

# SINGLE CELL IMAGING OF BAX TRANSLOCATION DURING APOPTOSIS INDUCED BY PHOTOFRIN-PDT

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Apoptosis is an important cellular event that plays a key role in the therapy of many diseases. The mechanism of the initiation and regulation of photodynamic therapy (PDT)-induced apoptosis is complex. Our previous study found that Photofrin was localized primarily in mitochondria, the primary targets of Photofrin-PDT. The key role of Bax in the mitochondria-mediated apoptosis has been demonstrated in many systems. In order to determine the role of Bax in the mitochondrion-mediated apoptosis induced by Photofrin-PDT, we used the GFP-Bax plasmid to monitor the dynamics of Bax activation after PDT treatment. With laser scanning confocal microscopy, we found that Bax did not translocate from the cytosol to mitochondria when the mitochondrial membrane potential ( $\Delta\Psi_m$ ) disappeared, measured by TMRM. Thus, for Photofrin-PDT, the commitment to cell death is independent of Bax activation.

*Keywords:* Photofrin-photodynamic therapy; mitochondria-mediated apoptosis; Bax; activation; translocation.

## 1. Introduction

Bax, a proapoptotic member of the Bcl-2 family, localizes largely in the cytoplasm but translocates to mitochondria and undergoes oligomerization to induce the release of apoptogenic factors in response to apoptotic stimuli. This process is probably a consequence of the exposure of its C-terminal membrane-seeking domain, facilitated by unknown cytosolic factors or by an increase in cellular pH.<sup>1–3</sup> Whereas in the cytosol Bax exists as monomers, the mitochondrion-inserted Bax is present as dimers and higher oligomers.<sup>4</sup> The membrane insertion and oligomerization of Bax is essential for the release of cytochrome c and apoptosis, as evidenced by the blockage of apoptosis in Bax mutants that have lost the capacity for mitochondrion insertion

due to deletion of the mitochondrion-targeting C-terminus.<sup>5</sup>

The key role of Bax in the mitochondrion-mediated apoptosis has been demonstrated in many systems. Bax translocation is essential during TNF $\alpha$  induced apoptosis, either promoted by Bid or BimL,<sup>6,7</sup> in tumor cells or in normal cells.<sup>6–8</sup> Bax translocation is necessary for UV-irradiation induced apoptosis, either independent of Bid or related to BimL and P53.<sup>9–11</sup> Also, PDT promotes Bax activation, which includes conformational change (exposure of an epitope detected by the 6A7 antibody) as well as translocation from the cytosol to mitochondria immediately or shortly after PDT.<sup>12–15</sup>

However, HF-LPLI promotes Bax activation after the mitochondrial depolarization and

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cytochrome c release, indicating Bax activation is a downstream event during HF-LPLI induced apoptosis.<sup>16</sup> HF-LPLI induces apoptosis through caspase-3 activation pathway but not caspase-8 pathway.<sup>17,18</sup>

PDT causes photo-oxidative damage to proteins and lipids that reside within a few nanometers of the photosensitizer binding sites.<sup>19–21</sup> Hence, the subcellular localization of a photosensitizer critically influences the kinetics and the regulatory pathways activated following PDT.<sup>22</sup> Photofrin is largely distributed in the cell mitochondria,<sup>23,24</sup> and Photofrin-PDT induces apoptosis through caspase-3 activation pathway but not caspase-8 pathway.<sup>23,25</sup> Here, we determine the role of Bax in the mitochondrion-mediated apoptosis induced by Photofrin-PDT.

## 2. Materials and Methods

### 2.1. Cell culture

ASTC-a-1 cells were grown on 22-mm culture glasses, in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc. USA) supplemented with 15% fetal bovine serum (FBS) (GIBCO Co. LTD), 50 units/ml penicillin, and 50  $\mu\text{g}/\text{ml}$  streptomycin, 5%  $\text{CO}_2$ , 95% air at 37°C in a humidified incubator.

### 2.2. Chemicals

The following fluorescent probes were used: MitoTracker Deeper Red 633 (100 nM) to stain mitochondria; TMRM to monitor  $\Delta\Psi_m$ . All the probes were purchased from Molecular Probes (MP, USA). The optimal incubation time for each probe was determined experimentally.

Lipofectamine<sup>TM</sup> 2000 reagent (Invitrogen life technologies, Inc. Grand Island, NY, USA) was used to transfect plasmid DNAs (pGFP-Bax) into cells. Cells were examined 36–48 h after transfection.

### 2.3. PF-PDT treatment

For irradiation of cells, a 633-nm He–Ne laser inside a confocal laser scanning microscope (LSM510-ConfoCor2) (Zeiss, Jena, Germany) was used in PF-PDT treatment. Laser irradiation was performed through the objective lens of the microscope. In this setup, only the cells under observation were irradiated by the laser. A minitype culture chamber with  $\text{CO}_2$  supply (Tempcontrol 37-2 digital, Zeiss,

Germany) was used in order to keep cells under normal culture conditions (37°C, 5%  $\text{CO}_2$ ) during irradiation. Under the PF-PDT treatment, cells were incubated in the dark with Photofrin in complete growth medium for 20 h, and then rinsed with PBS before irradiation. The cells in selected area were irradiated for 30 s with a laser dose of 5  $\text{J}/\text{cm}^2$  and Photofrin concentrations of 2.5  $\mu\text{g}/\text{ml}$ .

### 2.4. Imaging analysis of living cells

In order to capture image of single cells, the confocal laser scanning microscope system (LSM510-ConfoCor2) (Zeiss, Jena, Germany) was used. The system was equipped with a krypton–argon air-cooled laser ( $\text{Ar}^+$ ) (30 mW) and a He–Ne laser (HeNe) (5 mW) for excitation illumination. Cell images before and after laser irradiation were acquired with a Plan-Neofluar 100X/NA1.3, oil-immersed objective lens. Cells were maintained at 37°C, 5%  $\text{CO}_2$  during imaging with the minitype culture chamber with  $\text{CO}_2$  supply.

The following specific settings were used for light excitation and emissions: MitoTracker Deeper Red 633 (HeNe laser, Ex. 633 nm, Em. LP 650 nm, HFT UV/488/543/633 nm); GFP ( $\text{Ar}^+$  laser, Ex. 488 nm, Em. BP 500–550 nm, HFT UV/488/543/633 nm); TMRM ( $\text{Ar}^+$  laser, Ex. 543 nm, Em. LP560 nm, HFT UV/488/543/633 nm); Photofrin ( $\text{Ar}^+$  laser, Ex. 458 nm, Em. BP 600–650 nm, HFT 458/514 nm).

## 3. Results

To determine the subcellular localization of Photofrin, fluorescence emissions from the cells were captured using the LSM confocal microscope after the ASTC-a-1 cells were loaded with the photosensitizer for 12 h at 37°C in the dark. As shown in Fig. 1 (left panel), Photofrin displayed a punctuated pattern of fluorescence emission primarily in the perinuclear area. To assess whether Photofrin binds to the mitochondria, emission spectra of cells coincubated with MitoTracker Red, a mitochondrion-specific dye, were also obtained (Fig. 1, middle panel). In the overlay emission image (Fig. 1, right panel), the Photofrin fluorescence corresponded closely to that of MitoTracker Red, indicating the mitochondrial localization of Photofrin in the tumor cells.

To investigate the possibility of Bax translocation under PF-PDT treatment, a series of experiments were performed as follows. For monitoring

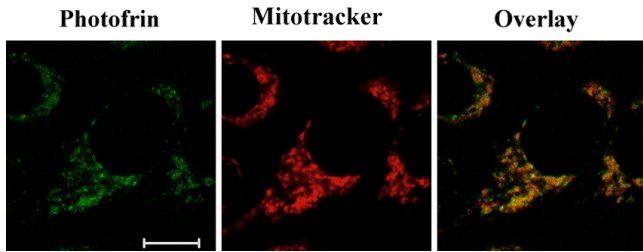


Fig. 1. Localization of Photofrin in ASTC-a-1 cells. Fluorescent images of ASTC-a-1 cells incubated with Photofrin for 20 h, then stained by MitoTracker for localization in mitochondria. Photofrin is largely distributed in the cell mitochondria. Bar = 10  $\mu$ m.

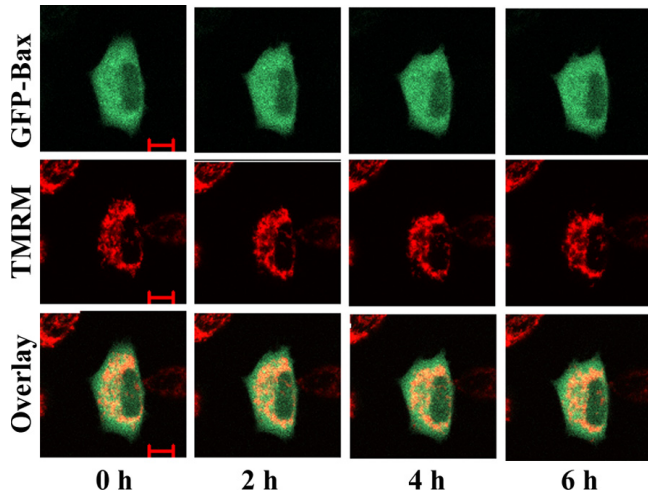


Fig. 2. Subcellular location of Bax in physiological condition. ASTC-a-1 cells were transfected with GFP-Bax and stained with TMRM for localizing Bax and mitochondrial membrane potential. Fluorescence images were acquired by confocal microscopy. Bar = 10  $\mu$ m.

the subcellular location of Bax in normal conditions, ASTC-a-1 cells were transfected with pGFP-Bax and stained with TMRM, and imaged by confocal microscopy. In control cells, GFP-Bax was largely cytosolic or loosely associated with mitochondria, as shown in Fig. 2, and mitochondrial membrane potential remained during detection. Cells treated with STS (1  $\mu$ M) for four hours were used as a positive control to show Bax translocation to mitochondria, and the mitochondrial depolarization occurred after Bax translocation (Fig. 3).

In order to know the relationship between mitochondrial depolarization and the changes of Bax's subcellular location under PF-PDT treatment, ASTC-a-1 cells transfected with pGFP-Bax and then labeled with TMRM were treated with PF-PDT, and monitored by confocal microscopy. Under PF-PDT treatment, when mitochondrial

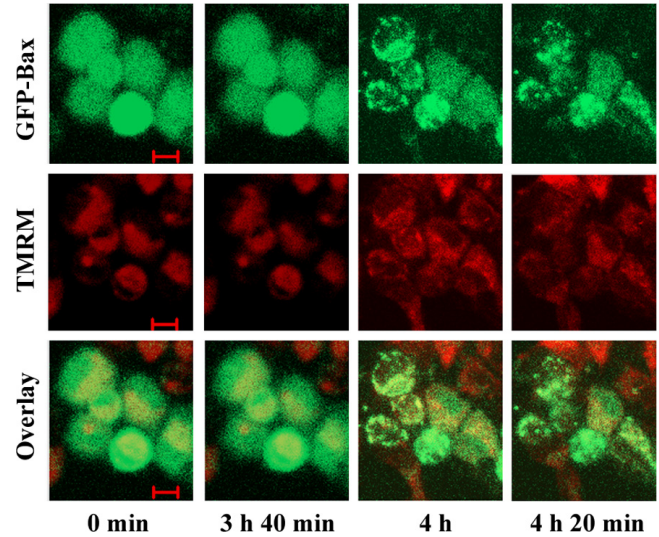


Fig. 3. Time sequence of Bax translocation and mitochondrial depolarization under STS treatment. ASTC-a-1 cells expressing GFP-Bax and labeled by TMRM were treated with STS. Bar = 10  $\mu$ m.

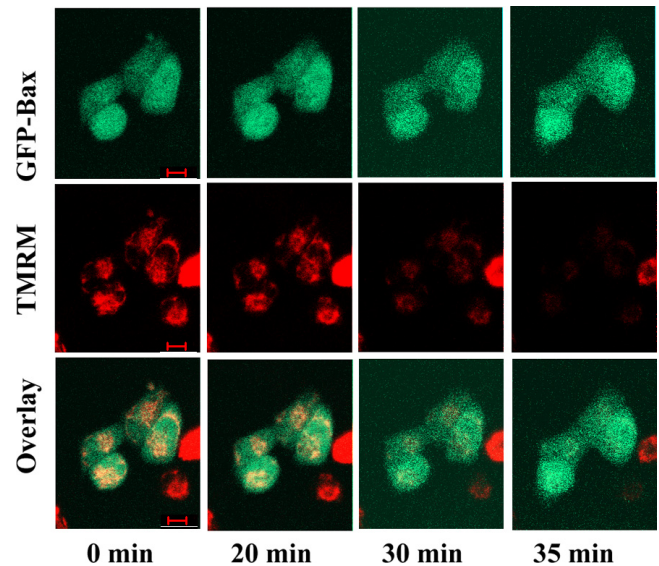


Fig. 4. Time sequence of Bax translocation and mitochondrial depolarization under PDT treatment. ASTC-a-1 cells expressing GFP-Bax and labeled by TMRM were treated with PDT. Bar = 10  $\mu$ m.

depolarization occurred, Bax translocation did not occur as manifested in Fig. 4. Taken together, these results indicate that Bax activation is not necessary for PF-PDT treatment which triggers apoptosis from mitochondria.

#### 4. Discussion

Since the first report of apoptosis in PDT-treated cells,<sup>26</sup> it has been found to be a prominent form

of cell death in response to PDT, evidenced by assays measuring either the fragmentation of DNA or the condensation of chromatin.<sup>20,27–32</sup> Apoptosis can be induced by the ligation of plasma membrane death receptors, which stimulate the “extrinsic” pathway, or by the perturbation of intracellular homeostasis, the “intrinsic” pathway.<sup>33</sup> Current evidence suggests that the most common pathway for apoptosis in PDT-treated cells involves mitochondria; however, other pathways, especially those through caspase-8, may also be important, particularly when the dominant pathway is suppressed.<sup>22</sup> PDT inflicts damage to cells largely via ROS. The ROS travels only about several tens of nanometers before reacting with a molecule.<sup>34</sup> It is clear that the subcellular localization of photosensitizer coincides with primary site of photodamage, and local photodamage to specific subcellular targets influences the kinetics and the regulatory pathways activated by PDT.<sup>22,35–37</sup>

Numerous reports have indicated mitochondria as important targets of PDT.<sup>22</sup> The binding of photosensitizers to mitochondria has been associated with the efficient induction of apoptosis.<sup>38–40</sup> In this study, we confirmed a strong mitochondrial localization of Photofrin in ASTC-a-1 cells, as shown in Fig. 1; the fluorescence emission of Photofrin corresponds spatially to the fluorescence emission of MitoTracker Red in the tumors. Furthermore, Photofrin-PDT induced apoptosis through caspase-3 activation pathway but not caspase-8 pathway,<sup>23,25</sup> so apoptosis may be triggered from mitochondria.

Bax is a proapoptotic member of the Bcl-2 family of proteins, which are implicated in the pathogenesis of cell death in an increasing number of models of apoptosis both *in vivo* and *in vitro*. Bax is constitutively present in many cell types that undergo apoptosis in response to a variety of stimuli. Translocation of preformed Bax from the cytosol to the mitochondria has been reported with a variety of apoptotic stimuli.<sup>4,41–44</sup>

Early reports show that there are two main mechanisms for mitochondrial permeabilization.<sup>45</sup> One is mediated by a long-lasting MPT demonstrated in the present studies. Another is mediated by proapoptotic members of Bcl-2 family, in which Bax/Bak is the most important member. Therefore, we tested the activity of Bax under the PF-PDT treatment. Results showed that during mitochondrial depolarization, Bax translocation did not occur as manifested in Fig. 4. These results

seemed to show that mitochondrial injury was independent of Bax activation.

Combining the results of present studies and the previous reports, we conclude the signaling pathway induced by PF-PDT: Light is absorbed by the photosensitizers, and this generates a great deal of ROS which is mainly produced in mitochondria other than cytosol. The ROS generation induces mitochondrial injury, and finally cell apoptosis. In particular, this injury is independent of Bax activation.

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