Journal of Cellular Physiology

Inhibitive Effects of Photofrin on Cellular Autophagy

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Depending on the circumstances, autophagy can be either a protective or damaging cellular process. The role of autophagy in photodynamic therapy (PDT), a photo-chemotherapy that utilizes light to activate a photosensitizer drug to achieve localized cellular damage, has been explored in recent years. It has been reported that autophagy in PDT is significantly influenced by the treatment protocol. In this work, the role of Photofrin, a well-established clinical photosensitizer, in regulating cellular autophagy was investigated. The effects of Photofrin on cellular autophagy induced by conventional starvation or rapamycin techniques were studied. By fluorescence imaging, Western blotting and cell viability assays, it was found that Photofrin can effectively inhibit cellular autophagy induced by starvation or rapamycin. This autophagy blocking is independent of the photosensitizing property of the drug. With Baf-A1, a well-established agent that inhibits autophagosome from fusing with lysosome, we also found that, the observed phenomenon is not due to accelerated degradation of existing autophagosomes, thus proving that the drug Photofrin alone, without light excitation, can truly block autophagy.
J. Cell. Physiol. 224: 414–422, 2010.

In eukaryotic cells, lysosome/vacuole-dependent pathways that contribute to the degradation of intracellular components are macroautophagy, microautophagy, and chaperon-mediated autophagy (Shintani and Klionsky, 2004). Macroautophagy (hereafter referred as autophagy in this report) can be either a protective or damaging cellular process that significantly influences the fate of a cell (Klionsky and Emr, 2000; Shintani and Klionsky, 2004; Levine and Yuan, 2005). On the one hand, autophagy can protect cells by enveloping and breaking down damaged large molecules and organelles, or by providing intracellular nutrients and energy to those cells under starvation by decomposing certain intracellular substances. On the other hand, under certain circumstances, autophagy can promote a so-called autophagy-death (Kanzawa et al., 2003; Shimizu et al., 2004; Gonzalez-Polo et al., 2005; Reef et al., 2006; Xu et al., 2006).

Photodynamic therapy (PDT) is a treatment modality that utilizes photosensitizers and light to achieve localized biological damage. Singlet oxygen ($^1O_2$) is highly cytotoxic and can oxidize proteins, lipids, DNA, or other biological molecules, and is commonly accepted as the predominant agent mediating PDT cellular damage (Pass, 1993; Fritsch et al., 1998). In tissues, $^1O_2$ typically has a short lifetime (<0.04 μsec) and a corresponding small radius of action (<0.02 μm) (Moan and Berg, 1991; Dougherty et al., 1998). It is well established that, depending on its molecular characteristics, each different photosensitizer has its unique dominant subcellular localization, for example, Lysyl chlorine p6 for lysosomes; hypericin for endoplasmic reticulum (ER); and lysosomes, monocationic porphyrin for membranes; and porphycene monomer for mitochondria (Dougherty et al., 1998). The subcellular localization of a photosensitizer is also influenced by factors such as the time period the drug has been co-incubated with/administered to the target. As the subcellular target of a PDT treatment is closely governed by the localization of the photosensitizer (Pass, 1993; Dougherty et al., 1998), it is not surprising that different photosensitizers may evoke different combinations of apoptosis, necrosis, and autophagy (Buytaert et al., 2007; Kessel, 2007). It has been reported in recent literature that autophagy can protect cells from death at lower PDT doses, but as PDT dose increases, cellular death occurs, typically by apoptosis (Kessel et al., 2006b). In the absence of apoptosis, cells can die by alternative available routes including autophagy (Lockshin and Zakeri, 2004; Buytaert et al., 2006a).

Photosensitizers are known to have dark pharmacotoxicity (effect on cells without being excited by light energy). Given appropriate conditions, it is likely that the effects of autophagy can be influenced by the pharmacotoxicity of a photosensitizer (Chen et al., 2009). Photofrin is a well-established clinical photosensitizer that can be predominantly localized in mitochondria by controlling its co-incubation/administration time (Triesscheijn et al., 2006; Wu et al., 2006). In this study, the effect of Photofrin alone on autophagy was investigated.

Many techniques exist for monitoring cellular autophagy (Klionsky et al., 2008). In this study, using GFP-LC3 labeled autophagosome fluorescence, the LC3 conversion (Western blotting) and the corresponding cell viability assay, it was found that Photofrin alone, without light excitation, can effectively inhibit cellular autophagy induced by starvation or rapamycin. As autophagy is a dynamic multi-step process (Klionsky and Emr, 2000; Klionsky et al., 2008), the effect of Photofrin on autophagy was also investigated by blocking the fusion of autophagosomes with lysosomes with Bafilomycin A1 (Baf-A1). It was found that Photofrin blocks the formation, rather than accelerates the degradation, of autophagosomes. As a supplementary study, the inhibitive properties of Photofrin on autophagy were also tested in in vitro PDT for its potential impact on biological outcomes of the treatment.

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

Contract grant sponsor: The National Basic Research Program of China;
Contract grant number: 2010CB732602.
Contract grant sponsor: The Program for Changjiang Scholars and Innovative Research Team in University;
Contract grant number: IRT0829.
Contract grant sponsor: The National Natural Science Foundation of China;
Contract grant numbers: 30870676, 30870658.

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Received 29 July 2009; Accepted 12 February 2010

Published online in Wiley InterScience (www.interscience.wiley.com.), 2 April 2010.
DOI: 10.1002/jcp.22137

Materials and Methods

Cell culture and transfection

Human lung adenocarcinoma (ASTC-a-1) and human cervical carcinoma (HeLa) cells were cultured in Dulbecco's modified Eagle medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μg/ml) in 5% CO_2, 95% air at 37°C in a humidified incubator.

The plasmid, rat microtubule-associated protein light chain 3 (LC3) inserted into pEGFP-C1 (Clontech, Palo Alto, CA, USA) and pBabe-puro vectors (GFP-LC3, a kind gift from Professor Marja Jäättelä, Apoptosis Department, Institute of Cancer Biology, Danish Cancer Society, Denmark) (Kabeya et al., 2004; Hoyer-Hansen et al., 2007), was transfected using the Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. ASTC-a-1 cells that stably expressed GFP-LC3 protein were selectively obtained for the study with G418 (0.8 mg/ml, Sigma–Aldrich, St Louis, MO).

Autophagy inducers and inhibitors

A cellular autophagy model was established by subjecting cells to either dual starvation of amino acid and serum, or 100 nM rapamycin (Alexis Biochemicals, Lausen, Switzerland). Both techniques are well established for autophagy induction.

To date, there are no clearly defined autophagy inhibitors. Most of the so-called autophagy inhibitors have complex effects on cellular pathways. Nevertheless, 3-methyladenine (3-MA, Sigma–Aldrich) and Baf-A1 (Sigma–Aldrich), are currently accepted as standard autophagy inhibitors and were used in the current study (5 mM and 10 nM, respectively). They were added to the culture 30 min before each corresponding experiment.

Cell viability assay

Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was used in the current study to evaluate cell viability after various treatments. Optical density (OD) was directly read using an automatic plate reader (Tecan infinite M200, Grodig, Austria) from each microplate well. The value was normalized to that of the untreated cells from the same plate.

Fluorescence imaging assays

Cells transfected with GFP-LC3 were observed by fluorescence imaging assays were used as an indicator to monitor the production of autophagosomes in real time. Briefly, cells were cultured on coverslips and maintained at 37°C and 5% CO_2 throughout each experiment in a mini-incubator (CTI-Controller 3700, Zeiss, Jena, Germany) built on the microscope stage. The time series of the fluorescence were imaged with a commercial laser scanning microscope combination system (LSM 510, Zeiss) equipped with a Plan-Neofluar 100×/1.3 NA Oil objective. The GFP fluorescence was excited at 488 nm with an Argon ion laser and the emission was recorded through a 500–550 nm band-pass filter. To minimize the undesired photodynamic effects caused by the 488 nm light used for image collection, a well-established singlet oxygen quencher, Dehydro-L-(+)ascorbic acid dimer (DHA, 3.5 mM, Sigma–Aldrich), was added to the samples that were used for monitoring the effect of Photofrin on the production of autophagosomes induced by starvation.

To identify the subcellular localization of Photofrin, cells were incubated with Photofrin (20 μg/ml, Axcan Pharma Ltd, Dublin, Ireland) in the dark for 16 h. Then, the cells were co-loaded with 100 nM MitoTracker deep red 633 (Molecular Probes, Eugene, OR, USA), which localizes in cell mitochondria, for 15 min. Prior to imaging, cells were washed three times with DMEM and maintained in the final fresh replacement of culture medium. Confocal images of Photofrin fluorescence from the cells were obtained using a 488 nm excitation light from an argon laser and a 600–650 nm band-pass barrier filter. Images of MitoTracker deep Red 633 fluorescence from the cells were obtained using a 633 nm excitation light from the He–Ne laser and a 650-nm long-pass filter. Control studies were performed to assure that there was no crossover of the signals. In specific, the fluorescence of MitoTracker is negligible when the excitation and imaging collection was set for Photofrin fluorescence, and vice versa.

Western blotting

Briefly, after a treatment of starvation for 1 h or rapamycin for 2 h, cells were washed three times with pre-cooled PBS and then harvested. The cells were lysed in a buffer containing 50 mM Tris (adjusted to pH 8.0 with HCl), 150 mM NaCl, 1% TritonX-100, 100 μg/ml PMSF, and 1× protease inhibitor cocktail set I (Calbiochem, San Diego, CA, USA). The samples were separated by 15% SDS–polyacrylamide gel electrophoresis and transferred onto PVDF membrane. The resulting membrane was blocked with 5% skim milk, incubated with designated primary and secondary antibodies. The following antibodies were used for immunoblot: anti-MAP1-LC3 (Novus Biologicals, Littleton, CO, USA), anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), IR-Dye®800CW anti-rabbit IgG (Rockland, Gilbertsville, PA), and Alexa Fluor 680® goat anti-mouse IgG (Molecular Probes). The signals were detected with an ODYSSEY Infrared Imaging System (LI-COR, Lincoln, NE, USA) and the gray value of protein bands quantified with Photoshop (Adobe, San Jose, CA) was used to determine the ratio of LC3-II/Actin.

PDT treatment

Since Photofrin is a clinical photosensitizer, the effects of its potential intrinsic autophagy blocking properties on treatment were investigated. Cells were cultured in 96-well microplates at a density of 5×10^3 cells/well or on coverslips for 12–24 h, then co-incubated with Photofrin (5 μg/ml) for 16 h. Prior to a light irradiation, cells were washed three times with DMEM and maintained in the final fresh replacement of culture medium. For PDT light irradiation, two types of light source were utilized. A semiconductor laser (NL-FBA-2.0-635, nLight Corporation, Shanghai, China, 635 nm with maximum output of 2 W) was used for cell viability assay, and a He–Ne laser (0.76 μW, 633 nm, built-in component of the laser scanning microscope system) was used for confocal study. The cells were irradiated at 10 mW/cm^2 for various times at room temperature.

Experimental procedures

To evaluate the effects of Photofrin alone on autophagy, cells were cultured in DMEM overnight to allow it to adhere, then co-incubated with Photofrin (5 μg/ml) for 16 h in the dark, and finally subjected to EBSS starvation, rapamycin, or PDT treatment, as described above. After the treatments, autophagy of the processed cells was directly or indirectly evaluated by using cell viability assay, fluorescence imaging technique, and Western blotting.

Data analysis

To evaluate the production of autophagosomes in the fluorescence imaging results, GFP-LC3 puncta were counted or the pixels of the GFP-LC3 conglomeration were assessed with Photoshop when Baf-A1 was used as an inhibitor. Each data set was collected from at least two independent experiments, with a minimum of 7 cells per experiment. The *P*-value was analyzed with a student *t*-test. The absolute number of LC3 puncta itself may not be the best presentation of autophagosomes, as there are reported false-positive in previous studies (Kuma et al., 2007). On the other hand, changes in the number of puncta, once a cell is subjected to autophagy, is a much more reliable indicator for the status of autophagosomes (Klionsky et al., 2008). Therefore, the increase of GFP-LC3 puncta after an autophagy inducer added was selected as the autophagy indicator for this article.

Results

Subcellular localization of Photofrin

Over years of research and clinical work, it has been well established that mitochondria are a significant binding site for Photofrin (Wilson et al., 1997). This requires a typical in vitro co-incubation/in vivo administration time of around 16–24 h (Wilson et al., 1997; Dougherty et al., 1998). The subcellular localization of Photofrin in our experiment was investigated with fluorescence imaging for the cell lines in use. As shown in Figure 1, after 16 h of co-incubation, the subcellular localization of Photofrin is closely related to that of mitochondria, while its presence in cell membrane is also demonstrated. This is consistent with previous reports by this and other laboratories (Wilson et al., 1997; Wu et al., 2006). Commercial Photofrin is purified to be around 85% oligomeric materials with a small percentage of non-fluorescent aggregates (Hennessey et al., 2003). It has been reported that some of the non-fluorescent aggregates may be decomposed into fluorescent components after being taken up by cells (Croce et al., 2002). Nevertheless, it cannot be completely excluded that certain non-fluorescent aggregates may still exist in the cells and their subcellular localization is not identified by the presented fluorescence images.

Effect of DHA on autophagy

In the current study, DHA was used to minimize the photodynamic effect produced by the scanning laser during confocal microscopy study. As Scherz-Shouval et al. (2007) reported, reactive oxygen species (ROS) are essential for autophagy by regulating Atg4. The potential effect of DHA on the production of autophagosomes induced by starvation or rapamycin was investigated as part of our preliminary study to establish the conditions for the experiments. As shown in Figure 2, the inhibitory effect of DHA on the autophagosome production was minimal under starvation conditions (Fig. 2A and B), while it was almost unaffected under rapamycin treatment (Fig. 2C and D). This minor difference is likely due to the fact that rapamycin is a much milder autophagy inducer compared to starvation, as reported by Kawai et al. (2006). Given the circumstances, in the following study, DHA was only used in the fluorescence imaging assay when the stimulator was starvation but not in any other studies. Furthermore, to exclude a role of DHA in autophagy inhibition process, the inhibitory effect of Photofrin on autophagy was confirmed with cell viability assay and Western blotting, free of DHA, and light irradiation.

Inhibitive effect of Photofrin on autophagy

Autophagosome images were collected from cells subjected to starvation or rapamycin. As shown in Figure 3, autophagosome formation increased within 20 min after starvation and reached its peak at 40 min. When the cells were pre-treated with Photofrin and then subjected to the same starvation process, there was little autophagosome formation during the same period of time (Fig. 3B and E). The absence of autophagosome formation continued for up to 150 min, suggesting that the effect of the Photofrin is not a mere delaying of the autophagosome formation. A similar autophagosome inhibition effect by Photofrin was also observed with rapamycin (Fig. 3D and F).

By utilizing Baf-A1, the inhibitive effect of Photofrin on autophagy was further verified. In our preliminary study, Baf-A1 alone had no significant effect on the GFP-LC3 puncta for the cells cultured in nutrient culture (data not shown). As Figure 4A and C shows, Baf-A1 resulted in an accumulation of GFP-LC3 labeled autophagosomes/autolysosomes by inhibiting the fusion of autophagosomes with lysosomes. However, when the cells were pre-treated with Photofrin, the extent of the accumulation was clearly reduced (Fig. 4B and E, D and G). This indicates that Photofrin inhibits the production, rather than accelerates the degradation, of autophagosomes.

As a secondary verification of the inhibitory effect of Photofrin on autophagy, Western blotting assay with an LC3 antibody was used to monitor the conversion of LC3-I to LC3-II after various treatments. As demonstrated in Figure 5, Photofrin could inhibit the conversion of LC3-I to LC3-II induced whether by starvation or by rapamycin treatment. With Baf-A1, the degradation of autophagosomes was reduced, thus resulting in an accumulation of LC3-II. By adding Photofrin into the above Baf-A1 study, there was a clear reduction of LC3-I to LC3-II conversion. This is an independent confirmation that Photofrin inhibits the production of autophagosomes rather than accelerates the degradation of autophagosomes. The results from the Western blotting experiments are in complete agreement with the results of fluorescence imaging.

The effects of starvation, Baf-A1, and Photofrin were investigated with CCK-8 assay. Cell viability evaluated 5 days after 20 or 24 h starvation decreased significantly in both ASTC-a-1 and HeLa cells, respectively. By treating the cells with Baf-A1 prior to the starvation, the cell viability further decreased (Fig. 6A). This clearly suggests that the inhibition of autophagy has reduced the self-protective capability of the cells. In Figure 6A, it is demonstrated that Photofrin alone, at the concentration used in the study, had negligible cytotoxicity.

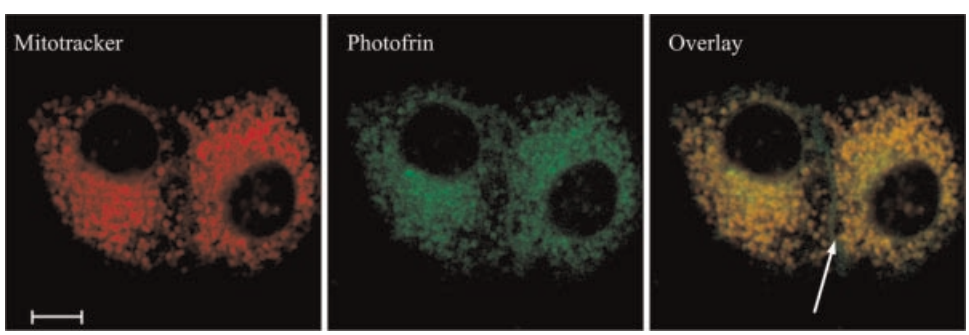

Fig. 1. Subcellular localization of Photofrin. Fluorescence imaging of ASTC-a-1 cells loaded with MitoTracker Red (left), Photofrin (middle), and corresponding overlay (right). The arrow indicates the position of cell membrane. Scale bar = 10 μm.

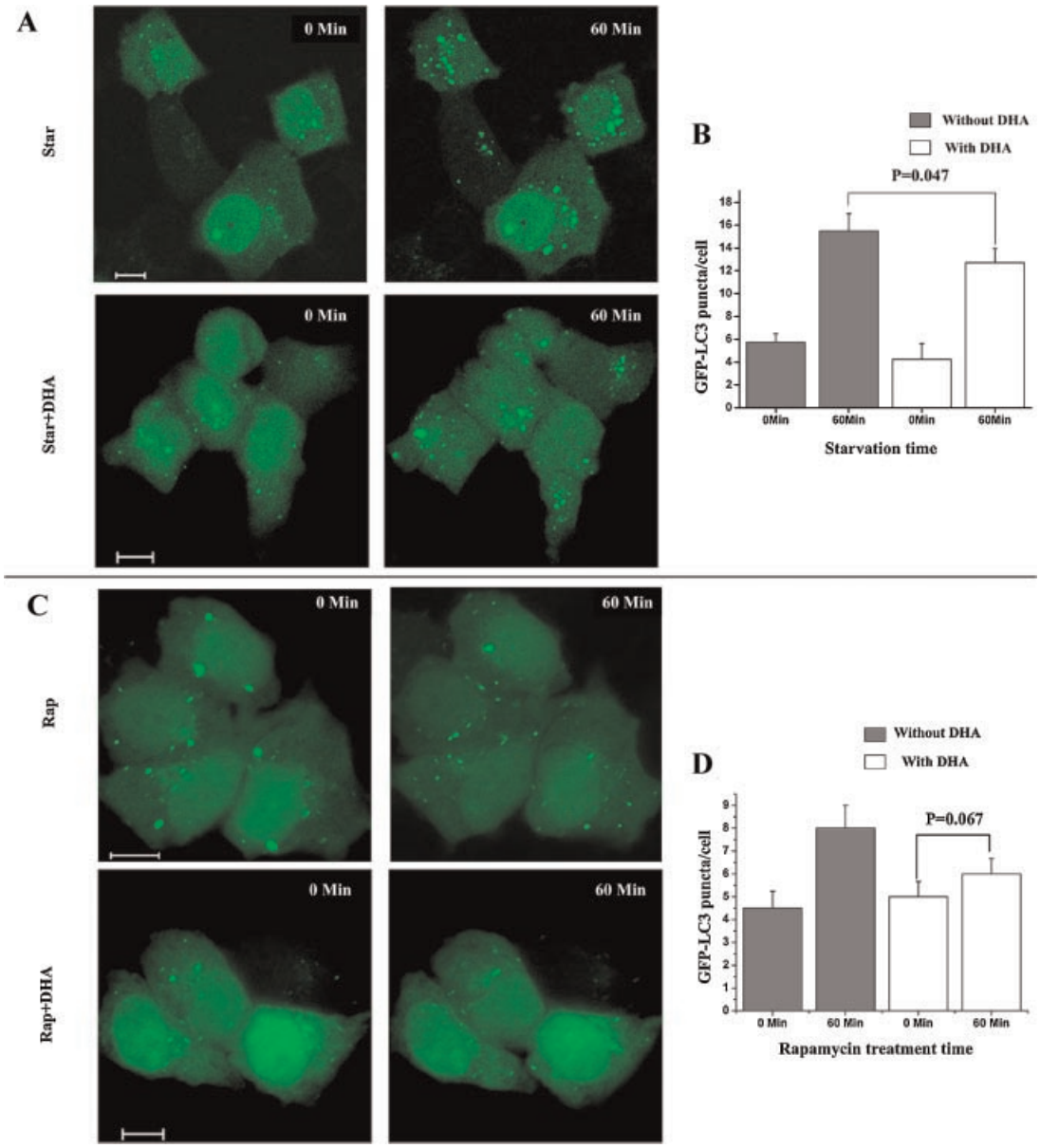

Fig. 2. Effect of DHA on autophagy. Effect of DHA on the production of autophagosomes induced by starvation (A,B) or rapamycin (C,D) in ASTC-a-1 cells, with B and D showing the corresponding numbers of GFP-LC3 puncta per cell. Each data is from two independently experiments, and at least 7 cells, *P*-value is evaluated by student *t*-test. Scale bar = 10 μm.

When the cells were co-incubated with Photofrin during the starvation, the viability of both cell lines was significantly reduced (Fig. 6A and B).

As described above, Photofrin can inhibit the formation of autophagosome and the conversion of LC3-I to LC3-II induced by EBSS starvation or rapamycin in ASTC-a-1 cells (Figs. 3–5). To test if this inhibitory effect is not a cell-type specific event, the same studies were repeated with HeLa cells. The results clearly demonstrate that the autophagy inhibition effect of Photofrin is also true for the HeLa cells, as shown in the supplemental Figures 1–3.

Autophagy in Photofrin-mediated PDT

The effect of 3-MA and Baf-A1 on Photofrin-mediated PDT, as evaluated with CCK-8 assay, is summarized in Figure 7. Each data set is normalized to that of untreated cells. The inhibitors showed little effect on the cell viabilities for the PDT treatment (One Way ANOVA followed by a post hoc Tukey's test). Figure 8 is a representative imaging sequence of in situ autophagosomes before and after a PDT treatment. These images demonstrate that, up to the death of the cells, there was no observable autophagosome formation.

Discussion

Depending on the circumstances, autophagy can serve as either a cellular survival or death pathway. For example, it is generally accepted that during nutrient deprivation and other forms of cellular stress including that from ROS, autophagy is pro-survival (Levine, 2005; Kaushik and Cuervo, 2006; Massey et al., 2006; Ogata et al., 2006). On the other hand, by utilizing

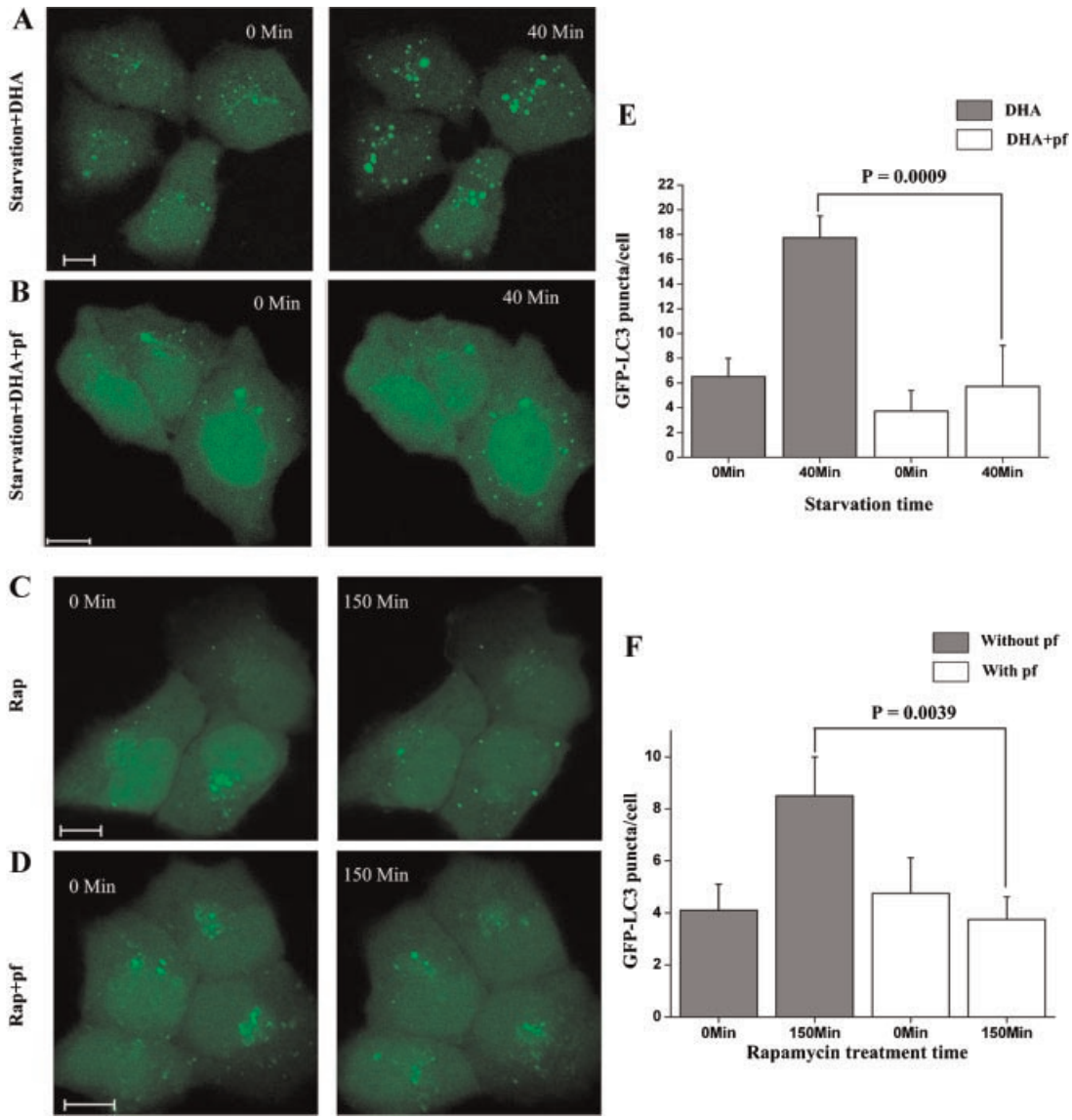

Fig. 3. Inhibitive effect of Photofrin on autophagy. Representative autophagosome fluorescence imaging sequences from ASTC-a-1 cells subjected starvation or rapamycin only (A and C) and that pre-treated with Photofrin followed by starvation or rapamycin (B and D), with E and F showing corresponding numbers of GFP-LC3 puncta per cell. Each data is from two independently experiments, and at least 7 cells, *P*-value is evaluated by student *t*-test. Scale bar = 10 μm.

the ER-associating photosensitizer hypericin, Buytaert et al. (2006a,b) report that, by inhibiting apoptotic pathways, HeLa cells experience a sustained autophagy that ultimately leads to cell death. Kessel et al. (2006a,b) report that, with yet another ER-associating photosensitizer porphycene CPO, autophagy in PDT can either promote cellular survival or death, depending on the extent of PDT damage. It is well established that photosensitizers with different subcellular localization have different mechanisms to destroy target cells (Pass, 1993; Dougherty et al., 1998). For photosensitizers from different pharmacological categories, the effects of autophagy and its role in cellular pathways are likely to be complex and need to be investigated accordingly.

In the current study, the most important report is that Photofrin itself, without light activation as in a typical PDT treatment, can be an effective autophagy inhibitor. With fluorescence imaging, we have observed that autophagosome formation can start as early as 20 min after cells are subjected to starvation. Autophagy plays a significant role in protecting cells from damage caused by starvation. This is evidenced by the fact that autophagy inhibitor Baf-A1 could induce additional cell damage during extended starvation. Although Photofrin could also reduce cell viability in cellular starvation, its mechanism is somewhat different from that of Baf-A1. By fluorescence imaging and Western blotting assay, it has been observed that Photofrin can reduce the GFP-LC3 puncta and LC3-II conversion induced by starvation and rapamycin, indicating that Photofrin possesses an autophagy inhibition property. However, in comparison with Baf-A1, Photofrin blocks autophagy by inhibiting the formation of autophagosomes, rather than inhibiting the fusion between lysosomes and autophagosomes.

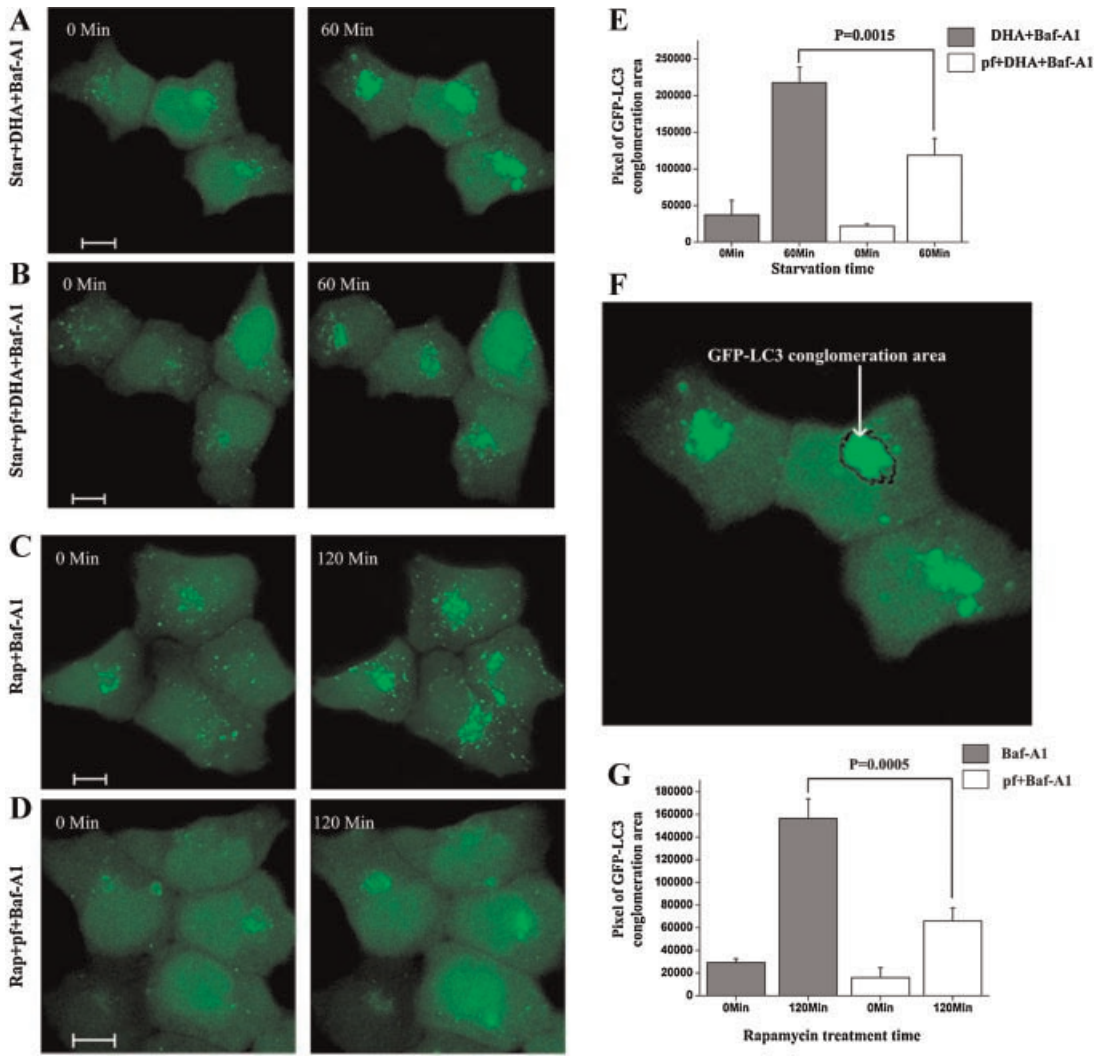

Fig. 4. Inhibitive effect of Baf-A1 on aggregates of autophagosomes. Baf-A1 inhibition of the degradation of autophagosomes induced by starvation (A and B) or rapamycin (C and D) when Photofrin was absent (A and C) or present (B and D) in ASTC-a-1 cells, with E and G showing corresponding numbers of pixel counts of GFP-LC3 conglomeration per cell. And F is a representative for analysis of the aggregates of autophagosones. Each data is from two independently experiments, and at least 7 cells, *P*-value is evaluated by student *t*-test. Scale bar = 10 μm.

Although we have demonstrated that the autophagy inhibition effect of Photofrin is independent of PDT, it is natural to test how Photofrin affects autophagy in PDT. The autophagy inhibition property of Photofrin was tested with in vitro PDT. Fluorescence imaging was used to monitor autophagosomes in situ during PDT treatment. Regardless of the irradiation light fluence, there was no autophagosome generation in the treated cells up to their PDT death. Furthermore, autophagy inhibitors 3-MA and Baf-A1 had little effect on the biological outcome of Photofrin-medicated PDT in vitro. This evidence further supports our hypothesis that the photosensitizer Photofrin itself is an effective inhibitor of autophagy. To the best of our knowledge, the current study is the first report of autophagy investigation with Photofrin. The absence of autophagy in Photofrin-mediated PDT is contradictory to what has been reported in other photosensitizers. Evidently, there are several literature reports that autophagy does occur after PDT mediated by various photosensitizers (Buytaert et al., 2006a; Kessel et al., 2006b; Kessel and Reiners, 2007; Xue et al., 2007). The lack of autophagy in Photofrin-mediated PDT is attributed to the fact that Photofrin itself may be an autophagy inhibitor. This difference between Photofrin and photosensitizers of other chemical nature may have an important impact on the future research of PDT.

The effect of Photofrin on autophagy is likely to be a complex issue and attributed to several factors that are yet to be explored, specifically that Photofrin alone can induce a number of enzymes that may impact cellular events (Gomer et al., 1991). One of the most probable mechanisms may relate to the fact that, given proper co-incubation/administration time, Photofrin is predominately localized on mitochondria. It has been traditionally assumed that mitochondria turnover is a result of cellular autophagy (Rodriguez-Enriquez et al., 2006; Kim et al., 2007). By blocking caspases in eukaryotic cells to prevent the cells from rapidly developing apoptosis, it was found that mitochondria could be selectively eliminated by autophagy (Xue et al., 2001; Tolkovsky et al., 2002). Similarly, by examining CHO-K1 cells transfected with a mitochondrial matrix green

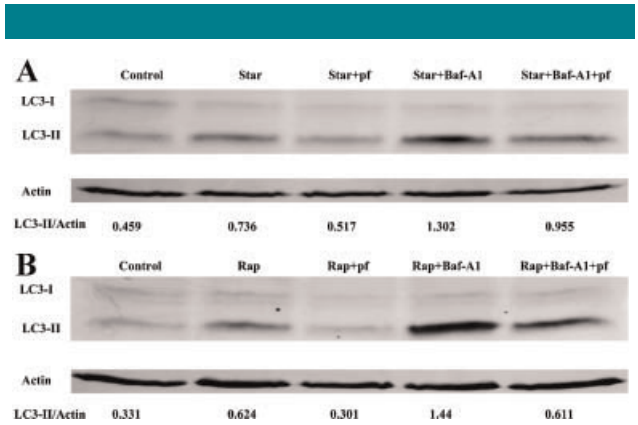

Fig. 5. Effects of different treatments on conversion of LC3-I to LC3-II. Western blotting assay to detect the LC3-I to LC3-II conversion after ASTC-a-1 cells were subjected to starvation (A) or rapamycin (B) combined with various treatments: Photofrin (pf), starvation (Star), rapamycin (Rap), and Bafilomycin-A1 (Baf-A1).

fluorescent protein (mtGFP), it was shown that the extent of autophagy induced via several different means is directly related to the degradation of mtGFP (Kawai et al., 2006). These results clearly demonstrate the involvement of mitochondria in autophagy, yet the actual role of mitochondria is not clearly defined. Combining the results of the current study that Photofrin can completely block autophagosome production and the known fact of its significant co-localization with mitochondria, it is reasonable to propose that mitochondria may be an active component in regulating autophagosome generation, rather than a mere downstream event of the process. Clearly, this speculation requires further in-depth investigation for its validation.

In conclusion, the current study has investigated the effect of a clinical drug Photofrin on autophagy. It is demonstrated that Photofrin has a clear inhibitive effect on cellular autophagy induced by various stimuli. Presently, most of the currently available autophagy inhibitors are not fit for in vivo applications. Although it has several known side effects such as prolonged skin sensitization, Photofrin is a well-established clinical drug with abundant pharmacokinetic data and application experience. It is reasonable to suggest that Photofrin may be a candidate as an autophagy inhibitor and it is justified for future in vivo research.

Acknowledgments

The authors thank Professor Marja Jäättelä (Apoptosis Department, Institute of Cancer Biology, Danish Cancer Society, Denmark) for kindly providing the GFP-LC3 plasmid and Professor Fred W. Hetzel (Radiation Oncology, University of Colorado, Denver, USA) for his expert advice and proof of the manuscript. The authors sincerely thank David T. Chen

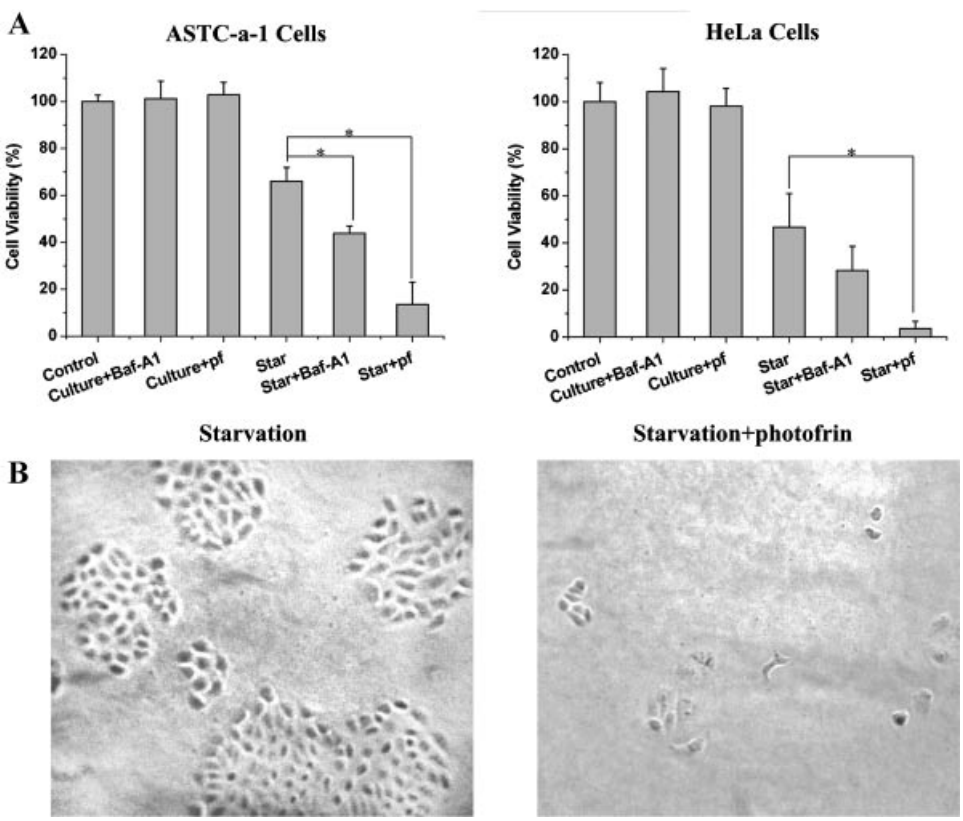

Fig. 6. Inhibitive effects of Photofrin and Baf-A1 on starved cell viabilities. (A) Cell viability assays (normalized to that of untreated cells) of ASTC-a-1 and HeLa cells subjected to combinations of Photofrin (pf), starvation (Star), and autophagy inhibitor Baf-A1. CCK-8 activity assay was conducted 5 days after starvation. Data are representative of four independent experiments. Star (*) indicates statistically significant difference between groups (student *t*-test, $P < 0.001$). (B) Optical microscopy image of cells (ASTC-a-1) 5 days after starvation with and without Photofrin.

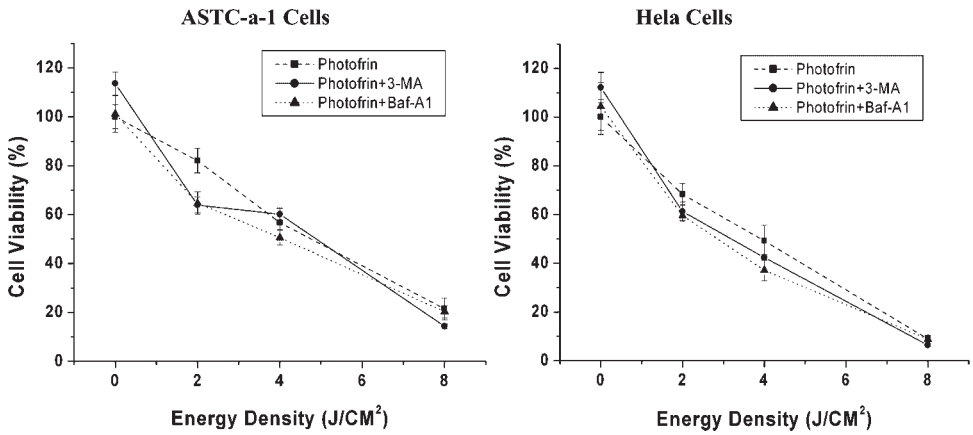

Fig. 7. Autophagy inhibitors have little effects on Photofrin-induced PDT. CCK-8 activity assay was conducted 12 h after PDT with a dose escalation. The data is normalized to that of untreated cells for each corresponding group. Each data point is from four independent experiments. There is no statistically significant difference among groups for either ASTC-a-1 or HeLa cells. (One Way ANOVA followed by a post hoc Tukey's test, $P > 0.05$).

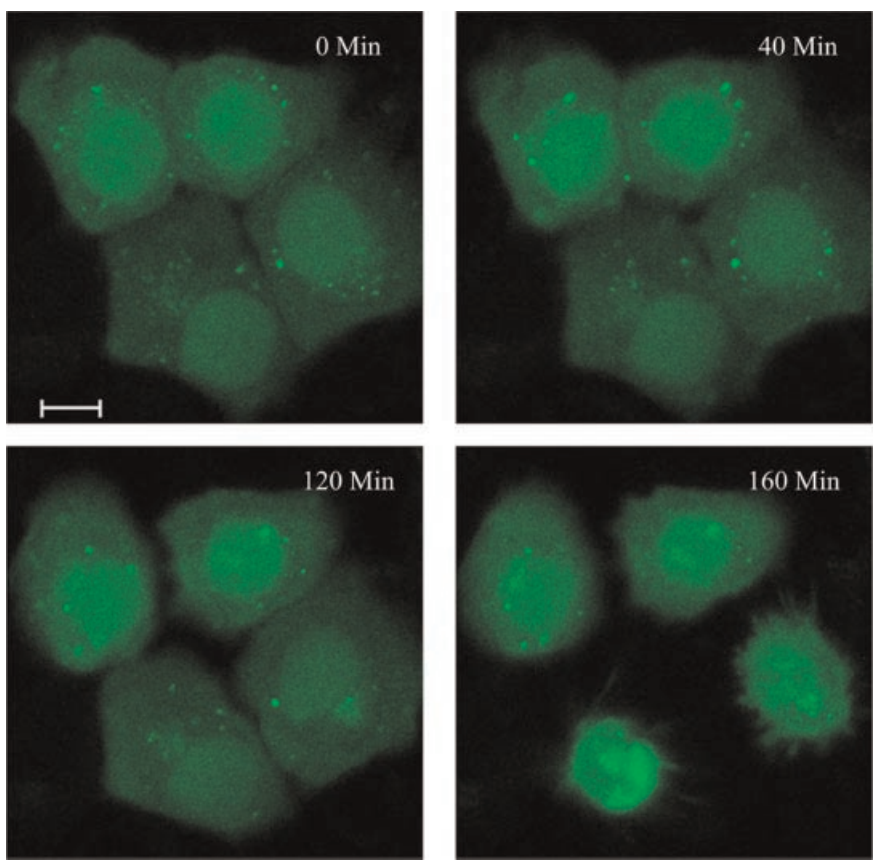

Fig. 8. No autophagosome formation in Photofrin-induced PDT. A representative in situ autophagosome fluorescence imaging sequence from a group of ASTC-a-1 cells before and after PDT treatment (Photofrin 5 μg/ml, 1 J/cm^2). Scale bar = 10 μm.

from Golden, Colorado, USA, for his editing and proofreading of the manuscript. This study was supported by the National Basic Research Program of China, contract grant no. 2010CB732602; the Program for Changjiang Scholars and Innovative Research Team in University, contract grant no. IRT0829; and the National Natural Science Foundation of China, contract grant nos. 30870676 and 30870658.

Literature Cited

Buytaert E, Callewaert G, Hendrickx N, Scorrano L, Hartmann D, Missiaen L, Vandenheede JR, Heirman I, Grooten J, Agostinis P. 2006a. Role of endoplasmic reticulum depletion and multidomain proapoptotic BAX and BAK proteins in shaping cell death after hypericin-mediated photodynamic therapy. FASEB J 20:756–758.

Buytaert E, Callewaert G, Vandenheede JR, Agostinis P. 2006b. Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum. Autophagy 2:238–240.

Buytaert E, Dewaele M, Agostinis P. 2007. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. Biochim Biophys Acta 1776:86–107.

Chen YJ, Huang WP, Yang YC, Lin CP, Chen SH, Hsu ML, Tseng YJ, Shieh HR, Chen YY, Lee JJ. 2009. Platonin induces autophagy-associated cell death in human leukemia cells. Autophagy 5:173–183.

Croce AC, Supino R, Lanza KS, Locatelli D, Baglioni P, Bottiroli G. 2002. Photosensitizer accumulation in spontaneous multidrug resistant cells: A comparative study with Rhodamine 123, Rose Bengal acetate and Photofrine. Photochem Photobiol Sci 1:71–78.

Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M, Moan J, Peng Q. 1998. Photodynamic therapy. J Natl Cancer Inst 90:889–905.

Fritsch C, Goerz G, Ruzicka T. 1998. Photodynamic therapy in dermatology. Arch Dermatol 134:207–214.

Gomer CJ, Luna M, Ferrario A, Rucker N. 1991. Increased transcription and translation of heme oxygenase in Chinese hamster fibroblasts following photodynamic stress or Photofrin II incubation. Photochem Photobiol 53:275–279.

Gonzalez-Polo RA, Boya P, Pauleau AL, Jalil A, Larochette N, Souquere S, Eskelinen EL, Pierron G, Saftig P, Kroemer G. 2005. The apoptosis/autophagy paradox: Autophagic vacuolization before apoptotic death. J Cell Sci 118:3091–3102.

Hennessey C, Murray M, Pfaff K, Raff B, Randall P, Skurzewski T, Weber M. 2003. Light-activated therapy: Photodynamic therapy. In: Prasad PN, editor. Introduction to biophotonics. Hoboken, New Jersey: John Wiley & Sons, Inc. pp 433-463.

Hoyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, Farkas T, Bianchi K, Fehrenbacher N, Elling F, Rizzuto R, Mathiasen IS, Jaattela M. 2007. Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Mol Cell 25:193–205.

Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T. 2004. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J Cell Sci 117:2805–2812.

Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I. 2003. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res 63:2103–2108.

Kaushik S, Cuervo AM. 2006. Autophagy as a cell-repair mechanism: Activation of chaperone-mediated autophagy during oxidative stress. Mol Aspects Med 27:444–454.

Kawai A, Takano S, Nakamura N, Ohkuma S. 2006. Quantitative monitoring of autophagic degradation. Biochem Biophys Res Commun 351:71–77.

Kessel D. 2007. PDT: Death pathways. Proc SPIE 6427:642702–642708.

Kessel D, Reiners JJ Jr. 2007. Apoptosis and autophagy after mitochondrial or endoplasmic reticulum photodamage. Photochem Photobiol 83:1024–1028.

Kessel D, Vicente MG, Reiners JJ Jr. 2006a. Initiation of apoptosis and autophagy by photodynamic therapy. Autophagy 2:289–290.

Kessel D, Vicente MG, Reiners JJ Jr. 2006b. Initiation of apoptosis and autophagy by photodynamic therapy. Lasers Surg Med 38:482–488.

Kim I, Rodriguez-Enriquez S, Lemasters JJ. 2007. Selective degradation of mitochondria by mitophagy. Arch Biochem Biophys 462:245–253.

Klionsky DJ, Emr SD. 2000. Autophagy as a regulated pathway of cellular degradation. Science 290:1717–1721.

Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, Bergamini E, Bi X, Biard-Piechaczyk M, Blum JS, Bredesen DE, Brodsky JL, Brumell JH, Brunk UT, Bursch W, Camougrand N, Cebollero E, Cecconi F, Chen Y, Chin LS, Choi A, Chu CT, Chung J, Clarke PG, Clark RS, Clarke SG, Clave C, Cleveland JL, Codogno P, Colombo MI, Coto-Montes A, Cregg JM, Cuervo AM, Debnath J, Demarchi F, Dennis PB, Dennis PA, Deretic V, Devenish RJ, Di Sano F, Dice JF, Difiglia M, Dinesh-Kumar S, Distelhorst CW, Djavaheri-Mergny M, Dorsey FC, Droge W, Dron M, Dunn WA Jr, Duszenko M, Eissa NT, Elazar Z, Esclatine A, Eskelinen EL, Fesus L, Finley KD, Fuentes JM, Fueyo J, Fujisaki K, Galliot B, Gao FB, Gewirtz DA, Gibson SB, Gohla A, Goldberg AL, Gonzalez R, Gonzalez-Estevez C, Gorski S, Gottlieb RA, Haussinger D, He YW, Heidenreich K, Hill JA, Hoyer-Hansen M, Hu X, Huang WP, Iwasaki A, Jaattela M, Jackson WT, Jiang X, Jin S, Johansen T, Jung JU, Kadowaki M, Kang C, Kelekar A, Kessel DH, Kiel JA, Kim HP, Kimchi A, Kinsella TJ, Kiselyov K, Kitamoto K, Knecht E, Komatsu M, Kominami E, Kondo S, Kovacs AL, Kroemer G, Kuan CY, Kumar R, Kundu M, Landry J, Laporte M, Le W, Lei HY, Lenardo MJ, Levine B, Lieberman A, Lim KL, Lin FC, Liou W, Liu LF, Lopez-Berestein G, Lopez-Otin C, Lu B, Macleod KF, Malorni W, Martinet W, Matsuoka K, Mautner J, Meijer AJ, Melendez A, Michels P, Miotto G, Mistiaen WP, Mizushima N, Mograbi B, Monastyrska I, Moore MN, Moreira PI, Moriyasu Y, Motyl T, Munz C, Murphy LO, Naqvi NI, Neufeld TP, Nishino I, Nixon RA, Noda T, Nurnberg B, Ogawa M, Oleinick NL, Olsen LJ, Ozpolat B, Paglin S, Palmer GE, Papassideri I, Parkes M, Perlmutter DH, Perry G, Piacentini M, Pinkas-Kramarski R, Prescott M, Proikas-Cezanne T, Raben N, Rami A, Reggiori F, Rohrer B, Rubinsztein DC, Ryan KM, Sadoshima J, Sakagami H, Sakai Y, Sandri M, Sasakawa C, Sass M, Schneider C, Seglen PO, Seleverstov O, Settleman J, Shacka JJ, Shapiro IM, Sibirny A, Silva-Zacarin EC, Simon HU, Simone C, Simonsen A, Smith MA, Spanel-Borowski K, Srinivas V, Steeves M, Stenmark H, Stromhaug PE, Subauste CS, Sugimoto S, Sulzer D, Suzuki T, Swanson MS, Tabas I, Takeshita F, Talbot NJ, Talloczy Z, Tanaka K, Tanaka K, Tanida I, Taylor GS, Taylor JP, Terman A, Tettamanti G, Thompson CB, Thumm M, Tolkovsky AM, Tooze SA, Truant R, Tumanovska LV, Uchiyama Y, Ueno T, Uzcategui NL, van der Klei I, Vaquero EC, Vellai T, Vogel MW, Wang HG, Webster P, Wiley JW, Xi Z, Xiao G, Yahalom J, Yang JM, Yap G, Yin XM, Yoshimori T, Yu L, Yue Z, Yuzaki M, Zabirnyk O, Zheng X, Zhu X, Deter RL. 2008. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4:151–175.

Kuma A, Matsui M, Mizushima N. 2007. LC3, an autophagosome marker, can be incorporated into protein aggregates independent of autophagy: Caution in the interpretation of LC3 localization. Autophagy 3:323–328.

Levine B. 2005. Eating oneself and uninvited guests: Autophagy-related pathways in cellular defense. Cell 120:159–162.

Levine B, Yuan J. 2005. Autophagy in cell death: An innocent convict? J Clin Invest 115:2679–2688.

Lockshin RA, Zakeri Z. 2004. Apoptosis, autophagy, and more. Int J Biochem Cell Biol 36:2405–2419.

Massey AC, Kaushik S, Sovak G, Kiffin R, Cuervo AM. 2006. Consequences of the selective blockage of chaperone-mediated autophagy. Proc Natl Acad Sci USA 103:5805–5810.

Moan J, Berg K. 1991. The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. Photochem Photobiol 53:549–553.

Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K, Shiosaka S, Hammarback JA, Urano F, Imaizumi K. 2006. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol 26:9220–9231.

Pass HI. 1993. Photodynamic therapy in oncology: Mechanisms and clinical use. J Natl Cancer Inst 85:443–456.

Reef S, Zalckvar E, Shifman O, Bialik S, Sabanay H, Oren M, Kimchi A. 2006. A short mitochondrial form of p19ARF induces autophagy and caspase-independent cell death. Mol Cell 22:463–475.

Rodriguez-Enriquez S, Kim I, Currin RT, Lemasters JJ. 2006. Tracker dyes to probe mitochondrial autophagy (mitophagy) in rat hepatocytes. Autophagy 2:39–46.

Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. 2007. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J 26:1749–1760.

Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y. 2004. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat Cell Biol 6:1221–1228.

Shintani T, Klionsky DJ. 2004. Autophagy in health and disease: A double-edged sword. Science 306:990–995.

Tolkovsky AM, Xue L, Fletcher GC, Borutaite V. 2002. Mitochondrial disappearance from cells: A clue to the role of autophagy in programmed cell death and disease? Biochimie 84:233–240.

Triesscheijn M, Baas P, Schellens JH, Stewart FA. 2006. Photodynamic therapy in oncology. Oncologist 11:1034–1044.

Wilson BC, Olivo M, Singh G. 1997. Subcellular localization of Photofrin and aminolevulinic acid and photodynamic cross-resistance in vitro in radiation-induced fibrosarcoma cells sensitive or resistant to Photofrin-mediated photodynamic therapy. Photochem Photobiol 65:166–176.

Wu Y, Xing D, Chen WR. 2006. Single cell FRET imaging for determination of pathway of tumor cell apoptosis induced by Photofrin-PDT. Cell Cycle 5:729–734.

Xu Y, Kim SO, Li Y, Han J. 2006. Autophagy contributes to caspase-independent macrophage cell death. J Biol Chem 281:19179–19187.

Xue L, Fletcher GC, Tolkovsky AM. 2001. Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis. Curr Biol 11:361–365.

Xue LY, Chiu SM, Azizuddin K, Joseph S, Oleinick NL. 2007. The death of human cancer cells following photodynamic therapy: Apoptosis competence is necessary for Bcl-2 protection but not for induction of autophagy. Photochem Photobiol 83:1016–1023.