Curcumin-ER Prolonged Subcutaneous Delivery for the Treatment of Non-Small Cell Lung Cancer

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Non-small-cell lung cancer therapy is a challenge due to poor prognosis and low survival rate. There is an acute need for advanced therapies having higher drug efficacy, low immunogenicity and fewer side effects which will markedly improve patient compliance and quality of life of cancer patients. The purpose of this study was to develop a novel hybrid curcumin nanoformulation (Curcumin-ER) and evaluate the therapeutic efficacy of this formulation on a non-small cell lung cancer xenograft model. Use of curcumin, a natural anticancer agent, is majorly limited due to its poor aqueous solubility and hence its low systemic bioavailability. In this paper, we carried out the nanoformulation of Curcumin-ER, optimized the formulation process and determined the anticancer effects of Curcumin-ER against human A549 non-small cell lung cancer using in vitro and in vivo studies. Xenograft tumors in nude mice were treated with 20 mg/kg subcutaneous injection of Curcumin-ER and liposomal curcumin (Lipocurc) twice a week for seven weeks. Results showed that tumor growth was suppressed by 52.1% by Curcumin-ER treatment and only 32.2% by Lipocurc compared to controls. Tumor sections were isolated from murine xenografts and histology and immunohistochemistry was performed. A decrease in expression of NFκB-p65 subunit and proliferation marker, Ki-67 was observed in treated tumors. In addition, a potent anti-angiogenic effect, characterized by reduced expression of annexin A2 protein, was observed in treated tumors. These results establish the effectiveness of Curcumin-ER in regressing human non-small cell lung cancer growth in the xenograft model using subcutaneous route of administration. The therapeutic efficacy of Curcumin-ER highlights the potential of this hybrid nanoformulation in treating patients with non-small cell lung cancer.

KEYWORDS: Curcumin, Curcumin-ER, Lipid-Polymer Nanoparticle, Non-Small Cell Lung Cancer, A-549.

INTRODUCTION
Lung cancer has one of the highest incidences of cancer among all the carcinomas worldwide. Non-small-cell lung cancer is often diagnosed at an advanced stage, with 70% of them resulting in metastases.¹ The first choice treatment in non-small cell lung cancer (NSCLC) is chemotherapy with gemcitabine, taxanes or vinorelbine, together with a platinum drug like cisplatin or carboplatin.²,³ Chemotherapy with drugs like cisplatin is associated with adverse side effects, such as anemia, neurotoxicity and nephrotoxicity.⁴ Poor prognosis and less than 15% of 5-year survival rate⁵ highlights the urgent need for new drugs or delivery systems against non-small-cell lung cancer.

Advancements in the field that are in use currently have included treatments to include less cytotoxic therapies specifically directed towards identifiable targets associated with tumor proliferation and progression. However, these treatment options are also plagued with immunogenic concerns and low bioavailability issues. For over a decade, polymeric nanoparticles have become highly sought as drug delivery systems due to their high structural integrity, stability during storage, ease of preparation, functionalization, and controlled release capability. In addition, incorporation of active targeting moieties makes them...
vastly attractive as targeted therapeutics.6 Another class of nanoparticles, liposomes, has long been thought to be the more ideal drug delivery system compared to polymeric nanoparticles as they have been shown to possess improved biocompatibility. This improved biocompatibility is because liposomes are often derived from biological membranes, but also can be prepared from both natural and synthesized phospholipids.7 The limitation with liposome delivery is that they are easily cleared by the reticular endothelial system (RES) leading to poor bioavailability.5 Further, liposomes suffer from lack of structural integrity which may result in erratic release of its contents and low storage life.8 To address the limitations of polymeric nanoparticles and liposomes, a new generation delivery system termed lipid–polymer nanoparticles has been developed.9 Traditionally, they have a polymer core encapsulating the therapeutic substances and a lipid layer enveloping the polymer core, which confers biocompatibility to the nanoparticle. In addition, the lipid layer functions to minimize leakage of the encapsulated content during the lipid–polymer nanoparticle preparation and helps slow down the polymer degradation rate.10

Curcumin is a natural anti-cancer agent extracted from the rhizome of turmeric (Curcuma longa Linn.) and has been much investigated for the past few decades.11–13 It has very low intrinsic toxicity along with a wide range of pharmacological activities including anti-cancer properties. Other research also reported that curcumin had anti-cancer effects via interference with the activity of the transcription factor NF-κB.14 Several phase I and phase II clinical trials with oral administration indicate that curcumin is quite safe and exhibits preventive and therapeutic efficacy.15–17 However, the main limitations of curcumin are its low aqueous solubility, inactivation in the gut wall, and hence reduced bioavailability.18

In the present study, we formulated a lipid-polymer hybrid nanoformulation encapsulating curcumin (Curcumin-ER) as a possible therapy against NSCLC. We characterized the hybrid nanoformulation for entrapment efficiency, drug loading, particle size, zeta potential, surface morphology and its in vitro release profile. Next, we determined the pharmacokinetics of Curcumin-ER in rats and carried out xenograft studies in subcutaneous A-549 tumor bearing mice during which we demonstrated improved cell uptake and anti-proliferative effects of Curcumin-ER. Finally, we conducted immunoblotting and immunohistochemical analysis in isolated tumors to determine the therapeutic efficacy of Curcumin-ER as a therapeutic for NSCLC.

MATERIALS AND METHODS

Materials
Poly(D,L-lactide-co-glycolide) 50:50; i.v. 0.77 dL/g (~0.5% w/v in chloroform at 30 °C); m.w. 124 kDa was purchased from Lakeshore Biomaterials (Birmingham, AL). Curcumin, 99.2% pure, was synthesized under GMP conditions by Sabinsa (NJ, USA) and obtained through SignPath Pharma Inc (PA, USA). The GMP grade Lipocurc was obtained from Polymun GmbH (Vienna, Austria). The Lipocurc was made with a 9:1 ratio of DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and DMPG (1,2-dimyristoyl-sn-glycero-3-phospho-rac-[1-glycerol]) with a curcumin content of 6.4 mg/ml. Ace-tonitrile, ethanol, nile red, sucrose, were purchased from Sigma Aldrich (St. Louis, MO). The human lung cancer cell line–A 549 was obtained from ATCC (Manassas, VA). RPMI 1640 and FBS were obtained from Gibco, Invitrogen (Carlsbad, CA). Gold anti-fade mounting agent with 4′-6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen (Carlsbad, CA). Double-distilled deionized water was used for all the experiments.

Preparation and Optimizations of the Curcumin-ER
Curcumin was dispersed in an organic phase containing PLGA in acetonitrile. The lipids, DMPC (1,2-dimyristoryl-sn-glycero-3-phosphocholine) and DMPG (1,2-dimyristoryl-sn-glycero-3-phospho-rac-[1-glycerol]) were used at a pre-determined ratio (7:3). There are previous reports of liposomes composed of the same lipids (i.e., DMPC and DMPG), which lacked general toxicity and decreased nystatin toxicity.19 Hence we chose to use the same combination of lipids but optimized the ratio in a previous study to obtain the optimal formulation. DMPG and DMPC were dissolved in 4% ethanol water. Both lipids were then mixed and heated to 50 °C. The organic phase mixture was subsequently added drop wise with constant stirring. The mixture was sonicated in sonication bath (FS 30, Fisher Scientific, PA, USA) for 5 min. The final mixture was stirred for 3 hours on magnetic stirrers to facilitate evaporation of the organic solvent from the formulation. Once the organic solvent was evaporated, the mixture was centrifuged and washed thrice at 4000 rpm for 15 minutes using Amicon filters with a 10 kDa cutoff. The nanoparticles so obtained were resuspended in a 10% (w/v) sucrose that acts as a cryoprotectant. Further, the nanoparticle-cryoprotectant mixture is flash frozen in liquid nitrogen for 5 minutes and then lyophilized for 48 hrs in a glass container.

For optimization of the nanoformulation, design of experiments (DOE) applying a 3-factor, 3-level Box-Behnken Design was used to establish the functional relationships between operating variables (factors) and their responses. The factors (operating variables) chosen were polymer concentration ($X_1$), amount of lipids ($X_2$) and molar ratio of lipids ($X_3$). The responses (dependent variables) studied were average Particle Size ($Y_1$), entrapment efficiency (EE) ($Y_2$) and drug loading (DL) ($Y_3$). The factors with their ranges and corresponding dependent responses are described in Supplement Table I. The nonlinear mathematical model generated by this design.
Table I. Factors and their levels in the Box-Behnken design.

<table>
<thead>
<tr>
<th>Independent factors</th>
<th>Unit</th>
<th>Minimum</th>
<th>Maximum</th>
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<tbody>
<tr>
<td>PLGA conc.</td>
<td>mg/ml</td>
<td>2.50</td>
<td>12.50</td>
</tr>
<tr>
<td>Total liquids</td>
<td>mg</td>
<td>1.00</td>
<td>8.00</td>
</tr>
<tr>
<td>DMPC/DMPG molar ratio</td>
<td>mole/mole</td>
<td>2.33</td>
<td>9.00</td>
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<th>Dependent factors</th>
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<tr>
<td>Particle size</td>
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<tr>
<td>Encapsulation efficiency</td>
<td>%</td>
<td>Maximize</td>
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<tr>
<td>Drug loading</td>
<td>%</td>
<td>Maximize</td>
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Cell Viability Assay in A549 Lung Cancer Cells

To determine the therapeutic efficacy of hybrid nanoparticles, MTT assay was carried out in A549 non small cell lung cancer cells as per the protocol described elsewhere.14

Liquid Chromatography Mass Spectrometry (LC-MS/MS)

An Agilent 1200 Series and Agilent 6410 Triple Quad LC/MS mass spectrometer with an ESI ion source were used for these analytical studies. The compounds were separated using a Thermo Aquasil C18, 50 × 4.6 mm, with a mobile phase of methanol (A): Acetonitrile/methanol/25 mM ammonium formate (80/10/10, v/v/v); (B): Acetonitrile in a run mode as follows: Isocratic, 95% of mobile phase A and 5% of mobile phase B 0 to 1.2 min to waste, at a solvent flow rate of 0.5 ml/min. The mass spectrometer was operated in negative ion mode: curcumin, 367.1 → 134.0 m/z, tetrahydrocurcumin 371.2 → 235.1 m/z and naproxen 229.1 → 169.9 m/z.

In Vivo Efficacy of Curcumin-ER in Lung Cancer Bearing Mice

Animal Models

Animal studies were performed according to a protocol approved by the IACUC committee at UNTHSC, Fort Worth, Texas.

Xenograft Study

Female athymic nude mice (nu/nu) (Harlan Inc. Indianapolis, In, USA) between 4–6 weeks old were used in each group (n = 10) for this study. Subcutaneous lung tumor xenografts were established by injecting A549 tumor cells in nude mice. A total of 3 × 10^6 A-549 cells (in log phase growth) in 50 μl RPMI were injected subcutaneously in the dorsal flank of the mice. Once adequate tumor volumes became established (Average ~ 50 mm^3), the animals were randomized and divided into three groups (n = 10/group). Each treatment group received subcutaneous Curcumin-ER or Lipocurc (20 mg/kg body weight, twice a week). Untreated animals served as the control group. Body weight and tumor size were measured with calipers three times a week. The tumor volume was calculated using the equation: volume (mm^3) = (length × width^2)/2. The total number of mice studied in each group was ten.

Histological Sections

Tumor tissue pre-fixed in formalin was paraffin-embedded, sectioned (3–5 μm), and stained with hematoxylin and eosin (H&E). Multiple sections were evaluated for changes in tumor cell cytology and evidence of toxicity.

Immunohistochemistry

To determine inhibition of the NFκB pathway in tumor cells by Lipocurc and Curcumin-ER, we assayed for the NFκB-p65 subunit. We determined levels of Annexin A2 (ANXA2) which plays a role in the regulation of cellular...
growth and signal transduction pathways, and SRC, a non-receptor tyrosine kinase protein in xenograft tissue with and without treatment. We also determined levels of Ki67 which has a role in cell proliferation. Tumors collected from the mice at the end of the study (7 weeks) were sectioned and examined. Immuno-histochemical studies were carried out using formalin-fixed, paraffin-embedded sections (5 μm), heat induced antigen retrieval and 1:50 to 1:200 concentrations of monoclonal antibody. Antibodies used for immunohistochemistry included antibody against annexin A2 (BD Bioscience, San Jose, CA, USA), NFκB-p65 (e Bioscience, San Diego, CA USA), ki67 (e Bioscience, San Diego, CA USA), Secondary antibody (either mouse or rabbit; Invitrogen, Grand island, NY, USA) was tagged to a fluorophore (Alexa 488: green; Alexa 594: red) and viewed under a confocal microscope (Zeiss LSM 510 META) attached to a Zeiss Axiovert 200 inverted microscope (Carl Zeiss Micro Imaging, Inc., Thornwood, NY, USA).

**Western Blot Analysis of Tissue Samples**

Isolated tumor samples were homogenized with lysis buffer with protease inhibitors added to prevent loss of protein in the samples. Western blot was run as per the protocol described elsewhere. Glyceraldehyde 3-phosphate

<table>
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<th>Table II. Predicted optimum formulation conditions selected for further studies.</th>
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<td><strong>Formulation factors</strong></td>
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<tr>
<td>PLGA conc.</td>
</tr>
<tr>
<td>Total liquids</td>
</tr>
<tr>
<td>DMPC/DMPG molar ratio</td>
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**Figure 1.** Curcumin-ER formulation and optimization: (A) Schematic representation of Curcumin-ER; (B) Three dimensional response surface plot showing the effect of molar ratio of lipids and PLGA concentration on particle size, encapsulation efficiency and drug loading keeping the total amount of lipids (mg) constant; (C) Physicochemical characterization of Curcumin-ER prepared under the predicted optimum conditions.
dehydrogenase (GAPDH) was used as a loading control for all the blots.

RESULTS AND DISCUSSION
Formulation, Optimization and Characterization of Curcumin-ER

The hybrid nanoformulation and optimization of Curcumin-ER was successful. Figure 1(A) shows the schematic representation of the nanoformulation. The formulation was optimized by varying the polymer concentration (range: 2.5–12.5 mg), total amount of lipids (range: 1–8 mg), different molar ratios of the lipids (range: DPMC/DMPG:2.33-9) (Tables: I–III). The response of these factors are presented as three-dimensional contour response surface graphs, depicted in Figure 1(B). The optimal formulation was achieved based on imposing constraints of minimum particle size maximum encapsulation efficiency and maximum drug loading. The optimum values for these variables for the optimized batch were found to be 7.5 mg/ml for PLGA concentration, 4.6 mg for total amount of lipids and a DPMC/DMPG molar ratio of 5.4 (Supplement Table II). Next, a Curcumin-ER batch with these contained predicted levels of formulation factors was prepared to verify the optimization design. Figure 1(C) shows average particle size of optimized batch of Curcumin-ER (henceforth represented as Curcumin-ER) to be 91.6 ± 3.1 nm and confirms a narrow size distribution. Measured size was presented as the average value of 20 runs. The zeta potential determines the surface charge in the nanoparticles and plays a role in intracellular uptake. Curcumin-ER had a zeta potential of −16.3 ± 1.0 mV. The entrapment efficiency was high at 93.7 ± 1.9% and drug loading was determined to be 1.27 ± 0.4%. These optimal experimental results were in good agreement with the predicted values as indicated by low bias % (Table III). Figure 2(A) illustrated the release kinetics profile and shows that it follows a biphasic pattern where ~15% of curcumin is released within an hour during the burst phase; ~50% of curcumin is then released from the formulation in about 10 hours to a total of about 70% in 50 hours.

<table>
<thead>
<tr>
<th>Response</th>
<th>Predicted value</th>
<th>Observed value</th>
<th>Bias (%)</th>
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<tr>
<td>Particle size (nm)</td>
<td>86.3</td>
<td>91.6</td>
<td>5.78</td>
</tr>
<tr>
<td>Drug loading (%)</td>
<td>1.30</td>
<td>1.27</td>
<td>−2.38</td>
</tr>
<tr>
<td>Encapsulation efficiency (%)</td>
<td>89.5</td>
<td>93.7</td>
<td>−4.48</td>
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Note: *Bias was calculated (observed value-predicted value)/observed value x 100.

Figure 2. In vitro evaluation of Curcumin-ER: (A) In vitro drug release kinetics of curcumin from Curcumin-ER; (B) Cell viability assay were performed on A 549 non small cell lung cancer cell line at 48 hrs; (C) Intracellular uptake of Curcumin-ER in A 549 lung cancer cell line (i-Magnification ×20 and ii and iii Magnification ×40). The error bars represent mean and standard deviations of experiments performed in triplicate.
In Vitro Evaluation of Curcumin-ER

Curcumin-ER enhanced the cytotoxicity of curcumin significantly as compared to free curcumin by inhibited growth of A-549 lung cancer cells. Cell viability assay results, post 48 hours, depicted that treatment with 38 µM concentration of Curcumin-ER resulted in more than 50% cell death (Fig. 2(B)). The IC$_{50}$ of free curcumin for 48 hour incubation was reported to be $\sim 70$ µM in A-549 cells.$^{22}$ This illustrates almost 46% reduction in the IC$_{50}$ value with Curcumin-ER which is a significant improvement in the efficacy of curcumin. This indicated that curcumin released from Curcumin-ER was functionally active and was able to bring about effective cell death in lung cancer cells.

Robust intracellular uptake indicated by red fluorescence was observed by confocal microscopy in A-549 cells incubated with nile red-labeled-Curcumin-ER (Fig. 2(C)). This increased intracellular uptake of Curcumin ER is critical as more uptakes will correspond to more Curcumin-ER accumulation and subsequent release of curcumin from the nanoparticles to elicit its anticancer action.

Subcutaneous Injection of Curcumin-ER Inhibits the Tumor Growth of Human Lung Cancer Xenograft

The in vivo anticancer activity of Curcumin-ER was investigated in female athymic nude mice bearing A549 subcutaneous lung tumor xenografts established by injecting A549 tumor cells in nude mice. Animals were randomly divided into groups ($n = 10$/group) and treated with Lipocurc and treated with Curcumin-ER subcutaneously. Animals were treated when average tumors were 50 mm$^3$ (day 0) twice in a week subcutaneously for a total of seven weeks (20 mg curcumin/kg of body weight/dose) and tumor growth monitored alternative days. A significant inhibition of tumor growth was observed in A549 tumor-bearing mice treated with Curcumin-ER; (E) Images showing the subcutaneous treatment of Lipocurc and Curcumin-ER in A549 lung cancer xenograft bearing mice.
xenograft lung tumors. Subcutaneous (SC) injections have found use in overcoming limitations of low systemic bioavailability by extending the release characteristics and thereby increasing systemic exposure of the drug.\textsuperscript{23} We compared the efficacy of Curcumin-ER and Lipocurc with untreated control animals. Our results show significant regression in tumor volume post treatment with subcutaneous injection of Curcumin-ER (20 mg/kg body weight, twice a week) compared to control and Lipocurc treatment (Fig. 3(A)). Figure 3(B) shows body weight of the animals during the entire treatment period. No weight loss or other signs of toxicity was evidenced in the mice treated with Curcumin-ER or liposomes. It is evident that Curcumin-ER was more effective in inhibiting tumor growth compared to Lipocurc treatment. At the end of the study period (7 weeks), there was 52.1 ± 7.2% decrease in tumor volume by the Curcumin-ER compared to 32.2 ± 6.8% by Lipocurc treatment. Tumors isolated from mice (from control and treatment groups) are shown in Figure 3(C). The average tumor weight of untreated control animals was found to be 182.54 mg while that of Curcumin-ER treated group was only 93.9 mg which represents a significant decrease of 48.5% (Fig. 3(D)). Figure 3(E) depicts representative images of A549 tumor bearing mice post subcutaneous injection with either Lipocurc or Curcumin-ER.

**Histology, Immunohistochemistry and Immunoblotting of Tumor Tissue**

**Histology**

Paraffin-embedded tissues were sectioned and stained with H&E to observe the \textit{in vivo} effect of Curcumin-ER and Lipocurc treatment compared to untreated controls. The tumor sections from the Curcumin-ER group showed...
Figure 6. (A) Immunohistochemistry images of isolated xenograft tumor tissue-sections showing expression of key proliferation marker, Ki67, post Lipocurc and Curcumin-ER treatment. Untreated tumor sections served as controls; (B) Graphical representation shows the quantification for expression in tissue sections. Results are expressed as mean ± SD. *p < 0.05, **p < 0.001.

Figure 7. (A) Immunohistochemistry images of isolated xenograft tumor tissue-sections showing expression of key angiogenesis marker, Annexin A2, post Lipocurc and Curcumin-ER treatment. Untreated tumor sections served as controls; (B) Graphical representation shows the quantification for expression in tissue sections. Results are expressed as mean ± SD. *p < 0.001.
Curcumin-ER treatment as compared to control tissues. These results show that curcumin encapsulated within hybrid nanoparticles is able to reach the cancer cells in active form where it blocks the activation of NFkB and inhibits translocation of NFkB from cytosol to nucleus, as seen from the decreased expression of NFkB-p65 subunit.24,25 We next determined the changes in expression levels of Ki67, a proliferation marker. Results illustrated that Curcumin-ER was able to significantly decrease the proliferation associated with cancer cells as seen by decreased expression levels of Ki67 in the animal xenograft models (Fig. 6). Further, curcumin has been shown to play a significant role in angiogenesis. We determined the in vivo anti-angiogenic effect of our Curcumin-ER by observing the expression levels of the protein, Annexin A2 (AnxA2), which is shown to regulate neoangiogenesis.26 Figure 7 reveals a significant decrease in AnxA2 expression in the tumor tissue sections post Curcumin-ER treatments as compared to untreated controls.

**Immunoblotting**

The immunoblot bands represent the amount of target proteins expressed in the tumor samples isolated from mice post-treatment with Curcumin-ER, Lipocurc or untreated control animals (Fig. 8). Curcumin-ER treatment brought about a reduction in the expression of target proteins (NFkB-p65, AnxA2) as compared to untreated samples. GAPDH expression (loading control) is not uniform in all the lanes. This has been adjusted by calculating the density of expression with respect to GAPDH density. There are almost equal loading of proteins in first two lanes. Figure 8 reveals a significant decrease in NFkB-p65 and AnxA2 expression in the tumor tissue sections post Lipocurc and Curcumin-ER treatments as compared to untreated controls.

**CONCLUSIONS**

In conclusion, Curcumin-ER was successfully formulated and showed high curcumin encapsulation with particle size in the range of 90 nm. The in vitro evaluation showed that Curcumin-ER showed improved cellular uptake and was able to decrease cell viability indicating therapeutic efficacy against NSCLC cancer cells. The tumor xenograft studies further illustrated the in vivo efficacy of Curcumin-ER by significantly decreasing tumor burden following subcutaneous administration of Curcumin-ER. The immunohistochemical and immunoblotting analysis of the isolated lung tumors confirmed the effect of Curcumin-ER in regressing tumorogenic and proliferation proteins. These results indicate that Curcumin-ER may be used as a therapeutic in the treatment of NSCLC.

**REFERENCES**


