**Small Molecule Therapeutics**

**Novel Curcumin-Loaded Magnetic Nanoparticles for Pancreatic Cancer Treatment**

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**Abstract**

Curcumin (CUR), a naturally occurring polyphenol derived from the root of *Curcuma longa*, has shown potent anticancer and cancer prevention activity in a variety of cancers. However, the clinical translation of CUR has been significantly hampered due to its extensive degradation, suboptimal pharmacokinetics, and poor bioavailability. To address these clinically relevant issues, we have developed a novel CUR-loaded magnetic nanoparticle (MNP-CUR) formulation. Herein, we have evaluated the *in vitro* and *in vivo* therapeutic efficacy of this novel MNP-CUR formulation in pancreatic cancer. Human pancreatic cancer cells (HPAF-II and Panc-1) exhibited efficient internalization of the MNP-CUR formulation in a dose-dependent manner. As a result, the MNP-CUR formulation effectively inhibited growth of HPAF-II and Panc-1 cells in cell proliferation and colony formation assays. The MNP-CUR formulation suppressed pancreatic tumor growth in an HPAF-II xenograft mouse model and improved the survival of mice by delaying tumor growth. The growth-inhibitory effect of MNP-CUR formulation correlated with the suppression of proliferating cell nuclear antigen (PCNA), B-cell lymphoma-extra large (Bcl-xL), induced myeloid leukemia cell differentiation protein (Mcl-1), cell surface-associated Mucin 1 (MUC1), collagen I, and enhanced membrane β-catenin expression. MNP-CUR formulation did not show any sign of hemotoxicity and was stable after incubation with human serum proteins. In addition, the MNP-CUR formulation improved serum bioavailability of CUR in mice up to 2.5-fold as compared with free CUR. Biodistribution studies show that a significant amount of MNP-CUR formulation was able to reach the pancreatic xenograft tumor(s), which suggests its clinical translational potential. In conclusion, this study suggests that our novel MNP-CUR formulation can be valuable for the treatment of pancreatic cancer.

**Introduction**

Pancreatic cancer ranks fourth in causing cancer-related deaths in the United States (1). Recent statistics estimated that 44,030 individuals were diagnosed with pancreatic cancer and 37,660 patients died in 2011 from this highly devastating disease (1). In spite of significant progress in the area of cancer therapeutics, pancreatic cancer still remains by and large untreatable with an extremely poor 5-year survival rate (1). Poor vasculature and the hyperfibrotic and desmoplastic tumor microenvironment of pancreatic cancer are primarily responsible for reduced uptake of chemotherapeutic drugs and low therapeutic outcome (2, 3). Gemcitabine is a standard chemotherapeutic drug used for the treatment of pancreatic cancer; however, survival is increased by only 5 to 12 months (4, 5). In addition, this therapeutic approach may lead to severe toxicity and may also result in chemo-resistance. Therefore, developing a safer and more efficient therapeutic modality to improve management of pancreatic cancer is highly desirable. Because of their potent anticancer activity, a few natural dietary ingredients are considered as complementary and alternative medicine by the National Cancer Institute for cancer prevention/treatment options. Treatment that can modulate tumor microenvironment using a natural dietary agent would be considered a novel therapeutic strategy.

Curcumin (CUR; chemical structure, Fig. 1A) is a widely known natural biactive polyphenolic component of turmeric. CUR is also a “Generally Recognized As Safe” compound by the Food and Drug Administration (FDA; ref. 6). CUR exhibits both anticancer and cancer prevention activities by suppressing key elements of initiation, promotion, and metastasis of a wide range of cancer(s) (7). The multitargeting role of CUR enables it to conduct a wide spectrum of actions against cancer(s) (8). Epidemiological
studies revealed that populations with CUR consumption have lower incidence and risk of various types of cancers including pancreatic cancer (9–11). Because CUR possesses exceptional therapeutic properties, more than 20 clinical trials are underway to assess CUR’s efficacy in cancer treatment(s) (as of January 2013). In vitro studies have concluded that CUR exhibits anticancer effects at micromolar concentrations. However, achieving these concentrations at the tumor site in humans is highly challenging and has not yet been accomplished due to its higher metabolic activity and low bioavailability (10, 12). CUR nanoformulations can be a useful delivery module (12). CUR nanoformulations have showed an enhanced delivery of CUR in biologically active form to various cancer cells (13). Although many nanotechnology investigations have primarily focused on either improving stability or bioavailability of CUR, limited studies have been pursued related to therapeutic effects on pancreatic cancer (14–18). To date, no study has reported on magnetic nanoparticle (MNP)-mediated CUR delivery for treatment of pancreatic cancer. Herein we report, for the first time, the effects of a novel CUR-loaded MNP (MNP-CUR) formulation (Patent# PCTUS2011/063723) on human pancreatic cancer cells in vitro and in vivo and elucidate its potential translational capabilities. In addition, we have used advanced and appropriate quantitative methods to study the efficacy of this novel formulation in both in vitro and xenograft mouse models.

Materials and Methods

Chemicals, reagents, and antibodies

All chemicals and reagents were acquired from Sigma-Aldrich Corporation and cell culture plastic ware and consumables were purchased from BD Biosciences, unless otherwise mentioned. The preparation and characterization of the MNP-CUR formulation was conducted following our optimized/patented composition protocol (19). Monoclonal antibodies against B-cell lymphoma-extra large (Bcl-xl; Abcam), induced myeloid leukemia cell differentiation protein (Mcl-1; Abcam), proliferating cell nuclear antigen (PCNA; Cell Signaling Technology), collagen I (Abcam), β-actin (Sigma), cell surface–associated Mucin 1 (MUC1; Cell Signaling Technology), and β-catenin (gift of Dr. K. Johnson, University of Nebraska Medical Center, Omaha, Nebraska) were used for immunohistochemistry and Western blot analyses.

Cancer cell lines and animals

HPAF-II and Panc-1 human pancreatic cancer cell lines were purchased from American Type Cell Culture (Manassas, VA), cultured as monolayer in 75 cm² culture flasks in Dulbecco’s Modified Eagle Medium (DMEM)-F12/DMEM medium (HyClone Laboratories, Inc.) and supplemented with 10% heat-inactivated FBS (Atlanta Biologicals), 1% penicillin, and 1% streptomycin (Gibco BRL) at 37°C in a humidified atmosphere (5% CO₂ and 95% air atmosphere). To maintain authenticity of the cell lines, frozen stocks were prepared from a parent stock and every 6 months a new frozen stock cell line was used for the experiments. All animal experiments were carried out using 6- to 8-week-old male athymic nude/nude mice (Harlan Laboratories) and all procedures were approved by the Sanford Research/University of South Dakota Institutional Animal Care and Use Committee.
Cellular uptake

Cellular uptake of MNP-CUR formulation in HPAF-II and Panc-1 cancer cells was evaluated by Prussian blue stain and 1,10-phenanthroline photometric method. For the Prussian blue staining experiment, 2 x 10^5 cells were seeded in 6-well plates in 2 mL medium and cells were allowed to attach to the plates overnight. These cells were treated with 25–to 200 µg MNP-CUR formulation for 6 hours. After treatment, cells were washed (with PBS), fixed (with methanol), and incubated subsequently with a mixture of 2% potassium ferrocyanide, 2% hydrochloric acid for 30 minutes, and nuclear fast red-aluminum sulfate solution (0.1%) for 5–to 10 minutes at 25°C. The representative images of internalized MNP-CUR in cancer cells were captured at 200 x magnification using an Olympus BX 41 Microscope (Olympus Corporation). For iron content estimation, pancreatic cancer cells (2 x 10^7) were treated with 100 µg MNP-CUR formulation, washed, and trypsinized using 0.25% Trypsin-EDTA, and cell pellets were washed twice with 1 x PBS, collected, and dissolved in hydrochloric acid to determine iron content using 1,10-phenanthroline photometric method as described in our recent publications (19, 20).

Cell proliferation

Cells (5 x 10^5) were seeded in 6-well plates in 2 mL media and allowed to adhere overnight. Cells were then incubated with 5, 10, and 20 µmol/L of free CUR or CUR equivalent MNP-CUR formulation for 2 days at 37°C. In this experiment, equivalent amounts of DMSO or MNPs (no CUR) in PBS served as controls for CUR and MNP-CUR, respectively. The media was removed after 2 days, cells were washed with PBS, trypsinized, and collected in culture medium and cell number was counted using a hemocytometer (21). Each concentration treatment was done at least 6 times.

Clonogenic assay

Cells (500) were seeded in 6-well plates in 2 mL growth media and incubated for 2–to 3 days to allow grow into colonies. Cells were treated with 2, 4, and 8 µmol/L free CUR or MNP-CUR for 7 days and then media without drugs for 7 days at 37°C. Subsequently, cells in all plates were gently rinsed with PBS, fixed with methanol, and stained with hematoxylin. A population of at least 50 cells was considered a colony and the number of colonies was counted and quantified according to our standard previously reported protocol (22).

In vivo antitumor activity and survival study

Approximately 6- to 8-week-old male athymic nude (nu/nu) mice were inoculated subcutaneously at their left flank with 5 x 10^5 HPAF-II human pancreatic cancer cells dispersed in a 200 µL solution of PBS and Matrigel (1:1 ratio; BD Biosciences; ref. 21). These mice were used to evaluate both antitumor activity and survival analysis. On day 13 (postinjection with cancer cells), the animals were randomly distributed into 4 groups (8 mice per group). Two treatment groups were administered intratumoral injections of 20 µg CUR dissolved in 100 µL of 0.1% Tween 20 or 20 µg equivalent CUR containing MNP-CUR dispersed in 100 µL PBS. Similarly, control groups were treated with either Tween 20 or empty MNPs (no CUR). The tumor size was measured on day 7, 13, 20, 24, 28, 32, 35, and 40 using a digital Vernier caliper. The tumor size data were presented up to day 28 because of statistical analysis consideration. The tumor volume was calculated using the ellipsoid volume equation: tumor volume (mm^3) = π/6 x L x W x H, where L is length, W is width, and H is height (21). The animals were sacrificed at the end of the treatment or when tumor volume reached 1,000 mm^3. Survival of mice was followed until day 40 and a plot was generated using Origin 6.1 software. Tumor tissues were collected for immunohistochemical and immunofluorescence analyses as well as the analysis of MNP-CUR accumulation.

Immunohistochemical and immunofluorescence analyses

Immunohistochemical and immunofluorescence analyses were conducted to investigate pathways that are involved in MNP-CUR–mediated suppression of pancreatic tumor growth. For immunohistochemistry analysis, 10% formalin fixed tumor tissues were processed as described earlier (21, 23) and tissue slides were incubated with primary antibodies against Bel-7XL (1:400), Mcl-1 (1:300), PCNA (1:2000), β-catenin (1:200), and MUC1 (1:250). Protein expression was detected using MACH 4 Universal HRP polymer detection kit (Biocare Medical) and 3,3’-diaminobenzidine (DAB substrate kit; Biocare Medical) as described earlier (21). Finally, slides were washed with water, counterstained with hematoxylin, dehydrated and mounted with Ecomount (Biocare Medical), immunostaining was assessed using an Olympus BX 41 Microscope (Olympus Corporation). Similarly for immunofluorescence analysis, rehydrated tissue slides were blocked using 4% normal donkey serum (Jackson ImmunoResearch Laboratories) followed by incubation with primary rat anti-mouse collagen I (1:200; Chondrex, Inc.) and mouse anti-human collagen I (1:200; Abcam). After washing with PBS, tissue slides were incubated with secondary antibodies Alexa-Fluor 488 and Alexa-Fluor 568 (1:200; Life Technologies). 4',6-Diamidino-2-phenylindole was used to visualize nuclei. A laser scanning confocal microscope (Nikon TIRF) was used with a 20× Apochromat objective and optical Z sections were taken at ~0.8 µm. Magnification, pinhole settings, laser, and detector gains, which were set below saturation were identical across samples (21). Western blot experiments and analyses were followed as described earlier (19).

Accumulation of MNP-CUR in pancreatic tumors

Paraffin-embedded tumor tissues were processed according to our protocol (21) and tissue sections were subsequently immersed in 10% potassium ferrocyanide and 10% hydrochloric acid solution for 20 minutes at 25°C.
and in 0.1% nuclear fast red-aluminum sulfate solution for 5 minutes at 25°C. Following dehydration, tissues were viewed and representative images captured at 400 x magnification using an Olympus BX 41 Microscope.

Bioavailability studies of MNP-CUR formulation

To determine bioavailability of CUR in blood serum in nu/nu mouse, 50 μg CUR in Tween 20 or CUR equivalent MNP-CUR formulation in PBS solution was administered intraperitoneally (i.p.) using a 30G needle. At predetermined time intervals (5, 10, 15, 30, 60, 120, 360, 720, and 1440 min), blood was drawn from tails and serum was immediately collected by centrifugation at 2,000 rpm for 5 minutes (Centrifuge 5415D; Eppendorf AG). Serum samples were stored at –80°C until further use. Serum samples (3–5 μL) were lyophilized using a Labconco Freeze Dry System (–48°C, 133 × 10⁻³ m Bar; Labconco) and CUR was extracted from serum by incubating 2 days in acetone/IR. CUR concentrations were determined using an UltiMate high-performance liquid chromatography (Dionex Corporation) equipped with UltiMate 3000 injector, RS variable wavelength detector, and an Acclaim polar advantage column of 3 μm 120 Å (4.6 x 150 mm). The mobile phase consisted of 1% citric acid:acetone/IR (50:50, v/v). Chromatograms were collected at 430 nm. Linear calibration curve for CUR under similar conditions was obtained in the range of 1 to 10 ng/mL.

Biodistribution studies

To examine MNP-CUR biodistribution in different organs in mice, tumor-bearing mice were injected intratumorally or i.p. with 20 μg CUR equivalent MNP-CUR formulations in 100 μL PBS solution using a 30G needle. After 24 hours, animals were euthanized and tumor, liver, spleen, and brain tissues were collected and stored at –80°C. Tissue homogenates were prepared by grinding at 8,000 rpm in PBS using a Power Gen 125 homogenizer (125 W, 115V, 50/60 Hz; Fisher Scientific) and lyophilized for quantitative analysis of iron of MNP-CUR. Known quantities of lyophilized tissue samples were dissolved in concentrated hydrochloric acid and iron content of MNPs was determined following 1,10-phenanthroline photometric method (24). The iron levels of control group mice (no MNP treatment) organs were recovered by centrifugation and observed for ultrastructural morphologies using TEM. The no-treatment group and dendrimer formulations were used as negative and positive controls, respectively.

Statistical analyses

Descriptive data are presented as the mean ± SEM. ANOVA models and t tests were used to evaluate differences in in vitro properties. Linear mixed regression analyses were conducted to determine the tumor volumes as a function of time. Transformations of continuous variables were conducted to meet model assumptions. Time to death (or euthanization) was used for Kaplan–Meier analysis and equality of survival was determined using log-rank analysis. All analysis was conducted using SAS 9.3 (SAS Institute, Inc.). P values of <0.05 were considered significant. All graphs were plotted using Origin 6.1 software.

Results

Optimal size of MNP-CUR formulation for cancer therapeutics

In this study we have investigated utilization of “triple crown MNPs” as a delivery vehicle for CUR (MNP-CUR; ref. 19). The optimized MNP-CUR was formulated with 810:300 mg Fe²⁺:Fe³⁺ salts, 200 mg cyclodextrin, 250 mg Pluronic F-127 (19) with 10% (w/w) CUR loading (Fig. 1A). An average individual MNP-CUR particle grain size of 10.5 ± 0.54 nm, observed under JEOL 1210 TEM (JEOL Ltd.; Fig. 1A), showed the ultra small particle size nature of this formulation. However, this formulation showed 109 nm particle size with ~0.99 mV ζ potential in dynamic light scattering analysis (19). These nanoparticle characteristics are suitable for optimal cancer therapeutics purpose.

MNP-CUR effectively internalizes in pancreatic cancer cells

Qualitative analysis of Prussian blue staining in HPAF-I1 and Panc-1 cells showed a dose-dependant uptake of MNP-CUR formulation (Fig. 1B). The uptake pattern was different in both cancer cells and this observation is consistent with uptake of other magnetic drug nanoformulations in cancer cells (26, 27). However, the amount of nanoparticle uptake in both cell lines was very close (i.e.,
54.06% and 53.86%) when incubated with 100 μg of MNP-CUR formulation (Supplementary Fig. S1A).

**MNP-CUR inhibits proliferation and clonogenic potential of pancreatic cancer cells**

In cell proliferation experiments, CUR and MNP-CUR exhibited a dose-dependent inhibition of cell proliferation (Fig. 1C). At all 3 tested concentrations, MNP-CUR had a very similar cytotoxicity on cancer cells as compared to free CUR treatment. This indicates the MNP-CUR formulation preserved the inherent biological activity of CUR. In addition, the effect of MNP-CUR was tested in the colony formation assay using HPAF-II and Panc-1 pancreatic cancer cells. CUR and MNP-CUR formulation effectively inhibited the clonogenic potential of HPAF-II and Panc-1 cancer cells compared to control-treated cells (Fig. 1D). In cell proliferation and colony formation assay, only ~53% internalized MNP-CUR and ~40% released CUR from MNP-CUR (19) caused cytotoxicity equivalent to free CUR.

**MNP-CUR decreases tumor growth and improves survival of tumor-bearing mice**

*In vivo* therapeutic efficacy of free CUR and the MNP-CUR formulation was examined in the HPAF-II tumor xenograft model following intratumoral administration. After cancer cells postinjection, on day 28, MNP-CUR treatment significantly inhibited tumor growth by 71.2% compared to empty MNP-treated controls, whereas the treatment with CUR inhibited tumor growth by 35.9% (Fig. 2A). Tumor size steadily increased in Tween 20 or MNP groups. A significant difference in tumor volume was observed for MNP versus MNP-CUR (P = 0.02), but not for Tween 20 versus CUR (P = 0.30). Higher tumor growth inhibition observed with MNP-CUR formulation may be attributed to long-term sustained release of CUR from MNP-CUR formulation within tumors. It was evident from the uptake experiment using flow cytometer that the MNP-CUR formulation can provide higher concentrations of CUR within cells compared to free CUR (Supplementary Fig. S1B). The flow cytometer measurements are based on the inherent fluorescence signals from the CUR in solution or CUR in nanoparticles. To prove that MNP-CUR remains in tumors after intratumoral injection and protects CUR’s biological activity, we examined the accumulation of MNP-CUR formulation on the histologic sections of tumor tissues by Prussian blue staining. Distinct Prussian blue stains were found throughout the region of the tumor(s) [Fig. 2B(a–e)]. Most of the tissues showed a similar pattern of Prussian blue staining but a few tumor slides revealed only peripheral stains [Fig. 2B(f)]. The observed patterns of nanoparticle existence in tumors are in accordance with a previous study (25). The Kaplan–Meier curves of mice treated with Tween 20, MNPs, CUR, and MNP-CUR. Data represents at least 6 mice/group (mean ± SEM). *P < 0.05.

**MNP-CUR formulation effectively modulates key oncogenic molecular targets**

A markedly decreased (~70%) staining of Bcl-xL and Mcl-1 was observed in both CUR- and MNP-CUR–treated animals compared to vehicle control groups (Fig. 3A). Interestingly, MNP-CUR–treated animals showed relatively less PCNA staining (~75%) compared to free CUR (~55%). In addition, we investigated the expression of a dual-function protein, β-catenin (a modulator of cell–cell adhesion and wnt signaling), in these tumor tissues. The deregulated expression or function of this protein results in decreased cell–cell adhesion due to loss of surface β-catenin and in enhanced cell proliferation due to the nuclear function of β-catenin as a cotranscription factor (16, 28). A distinct increase in membrane β-catenin was
detected in MNP-CUR–treated tumors (~80%) compared to free CUR–treated tumors (~60%) and control animals (Fig. 3A). MUC1 is a transmembrane O-glycosylated protein that is overexpressed in pancreatic cancer (29) and modulates various signaling pathways (including β-catenin), resulting in the overexpression of tumorigenic factors and formation of tumors. A striking downregulation (~75–80%) of MUC1 protein expression was observed upon CUR or MNP-CUR treatment (Fig. 3A). These results were also observed in vitro in HPAF-II cancer cell line models (Fig. 3B). To elucidate the role of CUR and MNP-CUR on tumor tissue fibrosis (desmoplasia), tumor tissues were double stained with anti-human collagen-I (green) and anti-mouse collagen-I (red). There was a marked reduction in both the human and mouse collagen levels in the tumors of mice treated with MNP-CUR (Fig. 4A), which implicated a decrease in host–tumor interactions. In vitro collagen production also significantly dropped to 24% to 25% upon treatment with MNP-CUR (Fig. 4B). CUR treatment did not efficiently regulate collagen production, that is, collagen production was ~71% to 84%. All together, these data indicate an enhanced efficacy of MNP-CUR treatment at the molecular level compared to control and free CUR.

**MNP-CUR increases bioavailability and tumor targeting**

Up to a 2.5-fold increase in bioavailability of CUR in blood plasma was observed with the MNP-CUR formulation as compared to free CUR (Fig. 5A). A considerable amount of CUR in MNP-CUR (1792.19 ± 644 ng) was observed in 1 mL serum at 6 hours whereas only...
766.54 ± 256 ng of CUR in Tween 20 was present. This increased serum bioavailability implies greater stability and prolonged sustained blood circulation of MNP-CUR under physiological conditions (30, 31), showing that MNPs possess strong properties as effective drug carriers.

To investigate key differences in biodistribution patterns of the MNP-CUR formulation injected intratumorally and i.p., the presence of MNPs was examined in tumor, liver, spleen, and brain during the 24 hours after administration (Fig. 5B). Other organs, including pancreas, exhibited very minimal or undetectable levels of MNPs (data not shown). Intratumoral administration obviously resulted in high concentrations of MNP-CUR in tumor (1.04 ± 0.48 mg Fe/g tissue), followed by liver (0.39 ± 0.17 mg Fe/g tissue), spleen (0.059 ± 0.2 mg Fe/g tissue), and brain (0.14 ± 0.02 mg Fe/g tissue). Interestingly, fairly high accumulation of MNP-CUR in tumor (0.48 ± 0.29 mg Fe/g tissue) as well as in spleen (0.78 ± 0.29 mg Fe/g tissue) was also achieved with i.p. administration. This observation was further confirmed by Prussian blue staining of tumor tissues. This data also indicate that MNP-CUR formulation is capable of targeting pancreatic tumors fairly well with the help of the "enhanced permeation and retention" (EPR) effect even with systemic (i.p.) administration (Fig. 5C).

MNP-CUR interaction with plasma proteins and RBCs

SDS-PAGE revealed the following order of quantity of bound plasma proteins on MNP-CUR: Transferrin > HSA > fibrinogen > IgG (Supplementary Fig. S2). Relative protein binding quantification by densitometry showed the protein affinity toward nanoparticles (Fig. 6A). This order variation occurred due to differences in the

![Figure 5](image-url)

**Figure 5.** Serum bioavailability and biodistribution of MNP-CUR formulation. A, comparative serum bioavailability of CUR and MNP-CUR formulations in a tumor-bearing mouse model. Enlarged 5, 10, 15, 30, 60, and 120 minutes bioavailability data area shown as inset. CUR and MNP-CUR (50 μg per mice) were administered via intraperitoneal injection and blood samples from tail bleed were collected at different time points. CUR levels in serum were determined using a Dionex HPLC instrument equipped with an Acclaim Polar Advantage II C18 column (3 μm 120 Å 4.6 × 150 mm) with the RS variable wavelength detector at 420 nm. B, comparative biodistribution in tumor, liver, spleen, and brain tissues after intratumoral and intraperitoneal administration of MNP-CUR formulation. Mice were treated with MNP-CUR particles (20 μg per mice) and iron content of particles in tissue was estimated using 1,10-phenanthroline colorimetric method. C, visual evidence of nanoparticles in tumor tissue was detected with Prussian blue staining method. Images were captured using an Olympus 1 x 171 microscope equipped with a DP71 camera (Olympus).

![Figure 6](image-url)

**Figure 6.** Hemocompatibility of MNP-CUR formulation. A, serum proteins adsorption of MNP-CUR formulation. MNP-CUR formulation (1 mg) was incubated with 100 μg plasma proteins (fibrinogen, IgG, transferrin, and HSA) for 2 hours, centrifuged, and bound proteins on NPs (palette) were estimated with the help of SDS-PAGE and Coomassie G-250 staining (Supplementary Fig. S2). Relative adsorbed protein density was quantified by densitometry using AlphaEase Fc software and data are presented as the mean of 3 repeats for each protein (mean ± SEM). *, P < 0.05 compared with fibrinogen and IgG. B, morphological observation of red blood cells (RBCs) upon treatment with MNP-CUR formulation. Control RBCs and RBCs treated with dendrimer formulation were considered to be negative and positive controls for hemolysis, respectively. TEM images of RBCs reveal bound/entrapped MNP-CUR nanoparticles (black circle in MNP-CUR) and secreted proteins and membrane damage (black arrows in dendrimer-treated group).
formation of protein corona on nanoparticles that resulted from nanoparticle–protein complex interplay factors (32). A similar protein binding order had been noticed in CUR assemblies (33). Human serum proteins have prevalent interactions with nanoparticles but higher aggregation of those complexes lead to opsonization; however, this was not observed with the MNP-CUR formulation.

Furthermore, we compared the hemocompatibility of MNP-CUR formulation with human RBCs (100 μmol/L CUR equivalent MNP-CUR formulation) to negative control (no treatment) and positive control (dendrimer formulation) groups. RBCs treated with MNP-CUR retained their morphology similar to negative control RBCs (Fig. 6B). This behavior is achieved due to the pluronic layers (bound polyethylene glycol chains) in the formulation. However, some nonspecific binding of MNP-CUR with RBCs was also observed (Fig. 6B, black circle), which is due to entrapment of nanoparticles in the cell pellet. In contrast, the dendrimer formulation caused secretion of hemoglobin and membrane proteins due to compromised RBC membranes and hemolytic activity (Fig. 6B, black arrows).

**Discussion**

Our systematic approach for CUR encapsulation in MNPs (MNP-CUR formulation) showed minimized uptake in RAW 247.1 cells (macrophages) which can prevent rapid clearance while enhancing synchronized internalization in cancer cells (19) including pancreatic cancer cells (Fig. 1B). The MNP formulation is also able to encapsulate other clinically relevant drugs such as doxorubicin and gemcitabine without significantly altering their structure. Effective internalization of MNPs in cancer cells is an important characteristic for drug delivery. A layer of bioadhesive material, Pluronic F127, on the MNPs greatly improved their cellular uptake and reduced particle aggregation (34). Sustained release of CUR from MNPs will not only improve anticancer efficacy but also help prevent relapse and drug resistance (35). Herein, we report for the first time, the antitumor efficacy of an MNP-CUR formulation in pancreatic cancer treatment using *in vitro* (Fig. 1C and D) and *in vivo* (Fig. 2A–C) models. Such equivalent biological activities against various cancer cells were observed for drugs in solution and MNP drug formulations (26, 36). As a drug carrier, MNPs can effectively increase the stability of drugs, protect them from degra-

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affects fundamental cellular processes that are essential for tumor progression, such as cell survival, apoptosis, and cellular invasion (44, 45). This data indicate that MNP-CUR efficiently inhibited the collagen I levels of both human and mouse origins within tumors (Fig. 4). This supports the finding that MNP-CUR induced a decrease in host–tumor interactions, thus may be involve in preventing pancreatic fibrosis.

The prolonged circulation time and an EPR effect of nanoformulations are general strategies used to target tumor(s). The MNP-CUR formulation showed a 2.5-fold increase in CUR concentration in serum (Fig. 5A). The preferential tumor accumulation of MNP-CUR (Fig. 5B and C) is anticipated due to leaky vasculature and lack of a lymphatic system in tumors. It is possible to further improve the accumulation of the MNP-CUR formulation by application of an external magnetic field gradient (46). In addition, in this study we observed an appropriate binding of transferrin and HSA proteins to the MNP-CUR nanoparticles to form a “hard corona” (Fig. 6A) which defines the long-lived equilibrium state of novel MNP-CUR nanoparticles (47). In general, enhanced adsorption of plasma proteins onto the surface of nanoparticles would lead to opsonization due to aggregation. However, we did not observe any aggregation property of MNP-CUR nanoparticles and hemotoxicity, indicating a long-term circulation of particles in the blood (Fig. 6A). Our particles gained this specific property due to the poly (propylene oxide) chain anchoring on the MNP-CUR particles gained this specific property due to the poly (propylene oxide) chain anchoring on the MNP-CUR formulation that minimizes the shear forces when nanoparticles are exposed to biological fluids. This observation was further confirmed by hemocompatibility of our formulation by evaluating RBC’s ultrastructural morpholology which suggested no signs of interaction of MNP-CUR with blood components and hemolysis (Fig. 6B).

Active and specific targeting of drug delivery systems has now become an interesting concept in cancer therapeatic research. The advantage of this MNP formulation is that CUR or other anticancer drug(s) can be loaded into a novel anti-MUC13 monoclonal antibody. Most other CUR nanoformulations either inefficiently load higher amounts of CUR or lack targeting chemistry. Our recent study has shown an overexpression of MUC13 in pancreatic cancer (21). A MUC13-targeted nanoformulation may lead to specific targeting of pancreatic cancer and possibly enhance further tumor uptake, thereby improving the efficacy of chemotherapeutic drugs while preserving imaging properties for simultaneous real-time monitoring of the disease condition.

Conclusions

Our data show that our MNP-CUR formulation can be efficiently internalized in human pancreatic cancer cells and induce potent anticancer effects. The in vitro tumor growth inhibition and improved survival rate show that the MNP-CUR formulation has superior anticancer activity compared to free CUR. In addition, the MNP-CUR formulation has exhibited an enhanced serum bioavailability of CUR along with appreciable tumor uptake and hemocompatibility. In conclusion, results presented in this study suggest that MNP-CUR is an excellent prospect for effective pancreatic cancer treatment/management because of enhanced bioavailability of CUR and its multifocal therapeutic effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M.M. Yallapu, S.C. Chauhan
Development of methodology: M.M. Yallapu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.M. Yallapu, M.C. Ebeling, S. Khan, V. Sundram, N. Chauhan, M. Jaggi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.M. Yallapu, M.C. Ebeling, V. Sundram, S.E. Puumala, M. Jaggi, S.C. Chauhan
Writing, review, and/or revision of the manuscript: M.M. Yallapu, M.C. Ebeling, S. Khan, V. Sundram, S.E. Puumala, M. Jaggi, S.C. Chauhan
Study supervision: S.C. Chauhan
Other: Performed mouse experiments with curcumin-loaded nanoparticles and sacrificed mouse and collected organs to study biodistribution of curcumin-loaded nanoparticles, B.K. Gupta

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