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Full Length Research paper

Preclinical biochemical studies using a novel 5-aminolevulinic acid ester derivative with superior properties for photodynamic therapy of tumors

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We have synthesized a novel 5-aminolevulinic acid (ALA) ester derivative from reacting ALA with β-citronellol and tested its potential for treatment of tumors by photodynamic therapy (PDT) in comparison to the widely used agents, ALA and Me-ALA (the methyl ester of ALA). The β-citronellol-ALA ester derivative was far superior to ALA in causing complete destruction of solid skin tumors after PDT treatment. *Ex vivo* PDT treatment of Ehrlich ascites carcinoma (EAC) cells indicated that the novel ester was effective in causing cell death and inhibition of cell growth, while flow cytometry studies revealed G0/G1 cell cycle arrest during the PDT-mediated response. Inoculation of healthy animals with EAC cell preparations extracted from tumor-bearing animals treated with various agents and then subjected to PDT treatment *ex vivo* revealed that the novel ester derivative was a significantly better agent than either ALA or Me-ALA at preventing or inhibiting growth of tumors in the inoculated animals. The findings suggest that the novel β-citronellol-ALA ester derivative offers a promising therapy for the treatment of apoptosis-reluctant tumors such as malignant tumors and may be superior to ALA or Me-ALA in PDT applications.

Key words: Cancer, PDT, ALA, β-citronellol-ALA ester derivative, flow cytometry, tumor apoptosis, ALA-PDT.

INTRODUCTION

Photodynamic therapy (PDT) is an established method for the treatment of neoplastic and non-neoplastic diseases. PDT is based on the use of photosensitizer-enhanced photochemical reactions leading to tumor destruction with high selectivity (Dougherty et al., 1998; Dougherty, 2002; Dougherty et al., 1978). The photosensitizers, located in the targeted tissue, can generate singlet oxygen ($^1O_2$) when activated by light. The generated singlet oxygen is a highly reactive oxidant that has the ability to react with lipids and proteins, leading to selective local destruction of cells (Korbelik and Cecic, 2003; Macdonald and Dougherty, 2001; Weishaupt et al., 1979; Nelson, 1987; Pass, 1993).

Provided that the photosensitizer is nontoxic, only the irradiated areas will be affected, even if the photosensitizer does bind to normal tissues. For successful PDT, the wavelength of light for photoactivation should be matched to the electronic absorption spectrum of the photosensitizer so that photons are absorbed by the photosensitizer and the desired photochemistry can occur (Nelson, 1987; Pass, 1993). During the past two decades, there has been much interest in a different approach to PDT where, instead of a preformed photosensitizer being administered, a precursor is administered, and the photosensitizer is synthesized *in situ* within the tumor (El-Far et al., 1990; Kennedy et al., 1990). This is the case with 5-aminolevulinic acid (ALA), a naturally occurring precursor in the biosynthetic pathway for heme production, the last step of which involves conversion of protoporphyrin IX (PpIX), a photosensitizing species, to heme (a nonphotosensitizing agent).

Porphyrins are specifically accumulated in tumor cells, either after exogenous administration or by endogenous synthesis. Therefore, their tumor-localizing abilities and photosensitivity properties have been extensively exploited

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for the detection and treatment of tumors. This has been reviewed by Batlle (1993), but it is appropriate to mention significant milestones here. By the end of the 1960s there was evidence that biosynthetic pathways were disturbed in neoplastic disease, while Rassetti et al. (1963) had reported preferential accumulation of porphyrins and their precursors, such as porphobilinogen (PBG) and (ALA), in gastrointestinal carcinomas compared to normal adjacent cells (Rassetti et al., 1963). We subsequently showed that the concentration of aminolevulinic acid synthetase (ALA-S), the rate limiting enzyme in the biosynthetic heme pathway, was significantly increased in bladder tumor tissues and in the leukocytes of patients with invasive bladder cancer when compared to normal controls (El-Far et al., 1986). We suggested that ALA-S activity may be of considerable aid in the diagnosis or confirmation of bladder cancer and developed a system to enhance biosynthetic production of porphyrins endogenously (Smith et al., 1979). Malik and Lugaci (1987) demonstrated selective destruction of friend erythroleukemic cells (CFLC) by endogenous porphyrins synthesized following supplementation of ALA followed by irradiation with light (Malik and Lugaci, 1987). El-Far et al. (1990) showed for the first time the biodisrtibution and selective *in vivo* tumor localization of endogenous porphyrins induced and stimulated by ALA as a newly developed technique and suggested its use for photodesrtuction of tumors by photodynamic therapy (PDT). This represented the first systemic experimental application of ALA for PDT using a bladder tumor model. We showed that *in vivo* administration of ALA can produce reasonable therapeutic favorable ratios of porphyrin concentration between cancerous cells and normal tissues. Furthermore, we have identified the type of porphyrins retained in tumor and other tissues. This was confirmed recently by others. We suggested and indicated that accumulated endogenous porphyrins which was stimulated and induced by supplementation of ALA can serve as tumor localizers as a novel approach towards photoradiation therapy of tumors.

Kennedy et al. (1990) described the first clinical application of this method, using topical application of ALA followed by photo activating light to treat selected superficial non-melanoma skin cancers (Kennedy et al., 1990). However, since PpIX accumulates in tumors after systemic administration of ALA (El-Far et al., 1990; Bedwell et al., 1993), it was not long before the use of systemic ALA was applied in humans (Grant et al., 1993).

Unfortunately, the topical application of ALA has very limited efficiency for treatment of deeply located or nodular lesions and there are limitations with systemic application (El-Far et al., 1994; El-Far et al., 1998). These limitations have led to the introduction of the more lipophilic methyl ester(s) as ALA (Me-ALA) for PDT, in particular, the use of ALA and some of its ester derivatives, mainly Me-ALA, for PDT of actinic keratosis (AK). Basal cell carcinoma (BCC) has been approved by the European Union (Me-ALA-PDT for BCC and AK in 2001) and the US Food and Drug Administration (ALA-PDT for AK in 1999) is now a well-established therapeutic option (Dognitz et al., 2008). However, there are still limitations with the use of Me-ALA, and these observations stimu-lated us to search for alternative derivatives of ALA that might offer better photosensitization and/or greater effec-tiveness for deeply located or nodular lesions, and to compare the new ALA-derivatives with ALA and Me-ALA only as they have been approved clinically by European Union and the US Food and Drug Administration. In an earlier study we modified and improved ALA-PDT by use of an ALA ester of the furocoumarin derivative alloimpe-ratorin, which we called Allo-ALA (El-Far et al., 1998). It was more effective than original native ALA in the destruction of large skin tumors by PDT, possibly because of improved penetration by the topically applied ALA ester derivative, of improved selectivity, and possibly because Allo-ALA incorporates a prenyl unit capable of being oxidized by singlet oxygen to an allylic hydrope-roxide that might contribute to the destruction of nearby cells through the generation of free radicals. However, some furocoumarins are known to induce apoptosis with-out the need for light (Pae et al., 2002) and this could complicate the interpretation. It was therefore of interest to investigate an alternative prenyl-derived ester of ALA for its effectiveness in PDT of tumors.

β-Citronellol is a relatively cheap, safe and readily available natural prenyl-derived alcohol. It possesses a long chain alkene unit that could play an important pharmacokinetic role, for example through lipophilicity or aggregation. Griesbeck and Bartoschek (2002) have reported ready photooxygenation of β-citronellol, to afford a mixture of hydroperoxides (Scheme 1) Griesbeck and Bartoschek,(2002) . We therefore decided to investigate the β-citronellol ester of ALA for PDT. The present study was designed to prepare ALA-β-citronellol ester deriveative and to examine its effect on tumor cells' photosensitization *in vivo* and *ex vivo*, and to examine possible molecular changes in the cell-cycle progression using flow cytometry.

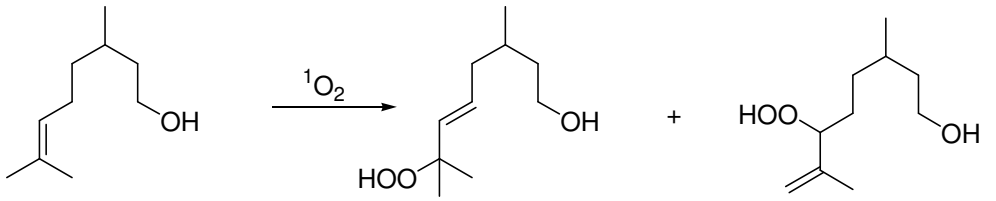
MATERIALS AND METHODS

Chemicals and chemical synthesis

Synthesis of 3, 7-dimethyloct-6-enyl 5-amino-4-oxopentanoate hydrochloride (ALA-β-citronellol ester derivative)

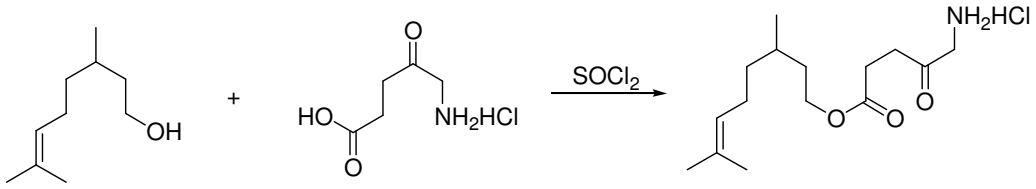
β-Citronellol-ALA ester derivative was synthesized using the basic technique of Takeya (1992) with modification, briefly by reacting ALA.HCl with (2 mL) β-citronellol in the presence of (0.17 mL) thionyl chloride (Scheme 2). The reaction mixture was stirred and refluxed until ALA.HCl was completely dissolved and reacted. The reaction was followed and confirmed by TLC (thin layer chromatography). The excess of solvent was evaporated under high vacuum. The product was obtained as amber oil.

Yield of β-Citronellol -ALA ester derivative was nearly 94% as established by TLC (single band). NMR, IR, MS spectra confirm presence of ester formation in the preparation with derivative of



β–Citronellol

Scheme 1. Photooxygenation of β.citronellol.



β–Citronellol ALA.HCl β–Citronellol-ALA ester

Scheme 2. Synthesis of β.citronellol-ALA ester.

ALA and β-Citronellol. The β-Citronellol-ALA ester derivative oil was directly used for PDT experiments.

Tumors and experimental PDT protocols

β-Citronellol-ALA ester derivative, ALA-HCl, Me-ALA and β-citronellol were separately dissolved in DMSO immediately before use and then diluted to the desired concentration using isotonic saline solution (1:1).

All experiments were performed with adult female BALB/c mice strain purchased from Theodore Bilharz institute, Giza, Egypt, with an average body weight of 20 to 25 g. Mice were housed in steel mesh cages and maintained for one week acclimatization periods on commercial standard diet and tap water *ad-libitum*. Ehrlich ascites carcinoma (EAC) line was kindly supplied from the National Cancer Institute, Cairo University, Egypt.

Ex-vivo irradiation and cell cycle distribution experiments

Ehrlich ascites carcinoma cells (EAC) were transplanted into female mice with age range of 6–7 weeks and weighing approximately 21 g. The implantation procedure is summarized as follows. Tumor was extracted from a donor mouse and EAC cells (2×10^6) were intraperitoneally (i.p.) transplanted using a 25 G needle into female Swiss albino healthy mice. The viability determination was done with trypan blue exclusion. On the 14th day after tumor inoculation, which was in the exponential growth phase, animals were divided. The first animal received i.p. ALA at a dose of 50 mg/kg body weight. The second animal received β-citronellol-ALA ester derivative at a dose of 25 mg/ kg, and another animal received β-citronellol-ALA ester derivative at a dose of 50 mg/ kg. The last animal, which served as a control, received isotonic saline solution / DMSO (1:1) (vehicle control). All animals were kept for 3 h of incubation, and then EAC cells were extracted from the peritoneal cavity of each animal to prepare *ex vivo*. Extracted EAC cells from each animal were divided into two equal portions and kept in separate tubes in the dark as homogeneous cell suspensions in 0.2 ml of isotonic saline solution. The first tube from each animal was kept in the dark and served as a control. The second tube from each animal was separately irradiated in a 7.5 mm glass tube. The light source used for these irradiations of Ehrlich ascites carcinoma cell suspensions consisted of a straight sodium lamp (Phillips, 220 V, 400 W); light intensity at the position of the cells was non-thermal, and the irradiation time was 90 s only at a distance of 30 cm according to Luksiene and de Witte (2003).

Flow cytometry protocol

Cells were fixed in 70% ice cold ethanol. Cells were washed prior to analysis using PBS (pH: 7.4), and incubated with RNAse A (100 µg/ml) and the DNA intercalating dye propidium iodide (20 µg/l) in citrate buffer (3.4 mM). Cell cycle analysis including DNA-content, DNA index, cellular proliferation, and apoptosis were measured using FACScalibur (Becton Dickson, Mountain View, CA) and Cellquest software (Becton Dickson, Mountain View, CA) with an excitation wavelength of 488 nm and emission above 580 nm. The cell cycle analysis was performed using Mod-Fit software. 15,000 cells were analyzed in each experiment.

Apoptosis detection in EAC cells

Cells were fixed and stained with propidium iodide and DNA content was revealed by flow cytometry, with apoptotic cells forming a sub-G1 peak. As a negative control, a sample with live cells was used. Up to 15,000 cells were analyzed using the Cellquest software (Krishan, 1975).

Tumor volume experiments

Animals subjected to EAC cells after 14 days were separately inoculated with appropriate chemical solutions [ALA-HCl, Me-ALA, a mixture of ALA.HCl and Me-ALA, β-citronellol (alone), β- citronellol

-ALA ester derivative and a mixture of ALA.HCl and β-citronellol (not ester)], all at a dose of 50 mg/kg, as described for the cell cycle analysis experiments. Following PDT treatment (also as described for the cell cycle analysis experiments), 0.2 ml of each irradiated cell suspension (2×10^6 cells) was separately inoculated into at least 5 healthy mice i.p. and tumor growth was measured after two weeks. The non-radiated cell suspension was also inoculated in at least 5 healthy mice i.p. and tumor growth was measured after two weeks. All experiments were repeated 3 times. At day 14 ascetics fluid was individually withdrawn from ether anaesthetized animals of both tumor-bearing non-treated (control), and tumor bearing treated groups using an 18 gauge needle. Individual ascetic fluid volume was measured.

Transplantation of solid tumor model

Ehrlich ascites carcinoma cells were collected from moderately growing 7 days old mice (donor). After doing appropriate dilutions the viability was checked as described above.

Solid tumors were induced by injecting 2×10^6 viable cells into the middle region area on the back of each mouse. After two weeks, the solid tumors were observed. None of the tumors exhibited necrosis when routinely examined at this specific time. The sizes of tumors were measured along three orthogonal diameters - length (L), width (W) and thickness (T) - every second day by using a micrometer. The tumor growth measurements were recorded and the volume was calculated according to the formula: $V = (L \times W \times T)/2$ (Hampson et al., 2001).

Experimental PDT protocol using solid tumors

The experimental groups consisted of 15 mice with tumors of 0.3-0.5 cm diameter; the tumor size was recorded as described before and the animals were then separated into three groups, which were treated as described below.

Group 1

This group consisted of 5 mice with tumors which received ALA formulation that contained 15 mg ALA.HCL dissolved in DMSO and isotonic saline solution (1:1). It was applied topically to cover completely each tumor in each animal after shaving the area and cleaning it. Every tumor received topically exactly 15 mg. All animals were kept in the dark for 4 h. They were then exposed to non-thermal photoactivating light according to our previous technique (El-Far et al., 1999) using a halogen lamp (250 V, 500 W) produced by Philips; the spectrum of light emitted from this source has wavelengths of less than 800 nm and greater than 400 nm. The temperature was measured to exclude any thermal effect during the experiment (El Far, 2009).

Group 2

This group consisted of 7 mice with tumors which received a β-citronellol-ALA ester derivative formulation which contained 15 mg β-citronellol-ALA ester derivative dissolved in DMSO and isotonic saline solution (1:1). It was applied topically to cover completely each tumor in each animal after shaving the area and cleaning it. Every tumor received topically exactly 15 mg. All animals were kept in the dark for 4 h. They were then exposed to non-thermal photoactivating light according to our previous technique (El-Far et al., 1999) using a halogen lamp under the same experimental conditions as above.

Group 3

This group consisted of 3 mice with tumors, which were treated identically to those in group 2 until the point of irradiation. They were then exposed to non-thermal photoactivating light under the same experimental conditions as above using a straight sodium lamp (Philips, 220 V, 400 W); the spectrum of light emitted from this source has wavelengths of less than 800 nm and greater than 400 nm.

Tumor response after PDT of solid tumors

After light exposure, the tumor size of each animal was measured three times every week; the results from each group were pooled to give mean growth curves. Measurements of the size of each tumor were carried out until animals were cured or died. At selected points of time after light exposure, photographs of the irradiated animals were taken to record visually the tumor size changes at specified time intervals.

Statistical significance

All values were expressed as mean ± SD. The statistical significance was determined using the one way ANOVA. The Statistical Package for Social Science (SPSS, Inc, Chicago, IL) was used for the statistical analysis. Statistical significance was considered to be $P < 0.05$.

RESULTS AND DISCUSSION

PDT effect on the cell-cycle distribution *ex-vivo*

Previous studies have shown that certain sensitizers, including 5-ALA.HCl and its hexyl ester derivative, are very rapid inducers of apoptosis (Kessel and Luo, 1998, 1999; Kessel et al., 1997). Apoptosis induction by photosensitizers primarily localized in the mitochondria has been attributed to the release of cytochrome c from mitochondria being a critical signal for the induction of apoptosis (Liu et al., 1996; Granville et al., 1998).

The data obtained in the present cell distribution study are presented in Table 1. They clearly indicate that the dominant subpopulation of the control cells remained in the G0/G1 phase after irradiation, whereas cells following 3 h incubation with 5- ALA and ALA-e (β-citronellol-ALA ester) and 90s irradiation *ex-vivo* exhibited a diminished G0/G1 phase and a highly significant increase in apoptotic cells (sub-G1) (Table 1). Moreover, there were many fewer cells in the cycle, indicating that some of them were dead. Table 1 suggests that cell death and especially apoptosis is induced from the G0/G1 phase of the cell cycle after PDT treatment.

Clearly, PDT treatment of EAC cells *ex-vivo* induced apoptosis very rapidly; within 90 s the proportion of apoptotic cells increased from 5% in the dark reaction upto 82% (~ a 16 fold increase) after exposure to irradiation following treatment with 25 mg/kg of our novel synthesized ALA ester. By comparison, the proportion of apoptotic cells was around 60% (a 12 fold increase) after exposure to irradiation following treatment with 50 mg/kg

Table 1. Flow cytometry results of *ex-vivo* PDT experiment.

	Sub-G1 %	SD	G0/G1 %	SD	S-phase %	SD	G2M %	SD
Dark								
Control	5.3	1.3	50.8	4.1	26.3	2.8	19.6	3.1
50 mg/kg 5-ALA	7.17	0.8	50.5	7.4	54.5	3.2	54.7	7.4
25 mg/kg ALA-e	6.7	0.7	49.7	7.9	15.9	1.9	20.8	2.4
50 mg/kg ALA-e	6.5	0.8	49.5	6.6	17	4.4	16.5	4.7
Irradiation								
Control	6.82	1.5	51.4	5.3	25.1	5.9	21.3	6.7
50 mg/kg 5-ALA	62.25	4.5	32.8	8.1	24.9	6.3	14.6	4.6
25 mg/kg ALA-e	82.31	3.1	14.6	8.5	7.5	4.3	2.37*	1.5
50 mg/kg ALA-e	64.27	4.9	24.9	4.6	2.1	8.7	1.15*	0.8

Table 2. Tumor volumes of mice treated with various chemicals (50 mg/kg) compared to a control group (no chemicals added, but exposed to the halogen lamp for 90 s).

Groups (n = Number of animals)	Tumor volume (ml) expressed as mean ± SD
Control (n=5)	4.56 ± 0.37
ALA (n=5)	2 ± 0.35*
β-Citronellol (n=5)	3.06 ± 0.26*
ALA β-citronellol ester derivative (n=10)	0.30 ± 0.26*
Me-ALA ester (n=5)	1.12 ± 0.16*
ALA+Me-ALA mixture (25 mg/kg each) (n=5)	1.76 ± 0.25*
ALA+β-citronellol mixture (25 mg/kg each) (n=5)	2.42 ± 0.29*

*Significant, P<0.05 for effect on treated groups *versus* the control group.

of either ALA-e or ALA.HCl.

In summary, the DNA cell cycle analysis of tumor cells in our *ex-vivo* experiments showed that our novel ALA ester is effective at inducing apoptosis and hinted that it might be more effective than ALA.HCl in this respect.

Anti-tumor effect and EAC tumor growth

The effect of the chemicals under consideration on EAC tumor growth was assessed by visual observation of changes as well as by determination of ascetics tumor volume of animals treated with the followings: ALA.HCl; ALA methyl ester; a mixture of ALA.HCl and ALA methyl ester; β-citronellol (alone); β-citronellol-ALA ester derivative; and a mixture of ALA.HCl and β-citronellol (not ester), using doses of 50 mg/kg, followed at 14 days post chemical treatment by irradiation *ex vivo* using a halogen lamp as described for the cell cycle distribution experiments. Samples of the fluids after irradiation were separately inoculated into healthy animals and tumor growth in these animals was monitored. The results for tumor volumes are shown in Table 2 and graphically in Figure 1. They reveal a significant decrease in tumor volume, due to inhibition of tumor growth in comparison to the control, in the cases of treatment with ALA.HCl, β-citronellol (alone) and a mixture of ALA.HCl and β-citronellol. Me-ALA and a mixture of ALA.HCl and Me-ALA showed an even greater reduction in tumor growth. However, the smallest ascetics tumor volume was observed in the case of treatment with ALA-β-citronellol ester derivative. After treatment with this novel ester the observed tumor volumes were close to zero in most cases, indicating a highly significant decrease in tumor growth; this reflects a dramatic anti-tumor activity of our novel ester (Figure 1). The tumor volume expressed as the mean value in the β-citronellol-ALA ester derivative group was 0.3 ± 0.26 ml, a decrease of 85% when compared to the mean tumor volume (2.0 ± 0. 35 ml) in the ALA.HCL group.

PDT of solid tumors

Tumors in the two groups of animals which were topically treated with 15 mg of our β-citronellol-ALA ester derivative and then exposed to broad band photoactivating light from either a halogen or sodium lamp, after an incubation period of 4 h, showed complete destruction of the tumors within 2-3 weeks post treatment. The tumors never re-grew or returned again during a follow up period of 2-3 months.

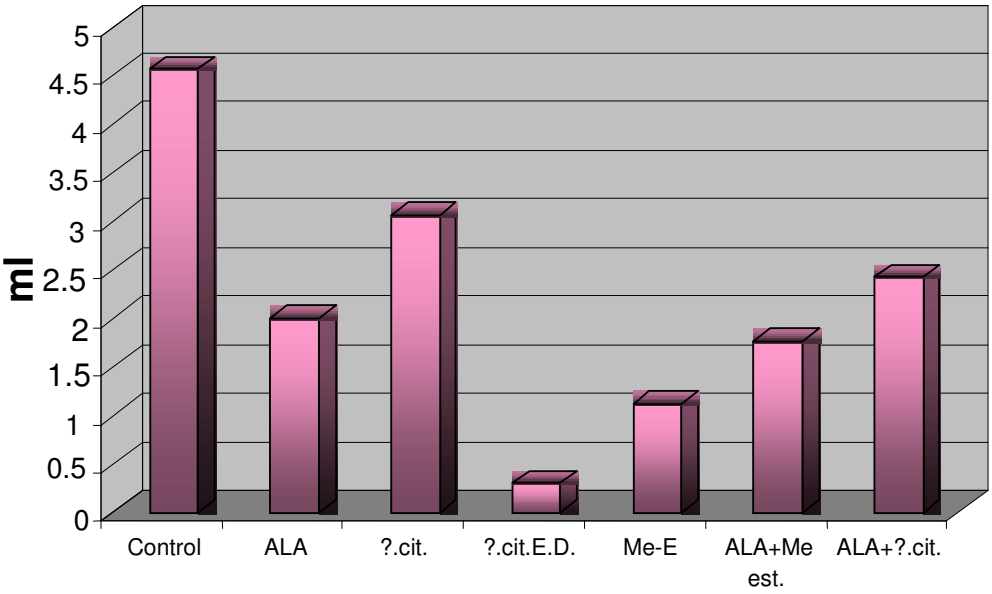


Figure 1a. The mean values of tumor volumes of mice treated with various agents compared to control group.

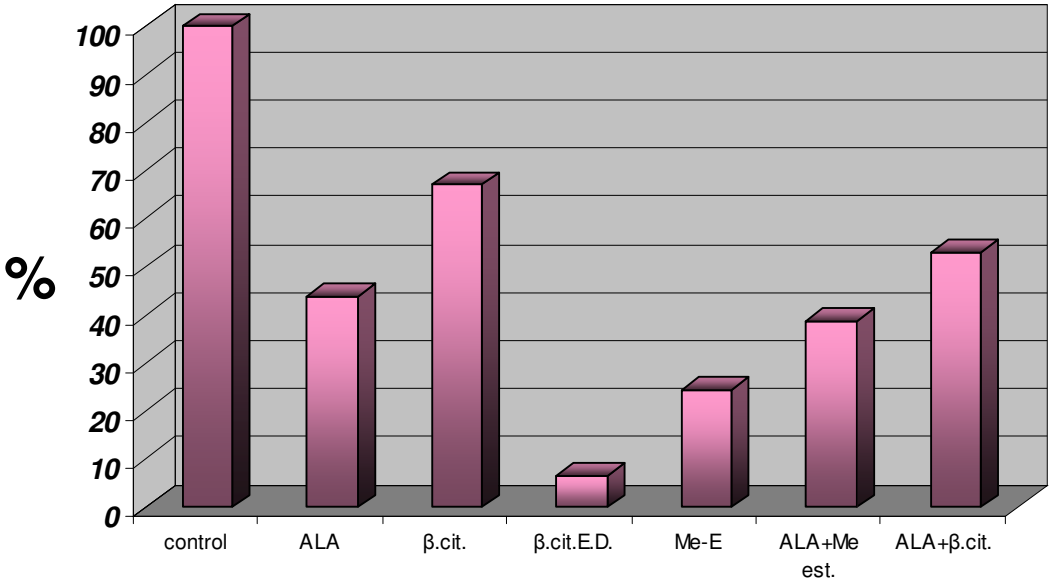


Figure 1b. The percent of tumor volumes of mice treated with various agents compared to control group.

Table 3. Tumor size of mice treated with ALA and ALA β-citronellol ester derivative (15 mg) compared to a control group (no chemicals) and exposed to halogen lamp.

Groups (n = Number of animals)	Tumor size (Cm^3) expressed as mean ± SD
Control (n=5)	0.481± 0.553
ALA (n=5)	0.123 ± 0.135*
ALA β.citronellol ester derivative (n=5)	0.018 ± 0.016*

*Significant, P<0.05 for effect on treated groups *versus* the control group up to 6 weeks, at week 7 tumors disappeared (complete response) only observed in ALA β.citronellol ester derivative.

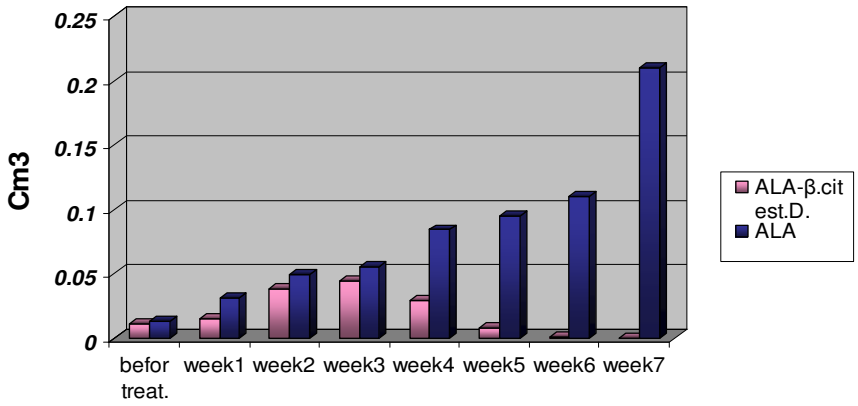


Figure 2a. The mean of tumor size of solid tumor of mice treated with ALA and β.citronellol- ALA ester derivative with dose of 15 mg and exposed to halogen lamp.

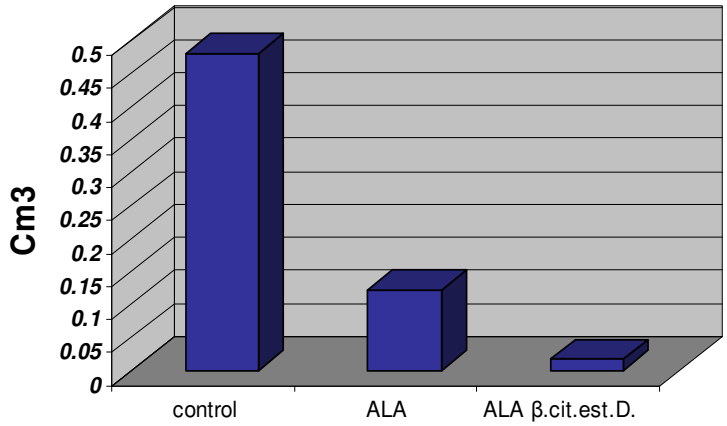


Figure 2b. The mean of tumor size of solid tumor of mice treated with ALA and β.citronellol- ALA ester derivative with dose of 15 mg and exposed to halogen lamp compared to a control group group up to 6 weeks, but in week 7 tumor size become zero in mice treated with β.citronellol- ALA ester derivative.

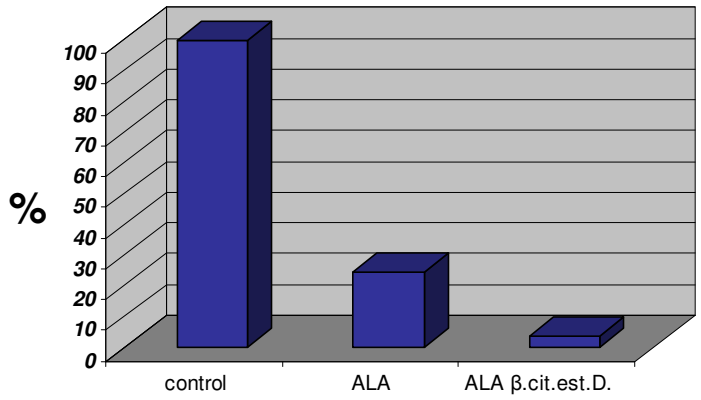


Figure 2c. The percent of decrease of tumor size of solid tumor of mice treated with ALA and β.citronellol- ALA ester derivative with dose of 15 mg and exposed to halogen lamp compared to a control group up to 6 weeks, but in week 7 tumor size become zero in mice treated with β.citronellol- ALA ester derivative.

Figure 3a. Effects of β.cit.ALA ester derivative on tumors of mice at first week post treatment.

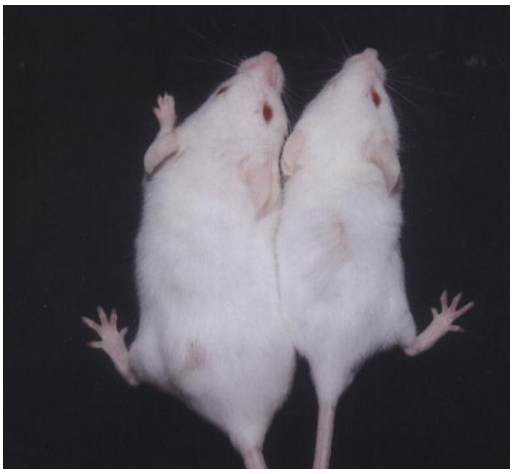


Figure 3b. Effects of β.cit.ALA ester derivative on tumors of mice at seven weeks post treatment, complete response observed only in this group of animals treated with our novel ALA-derivative.

On the other hand, the group of animals with solid tumors treated with 15 mg ALA.HCl showed only a partial response to the PDT treatment; in no case had complete destruction or disappearance of the tumors taken place. All tumors in this group of treated animals re-grew, with a significant increase in tumor size during the first week following PDT treatment. After this we observed an exponential re-growth and all animals died within 8 weeks. We thus conclude that topical application of 15 mg of β-citronellol-ALA ester derivative formulation to cover completely large tumors with a diameter not less than 0.5 cm, followed by exposure of the entire area to photoactivating light from either a halogen or sodium lamp, provides an excellent photodynamic effect in the treatment of solid tumors. It showed substantial superiority over a comparable formulation of parent ALA when used under the same experimental conditions. It should also be noted that the tumor sizes in the group of animals treated with ALA-PDT were less than those observed for tumors in a group of animals that received only light and no photosensitizer, which served as a control group (Table 3; Figures 2, 3, 4).

In this study, we showed significant improvement of anti-tumor activity due to chemical modification in the structure. This approach was also established not only in photosensitizers' development but also, in preparing novel promising other derivatives as chemotherapeutic anti-cancer agents (El-Far et al., 2009; Elmegeed et al., 2009).

Conclusions

The β-citronellol- ALA ester derivative is a novel and highly effective agent for PDT of tumors. *Ex vivo* studies show that it is highly effective at inducing apoptosis in

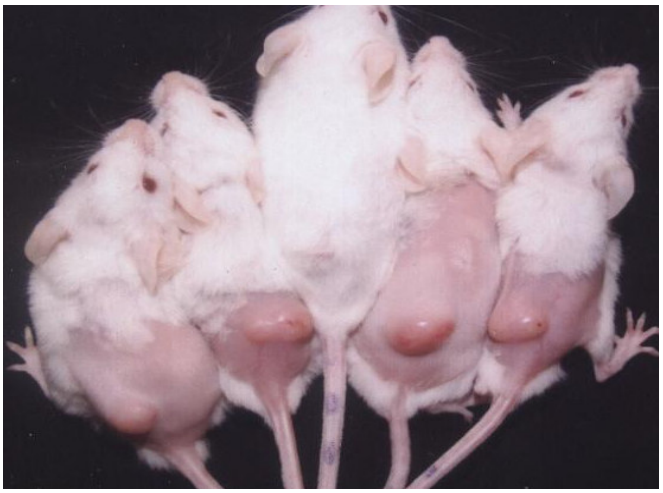


Figure 4a. Effects of ALA on tumors of mice at first week post treatment.

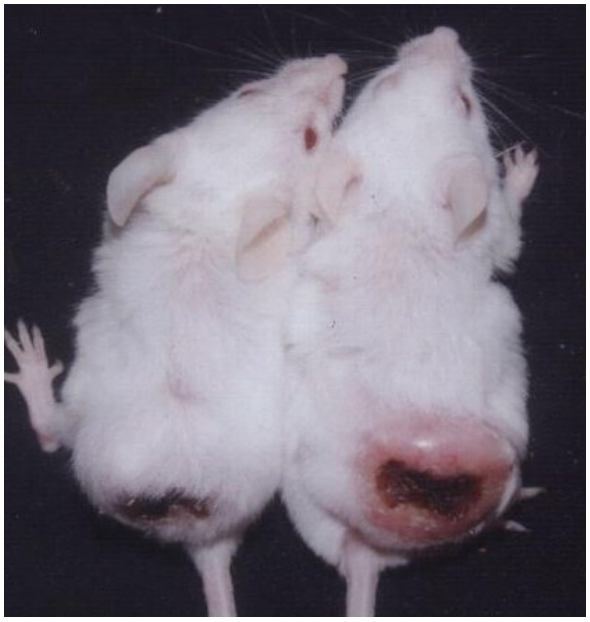


Figure 4b. Effects of ALA on tumors of mice at seven weeks post treatment.

EAC cells following irradiation of cells previously incubated with the relevant agents. It is substantially better than either ALA.HCl or ALA methyl ester at inhibitting growth of tumors involving EAC cells following inoculation of healthy animals with cell solutions that had been extracted from animals impregnated with EAC cells and incubated with the appropriate agents prior to irradiation with light. It is also substantially better and superior to ALA.HCl for destruction of large tumors following topical application to the skin covering solid tumors followed by irradiation with light. It is a very promising safe agent -as prepared from natural materials- for practical PDT. The photos showed impressive complete response in mice treated with our novel β-citronellol- ALA ester derivative.

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