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PII: S0344-0338(23)00672-6

DOI: https://doi.org/10.1016/j.prp.2023.154971

Reference: PRP154971

To appear in: *Pathology - Research and Practice*

Received date: 21 October 2023
Revised date: 19 November 2023
Accepted date: 21 November 2023

Please cite this article as: Gabriele De Rubis, Keshav Raj Paudel, Venkata Sita Rama Raju Allam, Vamshikrishna Malyla, Vetriselvan Subramaniyan, Sachin Kumar Singh, Nisha Panth, Gaurav Gupta, Philip M. Hansbro, Dinesh Kumar Chellappan and Kamal Dua, Involvement of osteopontin, EpCAM, estrogen receptor-alpha, and carbonic anhydrase IX protein in managing lung cancer via Berberine-loaded liquid crystalline nanoparticles, *Pathology - Research and Practice,* (2023) doi:https://doi.org/10.1016/j.prp.2023.154971

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Gabriele De Rubis[a,b,#], Keshav Raj Paudel[c,#], Venkata Sita Rama Raju Allam[d], Vamshikrishna Malyla[a,b,c], Vetriselvan Subramaniyan[e], Sachin Kumar Singh[b,f], Nisha Panth[c], Gaurav Gupta[g,h,i], Philip M. Hansbro[c], Dinesh Kumar Chellappan[j,], Kamal Dua[a,b,*]*

[a]Discipline of Pharmacy, Graduate School of Health, University of Technology Sydney, Sydney, NSW 2007, Australia

[b]Faculty of Health, Australian Research Centre in Complementary and Integrative Medicine, University of Technology Sydney, Ultimo, Australia.

[c]Centre of Inflammation, Centenary Institute and University of Technology Sydney, Faculty of Science, School of Life Sciences, Sydney, NSW 2007, Australia

[d]Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden.

[e]Pharmacology Unit, Jeffrey Cheah School of Medicineand Health Sciences, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 47500 Selangor Darul Ehsan, Malaysia

[f]School of Pharmaceutical Sciences, Lovely Professional University, Jalandhar-Delhi G.T Road, Phagwara, Punjab, India

[g]School of Pharmaceutical Sciences, Jaipur National University, Jagatpura, 302017, Jaipur, India.

[h]Center for Transdisciplinary Research, Saveetha Institute of Medical and Technical Science, Saveetha University, Chennai, India

[i]Uttaranchal Institute of Pharmaceutical Sciences, Uttaranchal University, Dehradun 248007, India

[j]Department of Life Sciences, School of Pharmacy, International Medical University, Bukit Jalil 57000, Kuala Lumpur, Malaysia

[#]First author with equal contribution

*Authors for correspondence:

Dr Dinesh Kumar Chellappan; Email: Dinesh_Kumar@imu.edu.my;

Dr Kamal Dua; Email: Kamal.Dua@uts.edu.au

Introduction

Lung cancer is the second most common type of cancer globally, with about 2.2 million lung cancer cases diagnosed in 2020, which lead to 1.7 million deaths worldwide [1], representing about one-fifth of the total amount of cancer-related deaths across the world [2]. Based on the histological characteristics, it is often diagnosed as one of two distinct types; non–small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), that contribute to 86% and 13% of the total lung cancer incidences respectively [3]. NSCLC is originated from lung epithelial cells and classified into several subtypes including squamous cell carcinoma, large cell carcinoma, and adenocarcinoma, depending on the specific cell subtype from which they originate [2]. SCLC is of neuroendocrine origin, and it is characterized by smaller cells compared to NSCLC. It is also more aggressive compared to NSCLC and has higher metastatic potential [4]. In more than 80% of the cancer cases cigarette smoke, use of tobacco-derived products and age are the major risk factors. Furthermore, several other risk factors namely, environmental pollution, occupational exposure to harmful irritants, family

history and epigenetic changes are also considered as triggering factors of lung cancer. Although early-stage lung cancer is mostly asymptomatic and difficult to diagnose, late-stage lung cancer is associated with various symptoms including fatigue, shortness of breath, chest pain, continuous cough, coughing up with blood and persistent chest infections [5].

Lung cancer is a heterogenous malignant disease with an altered cell cycle homeostasis resulting from the activation of various growth promoting-oncogenes and from the inhibition of the regulatory tumour suppressor genes. A precise understanding of this complex heterogenicity is therefore essential for the development of new therapeutic interventions that may target the genetic and molecular aberrations associated with lung cancer. Various tumour-associated biomarkers, genes, and proteins serve as novel therapeutic targets for the treatment of lung cancer [6].

Osteopontin (OPN), a secretory extracellular matrix glycosylated phosphoprotein, is one such prognostic biomarkers which is produced in several tissue-derived tumour cell lines and is implicated in cellular homeostasis including cell adhesion, cell migration, bone calcification, and tumour progression. OPN modulates various signalling pathways including immune response, cell adhesion, and migration to promote metastasis and tumorigenesis *via* binding to different OPN receptors like αvββ integrin and CD44. Interestingly, earlier studies had identified OPN as a prognostic biomarker in NSCLC. Although a positive correlation has been identified between increased OPN levels and SCLC, further studies are needed to identify its prognostic role in SCLC. Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein and is a prognostic biomarker which is quietly expressed in various tissue-derived human cancers including lung cancer. EpCAM exerts its

tumorigenesis activity by interacting with various cell adhesion and proliferative signalling mediators including claudins, integrins, CD44, E-cadherin, epidermal growth factor receptor (EGFR), and different mitogen-activated protein kinases like Wnt and Ras/Raf pathways, leading to enhanced cell adhesion and proliferation. Previous studies have reported EpCAM as a prognostic marker as studied in various *in vitro*, *in vivo* [7] and gene knockdown experiments. Carbonic anhydrase IX (CAIX) and Estrogen receptor alpha (ER-α) are two other diversified prognostic markers that play a major role in the progression of lung cancer. CAIX expression is increased in various types of human cancers, especially under hypoxic conditions, which is essentially one of the mechanisms of the tumour cells for survival. Increased CAIX expression raises the cellular pH by hydrolysing carbon dioxide to bicarbonate, promoting cell proliferation and metastasis formation [8]. ER-α promotes metastasis *via* a different mechanism by interacting with various coactivators resulting in the activation of transcription factors that are necessary for cell proliferation and tumorigenesis [9].

The current study attempts to investigate the biological role of these diverged prognostic markers in the human A549 adenocarcinoma lung cancer cell line, representing an *in vitro* model of NSCLC. The A549 cell line is a continuous tumor line derived from an adenocarcinoma patient with the characteristics of type II alveolar epithelial cells [10], and it represents one of the most commonly used lung cancer cell lines [11]. We have also investigated the therapeutic role of Berberine-loaded liquid crystalline nanoparticles (BBR-LCNs) in altering these markers in the lung cancer cell line. The advantage of encapsulating berberine in LCNs consists in the improvement of berberine's poor permeability, which could result in an improvement of its poor bioavailability and general pharmacokinetic characteristics [12]. The results of the

present study reinforce the notion thar nanoparticle-based advanced drug delivery systems are useful tools to improve the usually poor bioavailability of phytoceuticals such as berberine, which are often characterized by poor water solubility or permeability [13].

Materials and Methods

BBR-LCNs were prepared using the ultrasonication technique as described previously [12] . For protein array studies, A549 cells (1×10^5 cells/well) were seeded in 6-well plates and were then treated with 5 µM BBR-LCNs (treatment group) and media-only (control group) for 24 h. The cells were then collected and lysed with RIPA lysis buffer for the extraction of proteins (quantified by a BCA protein assay kit). 350µg of protein from each group were hybridized to the protein array blot and were processed according to the manufacturer's protocol. The protein array blot used is the Proteome Profiler Human XL Oncology Array (R&D Systems, Minneapolis, MN) , which allows the detection and relative quantitation of 84 cancer-related proteins simultaneously by hybridizing the protein extract to a membrane functionalized with the respective antibodies. The images were developed with a ChemiDoc MP system. ImageJ was used for pixel density analysis.

Results and Discussion

In terms of the findings, A549 cells were shown to express detectable levels of OPN (Fig 1A), EpCAM (Fig 1B), ER-α (Fig 1C) and CAIX (Fig 1D), suggesting the involvement of these biomarkers in maintaining cell proliferation and tumorigenic

activity of the adenocarcinoma lung cell line. Treating A549 cells with BBR-LCNs significantly reduced the expression of these biomarkers with reduced pixel density as compared to the control group (Fig 1A-1D). In particular: the expression of OPN was reduced by 37.0% (Fig 1A); the expression of EpCAM was reduced by 32.3% (Fig 1B); the expression of ER-α was reduced by 33.8% (Fig 1c); and the expression of CAIX was reduced by 40.1% (Fig 1D). These findings suggest that the BBR-LCN formulation may be a promising agent that could be used in its translation towards clinical use. However, further studies are required to understand the in-depth molecular mechanisms involved within the anti-cancer activity of these LCNs.

The *in vitro* study performed on the A549 cell line revealed that BBR-LCNs at 5 µM concentration significantly inhibited the expression of proteins namely, OPN, EpCAM, ER-α, and carbonic anhydrase IX. The selected BBR-LCNs concentration of 5 µM stems from our previous studies, where we found that BBR-LCNs at this equivalent free berberine concentration significantly inhibited A549 cells proliferation and migration [12], Without significantly impacting the viability of healthy cells such as 16HBE human non-cancerous bronchial epithelial cells and RAW264.7 mouse macrophages [15]. A previous study conducted by Qi et al., 2014, used berberine powder at a concentration of 20 µM in A549 cells for 24 h, where it showed inhibition of E-cadherin and Vimentin expression [16]. In comparison, the concentration used in our study was 4 times lower and furthermore had a significant effect in inhibiting lung cancer-related protein expression. In another study by Chen et al., 2019, the authors showed that free berberine powder exerted significant antiproliferative, anti-migration, and anti-colony formation activity on A549 cells, by inhibiting the expression of BCL-2 and Bax, at concentrations ranging from 40 to 120 µM [17]. The concentration of berberine which showed anticancer activity in our study was 8-fold to 24-fold lower

than that shown by Chen et al. This clearly explains the advantage of designing berberine into LCNs. Berberine is a biopharmaceutical classification system (BCS) class III molecules, characterized by high aqueous solubility but poor permeability. This translates into its poor oral bioavailability, which severely limits its clinical application [18]. Therefore, an advanced formulation of berberine such as BBR-LCNs was used to overcome this problem. Such formulations may hold the promise to serve as the ideal product that could exert better physicochemical properties along with its biological activity [19]. One of the possible mechanisms for BBR-LCNs' enhanced physicochemical properties could be due to an increased uptake of the LCN formulation by the A549 cells as compared to the berberine powder formulation which was reported in the previous studies [14, 20]. Thus, we can confirm that BBR-LCNs offer versatility in the management of chronic lung diseases, including lung cancer [21]. Among the proteins inhibited by berberine, EpCAM serves to be a crucial protein which is involved in lung cancer progression. The high expression of EpCAM in lung cancer cells compared to normal (healthy) lung epithelial cells is linked with lung cancer cell survival. Furthermore, knockdown of the *EpCAM* gene by RNAi mediated silencing results in a remarkable inhibition of lung cancer cell proliferation [22].

Several other proteins are also associated with poor prognosis or survival outcomes in lung cancer patients. OPN was found to be one of the key proteins that was highly expressed in surgically resected lung cancers as compared to normal tissues [23]. ER-α is another notable protein which is detected in the cytoplasm of around 73% of resected NSCLC specimens. ER-α expression is reported to be associated with poor prognosis [24]. Similarly, a study published by Kim et al., reported that CA IX protein expression was observed in almost 72% (54 out of 75) of stage I and II NSCLC patients, suggesting that CA IX might be an indicator of poor disease-free survival [25].

Treatment with 5 µM of BBR-LCN formulation significantly inhibited the protein expression of these prognostic markers demonstrating its potential *in vitro* anticancer activity in the A549 cell line.

Conclusions

The summary of our findings is represented in Figure 2. To conclude, our study addresses some of the crucial bottlenecks encountered while formulating drugs/compounds possessing low solubility, low bioavailability, or decreased uptake by cells. Such problems can be overcome by modifying the formulation using the approach of advanced nano-delivery systems such as LCNs. Berberine could be translated into a promising alternative approach for the management of lung cancer. However, more extensive, in-depth mechanism based *in vitro*, *in vivo,* and clinical studies must be conducted to validate the efficacy of berberine against lung cancer.

Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

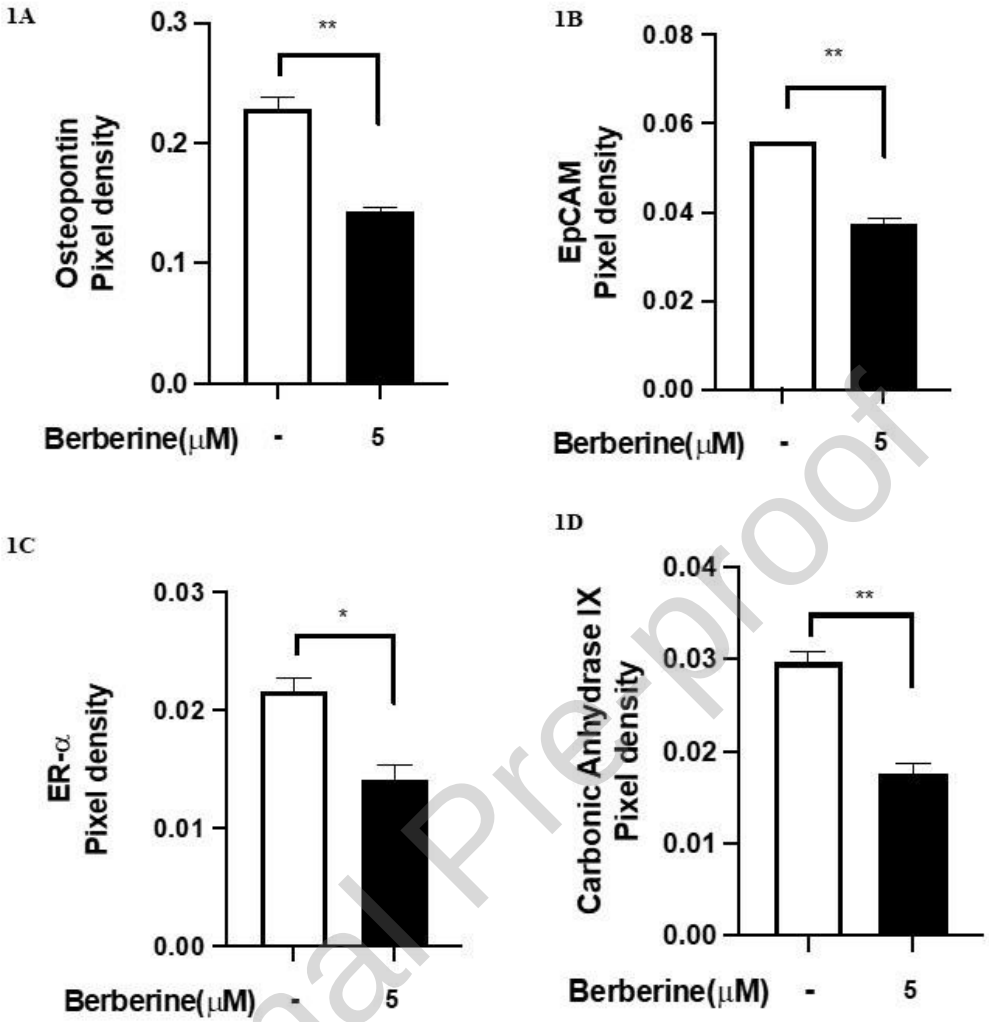

Figure 1: BBR-LCNs attenuate the normally expressed prognostic markers of A549 cell line

*A549 cells were treated with or without 5 μM Berberine-LCNs for 24 h, after which the protein was extracted. A protein array study was carried out using a human XL oncology array kit for A: Osteopontin, B: EpCAM, C: ER-α, and D: Carbonic anhydrase IX.The pixel density of each protein was compared with the control using ImageJ software. Statistical analysis was done with a two tailed unpaired t-test. *p < 0.05; **p < 0.01 versus control (media only/without Berberine).*

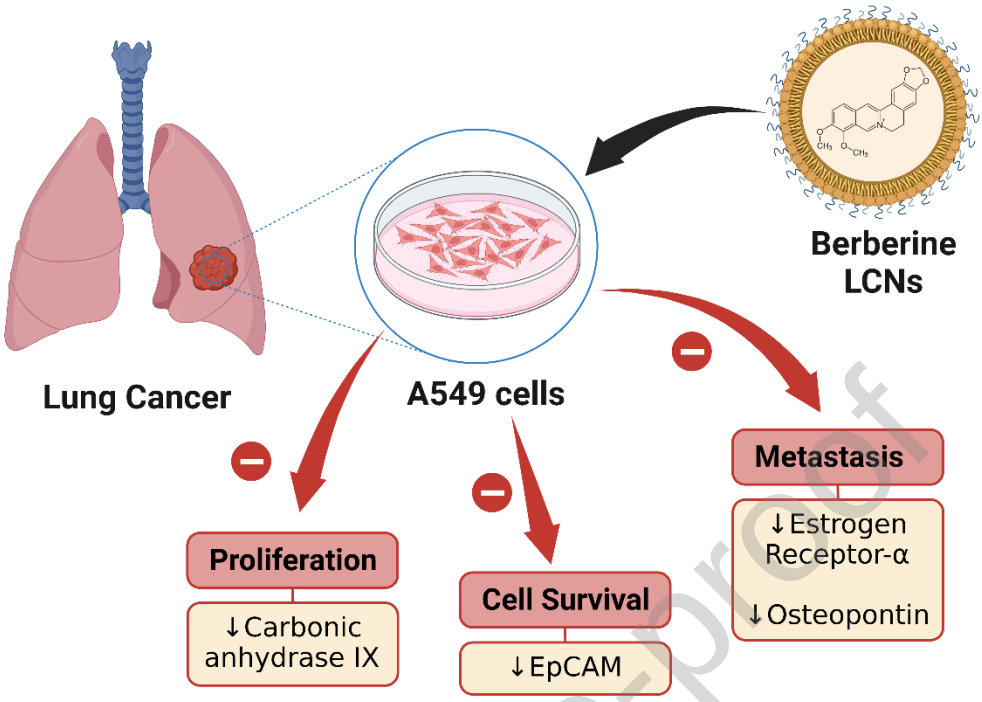

Figure 2. Mechanism involved in the anti-cancer activity of berberine-LCNs in A549 cell line

Progression of lung cancer is a result of alteration in the expression of various proteins involved in cancer cell survival, proliferation, and metastasis. EpCAM protein is involved in cell survival, while Carbonic anhydrase IX is involved in the proliferation of A549 cells. Estrogen receptor-alpha upregulates osteopontin expression to induce metastasis of lung tumour. Berberine-LCNs significantly inhibit these key protein expressions thereby suppressing the progression of lung cancer. Image generated with Biorender (www.BioRender.Com).

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CRediT authorship contribution statement

Gabriele De Rubis: Conceptualization, Data curation, Investigation, Methodology, Writing – Original Draft, Writing – Review and Editing; **Keshav Raj Paudel:** Conceptualization, Data curation, Investigation, Methodology, Writing – Original Draft, Writing – Review and Editing; **Venkata Sita Rama Raju Allam:** Conceptualization, Formal analysis, Methodology, Visualization; **Vamshikrishna Malyla:** Conceptualization, Formal analysis, Methodology, Visualization; **Vetriselvan Subramaniyan:** Conceptualization, Formal analysis, Methodology, Visualization, Writing – Review and Editing; **Sachin Kumar Singh:** Formal analysis, Investigation, Validation, Writing – Review and Editing; **Nisha Panth:** Conceptualization, Formal analysis, Methodology, Visualization; **Gaurav Gupta:** Formal analysis, Investigation, Validation, Writing – Review and Editing; **Philip Hansbro:** Conceptualization, Investigation, Resources, Supervision, Validation; **Dinesh Kumar Chellappan:** Conceptualization, Funding Acquisition, Investigation, Project Administration, Resources, Supervision, Visualization, Writing – Original Draft, Writing – Review and Editing; **Kamal Dua:** Conceptualization, Funding Acquisition, Investigation, Project Administration, Resources, Supervision, Visualization, Writing – Review and Editing

Declaration of Competing Interest

The authors of the manuscript “Involvement of osteopontin, EpCAM, estrogen receptor-alpha, and carbonic anhydrase IX protein in managing lung cancer via Berberine loaded liquid crystalline nanoparticles”, submitted to the journal “Pathology - Research and Practice”, have no conflict of interest to declare.