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A novel bioactive derivative of eicosapentaenoic acid (EPA) suppresses intestinal tumor development in *Apc*$^{\Delta 14/+}$ mice

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Summary:

A novel ionic derivative of EPA significantly suppresses intestinal tumor development in *Apc*$^{\Delta 14/+}$ mice, and the regression tree analyses to the global lipidomic data provide new insights into specific eicosanoid metabolites and their potential to exert control over early neoplasia.

Abstract

Familial adenomatous polyposis (FAP) is a genetic disorder characterized by the development of hundreds of polyps throughout the colon. Without prophylactic colectomy, most individuals with FAP develop colorectal cancer at an early age. Treatment with eicosapentaenoic acid (EPA) in the free fatty acid form (EPA-FFA) has been shown to reduce polyp burden in FAP patients. Since high-purity EPA-FFA is subject to rapid oxidation, a stable form of EPA compound has been developed in the form of magnesium l-lysinate bis-eicosapentaenoate (TP-252). We assessed the chemopreventive efficacy of TP-252 on intestinal tumor formation using *Apc*$^{\Delta 14/+}$ mice, and compared it to EPA-FFA. TP-252 was supplemented in a modified AIN-93G diet at 1, 2, or 4% and EPA-FFA at 2.5% by weight, and administered to mice for 11 weeks. We found that administration of TP-252 significantly reduced tumor number and size in the small intestine and colon in a dose-related manner and as effectively as EPA-FFA. To gain further insight into the cancer protection afforded to the colon, we performed a comprehensive lipidomic analysis of total fatty acid composition and eicosanoid metabolites. Treatment with TP-252 significantly decreased the levels of arachidonic acid (AA) and increased EPA concentrations within the colonic mucosa. Furthermore, a classification and regression tree (CART) analysis revealed that a subset of fatty acids,

including EPA and DHA, and their downstream metabolites, including PGE_3 and 14-HDoHE, were strongly associated with anti-neoplastic activity. These results indicate that TP-252 warrants further clinical development as a potential strategy for delaying colectomy in adolescent FAP patients.

Introduction

Familial Adenomatous Polyposis (FAP) is a rare genetic disease characterized by the formation of numerous colonic polyps occurring at an early age (1). FAP patients carry a germ-line mutation in the *Adenomatous Polyposis Coli* (*APC*) tumor suppressor gene. Upon loss of heterozygosity (LoH) of the wild-type allele (1), colon tumors develop rapidly throughout the colon. Screening, surveillance and prophylactic colectomy constitute the current standard of care for the management of FAP (2). Although a number of clinical and animal studies have demonstrated the limited chemopreventive efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) for the management of cancer risk in FAP patients (3-5), there are no FDA-approved drugs for the treatment of this disease. Given the significant likelihood of advanced neoplasia and the clinical consequences of colectomy in young FAP patients, the development of therapies to control colorectal polyp burden is strongly justified.

There is evidence from both clinical and animal studies that omega (ω)-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA), suppress intestinal polyp formation (6-8). Fini *et al.* (9) showed that a diet containing the free fatty acid form of EPA (EPA-FFA) reduced polyp burden in the $Apc^{Min/+}$ mouse model of FAP. Moreover, treatment of FAP patients for six months with EPA-FFA showed a 22% and 30% net reduction in adenomatous polyp number and size, respectively, in a randomized, double-blinded, placebo-controlled trial (7).

The underlying basis for the tumor suppressive activity of EPA has been attributed, in part, to its ability to act as a competitive inhibitor of AA oxygenation (10). Intake of EPA-FFA significantly increases the intestinal mucosal content of EPA, effectively displacing AA within membrane phospholipids (9). Both AA and EPA serve as substrates for the cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 (CYPs) enzymes and their respective synthases that collaborate in the formation of a complex array of bioactive lipid metabolites. Several metabolic products formed from AA, including prostaglandin E_2 (PGE_2), have been strongly associated with colorectal carcinogenesis (11). However, minor structural differences between AA and EPA lead to the synthesis of a distinct array of lipid metabolites, a shift that may contribute to the tumor suppressive properties of the ω-3 PUFAs (12).

Since high-purity EPA-FFA is subject to rapid oxidation, a novel ionic derivative of EPA has been developed in the form of magnesium l-lysinate *bis* eicosapentaenoate (TP-252; **Fig. 1A**). In the following study, we treated *Apc*$^{\Delta 14/+}$ mice, an established mouse model of human FAP (13-15), with a diet containing increasing concentrations of the drug to assess the potential efficacy of TP-252 against intestinal tumor development. Here we show that treatment with TP-252 significantly suppresses intestinal tumor development in *Apc*$^{\Delta 14/+}$ mice to a level comparable to that of EPA-FFA. To gain further insight into the cancer protection afforded to the colon, we performed a comprehensive lipidomic analysis of fatty acid composition and eicosanoid metabolites. Using the classification and regression tree (CART) method, these data suggest potential metabolic pathways associated with tumor protective mechanisms exerted by EPA. The data presented here provide justification for further clinical investigation of TP-252 as novel treatment for FAP patients.

Materials and methods

TP-252

TP-252 has been formulated as a unique derivative of EPA-FFA, designed to deliver therapeutic levels of EPA-FFA to the intestinal mucosa. The compound consists of magnesium L-lysinate*bis*eicosapentaenoate, an ionizable salt of eicosapentaenoic acid (EPA). As shown in **Figure 1A**, TP-252 uses an amine- based scaffold that links magnesium di-L-lysinate to two molecules of the free fatty acid form of EPA (EPA-FFA).

Animal treatment

Apc$^{\Delta 14/+}$ mice were kindly provided by Dr. Christine Perret at the Universite' Paris (15) and maintained in the animal facility at UConn Health. All mice were maintained in a light-cycled, temperature-controlled room and allowed free access to drinking water and diet *ad libitum*. All animal experiments follow the institutional and national guidelines for the care and use of animals, and were conducted with approval from the Center for Comparative Medicine (CCM) at UConn Health. Genotyping for *Apc* was performed using tail biopsies (15). Male and female *Apc*$^{\Delta 14/+}$ mice were included in each experimental group. Mice were housed individually and fed two pellets of diet per day (~5g). A modified AIN-93G diet with corn oil was used as a control. All mice were randomized at 4 weeks of age and fed control diet for one week. The experimental groups of mice were switched to diets supplemented with 1, 2 and 4% TP-252, or 2.5% EPA-FFA (w/w; Research Diets, Inc.; **Supplementary Table 1**) at 5 weeks of age. Each mouse had an average daily food intake of 2.5g. Since TP-252 has a 65% EPA

payload, average mice (25 g) consume ~0.7, 1.3 and 2.6g/kg body weight (*b.w.*) of TP-252 daily at 1, 2 and 4%, respectively. Similarly, daily consumption of 2.5% EPA-FFA yields an intake of 2.5g/kg of *b.w.* EPA-FFA diet was vacuum-packed for single use to avoid oxidative degradation of EPA. Mice were weighed once a week throughout the entire study period.

Tissue processing and analysis of tumor burden

Mice were sacrificed at 16 weeks of age, with the exception of two mice in the control group that were killed at 14 weeks of age. At sacrifice, the small intestine and colon were immediately flushed thoroughly with ice-cold PBS and slit-open longitudinally. Specimens were fixed flat in 10% neutral-buffered formalin and stored in 70% ethanol. Tissues were stained with 0.2% (v/v) methylene blue and the number and size of tumors (diameter) were scored under a dissecting microscope in a fully blinded manner. Tumor load per mouse was determined by using the tumor diameter to calculate the spherical tumor volume (mm^3), $V = (4/3) \times \pi \times r^3$.

Lipidomics Analysis

Proximal full-thickness colon specimens were cut in half and snap-frozen in liquid nitrogen in a Precellys tube (Cayman Chemicals). Global lipidomics analysis was performed at the Lipidomics Core at Wayne State University, as previously described (16). Briefly, samples were prepared for LC-MS analysis using C18 cartridges. HPLC was performed on a Prominence XR system (Shimadzu) using a Luna C18 column. HPLC eluate was directly introduced into the electrospray ionization source of a

QTRAP5500 mass analyzer (SCIEX) in the negative ionmode. Multiple Reaction Monitoring (MRM) was used to detect unique molecular ion-daughter ion combinations for each of the 125 transitions to monitor a total of 169 lipid mediators (**Supplementary Table 2**). Mass spectra for each detected lipid metabolite were recorded using the Enhanced Product Ion (EPI) feature to verify the identity of the detected peak. Data were collected and quantified using Analyst 1.6.2 (SCIEX) and MultiQuant (SCIEX) software, respectively. Correction for recovery efficiencies and relative quantitation of each analyte were performed using signals from each chromatogram corresponding to the spiked-in internal standards. Under standardized conditions of LC-MS quantitation, the detection limits for the eicosanoids are 1–2 pg on the column and the limit of quantitation is 5 pg at a signal-to-noise ratio of 3 (16).

Classification & Regression Tree (CART) analyses

We used the HPSPLIT procedure in SAS software (SAS Institute Inc., NC) to build a tree-based regression model to identify fatty acids that are the most important predictors of intestinal tumor numbers and colon tumor volume. We pooled data from the control, 4% TP-252 and 2.5% EPA-FFA, and omitted fatty acids or eicosanoids that had more than 40% of the data below the limit of detection. The rule generated at each step maximizes the class purity within each of the two resulting subsets. For example, the splits in the tree are based upon the tissue levels of fatty acids (ng/mg of protein) or eicosanoids (pg/mg of protein) identified as 'best-predictors', and the tree shows the predicted mean number or volume of intestinal tumors based on those splits.

Statistical analyses

For tumor and lipidomics analyses, GraphPad Prism V (GraphPad Software, Inc.) was used to perform statistical analyses. Comparisons of mucosal fatty acid and eicosanoid levels and of small intestine and colon tumor number and size were made using one-way ANOVA, with Bonferroni's *post-hoc* test for pairwise comparisons. For all statistical comparisons, a two-sided alpha level of significance of 0.05 was used.

Results

Treatment with TP-252 and EPA-FFA suppresses intestinal tumor development

To compare the effects of TP-252 with the previously reported efficacy of EPA-FFA on tumor development (9), $Apc^{\Delta 14/+}$ mice were examined after 11-weeks of treatment with EPA-FFA or TP-252. Mice treated with either EPA-FFA or TP-252 gained more body weight and showed a decrease in spleen weight, indicating reduced systemic inflammation associated with lessened disease severity (**Table 1**). In the small intestine, treatment with TP-252 caused a significant, dose-related reduction in tumor number (1.2-, 1.7-, and 2.1-fold at 1, 2 and 4%, respectively) equivalent to EPA-FFA (1.8-fold; **Table 1**). The protective effects of TP-252 were also evident in the incidence of tumors larger than 2-mm in diameter, which were significantly reduced with 2 and 4% TP-252 and EPA-FFA (2.5-, 4.0- and 11.9-fold, respectively; **Table 1**).

In the colon, TP-252 and EPA-FFA treatment resulted in a trend towards reduced tumor numbers, although the differences did not reach statistical significance due to the relative infrequency of colon tumors (**Table 1**). There was, however, a significant and dose-related reduction in colon tumor volume observed with 4% TP-252 (2.2-fold) and EPA-FFA (2.5-fold) compared to controls. Moreover, the frequency of colon tumors larger than 3-mm was significantly reduced with 1% (1.3-fold) and 4% (1.4-fold) TP-252, which was comparable to the effect observed with EPA-FFA (1.6-fold; **Table 1**). Most importantly, statistical analysis using a negative binominal model confirmed that the tumor protection observed with 4% TP-252 was as effective as that of EPA-FFA in both small intestine and colon (P=0.95).

The ANOVA results showed there were statistically significant treatment-related differences in tumor multiplicity in the small intestine both for gender (P=0.04) and for treatment (P>0.0001), but not for the gender/treatment interaction (P=0.13), indicating that the treatment effect was consistent for both male and female mice. For colon tumor multiplicity, there was a difference observed for gender (P=0.03), but not for treatment (P=0.23), and there was no suggestion of a gender/treatment interaction (P=0.79). For colon tumor volume, there were no differences observed for gender (P=0.28); however the treatment effect was significant (P=0.01), and there was no suggestion of a gender/treatment interaction (P=0.79). Importantly, the tumor protection observed with 4% TP-252 suggested a similarity of effectiveness when compared to that of EPA-FFA (P=0.95).

TP-252 treatment alters mucosal fatty acid profiles

Dietary supplementation with EPA has been shown to increase tissue levels of the ω-3 fatty acids, which are structurally distinct from the ω-6 fatty acids (**Fig. 1B**). To determine the effects of EPA-FFA and TP-252 on fatty acid composition within the colonic mucosa, total fatty acid profiles in aliquots of proximal colon tissue were analyzed by LC-MS, as described under Materials and Methods. As shown in **Figure 2A,** the tissue levels of AA were significantly reduced by TP-252 in a dose-related manner (1.5-, 1.7- and 2.6-fold at 1, 2 and 4%, respectively), and also by EPA-FFA treatment (3.3-fold; **Fig. 2A**). In addition, the levels of two AA metabolites, docosatetraenoic acid (DTA) and ω-6 docosapentaenoic acid (DPA), were significantly reduced in each of the treatment groups (**Fig. 2A**). As expected, EPA levels in the colon were markedly increased compared to controls in a dose-related manner (66-, 72- and 136-fold at 1, 2 and 4% TP-252; respectively) and 88-fold with EPA-FFA (**Fig. 2B**). Although it was not statistically significant, incorporation of EPA was higher in the 4% TP-252 group compared to the EPA-FFA (P=0.07). Furthermore, TP-252 significantly increased the levels of the EPA metabolites, ω-3 DPA (12-, 19- and 28-fold at 1, 2 and 4% TP-252, respectively) and docosahexaenoic acid (DHA) (2.6-, 2.9- and 2.5-fold at 1, 2 and 4% TP-252, respectively, **Fig. 2B**).

To elucidate the effect of EPA treatment on the *de novo* synthesis of fatty acids (**Fig. 1C**), we compared the tissue levels of several non-essential fatty acids, including palmitic acid, palmitoleic acids, stearic acids and oleic acids. As shown in **Supplementary Figure S1**, there were no significant treatment-related alterations to the levels of each of these non-essential fatty acids.

Eicosanoid profiles are significantly altered by changes to mucosal fatty acid composition

AA and EPA are the primary sources of a diverse array of eicosanoid metabolites generated through a complex network of enzymatic and non-enzymatic oxidation reactions (17). There are three major metabolic pathways that convert AA and EPA into their respective bioactive lipid metabolites (**Fig. 1B**). We first compared the effects of EPA-FFA and TP-252 on the formation of the COX metabolites; AA-derived series-2 prostanoids and EPA-derived series-3 prostanoids (**Fig. 1B**). As shown in **Figure 3A**, the mucosal levels of PGE_2 were reduced to a comparable extent by either 4% TP-252 (2.1-fold) or EPA-FFA (2.3-fold). In contrast, PGE_3 levels were markedly increased by treatment with TP-252 (up to 80-fold at 4%) and EPA-FFA (93-fold). Treatment with both TP-252 and EPA-FFA also affected the relative concentrations of the other COX-derived metabolites, including prostaglandin $F_3\alpha$ ($PGF_3\alpha$; **Fig. 3B**) and thromboxane B_3 (TXB_3; **Fig. 3C**). These levels were increased significantly relative to the corresponding levels of the AA-derived counterparts. Although prostaglandin D_2 (PGD_2) levels were modestly reduced by drug treatment, there was no corresponding increase in PGD_3 (**Fig. 3D**). Both forms of the J-series prostaglandins (PGJ_2/PGJ_3) were not significantly altered by EPA treatment (**Fig. 3E**).

The LOX-dependent pathways generate series-4 and series-5 leukotrienes and hydroxy-eicosatetraenoic acids (HETEs) and hydroxy-eicosapentaenoic acids (HEPEs) from AA or EPA, respectively (**Fig. 1B**) (18). As predicted from the observed tissue enrichment with EPA noted above (**Fig. 2B**), the levels of 12-HETE and 15-HETE were

significantly reduced, with the largest differences observed in the concentrations of 12-HETE in mice fed 4% TP-252 and EPA-FFA (3.7- and 4.1-fold, respectively; **Fig. 3F**). Consistent with these reduced concentrations, tissue levels of a series of EPA-derived HEPEs were increased to a significant extent by drug treatment (**Fig. 3F**). These changes were further reflected by an inverse relationship between LTB_4 and LTB_5, oxidized products of the 5/15-HETEs and 5/15-HEPEs, respectively (**Fig. 3G**).

Finally, we measured the levels of lipid metabolites generated by the activities of the CYP epoxygenases, focusing on epoxy-eicosatrienoic acid (EpETrE) and epoxy-eicosatetraenoic acid (EpETE), products of AA and EPA, respectively (17). These epoxygenase products are rapidly hydrolyzed and converted into dihydroxy-eicosatrienoic acid (DiHETrE) and dihydroxy-eicosatetraenoic acid (DiHETE). Similar to the changes observed for the COX/LOX metabolites described above, the tissue levels of the AA-derived metabolites were generally reduced, whereas EPA-derived lipid products were increased (**Fig. 3H & 3I**). Although 5,6-DiHETE has been identified as a ligand for the aryl hydrocarbon receptor (AhR) (19), which is an important signaling pathway for gastrointestinal homeostasis (20), its role in cancer has not been clearly defined.

DHA metabolites are increased with EPA treatment

DHA is a poor substrate for the COX enzymes and is primarily metabolized by the LOXs and CYPs, producing the hydroxy-docosahexaenoic acids (HDoHEs) (17). As shown in **Figure 4A,** a wide-ranging panel of HDoHEs was increased in the proximal colon upon treatment with either TP-252 or EPA-FFA. DHA and EPA can be further

metabolized *via* the LOXs to produce a series of resolvin Ds (RvD1-6) and resolvin Es (RvE1&3), respectively, which have potent immunomodulatory activities. Although colonic mucosal levels of EPA were significantly increased by treatment with TP-252 and EPA-FFA (**Fig. 2B**), the tissue concentrations of most of the assayed resolvins were at or below the level of detection (data not shown), with the exception of RvD5. As shown in **Figure 4B**, there was a moderate increase in RvD5 with 4% TP-252 and EPA-FFA.

CART analyses predicts lipid metabolites associated with tumor protection

To identify signature profiles of fatty acids, EPA treatment and tumor development, we built a tree-based regression model (CART) using data obtained from control, 4% TP-252 and EPA-FFA treated mice (n=53). The average tissue levels of CART predicted fatty acids and eicosanoids for each group are summarized in **Supplementary Table 3.** Here we show the results of these analyses for tumor multiplicity (small intestine) and tumor volume (colon). The impact of EPA treatment on colon tumor multiplicity is also reported in **Supplementary Figure S2**.

As shown in **Figure 5A**, the average tumor number in the small intestine was 42 in Node 0. The first split predicted that 68% of mice (36/53) had tissue levels of EPA higher than 52.9 ng/mg, which was correlated with lower tumor number (Node 2; avg. = 31). This node consisted of the EPA-treatment groups, highlighting the protection afforded by EPA. Node 2 was further subdivided into myristic acid and dihomo-g-linolenic acid (DGLA), indicating an average tumor multiplicity of 19 (Node 7; **Fig. 5A**). Each of the mice in the control group were associated with lower levels of EPA, with an average of 64 tumors (Node 1). This node was further bifurcated by cerotic acid, in

which mice were separated equally between higher (Node 3) and lower (Node 4) tumor multiplicity (avg. = 43 vs. 88; **Fig. 5A**).

Interestingly, the regression tree analysis produced a distinct lipid profile for colon tumor volume. As shown in **Figure 5B**, the first separation predicted that 87% of mice (46/53) with DHA levels exceeding 101.9 ng/mg had smaller tumors (avg. = 13 mm^3; Node 2), while the remaining 13% of mice had approximately three times larger tumors (Node 1). Since the average level of DHA is between 135.3 to 342.0 ng/mg across the three groups (**Supplementary Table 3**), a DHA cut-point above 101.9 ng/mg may be sufficient for cancer protection. The majority of EPA-treated mice segregated towards smaller colon tumors together with higher levels of DHA (**Fig. 5B**). Furthermore, suppression of colon tumor growth was associated with lower levels of erucic acid (Node 5; **Fig. 5B**). However, some additional benefit was correlated with palmitoleic acid and heptadecenoic acid (Node 8, 9; **Fig. 5B**). The majority of mice with DHA <101.9 ng/mg were found in the control group (Node 1). Further bifurcation generated two nodes based upon the levels of linoleic acid (LA), in which 57% of mice (4/7) had lower LA and smaller tumor size (Node 3; avg. = 17 mm^3).

As shown in **Figure 5C** and **5D,** CART analysis was utilized to stratify fatty acid-derived eicosanoid metabolites for their effects on tumorigenesis. For both small intestine and colon, there was a strong correlation between 14-HDoHE and tumor protection. The majority of mice had higher levels of 14-HDoHE, which were associated with both lower tumor multiplicity in small intestine and colon tumor volume (avg. = 32 and 11 mm^3, respectively) (Node 2; **Fig. 5C & 5D**). Similar to the results obtained with

fatty acid analysis, most EPA-treated mice had smaller tumors (number and volume) at the first separation. In further splits, tissue levels of 8-iso$PGF_2\alpha$/11b$PGF_2\alpha$ followed by 19,20-DiHDoPE were associated with lower tumor multiplicity in the small intestine (Node A; avg. = 20; **Fig. 5C**). Higher levels of PGE_3 correlated with tumor protection in the small intestine (Node 8; avg. = 28). Interestingly, treatment with TP-252 or EPA-FFA failed to suppress tumor development if PGE_3 levels were below a critical level (Node 7; avg. = 53). Control mice with lower levels of 14-HDoHE (Node 1) were separated by the levels of 13,14dh-15k-PGE_1 (Node 4), a breakdown product of PGE_1. As shown in **Figure 5D**, in addition to 14-HDoHE, suppression of colon tumor growth was strongly associated with 13-HODE (Node 5) and PGJ_2, with an average tumor volume of 2 mm^3 (Node 7; Fig. 5D). These particular nodes were comprised almost entirely of EPA-treated mice. The group with more PGJ_2 (Node 8) was further split by $PGF_2\alpha$, with colon tumor volume reduced to an average of 7 mm^3 (Node A; Fig. 5D).

Finally, mice with less 14-HDoHE (Node 1) were further separated by the levels of resolvin D5 (RvD5), with an average colon tumor volume of 72 mm^3 (Node 3) and 27 mm^3 (Node 4) (**Fig. 5D**). RvD5 is a DHA-derived specialized pro-resolving lipid mediator (SPM) with potent anti-inflammatory activity (21). Although the tissue levels of RvD5 (**Fig. 4B**) were not correlated with DHA (**Fig. 2B**), RvD5 was one of the strongest predictors of colon tumor size. The RvD5 cut-point (23.3 pg/mg) was much lower than the average levels of RvD5, ranging from 524.2 to 754.5 pg/mg (**Supplementary Table 3**), suggesting that even relatively small amounts of RvD5 may significantly contribute to tumor protection.

Discussion

TP-252, magnesium L-lysinate *bis* eicosapentaenoate, is a novel molecular entity that delivers beneficial levels of EPA-FFA to tissues and can be developed for the treatment of FAP. The present study extends earlier findings in $Apc^{Min/+}$ (9) and demonstrates that treatment with TP-252 significantly suppresses intestinal tumor development in a second *Apc* cancer model, $Apc^{\Delta 14/+}$ mice. As reviewed in-depth by Cockbain *et al.* (22), ω-3 PUFAs such as EPA elicit a wide array of anti-tumor activities, demonstrated in cell culture systems and in pre-clinical tumor models, as well as in human clinical trials. Although the precise mechanisms by which EPA suppresses tumor growth are not entirely understood, a unifying principle for its protective effects is largely attributed to its ability to act as a competitive inhibitor of AA, exerting its effects across a wide range of metabolic pathways. Our study uncovers a wide range of metabolic changes provoked by EPA that we believe may directly contribute to its tumor-suppressive properties.

Both AA and EPA are essential fatty acids that must be obtained from dietary sources. These fatty acids provide substrates to more than 20 individual receptor-mediated signaling cascades; however, subtle differences in the chemical structures of AA and EPA underscore their disparate cellular actions (22, 23). AA, which is derived largely from LA, is a 20-carbon ω-6 fatty acid with four double bonds (C20:4). On the other hand, EPA, which is ingested from fish oils or indirectly derived from α-linolenic acid (ALA), is a 20-carbon ω-3 fatty acid with five carbon double bonds (C20:5). An extra double bond in EPA results in the production of distinct set of metabolites, which may elicit anti-inflammatory properties. One key metabolic process that distinguishes

the biological actions of EPA from AA is its efficient conversion to DHA, a 22-carbon ω-3 fatty acid with six double bonds (C22:6), which gives rise to a wide range of bioactive lipid metabolites. Included among the DHA metabolites are the maresins and protectins, SPMs that play an important role in the resolution of inflammation (24). The role of these particular SPMs in intestinal carcinogenesis is presently unknown, but our data suggest potential contributions to the protection afforded by EPA.

Our study establishes that TP-252 is effective at delivering high concentrations of EPA into the colonic mucosa. Resulting concentrations are sufficient to markedly alter tissue fatty acid composition, causing a subsequent shift in the overall profile of downstream lipid metabolites. Using a CART analyses, we identified distinct lipid signatures that were associated with cancer protection. At the outset, tissue levels of EPA were strongly correlated with tumor multiplicity in the small intestine, providing an internal validation of our dietary protocol. In the colon, CART analysis identified DHA as the strongest predictor of tumor protection. The beneficial effects of DHA have been demonstrated earlier in several *Apc*-mutant mouse models (26, 27). However, the administration of EPA alone or as a fish oil preparation providing both EPA and DHA, indicated improved tumor protection (9, 26). The beneficial effects afforded by EPA, compared to DHA alone, may depend upon the model system employed. In the case of *Apc*-mutant mice, activation of the COX-2/PGE_2 axis is important in tumor growth that displacing AA with EPA, the direct source of prostaglandins, would be expected to exert a beneficial effect (13, 14, 28). While DHA is a structurally poor substrate for COXs, EPA can produce, for example, PGE_3 that can counteract the pro-tumorigenic effects of PGE_2. Therefore, EPA serves as a universal lipid precursor that provides substrate for a

diverse array of lipid metabolites, many of which may ultimately contribute to its anti-cancer activities.

DHA is formed by the actions of the elongases and delta-6 desaturases, followed by β-oxidation. These lipid-modifying enzymes play an important role in energy generation, particularly during cancer development (29). In fact, dysregulated lipid metabolism has been shown in a variety of cancers to directly influence tumor initiation and growth (30, 31). For example, melanomas, lung cancers (32) and breast cancers (33) have increased activity of the delta-6 desaturase that controls the *in situ* formation of AA directly within the tissue, thereby promoting cell proliferation and survival *via* elevated prostaglandin synthesis. Moreover, the selective inhibition of the delta-6 desaturase by SC-26196 reduces the number of intestinal tumors in $Apc^{Min/+}$ mice by 37% (34). In the present study, there is evidence for enhanced lipid metabolism influencing tumor development, demonstrated by the association between higher levels of cerotic acid and increased tumor multiplicity in the small intestine (**Fig. 5A**). Cerotic acid belongs to a family of very long chain saturated fatty acids (VLCFA, C26:0), and is a minor fatty acid component of human tissues (35). Increased serum levels of this fatty acid are often associated with coronary risk factors, such as metabolic syndrome, atherosclerosis and systemic inflammation (36). In fact, cerotic acid levels have recently been proposed as a metabolic serum marker for CRC, together with up-regulation of the elongases, *ELOVL1* and *ELOVL6*, within colon tumor tissues (37). In the present study, an involvment of cerotic acid was exclusively found within mice fed the control diet, suggesting that the fatty acid may be a lipid signature unique in the tumor-bearing $Apc^{\Delta 14/+}$ mice. Although the biological activity of cerotic acid towards colon carcinogenesis has not yet been defined, it is possible that EPA may exert control over

the synthesis and/or function of this fatty acid. For example, if we assume that dysregulated lipid metabolism is present in tumor-bearing mice, excess levels of EPA may facilitate the formation of DHA, which in turn produces metabolites that can shift early neoplastic growth towards a growth suppressive phenotype. In fact, eicosanoid metabolites most strongly associated with tumor protection were 14-HDoHE and 19, 20-DiHDoPE, DHA-derived metabolites formed by the actions of the LOX and CYP, respectively (**Fig. 5C & 5D**). Freeman *et al.* (38) recently showed that 14-HDoHE is a substrate for 15-PGDH, generating the electrophilic metabolite, 14-oxoDHA, which has been shown to inhibit LPS-induced pro-inflammatory cytokine expression in primary alveoloar macrophages. Moreover, DHA is metabolized by macrophage-derived 12-LOX to form 14-HDoHE, leading to the formation of maresins, a member of the SPM class of lipids (39, 40). SPMs elicit their effects in part by blocking neutrophil migration, while enhancing macrophage phagocytosis of apoptotic neutrophils, thus limiting neutrophil-mediated tissue damage (41). Several isomers of the hydroxy-DHA metabolites been reported to have bioactivity against tumor growth. For example, 4-HDoHE (4-HDHA) was shown to directly inhibit endothelial cell proliferation and angiogenesis *via* peroxisome proliferator-activated receptor γ (PPARγ), effects that are independent of its immunomodulatory activity (42). Moreover, as shown by O'Flaherty *et al.* (42), treatment of prostate cancer cells with the 17-series DHA metabolites, including 17-HDoHE (17-HDHA), significantly reduced cell proliferative activity. Although a wide range of anti-inflammatory activities for 14-HDoHE, 19,20-DiHDoPE and their direct metabolites have been established, the potential role of these bioactive lipid metabolites on colon carcinogenesis are presently unknown. To our knowledge, this is the first study to report the potential impact of these LOX and CYP metabolites on intestinal tumor development.

The influence on carcinogenesis of a number of metabolic products derived from AA *via* the COXs have been studied extensively (reviewed in (43)). In particular, our laboratory has validated a direct role for inducible PGE_2 in the initiation and promotion of intestinal cancer using a mouse genetic knockout model of *mPGES-1* (13, 14, 28). In the present study, CART analysis identified EPA-derived PGE_3 as one of the strongest predictors of tumor protection in small intestine. Emerging evidence suggests that the antagonistic effects of EPA on AA metabolism are due in part to their relative catalytic efficiency towards the COX/LOX/CYP enzymes and the binding affinities of the respective eicosanoid products for their cognate receptors (17, 44). For example, the relative activity of the COX enzymes towards EPA is only 10-30% of its activity towards AA (45). Furthermore, the downstream synthases, mPGES-1 and PGD synthase (PGDS), have three-fold less efficiency for generating PGE_3 and PGD_3 when EPA is the available substrate (45). Moreover, Wada *et al*. (45) demonstrated that PGE_3 is approximately 2- to 3-fold less effective than PGE_2 in binding efficiency to the EP1-3 receptors within the cell membrane fraction of human kidney cells (HEKs). Similarly, LTB_4, an AA-derived leukotriene that is a potent activator of neutrophils and eosinophils, is at least five times more active than its EPA counterpart, LTB_5 (46). Directly related to this observation, we found an increase in the levels of LTB_5 upoin treatment with either TP-252 or EPA-FFA, an effect that could potentially influence immune cell activation. Such an effect would likely contribute to the observed reduction in systemic inflammation found in the EPA-fed mice (**Table 1**). Taken together, our findings suggest that EPA-derived metabolites can markedly interfere with the formation of downstream AA-derived lipid mediators *and* simultaneously de-accelerate the rate of receptor-mediated activity that is dependent upon these eicosanoids. However, we cannot exclude the possibility

that many of the EPA-derived metabolites that were increased following EPA treatment may also independently contribute to its tumor suppressive properties.

In the present study, we selected the proximal colon for our lipidomic analysis because *Apc*$^{\Delta 14/+}$ mice do not typically develop tumors within this region of the intestine, thus circumventing the potential variability of these experimental endpoints associated with the presence of tumors. Thus we have provided a broad-based perspective of the metabolic changes associated with long-term EPA treatment to normal colonic mucosa in close proximity to *Apc*-initiated tumors. Recently, Djuric *et al.* (25) performed a lipidomic analysis of normal colonic mucosa and tumor tissue isolated from carcinogen-treated rats maintained on either a Western diet or a diet enriched in fish oils. Interestingly, the diets caused differential changes to fatty acid composition of the normal mucosa compared to tumor tissue. In the tumors, there was a distinct lipogenic phenotype that was absent in normal mucosa (25). Based upon these recent findings, future experiments may be warranted to investigate the effects of TP-252 on lipid metabolism directly within *Apc*-mutant tumors, data that would provide additional insight into the tumor protection associated with EPA treatment.

In summary, we have demonstrated the chemopreventive efficacy of a novel EPA derivative, TP-252, on intestinal tumor development in *Apc*$^{\Delta 14/+}$ mice. The protection afforded by TP-252 is comparable to that of EPA-FFA, the free fatty acid form of EPA that has been shown earlier in both clinical (7) and pre-clinical (9) studies to have therapeutic efficacy in FAP disease. Our comprehensive lipidomics analysis shows that treatment with TP-252 can simultaneously enhance both the incorporation of EPA into

colonic tissue, thereby displacing AA, while causing a pronounced metabolic redirection of fatty acids towards EPA-derived, anti-inflammatory lipid metabolites. Furthermore, the application of CART analyses to the global lipidomic data provide new insights into specific eicosanoid metabolites and their potential to exert control over early neoplasia. Based upon these promising pre-clinical findings, further studies are warranted to elucidate the exact mechanisms by which long-term treatment with this ω-3 fatty acid derivative may elicit its tumor protection, particularly in high-risk FAP patients.

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Figure Legends

Figure 1: Structure of TP-252 and fatty acid metabolizing pathways. (**A**) Structure of TP-252 showing an amine- based scaffold that links magnesium di-L-lysinate to the free fatty acid form of EPA (EPA-FFA). (**B**) Essential fatty acid synthesis pathways showing ω-6 and ω-3 series. A panel of eicosanoid metabolites synthesized *via* respective enzymes are also depicted. (**C**) Non-essential fatty acids synthesis pathways showing ω-7, ω-9 series and saturated fatty acid (SFA). Number of carbons and double bonds are denoted for each fatty acid. A panel of fatty acids is synthesized by a series of desaturation and elongation reactions. The respective enzymes that are responsible for generating lipid metabolites are depicted. Δ-6-d, delta-6-desaturase; Δ-9-d; delta-9-desaturase; ELOVL, elongase.

Figure 2: Changes in mucosal fatty acid profiles by dietary supplementation with TP-252 and EPA-FFA. A comprehensive lipidomic analysis of the proximal colon was performed as described under Materials and Methods. The data show alterations to the levels of AA and its fatty acid metabolites, DTA and ω-6 DPA (**A**), EPA and its fatty acid metabolites (**B**). Bars indicate means ± S.E.M. Asterisks indicate statistically significant differences in treatment groups relative to control; *=P<0.05, **=P<0.01, ***=P<0.001.

Figure 3: Changes in COX-, LOX- and CYP-generated eicosanoid metabolites by dietary supplementation with TP-252 and EPA-FFA. Colonic tissue levels of AA-derived eicosanoids and their EPA-derived counterparts show an inverse relationship with increasing concentrations of TP-252. Tissue levels of PGE_2 and PGE_3 (**A**), $PGF_2\alpha$ and $PGF_3\alpha$ (**B**), TXB_2 and TXB_3 (**C**), PGD_2 and PGD_3 (**D**), 15d-PGJ_2 and 15d-PGJ_3 (**E**), HETEs and HEPEs (**F**) LTB_4 and LTB_5 (**G**), AA-derived DiHETrEs (**H**) and EPA-derived 5,6-DiHETE (**I**). Bars indicate means ± S.E.M. Asterisks indicate statistically significant differences in treatment groups relative to control; *=P<0.05, **=P<0.01, ***=P<0.001.

Figure 4: DHA metabolites are increased by treatment with TP-252 or EPA-FFA. EPA treatment increases DHA metabolites. Tissue levels of DHA-derived metabolites (HDoHEs) (**A**) and RvD5 (**B**). Bars indicate means ± S.E.M. Asterisks indicate statistically significant differences in treatment groups relative to control; *=P<0.05, **=P<0.01, ***=P<0.001.

Figure 5: CART analyses identify distinct lipid metabolite profiles associated with tumor protection. Regression tree analysis predicts a set of fatty acids or eicosanoids

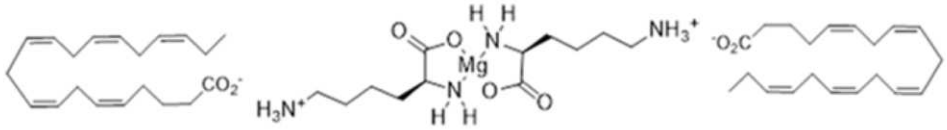
that are strongly correlated with the number or size of intestinal tumors. The splits in the trees are based upon the tissue levels of fatty acids (ng/mg of protein) or eicosanoids (pg/mg of protein) indicated above the nodes. Fatty acids associated with tumor multiplicity in the small intestine (**A**) and tumor size in the colon (**B**). Eicosanoids associated with tumor multiplicity in the small intestine (**C**) and tumor size in the colon (**D**). The relative thickness of the tree branch indicated in the diagram approximates the number of mice that are split into their respective nodes. N, number of animals; Avg, average number or size (mm^3) of tumors; Ctl, number of mice in the control group; TP, number of mice in the TP-252 treated group; EPA, number of mice in the EPA-FFA-treated group.

Supplementary Figure S1: Treatment with TP-252 and EPA-FFA does not modify the levels of fatty acids generated by *de novo* synthesis. A comprehensive lipidomic analysis of the proximal colon was performed as described under Materials and Methods. Tissue levels of palmitic acid, palmitoleic acid, stearic acid and oleic acids in each of the treatment group are shown. Bars indicate means ± S.E.M.

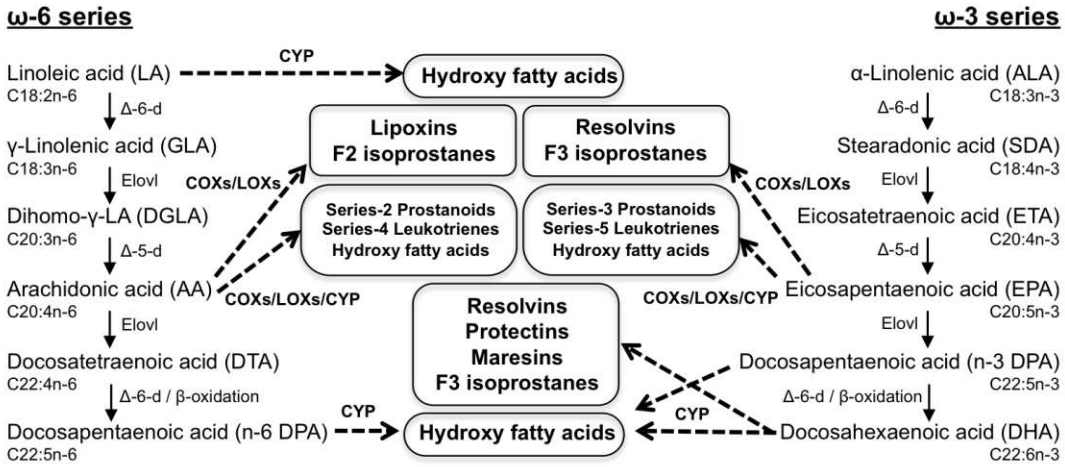
Supplementary Figure S2: CART analyses identify distinct lipid metabolite profiles associated with colon tumor numbers. Regression tree analyses predict a set of fatty acids or eicosanoids that are strongly correlated with the number of tumors in the colon. Fatty acids (**A**) and eicosanoids (**B**) associated with tumor multiplicity. N, number of animals; Avg, average number or size (mm^3) of tumors; Ctl, number of mice in control group; TP, number of mice in TP-252 treated group; EPA, number of mice in EPA-FFA treated group. The tissue levels of fatty acids (ng/mg of protein) or eicosanoids (pg/mg of protein) are indicated above the nodes.

Figure 1

A



B



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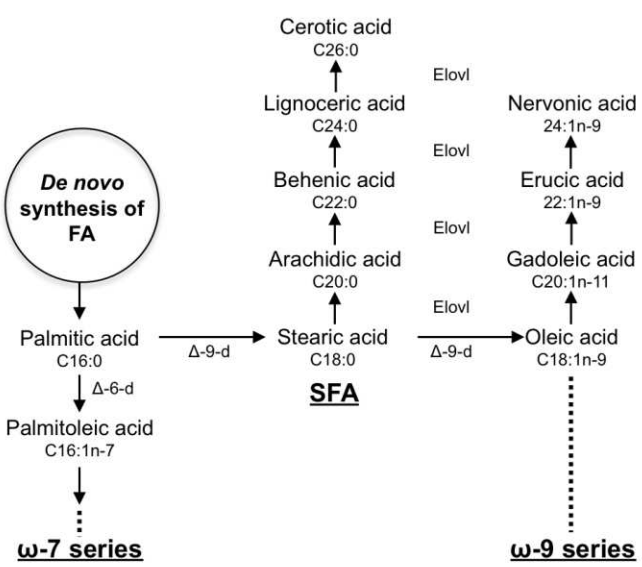

Figure 2

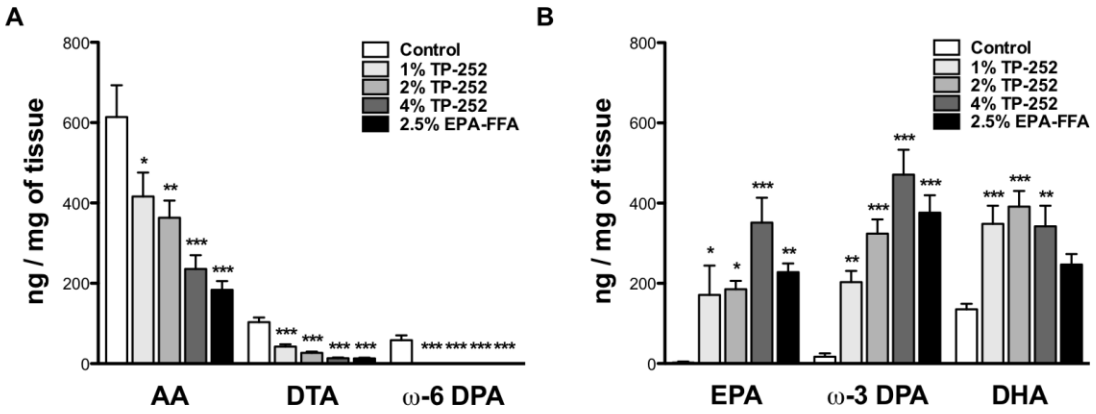

Figure 3

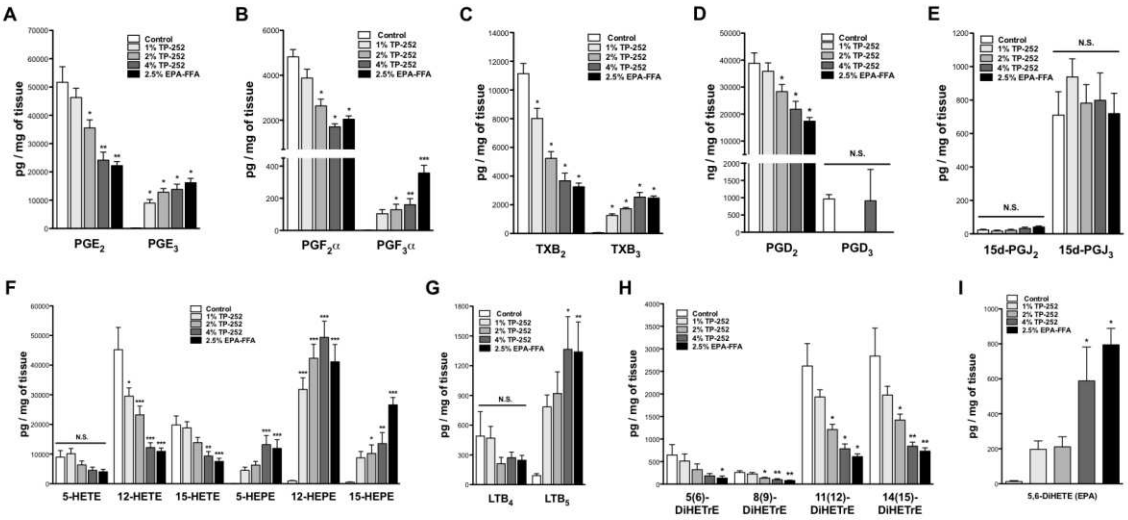

Figure 4

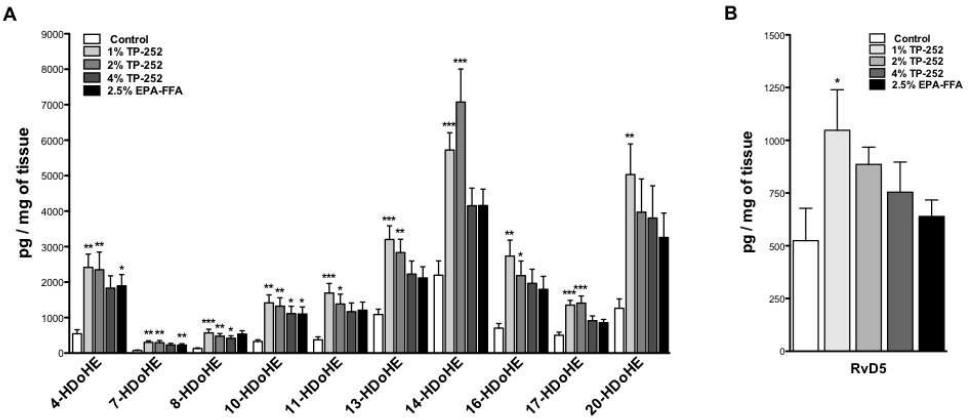

Figure 5