Liposomal curcumin with and without oxaliplatin: effects on cell growth, apoptosis, and angiogenesis in colorectal cancer

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Materials and Methods

Cell Lines

The human colorectal cancer cell lines Colo205 and LoVo cells were purchased from the American Type Culture Collection (Manassas, VA). Colo205 cells were grown in...
RPMI 1640 and LoVo cells were grown in DMEM-F12, both supplemented with 10% FCS. All media were purchased from Invitrogen (Carlsbad, CA).

**Lipids, Chemicals, and Curcumin**

1,2-Dimyristoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000], and 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) were obtained as a dry powder from Avanti Polar Lipids (Alabaster, AL). SyntheChol (synthetic cholesterol), curcumin, acetone, and tert-butanol were obtained from Sigma Chemical Co. (St. Louis, MO). Oxaliplatin (5 mg/mL; Sanofi-Synthelabo, Bridgewater, NJ) was purchased from Ben Venue Laboratories (Bedford, OH). For *in vivo* experiments, stock solution of oxaliplatin was diluted 5 to 10 times in 5% glucose solution for injection as recommended by the supplier.

**Liposomal Preparation**

The lipids used were 1,2-dimyristoyl-sn-glycero-3-phosphocholine/1,2-dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) (sodium salt) or a pegylated version of 1,2-dimyristoyl-sn-glycero-3-phosphocholine/cholesterol/1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000]. Different total lipid concentrations of liposomal curcumin, empty liposomes, and oxaliplatin or combinations thereof were incubated for 24 h and lyophilized to receive i.v. tail vein (40 mg/kg body weight) injections thrice weekly (this is the maximum volume that could be injected). Injections consisted of empty liposomes, liposomal curcumin, and oxaliplatin (i.p. 5 mg/kg), alone or in combination. Tumor size and body weight were measured with a caliper thrice weekly. The tumor volume was calculated using the following formula: volume = (length × width²) / 2, where width was the shortest measurement in millimeter.

**Histologic Sections**

Formalin-fixed tissue was paraffin embedded, sectioned (3–5 μm), and stained with H&E. Section was evaluated for tumor cell cytology, necrosis, and associated inflammatory cellular response.

**Immunohistochemistry for Angiogenesis**

To determine angiogenic effects, we assayed for CD31, basic fibroblast growth factor, vascular endothelial growth factor (VEGF), and interleukin-8 expression in xenograft tissue before and after treatment. Immunohistochemical studies were done by using formalin-fixed, paraffin-embedded sections (5 μm), heat-induced antigen retrieval (Dako Corp., Carpinteria, CA), and 1:50 to 1:200 monoclonal concentrations of antibody. The frozen sections were cut from snap-frozen tissue and embedded in OCT compound (Miles, Elkhart, IN). The detection system was the LSAB2 detection kit (Dako). The secondary antibody is a biotinylated antibody (Dako) and forms a complex with peroxidase-labeled streptavidin. Counterstaining was done by using Gill’s hematoxylin (Sigma Chemical). Negative controls were done by replacing the primary antibody with the biotinylated antibody.
anti-IgG1 (the same isotype with the primary antibody; Oncogene, Boston, MA).

**Antibodies**

Antibodies used for immunoblotting included monoclonal anti-tubulin antibody (Sigma Chemical), anti-PARP rabbit polyclonal (Cell Signaling, Beverly, MA), anti-VEGF monoclonal antibody (Oncogene), rat anti-mouse CD31 (BD PharMingen, San Diego, CA), and polyclonal anti-interleukin-8 antibody (Biosource, Camarillo, CA).

**Median Effect Analysis**

Median effect analysis (CalcuSyn version 2, Biosoft, Ferguson, MO) was used to determine interactions between liposomal curcumin and oxaliplatin. The CalcuSyn program provides a measure of the combined drug interaction by the generation of a combination index (CI) value. Dose-response interaction (antagonism, additive, or synergism) was expressed as a nonexclusive case. Combination indices were calculated according to Chou and Talalay (14). CI < 1 indicates synergism, CI > 1 indicates antagonism, and CI = 1 indicates that the two drugs are additive.

**Results**

**Liposomal Curcumin Internalizes in Cancer Cells as Easily as Free Curcumin**

Liposomal curcumin was expected to internalize into cells as easily as free curcumin (Fig. 1). Our experiments using light and fluorescence microscopy showed nearly complete internalization of pegylated liposomal curcumin into the colorectal cells after 2 h of treatment with a 10 μmol/L concentration. Internalization was comparable with that of free curcumin (Fig. 1).

**In vitro Growth Inhibitory Effect of Liposomal Curcumin Is Greater Than or Equal to That of Oxaliplatin**

After 72 h of incubation, the IC₅₀ of pegylated liposomal curcumin was less than that of oxaliplatin for LoVo cells as determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium salt assay (IC₅₀ for liposomal curcumin = 7.5 μmol/L; IC₅₀ of oxaliplatin was >40 μmol/L; Fig. 2). For Colo205 cells, the IC₅₀ of oxaliplatin was equivalent to that of pegylated liposomal curcumin. Pegylated liposomal curcumin had antiproliferative effects equivalent to those of free curcumin (Fig. 2).

**Figure 1.** Internalization of liposomal curcumin versus free curcumin. Light and fluorescence micrographs show representative internalization at 2 h after treatment with 10 μmol/L concentrations of pegylated liposomal curcumin versus free curcumin. Internalization is equal for both.

**Figure 2.** Inhibitory effect of liposomal curcumin and oxaliplatin on cell proliferation in colorectal cancer cells (A, Colo205 cells; B, LoVo cells). Points, mean of three independent experiments after 72 h of exposure to the above compounds; bars, SE. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium salt assay was used. Pegylated liposomal curcumin has growth-suppressive effects comparable with those of oxaliplatin in Colo205 cells. There was no significant difference between the effect of free curcumin and that of pegylated liposomal curcumin.
PARPcleavage after Liposomal Curcumin and Oxaliplatin Exposure

In vitro PARP is an enzyme involved in DNA damage and repair mechanisms, and cleavage reflects apoptosis. Figure 3 shows PARP cleavage as determined by Western blot analysis after 72 h of exposure of LoVo cells to pegylated liposomal curcumin, oxaliplatin, and the combination of these two drugs. Pegylated liposomal curcumin and oxaliplatin both induced PARP cleavage, but the combination of liposomal curcumin and oxaliplatin produced an increase in PARP cleavage in LoVo cells at 20 \( \mu \text{mol/L} \) (10 \( \mu \text{mol/L} \) of liposomal curcumin and 10 \( \mu \text{mol/L} \) of oxaliplatin) compared with that of 20 \( \mu \text{mol/L} \) of either drug alone (Fig. 3B).

PARP Cleavage after Liposomal Curcumin and Oxaliplatin Exposure In vitro

PARP is an enzyme involved in DNA damage and repair mechanisms, and cleavage reflects apoptosis. Figure 3 shows PARP cleavage as determined by Western blot analysis after 72 h of exposure of LoVo cells to pegylated liposomal curcumin, oxaliplatin, and the combination of these two drugs. Pegylated liposomal curcumin and oxaliplatin both induced PARP cleavage, but the combination of liposomal curcumin and oxaliplatin produced an increase in PARP cleavage in LoVo cells at 20 \( \mu \text{mol/L} \) (10 \( \mu \text{mol/L} \) of liposomal curcumin and 10 \( \mu \text{mol/L} \) of oxaliplatin) compared with that of 20 \( \mu \text{mol/L} \) of either drug alone (Fig. 3B).

Liposomal Curcumin Suppresses Colo205 and LoVo Tumor Growth in a Mouse Xenograft Model

Liposomal curcumin suppressed the growth of Colo205 and LoVo tumors in a murine xenograft s.c. model when compared with empty liposomes or saline (Fig. 4). Animals received 40 mg/kg of liposomal curcumin i.v. thrice weekly. The suppressive effects of liposomal curcumin were equal to or greater than those of oxaliplatin. No overt side effects were observed (but whether this molecule has toxicity in animals awaits formal toxicology studies). We also saw no significant changes in weight in liposomal curcumin-treated animals bearing xenografts compared with unimplanted animals treated with saline or empty liposomes.

Liposomal Curcumin Induces an Antiangiogenic Effect in Colo205 and LoVo Tumor Xenografts

It has been well documented that tumor growth and metastases require new vasculature. To determine whether the reduced growth of tumors after liposomal curcumin treatment of colorectal cancer xenografts was associated with an antiangiogenic effect, we examined the formation of neovasculature in Colo205 and LoVo tumor xenografts. Figure 5 shows that the number of cells stained with antibody against an endothelial cell marker (anti-CD31) was reduced in the treatment group tumor compared with the group treated with empty liposomes or saline. We also examined other critical angiogenic factors, such as VEGF, interleukin-8, and basic fibroblast growth factor, and found that levels were significantly reduced in the liposomal curcumin-treated group compared with the
controls (basic fibroblast growth factor levels were, however, unchanged in Colo205 cells). These results showed a potent antiangiogenic effect for liposomal curcumin.

Effect of Combinations of Liposomal Curcumin and Oxaliplatin
Combination therapy has been a cornerstone of treating cancer. We therefore examined the effect of combination of liposomal curcumin and oxaliplatin in vitro and in vivo. In vitro combinations of equimolar concentrations of oxaliplatin and liposomal curcumin resulted in no significant enhanced growth-inhibitory effect compared with that of either drug by itself (Fig. 2). However, Fig. 6 shows that combinations of liposomal curcumin and oxaliplatin at a 4:1 molar ratio resulted in synergy (median effect analysis). There was no synergy for the two drugs in vivo (Fig. 4).

Discussion
Plant-derived compounds have historically been some of the most commonly used and effective medicines. Several contemporary, evidence-based therapies, including chemo-therapeutic drugs, such as paclitaxel and Vinca alkaloids, are plant derived. Many plant-derived compounds have the advantage of having a favorable therapeutic-to-toxicity index.

Curcumin (diferuloylmethane) is a component of the root of the plant Curcuma longa. Centuries of culinary use in the Far East suggest that this phytochemical is safe to ingest. In recent years, there has been a growing interest in the antitumor activity of curcumin. Most of the studies used free curcumin, which is poorly absorbed in the gastrointestinal tract and is therefore limited in its clinical efficacy (14, 15). The highly hydrophobic nature of curcumin makes i.v. dosing impossible. Liposome encapsulation of curcumin
liposomal curcumin and oxaliplatin was synergistic. The fractional effects on cell death are calculated by following formula: (100 – alive cells) / 100. The CalcuSyn program was then used to calculate the two-drug interaction. CIs are presented as a function of fraction of cells affected by the cytotoxic effect. CI > 1.0 indicates antagonism, CI = 1.0 indicates additive effects, and CI < 1.0 indicates synergy. As shown, for our experiments, the 4:1 ratio of liposomal curcumin to oxaliplatin was synergistic.

Angiogenesis is integral to tumor growth and metastases. Of interest, in the current study, tumors from animals treated with liposomal curcumin showed an antiangiogenic effect, including attenuation of CD31 (an endothelial marker), VEGF, and interleukin-8 expression (Fig. 5).

There are now numerous publications documenting the potent preclinical antitumor effects of curcumin (4–10). In an early phase I trial in humans, curcumin has shown little, if any, side effects (14). Another pilot study, as well as preliminary results from our phase II study in pancreatic cancer, failed to show any toxicity even in large doses up to 8 g/d given p.o. (14–16). Given the low absorption of p.o. curcumin, we gave liposomal curcumin to animals in i.v. form. We have previously shown that this mode of administration effectively inhibits growth of pancreatic cancer xenografts (8). In both studies, there were no obvious host toxicities, although formal toxicology studies are still needed to rule out side effects in animals.

The molecular basis of tumor inhibition by curcumin is potentially attributable to its effects on transcription factors, apoptotic genes, angiogenesis regulators, and cellular signaling molecules. One of the chief mediators through which curcumin affects many of these pathways is nuclear factor-κB. The role of nuclear factor-κB in pathogenesis of colorectal cancer has been suggested (17). It has been shown to stimulate proliferation of colorectal cancer cells and enhance their survival through the regulation of antiapoptotic genes. A causative role of nuclear factor-κB in colorectal cancer has also been suggested (17) and it regulates the production of cyclooxygenase-2, a factor that is implicated in colon cancer pathogenesis (18).

The antiangiogenic effects of curcumin shown in the current study are consistent with those shown by Arbiser et al. (5), who documented that curcumin suppresses corneal neovascularization in mice. The results are also consistent with another study, which showed that curcumin treatment significantly reduces the tumor neocapillary density and expression of serum VEGF in hepatocellular carcinoma cells (19). Our own previously reported results in pancreatic cancer xenografts also confirm this action of curcumin (8).

Metastatic colorectal cancer is still a terminal illness for most patients. However, survival has increased from a few months to ~2 years (20) mainly because of the introduction of several new agents, each of which has a modest effect. Oxaliplatin, in combination with 5-fluorouracil, has been considered the standard first-line treatment for metastatic colorectal cancer (21). Oxaliplatin is generally well tolerated but can be associated with significant side effects (22) especially after cumulative dosing. This study establishes, for the first time, the comparable or greater growth-inhibitory and apoptotic effects of liposomal curcumin with oxaliplatin, with virtually no side effects for the former, at least in animals. Interestingly, a previous study showed synergism between curcumin and 5-fluorouracil against human colon cancer cells in vitro, associated with a 6-fold reduction of cyclooxygenase-2 protein expression (23). In our study, in vitro synergism was seen between liposomal curcumin and oxaliplatin at a 4:1 ratio, but there

Figure 6. Median effect analysis of the combination of oxaliplatin and liposomal curcumin on LoVo cell lines in vitro. Cells were incubated with pegylated liposomal curcumin and oxaliplatin at micromolar ratios of 4:1 for 72 h followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The fractional effects on cell death are calculated by following formula: (100 – alive cells) / 100. The CalcuSyn program was then used to calculate the two-drug interaction. CIs are presented as a function of fraction of cells affected by the cytotoxic effect. CI > 1.0 indicates antagonism, CI = 1.0 indicates additive effects, and CI < 1.0 indicates synergy. As shown, for our experiments, the 4:1 ratio of liposomal curcumin to oxaliplatin was synergistic.
was no substantial synergy \textit{in vivo}. However, the lack of overlapping side effects suggests that these two drugs could easily be combined.

Colorectal cancer is the second most frequently diagnosed cancer in the United States and incurs significant morbidity and mortality. There are multiple molecular and biochemical events involved in the pathogenesis of this disease. Drugs or combinations of agents affecting multiple pathways are therefore most likely to be effective. Curcumin is a natural plant product with an excellent safety profile that targets multiple signaling pathways. We have shown that it has antitumor and antiangiogenesis effects against colorectal cancer \textit{in vitro} and \textit{in vivo}, and these effects are equivalent or better than those of oxaliplatin, one of the most successful drugs available for treatment of this neoplasm. We are currently developing liposomal curcumin for introduction into the clinical setting.

\begin{thebibliography}{99}
\bibitem{7} Li L, Aggarwal BB, Shishodia S, Abbruzzese J, Kurzrock R. Nuclear factor-\(\kappa\)B and I\(\beta\)-kinase are constitutively active in human pancreatic cells, and their down-regulation by curcumin (diferuloylmethane) is associated with the suppression of proliferation and the induction of apoptosis. Cancer 2004;101:2351 – 62.
\end{thebibliography}


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