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Curcumin-Loaded Liposomes modulating the synergistic role of EpCAM and Estrogen Receptor Alpha in Lung Cancer Management

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Abstract

Lung cancer (LC) remains a leading cause of cancer-related mortality worldwide, necessitating the exploration of innovative therapeutic strategies. This study delves into the *in vitro* potential of liposomal therapeutics utilizing Curcumin-loaded PlexoZome® (CUR-PLXZ) in targeting EpCAM/TROP1 and Estrogen Receptor Alpha (ERα) signalling pathways for LC management. The prevalence of LC, particularly non-small cell lung cancer (NSCLC), underscores the urgent need for effective treatments. Biomarkers like EpCAM/TROP1 and ERα/NR3A1 play crucial roles in guiding targeted therapies and influencing prognosis. EpCAM plays a key role in cell-cell adhesion and signalling along with ERα which is a nuclear receptor that binds estrogen and regulates gene expression in response to hormonal signals. In LC, both often get overexpressed and are associated with tumour progression, metastasis, and poor prognosis. Curcumin, a phytochemical with diverse therapeutic properties, holds promise in targeting these pathways. However, its limited solubility and bioavailability necessitate advanced formulations like CUR-PLXZ. Our study investigates the biological significance of these biomarkers in the A549 cell line and explores the therapeutic potential of CUR-PLXZ, which modulates the expression of these two markers. An *in vitro* analysis of the A549 human lung adenocarcinoma cell line identified that CUR-PLXZ at a dose of 5 μM effectively inhibited the expression of EpCAM and ERα. This finding paves the way for targeted intervention strategies in LC management.

Keywords

Lung Cancer, Liposomes, Respiratory Diseases, Phytoceuticals, Curcumin

Introduction

Lung cancer (LC) is one of the most widely diagnosed malignancies globally, accounting for a large portion of cancer-related deaths (18.4%) each year [1]. The prevalence of LC varies by region, with greater incidences being observed in places with high tobacco use and exposure to environmental contaminants [2, 3]. Tobacco use is the key risk factor for LC, causing the unregulated growth of abnormal pulmonary cells in either of the two main types: non-small cell lung cancer (NSCLC), and small cell lung cancer (SCLC), which have unique cellular characteristics [4]. NSCLC, which is further classified as squamous cell carcinoma, lung adenocarcinoma, and large cell carcinoma, accounts for approximately 85% of all diagnosed LC cases worldwide [5]. However, LC's complexities transcend beyond histological categories, with wide intratumoral and intertumoral variability. This heterogeneity, shown as distinct genetic and molecular profiles within and between tumours, leads to resistance to treatment and presents significant challenges for therapeutic approaches [6]. However, genetic mutations do have an impact on the progression of LC, but a variety of other circumstances such as carcinogen exposure, genetic predisposition and environmental factors, do play a significant proliferative role [7]. Frequently involved mutations include genes such as EGFR, ALK, ROS1, BRAF, and KRAS, and result in the promotion of cancer growth and are often targeted for treatment. Meanwhile, inhibition of tumour suppressor genes (TP53, PTEN, RB1) and the expression of oncogenes (EGFR, ALK, ROS1, BRAF) result in abnormal signalling pathways that promote proliferation of cells and persistence in LC [8]. In recent years, it has been witnessed that oncological medicine advances have paved the path for targeted treatments and immunotherapies, which have significantly improved prognosis for LC patients.

Epithelial Cell Adhesion Molecule (EpCAM)/TROP1 and estrogen receptor alpha (ERα/NR3A1) proteins constitute significant biomarkers that influence the design of targeted therapies and have a substantial impact on the prognosis and treatment of LC [9]. EpCAM also referred to as TROP1/CD326, belongs to the GA733 protein family and is a type 1 transmembrane glycoprotein [10, 11]. Its overexpression is particularly common in epithelial carcinomas, such as human adenocarcinomas and squamous cell carcinomas. EpCAM functions as an intracellular signalling molecule, controlling a variety of activities including epithelial-to-mesenchymal transition (EMT), cancer stemness, cell proliferation, metabolism, angiogenesis, metastasis, chemotherapy/radiotherapy resistance, and immunomodulation [12]. EpCAM's transmembrane domain, which features a highly conserved valine-abundant and leucine-deprived helix, is highly expressed in epithelial-origin tumours. The EpCAM gene produces six transcript variations, with EpCAM-201 being the most common isoform, as verified by large-scale cancer transcriptome investigations [13]. EpCAM's existence in bodily fluids, which include circulating tumour cells (CTCs), cancer stem cells (CSCs), and exosomes from cancer patients' blood, highlights its possibility as a target for new therapeutic techniques [14]. EpCAM's abundance in both normal and malignant epithelial cells, together with its immunogenicity and pro-oncogenic properties, establishes it as a clinically relevant anti-

tumour target with intriguing translational implications [15]. Therapeutic techniques directed against EpCAM have great promise for prognosis, diagnostics, and therapeutic intervention in epithelial malignancies.

Additionally, estrogen, a versatile steroid hormone, plays an important role in lung development and function in both genders by activating its receptors (ER), which are widely expressed in lung epithelial cells [16]. Recent research suggests that the most potent estrogen form, 17-β-estradiol (E2), has an impact on LC proliferation. ERs have distinctive tissue distributions with Erα being mostly found in breast, ovarian, and endometrial tissues, while ERβ is highly expressed in ovaries and lungs [17]. ERβ is frequently identified in LC, particularly in adenocarcinoma, and its expression is significantly upregulated in NSCLC cell lines/tissues. In healthy lung tissue, ERβ maintains the extracellular matrix and ERα regulates alveolar count and surface area [18]. ERβ localises in mitochondria and affects bioenergetics and anti-apoptotic signalling in a ligand-dependent or -independent way [19]. This study intends to explore the biological significance of these different prognostic markers in the A549 adenocarcinoma lung cancer cell line.

Phytoceuticals such as curcumin (CUR) offer targeted approaches for addressing key molecules like EpCAM/TROP1 and ERα involved in cancer progression. These compounds have the potential to disrupt the expression of such proteins, thus preventing cancer growth and metastasis [20]. CUR has been intensively investigated for its capacity to affect numerous signalling pathways involved in cancer, including inflammation, apoptosis, and cell proliferation. It's a golden spice and a primary bioactive ingredient obtained from the *Curcuma longa L*. plant rhizome, and it has been shown to have both preventive and therapeutic benefits on LC cells. CUR has been investigated for its ability to regulate oestrogen signalling pathways, especially ERα activity [21]. It inhibits ERα expression and activity in breast cancer cells and may have comparable impacts on LC cells. Furthermore, CUR may prevent ERα+ lung tumours from growing and progressing by regulating ERα signalling [22].

Additionally, CUR has also been shown to reduce EpCAM/TROP1 expression in LC cells, possibly interfering with signalling pathways including the Wnt/β-catenin pathway, that aid tumour development and metastasis [23]. However, the specific mechanisms by which CUR targets EpCAM/TROP1 in LC cells remain unresolved and need more investigation. Our study intends to determine the biological importance of these prognostic indicators in the A549 adenocarcinoma lung cancer cell line, which is representative of NSCLC *in vitro* models. Additionally, we investigate the therapeutic potential of Curcumin-loaded liposomes (PlexoZome®) in modifying these indicators within the lung cancer cell line. Using the benefits of liposome-based drug delivery systems, such as enhanced bioavailability and pharmacokinetic features, our study aims to improve the therapeutic efficiency of Curcumin, a phytochemical with proven anticancer activities. In conclusion, our research provides insights into prospective avenues for targeted intervention techniques in lung cancer management, highlighting the potential of curcumin-loaded liposomes in inhibiting the expression of two key cancer biomarkers: EpCAM, and ER-α.

Materials and Methods

PlexoZome, a liposomal bilayer formulation containing curcumin and phosphatidylcholine (CUR-PLXZ), was developed utilising passive loading procedures. This formulation, sourced from Pharmako Biotechnologies Pty Ltd in Australia, was characterised before being delivered for our research project (proprietary data not shown). For the experiment, A549 cells were seeded at 2 × 10^5 cells per well in 6-well plates for protein array assays. Following the seeding of cells, they were treated with 5 μM CUR-PLXZ (treatment group) or medium alone (control group) for 24 hours [9]. After treatment, cells were collected and lysed in RIPA lysis solution (ThermoFisher Scientific, Australia) supplemented with protease inhibitors (Merck, Australia) to extract proteins. The cell debris was removed via centrifugation at 14,000 g for 15 minutes at 4 °C, and the supernatant was transferred to a fresh tube. Protein concentration was then measured with a BCA protein assay kit (ThermoFisher Scientific, Australia). Next, 300 μg of protein from each group were hybridised on the Proteome Profiler Human XL Oncology Array (R&D Systems, Minneapolis, MN) using the manufacturer's methodology [24-29]. This array detects and quantifies 84 cancer-related proteins at the same time by hybridising the protein extract to a membrane that has been functionalized with appropriate antibodies. A ChemiDoc MP system (BioRad, Australia) was used to create the images, and ImageJ software was used for pixel density analysis. Statistical analysis was performed using PRISM GraphPad software.

Result and Discussion

The study on A549 cells revealed detectable EpCAM/TROP1 and ER-α/NR3A1 protein expression, indicating their probable role in supporting cell proliferation and tumorigenic activity within the A549 cell line. Treatment with CUR-PLXZ resulted in a substantial decline in the expression of the aforementioned biomarkers compared tothe control group (Figure 1). In particular, EpCAM expression was lowered by 11.8% (Figure 1a), and ER-α expression was lowered by 6.1% (Figure 1b) upon treatment with CUR-PLXZ. These data emphasise the efficacy of CUR-PLXZ formulation as a potential therapeutic strategy for LC.

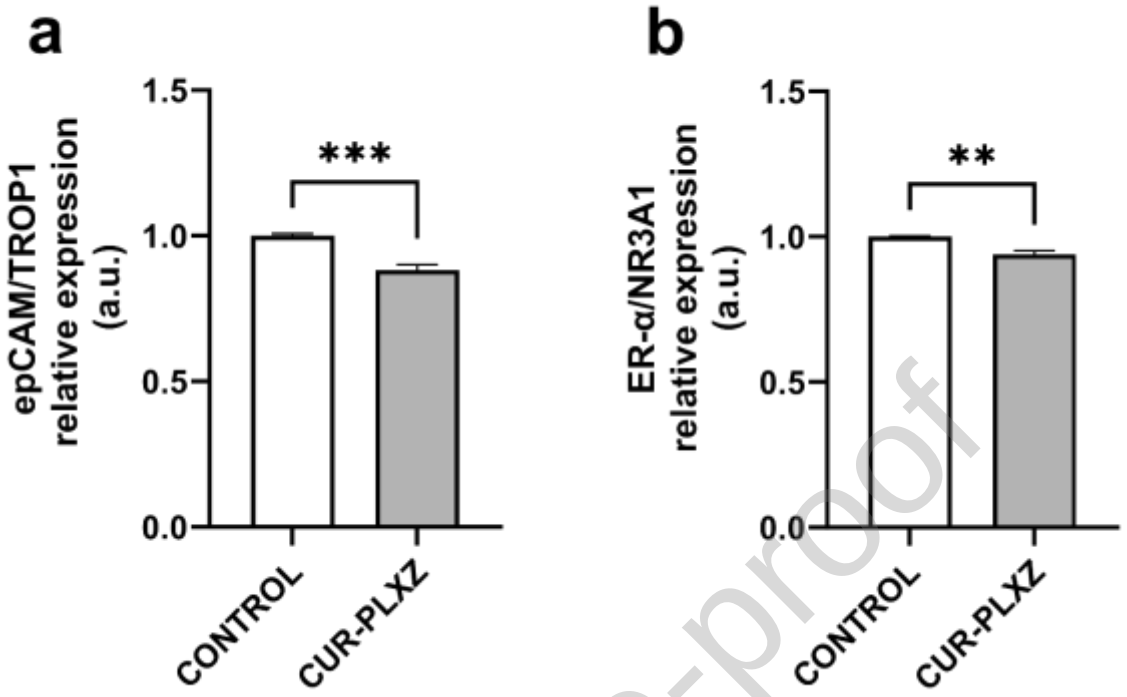

Figure 1 – CUR-PLXZ Downregulates the protein expression of EpCAM/TROP1 and ER-α/NR3A. A549 cells were treated with CUR-PLXZ corresponding to a final concentration of 5 µM curcumin for 24 hours. After treatment, the cells were lysed with RIPA buffer and 300 µg of clarified proteins were hybridized on a Proteome Profiler Human XL Oncology Array kit. The relative expression of EpCAM (**a**) and ER-α (**b**) is shown as the relative pixel density of the treated group vs the control untreated group. Statistical analysis was performed *via* unpaired t-test, using GraphPad PRISM software. **: $P < 0.01$; ***: $P < 0.001$; $n = 4$.

The *in vitro* analysis conducted on the A549 cell line identified that CUR-PLXZ at a dose of 5 µM effectively downregulated the expression of EpCAM and ER-α proteins, pointing to a promising therapeutic potential for the CUR-PLXZ formulation as a therapy that inhibits two fundamental malignancy markers of LC. Yao et al. (2015) found that adding 20 µM of CUR powder to A549 cells for 24 hours reduced cell growth and caused apoptosis in a dose- and time-dependent manner [30]. Curcumin at 20 µM significantly inhibited proliferation in A549 cells, with considerable variations after 24 hours of treatment. Also, research conducted by Wu et al. (2022), to counteract chemoresistance in LC cells by combining CUR with vincristine and docetaxel in A549 cell lines with different doses (20, 30, and 40 µM) for 48 hours caused apoptosis of cancer cells. It produced reactive oxygen species (ROS) and enhanced phosphorylation of proteins (ERK, p38 MAPK, eIF-2α) suggesting a similar possibility at the molecular level in our findings. Subsequent investigations to determine the amount of apoptosis induced by CUR treatment observed a significant increase in ROS levels that accelerated apoptosis by activating phosphorylated p38 MAPK (pro-apoptotic signal) in chemoresistant human LC cells [31]. Notably, the concentration used in our study was four to eight times lower than in the prior studies, but it still had a substantial effect on reducing the expression of LC-related proteins. This highlights the benefits of formulating CUR into

liposomes in counteracting the limitations stemming from its poor aqueous solubility. CUR belongs to the Biopharmaceutical Classification System (BCS) class IV, which is distinguished by poor solubility, oral bioavailability, fast metabolism into inactive metabolites, and rapid removal from the body, severely limiting its clinical value [32]. To overcome these limitations, an advanced formulation such as CUR-PLXZ was used. This formulation has the potential to provide superior physicochemical qualities as well as biological activity. One possible explanation for the improved characteristics of CUR-PLXZ is that the PLXZ formulation was more readily absorbed by A549 cells than CUR powder formulations reported in our earlier investigation thus, CUR-PLXZ's adaptability to manage LC has been confirmed. Among the proteins inhibited by CUR, EpCAM stands out as a key role in lung cancer growth. Its increased expression in LC cells compared to healthy lung epithelial cells correlates with improved cancer cell survival [33]. Furthermore, RNA interference (RNAi) is a powerful gene expression control mechanism in cells. The study highlights the role of EpCAM (Epithelial Cell Adhesion Molecule) in increasing LC cell development. EpCAM, a glycoprotein on cell surfaces, is more common in certain cancers like LC and its higher levels increase cell proliferation, invasion, and dissemination. Thus, focusing on EpCAM may be beneficial for cancer treatment. Small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) complementary to the mRNA of EpCAM are introduced in this manner. SiRNAs/shRNAs guide the RNA-induced silencing complex (RISC) to EpCAM mRNA, degrading it and therefore reducing the expression of EpCAM protein. A gene silencing study found that inhibiting EpCAM gene expression inhibits LC cell growth. This inhibition occurs because EpCAM promotes cell growth and viability. By reducing EpCAM levels, cancer cellsgrow more slowly, resulting in less cell division and slower tumour formation. RNA interference (RNAi)-mediated silencing of the EpCAM gene can reduce LC cell proliferation, suggesting its therapeutic potential in treating LC. This technique could enable the creation of novel RNAi-based medicines that target cancer-promoting genes like EpCAM. These findings further highlight the need to understand the development of cancer cells' molecular pathways and the potential of RNA interference (RNAi) technology to manipulate them for therapeutic purposes [34]. Several additional proteins have been linked to poor prognoses or survival rates in lung cancer patients. The results of the present study are summarised in Figure 2, which also has the role of a graphical abstract for this study.

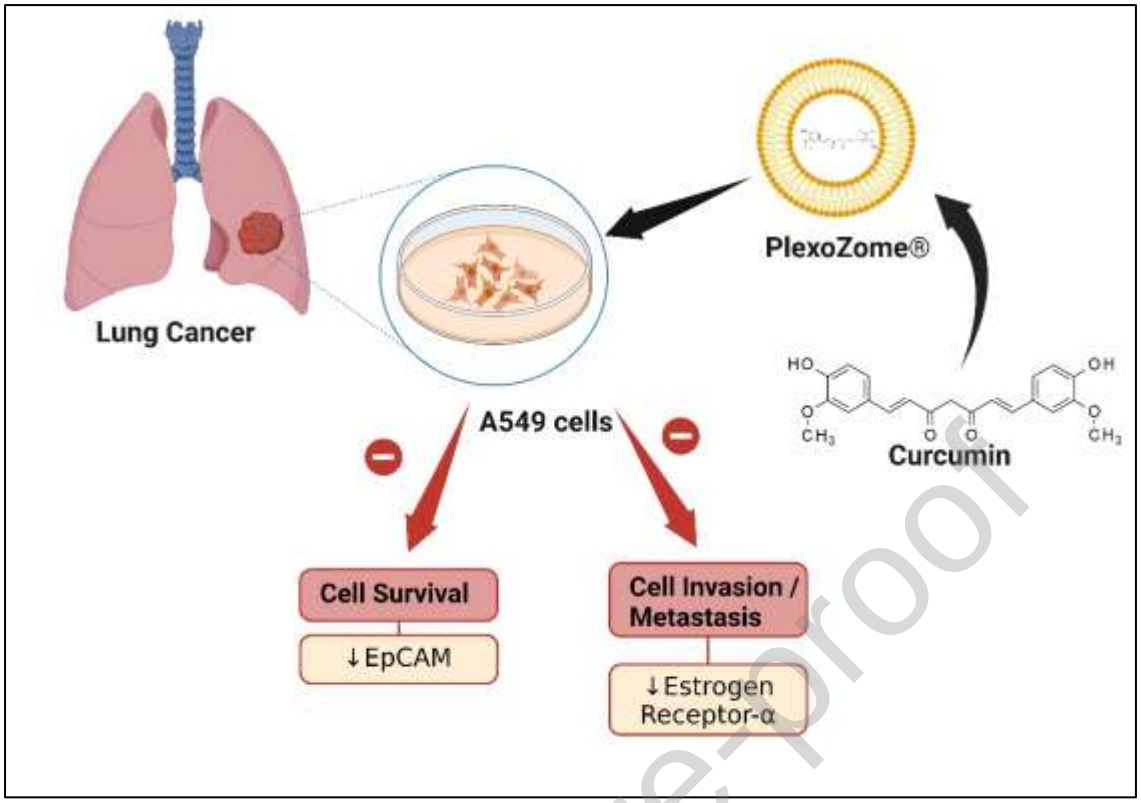

Figure 2: Curcumin-loaded liposomes downregulate the expression of two crucial proteins for lung cancer cell survival and invasion/metastasis: EpCAM, and ER-α, *in vitro* in A549 human lung adenocarcinoma cells.

Conclusion

In conclusion, our findings show CUR-PLXZ's potential as a promising treatment drug for LC. CUR-PLXZ suppresses the expression of essential proteins implicated in LC growth, such as EpCAM and ER-α, at substantially lower quantities than previously reported. The improved physicochemical features of CUR-PLXZ, which could be due to higher cellular absorption, highlight the drug's versatility and potential for therapeutic use. Our findings co-related the underlying mechanisms by which CUR-PLXZ may have induced the apoptosis in chemoresistant human LC cells, involving the activation of phosphorylated p38 MAPK via ROS modulation. This mechanistic insight provides valuable knowledge for the development of targeted therapies against LC, particularly in cases resistant to conventional treatments. Overall, our study contributes to the growing body of evidence supporting the potential of liposomal formulations of CUR (CUR-PLXZ) as effective anti-cancer agents. Further preclinical and clinical investigations are needed to validate the efficacy and safety of CUR-PLXZ in LC treatment along with its associated underlying mechanisms to improve patient outcomes and advance personalized therapeutic approaches for LC.

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Declaration of Competing Interest

The authors of the manuscript submitted to the journal “Pathology - Research and Practice”, have no conflict of interest to declare.