A hexane fraction of american ginseng suppresses mouse colitis and associated colon cancer: anti-inflammatory and pro-apoptotic mechanisms.

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory condition associated with a high colon cancer risk. We have previously reported that American Ginseng (AG) extract significantly reduced the inflammatory parameters of chemically induced colitis. The aim of this study was to further delineate the components of AG that suppress colitis and prevent colon cancer. Among five different fractions of AG (Butanol, Hexane, Ethylacetate, Dicholoromethane and Water), a Hexane Fraction has particularly potent anti-oxidant and pro-apoptotic properties. The effects of this fraction were shown in a mouse macrophage cell line (ANA-1 cells), in a human lymphoblastoid cell line (TK6), and in an ex-vivo model (CD4+/CD25- primary effector T cells). A key in vivo finding was that compared with the whole AG extract, the Hexane Fraction of AG was more potent in treating colitis in a dextran sulfate sodium (DSS) mouse model, as well as suppressing azoxymethane (AOM)/DSS-induced colon cancer. Furthermore, TUNEL labeling of inflammatory cells within the colonic mesenteric lymph nodes (MLN) was elevated in mice consuming DSS + the Hexane Fraction of AG. Results are consistent with our in vitro data, and with the hypothesis that the Hexane Fraction of AG has anti-inflammatory properties, and drives inflammatory cell apoptosis in vivo, providing a mechanism by which this fraction protects from colitis in this DSS mouse model. This study moves us closer to understanding the molecular components of AG that suppress colitis, and prevent colon cancer associated with colitis.

Key Words: Inflammation, Ginseng, Colitis, Hexane, Colon, Apoptosis
Introduction

Inflammatory Bowel Disease (IBD) [Ulcerative colitis (UC) and Crohn’s Disease (CD)] is a group of chronic disorders of unknown etiology characterized by inflammation in the gastrointestinal tract (1) and associated with an increased risk of colon cancer (2). The histopathogenesis of UC-associated colorectal cancer involves a stepwise progression from inflamed, hyperplastic epithelia, to flat dysplasia, to adenocarcinoma (3). Colitis-associated colorectal cancer (CAC) accounts for up to 5% of all colorectal cancers (4), and the incidence of CAC in UC patients increases with age. The azoxymethane (AOM)-tumor model has been used extensively to identify molecular mechanisms involved in the multistage progression of sporadic colorectal cancers (5). The addition of DSS to AOM has been used frequently because of its reproducibility, and the cyclical, dynamic nature of colitis replicates the flare ups characteristic of human UC (6-8).

American Ginseng (AG, *Panax quinquefolius*) is a perennial native of North America, and ginseng is one of the most popular medicinal herbs used in the world (9). AG has antioxidant properties, and targets many key players involved in inflammation, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (Cox-2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (10). In a series of studies, we have recently reported that AG suppresses the expression of inflammatory markers of colitis and prevents colon cancer associated with colitis (10-12). In general, active or inactive chemical entities obtained from ginseng species can be classified into five categories: saponins, polysaccharides, polyynes, flavonoids, and volatile oils (13). Ginseng’s saponins (generally called ginsenosides), and acidic polysaccharides of AG...
have been the main focus of its pharmacological activities (14-19). Water soluble polysaccharides also have medicinal properties, including immune-modulating and anti-proliferative effects (13). Other putative active components of AG, include polyacetylenes such as panaxynol and panaxydol, which are non-polar compounds (20). In contrast to ginsenosides and polysaccharides; polyynes, flavonoids, and volatile oils have been less studied, and therefore less is known about their medicinal properties. To further delineate the putative active components of AG against colitis, we have used Bioassay-Guided Fractionation. In doing so, we show here that a Hexane Fraction of AG is a potent anti-oxidant, can drive inflammatory cell apoptosis, and is more effective in its ability to ameliorate colitis and prevent colon cancer in mice compared with the whole AG extract.

Materials and Methods

Bioassay-Guided Fractionation of AG extract

The *P. quinquefolius* extract has been described previously in detail by our laboratory (10). For bioassay-guided fractionation, 10 gm of AG extract was dissolved in 150 ml of water and sequentially partitioned against 3 x 50 ml aliquots of: hexane, dichloromethane, ethyl acetate, water, and butanol. The fractions were reduced to near dryness on a vacuum centrifuge, freeze dried, and their respective dry weights determined: water fraction, 7.320 g (i.e., 73% of the original material); butanol fraction, 1.544 g; ethyl acetate fraction, 0.064 g; dichloromethane fraction, 0.062 g and hexane fraction, 0.044 g. Each fraction was then re-dissolved in a small volume of solvent to facilitate blending with the appropriate amount of maltodextrin to give a final weight of
10 g after a second round of evaporation by vacuum centrifuge and freeze drying. Thus, the original extract was subdivided based on polarity and reconstituted with maltodextrin to give an equivalent weight as the starting material for bioassay. All fractions were thoroughly vortexed to give a free flowing powder and split into two: one set was retained at NRC Canada as a reference; the other used for bioassay. Neat maltodextrin was used as a negative control.

**Analysis of the Hexane Fraction of AG**

Details are provided in the Supplementary Text.

**Fatty acid analysis by gas chromatography (GC)-mass spectrometry (MS) and flame ionization detector (FID)**

Details are provided in the Supplementary Text.

**Liquid Chromatography (LC)-UV analysis**

Details are provided in the Supplementary Text.

**Cell culture and treatment**

ANA-1 murine macrophage cells (a kind give from Dr. Michael Espey, National Cancer Institute, Bethesda, MD), TK6 lymphoblastoid cells (a kind give from Dr. Curtis Harris, National Cancer Institute, Bethesda, MD), and mouse primary CD4+/CD25-effector T cells were cultured and treated as described in detail in the Supplementary
Text. Although no authentication of the ANA-1 or TK6 cell lines was done by the authors, cells looked and behaved as we have observed for over a decade.

**DSS mouse model of colitis**

We followed our previous protocol for our DSS (MP Biomedicals, Solon, OH: 36 000-50 000 mw) mouse model of colitis (10). 11.9 mg/kg of whole AG extract or the Hexane Fraction of AG were dissolved in 100 µl 1x PBS per mouse and administered daily by oral gavage (per os: p.o.). 11.9 mg/kg daily, which is the human equivalent dose of 58 mg daily (21). Of note, currently the use of ginseng in human clinical trials can range anywhere from 200 mg to 9 g daily (22, 23). The control group of mice was given 100 µl of maltodextrin dissolved in 1x PBS by oral gavage. All procedures performed were in accordance with the Guide for care and Use of laboratory animals (National Research Council, Washington, DC) and approved by the Animal Resource Facility, University of South Carolina, Institutional Animal Care and Use Committee. Additional details are provided in the Supplementary Text. Supplementary Figure 1 outlines the time line of the protocol.

**Disease activity index (DAI)**

The DAI was calculated for each animal as done previously (12). Additional details are provided in the Supplementary Text.
Quantification of inflammation to examine effects on colitis

Paraffin embedded tissues were serially sectioned, and one section from each mouse was stained with H&E. Sections were microscopically examined for histopathologic changes using the system described in Supplementary Text, and as we described previously (12). Sections were evaluated independently by two blinded investigators (D.P. and A.C.).

AOM/DSS-induced colon cancer model

We carried out experiments with the AOM/DSS model of colitis-driven colon cancer as we have described previously (24). 11.9 mg/kg of the Hexane fraction of AG, whole AG extract and vehicle groups (1x PBS), were given to the mice at day 14 (after AOM and first week of DSS) by oral gavage and continued daily throughout the course of the experiment. The mice were euthanized at day 35 (1 ½ cycles), and day 50 (2 cycles. Additional details are provided in the Supplementary Text. Supplementary Figure2 outlines the time line of the protocol.

Definition of terms to quantify the effects of treatment on pre-cancerous and cancerous lesions in the AOM/DSS mouse model.

All lesions were examined blindly by a trained pathologist, specializing in mouse tissues. Details are provided in the Supplementary Text.
Immunohistochemical staining, western blot analysis and antibodies, real-time PCR, Annexin V and TUNEL assays

We followed the same protocol as outlined by our group previously (24, 25). Additional details are provided in the Supplementary Text.

Statistical analysis

Statistical analysis was done using one-way ANOVA with Scheffe's post hoc test for TUNEL scores or the Kruskal-Wallis test when comparing histology inflammatory scores. A two-way ANOVA for repeated measures was used to test for group and time effects on clinical data (e.g., DAI) over successive days of observation. For flow cytometry data, differences between groups were compared using a two-tailed paired Student's t test or an unpaired Mann-Whitney U test. Results were analyzed using the Stat-View II statistical program (Abacus Concepts, Inc.) and Microsoft Excel (Microsoft) for Macintosh computers. Single-factor variance ANOVA analyses were used to evaluate groups. A Fisher’s exact test was used to test the significance of association between treatments and classifications (inflammation, ulceration, polyps, low grade dysplasia, high grade dysplasia and adenocarcinomas). The P value chosen for significance in this study was 0.05.

Results

The Hexane Fraction of AG suppresses iNOS and Cox-2 expression

We have previously shown that whole AG extract suppresses the expression of inflammatory markers in ANA-1 mouse macrophages (10). To better delineate the active
ingredients in AG, we first screened ANA-1 mouse macrophage cells for suppression of interferon gamma (IFN-γ)-induced iNOS expression by various AG fractions obtained through Bioassay-Guided Fractionation. Interestingly only the Hexane Fraction of AG (260 µg/ml) was able to suppress the induction of iNOS protein to an extent similar to that of the whole AG extract (Figures 1A and 1C and Supplementary Figure 3). To confirm these anti-inflammatory properties, we also examined Lipopolysaccharide (LPS)-induced expression of Cox-2 protein in the ANA-1 cells. Figures 1B and 1C indicate Cox-2 protein expression was also suppressed by the Hexane Fraction of AG. To determine whether the Hexane Fraction of AG regulates iNOS and Cox-2 expression at the transcriptional level, we carried out Real-Time PCR analysis of these two genes. Figures 1D and 1E indicate the Hexane Fraction of AG (260 µg/ml) suppresses the induced transcription of both iNOS and Cox-2, respectively.

The Hexane Fraction of AG induces apoptosis in inflammatory cells

We have previously shown that AG drives apoptosis of inflammatory cells (12), providing a mechanism by which AG suppresses inflammation associated with colitis. To further delineate the active ingredient responsible for apoptosis, and complement our screen of AG fractions (Figure 1, and Supplementary Figure 3), we treated TK6 cells with the increasing concentrations (0 - 1000 µg/ml) of whole AG extract and the Hexane Fraction of AG for 24 hrs. Results are shown in Supplementary Table I, and Supplementary Figure 4A. Interestingly, although the whole AG fraction had a modest effect on apoptosis of these cells [3.4-fold increase in number of cells undergoing early apoptosis when exposed to 1000 µg/ml; consistent with our previous findings (12)], there
was extensive apoptosis (10.4-fold increase in number of cells undergoing early apoptosis when exposed to 1000 µg/ml) induced by the Hexane Fraction of AG. Notably, although there was also a modest induction of apoptosis (6.1-fold increase in number of cells undergoing early apoptosis when exposed to 1000 µg/ml) by the Butanol Fraction of AG, there was little to no apoptosis caused by all other AG fractions (Supplementary Table II).

To complement results with Annexin V, we carried out another dose-response experiment with the Hexane Fraction of AG, and processed cells for western analysis. Results suggest apoptotic markers, including p53, phospho-serine-15, PUMA, and cleaved PARP, are induced by the Hexane Fraction of AG in TK6 lymphoblastoid cells (Supplementary Figures 5 and 6). Interestingly, the oncogenic phosphatase, wild-type p53-induced phosphatase (Wip1), is decreased by the Hexane Fraction of AG, correlating with induction of p53 phosphorylation (Supplementary Figure 6), consistent with its’ (Wip1) capacity to dephosphorylated and deactivate p53 (26).

The Hexane Fraction of AG induces apoptosis in CD4+/CD25- effector T cells

Overly aggressive CD4+/CD25- T cells are thought to contribute to colitis, and defects in mucosal T-cell apoptosis are likely to be critical in the pathogenesis of colitis (12, 27, 28). We therefore isolated CD4+/CD25- effector T cells from spleens of C57BL/6 mice, then exposed unactivated or activated (pre-incubated for 12 hr with 2.5 µg/ml Concanavalin A) cells to either whole AG extract or the Hexane Fraction of AG (0 - 300 µg/ml). Supplementary Table III and Supplementary Figure 3B show the Hexane Fraction of AG induces apoptosis of CD4+/CD25- effector T cells to a similar extent to
The Hexane Fraction of AG suppresses inflammation in the DSS model of colitis

We have previously shown that the whole AG extract can be used to prevent and treat mouse colitis (10-12). The mechanism is at least in part due to the induction of inflammatory cell apoptosis (12). Given our in vitro results indicating substantial anti-inflammatory and pro-apoptotic properties of the Hexane Fraction of AG (Figure 1; Supplementary Figures 4 - 6; Supplementary Tables I and III), we hypothesized that this fraction can be used to treat DSS-induced mouse colitis. Here, mice were given 1% DSS for 1.5 cycles (7 days DSS, 7 days water and 7 days DSS), then fed vehicle control (1x PBS by oral gavage), the whole AG extract (11.9 mg/kg/day by oral gavage) or the Hexane Fraction of AG (11.9 mg/kg/day by oral gavage) for the duration of the experiment (outlined in Supplementary Figure 1). Figure 2 shows results. The colons were graded for histology scores as described in Methods and Supplementary Text. 1% DSS stimulates colitis. When mice were fed the Hexane Fraction of AG, there was a significant reduction in colon inflammation (“histology score”) at 3.5 cycles. Although there was also significant suppression of colitis at 5.5 cycles, the reduction was less than that at 3.5 cycles, but more than that of the whole AG extract at 5.5 cycles. Representative hematoxylin and eosin sections are shown.

Mouse colon length shrinks with stress, inflammation and ulceration (10). Therefore, as an additional indicator of inflammation and inflammatory stress, mouse
Colon lengths were measured. The control group had an average colon length of 8.5 ± 0.5 cm. There was a significant decrease in the length of the colon from 1.5 cycle DSS group (7.3 ± 0.2 cm) and 3.5 Cycles DSS group (7.3 ± 0.3 cm). In contrast, there was no significant decrease in colon length in the 3.5 cycles DSS + the Hexane Fraction of AG group (8.1 ± 0.2 cm). Similarly, in the 5.5 cycle groups, there was a significant decrease in colon length in the DSS only group (7.3 ± 0.4 cm) compared with the DSS + whole AG extract group (8.4 ± 0.3 cm) and the DSS + the Hexane extract of AG group (8.6 ± 0.2 cm). This is consistent with the hypothesis that the Hexane Fraction of AG is a potent anti-inflammatory agent in the DSS mouse model of colitis.

The DAI, which monitors weight loss, stool consistency, and blood in the stool as a measure of disease severity, was also scored DAI for each animal at 0, 1.5 cycles, 3.5 cycles, and 5.5 cycles. As shown in Supplementary Figure 7, the DAI increased with 1% DSS exposure, but this was suppressed by both the whole AG extract and the Hexane Fraction of AG. Significance (p < 0.05) was reached at Day 21, and continued until the end of the experiment.

Markers of inflammation and inflammatory stress are reduced in DSS + Hexane Fraction of AG-treated mice.

To further assess the impact of the Hexane Fraction of AG on inflammatory markers in vivo, we examined iNOS, Cox-2 and p53 expression. Immunohistochemical staining was accomplished by rocking slides using the Antibody Amplifier™ (ProHisto, LLC) to ensure even, consistent, sensitive and reproducible staining. Figure 3A shows representative sections of each endpoint as indicated. Figure 3B shows quantification of...
each endpoint. Overall, iNOS, Cox-2, and p53 levels were elevated in DSS-treated mice, with most staining appearing in the inflammatory cells. iNOS and Cox-2 staining were statistically significantly reduced in the DSS + Hexane Fraction of AG-treated mice; there was also a trend to decreasing p53 levels. Such results reflect a reduction in the number of inflammatory cells (that otherwise are expressing these inflammatory markers), and complement our H&E pathology results.

The Hexane Fraction of AG stimulates apoptosis of lymphocytes \textit{in vivo}

To examine the effects of the Hexane Fraction of AG on apoptosis \textit{in vivo}, we carried out a TUNEL assay on serial sections used for quantifying inflammation (Figure 2, 3.5 cycles). As shown in Figure 4, there was significantly higher Immunoreactivity Score (IRS) (i.e. TUNEL label) in both epithelium (Figure 4A, C) and the MLN (Figure 4B, D) of mice treated with DSS, compared with water-treated mice. The IRS in the epithelial cells decreased when they were treated with both DSS + Hexane Fraction of AG. This observation is consistent with data from the inflammatory index (Figure 2), indicating that the Hexane Fraction of AG protects epithelial cells from DNA damage \textit{in vivo}. Alternatively, in the MLNs, there was an increase in IRS in the MLNs in mice when treated with DSS + Hexane Fraction of AG. Such results are consistent with our \textit{in vitro} data, and with the hypothesis that the Hexane Fraction of AG drives apoptosis in inflammatory cells \textit{in vivo}, providing a mechanism by which the Hexane Fraction of AG protects from colitis in this DSS mouse model.
The Hexane Fraction of AG suppresses colon cancer associated with colitis

We have shown that the Hexane Fraction of AG suppresses DSS-induced colitis (Figure 2). Mechanistically, this appears to be at least in part by the ability of this fraction to induce apoptosis of lymphocytes (Figure 4; Supplementary Tables I and III). Because both mice and humans with chronic colitis are at a high risk for colon cancer, we next tested the hypothesis that the Hexane Fraction of AG prevents the onset of colon cancer in a mouse model of colitis-driven colon cancer. Tables I and II show results that are consistent with this hypothesis. We first examined the levels of inflammation, ulceration, pre-cancerous and cancerous lesions at an intermediate point during the experiment (day 35). As shown in Table I (A and B), both the Hexane Fraction of AG and the whole AG extract reduced the severity of microscopic lesions. There was a significant reduction in total number of inflammatory/ulcerative lesions from 44 in the control group (AOM + DSS + 1x PBS, p.o.) to 24 and 28 in the AG (AOM + DSS + AG, p.o.) and the Hexane Fraction of AG (AOM + DSS + Hex AG, p.o.), respectively. There was also a shift in the severity of lesions, with more lesions being classified as mild inflammatory lesions in the AG and Hexane Fraction of AG groups. There was also a drop in the number of ulcerative lesions compared with the control group; with a greater drop in the Hexane Fraction of AG (36.4% vs. 7.2%) than the AG (36.4% vs. 16.7%) group (Table IA). Similarly, there was a significant reduction in the total number of pre-cancerous/cancerous lesions from 11 in the control group (AOM + DSS + 1x PBS, p.o.) to 1 and 0 in the AG (AOM + DSS + AG, p.o.) and the Hexane Fraction of AG (AOM + DSS + Hex AG, p.o.), respectively. Interestingly, most lesions (91%) in the AOM + DSS
PBS (control) group were of high grade dysplasia or invasive adenocarcinoma (Table IB).

We next examined levels of inflammation, ulceration, pre-cancerous and cancerous lesions at a later time point during the experiment (day 50). As shown in Table II (A and B), both the Hexane Fraction of AG and the whole AG extract reduced the severity of microscopic lesions. There was a significant reduction in the total number of inflammatory/ulcerative lesions from 22 in the control group (AOM + DSS + 1x PBS, p.o.) to 14 and 17 in the AG (AOM + DSS + AG, p.o.) and the Hexane Fraction of AG (AOM + DSS + Hex AG, p.o.), respectively. There was again a shift in the severity of lesions, with 2.7 and 4.2 fold lesions being classified as mild inflammatory lesions in the AG group vs. the control (PBS) group and the Hexane Fraction of AG group vs. the control (PBS) group, respectively. There was also a dramatic drop in the number of ulcerative lesions compared with the control group compared with the Hexane Fraction of AG (27.3% vs. 5.8%) than the AG (27.3% vs. 0%) group (Table IIA). At the 50 day period, there was not as dramatic of a drop in the total number of pre-cancerous or cancerous lesions. However, similar to the 35 day time-point, the severity of lesions was reduced at 50 days. More lesions (33%) in the AOM + DSS + PBS (control) group were of high grade dysplasia or invasive adenocarcinoma. Almost half (17%) of the AG fed group, and only 4% of the Hexane Fraction of AG group were in this classification (Table IIB).
Preliminary chemical analysis of the hexane fraction of AG

Given the potency of the Hexane Fraction of AG, we initiated experiments to better understand the components of this fraction. The amounts of fatty acids determined from the analysis of the Hexane Fraction of AG are given in Supplementary Table IV, and account for greater than 40% w/w of the total extract. Strikingly, linoleic acid (18:2n6) was the major fatty acid, accounting for approximately 50% of the total fatty acids detected, followed by palmitic (16:0) and palmitoleic (16:1) acids. LC-MS analysis did not detect either protopanaxdiol or protopanaxtriol; however, low levels of ginsenosides Re, F11, Rb1 and Rd were found, but amounted to less than 0.1% w/w of the Hexane Fraction of AG.

Descriptive LC-UV Diode Array Detector analysis of the Hexane Fraction of AG gave 3 major UV active peaks, one eluting at 22.7 min with UV maxima at 220, 230, 243 and 257 nm and another eluting at 26.7 min with UV maxima at 230, 243 and 257 nm which match the UV maxima reported for the polyacetylenes, panaxydol and falcarinol respectively (29). The third peak eluting at 20.7 min also exhibited multiple UV maxima at 215, 242, 255, 269 and 284 nm may be a related compound. Confirmation of identity and precise quantification of these compounds awaits individual isolation and structural elucidation, which is currently underway and will be reported in detail separately.

Discussion

We have previously shown that whole AG root extract suppresses colitis, and prevents colon cancer associated with colitis in mice (10-12). To better delineate the ingredients responsible for these findings, we carried out Bioassay-Guided Fractionation,
using multiple solvents. Interestingly, it appears one fraction (the Hexane Fraction of AG) is particularly potent in its’ anti-inflammatory and pro-apoptotic properties. As well, this Hexane Fraction of AG appears to be more effective than the whole AG extract in treating DSS-induced mouse colitis, and modestly more effective at reducing the number and severity of pre-cancerous and cancerous lesions of the colon in the AOM/DSS mouse model.

Specifically, from our in vitro results, the Hexane Fraction of AG was most effective in suppressing IFNγ-induced expression of iNOS in ANA-1 mouse macrophages (Figures 1A, 1C, 1D and Supplementary Figure 3). iNOS, which is responsible for the high-output production of NO, is up-regulated within the inflammatory infiltrate of the lamina propria and in the cytoplasm of the epithelial cells in patients with IBD (30). Large amounts of Cox-2 have also been found in inflamed areas, producing most of the prostaglandins (PG) (31) and it has been reported that the increased PG production during acute colitis is dependent upon the activity of Cox-2 (32-34). Therefore, suppression of the inflammatory response may be reached through the inhibition of prostaglandin E2 (PGE2) production and Cox-2 activation (31). Compared to untreated cells, Cox-2 expression was also suppressed by the Hexane Fraction of AG in ANA-1 cells (Figures 1B, 1C and 1E). However Cox-2 protein expression was affected minimally by the whole AG extract (Figures 1B and 1C). Interestingly, Ichikiwa et al. have reported that AG extract has minimal effects on Cox-2 protein expression in Raw 246.7 murine macrophages (35). Jeong et al. have also reported ginsenoside Rd induces Cox-2 expression in Raw 264.7 cells and other ginsenosides (Rg1, Rg3, Rb1, and Re) did not induce Cox-2 (36). Our original AG extract supplied to us by the Canadian
Phytopharmaceutical Corporation contains 23.5 mg/g of Rd. This is consistent with the hypothesis that Rd may be one of the ingredients that prevents the whole AG extract from suppressing Cox-2 expression, as the Hexane Fraction of AG has a minimal ginsenoside content, including very little Rd (Supplementary Table IV). Also, at this time, we can only speculate on the specific molecules targeted by the Hexane Fraction of AG. Because Cox-2 and iNOS transcription is regulated by Signal Transducers and Activators of Transcription-1 (STAT-1), Hypoxia Inducible Factor-1α (HIF1α), NF-κB and Interferon Regulatory Factor-1 (IRF-1) (37-42), such molecules remain candidates. There also may be indirect mechanisms, such as targeting growth factors, including tumor necrosis factor alpha (TNFα) and IFN-γ, both of which regulate iNOS and Cox-2 levels (43). Another hypothesis takes into account the Fatty Acid content of the Hexane Fraction of AG (Supplementary Table IV). Fatty acids are known to readily react with nitric oxide species to form nitro-fatty acid derivatives (NO₂-FA) (44). NO₂-FA signal through anti-inflammatory mechanisms that inhibit neutrophil activation, platelet aggregation, and macrophage activation (45). It is therefore possible that the formation of NO₂-FA plays a key role in the anti-inflammatory properties of the Hexane Fraction of AG. We are currently trying to better understand these mechanisms and will report results in future studies. Nevertheless, the finding that the Hexane Fraction of AG is effective in suppressing both iNOS and Cox-2 expression in vitro led us to hypothesize that this fraction could be potent in suppressing mouse colitis. Results are consistent with this hypothesis (Figure 2).

In IBD, lymphocytes (both B and T cells) infiltrate the mucosa to eradicate the foreign antigen (46, 47). Once the antigen has been eliminated, T lymphocytes of
intestinal mucosa require a mechanism to attenuate the local immune response (12), and the failure to do so results in chronic immunogenic reactions. A key mechanism of immune suppression is the apoptosis of overly aggressive effector T cells and we have shown that the whole AG extract induces apoptosis of such cells (12). In this study, we show that the Hexane Fraction of AG and to a lesser extent, the Butanol Fraction of AG also have pro-apoptotic properties. The whole AG extract has only a modest impact on TK6 apoptosis (Supplementary Table I) but is as potent as the Hexane Fraction of AG in CD4+/CD25- effector T cells (Supplementary Table III). Due to the potential impact of differential cellular sensitivity to apoptosis, one of several hypotheses’ being explored is that this may be a result of the heterogeneity of the potency of AG based on the cell type. This is consistent with other studies that have reported pro-apoptotic properties of some of the ingredients we have determined to be in the Hexane Fraction of AG (48-54).

Another finding that deserves further attention is that AG (whole AG extract and the Hexane Fraction of AG) both suppresses iNOS and induces apoptosis. This is especially apparent in ANA-1 mouse macrophages, where we measured both iNOS expression (Figure 1) and apoptosis by TUNEL labeling (Supplementary Figure 8 and Supplementary Table V). Although this is consistent with studies finding suppression of iNOS induces apoptosis (55), other groups have found an induction of apoptosis by nitric oxide from iNOS in T cells (56). These findings may again be explained by cell type selectivity, as well as many other factors such as NO output by iNOS. Although our findings here indicate both an anti-inflammatory and pro-apoptotic effect of AG, it is likely that there are mechanisms of apoptosis by AG other than through iNOS in T cells, which we are exploring.
It is currently unclear which component(s) in the Hexane Fraction of AG suppresses colitis and drives apoptosis of inflammatory cells. While we are subfractionating the Hexane Fraction of AG to address this question, at this time we can only speculate. Full details of the chemical analysis and spectroscopic identification of the major components of the hexane fraction will be reported in a separate manuscript. However, from our initial analysis (Supplementary Table IV), it is unlikely that the ginsenosides are responsible, since these comprise a very minor portion of this fraction. More likely are either the fatty acids or the polyacetylenes which, combined, comprise approximately 70% of the total hexane fraction by weight (Supplementary Table IV). Conjugated linoleic acid, for example, has been shown by others to attenuate colitis in animals (57-59). However, in humans, linoleic acid (18:2n6), the fatty acid found in greatest abundance in the Hexane Fraction of AG, may exacerbate colitis (60). Others have shown fatty acids such as oleic acid have no effect in suppressing colitis (61). The effects of the other fatty acids we detected in the Hexane Fraction of AG on colitis are, to our knowledge, unknown, but worth exploring. Interestingly, trilinolein, a triglyceride isolated from *P. notoginseng* where glycerol is esterified at all three positions with linoleic acid (18:2n6) has been shown to have antioxidant and cardio-protective effects in animal models (62). The high relative levels of 18:2n6 found in the Hexane Fraction of AG after transesterification suggest that trilinolein may be present in AG also. Eight polyacetylenes have now been reported from AG (63) a class of compound with potent anti-inflammatory activities (64). Based on LC-UV, $^1$H-NMR and high resolution mass spectra, three C$_{17}$ polyacetylenes: panaxydiol, panaxydol and panaxynol were identified and comprised over 25% of the Hexane Fraction of AG (Supplementary Table IV).
Therefore this class of compound is another candidate responsible for the observed activity against colitis.

In the DSS model of colitis, the Hexane Fraction of AG was found to be very effective in suppressing colon inflammation (Figure 2). At 3.5 Cycles, the DSS + Hexane Fraction of AG group was able to reverse the inflammation to almost basal levels. At 5.5 cycles, the Hexane Fraction of AG was less potent against colitis than it was at 3.5 cycles. One reason for this observation is that the increased cycles of DSS (2 more cycles) was able to cause much more damage to the colon to the point where complete recovery was unattainable. Regardless, at 5.5 cycles, the Hexane Fraction-treated mice had significantly less colon inflammation than the DSS only treated groups of mice. It therefore appears that the Hexane Fraction of AG is more effective during the short-term inflammation (acute colitis) than the long-term inflammation (chronic colitis). It also appears to be more potent than the whole AG extract (Figure 2). Interestingly, others have found an n-Hexane extract of red ginseng is particularly potent in inhibiting the growth of human lung tumor xenografts in nude mice (65).

Many studies have shown anti-cancer effects of AG in vitro and in vivo, which we have described in detail previously (11). As an extension of that study, it appears that although the Hexane Fraction of AG has a similar potent effect of suppressing colon cancer associated with colitis in the AOM/DSS model, the severity of pre-cancerous and cancerous lesions is modestly reduced with the Hexane Fraction of AG compared with the whole AG extract (Tables I and II). Similar to the colitis data (Figure 2), the reduction in severity of such lesions is greater at the earlier time period (35 days) than at 50 days. Again, one reason for this observation is that the increased time was able to
cause much more damage to the colon to the point where complete recovery was unattainable.

In summary, we have identified through various endpoints that the Hexane Fraction of AG is at least one component of AG extract responsible for the suppression of DSS-induced colitis, and apoptosis of inflammatory cells is a mechanism by which it acts. This Hexane Fraction of AG is also modestly more potent than the whole AG extract in suppressing the severity of AOM/DSS-induced colon cancer. This finding represents a significant advancement in the field, since it has previously been thought that ginsenosides, extremely minor elements of this fraction, are key anti-inflammatory and anti-cancer agents in AG (66). To this end, it is currently unclear what component within the Hexane Fraction of AG suppresses colitis and colon cancer associated with colitis. However, many of the fatty acids detected in our Hexane Fraction of AG, can induce apoptosis in various cell types (48-54), and conjugated linoleic acid and oleic acid has been shown to suppress colitis in other studies (57-59, 61). This is consistent with the hypothesis that at least one of these ingredients may be responsible for the activity of AG root extract against colitis and associated colon cancer. Further Bioassay-Guided Fractionation of the Hexane extract of AG is ongoing to extend these current results to further pinpoint this active ingredient(s).

Acknowledgements

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References


17. Park E, Hwang I, Song JY, Jee Y. Acidic polysaccharide of Panax ginseng as a defense against small intestinal damage by whole-body gamma irradiation of mice. Acta Histochem. 2011;113:19-23.


Figure Legends

Figure 1. The Hexane Fraction of AG suppresses the induced expression of iNOS and Cox-2 at the protein and mRNA level in ANA-1 mouse macrophages. (A) Effect of whole AG extract and the Hexane Fraction of AG on IFN-γ-induced iNOS protein expression. The murine macrophage cell line (ANA-1 cells) was incubated for 12 hr with No AG (media only), the whole AG extract (260 µg/ml), or the indicated AG Fraction (260 µg/ml), washed, then exposed to IFN-γ (100 U/ml) for 0, 2, 4 and 8 hrs. Cell lysates were analyzed by western blot analysis. C+, indicates the positive control, which was an archived ANA-1 cell lysate previously induced by IFN-γ, and known to have iNOS induction. (B) Effect of whole AG extract and the Hexane Fraction of AG on LPS-induced Cox-2 protein expression. Cells were treated as was described in (A). Numbers under the bands indicate densitometry values as a ratio relative to control (time 0 hr) for each treatment. (C) Densitometric quantification of iNOS and Cox-2 bands shown in (A) and (B), respectively, and adjusted for Actin levels. (D) Effect of the Hexane Fraction of AG on IFN-γ-induced iNOS mRNA expression. Cells were treated as was described in (A). (E) Effect of whole AG extract and the Hexane Fraction of AG on LPS-induced Cox-2 mRNA expression. All treatments were repeated 3 times to ensure consistency. *, indicates significant (p < 0.05) reduction in mRNA expression, relative to the untreated sample (no AG).

Figure 2. Effects of whole AG extract (AG) and the Hexane Fraction of AG on the colon histology score in the DSS mouse model of colitis. Results suggest the Hexane Fraction of AG is more potent in treating colitis than the whole AG extract. Values
Figure 3. iNOS, Cox-2 and p53, markers of inflammation and inflammatory stress, are reduced in DSS + Hexane Fraction of AG-treated mice. Tissues from experiments performed for Figure 3 (3.5 Cycles) were examined for iNOS, COX-2, and p53 by immunohistochemistry, using the Antibody Amplifier™ (ProHisto, LLC) rocked on a laboratory rocker to ensure even staining and reproducible results. (A) Representative staining of indicated end points in serial sections from water (n = 11), DSS (n = 15) and DSS + Hexane Fraction of AG (n = 11) groups. Positive staining is brown colored. 100X magnification. (B) Quantification of indicated end points. All three markers were elevated in the DSS-treated group and suppressed when the DSS-treated group was fed the Hexane Fraction of AG. p-values are indicated.

Figure 4. Effects of the Hexane Fraction of AG on apoptosis in cells of the epithelium (A, C) and the MLNs (B, D). (A) IRS (TUNEL staining) in epithelial cells of indicated groups. (B) IRS (TUNEL staining) in MLNs cells of indicated groups. (C) Quantification of staining in the epithelium. (D) Quantification of staining in the MLNs.
**Table I.** Percentage of inflammatory and ulcerative lesions (A) and of pre-cancerous and cancerous lesions (B) in mice treated with AOM/DSS ± AG ± Hexane fraction of AG (Hex AG) at days 35.

**A. Day 35: Analysis of Inflammatory and Ulcerative Lesions**

<table>
<thead>
<tr>
<th>Group</th>
<th># of Animals</th>
<th>Total # of Inflammatory/ Ulcerative Lesions</th>
<th>Inflammatory Lesions</th>
<th>Ulcerative Mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mild</td>
<td>Severe</td>
</tr>
<tr>
<td>PBS</td>
<td>4</td>
<td>44</td>
<td>45.4%</td>
<td>18.2%</td>
</tr>
<tr>
<td>AG</td>
<td>4</td>
<td>24c</td>
<td>62.5%</td>
<td>20.8%</td>
</tr>
<tr>
<td>Hex AG</td>
<td>4</td>
<td>28c</td>
<td>71.4%</td>
<td>21.4%</td>
</tr>
</tbody>
</table>

* a, Whole American Ginseng Extract  
* b, Hexane Fraction of American Ginseng  
* c, p < 0.05.

**B. Day 35: Analysis of Pre-cancerous and Cancerous Lesions**

<table>
<thead>
<tr>
<th>Group</th>
<th># of Animals</th>
<th>Total # of Pre-Cancerous/ Cancerous Lesions</th>
<th>Polyps</th>
<th>Non-Invasive Adenomas Low Grade Dysplasia</th>
<th>High Grade Dysplasia</th>
<th>Invasive Adeno-Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>4</td>
<td>11</td>
<td>0%</td>
<td>9%</td>
<td>82%</td>
<td>9%</td>
</tr>
<tr>
<td>AG</td>
<td>4</td>
<td>1a</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Hex AG</td>
<td>4</td>
<td>0a</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

* a, p < 0.05.
Table II. Percentage of inflammatory and ulcerative lesions (A) and of pre-cancerous and cancerous lesions (B) in mice treated with AOM/DSS ± AG ± Hexane fraction of AG (Hex AG) at day 50.

### A. Day 50: Analysis of Inflammatory and Ulcerative Lesions

<table>
<thead>
<tr>
<th>Group</th>
<th># of Animals</th>
<th>Total # of Inflammatory/ Ulcerative Lesions</th>
<th>Inflammatory Lesions</th>
<th>Ulcerative Mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mild</td>
<td>Severe</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
<td>22</td>
<td>18.2%</td>
<td>54.5%</td>
</tr>
<tr>
<td>AG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9</td>
<td>14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>Hex AG&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10</td>
<td>17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>76.6%</td>
<td>17.6%</td>
</tr>
</tbody>
</table>

<sup>a</sup>, Whole American Ginseng Extract  
<sup>b</sup>, Hexane Fraction of American Ginseng  
<sup>c</sup>, p < 0.05.

### B. Day 50: Analysis of Pre-cancerous and Cancerous Lesions

<table>
<thead>
<tr>
<th>Group</th>
<th># of Animals</th>
<th>Total # of Pre-Cancerous/ Cancerous Lesions</th>
<th>Polyps</th>
<th>Non-Invasive Adenomas</th>
<th>Invasive Adenoma-Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low Grade Dysplasia</td>
<td>High Grade Dysplasia</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
<td>27</td>
<td>0%</td>
<td>67%</td>
<td>26%</td>
</tr>
<tr>
<td>AG</td>
<td>9</td>
<td>18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0%</td>
<td>83%</td>
<td>11.5%</td>
</tr>
<tr>
<td>Hex AG</td>
<td>10</td>
<td>25</td>
<td>8%</td>
<td>83%</td>
<td>4%</td>
</tr>
</tbody>
</table>

<sup>a</sup>, p < 0.05.
A. Epithelium TUNEL

- Water
- DSS
- DSS + Hexane Fraction of AG

B. MLN TUNEL

- Water
- DSS
- DSS + Hexane Fraction of AG

C. Epithelium Quantification

- TUNEL Immunoreactivity Score (Epithelial Cell Apoptosis)
- Water
- DSS
- DSS + Hexane Fraction of AG

D. MLN Quantification

- TUNEL Immunoreactivity Score (Inflammatory Cell Apoptosis)
- Water
- DSS
- DSS + Hexane Fraction of AG
Deepak POUDYAL, Phuong Mai Le, Tia DAVIS, et al.

A hexane fraction of american ginseng suppresses mouse colitis and associated colon cancer: anti-inflammatory and pro-apoptotic mechanisms.


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