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Mechanism of cell death mediated by a BF₂-chelated tetraaryl-azadipyrrromethene photodynamic therapeutic: dissection of the apoptotic pathway *in vitro* and *in vivo*

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Abstract

Photodynamic therapy (PDT) is an established treatment modality for cancer. ADPM06 is an emerging non-porphyrin PDT agent which has been specifically designed for therapeutic application. Recently, we have demonstrated that ADPM06-PDT is well tolerated *in vivo* and elicits impressive complete response rates in various models of cancer when a short drug-light interval is applied. Herein, the mechanism of action of ADPM06-PDT *in vitro* and *in vivo* is outlined. Using a drug and light combination that reduces the clonogenicity of MDA-MB-231 cells by >90%, we detected a well-orchestrated apoptotic response accompanied by the activation of various caspases *in vitro*. The generation of reactive oxygen species (ROS) upon photosensitizer irradiation was found to be the key instigator in the observed apoptotic response, with the endoplasmic reticulum (ER) found to be the intracellular site of initial PDT damage, as determined by induction of a rapid ER stress response post-PDT. PDT-induced apoptosis was also found to be independent of p53 tumor suppressor status. A robust therapeutic response *in vivo* was demonstrated, with a substantial reduction in tumor proliferation observed, as well as a rapid induction of apoptosis and initiation of ER stress, mirroring numerous aspects of the mechanism of action of ADPM06 *in vitro*. Finally, using a combination of ¹⁸F-labeled 3'-deoxy-3'-fluorothymidine (¹⁸F-FLT) nuclear and optical imaging, a considerable decrease in tumor proliferation over 24-hr in two models of human cancer was observed. Taken together, this data clearly establishes ADPM06 as an exciting novel PDT agent with significant potential for further translational development.

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Additional Supporting Information may be found in the online version of this article.

Keywords

BF₂-chelated tetraaryl-azadipyromethene; photodynamic therapy; endoplasmic reticulum stress; optical imaging; ¹⁸F-FLT nuclear imaging

PDT is an established treatment modality for cancer, which utilizes a photosensitive compound accompanied by low-energy light of a specific wavelength. The combination of these two independently nontoxic components produces extensive cellular, vascular and tissue damage in an oxygen-dependent manner, *via* reactive oxygen species (ROS) generation, resulting in tumor ablation.¹

Following the first report of PDT-induced apoptosis, two decades ago,² the complex pathways leading to cell death resulting from PDT using an array of photosensitizers has been extensively studied.^{3–5} The cellular response to PDT is highly complex and differs dramatically depending on a number of factors such as cell genotype,^{6,7} the photosensitizer used, the dose and light fluence applied^{8–10} and the intracellular localization of the photosensitizer.^{11,12} To enable further translational development of photosensitizers as therapeutic agents, it is of paramount importance to comprehend the complex biochemical and biological network of events that lead to PDT-induced cell death. In addition, understanding the mechanism of action of PDT agents offers significant potential for optimization of current PDT protocols by means of combination therapies with existing clinically approved therapeutics.

Previously, we have reported the discovery of a new class of modifiable non-porphyrin-based PDT agents, namely, the BF₂-chelated tetra-arylazadipyromethenes (ADPMs). We published data revealing the photophysical characteristics of the ADPM family,¹³ as well as fundamental *in vitro* biological characteristics, such as cytotoxicity and activity in hypoxia, of the lead therapeutic, ADPM06.¹⁴ Recently, we have reported *in vivo* efficacy of ADPM06 using a short drug-light interval, as well as biodistribution studies and elucidation of *in vivo* mechanism of action using a multimodality imaging approach.¹⁵ Importantly, we have found both ¹⁸F-fluorodeoxyglucose (FDG) nuclear imaging and magnetic resonance imaging to be useful biomarkers of therapeutic response post-ADPM06-PDT. Following these studies examining *in vivo* characteristics of this novel photosensitizer, we have now undertaken a comprehensive investigation of the mechanism of action of ADPM06-PDT *in vitro*. In addition, we have used a combination of optical imaging, immunohistochemistry (IHC) and Western blot analysis to examine early tumor response following ADPM06-PDT and to determine early markers of tumor cell death. Finally, similar to our previous publication reporting the use of ¹⁸F-FDG nuclear imaging to monitor early response to PDT, here, we have again utilized a nuclear imaging approach, this time using the proliferation tracer ¹⁸F-labeled 3'-deoxy-3'-fluorothymidine (¹⁸F-FLT) in two preclinical models of human cancer to further assess the mechanism of action of ADPM06-PDT *in vivo*.

Experimental Procedures

Photosensitizer formulation

The synthesis and formulation of ADPM06 in a mixture of Cremophor EL (CrEL)/1,2-propanediol for *in vitro* studies has been described previously.¹³ The quantity of CrEL/1,2-propanediol in an assayed photosensitizer solution was always less than 0.03%. For *in vivo* studies, ADPM06 was formulated in a CrEL solution without 1,2-propanediol.

Cells

MDA-MB-231 tumor cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich, Steinheim, Germany) supplemented with 10% fetal calf serum (FCS), 50 units/mL penicillin, 50 µg/mL streptomycin and 1% L-glutamine. Phoenix-Ampho cells were provided by Prof. Garry P. Nolan, Stanford University, California, and were maintained in DMEM as above. HCT116 p53+/+ and HCT116 p53-/- cells were both generous gifts from Prof. Bert Vogelstein, Johns Hopkins Medical Institutions, Baltimore, MD, and were maintained in McCoy's 5A medium (Sigma-Aldrich) containing all the supplements listed above. U87-TGL glioma cells that are transduced with a triple fusion reporter gene (TGL) have been described previously.¹⁶ MDA-MB-231 luciferase expressing cells (MDA-MB-231-luc-D3H1) were obtained from Caliper Life Sciences (Caliper, Alameda, CA) and maintained in minimum essential medium (MEM) supplemented with 10% FCS, 1% non-essential amino acids, 50 units/mL penicillin, 50 µg/mL streptomycin, 1% sodium pyruvate and 1% L-glutamine. Cells were maintained in 5% CO₂, 21% O₂ at 37 C. Cell lines were routinely tested for mycoplasma contamination.

MTT cell viability assay

Cellular viability was assessed using the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay. Media containing ADPM06 (1×10^{-5} – 1×10^{-9} M) were administered to cells and allowed to uptake for 3-hr, the time interval required to obtain maximal intracellular uptake of ADPM06 *in vitro*. Media containing ADPM06 was removed and cells washed in PBS before irradiation with 16 J/cm² light (fluence rate 92.8 mW/cm²) using a Waldmann PDT 1200 L light system (Waldmann Medizintechnik, Villingen-Schwenningen, Germany) with a bandwidth of 600–750 nm. MTT solution (5 mg/mL) (Sigma-Aldrich) was added 48-hr following PDT with absorbance of the DMSO-solubilized formazan crystals measured at 570 nm using a Wallac 1420 Multilabel HTS plate reader (Wallac, Gaithersburg, MD).

Clonogenic assay

MDA-MB-231 cells were treated with either 15 or 150 nM ADPM06 for 3-hr before irradiation with 8 or 16 J/cm² light. Directly following PDT, cells were seeded in triplicate in 6-well plates at densities of 1,000 and 200 cells/well for MDA-MB-231 and HCT116 cells, respectively. Cells were allowed to form colonies for approximately 3-weeks for MDA-MB-231 cells, 10-days for HCT116 cells. Cells were fixed in 10% neutral-buffered formalin (Sigma-Aldrich) for 15-min at room temperature. Fixing agent was removed, and

plates were allowed to air dry, before cells were stained with a 0.25% solution of crystal violet.

Apoptosis assay

Cell death was assessed post-PDT using the Vybrant Apoptosis Assay Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Treated and control samples were analyzed using Summit software version 4.2 on a CyanADP flow cytometer (DakoCytomation, Cambridge, UK). All flow cytometry was carried out in the Flow Cytometry Core Facility at UCD Conway Institute.

Western blot analysis

For Western blot analysis of PDT treated cells, cells were lysed following addition of radioimmunoprecipitation assay (RIPA) buffer. Cells were then sonicated at 15 microns for 20 s using a Soniprep 150 (MSE, London, UK). Protein was extracted from snap-frozen tumor samples using a Micro-Dismembrator (Braun Biotech, Aylesbury, UK). Powdered tissue was resuspended in RIPA buffer and sonicated as described above. Protein levels were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Protein samples were separated by SDS-PAGE under reducing conditions. Resolved proteins were transferred to methanol-activated polyvinylidenedifluoride (PVDF) membrane (Millipore, Bedford, MA) in transfer buffer. Membranes were blocked in 5% nonfat milk or 5% bovine serum albumin (BSA) for 1-hr at room temperature. Primary antibody was applied to membranes overnight at 4°C (anti-cleaved caspase 3, 7, 8, 9 and poly ADP-ribose polymerase (PARP), anti-BiP (1:1000) (Cell Signaling Technology, Danvers, MA); antiphospho eIF2 α (1:1000) (Millipore)). Membranes were washed in Tris Buffered Saline containing 0.1% Tween 20 (TBS-T), incubated for 1-hr with horseradish peroxidase (HRP)-conjugated secondary antibody, washed again in TBS-T before HRP detection using enhanced chemiluminescence (ECL) Western blotting substrate (Pierce). Chemiluminescent signal was detected by autoradiography using X-ray film. Membranes were stripped and reprobed with anti- β -actin (1:1000) or GAPDH (1:1000) (Santa-Cruz Biotechnology, Santa Cruz, CA) as a loading control.

Reverse transcription and X-box protein 1 (XBP1) detection

Total RNA was extracted from control and PDT treated MDA-MB-231 cells using the TRI Reagent method. For reverse transcription PCR, RNA samples were first treated with DNase I. cDNA synthesis was then performed using Superscript II reverse transcriptase (Invitrogen). PCR was performed using Taq DNA polymerase (New England Biolabs, Ipswich, MA) with a pair of primers corresponding to human XBP1, both unspliced (442 bp) and spliced (416 bp) transcripts: 5'-CCTTGTAAGTTGAGAACCAGG-3' (XBP1_forward), 5'-GGGGCTTGGTATATATGTGG-3' (XBP1_reverse). XBP1 mRNA levels was normalized to 18S ribosomal RNA (rRNA) levels.

Detection of intracellular ROS

Intracellular ROS were detected using the non-fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCDHF) (Sigma-Aldrich). Cells were incubated with

10 μ M DCDHF 20-min before irradiation for samples analyzed immediately postirradiation or 20-min before flow cytometric analysis for all other treated samples. Cells were trypsinized, centrifuged and resuspended in PBS before flow cytometric analysis of intracellular fluorescent DCF.

Retroviral transduction

A stable MDA-MB-231 cell lines capable of triple fusion reporter expression was generated *via* retroviral transduction. The triple fusion reporter (SFG-nTGL) consisting of green fluorescent protein (GFP), luciferase and herpes simplex virus 1 thymidine kinase (HSV1-tk) has been described previously.¹⁶ Briefly, viral particles were produced by transient calcium phosphate transfection of Phoenix-Ampho cells with the SFG-nTGL vector. Transfected cells were incubated for 48-hr after which medium containing the retroviral particles was removed and passed through a 0.45-micron filter. Viral supernatant was placed directly on subconfluent MDA-MB-231 cells and centrifuged at 1,800 rpm for 45-min. Cells were incubated for 24-hr before washing with PBS and refreshing the media. Transduced cells were analyzed via fluorescent-activated cell sorting (FACS) using a BD FACSAria Cell Sorter to collect the positively transduced cells (MDA-MB-231-TGL) according to their GFP content.

Animal models

All animal experiments were licensed by the Department of Health and Children, Ireland, and specific protocols reviewed by the Animal Research Ethics Committee at UCD. Female Balb C nu/nu mice were received to the SPF-grade Conway Institute Biotechnical Services Xenograft Facility at 4–6 weeks (Harlan, UK). Mice were anesthetized using isoflurane and 5×10^6 MDA-MB-231-TGL, U87-TGL or 2×10^6 MDA-MB-231-luc-D3H1 cells (Caliper) were injected subcutaneously in 100- μ L PBS/Matrigel (50:50) into the right forelimb. For ^{18}F -FLT PET studies, MDA-MB-231-TGL or U87-TGL tumor cells were inoculated subcutaneously into both right and left forelimb areas on mice.

In vivo PDT protocol

ADPM06 in PBS/CrEL solvent was injected at a dose of 2 mg/kg in 0.3 mL solution *via* the lateral tail vein. PDT was performed <5-min following injection to take advantage of maximal ADPM06 concentrations present within tumor tissue at this time as previously described using *in vivo* biodistribution studies.¹⁵ Irradiation was performed using a 670-nm light beam delivered *via* a fiber optic by a diode laser (B&W Tek., Newark, DE). Mice were anesthetized as described above, and the light beam was spread uniformly over the tumor area and maintained for 215 s (total fluence 150 J/cm² and fluence rate 700 mW/cm²).

Optical imaging

Optical imaging was performed using an IVIS Spectrum imaging system (Caliper). Trireporter and luciferase-expressing breast and glioma tumors were imaged 12-min following intraperitoneal administration of luciferin substrate (150 mg/kg; Caliper). Images and measurements of bioluminescent signals were acquired and analyzed using Living Image Software v3.2 (Caliper).

Positron emission tomography imaging with ^{18}F -FLT

^{18}F -FLT was prepared as previously described.¹⁷ MicroPET imaging was performed 2-hr after tail-vein administration of 7.4 MBq (200 μCi) of ^{18}F -FLT to each animal. PET imaging was performed using a FOCUS 120 microPETTM scanner (Siemens Preclinical Solutions, Knoxville, TN). At least 10 million coincidence events were acquired per study using a 350–750 keV energy window and a 6-nsec timing window. List-mode data were sorted into sinograms by Fourier rebinning and reconstructed by filter back-projection without attenuation or scatter correction. Count data in the reconstructed images were converted to activity concentration (*i.e.*, % of the injected dose per cm^3) based on a system calibration factor determined using an ^{18}F -filled mouse-size phantom). Visualization and analyses of images were carried out using AsiPROTM software (Siemens). Radioactivity concentration in tissue was calculated from the microPET images using maximum pixel values.

Immunohistochemistry

Control and treated tumors were fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 24-hr. Tissue was then processed using a Leica TP1020 tissue processor (Leica Microsystems, Wetzlar, Germany). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using an Apoptag Peroxidase *In situ* Apoptosis Detection Kit (Millipore) according to manufacturer's instructions. Slides were scanned at $\times 20$ magnification using a ScanScope XT Digital Slide Scanner (Aperio Technologies, Vista, CA). Quantification of TUNEL-staining in tumor sections was performed using a nuclear algorithm (Aperio).

Results

ADPM06-PDT induces apoptosis and involves caspase enzymatic activity

Clonogenic assays were used to determine the long-term survival of MDA-MB-231 cells post-PDT using various ADPM06 doses and light fluences (Fig. 1*a*). A $>90\%$ reduction in clonogenic ability was observed in samples treated with 150 nM ADPM06+16 J/cm^2 light when compared with ADPM06 alone, light alone and untreated control samples. This ADPM06 and light combination was therefore used for all further experiments. In addition, ADPM06-mediated cytotoxicity was found to be independent of p53 tumor suppressor status (Supporting Information Fig. 1).

The extent and mode of cell death induced by ADPM06-PDT was assessed using the Vybrant Apoptosis Assay which utilizes two fluorescent nucleic acid stains, Yo-Pro-1 and PI, to distinguish viable, apoptotic and necrotic cell populations by flow cytometry. Results reveal that ADPM06-PDT induces a time-dependent increase in apoptosis (Fig. 1*b*). Apoptosis was detected within 2-hr of PDT and apoptotic levels peaked at 4–8 hr with between 47 and 51% apoptosis detected. At 12- and 24-hr post-PDT, the apoptotic population declined and cells became necrotic as indicated by a PI positive Yo-Pro-1 negative population. Control, ADPM06-only and light-only samples showed minimal staining with either nucleic acid stain and displayed viability of $>90\%$.

The involvement of caspases during apoptosis induced by ADPM06-PDT was investigated by Western blotting using antibodies to detect the cleaved activated caspases (Fig. 1c). Proteolytic processing of the initiator caspase 9 into its active form was observed for 2-hr following ADPM06-PDT, with cleavage of caspase 8 detected 4-hr following treatment. In addition, the activation of executioner caspases 7 and 3 were detected 2- and 4-hr post-PDT, respectively. Finally, we detected cleavage of the caspase substrate, PARP, in response to PDT. Overall, this data suggests that ADPM06 induces a robust and well-orchestrated apoptotic response, which is accompanied by activation of members of the caspase family and their downstream substrate.

ADPM06-PDT induces ER stress and unfolded protein response

Confocal microscopy revealed a localization of ADPM06 to the endoplasmic reticulum (ER) compartment of MDA-MB-231 cells (Supporting Information Fig. 2), as well as initiation of a robust ER stress response following ADPM06-PDT (Fig. 2). During ER stress, the unfolded protein response (UPR) initiates intracellular signal transduction pathways, which inhibit translation of proteins and upregulate the expression of a number of UPR genes in an attempt to regain protein homeostasis and normal ER function. However, if ER homeostasis cannot be restored, programmed cell death is initiated.¹⁸ Following ADPM06-PDT, a rapid processing of XBP1 mRNA occurs resulting in the removal of an intron from the mRNA in a spliceosome-independent manner, a post-transcriptional modification catalyzed by the action of activated inositol-requiring protein 1 (IRE1). This then leads to transcription of XBP1s, a potent transcriptional activator of UPR genes. RT-PCR analysis revealed that splicing of XBP1 was evident as early as 30-min following ADPM06-PDT revealing an early involvement of the IRE1-XBP1 arm of the UPR in ADPM06-PDT (Fig. 2a).

A second arm of the UPR was also found to be activated in response to PDT. Western blot analysis revealed a phosphorylation of the α -subunit of eukaryotic translation initiation factor-2 (eIF2 α) at Ser51. Phosphorylation was detected 4-hr post-PDT and peaked at 12-hr post-PDT (Fig. 2b). This suggests the activation of the ER-localized type I transmembrane protein PERK (protein kinase RNA-like ER kinase), which phosphorylates eIF2 α thereby reducing the ER load by decreasing global protein synthesis within the cell, as well as potentially inducing cell-cycle arrest.

Additionally, a distinct increase in expression of the ER chaperone immunoglobulin binding protein (BiP) was detected 8-hr post-PDT and increased in a time-dependent manner (Fig. 2b).

Additionally, a distinct increase in expression of the ER chaperone immunoglobulin binding protein (BiP) was detected, which increased with time 8-hr post-PDT (Fig. 2b). BiP is a chaperone that is upregulated because of transcriptional activation by XBP1s in an attempt to attenuate the UPR and maintain cell survival under stressful conditions. Overall, these results suggest that ADPM06-PDT induces an ER stress response in MDA-MB-231 cells.

ADPM06-PDT-induced apoptosis involves the generation of ROS

To determine the degree of intracellular oxidative stress following ADPM06-PDT, the formation of DCF, a fluorescent oxidation product derived from non-fluorescent DCDHF,

was measured by flow cytometry using MDA-MB-231 cells subject to PDT treatment (Fig. 3a). DCDHF was administered to cell cultures 20-min before irradiation or at appropriate time points during the last 20-min of incubation before cell harvesting. The cell permeable DCDHF accumulates in the cytosolic compartment of cells, where it is deacetylated by intracellular esterases allowing it to react with oxidizing species, thereby converting it to DCF.¹⁹ Intracellular ROS generation was measured following treatment with 150 nM ADPM06+16 J/cm² light from 5-min to 4-hr post-PDT. A statistically significant increase in oxidative stress was detected promptly post-PDT, with peaks in DCF fluorescence evident 30-min and 4-hr post-PDT (Fig. 3a). The 30-min peak in DCF fluorescence (73%) is most likely due to the presence of oxygen radicals formed following interaction with highly reactive singlet oxygen, which itself is produced during irradiation. As this fluorescent probe can detect both ROS and reactive nitrogen species, with the extent of conversion from DCDHF to DCF being highly dependent on peroxidase activity, the 4-hr peak in DCF fluorescence (65%) may be associated with the presence of lipid oxidation products or possibly an initiation of apoptosis.²⁰ No change in oxidative stress was observed between untreated, ADPM06-only and light-only treated samples. The robust generation of reactive species by ADPM06-PDT is attributable to the presence of bromine atom substituents on the photosensitizer, a structural modification exploited when designing ADPM06.¹³

To confirm that the oxidative stress detected is directly associated with cell-death induced by ADPM06-PDT, we pretreated cells with a potent ROS scavenger and glutathione precursor, N-acetylcysteine. Results show that while ADPM06 induces loss of viability in a dose-dependent manner, the pre-treatment of cells with NAC leads to a significant decrease in death induced by ADPM06-PDT. Specifically, an eightfold difference in EC₅₀ between PDT-treated and PDT+NAC-treated cells was obtained across all replicates ($p < 0.05$). A significant difference in percentage viability was also observed between PDT alone and PDT +NAC treatment groups over a range of ADPM06 concentrations ($p < 0.05$).

Flow cytometric analysis of cell-death was also used to determine the degree of protection conferred by NAC (Fig. 3c). MDA-MB-231 cells treated with ADPM06 PDT alone (150 nM ADPM06+16 J/cm²) or PDT+NAC were stained using the Vybrant apoptosis assay kit as described above. Treated and control cells were analyzed 24-hr post-irradiation. As expected, PDT reduced the cell viability by 50%, whereas a pretreatment of cells with NAC before PDT conferred complete protection against cell-death. No significant difference in the percentage of viable cells was found in NAC+light-treated samples when compared with untreated, ADPM06-only and light-only controls.

Rapid reduction in tumor bioluminescence *in vivo* post-ADPM06-PDT

A rapid reduction in bioluminescence from luciferase-expressing MDA-MB-231 cells was observed between 1- and 4-hr post-PDT (2 mg/kg ADPM06+150 J/cm² light) using a short drug-light interval (Fig. 4a). A >1,000-fold reduction in bioluminescence was observed in animals 1-hr post-PDT when compared with luciferase activity before PDT. A >1,500- and >2,500-fold decrease in bioluminescence was observed in animals 2- and 4-hr post-PDT, respectively (Fig. 4b). This reduction in bioluminescence can be correlated with a prompt initiation of cell-death, as determined by a combination of Western blot and IHC analysis

(Figs. 4c and 4d), which may result from a combination of damage to the tumor microvasculature and direct tumor cell-death following PDT.

Western blot analysis of BiP and phospho-eIF2 α in tumor tissue 1-, 2- and 4-hr post-PDT revealed a rapid induction of ER stress in malignant tissue, which correlates with the observed mechanism of action of ADPM06 within *in vitro* models (Fig. 4c). An upregulation of BiP was apparent 2-hr following PDT with levels increasing further 4-hr post-treatment, indicating an induction of ER stress *in vivo*. In addition, phosphorylation of eIF2 α was also evident 2-hr post-PDT, with a marked increase in phosphorylation by 4-hr.

TUNEL staining of tumor sections was used for detection of apoptotic cells post-PDT (Figs. 4d and 4e). A ~25% apoptotic population was observed in tumor samples 1- and 2-hr post-PDT, with a distinct increase in apoptosis to 41% at 4-hr post-PDT. Levels of apoptosis in control tissue were <5%.

Evaluation of ^{18}F -FLT for monitoring tumor response to ADPM06-PDT

Aberrant tumor-cell proliferation is a well-characterized hallmark of cancer attributable to the ability of tumor cells to be self-sufficient in terms of growth signals, having a dramatically reduced sensitivity to antigrowth signals and showing a decrease in the rate of cell attrition, mainly due to an evasion of apoptosis.²¹ Currently, the most broadly used radiotracer for PET imaging of tumor proliferation is ^{18}F -FLT,²² which is phosphorylated by cytosolic thymidine kinase-1, a key enzyme of the DNA salvage pathway that is upregulated before and during S-phase of the cell-cycle. The phosphorylated FLT is metabolically trapped within the cell, resulting in its accumulation.

We have previously demonstrated that ^{18}F -FDG imaging of rat MatIIB tumors shortly following ADPM06-PDT represents a clinically relevant biomarker of response.¹⁵ The aim of this study was to examine further tumor response to PDT, using non-invasive ^{18}F -FLT-PET imaging, in an attempt to identify a second clinically relevant radiotracer that may synergistically complement ^{18}F -FDG-PET results. Combination of both FDG and FLT data may prove useful for extensive monitoring of response to therapy, as well as in prediction of prognosis.

A marked reduction in ^{18}F -FLT was observed in PDT-treated breast (Fig. 5a) and glioma (Fig. 5d) tumors 4-hr postirradiation, which diminished further 24-hr post-treatment, indicative of a decrease in tumor-cell proliferation. ADPM06-only- and light-only-treated tumors showed an increase in ^{18}F -FLT activity at both time points compared to pretreatment activity indicating that each nontoxic component alone has no effect on the proliferation status of breast or glioma tumors. The percentage of injected dose of ^{18}F -FLT per gram of tumor tissue (%ID/g) decreased rapidly following PDT in both MDA-MB-231-TGL (Fig. 5b) and U87-TGL (Fig. 5e) tumors, while a steady increase in ^{18}F -FLT was observed in ADPM06-only treated tumors over the 24-hr, thereby, emphasizing the negligible effect of the photosensitizer alone on proliferation of both tumor types.

To further assess the therapeutic response to ADPM06-PDT in both trireporter expressing tumors, optical imaging was performed pre- and post-PDT to assess the effect of ADPM06-

PDT on tumor luciferase-activity over a 96-hr period (Figs. 5c and 5f). A rapid reduction in tumor bioluminescence was observed 4-hr post-PDT, which was demonstrated earlier in Figure 4a. The decrease in bioluminescence was maintained up to 96-hr post-PDT in U87-TGL cells, with the formation of an eschar as soon as 4-hr post-PDT. A small increase in luciferase activity was detected in MDA-MB-231-TGL tumors 24- and 48-hr post-PDT; however, this signal diminished once more by 96-hr post-PDT. Complete response rates of 100% and 66% were demonstrated in breast cancer and glioma models, respectively, with no tumor recurrence observed 5-months post-PDT.

Discussion

Despite the vast array of porphyrin photosensitizers that currently exist for the treatment of cancer, several disadvantages exist, with photosensitivity being the predominant drawback experienced. Therefore, significant potential exists for the development of non-porphyrin photosensitizers, which can easily be fine tuned for specific photochemical and photobiological requirements, such as wavelength absorption and ROS generation. However, in contrast to porphyrin-based PDT, the development of non-porphyrin photosensitizers in the oncology arena has lagged considerably.²³

Previously, we have reported the development of an exciting new class of non-porphyrin PDT agent.¹³ This family of compounds are generated from readily available precursors and may be sequentially modified, allowing for the refinement of essential therapeutic parameters to match the specific therapeutic target of choice by straightforward functional group manipulation of the core photosensitizer scaffold. Recently, we have demonstrated that ADPM06-PDT is well tolerated *in vivo* and elicits impressive complete response rates in various models of cancer utilizing a short drug-light interval.¹⁵ Moreover, we have established ¹⁸F-FDG as an important biomarker of therapeutic response. Following these observations, we have now undertaken studies to dissect the cell-death pathway initiated post-ADPM06-PDT and identify key mediators of this response. We have shown that a combination of 150 nM ADPM06+16 J/cm² light significantly reduces the clonogenic potential of MDA-MB-231 cells and induces a robust apoptotic response, which had initiated 2-hr post-PDT. This apoptotic response is accompanied by cleavage of a number of initiator and executioner caspases detected by 4-hr post-PDT. Importantly, we have shown that the intracellular site of PDT-induced damage is the ER, with a splicing of XBP1 mRNA observed as soon as 30-min following PDT. Upregulation of BiP expression and phosphorylation of eIF2 α were also observed, suggesting that a robust ER stress response is initiated in an attempt to curtail the UPR following ADPM06-PDT. As a result of the mitochondrial-mediated activation of caspase 9, as well as the induction of ER stress post-ADPM06-PDT, future studies will examine potential crosstalk between the mitochondrial and ER compartments. The rapid generation of ROS post-PDT was revealed as the key instigator of the apoptotic response, as inclusion of the ROS scavenger, NAC, was found to confer complete protection to cells following ADPM06-PDT.

Following *in vitro* characterization of the mechanism of action of ADPM06-PDT, the efficacy and *in vivo* mechanism of action was subsequently investigated. Use of a short drug-light interval *in vivo* using 2 mg/kg ADPM06 followed immediately by light irradiation

with 150 J/cm² has revealed a rapid reduction in tumor-specific luciferase activity as early as 1-hr post-PDT, with levels decreasing further 4-hr post-PDT. Further examination of this rapid response to ADPM06-PDT has revealed an initiation of apoptosis *in vivo*, as well as induction of an ER stress response, which correlates well with numerous aspects of the observed mechanism of action of ADPM06 within *in vitro* models. Future studies will focus on enhancement of the PDT-induced ER stress, systemic immunity and possible potentiation of treatment efficacy, using combination therapy with ER stress inducers such as bortezomib, as has recently been demonstrated.²⁴

Further examination of the therapeutic response to PDT, using a combination of ¹⁸F-FLT nuclear and optical imaging, has revealed a substantial decrease in tumor proliferation over a 24-hr period in both breast and glioma models of cancer. We have previously demonstrated that ¹⁸F-FDG imaging of rat MatIIB tumors shortly following ADPM06-PDT represents a clinically relevant biomarker of response.¹⁵ Combination of data from these two clinically relevant radiotracers may synergistically complement one another and may prove useful for extensive monitoring of response to therapy, as well as in prediction of prognosis.

In summary, we have shown that ADPM06-PDT induces a well-orchestrated apoptotic response instigated by ROS and commencing in the ER with a rapid initiation of the UPR post-PDT. A robust therapeutic response has been shown *in vivo* with a substantial reduction in tumor proliferation observed, as well as a rapid induction of apoptosis and initiation of an ER stress response. There are numerous reasons as to why a prompt initiation of ER stress following ADPM06-PDT using a short drug-light interval, which primarily induces a vascular-specific response *in vivo*, was observed. A rapid uptake of ADPM06 directly in tumor cells during the irradiation process may explain the observed response or, perhaps, a prompt increase in tumor hypoxia resulting from vascular damage may play a role in the initiation of ER stresses. A better understanding of this *in vivo* response is currently being investigated. Moreover, examination of *in vivo* efficacy utilizing more clinically applicable PDT protocols is currently ongoing. Taken together, the data presented here clearly establishes the novel non-porphyrin photosensitizer, ADMP06, as an exciting new therapeutic with significant potential for further translational development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

ADPM06-PDT induces apoptosis and involves caspase enzymatic activity. (a) Loss of clonogenicity in MDA-MB-231 cells treated with varying ADPM06 doses and light fluences. A statistically significant ($p < 0.001$) >90% loss in clonogenic potential was observed in cells treated with 150 nM ADPM06+16 J/cm², the drug and light combination used for all further *in vitro* work. Error bars represent standard error within triplicate experiments, with each individual experiment containing three internal replicates. (b) Examination of the mode of cell-death following ADPM06-PDT. Percentages of cell

populations in early apoptosis, late apoptosis and necrosis are shown. (c) Effect of ADPM06-PDT on caspase enzymatic activity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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Figure 2. ADPM06-PDT induces ER stress and UPR. (a) Examination of ER stress-induced splicing of XBP1 mRNA (XBP1s) post-ADPM06-PDT, as well as (b) Western blot analysis of BiP expression and (c) phosphorylation of eIF2α. Tunicamycin treatment of cells (10 μM for 16-hr) was used as a positive control for induction of UPR.

Figure 3.

The ROS scavenger, NAC, inhibits ADPM06-induced cell-death. (a) Determination of intracellular oxidative stress following ADPM06-PDT. Percentages of PDT-treated DCF positive cells, when compared with DCF only treated cells, outlined for each time point ($p < 0.001$). (b) Changes in cell viability, as assessed by MTT assay, of PDT+NAC treated cells compared to PDT-treatment only. Error bars represent standard error within triplicate experiments, with each individual experiment containing three internal replicates. (c) Flow cytometric analysis of PDT treated cells (150 nM ADPM06+16 J/cm² light), in the presence or absence of NAC, 24-hr following irradiation using Yo-Pro-1 and PI staining. Percentages of cells in early apoptosis, late apoptosis and necrosis shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4.

Optical imaging, Western blot and immunohistochemical analysis of PDT-treated tumors. (a) Optical imaging of mice bearing luciferase-expressing MDA-MB-231 cells before and 1-, 2- and 4-hr following PDT (2 mg/kg ADPM06+150 J/cm² light). Quantification of bioluminescent signal pre- and post-treatment is shown in photons/sec/cm²/steradian. (b) Fold difference in bioluminescent signal pre- and post-PDT up to 4-hr post-treatment. Error bars represent standard error among three mice at each time point. (c) Western blot analysis of ER stress markers, BiP and phosphorylated eIF2α, in tumor cell lysates. (d) Immunohistochemical analysis of control and PDT-treated tumors using TUNEL staining.

(e) Automated analysis of TUNEL nuclear staining in PDT-treated tumors sections over 4-hr period. Tumor sections from three individual animals were analyzed for each time point shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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Figure 5.

Evaluation of ^{18}F -FLT for monitoring tumor response to ADPM06-PDT. (a) ^{18}F -FLT nuclear imaging of subcutaneously implanted MDA-MB-231-TGL and (d) U87-TGL tumors pre- and post-PDT treatment using 2 mg/kg ADPM06+150 J/cm² light. Nuclear imaging at selected time points before and after ADPM06-PDT, illustrating the change in tumour proliferation. Light-only treated tumors imaged 24-hr post-irradiation. White arrows, control tumor; red arrows, treated tumors. (b) % ID ^{18}F -FLT/gram tissue in MDA-MB-231-TGL and (e) U87-TGL tumors post-ADPM06-PDT. Error bars represent standard error among five mice at each time point. (c) Optical imaging of luciferase activity of MDA-MB-231-TGL

and (f) U87-TGL tumors prior and up to 96-hr post-PDT treatment. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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