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Combination of atorvastatin with sulindac or naproxen profoundly inhibits colonic adenocarcinomas by suppressing the p65/β-catenin/cyclin D1 signaling pathway in rats

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Abstract

Evidence supports the protective role of non-steroidal anti-inflammatory drugs (NSAIDs) and statins against colon cancer. Experiments were designed to evaluate the efficacies atorvastatin and NSAIDs administered individually and in combination against colon tumor formation. F344 rats were fed AIN-76A diet and colon tumors were induced with azoxymethane (AOM). One week after the second AOM-treatment groups of rats were fed diets containing atorvastatin (200 ppm), sulindac (100 ppm) or naproxen (150 ppm), or their combinations with low-dose atorvastatin (100 ppm) for 45 weeks. Administration of atorvastatin at 200 ppm significantly suppressed both adenocarcinoma incidence (52% reduction, $p=0.005$) and multiplicity (58% reduction, $p=0.008$). Most importantly, colon tumor multiplicities were profoundly decreased (80–85% reduction, $p<0.0001$) when given low-dose atorvastatin with either sulindac or naproxen. Also, a significant inhibition of colon tumor incidence was observed when given a low-dose atorvastatin with either sulindac ($p=0.001$) or naproxen ($p=0.0005$). Proliferation markers, proliferating cell nuclear antigen, cyclin D1 and β-catenin in tumors of rats exposed to sulindac, naproxen, atorvastatin, and/or combinations showed a significant suppression. Importantly, colon adenocarcinomas from atorvastatin and NSAIDs fed animals showed reduced key inflammatory markers, inducible nitric oxide synthase and cyclooxygenase-2, phospho-p65, as well as inflammatory cytokines, TNF-α, IL-1β, and IL-4. Overall, this is the first report on the combination treatment using low-dose atorvastatin with either low dose sulindac or naproxen, which greatly suppress the colon adenocarcinoma incidence and multiplicity. Our results suggest that low-dose atorvastatin with sulindac or naproxen might potentially be useful combinations for colon cancer prevention in humans.

Keywords

atorvastatin; NSAIDs; colon cancer; β-catenin; cyclin D1; p65

INTRODUCTION

Colorectal cancer is one of the major leading causes of death from cancer in the United States as well as in the worldwide. It is predicted to be responsible for the death of almost 50,000 people each year in the United States alone [1,2]. Because the majority of the cause of colon cancer is attributable to lifestyle, diet, and genetic factors, there has been increasing awareness and focus on the prevention of colon cancer [3,4]. In particular, the presence of chronic inflammatory conditions in the colonic environment has been implicated in the development of colorectal cancer, and treatment regimens against inflammatory markers have reduced the risk of colon cancer [5–7].

Increased aberrant expression of inflammatory genes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenases (COXs), has been shown in the azoxymethane (AOM)-induced colon cancer model from the early stage of hyperplastic aberrant crypt foci (ACF) to late stage adenocarcinoma [8–14]. Since many studies report that selective iNOS and COX-2 inhibitors exerted suppressive effects against colon cancer [8,11,15–22], there is a rationale for investigate the ability of the combination of low dose atorvastatin and non-steroidal anti-inflammatory drugs (NSAIDs) to inhibit iNOS and COX-2 in a colon cancer model where inflammatory genes play a key role in carcinogenesis.

Evidence supports the protective role of NSAIDs and statin [15,16,23–27]. In our earlier study, we showed that statins such as atorvastatin (Lipitor® as well as anti-inflammatory drugs (NSAIDs) as effective agents in suppressing colon cancer in animals [7,16,24]. AOM-induced tumors result from mutations in the Wnt/ β -catenin pathway [28–30]. Aberrant expression of β -catenin can be regarded as a key event during colorectal tumorigenesis [31] and is linked to the increased transcription of a number of genes, such as cyclin D1 [32,33]. Cyclin D1 is overexpressed in patients with adenomatous polyps, primary colorectal adenocarcinoma, and familial adenomatous polyposis [32,34]. Cyclin D1 is a target gene of the Wnt signaling pathway [35], and mutations in this pathway are responsible for approximately 90% of colorectal cancer [36]. Mutations in genes belonging to the Wnt pathway, such as inactivating mutations in the adenomatous polyposis coli (APC) gene or activating mutations in β -catenin, result in the nuclear accumulation of β -catenin and subsequent complex formation with T-cell transcription factor/lymphoid enhancing factor (TCF/LEF) transcription factors to activate gene transcription [37]. TCF/LEF binding sites on promoters of cell proliferation genes, such as cyclin D1 and c-MYC [35,38], thus serve to transmit the aberrant mutations to tumorigenic signals within the colonic crypts.

As discussed above, we and others have shown the chemopreventive effects of statins and number of NSAIDs [7,16,20,23,24,39]. However, many of these studies utilized higher dose levels and also no efficacy data on the most commonly used NSAID, naproxen, in the colon cancer model is available. Importantly, our aim to establish whether low-dose combinational approaches targeting different pathways would be ideal for human colorectal cancer prevention. Thus, in the present study, experiments were designed to evaluate the efficacies of atorvastatin and NSAIDs, sulindac and naproxen, administered individually and in combination against colon tumorigenesis. We evaluated the chemopreventive potential of a low dose atorvastatin in combination with NSAIDs with colonic tumor formation as the endpoint, and further determined the action of atorvastatin and in combination with NSAIDs in regulating the expression of key protein markers and signaling pathway during colon carcinogenesis.

MATERIALS AND METHODS

Compounds

Atorvastatin, sulindac, and naproxen (Fig. 1) were provided by the DCP Repository at the National Cancer Institute (Bethesda, MD). *L*-[³H]-arginine to *L*-[³H]-Citrulline was obtained from New England Nuclear (Boston, MA).

Animals, diet and *in vivo* experimental procedures

Weanling male F344 rats obtained from Charles River Breeding Laboratories (Kingston, NY) were randomly distributed by weight into control and experimental groups. Animals had access to food and water at all times. Food cups were replenished with fresh diet twice weekly. Experimental diets were purchased from Research Diets (New Brunswick, NJ) and stored at 4°C. Beginning at 5 weeks of age, all rats were fed the modified American Institute of Nutrition-76A (AIN-76A) diet. At 7 weeks of age, the animals were given s.c. injections of azoxymethane (AOM, CAS no. 25843-45-2; Ash Stevens, Detroit, MI) at a dose rate of 15 mg/kg body weight or saline as solvent control once weekly for 2 weeks. One week after the second AOM-treatment, groups of rats were fed AIN-76A diet containing atorvastatin (200 ppm), sulindac (100 ppm) or naproxen (150 ppm), or their combinations with low-dose atorvastatin (100 ppm) for 45 weeks. At autopsy, animals were sacrificed by CO₂ asphyxiation and the colon was removed, rinsed in PBS, opened longitudinally and flattened on a filter paper. The location and size of each tumor was noted. Mucosal scrapings were collected and stored at -80°C for further analysis. Tumors were removed, fixed in 10% buffered formalin for 24 h and transferred to 80% ethanol for histopathologic analysis.

Histopathology and immunohistochemistry

The tumor tissues were dehydrated, embedded in paraffin and cut into 4 μm thick sections. For histopathology, the sections were hydrated and stained with hematoxylin and eosin according to the standard protocol. The stained sections were analyzed for tumor grades by a pathologist. For immunohistochemical analysis, only non-invasive adenocarcinomas were selected for the evaluation of protein markers. The detailed procedures for immunohistochemical analysis are reported previously [40]. The primary antibodies against proliferating cell nuclear antigen, PCNA (1:1500 diluted, BD PharMingen, San Diego, CA), cyclin D1 (1:500 diluted), β-catenin (1:500 diluted), p-p65 (1:250 diluted), iNOS (1:500 diluted) (Santa Cruz Biotechnology, Santa Cruz, CA), and COX-2 (1:200 diluted, Cayman chemical, Ann Arbor, MI) were treated on the sections. The images were taken randomly at 400× using Zeiss AxioCam HRc camera fitted to a Zeiss Axioskope 2 Plus microscope. For β-catenin quantification, Image Pro 6.2 Plus (Media Cybernetics, INC., Bethesda, MD) was used to obtain the IOD (Integrated optical density= average intensity/density of each object) values.

Measurement of iNOS activity

iNOS activities were determined in colonic tumor samples of rats exposed to various experimental diets. Conversion of *L*-[³H]-arginine to *L*-[³H]-Citrulline was measured by a modification described previously [7]. iNOS activity is expressed as pmol *L*-[³H]-citrulline/mg protein/min.

Measurement of cytokine production by ELISA

Colonic mucosa samples were homogenized in a PBS based buffer solution (PBS, 0.4 M NaCl, 10 mM EDTA, 0.1 mM PMSF, 0.1 M Benzethonium ion, 0.5% BSA, 3.0% Aprotinin, 0.05% Tween 20) on ice using a Tekmar Tissuemiser (Fisher Scientific International, Inc., Pittsburgh, PA). The homogenized solution was centrifuged at 10,000 rpm, 4°C for 10 min.

The supernatant was collected for determination of protein concentration and stored at -20°C . For determination of the levels of IL-1 β , IL-4 and TNF- α , tissue homogenates were normalized down to a concentration of 1.0 mg/ml of total protein, and then diluted 10-fold in diluent buffer for analysis, following the manufacturer's protocols. Invitrogen Immunoassay kits (BioSource International Inc., Carmillo, CA) were used to determine the levels of IL-1 β (cat. KRC0012), IL-4 (cat. KRC0042) and TNF- α (cat. KRC3012).

Statistical analysis

Statistical significance was analyzed using the Student's *t*-test or ANOVA test followed by Tukey's multiple comparison test. Tumor incidence was analyzed by two-tailed Fisher's exact probability test.

RESULTS

General Observations

Body weights of animals fed the experimental diets containing atorvastatin, sulindac or naproxen individually or in combination were comparable to those fed the control diet throughout the study, indicating that the dose of atorvastatin, sulindac or naproxen used did not cause any overt toxicity. The maximum tolerated dose (MTD) for each agent was previously determined (sulindac ~400 ppm, naproxen ~700 ppm, and atorvastatin >600 ppm). Therefore, the doses were determined based on the information with these agents in AIN-76A diet on the F344 rats [16,20,24,25]. In the present study, we used the lower MTD doses of sulindac (~25% MTD), naproxen (~20% MTD), and atorvastatin (~30% and 15%), respectively. Importantly, administration of these dose levels would produce plasma area under the curve (AUC) levels in rats that would somewhat equal the plasma AUC levels of humans given low to mid doses of these agents.

A low dose atorvastatin with sulindac or naproxen reduces tumor incidence and tumor multiplicity in azoxymethane (AOM)-injected rats

The effects of administration of a low dose atorvastatin with sulindac or naproxen on AOM-induced colon tumorigenesis were evaluated, and the results are summarized in Table 1. None of the rats in the saline groups (without AOM injection, $n=6$ per group) developed tumors when autopsied at week 45 (data not shown). Most of AOM-treated control-diet fed rats developed adenocarcinomas at 45 weeks. Histopathological analysis by hematoxylin/eosin (H & E) staining revealed > 90% of the tumors from the control group as adenocarcinomas (AC), and the remaining <10% were as carcinoma in situ (CIS). Approximately 95% of the total ACs belonged to the non-invasive adenocarcinoma (NIA) grade, while the remaining 5% was invasive adenocarcinoma (IA) (Table 1). Administration of sulindac and naproxen individually had modest inhibitory (~25% incidence and ~33% multiplicity) effect on colon adenocarcinomas. However, atorvastatin (200 ppm) significantly suppressed both colon tumor incidence (52% reduction, $p=0.005$) and multiplicity (58% reduction, $p=0.008$). Most importantly, total colon tumor incidence was significantly decreased when rats were given low-dose atorvastatin with either sulindac (52% reduction, $p=0.001$) or naproxen (63% reduction, $p=0.0005$), respectively (Table 1). Colon tumor multiplicities were also profoundly reduced when rats were given low-dose atorvastatin with either sulindac or naproxen (80-85%, $p<0.0001$) (Table 2).

A low-dose atorvastatin, in combination with sulindac or naproxen, decreases cell proliferation markers, proliferating cell nuclear antigen (PCNA), β -catenin and cyclin D1, in the colon adenocarcinomas

As shown in Fig. 2A (first row), the histological evaluation revealed that the majority of tumors were non-invasive adenocarcinomas. The expression of PCNA, a marker for cell proliferation, was determined in the adenocarcinomas from the control and treatment groups. The colon tumors from a low-dose atorvastatin, in combination with sulindac or naproxen, fed group showed significant reduction of PCNA nuclear staining, compared to the control group (Fig. 2A, second row). Aberrant expression of β -catenin can be regarded as a key event during colorectal tumorigenesis and is linked to the increased transcription of a number of genes, such as cyclin D1 [32,33]. β -Catenin was identified along the membrane of the epithelial cells in the control group. Compared to the control, all treatment groups showed marked inhibition of β -catenin membrane staining: sulindac (35.6% inhibition), naproxen (41.6% inhibition), atorvastatin (41.7% inhibition), atorvastatin + sulindac (59.4% inhibition), and atorvastatin + naproxen (54.6% inhibition). (Fig. 2A, third row). The colonic crypt cells in the control group showed homogeneous and intense staining for β -catenin in the cytosol as well as in the membrane, with lower and scattered staining in the nucleus. In contrast, the tumors from the treatment groups had no observable nuclear staining. Furthermore, the cytoplasmic expression of β -catenin was also markedly inhibited by the treatment with atorvastatin alone and in combination with sulindac or naproxen (Fig. 2A, third row). Since cyclin D1 is a downstream signaling target of β -catenin, and overexpression of cyclin D1 is reported in patients with colorectal tumors where its lowering has therapeutic significance [32,33], we determined whether treatment reduces cyclin D1 levels in colon tumors. Positive brownish staining of cyclin D1 in the control group predominantly localized in both the cytoplasm and nucleus. Administration of a low dose of atorvastatin, in combination with sulindac or naproxen, markedly reduced the staining for cyclin D1 in both the cytoplasm and the nucleus (Fig. 2A, fourth row).

A low-dose atorvastatin, in combination with sulindac or naproxen, reduces the expression of inflammatory enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and decreases nuclear staining of phospho-p65 in colon adenocarcinomas

Overexpression of inflammatory markers is a hallmark of colorectal tumors [41,42]. This knowledge led us to examine the effects of long-term feeding of treatment with atorvastatin and NSAIDs on the inflammatory markers in the AOM-injected rats. There was significant inhibition of the expression of iNOS and COX-2 proteins within the crypts in the adenocarcinomas from the treatment groups, compared to those from the control group (Fig. 2B). We also determined the effects of each treatment on a key NF- κ B signaling molecule, p65, because NF- κ B is an upstream factor of both iNOS and COX-2 transcription, and it is critical in the tumorigenesis where ablation of the proteins in this pathway caused the regression of tumors in animal models [43]. The activated form of NF- κ B subunit p65, i.e. phospho-p65, is markedly reduced in the nucleus of the colon tumors from the treatment groups, when compared to those from the control group (Fig. 2B). In addition, significant inhibition of the iNOS enzyme activity was shown in tumors from naproxen, atorvastatin and combination treatment groups. Importantly, combinational treatment of atorvastatin with sulindac or naproxen showed maximal inhibition on iNOS enzyme activity, compared to single agents (Fig. 3). In summary, colon adenocarcinomas from atorvastatin and NSAIDs fed animals showed reduced expression of key inflammatory markers as well as nuclear staining for phospho-p65, a key molecule in the NF- κ B pathway.

A low-dose atorvastatin, in combination with sulindac or naproxen, inhibits colonic mucosal levels of cytokines TNF α , IL-1 β and IL-4

Inflammatory cytokines are found to be present in human cancers including those of the colorectum, breast, prostate and bladder [44,45]. The action of cytokines to facilitate carcinogenesis is multi-fold: DNA damage by reactive oxygen species (ROS) and reactive nitrogen species (RNS); inhibition of DNA repair by ROS; functional inactivation of tumor suppressor genes; tissue remodeling via activation of matrix metalloproteinases (MMPs); stimulation of angiogenesis and control of cell adhesion molecules [45]. ELISA conducted for inflammatory cytokines on mucosal scrapings derived from the AOM injected rats are shown in Table 2. Sulindac treatment alone strongly inhibited the production of cytokines, TNF α by 36.2% ($p=0.004$), IL-1 β by 26.6% ($p=0.04$) and IL-4 by 34.0% ($p=0.03$). More importantly, administration of a low-dose atorvastatin, in combination with sulindac or naproxen, significantly lowered the levels of cytokines in the colon; TNF α by 36.8% ($p=0.03$) and 33.3% ($p=0.01$); IL-1 β by 35.7% ($p=0.01$) and 31.7% ($p=0.03$); IL-4 by 39.9% ($p=0.03$) and 40.4% ($p=0.01$), respectively.

DISCUSSION

This is the first report on the combination treatment using low-dose atorvastatin with either low dose sulindac or naproxen, which greatly suppress the colon adenocarcinoma incidence and multiplicity. Our results suggest that decreased inflammatory cytokines and signaling molecules, particularly inhibition of nuclear p65, β -catenin and cyclin D1, are responsible for suppression of colonic adenocarcinomas. The present study is an extension of our previous work, which identified atorvastatin, sulindac and naproxen as effective agents in suppressing colon cancer in animals [7,16,24]. The results from the current research conducted in colon cancer reveal that administration of a low dose of atorvastatin, in combination with sulindac or naproxen, reduces the colon tumor multiplicity and regulates intermediate signaling pathways of proliferation and inflammation in the colon (Fig. 4).

A comparison of tumor numbers across the different grades of tumor shows an overall reduction by the treatment with a low dose of atorvastatin, in combination with sulindac or naproxen. Importantly, statistical analysis on tumor data revealed a profound inhibitory effect of low-dose atorvastatin with either sulindac or naproxen on adenocarcinomas. Unlike the APC^{Min/+} mice, where most of tumors are localized to the small intestine and those are predominantly adenomas, whereas the AOM-induced rat intestinal tumors mostly localized to distal colon and adenocarcinomas, similar to human etiology. Thus, the results of our present study provide potential significance for human clinical trials. Also, it is important note that in our previous studies we have shown that 150 ppm atorvastatin inhibits colon adenocarcinoma incidence and multiplicity by (~34% inhibition, $p\leq 0.05$); whereas in this study use of 200 ppm of atorvastatin suppressed >52% incidence ($p=0.005$) and >58% multiplicity ($p<0.008$) of colon adenocarcinomas. These results suggest that a modest dose increase atorvastatin (from 150 ppm to 200 ppm) significantly enhance the chemopreventive efficacy.

AOM-induced tumors result from mutations in the Wnt/ β -catenin pathway [28] as does the APC^{Min/+} model. However, unlike the APC^{Min/+} model, AOM-induced tumors are caused by mutations in the β -catenin gene [29,30]. These mutations result in β -catenin stabilization and aberrant expression of β -catenin, which is considered as a key event during colon tumorigenesis [31]. Immunohistochemical analysis revealed abundance of β -catenin mostly in the cytoplasm and relatively low nuclear staining in the adenocarcinomas of rats injected with AOM, while administration of a low dose of atorvastatin, in combination with sulindac or naproxen, markedly reduced the staining for β -catenin in both the cytoplasm and the nucleus (Fig. 2A).

Cyclin D1 is a very well-known cell cycle protein targeted by β -catenin [35] and is known to be overexpressed in colonic tumors [32,34]. c-MYC is yet another important protein for cell proliferation regulated by β -catenin and Wnt pathway [38]. These observations on cyclin D1 were corroborated by the potency of a low dose of atorvastatin, in combination with sulindac or naproxen, to affect the β -catenin levels in the colon tumors. In our studies, we identified a low dose of atorvastatin, in combination with sulindac or naproxen, to significantly lower the levels of cyclin D1 in the colon tumors induced with AOM (Fig. 2A). More importantly, nuclear levels of β -catenin and cyclin D1 are reported to play more important role in tumorigenesis than the total protein levels [46,47]. In our studies, a low dose of atorvastatin, in combination with sulindac or naproxen reduced the levels of cyclin D1 and β -catenin in the nucleus (Fig. 2A).

In addition to the effects on β -catenin and cell proliferation, our results indicate the anti-inflammatory property of a low dose of atorvastatin, in combination with sulindac or naproxen. We observed marked reduction in the staining intensities for iNOS, COX-2 and phospho-p65 markers as well as for the iNOS enzyme activity in colon tumors from the combination treatment groups (Figs. 2B and 3). Mucosal levels of inflammatory cytokines, such as TNF- α , IL-1 β and IL-4, were also significantly down-regulated by a low dose of atorvastatin, in combination with sulindac or naproxen (Table 2). Several anti-inflammatory agents that target the nitric oxide or the prostaglandin pathway are reported to present chemopreventive action in the colon [4,48,49]. A clinical trial on celecoxib, the selective COX-2 inhibitor, at a dose of 400 mg daily, reduced advanced adenoma formation in the colon by almost 50% compared to the placebo through a 3-year treatment period [50]. In addition to antiinflammatory and antiproliferative mechanisms AOM treatment may also generate oxidative stress and significant genotoxicity by inducing methyl-DNA adducts; however, these processes may significantly subside within 12–18 hours after the AOM treatment. In this study, we administered chemopreventive agents one week after the carcinogen treatment and thus possibility of AOM's carcinogenic action via inhibition of oxidative stress or DNA adducts is very minimal to none by the chemopreventive agents. Promising results with other agents such as the use of low concentrations of difluoromethylornithine (DFMO) and sulindac as chemopreventive agents in colorectal cancer highlight the potential role of inflammation in its pathogenesis and the importance of combination strategies [49].

In conclusion, a low dose of atorvastatin, in combination with sulindac or naproxen, inhibits profoundly colon tumorigenesis by regulating the p65/ β -catenin/cyclin D1 signaling pathway and the inflammatory responses. Overall, the data indicate that a low dose of atorvastatin, in combination with sulindac or naproxen, holds great promise in the field of colon cancer chemoprevention in humans.

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Fig. 1. Structures of sulindac, naproxen, and atorvastatin

Fig. 2.

Fig. 2A. A low dose atorvastatin in combination with sulindac or naproxen inhibits cell proliferation in colon adenocarcinomas. H & E staining (first row) and PCNA staining (second row) of the colon tumors. β -Catenin (third row) and cyclin D1 (fourth row) staining was high in the cytosol and also present in the nucleus to a lower extent, while nuclear staining was predominant with PCNA. Colon tumor sections were processed and incubated with the respective primary antibodies as described in the Materials and Methods. B. A low dose atorvastatin in combination with sulindac or naproxen reduces the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and decreases nuclear staining of phospho-p65 in colon adenocarcinomas. The colon tumor sections were processed and incubated with the respective primary antibodies as explained in the Materials and Methods. iNOS and COX-2 showed cytoplasmic staining, while nuclear staining was predominant with phospho-p65. $n=3$ per group for each analysis. A representative section is shown. Image magnification, 400 $\times$.

Fig. 3. Atorvastatin alone or in combination of sulindac or naproxen significantly suppress the iNOS enzyme activity in colon adenocarcinomas

The colon tumor samples were homogenized and cytosolic extracts were subjected to assay for iNOS activity. The iNOS activity is shown as mean $\pm$ S.E. (n= 6-8 per group). Control (CON); sulindac (SUL); naproxen (NAP); atorvastatin (ATO); sulindac + atorvastatin (SUL +ATO) and naproxen + atorvastatin (NAP+ATO).

Fig. 4. The β -catenin/Wnt and NF- κ B pathways and their downstream targets in colon cancer APC gene or activating mutations in β -catenin, result in the accumulation of β -catenin and subsequent complex formation with TCF/LEF transcription factors. Excessive β -catenin can interact with TCF to activate transcription of proliferating genes, such as c-Myc and cyclin D1, in the colon. Inflammatory cytokines activate NF- κ B by releasing p65, which is then translocated to the nucleus, leading to increased transcription of target genes, such as iNOS, COX-2, TNF- α interleukins, and cyclin D1. Atorvastatin, in combination with sulindac or naproxen, targets the NF- κ B and β catenin/Wnt pathways and inhibits downstream signaling, iNOS, COX-2, cyclin D1 and others.

Table 1

Chemopreventive effects of atorvastatin, sulindac, naproxen alone or combination of low-dose atorvastatin either sulindac or naproxen on azoxymethane (AOM)-induced colon adenocarcinoma incidence and multiplicity in male F344 rats.

Experimental Group*	Number of rats at autopsy	Tumor Incidence (% of rats with adenocarcinomas)**	% inhibition	Tumor multiplicity (adenocarcinomas/rat) (Mean $\pm$ SE)***	% inhibition
AOM-Control(AIN-76A) diet	31	23/31 (74.2%)		1.77 $\pm$ 0.31	
AOM-Sulindac(100 ppm)	33	19/33 (57.6%)	22.4%	1.15 $\pm$ 0.26	35.0%
AOM-Naproxen(150 ppm)	30	17/30 (56.7%)	23.6%	1.23 $\pm$ 0.25	30.5%
AOM-Atorvastatin(200 ppm)	31	11/31 (35.5%) ($P=0.005$)	52.2%	0.74 $\pm$ 0.19 ($P=0.008$)	58.2%
AOM-Atorvastatin(100) + Sulindac(100)	32	10/32 (31.3%) ($P=0.001$)	57.8%	0.31 $\pm$ 0.09 ($P_1<0.0001$; $P_2=0.005$)	82.5%
AOM-Atorvastatin(100) + Naproxen(150)	33	9/33 (27.3%) ($P=0.0004$)	63.2%	0.27 $\pm$ 0.08 ($P_1<0.0001$; $P_2=0.004$)	84.8%

* Test agents were administered in the diet following the second AOM or saline treatment and continuously thereafter for the duration of the experiment which is 45 weeks from the start of AOM or saline treatment.

** Tumor incidence was analyzed by two-tailed Fisher's exact probability test in comparison to the control group.

*** Statistical significance was analyzed using the Student *t*-test. P₁ is the value for the comparison of rats treated with chemopreventive agents with control rats; P₂ is the value for the comparison of rats treated with combination of low dose atorvastatin (100 ppm) with rats treated with either sulindac or naproxen alone.

Atorvastatin in combination with sulindac or naproxen decreases mucosal and colonic tumor levels of the pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-4

Table 2

Experimental Group*	TNF- α (pg/mg)	IL-1 β (pg/mg)	IL-4(pg/mg)
AOM-Control (AIN-76A) diet	1182.9 $\pm$ 114.9	2194.7 $\pm$ 209.4	321.0 $\pm$ 35.6
AOM-Sulindac (100 ppm)	754.1 $\pm$ 64.7 (P =0.004)	1610.0 $\pm$ 173.0 (P =0.04)	212.4 $\pm$ 26.4 (P =0.02)
AOM-Naproxen (150 ppm)	910.7 $\pm$ 85.5	1917.9 $\pm$ 316.6	292.5 $\pm$ 51.1
AOM-Atorvastatin (200 ppm)	812.8 $\pm$ 117.7 (P =0.03)	1431.3 $\pm$ 195.1 (P =0.01)	186.2 $\pm$ 30.8 (P =0.01)
AOM-Atorvastatin (100) + Sulindac (100)	747.7 $\pm$ 109.2 (P =0.03)	1410.9 $\pm$ 200.7 (P =0.01)	193.9 $\pm$ 39.2 (P =0.03)
AOM-Atorvastatin (100) + Naproxen (150)	759.7 $\pm$ 205.0 (P =0.01)	1499.9 $\pm$ 226.7 (P =0.03)	191.7 $\pm$ 29.8 (P =0.01)

* The mucosa samples were homogenized and assayed by ELISA for the different cytokines, as described under Materials and Methods section. Colon mucosa samples were randomly selected from each group and cytokine levels were analyzed (n=12). The mean $\pm$ S.D. values are shown.