Efficacy of Liposomal Curcumin in a Human Pancreatic Tumor Xenograft Model: Inhibition of Tumor Growth and Angiogenesis

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Abstract. *Background: Liposome-based drug delivery has been successful in the past decade, with some formulations being Food and Drug Administration (FDA)-approved and others in clinical trials around the world. The major disadvantage associated with curcumin, a potent anticancer agent, is its poor aqueous solubility and hence low systemic bioavailability. However, curcumin can be encapsulated into liposomes to improve systemic bioavailability. Materials and Methods: We determined the antitumor effects of a liposomal curcumin formulation against human MiaPaCa pancreatic cancer cells both in vitro and in xenograft studies. Histological sections were isolated from murine xenografts and immunohistochemistry was performed. Results: The in vitro (IC_{50}) liposomal curcumin proliferation-inhibiting concentration was 17.5 μM. In xenograft tumors in nude mice, liposomal curcumin at 20 mg/kg i.p. three-times a week for four weeks induced 42% suppression of tumor growth compared to untreated controls. A potent antiangiogenic effect characterized by a reduced number of blood vessels and reduced expression of vascular endothelial growth factor and annexin A2 proteins, as determined by immunohistochemistry was observed in treated tumors. Conclusion: These data clearly establish the efficacy of liposomal curcumin in reducing human pancreatic cancer growth in the examined model. The therapeutic curcumin-based effects, with no limiting side-effects, suggest that liposomal curcumin may be beneficial in patients with pancreatic cancer.*

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Pancreatic cancer is a disease with a very poor prognosis. In most cases, a mortality rate of near 99% is seen within five years following detection (1, 2). This malignancy is highly resistant to chemotherapy and the only curative therapy is early detection and complete surgical resection. Prolongation of survival of patients with unresectable cancer with other treatment modalities remains limited. One of the chemotherapeutic agents currently FDA-approved for treatment of pancreatic cancer is gemcitabine, which confers minimal improvement in survival time (3). There is an unmet need to improve survival and disease control of pancreatic cancer. This may be addressed by developing novel chemotherapeutic agents that target the molecular pathways contributing to tumor growth.

Natural herbal extracts have been investigated for a plethora of diseases for a very long time. One such natural extract is turmeric (containing the active component, curcumin) derived from the roots of *Curcuma longa* Linn. Curcumin has been reported to exhibit antioxidant, anti-inflammatory, antimicrobial and anticancer activity *in vitro* and in animal models of disease (4-12). Its anticancer activity is exerted through modulation or inhibition of multiple molecular pathways (13-16). Notably, curcumin is a potent inhibitor of (NFκB), a transcription factor found to play a role in the pathogenesis of pancreatic cancer, as well as other malignancies (17). Curcumin has shown dose-dependant chemopreventive and chemotherapeutic effects in numerous carcinogenesis models and pre-clinical trials (18). However, the extract of the root, containing several curcuminoids as well as curcumin, when administered orally has limited bioavailability and solubility in aqueous fluids

(19). This can be circumvented by designing novel formulations allowing parenteral drug delivery.

Designing a nanoparticle-based drug delivery system that enhances the solubility of curcumin in an aqueous phase medium may significantly improve its potential clinical applications (20). In this direction, various methods, including curcumin complexes of surfactant, protein, phospholipid, and chitosan, and other polymer nanoparticle-mediated curcumin delivery approaches have been developed (19, 21-25). Among polymeric nanoparticle-based approaches, biodegradable polymer nanoparticles offer promising enhanced therapeutic performance of anticancer drugs by increasing their bioavailability (18, 19, 26, 27). Studies have also shown that incorporation of curcumin into a membrane strongly enhances its stability and has a strong impact on the efficacy of liposome-bound curcumin (22, 28, 29).

In the present study, we investigated the *in vitro* and *in vivo* antitumor activity of liposomal curcumin against human pancreatic cancer cells. Our data illustrate that the liposomal curcumin formulation inhibited xenoplanted pancreatic tumor growth in mice. Immunohistochemistry of tumor tissues of liposomal curcumin treated mice exhibited reduced expression of (NFκB), proteins downstream in its pathway, and reduced angiogenesis associated due to mitigation of vascular endothelial growth factor receptor 2 (VEGFR2) expression.

Materials and Methods

Cell culture. The human pancreatic cell line MiaPaCa was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured using Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, pyruvate, glutamine, and supplemented with 10% fetal bovine serum (FBS) purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

Liposomal curcumin. (GMP) Grade curcumin (99.2% pure) was synthesized by Sabinsa Inc, Princeton, NJ, USA, and the liposomal formulation was generated at Polymun GmbH (Vienna, Austria). The liposomes were made up of a 9:1 ratio of 1,2-dimyristoil-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phospho-rac-[1-glycerol] (DMPG) and reconstituted buffer (20 mM sodium acetate/300 mM sucrose, pH 5.0). The curcumin content of the final liposomal curcumin formulation was 6.4 mg/ml.

Characterization of liposomal curcumin. Characterization of liposomal curcumin was carried out to define reproducibility in terms of *in vitro* and *in vivo* parameters. The formulation was characterized for particle size, zeta potential and surface morphology. Particle size distribution was determined by differential light scattering using Nanotrac (Microtrac Inc., Montgomeryville, PA, USA). The formulated product was dispersed in aqueous buffer and then measured for particle size. The results are reported as the average of five runs with triplicate samples. Zeta potential was determined by Zeta PALS (Brookhaven instruments Corporation, NY, USA). The surface morphology of liposomal curcumin was studied using atomic force microscopy (AFM). A small quantity of aqueous solution of the formulation was placed on a glass slide and air dried. It was then loaded into an AFM instrument (TMX 2100 Explorer SPM; Veeco, Plainview, NY,USA), equipped with AFM dry scanner.

Cell viability. To determine the inhibitory effect of liposomal curcumin on cell growth and cell viability, a methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was carried out with the pancreatic cancer cell line MiaPaCa. MTT is a yellow compound which is taken up by living cells and cleaved to yield a purple-colored formazan product. This colorimetric change can be measured using a plate reader (BioTek, Synergy 2 plate reader, VT, USA) and indicates the number of viable cells, thereby reflecting cell proliferation and survival. For this assay, 2000 cells/well were plated in a 96-well plate and were treated with 0 to 30 μM of liposomal curcumin. The assay was terminated after 48 hours' exposure. Relative growth inhibition was measured by comparison with untreated control cells. All experiments were performed in triplicate and repeated twice for statistical analysis. Results are expressed as the mean±S.D.

Animals. Female athymic *nu/nu* mice (3-5 weeks old) obtained from Harlan (Indianapolis, IN, USA) were maintained in micro-isolator units (5 animals/cage). Animals were given a commercial diet and water. Mice were quarantined for one week before experimental procedures. All animal experiments were performed at the UNT Health Science Center under a protocol approved by the Institutional Animal Care and Use Committee of UNT Health Science Center (2011/12-29-A04).

Xenograft study. A total of 3×10^6 MiaPaCa cells (in log phase growth) in 50 μl DMEM were injected subcutaneously in the dorsal flank of the mice. Once adequate tumor volumes became established (minimum 50 mm^3), the animals were randomized and divided into two groups. One group received intraperitoneal liposomal curcumin (20 mg/kg body weight, three times a week) and the other one was 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 twenty. Each group was studied twice.

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 liposomal curcumin, we assayed for the NFκB-p65 subunit, and downstream proteins signal transducer and activator of transcription 3 (STAT3) and matrix metallopeptidase 9 (MMP9) in the NFκB signaling pathway. We determined anti-angiogenic effects of liposomal curcumin by changes in expression of VEGFR2. We also 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. Tumors collected from the mice at the end of the study (31 days) were sectioned and examined. Immunohistochemical studies were carried out using formalin-fixed, paraffin-embedded sections (5 μm), heat-induced antigen retrieval and 1:50 to 1:200 concentrations of

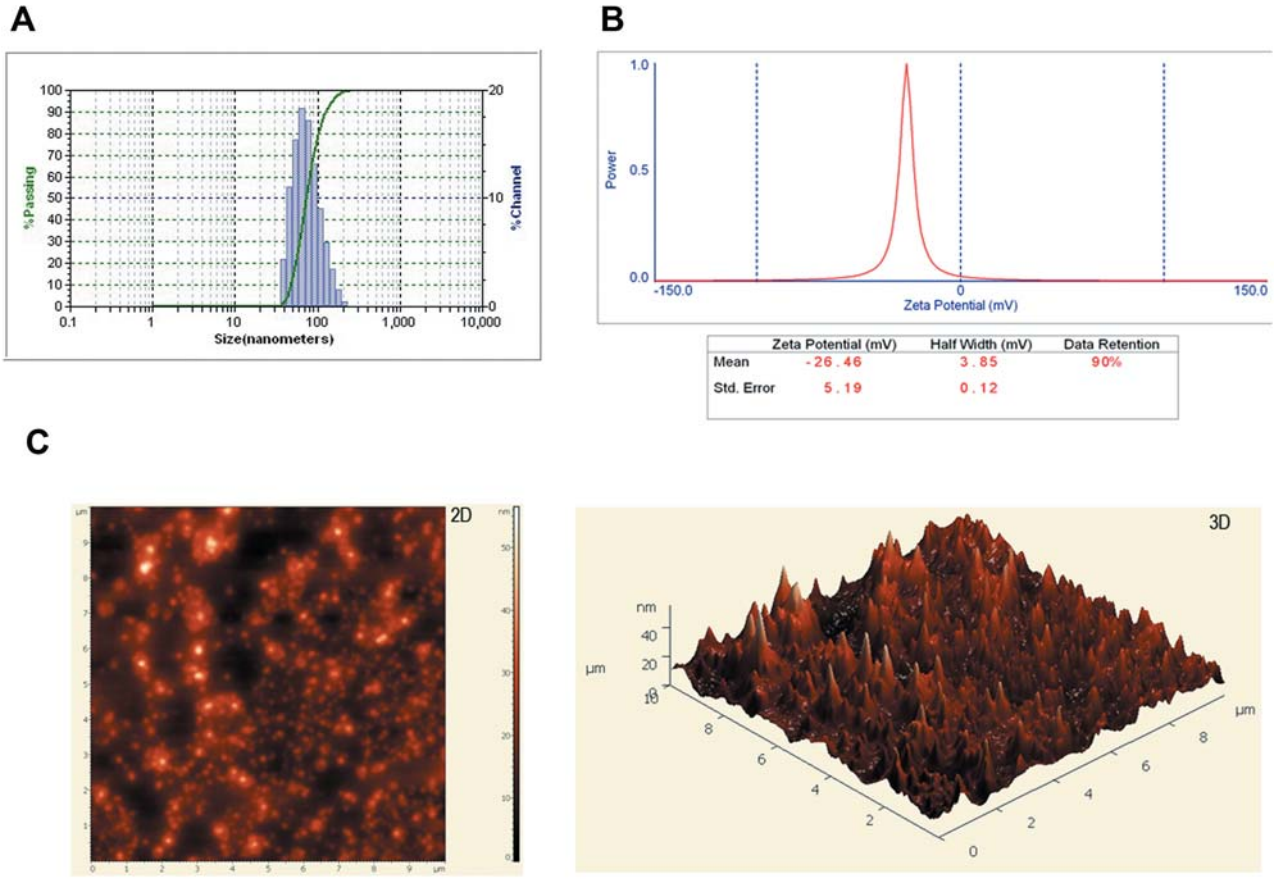

Figure 1. *Characterization of liposomal curcumin: A: Particle size distribution; B: zeta potential; C: atomic force microscopy: 2D and 3D views.*

monoclonal antibody. Antibodies used for immunohistochemistry included antibody against annexin A2 (BD Bioscience, San Jose, CA, USA), NFκB-p65 (Cell Signaling Technology, Danvers, MA, USA), MMP9 (Cell Signaling Technology), SRC (Cell Signaling Technology), STAT3 (LifeSpan BioSciences, Inc., Seattle, WA, USA) and VEGFR2 (Cell Signaling Technology). 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).

Results and Discussion

Liposomal curcumin was tested for its effect on pancreatic cancer. The formulation was characterized for particle size, zeta potential and surface morphology. The average particle size of the curcumin liposomes was 72.90 nm (Figure 1A). The zeta potential or total charge on the surface of the liposomes was -26.46±5.16 mV (Figure 1B). Surface morphology of the liposomes by AFM revealed them to have a spherical shape and unimodal distribution of particle size of the liposomes (Figure 1C).

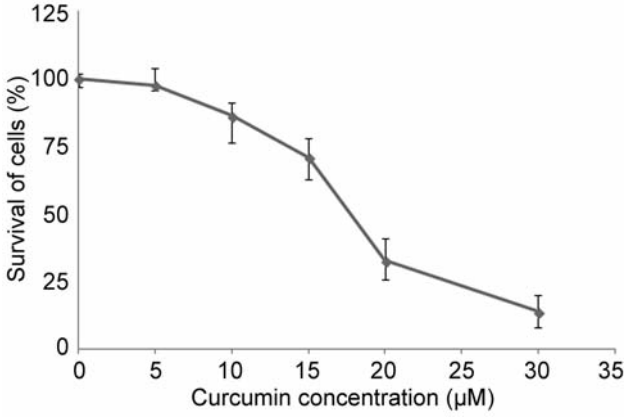

Figure 2. *Cell viability assay on pancreatic cancer cell lines at 48 hours. The half-maximal inhibitory concentration (IC_{50}) concentration of liposomal curcumin is 17.5 µM for MiaPaCa cells.*

Liposomal curcumin inhibits proliferation/survival of MiaPaCa Cells. We studied the *in vitro* effect of liposomal curcumin on cell proliferation of the human pancreatic cell

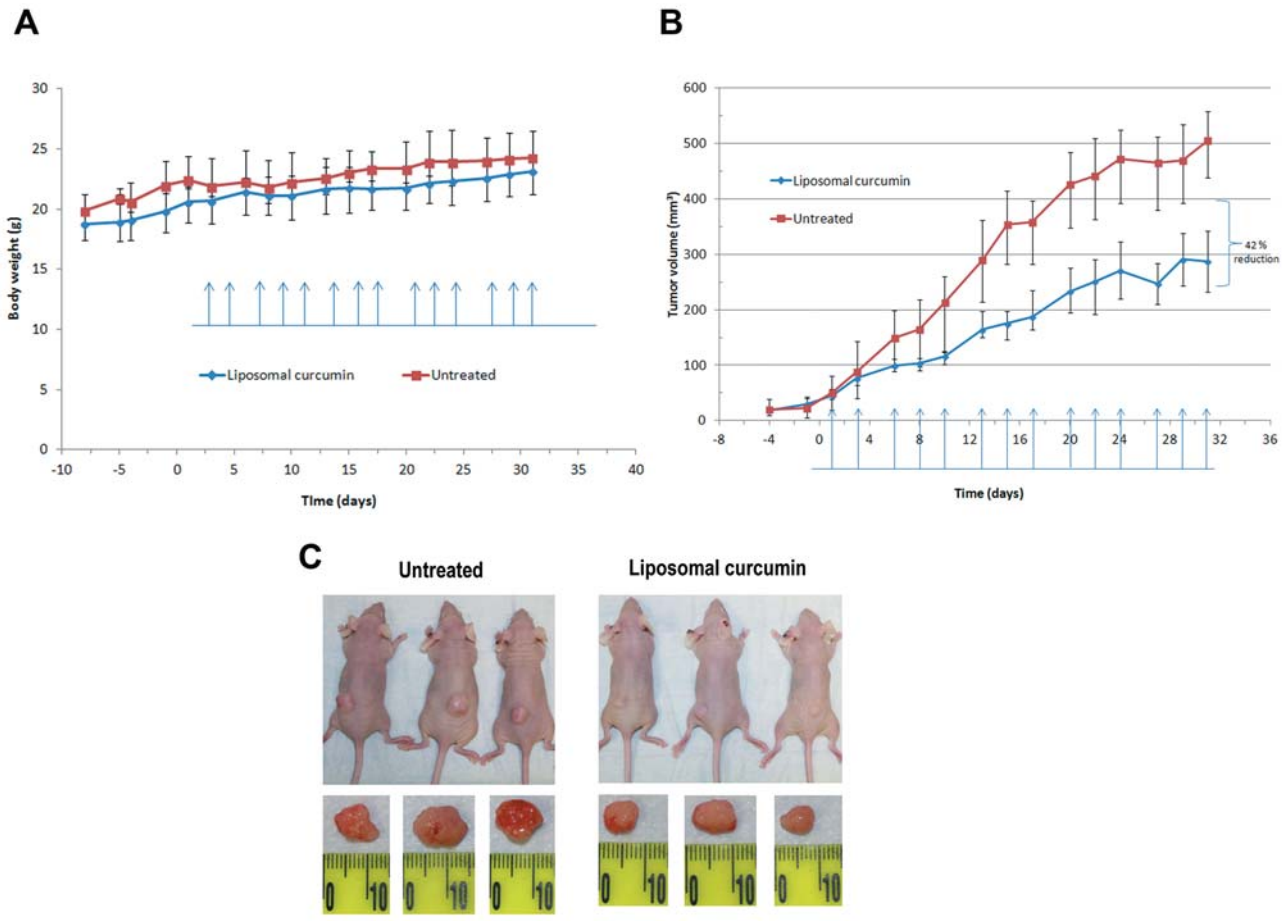

Figure 3. *In vivo efficacy of liposomal curcumin (20 mg/kg, thrice weekly) on pancreatic tumor xenografts over the duration of study: A: Body weight; B: mean xenograft tumor volume; C: mice bearing tumor and extracted tumors.*

line MiaPaCa for 48 hours. The MTT assay results revealed that liposomal curcumin has an antiproliferative effect in a concentration-dependent manner (Figure 2). The half-maximal inhibitory concentration (IC_{50}) for liposomal curcumin in MiaPaCa cells was found to be 17.5 μM. Our result is in accordance with similar studies on pancreatic cancer cells wherein the IC_{50} concentration range of liposomal curcumin extract was reported between 2.0 μM for Capan-1 to 37.8 μM for Capan-2 cells following a 72-hour exposure (16). In another study on human colorectal cancer cell lines Colo205 and LoVo, the IC_{50} of pegylated liposomal curcumin, was 30 μM and 7.5 μM respectively after 72 hours of incubation (30); the IC_{50} for free curcumin in their study was observed at 40 μM and 10 μM respectively in the colorectal cancer cell lines (30). Comparing our results of liposomal curcumin with other novel curcumin delivery systems which are in pre-clinical or clinical testing, the IC_{50} for nanocurcumin (polymeric nanoparticle-based system) was found to be higher than 20 μM in MiaPaCa cells after 72-hour treatment (25). In another study, the IC_{50} of curcumin-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles was reported to be between 20 μM to 22.5 μM for prostate cancer cells PC3, Du145 and LNCaP after 72-hour treatment, while that of free curcumin ranged from 32 μM to 34 μM among the cancer cell lines (19). These results are substantially higher than the IC_{50} value we recorded for our liposomal curcumin after 48-hour treatment.

Liposomal curcumin inhibits pancreatic tumor xenograft growth in murine models. Female athymic nude mice were injected subcutaneously with MiaPaCa cells to form tumor xenografts. For the subsequent 31 days, their body weight was measured thrice weekly. Synthesized liposomal curcumin administered *via* bolus intraperitoneal injections thrice a week at a dose of 20 mg/kg body weight induced an overall decrease of 42% in tumor growth (Figure 3A-C) compared to untreated controls. No significant changes in body weights were observed in either group during the study period.

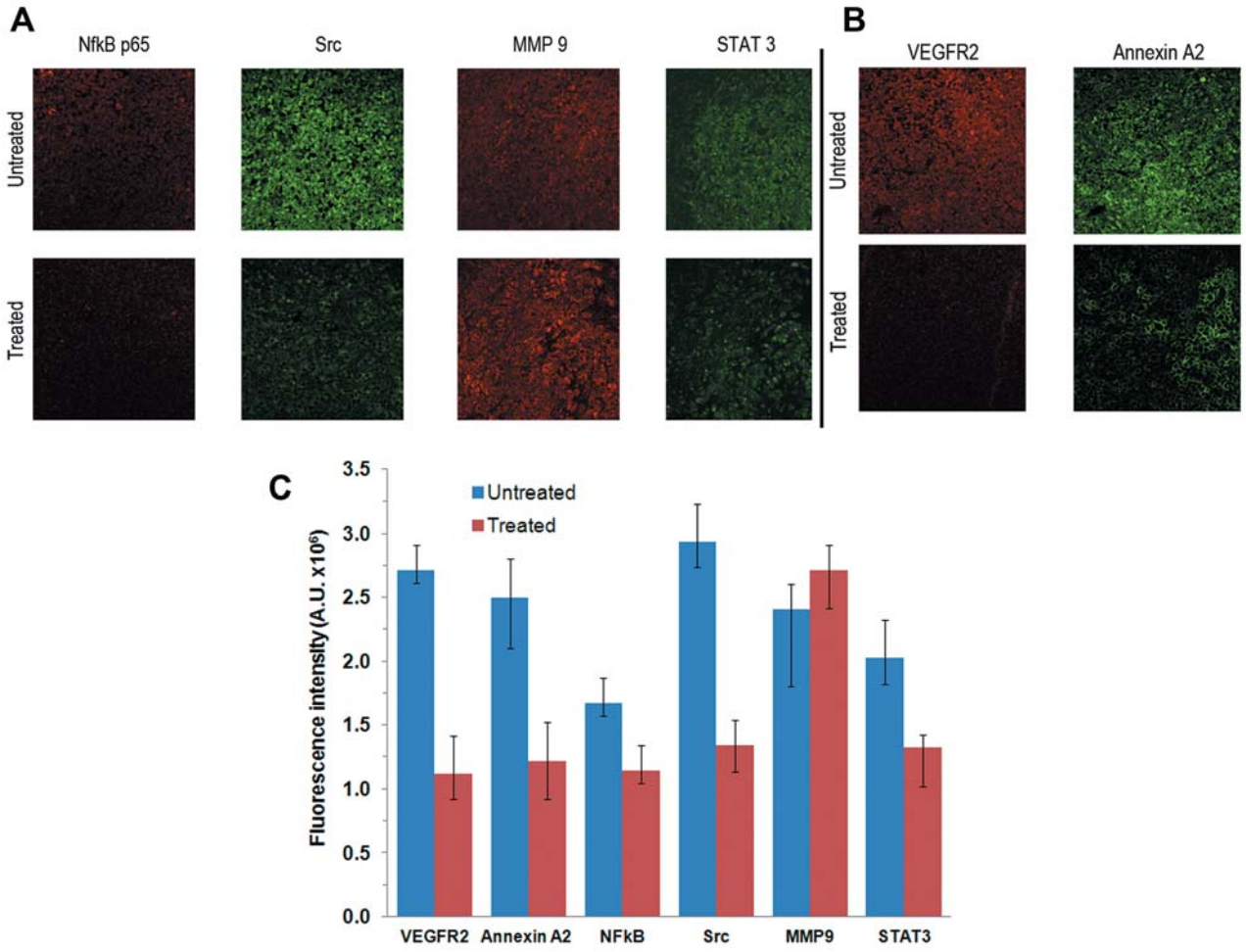

Figure 4. *Immunohistology of pancreatic xenograft tumor sections post liposomal curcumin treatment at a dose of 20 mg/kg, thrice weekly: A: Tumor markers; B: angiogenesis markers. Untreated tumor sections served as controls.*

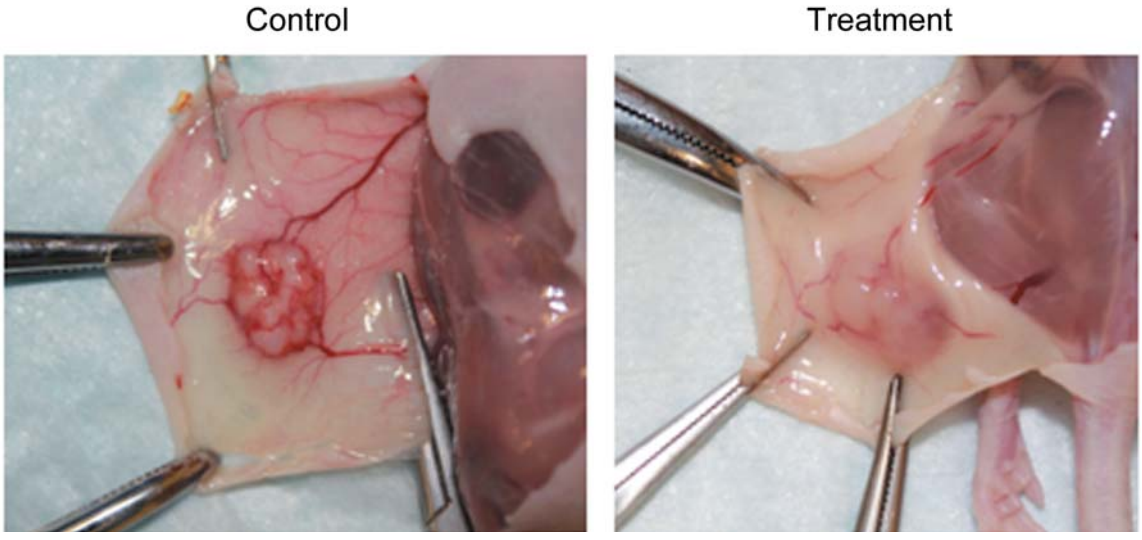

Figure 5. *Antiangiogenic effect of liposomal curcumin treatment as seen by reduced number of blood vessels formed surrounding the tumor.*

In a separate study of liposomal curcumin extract at a dose of 40 mg/kg intravenously thrice weekly in murine subcutaneous xenografts of colorectal tumors Colo205 and LoVo, growth was suppressed by 50-66% compared to mice treated with empty liposomes (30). There were no significant changes in weight in liposomal curcumin-treated animals bearing xenografts compared with unimplanted animals treated with saline or empty liposomes. In another study of pancreatic cancer tumors in murine models, liposomal curcumin extract at a dose of 40 mg/kg of liposomal curcumin intravenously three times weekly suppressed the growth of both BxPC-3 and MiaPaCa2 tumors by 60-77% (16). Both these studies were carried out for 17-20 days. In this study, synthesized liposomal curcumin at half the dose reported (20 mg/kg) *via* intraperitoneal injections thrice a week for 30 days, induced 42% tumor growth suppression, similar to a study on pancreatic cancer xenografts with the same synthesized liposomal curcumin at 20 mg/kg: a comparable 50% tumor regression was observed (31). This study differed from our study in their intravenous route of administration.

Immunohistochemistry. Immunohistochemical data revealed reduced expression of NFκB-p65, STAT3 and SRC in tumor tissue following liposomal curcumin treatment when compared with tissues from untreated control mice. (Figure 4A and B). Difference of MMP9 expression due to curcumin was minimal which may be explained by the observation that any decrease in MMP9 expression may begin as early as 6 hours after the last dose and persist only up to 72 hours from the last dose (32). In this experiment, these studies were carried out on tissues obtained from mice sacrificed 72 hours after the last dose of chemotherapy.

The antiangiogenic effect of the liposomal curcumin treatment was secondary to the decrease in VEGFR2 expression. Since synthesized liposomal curcumin treatment induces a clear reduction in the expression of ANXA2 it indicates that curcumin has an important role in reducing angiogenesis and migration/ invasion (33).

Liposomal curcumin inhibits angiogenesis in vivo. To determine whether the reduced growth of tumors after liposomal curcumin treatment of mice with pancreatic cancer xenografts was associated with an antiangiogenic effect, we examined the formation of neovasculature in the tumor xenografts. Our data reveal increased angiogenesis associated with untreated tumor (Figure 5). By contrast, a decrease in the number of blood vessels in tumor from mice treated with liposomal curcumin can be observed. This is consequent to a decrease in the expression of VEGFR2 (Figure 4B). Similar antiangiogenic effects of liposomal curcumin have been reported in both BxPC-3 and MiaPaCa2 tumor xenografts, where decreased expression of cluster of differentiation 31 (CD31) and VEGF were observed (30). In a previous study, the antiangiogenic effect of curcumin was clearly demonstrated when the peritoneal lining of ehrlich ascites tumor (EAT)-bearing mice treated with vehicle (0.1% DMSO) or free curcumin was inspected for angiogenesis. Inhibition of angiogenesis, represented as reduction in vessel density and the number of smaller capillaries, in curcumin-treated mice was successfully demonstrated using a chorioallantoic membrane (CAM) assay (34). In another study, similar antiangiogenic activities of curcumin analogs were demonstrated using CAM assay where a decrease in vessel density and fewer capillaries were observed (35). Both these studies also reported a decrease in VEGF expression.

Conclusion

This study focused on the *in vitro* and *in vivo* evaluation of a liposomal formulation of synthesized curcumin in human pancreatic cancer cells. These data illustrate that the liposomal curcumin formulation inhibited pancreatic tumor growth in murine xenograft models by 42%. Immunohistochemistry of tumor tissues showed reduction in expression of NFκB, proteins, SRC and ANXA2 downstream in its pathway. These antitumor effects were accompanied by an antiangiogenic response associated with reduced VEGFR2 expression. No evident toxicity in mice was observed with liposomal curcumin treatment. These data suggest that liposomal curcumin may be used either alone or as an adjuvant therapy for pancreatic cancer.

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