Combination of Chemopreventive Agents in Nanoparticles for Cancer Prevention

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Abstract
Carcinogenesis involves multiple genetic and epigenetic alterations, and a single chemopreventive agent may not be sufficient to prevent these events. Therefore, the use of a combination of agents is an attractive approach for cancer chemoprevention. In this issue of the journal, Prabhu and colleagues examined a combination of aspirin, curcumin, and sulforaphane for the prevention of pancreatic cancer in hamsters (beginning page 1015). The novelty of this work is that when aspirin and curcumin were incorporated in nanoparticles and administered orally, in combination with sulforaphane, the effective dosages were decreased by 10-fold in comparison with the free form mixture. In this commentary, the possible mechanisms of synergistic action among multiple chemopreventive agents and the use of stable nanoparticles for oral delivery are discussed. Also discussed is the importance of measuring tissue levels of the chemopreventive agents to understand the mode of action of these nanoparticles and to avoid toxicity.

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Introduction
Cancer chemoprevention may need to prevent the aberration of multiple molecular alterations, and the use of a combination of agents has been discussed extensively as a promising approach. However, this area is not as well studied as combinational therapy. Because of the heterogeneity and genomic instability of cancer, a single therapeutic agent is often insufficient and the recurrent cancer is subsequently much harder to treat therapeutically. However, the use of a combination of different drugs that target multiple pathways has been considered as an effective strategy for cancer therapy (1, 2). Indeed, clinical studies have showed the potential of this approach. For example, when patients with metastatic breast cancer were given a combination of trastuzumab (monoclonal antibody against HER2) and paclitaxel, improved clinical responses and survival rate were observed (3). Several preclinical and clinical studies also showed that the combination of phosphinositide 3-kinase (PI3K)/AKT pathway inhibitors with other agents, such as taxanes, improved the clinical responses by generating a synergistic effect in triggering cancer cell death, blocking negative feedback, and reducing side effects (4).

Nanoparticles have been vigorously investigated in recent years to improve drug delivery and to reduce side effects or toxicity. For example, abraxane, the U.S. Food and Drug Administration (FDA)–approved nanoformulation of paclitaxel bound on albumin, has been shown to be more effective and have less side effects than standard paclitaxel in treating breast cancer (5). Several related nanoformulations have been further developed and shown to be more efficacious in cancer therapy; the drugs presumably have increased accumulation in local tissues, extended half-life, increased solubility, and less premature release in circulation (6). The emerging approach of nanotechnology-based combinational drug delivery has also been discussed (2).

For cancer prevention, nanoformulations of curcumin and other agents have been developed to overcome the low bioavailability of these chemopreventive agents (7–10). The use of nanotechnology for the delivery of combined chemopreventive agents, however, has not been fully explored.

Combination of Aspirin and Curcumin in Nanoparticles in Pancreatic Cancer Prevention
In this issue of the journal, Grandhi and colleagues reported a novel, combinatorial nanotechnology-based oral delivery of chemopreventive agents in the suppression of pancreatic carcinogenesis induced by N-nitrosobis(2-oxopropyl)amine in hamsters (11). These authors used a combination of aspirin, curcumin, and sulforaphane (ACS) and found that these agents significantly reduced the tumor incidence, tumor multiplicity, and severity of histologic lesions. When aspirin and curcumin were delivered together in solid–lipid nanoparticles (SLN) in combination with sulforaphane in solution to hamsters, by daily oral gavage, the effective inhibitory...
dosages were reduced by a factor of 10 as compared with the ACS mixture in free forms. For example, with the medium levels of ACS, the dose of aspirin was reduced from 70 to 7 mg/kg and that of curcumin from 150 to 15 mg/kg. The comparison was also made at a higher and a lower dosage level of ACS. The results provide a proof-of-concept for the use of an oral, low-dose nanotechnology-based combinational treatment regimen for long-term cancer chemoprevention (11).

The nanoparticles were prepared with stearic acid using a hot melt, oil-in-water emulsion technique. The SLNs consist of a solid–lipid core and are stabilized by surfactants (12). The surfactant used in this study was proloxamer, a triblock polymer of polyethylene glycol (PEG)-polypropylene glycol (PPG)–PEG. The chemopreventive agents in the SLNs are thought to be absorbed via the lymphatic circulation, hence avoiding presystemic first-pass metabolism and increasing their systemic bioavailability (11, 13, 14). It would be interesting to determine the quantities of intact SLNs that actually enter the lymph and blood. If they remain intact when absorbed, the SLN approach would reduce the possible gastrointestinal irritation caused by aspirin and other agents.

**Synergistic Actions from Combination of Chemopreventive Agents**

Cancer chemoprevention may involve the prevention of aberrations of different metabolic and signaling pathways. The use of a combination of agents, especially chemopreventive agents that can affect multiple targets, has been considered as an attractive approach. In general, the combination of agents that affects different functional pathways may have the opportunity to generate additive or synergistic activity. The study by Grandhi and colleagues (11) is an example of such an approach. The cancer-preventive activities of aspirin, curcumin, and sulforaphane have been studied extensively. Aspirin is noted for its inhibition of cyclooxygenases and anti-inflammatory activity (15). Curcumin is known to inhibit NF-kB, though many other mechanisms of action have also been proposed (16). The induction of phase II xenobiotic-metabolizing enzymes, antioxidant enzymes, and other detoxification enzymes has been shown to be a major mechanism of action for sulforaphane; however, other mechanisms have also been suggested (17). In the study by Grandhi and colleagues (11), it is not known whether these three agents produce a synergistic or additive effect when used together, even though the mechanisms of their actions have been studied previously in pancreatic cell lines by the same research group (18, 19). It would be a challenge in future studies to elucidate the specific targets or pathways that are affected by these agents, as well as the mechanisms by which the synergy is generated.

A good example for showing synergistic effect between two agents was recently provided by Kumazoe and colleagues (20). These authors showed that (−)-epigallocatechin 3-gallate (EGCG) activated 67-kDa laminin receptors in multiple myeloma cells. This resulted in elevated cGMP levels, which initiated apoptosis through the activation of PKCδ and acid sphingomyelinase in a novel death pathway. However, EGCG alone was not very effective in killing the cancer cells, because these cells also overexpressed phosphodiesterase 5 (PDE5), a negative regulator of cGMP. When a PDE5-selective inhibitor, vardenafil, was also added to cultured cells, it synergized with EGCG to reduce the IC_{50} of EGCG from 23.2 to 1.4 μmol/L. Such synergy was also observed in xenograft tumors (20).

In general, it is easier to study the synergistic actions in cell lines and subsequently verify the results in animal models. This approach was used in our studies concerning the synergistic action of EGCG and atorvastatin in inhibiting chemically induced lung carcinogenesis in mice (21). The inhibitory action was associated with the synergistic induction of apoptosis. For mechanistic studies, we used lung cancer cell lines and also showed synergistic action between EGCG and atorvastatin in the inhibition of cell growth and induction of apoptosis. Furthermore, we showed that the induction of apoptosis by this combination was associated with downregulation of an antiapoptotic protein, MCL1, which was then confirmed in mouse lung tissues (21). In this case, we believe that similar mechanisms are involved in vitro and in vivo, however, how these two agents act synergistically to affect this protein and induce apoptosis is still unclear. In many other situations, the results of in vitro studies are very different from those obtained in vivo. Interactions among three chemopreventive agents have not been extensively studied. In a previous study, strong synergistic action was observed between atorvastatin and γ- or δ-tocotrienol in the inhibition of colon cancer growth, possibly through the actions of γ-tocotrienol in attenuating the induction of β-hydroxy-β-methylglutaryl coA (HMG–CoA) reductase by atorvastatin and enhancing the reduction of membrane-bound RhoA by atorvastatin (22). Furthermore, the combination of these two agents with celecoxib generated additional synergy. However, whether these actions occur in vivo remains to be showed.

**Oral Delivery of Nanoparticles**

For cancer chemopreventive nanoparticles, which need to be consumed daily, oral delivery is desirable. The particles should be stable with a reasonable shelf life, and more importantly, the particles should be stable under the gastric environment (high ionic strength and low pH). Sutaria and colleagues previously made SLNs containing either aspirin or curcumin (18). The nanoparticles ranged 150 to 250 nm in size, had sustained release of the drug over a 96-hour period, and were stable over a 3-month storage period at room temperature. It would be interesting to know the physical characteristics of the nanoparticles when both compounds are incorporated into these particles. Although SLNs are generally considered to be stable, there are limitations in their stability (12). In future applications of SLNs, the heat stabilities of the chemopreventive agents and the stability
of the nanoparticles during storage and in the stomach are important issues for consideration. The drugs in SLNs have been reported to be absorbed through the lymphatic system and then go into systemic circulation, bypassing the first-pass metabolism in the intestine and liver (Fig. 1; refs. 13, 14). It is desirable to have stable SLNs that can be absorbed intact in the intestine and enter into lymph and blood (14). This would increase the bioavailability of the chemopreventive agents tremendously.

There are other approaches in making oral delivery nanoparticles with dietary chemopreventive agents; these include polymer nanoparticles, micelles, and liposomes. For example, in a study by Hu and colleagues (10), a food grade polymer nanoparticle (150 ± 4.3 nm with a surface charge of 32.2 ± 3.3 mV) was fabricated through the electrostatic interaction between chitosan and milk casein phosphopeptides (CPP) for encapsulation and oral delivery of EGCG. The encapsulation efficiency ranged from 70.5% to 81.7%, with sustained release of the EGCG over a 96-hour period. The nanoparticles are able to enhance the intestinal absorption of EGCG significantly in an in vitro Caco-2 cell monolayer assay, possibly due to intracellular delivery of EGCG through direct cellular uptake of the nanoparticles as well as paracellular transport of EGCG due to opening of the cellular tight junctions. Furthermore, after surface cross-linking with genipin (a nontoxic cross-linking reagent from gardenia fruits), the chitosan–CPP nanoparticles can withstand the harsh pH and digestion enzyme conditions in gastrointestinal fluids. The release of EGCG is dependent on the degradation of chitosan chains by a number of enzymes in human serum and cells, and it could be regulated through adjusting the extent of cross-linking (23). These nanoparticles are nontoxic and could complement SLNs in the delivery of polar and charged molecules.

**Figure 1.** Structures of aspirin, curcumin, and sulforaphane and a cartoon illustrating possible absorption of aspirin and curcumin in solid-lipid nanoparticles in combination with sulforaphane.

**Tissue Levels of Chemopreventive Agents, Safety, and Other Concerns**

Once a stable, orally deliverable nanoformulation is developed, it would be important to study the tissue levels of these agents after short- and long-term administration of the nanoparticles. These studies are extremely important for understanding the biologic fate of these particles and the concentration of the chemopreventive agents at the site of action. It will also help researchers to predict the organ sites where these agents would be effective. A good example of such tissue level study has been provided by Veeranki and colleagues, in which the tissue levels of sulforaphane were studied in detail (24).

Although many of the cancer-chemopreventive agents are considered safe, aspirin is known to cause gastrointestinal bleeding in some individuals (15). Consumption of high doses of curcumin and concentrated green tea has also been reported to produce gastrointestinal irritation. Liver toxicity has been experienced by some individuals who took large quantities of green-tea extract-based dietary supplements for weight reduction purpose (25). EGCG is a major active constituent of green tea extract and has been shown to induce liver toxicity in rodents when administered in large doses (26). Incorporating chemopreventive agents into nanoparticles at lower doses is expected to eliminate or diminish these problems.

Dietary-derived chemopreventive agents, such as curcumin and EGCG, are considered nontoxic because of their low bioavailability. However, once the bioavailabilities of these agents are enhanced markedly by the use of nanoparticles, caution should be applied to avoid reaching toxic
levels. It is also interesting that drugs in SLNs, because of the lipophilicity, may cross the blood and brain barrier. This may cause these agents to be more effective in preventing neurologic disease; but at high levels, they may induce neurotoxicity. Therefore, studies on tissue levels are important.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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