Effects of vandetanib on adenoma formation in a dextran sodium sulphate enhanced *Apc*$^{MIN/+}$ mouse model

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Abstract. The *Apc*$^{MIN/+}$ mouse is a well-characterised model of intestinal tumourigenesis in which animals develop macroscopically detectable adenomas. However, most of the adenomas are formed in the small intestine and resolution of events in the colon, the most relevant site for human disease, is limited. Inducing colitis with dextran sodium sulphate (DSS) can selectively enhance the development of lesions in the colon. We demonstrated that a DSS pre-treatment is well tolerated and effective at inducing colon adenomas in an *Apc*$^{MIN/+}$ mouse model. We then investigated the effect of inhibiting vascular endothelial growth factor (VEGFR)- and epidermal growth factor receptor (EGFR)-dependent signalling pathways on the development of adenomas induced in DSS-pretreated (DSS/*Apc*$^{MIN/+}$) or non-DSS-pretreated (*Apc*$^{MIN/+}$) mice using vandetanib (ZD6474), a potent and selective inhibitor of VEGFR and EGFR tyrosine kinase activity. Eight-week old *Apc*$^{MIN/+}$ mice were given either drinking water or 1.8% DSS and then vandetanib (ZD6474) (50 mg/kg/day) or vehicle by oral gavage for 28 days and sacrificed 24 h after the last dose and assessed for adenoma formation in the intestines. DSS pre-treatment was well tolerated and significantly enhanced formation of adenomas in the colon of control *Apc*$^{MIN/+}$ mice. Vandetanib treatment significantly reduced adenoma formation in the small intestine by 68% (P=0.001) and the colon by 77% (from 13.8 to 3.1, P=0.01) of DSS-pretreated *Apc*$^{MIN/+}$ mice. In the *Apc*$^{MIN/+}$ group, vandetanib also reduced the mean number of adenomas in the small intestine by 76% (P<0.001) and in the colon by 60% (from 3.9 to 1.5, P=0.1). DSS-pre-treatment increased the resolution of the model, allowing us to confirm statistically significant effects of vandetanib on the development and growth of colon adenomas in the *Apc*$^{MIN/+}$ mouse. Moreover these preclinical data provide a rationale for studying the effects of vandetanib in early stages of intestinal cancer in the clinic.

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Introduction

The link between inflammatory bowel disease (IBD) and colorectal cancer is well established, and individuals with ulcerative colitis are known to be at increased risk of developing colon cancer (1). The importance of various associated inflammatory pathways (2,3) has been demonstrated by the anti-inflammatory COX-2 inhibitor, celecoxib, which reduced the number of colorectal adenomas in patients with familial adenomatous polyposis (4).

There are several animal models of intestinal inflammation (5). Dextran sodium sulphate (DSS), a synthetic, sulphated polysaccharide, is a known irritant of the murine intestinal tract and is effective in inducing colitis through effects on the intestinal epithelium (6). The patho-physiological events that follow exposure to DSS appear clinically and histologically comparable to those observed in human ulcerative colitis (7,8).

DSS treatment causes chronic colitis characterised by an increased production of pro-inflammatory cytokines, lymphoid hyperplasia, inflammatory cell infiltration, focal crypt damage and epithelial injury and ulceration. Damage to the colon occurs within 2-3 days, becomes more pronounced with time, and is proportional to the dose of DSS (9). The number of intestinal tumours produced with DSS alone is relatively low, but can be greatly enhanced if DSS is given after a chemical carcinogen (10-12).

DSS can also enhance the number of tumours in the colon of the *Apc*$^{MIN/+}$ mouse (13), which carries a germ-line mutation in the *Apc* gene similar to that found in patients with human familial adenomatous polyposis. There is a close association between the *Apc*$^{MIN/+}$ mouse model and human colorectal cancer (14,15). Therefore, *Apc*$^{MIN/+}$ mice have been used extensively as a preclinical model of intestinal cancer, although the adenomas occur predominantly in the small

intestine. Although adenoma numbers in the colon of *Apc*$^{MIN/+}$ mice can be assessed to give an indication of the effect of a treatment, colon adenoma numbers are usually very low and variable from mouse to mouse. This means that demonstrating statistically significant effects of therapeutic interventions in the colon is difficult to achieve with group sizes that are practicable for routine experiments. It was anticipated that DSS treatment would augment adenoma development in the colon of *Apc*$^{MIN/+}$ mice making this model more amenable to therapeutic studies evaluating effects on colon tumourigenesis, and thereby more relevant to human disease.

Activation of the epidermal growth factor receptor (EGFR) signalling pathway is a key process in the development and progression of many tumours, including colorectal cancer (16). EGFR activity is an important regulator of epithelial cell proliferation, survival and invasiveness (17). *Apc*$^{MIN/+}$ mice bearing an EGFR mutation with a marked reduction in EGFR tyrosine kinase activity had a 90% reduction in intestinal adenoma number compared with *Apc*$^{MIN/+}$ mice expressing the normal EGFR (18). In addition, once beyond microscopic size, continued growth of solid tumours is highly dependent on angiogenesis to provide an adequate supply of oxygen and nutrients (19). Vascular endothelial growth factor (VEGF) is considered a pivotal pro-angiogenic factor, promoting endothelial cell proliferation, migration and survival by binding to, and activating, VEGF receptor-2 (VEGFR2) on tumour endothelial cells (20).

Vandetanib (ZD6474) is a potent, selective, orally active, small-molecule inhibitor of both VEGFR-2 and EGFR tyrosine kinase activities (21) that has demonstrated *in vivo* efficacy in a histologically diverse panel of established human tumour xenografts and orthotropic models (21,22). Previous studies were able to demonstrate that vandetanib treatment could significantly reduce tumour burden in the small intestine, but not in the colon of *Apc*$^{MIN/+}$ mice (23). In the present study, we evaluated the anti-tumour effects of vandetanib on adenomas induced by DSS pre-treatment in *Apc*$^{MIN/+}$ mice.

Materials and methods

Materials. Chemicals were purchased from Sigma unless otherwise stated. ZD6474 [N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl) methoxy]quinazolin-4-amine was synthesized by AstraZeneca Pharmaceuticals as previously described (32).

Animals. *Apc*$^{MIN/+}$ heterozygous mice were originally obtained as a gift from Amy R. Moser (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, USA). All breeding was by brother-sister mating within our C57BL/6J colony. All procedures were approved by the Cancer Research UK Animal Ethics Committees and covered by the appropriate licences under the Home Office Animal Procedures Act, 1986.

Genotyping of *Apc*$^{MIN/+}$ mice was carried out using reagents described elsewhere (24). DSS dose-response studies where conducted in four groups of *Apc*$^{MIN/+}$ mice (n=6/group, mixed sex, 6-weeks of age) given drinking water alone or drinking water supplemented with 1.2, 1.8 or 2.4%, DSS (MP Biomedicals Inc., Irvine, CA, USA) for 4 days. Mice were sacrificed 3 weeks later (25).

Vandetanib treatment. *Apc*$^{MIN/+}$ mice were divided into four groups of mice (n=7-8/group, mixed sex, 8-weeks of age). Group 1: drinking water alone from day 1; drug vehicle alone (1% polysorbate, Sigma-Aldrich) by oral gavage for 28 days from day 7. Group 2: drinking water alone from day 1; vandetanib (50 mg/kg/day, 0.1 ml/10 g body weight) by oral gavage for 28 days from day 7. Group 3: DSS (1.8%) in the drinking water days 1-4; drinking water alone from day 5; drug vehicle alone by oral gavage for 28 days from day 7. Group 4: DSS (1.8%) in the drinking water days 1-4; drinking water alone from day 5; vandetanib (50 mg/kg/day, 0.1 ml/10 g body weight) by oral gavage for 28 days from day 7. Mice were sacrificed 24 h after the last dose of vehicle/vandetanib.

Adenoma measurements. Mice were sacrificed by CO_2 inhalation and cervical dislocation. The entire small bowel along with the entire colon, was dissected longitudinally using a recently described cutting guide (26), spread onto filter paper, fixed in Carnoy's fluid for 3 h and subsequently stored in 70% ethanol. For adenoma counts a calibrated dissecting microscope (x20 magnification) was used to assess the number and diameter of adenomas in the small and large intestines. Adenoma volume was derived from adenoma diameter, and tumour burden was calculated as the product of adenoma number and adenoma volume (27).

ß-catenin. ß-catenin was detected with a mouse anti-ß-catenin monoclonal primary antibody (DakoCytomation Ltd., Ely, UK) and a standard streptavidin-biotin system visualized with 3,3'-Diaminobenzidine (DAB). Ten high-power fields (x40) (from 7-8 small intestines/group) were assigned a score for the proportion and intensity of staining in the nuclei: for proportion: 0, no staining; 1, >0-33%; 2, >33-66%; and 3, >66-100% stained. For intensity: 0, no staining; 1, >0-33% intensely stained (i.e., remainder are weakly stained or not stained); 2, >33-66%; and 3, >66-100% intensely stained.

Statistical analysis. Results are presented as group mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) used to test of difference amongst groups and if appropriate, Dunnet's test was performed. Two-way analysis was used to determine the effects of DSS and vandetanib and if there was any interaction between these factors. All statistics were performed using Minitab Statistical Software, Release 10.5 Xtra (Minitab Ltd., Coventry, UK).

Results

DSS dose-response. Four out of six mice on the highest concentration of DSS tested (2.4%) experienced rectal bleeding and were sacrificed early. There was an increase in the number of adenomas throughout the small intestine at DSS concentrations of 1.2 and 1.8%, but lower adenoma counts were seen at 2.4% (Fig. 1). The colon demonstrated a progressive dose-response effect to DSS with a 4-, 8- and 11-fold increase in adenoma count at 1.2, 1.8 and 2.4% DSS, respectively. The diameters of the lesions were not changed after DSS treatment (data not shown) therefore, the effects of DSS on tumour burden directly reflected the effects of DSS on adenoma number (data not

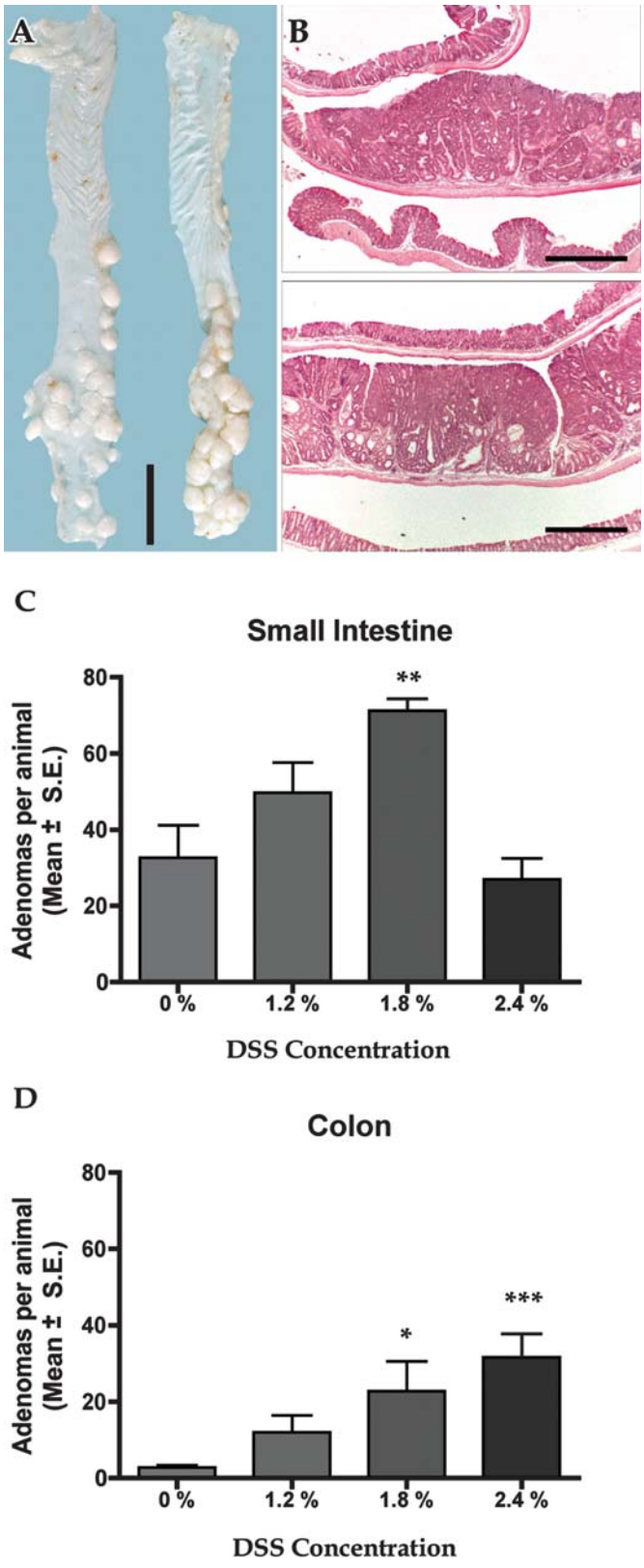

Figure 1. Effects of DSS on adenoma development in the *Apc*$^{MIN/+}$ mice. (A) Colon of 6-week old *Apc*$^{MIN/+}$ mice exposed to DSS (2.4%) in the drinking water for 4 days and sacrificed 3 weeks later (scale 1 cm). (B) H&E stained section of colon of 6-week old *Apc*$^{MIN/+}$ mice exposed to DSS (1.8%) in the drinking water for 4 days and sacrificed 3 weeks later (scale 500 μm). Adenoma development with increasing concentrations of DSS in the small intestine (C) or colon (D) of *Apc*$^{MIN/+}$ mice. N=6 per group (0, 1.2, 1.8% DSS) or n=4 per group (2.4% DSS). *P<0.05, **P<0.01, ***P<0.001 effects of DSS compared with controls (0% DSS).

shown). Based on these findings we further evaluated 1.8% DSS in the drinking water.

Effects of 1.8% DSS treatment on adenoma formation in Apc$^{MIN/+}$ mice. Compared with drinking water alone, administering 1.8% DSS in the drinking water for 4 days significantly increased mean adenoma numbers in the colon (3.9±1.1 to 13.8±3.9, P=0.02) but not in the small intestine (34.1±4.2 compared with 36.0±6.2) (Fig. 2A). In addition, pre-treatment with 1.8% DSS led to a 2-fold increase in adenoma diameter in the colon (0.68±0.1 compared with 1.3±0.2 mm, P=0.004) but there was no significant effect on adenoma diameter in the small intestine (Fig. 2B). Overall, DSS pre-treatment produced a marked increase in overall mean tumour burden in the colon (1.8±1.0 compared with 44.4±14.6 mm^3, P=0.01) but not the small intestine (23.9±6.6 compared with 34.7±8.5 mm^3, P=NS) (Fig. 2C).

Effects of vandetanib on adenoma development in the small intestine of Apc$^{MIN/+}$ mice. In the small intestine, compared with vehicle treated animals, treatment with vandetanib (50 mg/kg/day, 21 days) in *Apc*$^{MIN/+}$ mice markedly reduced the mean number of adenomas by 76% from 34.2 to 8.3 (P<0.001), the mean adenoma diameter by 42% from 1.10 to 0.64 mm, (P<0.001) and overall tumour burden by 95% from 23.9 to 1.1 mm^3 (P<0.05) (Fig. 2A).

In DSS pretreated animals, vandetanib reduced mean adenoma number by 68% from 36.0 to 11.4 (P=0.006), mean adenoma diameter by 39% from 1.22 to 0.75 mm, (P=0.009) and overall tumour burden by 93% from 34.7 to 2.6 mm^3 (P<0.05) (Fig. 2A).

Effects of vandetanib on adenoma development in the colon of Apc$^{MIN/+}$ mice. In the colon of *Apc*$^{MIN/+}$ mice, vandetanib treatment produced non-significant reductions in mean adenoma number, diameter, and overall tumour burden (Fig. 2).

In DSS pretreated *Apc*$^{MIN/+}$ mice, vandetanib produced statistically significant effects on colon adenoma: there was a 77% decrease in mean adenoma number from 13.8 to 3.1 (P=0.04), a 47% reduction in mean adenoma diameter (1.28 and 0.68 mm, P<0.05), resulting in a marked 96% reduction in tumour burden from 44.4 to 1.6 mm^3 which reached (P<0.05) (Fig. 2). A two-way ANOVA analysis of the effects of vandetanib treatment and DSS treatment, for both the small intestine and colon showed no significant interaction between these two factors (P=0.67 and P=0.12, respectively).

DSS effect on ß-catenin staining. Compared with drinking water alone, DSS treatment had no effect on the intensity of ß-catenin staining or on the proportion of cells in adenomas that stained positive for nuclear ß-catenin (Fig. 3). Compared with vehicle treated animals, vandetanib treatment significantly reduced the proportion of the cells showing nuclear ß-catenin positivity in *Apc*$^{MIN/+}$ mice (2.4±0.1 and 1.3±0.2, P=0.010) in line with a previous report (28). However, in *Apc*$^{MIN/+}$ mice pretreated with DSS the effect of vandetanib in reducing the proportion of nuclei with ß-catenin positivity did not reach statistical significance (2.2±0.3 and 1.7±0.2, P=NS) (Fig. 3). Vandetanib treatment had no effect on the intensity of ß-catenin

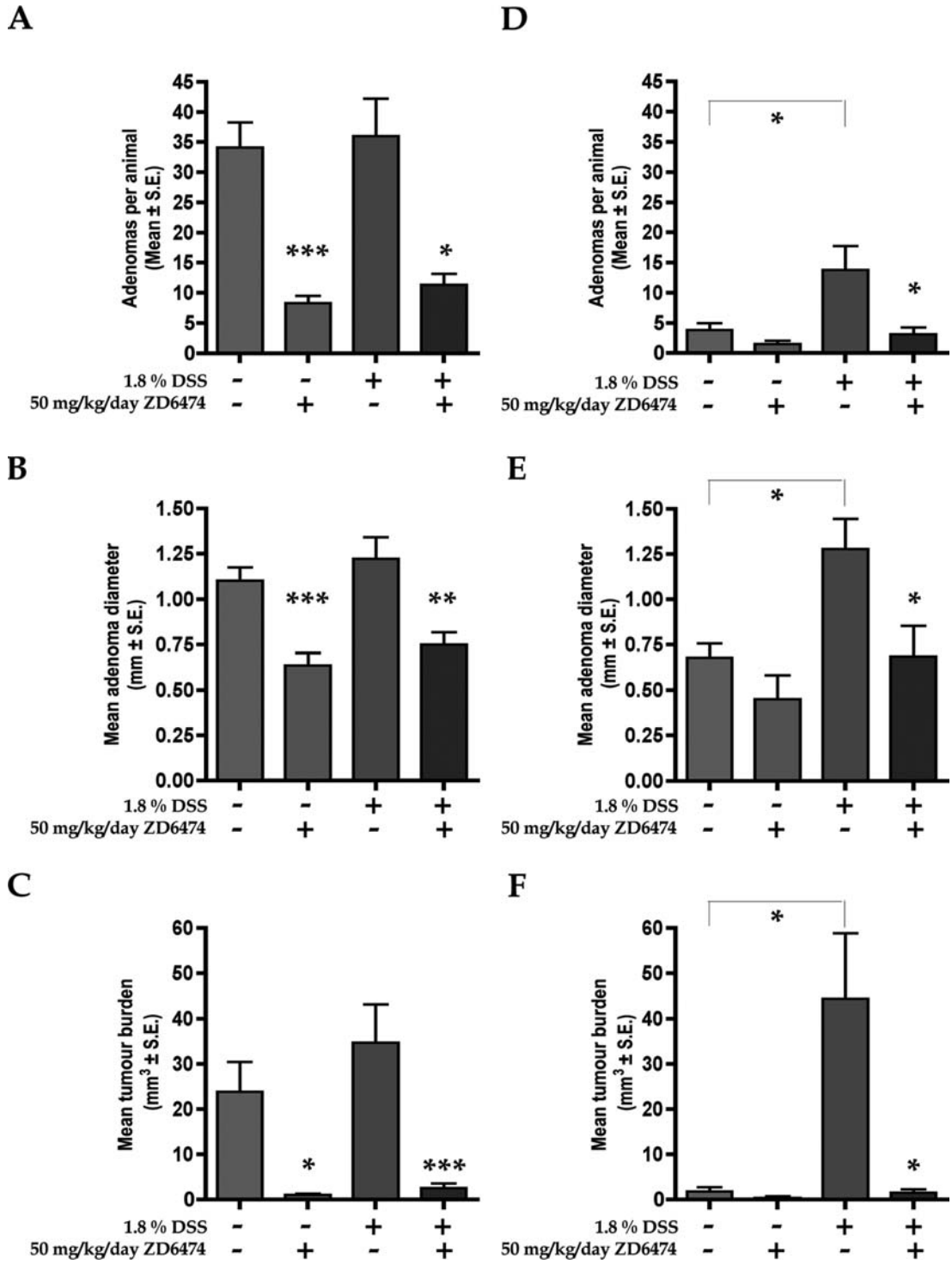

Figure 2. Effects of vandetanib (ZD6474) on adenoma development in the *Apc*$^{MIN/+}$ mice. Mice were pre-treated with DSS (1.8%) in the drinking water for 4 days followed by a 2-day interval by vandetanib (50 mg/kg/day p.o.) for 28 days. Control animals received drinking water alone and/or drug vehicle alone. Mean (± SEM) adenoma number (A and D), adenoma diameter (B and E), and tumour burden (C and F), are shown for small intestine (A-C) and colon (D-F), respectively. N=7-8 per group. *P<0.05, **P<0.01, ***P<0.001 effects of vandetanib compared with relevant control (0% or 1.8% DSS). Linkers show significant effects of DSS in groups not treated with vandetanib.

staining in either the *Apc*$^{MIN/+}$ or the DSS/*Apc*$^{MIN/+}$ treatment groups (Fig. 3).

Discussion

Although the predominant site of adenoma formation in *Apc*$^{MIN/+}$ mice and in *Apc*-deficient humans is different, the type of adenoma (intravillous adenoma) found is similar (29). The use of chemically-induced colitis as a carcinogen or pro-carcinogen has recently gained impetus (12). This is in part due to the appreciation of the close links between inflammation and cancer (1,30), and also because the model of induced colitis yields more colonic lesions and adenomas over a shorter time period than is required with standard carcinogenesis

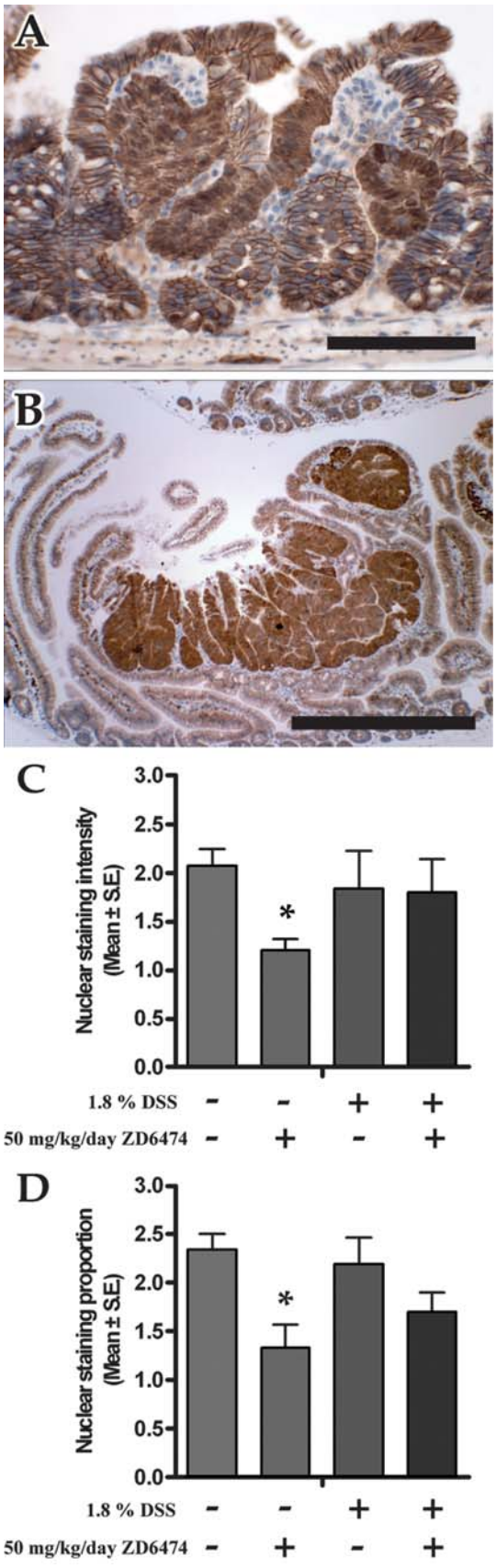

Figure 3. Effects of vandetanib (ZD6474) on ß-catenin staining. Immunological staining for ß-catenin in control *Apc*$^{MIN/+}$ mice and *Apc*$^{MIN/+}$ pre-treated with DSS (1.8%). (A) Whole small intestinal adenoma in *Apc*$^{MIN/+}$ control mouse and (B) in *Apc*$^{MIN/+}$ pre-treated with DSS (1.8%) (scale 100 and 500 μM respectively). Mean ß-catenin staining intensity (C) or proportion (D). N=7-8 per group. P-values are shown for effects of vandetanib compared with relevant control (0% or 1.8% DSS).

models (12). In the pesent study in *Apc*$^{MIN/+}$ mice, the yield of colon adenomas was greatly enhanced in a relatively short time, and the effect appeared to be somewhat selective for the colon compared with the small intestine.

The *Apc*$^{MIN/+}$ mice used in the present work appeared more sensitive to DSS treatment compared with previous published data which used multiple cycles of 4% and over of DSS in drinking water (13). Although strains of mice can have widely different susceptibilities (31) both studies used the same mouse strain (C57BL/6J).

Differences in gut micro-flora can have a significant impact on the local inflammatory environment and subsequent response to gastrointestinal irritants. We speculate that in the present study, the effectiveness of the relatively low dose of DSS (1.8%) versus previous studies (13); may in part reflect differences in animal housing (cages etc) and thus the specific pathogen-free conditions between the two animal facilities.

The results of the present study demonstrate that in the DSS-*Apc*$^{MIN/+}$ mouse model, vandetanib treatment significantly reduced adenoma number, size and burden in both the small intestine and, most importantly, in the colon suggesting an important role for EGFR and VEGFR signalling in adenoma development in the colon. Notably, with groups of 7-8 animals in the present study or with groups of 12 animals in a previous study (28), the effect of vandetanib in reducing colon adenomas in *Apc*$^{MIN/+}$ mice did not reach statistical significance. In contrast, in the DSS-pretreated animals the effects of vandetanib treatment in the colon were both substantial and statistically significant. Therefore, using DSS-pretreated *Apc*$^{MIN/+}$ mice has the potential to significantly reduce the number of animals needed to detect a treatment effect against colon adenomas in future therapeutic studies. It is of interest that the effects of vandetanib in reducing adenoma burden were of broadly similar magnitude in both the small intestine and the colon of DSS-pretreated *Apc*$^{MIN/+}$ mice, indicating that the development and growth of adenomas at both these sites may be underpinned by similar biological processes, including activation of both EGFR and VEGFR-2 signalling.

It has been shown previously that nuclear ß-catenin staining is markedly increased in adenoma compared with non-adenoma tissue from the intestines of *Apc*$^{MIN/+}$ mice (28) and in the present study we showed that nuclear ß-catenin staining was similarly high irrespective of DSS-pretreatment. Vandetanib reduced adenoma ß-catenin nuclear staining (both intensity and proportion) in *Apc*$^{MIN/+}$ mice, in line with previous observations (28), but interestingly vandetanib had no significant effect following DSS-treatment. At present, the relevance of this observation is uncertain, but it may indicate that subtle changes in the underlying biology of colon adenomas may occur following DSS treatment, but this would require further work to confirm.

In conclusion, DSS-pretreatment significantly and selectively augments the formation of adenomas in the colon of *Apc*$^{MIN/+}$ mice allowing more efficient use of this model of early disease to evaluate novel therapeutics. Vandetanib significantly reduced tumour burden in the colon of DSS-pretreated *Apc*$^{MIN/+}$ mice providing a scientific rationale for studying the effects of dual VEGFR and EGFR signalling inhibition in early stages of intestinal cancer in the clinic.

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