Published in final edited form as:

Cancer Prev Res (Phila). 2015 April ; 8(4): 327–337. doi:10.1158/1940-6207.CAPR-14-0380.

Improved innate immune responses by Frondanol® A5, a sea cucumber extract, prevent intestinal tumorigenesis

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Abstract

Sea cucumbers are a source of anti-bacterial, anti-inflammatory, and anti-cancer compounds. We show that sea cucumber extract Frondanol® A5 is capable of enhancing innate immune responses and inhibiting intestinal tumors in APC^{Min/+} mice. APC^{Min/+} mice were fed semi-purified diets containing 0, 250, or 500 ppm Frondanol® A5 for 14 weeks before we assessed intestinal tumor inhibition. Dietary Frondanol® A5 suppressed small intestinal polyp sizes and formation up to 30% ($p<0.02$) in males and up to 50% ($p<0.01$) in females. Importantly, 250 and 500 ppm Frondanol® A5 diet suppressed colon tumor multiplicities by 65% ($p<0.007$) and 75% ($p<0.0001$), compared with untreated male APC^{Min/+} mice. In female APC^{Min/+} mice, both dose levels of Frondanol® A5 suppressed colon tumor multiplicities up to 80% ($p<0.0001$). Isolated peritoneal macrophages from treated mice showed increased phagocytosis efficiency (Control 24% Vs treated 50%; $p<0.01$) and an increase in GILT mRNA expression, indicating increased innate immune responses by these cells in treated animals. Similarly, we observed an increase in GILT expression in treated tumors, compared with untreated tumors. Furthermore, an increase in GCSF cytokine, a decrease in inflammatory cytokines and marker 5-LOX, its regulator FLAP, proliferation (PCNA), and angiogenesis (VEGF) markers was observed in treatment groups. These data suggest that Frondanol® A5 decreased inflammatory angiogenic molecules and increased GILT expression and macrophage phagocytosis. These decreases may have improved the innate immune systems of the treated mice, thus aiding in inhibition of intestinal tumor formation. These results suggest that Frondanol® A5 exhibits significant chemopreventive potential against intestinal tumorigenesis.

Keywords

APC^{Min/+} mice; colon cancer; chemoprevention; sea cucumber; inflammation

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No potential conflicts of interest

Introduction

Colorectal cancer (CRC) is the third leading cause of death among all cancers, with an estimated 1,665,540 new cases and 585,720 deaths in the U.S. this year (1). CRC can arise due to germ line or somatic mutations, environmental factors, or dietary lifestyle. In most industrialized countries, the rate of CRC is high. CRC occurrence varies by 25-folds worldwide, based on the adoption of western diets and lifestyles (2). Epidemiological evidence suggests that western diets are poor in vegetables, fruits, fiber, whole grains, and omega 3s (3). Published epidemiological data suggest that long-lasting chronic inflammatory conditions produce deregulated inflammatory cytokines that promote the initiation or transformation of cells into highly proliferative cancer cells of many types, including CRC (4). Several studies from our lab and others have shown that Non-Steroidal Anti-inflammatory agents (NSAIDs), such as celecoxib, aspirin, sulindac, naproxen and licoferone, and both selective and non-selective COX-2 inhibitors have chemopreventive effects against CRC (5). These results emphasize that inhibition of inflammation is a promising approach for CRC prevention. Though NSAIDs show better chemopreventive effects, their use is restricted due to known gastrointestinal or thrombotic side effects. Hence, the development of safer natural agents that show similar inhibitory effects is warranted.

Sea cucumbers are marine animals that are rich sources of novel biologically active molecules, including compounds which display anti-microbial, anti-inflammatory, and anti-cancer effects, such as triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycan, sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, peptides, and lectins (6). Previous research by us and others showed that different sea cucumber extracts displayed anti-inflammatory, anti-proliferative, and anti-cancer properties in various human and mouse cancer cell lines, and in a rat model of colon carcinogenesis (7 – 12). It is well evidenced that CRC tumors are strongly associated and sustained by chronic inflammation. CRC is modulated by immune cells' innate and adaptive responses in the tumor microenvironment, which usually favors tumor cell growth and development (13).

Colonic macrophages, part of the innate immune responses, normally mediate nonspecific effector functions against microbial infections and during neoplasia. The macrophages may have pro- or anti-tumorigenic roles in CRC. Macrophages may produce either anti-inflammatory or pro-inflammatory cytokines. These functions change based on the stimulating agent. IFN- γ and GM-CSF are reported to influence macrophage functions to overcome an immune-suppressive tumor microenvironment towards increased anti-tumor activities (14). Thus, it is evident that innate immune cells orchestrate an inflammatory environment which can be modulated with the use of natural agents, such as Frondanol® A5, to inhibit CRC proliferation and growth.

Gamma-interferon-inducible lysosomal thiol reductase (GILT) helps in the reduction of protein disulfide bonds for MHC-class-II presentation and processing (to CD4⁺ T cells) for immune responses against that antigen (15). GILT is usually present in antigen presenting cells (APC). Its antigen presentation in tumor cells may help influence immune T cell

responses against tumors. Low GILT expression in tumors may explain the tumors' ability to invade the immune defense. Using agents to increase or restore GILT expression may enhance immune responses against CRC. Hence, in the present study, we investigated whether Frondanol® A5 has efficacy against CRC in APC^{Min/+} mice and we analyzed the modulation of circulatory cytokines and inflammatory markers in intestinal tumors. Further, we isolated peritoneal macrophages from mice to test if Frondanol® A5 treatment *in vitro* enhanced macrophage phagocytosis. We evaluated isolated peritoneal macrophages and intestinal tumors for GILT mRNA expression following Frondanol® A5 treatment. Results from these experiments suggested that Frondanol® A5, 1) has chemopreventive efficacy against intestinal tumors in APC^{Min/+} mice, 2) inhibits inflammatory cytokines, and 3) validates the mechanism that enhances phagocytosis.

Materials and methods

Mice and Experimental Design

Male APC^{Min/+} (C57BL/6J) and female wild-type littermate mice were purchased from The Jackson Laboratory (Bar Harbor, ME) as founders. We established our own breeding colony in the OUHSC rodent barrier facility and genotyped the animals as described before (16). For Frondanol® A5 composition (Fig. 1A), detailed experimental design, (Fig. 1B) and sample collection please see supplementary data.

Intestinal Tumor Evaluation

Intestinal tumor evaluation and sample preservation for various analyses was performed as discussed previously (16). For detailed information please see supplementary data.

Inflammatory Cytokines

Inflammatory cytokine levels were determined in serum by ELISA (SA Biosciences and Biolegend) per the manufacturers' instructions. For additional description please see supplementary data.

Giemsa Staining

Intestinal tissue samples were stained using a standard Giemsa staining protocol, (17) to observe the changes in types of cells in the treatment groups compared with control groups.

Isolation of Murine Peritoneal Macrophages

After the completion of the experimental period of treatments, animals were killed. Macrophages were then harvested by peritoneal lavage as described elsewhere (18). Please see supplementary data for the brief protocol.

Phagocytosis assays

Macrophage cultures were established and yeast phagocytosis assays were performed as described previously (19,20). The procedure is briefly described in supplementary data. The cells were observed at 1000× magnification under an oil immersion microscope. The

internalized yeast cells were differentiated with tannic acid treatment showing a light pink stain from deep violet staining for adherent yeast cell (21,22).

In all cases, the rate of phagocytosis was determined by counting the number of cells which had internalized at least one particle per high power field (1000 $\times$, oil immersion) as a ratio of the total number of cells per field. Over 100 fields were enumerated to give the “phagocytosis rate” for a typical experiment. Phagocytosis indices were also measured, since we could count up to 8 engulfed intracellular yeast cells. Phagocytes that contained 1 or 2 and 3 or more yeast cells were measured by examining ~200–300 macrophages over 100 fields. The data was analyzed statistically. To assess the statistical variability, at least three separate experiments were performed. The mean and *SE* were calculated for each of the experiments. The number of times that each experiment was repeated is stated in the corresponding figure legends.

Histological Staining

Immunohistochemical and immunohistofluorescence staining of intestinal samples were performed as described previously (16,23). Please see supplementary data for additional information.

5-LOX, FLAP, VEGF, and GILT mRNA Expression

RNA isolation from intestinal tumors, reverse transcription for cDNA, and PCR were performed and analyzed as described previously (16). Please see supplementary table 1 for primer information. Results were expressed as a fold difference in gene expression.

Statistics

Data were described as mean $\pm$ *SEM*. Statistical analysis was done with GraphPad Prism software 5.1 (GraphPad Software, Inc., San Diego, CA). Comparisons were made between APC^{Min/+} control and Frondanol[®] A5-fed mice using two-tailed Student's t-tests.

Results

Frondanol[®] A5 suppresses intestinal tumor formation in APC^{Min/+} mice

Dietary administration of Frondanol[®] A5 produced no toxicities in animals. The weekly body weights of treated mice were greater than the body weights of control untreated mice, indicating the suppressive effect of this agent on intestinal tumors (Fig. 1C and D). Animals treated with Frondanol[®] A5 appeared to be normal, without any demonstration of discomfort or toxicity.

To determine whether Frondanol[®] A5 plays an inhibitory role in the APC-deficient conditions of colorectal cancer, we studied the efficacy of Frondanol[®] A5 in APC^{Min/+} mice at two dose levels. After 14 weeks of feeding with Frondanol[®] A5, at the age of 20 weeks, the number of SIPs and CTs decreased significantly in both male and female mice (Fig. 2). The number of SIPs and CTs in male mice at 20 weeks of age was 59.0 $\pm$ 4.0 and 2.22 $\pm$ 0.44, respectively (Fig. 2A and B). The number of SIPs and CTs in female mice at 20 weeks of age was 78.4 $\pm$ 9.4 and 2.43 $\pm$ 0.37, respectively (Fig. 2C and D).

Dietary administration of 250 and 500 ppm of Frondanol® A5 suppressed small intestinal polyp formation up to 28% ($p<0.02$) in males (Fig. 2B) and up to 50% ($p<0.01$) in females (Fig. 2D). Polyp size also significantly decreased in both genders. In the mice fed Frondanol® A5, the numbers of small, medium, and large polyps on the small intestines were significantly decreased by 19.0% male - 0% female ($p<0.05$ vs. the untreated control) small, 21.0% male - 53% female ($p<0.01$ vs. the untreated control) medium, and 19.00% male - 81.5% female ($p<0.01$ vs. the untreated control) large, shown in Fig. 2E-F. We observed an increase in small polyps in female mice upon treatment, indicating the growth arrest of these polyps, which might have progressed into medium and large polyps. Importantly, 250 and 500 ppm Frondanol® A5 diet suppressed CTs multiplicities by 65% ($p<0.007$) and 75% ($p<0.005$) compared with control diet-fed male APC^{Min/+} mice (Fig. 2B) in a dose-dependent manner. Similarly, in female APC^{Min/+} mice, both dose levels of dietary Frondanol® A5 suppressed colon tumor multiplicities up to 80% ($p<0.0001$; Fig. 2D). Further histological analysis of the intestinal tumors was performed by a pathologist blinded to the experimental groups. The total high grade dysplasia (HGD) observed in untreated control APC^{Min/+} mice was limited to few intestinal tumors. With both doses of Frondanol® A5 treatments, we observed about 90% reduced HGD as compared to untreated control diet fed APC^{Min/+} mice HGD of intestinal tumors. Overall, our results suggest that dietary Frondanol® A5 significantly reduced the HGD in intestinal tumors.

Frondanol® A5 altered circulating inflammatory cytokines

We evaluated Frondanol® A5 to determine its inhibitory effects on inflammatory cytokines in the circulation of mice. In both genders, Frondanol® A5 significantly decreased several inflammatory cytokines: Interleukin (IL)-1A 98% - 87.9%, IL-1B 98% - 93%, IL-2 98% - 99%, IL-4 90% - 95.9%, IL-6, 30.9% - 54.31%, IL-10 57% - 73%, IL-12 62% - 55.4%, IL-17A 53.4%, IFN- γ 46.4%, and TNF- α , 94.8% - 94.7%, in male and female mice, respectively ($p<0.0001$ vs. the untreated control). These cytokines were upregulated in male and female untreated control APC^{Min/+} mice (Fig. 3 A and B). Upon treatment, we observed increases in G-CSF 60.6% ($p<0.002$ vs. the vehicle control) and GM-CSF 60.7% ($p<0.001$ vs. the untreated control) in male mice. In female mice, we observed a non-significant increase in IL17A (30%) and G-CSF (31.7%).

We then evaluated whether Frondanol® A5 had inhibitory effects on the mRNA of inflammatory and angiogenic biomarkers in adenomatous polyps.

Decreased inflammatory and angiogenic biomarkers and proliferation in treated intestinal tumors

We evaluated the inhibitory effects of Frondanol® A5 on mRNA expression analysis of inflammatory markers (5-LOX, FLAP) and an angiogenesis marker (VEGF) in intestinal tumors. Decreased mRNA expression analysis showed that Frondanol® A5 inhibits mRNA expression of 5-LOX and its regulator, FLAP (Fig. 4A and B). We compared the density of mRNA expression in these intestinal tumors. We observed a significant decrease in the levels of 5-LOX ~85% ($p<0.02$), FLAP >50% (not significant), and VEGF (48% for VEGF₁₈₈, $p<0.05$; 60% for VEGF₁₆₄, $p<0.05$) in small intestinal adenomatous polyps after treatment with Frondanol® A5 (Fig. 4A). Similarly, in Frondanol® A5-treated colon tumors, the levels

of 5-LOX, FLAP, and VEGF were significantly reduced >75.1% ($p<0.05$ vs. the untreated control), 95.7% ($p<0.0001$ vs. the untreated control), and VEGF isoforms, VEGF₁₆₄, 98.7% ($p<0.0001$ vs. the untreated control), and VEGF₁₈₈ 99.7% ($p<0.001$ vs. the untreated control; Fig. 4B). A similar inhibitory effect on these biomarkers was observed with both of the doses tested for protein expression in intestinal tumors, as evaluated by immunohistofluorescence staining (Fig. 4C, D and E). After Frondanol® A5 treatment, the protein levels of VEGF and 5-LOX in the adenomatous intestinal polyps were significantly reduced by 90.8% and 60.5%, respectively, $p<0.01$ (Fig. 4C). We observed significantly decreased protein expression of PCNA in treated intestinal tumors, suggesting inhibition of proliferation in treated samples (Supplementary Fig. 1A, B and C).

Frondanol® A5 increased antigen presenting cells and phagocytosis in peritoneal macrophages with an increase in GILT expression

Giemsa staining of CTs from Frondanol® A5-treated mice showed an increase in macrophages (Supplementary Fig. 2A). To be more specific, SIPs and CTs were stained for immunohistofluorescence with macrophage marker CD163, which is highly expressed by mature tissue macrophages (24). We observed a clear red fluorescent stain, corresponding to macrophages, in intestinal tumors. Frondanol® A5 significantly increased peritoneal macrophages by ~50% and intratumoral macrophages by ~45% in intestinal tumors, $p<0.01$ (Supplementary Fig. 2A and B).

To evaluate if the increase in macrophages had an effect on macrophage phagocytosis, we isolated peritoneal macrophages from treated and untreated animals, analyzed GILT expression, and performed an *in vitro* phagocytosis assay. To confirm increased phagocytosis, treated and untreated peritoneal macrophages, SIPs, and CTs were analyzed for GILT expression. As innate immune recognition/responses trigger secretion of lysosomal enzymes, macrophages accumulate GILT and mature into phagolysosomes. Peritoneal macrophages were successfully grown *in vitro* (Fig. 5A). A significant 100% increase in GILT was observed in peritoneal macrophages isolated from treated animals (Fig. 5B). We also observed a similar, significant increase of GILT mRNA by 50% ($p<0.01$) and 30% ($p<0.01$) in CTs and SIPs, respectively (Fig. 5C). As GILT mRNA expression increased in tumors, we analyzed for IFN- γ expression in CTs and SIPs, since IFN- γ is reported to induce GILT. Though the increase was not significant, Frondanol® A5 treatment increased IFN- γ protein expression in CTs and SIPs (Fig. 5C). We performed an *in vitro* phagocytosis assay to further understand whether Frondanol® A5 enhances the innate immune response in the macrophages.

In vitro phagocytosis assay analysis revealed a significant increase in phagocytes in treated samples compared with untreated samples (~50%, $p<0.001$; Fig. 5D-G). Phagocytic index measurements also indicated a significant increase in ingestion of yeast particles by phagocytes isolated from animals treated with Frondanol® A5 (~56% with 1 or 2 yeast cells/ ϕ , $p<0.01$, Fig. 5F; ~38% with 3 or more yeast cells/ ϕ , $p<0.01$; Fig. 5G). The results of this experiment clearly demonstrate that Frondanol® A5 significantly increases macrophage function, which significantly contributes to the hosts' innate immune responses. This improved immune response is reflected by a decrease in intestinal tumors.

Frondanol® A5 increased apoptosis in treated intestinal tumors

Intestinal tumors from the treated group displayed markedly increased Annexin V staining compared with tumors from non-treated groups (Fig. 6A and B). The increased Annexin V staining suggests induction of apoptosis in the tumors treated by Frondanol, correlating with the induction of apoptosis in colon cancer cells as reported in our previous publication with this agent. Significantly increased apoptotic cells in treated tumors that have bound Annexin V-Alexa Fluor 488 showing green staining in the plasma membrane of cells was observed compared to untreated tumors (SIPs, ~70%, $p<0.01$; Fig. 5D-G; CTs, ~75%, $p<0.01$) (Fig. 6A and B).

Discussion

Most cancers arise from increased inflammation and hampered innate immunity against oncogenic transformation. Epidemiologic data has shown that, aside from genetic predispositions, CRC is mainly influenced by or associated with food intake. Many researchers have suggested that the most effective agents for CRC prevention are derived from natural food sources. Though ongoing research continues to explore CRC prevention, no natural agent has been approved for CRC prevention to date. The present study showed that sea cucumber extract Frondanol® A5 reduces the number of large polyps in the small intestine and colonic tumors of APC^{Min/+} mice. Though we observed a significant inhibition of intestinal tumors, the percent inhibition of intestinal tumors differed in male and female mice. The differential hormonal effects in male and female mice might influence the sensitivity and resistance of tumors towards treatments, a finding which we reported in our previously published papers examining different agents in APC^{Min/+} mice models (16). We strongly believe that hormonal differences in these mice might have played a role in the effects of Frondanol® A5 on small intestinal tumor formations. The literature and experimental observations of the differences in male and female responses to certain chemoprevention drugs indicate that different genders may need to be dosed differently in order to obtain optimal chemoprevention activity in each gender. The inhibitory effect on intestinal tumors by Frondanol® A5 was primarily due to the inhibition of inflammatory cytokines, biomarkers, angiogenic markers, tumor cell proliferation, and apoptosis. We also found an increase in innate immune responses in CTs and SIPs from treated animals. In APC^{Min/+} mice, Frondanol® A5 activates macrophages, which in turn mediate antigen presentation, with an increase in GILT expression, formation of polyps, and inhibition of large polyp growth. Chemopreventive drugs and “nutraceuticals” are important and promising, if they prevent the growth of the existing tumors (25). The protective effect of Frondanol® A5 in an APC^{Min/+} mouse model against intestinal tumorigenesis, through inhibition of cell proliferation and induction of apoptosis, is consistent with the earlier reports of other naturally occurring dietary phytochemicals against tumor formation (26). In the present study, Frondanol® A5 was observed to be a promising agent possessing strong chemopreventive abilities in inhibiting polyp formation and growth.

Sea cucumbers are commonly used in Asian traditional medicine. These marine animals are reported to contain compounds that inhibit arthritis, inflammation, and cancers (27 – 31). Sea cucumbers have also been described to interfere with several tumor pathways and

improve immune responses in other cancers [32]. We have previously shown that Frondanol® A5 reduces the number of azoxymethane-induced aberrant crypt foci in a rat model of colon carcinogenesis. The reduction primarily occurs through growth inhibition and induction of apoptosis (12). Further, Collin et al. demonstrated that Frondoside A, a component of Frondanol® A5, inhibited microtubule formation in an angiogenesis assay, and also stimulated innate immunity *in vivo* (33-34). Dietary supplementation of Frondanol® A5 in combination with other natural agents enhanced its anti-inflammatory properties (35). Previous studies with Frondanol® A5 showed that it displays multi-mechanistic potential due to the presence of several active agents. In our present study with APC^{Min/+} mice, the data suggest decreased proliferation and angiogenesis due to the compound's inhibitory effects on circulating inflammatory cytokines and inflammatory and angiogenic biomarkers in intestinal tumors, with an accompanying increase in innate immune response due to increased macrophage phagocytosis and GILT expression.

PCNA, a marker of cell division (36), is often correlated with stage of malignancy, infiltration, metastases, and survival. Normal intestinal mucosa show ~30% PCNA expression, compared with 72% expression in carcinoma cells. PCNA is also highly expressed in higher grade tumors (37). We have observed decreased PCNA protein expression in intestinal tumors treated with Frondanol® A5. A decreased VEGF isoform was also observed. The role of VEGF in angiogenesis is well documented, and primarily regulated by pro-inflammatory cytokines such as IL6, TNF- α , and IL1. In the present study, IL10 was observed to be downregulated in serum samples from treated mice. Though studies have shown that IL10 is anti-inflammatory under IBD conditions, IL10 is reported to play both pro-tumoral and anti-tumoral roles. We observed (unpublished data) and others reported that IL10 assists in inducing Tolerogenic T cells, which aid in the evasion of immune responses against tumor formation. In this context, we have previously shown that IL-10 is also reduced by licofelone in this mouse model (39). Similarly, in the present study we have seen that Frondanol® A5 decreased IL-10 expression and improved innate immune responses against tumor formation. Overexpression of 5-LOX has been implicated in inflammatory diseases and many cancers (38, 39). Previous studies have shown that inhibition of the 5-LOX metabolite leads to apoptosis in prostate and colon cancers (40). Frondanol® A5-treated tumors showed a significant decrease in 5-LOX and its activator protein. These results suggest that Frondanol® A5 possesses anti-inflammatory properties. The sources of inflammatory molecules are reported to be macrophages and DCs (41). We observed a decrease in isoforms of VEGF transcription and pro-inflammatory cytokines in Frondanol® A5-treated tumors. Pro-inflammatory cytokines seem to serve a role in tumor development rather than tumor evasion. However, in the present study, we observed a decrease in pro-inflammatory cytokines with an increase in G-CSF and GM-CSF.

Enhancement of macrophage effector functions in neoplastic disease may require stimulation with an activating agent. In the present study, we determined that Frondanol® A5 can increase GM-CSF and G-CSF protein expression in APC^{Min/+} animals. These increased protein expressions have stimulated monocytes/macrophages to become tumoricidal against intestinal tumors. GM-CSF was reported to potentiate antibody responses *in vitro* by activating murine splenic macrophages (14). Grabstein et al. reported that GM-CSF stimulates tumoricidal activity against the malignant melanoma cell line A375 (14). In the

present study, Frondanol® A5 increased macrophage phagocytosis. This finding was confirmed in *in-vitro* studies and evidenced by a decrease in intestinal tumors in treated animals. We observed an increase in intratumor CD163, which was used to identify mature macrophages in tumor tissues upon Frondanol® A5 treatment. CD163 is reported to be part of the innate immune system with a role in the resolution of inflammation and suppression of inflammatory responses (42 – 44). CD163 expression was reported to be associated with early recurrence and reduced survival time in patients with rectal cancer; CD163 expression was mostly observed after irradiation (45). Our data clearly suggest that Frondanol® A5 treatment decreased inflammation, angiogenesis, and tumor formation.

CD163 may have a different role as a phagocytic inducer in macrophages. The CD163 that is expressed on macrophages internalizes the complex hemoglobin/haptoglobin by endocytosis. Toxic and proinflammatory molecules are often removed from the circulation and from local sites of inflammation by CD163 (46). It is reported that upregulation of CD163 attenuates inflammation, either in its soluble form via cytokines or by increasing phagocytosis of macrophages. Our data suggest that the increased expression of CD163 within tumors might have inhibited tumor growth by increased phagocytosis by macrophages. The mechanism(s) of macrophage-induced killing of tumor cells has not yet been explained. Macrophages might be diverted towards anti-tumor activities, rather than positively influencing tumor growth by overproduction of inflammatory cytokines and biomarkers.

Levels of GILT expression are often associated with poor patient survival, indicating an impaired presentation of class-II-restricted peptides (47). Further, lower GILT expression reduced CD4⁺ T cell-mediated responses in *in vivo* mouse models (48 – 50). Our results with Frondanol® A5 demonstrate that GILT expression levels in peritoneal macrophages and in tumors are correlated with the total tumor outcome in APC^{Min/+} mice. The combination of various bioactive molecules in Frondanol® A5 has produced a decrease in inflammatory markers and an increase in GILT expression.

First, we observed significantly increased peritoneal and intra-tumoral macrophages in the treated mice. Second, the macrophages isolated from treated animals possess greater capacity to engulf the antigens showing enhanced phagocytosis than do macrophages isolated from untreated animals. Further, the macrophages showed increased GILT expression in the treatment groups leading to a modulation of inflammatory cytokines in the treated tumors. Also, a significant increase of GILT mRNA was observed in CTs and SIPs (Fig. 5C). As GILT mRNA expression is increased in tumors, we analyzed for IFN- γ expression in CTs and SIPs, since IFN- γ is reported to induce GILT. Though the increase was not significant, Frondanol® A5 treatment increased IFN- γ protein expression in CTs and SIPs. This partly explains the mechanistic axis of macrophages-GILT-IFN γ -Phagocytosis. Thus, the main mechanism by which Frondanol® A5 inhibits intestinal tumors is through an increase in innate immune responses against tumors and by inducing apoptosis in tumors. These findings may help in designing drugs or natural agents to recruit or divert macrophage cells towards effector cells, eliminate transformed or tumor cells, and inhibit tumor growth.

In summary, Frondanol® A5 exhibited anti-inflammatory, anti-proliferative, and anti-angiogenic effects. This sea cucumber extract also enhanced innate immunity against intestinal tumors by improving macrophage phagocytosis in APC^{Min/+} mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Ms. Kathy Kyler, Staff Editor, for editorial assistance with this article. The authors also thank Ms. Misty Brewer with breeding and feeding of animals and the OUHSC Rodent Barrier Facility staff for the valuable support in bioassay studies.

Grant support: Dr. Rao CV: N01-CN-53300 and R01CA094962.

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Figure 1.

(A) Active ingredients in sea cucumber extract Frondanol® A5. Fucosylated Chondroitin Sulphate, 12-Methyltetradecanoic acid, and Frondoside A (B) Experimental design for Frondanol® A5 dietary feeding in APC^{Min/+} mice from 6 weeks of age until termination. AIN76A was the control diet. The experimental groups (diet + drug) and total number of animals per group are listed. (C) Male APC^{Min/+} mice were exposed to two different doses of Frondanol® A5. Changes in body weights were recorded every week from 6 weeks of age to 20 weeks of age ($n=10$, Mean $\pm$ SEM). (D) Female APC^{Min/+} mice were exposed to two different doses of Frondanol® A5. Changes in body weights were recorded every week from 6 weeks of age to 20 weeks of age ($n=10$; Mean $\pm$ SEM). We observed statistically significant differences in body weight gains with both genders.

Figure 2.

Efficacy of Frondanol® A5 on intestinal tumors of male and female APC^{Min/+} mice. (A) Inhibition of total small intestinal polyps (SIPs) formation in APC^{Min/+} male mice by 250 and 500 ppm of Frondanol® A5. Data are means $\pm$ SE of ten animals. Control and treated groups are significantly different from one another ($P<.02$). (B) Average number of colon tumors per mouse in control and treated APC^{Min/+} male mice. We observed a significant inhibition of colon tumors with both low-dose ($P<.0078$) and high-dose ($P<.005$) Frondanol® A5. (C) Inhibition of total small intestinal polyps (SIPs) formation in APC^{Min/+} female mice by 250 and 500 ppm of Frondanol® A5. Control and treated groups are significantly different from one another (Low dose $P<.01$, high dose $P<.007$) per treatment group. (D) Average number of colon tumors per mouse in control and treated APC^{Min/+} female mice. We observed a significant inhibition of colon tumors with both low-dose ($P<.0001$) and high-dose ($P<.0003$) Frondanol® A5. (E) The size distribution of polyps in small intestines of male mice. (F) The size distribution of polyps in small intestines of female mice. For (E) and (F) * $p<0.05$; ** $p<0.001$ versus the unfed control; Data are means $\pm$ SE of ten animals per treatment group.

Figure 3.

Effect of 500 ppm Frondanol[®] A on inflammatory cytokines in serum samples from treated (High Dose) and untreated APC^{Min/+} male (A) and female (B) mice (N=3 / group), as analyzed by ELISA. All assayed cytokines, except G-CSF and GM-CSF, were decreased by Frondanol[®] A5. IL-2, IL-4, and TNF- α were lowest/undetectable in sera from treated animals.

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Figure 4.

Modulatory effects of Frondanol® A5 on 5-LOX, FLAP, and VEGF mRNA expression in CTs (A) and SI polyps (B) of treated (High Dose) and untreated APC^{Min/+} male mice. We observed significantly decreased expression of 5-LOX, FLAP, and VEGF mRNA (VEGF₁₈₈ and VEGF₁₆₄) variants after Frondanol® A5 treatment (500 ppm). Decreased 5-LOX and VEGF protein expression in the nucleus of Frondanol® A5-treated (500 ppm) intestinal tumor cells. Serial paraffin sections of colon tumors (C) and intestinal (D) from APC^{Min/+} were subjected to immunohistochemical analysis using an anti-5-LOX and anti-VEGF polyclonal antibody. A marked decreased accumulation of 5-LOX and VEGF is clear in the cytoplasm and nucleus of tumor cells in treated animals, compared with tumor cells from control animals.

Figure 5.

In vitro phagocytosis of yeast cells by peritoneal macrophages from untreated and treated mice (500 ppm). Continuous feeding of Frondanol® A5 at the tumor initiation stage led to an increase in phagocytosis by peritoneal macrophages *in vivo*. Mice were exposed to Frondanol® A5 diet or control diet for 14 weeks and then necropsied. Peritoneal macrophages were harvested, plated on glass slides in petri plates, and examined by microscopy to evaluate the presence of internalized dead yeast cells. These peritoneal macrophages were evaluated for GILT mRNA expression. (A) Microscopic picture of adhered macrophages isolated from mice after 18 h incubation. (B) mRNA expression of GILT in control and Frondanol® A5-treated macrophages. (C) mRNA expression of GILT and IFN- γ in control and Frondanol® A5-treated CTs and SIPs. (D) Phagocytosis was determined after staining with tannic acid to differentiate adherent yeast cells from those internalized by macrophages. Microscopic image representing the control and Frondanol® A5-treated macrophages after phagocytosis with yeast cells. The magnified image displaying the internalized yeast cells in Frondanol® A5-treated (500 ppm) sample. Arrow indicates internalized yeast. (E) The percentages of phagocytes in control and treated macrophage populations were determined. (F) The percentages of phagocytes with one or two yeast cells in control and treated macrophage populations. (G) The percentages of phagocytes with three or more yeast cells in control and treated macrophage populations. The same slides for analyzing the percentages of phagocytosis are used for these

measurements. Quantification of phagocytosis; * $P<0.05$; $n=3$, separate experiments with three mice per experiment, over 100 macrophages per mouse.

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Figure 6.

IHF staining for Annexin 5 was done for apoptotic cells in intestinal tumors from APC^{Min/+} mice fed control diet or treated with Frondanol[®] A5 (500 ppm). (A) A significant induction of apoptosis (green staining) was observed in polyps of treated mice compared with untreated mice. A significant difference was observed in apoptotic index between Frondanol-treated (500 ppm) and control group polyps. (B) A significant induction of apoptosis (green staining) was observed in CTs of treated mice compared with untreated mice. A significant difference was observed in apoptotic index between Frondanol-treated (500 ppm) and control group CTs.