.L. designed and performed chemical synthesis and characterization of the compounds. Y.F. performed and analyzed the experiments. J-.S.L. performed the data analysis and wrote the manuscript. M.V. supervised the overall project.

Competing interests
The University of Edinburgh has submitted the patent application “Small molecule photosensitizers for photodynamic therapy” (ID: WO2020/187913) covering some of the work described in this manuscript, where S.B., F.d.M., and A.F. are named inventors. The remaining authors declare no competing interests.

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