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Therapeutic enhancement of vascular-targeted photodynamic therapy by inhibiting proteasomal function

Zhuzhu Li, Priyanka Agharkar, Bin Chen *

Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy, University of the Sciences, Philadelphia, PA 19104, USA

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ABSTRACT

Vascular-targeted photodynamic therapy (vPDT) is a novel vascular targeting modality based on site-directed delivery of a photosensitizer to tumor vasculature, which induces reactive oxygen species (ROS)-mediated vascular effects upon light activation. To enhance the therapeutic outcome of vPDT, we combined proteasomal inhibitor bortezomib and vPDT using photosensitizer verteporfin in the present study. We found that bortezomib in combination with verteporfin-PDT induced more accumulation of ubiquitinated proteins and apoptosis in endothelial cells than each individual treatment. The combination therapy also enhanced vPDT-induced inhibition in tumor growth. These results indicate that bortezomib can be used together with verteporfin-PDT for enhanced treatment outcome.

1. Introduction

Photodynamic therapy (PDT) utilizes a photosensitizing agent, light, and oxygen to generate reactive oxygen species (ROS), which induces cytotoxicity though oxidative damages to cellular macromolecules [1]. PDT has emerged as an established cancer treatment modality for skin, head and neck and esophageal cancers, and is currently under clinical investigation for other types of cancer including brain, prostate, breast and ovarian cancers [1,2]. An important target of PDT is tumor vasculature because numerous studies have shown that vascular effects contribute significantly to tumor control by PDT [3]. To further enhance PDT-mediated vascular effects, light activation can be performed at a time when photosensitizer molecules are primarily confined within tumor vasculature or PDT is performed using a photosensitizer that is able to selectively localize to tumor vasculature [4]. This PDT regimen is termed as vascular-targeted PDT (vPDT) to highlight its primary target.

Photosensitizer verteporfin has been explored for targeting tumor vasculature [5,6]. Its clearance from the blood after intravenous injection follows a two-phase exponential decay mode with a half-life of less than 20 min for the first phase and a half-life of less than 8 h for the second slower phase [7]. Because of this rapid blood clearance, light needs to be delivered shortly after injection to activate verteporfin molecules while they are still in the vascular compartment. Thus, a drug-light interval of 15 min is commonly used to target blood vessels for the treatment of age-related macular degeneration (AMD) and tumors. Since there is a very low amount of verteporfin present in tumor parenchymal compartment at this time, direct phototoxicity to tumor cells is expected not to be significant [5].

The mechanism of vPDT-induced vascular disruption is not yet fully understood. Intravital fluorescence microscopic studies by us [6,8] and others [9,10] have demonstrated that vPDT with different photosensitizers rapidly induces vascular permeability increase, thrombus formation, blood vessel occlusion and vascular function shutdown. As a result of tumor perfusion disruption, reduction in tumor oxygenation, metabolism and cell survival is commonly observed after vPDT treatment [10,11]. All these studies support the notion that vPDT causes direct damage to endothelial cells, resulting in subsequent vascular function disruption. PDT using photosensitizer verteporfin has been reported to rapidly induce endothelial cell morphological changes and apoptosis both *in vitro* [6,12] and *in vivo* [13]. Using a high resolution intravital fluorescence microscopic imaging system, death of endothelial cells has been observed after vPDT with verteporfin in live animals [14].

Although the molecular mechanisms underlying endothelial cell damages induced by verteporfin-PDT are not fully understood,

* Corresponding author. Tel.: +1 215 596 7481; fax: +1 215 895 1161.
E-mail address: b.chen@usciences.edu (B. Chen).

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ROS-mediated oxidative stress is presumably an important contributor. PDT has been shown to induce protein carbonylation, a common biomarker indicating protein oxidation [15,16]. Oxidized proteins depend on molecular chaperones such as heat shock proteins (HSP) and endoplasmic reticulum (ER) chaperone immunoglobulin heavy chain-binding proteins (BiP) to restore their conformation and function. PDT using verteporfin as well as other photosensitizers has been known to induce the expression of different isoforms of HSP [17–22], which suggests the existence of oxidative stress. Recent studies further demonstrate that PDT-induced oxidized proteins cause ER stress, which contributes to PDT-induced cell death [15,23,24].

Because cell survival depends on efficient restoration and/or degradation of ROS-modified proteins, strategies targeting protein folding and degradation have been used to enhance PDT response. For instance, targeting HSP-90 has been shown to enhance the outcome of PDT [19]. Because ubiquitin–proteasome system plays an important role in protein degradation, inhibiting proteasomal function has been demonstrated to potentiate tumor response to PDT by inducing the accumulation of oxidized proteins [15]. The goal of the present study was to determine whether inhibiting proteasomal function can improve endothelial cell and tumor response to vPDT with photosensitizer verteporfin. By reversibly inhibiting the active sites in the 20S proteasome to induce cell growth inhibition and even death, bortezomib has been approved for the treatment of multiple myeloma [25]. Photosensitizer verteporfin has received approval for the treatment of age-related macular degeneration by targeting neovascular formation [26]. The effectiveness of combination of bortezomib and verteporfin-mediated PDT were evaluated in both endothelial cells *in vitro* and a mouse tumor model *in vivo*.

2. Materials and methods

2.1. Drugs

Verteporfin (benzoporphyrin derivative in a lipid formulation, vert) was a generous gift from QLT Inc. (Vancouver, Canada). A stock saline solution of verteporfin was reconstituted according to the manufacturer's instructions and stored at 4 °C in the dark. Bortezomib (bort) was purchased from LC Laboratories (Wuburn, MA) and dissolved in DMSO. Bortezomib stock solution was filter-sterilized and stored in a freezer.

2.2. Cell culture

SVEC4-10 (SVEC) mouse endothelial cells and PC-3 human prostate cancer cells, obtained from American Type Culture Collection (Manassas, VA), were maintained in RPMI 1640 with glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 100 units/mL penicillin/streptomycin (Mediatech) at 37 °C in a 5% CO_2 incubator. Cells were used for experiments when they reached 70–80% confluence.

2.3. Cell treatment and viability assay

Effect of treatments on cell viability was determined by CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS assay; Promega, Madison, WI), which is based on the conversion of a tetrazolium substrate into water soluble formazan by dehydrogenase enzymes in metabolically active cells. Briefly, SVEC cells were implanted in 96-well plates and allowed to adhere for overnight. Cells were then treated with PDT only, bortezomib only and the combination of PDT and bortezomib. PDT was performed as described previously [27]. Cells were treated with an irradiance of 5 mW/cm^2 light at 690-nm wavelength for 100 s at 15 min after incubation with 200 or 400 ng/mL dose of verteporfin. Bortezomib dissolved in DMSO was added into cell culture medium for treatment. The final DMSO concentration was less than 0.1%. For combination therapy, bortezomib was added into medium at 12 h before PDT. At 48 h after treatment, absorbance at 492 nm wavelength was measured with a microplate reader. Cell viability was estimated by normalizing the absorbance of treated wells to the control wells. Each experimental condition was assessed in duplicate and experiments were repeated 3 times.

2.4. Clonogenic assay

Cell proliferation was assessed by clonogenic assay, as described before [28]. Cells were treated with bortezomib alone, PDT alone and the combination of bortezomib and PDT. Immediately after treatments, cells were trypsinized and known numbers of cells were seeded in cell culture dishes. After 7 days' incubation, cell

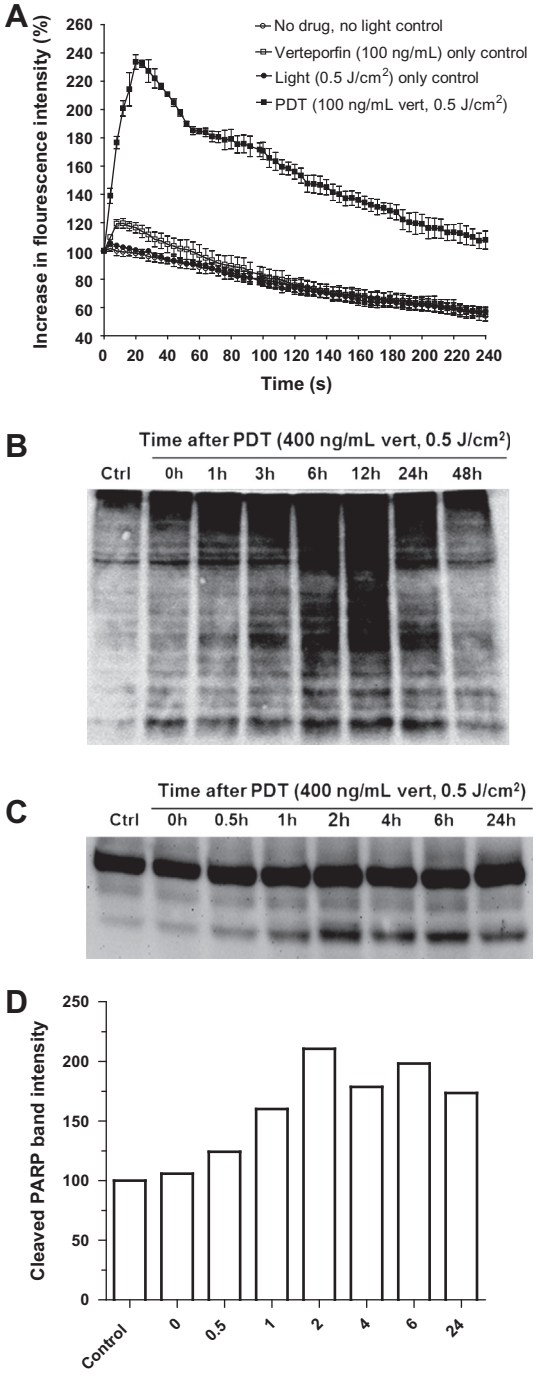

Fig. 1. Effects of PDT with verteporfin on ROS production, protein ubiquitination and apoptosis in SVEC endothelial cells: (A) SVEC cells were treated with verteporfin-PDT as described in the Materials & Methods and imaged for ROS production during and after light treatment using CM-H_2DCFDA fluorogenic ROS probe. Zero time point indicates the time right before turning on the laser and 100 s time point is when laser was turned off. Each value represents mean ± SE of three experiments. (B and C) SVEC cell lysates were prepared at indicated times after verteporfin-PDT and probed by western blot for the detection of ubiquitinated proteins (B) and apoptotic marker PARP cleavage (the lower band) (C). (D) Quantification of cleaved PARP band density.