Systemic Administration of Polymeric Nanoparticle-Encapsulated Curcumin (NanoCurc) Blocks Tumor Growth and Metastases in Preclinical Models of Pancreatic Cancer

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Abstract

Curcumin or diferuloylmethane is a yellow polyphenol extracted from the rhizome of turmeric (*Curcuma longa*). A large volume (several hundreds) of published reports has established the anticancer and chemopreventative properties of curcumin in preclinical models of every known major cancer type. Nevertheless, the clinical translation of curcumin has been significantly hampered due to its poor systemic bioavailability, which mandates that patients consume up to 8 to 10 g of the free drug orally each day to achieve detectable levels in circulation. We have engineered a polymeric nanoparticle encapsulated curcumin formulation (NanoCurc) that shows remarkably higher systemic bioavailability in plasma and tissues compared with free curcumin upon parenteral administration. In xenograft models of human pancreatic cancer established in athymic mice, administration of parenteral NanoCurc significantly inhibits primary tumor growth in both subcutaneous and orthotopic settings. The combination of parenteral NanoCurc with gemcitabine results in enhanced tumor growth inhibition versus either single agent, suggesting an additive therapeutic influence *in vivo*. Furthermore, this combination completely abrogates systemic metastases in orthotopic pancreatic cancer xenograft models. Tumor growth inhibition is accompanied by significant reduction in activation of nuclear factor-κB, as well as significant reduction in expression of matrix metalloproteinase-9 and cyclin D1, in xenografts treated with NanoCurc and gemcitabine. NanoCurc is a promising new formulation that is able to overcome a major impediment for the clinical translation of curcumin to cancer patients by improving systemic bioavailability, and by extension, therapeutic efficacy. *Mol Cancer Ther; 9(8); 2255–64. ©2010 AACR.*

Introduction

Curcumin or diferuloylmethane is a yellow polyphenol extracted from the rhizome of turmeric (*Curcuma longa*), a plant grown in tropical Southeast Asia (1). Enthusiasm for curcumin as an anticancer agent evolved based on the wealth of epidemiologic evidence suggesting a correlation between dietary turmeric and low incidence of gastrointestinal mucosal cancers in Southeast Asian populations (2). A large volume of experimental data has established the therapeutic efficacy of curcumin in preclinical models (principally, in cell lines) derived from a variety of solid tumors like pancreatic, colorectal, lung, breast, prostate, and hepatocellular carcinoma, among others (selected reviews include refs. 3–5). Equally important, free curcumin was shown not to be cytotoxic to normal cells, including hepatocytes, mammary epithelial cells, kidney epithelial cells, lymphocytes, and fibroblasts, at the dosages required for therapeutic efficacy against cancer cell lines; these *in vitro* findings are underscored by the limited human clinical trials done with oral curcumin, wherein doses up to 8 g per day have had minimal adverse effects, even to the highly exposed gastrointestinal mucosa (6, 7). In addition to a possible role in the therapy of established tumors, studies in numerous experimental (chemical) carcinogenesis models have confirmed that curcumin can ameliorate the progression to cancer in a variety of organ sites, reiterating its potential for chemoprevention (8, 9).

Despite these encouraging results, the promise of curcumin in the clinic has never been fully realized. The single most important reason for this "benchside to bedside" disconnect has been the poor bioavailability of curcumin, such that its therapeutic effects are essentially limited to the tubular lower gastrointestinal tract

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-10-0172

(i.e., colorectum; refs. 10, 11). In a phase I clinical trial, patients with hepatic colorectal cancer metastases were administered 3,600 mg of oral curcumin daily, and curcumin and its glucoronide and sulfate conjugates were detected in low nanomolar concentrations in the peripheral blood or portal circulation (12). In another phase I study, patients were required to partake 8,000 mg of free curcumin orally per day to achieve detectable systemic levels; beyond this dose, tolerability of the formulation was unacceptable to patients (13). In the few curcumin clinical trials that are currently active for visceral cancers (http://www.clinicaltrials.gov), patients have to partake as much as 8 to 10 g of oral curcumin per day. There is little doubt that despite the absence of dose-limiting toxicity, such high doses severely affect patient compliance due to a metallic aftertaste and associated gastrointestinal discomfort. In view of these issues, there has been a considerable interest in developing formulations that allow for improved systemic bioavailability. We envisioned that nanoparticle-mediated drug delivery could be useful for harnessing the full potential of curcumin in the clinical arena.

In our previous proof-of-principle report, we showed the comparable efficacy of curcumin loaded within polymeric nanoparticles (NanoCurc) to that of free curcumin *in vitro*, thereby confirming that the biological activities of curcumin are retained upon nanoencapsulation (14). Here, we have assessed the bioavailability, toxicity, and *in vivo* therapeutic efficacy of parenteral NanoCurc, either as a single agent or upon combination with the antimetabolite gemcitabine, in xenograft models of pancreatic cancer. We selected pancreatic cancer as our disease model in light of the uniformly dismal prognosis of this malignancy and the dire need for developing more effective therapies, particularly for combating systemic metastases that are present in the overwhelming majority of patients (15). With a median survival of ~5 to 6 months for most individuals with advanced pancreatic cancer, current chemotherapeutic modalities (including gemcitabine, the standard-of-care agent) have had minimal success in ameliorating the poor survival outcomes for this disease. Our results confirm that parenteral NanoCurc significantly reduces primary tumor growth, as well as systemic metastases, and potentiates the effects of gemcitabine in both subcutaneous and orthotopic xenograft models. Further, we show that the potent *in vivo* effects of NanoCurc are observed at dosages that are ~20-fold lower than that previously published with free curcumin for antitumor efficacy in pancreatic cancer xenograft models (1 g/kg per day, albeit administered through the oral route; ref. 16), underscoring the translational relevance of this novel nanoformulation for cancer therapy.

Materials and Methods

Materials

Ultrapure curcumin (>99% diferuloylmethane) was purchased from Sabinsa Corporation; this source of curcumin had been used for both preclinical and clinical studies in the past (7, 12). Monomers for polymer nanoparticle synthesis, specifically *N*-isopropylacrylamide (NIPAAM), vinylpyrrolidone (VP), and acrylic acid (AA), were obtained from Sigma-Aldrich. Reagents for the polymerization step, including *N*,*N*′ methylene-bis-acrylamide, ammonium persulfate, and ferrous sulfate were also procured from Sigma. Gemcitabine (NetQem LLC) was stored at 4°C and dissolved in sterile NaCl (0.9% w/v) on the day of use. Reagents used for Western blot and immunohistochemistry were obtained from Invitrogen. Polyclonal antibodies against the p65 subunit of nuclear factor-κB (NF-κB), cyclin-D1, and matrix metalloproteinase-9 (MMP-9) were obtained from Cell Signaling. Anti–β-actin antibody was from Santa Cruz Biotechnology. *In vivo* xenograft studies were conducted using the low-passage metastatic human pancreatic cancer cell line, Pa03C (also known as LZ10.7; ref. 17). This cell line was part of the pancreatic cancer genome-sequencing effort, and harbors somatic mutations of both *TP53* and *KRAS2* (18).

Synthesis of NanoCurc

Polymer nanoparticles composed of NIPAAM, VP, and AA were synthesized through a free radical reaction, according to the detailed synthesis method we previously described (14, 19). The predistilled monomers of NIPAAM, VP, and AA are mixed together in a molar ratio of 60:20:20, respectively, hence the acronym "NVA622" for the resulting polymer. Polymerization was done for 24 hours at 30°C under an inert (nitrogen) atmosphere, using ammonium persulfate and ferrous sulfate as initiator and activator, respectively. After complete polymerization, the total aqueous solution of polymer was purified using dialysis, and then lyophilized for postloading of curcumin, as described (14). Typically, a 10 mL stock solution of polymeric nanoparticles (100 mg) was slowly mixed with 150 μL of curcumin solution in chloroform (10 mg/mL), and gently stirred for 15 to 20 minutes on low heating, to load curcumin and evaporate chloroform simultaneously. The resulting solution, corresponding to 1.5% (w/w) loading of curcumin in nanoparticles, was then snap frozen on a dry ice/acetone bath and lyophilized. The lyophilized NanoCurc powder is stored at 4°C until further use, whereupon simple reconstitution in an aqueous phase is required before parenteral administration.

Pharmacokinetic analyses of parenteral NanoCurc compared with free curcumin

To compare the *in vivo* pharmacokinetics of NanoCurc versus free curcumin suspended in corn oil, two cohorts of four mice each were administered a single intraperitoneal (i.p.) injection of either formulation, at an equivalent curcumin dose of 25 mg/kg. Pharmacokinetics data were analyzed by noncompartmental methods (WinNonlin Professional, version 5.2 software, Pharsight Corporation). Additional methodological details are available in Supplementary Data.

Establishment and treatment of Pa03C subcutaneous xenografts

All small animal (mouse) experiments described here conformed to the guidelines of the Animal Care and Use Committee of Johns Hopkins University. Mice were maintained in accordance to the guidelines of the American Association of Laboratory Animal Care. To generate subcutaneous Pa03C xenografts, flanks of 5- to 6-week-old male athymic *nu/nu* mice (Harlan Laboratories) were injected with 2.5×10^6 Pa03C cells suspended in a total volume of 200 μL [PBS/Matrigel (BD Biosciences), 1:1 (v/v), prechilled to 4°C]. One week after the injection of tumor cells, subcutaneous tumor volumes (V) were measured with digital calipers (Fisher Scientific) and calculated using the formula $V = 1/2(ab^2)$, where a is the biggest and b is the smallest orthogonal tumor diameter (17, 20). Twenty mice with successfully engrafted Pa03C xenografts were then randomized into four cohorts of five animals each and administered one the following regimens (Supplementary Fig. S1A): (*a*) void NVA622 polymeric nanoparticles, (*b*) single-agent NanoCurc at a dose of 25 mg/kg i.p. twice daily, (*c*) single-agent gemcitabine at a dose of 20 mg/kg i.p. twice weekly, or (*d*) the combination of NanoCurc (25 mg/kg i.p. twice daily) and gemcitabine (20 mg/kg i.p. twice weekly). The daily dose of NanoCurc was selected based on maximal tolerated volume of i.p. injection over a 24-hour period in mice. Gemcitabine was administered for a cycle of 2 weeks (i.e., total of four doses of the drug), with 1 additional week of NanoCurc in both single-agent and combination arms. Tumor size and body weight were measured once weekly. At the culmination of treatment, visceral organs and tumor tissues were harvested and either preserved in 10% neutral buffered formalin for histology and immunohistochemical studies or snap frozen for pharmacokinetics and pharmacodynamic analyses (see Supplementary Methods). Intratumoral curcumin concentrations were estimated by liquid chromatography-tandem mass spectrometry (LC-MS/MS), as previously described (21).

Establishment and treatment of Pa03C orthotopic xenografts

The generation of orthotopic Pa03C human pancreatic cancer xenografts by surgical implantation in athymic mice has been described previously by our group (17, 20). Briefly, subcutaneous xenograft tumors (Pa03C) were harvested under sterile conditions and minced into 1 mm^3 cubes for orthotopic implantation. A small pocket was prepared inside the pancreas, into which one of the previously prepared fresh tumor chunks was inserted. Three weeks after surgical orthotopic implantation, the presence of "primary" tumors was confirmed by ultrasound scan (Vevo660, VisualSonics) and measured in three orthogonal axes, *a*, *b*, and *c*; tumor volumes were determined as $V = (abc)/2$, as described (17, 20). Twenty-eight mice with demonstrable primary xenografts were then randomized into four cohorts, with seven mice per arm, as follows (Supplementary Fig. S1B): (*a*) void NVA622 polymeric nanoparticles, (*b*) single-agent NanoCurc at a dose of 25 mg/kg i.p. twice daily, (*c*) single-agent gemcitabine at a dose of 20 mg/kg i.p. twice weekly, or (*d*) combination of NanoCurc (25 mg/kg i.p. twice daily) and gemcitabine (20 mg/kg i.p. twice weekly). Both NanoCurc and gemcitabine were administered for a period of 3 weeks.

At the culmination of therapy, the mice were euthanized; spleen, liver, kidneys, intestine, peritoneum, and lungs were inspected for grossly visible metastases. In addition to macroscopic inspection, visceral organs (including any enlarged lymph nodes) and tumor tissues were harvested and preserved in 10% neutral buffered formalin for histology and immunohistochemical studies, including a rigorous effort to detect any organ-specific micrometastases. Additionally, tumor tissues were snap frozen for pharmacokinetics and pharmacodynamic analyses (see Supplementary Methods). Intratumoral curcumin concentrations were estimated by LC-MS/MS (21).

Results

NanoCurc readily overcomes the bioavailability pitfall of free curcumin *in vivo*

To improve the systemic bioavailability of free curcumin, the free compound was encapsulated in NVA622 polymeric nanoparticles (henceforth referred to as NanoCurc). We compared the bioavailability of NanoCurc to free curcumin dissolved in corn oil, following administration of a single dose of either formulation (equivalent to 25 mg/kg of curcumin) through the i.p. route in non–tumor-bearing mice (Fig. 1A). Relevant pharmacokinetic parameters, including C_{max}, T_{max}, and area under the curve (AUC), were calculated and compared for both formulations (see Supplementary Methods). As evident from the figure, marked differences in the bioavailability of curcumin were observed. NanoCurc showed sustained plasma concentrations of curcumin with a T_{max} of 2.75 ± 1.50 hours and C_{max} of 17,176 ± 5,176 ng/mL, whereas mean plasma levels of free curcumin in corn oil were barely detectable above the LC-MS/MS limit of quantitation. A large difference in the AUC values were also observed, with NanoCurc having an AUC of 153,865 ± 30,445 ng h/mL ($n = 4$) and free curcumin in corn oil having an AUC of 445 ng h/mL ($n = 1$; in the remaining three mice, curcumin concentrations were below the detection limit). In addition to this single dose study, we also determined steady-state levels of curcumin in plasma, as well as in tissue samples, following the administration of parenteral NanoCurc (25 mg/kg equivalent curcumin, twice daily i.p.) to non–tumor-bearing mice for 4 weeks (Fig. 1B). Curcumin was readily detectable in all visceral tissues, as well as in plasma at the end of 4 weeks of therapy. Body weights were also monitored on a weekly basis to observe for any signs of systemic toxicity (Fig. 1C). No evidence of loss in body weight, or behavioral abnormalities, was found during the course of treatment. Histologic evaluation of major visceral organs (kidneys, pancreas, lungs, liver, spleen, and intestines) did not

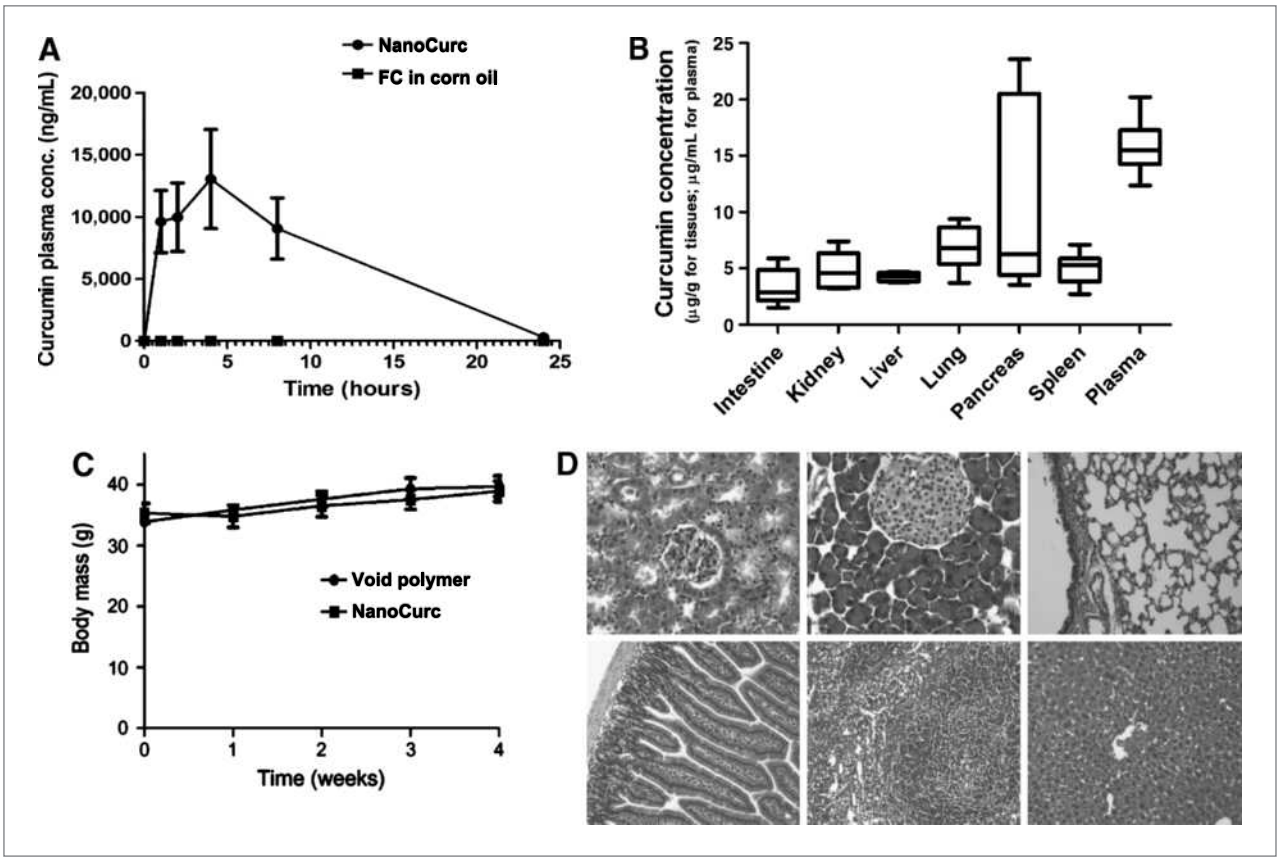

Figure 1. *In vivo* pharmacokinetics, biodistribution, and toxicity analysis of NanoCurc. A, comparative pharmacokinetics of parenteral NanoCurc versus free curcumin in corn oil, administered in non–tumor-bearing mice. Plasma concentrations of curcumin (ng/mL) were determined at 1, 2, 4, 8, and 24 hours after single i.p. administration of either formulation, at an equivalent dose of 25 mg/kg curcumin. See text for analytical details. B, tissue and plasma levels of curcumin assessed following necropsy in non–tumor-bearing mice receiving 4 weeks of parenteral NanoCurc. Curcumin levels were measured by LC-MS/MS and are expressed as μg/g for tissues and in μg/mL for plasma. Horizontal line indicates mean of levels measured in three mice. The anatomic site is indicated on the *X* axis. C, no significant alterations in body mass (g) are observed in cohorts of non–tumor-bearing mice ($n = 3$) receiving either NanoCurc or void NVA622 polymer for 4 weeks. D, histopathologic assessment of visceral tissues (clockwise from top left: kidney, pancreas, lung, liver, spleen, and intestine) obtained at necropsy from mice receiving 4 weeks of NanoCurc showing no abnormalities.

show any overt microscopic abnormalities (Fig. 1D). These pilot experiments confirmed the enhanced systemic bioavailability of NanoCurc compared with free curcumin, as well as the absence of overt toxicity in mice, allowing us to proceed to *in vivo* therapeutic efficacy trials in xenograft models of pancreatic cancer.

NanoCurc inhibits the growth of subcutaneous Pa03C xenografts and potentiates the effects of gemcitabine in this setting

The *in vivo* therapeutic efficacy of NanoCurc on tumor growth was assessed either alone or in combination with gemcitabine in subcutaneous Pa03C xenografts. When compared with animals treated with void NVA622 polymer, there was significant tumor growth inhibition in mice receiving single-agent NanoCurc (~50% reduction in mean tumor volume, $P < 0.01$; Fig. 2A and B). Single-agent gemcitabine was very effective in inhibiting Pa03C tumor growth in the subcutaneous milieu, and superfluously, there was no significant difference in mean tumor volumes between this cohort versus mice receiving combination therapy with NanoCurc and gemcitabine. However, a readout of "mean tumor volume" per se greatly underestimated the effects of combination therapy, as four of five xenografts underwent complete histologic regression in this cohort (see the inset with the magnified *Y* axis in Fig. 2B, highlighting the onset of tumor regression in the combination therapy cohort during week 3). Thus, only a single residual xenograft "nubbin" was observed at the culmination of therapy in the combination group, illustrated in the photomicrograph panel on the right (Fig. 2A). Plasma curcumin measured using samples obtained at necropsy confirmed comparable levels of drug between single-agent and combination arms (Supplementary Fig. S2).

NanoCurc inhibits the growth of Pa03C orthotopic xenografts and abrogates systemic metastases upon combination with gemcitabine

Although subcutaneous pancreatic cancer xenografts provide preliminary insights into therapeutic efficacy,

they do not recapitulate one of the most critical features of the cognate human disease, namely systemic metastases (17, 20). Moreover, the subcutaneous milieu may not accurately reflect the microenvironment of the pancreas nor simulate the drug distribution kinetics observed in the specific organ (likely accounting for the observed efficacy of even single-agent gemcitabine in this setting; see above; ref. 22). In this respect, intrapancreatic orthotopic xenografts have been considered to be more biologically relevant (23, 24). Therefore, we investigated the efficacy of NanoCurc as a single agent, or in combination with gemcitabine, in orthotopically implanted Pa03C pancreatic cancer xenografts. Compared with void NVA622 polymer–treated mice, there was significant reduction (**, $P < 0.01$) in growth of the primary tumor in mice receiving either NanoCurc or gemcitabine alone (Fig. 3A

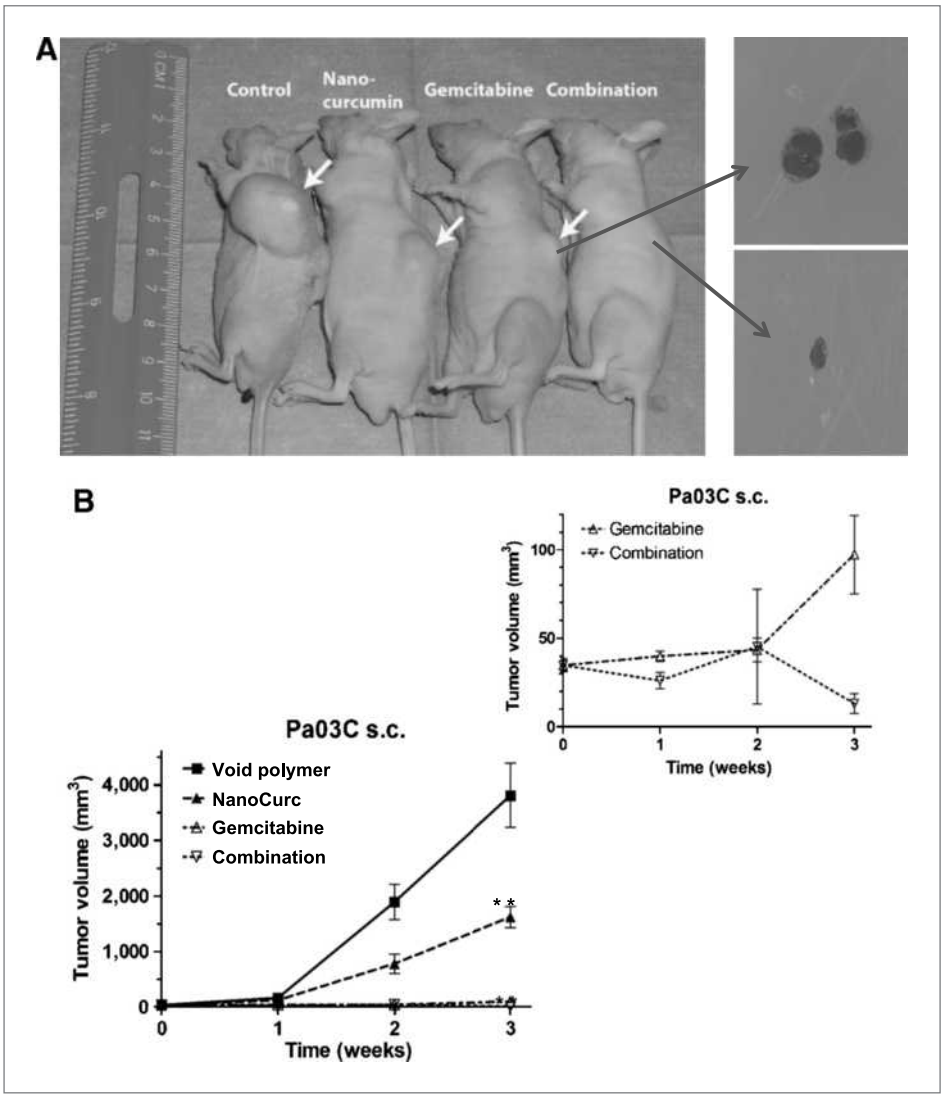

Figure 2. Parenteral NanoCurc significantly inhibits the growth of subcutaneous (s.c.) pancreatic cancer xenografts, and therapeutic efficacy is further potentiated by the combination with gemcitabine. A, subcutaneous xenografts were established using the low-passage Pa03C (LZ10.7) human pancreatic cancer cell line, and mice were randomized to four arms, including control (void NVA622 polymer), NanoCurc, gemcitabine, and the combination of NanoCurc and gemcitabine. Treatment was culminated at 3 weeks. Representative xenografted mice from each of the four arms are illustrated. Whereas single-agent NanoCurc significantly blocked tumor growth compared with void NVA622 arm (see B), the results were even more striking upon administration with gemcitabine, wherein four of five xenografts showed complete regression in the combination arm. Photomicrographs to the right depict a representative xenograft from the gemcitabine arm and the single residual nubbin from the combination therapy group. B, graphical depiction of the tumor volumes in the four arms, over the 3-week time course of therapy. Single-agent NanoCurc shows significant reduction in tumor volume compared with void NVA622 (control) arm at 3 weeks (**, $P < 0.01$). No significant difference is observed in the tumor volumes between single-agent gemcitabine and combination arms; however, these data underestimate the effect of combination therapy, as four of the five xenografts had undergone complete histologic regression (see A) and only a residual nubbin of tumor from a single xenograft was available for measurement. The inset shows the comparative tumor volume data for gemcitabine and combination arms using a magnified *Y* axis, which illustrates the clear separation in growth curves between the two arms during the 3rd week.

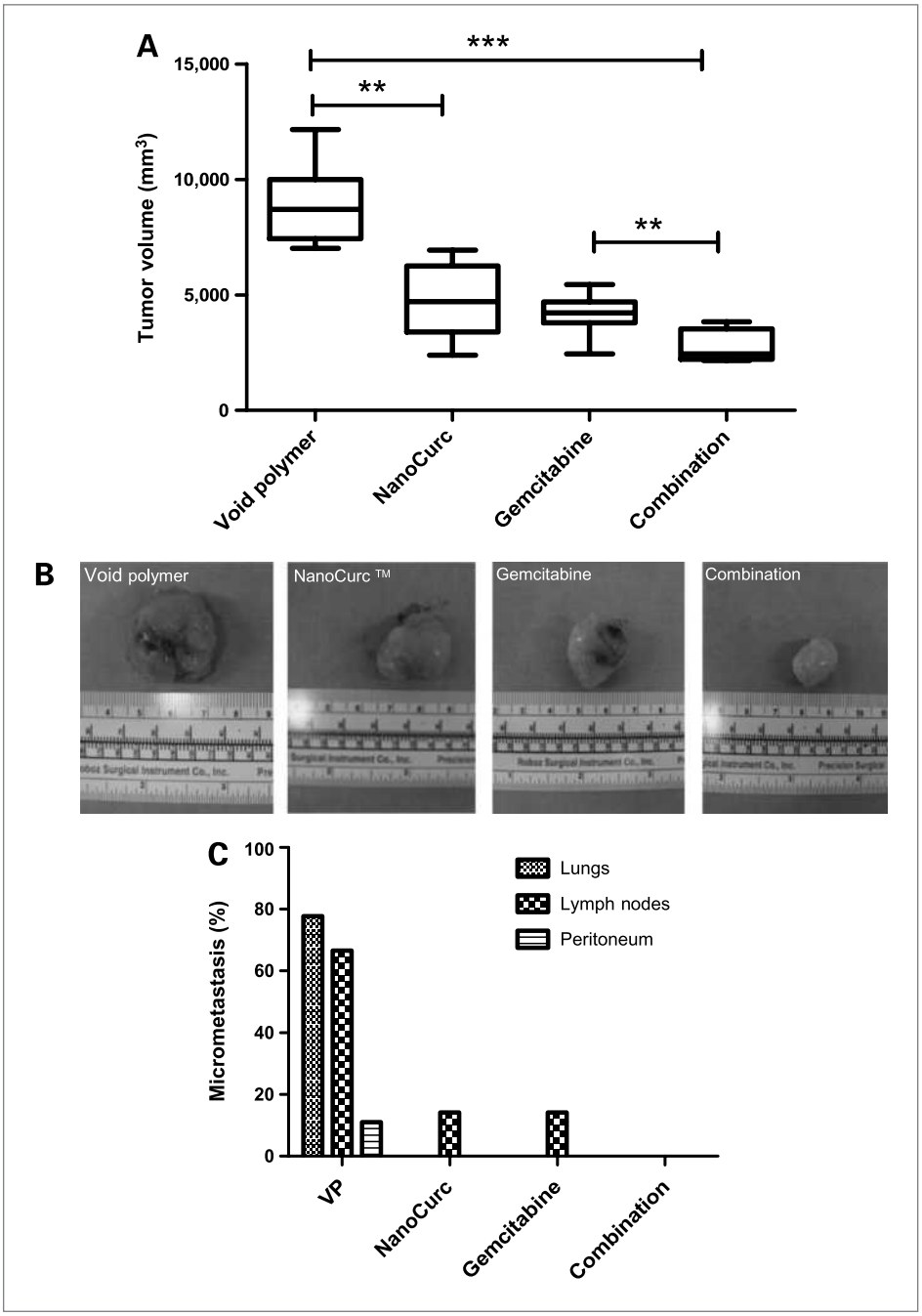

Figure 3. Parenteral NanoCurc significantly inhibits the growth of orthotopic pancreatic cancer xenografts, including the abrogation of systemic metastases upon combination with gemcitabine. A, graphical illustration of tumor volumes for orthotopic Pa03C pancreatic cancer xenografts treated with NanoCurc, gemcitabine, or the combination, compared with void NVA622 polymer. Single-agent NanoCurc leads to significant inhibition of the primary tumor volume compared with void polymer (**), and the effect is further accentuated upon addition of gemcitabine (***). Note that the combination also has a more significant effect on tumor volume compared with single-agent gemcitabine (**). All treatments were carried out for a period of 3 weeks; horizontal lines represent the average of measurements in seven mice per arm. B, representative excised Pa03C xenografts from the control (far left) and three treatment arms (NanoCurc, gemcitabine, and combination, respectively) are designated. C, control mice receiving void polymer (VP) show extensive micrometastases to the lungs, lymph nodes, and peritoneum (also see Supplementary Fig. S3). Both single-agent therapy arms show considerable reduction in micrometastatic disease (albeit still present in the lymph nodes), whereas the combination arm shows complete abrogation of micrometastatic disease in all examined viscera.

and B). The effects of the two single agents were comparable, and no significant difference was noted. In contrast, there was further significant accentuation of growth inhibition when the two agents were combined (**, $P < 0.01$ compared with either single agent; ***, $P < 0.001$ compared with control arm), confirming the prior observations that curcumin can have an additive effect with conventional chemotherapeutics *in vivo* (5, 25–28), including potentiating the effects of gemcitabine against pancreatic cancer cells (16).

Apart from significantly inhibiting primary tumor growth, NanoCurc also showed profound effects on metastatic tumor spread (Fig. 3C). In the control arm, five of seven (71%) mice exhibited histologically confirmed metastases to the lungs and regional lymph nodes (Supplementary Fig. S3), with one of seven (14%) mice also demonstrating peritoneal metastases. Both NanoCurc and gemcitabine administered as single agents led to striking and comparable reduction in metastases, with only lymph node metastases seen in one of seven (14%)

mice, and absence of lung or peritoneal disease. In the combination group, complete abrogation of systemic metastases was observed in all microscopically examined viscera. Of note, whereas our results mirror those previously observed by combining free curcumin with gemcitabine in preclinical models (16), the reduction in primary tumor growth and abrogation of systemic metastases was observed at a NanoCurc dose that was ~20-fold lower than used previously with free curcumin (1 g/kg per day, albeit through the oral route).

NanoCurc downregulates activation of NF-κB in subcutaneous and orthotopic pancreatic cancer xenograft models

NF-κB is a transcription factor that activates cell survival pathways in cancer cells and renders them resistant to conventional cytotoxic agents (29, 30). NF-κB is constitutively active in human pancreatic cancer (31). Prior studies have established that free curcumin is a potent inhibitor of NF-κB activity in cancer cells (25, 26, 32, 33), which was also confirmed previously by our group *in vitro* in pancreatic cancer cell lines using nanoparticulated curcumin (14). We now show that systemic NanoCurc also blocks NF-κB activation *in vivo*, in both subcutaneous and orthotopic settings. Two different assay methods were used to enhance the stringency of our observations. In subcutaneous Pa03C xenografts, NF-κB activity was assessed by quantitative immunohistochemistry for nuclear localization of p65, the principal subunit protein of NF-κB. Quantification of nuclear p65 labeling showed significant reduction in NanoCurc-treated xenografts versus those treated with void NVA622 polymer (Fig. 4A). Of note, we were only able to compare the NanoCurc-treated group with the control arm due to the lack of availability of sufficient tumor material in the combination group. The gold standard for assessing NF-κB activity is to determine its DNA-binding capacity in an electrophoretic mobility shift assay (EMSA; ref. 34). We had sufficient tumor material from all four arms in the orthotopic P03C study to perform EMSA, and nuclear extracts were obtained from two representative xenografts in each cohort (see Supplementary Methods); intratumoral curcumin concentrations were assessed to determine whether there was a dose-dependent effect of curcumin in the tumor milieu on NF-κB activation. As seen in Fig. 4B, there was a downregulation in the

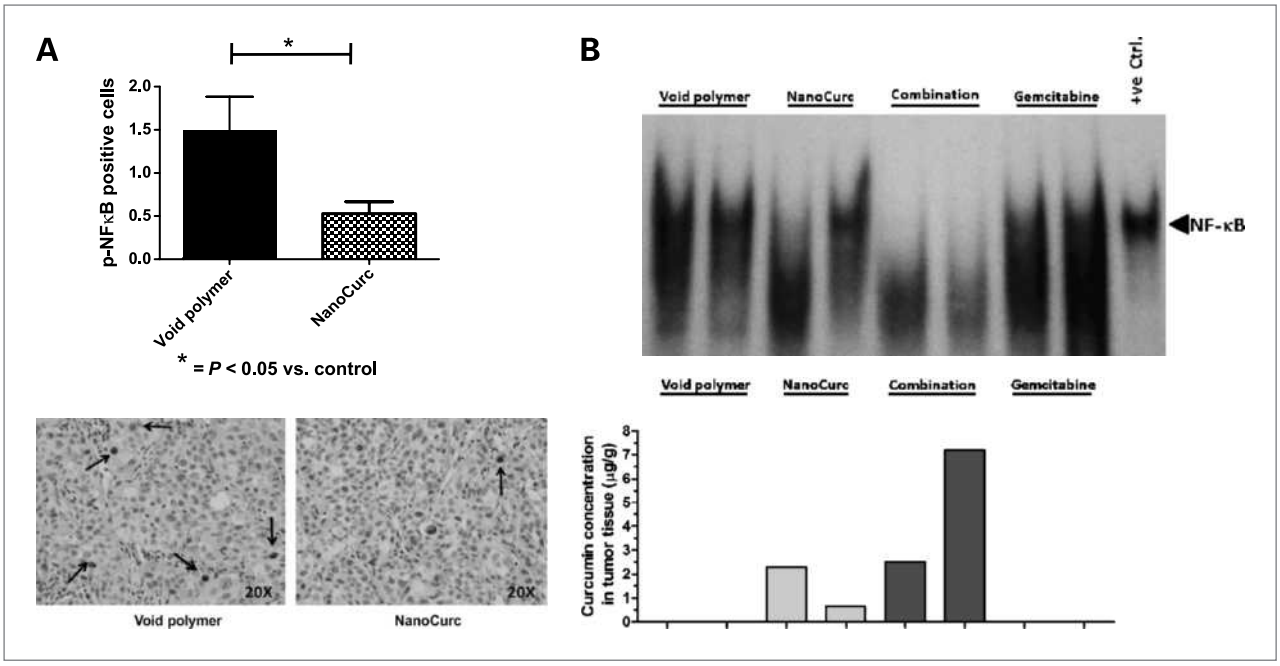

Figure 4. NanoCurc inhibits NF-κB activation in both subcutaneous and orthotopic pancreatic cancer xenografts. A, in subcutaneous Pa03C xenografts, immunohistochemistry for nuclear p65 (active NF-κB) shows significant reduction in nuclear labeling in NanoCurc-treated xenografts (hatched bar) compared with those receiving void NVA622 polymer (black bar). The *Y* axis designates number of cells with nuclear p65 staining per high power field (40×) over an average of 10 randomly selected fields. Bottom, photomicrograph of p65 immunohistochemistry in the two arms at the given (40×) objective. Please note that due to only a single residual nubbin of tumor in the combination therapy arm, immunohistochemistry could not be reliably performed in this case. B, EMSA was performed as a gold standard for NF-κB activity in orthotopic Pa03C xenografts, treated with vehicle (lanes 1 and 2), NanoCurc (lanes 3 and 4), combination (lanes 5 and 6), and gemcitabine (lanes 7 and 8); lane 9 is a Jurkat cell line nuclear lysate with activated NF-κB (positive control). The intratumoral curcumin concentrations for each pair of xenografts in each arm are represented at the bottom (*Y* axis equals concentration in μg/g of tissue). Reduction in DNA-binding ability of NF-κB (as indicated by gel shift) is observed in both xenografts in the combination arm (lanes 5 and 6), as well as in one of two xenografts receiving single-agent NanoCurc (lane 3), all three of which have robust levels of intratumoral curcumin (>2.5 μg/g of tissue). In contrast, one of the two xenografts receiving single-agent NanoCurc (lane 4) with retained NF-κB activity also shows ~4-fold lower intratumoral curcumin concentration (~0.6 μg/g). Expectedly, no reduction in DNA-binding ability is seen for the xenografts treated with vehicle or single-agent gemcitabine only.

DNA-binding capacity of NF-κB in both xenografts receiving combination therapy (lanes 5 and 6) and in one of two xenografts receiving single-agent NanoCurc (lane 3)—all three had robust intratumoral curcumin levels (>2.5 μg/g of tissue). In contrast, the second xenograft administered single-agent NanoCurc (lane 4), which retained NF-κB activity, had ~4-fold lower curcumin levels in the tumor than its counterpart. Thus, the anticancer effects of systemic NanoCurc seem to be mediated through the same major intracellular pathways as free curcumin.

NanoCurc in combination with gemcitabine inhibits cyclin D1 and MMP-9 in orthotopic Pa03C xenografts

Curcumin has pleiotropic effects on cancer cells, one of which is inhibition of proliferation through downregulation of cyclin D1, as previously described (35, 36). In orthotopic Pa03C xenografts, we did not observe a significant difference in *CCDN1* (gene encoding cyclin D1) transcripts measured by quantitative reverse transcriptase-PCR in either single-agent therapy arm compared with the void polymer–treated control xenografts (Supplementary Fig. S4A); in contrast, however, we detected a significant reduction ($P < 0.01$) in *CCDN1* expression in xenografts administered the combination of NanoCurc and gemcitabine. We then assessed for expression of the *MMP9* transcripts, as this has been previously identified as another bona fide target of curcumin in cancer cells (37–39). Comparable to CCDN1, we observed a significant reduction in MMP9 transcripts in the combination therapy arm ($P < 0.01$) when compared with the control xenografts and either single-agent tumor cohort (Supplementary Fig. S4B). mRNA data were confirmed at the protein level using representative xenografts from each of the four arms (Supplementary Fig. S4C). The results were particularly striking for MMP-9, where we observed near-total abrogation of this MMP in the combination therapy group. It is unclear why the effects on curcumin targets is significantly different in the combination therapy arm compared with xenografts receiving single-agent NanoCurc. It is possible that the enhanced MMP-9 downregulation represents an additive influence of gemcitabine itself, which has been shown to modestly reduce MMP levels in pancreatic cancer xenografts when used as a single agent (40). This effect could well be independent of NF-κB. Similarly, gemcitabine as an antimetabolite likely has NF-κB–independent effects on cell cycling, and hence, on the expression of cyclin D1, which could explain the additive effects observed in combination therapy. Finally, we performed studies on proliferation and microvessel density in the treated xenografts, which are presented in Supplementary Results (see Supplementary Fig. S5).

Discussion

In this article, we describe the *in vivo* application of a polymeric nanoparticle encapsulated formulation of curcumin (NanoCurc) in preclinical models of pancreatic cancer. Parenteral NanoCurc is able to overcome one of the primary pitfalls of free curcumin, namely suboptimal systemic bioavailability (10), without compromising the therapeutic efficacy of this promising natural compound. We show that single-agent NanoCurc inhibits the growth of pancreatic cancer xenografts in both subcutaneous and orthotopic settings, and in particular, we confirm the ability of NanoCurc in potentiating gemcitabine efficacy, as evidenced by histologic regression in subcutaneous xenografts and complete abrogation of metastases emanating from orthotopic tumors upon combination therapy. NanoCurc seems to function through blockade of the same major intracellular pathways (e.g., NF-κB) in cancer cells as free curcumin (1, 4). The readily demonstrable levels of tissue curcumin in most visceral organs suggest that this formulation could potentially be used for treatment of visceral malignancies, including metastases, beyond the tubular gastrointestinal tract. Although NanoCurc is not orally bioavailable and must be administered as an injectable formulation, this would not impede its applicability to patients with established cancers because the majority of chemotherapeutics are parenterally administered. In fact, one of the most widely used nanoparticle formulations of an anticancer agent (*nab*-paclitaxel or Abraxane) is administered solely through the parenteral route (41). Our pilot toxicity experiment, albeit not "regulatory grade" by any means, provides some degree of reassurance that the formulation and dosing regimen used does not entail obvious systemic adverse effects. NanoCurc can be readily stored at room temperature as a lyophilized powder, and can also be transported as such, requiring only reconstitution in an aqueous phase at the point of destination, all of which should facilitate eventual application in a clinical setting.

Although our prior report on polymeric nanoparticle-encapsulated curcumin was the first attempt at engineering a nanoparticle formulation of this compound (14), multiple investigators have synthesized a variety of curcumin nanoparticles since that time (42–50); of note, all but two (47, 50) have restricted their observations to the *in vitro* setting, and none have done *in vivo* xenograft treatment studies. Thus, to the best of our knowledge, our study is the first to rigorously show that curcumin nanoencapsulation has tangible therapeutic effects *in vivo* when used as either single agent or in combination with a conventional antimetabolite. We emphasize that our study neither shows nor claims the superiority of NanoCurc over any given curcumin nanoparticle formulation, and it is likely that many of the published platforms will have "niche" applications in cancer. For example, some of the recent curcumin nanoparticles have sustained-release properties (45, 50) and could be applicable in the setting of chemoprevention (analogous to a "patch" or "depot" preparation). Most importantly, our study reinforces the emerging notion that nanotechnology provides a highly appropriate

avenue for harnessing the full potential of promising, yet poorly bioavailable, natural anticancer agents like curcumin (10, 11).

Disclosure of Potential Conflicts of Interest

NanoCurc is a registered trademark of SignPath Pharmaceuticals, Inc., Quakertown, PA. An. Maitra is a member of the scientific advisory board of SignPath Pharma, Inc., and any conflicts of interest under this arrangement are handled in accordance with the Johns Hopkins University Office of Policy Coordination guidelines. SignPath Pharma has provided partial support for these studies through offsetting the costs of polymer synthesis. S. Bisht, G. Feldman, and An. Maitra have filed a patent application (U.S. 2008/0107749) that is relevant to the formulation described in this article. A report of invention to this effect has been filed with Johns Hopkins Technology Transfer and licensed by SignPath Pharma.

Grant Support

Supported by R01CA113669 (An. Maitra), R01CA13767 (An. Maitra), P01CA134292 (An. Maitra), P30CA069773 (M.A. Rudek), Flight Attendants Medical Research Institute (FAMRI; An. Maitra and M.A. Rudek), Johns Hopkins CTSA Institute for Clinical and Translational Research (UL1RR025005; M.A. Rudek), The Sol Goldman Pancreatic Cancer Research Center (An. Maitra), Michael Rolfe Foundation for Pancreatic Cancer Research (An. Maitra), the German Academic Exchange (DAAD) postdoctoral program (G. Feldmann), and SignPath Pharmaceuticals, Inc (An. Maitra).

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Received 02/22/2010; revised 05/13/2010; accepted 05/28/2010; published OnlineFirst 07/20/2010.

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