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 antibacterial, anti-inflammatory, and anticancer compounds. We show that a sea cucumber extract Frondanol A5 is capable of enhancing innate immune responses and inhibiting intestinal tumors in APCMin/+ mice. APCMin/+ 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 APCMin/+ mice. In female APCMin/+ 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 G-CSF cytokine, a decrease in inflammatory cytokines and marker 5-LOX, its regulator FLAP, proliferation (PCNA), and angiogenesis (VEGF) markers were 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. Cancer Prev Res; 8(4); 327–37. ©2015 AACR.

Introduction

Colorectal cancer is the third leading cause of death among all cancers, with an estimated 1,665,540 new cases and 585,720 deaths in the United States this year (1). Colorectal cancer can arise due to germline or somatic mutations, environmental factors, or dietary lifestyle. In most industrialized countries, the rate of colorectal cancer is high. Colorectal cancer occurrence varies by 25-fold worldwide, based on the adoption of western diets and lifestyles (2). Epidemiologic evidence suggests that western diets and lifestyles have contributed to a significant increase (up to 50%) in colorectal cancer incidence (1). It is well evidenced that colorectal cancer is strongly associated and sustained by chronic inflammation. Colorectal cancer is modulated by immune cells’ innate and adaptive responses in the tumor microenviroment, which usually favors tumor cell growth and development (13).
Colonic macrophages, part of the innate immune responses, normally mediate nonspecific effectors functions against microbial infections and during neoplasia. The macrophages may have pro- or antitumorigenic roles in colorectal cancer. Macrophages may produce either anti-inflammatory or proinflammatory cytokines. These functions change based on the stimulating agent. IFNγ and GM-CSF are reported to influence macrophage functions to overcome an immunosuppressive tumor microenvironment toward increased antitumor activities (14). Thus, it is evident that innate immune cells orchestrate an inflammatory environment that can be modulated with the use of natural agents, such as Frondanol A5, to inhibit colorectal cancer 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 colorectal cancer. Hence, in this study, we investigated whether Frondanol A5 has efficacy against colorectal cancer in APCMin/+ mice and we analyzed the modulation of circulating cytokines and inflammatory markers in intestinal tumors. Furthermore, we isolated peritoneal macrophages from mice to test whether 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 (i) has chemopreventive efficacy against intestinal tumors in APCMin/+ mice, (ii) inhibits inflammatory cytokines, and (iii) validates the mechanism that enhances phagocytosis.

Materials and Methods

Mice and experimental design

Male APCMin/+ (C57BL/6J) and female wild-type littermate mice were purchased from The Jackson Laboratory as founders. We established our own breeding colony at the University of Oklahoma Health Sciences Center (OUHSC; Oklahoma City, OK) 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 manufacturer’s 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 ×1,000 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 that had internalized at least one particle per high power field (×1,000, 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, because we could count up to eight engulfed intracellular yeast cells. Macrophages that contained 1 or 2 and 3 or more yeast cells were measured by examining approximately 200 to 300 macrophages over 100 fields. The data were 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.

Histologic 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 S1 for primer information. Results were expressed as a fold difference in gene expression.

Statistical analysis

Data were described as mean ± SEM. Statistical analysis was done with GraphPad Prism software 5.1 (GraphPad Software, Inc.). Comparisons were made between APCMin/+ control and Frondanol A5-fed mice using the two-tailed Student t tests.

Results

Frondanol A5 suppresses intestinal tumor formation in APCMin/+ mice

Dietary administration of Frondanol A5 produced no toxicities in animals. The weekly body weights of treated mice
Frondanol A5 Prevents Intestinal Tumorigenesis

Figure 1.
A, active ingredients in sea cucumber extract Frondanol A5. Fucosylated chondroitin sulfate, 12-methyltetradecanoic acid, and Frondoside A. B, experimental design for Frondanol A5 dietary feeding in APCmin/C6 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 APCmin/C6 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 ± SEM); D, female APCmin/C6 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 ± SEM). We observed statistically significant differences in body weight gains with both genders.

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 APCmin/+ mice at two dose levels. After 14 weeks of feeding with Frondanol A5, at the age of 20 weeks, the number of small intestinal polyps (SIP) and colon tumors (CT) 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 ± 4.0 and 2.22 ± 0.44, respectively (Fig. 2A and B). The number of SIPs and CTs in female mice at 20 weeks of age was 78.4 ± 9.4 and 2.43 ± 0.37, respectively (Fig. 2C and D).

Dietary administration of 250 and 500 ppm of Frondanol A5 suppressed SIP 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% in male and 0% in female (P < 0.05 vs. the untreated control), 21.0% in male and 53% in female (P < 0.01 vs. the untreated control), and 19.00% in male and 81.5% in female (P < 0.01 vs. the untreated control), respectively (as shown in Fig. 2E and 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 CT multiplicities by 65% (P < 0.007) and 75% (P < 0.005) compared with control diet-fed male APCmin/+ mice (Fig. 2B) in a dose-dependent manner. Similarly, in female APCmin/+ mice, both dose levels of dietary Frondanol A5 suppressed CT multiplicities up to 80% (P < 0.0001; Fig. 2D). Further histologic 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 APCMin/þ mice was limited to few intestinal tumors. With both doses of Frondanol A5 treatments, we observed about 90% reduced HGD compared with untreated control diet–fed APCMin/þ 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%, IL1B 98%–99%, IL2 98%–99%, IL4 90%–95.9%, IL6, 30.9%–54.31%, IL10 57%–73%, IL12 62%–55.4%, IL17A 53.4%, IFNγ 46.4%, and TNFα, 94.8%–94.7%, in male and female mice, respectively (P < 0.0001 vs. the untreated control).
Frondanol A5 inhibits mRNA expression of 5-LOX and its regulator, FLAP. Decreased mRNA expression analysis showed that Frondanol A5 inhibited mRNA expression of 5-LOX and its regulator, FLAP (Fig. 4A and B). A similar inhibitory effect on these biomarkers was observed with both of the doses tested for protein expression in intestinal tumors, as evaluated by immunohistochemistry staining (Fig. 4C–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 proliferating cell nuclear antigen (PCNA) in treated intestinal tumors, suggesting inhibition of proliferation in treated samples (Supplementary Fig. 5A–5C).

Frondanol A5 increased APCs 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. S2A). To be more specific, SLPs and CTs were stained for immunohistochemistry 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 approximately 50% and intratumoral macrophages by approximately 45% in intestinal tumors (P < 0.01; Supplementary Fig. S2A and S2B).

To evaluate whether 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, SLPs, 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 SLPs, respectively (Fig. 5C). As GILT mRNA expression increased in tumors, we analyzed for IFNγ expression in CTs and SLPs, because IFNγ is reported to induce GILT. Although the increase was not significant, Frondanol A5 treatment increased IFNγ protein expression in CTs and SLPs (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. 5E; ~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 with 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 have shown that, aside from genetic predispositions, colorectal cancer is mainly influenced by or associated with food intake. Many researchers have suggested that the most effective
agents for colorectal cancer prevention are derived from natural food sources. Although ongoing research continues to explore colorectal cancer prevention, no natural agent has been approved for colorectal cancer prevention to date. This study showed that sea cucumber extract Frondanol A5 reduces the number of large polyps in the small intestine and colonic tumors of APCMin/+ mice. Although we observed a significant inhibition of intestinal tumors, the percentage 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 toward treatments, a finding which we reported in our previously published articles examining different agents in APCMin/+ 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 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 APCMin/+ 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 APCMin/+ 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 this 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 (9, 27–30). Sea cucumbers have also been described to interfere with several tumor pathways and improve immune responses in other cancers (31). We have previously shown

**Figure 4.** (Continued.) 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.
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). Furthermore, Collin and colleagues (32, 33) demonstrated that Frondoside A, a component of Frondanol A5, inhibited microtubule formation in an angiogenesis assay, and also stimulated innate immunity in vivo. Dietary supplementation of Frondanol A5 in combination with other natural agents enhanced its anti-inflammatory properties (34). Previous studies with Frondanol A5 showed that it displays multi-mechanistic potential due to the presence of several active agents. In this study with APCMin/+ 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 (35), is often correlated with stage of malignancy, infiltration, metastases, and survival. Normal intestinal mucosa show approximately 30% PCNA expression, compared with 72% expression in carcinoma cells. PCNA is also highly expressed in higher grade tumors (36). 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 proinflammatory cytokines such as IL6, TNFα, and IL1. In this study, IL10 was observed to be downregulated.
in serum samples from treated mice. Although studies have shown
that IL10 is anti-inflammatory under inflammatory bowel disease
conditions, IL10 is reported to play both protumoral and antitu-
moral 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 IL10 is also reduced by
licofelone in this mouse model (37). Similarly, in this study, we
have seen that Frondanol A5 decreased IL10 expression and
improved innate immune responses against tumor formation.
Overexpression of 5-LOX has been implicated in in-
flammatory diseases and many cancers (37, 38). Previous studies have shown that inhibition of the 5-LOX metabolite leads to apoptosis in prostate and colon cancers (39). Frondanol A5—treated tumors showed a significant decrease in 5-LOX and its activator protein. These results suggest that Frondanol A5 possesses anti-inflammato-
ry properties. The sources of inflammatory molecules are
reported to be macrophages and DCs (40). We observed a decrease in isoforms of VEGF transcription and proinflammatory cytokines in Frondanol A5—treated tumors. Proinflammatory cytokines seem to serve a role in tumor development rather than tumor evasion. However, in this study, we observed a decrease in proinflamma-
tory 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 this
study, we determined that Frondanol A5 can increase GM-CSF
and G-CSF protein expression in APCMin/+ 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 and
colleagues (14) reported that GM-CSF stimulates tumoricidal
activity against the malignant melanoma cell line A375. In this
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 (41–43). CD163
expression was observed to be associated with early recurrence
and reduced survival time in patients with rectal cancer; CD163
expression was mostly observed after irradiation (44). Our data
clearly suggest that Frondanol A5 treatment decreased in-
flammation, 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 (45). 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 toward antitumor 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 (46). Furthermore, lower GILT expression reduced CD4+ T cell–mediated responses in vivo mouse models (47–49). 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<sup>Min</sup>/<sup>+</sup> 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 intratumoral 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. Furthermore, 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 IFNγ expression in CTs and SIPs, because IFNγ is reported to induce GILT. Although the increase was not significant, Frondanol A5 treatment increased IFNγ protein expression in CTs and SIPs. This partly explains the mechanistic axis of macrophages—GILT–IFNγ–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 toward effector cells, eliminate transformed or tumor cells, and inhibit tumor growth.

In summary, Frondanol A5 exhibited anti-inflammatory, anti-proliferative, and antiangiogenic effects. This sea cucumber extract also enhanced innate immunity against intestinal tumors by improving macrophage phagocytosis in APC<sup>Min</sup>/<sup>+</sup> mice.

Disclosure of Potential Conflicts of Interest
P.D. Collin is Director Coastside Bio Resources; reports receiving a commercial research grant from Maine Technology Institute; has received other commercial research support from National Cancer Institute; and has ownership interest (including patents) in U.S. Patent Office. No potential conflicts of interest were disclosed by the other authors.

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Other [designed, produced, and trademarked Frondanol A5 to be a cancer-preventive agent for various cancers with grant support (RAPID Program/2001) from the NCI, Division of Cancer Prevention, wherein 5-lipoxygenase and compromised immunity contribute to the pathologic condition]: P.D. Collin

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