Effect of vitamin E succinate as a differentiation agent on the efficacy of 5-ALA-PDT on prostate cancer cells in culture

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ABSTRACT

Photodynamic Therapy (PDT) using 5-aminolevulinic acid (5-ALA)-induced protoporphyrin IX (PpIX) has been considered as a new method for treating neoplasms. However, ALA-PDT is suboptimal for thick tumors. Searching for new approaches, we investigated the effect of adding differentiation therapy (DT) with Vitamin E succinate (VES) to PDT in human prostate LN-CaP-FGC10 cancer cells *in vitro*. The purpose of DT was to reverse the lack of differentiation in cancer cells and to enhance the effectiveness of ALA- dependent PDT.

Three groups of cells were grown on RPMI1640 culture medium supplemented with 10% FBS. The cells included: ALA-PDT cells, which received 0.3 mM ALA for 4 hours at dark, and exposed to 532 nm, 50 mW Nd-YAG laser beam for 3 min; DT+ALA-PDT cells, which received 6 µg/ml VES for 24, 48 and 72 hours, followed by the addition of 0.3 mM ALA for 4 h and exposure to Nd-YAG laser beam for 3 min; control cells which were untreated. After 24 h, the percentage of cell viability was determined by MTT assay. Accumulation of PpIX was measured by spectrophotometry and fluorescent microscopy. Mechanism of induced cell death was investigated via Hoechst staining. The combination of both factors (VES and 5-ALA) lead to a significant increase in cell death after 72 h. Induction of differentiation augmented PpIX accumulation in cells treated with ALA. Elevated intracellular PpIX levels resulted in an enhanced lethal photodynamic sensitization of VES plus ALA-treated cells after 72 h. Apoptotic cell death by both ALA-PDT and VES-ALA-PDT was confirmed by Hoechst staining. Our data suggest that VES used in combination with 5-ALA may provide a new combinatorial approach for treating certain cancers.

Keywords*:* Photodynamic therapy; differentiation therapy; vitamin E succinate; apoptosis

INTRODUCTION

Prostate cancer is the most common type of cancer among men of 65 years and older. It is also the second leading cause of cancer-related deaths in men. There are a variety of cancer treatments, including hormone therapy, radio therapy, chemo therapy and surgery (radical prostatectomy). However, none of these approaches lead to an absolute cure, and the number of treatment failures remain unacceptable [1, 2]. Photodynamic therapy (PDT) is an experimental cancer treatment modality, in which systemic administration of a tumor-localizing photosensitizer (Ps) is followed by irradiation of the tumor with visible light of an appropriate wavelength. The photochemical interaction of Ps-light and the molecular oxygen results in the production of reactive oxygen species (ROS) [3, 4].

Damage to organelles within the malignant cells and to stromal elements of the tumors lead to tumor ablation [5, 6]. Membranous organelles, including mitochondria, plasma membrane and lysosomes have been suggested as the major targets of PDT damage [7]. Since cancer cells administered by the pro-drug 5-ALA (5-

aminolevulinic acid) exhibit enhanced capacity tumors, this enhanced capacity was recently exploited clinically for PDT and photodiagnosis [8, 9]. Fluorescent microscopy data have indicated that due to the localization of PpIX in mitochondria, the primary cause of cell death following PDT is mitochondrial phototoxicity [10]. However, ALA-PDT is suboptimal for thick or refractory tumors. So, new modalities are needed, including ways to increase the clinical responsiveness of tumor cells to ALA-PDT [11]. One promising approach to improve the efficacy of ALA-PDT cell death is to alter the biological responses of the target cancer cells in a manner that would enhance susceptibility to ALA-mediated PDT [12]. Differentiation therapy (DT), as an alternative modality, can help increase responses of cancer cells to PDT, since malignant cells typically do not progress through the normal process of growth arrest. Differentiation, apoptosis and cellular proliferation of cancer cells are due to a block in maturation. DT can reactivate the tumor cell differentiation program, forcing them to resume maturation and eventually undergo normal apoptosis [12, 13]. Vitamin E succinate (VES) (α-tocopherol succinate = αTS) is the most active form of vitamin E compared to α-tocopherol, α-tocopherol acetate and α-tocopherol nicotinate. α-TS induces differentiation, inhibits proliferation and causes apoptosis in cancer cells without affecting the proliferation of most normal cells [14, 15, 16]. It has been proven that VES can cause LN-CaP cell growth arrest at G1 phase of the cell cycle [16]. Since VES induces cellular differentiation in many tumor cells, and agents capable of inducing differentiation will also cause elevated synthesis of PpIX after ALA administration in epithelial cells of the skin and prostate [17], we utilized a combination regimen involving DT, and using VES, followed by ALA-PDT in order to enhance the efficacy of ALA-PDT cell death. Our findings indicated that using VES for 72 h may represent a useful adjunct to ALA-PDT in managing prostate cancer.

MATERIALS AND METHODS

ALA and Vitamin E Succinate (VES) were purchased from Sigma (USA). Hoechst 33432 was obtained from Sigma-Aldrich (USA). ALA was dissolved in Phosphate Buffer Saline (PBS) for PpIX synthesis both *in vitro* and in solid (pH 7.2) and sterilized with a 0.2 µm filter, and VES was dissolved in absolute ethanol.

Cell Culture

LN-CaP-FGC10 cell line, a human prostate cancer cell line, was obtained from the Pasteur Institute of Iran and grown in RPMI1640 medium containing 10% Fetal Bovine Serum (FBS) (Gibco, USA) supplemented with L-glutamine (Sigma, USA) and 1% antibiotic (Penicilin-Stereptomycin) at 37 °C in a humidified, 5% CO_2 incubator. Cells were maintained subconfluent and passaged twice weekly. All experiments were repeated for at least three different times.

Differentiation therapy

LN-CaP-FGC10 cells were plated in 24-well plates at a density of 2×10^4 cells per well. After 24 h, the medium was changed with one containing 10% FBS (GIBCO, USA). Then, VES (as a differentiation agent) was diluted in the medium and immediately added to the cells, incubated for 24, 48 and 72 h. Controls were untreated and grown in RPMI1640 medium with 10% FBS.

Measurement of PpIX synthesis

Cells were seeded at a density of 2×10^4 cells/well in 24-well plates. The medium was replaced after 24 hours. Five groups of cells included: the control group (untreated); ALA group (cells were resuspended in 1 ml fresh serum-free medium containing 0.3 µM ALA and incubated for 4 h in the dark; Three different VES-ALA groups (following DT with vitamin E for 24, 48, and 72 h, cells were treated with 1 ml fresh medium containing 0.3 µM ALA and incubated for 4 h in the dark). Cells in each group were then solubilized in 0.1 N NaOH containing 1% Sodium Dodecyl Sulfate (SDS). Next, the cell lysates were submitted to fluorescence spectrophotometry (Cary Eclips, Australia) (excitation 400 nm, emission 580-720 nm, peak 630 nm).

Analysis of PpIX in living cells by fluorescence microscopy

Sterilized coverslips were placed in each well of a 12-well plate, and cells were placed on the coverslips. The medium was replaced after 24 h. Three groups including control, ALA and VES-ALA-72 h were included. Fluorescence of PpIX was analyzed by fluorescence microscopy (Zeiss, West Germany) using a green filter (excitation 480 nm).

In vitro Photodynamic treatment

For phototoxicity, cells were incubated in a serum-free medium containing 0.3 µM ALA. After 4 h, cells were exposed to 532 nm, 50 mW continuous wave Nd-YAG laser (class 3B, 400-700 nm, Germany). To distribute the laser beam (uniform irradiation), a 10 cm lens was utilized.

MTT viability assay

Mitochondrial dehydrogenase activity was quantified by MTT assay. To evaluate PDT effect on cell survival, 0.5 mg/ml MTT (3-(4,5-dimethylthiazol)-2,5-diphenyl-2*H*-tetrazolium bromide) was added to each well after 24 h post-treatment and incubated at 37 ˚C for 3-4 h. Isopropyl alcohol containing 0.04 mM HCl was added and incubated for 5 h. The optical absorbance was determined at 570 nm in a spectrophotometer. Percentage of cell viability was calculated as(optical dencity of treated cell/optical dencity of control)×100.

Morphological/Cytological analysis

Before and after each treatment, cells in all groups were scored based on their morphological appearance using an invert microscope.

Assessment of apoptosis and necrosis in ALA-PDT and VitE-ALA-PDT by Hoechst staining of cells

Hoechst 33342 stains the nucleus of viable and apoptotic cells, which are distinguished by their morphology. For this procedure, three groups of cells (Control, ALA-PDT and VES-ALA-PDT) were seeded in sterilized coverslips, as indicate above. After 24 h, cells were stained with Hoechst dye (5 µg/ml). Then, coverslips were placed on glass slides and fluorescence was monitored through a green filter (450-490 nm) under a fluorescent microscope. Cells with fragmented nuclei were scored as apoptotic.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made by the analysis of variance (ANOVA) (InStat), and when significant differences were observed, a *P*-value of 0.05 was considered as statistically significant.

RESULTS

Effect of ALA-PDT and DT(VES)-ALA-PDT on viability of LN-CaP-FGC10 cells

Our results indicated that ALA-PDT with 0.3 µM ALA followed by Nd-YAG laser beam (532 nm, 50 mW) treatment reduced cell survival to 58.5%. Cell viability in VES-ALA-PDT after 24, 48 and 72 h were 51%, 44.5% and 29%, respectively, while a 72 h DT with 6 µg/ml VES was the most effective regimen in reducing cell survival (Figure1).

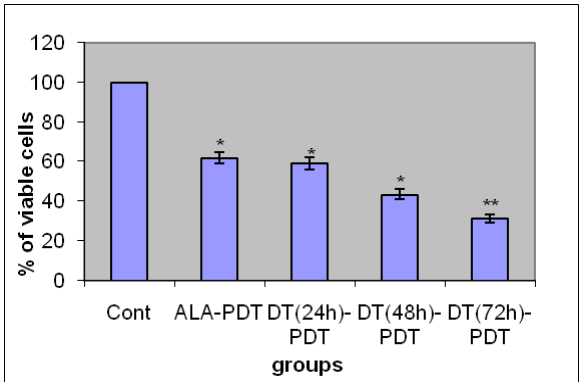

Figure 1. Phototoxicity effect of ALA-PDT and VES-ALA-PDT on cell survival as compared to control using MTT assay. DT groups received 6µg/ ml VES for 24, 48 and 72h, followed by 0.3mM ALA for 4h at dark, and Nd-YAG laser beam (532nm,50mW for 3 min). (**P*<0.05, ***P*<0.01, ****P*<0.001). Mean±SEM.

72 h VES significantly enhances ALA-induced PpIX in LN-CaP-FGC10 cells

To evaluate the effect of VES as a differentiation agent on ALA-PDT treatment, LN-CaP-FGC10 cells were incubated with 6 µg/ml VES for 24, 48 and 72 h.

Spectrophotometric quantification of PpIX demonstrated that ALA-PDT efficiency was enhanced significantly (P<0.001) after 72 h of incubation with VES (Figure 2).

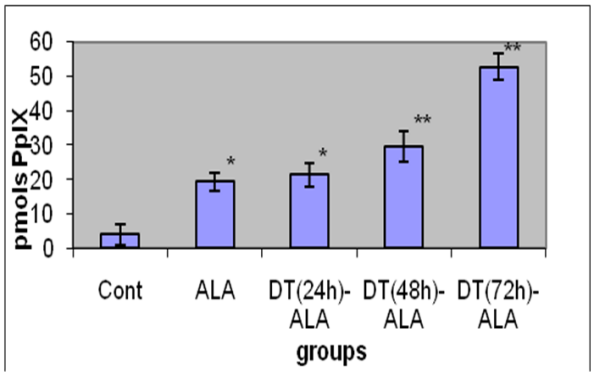

Figure 2. Time dependent VES-induced PpIX accumulation. Cells in DT groups were exposed to 6µg/ml VES for 24, 48 and 72h, followed by 0.3mM ALA for 4h at dark. (*P<0.05, **P<0.01, ***P<0.001). Mean±SEM.

VES-treated living cells were also analyzed under a fluorescent microscope for PpIX accumulation. The highest PpIX fluorescence was obtained after a 72 h treatment with 6 µg/ml VES (Figure 3).

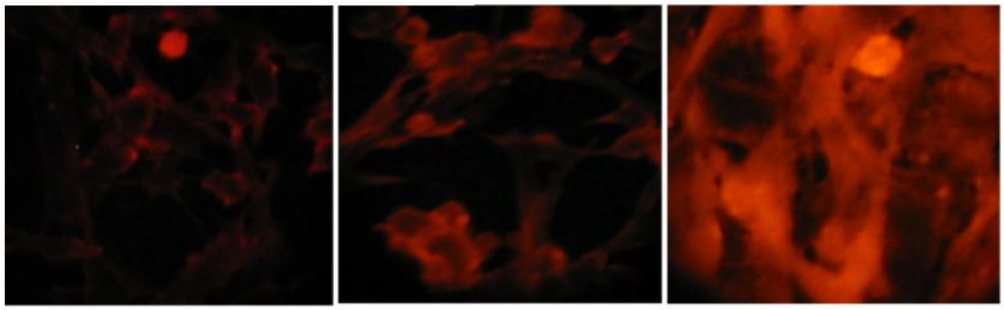

Figure 3. PpIX fluorescence in living cells. 24 hours after cultured cells were incubated with a medium containing VES (6µg/ml) (for 72h) or without VES, followed by 0.3mM ALA for 4h at dark. Fluorescence of PpIX was analyzed by a fluorescent microscope.

Effect of ALA-PDT and VES-ALA-PDT on cell morphology

Before and after treatments, LN-CaP-FGC10 cells were examined under an invert microscope. 24 hours after the differentiation therapy started, cells showed slight elongation, and these changes in cell shape were further pronounced in 72 h. ALA-PDT and VES-ALA-PDT resulted in the deformation of cell shape (Figure 4).

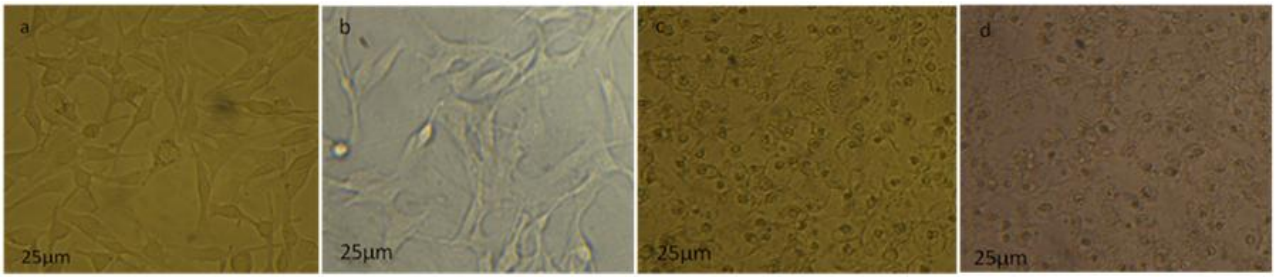

Figure 4. Inverted photomicrographs of LN-CaP-FGC10 cells. a) cells without treatment. b) cells after a 72h differentiation therapy. c) cells after administration of 0.3 mM ALA and 532nm Nd-YAG laser beam. d) cells after VES (72h) ALA-PDT (6µg/ml VES for 72h, 0.3 mM ALA, 532nm Nd-YAG laser beam). Arrows in b indicate differentiating cells. Cell shape deformation is obvious in c and d.

Analysis of cell death induced by ALA-PD and VES-ALA-PDT

To determine whether VES treatment along with ALA-PDT induces apoptosis in LN-CaP-FGC10 cells, Hoescht staining was performed and cells were examined under a fluorescent microscope. Data from Hoescht staining revealed normal chromatin in control group, whereas in ALA-PDT and VES-ALA-PDT groups condensed and fragmented nuclei (two important characteristics of apoptosis) were observed (Figure 5).

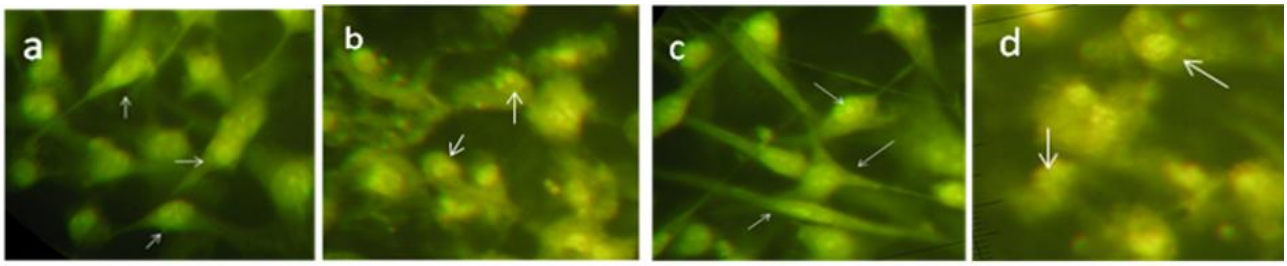

Figure 5. Fluorescence micrografs of LN-CaP-FGS-10 cells stained by Hoescht dye. a and c) cells with normal chromatin (arrows). b and d) cells after ALA-PDT and VES-ALA-PDT treatment, respectively. Arrows show condensed and fragmented apoptotic nuclei.

DISCUSSION

The basis for photodynamic therapy is a selective accumulation of a certain photosensitizing agent in neoplastic tissue without affecting the healthy tissue. Activation of the photosensitizer by a specific wavelength of light could result in the production of cytotoxic singlet oxygen and ROS, leading to the death of tumor cells. PDT using 5-ALA as a pro-drug that is converted to an intracellular Ps (PpIX) is widely used for treatment of rapidly proliferating cells, especially the solid tumors. However, ALA-mediated PDT is suboptimal for treatment of deeper tumors, and the number of treatment failures remains unacceptable. Cancer development and progress is a complex process [4, 18, 19, 20]. In malignant cells, genomes are changed and mechanisms leading to apoptosis are defective. *In vitro* and *in vivo* studies as well as various clinical trials are making it increasingly evident that no single therapeutic modality could be a likely curative. So, a combination regimen is more likely to destroy malignant cells [12, 21]. In this study, we demonstrated a combination treatment utilizing differentiation therapy and PDT in order to enhance the cytotoxic effects on prostate cancer cells. ALA-induced PpIX production was markedly increased in LN-CaP cells after treatment with the differentiation agent VES, the most effective form of vitamin E, resulting in a significant enhancement of phototoxicity in the

differentiated cells. While ALA-PDT reduced LN-CaP cell survival to 58.5%, DT with VES for 24, 48 and 72 h followed by ALA-PDT reduced LN-CaP cell survival to 51%, 44.5% and 29%, respectively. A marked reduction in cell viability was observed after 72 h of DT with VES followed by ALA-PDT.

Since the arrest of cell cycle is a prerequisite for differentiation, and differentiation eventually results in cell death in many systems, these associations are utilized by DT, restoring the pathways lost in malignant cells [12]. Application of ATRA (all-trans retinoic acid) as a differentiating agent for treatment of APML (acute promyelocyte leukocyte) was the typical clinical DT program used by Breitman et al [22] to induce differentiation of the cancer cells leading to their apoptosis.

As described earlier, four parameters are required for an effective PDT: the photosensitizing agent, light, oxygen and cellular physiology. By proper manipulation of the cellular metabolic state, we obtained a higher level of PpIX. This increase in the cellular PpIX content led to an improved photodynamic sensitization. Several other agents such as methotrexate, androgens, vitamin A and analogs of vitamin D also promote cellular differentiation, and by enhancing PpIX accumulation in LN-CaP cells improve photodynamic efficacy [11, 12]. Schwartz et al [23] used Hexa Methylen Bis Acetamide (HMBA) and butyrate followed by ALA-PDT and showed that HMBA and butyrate increase the rate of ALA-PDT- induced cell death. They further demonstrated that HMBA was a prominent inducer of PpIX accumulation. Esquent et al [24] used R1881 as a modulator of differentiation in LN-CaP cells. This agent arrested cell growth by reducing DNA synthesis. However, not all differentiating agents are capable of elevating PpIX formation. For instance, Schoenfield et al [25] suggested that DT treatment with DMSO had no stimulatory effect on PpIX synthesis. Almedia et al [26] reported that cell death induced by PDT is due to apoptosis or necrosis depending on the cell genotype, the photosensitizer and the PDT dose. However, even for a specific Ps used for PDT, the unique type of cell death is difficult to determine [27].

Accumulation of PpIX in differentiated cancer cells may be due to several factors: a) an increased uptake of ALA, b) a higher metabolic activity in tumor cells, c) a reduced efflux of PpIX into the medium, d) an increase in the mRNA of haem synthetic enzyme and e) a lower enzymatic activity of ferrochelatase [17, 28]. Our results demonstrated a high level of induction of apoptosis by ALA-PDT as revealed by Hoechst staining using fluorescence microscopy. Cells with condensed and fragmented nuclei (two important characteristics of apoptosis) were considered as apoptotic.

In summary, our results suggest that VES(72 h)-ALA-PDT by elevating the production of endogenous PpIX can enhance the efficacy of PDT in killing cells. Our data support the importance of differentiation in ALA-mediated PpIX formation, making the future clinical protocols less toxic for normal tissues and more efficient for prostate cancer treatment.

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