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# Murine colitis treated with multitargeted tyrosine kinase inhibitors

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ABSTRACT

*Background:* Angiogenesis, a known pathogenic component of neoplastic and nonneoplastic diseases, serves as a therapeutic target. Vascular endothelial growth factor (VEGF) and angiogenesis are clinically elevated in inflammatory bowel disease. By targeting vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) with receptor tyrosine kinase inhibitors in a murine model of colitis, we hypothesize that angiogenesis will be suppressed and disease severity improved.

*Methods and methods:* Sorafenib, sunitinib, and axitinib were administered by oral gavage in a murine model of dextran sodium sulfate (DSS) colitis. Inflammation score, microvessel density (MVD), and gene expression of VEGF, VEGFR, platelet-derived growth factor, PDGFR, Ang-2, and epidermal growth factor receptor was assessed.

*Results:* Inflammation and MVD were elevated in groups receiving DSS, but were similar between DSS-only and treatment cohorts. Unexpected weight loss was present in the gavaged groups *versus* DSS only. In treated groups, VEGFR was significantly decreased ($P = 0.002$) and VEGF gene expression trended down ($P = 0.213$) *versus* DSS only. Neither the platelet-derived growth factor/PDGFR pathway nor the alternative pathways, Ang-2 and epidermal growth factor receptor, were significantly changed from DSS control in treatment cohorts.

*Conclusions:* This study confirms the association between inflammation and MVD. Anti-angiogenic receptor tyrosine kinase inhibitors suppressed the VEGF/VEGFR pathway but the expected decrease in colonic MVD did not follow, suggesting possible involvement of other angiogenic pathway(s). In the DSS model of colitis, vehicle selection and mouse strain can impact disease response.



## 1. Introduction

Physiologic angiogenesis is essential in wound healing and reproductive cycle, whereas pathologic angiogenesis is a contributing component of neoplastic diseases and chronic inflammatory conditions [1,2]. A number of angiogenic factors and their pathways have been described, some of which have proven as therapeutic targets, such as PDGF/PDGFR (platelet-derived growth factor/receptor) and VEGF/VEGFR (vascular endothelial growth factor/receptor) [2,3]. Many anti-VEGF agents are used to treat neoplastic and nonneoplastic diseases, most notably colorectal cancer and age-related macular degeneration [4,5].

Currently, aggressive therapy for severe inflammatory bowel disease (IBD) includes infliximab, an antitumor necrosis factor alpha, which is known to reduce inflammation and

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improve disease severity. Recent studies have reported an unexpected effect in responding patients, a decrease in mucosal angiogenesis [6]. Further supporting the role of angiogenesis in IBD, patients with active disease have elevated angiogenesis and VEGF levels, whereas patients in remission have diminished levels [7–9]. Not only does this support angiogenesis as a component of IBD it proposes the VEGF pathway as a key controller. Similar findings have been published in murine models of colitis, again demonstrating elevated inflammation-associated angiogenesis and VEGF [7,10,11].

Antiangiogenic receptor tyrosine kinase inhibitors (RTKIs) are widely used in the treatment of cancer [12,13]. RTKIs target multiple receptors that broadly inhibit angiogenesis. Sorafenib, sunitinib, and axitinib are RTKIs that target VEGFR-1, -2, -3, PDGFR, and c-kit, with sorafenib additionally targeting Flt-3 and Raf and sunitinib targeting Flt-3 and Ret [12,13]. These small molecule RTKIs competitively inhibit autophosphorylation, thereby preventing the signal transduction to target proteins [14]. This disrupts the downstream, angiogenic effect of the targeted receptors, most notably VEGFR and PDGFR. We hypothesize that angiogenesis can be suppressed in a model of experimental murine colitis by targeting angiogenic pathways using multitargeted RTKIs.

## 2. Methods and materials

### 2.1. Murine colitis model

Experimental colitis was induced by a 7-d course of 1% (wt/vol) dextran sodium sulfate (DSS) supplemented *ad libitum* in drinking water of 6–8 wk old male C57BL/6 mice (Harlan Laboratories, Indianapolis, IN). Male mice were used to limit genetic variability based on gender and because of their enhanced inflammation in the DSS model [15,16]. Clinical signs and symptoms of disease were monitored daily. All experiments were performed in accordance with our Institutional Animal Care and Use Committee (IACUC #2D04026).

### 2.2. Administration of antiangiogenic RTKIs

Animals were divided into wild type (WT), DSS only, vehicle, sorafenib (60 mg/kg), sunitinib (80 mg/kg), and axitinib (5 mg/kg) cohorts (n = 4–5 per group) [17–19]. Drugs (LC Laboratories, Woburn, MA) were reconstituted with 0.5% of carboxymethyl cellulose (CMC; Sigma–Aldrich, St. Louis, MO) and dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ) to produce a soluble form (0.25% of CMC and 7%–18% of DMSO). On day 3 after the initiation of DSS, when inflammation ensues, mice in the treated and vehicle cohorts received their respective regimen by daily oral gavage (0.1 mL) until necropsy at day 10.

### 2.3. Histopathologic colitis scoring

Fixed tissues were preserved in 4% of paraformaldehyde overnight, followed by serial alcohol dehydrations. Hematoxylin and eosin–stained colons were evaluated by light microscopy at ×40 magnification to score the four consecutive fields proximal to the anorectal junction. Using an accepted colitis scoring method [20,21], two blinded observers graded the tissues for a cumulative score (range, 0–18) in the following categories: (1) percent area involved, (2) ulcerations, (3) crypt loss, (4) edema, and (5) immune cell infiltration.

### 2.4. Endothelial immunohistochemistry and microvessel density

Tissue sections were deparaffinized and rehydrated. Then, a citrate-based epitope enhancement was performed with antigen retrieval solution (Dako, Carpinteria, CA), followed by protein blocker solution (Dako). Slides were incubated with MECA-32 (endothelial stain) primary monoclonal antibody (1:10; Developmental Studies Hybridoma Bank, Iowa City, IA). Alexa 488–tagged fluorescent secondary antibody (1:200; Invitrogen, Carlsbad, CA) was then applied, followed by mounting with 4′,6-diamidino-2-phenylindole Vectashield (Vector Laboratories, Burlingame, CA).

Specimens were microphotographed for 10 consecutive high-power (×200) fields starting at the anorectal junction using fluorescent microscopy (Nikon 90i; Nikon Instruments, Inc, Melville, NY). The average mucosal microvessel density (MVD) per high-powered field ($\mu m^2$) was quantitated with NIS-Advanced Elements software (Nikon Instruments, Inc).

### 2.5. Colonic angiogenic gene expression

Colonic specimens frozen in RNAlater (Sigma Life Science) were analyzed for VEGF-A, VEGFR2, PDGF-β, PDGFR, angiopoetin 2 (Ang-2), and epidermal growth factor receptor (EGFR) gene expression. After homogenization, RNA was extracted from tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instruction. Conversion to complementary DNA was performed with the high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Using SYBR Green detection with β-2-Microglobulin (RealTimePrimers.com, Elkins Park, PA) as the internal reference gene, quantitative reverse-transcription polymerase chain reaction was completed by StepOnePlus Real-Time PCR System (Applied Biosystems). The following polymerase chain reaction primers were used: VEGF-A forward 5′ TCT GCT CTC CTT CTG TCG TG 3' & reverse 5′ ACT GGA CCC TGG CTT TAC TG 3′, VEGFR2 forward 5′ CGT TGT ACA AAT GTG AAG C 3' & reverse 5′ CAC AGT AAT TTC AGG ACC C 3′, PDGF-β forward 5′ TTC CTC TCT GCT ACC TG 3' & reverse 5′ GAG TGG TCG CTC AGC ATT TC 3′, PDGFR forward 5′ATG AGG TCC AGC TCT CCT TC 3' & reverse 5′CAT TGG CAG GGT GAC TCT C 3′, Ang-2 forward 5′ GCA CAA AGG ATT CGG ACA AT 3' & reverse 5′ AAG GAC CAC ATG CGT CAA A 3′, and EGFR forward 5′ CTG CCA GAA TGT GAG CAG AG 3' & reverse 5′ TCC ACA AAC TCC CTT GGT TC 3' (Integrated DNA Technologies, Coralville, IA). Relative quantification used the $2^{-\Delta\Delta Ct}$ method to yield results as fold change expression of target gene relative to internal control.

### 2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc, La Jolla, CA). Continuous data were