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Promotion of Pro-Apoptotic Signals by Lysosomal Photodamage

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Abstract

We previously reported that low level lysosomal photodamage enhanced the efficacy of subsequent mitochondrial photodamage, resulting in a substantial promotion of apoptotic cell death. We now extend our analysis of the sequential PDT protocol to include two additional lysosomal-targeting photosensitizers. These agents, because of enhanced permeability, are more potent than the agent (N-aspartyl chlorin E6, NPe6) used in the initial study. Addition of the cell-permeable cysteine protease inhibitor E-64d and calcium chelator BAPTA-AM almost completely suppressed sequential PDT-induced loss of mitochondrial membrane potential, activation of procaspases-3 and -7 and loss of colony formation. These inhibitors did not, however, suppress the pro-apoptotic effect of a BH3 mimetic or mitochondrial photodamage. Knockdowns of ATG7 or ATG5, proteins normally associated with autophagy, suppressed photodamage induced by the sequential PDT protocol. These effects appear to be independent of the autophagic process since pharmacological inhibition of autophagy offered no such protection. Effects of ATG7 and ATG5 knockdown may reflect the role that ATG7 plays in regulating lysosome permeability, and the likelihood that a proteolytic fragment of ATG5 amplifies mitochondrial pro-apoptotic processes. Our results suggest that low-dose photodamage that sequentially targets lysosomes and mitochondria may offer significant advantages over the use of single photosensitizers.

Abstract

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INTRODUCTION

Use of photosensitizing agents to sensitize neoplastic tissues to light is termed photodynamic therapy (PDT) (1,2). Since reactive oxygen species (ROS) formed upon irradiation of photosensitized cells have a very short (microsecond) half-life, photodamage is confined to regions where photosensitizers initially localize. Several photosensitizers have been identified that preferentially localize to specific organelles such as lysosomes, mitochondria, golgi and the endoplasmic reticulum (3–5). Photodamage can thereby be directed to specific sub-cellular loci.

We recently reported that low level lysosomal photodamage markedly enhanced subsequent photokilling by PDT targeted to mitochondria (6); the optimal effect occurred when lysosomal photodamage preceded mitochondrial photodamage. This phenomenon was not related to increased ROS formation in mitochondria, but appeared to involve an enhancement of the pro-apoptotic signal(s) resulting from mitochondrial photodamage. This report describes the result of subsequent investigations into the nature of this effect. Initial studies were carried out with the lysosomal and mitochondrial sensitizers NPe6 (N-aspartyl chlorin e6) and BPD (benzoporphyrin derivative), respectively (6). Although effective in the sequential protocol, NPe6 is poorly accumulated by cells, necessitating the use of a high extracellular concentration. In this study we show that the photosensitizers 5-ethylamino-9-diethylaminobenzo [a]phenothiazinium chloride (EtBNS) and the galactose conjugate of 3-(1-hexyloxyethyl)-3-devinyl pyropheophorbide-*a* (HPPHgal) preferentially localize to lysosomes and can substitute for NPe6 in the sequential PDT protocol at much lower concentrations. Studies employing combinational treatment with a cysteine protease inhibitor and a calcium chelating agent, and knockdowns of two autophagy-related proteins, provide clues to an explanation for the enhanced efficacy of the sequential PDT protocol.

MATERIALS AND METHODS

Chemicals and supplies

NPe6 was provided by Dr. Kevin M. Smith, Louisiana State University. BPD (benzoporphyrin derivative, Verteporfin) was purchased from VWR (Cat No 1711461). EtBNS was prepared as described by Clapp et al (7) and provided by Dr. Conor Evans (Harvard Medical School\Massachusetts General Hospital). The galactose conjugate of 3-(1-hexyloxyethyl)-3-devinyl pyropheophorbide-*a* (HPPHgal) was provided by Dr. R.K. Pandey, Roswell Park Cancer Institute (8). Other reagents were obtained from Sigma-Aldrich and were of the highest available purity. Fluorescent probes were provided by Life Technologies, Inc.

Cell culture and clonogenic assays

Conditions used for the growth of murine hepatoma 1c1c7 cells and clonogenic assays have been described (9). 1c1c7 variants expressing greatly reduced levels of ATG7 and ATG5, due to stable expression of ATG7 or ATG5 shRNA, were prepared as previously reported (9).

PDT protocols

Cells cultured in plastic dishes or on cover slips maintained in plastic dishes were incubated at 37°C with 40 μ M NPe6 or 0.25 μ M EtNBS for 1 h, or with 0.9 μ M HPPHgal for 24 h. Where indicated, 0.5 μ M BPD was added concurrently with NPe6 and EtNBS, or during the final hour of HPPHgal incubation. The medium was then replaced and the dishes irradiated using a 600-watt quartz-halogen source filtered through 10 cm of water to remove wavelengths of light > 900 nm. The bandwidth was further confined by interference filters (Oriel, Stratford CT): 660 $\pm$ 10 nm, 90 mJ/sq cm for NPe6; 650 $\pm$ 10 nm, 270 mJ/sq cm for EtNBS; 660 $\pm$ 10 nm, 180 mJ/sq cm for HPPHgal. Where specified, this was directly followed by irradiation at 690 $\pm$ 10 nm, 37.5 mJ/sq cm, for initiation of BPD-induced photodamage. The duration between irradiations did not influence cell killing provided the second irradiation was within 2 to 20 minutes of the first irradiation. Potentiation of cell killing progressively declined if the interval exceeded 20 minutes. In some experiments the cysteine protease inhibitor E-64d (10 μ M), the calcium chelator BAPTA-AM (10 μ M) or both, or the indocarbazole Gö6976 (1 μ M) were added with BPD. The latter agent is an inhibitor of a PI3 kinase needed for initiation of autophagosome development (10).

Microscopy protocols

Photodamage to mitochondria resulting in loss of membrane potential (Ψ_m) was assessed using MitoTracker Orange (MTO), as previously described (6,11,12). Alkalinization of late endosomes/lysosomes as a consequence of lysosomal photodamage was assessed using LysoTracker Green (LTG), as previously described (6). After irradiation, cells were incubated for 10 min at 37°C with 200 nM MTO and 100 nM LTG. The medium was replaced and the dishes were held briefly at 15°C before microscopy. Chilling was necessary to prevent the recovery of Ψ_m which can occur if the cells are not chilled, even in lethally-irradiated cultures (11). Images were acquired with a Nikon E-600 microscope and a Rolera

EM-CCD camera with MetaMorph software (Molecular Devices, Sunnyvale CA). MTO fluorescence was detected using 510–560 nm excitation and measuring emission at wavelengths > 590 nm). LTG fluorescence was detected using 450–490 nm excitation and measuring emission at wavelengths > 515 nm). A 650 nm low-pass filter was placed in the emission beam to prevent photosensitizer fluorescence from reaching the CCD camera. Photosensitizer fluorescence was detected using the 400–440 nm excitation and assessing emission at wavelengths > 650 nm. At least 3 images were acquired for each sample with typical representations shown here.

DEVDase assays

Cells were collected 2 h after irradiation and assayed for DEVDase activity (12). This activity reflects the activation of pro-caspases 3 and/or 7, a hallmark of apoptosis. A kit provided by Invitrogen (cat. no. E13184) was used for this purpose. Each assay was performed in triplicate. Enzyme specific activities are reported as nmol product/min/mg protein. The Micro Lowry assay was used to estimate protein concentrations, using bovine serum albumin as the standard.

RESULTS

Localization of photosensitizing agents

Fluorescence microscopy confirmed our previous reports (5,13) that NPe6 colocalizes (indicated by fluorescent yellow puncta) in 1c1c7 cells almost exclusively with LTG, a fluorescent reporter that accumulates in acidic organelles (Fig. 1). The photosensitizers EtNBS and HPPHgal also showed a high degree of co-localization with LTG (Fig. 1).

Specificity of NPe6, HPPHgal and EtNBS-mediated photodamage

We established photodynamic doses of the individual photosensitizers that had marginal effects on procaspase-3/7 activation (Table 1), and no detectable effect on mitochondrial membrane potential or the lysosomal pH gradient, as assessed by MTO and LTG labeling patterns (Fig. 2A). Moreover, there was no significant photokilling (Fig. 3) by any individual photosensitizers.

Effects of the sequential PDT protocol

The sequential protocol involved incubation of 1c1c7 cells with the lysosomal-targeting agents + BPD, followed by sequential irradiation at 650 nm (EtNBS) or 660 nm (HPPHgal, NPe6), then at 690 nm (BPD). Whereas none of the photosensitizers alone had an effect on Ψ_m , a substantial loss of Ψ_m occurred when each of the three lysosomal photosensitizers was used in the sequential PDT protocol (Fig. 2B). Loss of Ψ_m was subsequently accompanied by the activation of procaspases-3 and -7 (Table 1), indicative of an apoptotic outcome. The sequential PDT protocol, irrespective of which lysosomal photosensitizer was employed, resulted in synergistic killing, as scored in colony formation assays (Fig. 3). An appreciation of how well the sequential protocol promotes PDT efficacy can be obtained by comparing dose-response curves for BPD PDT alone with BPD PDT following prior low-dose NPe6 photodamage (Fig. 4). The 'BPD' curve represents photokilling by BPD alone,

as a function of the light dose. Prior photodamage by an LD₅₋₁₀ PDT dose of NPe6 resulted in a significant enhancement of photokilling.

Role of autophagy and effects of ATG knockdown

PDT with either mitochondrial or lysosomal photosensitizers can initiate autophagy (3). The induction of autophagy in PDT protocols has been reported to both enhance and inhibit cell death (3,9,14). As an approach to addressing the role of autophagy we examined 1c1c7 variants that are unable to form autophagosomes because of deficiencies in ATG7 or ATG5. Unlike the parental cell line, the ATG7 and ATG5 KD lines did not exhibit a loss of Ψ_m following sequential PDT (Fig. 5). ATG5 deficiency also protected against the loss of viability (Fig. 3). While this might be interpreted as indicative of a role for autophagy, treatment of wild type cells with the PI3 kinase inhibitor Gö6976 did not preserve Ψ_m (Fig. 6). Since this kinase is required for development of the phagophore, the precursor of the autophagosome (10), it appears that autophagy-related processes do not contribute to the synergistic killing observed in the sequential PDT protocol. Furthermore, the results with Gö6976 suggest that the protective effects of ATG5 and ATG7 knockdown are independent of a role in the autophagic process.

We recently reported that a deficiency in ATG7, but not ATG5, suppressed the permeabilization of 1c1c7 lysosomes by NPe6 PDT and the release of lysosomal proteases (15). The finding that ATG7-deficiency suppressed the loss of Ψ_m in the sequential PDT protocol suggests that a lysosome-derived factor contributes to the observed synergism. Addition of E-64d, an inhibitor of cysteine proteases such as lysosomal cysteine cathepsins, partially suppressed the activation of procaspase-3 and -7 in wild type 1c1c7 cultures. This inhibition was further enhanced by addition of the calcium-chelating agent BAPTA-AM. This inhibitor combination was somewhat less effective at suppressing DEVDase activation when NPe6 or HPPHgal was substituted for EtNBS (Fig. 7). Substitution of N-acetyl-pepstatin A, an inhibitor of lysosomal aspartate protease cathepsin A, for E-64d did not inhibit DEVDase activity (data not shown).

In addition to its role in autophagy, ATG5 appears to have proapoptotic activity. Yousef *et al.* (16) reported that a calpain-derived ATG5 cleavage fragment interacted with the mitochondrial membrane, and potentiated the induction of apoptosis by other agents that cause mitochondrial dysfunction. Many of the isoforms of calpain requires calcium for maximum activity (17), and E-64d is an inhibitor of calpain. As indicated in Fig. 7, the calcium chelating agent BAPTA-AM had only a slight effect on DEVDase activity using the EtNBS-BPD combination unless supplemented with E-64d.

Effects of inhibitors on initiation of apoptosis. To discover whether the combination of E-64d + BAPTA-AM could prevent any apoptotic response, we examined the effect of these inhibitors on the induction of apoptosis by the BH3 mimetic ABT737 and high-dose PDT using BPD alone. No suppression of procaspase 3/7 activation was observed (Table 2).

DISCUSSION

We previously described the ability of sequential lysosomal/mitochondrial photodamage to promote PDT efficacy, using NPe6 and BPD as the photosensitizing agents (6). While NPe6 is a potent lysosomal photosensitizer *in vitro*, uptake by cells and tissues is poor, requiring the use of an extracellular concentration of 40 μ M in our culture model. In this report, we show that HPPHgal or EtNBS can substitute for NPe6. Although the three photosensitizers are structurally different, and we have no information on their mode of uptake or duration of retention, all three agents show an affinity for lysosomal structures as revealed by co-localization analyses, and were effective at potentiating subsequent mitochondrial photodamage. However, EtNBS and HPPHgal were effective at sub-micromolar levels in the sequential PDT protocol, which represents an advantage for clinical protocols.

An early effect of mitochondrial photodamage is loss of Ψ_m . Loss of Ψ_m is often associated with induction of the mitochondrial permeability transition (MPT), which leads to the release of cytochrome c, formation of the apoptosome, and subsequent activation of initiator and executioner caspases. The conditions used for BDP PDT were insufficient to induce loss of Ψ_m . However, when coupled with prior low-level lysosomal damage, we observed a substantial loss of Ψ_m and a promotion of photokilling. Although the absorption spectra of NPe6 and BPD exhibit minimal overlap (6), and we employed interference filters to further limit overlap, we cannot unequivocally eliminate the possibility that some photoactivation of BPD occurred during photoactivation of the lysosomal sensitizers. If this occurred, however, it had to have been inconsequential. No loss of Ψ_m occurred if we sequentially irradiated BPD-loaded cultures (no NPe6 present) with the light-dose conditions used for NPe6 activation, followed by the normal light-dose conditions used for BPD photoactivation (Kessel, unpublished data). Hence, if the conditions used for the photoactivation of the lysosomal sensitizers also caused some photoactivation of BPD, the effects of this activation were insufficient to prime mitochondria to the effects of subsequent intentional mitochondrial photodamage.

How might lysosomal photodamage potentiate BPD-mediated mitochondrial photodamage? Although lysosome-derived iron can potentiate mitochondrial photodamage (18,19), the conditions used for NPe6-mediated lysosomal photodamage in the sequential PDT protocol do not trigger the release of lysosomal Fe^{++} stores in 1c1c7 cultures (6). A second lysosome-derived potentiator may be hydrolases. We have documented that lysosomal proteases can facilitate lysosomal dysfunction and trigger the intrinsic apoptotic pathway (5). Conditions used here have no detectable effect on lysosomal pH, as assessed with LTG (Fig. 2A). Confocal colocalization analyses of Lamp1 (a lysosomal marker) and cathepsin A in 1c1c7 cultures treated with NPe6/PDT LD₃₀ conditions indicate that loss of the lysosomal pH gradient and the release of cathepsins is sequential, with there being a 30 to 60 minute lag before there is a significant release of cathepsin A into the cytosol (Reiners, unpublished data). Although we are using less than LD₃₀ conditions in the sequential PDT protocol, we cannot eliminate the possibility of rapid leakage of some lysosomal hydrolases in our protocol. Indeed, the findings that loss of Ψ_m was muted by treatment with E-64d, a potent inhibitor of lysosomal cysteine cathepsins, and was suppressed in ATG7 KD cells, a condition known to inhibit lysosome permeabilization by PDT in 1c1c7 cells (15), suggest

that lysosome-derived products contribute to the potentiation of mitochondrial photodamage.

It has been reported that ATG5 can be cleaved by calpain to yield a proapoptotic 24 kDa fragment that binds to mitochondria-associated Bcl-XL, resulting in cytochrome c release (16). Many observations reported here are consistent with such a model. Calpain is a calcium-activated protease (17), and inclusion of the calcium chelator BAPTA-AM weakly suppressed procaspase activation in the sequential PDT protocol. The cysteine protease inhibitor E-64d, an inhibitor of calpain activity, also modestly suppressed procaspase activation in the sequential PDT protocol. However, the combination of E-64d + BAPTA-AM strongly suppressed the pro-apoptotic effect of sequential PDT without affecting apoptosis induced by a BH3 mimetic or BPD PDT (Table 2). While we could detect the ATG5-ATG12 conjugate by western blotting, and ATG5-ATG12 content rapidly and significantly decreased after the sequential PDT protocol, we have been unable to detect the putative 24 kDa ATG5 fragment (Kessel, unpublished data). Hence, the mechanism by which ATG5 deficiency suppresses sequential PDT-induced phototoxicity is not yet fully delineated.

Mitochondria have a considerable capacity for calcium uptake, and excessive calcium can lead to mitochondrial dysfunction and induction of MPT (20). In part, this dysfunction has been attributed to calcium activation of the mitochondrial protease calpain 10, and its induction of respiratory dysfunction via cleavage of components of Complex I (21). Interestingly, calpain 10 is not inhibited by the calpain inhibitor PD150606 (21). Although potentially consistent with the cytoprotection afforded by BABTA-AM treatment, we recently reported that treatment with Ru360, which inhibits mitochondria uptake of both calcium and iron, offers no protection in the sequential PDT protocol using NPe6 as the lysosomal sensitizer (6). Hence, we do not believe that processes associated with the influx of calcium into mitochondria are responsible for the observed synergy in our sequential PDT model.

In 1996 Cincotta *et al.* (22) described an *in vivo* PDT study employing a combination of two photosensitizers used in this study, EtBNS and BPD, to treat s.c. growing xenografts of EMT-6 fibrosarcomas. The objective was to determine whether the known effects of BPD on tumor vasculature could potentiate direct tumor cell kill by EtNBS (23). The combination did result in a synergistic effect and eradication of comparatively large tumors (22). The outcome was clearly superior to the effects of either photosensitizer alone but the basis was unclear since the expected vascular photodamage was not detected. We propose that the observed synergy in the Cincotta *et al.* study lies in the ability of low-dose lysosomal photodamage to initiate a pro-apoptotic process that amplifies mitochondrial photodamage.

In addition to the present study, synergistic tumor killing has been reported to occur in low-dose PDT protocols involving the lysosomal photosensitizer meso-tetrakis(r-N-methylpyridyl)porphine and the golgi photosensitizer zinc(II)-phthalocyanine (24,25). In these studies a single light source was used for the activation of both sensitizers. Hence, activation of the two sensitizers was simultaneous, as opposed to sequential. The basis for the observed apoptosis in these studies was attributed to activation of the intrinsic (*i.e.*,

mitochondrial) apoptotic pathway. What is not clear is whether the above combinational protocol enhanced the production of lysosomal or golgi pro-apoptotic factors that affected mitochondria, or if the treatment primed the mitochondria to be more responsive to putative apoptotic factors. Irrespective of the answer, these latter studies coupled with our results suggest that low level lysosomal photodamage can enhance photokilling in combinational PDT protocols. Although our studies employed a hepatoma cell line, which is not a neoplasia normally targeted by PDT in the clinic, our study, in conjunction with others (22,24,25), suggest that low-dose sequential PDT should be applicable to the treatment of a wide variety of tumor types.

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Figure 1.

Sub-cellular localization of photosensitizers. Ic1c7 cultures were incubated with 40 μ M NPe6 or 0.25 μ M EtNBS for 1 h, or 0.9 μ M HPPHgal for 24 h, then resuspended in fresh medium and incubated for 10 min with 200 nM LTG. The left panels show the distribution of photosensitizer (PS) fluorescence, and center panels show LysoTracker Green (LTG). An overlay is shown at the right where co-localization is indicated by yellow pixels.

Figure 2.

Effects of photodamage on patterns of MTO and LTG fluorescence. A. 1c1c7 cultures were sensitized with individual photosensitizers, irradiated and then labeled with fluorescent probes. Concentrations and times: 0.5 μ M BPD for 1 h, 0.25 μ M EtNBS for 1 h EtNBS, 0.9 μ M HPPHgal for 24 h, 40 μ M NPe6 for 1 h. Irradiation: 660 ± 10 nm (NPe6 and HPPHgal), 650 ± 10 nm (EtNBS) at 90, 180 and 270 mJ/sq cm, respectively. For BPD, the light dose was 37.5 mJ/cm sq at 690 nm. B. Cells were treated with lysosomal photosensitizers and BPD, irradiated as indicated for the lysosomal sensitizers, then given an additional period of irradiation (690 nm, 37.5 mJ/ cm sq). Data represent mean $\pm$ SD for three independent experiments

Figure 3. Clonogenic analyses of photokilling by sequential PDT using three different lysosomal photosensitizers. 1c1c7 cultures were subjected to the PDT conditions specified in the legend to Fig. 2. Studies with an ATG5 knockdown were carried out only with NPe6 + BPD. Data represent mean $\pm$ SD for three independent experiments

Figure 4.

Clonogenic analyses of dose-response experiments showing the ability of low-dose lysosomal photodamage to potentiate photokilling by BPD. 1c1c7 cultures were incubated for 1 h with BPD (0.5 μ M) alone or BPD + NPe6 (40 μ M). The medium was then replaced and the cultures irradiated with varied light doses at 690 nm. For the combination protocol, there was an initial irradiation at 660 nm (90 mJ/cm sq). Data represent mean $\pm$ SD for three determinations.

Figure 5.

Effects of sequential PDT on MTO fluorescence in autophagy-deficient cell lines. Wild type, ATG5 and ATG7 knockdown derivatives of 1c1c7 cells were sensitized with NPe6 and BPD, and then irradiated (90 mJ/sq cm @ 660 nm, then 37.5 mJ/sq cm @ 690 nm). Effects on Ψ_m were examined 10 min after irradiation by monitoring MTO fluorescence. Data are representative of three independent experiments.

Figure 6.

Effects of sequential PDT on MTO fluorescence in cultures pretreated with the PI3 kinase inhibitor Gö6976. Wild-type 1c1c7 cells were photosensitized with NPe6 and BPD and irradiated as described in the legend to Fig. 4. Gö6976 (10 μ M) was added with the sensitizers. Effects on Ψ_m were examined 10 min after irradiation by monitoring MTO fluorescence. Data are representative of three independent experiments.

Figure 7.

Effects of E-64d and BAPTA-AM on activation of procaspases-3/7 as determined by DEVDase assays. 1c1c7 cultures were treated with photosensitizers and irradiated as specified in Fig. 2. A: Effects of EtNBS and BPD PDT; B: effects of E-64d, BAPTA-AM or both on EtNBS/BPD PDT; C: effects of E-64d + BAPTA on NPe6/BPD PDT; D: effects of E-64d + BAPTA on HPPHgal/BPD PDT. Cultures were harvested 2 h after irradiation for assay of DEVDase activity. Data represent mean $\pm$ SD for three determinations.

Table 1

DEVDase activity

Photosensitizer	Wavelength (nm)	DEVDase activity
Control	none	0.08 ± 0.01
BPD	690	0.19 ± 0.04
NPe6	660	0.10 ± 0.03
EtNBS	650	0.15 ± 0.02
HPPHgal	660	0.11 ± 0.03
NPe6 + BPD	660 → 690	2.5 ± 0.13 *
EtNBS + BPD	650 → 690	2.7 ± 0.10 *
HPPHgal + BPD	660 → 690	2.4 ± 0.16 *

1c1c7 cultures were treated with 40 μM (NPe6), 0.25 μM (EtNBS), 0.9 μM (HPPHgal) and/or 0.5 μM (BPD) for 1 h, except for HPPHgal (16 h), before being irradiated as noted in the table. Light doses are indicated in the text. DEVDase activity was measured 2 h after irradiation. DEVDase units = nmol product/min/mg protein. Values represent mean ± SD for three determinations.

* Statistically different from controls ($p < 0.05$).

Table 2

Effects of inhibitors on apoptotic responses

Condition	Additions	DEVDase Activity
Control	None	0.04 ± 0.003
ABT-737	None	2.4 ± 0.3 [*]
	BAPTA-AM + E-64d	2.3 ± 0.2 [*]
BPD-PDT	none	2.1 ± 0.3 [*]
	BAPTA-AM + E-64d	2.2 ± 0.2 [*]

1c1c7 cultures were incubated with ABT-737 for 2 h. Alternatively, cells were incubated with 0.5 μ M BPD for 1 h. The medium was replaced and cultures were irradiated (690 nm, 135 mJ/sq cm). E-64d and BAPTA-AM (10 μ M each) were present where specified. DEVDase was measured 2 h after ABT-737 addition or irradiation. DEVDase units = nmol product/min/mg protein. Data represent mean $\pm$ SD for three determinations.

* Statistically different from control ($p < 0.05$).