α-Lipoic Acid Induces Intrinsic Apoptosis in Human Oral Squamous Carcinoma Cells

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Research Article

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Abstract

Oral squamous cell carcinoma is one of the leading cancers in India and it is responsible for significant morbidity and mortality. α-lipoic acid, a co-factor for several metabolic enzymes, suppresses the tumor growth. In this study, we investigated the α-lipoic acid-induced cytotoxicity and apoptosis in human oral squamous carcinoma (SCC-25) cells. α-lipoic acid treatments were given to SCC-25 cells for 24 h and cell proliferation was evaluated by MTT assay. The reactive oxygen species expression was examined by dichloro-dihydro-fluorescein diacetate assay. Apoptosis-related morphological changes were detected by dual staining. Cytochrome c and RAS (H-Ras) expression was measured by dual staining and RT-PCR respectively. Intrinsic apoptosis-related markers are analyzed using qPCR. α-lipoic acid inhibited SCC-25 cell proliferation in a concentration-dependent manner. This treatment also increased intracellular reactive oxygen species expression and the percentage of apoptotic cells (up to 70% of the cell population). Dual staining further confirms cytochrome c cytosolic expression. The oncogene H-Ras protein and gene expression was also down-regulated upon α-lipoic acid treatment in SCC-25 cells. qPCR analysis further confirms α-lipoic acid-induced an upregulation of bax, Apaf-1, caspase 3 and −9, pro-apoptotic gene expressions and downregulation of bcl-2, an anti-apoptotic gene expression. The present results suggest that α-lipoic acid has cytotoxic and pro-apoptotic potential and it also downregulates H-Ras oncogene expression in human oral squamous carcinoma cells. α-lipoic acid may have promising role in the treatment of human oral squamous carcinoma.

Introduction

Oral cancer causes 50–70% of total cancer mortality in India and it ranks top three in cancer incidence among Asian countries [1–3]. Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity represents 80 to 90% of all malignancies and the fifth most common type of cancer in the world [4, 5]. Numerous etiological factors like tobacco, smoking, alcohol consumption alone or with chewing tobacco, and betel quid have been associated with OSCC, these potential carcinogens responsible for the high occurrence of OSCC.6 Current treatment modalities employed in OSCC patients are chemo/radiotherapy, surgery, targeted therapy using epidermal growth factor receptor inhibitors and cyclooxygenase-2 inhibitors, and photodynamic therapy [6]. Despite great progress in chemotherapy, radiotherapy, and targeted therapy in the last three decades, the prognosis of OSCC is poor due to aggressive local invasion and metastasis, leading to high recurrence [7]. Survival rates of OSCC have also no improvement in recent years due to surgical mortality, resistance in chemo/radiotherapy [8]. It was reported that more than 20 oral cytotoxic drugs are available for oral cancer. Cytotoxic drugs like methotrexate, 6-mercaptopurine, and busulphan are widely used in oral chemotherapy [9]. These anticancer drugs have a narrow clinical setting in which their use is indicated and in addition, radiotherapy is also reported to induce certain side effects predominantly nausea, alopecia, vomiting, indigestion and metallic taste [9]. Therefore, there is an urgent need for the identification of a novel anticancer drug with minimal or no side effects. To overcome these side effects caused by cancer
chemotherapy, various new synthetic and naturally derived compounds are being widely investigated for their therapeutic potential against OSCC [10–12].

α-lipoic acid (ALA, thioctic acid (5-(1,2-dithiolan-3-yl)pentanoic acid) is a usually considered as "universal antioxidant". It is a naturally occurring antioxidant found in foods like meat, spinach, and cabbages [13–15]. It is synthesized in small amounts by plants and animals, including humans and it functions as an essential co-factor for several mitochondrial multi-enzyme complexes involved in energy metabolism [16]. It has been used as one of the dietary supplements to prevent and treat many diseases, including stroke, diabetes, neurodegenerative and hepatic disorders [13]. In experimental studies, ALA has shown beneficial effects against cardiovascular dysfunction [17], sepsis [18], endothelin dysfunction and oxidative stress [19], testicular dysfunction [20] and liver dysfunction [21]. Recently, ALA has been clinically tested for its beneficial effects in patients with fibromyalgia pain [14], multiple sclerosis [22], obesity [23], β-thalassemia [24], diabetes [25], non-alcoholic fatty liver disease [26]. In the cancer perspective, the anticancer potential of ALA was investigated against a variety of cancer cell lines including lung neuroblastoma, breast, leukemia and hepatoma [27–31] Clinically, ALA was undergone a phase II clinical trial against anticancer drug-induced alopecia in breast cancer patients [32] and chemotherapy-induced peripheral neuropathy [33] Therefore, it is reasonable to assume that ALA could be an effective agent against OSCC. Further, the ALA effect on SCC-25 human oral squamous carcinoma (OSCC) cell line has not been studied. Hence, in this study, we investigated the cytotoxic potentials of ALA on SCC-25 cells.

**Materials And Methods**

**Chemicals**

α-lipoic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT), dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co. (Chennai, India). The other chemicals used in this study were purchased locally and were of analar grade.

**Cell cultures and treatment**

The SCC-25 human oral squamous carcinoma (OSCC) cell line was procured from ATCC. Cells were maintained in Dulbecco’s Minimum Essential Media and Ham’s F-12 (1:1 ratio) supplemented with 10% fetal bovine serum (FBS), with penicillin (100 units/ml) and streptomycin (100 µg/ml). Cells were cultured in a humidified atmosphere with 5% CO2 at 37°C. Cells were grown in 75 cm2 culture flasks and after a few passages, cells were seeded for experiments. The experiments were done at 70 to 80% confluence. Upon reaching confluence, cells were detached using 0.05% Trypsin-EDTA solution.

ALA was dissolved in 0.1% DMSO (v/v). SCC-25 cells were plated at 10,000 cells/cm2. After 24 h, cells were fed with fresh expansion culture medium supplemented with different final concentrations of ALA (3, 6 and 9 mM) or the corresponding volumes of the vehicle. After 24 h of treatment, cells were collected
by trypsin application. Total cell number was determined by counting each sample in triplicate under inverted microscope.

**MTT assay**

Cytotoxic effect of ALA on SCC-25 cells was assessed by MTT assay [34] Cells were plated in 96-well plate at a concentration of 5 × 10⁴ cells/well. After 24 h, cells were fed with fresh expansion culture medium supplemented with different final concentrations of ALA (3, 6 and 9 mM) and incubated for 24 h. Untreated cells served as control and received only 0.1% DMSO. At the end of treatment period, media from control, ALA treated cells was discarded and 50 µl of MTT (0.5 mg/ml) in phosphate-buffer saline (PBS) was added to each well. Cells were then incubated for 4 h at 37°C in CO2 incubator. MTT was then discarded and the colored crystals of produced formazan were dissolved in 150 µl of DMSO and mixed effectively. The purple blue formazan dye formed was measured using an ELISA reader (BIORAD) at 570 nm.

**Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay**

The formation of ROS was measured using a non-fluorescent probe, DCFH-DA assay [35]. The expression of intracellular reactive oxygen species (ROS) was estimated in the control and ALA-treated OSCC. Briefly, an aliquot of 8 ×10⁶ cells/ml was made up to a final volume of 2 ml in normal PBS (pH 7.4). Then, 1 ml aliquot of cells was taken, to which 100 µl of DCFH-DA (10 µM) was added and incubated at 37 °C for 30 min. Fluorescent measurements were made with excitation and emission filters were set at 485 ± 10 nm and 530 ± 10 nm respectively (Perkin Elmer Multimode reader). Stained cells from each group were examined and imaged under Nikon fluorescence inverted microscope.

**Acridine orange/ethidium bromide (Dual staining)**

Acridine orange/ethidium bromide orange staining was carried out by the method of Gohel et al. [36]. SCC-25 cells were plated at a density of 1×10⁴ in 48-well plates. They were allowed to grow until they are 70–80% confluent. After 24 h, the cells were treated with different concentrations of ALA. The culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature. Then equal volumes of cells from control and ALA treated were mixed with 100 µl of dye mixture (1:1) of ethidium bromide and acridine orange and viewed immediately under Nikon inverted fluorescence microscope (Ti series) at 10x magnification. A minimum of 300 cells was counted in each sample at two different fields. The percentage of apoptotic cells was determined by [% of apoptotic cells = (total number of apoptotic cells/total number of cells counted) ×100].

**Double immunofluorescence staining**

SCC-25 cells were plated at a density of 1×10⁴ on glass cover slips in 48-well plates. After 24 hours post-plating, cells were incubated in the presence of increasing doses of ALA for 24 h. At the end of exposure, the cells were washed twice with D-PBS and then fixed using 4% formalin in PBS for 20 min at room temperature. After fixation, cells were washed twice with PBS and incubated with hydrogen peroxide
(3.3%) for 3 min to inactivate the endogenous peroxidase activity. Thereafter, fixed cells were permeabilized after 10 min incubation with D-PBS containing 1% Triton X-100 at room temperature. Permeabilized cells were washed twice with D-PBS and incubated with blocking solution (1% BSA in PBS) for one hour at room temperature. Primary antibody of monoclonal mouse caspase-3 diluted at 1:150 in 0.1% BSA was incubated with the cells for one hour at room temperature. After three washes with D-PBS, nuclear counterstaining was performed using propidium iodide. Preparations were then mounted for microscopic analysis.

**Gene expression analysis of H-Ras**

Total RNA was extracted by trizol reagent according to the standard protocol. Concentration of the extracted RNA was determined and the integrity of RNA was visualized on a 1% agarose gel using a gel documentation system (BioRad, Hercules, CA). The first strand of cDNA was synthesized from 1 µg of total RNA by reverse transcriptase using M-MLV (Promega, Madison, WI) and oligo (dT) primers (Promega) according to the manufacturer's protocol. Then, 2 µl of template cDNA was added to the final volume of 20 µl of reaction mixture. RT-PCR cycle parameters included 10 min at 95° C followed by 40 cycles involving denaturation at 95° C for 15 sec, annealing at 60° C for 20 sec, and elongation at 72° C for 20 sec. The sequences of the specific sets of HRAS and GAPDH used in this study were taken from literatures. Expressions of selected genes were normalized to the GAPDH gene, which was used as an internal housekeeping control. All the RT-PCR experiments were performed in triplicate.

**Protein expression analysis of H-Ras**

SCC-25 cells were treated with different ALA concentrations for 24 h. After treatment, cells were washed once with PBS and were harvested in RIPA buffer (Sigma Aldrich, USA) and the whole cell lysates were mixed with loading buffer containing sodium dodecyl sulphate (SDS) (Thermo Scientific, USA). Then, this mixture was heated at 95oC for 5min. After protein quantification, 50 µg of protein samples were subjected to 10% SDS-polyacrylamide gel electrophoresis with 120 v for 90 min. The gel was then electro-transferred to polyvinylidene difluoride membranes and was blocked for 2 h with 5% non-fat milk in PBS at room temperature. The primary antibodies were diluted in blocking buffer (1:1000) and H-Ras antibody (monoclonal, IgG1, BioRad, Chennai, India) were incubated with membranes overnight at 4oC. After incubation, membranes were incubated with corresponding horseradish peroxidase conjugated secondary antibodies at a dilution of 1:2000 at room temperature for 2 h. The membranes were then developed using the Pierce ECL plus western blotting substrate (Thermo Scientific, USA) followed by quantification using the NIH ImageJ analysis software.

**Gene expression analysis by real time PCR**

The modulation of apoptosis related gene expressions were analyzed by real time PCR. Total RNA was extracted from ALA treated and control cells. The purity and concentration of total RNA was measured using Nanodrop and 2 µg of RNA used for cDNA conversion using commercial cDNA conversion Kit (high cDNA, Applied Biosystem). The targeted genes Bax, Bcl-2, caspase 3 and 9, and Apaf −1ene were
amplified using gene specific primer sequences. B-actin used as internal control. Real time PCR reaction was performed in AbiprismIM 7700 PCR machine (Applied Biosystem). The real time PCR results were calculated by 2−ΔΔCT method and results are expressed as fold change.

**Statistical analysis**

Data were expressed as mean ± S.E.M and analyzed by Tukey’s test to determine the significance of differences between groups. A p value < 0.05, 0.01 or/and 0.001 was considered to be significant.

**Results**

**ALA-induced cytotoxicity in SCC-25 cells**

In this study, to find out the exact range of effective cytotoxic concentration of ALA in the SCC-25 cell line, initially, we have treated cells with ALA concentration ranges 2.5, 5, 7.5, 10, 12.5 and 15 mM. ALA treatments for 24 h caused a significant increase in the cytotoxicity of SCC-25 cells. The IC50 of ALA was found to be 9 mM in SCC-25 cells. Hence, further analyses were carried out with the concentration below the inhibitory concentration (< 9 mM) (Fig. 1A). The control and ALA treated cells displayed typical cancer morphology (Fi. 1B).

**ALA-induced ROS generation in SCC-25 cells**

To find out the exact reason behind the cytotoxic potential of ALA, we investigated the intracellular ROS expression. ALA treatments induced intracellular ROS expression in a concentration-dependent manner when compared to control cells. In this study, SCC-25 cells treated with 9 mM ALA show high fluorescent intensity, which indicates significant ROS generation and oxidative stress upon ALA treatment (Fig. 2A). Quantitative analysis also revealed the dose-dependent significant increase in ROS expression (Fi. 2B).

**ALA-induced apoptosis-related morphological changes in SCC-25 cells**

In order to ascertain whether intracellular ROS is responsible for apoptosis, we performed dual staining with AO/EB to investigate the apoptosis related morphological changes in SCC-25 cells. The untreated (control) cells shows completely absent from AO/EB staining which indicates the viable nature of control cells. In the experimental groups, ALA treatments caused SCC-25 cells positive to AO/EB staining. The presence of yellowish-green fluorescent color indicates the presence of early apoptotic cells. While cells with DNA damage stains red-colored ethidium bromide and are late apoptotic cells (Fig. 3A). The quantification of apoptotic cells further confirms the presence of a high percentage of apoptotic cells only in the experimental group. The percentage of apoptotic cells were increased (< 70%) significantly (P < 0.001) upon ALA treatments in SCC-25 cells (Fig. 3B).

**ALA induced cytosolic cytochrome c expression in SCC-25 cells**
Further, to explore mechanism behind the earlier mitochondrial apoptosis induced changes in SCC-25 cells, we investigated the cytosolic expression of caspase-3 by double staining with the maximum cytotoxic concentration of ALA on SCC-25 (i.e., 9 mM). Propidium iodide was used as a nuclear stain to differentiate the cytosolic cytochrome c expression. ALA treatment at the maximum concentration used in this study induced cytochrome c expression only in the cytoplasm whereas propidium iodide expression was confined to the nucleus. The control cells did not show any positivity to the above fluorescent staining (Fig. 4).

**ALA treatments downregulated H-Ras gene and protein expressions**

In this study, we also investigated the proto-oncogene H-Ras expression at gene and protein levels. ALA treatments significantly inhibited the H-Ras gene/protein expressions especially at the high concentrations used in this study. It has to emphasize that at low concentration i.e., 3 mM, ALA treatment did not cause any significant alterations in the H-Ras gene and protein expressions in SCC-25 cells. In higher concentrations (6 and 9 mM), ALA treatments significantly downregulated the H-Ras gene expression and however, protein expression was also prominently decreased (P < 0.01) only at 9 mM of ALA treatment in SCC-25 cells (Fig. 5A, B).

**ALA treatments caused the modulation of apoptosis-related gene expressions**

Further, to explore mechanistic reason behind the cytotoxic effect of ALA, we investigated apoptosis-related marker gene expressions pertaining to the mitochondrial pathway. ALA treatments caused significant (p < 0.001) increase in bax, Apaf-1, caspases 3 and −9 gene expressions with concomitant downregulation of (p < 0.01 vs 3 mM; p < 0.001 vs 6 and 9 mM) bcl-2 expression (Fig. 6).

**Discussion**

*In vivo* experimental studies, ALA, a co-factor for mitochondrial metabolic enzymes is shown to have a beneficial effect against various diseases [37–40]. ALA treatments *in vitro* have shown to cause significant cytotoxic effects in various cancer cell lines other than OSCC cells [30, 41–43]. In this study, ALA induced cytotoxicity of OSCC cells and our current results are in line with the above reports. Induction of intracellular ROS generation by natural agents or anticancer compounds is one of the prime reasons for their cytotoxic effect in cancer cells [44, 45]. Studies have also demonstrated the ROS inducing potential of ALA in cancer the cell line [41, 46]. Taken together, these results suggest that ALA could be a cytotoxic effect to OSCC cells possibly due to its ROS-inducing potential in cancer cells. AO/EB staining is one of the gold standard methods to differentiate early and late apoptotic cells [47]. The EB enters only in the cells that are in the late apoptotic stage due to nuclear membrane damage and hence these cells appear in red. While early apoptotic cells appear in yellowish-green. Control cells appeared as intense green color indicates the viable nature [48, 49]. In this study, ALA treatments caused early apoptosis and significant alteration in the OSCC cell membrane which was confirmed by the presence of yellowish-green colored cells. Further, intracellular ROS generation is also implicated in apoptosis-related morphological changes regardless of cancer cell lines [42, 50]. In light of the above studies, perhaps excessive ROS
generation by ALA might be responsible for the cytotoxic effect and oxidative stress-induced membrane damage associated with apoptosis in OSCC cells.

Upregulation of Bax and down-regulation of Bcl-2 is often observed during intrinsic apoptosis. Dissipation of mitochondrial membrane potential due to the accumulation of intracellular ROS can cause cytochrome c release into the cytosol. In the cytosol, cytochrome c form apoptosis that activates executioner caspases such as caspase 9 and −3. In a previous study, ALA is shown to induce caspase-3 expression in cancer cells and its expression is implicated for molecular target in the apoptosis induction [30]. Therefore, we analyzed the caspase-3 expression. Caspase-3 is localized in the cytosol and not actively expressed in normal cells. Any pro-apoptotic signal due to stress or ROS accumulation can cause cytochrome C release from mitochondria. In the cytosol, cytochrome C forms apoptosome complex with caspase-9 and Apaf-1 and this complex then activates the caspase-3 that triggers the apoptosis [47, 50]. ALA treatments enhanced bax, Apaf-1, caspase 9 and cytosolic caspase-3 expressions further confirm the apoptosis induction perhaps through the mitochondrial pathway in OSCC cells. RAS protein (H-Ras) is small GTPases that cycles between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound conformations [51]. The RAS signaling pathway is activated by several cellular stimuli regulating various physiological functions such as cell growth, cell survival, cell cycle progression, protein translation, and metabolism [52]. Overexpression of H-Ras is shown to promote proliferation, metastasis and angiogenesis of cancer cells [53, 54]. ALA treatment caused significant downregulation of proto-oncogene H-Ras expression at gene and protein level and these results further indicate the tumor suppressive effect of ALA in OSCC cells. Moreover, it is worth noting that ALA is shown to attenuate radiation-induced oral mucositis [55] and cisplatin [56], cyclophosphamide [57] doxorubicin-induced toxicities [58]. In view of the above results and our current findings suggests that ALA may be useful as a therapeutic adjuvant along with other anticancer cancer drugs to minimize their side effects. The possible ALA-induced apoptotic effect is reported in Fig. 7.

Conclusions

Our present results suggest that ALA is cytotoxic to SCC-25 cells. The intracellular ROS generation was the primary reason for ALA-induced cytotoxicity and oxidative stress-related morphological damage. Further, ALA treatments induced prominent cytosolic expression of caspase 3, an important indicator of early apoptotic signal and downgraded the H-Ras expressions at gene and protein level in SCC-25 cells providing more basis for ALA use as antitumor agents in cancer therapy.

Declarations

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NIL

Conflicts of interest
The author declares no conflict of interest.

Availability of data and material

Data available with the corresponding author. It will be shared upon reasonable request

Code availability (software application or custom code)

Not available

Authors' contributions

DR, performed research and written the draft manuscript; DE, conceived the idea, performed research and corrected the manuscript; PE, performed research and corrected the manuscript

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

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**Figures**
Figure 1

Anti-proliferative effect of α-lipoic acid (ALA) in oral squamous carcinoma cells (SCC-25) cells. Cells treated with different concentrations of ALA (A) Morphology of OSCC. (B) Cytotoxic effect. Values are expressed as Mean ± SEM (n = 3). *** P < 0.001.
Figure 2

Intracellular ROS analysis by dichlorofluorescein staining assay. A. Representative fluorescent microscopic images of control and ALA treated OSCC cells B. Quantification of ROS expression.

Figure 3

Apoptosis related morphology (A) Representative images of control and ALA treated OSCC cells stained with ethidium bromide and acridine orange. Blue and white arrow heads indicate early and late apoptotic cells respectively. A-Control, B, C and D – ALA 3, 6 and 9 mM treatments respectively. (B) Quantification of percentage of apoptotic cells. Values are expressed as mean ± SEM (n=3). Apoptotic cells were individually calculated as percentage of apoptotic cells relative to the total number of cells in each random field and represented the average of three independent experiments ± SEM. ***p<0.001.
Figure 4

Cytochrome C expression by dual staining. Representative immunestaining images of control OSCC and ALA treated (9 mM) cells. Cells were treated with caspase-3 and propidum iodide nuclear staining. Merged image confirm the expression of cytochrome c in cytosol (green) and nucleus stains only with propidium iodide (PI) (red).
Figure 5

Expression of HRAS (A) HRAS gene expression (B) HRAS protein expression. 1-DNA/protein Ladder, 2 - Control, 3, 4 and 5 – ALA 3, 6 and 9 mM treatments respectively (C) Quantification of HRAS gene expression (D) Quantification of HRAS protein expression. Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed by One way ANOVA followed by Tukey’s test. *P < 0.05; **P < 0.01 and ***P<0.001 as considered significant.
Intrinsic apoptosis pathways-related marker gene expression Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed by One way ANOVA followed by Tukey’s test. bP < 0.01 and aP<0.001 considered as significant. β‐actin was used as internal control for normalization.

Figure 6
Figure 7

Possible pro-apoptotic mechanism of α-lipoic acid (ALA) in oral cancer cells. N, nucleus; M, mitochondria

 Supplementary Files

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