Pulmonary delivery of bedaquiline-loaded cubosomes for non-small cell lung cancer (NSCLC) treatment

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Summary

Non-small cell lung cancer (NSCLC) is the leading cause of cancer deaths globally. The available treatment options are limited by adverse effects and development of drug resistance. Hence, newer drug candidates and drug delivery systems that have minimal adverse effects with significant anticancer efficacy are required. For NSCLC treatment, inhalation route of drug delivery is highly beneficial as it delivers drugs directly into the lungs, requires lower doses, and limits systemic toxicity. Bedaquiline (BQ), an anti-tuberculosis drug has previously shown excellent anti-cancer efficacy, but the drug's poor aqueous solubility limits its delivery via the lungs. In this project, we developed inhalable BQ-loaded cubosome (BQLC) nanocarriers for NSCLC treatment. The BQLC were prepared using a solvent evaporation technique and displayed a particle size of 150.2 ± 5.1 nm, zeta potential of (+) 35.4 ± 2.3 mV, and encapsulation efficiency of 51.85 ± 4.83%. The solid-state characterization (DSC and PXRD) confirmed drug encapsulation within the cubosomes. The BQLC nanocarriers showed excellent aerodynamic properties after nebulization (MMAD of 4.21 ± 0.53 µm and FPF > 75%). The BQLC displayed enhanced cytotoxicity with a ~3-fold reduction in IC_{50} compared to free BQ in NSCLC (A549) cells, after 48 h treatment. Additionally, 3D-tumor simulation studies established the anticancer efficacy of cubosomal nanocarriers as compared to free BQ.

Key Message

This is the first study exploring the potential of cubosomes for inhaled therapy. Further, the results suggest that bedaquiline-loaded cubosomes could be a promising NSCLC treatment with optimum particle characteristics, excellent aerosolization performance, and enhanced anti-cancer activity.

Introduction

Lung cancer is one of the leading causes of cancer related deaths worldwide with more than 85% of the cases associated with non-small cell lung cancer (NSCLC). The low overall 5-year survival rate of ~23% for NSCLC patients prompts the need for new treatments. There is an urgent need for new treatment strategies with enhanced anticancer activity and minimal toxicity to healthy cells. Drug repurposing significantly shortens the drug development timeframe and helps identify promising drug candidates for NSCLC treatment with limited cytotoxicity. We and others have previously shown that bedaquiline (BQ), an anti-tuberculosis drug, has potent anti-cancer efficacy against NSCLC [1,2]. However, the traditional route of anticancer therapy (oral or parenteral) often leads to systemic toxicity, frequent dosing, and low drug concentrations at tumor site in the lungs. Hence, inhalation therapy of the anticancer agents can be a promising alternative to other delivery routes as the drug gets deposited directly into the lungs with potentially reduced systemic side effects and reduced dosing. Further, to increase the efficacy and enable pulmonary delivery of bedaquiline, we used cubosomes as drug delivery carriers. Cubosomes are a lipidic nanocarrier system with cubic inner crystalline structure formed by the self-assembly of an amphiphilic molecule in excess water. Cubosomes can load both hydrophilic and hydrophobic drugs, offer high thermodynamic stability, robust physical structure, and improved drug loading compared to conventional liposomes [3]. In this study, we developed bedaquiline-loaded cubosomes (BQLC) capable of providing localized inhalation therapy against NSCLC. The BQLC was fabricated using single-emulsion solvent evaporation method and characterized regarding their size, surface potential, morphology, and encapsulation efficiency. The solid-state characterization was performed to validate the encapsulation of the BQ within cubosomes. The aerosolization performance was optimized for BQLC formulation using NGI after nebulization. Further, *in vitro* anticancer activity of the BQLC and free BQ was estimated by MTT assay and 3D tumor simulation studies on NSCLC (A549) cells.

Experimental Methods

Preparation of BQLC formulation: A single emulsion solvent evaporation method was used to formulate BQLC [4]. Briefly, BQ (5 mg), glyceryl monooleate (GMO, 100 mg), Poloxamer 188 (P188, 20 mg), and propylene glycol (270 mg) were dissolved in 5 mL of chloroform and stirred overnight to facilitate solvent evaporation. After, distilled water (1 mL) was added to the lipid precursor and probe sonicated to obtain BQLC formulation. The free drug in the formulation was separated by dialysis using dialysis cassettes (Thermo-Scientific, Waltham, MA, USA) in an aqueous system.

Formulation characterization: The encapsulation efficiency and drug loading were reported based on the amount of BQ entrapped, analysed using an established high performance liquid chromatography (HPLC) method after direct vesicle lysis of BQLC. Particle size, poly dispersity index (PDI), and zeta potential were measured using dynamic light scattering, Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). The morphology of the cubosomes was visualized using cryogenic transmission electron microscopy (cryoTEM), Titan Halo 80-300 (FEI, Hillsboro, OR, USA). The thermograms of BQ, BQLC (freeze-dried), and blank cubosomes (freeze-dried) were generated using differential scanning calorimetry instrument, DSC 6000 (PerkinElmer, Waltham, MA, USA) over a range of 20-260 °C with a sealed empty aluminum pan serving as a reference. Powder X-ray diffraction (PXRD) spectroscopy was performed using XRD-6000 (Shimadzu, Kyoto, Japan) in the range of 10-80° at a scanning speed of 2° (2θ)/min.

Aerosolization Performance: The aerosolization performance of BQLC was evaluated by Next Generation Impactor (NGI) M-170 (MSP Corporation, Minneapolis, MN, USA), using previously published method [5]. Prior to the run, the NGI was cooled by refrigerating at 4 °C for 90 min to minimize heat evaporative effects. Briefly, 2 mL of formulation was nebulized with PARI LC PLUS® nebulizer and passed through induction port into the NGI using HCP5 Vacuum Pump (Copley Scientific, Nottingham, UK) at a flow rate of 15 L/min for 4 min. A lysis solvent (binary mixture of acetonitrile and water) was used to collect the deposited samples from the NGI stages and evaluated using HPLC. Mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated using log-probability analysis (n=3). The fine particle fraction (FPF, %) was defined as fraction of emitted dose deposited in the NGI from stage 3.

In vitro anticancer activity: The *in vitro* cytotoxicity of the free BQ and BQLC on A549 cells was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A549 cells (5 x 10^3 cells/well) were seeded in 96-well tissue culture plates, incubated overnight, and treated with different concentrations of BQLC, blank cubosomes, or BQ for 48 h. After, MTT was added to the wells and the formed formazan crystals were solubilized using dimethyl sulfoxide and the absorbance was measured at 570 nm using a plate reader, Synergy H1 (BioTek, Winooski, VT, USA). The cell viability was calculated as the ratio of absorbance between treatment groups and untreated control wells. *In vitro* tumor simulation studies were performed to better mimic *in vivo* tumor conditions and involves growing cells as a 3D spheroid. Briefly, cells were seeded at a density of 750 cells/well in an ultra-low attachment 96-well U bottom plate (Corning®, Kennebunk, ME, USA) and grown for 3 days before treatment. The multiple doses regimen involved treating cells every 3 days until day 15 with BQLC or BQ (15 μM; IC_{50} concentration). The optical imaging of tumors (n = 6) was carried out using an inverted Leica microscope (Leica Microsystems, Chicago, IL, USA) and the spheroid diameter (d) was calculated using an ImageJ software.

Results and Discussion

A.

Formulation Characterization				
Particle Size	PDI	Zeta Potential	Encapsulation Efficiency	Drug Loading
150.2 ± 5.1 nm	0.24 ± 0.02	(+) 35.4 ± 2.3 mV	51.85 ± 4.83 %	20.45 ± 3.55 µg/mg

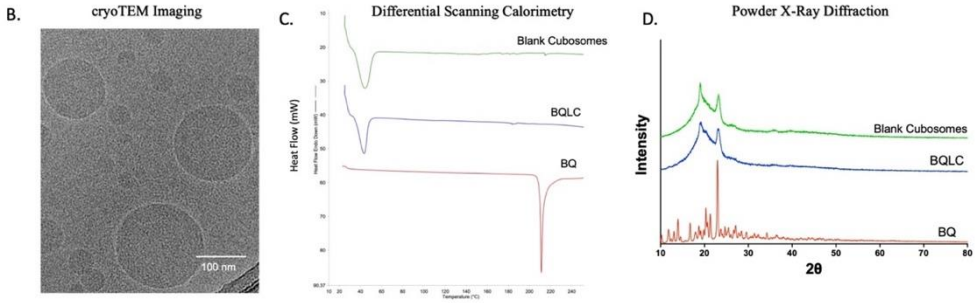


Figure 1. Physicochemical characterization of BQLC A. Formulation characterization. B. Morphological analysis of cubosomes depicted by cryoTEM images. C. Thermograms of BQ, BQLC and blank cubosomes generated by differential scanning calorimetry (DSC). D. Diffraction patterns of BQ, BQLC and blank Cubosomes obtained using powder X-ray diffraction (PXRD).

The BQLC were successfully fabricated with a particle size of 150.2 ± 5.1 nm and a PDI of 0.24 ± 0.02. The developed nanocarriers were cationic with a zeta potential of (+) 35.4 ± 2.3 mV, encapsulation efficiency of 51.85 ± 4.83 % w/w and drug loading of 20.45 ± 3.55 µg of BQ per mg of lipid content (Fig. 1A). The cryoTEM micrographs depicted nearly spherical cubosomes with irregular polyangular shapes (Fig. 1B). The BQ thermograms showed an endothermic melting peak at 215 ºC. Blank cubosomes consisting of a lipid and surfactant displayed a glass transition peak at 40 ºC, corresponding to GMO melting point as per manufacturers datasheet. The BQLC thermogram was similar to blank cubosomes with absence of BQ melting peak suggesting successful encapsulation in cubosomes (Fig. 1C). Diffractometry studies revealed BQ characteristic peaks over 10º to 30º 2θ range (Fig. 1D). Blank and BQ-loaded cubosomes showed characteristic peaks at 18º and 23º 2θ values, corresponding to a partially ordered structure of P188 [6] and absence of BQ characteristic peaks between 10°– 30° 2θ values further confirming the successful encapsulation of BQ.

A.

Aerosolization Performance		
Formulation	Undiluted	Diluted
MMAD (µm)	5.12 ± 0.12	4.21 ± 0.53
GSD	2.20 ± 0.03	2.23 ± 0.07
FPF (%)	67.97 ± 1.40	78.94 ± 5.66

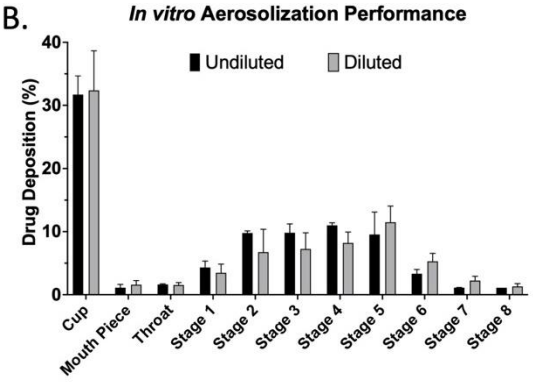


Figure 2: A. *In vitro* aerosolization performance of BQLC after nebulization for diluted and undiluted formulations. B. Stage-wise drug deposition (%) of BQLC in NGI. (Mean ± SD, n = 3).

The aerosolization performance (Fig. 2A & B) of cubosomes was assessed using NGI. The prepared BQLC (referred to as undiluted BQLC) formulation demonstrated MMAD of 5.12 ± 0.03 µm with FPF of 67.97 ±1.40%. The developed cubosomal formulations were viscous in nature and resulted in higher MMAD, as increased viscosity increases MMAD as well as impacts aerosol deposition for jet nebulizers [7]. So, to reduce the viscosity of the formulation, BQLC was diluted with water (1:1). The diluted BQLC displayed enhanced aerosolization performance with MMAD of 4.21 ± 0.53 µm, GSD of 2.23 ± 0.07 and FPF of 78.94 ± 5.66%. Hence, the aerosolization performance suggests BQLC can be administered *via* inhalation route, facilitating local delivery to the deep lungs, thus resulting in reduced exposure to other organs and limiting adverse events.

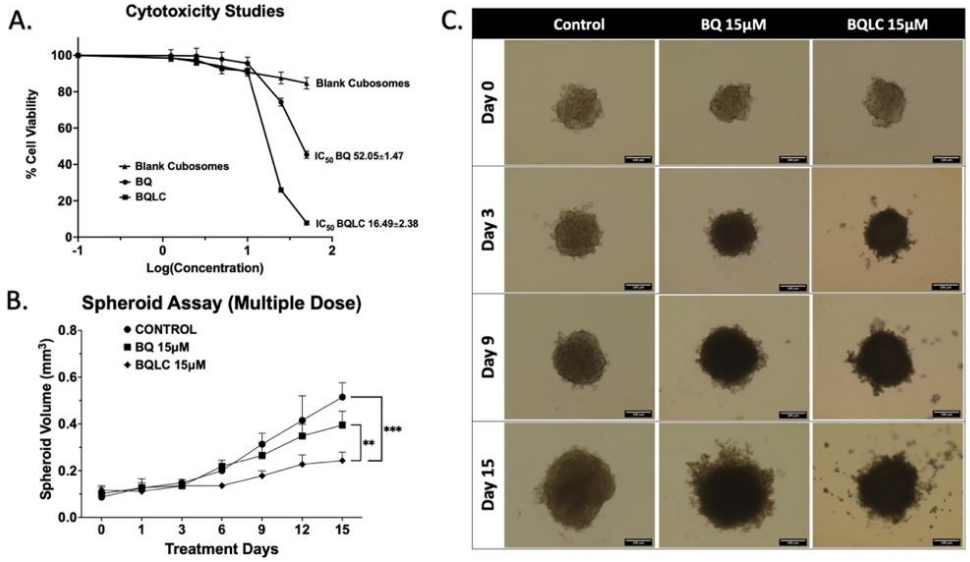


Figure 3: A. Cytotoxicity studies after 48 h treatment with BQ, BQLC and blank cubosomes determined using MTT assay in A549 cells. B. Spheroid growth characterization of A549 cells after multiple doses of BQ and BQLC treatments. C. Representative images of spheroids after multiple dose treatment demonstrating tumor suppression at days 0, 3, 9 and 15. (Mean ± SD, n = 6). Statistical analysis (two-way ANOVA) was performed using Prism software (GraphPad, USA). Scale bar = 200 µm. ** p < 0.01, *** p < 0.001.

The IC_{50} of free BQ (52.05 ± 1.47 µM) was significantly reduced using the BQLC formulations (16.49 ± 2.38 µM; $p < 0.0001$) as shown in Fig. 3A. A ~3-fold lower IC_{50} of cubosomal nanoparticles, compared to the free drug, could be attributed to enhanced solubility of BQ and rapid internalization of cubosomes (data not shown). The safety of nanocarriers (blank cubosomes) was ascertained with 84.67 ± 3.11 % of cell viability when used around the IC_{50} concentrations (0.5 mg of formulation). The 3D-spheroids resemble the appearance of a tumor *in vivo* and better mimic the tumor behaviour providing physiologically relevant results [8]. The BQLC showed a significant inhibition in spheroid volume, compared to free BQ ($p < 0.01$) and control ($p < 0.001$), as shown in Fig. 3B & C. The spheroid volume of untreated group was 0.51 ± 0.06 mm^3, a 4-fold increase in spheroid volume from day 1 (0.12 ± 0.03 mm^3). The spheroids treated with 15 µM BQLC exhibited a 0.24 ± 0.04 mm^3 spheroid volume with significant inhibition as paralleled to 0.40 ± 0.05 mm^3 of 15 µM free BQ. The physicochemical properties of nanocarriers, such as small particle size and positive surface charge are crucial for effective tumor penetration, and it can be concluded that BQLC having these characteristics facilitates the entry of drug into solid tumor core which could be inaccessible to free BQ. Therefore, our results suggest that BQLC can be a promising strategy to repurpose BQ for NSCLC treatment at reduced dose. Moreover, the ability to deliver these cubosomes locally and directly to the primary tumor site in the lungs may further reduce the required dose and decrease systemic side effects compared to systemic delivery.

Conclusion

This is the first study focusing on inhaled delivery of bedaquiline-loaded cubosomal nanocarriers for the treatment of NSCLC. The developed BQLC nanocarriers showed suitable particle characteristics and efficient drug entrapment. Further, upon nebulization the cubosomal nanocarriers demonstrated suitable aerodynamic properties (MMAD and FPF) for inhalation delivery. The positively charged BQLCs enhanced cytotoxicity compared to free BQ in A549 cells. The ability of the BQLC to inhibit tumor growth in 3D spheroid studies further provides preliminary evidence for translation of developed drug delivery system to a preclinical setup.

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References

1. Parvathaneni V, Elbatanony RS, Goyal M, Chavan T, Vega N, Kolluru S, Muth A, Gupta V, Kunda NK: Repurposing Bedaquiline for Effective Non-Small Cell Lung Cancer (NSCLC) Therapy as Inhalable Cyclodextrin-Based Molecular Inclusion Complexes. *International Journal of Molecular Sciences* 2021;22(9):4783 (1-18).

2. Wu X, Li F, Wang X, Li C, Meng Q, Wang C, Huang J, Chen S, Zhu Z: Antibiotic bedaquiline effectively targets growth, survival and tumor angiogenesis of lung cancer through suppressing energy metabolism. *Biochem Biophys Res Commun* 2018;495(1):267-272.

3. Chang C, Meikle TG, Drummond CJ, Yang Y, Conn CE: Comparison of cubosomes and liposomes for the encapsulation and delivery of curcumin. *Soft Matter* 2021;17(12):3306-3313.

4. Rizwan SB, Assmus D, Boehnke A, Hanley T, Boyd BJ, Rades T, Hook S: Preparation of phytantriol cubosomes by solvent precursor dilution for the delivery of protein vaccines. *Eur J Pharm Biopharm* 2011;79(1):15-22.

5. Vartak R, Patil SM, Saraswat A, Patki M, Kunda NK, Patel K: Aerosolized nanoliposomal carrier of remdesivir: an effective alternative for COVID-19 treatment in vitro. *Nanomedicine* 2021.

6. Dong W, Su X, Xu M, Hu M, Sun Y, Zhang P: Preparation, characterization, and in vitro/vivo evaluation of polymer-assisting formulation of atorvastatin calcium based on solid dispersion technique. *Asian J Pharm Sci* 2018;13(6):546-554.

7. Broniarz-Press L, Ochowiak M, Matuszak M, Włodarczak S: The effect of shear and extensional viscosity on atomization in medical inhaler. *International Journal of Pharmaceutics* 2014;468(1):199-206.

8. Shukla SK, Kulkarni NS, Farrales P, Kanabar DD, Parvathaneni V, Kunda NK, Muth A, Gupta V: Sorafenib Loaded Inhalable Polymeric Nanocarriers against Non-Small Cell Lung Cancer. *Pharm Res* 2020;37(3):67 (1-19).