

Dual inhibition of VEGFR and EGFR signaling reduces the incidence and size of intestinal adenomas in *Apc*^{Min/+} mice

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Abstract

Both the epidermal growth factor (EGF) and the vascular endothelial growth factor (VEGF) pathways are associated with intestinal cancer, and therapeutic approaches targeting either EGF receptor (EGFR) or VEGF receptor (VEGFR) signaling have recently been approved for patients with advanced colorectal cancer. The *Apc*^{Min/+} mouse is a well-characterized *in vivo* model of intestinal tumorigenesis, and animals with this genetic mutation develop macroscopically detectable adenomas from ~6 weeks of age. Previous work in the *Apc*^{Min/+} mouse has shown that therapeutic approaches targeting either VEGFR or EGFR signaling affect predominantly the size or number of adenomas, respectively. In this study, we have assessed the effect of inhibiting both these key pathways simultaneously using ZD6474 (Vandetanib, ZACTIMA), a selective inhibitor of VEGFR and EGFR tyrosine kinases. To assess the effects of ZD6474 on early- and later-stage disease, treatment was initiated in 6- and 10-week-old *Apc*^{Min/+} mice for 28 days. ZD6474 markedly reduced both the number and the size of polyps when administered at either an early or a later stage of polyp development. This reduction in both adenoma number and size resulted in a total reduction in tumor burden in the small intestine of

nearly 75% in both studies ($P < 0.01$). The current data build on the concept that EGFR-dependent tumor cell proliferation and VEGF/VEGFR2-dependent angiogenesis and survival are distinct key mechanisms in polyp development. Pharmacologic inhibition of both signaling pathways has significant antitumor effects at both early and late stages of polyp development. Therefore, targeting both VEGFR- and EGFR-dependent signaling may be a beneficial strategy in early intestinal cancer. [Mol Cancer Ther 2008;7(3):590–8]

Introduction

The third most common cause of cancer-related death in western countries is colorectal cancer, which accounts for 550,000 annual deaths worldwide (1). Tumor development in colorectal cancer is characterized by cells within the normal mucosa acquiring multiple genetic mutations. Targets for somatic genetic “hits” during the early stages of colorectal cancer tumorigenesis include the oncogene *KRAS* and the tumor suppressor genes *adenomatous polyposis coli* (*APC*), *SMAD4*, and *TP53* (2, 3). Somatic mutations in the *APC* gene occur in the majority of sporadic colorectal cancers; in addition, germ-line mutations in the *APC* gene result in the hereditary cancer syndrome of familial adenomatous polyposis (4).

The *Apc*^{Min/+} mouse was derived by chemical mutagenesis with ethylnitrosourea (5) and is heterozygous for a germ-line mutation in the *Apc* gene similar to that found in patients with human familial adenomatous polyposis. Recent studies support an association between the *Apc*^{Min/+} mouse model and human colorectal cancer (6–8). In *Apc*^{Min/+} mice, adenomas arise stochastically following loss of heterozygosity of *Apc* and numerous macroscopic adenomas, predominantly in the upper gastrointestinal tract, are present by age 6 weeks. *Apc*^{Min/+} mice usually die by age 120 days due to intestinal occlusion and blood loss (9). The *Apc* protein normally acts as an inhibitory component of the Wnt/ β -catenin signal transduction pathway. However, mutation of *Apc* produces a truncated *Apc* protein that fails to bind β -catenin. This allows β -catenin to migrate to the nucleus where it activates gene transcription and cell division through binding to T-cell factor (3).

Tumor growth is highly dependent on angiogenesis to ensure an adequate supply of oxygen and nutrients (10), and vascular endothelial growth factor (VEGF)-A is considered to be a critical factor in promoting the angiogenic cascade through binding to VEGF receptor (VEGFR) 2 (11). Evidence for the role of neovascularization in the development of colorectal cancer has been obtained from clinical observations (12) and animal models (13).

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Hanrahan et al. detected a correlation between tumor grade and expression of several members of the VEGF family. These data suggested VEGF signaling may be important in early tumorigenesis, at the stage of adenoma formation (14). In another study of human colon cancer tissue, Easwaran et al. showed a direct correlation between activation of β -catenin signaling and up-regulation of VEGF, implying that β -catenin signaling may be involved in the regulation of angiogenesis in colon cancer (15). When the $Apc^{Min/+}$ mouse was crossed with a mouse null for the endogenous antiangiogenic protein thrombospondin 1, the resulting offspring ($Apc^{Min/+}/TSP1^{-/-}$) developed more polyps, with larger diameters, compared with controls (16). Data obtained from another mouse model of intestinal adenomas (Apc^{1716}) are also consistent with an association between VEGF and polyp development, where VEGF levels were elevated in polyps compared with epithelial cells from control gastrointestinal tracts (17).

Ligand-dependent activation of the epidermal growth factor (EGF) receptor (EGFR) signaling pathway is also a key process in the development and progression of many human tumors, including colorectal cancer (18). EGFR activity is an important regulator of epithelial cell proliferation, survival, and invasiveness (19) as well the expression of proangiogenic factors such as VEGF (20). Indeed, inhibition of EGFR signaling is a promising therapeutic approach in colorectal cancer (21, 22). In $Apc^{Min/+}$ mice, there was increased activation of the EGFR-phosphatidylinositol 3-kinase-Akt signaling pathway in adenomas relative to normal enterocytes (23) and pharmacologic inhibition of EGFR tyrosine kinase activity significantly reduced polyp number (21, 24). The potential importance of EGFR signaling was also shown by backcrossing $Apc^{Min/+}$ mice with mice bearing an EGFR mutation ($EGFR^{wa2}$) that results in a marked reduction in EGFR tyrosine kinase activity (24). The offspring had a marked (90%) reduction in intestinal polyp number compared with $Apc^{Min/+}$ mice expressing wild-type EGFR.

The aim of the current study was to investigate the therapeutic potential of inhibiting both VEGFR- and EGFR-dependent signaling pathways in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis. We used ZD6474, a potent, orally bioavailable, small-molecule inhibitor of VEGFR2 and EGFR tyrosine kinase activity (25). ZD6474 has *in vivo* efficacy against a histologically diverse range of human xenograft models, including tumors of colorectal origin (25, 26).

Materials and Methods

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 described previously (27).

Animals

$Apc^{Min/+}$ heterozygous mice were originally obtained as a gift from Amy R. Moser, (McArdle Laboratory for Cancer

Research, University of Wisconsin-Madison). Male mice were backcrossed to female C57BL/6J and the resultant embryos were transferred by aseptic hysterectomy to foster mothers in specific pathogen-free isolators. All breeding was subsequently by brother-sister mating with the C57BL/6J colony at Cancer Research UK. All procedures, including mutant and transgenic breeding of $Apc^{Min/+}$ mice, were approved by the Cancer Research UK and Imperial College School of Medicine Animal Ethics Committees and covered by the appropriate licenses under the Home Office Animal Procedures Act 1986.

Genotyping

DNA was extracted from mice tail-snips using a DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions, and PCR was used to identify mice carrying the $Apc^{Min/+}$ mutation. The general PCR conditions, including primer pairs and amplification, have been described previously for detection of the Apc mutation (28).

Experimental Protocol

Early Intervention Study. Recently weaned (6-week-old) $Apc^{Min/+}$ mice were given ZD6474 p.o. (12.5, 25, or 50 mg/kg/d) or vehicle only.

Late Intervention Study. Ten-week-old $Apc^{Min/+}$ mice were given ZD6474 (50 mg/kg/d) or vehicle. Each study included 12 animals per group (mixed sexes) and ZD6474 was given once daily at 0.1 mL/10 g body weight for 28 days by oral gavage. For separate histologic evaluation, satellite groups of 6- and 10-week-old mice received 50 mg/kg/d ZD6474 or vehicle ($n = 4-6$ per group). Twenty-four hours after the last day of ZD6474 dosing, mice were injected i.p. with vincristine sulfate (1 mg/kg; Mayne Pharma Plc) to induce metaphase arrest 2 h before being killed humanely and with bromodeoxyuridine (BrdUrd; to label DNA-synthesizing cells at 50 mg/kg i.p.) 1 h before being killed humanely.

Macroscopic Assessment of Tumor Burden

At necropsy, organs and intestinal tract was dissected and processed as described previously (29). Briefly, the small intestine was divided into three equal sections: proximal, middle, and distal. These sections, as well as the entire colon, were then dissected longitudinally using a recently described cutting guide (30) and fixed in Carnoy's fluid for 2 h and stored in 70% ethanol. For macroscopic polyp counts assessed with a dissecting microscope (at $\times 20$ magnification), the number and size (mean of two largest diameters measured with digital calipers) of polyps in the small and large intestines were determined. Polyp volume was derived from polyp diameter; consistent with their histologic appearance, a hemispherical shape was assumed in the small bowel polyps and a spherical shape for the polyps in the colon. Tumor burden was calculated as the product of polyp number and polyp volume (31).

Histologic Evaluation

Dissected gastrointestinal tracts from the satellite groups of mice were used for histologic evaluation of microscopic polyp counts and immunohistochemistry. The tissues were fixed in neutral-buffered formalin for 24 h, transferred to 70% ethanol, and then rolled and processed to paraffin

blocks in a standard manner. Step sections (3 μm) were stained with H&E and scored morphologically for micropolyps. Micropolyps were assigned to a category from 1 to 4: polyps that remained within the limits of a single villus were classified as category 1; those within the space of >1 to 5 villi were classified as category 2, >5 to 10 villi as category 3, and >10 villi as category 4 (32).

von Willebrand Factor (Factor 8). von Willebrand factor (vWF) was detected in Carnoy's fixed tissue ($n = 6$ per group) using a rabbit anti-human vWF polyclonal primary antibody (DakoCytomation) and developed in 3,3'-diaminobenzidine. After counterstaining with hematoxylin, a quantitative "point counting" method was used. Ten high-power fields (five from both normal and tumor tissue) from each of the six mice per group (control and treated) were scored for "hits" using a 50-point Weibel 2 graticule, with the number of hits being proportional to the volume fraction (33).

Bromodeoxyuridine. BrdUrd was detected in Carnoy's fixed tissues ($n = 6$ per group) using sequential incubation with a mouse anti-BrdUrd monoclonal primary antibody (Abcam), a biotin-conjugated rabbit anti-mouse secondary antibody, and streptavidin-peroxidase system was developed in 3,3'-diaminobenzidine. Thirty well-orientated crypts per animal were scored, and each crypt was scored from the crypt base to either the crypt villus junction or the top of the colonic crypt. The presence of labeled and metaphase cells and the crypt length (in cells) were recorded (34). All tissues were scored "blind," in that the person reading the slides was unaware of the group to which the slides belonged.

β -Catenin. β -Catenin was detected in Carnoy's fixed tissue ($n = 6$) with a mouse anti- β -catenin monoclonal primary antibody (DakoCytomation) and a standard streptavidin-biotin system visualized with 3,3'-diaminobenzidine. Ten high-power fields ($\times 40$) were assigned a score for the proportion and intensity of staining in both the cytoplasm and the nuclei: For proportion: 0, no staining; 1, 0-33% stained; 2, >33-66% stained; 3, >66-100% stained. For intensity: 0, no staining; 1, 0-33% intensely stained (that is, remainder are weakly stained or not stained); 2, >33-66% intensely stained; 3, >66-100% intensely stained.

CD31. Immunostaining was done with 4- μm sections of formalin-fixed paraffin-embedded intestinal tissue from the late intervention study ($n = 4$; ref. 29). Epitope retrieval was carried out using DakoCytomation antigen retrieval buffer in a 2100-retriever pressure cooker (PickCell Laboratories). Staining for CD31 used anti-mouse CD31 (Santa Cruz) along with an avidin-biotin block (DakoCytomation). Detection was achieved using a standard streptavidin-biotin system (Vectastain ABC-Elite solution) visualized with 3,3'-diaminobenzidine (DakoCytomation). CD31 analysis was done blind using a Automated Cellular Imaging System (ChromaVision Medical Systems) to measure CD31⁺ vessel number and CD31 staining area for each tissue region selected. Briefly, each tissue section was scanned into the Automated Cellular Imaging System at $\times 10$ magnification. An analysis threshold was set and

applied to all sections within the study. Tumor regions ($n = 3-7$ depending on whether treated or control samples) and normal regions ($n = 7$) within each section were selected and analyzed.

Phospho-EGFR. Immunostaining was done on formalin-fixed paraffin-embedded intestinal sections from the late intervention study. Antigen retrieval was carried out with high pH target retrieval solution (DakoCytomation) using the using a 2100-retriever pressure cooker (PickCell Laboratories). Endogenous peroxidase activity was blocked using a peroxidase block (DakoCytomation) and then incubated with phospho-EGFR (Tyr⁹⁹²) in 0.05% TBS-Tween at 4°C overnight. Isotype controls were also included. Subsequent staining followed with an anti-rabbit peroxidase-labeled polymer visualized in 3,3'-diaminobenzidine (DakoCytomation).

In situ Hybridization for VEGFR2. VEGFR2 mRNA from formalin-fixed tissue (distal small bowel, $n = 4$ per group) was specifically localized using an antisense riboprobe that was synthesized with T7 RNA polymerase using [³⁵S]UTP (~800 Ci/mmol; Amersham-GE) and a plasmid prepared from IMAGE clone 5359101 (MRC Geneservice) and consecutive procedure as described before (35). Signal above the muscular layer (that is, mucosa, muscularis mucosa, and submucosa) was scored in a blinded fashion and semiquantitative analysis was used to rank sections in order of staining intensity.

Statistical Analysis

All results are presented as group mean \pm SE. One-way ANOVA was used to test for any effect of treatment, and if appropriate, Dunnett's test was applied. All statistics were done using Minitab Statistical Software Release 10.5 Xtra (Minitab).

Results

Macroscopic and Histologic Characteristics of the *Apc*^{Min/+} Mouse

Apc^{Min/+} mice develop numerous spontaneous adenomas that were predominantly located in the upper gastrointestinal tract (Fig. 1). Histologic analysis of polyps using an antibody for vWF revealed strong staining in both small (categories 1 and 2) and large (categories 3 and 4) micropolyps near the luminal surface, showing that angiogenesis occurs during micropolyp formation in *Apc*^{Min/+} mice (data not shown).

Early Intervention Study

In vehicle-treated animals, macroscopic analysis showed a large number of polyps in the small bowel with fewer, but larger, polyps in the colon (Fig. 2A). In the small bowel, ZD6474 treatment resulted in significantly fewer polyps per animal [mean \pm SE polyp number, 90.9 \pm 20.7 versus 49.5 \pm 12.9 in control and (50 mg/kg/d) treated, respectively; $P = 0.033$]. Mean polyp diameter was also significantly lower at all doses of ZD6474 tested (12.5-50 mg/kg/d). These effects of ZD6474 on polyp number and/or diameter produced a significant lower polyp burden throughout the small bowel across the dose range, including a 75%

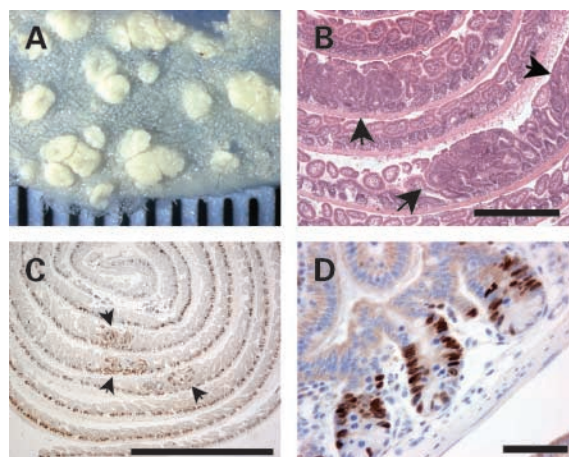


Figure 1. **A**, macrophoto of small intestine from *Apc*^{Min/+} mouse. Bar, 1 mm. **B**, H&E-stained "Swiss roll" of small intestine. Arrows, polyps. Bar, 200 μ m. **C**, BrdUrd immunohistochemistry of "Swiss roll" of small intestine. Arrows, polyps. Bar, 500 μ m. **D**, BrdUrd immunohistochemistry of small intestinal crypts. Bar, 50 μ m.

decrease at 50 mg/kg/d ($P < 0.005$). The effects were qualitatively similar in distal, middle, and proximal sections of the colon (data not shown). In the colon, 50 mg/kg/d ZD6474 resulted in fewer polyps (3.46 ± 0.98 versus 0.83 ± 0.34 in control and treated, respectively; $P = 0.019$), although there was no statistically significant effect on polyp diameter or burden. There was some indication of a dose-response effect in the polyp diameter, but the burden reduction was near maximal in the 12.5 mg/kg group.

Detailed histologic evaluation of H&E-stained intestines showed that ZD6474 treatment markedly reduced both the number and the size of micropolyps in the small bowel of *Apc*^{Min/+} mice; the larger category 3 or 4 polyps were absent, and in addition, there were significant fewer of the smaller category 1 or 2 polyps compared with controls (Fig. 3).

Apc^{Min/+} mice with a large polyp burden develop anemia due to excessive blood loss from the intestine, and onset of anemia in this model is indicated by splenomegaly. The spleens of vehicle-treated mice were enlarged, but ZD6474 treatment, in a dose-related manner, resulted in a lower spleen weight. The vehicle-treated mice had a mean spleen weight of $0.63 \pm 0.09\%$ (of body weight), the 12.5 mg/kg were $0.50 \pm 0.09\%$ ($P < 0.05$), the 25 mg/kg were $0.39 \pm 0.02\%$ ($P < 0.05$), and the 50 mg/kg were $0.36 \pm 0.018\%$ ($P < 0.01$), respectively.

Late Intervention Study

ZD6474 treatment resulted in markedly fewer macropolyps in the small bowel (62.6 ± 11.7 versus 30.3 ± 4.6 in controls and treated, respectively; $P < 0.01$; Fig. 2B), with the greatest effect observed in the distal section. Polyp diameter was also smaller in ZD6474-treated mice, resulting in a significantly lower polyp burden in the small bowel (43.9 ± 10.4 versus 12.1 ± 2.4 mm³ in controls and treated, respectively; $P < 0.01$). In the colon, there were far fewer

polyps than in the small bowel and ZD6474 did not reduce polyp burden compared with control.

As in the early intervention study, the number and size of micropolyps in the small bowel were reduced dramatically following treatment with ZD6474 (Fig. 3). The most pronounced effects of ZD6474 were on large micropolyps, with 69% and 96% reductions in the number of categories 3 and 4 polyps, respectively. A similarly marked inhibition of micropolyp count and size was observed in the colon.

Pharmacodynamic Changes

The results from analyses of pharmacodynamic markers of tumorigenesis and angiogenesis are summarized in Table 1. In nonadenoma tissue, ZD6474 treatment resulted in significantly fewer VEGFR2 mRNA *in situ* hybridization-positive cells than in vehicle-treated controls (Fig. 4; Table 1). In addition, there was a higher BrdUrd labeling index and metaphase index following ZD6474 treatment, although microvessel density (by both vWF and CD31 immunohistochemistry), β -catenin staining, and the number of metaphases/crypt and cells/crypt were not significantly affected by ZD6474 treatment. It should be noted that due to low intensity/proportion of nuclear staining it was not possible to unequivocally rule out an effect of ZD6474 on β -catenin staining and intracellular distribution in nonadenoma tissue.

The number of cells per polyp was significantly lower in animals treated with ZD6474 compared with vehicle-treated controls. Metaphase and BrdUrd labeling indices were lower in adenomas than in nontumor crypts, and these were unaffected by ZD6474 treatment despite the smaller tumor size. Microvessel density (measured by either vWF or CD31 staining) was similar in adenoma and nontumor tissue, and this was not affected by ZD6474 treatment (Table 1). The intensity of nuclear β -catenin staining and the proportion of cells with nuclear β -catenin staining were markedly higher in adenoma tissue compared with nonadenoma tissue (Table 1). In ZD6474-treated animals, the nuclear β -catenin staining in adenomas was significantly lower than in vehicle-treated controls (Fig. 4; Table 1).

Membrane-associated phospho-EGFR staining was apparent in adenomas of vehicle-treated animals (Fig. 5). In contrast, animals treated for 4 weeks with ZD6474 (50 mg/kg/d) showed no evidence of membrane staining of phospho-EGFR, although there was still some residual staining of the basement membrane.

Discussion

The results of this study show that pharmacologic inhibition of VEGFR and EGFR tyrosine kinase activity with ZD6474 reduces macropolyp number, size, and burden in the small bowel in the *Apc*^{Min/+} mouse model of familial adenomatous polyposis. Micropolyps, the putative precursors to macropolyps, were also decreased in number and size by ZD6474 treatment. Qualitatively similar results were obtained when ZD6474 was given as once-daily oral administration for 28 days to 6-week-old

mice (early intervention) or 10-week-old mice (late intervention). In the early intervention study, ZD6474 also significantly inhibited polyp number in the colon, a finding that is notable given the initial low polyp count in the colon.

The *Apc*^{Min/+} mouse is one of several animal models of human gastrointestinal cancer, and it is a widely used model for the study of various factors on the early stages of intestinal cancer (8, 36). From the age of 6 weeks, *Apc*^{Min/+} mice have macroscopically detectable adenomas

(5). Similarly, in man, early inactivation of the *Apc* tumor suppressor gene usually occurs in both sporadic colorectal cancer and familial adenomatous polyposis (37). However, the differences between animal models and human disease should be considered when interpreting data. The predominant site of polyp formation in *Apc*^{Min/+} mice is the small intestine, whereas polyp formation in *Apc*-deficient humans is mainly confined to the colon. Nevertheless, the types of adenomas (intravillous adenoma) found in the small bowel are remarkably similar in mice and men (38).

A 'Macropolyps' early intervention

B 'Macropolyps' late intervention

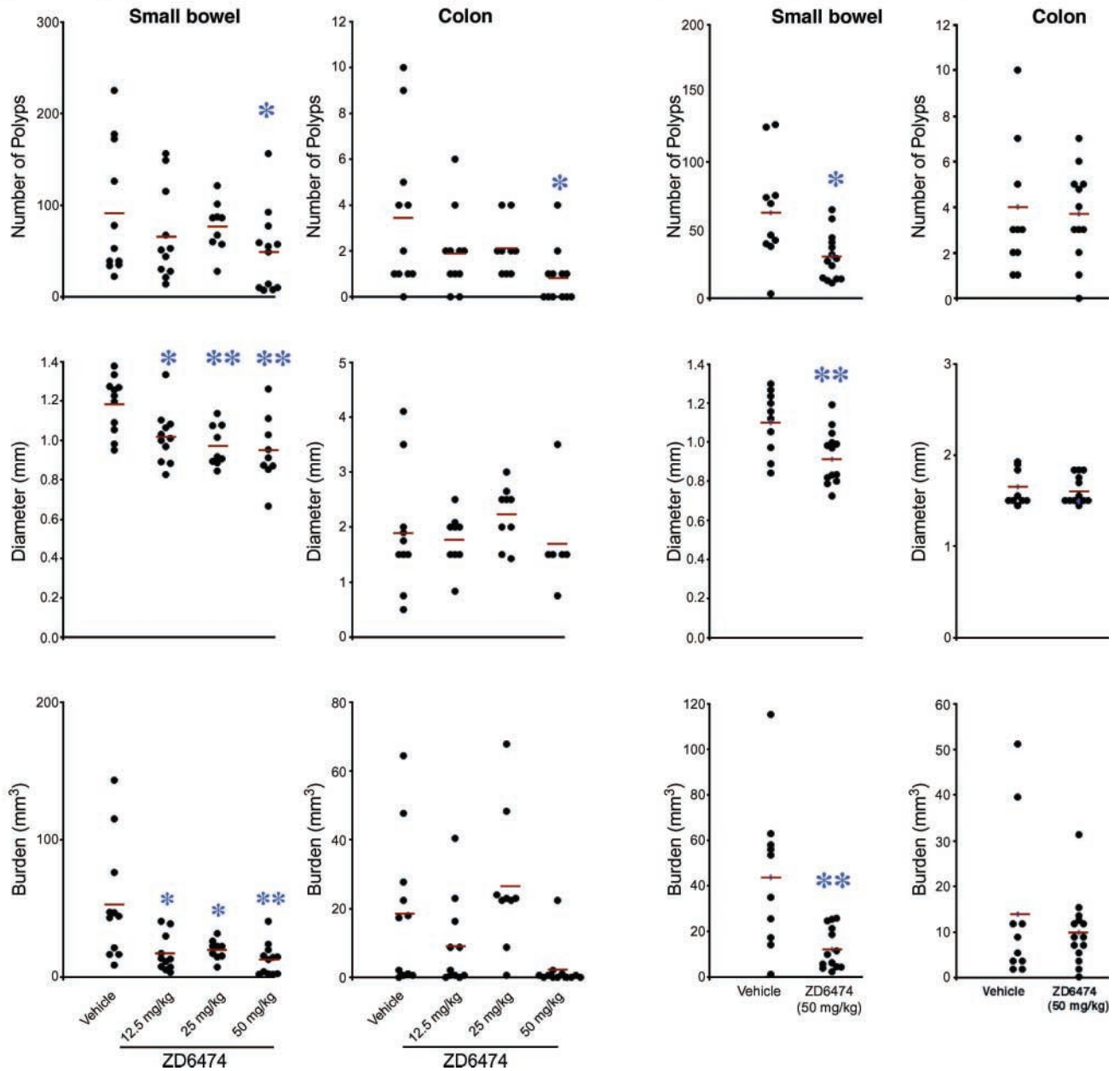


Figure 2. Polyp number (macropolyps), diameter, and burden in small intestine and colon of the (A) early and (B) late intervention studies. *, *P* < 0.05; **, *P* < 0.01 versus the vehicle-treated control.

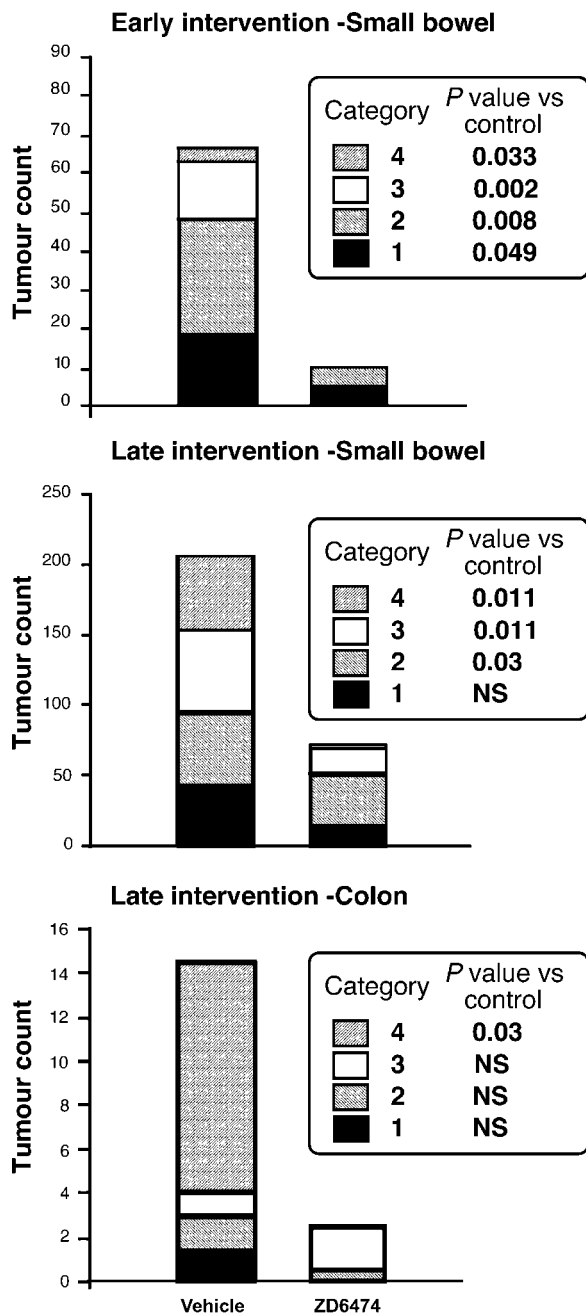


Figure 3. Micropolyp data. Category 1, polyps that remained within the limits of a single villus; category 2, those within the space of >1 to 5 villi; category 3, >5 to 10 villi; category 4, >10 villi.

The aim of conducting 6- and 10-week intervention studies was to assess the effect of ZD6474 on early and more established tumors, respectively. A comparison of micropolyp formation in these two studies confirms that the *Apc*^{Min/+} model is one of progressive tumorigenesis (Figs. 2 and 3; Table 1). The small bowels of older mice had more micropolyps, and a greater proportion of these were classified as category 3 or 4. However, ZD6474 showed

profound antitumor effects in both studies. This suggests that the development of both nascent and established adenomas depends, at least in part, on aberrant signaling pathway(s) that are sensitive to ZD6474.

The relative contribution of anti-VEGFR and anti-EGFR mechanisms to the antitumor activity of ZD6474 observed in this model has not been determined. However, the data are consistent with a mode of action that is antiangiogenic, at least in part. Tumor angiogenesis is stimulated when tumors grow beyond a certain size, and this angiogenic switch is common to both benign adenomas and malignant adenocarcinomas (39).

The angiogenic switch occurs at an early, premalignant stage of tumor development and VEGF signaling is thought to play a key role in the process (40). Recent data (41) have shown that highly selective inhibition of VEGF-A signaling prevents the angiogenic switch in *Apc*^{Min/+} mice and that this is associated with a reduction in polyp diameter but with no effect on polyp number. This is consistent with the hypothesis that inhibition of VEGF-A signaling is sufficient to inhibit growth of established intestinal adenomas through inhibition of angiogenesis but that VEGF-A signaling is not important in the establishment of early adenomas before the angiogenic switch (41). Interestingly, an elegant genetic study of *Apc*^{Min/+} mice with wild-type or reduced EGFR activity showed that the establishment of intestinal tumors, but not the rate of polyp growth, was dependent on EGFR activity (24). This finding was

Table 1. Tissue analyses from late intervention study (mean \pm SE)

	Treatment group		P
	Control	ZD6474 (50 mg/kg/d)	
Nonadenoma			
Cells per crypt	33.8 \pm 1.2	31.5 \pm 1.1	NS
Metaphases per crypt	3.2 \pm 0.2	3.8 \pm 0.2	NS
Metaphase index (%)	9.5 \pm 0.3	12.0 \pm 0.6	0.004
BrdUrd per crypt	8.1 \pm 0.3	9.2 \pm 0.4	0.05
Labeling index (%)	24.1 \pm 0.6	29.1 \pm 0.6	0.001
Vessel density (vWF)	1.33 \pm 0.38	1.73 \pm 0.27	NS
Vessel density (CD31)	68.7 \pm 15.9	77 \pm 29.2	NS
β -catenin nuclear intensity	0.07 \pm 0.07	0	
β -catenin nuclear proportion	0.03 \pm 0.03	0	
VEGFR2 <i>in situ</i> hybridization cells per villus	7.35 \pm 0.64	2.76 \pm 0.32	0.001
Adenoma			
Cells per polyp	426.5 \pm 91.4	191.9 \pm 23.5	0.038
Metaphases per polyp	24.4 \pm 6.0	10.8 \pm 2.1	NS
Metaphase index (%)	1.86 \pm 0.13	1.85 \pm 0.30	NS
BrdUrd per polyp	139.5 \pm 27.1	62.5 \pm 8.7	0.027
Labeling index (%)	11.0 \pm 0.50	10.77 \pm 0.54	NS
Vessel density (vWF)	1.60 \pm 0.33	2.27 \pm 0.35	NS
Vessel density (CD31)	61.6 \pm 11.4	86 \pm 25.4	NS
β -catenin nuclear intensity	1.60 \pm 0.17	1.07 \pm 0.11	0.03
β -catenin nuclear proportion	1.97 \pm 0.22	1.03 \pm 0.14	0.01

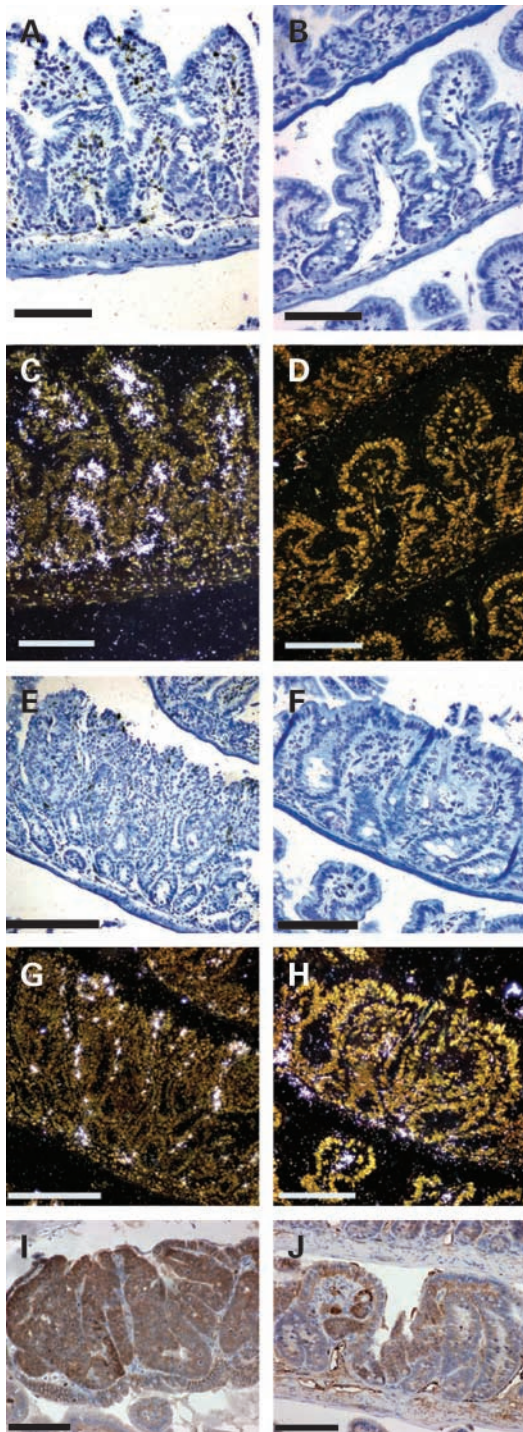


Figure 4. Effects of ZD6474 on VEGFR2 mRNA and β -catenin expression. Small intestine showing localization of VEGFR2 mRNA using *in situ* hybridization (white areas in dark-field illumination) from control (**A**) and ZD6474-treated (**B**) animals. **C** and **D**, corresponding dark-field images. Note the marked reduction in the signal (silver grains) in the treated mice and the location of the signal in the lamina propria of the villi. **E** to **H**, as above, but for polyps from control and treated groups, reduction of signal also occurs in the treated groups. Immunohistochemistry of β -catenin expression and localization in ZD6474-treated groups (**J**) was significantly reduced when compared with control (**I**). Bar, 100 μ m.

supported by pharmacologic data from 12-week-old animals that showed a reduction in polyp number, but no effect on polyp diameter, following treatment with an EGFR tyrosine kinase inhibitor. It has been shown that APC deficiency is associated with an increase in EGFR activity (23) and that EGFR signaling is required for microadenoma formation following azoxymethane treatment (42), implicating this signaling pathway in the earliest stages of colon carcinogenesis. EGFR is a transmembrane receptor tyrosine kinase characterized by a “cobblestone” appearance on immunohistochemical sections due to its localization to the plasma membrane. Intracellular staining of EGFR may also be detected to variable extents in tumor and normal tissues, possibly reflecting receptor recycling and/or intracellular compartmentalization. In the current study, strong phospho-EGFR activity was seen on the cell membrane and was dramatically reduced by ZD6474. Because of the technical limitations of the available antibodies, it is currently only possible to determine phospho-VEGFR2 levels by immunohistochemistry in situations where there is a high level of activity of the receptor, such as the lung, where inhibition of phospho-VEGFR2 by ZD6474 has been shown (43). The expression levels of VEGFR2 and pVEGFR2 in the intestinal epithelia are relatively low (41).

ZD6474 reduced both polyp number and diameter in both early and late intervention studies. These data are consistent with the hypothesis that EGFR signaling is required during the establishment of intestinal adenomas but is less important during their subsequent growth.

It is tempting to speculate that the reduction in polyp size and polyp number seen in the present study following treatment with ZD6474 is due to inhibition of both EGFR and VEGFR signaling, affecting both the establishment and the growth of adenomas in the intestine of $Apc^{Min/+}$ mice. However, in contrast to the recent data selectively inhibiting VEGF-A signaling (41), ZD6474 has the potential to inhibit signaling through VEGFR1, VEGFR2, and VEGFR3. Therefore, it is not possible to rule out an important role for other VEGF ligands (VEGF-B, VEGF-C, VEGF-D and placenta growth factor) or signaling through VEGFR3 in the establishment intestinal adenomas because these would not be affected by inhibiting VEGF-A signaling alone. Indeed, in a previous study with a highly selective inhibitor of VEGFR1, VEGFR2, and VEGFR3 signaling with no activity against EGFR signaling, there was a reduction in polyp number and diameter in a 6-week intervention study but only a reduction in polyp diameter in a 10-week intervention study, suggesting that VEGFR signaling through ligands other than VEGF-A may play a role in the development of at least some adenomas in $Apc^{Min/+}$ mice (29).

In the present study, microvessel density measured by vWF staining was not affected by ZD6474 treatment, which was confirmed by CD31 staining. However, in vehicle-treated $Apc^{Min/+}$ mice, the microvessel density in adenomas was not significantly different from that in the surrounding intestine, so a reduction in microvessel density in the adenomas of ZD6474-treated mice was not

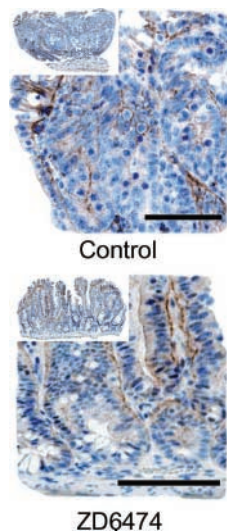


Figure 5. Immunohistochemical staining for membrane-associated phospho-EGFR staining from treated and control polyps from the late intervention study. Bar, 650 and 700 μm for the control and treated, respectively.

anticipated. It has been recognized that changes in microvessel density may not be a good way to assess the efficacy of antiangiogenic therapies (44). Intriguingly, we did observe a significantly lower level of VEGFR2 expression in nonadenoma tissue measured by *in situ* hybridization. The mechanism underlying this effect is not known, but reduced vascular expression of VEGFR2 mRNA (29) or protein (45) has been observed with other potent inhibitors of VEGFR2 signaling. In the present study in *Apc*^{Min/+} mice, ZD6474 treatment was associated with significantly lower levels of nuclear β -catenin compared with vehicle-treated controls. It has been shown that EGFR signaling can affect intracellular distribution of β -catenin (46), so a complex interrelationship may exist between β -catenin and EGFR signaling pathways in APC-deficient cells and may provide the mechanistic framework for the antitumor effects of EGFR signaling inhibitors in this model of early colorectal cancer.

ZD6474 treatment led to pronounced reductions in spleen weight. Because spleen weight reflects blood loss and tumor burden in the *Apc*^{Min/+} phenotype, spleen size may have potential as a surrogate marker of treatment efficacy in this tumor model (47).

Surgery and adjuvant chemotherapy with agents, such as 5-fluorouracil, are the mainstay of treatments for colorectal cancer, but the prognosis for patients remains poor. However, the number of available therapeutic options has been expanded recently with the advent of molecular-targeted agents. The development of these agents has evolved from a better understanding of the molecular mechanisms underlying cancer, including changes that occur at the premalignant stage of tumorigenesis. By simultaneously targeting VEGFR-dependent tumor angiogenesis and EGFR-dependent tumor cell proliferation,

ZD6474 offers the potential advantage of inhibiting two key pathways in tumor growth and development. Targeting VEGFR activity in endothelial cells offers potential advantages compared with agents that only target tumor cells directly. These include a reduced probability for the emergence of drug-resistant clones, because normal endothelial cells lack the genetic instability of cancer cells, as well as a possible amplification of any antitumor effect, because each capillary in a tumor supplies many hundreds of tumor cells. ZD6474 is currently in phase III clinical development, and promising efficacy has been observed in recurrent non-small cell lung cancer (48).

In conclusion, the current data suggest that ZD6474 is effective at different stages of adenoma development, with the potential to target EGFR signaling during the establishment of adenomas before the angiogenic switch and to target VEGFR signaling and blood vessel growth in established adenomas. These data provide a scientific rationale for studying the effects of inhibition of these pathways in both early and late stages of intestinal cancer in the clinic. These results are particularly timely as they follow recent reports of clinical efficacy in colorectal cancer with targeted inhibitors of VEGF-A (49) and EGFR (50) signaling.

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