Chemoprevention of Colon Cancer in a Rat Carcinogenesis Model Using a Novel Nanotechnology-Based Combined Treatment System

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Abstract

Colorectal cancer (CRC) is the third most common cause of cancer death in the United States, accounting for approximately 51,000 deaths each year. We have previously shown in vitro chemopreventive effects of mixtures of aspirin, folic acid, and calcium (AFAC) on colon cancer cell lines. The objective of the present study was to evaluate the in vivo effects of orally administered, colon targeted chemopreventive combination regimens on the inhibition of aberrant crypt foci (ACF) in a rat model of colon carcinogenesis using (i) unmodified (free drug) combinations of AFAC and (ii) nanoparticle-encapsulated combinations of the same agents. A 14-week animal study was conducted in three phases to determine an optimal effective dose from AFAC combinations and evaluate the efficacy of nanotechnology-based chemopreventive regimens administered in combined (mixtures) and individual (single entity) forms. ACF inhibition when compared with azoxymethane-treated rat control group was significant in both the unmodified and the modified nanoparticle-mediated chemopreventive regimens, showing a range of 31% to 38% ($P < 0.05$) and 50% to 75% ($P < 0.001$) reduction, respectively, in the number of ACFs. In addition, the nanoparticulate combination regimens of AFAC showed a 2-fold increase in suppression of ACF compared with free drug mixtures. Individual administration of nanoparticle-encapsulated drugs showed no significant effect on the reduction of ACF. Histochemical analysis provided further confirmation of chemopreventive effects, showing a significant reduction in cell nuclear proliferation. Overall, our results provide a strong proof of concept using nanoparticle-mediated combination treatment in the chemoprevention of colon cancer.

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Introduction

It was estimated that colon and rectal cancer would affect over 142,000 Americans in 2010 and result in the deaths of approximately 51,000 patients from this disease, making it the third most common cancer in both men and women in the United States (1). Lately, cancer chemoprevention has received significant attention, as a growing body of evidence has emerged with the promise of chemopreventive agents being successfully used in the prevention of tumors (2–4). Cancer chemoprevention is an approach in which dietary or synthetic chemical agents are used to prevent cancer in normal and/or high-risk populations (5). Many compounds have been extensively studied for their chemopreventive effects. Among them, aspirin, folic acid, and calcium have shown promise when used on an individual basis in the prevention of colon cancer (6–8). Aspirin has shown high effectiveness as a chemopreventive agent against colon cancer (9). The mechanism of inhibition involves the suppression of overexpressed COX-2 enzyme, which leads to the suppression of colonic polyp formation (10,11). However, aspirin is known to cause complications, such as gastric mucosal injury, bleeding, and anti-platelet effects, in patients who frequently use high doses (12, 13). For this reason, physicians are reluctant to recommend this drug for regular use as a preventive measure toward colon cancer in the general population. Thus, further determination of the minimally effective dose, perhaps in combination with folic acid and calcium, is needed to balance benefits with potential toxic risks of long-term administration (14).

Folic acid is essential for DNA synthesis and DNA methylation (15). Folic acid is provided in diet from major sources including citrus fruits, dark green vegetables, and dried beans or by synthetic supplementation. Colorectal adenomas were shown to be decreased by 31% in epidemiologic studies after prolonged folate supplementation of...
400 µg/d. Maintaining adequate levels of folic acid ensures protection of genes from mutation leading to colon cancer (8, 16–18).

Among nonpharmacologic agents, calcium supplementation has emerged as a promising candidate for chemoprevention. Prior studies have suggested an inverse association between calcium intake and the occurrence of colorectal neoplasia (19). A mechanism by which it affects the intestinal epithelium is through activation of a calcium-sensing receptor with resultant growth inhibition and differentiation of transformed cells (20, 21). Because of the abundant availability of calcium, low cost, and its potential effectiveness in preventing colorectal cancer (CRC), calcium is a good candidate for further studies in chemoprevention.

We have recently shown that combinations of aspirin, folic acid, and calcium (AFAC) significantly reduced cell viability in HT-29 and SW-480 colon cancer cell lines (22). Other research groups have shown similar results whereby administering combinations of low doses of other chemopreventive agents provided increased efficacy and minimized toxicity (23, 24). In addition, we were the first to report the development of novel nanotechnology-based formulations encapsulating aspirin and folic acid with small particle sizes (average size: 120 nm) and high encapsulation efficiencies (85%), for targeted delivery to the colon (22, 25). For the current study, using male Sprague-Dawley rats, we report AFAC, in unmodified (free drug) form and nanoparticle-encapsulated combination forms, exerting significant effects in the colonic epithelia for the chemoprevention of colon cancer. To our knowledge, this is a first proof-of-concept report using nanotechnology-based chemopreventive regimens of AFAC combinations in the suppression of colon cancer.

Materials and Methods

Materials

Aspirin, folic acid, methylene blue, 10% buffered formalin, and dichloromethane were purchased from Sigma and calcium carbonate (calcium) was supplied by PCCA (Houston, TX). The carcinogen azoxymethane was obtained from Spectrum Chemicals. Male Sprague-Dawley rats were supplied by Harlan and disposable plastic cages for the rats were purchased from Innovive. Hematoxylin and eosin (H&E) staining was conducted at the Histo-Scientific Research Laboratories. Animal diet and water were provided ad libitum to the rats. The animal study protocol was approved by the Western University of Health Sciences Institutional Animal Care and Use Committee before the initiation of the experiments. For the preparation of nanotechnology-based chemopreventive regimens, the biodegradable polylactide-co-glycolide (PLGA; 50:50) copolymer was purchased from Durect Corporation. Low molecular weight Eudragit S 100, an enteric coating polymer, was gifted by Degussa.

Methods

Rat study design. Seven-week-old male Sprague-Dawley rats weighing approximately 225 to 300 g were randomized in groups (n = 6) and placed in disposable plastic cages. At 8 weeks old, azoxymethane (15 mg/kg) was injected subcutaneously into rats once weekly for 2 consecutive weeks. For the next 14 weeks, rats were dosed every 24 hours by oral gavage with freshly prepared chemopreventive regimens as per the following protocol:

Set 1: As indicated in Table 1, this set consisted of 7 groups (G1–G7) of rats treated with high to low dose ranges of plain, unmodified mixtures (AFAC) starting the day after the first azoxymethane inoculation (G3–G7). The purpose of these studies was to determine a minimally effective chemopreventive dose for aberrant crypt foci (ACF) suppression. Two groups of control animals, saline (G1) and azoxymethane-treated (G2), serving as (−) and (+) controls, respectively were also included. The highest dose selected for aspirin, folic acid and calcium regimen was 400, 32, and 650 mg/kg (G3), respectively. On the basis of previous studies in the literature, the highest dosage for aspirin was determined to be 400 mg/kg (26). By drawing a correlation to cell line studies (22), the high dose levels for folic acid was calculated to be 32 mg/kg and for calcium 650 mg/kg. Other doses were calculated as 1 of 3 (G4), 1 of 10 (G5), 1 of 30 (G6), and 1 of 100 (G7) of the highest dose to obtain a wide range of doses (Table 1).

Set 2: On the basis of results obtained from set 1 studies and subsequent dose optimization, a comparative study between mixtures of unmodified free drugs and drugs encapsulated in nanoparticles was conducted (set 2; Table 1) at lower doses. Three groups of rats (G10–G12) received unmodified combinations of AFAC whereas another 3 groups of rats (G13–G15) received combinations of drug-loaded nanoparticles. Among the 3 chemopreventive agents, only aspirin and folic acid were encapsulated within nanoparticles. Calcium was not encapsulated, as it has previously been shown to be a virtually nontoxic compound with no side effects even when ingested at very high doses (27).

Set 3: Animals in this final set of studies (G18–G20; Table 1) received single doses of low concentrations of chemopreventive agents encapsulated within polymer nanoparticles. The last set (G21) received a combination of the 3 agents. This study was conducted to confirm our hypothesis that any inhibition response of nanoparticle drug combinations on ACFs observed from the previous studies (set 2) were based on a combined additive response and not as a result of the effect of individual chemopreventive agents.

Colon harvesting and staining. At the end of each study, the colons were extracted from the sacrificed rats, cleaned with phosphate buffer solution (PBS), and cut into the proximal, middle, and distal regions of the large intestine.
A subjective examination of other organs (stomach, small intestine, liver, and kidney) was conducted to observe for signs of toxicity from the chemopreventive regimen. To quantitate the ACF, methylene blue staining was conducted by dipping the colonic segments in 10% formalin buffer fixative solution for 24 hours followed by methylene blue dye (0.1% w/v) staining for 20 to 30 minutes. A light microscope with a 40× magnification was used to quantitate ACFs on the colon. Aberrant crypts were identified as increased distance from the basal to lamina surface of cells, easily discernible pericryptal zone, split-like opening in the centre, enlarged size, and deeper staining. All colon segments were scored by the primary investigator responsible for these studies and subsequently by a trained laboratory assistant who was blinded to the study.

**Dose–response curve.** Dose–response curves were used to assess the inhibition of ACF per treatment dose after completion of studies from set 1. A maximum response was defined as 100% inhibition of ACFs whereas a minimum response consisted of the groups with most number of ACFs, when compared with the azoxymethane-treated control group. The azoxymethane-treated control group was expected to present the highest number of ACFs as a result of the carcinogenic effect of azoxymethane on the colon. Subsequently, a probit analysis (28) was conducted to calculate the effective dose (ED$_{50}$) from the range of treatment doses tested.

**Histochemical analysis.** Standard protocol for H&E staining was followed (29, 30). Four- to 5-μm thick longitudinal sections of the distal colonic segments were cut from the paraffin block using a cryostat and subsequently de waxed in several baths of xylene and hydrated through graded alcohols to ultrapure water. Thereafter, the sectioned tissue was stained with hematoxylin and washed with distilled water. Tissues were clarified by a “blueing” step using 1% HCl and ammonium hydroxide to provide a better contrast. An eosin stain was applied and samples were dehydrated in 95% alcohol and absolute alcohol followed by clearing in xylene. Tissues were mounted on the slides and evaluated microscopically at 40× magnification.

**Preparation of colon targeted novel nanotechnology-based chemopreventive formulations.** Aspirin and folic acid were encapsulated within PLGA copolymer using a previously reported method (22). Briefly, aspirin and folic acid suspended in distilled water were emulsified in organic solution of dichloromethane containing PLGA (50:50) copolymers. The emulsion was probe sonicated and added dropwise to an outer aqueous 2% polyvinyl alcohol solution. The dichloromethane solvent was evaporated, resulting in the precipitation of drug-loaded nanoparticles in the aqueous phase. Subsequently, ultracentrifugation at 10°C (34,500 × g for 25 minutes) was conducted to recover the nanoparticles. Finally, the nanoparticles were freeze dried and stored at 4°C until use. For the purposes of targeting to

### Table 1. Treatment plan showing groups of Sprague-Dawley rats treated with unmodified and nanoparticle forms of chemopreventive agents aspirin, folic acid, and calcium at various doses

<table>
<thead>
<tr>
<th>Set</th>
<th>Group (n = 6)</th>
<th>Treatment plan</th>
<th>Dose, mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Set 1 (unmodified combination)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>G1</td>
<td>Saline (control)</td>
<td>0</td>
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<tr>
<td>G2</td>
<td>AOM (control)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>ASP + FA + Ca</td>
<td>400 + 32 + 65</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>ASP + FA + Ca</td>
<td>133 + 11 + 216</td>
<td></td>
</tr>
<tr>
<td>G5</td>
<td>ASP + FA + Ca</td>
<td>40 + 3.2 + 65</td>
<td></td>
</tr>
<tr>
<td>G6</td>
<td>ASP + FA + Ca</td>
<td>13 + 1.1 + 22</td>
<td></td>
</tr>
<tr>
<td>G7</td>
<td>ASP + FA + Ca</td>
<td>4 + 0.32 + 6.5</td>
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<tr>
<td><strong>Set 2 (unmodified and modified combination)</strong></td>
<td></td>
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<tr>
<td>G8</td>
<td>Saline (control)</td>
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<tr>
<td>G9</td>
<td>AOM (control)</td>
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<tr>
<td>G10</td>
<td>ASP + FA + Ca</td>
<td>133 + 11 + 216</td>
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<tr>
<td>G11</td>
<td>ASP + FA + Ca</td>
<td>80 + 6.4 + 130</td>
<td></td>
</tr>
<tr>
<td>G12</td>
<td>ASP + FA + Ca</td>
<td>40 + 3.2 + 65</td>
<td></td>
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<tr>
<td>G13</td>
<td>ASP (NP) + FA + Ca</td>
<td>133 + 11 + 216</td>
<td></td>
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<tr>
<td>G14</td>
<td>ASP (NP) + FA + Ca</td>
<td>80 + 6.4 + 130</td>
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<tr>
<td>G15</td>
<td>ASP (NP) + FA + Ca</td>
<td>40 + 3.2 + 65</td>
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<tr>
<td><strong>Set 3 (modified combination)</strong></td>
<td></td>
<td></td>
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<tr>
<td>G16</td>
<td>Saline (control)</td>
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<td></td>
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<tr>
<td>G17</td>
<td>AOM (control)</td>
<td>0</td>
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<tr>
<td>G18</td>
<td>ASP (NP)</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>G19</td>
<td>FA (NP)</td>
<td>3.2</td>
<td></td>
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<tr>
<td>G20</td>
<td>Ca</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>G21</td>
<td>ASP (NP) + FA + Ca</td>
<td>40 + 3.2 + 65</td>
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</table>

**Abbreviations:** ASP, aspirin; AOM, azoxymethane; FA, folic acid; Ca, calcium; NP, nanoparticles.
the colon, mixtures of freeze-dried aspirin and folic acid nanoparticles were coated with Eudragit S 100 (1% in methanol w/v). Particle sizing and drug encapsulation efficiency measurements were conducted and optimized (22).

Rat cecal content studies. To isolate the cecal contents, a previously published procedure was used (31). Briefly, Sprague-Dawley rats were sacrificed and the cecum was isolated and immediately transferred into a buffer medium at pH of 6.8. The cecal bags were opened, contents weighed individually, then pooled and suspended in buffer continuously bubbled with CO2 to maintain anerobic conditions. These were finally added to the dissolution media to give a final cecal dilution of 2%.

A 48-hour drug release study was carried out in the presence of CO2. At different time intervals, 5 mL sample was withdrawn from the dissolution medium and analyzed by UV-visible spectrophotometry at wavelengths of 233 nm for aspirin and 277 nm for folic acid. The sample was replaced by fresh dissolution medium. All runs were conducted in triplicate.

Statistical analysis
A 2-way ANOVA followed by a Bonferroni post hoc analysis using GraphPad prism software was used for statistical comparison of results. Data were tested for significant difference on the frequency of ACFs along the different segments of colon. A value of \( P < 0.05 \) was considered significant.

Results

General observations
A steady increase in mean body weight of the rats during the 14-week study was shown. All animals were under continuous observation for signs of distress, weakness, and other possible side effects. For rats in set 1, the mean body weights were lower (243 g) at the start of the experiments, in comparison with groups 2 (305 g) and 3 (317 g). By the end of the study, mean body weight for animals in their respective groups increased proportionately from their original weight (set 1, 414 g; set 2, 445 g; and set 3, 427 g). On average, the weight gain of approximately 50% was seen across all sets of rats tested, indicating overall good health of the rats during the study (see Supplementary Fig. S).

No signs of toxicity in organs (stomach, intestine, liver, and kidney) from the azoxymethane inoculation and chemopreventive regimens were evident after the 14-week duration of study.

Unmodified combinations of chemopreventive agents significantly inhibit ACF formation
In general, ACFs were more pronounced in the distal than in the proximal or the middle part of the colon, consistent with previous reports (32, 33). Thus, our results in this report focus primarily on the inhibition of ACF formation in the distal region of the rat colon.

Figure 1 shows azoxymethane-treated rats (G2) from set 1 which received unmodified combinations of AFAC in a wide range of doses (G3–G7). Saline (G1) and azoxymethane (G2) control groups were included for comparison. The objectives of this study were 2-fold: (i) to show the effectiveness of combined treatment on azoxymethane-treated rats when treated with unmodified forms of chemopreventive agents, AFAC, and (ii) to optimize the dose for subsequent design of drug-loaded PLGA nanoparticles targeted to the colon.

As shown in Figure 1, all treatment doses showed a wide range of effectiveness in the distal colon with significant inhibition of ACF formation observed in all treatment doses, except one. These observations provide first evidence of the effect of combined treatment using this particular regimen on the chemoprevention of colon cancer. As expected, the azoxymethane control group (G2) showed the maximum number of ACFs (~30) in the distal colon. Rats treated with unmodified chemopreventive regimens exhibited a dose-dependent response with strongest inhibition observed for the highest dose used (G3), progressively decreasing in effect as the dosing strengths were reduced (G4–G7). Animals in group G3, the highest dose regimen, showed maximum inhibition of ACFs with an average of 2.67 crypts, representing a 96% decrease in ACF formation when compared with the azoxymethane control group. Similarly, groups G4 to G7 showed decrease in the ACF count to approximately 10 (G4), 14 (G5), 17 (G6), and 20 (G7) ACFs, representing a decrease of 80%, 54%, 42%, and 37%, respectively, thus showing the dose-dependent inhibitory effect of the triple
to determine the effective dose (ED50) from the sigmoid response (96%, G3). A probit analysis was conducted for the least responsive dose (12%, G7) with that of maximum inhibition was observed when comparing the chemopreventive regimens (Fig. 2). An 8-fold difference in inhibition was found when comparing the proximal or middle sections of the colon whereas significant differences were found toward the distal end. Rats in set 1 showed the following results: G3 (t = 4.23, P < 0.001), G4 (t = 3.15, P < 0.01), G5 (t = 3.18, P < 0.01), and G6 (t = 2.49, P < 0.05) were significantly different from the G2 (azoxymethane control group). However, G7 (t = 2.2, P > 0.05) was not significantly different from the azoxymethane group, despite showing a decrease in the number of ACFs when compared with control.

A plot of ACF inhibition (%) versus dose administered (mg/kg) for the unmodified chemopreventive combinations (set 1) showed a sigmoidal curve showing increased inhibition of ACFs with increasing doses of chemopreventive regimens (Fig. 2). An 8-fold difference in inhibition was observed when comparing the least responsive dose (12%, G7) with that of maximum response (96%, G3). A probit analysis was conducted to determine the effective dose (ED50) from the sigmoid dose–response curve and analyzed by regression either through least squares or maximum likelihood (Fig. 2 inset). Percentage of inhibition response calculated previously was transformed into probits using Finney’s table (28), where a 50% response corresponded to a 5 on the probit scale (y-axis). Extrapolating to the log dose values on the x-axis obtained the corresponding dose value of approximately 120 mg/kg. This dosage value was deemed closest to the combined AFAC dose administered to rats in G5 of set 1 studies (108 mg/kg).

Both unmodified and nanoparticle-mediated chemopreventive regimens significantly inhibit ACF formation in rat colon

On the basis of results obtained from set 1 studies and subsequent calculations of the ED50 dose, a new set of animal studies was conducted using optimized doses of unmodified chemopreventive combination regimens (Table 1; G10–G12). Two of these selected doses were similar to the previous studies in set 1 (G4 and G5). In addition, an intermediate dose was selected to assess the effect of this new dosage regimen on colonic ACFs. Subsequently, 3 additional groups using modified, drug-loaded nanoparticles combination regimen (Table 1; G13–G15) at equivalent doses were used.

The purpose of these studies (set 2) were (i) to further optimize the unmodified dosage regimens to enable the identification of the lowest dosing strength that would significantly decrease the incidence of colon ACFs in the rats and (ii) to observe and report, for the first time, the effect of colon targeted nanotechnology-based formulations on the inhibition of ACFs in the colon. As before, a new saline (G8) and azoxymethane-treated control (G9) was maintained for this group of animals. As shown in Figure 3A, most of the ACFs were again present in the distal portion of the rat colon though there was some evidence of ACF formation in the proximal and middle colon regions. However, as no significant differences in the inhibition of ACF formation were apparent from the proximal and middle colon, only the distal colonic ACFs were measured wherein the response of the treatment doses were all significant at the dose administered. As expected, the azoxymethane treatment set (G9) showed the maximum number of ACFs in the distal colon (~45). Treatment with unmodified chemopreventive combinations resulted in 38% (G10), 31% (G11), and 31% (G12) reduction in the number of ACFs in the distal colon. This trend was similar to our observations from the previous set of studies in set 1. However, the new intermediate dose (G11) did not show any difference in inhibition when compared with the low dose (G12; Fig. 3A), indicating that both doses have equivalent effect on the inhibition of ACF formation. A statistical 2-way ANOVA followed by the Bonferroni post hoc test revealed a significant treatment effect in the distal colon. The highest dose group G10 (360 mg/kg) was found to be the most effective chemopreventive combination in this group (set 2). G10 with a difference of 16 ACFs (t = 3.33, P < 0.01) from the azoxymethane control was most effective followed by G11 (216 mg/kg) and G12 (108 mg/kg) which were almost similar in effect with a difference of 12 crypts (t = 2.5, P < 0.05) each.

In comparison, Figure 3B shows the effect of a modified chemopreventive regimen where aspirin and folic acid were encapsulated in PLGA 50:50 polymer nanoparticles (20) and mixed with free calcium and administered to rats in set 1...
When compared with the azoxymethane-treated group (G9), significant differences were observed wherein treatment with the modified form of G13 (360 mg/kg) showed a significant inhibition of ACFs in the distal colon by approximately 75%. Similarly, the 2 other groups G14 (216 mg/kg) and G15 (108 mg/kg) each showed considerable ACF inhibition of over 50% when compared with the azoxymethane control. These findings, reported here for the first time, show that the PLGA 50:50 polymer-encapsulated nanoparticle regimens were significantly more effective in chemoprevention than their unmodified counterparts. The G13 dosage group which was most effective in the inhibition of ACF formation showed the maximum difference of 32.8 ACFs ($t = 4.73, P < 0.001$) when compared with G9 (azoxymethane control). It was followed by G14 and G15 with a difference of 23.2 ACFs ($t = 3.35, P < 0.01$) and 23.4 ACFs ($t = 3.38, P < 0.01$), respectively, from G9 (azoxymethane control).

A comparison of dose responses from unmodified chemopreventive regimens (G10, G11, and G12) to that of the nanoparticle set (G13, G14, and G15) showed that at the highest dose (360 mg/kg) for both groups, namely G10 (unmodified) and G13 (nanoparticle encapsulated), respectively, the average ACF count was observed as approximately 40 and 25, respectively, representing a 1.6-fold increase in the efficacy of the nanoparticle-modified regimen (Fig. 3). Similarly, at 208 mg/kg dose of unmodified (G11) and modified (G14) regimens showed similar results of approximately 48 and 28 ACFs, a 1.7-fold increase in efficacy again with the novel nanoparticle regimens. Finally, at the lowest dose (108 mg/kg) administered, G12 showed an average ACF count of 59 whereas the modified G15 group showed 33 ACFs and hence an approximate increase of 1.8-fold over the unmodified regimen. Thus, the nanoparticle-based treatment regimen showed better inhibition of ACF formation not only in comparison within its own group but also between treatment groups.

Histochemical analysis of distal colonic tissues samples qualitatively analyzed the cellular morphology of the saline and azoxymethane-treated samples from those that received a modified nanoparticle combined treatment regimen. The results obtained from these studies (Table 1; set 2, G13–G15) provided further evidence of the effect of nanoparticle-based combined treatment regimens of aspirin, folic acid, and calcium on inhibition of ACF formation. As shown in Figure 4, the azoxymethane control group showed a high concentration of cell nuclei stained by H&E as noted by the occurrence of a densely stained band. In comparison, the treatment sets (G13–G15) showed decreasing levels of nucleus staining as the dosing strength reduced from a higher dose (360 mg/kg) to the lowest dose level (108 mg/kg). However, even at the lowest dose, significant reduction in nuclear proliferation was observed compared with the azoxymethane control, thus confirming the positive effect of nanoparticle drug combinations on ACF of the rat distal colon. These results provided confirmation on the efficacy of the treatment regimen in controlling cellular proliferation.

**Individual administration of nanoparticle-mediated chemopreventive agents has no effect on ACF inhibition**

The purpose of the final set of studies (set 3, G16–G21) were (i) to study the effect of individually administered nanoparticle formulations of aspirin, folic acid, and free calcium on rats treated with azoxymethane and saline as control and (ii) to confirm that the inhibitory effect on ACF formation observed from previous studies was a result of an additive/synergistic effect of the triple combination and not due to the action of any one chemopreventive agent used. The dosage regimen selection (G15) was based on the lowest combined dose showing a significant effect in the inhibition of ACF formation in the distal colon. Thus,
Individual concentrations of PLGA 50:50 encapsulated aspirin (G18, 40 mg/kg) and folic acid (G19, 3.2 mg/kg) nanoparticles and free calcium (G20, 65 mg/kg) were administered to azoxymethane-treated rats. A combination dose (set 2) was also administered to replicate the observations from the previous set of studies. Individually, none of the chemopreventive agents (aspirin, folic acid or calcium) showed any effect on the reduction of ACFs, though calcium seemed to have an effect in the distal colon, however, this was not statistically significant. When administered in combination, the distal colonic segments showed significant inhibition of ACFs. The effect of the treatment combination was similar to that observed from previous studies wherein approximately 50% inhibition was evident when compared with the azoxymethane-treated control group. These studies confirmed that individual administration of a chemopreventive drug at a low dose had no effect on ACF inhibition whereas a significant additive chemopreventive response was evident when administered in combination.

**Rat cecal content studies**

Aspirin release from PLGA nanoparticles in rat cecal medium was significantly increased when compared with release of the same drug from a medium without cecal content (PBS). Over a 48-hour period, approximately 80% of total aspirin was released from the cecal medium as compared with only 55% from the PBS dissolution medium. In case of folic acid release, there was not much difference in the extent (80%) however, the rate of release from PLGA nanoparticles was increased when compared with release from the PBS dissolution medium.

**Discussion**

Recently, the term “nanochemoprevention” has been coined to show the novel approach of using nanotechnology-based regimens for the prevention of cancer (34–36). Successful encapsulation of a green tea extract and its subsequent increase in effectiveness over unencapsulated treatment regimens for the chemoprevention of prostate cancer was reported. Other recent studies have shown the benefits of encapsulating curcumin (37, 38) and selenium (39) for anticancer therapy and prevention. Overall, the use of nanotechnology in cancer chemoprevention is still very limited. However, with more reports in this area being published, this is expected to change.

There is increasing interest in the use of combinations of low doses of chemopreventive agents that differ in mode of action.
action. This approach provides means of obtaining increased efficacy and minimized toxicity when a promising chemopreventive agent may show toxic effects at higher doses (40). Studies using calcium/vitamin D combination have shown to exert an additive effect, leading to the conclusion that the 2 agents work together and not separately, in the reduction of the risk of CRC (41). Recently, the combined use of low-dose mixtures of atorvastatin, aspirin, and celecoxib showed significant inhibition of colon carcinogenesis in comparison with individual doses of the same drug (42). Another study using piroxicam, another nonsteroidal anti-inflamatory drug (NSAID), combined with difluoro-ornithine showed increasing effectiveness in inhibiting incidence and multiplicity of colon adenocarcinomas than administration of individual compounds at higher levels (43). More recently, combinations of COX-2 inhibitors and oxaliplatin, an anticancer agent, showed increased growth inhibition and death in human colon cancer cells (44). The use of naproxen (an NSAID) and its combination with nitrous oxide has also been shown to be effective against colon and urinary bladder cancer (45).

Previously, no group has investigated the combined treatment effects of aspirin with folic acid and calcium on the prevention of CRC. Our present study, an extension from our previous findings from in vivo studies (22), provides strong evidence for the successful use of nanotechnology-based formulations on the combined chemoprevention of colon cancer in the animal model. Because aspirin, folic acid, and calcium have different mechanisms of action, it was hypothesized that a combination effect of all these entities, administered in relatively low doses, would be additive for the prevention of CRC and would also be expected to exhibit minimal toxicity because of the targeting action creating a local effect with minimal systemic absorption. With the encouraging results from this study, we have established the importance of using a novel nanotechnology-based drug delivery system to ultimately target the human colon for chemopreventive treatment of colon cancer.

The PLGA 50:50 polymer is best known for its controlled release effect of drug over a prolonged period of time (46). Previous studies conducted in our laboratory (22, 25) measured the drug release kinetics of aspirin and folic acid from PLGA-based nanoparticles for a 72-hour period, indicating the controlled release of drugs encapsulated within polymer nanoparticles. The use of Eudragit S 100, a colon targeted class of polymer imparts site specificity to the drug-loaded nanoparticles because of a pH-sensitive solubility profile. The polymer dissolves at the colonic pH (≥7.0) but resists lower acidic pH (1–3) activity in the stomach or the small intestine (8). Upon transit into the colon, the Eudragit S 100 polymer dissolves thus exposing the drug-encapsulated PLGA 50:50 nanoparticles within the colonic environment. From our studies on drug release from rat cecal contents, a significant difference in the extent of release of aspirin at the end of the 48-hour run was observed; however, folic acid release was very similar with or without rat cecal contents. The increased rate of release of both chemopreventive agents was probably due to the presence of cecal enzymes which rapidly hydrolyze the polymer backbone, resulting in a faster release of drug from the matrix of the polymer shell (47).

The PLGA polymer–encapsulated drug is released over time, initially because of simple diffusion effects and subsequently because of hydrolysis of the polymer backbone leading to degradation of the polymer, allowing the remainder of the encapsulated drugs to be released. This ensures a prolonged local presence of chemopreventive agents and hence could explain the higher efficacy of these regimens than their unmodified counterparts. The controlled release of aspirin and folic acid from nanoparticle-based formulations and the presence of calcium ensure a sustained, subtoxic presence of chemopreventive agents to exhibit an additive chemopreventive effect. Nanoparticles due to their small size and large surface area are able to penetrate the mucous cell lining and improve bioavailability. Site-specific drug-loaded nanoparticles were successfully shown as chemopreventive regimens, increasing the amount of drug reaching the gut and showing a significantly different effect than plain unmodified drugs.

The most compelling evidence for our hypothesis was the results from set 2. Compared with azoxymethane-treated controls, both the unmodified and the encapsulated regimens administered at different dosage strengths displayed significant differences in ACF inhibition. However, the use of nanoparticle-based formulations proved to be more effective in their chemopreventive action opening the possibility of optimizing the dosage strengths at lower concentrations. Results from the in vivo studies presented here corroborate our earlier findings (22, 25) that a combined regimen may be more effective in the chemoprevention of colon cancer than individual drug administration. For the first time, we have been able to show in vivo that specific chemopreventive agents (aspirin and folic acid) when encapsulated within polymer-based nanoparticles and administered in combination doses with calcium are more effective in inhibition of ACF formation than when administered individually.

In summary, our study provides clear evidence of 2 important aspects in the chemoprevention of colon cancer: (i) combination treatment plays a significant role in the suppression of ACFs in the rat colon and (ii) the use of targeted nanotechnology-based combined regimens are more effective than the unmodified mixtures of chemopreventive agents in the chemoprevention of colon cancer. The drug delivery system used in this study has provided new information on the sustained release of chemopreventive agents in the colon and their effectiveness in CRC prevention and has potentially opened the doors for further research into these nanoscaled carriers for the chemoprevention of colon cancer. More studies are needed to determine the utility of this novel technology and its potential impact on chemoprevention as a whole. In addition, pharmacokinetic data on the release of aspirin and
folic acid from PLGA nanoparticles would be helpful in designing nanotechnology-based regimens for eventual use in humans. From the current study, this novel approach of delivering agents for chemoprevention of cancer appears to be promising.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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