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AVX-470: A Novel Oral Anti-TNF Antibody with Therapeutic Potential in Inflammatory Bowel Disease

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Abstract

Background—Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the GI tract that is currently treated with injected monoclonal antibodies specific for tumor necrosis factor (TNF). We developed and characterized AVX-470, a novel polyclonal antibody specific for human TNF. We evaluated the oral activity of AVX-470m, a surrogate antibody specific murine TNF, in several well-accepted mouse models of IBD.

Methods—AVX-470 and AVX-470m were isolated from the colostrum of dairy cows that had been immunized with TNF. The potency, specificity and affinity of both AVX-470 and AVX-470m were evaluated *in vitro* and compared with infliximab. AVX-470m was orally administered to mice either before or after induction of colitis and activity was measured by endoscopy, histopathology, immunohistochemistry and quantitative measurement of mRNA levels. Colitis was induced using either 2,4,6-trinitrobenzene sulfonate (TNBS) or dextran sodium sulfate (DSS).

Results—AVX-470 and AVX-470m were shown to be functionally comparable *in vitro*. Moreover, the specificity, neutralizing potency and affinity of AVX-470 were comparable to infliximab. Orally administered AVX-470m effectively reduced disease severity in several mouse models of IBD. Activity was comparable to that of oral prednisolone or parenteral etanercept. The antibody penetrated the colonic mucosa and inhibited TNF-driven mucosal inflammation with minimal systemic exposure.

Conclusions—AVX-470 is a novel polyclonal anti-TNF antibody with an *in vitro* activity profile comparable to that of infliximab. Oral administration of a surrogate antibody specific for mouse TNF is effective in treating mouse models of IBD, delivering the anti-TNF to the site of inflammation with minimal systemic exposure.

Keywords

tumor necrosis factor; ulcerative colitis; polyclonal; gut-targeted

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Conflicts of Interest The remaining authors disclose no conflicts.

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract that affects over 5 million people in North America and Europe, and its increasing incidence and prevalence in many regions of the world point to its emergence as a global disease¹. The two forms of IBD, ulcerative colitis and Crohn's disease, are caused by dysregulated intestinal immune homeostasis resulting in mucosal inflammation²⁻⁴. Increased levels of both soluble and membrane-bound forms of tumor necrosis factor (TNF) are believed to play a central pathogenic role in IBD⁵. TNF is thought to be at the top of a cascading network of cellular pathways that result in inflammation and tissue destruction in IBD and other autoimmune diseases⁶.

Monoclonal anti-TNF antibodies, most notably infliximab and adalimumab, have transformed the treatment of IBD⁷. Despite their impressive efficacy, the use of these antibodies is associated with an increased risk of serious side effects, including the reactivation of tuberculosis, opportunistic infections and a long-term risk of malignancy. These side effects result from systemic immunosuppression as a consequence of the inhibition of TNF's host defense functions^{8,9}. An ideal anti-TNF antibody therapy for IBD would deliver antibodies directly to the site of inflammation in the gut while avoiding systemic exposure and immunosuppression. To this end, we set out to develop an orally-delivered anti-TNF antibody.

Breakdown of an antibody therapeutic in the hostile environment of the gut is a central challenge to the use of an oral biologic. We reasoned that bovine antibodies from milk or colostrum might be suitable for oral delivery by virtue of their known stability to digestion in the gastrointestinal tract¹⁰. A second challenge to the use of an oral biologic arises from the need to penetrate the gut wall to reach the site of inflammation. We reasoned that the increased mucosal permeability seen in IBD patients¹¹ may allow an orally-dosed antibody to diffuse to the site of TNF production in the gut mucosa.

A novel antibody therapeutic, AVX-470, was generated by purifying immunoglobulin (Ig) from the colostrum of cows immunized with recombinant human TNF. The *in vitro* activity of this bovine polyclonal anti-TNF antibody was defined and compared with the activity of infliximab. A surrogate antibody specific for murine TNF, AVX-470m, was generated in parallel and tested in three well-established mouse models of IBD in which anti-TNF antibodies are known to be active: preventative models of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis and acute dextran sodium sulfate (DSS)-induced colitis, as well as a treatment model of established DSS-induced colitis^{12,13}. In all studies, the primary assessment of disease activity was video endoscopy, a method that provides a robust clinical readout of disease severity¹⁴. Oral administration of AVX-470m was shown to inhibit gut inflammation and disease in these mouse models of IBD without significant systemic exposure. These data suggest that orally delivered AVX-470 antibody may be effective as a first line therapy for patients with IBD.

MATERIALS AND METHODS

AVX-470 and AVX-470m Production

AVX-470 and AVX-470m were produced from the colostrum of cows that had been immunized with recombinant human or murine tumor necrosis factor (TNF), respectively. Colostrum was collected, fat was removed by centrifugation and casein was removed by acid precipitation and filtration. The immunoglobulin fraction was enriched using sequential anion and cation exchange chromatography followed by tangential flow filtration. Control immunoglobulin (Ig) was purified in parallel from the colostrum of cows that had not been immunized with TNF. The purification process results in antibodies containing IgG₁ (the

dominant isotype in bovine colostrum), IgG₂, IgM and IgA. AVX-470 does not appreciably recognize TNF from non-primate species (Figure, Supplemental Digital Content 1, <http://links.lww.com/IBD/A238>; Tables, Supplemental Digital Content 2, <http://links.lww.com/IBD/A239>, TNF ELISA and neutralization data), and the surrogate antibody AVX-470m, specific for mouse TNF, was used in mouse model efficacy studies.

AVX-470 and AVX-470m TNF-Binding and Neutralization Activities

The ability of AVX-470 and AVX-470m to bind to TNF was measured in a direct enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with human or mouse TNF at 1 µg/mL and binding was detected using horseradish peroxidase-conjugated sheep anti-bovine antibody (Bethyl Laboratories, Montgomery, TX) using standard techniques. Titers, expressed as µg/mL, were defined as the concentration of test article resulting in an absorbance value of 0.2. The ability of antibody to neutralize TNF was determined using the murine fibroblast cell line L929. These cells are killed by TNF, especially in the presence of actinomycin D, and a neutralizing anti-TNF antibody will prevent this cytotoxicity¹⁵. IC₅₀ values were calculated as the concentration of AVX-470/AVX-470m resulting in 50% inhibition of TNF-mediated killing of L929 cells.

Preparation of affinity-purified AVX-470A and AVX-470mA

An affinity matrix was prepared by coupling 3 mg recombinant human TNF (Cell Sciences, Inc., Canton, MA) to 0.5 ml Affigel-10 (BioRad Laboratories, Hercules, CA) per the manufacturer's instructions. AVX-470A was produced by application of AVX-470 to the column in PBS and elution with 50 mM citric acid/100 mM NaCl, pH 2.0. A similar process was used to prepare a murine TNF affinity column and to purify AVX-470mA.

Affinity of AVX-470A and AVX-470mA for TNF by Competition ELISA

Serial dilutions of human or mouse TNF were incubated with defined levels of anti-TNF antibodies or control bovine Ig. The levels of TNF-specific antibodies used in these assays were previously determined to be in the linear portions of titration curves in direct ELISAs: AVX-470A (35 ng/mL), AVX-470mA (50 ng/mL), infliximab (Janssen Biotech, Inc., Horsham, PA, 2 ng/mL) and TN3 (TN3-19.12, BD Pharmingen, 10 ng/mL). The antibody/TNF dilution mixtures were then added to the TNF-coated plates which were incubated, washed, incubated with horseradish peroxidase-labeled anti-bovine, human or hamster IgG secondary antibodies, washed and read on a plate reader. Data were analyzed using Gen5 Data Analysis Software and affinities were estimated as dissociation constants (K_D) calculated from the amount of soluble TNF required to inhibit 50% of the binding of the antibodies to plate-bound TNF.

Mouse models of colitis

Animal experiments were conducted in the AAALAC-approved facility of Biomodels, LLC with ethical review approval from Biomodels' Institutional Animal Care and Use Committee. Colitis was induced in male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) by exposure to 3% DSS (MP Biomedicals, Solon, OH, MW 36-50,000) in drinking water from Day 0 to Day 5¹² or by intrarectal administration of 4 mg TNBS (Sigma-Aldrich Corp., St. Louis, MO) in 50% ethanol on Day 0. Treatments with vehicle (saline), AVX-470m (1 – 10 mg/day), control Ig (3 or 10 mg/day), or prednisolone (3 mg/kg/day) (Hi-Tech Pharmacal Co., Amityville, NY) were administered in volumes of 0.2 mL/mouse twice a day by oral gavage. Treatments with etanercept (10 mg/kg) (Amgen, Inc., Thousand Oaks, CA) were administered by intraperitoneal injection every other day. In prevention studies, treatment began one day before the induction of colitis and continued

through day 3 for TNBS colitis or through day 12 for DSS colitis. In treatment studies, treatment began on day 6 and continued through day 19.

Colitis severity was assessed using video endoscopy (Karl Storz Endoskope, Tuttlingen, Germany) a method that provides a robust clinical readout of disease severity^{14,16}. Images were scored by a blinded observer: 0 = normal, 1 = loss of vascularity, 2 = loss of vascularity and friability, 3 = friability and mucosal erosions, and 4 = ulcerations and bleeding.

Measurement of Bovine Ig in Serum

Sera were assayed for bovine Ig levels by ELISA (detection limit 0.25 µg/mL at 1:25 dilution of serum). Of the 8% positive sera, the range of bovine Ig levels was 0.3 – 7 µg/mL. Neutralizing anti-TNF antibody levels are estimated based on 0.2% of AVX-470m Ig active in the L929 assay.

Histopathology

Colons were removed at terminal sacrifice (distal and proximal portion), processed, and sections were H&E stained and examined by a qualified examiner blinded to the treatment groups. Tissues were scored for inflammation, edema, and mucosal necrosis on a scale of 0–4. Edema: 0 = normal/no changes; 1 = rare foci, minimal; 2 = scattered regions, or mild diffuse edema, 3 = numerous regions or moderate diffuse edema; 4 = marked diffuse edema. Inflammation: 0 = normal/no changes; 1 = rare foci, minimal infiltration of inflammatory cells; 2 = scattered aggregates or mild diffuse infiltrates of inflammatory cells; 3 = Numerous aggregates or moderate diffuse infiltrates of inflammatory cells; 4 = Marked diffuse infiltrates of inflammatory cells. Mucosal Necrosis: 0 = normal/no changes; 1 = < 25%; 2 = 26–50%; 3 = 51–75%; 4 = > 76% of the mucosa affected.

Immunohistochemistry (IHC)

Four to six sections of each colon were cut and mounted on a single slide and stained using standard procedures. Primary antibodies were from Abbiotec LLC (San Diego, CA) and secondary antibodies and detection reagents were from Vector Laboratories (Burlingame, CA). Sections were counter stained with hematoxylin, dehydrated and mounted on slides. The staining of the entire section was examined at ×400 magnification by a qualified examiner, who was blinded to the treatment groups. The degree of staining was scored on a scale of 0–4 as described^{17,18} (where 0 = no staining, 1 = < 25% 2 = 26–50%; 3 = 51–75%; 4 = >75% of the section with positive staining).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Frozen mouse colon tissue was weighed and pulverized and a 50 mg portion was homogenized. Total RNA was extracted using a Rneasy Midi-Kit (Qiagen, Inc., Valencia, CA). One-Step qPCR was carried out using the Brilliant II 1-Step qRT-PCR Master Mix Kit (Agilent Technologies, Inc., Santa Clara, CA) and Taqman primer/probe kits (Life Technologies Corp., Grand Island, NY). Fifty ng of total RNA were loaded per well. Samples from individual mice were run in duplicate. Data were analyzed using Agilent MxPro QPCR software (Version 4.10d). Samples were normalized to hypoxanthine-guanine phosphoribosyltransferase (Life Technologies, Corp.) and expression of the gene of interest was calculated relative to untreated mice as described¹⁹ and reported as “Relative mRNA Expression.”

Statistical Analysis

The statistical significance of the differences between groups in analysis of colitis, IHC, qPCR and bovine Ig ELISA data was determined using Tukey-Kramer's multiple comparison tests following one-way analysis of variance. Data were expressed as Mean $\pm$ SEM. $P < .05$ was considered statistically significant.

RESULTS

Comparative *in vitro* pharmacology of AVX-470m, AVX-470 and infliximab

The activity of both AVX-470 (specific for human TNF) and AVX-470m (specific for murine TNF) was measured in a direct ELISA (Figs. 1A,B) and the titer for binding to human and mouse TNF, respectively, was found to be 1.2 $\mu\text{g/mL}$ for AVX-470 and 4.3 $\mu\text{g/mL}$ for AVX-470m. A low level of binding to human and mouse TNF was observed with control Ig, reflecting nonspecific binding in this ELISA. Next the TNF-binding antibody fractions of AVX-470 and AVX-470m, termed AVX-470A and AVX-470mA, respectively, were isolated by affinity chromatography on TNF-Affigel columns. The affinity of these antibodies for TNF was measured using two different methodologies: competition ELISA²⁰ and surface plasmon resonance. Using the competition ELISA, AVX-470A had an average affinity for human TNF of 260 pM, comparable to the affinity of 280 pM measured for the marketed anti-TNF monoclonal antibody infliximab (Fig. 1C and Table, Supplemental Digital Content 3, <http://links.lww.com/IBD/A240>). The polyclonal AVX-470A displayed a broader inhibition curve than did the monoclonal antibody infliximab, indicative of a broad range of antibodies directed against multiple epitopes on TNF. A similar profile for AVX-470m was seen compared with TN3, a high-affinity monoclonal anti-mouse TNF antibody²¹ (Fig. 1D and Table, Supplemental Digital Content 3, <http://links.lww.com/IBD/A240>). The average affinity of AVX-470m for mouse TNF was 550 pM. High affinities were also seen when the same set of antibodies was immobilized on biosensor chips and analyzed by surface plasmon resonance, (Figure, Supplemental Digital Content 4, <http://links.lww.com/IBD/A241>); i.e., 95 pM for AVX-470A, 430 pM for infliximab and 120 pM for AVX-470mA. In addition, the on-rates and off-rates for AVX-470A and AVX-470mA (Table 1 and Table, Supplemental Digital Content 5, <http://links.lww.com/IBD/A242>) were comparable to those of high-affinity monoclonal anti-TNF antibodies²². Thus, by two different methodologies, the affinities of AVX-470, infliximab and AVX-470m for TNF were within an order of magnitude of each other. In addition, neither AVX-470 nor AVX-470m bound to lymphotoxin, TRAIL, IL-1 β , IL-6 or VEGF (data not shown), demonstrating specificity for TNF within this panel of cytokines.

In vitro TNF neutralization was analyzed using a standard L929 cell-based assay that is sensitive to both human and mouse TNF²³. Both antibodies potently neutralized TNF with an IC₅₀ of 22 $\mu\text{g/mL}$ (AVX-470 with human TNF; Fig. 1E) and 133 $\mu\text{g/mL}$ (AVX-470m with murine TNF; Figure, Supplemental Digital Content 6, <http://links.lww.com/IBD/A243>). These data suggest that AVX-470m is about 6-fold less potent than AVX-470, with the caveat that direct quantitative comparisons are difficult because different concentrations of human and mouse TNF are used in the L929 assays. The potency of affinity-purified AVX-470A was comparable to that of infliximab in the L929 assay (50 ng/mL vs 56 ng/mL; Fig. 1F), and about 6-fold more potent than AVX-470mA (340 ng/mL; Table 1). In addition to TNF neutralization, it is thought that infliximab induces apoptosis of inflammatory leukocytes in IBD²⁴, and AVX-470 and infliximab were compared for their ability to induce apoptosis of activated human PBMC. Both agents induced higher levels of early and late apoptotic cells above the background levels seen with untreated or control Ig-treated cells (Figure and Table, Supplemental Digital Content 7, <http://links.lww.com/IBD/A244>).

A direct side-by-side comparison of AVX-470 and the marketed monoclonal anti-TNF infliximab (Table 1), demonstrate that the two antibodies have comparable specificity, TNF-binding affinity, TNF-neutralizing activity and apoptosis-inducing activity, supporting the therapeutic potential of AVX-470 in IBD. Moreover, AVX-470 and AVX-470m are comparable in specificity, TNF-binding affinity and TNF-neutralizing activity, indicating that AVX-470m is an appropriate surrogate to use in mouse models of IBD.

Oral bovine anti-TNF antibody therapy in mouse IBD models

Colitis was induced using either TNBS or DSS and disease severity was assessed by video endoscopy. Upon endoscopy, TNBS-induced colitis presented with patchy areas of active inflammation and associated loss of mucosal integrity (bleeding) (Fig. 2A), a phenotype that would be expected from a mouse model of Crohn's disease based on disease presentation in patients. DSS-induced colitis, a model of ulcerative colitis, displayed a much more diffuse disease phenotype with more uniform presentation of inflammation and associated loss of mucosal integrity (Fig. 2B). Oral treatment with AVX-470m, starting before the induction of colitis, significantly reduced endoscopy scores in both the TNBS and DSS colitis models (Fig. 2C,D). The responses were dose-dependent, with greater activity seen with 10 mg/day than with lower doses of AVX-470m. No activity was seen in either model with control Ig, suggesting that the observed activity was the result of the anti-TNF component of AVX-470m.

AVX-470m was administered to animals with established disease in a DSS colitis model. As shown in Fig. 2E and Figure, Supplemental Digital Content 8, <http://links.lww.com/IBD/A245>, mice treated with 3 mg/day or 10 mg/day AVX-470m displayed significantly reduced disease severity on Day 12 and Day 19. Prednisolone, used as a positive control, effectively reduced disease severity by endoscopy while no activity was seen following dosing with 10 mg/day control Ig. In addition, AVX-470m was comparable to or superior to systemically administered etanercept, the only marketed human TNF antagonist which is known to neutralize murine TNF in animal models and to be effective in rodent models of IBD²⁵.

In four experiments encompassing the TNBS and DSS colitis prevention models and two established DSS colitis experiments, a linear dose-response was seen across the dose range of 1 – 10 mg/day at the peak of disease (Figure, Supplemental Digital Content 9, <http://links.lww.com/IBD/A246>). No further increase in activity was seen at 30 mg/day in an established DSS colitis experiment (data not shown). AVX-470m also inhibited the effect of DSS on colon weight, colon length and body weight (Figures, Supplemental Digital Content 10 and 11, <http://links.lww.com/IBD/A247> and <http://links.lww.com/IBD/A248>), clinical parameters known to relate to disease severity in this established DSS colitis model¹³, thus corroborating the endoscopy data. Prednisolone, while effectively reducing disease severity as assessed by endoscopy, displayed only modest activity on colon length or colon weight and had no effect on body weight loss.

Penetration of AVX-470m into colonic tissue

Colon tissue sections were analyzed by immunohistochemistry (IHC) and strong staining for bovine Ig was seen in tissues from mice with DSS colitis treated with AVX-470m. This was not a function of the specificity of the antibody, as comparable staining was seen with control Ig (Fig. 3A). Significant staining was seen using detection antibodies specific for either the F(ab')₂ or Fc regions of bovine IgG (data not shown), suggesting that a significant proportion of IgG molecules reaching and penetrating the colon were intact. Bovine Ig was detected in the lamina propria, mucosa and muscularis mucosa regions, with lower levels of staining in the submucosa. These are the areas associated with inflammation and TNF production in IBD²⁶. In contrast to the results seen in mice with colitis, bovine Ig was not

detected in the colon tissues of normal mice administered AVX-470m by repeated oral gavage (twice per day dosing for 28 days) (Fig. 3A), demonstrating that antibody penetration into the colon tissue was only seen in an inflamed gut.

Minimal systemic exposure to AVX-470m

Systemic exposure following oral administration was assessed by measuring serum levels of bovine Ig using a specific ELISA. Only 22 of 268 sera from mice with either TNBS- or DSS-colitis treated with oral AVX-470m or control Ig had levels of bovine Ig above the lower limit of detection (0.25 µg/mL at a 1:25 dilution of serum) (Fig. 3B); all levels were low (mean of positive samples = 6.8 µg/mL) and the possibility that these positive samples were due to oral gavage injuries resulting in tracheal tears could not be ruled out. A similar rate of serum exposure of bovine Ig was seen in normal mice following repeated oral dosing (not shown), indicating that the antibody was not entering the circulation as a result of colonic inflammation. These data demonstrate that there is minimal systemic exposure after oral dosing of mice with AVX-470m or control Ig.

Reduction of inflammation and TNF-driven pathways by AVX-470m in DSS-induced colitis

By histology, reductions in DSS-induced inflammation, edema, and mucosal necrosis were seen in colon sections from mice treated with 10 mg/day AVX-470m (Fig. 4A and Figure, Supplemental Digital Content 12, <http://links.lww.com/IBD/A249>). Similar histological data were seen in the TNBS colitis model where AVX-470m inhibited inflammation, edema and mucosal necrosis at the peak of disease on Day 5 (Figure, Supplemental Digital Content 13, <http://links.lww.com/IBD/A252>). As with the endoscopy measure, no activity was seen from control Ig.

Using IHC, there was a significant increase in staining for the macrophage marker CD68 in colon tissues in DSS colitis compared with normal mice (Fig. 4B). This CD68 staining was dramatically reduced following treatment with AVX-470m, but not with control Ig (Fig. 4B,C). Furthermore, a reduction was seen in the neutrophil granulocyte marker myeloperoxidase (MPO), but not in the T-cell marker CD3, following treatment with AVX-470m (Fig. 4C). These data are consistent with the finding that granulocytes and macrophages were the dominant inflammatory cells seen in DSS-induced colitis by histology (not shown).

Expression of TNF and markers of TNF-driven pathways was examined in colon tissue. TNF and other inflammatory markers were upregulated in the colons of mice with colitis. AVX-470m treatment resulted in a strong reduction in colonic TNF protein expression (Figs. 4C,D), as well as several downstream cytokines in TNF pathways such as IFN γ , IL-1 β , IL-6 and IL-12/IL-23 (Fig. 4C and Figure, Supplemental Digital Content 14, <http://links.lww.com/IBD/A250>). Using quantitative polymerase chain reaction (qPCR), significant reductions were also seen in messenger RNA (mRNA) levels of TNF, IL-1 β , IL-6, IL-12B (IL-12/IL-23 p40) and matrix metalloproteinase (MMP)-9, but not of intercellular adhesion molecule (ICAM)-1, TNFR1 or TNFR2 (Fig. 4E and Figure, Supplemental Digital Content 15, <http://links.lww.com/IBD/A251>). These results demonstrate that AVX-470m treatment inhibited expression of both TNF protein and mRNA, as well as several inflammatory cytokines and proteins known to be downstream of TNF⁶. Control Ig had no effect on inflammatory markers.

DISCUSSION

The data presented here show that AVX-470, a bovine colostral anti-TNF antibody, binds to and neutralizes TNF with an in vitro profile comparable to that of infliximab. AVX-470m, a

surrogate of AVX-470 that is specific for murine TNF, can be delivered orally for the treatment of colitis in well-accepted mouse models of IBD. As in IBD patients, the colonic mucosal lining in mice with DSS-induced or TNBS-induced colitis has increased permeability due to disease-related epithelial cell damage¹¹. Active AVX-470m reaches the colon and penetrates into the colonic mucosa where it inhibits TNF and TNF-driven pathways of inflammation. Minimal amounts of bovine immunoglobulin reach the systemic circulation. A clinical trial with oral AVX-470 in ulcerative colitis patients is in progress (Clinicaltrials.gov identifier NCT01759056). Based on the murine colitis data presented here, we hypothesize that AVX-470 will have comparable efficacy to the marketed injected anti-TNF antibodies but with minimal systemic immunosuppression.

Three previous lines of research formed the basis of the development of AVX-470 for IBD. First, there was anecdotal evidence in the literature that local inhibition of TNF in the gastrointestinal tract may be effective in treating IBD. Case studies demonstrate that local perirectal injection of a monoclonal anti-TNF antibody is effective in treating fistulizing Crohn's disease²⁷. In addition, Worledge et al. demonstrated that oral delivery of an avian anti-TNF IgY preparation treated TNBS-induced colitis in rats²⁸; unfortunately, avian antibody does not appear to have sufficient stability in the GI tract to make these data generalizable to humans, and this project was never further developed. These data support the hypothesis that the gut is the primary source of TNF in IBD and that local inhibition is effective in treating disease.

Second, previous reports have supported the use of orally administered antibodies from bovine colostrum for the treatment of human disease²⁹. These prior studies all utilized antibodies designed to work within the lumen of the gut and were directed against infectious disease indications. These studies established an early safety and efficacy database for bovine antibodies, and lend support to the use of bovine colostrum antibodies as gut-targeted therapeutics.

Third, there is extensive literature on the increase in gastrointestinal permeability associated with IBD¹¹. We reasoned that this increased permeability due to gut inflammation could be used to drive delivery of antibody to the site of disease. This delivery mechanism is analogous to Enhanced Permeation and Retention, the mechanism thought to be operating in the delivery of therapeutics to solid tumors³⁰. Selectively increased vascular permeability around a solid tumor results in increased drug access and impaired lymphatics prevent the therapeutic from being cleared. In the case of AVX-470, the leaky gut permits the antibody to reach the site of inflammation while the lymphatic dysfunction associated with IBD³¹ prevents the antibody from being rapidly cleared.

One potential difficulty in the use of any mouse model to study an orally administered antibody arises from the differential expression of neonatal Fc receptors (FcRn) in the intestine of mice and humans. The intestinal FcRn mediates the transcytosis of immunoglobulin across the intestinal epithelium. Humans express these receptors throughout life, while mice only express these receptors until they are weaned³². However, this is not a confounding factor in using bovine immunoglobulins to treat human disease, as the human FcRn does not bind to bovine immunoglobulin³³. Therefore, AVX-470m would not be expected to be taken up by FcRn in the mouse colitis studies because the receptor is not expressed in the intestine of adult mice, while AVX-470 would not be expected to be taken up by FcRn in humans because bovine immunoglobulin does not bind to the receptor.

Gut-targeted therapeutics - orally administered, minimally absorbed drugs that are designed to act locally in the gastrointestinal tract - are of increasing interest and importance³⁴. Most recently, the minimally absorbed peptide therapeutic linacotide was approved for the

treatment of irritable bowel syndrome³⁵. The data presented here suggest that bovine colostral antibodies may form the basis for a class of molecules that allow the safety and specificity of antibody therapeutics to be brought to bear on gastrointestinal and metabolic diseases through local delivery of antibody to the gastrointestinal tract.

The data presented here demonstrate that an orally-delivered anti-TNF antibody reaches the inflamed colon in mouse models of IBD and neutralizes local TNF, leading to a significant reduction in disease severity. These results suggest that oral dosing with AVX-470 in patients with IBD may also lead to neutralization of TNF in the gut. The lack of systemic exposure to the antibody is expected to minimize systemic immunosuppression, potentially creating a safer version of the anti-TNF therapeutics that are already known to be effective in the treatment of both ulcerative colitis and Crohn's disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

TNF-binding potency, TNF-binding affinity and TNF-neutralizing potency of AVX-470 and AVX-470m. (A) TNF-binding potency of AVX-470 and control Ig measured in duplicate wells by ELISA on rhTNF-coated plates. Representative experiment. AVX-470 mean titer = 1.2 μg/mL in 8 experiments. (B) TNF-binding potency of AVX-470m and control Ig measured in duplicate wells by ELISA on rmTNF-coated plates. AVX-470m mean titer = 4.3 μg/mL in 3 experiments (C) TNF-binding affinity in a representative experiment of AVX-470A affinity-purified antibody and infliximab for human TNF analyzed in duplicate wells by competition ELISA. IC₅₀ concentration is an estimate of affinity. AVX-470A mean IC₅₀ = 260 pM from 2 experiments; infliximab mean IC₅₀ = 280 pM from 2 experiments. (D) Affinity of AVX-470mA affinity-purified antibody and TN3 antibody for murine TNF in a representative experiment analyzed in duplicate wells by competition ELISA. AVX-470mA IC₅₀ = 550 pM; TN3 IC₅₀ = 26 pM. Means from 2 experiments. (E) TNF-neutralization potency of AVX-470 measured in an L929 cell-based assay. Representative

experiment. AVX-470 mean $IC_{50} = 22 \mu\text{g/mL}$ over 31 experiments (**F**) TNF-neutralization potency of AVX-470A and infliximab measured in an L929 cell-based assay. Representative of 2 separate experiments. AVX-470A mean $IC_{50} = 50 \text{ ng/mL}$; infliximab mean $IC_{50} = 56 \text{ ng/mL}$.

Figure 2.

Oral AVX-470m efficacy in murine IBD models as assessed by endoscopy. **(A)** Representative endoscopic images in TNBS-colitis (Day 5). Preventative treatment with saline, AVX-470m (10 mg/day shown) or control Ig (3 mg/day). **(B)** Representative endoscopic images in DSS-colitis (Day 12). Preventative treatment with saline, AVX-470m (10 mg/day shown) or control Ig (3 mg/day). **(C)** Endoscopy scores in TNBS-colitis (Day 5). 8–12 mice per group. *, $P < .05$ compared with saline-treated group. **(D)** Endoscopy scores in DSS-colitis prevention (Day 12). 10–12 mice per group. *, $P < .05$ compared with

control Ig group. (**E**) Endoscopy scores in established DSS-colitis study (Day 19). 12–15 mice per group. *, $P < .05$ compared with salinetreated group.

A **B** **Figure 3.**

Oral bovine Ig localizes in colon tissues, but with minimal systemic exposure, in mice with DSS-induced colitis. **(A)** Representative sections of colon tissues showing localization of bovine Ig by IHC (brown staining). Colon sections were from mice with DSS-induced colitis on Day 19 treated with AVX-470m (10 mg/day) or control Ig (3 mg/day) or from normal mice without colitis (top right panel) treated for 28 days with AVX-470m (10 mg/day). Colon sections show mucosa and lamina propria regions except lower left panel shows muscularis mucosa (top) and submucosa (bottom) regions. **(B)** Pie chart of sera from oral bovine Ig-treated mice showing the percentage positive for bovine Ig by ELISA. Sera were obtained from individual mice on the day of sacrifice from TNBS and DSS colitis prevention studies and three established DSS-induced colitis studies with oral administration of AVX-470m or control Ig.

Figure 4.

Oral AVX-470m inhibits colon inflammation and inflammatory marker expression in established DSS-induced colitis. **(A)** H&E stained colon sections were scored for the presence of inflammatory aggregates and the degree of diffuse inflammation on a scale of 0–4; magnification 400 $\times$. **(B)** Representative IHC staining of Day 19 colon sections for the macrophage marker CD68. Shown are representative sections from normal mice (No DSS), or mice treated with saline, AVX-470m or control Ig. **(C)** IHC scores for the saline and AVX-470m (10 mg/day) treatment groups on a scale of 0–4 for a panel of inflammation markers: MPO (myeloperoxidase, a neutrophil granulocyte marker); CD3, a pan T-cell

marker; and cytokines TNF, IFN γ , IL-1 β , IL-6 and IL-12p40 (which detects IL-12 and IL-23). **(D)** Representative IHC staining for TNF in Day 19 colon sections. Shown are representative sections from normal mice (No DSS), or mice treated with saline, AVX-470m or control Ig. **(E)** Relative mRNA expression levels of a panel of inflammation markers as measured by qRT-PCR for the saline and AVX-470m (10 mg/day) treatment groups. *, $P < .05$ for treatment groups compared with the saline-treated group. Results are expressed as mean $\pm$ SEM.

Table 1

Comparative in vitro TNF pharmacology of infliximab, AVX-470 and AVX-470m

Parameter	Infliximab	AVX-470/470A*	AVX-470m/470mA*
Cytokine Specificity	Human TNF	Human TNF	Murine TNF
TNF Binding Affinity (Competitive ELISA)	$K_D = 2 \times 10^{-10}$ M Monoclonal	$K_D = 2 \times 10^{-10}$ M Broad Range	$K_D = 5 \times 10^{-10}$ M Broad Range
TNF Binding Affinity (Surface Plasmon Resonance)	$K_D = 4 \times 10^{-10}$ M	$K_D = 1 \times 10^{-10}$ M	$K_D = 1 \times 10^{-10}$ M
TNF Binding On-Rate (Surface Plasmon Resonance)	$k_a = 3.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	$k_a = 5.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	$k_a = 2.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$
TNF Binding Off-Rate (Surface Plasmon Resonance)	$k_d = 1.3 \times 10^{-4} \text{ s}^{-1}$	$k_d = 4.9 \times 10^{-5} \text{ s}^{-1}$	$k_d = 3.3 \times 10^{-5} \text{ s}^{-1}$
TNF Neutralization Potency (L929)	$IC_{50} = 56 \text{ ng/mL}$	$IC_{50} = 50 \text{ ng/mL}$	$IC_{50} = 340 \text{ ng/mL}$
Apoptosis Induction	Activated Human PBMC	Activated Human PBMC	Not tested

* AVX-470 or AVX-470m were used to measure cytokine specificity and apoptosis, whereas AVX-470A and AVX-470mA were used to measure all other parameters.