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RESEARCH ARTICLE

L-ascorbic acid Synergizes with Paclitaxel to Enhance Anti-cancer Efficacy in Skin Cancer Cells

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Abstract

Background: Skin cancers with two major subtypes, melanoma and non-melanoma cases are rising worldwide. Paclitaxel has been used as an advanced stage therapeutic option in skin cancers, however, therapy resistance and metastasis are a limitation to its efficacy. L-ascorbic acid on the other hand is less toxic and has been demonstrated to possess both anti-cancer and pro-immune activities in clinical and preclinical settings. **Methods:** The A375, melanoma cell line and the A431, non-melanoma cell line were used to determine efficacy between single agent paclitaxel and combination with L-ascorbic acid by multiple cells-based functional assays including cytotoxicity, invasiveness, cancer stem cell specific functions and molecular assessments by quantitative PCR. Low glucose conditions and/or adaptations were also used to determine the impact of differential glucose concentrations on drug combination efficacy. **Results:** Functional and transcriptomics data demonstrated that compared to single agent paclitaxel, the combination of paclitaxel with L-ascorbic acid restricted A375 and A431 cell growth potential, cellular invasiveness and cancer stem cell like-cells. Low glucose conditions further enhanced the superiority of the combination therapy. **Conclusion:** This study reveals that L-ascorbic acid may improve the current therapeutic limitations associated with paclitaxel in skin cancers. Importantly, this combination is equally effective in both melanoma and non-melanoma based *in vitro* cell model systems.

Keywords: Skin cancers- melanoma- non-melanoma- LAA- paclitaxel- combination therapy- CD44- GLUT 1- PI3K

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Introduction

Skin cancers are the fifth most commonly diagnosed cancer worldwide with two major subtypes; melanoma and non-melanoma [1]. Comparatively, non-melanoma constitutes the majority of the skin cancers, but are mostly benign or remain less aggressive, if treated early; whereas melanoma represents approximately 20% of the total skin cancer incidences but is responsible for approximately 80% skin cancer related death due to its aggressiveness [2]. Higher incidence of skin cancers is primarily confined to Caucasians with North America, Northern Europe and Australasia exhibiting a 4-6% annual increase in incidence rate over the last 25 years [3].

Currently, for skin cancer management, the most common treatment strategies include surgery, chemo-/radio-therapy, targeted-/immune-therapy [4]. Surgery and radio-therapy is restricted to locally advanced disease conditions whereas targeted/immune-therapy can be limited by poor bioavailability and high treatment cost. In addition, a plethora of chemotherapeutic agents such as Dacarbazine, Temozolomide, Nab-paclitaxel, Paclitaxel, Cisplatin and Carboplatin are being used for the treatment of skin cancers where they can either be used as monotherapy or in combination with other agents [5].

Unfortunately, currently available therapies are associated with poor prognosis and long-term side effects along-with the development of resistance to these drugs. Although, paclitaxel is not typically a first-line treatment for skin cancer, in certain cases, paclitaxel may be considered as a treatment option for advanced or metastatic skin cancer, particularly if other treatments have

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not been effective [6]. Several studies have highlighted the effectiveness of paclitaxel either as monotherapy or in combination with other agents for managing metastatic melanoma. Gebhardt et al proposed that low dose paclitaxel combined with immunotherapy could further improve therapeutic outcomes in some advanced melanoma patients [6]. In addition, a peptide pulsed dendritic cells vaccine in combination with Carboplatin and paclitaxel chemotherapy exhibited superiority over standard regimens for advanced melanoma [7]. Importantly, both intravenous and topical based paclitaxel has also been shown to be efficacious in non-melanoma skin cancers [8, 9].

Vitamin C (i.e. L-ascorbic acid (LAA)), is a potent anti-oxidant and exhibits anti-tumour properties in various preclinical studies [10]. In the clinical setting, high dose intravenous LAA was demonstrated to be safe however showed inconsistent results in terms of efficacy in advanced stage cancers. Severe LAA deficiency (plasma concentration < 11µmol/L) is significantly increased in advanced stage cancer patients [11]. Furthermore, LAA has exhibited promising signs of efficacy in combination with various chemotherapy agents and can decrease therapy mediated complications in advanced disease conditions [11]. LAA induced epigenetic reprogramming and enhanced immune functions also resulted in decreased tumour growth in preclinical melanoma models [11, 12].

In this study, we evaluated whether paclitaxel in combination with LAA could further improve the efficacy of paclitaxel alone in skin cancer using *in vitro* models.

Materials and Methods

Cell culture

All cell lines used in this study were procured and authenticated by STR profiling from National Centre for Cell Science, Pune, India. A375 and A431 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 25mM glucose and 10% FBS and incubate under 37 ^{0}C/ 5% CO_2 incubator. For differential glucose adaptation purposes, a fraction of cells from 25mM glucose (High Glucose, HG) conditions were continuously cultured in 5mM glucose (Low Glucose, LG) containing media for at least 8 weeks (Supplementary Figure 1). U937 cells were maintained in RPMI media supplemented with 25mM glucose and 10% FBS and incubate under 37 ^{0}C/ 5% CO_2 incubator.

Reagents

DMEM and FBS (Himedia, IN), MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Toronto Research Chemicals, CA), poly-HEMA (2-hydroxyethyl methacrylate), LAA, TRI reagent and Geltrex™ Matrix (Merck, US), paclitaxel (SPAL, IN), cDNA synthesis kit (Biorad, US) and SYBR green (Genei, IN).

Cell based functional assays

For MTT and apoptosis assay, 3 x 10^3 cells were seeded in a 96 well plate format and after 48h, exposed to either paclitaxel and/or LAA for a further 48h drug exposure. Cell viability was measured by MTT based colorimetric readouts. Apoptosis was assessed using Hoechst 33342 staining, followed by counting of apoptotic nuclei under UV illumination. For Colony forming assay, 4 x 10^4 cells were seeded in a 24 well plate format and after 48h, exposed to either paclitaxel and/or LAA for a further 48h.Cells were then fixed and stained with 0.1% crystal violet. After image acquisition, the corresponding colonies were dissolved in 20% acetic acid, and the absorbance was measured at 595 nm for colorimetric analysis. For the sphere-formation assay, 2 x 10^3 cells were seeded in conditioned media in poly-HEMA-coated, low-attachment 96-well plates. After 48-72 hours, once spheres became visible, the wells were treated with paclitaxel and/or LAA. Following 48 hours of drug exposure, sphere sizes were measured using the Cytation 5 (BioTek, USA) imaging platform. For the cell invasion assay, 2.5 x 10^4 cells were seeded in 1% FBS medium containing paclitaxel and/or LAA on the inner side of 8 µm Boyden chamber inserts (24-well format), with 10% FBS medium placed in the lower chamber as the chemoattractant. After 24 hours, the invaded cells were stained with Hoechst 33342 and subsequently counted under UV illumination. For the immune cell co-culture assay, U937 cells (effectors, E), with or without prior exposure to LAA, were co-cultured with the respective cancer cells (targets, T) at an E:T ratio of 10:1 for 24 hours, followed by MTT-based assessment. To determine synergistic or additive effects between paclitaxel and LAA, drug interactions were analysed as previously described [13]. In brief, for both A375 and A431 HG and LG cells, the observed survival, corrected for the baseline viability of each sample, was plotted against the expected survival, which was calculated from the product of the viability fractions of samples treated individually with paclitaxel and LAA [(paclitaxel × LAA)/100]. The diagonal XY line, represents the condition where the observed survival equals the predicted survival. Points falling below this line indicate synergistic interactions (observed survival < expected survival), while points above the line indicate additive interactions (observed survival > expected survival).

Quantitative PCR

Quantitative PCR (qPCR) was performed as described earlier [14]. In brief, 2 × 10^5 cells were seeded, and after 48 hours of incubation, they were treated with paclitaxel and/or LAA for 18 hours. Subsequently, qPCR was performed using SYBR Green, and data were analyzed using the ddCt method. Beta-actin (β-Actin) was used as a housekeeping gene. Primer sequences used are listed below in Table 1.

Statistics

Statistical analyses were performed using GraphPad Prism (GraphPad, US). All assays were performed in triplicate with at least two technical repeats (unless otherwise indicated) and the data are described as the mean ± standard error. Statistical significance was determined either by one-way Analysis of Variance (ANOVA) followed by Bonferroni's post-hoc test or unpaired two

Table 1. The List of Primer Sequences

Gene	Forward Primer	Reverse Primer
VEGFA	5'-TACCTCCACCATGCCAAG-3'	5'-GGTACTCCTGGAAGATGTC-3'
LDHA	5'-GAGTGGAATGAATGTTGCTGGTGTC-3'	5'-CCAGGATGTGTAGCCTTTGAGTTTG-3'
CD44	5'- ACACCATGGACAAGTTTTGGTG-3'	5'-CTGCAGGTCTCAAATCCGATG-3'
GLUT1	5'-ATTGGCTCCGGTATCGTCAAC-3'	5'-GCTCAGATAGGACATCCAGGGTA-3'
TWIST1	5'-GTCCGCAGTCTTACGAGGAG-3'	5'-GCTTGAGGGTCTGAATCTTGCT-3'
SLC7A5	5'-GGACTTCGGGAACTATCACC-3'	5'-GAACAGGGACCATTGACGG-3'
PI3K	5'-CCACGACCATCATCAGGTGAA-3'	5'-CCTCACGGAGGCATTCTAAAGT-3'
β-Actin	5'-ATGATATCGCCGCGCTCG-3'	5'-CGCTCGGTGAGGATCTTCA-3'

tailed student's-T-test (as stated in the Figure legends). P value of ≤0.05 was considered as statistically significant.

Results

LAA improves paclitaxel mediated cytotoxicity in skin cancer in vitro models

To evaluate whether LAA enhances paclitaxel sensitivity in A375 and A431 cells, IC_{50} concentrations of each drug (paclitaxel ~10 nM for both A375 and A431; LAA ~300 µM for both cell lines), as determined in Supplementary Figure 2, were applied either individually or in combination. Cytotoxicity was assessed using MTT-based cell viability assays and Hoechst 33342-based apoptosis measurements. Compared with paclitaxel alone, the combination treatment resulted in significantly reduced cell viability (A375: 51% ± 2.8 vs. 20% ± 0.3, $p < 0.001$; A431: 43% ± 1.7 vs. 31% ± 0.68, $p = 0.008$) (Figure 1A) and markedly increased apoptosis (A375: 1.3 ± 0.2-fold vs. 5.8 ± 1.5-fold, $p = 0.02$; A431: 2.6 ± 0.3-fold vs. 4.9 ± 0.6-fold, $p = 0.02$) (Figure 1B). Additionally, LAA and paclitaxel exhibited synergistic effects in both high-grade (HG) and low-grade (LG) A375 cells, while demonstrating additive interactions in A431 HG and LG cells (Figure 1C). Collectively, these findings suggest that combining LAA with paclitaxel may offer a promising therapeutic strategy, particularly in advanced skin cancer settings.

LAA augments the anti-cancer efficacy of paclitaxel in skin cancer in-vitro models

To assess whether LAA could enhance paclitaxel's efficacy in suppressing skin cancer growth, colony formation assay was conducted. The combination treatment resulted in significantly lower colony-forming efficiency compared to paclitaxel alone (A375: 59% ± 4 vs. 16.3% ± 3.2, $p = 0.0004$; A431: 66% ± 4 vs. 26% ± 0.4, $p < 0.0001$) (Figure 2A and Supplementary Figure 3).

Next, to evaluate the effect of the combination on treatment-resistant, cancer stem cell–like populations, sphere formation assays were performed. The combination treatment produced smaller spheres than paclitaxel alone (A375: 40% ± 0.8 vs. 22% ± 0.9, $p = 0.0002$; A431: 49% ± 3.2 vs. 37% ± 1.7, $p = 0.008$) (Figure 2B and Supplementary Figure 4).

Furthermore, in a cell invasion assay, the combination of

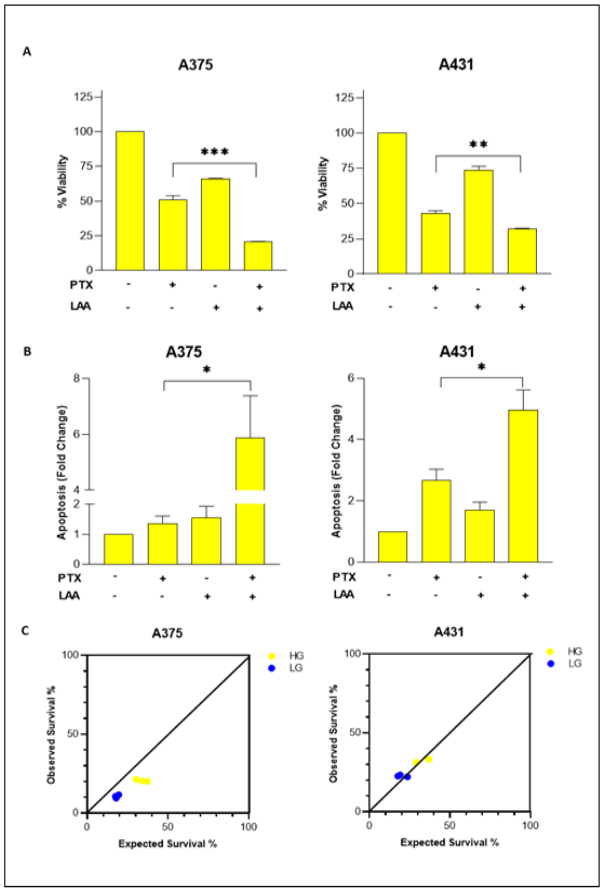

Figure 1. Compared to single agent, combination of paclitaxel and L-ascorbic acid treatment exhibited lowest cell survival in both A375 and A431 skin cancer *in vitro* models. Both A375 and A431 cells were treated either with paclitaxel (10nM) or L-AA (300µM) or combinations for 48h followed by (A) MTT based quantitative cell viability assessments and (B) Hoechst 33342 dye based apoptotic nucleus staining. Untreated group was used as a control (100% for panel A or 1 for panel B) in respective cell line and all other treatment groups are compared against respective control groups. (C) Synergistic interactions between paclitaxel and L-AA were evaluated in both A375 and A431 HG and LG adapted cells and as described in the Materials & Methods section. XY line, observed survival= expected survival. Points below the line represents synergistic interactions; points above the line represents additive interactions. Quantitative results are average of two independent experiments with n=3 replicates. One way ANOVA followed by multiparametric test was performed for statistical evaluation ***; P<0.001; **; p<0.01 and *; p<0.05.

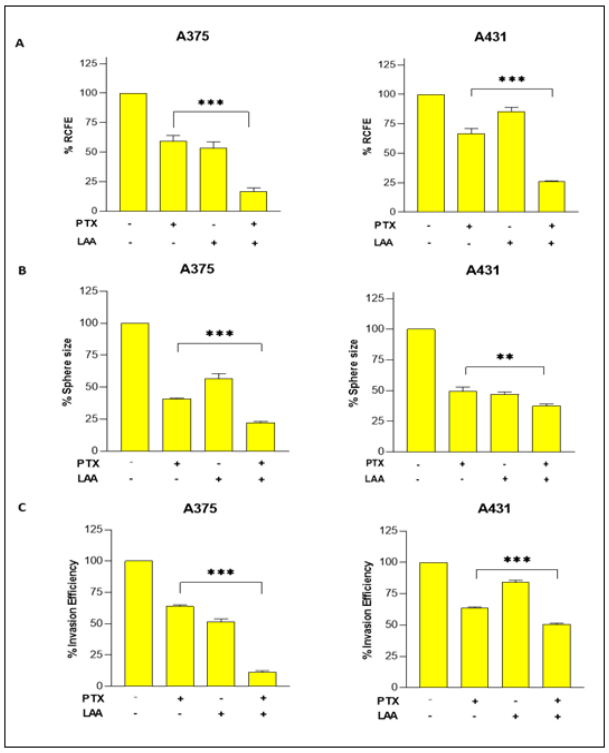

Figure 2. Compared to single agent, combination of paclitaxel and L-ascorbic acid treatment exhibited enhanced therapeutic efficacy in both A375 and A431 skin cancer *in vitro* models. (A) Post cell seeding and cell growth observations, both A375 and A431 cells were treated either with paclitaxel (10nM) or L-AA (300µM) or combinations for 48h followed by 0.1% crystal violet staining and quantitative assessments by dissolving crystal violet stains using 20% acetic acid and colorimetric measurement at 595nm. % RCFE= (Total no. of colonies in the well/ Total no. of cells seeded) X 100. RCFE: Relative Colony Forming Efficiency. (B) Both A375 and A431 cells were seeded in low attachment plate in respective conditioned media for 72-96h till compact sphere formation was observed and then treated either with paclitaxel (10nM) or L-AA (300uM) or combinations for another 48-72h followed by microscopic observation of sphere sizes across the groups. (C) Both A375 and A431 cells were seeded in the inner side of 8µM Boyden chamber inserts with 1% FBS media with/out drug/s. After 24h invaded cells were fixed, stained with Hoechst dye to stain the nucleus and counted. % Invasion efficiency = Total number of cells invaded on the outer side of the inserts/total number of cells seeded at 0h X100. Untreated group was used as a control (100%) in respective cell line and all other treatment groups are compared against respective control groups. Quantitative results are representative of two independent experiments with at least n=2 replicates. One way ANOVA followed by multiparametric test was performed for statistical evaluation ***; P<0.001 and **; p<0.01.

paclitaxel and LAA markedly reduced invasion efficiency compared to paclitaxel alone (A375: 63.8% ± 1.2 vs. 11.5% ± 1.1, p < 0.0001; A431: 63.7% ± 0.7 vs. 50.7% ± 0.7, p < 0.0001) (Figure 2C and Supplementary Figure 5).

Previous clinical findings suggest that reduced plasma serum LAA levels in various cancer stages and conditions correlates with poorer survival [15]. High dose LAA treatment in various clinically relevant advanced disease conditions, including skin cancers exhibits minimum toxicity with some improvement in clinical manifestations [16]. Moreover, paclitaxel induced systemic toxicity has been well documented [17]. The current study opens up the possibility of clinical utilization of LAA and paclitaxel as a superior choice in an advanced skin cancer cases and may allow for lower drug doses to be used in patients to reduce overall toxicity.

Low glucose conditions further enhance combination treatment-mediated cytotoxicity in skin cancer cells

A low-glucose (LG) environment has been shown to enhance paclitaxel's therapeutic efficacy in in-vitro solid tumor models [18]. Consistent with this, LG adaptation led to increased basal cytotoxicity in both cell lines (Figure 3A). Notably, under LG conditions, the combination treatment produced the lowest cell viability compared to paclitaxel alone (A375: 36.8% ± 1 vs. 10.5% ± 0.5, p < 0.0001; A431: 41% ± 1.4 vs. 22.7% ± 0.3, p = 0.0002) and the highest apoptosis levels (A375: 6.8 ± 1.1-fold vs. 16.7 ± 1.9-fold, p = 0.001; A431: 2.6 ± 0.1-fold vs. 6 ± 0.1-fold, p < 0.0001) in both skin cancer cell lines (Figure 3).

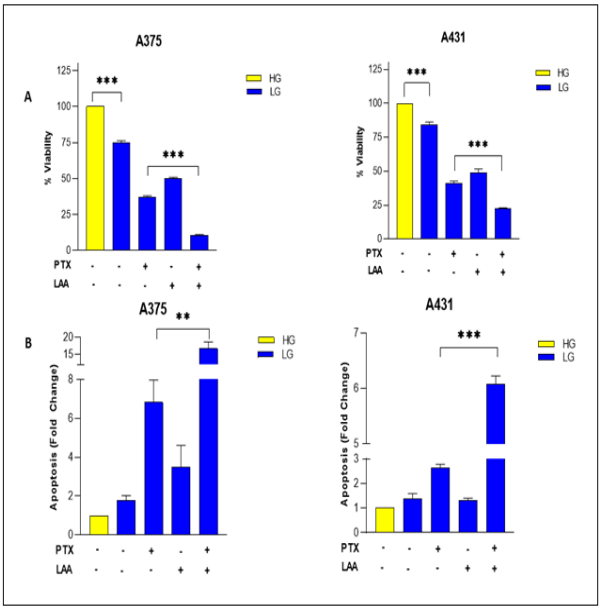

Figure 3. Low glucose conditions further enhances combinatorial treatment efficacy by exhibiting lowest cell survival in both A375 and A431 skin cancer *in vitro* models. Post cell seeding of both A375 and A431 HG and LG adapted cells, only LG adapted cells were treated either with paclitaxel (10nM) or L-AA (300µM) or combinations for 48h followed by (A) MTT based quantitative cell viability assessments; and (B) Hoechst 33342 dye based apoptotic nucleus staining. Untreated group was used as control in respective HG and LG adapted cell line and all other treatment groups are compared against respective HG control groups (100% for panel A or 1 for panel B). Quantitative results are average of two independent experiments with n=3 replicates. One way ANOVA followed by multiparametric test was performed for statistical evaluation ***; P<0.001 and **; p<0.01.

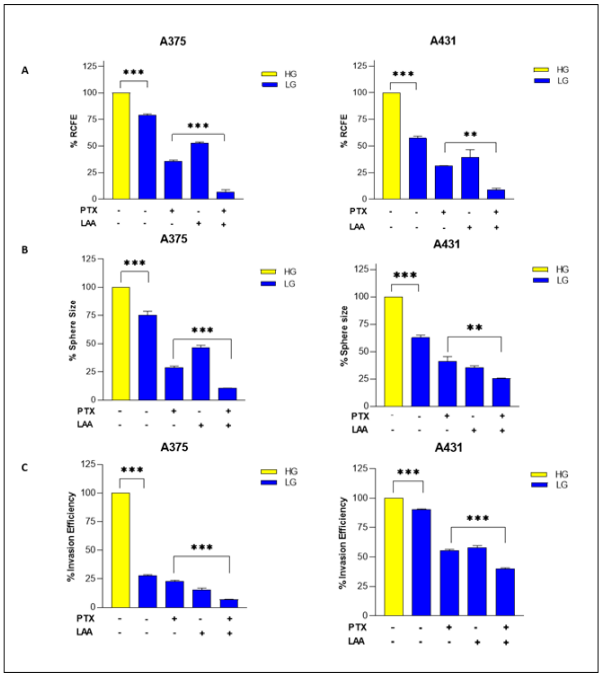

Figure 4. Low glucose conditions further enhances combinatorial treatment efficacy in both A375 and A431 skin cancer *in vitro* models. (A) Post cell seeding and upon observation of cell growth of both A375 and A431 HG and LG adapted cells, only LG adapted cells were treated either with paclitaxel (10nM) or L-AA (300µM) or combinations for 48h followed by 0.1% crystal violet staining and quantitative assessments by dissolving crystal violet stains using 20% acetic acid and colorimetric measurement at 595nm; % RCFE= (Total no. of colonies in the well/ Total no. of cells seeded) X 100. RCFE:Relative Colony Forming Efficiency. (B) Both A375 and A431 HG and LG adapted cells were seeded in low attachment plate in respective conditioned media for 72-96h till compact sphere formation was observed and then only LG group cells were treated either with paclitaxel (10nM) or L-AA (300µM) or combinations for another 48-72h followed by microscopic observation of sphere sizes across the groups. (C) Both A375 and A431 HG and LG adapted cells were seeded in the inner side of 8µM boyden chamber inserts with 1% FBS media and only the LG cell inserts were treated either with paclitaxel (10nM) or L-AA (300µM) or combinations for 18-24h with/out drug/s. After 24h invaded cells were fixed, stained with Hoechst dye to stain the nucleus and counted. % Invasion efficiency = Total number of cells invaded on the outer side of the inserts/total number of cells seeded at 0h X100. For above all experimental conditions, untreated HG adapted cell group was used as a control (100%) in respective cell line and other treated groups are compared against respective HG control groups. Quantitative results are average of two independent experiments with at least n=3 replicates. One way ANOVA followed by multiparametric test was performed for statistical evaluation ***; P<0.001 and **; p<0.01.

Low-glucose conditions elicit the strongest inhibitory effects of the combination therapy on cancer stemness, clonogenicity, and invasiveness in skin cancer cells

Consistent with the cytotoxicity results, LG conditions markedly reduced colony-forming efficiency (A375: 100% vs. 79 ± 1.2%, $p < 0.0001$; A431: 100% vs. 57 ± 1.9%, $p < 0.0001$), sphere size (A375: 100% vs. 75 ± 3.3%, $p < 0.0001$; A431: 100% vs. 62.7 ± 2.3%, $p < 0.0001$), and invasion efficiency (A375: 100% vs. 27.9 ± 0.7%, $p < 0.0001$; A431: 100% vs. 90 ± 0.3%, $p = 0.0005$) at baseline in both cell lines (Figure 4).

Under LG conditions, the combination treatment further reduced colony-forming efficiency compared to paclitaxel alone in A375 (35 ± 1% vs. 6.6 ± 2.1%, $p < 0.0001$) and A431 (31 ± 0.3% vs. 9 ± 1.3%, $p = 0.008$) cells (Figure 4A and Supplementary Figure 3). Similarly, sphere formation was lowest with the combination treatment compared to paclitaxel alone in both A375 (28.7 ± 1.2% vs. 10 ± 0.1%, $p < 0.0001$) and A431 (41.3 ± 4.2% vs. 25.5 ± 0.3%, $p = 0.001$) cells (Figure 4B and Supplementary Figure 4).

In the invasion assay, the combination also yielded the lowest invasion efficiency compared to paclitaxel alone in A375 (22.8 ± 0.9% vs. 6.9 ± 0.2%, $p < 0.0001$) and A431 (55.5 ± 0.9% vs. 40 ± 0.5%, $p < 0.0001$) cells (Figure 4C and Supplementary Figure 5).

Taken together, LG conditions enhanced the combinatorial drug response, amplifying the overall anti-cancer effects. These findings align with previous reports showing that paclitaxel displays greater activity under LG conditions [18] and that LAA can inhibit glucose utilization pathways to exert anti-cancer effects [19].

3.5 LAA enhances immune functions, promoting a stronger anti-tumour immune response in a skin cancer-immune cell co-culture model

Next, to evaluate whether LAA can enhance anti-tumor immune activity within the tumor microenvironment (TME), U937 cells (a pro-monocytic model) were pre-treated with LAA (300 µM) and subsequently co-cultured directly with HG- and LG-adapted A375 and A431 tumor cells to assess cytotoxicity. Compared with untreated U937 cells, LAA-treated U937 cells demonstrated significantly greater tumor-killing capacity

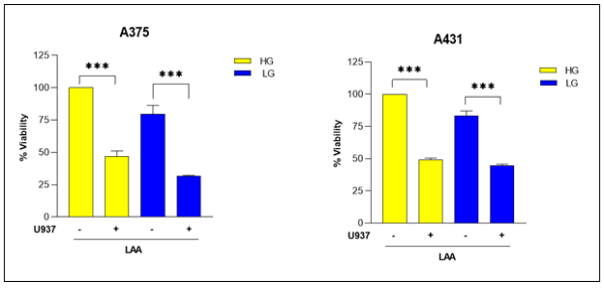

Figure 5. Exposures of L-AA in U937 cells resulted in an enhanced anti-tumour activity in both HG and LG conditions with decreased cell viability in skin cancer *in vitro* co-culture models. U937 cells (pro-monocytic model) with/out exposures to L-AA (300µM) for 24h followed by U937 and tumour cells (A375 HG and LG adapted or A431 HG and LG adapted) co-culture (Target (cancer cells): Effector (U937) =1:10) for 24h durations and further (A) MTT based cell viability determinations (direct co-culture) was performed. Respective HG cells without L-AA exposure group was considered as control (100%) and other treated groups (both HG and LG) were compared against their respective HG control groups. Quantitative results are average of two independent experiments with n=3 replicates. ***; P<0.001.

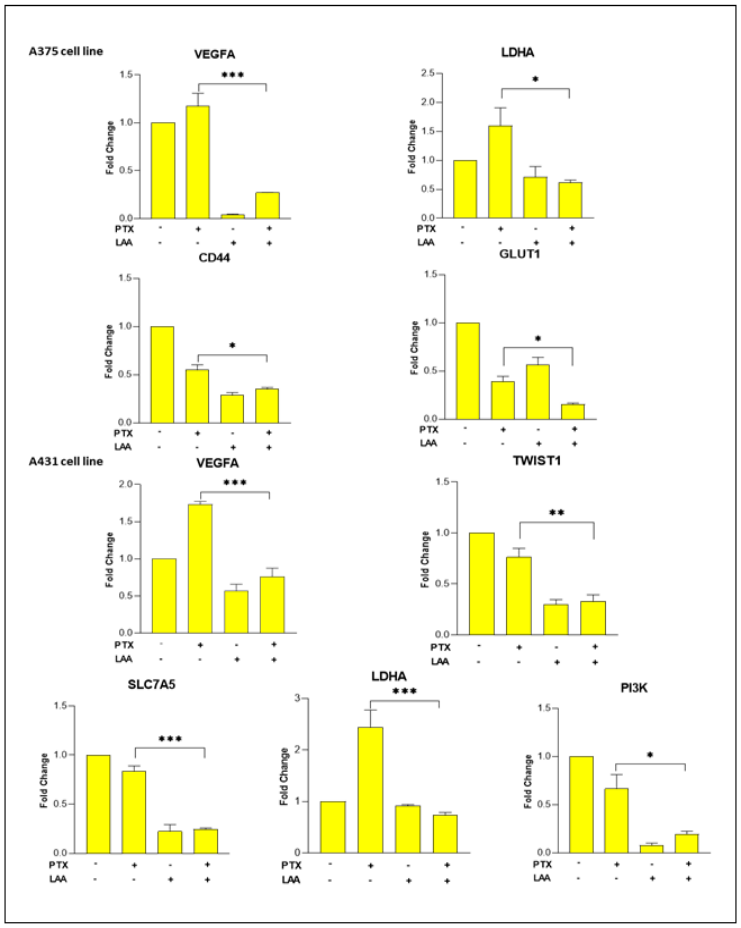

Figure 6. Paclitaxel with L-AA combination treatment resulted in altered gene expression signatures in skin cancer *in vitro* cell models. Both A375 and A431 HG cultured cells were treated either with paclitaxel (10nM) or L-AA (300uM) or combinations for 18h followed by RNA extraction, cDNA conversion and qPCR with a sets of metastatic and metabolism specific primers (Vascular Endothelial Growth Factor A (*VEGF-A*), Lactate Dehydrogenase A (*LDHA*), *CD44* and Glucose transporter 1 (*GLUT1*) for A375 cells; *VEGFA*, *TWIST1*, Solute carrier family 7 member 5 (*SLC7A5*), *LDH-A*, and phosphatidylinositol 3-kinase (*PI3K*) for A431 cells). qPCR was analyzed with ddCT methods and untreated group was used as a control and a baseline for other drug mediated fold expression calculations. B-Actin was used as a housekeeping gene. Results are interpreted from n=3 replicates. One way ANOVA followed by multiparametric test was performed for statistical evaluation ***; P<0.001, **; p<0.01 and *; p<0.05.

under both HG (A375: 100% vs. 46.8 ± 4.3%, p < 0.0001; A431: 100% vs. 49 ± 1.3%, p < 0.0001) and LG conditions (A375: 79.8 ± 6.5% vs. 31.6 ± 0.7%, p = 0.001; A431: 83.2 ± 3.7% vs. 44.2 ± 1.2%, p < 0.0001) (Figure 5).

Similarly, conditioned media from LAA-treated U937 cells also reduced tumor cell viability in indirect co-culture assays, though to a lesser extent (Supplementary Figure 6A). Consistent with previous reports [20], LAA-treated U937 cells in co-culture showed reduced levels of pro-inflammatory cytokines *IL-6* and *CXCL-10* (Supplementary Figure 6B).

Paclitaxel-induced immune suppression within the TME [21], as well as LAA-mediated immune activation observed in both clinical [22] and preclinical studies [23], are well documented. Consistent with these findings, our U937-tumor cell co-culture experiments showed that LAA enhances U937 immune activity under both HG and LG conditions (Figure 5). Moreover, LAA treatment substantially reduced pro-inflammatory cytokine responses within the TME, which may further potentiate anti-tumor immune activity [24].

LAA-paclitaxel combination treatment suppresses metastasis and stemness-associated gene expression in skin cancer cells

To correlate the functional assessment data with the combinatorial treatment of LAA and paclitaxel in both A375 and A431 cells, quantitative PCR (qPCR) was performed. Lactate dehydrogenase A (*LDHA*) and vascular endothelial growth factor A (*VEGFA*) are well-established contributors to cancer progression and metastasis across several tumor types, including skin cancer [25, 26]. Relative to untreated controls, paclitaxel alone induced an upregulation of *VEGFA* (A375: 17%; A431: 73%, p <0.001) and *LDHA* (A375: 60%; A431: 143%, p <0.001) (Figure 6). However, compared to paclitaxel monotherapy, the combination treatment led to a significant reduction in both VEGFA (A375: 76.99%, p < 0.0001; A431: 56%, p = 0.0001) and *LDHA* expression (A375: 61%, p = 0.03; A431: 69%, p = 0.0007).

CD44, a recognized stem cell marker across multiple cancers [27], and *GLUT1*, whose elevated expression is associated with poor prognosis [28], were also examined. In A375 cells, the combination treatment significantly

downregulated *CD44* (36%, $p < 0.01$) and *GLUT1* (61%, $p = 0.03$) compared to paclitaxel alone.

Additionally, TWIST1 (a key metastatic regulator [29]), *SLC7A5* (an amino acid transporter linked to aggressive disease [30]), and PI3K (a central player in survival and metastasis signalling [31]) were substantially reduced in A431 cells following combination treatment relative to paclitaxel monotherapy, TWIST1 (56%, $p = 0.004$), *SLC7A5* (70%, $p < 0.0001$), and PI3K (71%, $p < 0.01$) (Figure 6). Similar downregulatory trends, though not statistically significant, were observed for HIF-1α, *GLUT1*, MMP9, and *CD44* (Supplementary Figure 7).

Collectively, these gene expression results strengthen the evidence that the LAA-paclitaxel combination more effectively suppresses pathways associated with invasiveness, stemness, and metastatic potential. Notably, this therapeutic advantage is observed in both melanoma and non-melanoma skin cancer models.

Discussion

Chemotherapy plays a significant role in the clinical management of skin cancers (including melanoma and non-melanoma). However, the major therapeutic challenges to tackle skin cancers spread and metastasis arise from the limited therapeutic efficacy due to poor bioavailability, non-specificity, severe side effects of the drug/s and the possibility of development of therapy resistance [4]. Although paclitaxel is not the first line of therapeutic agent for malignant melanoma, it has shown a response rate of 18–20% in advanced metastatic malignant melanoma [32]. Utilization of paclitaxel either in single or in combination therapy in progressive non-melanoma skin cancers has also demonstrated clinical efficacy, but only in limited instances [33, 34].

Previous clinical findings suggest that reduced plasma LAA levels in various cancer stages and conditions correlate with poorer patient survival [15]. High dose LAA treatment in various clinically relevant advanced disease conditions, including skin cancers exhibits minimum toxicity with some improvement in clinical manifestations [16]. In contrast, paclitaxel-induced systemic toxicity has been well documented [17]. Recently, it has also been reported that reducing the dose of paclitaxel and combining chemotherapy with other treatment modalities like LAA can reduce chemotherapy-induced toxicity, while simultaneously improving therapeutic efficacy, in part by altering the differentiation and functional activity of regulatory and effector immune cell populations [35].

In the current study, it was demonstrated that the combination of paclitaxel and LAA provides synergistic and/or additive functions in restricting skin cancer growth and progression utilizing two *in vitro* skin cancer models. These findings are in accordance with a previous report where paclitaxel in combination with metformin exhibited better anti-cancer functions in a melanoma *in vitro* model [36]. Interestingly, LAA has also been shown to be useful in combination with paclitaxel by alleviating chemotherapy-induced cytotoxicity, and improving clinical outcomes, including higher survival rate and lesser side effects in breast cancer [37]. Moreover, the current report also highlights that limited glucose availability, or low-glucose adaptation conditions may further enhance the therapeutic potential of LAA and paclitaxel mediated combinatorial approach in skin cancers. In a similar line, both preclinical and clinical evidences suggest that targeting glucose metabolic pathways alongside chemotherapy could further provide better therapeutic efficacy in various cancer types [38].

The selective anti-cancer effects of LAA include modulation of cellular redox potential, alteration of glucose metabolism and hypoxic microenvironment and regulation of epigenetic factors to stimulate immune functions [39]. Notably, paclitaxel treatment also impacts cellular redox state [40], modulates glucose metabolism [41] and influences the tumour immune microenvironment [21]. Hence, utilization of LAA could complement paclitaxel limitations and even could enhance its therapeutic potential within the tumour microenvironment.

In summary, the current study opens up the possibility of clinical utilization of LAA and paclitaxel as a superior therapeutic strategy in advanced skin cancer cases, potentially allowing for lower drug does and reduced overall toxicity.

In conclusion, the above data paved a new avenue for improving paclitaxel efficacy while minimizing the physiological toxicity associated with the second drug. This study indicates that the current limitations associated with paclitaxel therapy in skin cancer may be overcome by combining it with LAA. To our knowledge, this study is the first to demonstrate that combining LAA with paclitaxel provides superior anti-cancer effects compared to paclitaxel alone in preclinical skin cancer models.

Author contributions

Rajendra Marathe: Data curation; Formal analysis; Investigation; Methodology; Software; and Roles/Writing - original draft. Pawan Pawar: Data curation; Formal analysis; Investigation; Methodology; Visualization. Bharti Rathod: Data curation; Formal analysis; Investigation; Methodology; Visualization. Preeti Singh: Data curation; Methodology. Sudip Konar: Formal analysis. Omkar Wakale: Investigation; Software. Debjani Dasgupta: Resources and Writing - review & editing. Matthew D. Blunt: Data curation; Formal analysis; Methodology; Validation and Writing - review & editing. Arindam Banerjee: Conceptualization; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Roles/Writing - original draft; and Writing - review & editing.

Ethics statement

This study did not include any human participants or animal subjects; therefore, neither ethical approval nor informed consent was necessary.

Data availability statement

Data will be provided upon request for reasonable purposes by the corresponding author.

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Declaration of Competing Interest

The authors (PP, BR, SK, OW and AB: employees of LifeSenz Cancer Research Labs. RM, and PS: Ex-employee of LifeSenz Cancer Research Labs. DD: Professor at D.Y Patil deemed to be University, Navi Mumbai. MDB: Employee of University of Southampton, UK), confirm that they have no financial or personal conflicts of interest that could have influenced the research presented in this manuscript.

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