Multitargeted Low-Dose GLAD Combination Chemoprevention: A Novel and Promising Approach to Combat Colon Carcinogenesis[1,2]

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Abstract

Preclinical studies have shown that gefitinib, licofelone, atorvastatin, and α-difluoromethylornithine (GLAD) are promising colon cancer chemopreventive agents. Because low-dose combination regimens can offer potential additive or synergistic effects without toxicity, GLAD combination was tested for toxicity and chemopreventive efficacy for suppression of intestinal tumorigenesis in adenomatous polyposis coli (APC)$^{Min/+}$ mice. Six-week-old wild-type and APC$^{Min/+}$ mice were fed modified American Institute of Nutrition 76A diets with or without GLAD (25 + 50 + 50 + 500 ppm) for 14 weeks. Dietary GLAD caused no signs of toxicity based on organ pathology and liver enzyme profiles. GLAD feeding strongly inhibited (80–83%, $P < .0001$) total intestinal tumor multiplicity and size in APC$^{Min/+}$ mice (means ± SEM tumors for control vs GLAD were 67.1 ± 5.4 vs 11.3 ± 1.1 in males and 72.3 ± 8.9 vs 14.5 ± 2.8 in females). Mice fed GLAD had >95% fewer polyps with sizes of >2 mm compared with control mice and showed 75% and 85% inhibition of colonic tumors in males and females, respectively. Molecular analyses of polyps suggested that GLAD exerts efficacy by inhibiting cell proliferation, inducing apoptosis, decreasing β-catenin and caveolin-1 levels, increasing caspase-3 cleavage and p21, and modulating expression profile of inflammatory cytokines. These observations demonstrate that GLAD, a novel cocktail of chemopreventive agents at very low doses, suppresses intestinal tumorigenesis in APC$^{Min/+}$ mice with no toxicity. This novel strategy to prevent colorectal cancer is an important step in developing agents with high efficacy without unwanted side effects.

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Introduction

Colorectal cancer is the third most common cause of cancer deaths in the United States [1]. Globally, about 1.24 million cases and 610,000 deaths were reported in 2008 from colorectal cancers (CRCs) [2]. Identifying strategies that interrupt the process of carcinogenesis without causing undue side effects is critical to long-term successful application of chemoprevention to high-risk populations. Chemoprevention of cancer is a strategy that employs treatments during the stages of carcinogenesis before the development of invasive cancer. Chemoprevention has emerged as a pragmatic approach to reduce the risk of various cancers including CRC [3].

Use of animal models in which disease progression can be followed allows testing of chemopreventive agents. The adenomatous polyposis coli (APC)$^{Min/+}$ mouse, one of the most studied models of intestinal tumorigenesis, harbors a dominant germ-line mutation in the *APC* gene at codon 850, the mouse homologue of a similar mutation in human patients with familial adenomatous polyposis [4,5]. APC$^{Min/+}$ mice develop multiple adenomas in the intestinal tract, primarily in the small intestine (SI) with fewer in the colon [5]. Thus, the APC$^{Min/+}$ mouse model is extensively used in both mechanistic and chemoprevention/intervention efficacy studies [5,6].

Drug development has led to discovery of potential chemopreventive agents that are effective at the preclinical and clinical levels [7–14]. For example, anti-inflammatory agents that target cyclooxygenase-2 (COX-2), such as celecoxib, are noteworthy because of their clinical efficacy in the prevention of polyp formation [12]. However, recent 5-year efficacy and safety analysis of adenoma prevention with celecoxib suggests a significant interaction between celecoxib treatment and cardiovascular and thrombotic events for those reporting a baseline history of atherosclerotic heart disease [12]. Overall, targeting COX-2 for colon cancer prevention is still valid, but use of higher doses of COX-2 inhibitors in individuals at high risk for colon cancer and, more so, in those at high risk for atherosclerotic events carries significant risk and indicates a need for new approaches to colon cancer prevention and treatment. Similarly, clinical use of the epidermal growth factor receptor (EGFR) inhibitor gefitinib and the selective ornithine decarboxylase (ODC) inhibitor D,L-α-difluoromethylornithine (DFMO) as anticancer agents is associated with skin and ototoxicity, respectively [15,16].

Recently, focus has been directed at the strategy of combining several chemopreventive agents at low doses to achieve greater inhibition of carcinogenesis [17–19]. Combining agents that work by different mechanisms has the potential of providing additive or synergistic effects, and lowering doses of individual agents in a combination offers the prospect of reduced toxicities [17–21]. Combinations of agents targeting polyamine synthesis and inflammation for chemoprevention of colon and intestinal carcinogenesis have been evaluated in several rodent models [18]. DFMO has been tested alone and in combination with several nonsteroidal anti-inflammatory drugs (NSAIDs), including piroxicam [22], aspirin [23], celecoxib [24], and sulindac [25]. Polyamines contribute to inflammatory responses by mechanisms in addition to those affecting tissue arginine levels. Polyamines also can influence the expression of the proinflammatory gene *COX-2* by a post-transcriptional mechanism [18]. The combinations of DFMO with NSAIDs have proven to be potent inhibitors of colon and intestinal polyp formation both in rodents and in humans [22–27].

The activation of EGFR results in promotion of growth through transcription of the *COX-2* gene and inhibition of apoptosis [28]. Similarly, the COX-2 signaling pathway activates EGFR phosphorylation and EGFR transcription [28]. Because both EGFR and COX-2 pathways are involved in cell growth and modulation of apoptosis, improved inhibition of these pathways by combination inhibitor regimens could partly account for the observed potentiation of the effects of the EGFR inhibitor erlotinib by the COX-2 inhibitor celecoxib [28].

Several studies suggest that statins [3-hydroxy-3-methylglutaryl CoA reductase (HMGR) inhibitors] suppress chemically induced colon carcinogenesis in animal models [29,30]. Clinical observations show an inverse relationship between the use of statins and the reduction of colon cancers [31]. In two large clinical trials involving patients with coronary artery disease, use of the statins led to a 43% [32] and a 19% [33] reduction, respectively, in the number of newly diagnosed cases of colon cancer during a 5-year follow-up period. In the same study, 83% of patients in both the pravastatin group and placebo group were given a daily dose of aspirin. Only patients taking pravastatin along with aspirin showed a greater reduction in the incidence of new cases of colon cancer, suggesting a possible synergistic effect of HMGR inhibitors with NSAIDs in colon cancer reduction [32]. In support of potential synergy between these agents, we previously showed inhibition of colon carcinogenesis in rodent models with a combination of low doses of statins and NSAIDs [19,34,35].

Preclinical studies have shown previously that the EGFR inhibitor gefitinib, the novel COX-lipoxygenase (LOX) inhibitor licofelone, the HMGR inhibitor atorvastatin, and the ODC inhibitor DFMO are all promising colon cancer chemopreventive agents (GLAD) [13,18,35,36]. Each has been shown to be effective as a single agent and in combinations, by targeting critical pathways of colon carcinogenesis, and each is currently in clinical use or in trials of various phases for treatment of colon or lung cancers, cholesterol lowering, or arthritis. In the present study, we tested a very low dose [≤10% of the maximum tolerated dose (MTD)] combination of these four agents for prevention of colon cancer.

Materials and Methods

Chemicals

All the chemopreventive agents (GLAD; Figure 1) were kindly provided by the National Cancer Institute's Chemopreventive Drug Repository (Rockville, MD). Primary antibodies to proliferating cell nuclear antigen (PCNA), caveolin-1, p21, and β-catenin were from Santa Cruz Biotechnology (Santa Cruz, CA) caspase-3 and β-actin were from Cell Signaling Technology (Danvers, MA) HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology. Multi-Analyte ELISArray Kit was from SA Biosciences (Frederick, MD).

Breeding and Genotyping of APC$^{Min/+}$ Mice

All animal experiments were performed in accordance with the institutional guidelines of the American Council on Animal Care and were approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center (OUHSC). Male APC$^{Min/+}$ (C57BL/6J) and female wild-type littermate mice initially were purchased from The Jackson Laboratory (Bar Harbor, ME) as founders, and our own breeding colony was established in the OUHSC Center Rodent Barrier Facility and genotyped according to the vendor's instructions. All mice were housed three per cage in ventilated cages under standardized conditions (21°C, 60% relative humidity, 12-hour light/12-hour dark cycle, 20 air changes per hour). All mice were allowed *ad libitum* access to the respective diets and automated tap water purified by reverse osmosis.

Diets

All ingredients for the semipurified diets were purchased from Bioserv (Frenchtown, NJ) and stored at 4°C before diet preparation. Diets were based on the modified American Institute of Nutrition 76A (AIN-76A) diet. GLAD was premixed with a small quantity of diet and then blended into bulk diet using a Hobart mixer. Both control and experimental diets were prepared weekly and stored in a cold room. In this study, experimental diets were prepared with AIN-76A diet containing 0 or 25 ppm gefitinib + 50 ppm licofelone + 50 ppm atorvastatin + 500 ppm DFMO (Figure 2*A*).

Bioassay: Intestinal Tumorigenesis in APC^Min/+ Mice

The antitumor efficacy of GLAD was assessed in male and female APC$^{Min/+}$ mice according to the experimental protocol summarized in Figure 2B. Five-week-old male and female mice were randomized for age and average body weights in each group (C57BL/6 or APC$^{Min/+}$ mice, 10 per group), and mice were fed the AIN-76A diet for 1 week. At 6 weeks of age, mice were fed control or GLAD experimental diets until termination of the study. Body weight, animal behavior, and food and fluid consumption were monitored weekly for signs of weight loss, lethargy, or decreased consumption that might indicate intestinal obstruction or anemia. Mice were checked routinely for any other abnormalities. After 14 weeks of feeding, at 20 weeks of age, all mice were killed by CO_2 asphyxiation, blood was collected by heart puncture, and serum was separated by centrifugation and stored at –80°C until further analysis. This termination time was chosen to minimize the risk of intercurrent mortality caused by severe progressive anemia, rectal prolapse, or intestinal obstruction, which usually occurs among Min mice at older than 20 weeks of age.

After necropsy, the entire intestinal tract was harvested, flushed with 0.9% NaCl, and opened longitudinally from the esophagus to the distal rectum. The tissue was flattened on filter paper to expose the tumors and briefly frozen on dry ice to aid visual scoring of tumors. The number, location, and size of visible tumors in the entire intestine were determined under a dissection microscope (5×). All tumors were scored and subdivided by location (duodenal, jejunal, ileum, and colon) and size (>2, 1–2, or <1 mm in diameter). This procedure was completed by two individuals, who were blinded to the experimental group and the genetic status of the mice. Colonic and other SI tumors that required further histopathologic evaluation were fixed in 10% neutral-buffered formalin and embedded in paraffin blocks. In addition, multiple samples of tumors from the intestines were harvested and stored in liquid nitrogen for molecular analysis.

Assessment of Liver Enzymes and Packed Cell Volume

Liver enzymes in serum were quantified by the Veterinary Associates Laboratory (Edmond, OK) using Pointe Scientific Reagents (Pointe Scientific, Canton, MI) and a Hitachi 717 chemistry analyzer, as per the manufacturer's instructions. For packed cell volume (PCV)/hematocrit measurement, blood was sampled by cardiac puncture with a 21-gauge needle attached to a 1-ml syringe and dispensed into a plastic microfuge tube on ice. Microhematocrit tubes containing ammonium heparin were then placed in the microfuge tubes and centrifuged in a hematocrit centrifuge for 5 minutes.

Immunohistochemistry

To evaluate the effect of GLAD, we assessed the PCNA, p21, and caveolin-1 expression in intestinal tumor tissue sections by immunohistochemistry. Briefly, paraffin sections were deparaffinized in xylene and rehydrated through graded ethanol solutions to phosphate-buffered saline (PBS). Antigen retrieval was carried out by heating sections in 0.01 M citrate buffer (pH 6) for 30 minutes in a boiling water bath. Endogenous peroxidase activity was quenched by incubating in 3% H_2O_2 in PBS for 5 minutes. Nonspecific binding sites were blocked
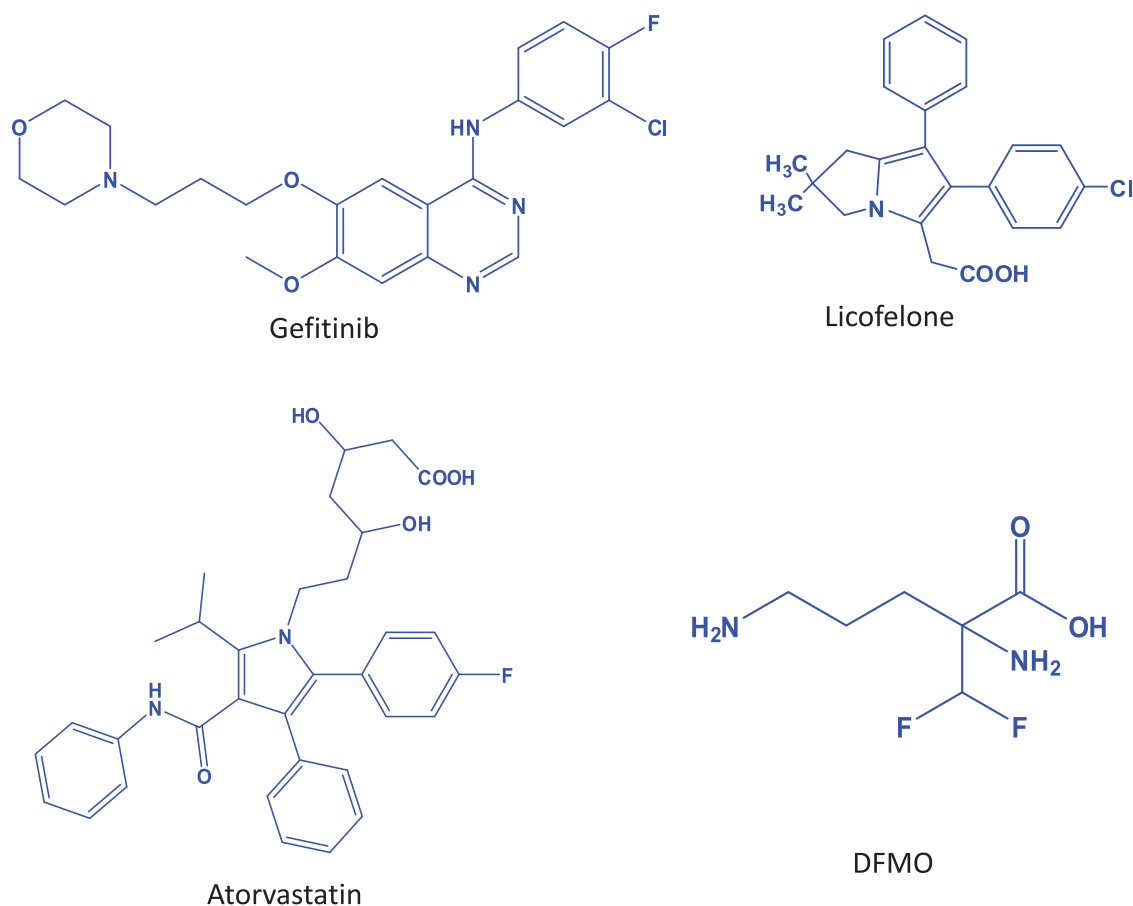

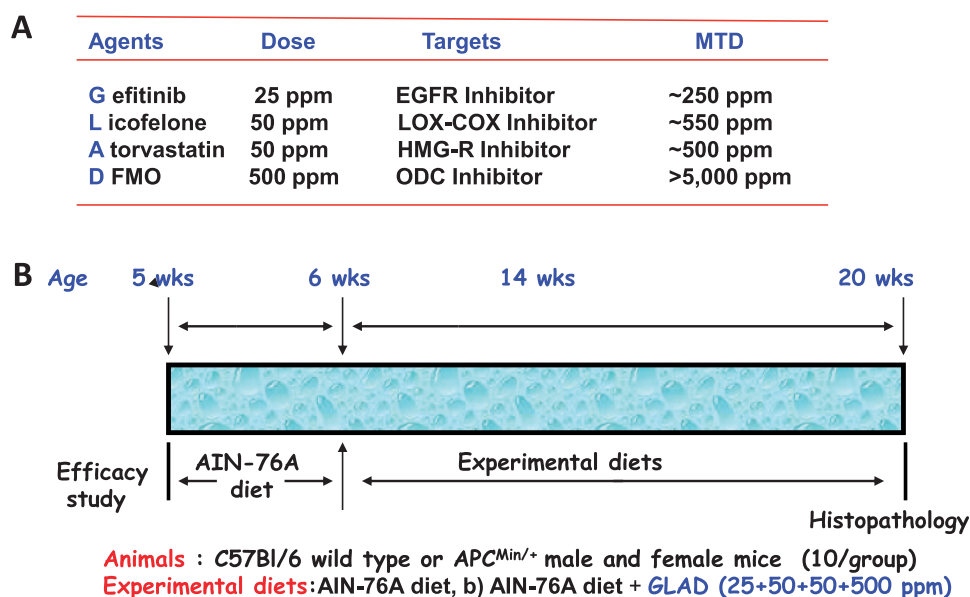**Figure 1.** Chemical structures of GLAD agents.

Figure 2. (A) Doses and molecular targets of GLAD agents. (B) Experimental design for evaluation of the chemopreventive efficacy of GLAD administered in the diet from 6 weeks of age to the end of the experiment. Modified AIN-76A was the control diet. The study was terminated after 100 days. For details about animals and treatments, see Materials and Methods section.

using protein block for 20 minutes. Sections then were incubated overnight at 4°C with 1:300 dilutions of mouse monoclonal antibodies against PCNA, p21, and rabbit polyclonal antibody against caveolin-1 (Santa Cruz Biotechnology). After several washes with PBS, the slides were incubated with secondary antibody for PCNA, p21, and caveolin-1 for 2 hours. The color reaction was developed with 3,3'-diaminobenzidine, according to the manufacturer's instructions given in the kit supplied by Zymed Laboratories (Camarillo, CA). Non-immune rabbit Igs were substituted for primary antibodies as negative controls. Scoring, using light microscopy at ×400 magnification, was performed by two investigators blinded to the identity of the samples. Cells with brown nuclei were considered positive. The proliferation index was determined by dividing the number of positive cells per polyp (upper, middle, and lower) and multiplying by 100.

Western Blot Analysis of Protein Expression

Intestinal polyps from mice were homogenized and lysed in ice-cold lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 1× protease inhibitor cocktail (Sigma, St Louis, MO)]. After a brief vortexing, the lysates were separated by centrifugation at 12,000g for 15 minutes at 4°C, and protein concentrations were measured with the Bio-Rad Protein Assay reagent (Hercules, CA). Proteins (50 μg/lane) from an aliquot were separated with electrophoresis through 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% milk powder, membranes were probed for expression of PCNA, p21, caspase-3, and β-actin in hybridizing solution (1:500 in TBS–Tween 20 solution) using the respective primary antibodies and then probed with their respective HRP-conjugated secondary antibodies. Detection was performed using the SuperSignal West Pico Chemiluminescence procedure (Pierce, Rockford, IL). The bands were captured on Ewen Parker Blue sensitive X-ray films and analyzed by densitometry.

Reverse Transcription–Polymerase Chain Reaction for p21 and β-Catenin mRNA Expression

Total RNA from intestinal polyp samples was extracted using the TRIzol RNA Kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Equal quantities of DNA-free RNA were used in reverse transcription (RT) reactions for making cDNA using SuperScript Reverse Transcriptase (Invitrogen). RT–polymerase chain reactions (PCRs) were performed for p21 and β-catenin using the Taq polymerase, 10 mM deoxyribonucleotide triphosphates (dNTPs), respective primers, and buffers from Invitrogen. For p21, denaturation at 94°C for 3 minutes was followed by 35 cycles at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 45 seconds. Oligonucleotide primer sequences used for p21 were given as follows: sense, 5'-TCCTGGTGATGTCCGACCTG-3'; antisense, 5'-TCCGTTTT-CGGCCCTGAG-3'. For β-catenin, denaturation at 94°C for 3 minutes was followed by 35 cycles at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 45 seconds. Oligonucleotide primer sequences used for the *β-catenin* gene were given as follows: sense, 5'-CGTCAGTGCAGGAGGCCGAG-3'; antisense, 5'-TCCTCA-GGGTTGCCCTTGCCA-3'. The PCR products were visualized and photographed under UV illumination.

Apoptosis Assay

Paraffin sections of 5-μm thickness were mounted on slides and rehydrated. They were stained using the terminal deoxynucleotidyl transferase–dUTP nick end labeling (TUNEL) method using the Fragment End Labeling DNA Fragmentation Detection Kit (Calbiochem, Billerica, MA) following the manufacturer's instructions to detect apoptotic nuclei. Terminal deoxynucleotidyl transferase binds to exposed ends of DNA fragments generated in response to apoptotic signals and catalyzes the template-dependent addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides are detected using streptavidin-HRP conjugate. Diaminobenzidine reacts with the labeled sample to generate an insoluble colored product at the site of DNA

fragmentation. Counterstaining with methyl green aids in the morphologic evaluation and characterization of normal and apoptotic cells. Stained apoptotic epithelial cells (a minimum of 10 microscopic fields per section) were counted manually in a single-blind fashion.

Inflammatory Cytokine Assay

Determination of inflammatory cytokine levels in serum was evaluated by ELISA (SA Biosciences) as per the manufacturer's instruction and our previous publications [13]. The Mouse Inflammatory Cytokines and Chemokines Multi-Analyte ELISArray Kit analyzes a panel of 12 proinflammatory cytokines in serum all at once using an ELISA protocol under uniform conditions. The cytokines and chemokines included in this array are interleukin 1A (IL-1A), IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, interferon-γ, tumor necrosis factor–α (TNF-α), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Results are expressed as nanogram per milliliter of serum. Determination was carried out in triplicate from each sample.

Sample Size and Statistical Analyses

For cellular and molecular outcome parameters, a sample size of six (depending on marker variability) per treatment was calculated to be adequate to produce effects that are statistically distinguishable. All results are expressed as means ± SE. Differences in body weights were analyzed by analysis of variance, and differences in tumor multiplicity and volume were determined by Student's t test. Differences were considered significant at the $P < .05$ level. All statistical analysis was performed in GraphPad Prism Software 5.0 (GraphPad Software, Inc, San Diego, CA).

Results

GLAD Lacks Overt Toxicity

Wild-type C57Bl/6 mice fed the GLAD diet did not show any overt toxicity or body weight loss. As expected, control diet–fed APC$^{Min/+}$ mice of both genders began to lose body weight at approximately 13 weeks of age because of intestinal obstruction and progressive anemia. APC$^{Min/+}$ mice fed the GLAD diet showed a steady increase in body weights similar to the wild-type mice with no noticeable signs of toxicity. Statistically significant ($P < .05$) differences in body weights were observed between the dietary groups (Figure 3, A and B). Our studies and other reports have shown that these agents (GLAD) administered to wild-type mice (at up to 10% MTD in the diet) for 6 weeks have not caused any observable toxicity or significant body weight loss (data not shown). Thus, GLAD doses applied in the efficacy studies were free from overt toxicity. APC$^{Min/+}$ mice fed the control diet showed alterations in most liver enzymes over the course of the 14-week experimental period (Figure 3C). We observed a several fold increase in the aspartate
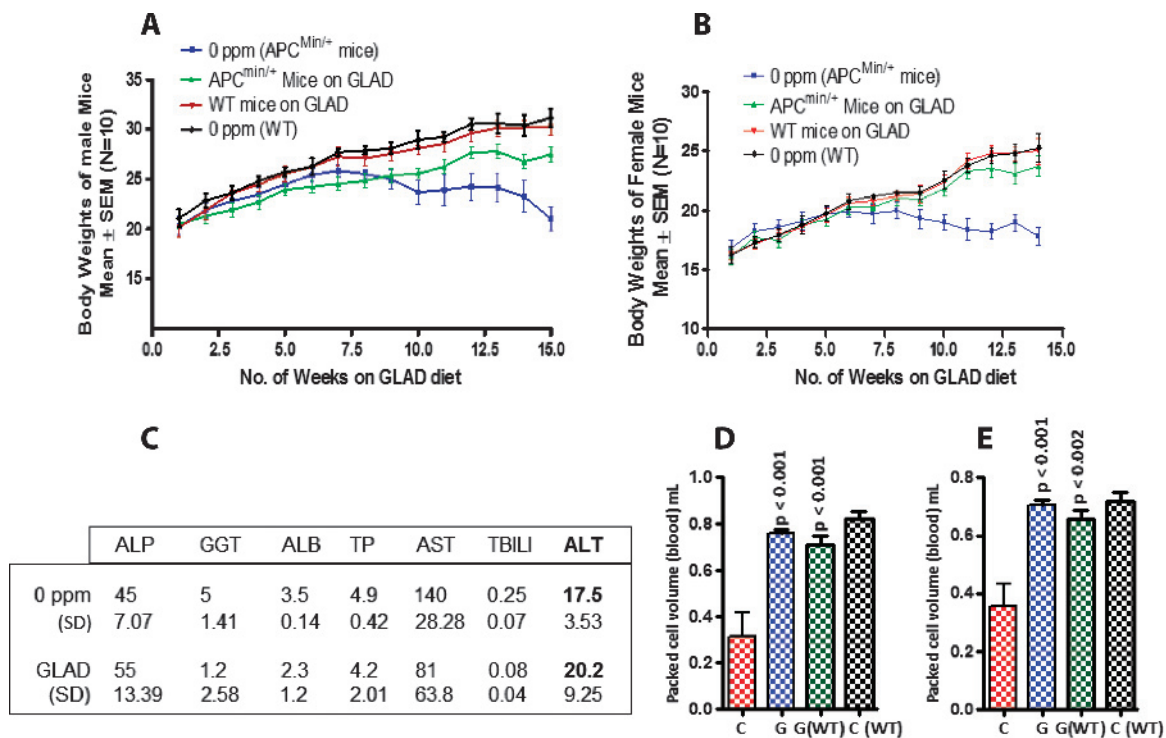

	ALP	GGT	ALB	TP	AST	TBILI	ALT
0 ppm	45	5	3.5	4.9	140	0.25	**17.5**
(SD)	7.07	1.41	0.14	0.42	28.28	0.07	3.53
GLAD	55	1.2	2.3	4.2	81	0.08	**20.2**
(SD)	13.39	2.58	1.2	2.01	63.8	0.04	9.25

Figure 3. Male and female C57BL/6J and APC$^{Min/+}$ mice (10 per group) were fed control diet or diet containing GLAD (25 + 50 + 50 + 500 ppm, respectively) for 100 days and body weights are plotted for males (A) and females (B). No significant differences were observed in the wild-type mice fed control or GLAD diet. Statistically significant differences in body weight between GLAD-treated and control groups were observed for APC$^{Min/+}$ mice. GLAD-treated animals were found to gain weight by the end of the study. (C) Liver enzyme profiles in the serum of APC$^{Min/+}$ mice fed control or GLAD diets. Serum from GLAD-fed animals showed marked decrease in aspartate aminotransferase and increased alanine aminotransferase levels. (D and E) PCVs of male or female wild-type and APC$^{Min/+}$ mice fed either control or GLAD diets. A significant increase in PCV was observed in treated *versus* untreated APC$^{Min/+}$ mice. The PCV of GLAD-treated APC$^{Min/+}$ mice is comparable to that of the control and treated wild-type mice.
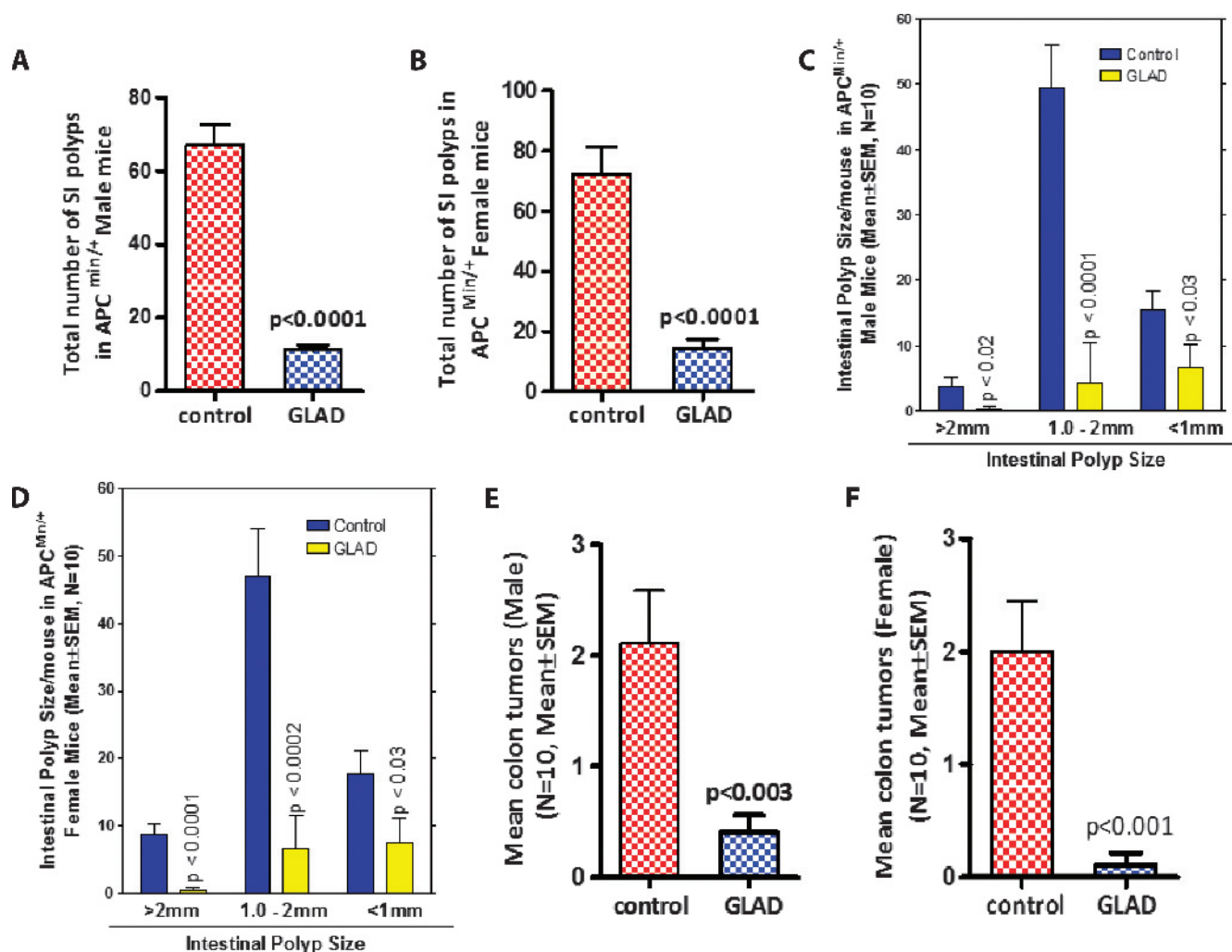
Figure 4. (A) Inhibition of total SI polyp formation in male APC$^{Min/+}$ mice by GLAD. Values are means ± SEM of 10 animals per treatment group. Control and treated groups are significantly different from one another ($P < .0001$). (B) Inhibition of total SI polyp formation in female APC$^{Min/+}$ mice by GLAD. Values are means ± SE of 10 animals per treatment. Control and treated groups are significantly different from one another ($P < .0001$). (C and D) Polyp sizes in the SI of APC$^{Min/+}$ mice. Intestines were divided into sections, examined under a stereomicroscope, and the size of polyps was determined. Values are given as means ± SE of 10 animals per treatment. Tumors greater than 2-mm diameter were suppressed by >95% ($P < .02–.0001$) in GLAD-treated mice. (E) Average number of colon tumors per mouse in control and treated male APC$^{Min/+}$ mice. A significant ($P < .003$) inhibition of colon tumors was observed with GLAD treatment. (F) Average number of colon tumors per mouse in control and treated APC$^{Min/+}$ female mice. A significant ($P < .001$) inhibition of colon tumors was observed with GLAD treatment.

aminotransferase/alanine aminotransferase ratio in APC$^{Min/+}$ mice fed the control diet, whereas this ratio was reduced by 50% in APC$^{Min/+}$ mice fed the GLAD diet, indicating less liver damage (Figure 3C). The APC$^{Min/+}$ mice fed the GLAD diet had no significant anemia, and PCV of these mice is comparable to that of wild-type mice fed GLAD (Figure 3, D and E). These results clearly show that GLAD, at the tested doses, lacks overt toxicity.

Chemopreventive Efficacy of GLAD in APC$^{Min/+}$ Mice

To assess the effects of GLAD on intestinal tumor formation in APC mutant transgenic mice, we examined the polyp number and size in different regions of the SI and colon. GLAD treatment of APC$^{Min/+}$ mice resulted in a strong inhibition of intestinal tumorigenesis in terms of decreased polyp number and size in the SI (Figure 4, A–D). Male mice fed control and GLAD diets developed

67.1 ± 5.4 and 11.3 ± 1.1 (means ± SEM), respectively; females developed 72.3 ± 8.9 and 14.5 ± 2.8 SI polyps, without and with GLAD, respectively. GLAD caused 83% or 80% ($P < .0001$) inhibition of intestinal tumors in male and female APC$^{Min/+}$ mice, respectively. Specifically, control APC$^{Min/+}$ mice developed, on average, 3.7, 49.4, and 15.57 polyps of >2 mm, 1 to 2 mm, and <1 mm sizes, respectively, in males and 8.7, 47, and 17.7 polyps of those sizes in females. GLAD diet feeding for 14 weeks significantly decreased polyp numbers and sizes in the SI (Figure 4, A–D). Mice fed GLAD had >95% fewer SI polyps of a size of >2 mm compared with control mice ($P < .02–.0001$) (Figure 4, C and D). GLAD also significantly decreased the number of colonic polyps in APC$^{Min/+}$ mice (by 75% and 85% in males and females, respectively; $P < .03–.001$; Figure 4, E and F). The GLAD cocktail also decreased the size of colonic polyps by 46% and 56% in male and female mice, respectively.

GLAD Feeding Inhibits Proliferation and Induces Apoptosis in Intestinal Polyps of APC$^{Min/+}$ Mice

To assess whether GLAD efficacy is associated with *in vivo* antiproliferative and proapoptotic effects, SI polyps and colon tumors were analyzed for PCNA and TUNEL, widely used markers for cell proliferation and apoptosis, respectively, by either immunostaining or immunoblot analysis. Microscopic examination of tissue sections showed a decrease in PCNA-positive cells (Figures 5A and 6A) but an increase in TUNEL-positive cells (Figure 6B) in intestinal polyps from APC$^{Min/+}$ mice fed GLAD diet compared with control diet. Qualitative microscopic examination of PCNA-stained sections showed a substantial decrease in PCNA-positive cells in the intestinal polyps from GLAD-fed mice compared with the untreated controls. Quantification of PCNA staining showed 72% (SI polyps) and 83% (colon tumors) ($P < .0001$) decrease in proliferation indices in colon tumors from GLAD-fed mice compared with controls. TUNEL-positive cells increased by 2.5-fold ($P < .0001$) (Figures 6B and W2). These results were confirmed further by immunoblot analysis (Figure 5A), with β-actin as a loading control. Together, these results show *in vivo* antiproliferative and proapoptotic effects of GLAD in polyps, supporting its chemopreventive efficacy against spontaneous intestinal tumorigenesis in APC$^{Min/+}$ mice.

GLAD Decreases Caveolin-1 and β-Catenin and Increases p21 and Caspase-3 in Intestinal Polyps of APC$^{Min/+}$ Mice

Alteration in the β-catenin pathway due to loss of APC function has been implicated in CRC initiation and progression [37]. β-Catenin and caveolin-1 have important roles in cell cycle progression. Expression of these two proteins was analyzed in SI polyps and colon tumors by immunohistochemistry or RT-PCR (Figures 5B and 6A). A significant decrease in caveolin-1 and β-catenin protein expression was observed in intestinal polyps from GLAD-treated mice (Figure 5B). Dietary administration of GLAD also resulted in a significant increase in p21 expression and caspase-3 cleavage, indicators of apoptosis, in intestinal polyps compared with control polyps as observed with immunostaining, immunoblot analysis, and/or RT-PCR (Figures 5C and 6A). Collectively, these results correlate with the inhibition of proliferation and increase in apoptosis.

Modulation of Inflammatory Cytokines

To examine GLAD effect on expression of various circulating cytokines, we screened serum from both control and GLAD-fed APC$^{Min/+}$ mice with an inflammatory cytokine array (Figure W1). Among 12 cytokines tested, GLAD significantly ($P < .05$ to $P < .0001$) decreased circulating levels of 10 and increased the expression of one compared with
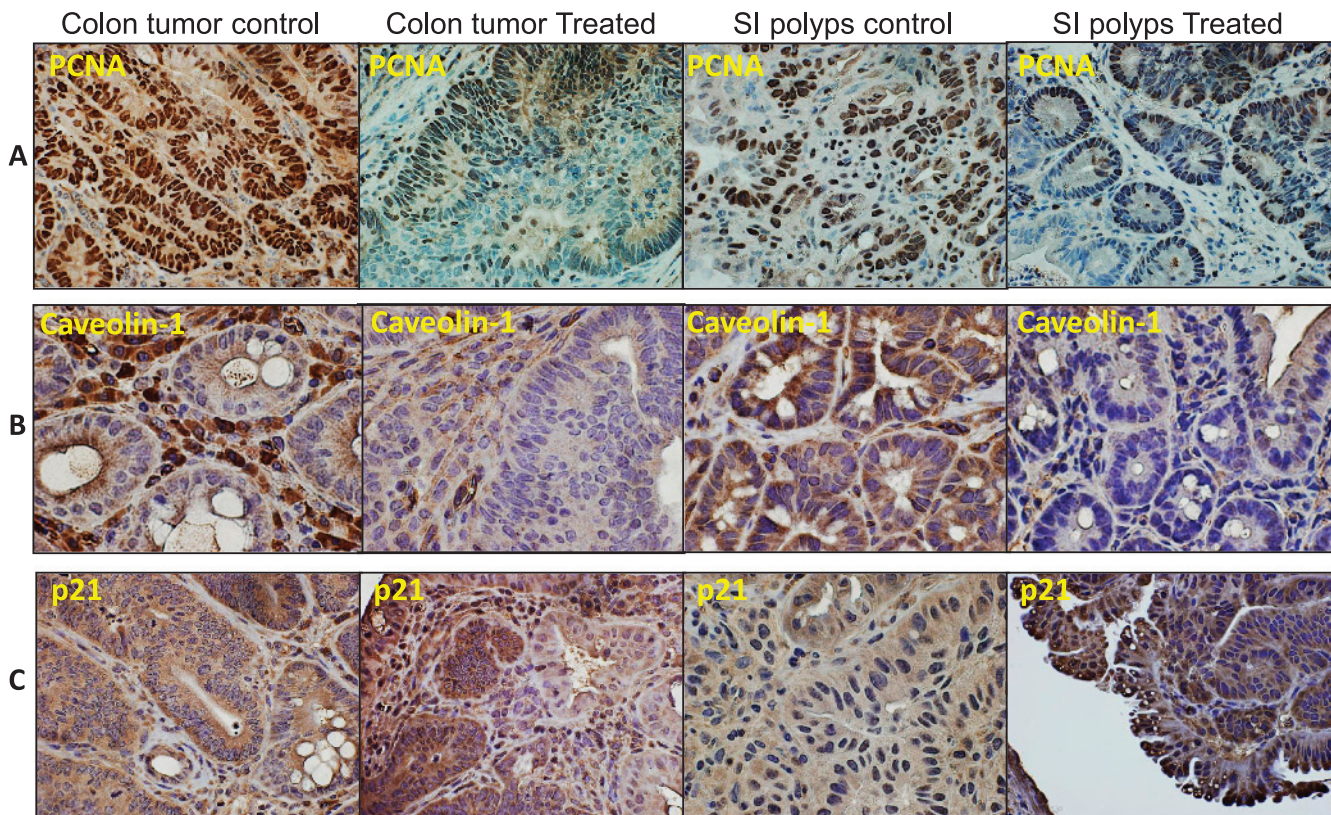

| | Colon tumor control | Colon tumor Treated | SI polyps control | SI polyps Treated |

Figure 5. (A) Serial paraffin sections of SI and colon from APC$^{Min/+}$ mice were subjected to immunohistochemical analysis using an anti-PCNA monoclonal antibody. Intense positive staining for PCNA in the tumor region of control animals was observed. Staining for PCNA was decreased in the nuclei of SI polyps and colon tumors from GLAD-treated animals. (B) Immunohistochemical staining for caveolin-1 expression in SI polyps and colon tumors from APC$^{Min/+}$ mice fed control diet or treated with GLAD. Marked decrease in the expression of caveolin-1 was observed in SI polyps and colon tumors in treated animals compared with that in control animals. (C) Immunohistochemical staining for p21 expression in SI polyps and colon tumors from APC$^{Min/+}$ mice fed control diet or treated with GLAD. Marked increase in the expression of p21 was observed in SI polyps and colon tumors in treated animals compared with that in control animals. All images are at ×60 magnification; p21-stained–treated SI polyp image is taken at ×40 magnification.
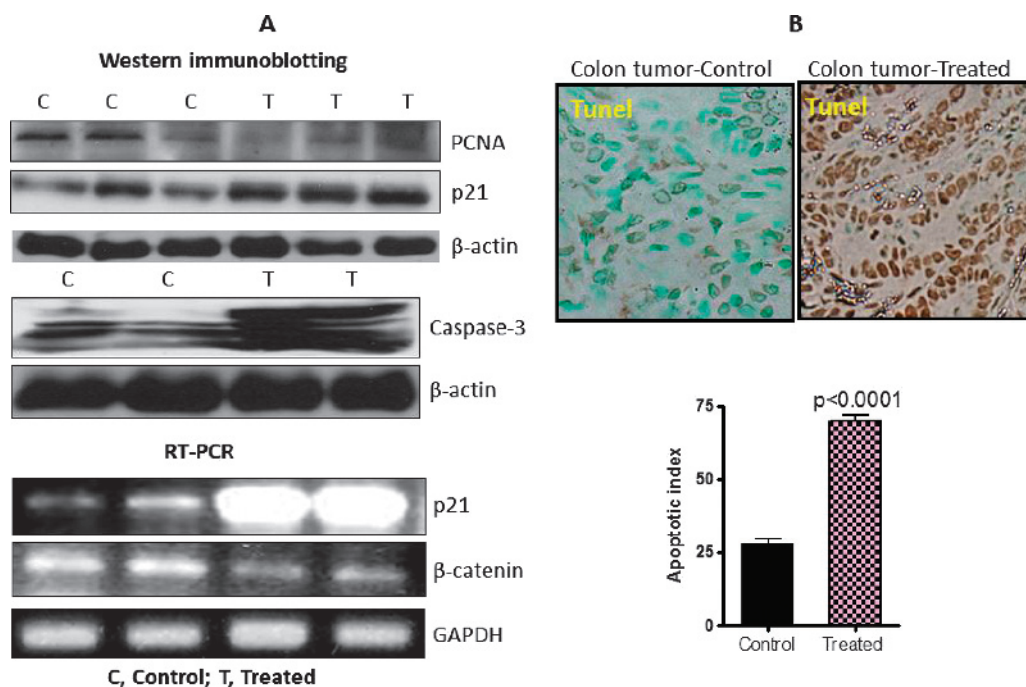
Figure 6. (A) Modulatory effects of GLAD on PCNA, p21, caspase-3, and β-catenin protein or mRNA expression in intestinal polyps of treated and untreated APC$^{Min/+}$ mice. A significant suppression of PCNA and β-catenin protein expression was observed upon GLAD treatment in APC$^{Min/+}$ mice. The protein and mRNA expression of p21 was increased with GLAD treatment. Caspase-3 cleavage was significantly higher in the GLAD-treated SI polyps compared to untreated polyps. (B) TUNEL assay for apoptotic cells in colon tumors from APC$^{Min/+}$ mice fed control diet or treated with GLAD. A significant induction of apoptosis was observed in colonic tumors of treated mice compared with those of untreated mice. A significant difference was observed in apoptotic index between GLAD-treated and control groups. Tumors from treated mice showed approximately two-fold ($P < .0001$) induction of apoptosis compared with tumors from control mice.

control (Figure W1). G-CSF was observed to increase by approximately 37%, but no significant difference was observed in the expression of GM-CSF (Figure W1).

Discussion

Preclinical, clinical, and epidemiological studies have shown clearly that chemopreventive agents are effective for CRC. The potential role of COX-2, 5-LOX, ODC, and EGFR signaling is well established in colon carcinogenesis. Targeting of individual implicated enzymes shows promising results; however, toxicity is a problem with higher doses of many single agents. NSAIDs and COX-2–selective inhibitors have been tested widely for CRC prevention. However, the gastrointestinal and cardiovascular toxicities exhibited by these agents have prompted the search for novel approaches or agents with similar or higher efficacies but devoid of unwanted side effects. Similarly, the EGFR inhibitor gefitinib has shown chemopreventive efficacy, but higher doses of gefitinib in humans result in diarrhea, skin rash, and weight loss [15,38]. In an attempt to limit toxicities, we have tested a low-dose, multiagent combination consisting of gefitinib, licofelone (a novel dual COX-LOX inhibitor), atorvastatin (an HMGR inhibitor), and DFMO (an ODC inhibitor) at very low doses (≤10% MTD) as a new chemoprevention strategy for colon cancer. Here, we showed that feeding of the GLAD combination in low dose decreases spontaneous intestinal tumorigenesis in APC$^{Min/+}$ mice, a genetically predisposed animal model of human familial adenomatous polyposis. The key findings of this study are given as follows:

1) GLAD significantly reduced the number as well as the size of SI polyps and colonic tumors in male and female APC$^{Min/+}$ mice without any toxicities; 2) the chemopreventive effect of GLAD was associated with a decrease in proliferation and an increase in apoptosis indices in polyps; and 3) GLAD decreased β-catenin and caveolin-1 levels in intestinal polyps and decreased various proinflammatory cytokines in serum. These results, together with earlier findings with these agents tested individually [13,24,35,39–42], strongly support the chemopreventive efficacy of GLAD in this preclinical animal model of CRC, suggesting the potential of this regimen for chemoprevention of human CRC.

The efficacy of GLAD in decreasing the number and size of SI polyps in both male and female APC$^{Min/+}$ mice is comparable to, or better than, that of the individual agents at high doses or of combinations of only two GLAD agents (Table W1). For example, previous studies with APC$^{Min/+}$ mice have shown that DFMO, at 0.5% to 2% in drinking water (~equivalent to 5000 and 20,000 ppm in the diet), suppressed SI and colonic tumor formation by 25% to 53% and 8%, respectively, compared with that in mice fed control diet [35,39–42]. The combination of DFMO with piroxicam suppressed SI polyps by only 11%, and DFMO with arginine caused about 44% suppression of colonic tumors (Table W1) [39]. Gefitinib (10 mg/kg body weight, i.e., ~equivalent to 200 ppm in the diet) caused about 71% inhibition of tumor multiplicity in azoxymethane-induced colon cancer in rats [42]. We previously have shown that a combination of low doses of statin and sulindac or naproxen suppressed azoxymethane-induced colonic aberrant crypt foci formation in rats more effectively than each compound alone [19]. In addition, the

combination of 100 ppm atorvastatin and 300 ppm celecoxib in the diet significantly suppressed the intestinal polyps compared with the control group [35]. In comparison, the GLAD combination, with very low doses ($\leq$10% MTD) of each agent, suppressed SI and colon tumors by 85% in APC$^{Min/+}$ mice and caused a significant decrease in the size of SI and colonic polyps in both male and female mice (Figure 4). Collectively, these results support additive to synergistic activity of the agents in the low-dose GLAD combination with efficacy comparable to, or even better than, that with the high-dose individual agents.

Overexpression of β-catenin is associated directly with increased proliferative index in CRC and results in a more aggressive cancer phenotype. A direct correlation between β-catenin signaling and regulation of angiogenesis and tumor growth also has been shown [19,37]. In the present study, APC$^{Min/+}$ mice showed an increased level of β-catenin together with increased expression of caveolin-1 in polyps. Expression of both proteins was decreased significantly by GLAD treatment, consistent with previous observations with several of single agents [13,19].

Various inflammatory cytokines also are associated with growth and development of CRC. The levels of circulating IL-6, IL-8, M-CSF, and the IL-1 receptor antagonist significantly increase with the clinical stage of CRC [43,44]; and increased levels of IL-6, TNF receptor type I, soluble IL-2 receptor a, and TNF-α have been observed with increasing tumor grade and bowel wall invasion [43,44]. Some of these cytokines can be modulated by COX-2 and some of them also are regulated by the β-catenin pathway [45,46]. Induction of COX-2 is also regulated by TNF-α and IL-1β [45,46]. Tumor-promoting roles of TNF-α, interferon-γ, IL-1α, IL-1β, and IL-6 during cancer development are well documented [47]. IL-1β has been shown to enhance the production of vascular endothelial growth factor through IL-2, which was shown to induce angiogenesis in colon cancer cells [48]. TNF-α and IL-1β are key cytokines involved in inflammation, immunity, and cellular organization [49]. In a previous study, we observed that licofelone treatment alone led to significant decreases in most proinflammatory cytokines [13]. Therefore, we also examined the GLAD effect on the serum inflammatory cytokine profile using cytokine array analysis. The expression of TNF-α and IL-1β proteins was substantially upregulated in the serum of control APC$^{Min/+}$ mice compared with that in wild-type mice. GLAD significantly decreased the levels of the tumor-promoting and proinflammatory cytokines in the serum of APC$^{Min/+}$ mice (Figure W1). Thus, the suppression of TNF-α and IL-1β expression by GLAD may contribute to the low frequency of polyps observed in this study. Collectively, these results suggest that the GLAD combination may exert some of its chemopreventive effects through its immunomodulatory activities.

Studies are needed to evaluate the appropriate doses for clinical settings. Careful statistical approaches like factorial designs involving multiple combinations must be used along with individual agent controls in the experiments to optimize the doses of these multiple agents for combination usage. The factorial design is a natural choice for testing multiple treatment modalities in the same prevention setting because it allows the assessment of the drug effect for each single modality, as well as that of the combinations [50]. Factorial designs are efficient in estimating the chemoprevention effect when there is a positive interaction (synergistic effect) or no interaction between tested agents. The interaction can be quantified and the chemopreventive effects associated with paired modalities can also be estimated [48]. If agents have different toxicity profiles, combination of the agents (e.g., in a factorial design) can increase efficacy without increasing toxicity. The Physicians' Health Study (aspirin and beta-carotene) and the Alpha-Tocopherol, Beta-Carotene Trial are few examples of the successful implementation of factorial designs. Collectively, the results presented here support further development of GLAD and other multitargeted, multiagent combinations in chemoprevention and treatment of colon cancers.

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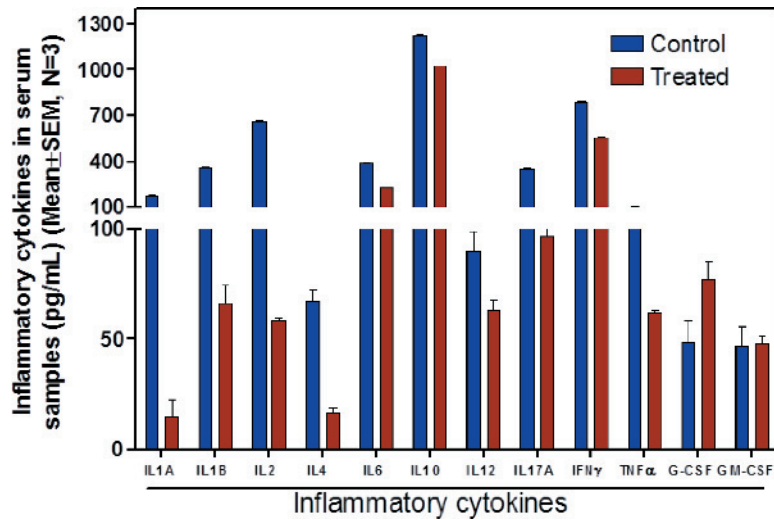
Figure W1. Effect of GLAD on inflammatory cytokines in serum samples from treated and untreated APC$^{Min/+}$ mice as analyzed by ELISA. All assayed cytokines except G-CSF and GM-CSF were decreased by GLAD.
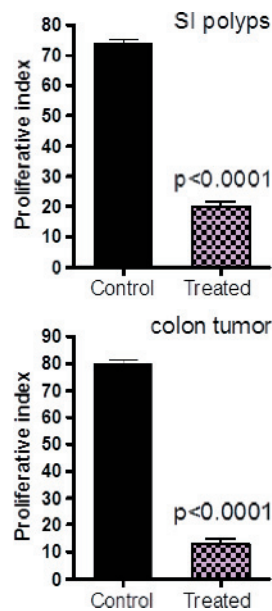

Figure W2. Immunohistochemical staining for PCNA in intestinal tumors from APC$^{Min/+}$ mice fed control diet or treated with GLAD. A significant difference ($P < .0001$) was observed in proliferative index between GLAD-treated and control group polyps.

Table W1. The Effects of Individual and Dual Combinations of Chemopreventive Agents in APC$^{Min/+}$ Mice.

Agents and Doses	No. of SI Polyps Control	No. of SI Polyps Treated	% Efficacy* (∼ % Inhibition)	Colon Polyps Control	Colon Polyps Treated	% Efficacy* (∼ % Inhibition)	Reference
DFMO, 1%	42	24	42	–	–	–	[37]
DFMO, 2%	45	21	53	3.5	3.2	8	[38]
DFMO, 0.5%	41	24	41	0.5	0.7	–40	[39]
DFMO, 0.5%	32	24	25	–	–	–	[24]
Lipitor, 100 ppm	40	27	32	1.4	0.6	57	[33]
Licofelone, 150 ppm	48	17	64	1.8	0.5	72	[13]
Licofelone, 300 ppm	48	8	83	1.8	0	100	[13]
DFMO (0.5%) + celecoxib (500 ppm)	32	4	87	–	–	–	[24]
Lipitor (100 ppm) + celecoxib (300 ppm)	40	6	85	1.4	0.1	92	[33]
DFMO (0.5%) + arginine	47	56	–19	0.9	0.5	44	[39]
DFMO (1%) + piroxicam (50 ppm)	42	11	11	–	–	–	[37]

*The % efficacy of the agents determined by the authors on the basis of the published studies indicated in the references.