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Differential preventive activity of sulindac and atorvastatin in Apc$^{+/Min\text{-}FCCC}$ mice with or without colorectal adenomas

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ABSTRACT

Objective The response of subjects to preventive intervention is heterogeneous. The goal of this study was to determine if the efficacy of a chemopreventive agent differs in non-tumour-bearing animals versus those with colorectal tumours. Sulindac and/or atorvastatin was administered to Apc$^{+/Min\text{-}FCCC}$ mice with known tumour-bearing status at treatment initiation.

Design Male mice (6–8 weeks old) underwent colonoscopy and received control chow or chow with sulindac (300 ppm), atorvastatin (100 ppm) or sulindac/atorvastatin. Tissues were collected from mice treated for 14 weeks (histopathology) or 7 days (gene expression). Cell cycle analyses were performed on SW480 colon carcinoma cells treated with sulindac, atorvastatin or both.

Results The multiplicity of colorectal adenomas in untreated mice bearing tumours at baseline was 3.6-fold higher than that of mice that were tumour free at baseline (P=0.002). Atorvastatin completely inhibited the formation of microadenomas in mice that were tumour free at baseline (P=0.018) and altered the expression of genes associated with stem/progenitor cells. Treatment of tumour-bearing mice with sulindac/atorvastatin led to a 43% reduction in the multiplicity of colorectal adenomas versus untreated tumour-bearing mice (P=0.049). Sulindac/atorvastatin increased the expression of *Hoxb13* and *Rprm* significantly, suggesting the importance of cell cycle regulation in tumour inhibition. Treatment of SW480 cells with sulindac/atorvastatin led to cell cycle arrest (G0/G1).

Conclusions The tumour status of animals at treatment initiation dictates response to therapeutic intervention. Atorvastatin eliminated microadenomas in tumour-free mice. The tumour inhibition observed with Sul/Atorva in tumour-bearing mice was greater than that achieved with each agent.

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer-related mortality in the USA.[1] Development of preventive interventions for individuals at high risk for CRC has been hindered by the discovery that the most efficacious agents identified, including cyclo-oxygenase inhibitors, cause intolerable side effects.[2 3] Use of cyclo-oxygenase inhibitors, in combination with low-dose agents with distinct mechanisms of action, has yielded promising data.[4] In many cases, the antitumour

Significance of this study

What is already known on this subject?

- Sulindac reduces the multiplicity of small intestinal tumours in Apc^{Min} mice.
- Conflicting data exist regarding the effect of atorvastatin on intestinal polyps in Apc^{Min} mice.
- GI, renal and cardiovascular toxicities are associated with extended use of sulindac.
- Patients with familial adenomatous polyposis who have undergone colectomy but have an intact rectum can develop rectal cancers even after long-term use of sulindac.
- Epidemiological data indicate that the response to preventive interventions is heterogeneous.

What are the new findings?

- The colonic tumour status (tumour free and tumour bearing) of Apc$^{+/Min\text{-}FCCC}$ mice at treatment initiation dictates response to therapeutic intervention.
- Atorvastatin dramatically inhibits the formation of microadenomas, but only in Apc$^{+/Min\text{-}FCCC}$ mice that are tumour free at baseline, leading to a corresponding reduction in the incidence of colon adenomas.
- Administration of sulindac and atorvastatin in combination reduces the multiplicity of colon adenomas only in Apc$^{+/Min\text{-}FCCC}$ mice with adenomas at baseline.
- Atorvastatin modulates the expression of genes associated with stem/progenitor cells, and atorvastatin and sulindac in combination alter cell cycle progression (cells accumulate in G0/G1) and the expression of associated genes.

How might it impact clinical practice in the foreseeable future?

- Atorvastatin may provide protection against the formation of early colorectal neoplasias (microadenomas) in subjects without a history of colorectal adenomas.
- A prior history of the presence or absence of adenomas in subjects/patients should be considered when prescribing specific preventive interventions.

activity produced by the drug combination is greater than that observed with each agent alone. Despite this success, variability in response to therapy remains an issue and has been attributed in part to genetic polymorphisms[5 6] and biomarker levels at baseline.[7] Much less attention has been given to the role of the colonic mucosa (naïve vs history of adenomas/cancer) in dictating which high-risk subjects will gain the most benefit from chemopreventive intervention.

The ability of the non-steroidal anti-inflammatory drug (NSAID) sulindac to induce the regression of intestinal polyps is well documented.[8] However, studies to assess the antitumour efficacy of sulindac in subjects with familial adenomatous polyposis (FAP)[9] or with sporadic colorectal adenomas[10] have yielded conflicting data. Enthusiasm for the further development of this class of drugs for cancer prevention has been dampened by the GI and cardiovascular toxicities associated with extended use and the questionable preventive benefits of long-term sulindac use.[8 11] To circumvent these toxicities, sulindac was administered in combination with the ornithine decarboxylase inhibitor difluoromethylornithine.[4] This treatment lead to a 70% decrease in colorectal adenomas and a 92% reduction in advanced adenomas in patients with sporadic disease. Based on these data, significant effort has been invested in evaluating the benefit of using chemopreventive agents in combination.

Statins are widely prescribed to lower cholesterol levels and also exhibit chemopreventive activity against CRC.[12] Use of statins in combination with NSAIDs leads to enhanced antitumour efficacy.[13] Administration of low-dose atorvastatin plus sulindac to azoxymethane (AOM)-treated rats led to a 80%–85% reduction in the multiplicity of colon adenocarcinomas and several oncogenic biomarkers.[14] A similar decrease in colonic aberrant crypt foci (ACF) was observed when rats were exposed to lovastatin in combination with sulindac.[13–15] Combined use of statins and low-dose aspirin in a population-based, case–control study for ≥5 years led to a 62% decrease in CRC risk, a result greater than that observed with either agent alone.[16] Sulindac and atorvastatin, as single agents, failed to inhibit rectal ACFs in patients with previously resected multiple/advanced adenomas or colon cancer.[17] A clinical assessment of the combination therapy has not been performed.

Emerging preclinical data indicate that the efficacy of a chemopreventive agent is dictated in part by the point of intervention in the carcinogenesis process. Vitamin D affords protection against CRC if given early but is ineffective when administered later in the disease process, due to repression of the vitamin D receptor.[18] Likewise, oral administration of folic acid to mice that are highly susceptible to developing intestinal tumours caused a 2.8-fold decrease in colonic ACFs when given prior to ACF formation, a response not observed when mice with ACFs were treated.[19] These data indicate that initiating events in the background mucosa play an important role in determining the ability of a chemopreventive regimen to inhibit CRC.

The $Apc^{+/Min\text{-}FCCC}$ mouse model, bearing a germline adenomatous poly-posis coli (*Apc*) mutation, represents a clinically relevant system in which to assess the ability of agents to inhibit spontaneous colorectal adenomas.[20] Unlike conventional multiple intestinal neoplasia (Min) mice,[21] this strain develops multiple colon adenomas (3.7±0.3, mean±SEM) and has an extended life span. Determination of the colon tumour status of each animal at the time of treatment initiation, using endoscopic methods,[22] provides a unique opportunity to compare the ability of agents to prevent the formation of new colon lesions and alter the growth of established adenomas in a single experiment.

The goal of the present study was to determine if the efficacy of a chemopreventive agent differs in naïve non-tumour-bearing animals versus those with established colorectal tumours. Sulindac and/or atorvastatin was administered to $Apc^{+/Min\text{-}FCCC}$ mice known to be tumour free or tumour bearing at treatment initiation. The results demonstrate that the chemopreventive activity of each regimen varies depending on whether the animal is tumour free or tumour bearing at baseline. These data stress the importance of knowing the tumour status of an animal prior to therapy and provide invaluable insight into which subpopulations of high-risk subjects will benefit most from treatment with sulindac and/or atorvastatin.

MATERIALS AND METHODS

Animals and diets

C57BL/6J male $Apc^{+/Min\text{-}FCCC}$ mice (Fox Chase Cancer Center (FCCC))[20] were maintained on a Teklad 2018SX diet (Envigo). Atorvastatin calcium was a gift from Pfizer, and sulindac was obtained from the National Cancer Institute's Chemopreventive Drug Repository. The sulindac dose (300 ppm) was selected based on its lack of toxicity (Sørensen *et al*[23] and our unpublished data). Atorvastatin (100 ppm) has been shown to decrease intestinal tumours in Min mice.[15] Based on an intake of 4 g chow/day/mouse, the selected atorvastatin dose (100 ppm) is less than the maintenance dose (80 mg) in humans.[24] Likewise, sulindac at 300 ppm is less than the daily dose prescribed to patients with FAP (300 mg). All experiments were approved by the Institutional Animal Care and Use Committee at FCCC. Transcript Profiling: GEO accession number GSE81375.

Colonoscopic examinations

$Apc^{+/Min\text{-}FCCC}$ mice were subjected to colonoscopy (6–8 weeks of age) prior to drug administration (figure 1A). Food was replaced with Pedialyte overnight, and colonoscopies were performed using a veterinary endoscope (1.5 mm outer diameter) (Karl Storz Veterinary) with a 0° viewing angle.[22]

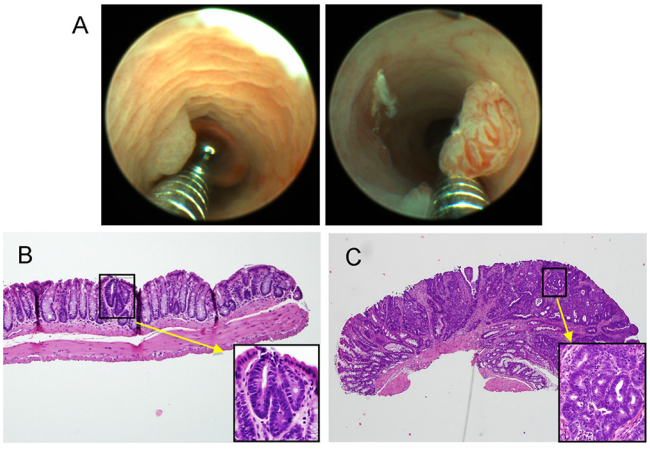

Figure 1 Representative colon lesions in $Apc^{+/Min\text{-}FCCC}$ mice. (A) Images of adenomas protruding from the wall of the colon, obtained using a rigid bore endoscope. Forceps (1 mm in diameter) are included for size comparison. (B) Microadenoma consisting of three crypts (100× view). (C) Adenoma with characteristic irregular crypt structure (40× view). Inserts in (B) and (C) are respective high power views (400×). Images in (B) and (C) are of mice that completed the treatment regimen (14 weeks).

Experimental design

Mice were categorised as tumour free or tumour bearing at baseline based on colonoscopy results and assigned to groups (n=23/group): untreated chow or chow supplemented with sulindac (300 ppm), atorvastatin (100 ppm) or sulindac/atorvastatin (Sul/Atorva, 300 ppm/100 ppm) (online supplementary figure 1). For the drug efficacy study, animals were treated for 14 weeks and body weights were recorded weekly.

At the time of euthanasia, the entire small intestine and colon were examined grossly. The location of each colonic lesion was recorded and its size measured using callipers. Tumour volume (assuming an ellipsoid) was calculated (height×width×length×π/6).

Individual colonic lesions (2–3 mm) were embedded in optimum cutting temperature (OCT) media (Thermo Scientific). Tumours >3 mm were cut in half, with half frozen in OCT and half fixed in 10% buffered formalin. The remaining colon was fixed, cross-sectioned at 2 mm intervals and submitted for histopathological review.

The acute effect of Sul/Atorva (300 ppm/100 ppm) and atorvastatin (100 ppm) on genome-wide gene expression in colon adenomas and the normal colonic epithelium, respectively, was examined (n=4/group). Tumour-free and tumour-bearing mice were treated with atorvastatin; tumour-bearing mice were administered Sul/Atorva. Untreated tumour-free and tumour-bearing mice of a similar age served as controls. After 7 days of treatment, colon tumours and normal colon tissue were excised and stored in OCT at −80°C.

Histopathology

Formalin-fixed and frozen tissues were sectioned and stained with H&E. Pathological reviews were conducted in a blinded manner. Tumours were classified as adenomas (>4 dysplastic crypts) or microadenomas (1–4 dysplastic crypts) (figure 1B,C). This definition is consistent with the criteria for adenomas established by a leading panel of intestinal pathologists.[25] The mean diameter of a colon microadenoma is <300 µm.[26] The total number of adenomas included adenomas plus microadenomas. Only adenomas in the colon/rectum were confirmed histopathologically. The multiplicity of small intestinal lesions was based on gross counts.

Microarray analyses and gene validation

Cryosections (6 µm) were cut and stained with H&E. Colonic epithelial cells (tumour: Sul/Atorva; normal: atorvastatin) were laser microdissected using a Leica 6500 system and placed in PicoPure extraction buffer (Life Technologies) at 42°C for 30 min. RNA was extracted using the PicoPure isolation kit and evaluated using an Agilent Bioanalyzer 2100. Total RNA (10 ng) was subjected to linear RNA amplification (two rounds) and amino allyl labelled (Life Technologies). Samples were hybridised to Mouse Whole Genome 4×44K microarrays (Agilent), washed and scanned.

Genes of interest were validated in independent microdissected preparations. Samples with intact 18S and 28S ribosomal RNA were reverse transcribed and subjected to real-time quantitative PCR (RT-qPCR) using TaqMan Universal PCR MasterMix gene-specific primers (Life Technologies) (online supplementary table 1). Amplification products were monitored using an ABI7900 Sequence Detection System and quantified using the comparative $\Delta\Delta C_t$ method.

Ki-67 immunohistochemistry

Formalin-fixed paraffin-embedded colon tissue was incubated with Ki-67 antibody (1:400 overnight at 4°C, Cell Signaling Technology) and processed using the Vectastain ABC Kit (Vector Laboratories). The number of Ki-67 positive cells in 20 normal crypt columns/animal (400×) was counted and expressed as a labelling index (Ki-67 positive cells/total number of cells).

Cell cycle analyses

SW480 colon carcinoma cells were incubated with dimethyl sulfoxide, sulindac (30, 60 and 120 µM), atorvastatin (0.1, 0.5, 1 µM) or Sul/Atorva. The doses reflect plasma concentrations in humans.[27 28] After 48 hours of treatment, the cells were washed and fixed in 70% ethanol overnight. Cells were rinsed in phosphate-buffered saline, resuspended in 0.5 mL FxCycle PI/RNase Staining Solution (Life Technologies) and incubated at room temperature for 30 min. DNA content was determined using a BD LSR II Flow Cytometer and FlowJo software.

Statistical analysis

Variance stabilising and normalising transformations were applied to tumour multiplicity and body weight data (mean±SEM). Comparisons were made using the Mann-Whitney test. Tumour incidence was compared using the two-sample test of proportions. Ki-67 staining and cell cycle data (mean±SEM) were evaluated using the Student's t-test. All tests were two sided and used a type I error of 5% to determine significance.

Raw data from Agilent microarrays were background corrected and quantile normalised across experimental conditions.[29] The limma methodology (Linear Models for Microarray Data)[30] was applied to the $\log_2$-transformed expression data to identify genes differentially expressed in each comparison. The limma module in the Open Source R/Bioconductor Package[31] was used in computations. Differentially expressed genes were identified based on statistical and biological significance. Statistical significance (P values) was adjusted to account for multiple testing using the Benjamini-Hochberg false discovery rate approach.[32] Biological significance was defined as ≥2-fold change in expression computed as the ratio of mean expression profiles between two groups. The enriched canonical pathways and interaction networks of significant genes were generated using the Ingenuity Pathway Analysis Suite (QIAGEN). Heat maps of the expression of genes in the enriched canonical pathways were generated using Java TreeView (V.1.1.6r4).

RESULTS

Drug tolerance

Body weights did not differ significantly among the treatment groups at the time of study entry and prior to week 13, but then began to diverge (online supplementary figure 2). At week 14, the body weights of animals administered sulindac were higher than those of atorvastatin-treated (P=0.018) and untreated control (P=0.024) animals.

Gross small intestinal tumours

The impact of sulindac and/or atorvastatin on small intestinal tumours was evaluated at week 14. The multiplicity of gross small intestinal tumours in mice receiving 300 ppm sulindac (22.7±3) was reduced 54% and 59% vs that of untreated controls (49.5±4, P≤0.001) and mice treated with atorvastatin (54.3±6, P≤0.001), respectively (figure 2A). Use of Sul/Atorva failed to confer any additional antitumour activity over sulindac alone. However, the multiplicity of gross small intestinal tumours

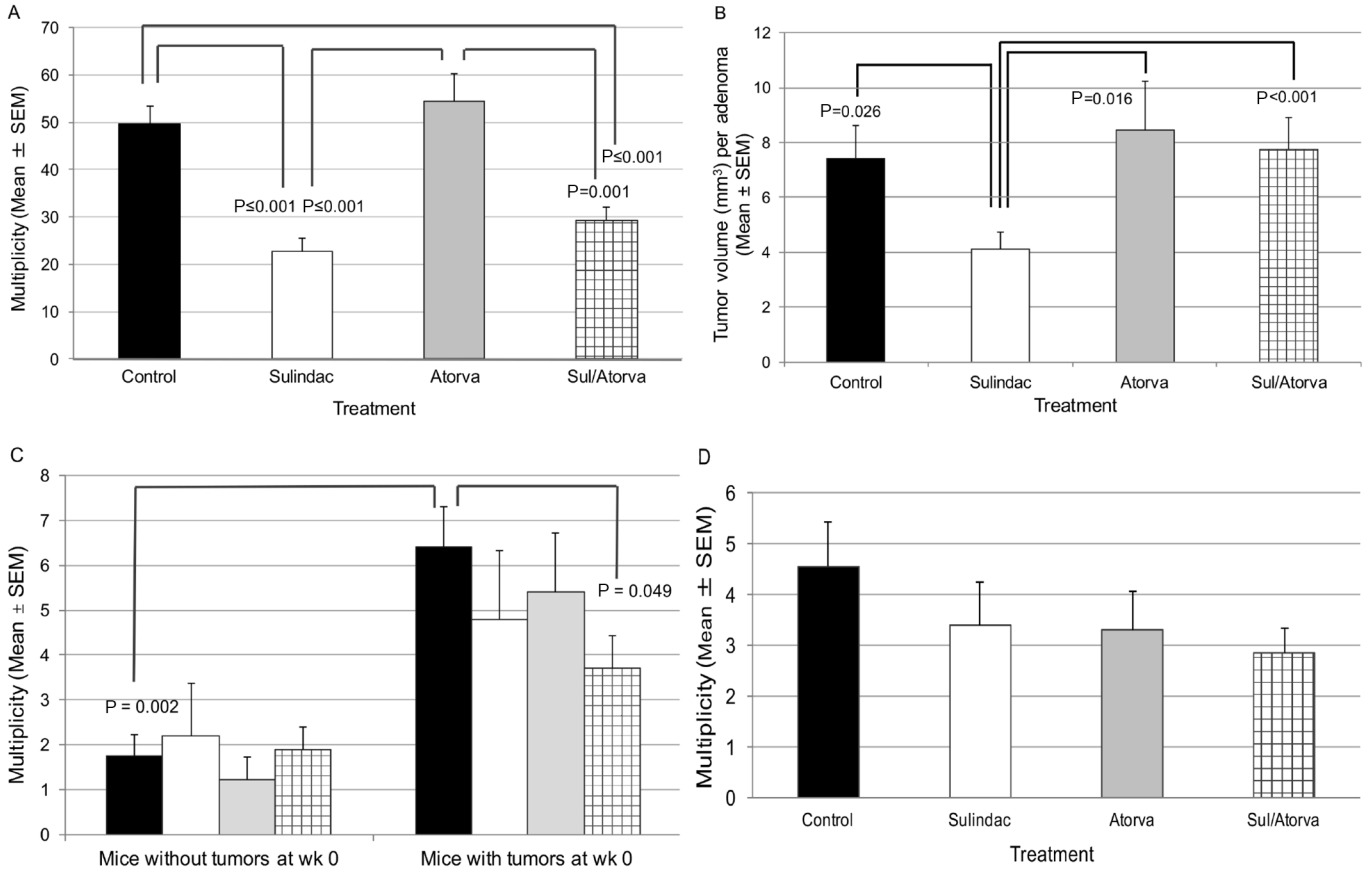

Figure 2 Multiplicity of intestinal adenomas in Apc$^{+/Min\text{-}FCCC}$ mice following drug exposure. All evaluations were conducted after 14 weeks of drug treatment. (A) Multiplicity of gross small intestinal adenomas per treatment group, irrespective of tumour status at baseline. (B) Volume of histopathologically confirmed colon tumours by treatment group, irrespective of tumour status at baseline. (C) Multiplicity of colorectal adenomas in mice by treatment group, as defined in (A), stratified for the presence or absence of colon tumours at the time of treatment initiation (week 0). (D) Multiplicity of colorectal adenomas in all mice by treatment group (P>0.05), irrespective of tumour status at baseline. Results are expressed as the mean±SEM per group. The brackets denote group comparisons that achieved statistical significance using the Mann-Whitney test (tumour multiplicity and volume). Atorva, atorvastatin; Sul, sulindac; wk, week.

in mice receiving Sul/Atorva (29.2±3) was reduced 41% and 46% compared with that of untreated controls (P≤0.001) and mice treated with atorvastatin (P=0.001), respectively. The multiplicity of gross small intestinal tumours in untreated and atorvastatin-treated animals did not differ significantly (online supplementary table 2).

Colorectal adenomas

The incidence of colon adenomas in tumour-free mice was 87.5%, a percentage consistent with previous findings from this group.[20] The effect of each agent on the size of colorectal adenomas was assessed at the time of euthanasia. Only mice with gross colon tumours were included in the analysis. Administration of sulindac alone led to a 44.5% decrease in tumour volume as compared with untreated controls (P=0.026) (figure 2B), an effect observed in tumour-free or tumour-bearing animals at baseline (online supplementary figure 3). Atorvastatin±sulindac had no effect on colon tumour volume as compared with controls (P>0.05).

The multiplicity of total colonic adenomas (including microadenomas) was assessed independently for animals that were tumour free or tumour bearing at study enrolment (week 0). An association was observed between baseline tumour status and the number of colorectal adenomas a mouse developed, irrespective of treatment (figure 2C). The mean multiplicity of colorectal adenomas in untreated mice that were tumour bearing at baseline was 3.6-fold higher (6.4±1.2) than that of mice found to be tumour free at week 0 (1.8±0.9, P=0.002).

Therapeutic response differed, depending on the tumour-bearing status of the animal at study entry (figure 2C). Sulindac, atorvastatin and Sul/Atorva failed to alter the multiplicity of colorectal adenomas in mice that were tumour free at baseline as compared with controls. In contrast, administration of Sul/Atorva to tumour-bearing mice led to a 43% reduction in the multiplicity of colorectal adenomas (Sul/Atorva: 3.7±0.7; untreated controls: 6.4±1.2) (P=0.049). Neither sulindac nor atorvastatin alone had any effect on colorectal tumour multiplicity. Of note, the antitumour effect of all agents was not detectable when the data for tumour-free and tumour-bearing animals were combined for analysis (figure 2D), a standard practice used when assessing therapeutic efficacy in models of spontaneous tumorigenesis.

To investigate the mechanisms responsible for the antitumour activity of Sul/Atorva, genome-wide expression profiling was conducted using neoplastic colonic epithelial cells from tumour-bearing mice (untreated vs Sul/Atorva for 7 days). *Hoxb13*, *Fxyd4*, *Col17a1*, *Rprm* and *Mt4* were among the most differentially expressed genes (Sul/Atorva vs untreated controls; $\log_2$ fold change 5.17–6.75). The involvement of *Hoxb13* and *Rprm* in cell cycle checkpoint control is well documented.[33 34]

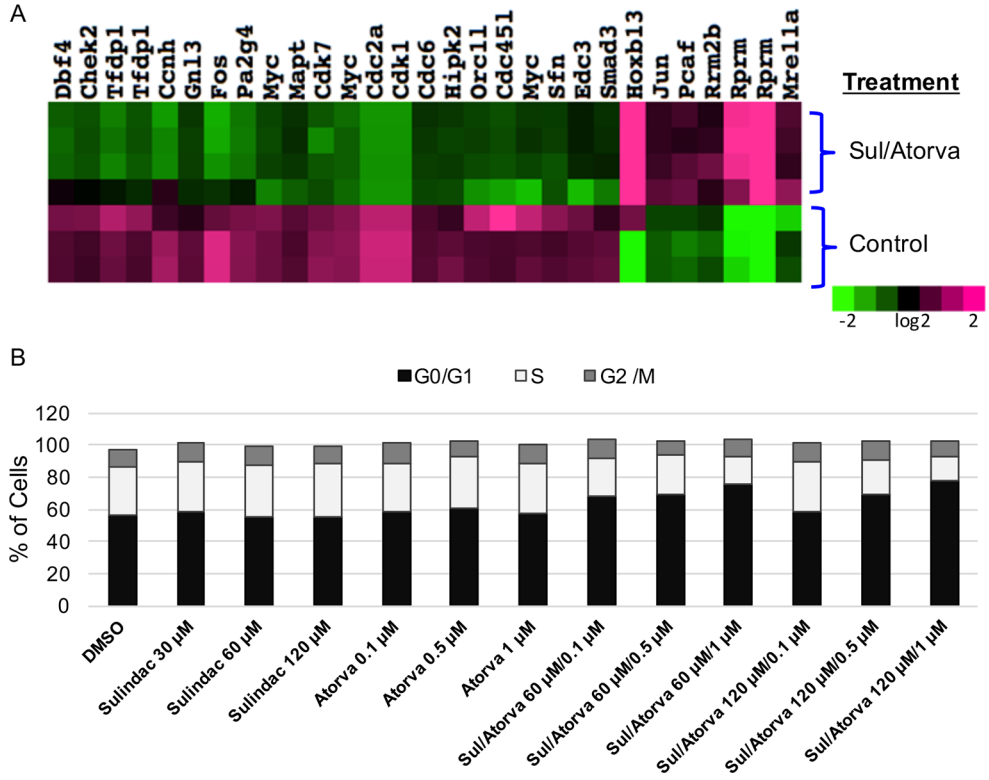

Figure 3 Effect of sulindac and atorvastatin on the cell cycle. (A) Heat map of cell cycle regulatory genes that are differentially expressed in untreated tumour-bearing mice versus those treated with Sul/Atorva. Neoplastic colonic epithelial cells were laser microdissected for microarray analyses. The intensity of the colour indicates the degree of upregulation (magenta) or downregulation (green) when the data are expressed as a ratio (Sul/Atorva vs control). Multiple listings of a gene reflect the analysis of several probes for the same gene. (B) Sul/Atorva induces cell cycle arrest. SW480 human colon carcinoma cells were treated with various doses of sulindac, atorvastatin or Sul/Atorva for 48 hours, stained with propidium iodide and analysed by flow cytometry for DNA content. Histograms were generated using FlowJo software. Values from a representative experiment are presented, with similar results obtained in two independent experiments. Atorva, atorvastatin; DMSO, dimethyl sulfoxide; Sul, sulindac.

Canonical pathway enrichment analyses identified additional cell cycle regulatory genes as being differentially expressed in control versus Sul/Atorva-treated tumours (figure 3A). Six genes (three upregulated and three downregulated) were selected for validation by RT-qPCR (table 1). Five of the six genes assayed were validated by RT-qPCR, with the same trend of altered expression observed for each after Sul/Atorva treatment.

To further evaluate the effect of Sul/Atorva on cell cycle progression, SW480 cells were treated with varying doses of sulindac and/or atorvastatin (online supplementary table 3). No difference was observed in the percentage of cells in each phase of the cell cycle following treatment with each agent alone (figure 3B). However, exposure to Sul/Atorva (sulindac: 60 or 120 µM; atorvastatin: 0.5 or 1 µM) increased the percentage of SW480 cells in G0/G1 (up to 22%) and reduced the number in S phase (up to 18%) (figure 3B) (P<0.004).

Table 1 Genes selected for validation by RT-qPCR from microarray data comparing the gene expression profile of Sul/Atorva-treated adenomas versus untreated adenomas

Gene*	Microarray $\log_2$ fold change	P value	RT-qPCR validation correlation†
Hoxb13	6.74	0.004	0.94
Rprm	5.17	<0.001	
Mre11a	1.55	0.002	
Cdk1	−2.70	<0.001	
Cdk7	−1.75	<0.001	
Myc	−1.83	<0.001	

*False discovery rate for these genes was ≤0.033.
†Spearman rank correlation of fold change in mRNA expression determined by microarray versus RT-qPCR.
mRNA, messenger RNA; RT-qPCR, real-time quantitative PCR.

Microadenomas

Because microadenomas are direct precursors of colon tumours in $Apc^{+/Min}$ mice[35] and early intervention is most efficacious, the effect of tumour-bearing status and drug treatment on the formation of colorectal microadenomas was evaluated. Mice with colon tumours at baseline had approximately twice as many colonic microadenomas as mice that were tumour free at baseline, irrespective of treatment (figure 4A). Most notable was the complete absence of microadenomas in 'tumour-free' mice administered atorvastatin (P=0.007 for atorvastatin-treated vs untreated control tumour-free mice). This finding was not observed in animals with colon tumours at baseline. As expected, tumour-free atorvastatin-treated mice (week 0) also had the lowest incidence of colorectal adenomas of all groups at week 14 (figure 4B). In addition, treatment of tumour-free mice with atorvastatin reduced the Ki-67 labelling index of the normal colon to 50% of that of untreated controls (8±1.3 vs 16±3.8, respectively) (figure 4C).

To investigate the basis for the potent ability of atorvastatin to inhibit microadenomas, genome-wide expression profiling was conducted using normal colonic epithelial cells from untreated tumour-free mice and atorvastatin-treated

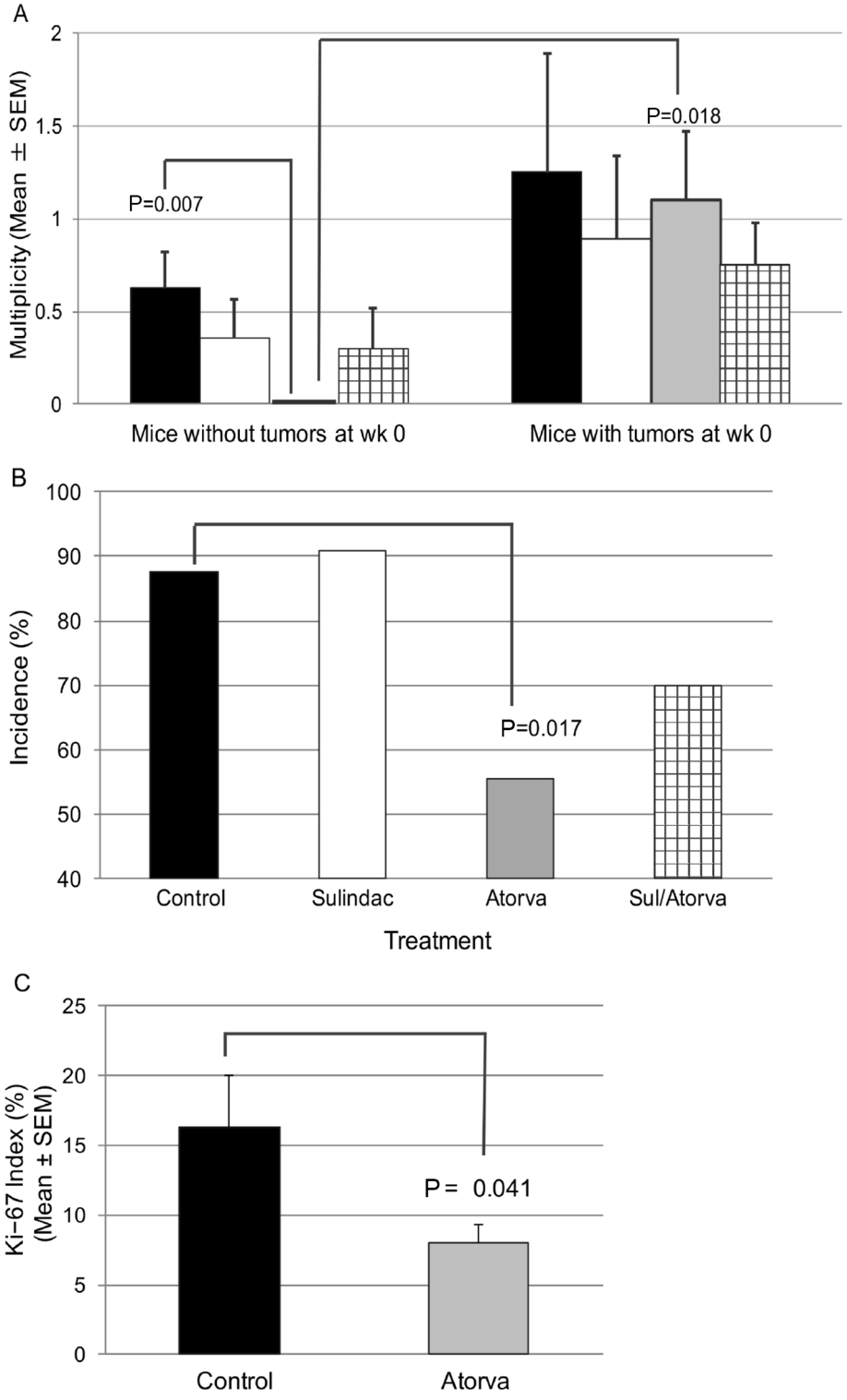

Figure 4 Multiplicity of colonic microadenomas and colon tumour incidence in $Apc^{+/Min\text{-}FCCC}$ mice, following drug exposure. (A) Multiplicity of colorectal microadenomas (≤4 crypts) in mice by treatment group (as defined in (B)), stratified for the presence or absence of colon tumours at the time of treatment initiation (week 0). (B) Incidence of colon tumours (adenomas+microadenomas) per treatment group among mice that were tumour free at baseline. All colon tumours were confirmed histopathologically. Results are expressed as the mean±SEM per group. The brackets denote group comparisons that achieved statistical significance using the Mann-Whitney test (tumour multiplicity) and the two-sample test of proportions (tumour incidence). Atorva, atorvastatin; Sul, sulindac; wk, week.

tumour-free and tumour-bearing mice (atorvastatin 100 ppm for 7 days). Comparison of the expression profile of the normal colonic mucosa from tumour-free untreated versus atorvastatin-treated mice yielded only nine genes that were differentially expressed, without enrichment for any specific pathway (data not shown). In contrast, when the gene expression profile of atorvastatin-treated tumour-free and tumour-bearing mice was compared, integrin and TNFR2 signalling

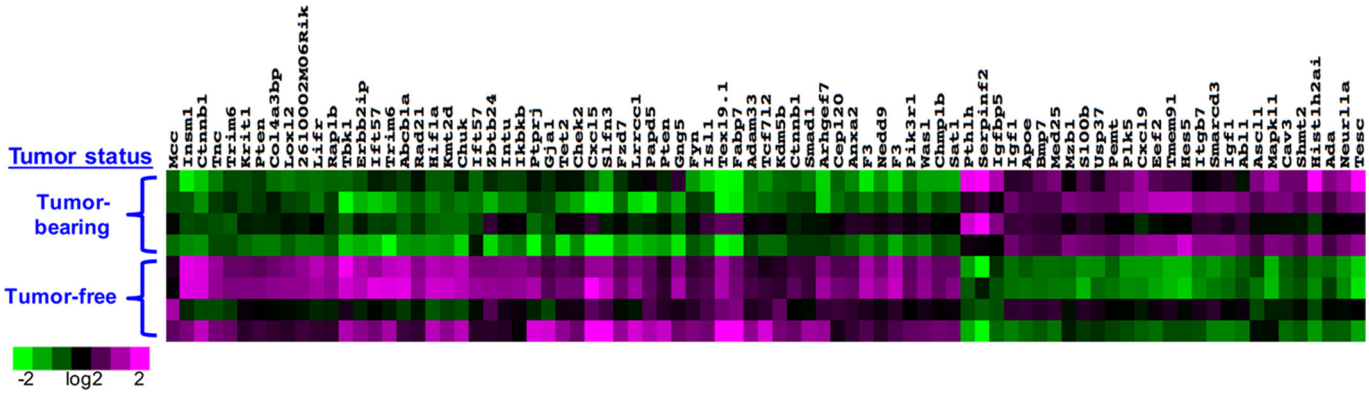

Figure 5 Heat map of stem/progenitor cell genes differentially expressed in atorvastatin-treated, tumour-free versus tumour-bearing mice. Normal colonic epithelial cells were laser microdissected from crypts for microarray analysis. The intensity of the colour indicates the degree of upregulation (magenta) or downregulation (green) when the level of expression in tumour-free versus tumour-bearing mice is expressed as a ratio.

and stem cell pluripotency were among the top canonical pathways identified. Because of the critical role of stem/progenitor cells in microadenoma formation,[36] additional analyses were performed on genes in that category. As shown in figure 5, genes were differentially expressed: *Cxcl5*, *Slfn3*, *Tbk1*, *Fabp7*, *Ctnnb1* and *Trim6* were upregulated and *Tesc*, *Pthlh*, *Hes5*, *Mapk11* and *Igf1* were downregulated in atorvastatin-treated normal colon cells from tumour-free versus tumour-bearing mice. Eight genes (four upregulated and four downregulated) were selected for validation by RT-qPCR (table 2). Seven of the eight genes assayed were validated, with the same trend of altered gene expression observed in atorvastatin-treated tumour-free versus tumour-bearing mice. Thus, the potent chemopreventive activity of atorvastatin against microadenomas formation in tumour-free mice may be attributed to its ability to modify stem/progenitor cells and/or cell proliferation in the normal colonic mucosa.

DISCUSSION

Results from this study demonstrate for the first time that the efficacy of an agent against CRC varies depending on the presence or absence of colorectal adenomas at the time of treatment initiation. Atorvastatin alone eliminated microadenomas and decreased the incidence of colon tumours in mice confirmed to be tumour free at baseline, while sulindac alone reduced tumour volume. In contrast, the multiplicity of colon adenomas was decreased significantly only in tumour-bearing mice treated with Sul/Atorva. These data demonstrate that the efficacy of agents can be improved by targeting lesions within a defined period of peak responsiveness during disease progression. The hetero-geneity in CRC risk observed among statin users could be linked to variability in the time of treatment initiation. The protective effects of statins against CRC are most prevalent among subjects without a history of colon polyps.[37 38] A nested case–control study revealed a significant inverse association between risk of CRC and statin-filled prescriptions among veterans without a history of colon polyps, with a 14% reduction in risk noted after adjusting for confounders including NSAID use.[38] Secondary analyses of data from three large chemoprevention trials failed to identify any association between statin use and recurrence of multiple or advanced adenomas.[39] Likewise, statin use did not prevent adenoma recurrence among male veterans undergoing surveillance colonoscopy.[40] These observations are consistent with the ability of atorvastatin to decrease the incidence of colorectal adenomas in mice that were tumour free at baseline, while failing to reduce the multiplicity of total adenomas in tumour-bearing mice.

Administration of atorvastatin to tumour-free mice led to a significant decrease in the multiplicity of colon microadenomas, a response absent in tumour-bearing mice. Microadenomas, dysplastic ACFs,[41] are direct precursors of colon adenomas[35] and represent the earliest lesions that can be monitored histopathologically to assess chemopreventive response. At 7 weeks of age (treatment initiation), 45% of the tumour-free Apc$^{+/\text{Min-FCCC}}$ mice possessed microadenomas, with an average multiplicity of 0.63 (unpublished data). After 14 weeks of atorvastatin treatment, no microadenomas were observed in mice that were tumour free at baseline. As expected, early elimination of microadenomas led to a reduction (32%) in the incidence of colon adenomas at 14 weeks in these animals versus untreated tumour-free mice. These data are consistent with an evaluation of the effect of lovastatin±sulindac sulfone on chemically induced ACF.[42] When treatment was begun during the initiation phase of carcinogenesis, animals receiving lovastatin±high-dose sulindac sulfone exhibited the lowest multiplicity of ACF as compared to controls. However, administration of drug postinitiation decreased the multiplicity of ACFs only in mice receiving lovastatin plus high-dose sulindac sulfone. These data indicate that the antitumour activity of the statin is limited to the early stage of tumorigenesis, with combination

Table 2 Genes selected for validation by RT-qPCR from microarray data comparing the gene expression profile of atorvastatin-treated normal colonic epithelial cells from tumour-free versus tumour-bearing mice

Gene*	Microarray $\log_2$ fold change	P value	RT-qPCR validation correlation†
Cxcl5	3.13	0.004	0.9
Fabp7	2.46	0.004	
Ctnnb1	2.23	0.002	
Pten	1.09	0.003	
Tesc	−2.38	0.002	
Pthlh	−1.96	0.002	
Mapk11	−1.61	0.005	
Igf1	−1.24	0.005	

*False discovery rate for these genes was 0.379.
†Spearman rank correlation of fold change in mRNA expression determined by microarray versus RT-qPCR.
mRNA, messenger RNA; RT-qPCR, real-time quantitative PCR.

therapy (statin plus NSAID) being most effective in inhibiting tumour growth following initiation. Similar to the present study, the chemopreventive activity of atorvastatin depended on the characteristics of the colonic mucosa of the target population.

Stem/progenitor cells are critical for the initiation of intestinal adenomas. Deletion of *Apc* in colon stem cells leads to transformation in *Lgr5* knockin mice.[36] Transformed cells remain at the bottom of the crypt, promoting the growth of microadenomas and eventually macroscopic adenomas.[36] The percentage of cells positive for CD44 staining, a cancer stem cell marker, increases progressively as the normal colonic mucosa transitions to neoplasia in humans (normal mucosa: 27.2%; ACF: 35.2%; CRC: 71.9%).[43] Thus, agents that modify the expression profile of stem/progenitor cells could have a major effect on the initiation of colonic tumours. Atorvastatin enhanced the pluripotency of stem cells and negatively regulated cell cycle progression via genes such as *Mcc*,[44] *Pten*,[45] *Trim6*[46] and *Krit1*[47] in tumour-free mice. Atorvastatin also decreased the expression of genes that promote cell proliferation (*Tesc*[48] and *Igf1*)[49] in treated tumour-free versus tumour-bearing mice. These microarray data demonstrate, once again, the importance of selecting a specific population prior to chemopreventive agent administration to achieve maximal therapeutic benefit and provide strong support for the use of precision medicine in CRC prevention.

Sulindac reduced the mean multiplicity of microadenomas in tumour-free and tumour-bearing mice, although not significantly. In a clinical setting, administration of sulindac to patients without polyps or those who had undergone polypectomy led to a significant reduction in ACFs.[50] Possible explanations for the differential effect of sulindac on these precursors includes: (1) sulindac may be more effective in inhibiting non-dysplastic ACFs. Only 10%–22% of human ACFs have dysplasia,[51 52] while dysplasia was a requirement for the classification of microadenomas in the present study. Of note, the percentage of dysplastic ACFs was not reported in the sulindac trial.[50] (2) The differential response of microadenomas to sulindac may be attributed to the distinct gene mutation profile of mice versus humans. In non-FAP cases, *K-RAS* mutations, which are absent in murine microadenomas,[53] were present in 82% of non-dysplastic and 63% of dysplastic ACFs. In addition, *APC* mutations and β-catenin accumulation, hallmarks of early colon lesions in $Apc^{+/Min\text{-}FCCC}$ mice, were not detected in non-FAP ACFs (non-dysplastic or dysplastic).[54] Expression of activated *K-RAS* caused human CRC cells treated with either sulindac sulfide or sulfone to undergo apoptosis earlier than cells without activated *K-RAS*.[55] Additional studies in genetically defined mice are needed to correlate the mutation status of early colon lesions with chemopreventive response to sulindac.

Sul/Atorva caused induction of *Hoxb13* and *Rprm*. Expression of Hoxb13 is diminished or lost in 62% of human CRCs.[56] Hoxb13 downregulates TCF4 and its target c-Myc and consequently inhibits β-catenin/T-cell factor (TCF)-mediated signalling.[56] Cellular staining of both β-catenin and its downstream target cyclin D1 was reduced in AOM-induced colon adenocarcinomas from rats treated with Sul/Atorva.[14] Overexpression of Hoxb13 in prostate cancer cells triggers G1 arrest by decreasing cyclin D1 levels via enhanced ubiquitination and degradation.[33] *Rprm* is a p53-inducible gene whose overexpression leads to cell cycle arrest at G2/M via inhibition of CDK1 activity and nuclear translocation of cyclin B1.[34] Colony formation and anchorage-independent growth are inhibited in cells overexpressing Rprm; loss of expression is common in gastric cancer.[57] The ability of sulindac and atorvastatin, as single agents, to modulate cell cycle checkpoints and proliferation has been reported at higher doses (250–500 μM sulindac[58] and 10 μM atorvastatin).[59] Treatment of AOM-treated rats with Sul/Atorva led to a significant reduction in nuclear PCNA in colon adenocarcinomas.[14] Exposure of SW480 cells to lower doses of Sul/Atorva (maximum of 120 μM for sulindac and 1 μM for atorvastatin) in the present study resulted in G0/G1 accumulation, a response not seen with each agent alone. The observation that Sul/Atorva alters the expression of *Hoxb13* and *Rprm* and arrests cells in G0/G1 is novel.

In summary, results from the present study demonstrate for the first time that the response of mice to sulindac, atorvastatin and Sul/Atorva is dictated by their tumour status at treatment initiation. Atorvastatin alone completely inhibited colorectal microadenomas in $Apc^{+/Min\text{-}FCCC}$ mice that were tumour free at baseline, most likely via modification of stem/progenitor cells. The tumour inhibition afforded by Sul/Atorva was greater than that observed with either agent alone. The strong correlation observed between therapeutic responses in mice and humans again confirms the clinical relevance of the $Apc^{+/Min\text{-}FCCC}$ mouse strain.

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