Vidofludimus Inhibits Colonic Interleukin-17 and Improves Hapten-Induced Colitis in Rats by a Unique Dual Mode of Action

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ABSTRACT

Vidofludimus (Vido) is a novel oral immunomodulatory drug that inhibits dihydro-orotate dehydrogenase and lymphocyte proliferation in vitro. Vido inhibits interleukin (IL)-17 secretion in vitro independently of effects on lymphocyte proliferation. Our primary goal was to evaluate the in vivo effects of Vido on IL-17 secretion and the parameters of trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats. To further delineate the mechanism of action for Vido, rats were dosed concomitantly with uridine (Uri). Young Wistar rats received a 150-μl enema of either phosphate-buffered saline (PBS) or TNBS on study day 1. The ex vivo effects of Vido on 24-h colonic IL-17 secretion were determined by using colonic strips from PBS- or TNBS-treated rats. Some rats were dosed with vehicle, Vido, or Vido + Uri for 6 days. On day 6, the parameters of colitis were determined from colonic tissue. These parameters included macroscopic, histological, and transcription factor measurements, IL-17 production, and numbers of CD3+ T cells. Ex vivo Vido completely blocked IL-23 + IL-1β-stimulated secretion of IL-17 by colonic strips. In vivo Vido treatment alone most effectively reduced macroscopic and histological pathology and the numbers of CD3+ T cells. In contrast, similarly reduced nuclear signal transducer and activator of transcription 3 (STAT3) binding and IL-17 levels were observed from animals treated with Vido alone and Vido + Uri. Vido improves TNBS-induced colonic inflammation by a unique dual mode of action: 1) inhibiting expansion of colonic T lymphocytes, and 2) suppressing colonic IL-17 production, which is independent from the control of T-lymphocyte proliferation, by inhibition of STAT3 and nuclear factor-κB activation.

Introduction

More optimal pharmacological approaches are still needed for the treatment of inflammatory bowel disease (IBD), in particular for patients with Crohn’s disease (CD) (Holtmann and Neurath, 2006; Lanzarotto et al., 2006). Some patients with moderate to severe CD are intolerant and/or nonresponsive to azathioprine/6-mercaptopurine and anti-tumor necrosis factor-α antibodies (Prajapati et al., 2003; Holtmann et al., 2008). Therefore, other immunomodulatory drugs [including vidofludimus (Vido)] have been investigated in clinical trials for the treatment of IBD (Prajapati et al., 2003; Holtmann et al., 2008; Herrlinger et al., 2011). Vido [2-(3-Fluoro-3’-methoxybiphenyl-4-ylcarbamoyl)cyclopent-1-enecarboxylic acid (4SC-101)] is a potent inhibitor of dihydro-orotate dehydrogenase (DHODH), which is a key enzyme involved in pyrimidine biosynthesis (Fitzpatrick et al., 2010a; Kulkarni et al., 2010). Most mammalian cells recruit uridine by recycling through a salvage pathway. In contrast, activated lymphocytes synthesize 4- to 8-fold more pyrimidines and use the de novo biosynthetic pathway (Rückemann et al., 1998; Fox et al., 1999). Because of its capability to inhibit DHODH, Vido can inhibit leukocyte proliferation (Fitzpatrick et al., 2010a; Kulkarni et al., 2010). However, we

AABBREVIATIONS: IBD, inflammatory bowel disease; CD, Crohn’s disease; IL, interleukin; TNBS, trinitrobenzene sulfonic acid; PBS, phosphate-buffered saline; Vido, vidofludimus; Uri, uridine; STAT, signal transducer and activator of transcription; DHODH, dihydro-orotate dehydrogenase; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; PBMC, peripheral blood mononuclear cell; NF-κB, nuclear factor-κB; MPO, myeloperoxidase; 4SC-101, 2-(3-Fluoro-3’-methoxybiphenyl-4-ylcarbamoyl)cyclopent-1-enecarboxylic acid.
showed previously that Vido inhibits IL-17 secretion by peripheral blood mononuclear cells (PBMCs) independently of its effects on cellular proliferation (Fitzpatrick et al., 2010a).

Specifically, we found that the inhibitory effects of Vido on cellular proliferation could be reversed in the presence of exogenous uridine (Uri) (Fitzpatrick et al., 2010a). In contrast, Uri could not reverse the inhibitory effect of Vido on phytohemagglutinin-stimulated IL-17 secretion by PBMCs. Moreover, we also reported that Vido attenuates IL-17 secretion from cytokine-stimulated murine splenocytes, by inhibiting STAT3 and NF-κB signaling pathways in these cells (Fitzpatrick et al., 2011b). As a whole, these data suggest that Vido may have a dual mode of immunomodulatory action in vitro: 1) inhibition of leukocyte proliferation, and 2) inhibition of IL-17 secretion, which is unrelated to leukocyte proliferation (Fitzpatrick et al., 2010a, 2011b).

Of relevance to this article, we also reported that Vido could effectively improve both chronic dextran sulfate sodium-induced colitis and acute hapten [trinitrobenzene sulfonic acid (TNBS)]-induced colitis in mice (Fitzpatrick et al., 2010a). Moreover, Vido attenuated the activation of STAT3 and NF-κB signaling pathways in the colons of TNBS-treated mice and also inhibited the production of IL-17 (Fitzpatrick et al., 2010a, 2011b). These anticolitis actions of Vido may reflect the inhibition of leukocyte proliferation and/or the direct inhibition of IL-17, by modulating relevant intracellular signal transduction systems (Fitzpatrick et al., 2010a, 2011b).

With this background information, our primary goal was to evaluate the effects of Vido on colonic IL-17 secretion (ex vivo and in vivo) and the pharmacological effects on various parameters of TNBS-induced colitis in rats. Included in these parameters was the measurement of CD3+ T-cell numbers in the rat colon. To help further delineate the mechanisms of anticolitis action for Vido, rats were dosed in the presence or absence of exogenous Uri. Other investigators have used this approach to examine the in vivo mode of action for leflunomide (another DHODH inhibitor) in rats (Chong et al., 1999).

Materials and Methods

**Key Chemicals.** Vidofludimus was provided by 4SC AG (Planegg-Martinsried, Germany). The chemical structure of this compound can be found in a prior publication (Fitzpatrick et al., 2010a). Re-combinant rat IL-1β and IL-23 were obtained from R&D Systems (Minneapolis, MN). Key reagents for the myeloperoxidase (MPO) assay such as 3,3′,5,5′-tetramethylbenzidine, hexadecyltrimethylammonium bromide, and N,N-dimethylformamide were obtained from Sigma (St. Louis, MO). TNBS and uridine were also obtained from Sigma. The rat IL-17 enzyme-linked immunosorbent assay kit was obtained from ebioscience (San Diego, CA). The CD3 and proliferating cell nuclear antigen (PCNA) antibodies were obtained from Abcam Inc. (Cambridge, MA). The Vectastatin Elite Kit, which was used for immunohistochemistry studies, came from Vector Laboratories (Burlingame, CA). The ApopTag peroxidase in situ apoptosis detection kit and Proteinase K were obtained from Millipore Corporation (Billerica, MA).

**Animals.** Timed pregnant female Wistar rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Animals were housed under standard conditions with a 12-h light/dark cycle. Both male and female weanling rats were used in these studies. The average weight of these rats on postnatal day 23 was 62.1 ± 0.9 g.

**Colonic Organ Culture for Determining IL-17 Secretion.** Young rats (4 weeks old; n = 3 per group) that had been treated with one intracolonic treatment of PBS or TNBS (see TNBS-Induced Colitis in Rats and Methods) were used for colonic organ culture experiments as described previously (Tanabe et al., 2008). In brief, 5 days after the intracolonic instillation of PBS or TNBS multiple distal colonic strips (n = 11–12 per treatment group) of standard size (3 mm) were cultured for 24 h in culture medium that contained an antibiotic-antimycotic cocktail (Invitrogen, Carlsbad, CA). This antibiotic-antimycotic solution contained 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B. These colonic strips were treated with: no cytokines, cytokine treatment [rat IL-23 (10 ng/ml) + rat IL-1β (10 ng/ml)], or cytokine treatment + Vidofludimus (25, 50, or 100 μM). IL-17 secretion was measured from the culture medium by enzyme-linked immunosorbent assay.

**TNBS-Induced Colitis in Rats.** Essentially, we used the acute version of the TNBS-induced colitis model as described previously (Fitzpatrick et al., 2010b). In brief, on postnatal day 22 newly weaned rats (both sexes) were dosed with vehicle (Phosal 50 PG orally or 0.9% saline intraperitoneally), uridine (500 mg/kg i.p.), vidofludimus (60 mg/kg p.o.), or vidofludimus (60 mg/kg p.o.) + uridine (500 mg/kg i.p.). There were four to eight rats in each treatment group. The dose volume for Phosal 50 PG (4SC AG) and other oral treatments was 5 ml/kg. The dose volumes for saline and uridine treatments were also 5 ml/kg. These animals were dosed for 6 days (postnatal days 22–27). On postnatal day 23 rats received a 150-μl enema of either PBS or TNBS (8 mg in 40% ethanol/PBS). On day 28 rats were euthanized by exposure to carbon dioxide. The colons were collected for the measurement of various colitis indices (see Macroscopic Colonic Score, Colonic Histology Score and Colonic MPO and IL-17 Assays sections in Materials and Methods) as described previously (Fitzpatrick et al., 2010a,b).

**Macroscopic Colonic Score.** The macroscopic scores were determined by the method of Reuter et al. (1996) as described previously (Fitzpatrick et al., 2010a). The scoring system involves a combination of various features: ulceration, adhesions, colonic thickness, and loose stool/diarrhea. The macroscopic ulcer component was scored according to the following criteria: 0, normal appearance; 1, focal hyperemia, no ulcers; 2, ulceration without hyperemia or bowel wall thickening; 3, ulceration with inflammation at one site; 4, ulceration/inflammation at two or more sites; 5, major sites of damage extending >1 cm along the length of colon, and 6 to 10, when damage extends >2 cm along the colon, the score is increased by 1 for each additional centimeter of involvement (Reuter et al., 1996; Fitzpatrick et al., 2010a).

**Colonic Histology Score.** For the colonic histology evaluations, we used two segments (both approximately 1.5 cm in length) of the distal colon from each animal. Therefore, these samples encompassed relevant areas of macroscopic colonic damage if present (Fitzpatrick et al., 2010a). Histological evaluations were performed on coded hematoxylin and eosin-stained slides. Two slides from the distal colon were examined per animal. The colonic histology scores were determined by the method of Elson et al. (1996) on a 12-point severity scale as described previously (Fitzpatrick et al., 2010a).

**Colonic MPO and IL-17 Assays.** These parameters were measured by standard methods as described previously (Fitzpatrick et al., 2010a,b). These results were expressed as the fold change compared with the PBS treatment group.

**Immunohistochemistry.** Generally, we followed the procedures for immunohistochemistry with colonic tissue samples as described previously (Fitzpatrick et al., 2010a,b). For determining the number of CD3-positive T cells, we used coded slides and counted (at a magnification of 600×) the number of positively dianisobenzidine-stained cells within six areas of the colonic lamina propria and submucosa. The average number of positively stained cells was then determined for each slide (i.e., colonic specimen). Subsequently, the results were expressed as the mean number of CD3+ cells per individual treatment group.
For examining leukocyte proliferation, we used the PCNA immunohistochemistry method. We evaluated two slides per treatment group. This method has been used previously in conjunction with the rat TNBS colitis model (Nakase et al., 2001).

**Assessment of Apoptosis (TUNEL Method).** The TUNEL method was used to determine leukocyte apoptosis in the colonic lamina propria and submucosa. Generally, we used the method as described previously (Fitzpatrick et al., 2011a). This method has been used previously in conjunction with the TNBS colitis model (Wittig et al., 2000). In brief, slides (two per treatment group) were immersed in xylene to remove paraffin. After washes in successively lower concentrations of ethanol, the sections were permeabilized with 0.3% Triton X-100/PBS, followed by Proteinase K. Subsequently, the sections were quenched in 3% hydrogen peroxide, followed by successive applications of various components (equilibration buffer, terminal deoxynucleotidyl transferase, and antidigoxigenin conjugate) provided with the apoptosis detection kit. After application of dianaminobenzidine substrate (10 min) and brief counterstaining with Gill’s hematoxylin (30 s), aqueous mounting solution was applied to the slides.

**Western Blot Analysis.** Nuclear extracts from the distal colon were used for assessing NF-κB p65 expression. A protein determination was done with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Overall, we followed the Western blot procedure described previously (Fitzpatrick et al., 2008). For these Western blot analyses, we used 40 μg of protein. We used a rabbit polyclonal antibody from Abcam, Inc. Relative densitometry analyses were performed on the p65 and actin bands with the QuantiScan software program (Biosoft, Cambridge, UK). The ratios of p65 to actin band densities were determined for each colonic sample. The colonic IL-17 results were expressed as the fold change compared with the value from the PBS treatment group (Fitzpatrick et al., 2008).

**Nuclear Binding of STAT3.** Nuclear extracts from the colon were prepared as described previously (Fitzpatrick et al., 2008). We used a TransAM-STAT3 assay kit from Active Motif Inc. (Carlsbad, CA), which measures the nuclear binding of STAT3 to a consensus binding site (5′-TTCGGAA-3′). For the assay, we used 20 μg of protein from colonic nuclear extracts. The results are expressed as the absorbance at 450 nm.

**Statistical Analyses.** The data were analyzed by Prism (GraphPad Software Inc., San Diego, CA). Specifically, for the IC₅₀ determination (colonic organ culture experiment) we used a nonlinear regression analysis. Almost all of the measured parameters (TNBS colitis model) were analyzed by one-way analysis of variance, followed by Bonferroni’s multiple comparison test. The exception was the colonic ulcer score data. Those data were analyzed by the Kruskal-Wallis test, followed by the Dunn’s multiple comparison test. A difference of p < 0.05 was considered significant for all statistical analyses.

**Ethical Considerations.** The rat TNBS colitis protocol was approved by the Institutional Animal Care and Use Committee at the Penn State College of Medicine.

### Results

**Vidofludimus Attenuates IL-17 Secretion from Colonic Strips.** As shown in Fig. 1, on day 5 after the intracolonic administration of TNBS to rats the ex vivo secretion of IL-17 was increased 5.6-fold (compare basal IL-17 secretion levels in Fig. 1). Vido (100 μM) inhibited basal IL-17 secretion from the colonic strips of TNBS-treated mice (Fig. 1B). It is noteworthy that cytokine (IL-1β + IL-23) stimulation resulted in a dramatic up-regulation of IL-17 secretion in colonic strips from either PBS- or TNBS-treated animals (Fig. 1, red bars). It is noteworthy that Vido dose-dependently inhibited cytokine-stimulated secretion by colonic strips from PBS- or TNBS-treated rats. The calculated IC₅₀ values were 26.4 and <25 μM for inhibiting IL-17 secretion from the colonic strips of PBS- and TNBS-treated rats, respectively (Fig. 1).

**Oral Administration of Vidofludimus Alone Most Effectively Improves Many Parameters of TNBS-Induced Colitis in Rats.** The measured parameters of colitis were similar in rats treated with vehicle by the intraperitoneal or orogastric routes. Therefore, the data from these animals were combined for subsequent statistical analyses. In most of the treatment groups rats gained weight over the 6-day study period. However, the mean body weight gain was less in TNBS-treated animals compared with rats that received PBS.
enemas. It is noteworthy that Uri/TNBS-treated rats showed a slight mean weight loss during the study period. Specifically, weight changes (grams/6 days) were: 21.8 ± 1.4 (vehicle/PBS; p < 0.05 versus vehicle/TNBS), 5.8 ± 3.5 (vehicle/TNBS), 9.3 ± 4.4 (Vido/TNBS), -0.5 ± 3.5 (Uri/TNBS), and 7.3 ± 5.0 (Vido + Uri/TNBS).

Representative pictures of the distal rat colon are shown in Fig. 2. There was no ulceration in the colon of an animal given PBS by enema (Fig. 2A). In contrast, Fig. 2B shows a prominent area of ulceration (boxed area) in the colon of a vehicle/TNBS-treated rat. In contrast, smaller areas of ulceration were evident in the colons of animals treated with Vido ± Uri (Fig. 2, C and D, arrow and boxed area, respectively). Figure 3A shows the quantification of the colonic ulcer score data. Specifically, these ulcer scores were: 0.3 ± 0.3 (vehicle/PBS), 6.0 ± 0.3 (vehicle/PBS), 3.2 ± 0.6 (Vido/TNBS), 5.4 ± 0.4 (Uri/TNBS), and 4.5 ± 0.8 (Vido + Uri/TNBS). A significant reduction (p < 0.05 versus vehicle/TNBS) in colonic ulceration was found with Vido treatment. In contrast, colonic ulceration in Vido + Uri/TNBS-treated rats (Fig. 3A, pink bar) was reduced but not to the degree seen in rats treated with Vido/TNBS (Fig. 3A, green bar). Uri/TNBS treatment (Fig. 3A, blue bar) was ineffective for reducing colonic ulceration.

Colonic thickening is also evident in this tissue specimen from the vehicle/TNBS-treated rat (Fig. 2). In parallel with the colonic thickening noted in vehicle/TNBS-treated rats, the mean colonic weight was also significantly increased (p < 0.05) in those animals compared with the vehicle/PBS cohort. The mean colonic weight values (grams/5 cm of colon) were: 0.24 ± 0.01 (vehicle/PBS), 1.01 ± 0.25 (vehicle/TNBS), 0.55 ± 0.09 (Vido/TNBS), 1.14 ± 0.29 (Uri/TNBS), and 0.58 ± 0.18 (Vido + Uri/TNBS).

Fig. 2. Representative pictures of the distal rat colon on postnatal day 28, 5 days after animals received either intracolonic PBS or TNBS (8 mg in 40% ethanol) enemas. A, the colon of a rat from the vehicle/PBS treatment group showed a normal macroscopic appearance. B, a prominent area of ulceration (boxed area) was evident in the colon of a vehicle/TNBS-treated rat. C and D, in contrast, smaller areas of ulceration were evident in the colons of animals from the Vido/TNBS treatment group (arrow in C) and the Vido + Uri/TNBS treatment group (boxed area; D).

Fig. 3. Colonic ulcer scores (A) and macroscopic colonic scores (B) were lower in rats treated with Vido + Uri/TNBS. A, colonic ulcer scores were: 0.3 ± 0.3 (vehicle/PBS), 6.0 ± 0.3 (vehicle/PBS), 3.2 ± 0.6 (Vido/TNBS), 5.4 ± 0.4 (Uri/TNBS), and 4.5 ± 0.8 (Vido + Uri/TNBS). B, total macroscopic colonic scores (representing a combination of ulceration, adhesions, colonic width, and presence of loose stool/diarrhea) were: 1 ± 1 (vehicle/TNBS), 125 ± 31 (vehicle/TNBS), 43 ± 17 (Vido/TNBS), 124 ± 34 (Uri/TNBS), and 72 ± 26 (Vido + Uri/TNBS). *, p < 0.05 versus the vehicle/TNBS treatment group.
Therefore, treatment with Vido alone reduced the colonic weight. However, because of some variability in vehicle/TNBS-treated rats, statistical significance was not attained in the Vido treatment groups. Figure 3B shows the total macroscopic colonic score data. Those data showed a similar pattern to the colonic ulcer data. Total macroscopic scores (representing a combination of ulceration, adhesions, colonic width, and presence of loose stool/diarrhea) were: 1 ± 1 (vehicle/TNBS), 125 ± 31 (vehicle/TNBS), 43 ± 17 (Vido/TNBS), 124 ± 34 (Uri/TNBS), and 72 ± 26 (Vido + Uri/TNBS). A significant reduction (p < 0.05 versus vehicle/TNBS) in the macroscopic colonic score was found with Vido/TNBS treatment (Fig. 3B, green bar). As shown in Fig. 4B, intracolonic administration of TNBS to rats resulted in colonic epithelial damage and ulceration (large arrow). There was evidence of prominent leukocyte influx throughout the mucosa and submucosa, which extended to the muscularis propria (Fig. 4B, small arrow). Histological pathology was less prominent in the colons of rats treated with Vido/TNBS or Vido + Uri/TNBS (Fig. 4, C and D, respectively). The calculated colonic histology scores (0–12 scale; Fig. 4E) were: 4.7 ± 0.7 (vehicle/PBS), 10.0 ± 0.4 (vehicle/TNBS), 7.6 ± 0.5 (Vido/TNBS), 8.9 ± 0.7 (Uri + TNBS), and 8.1 ± 0.7 (Vido + Uri/TNBS) (p < 0.05 versus vehicle/TNBS for Vido/TNBS and Vido + Uri/TNBS treatment groups).

Colonic MPO was measured as an indicator of neutrophil influx into the colon. Normalized MPO values (fold changes versus PBS) were: 1.0 ± 0.1 (vehicle/PBS), 7.5 ± 0.9 (vehicle/TNBS), 4.5 ± 0.8 (Vido/TNBS), 6.7 ± 0.7 (Uri/TNBS), and 3.0 ± 0.7 (Vido + Uri/TNBS) (p < 0.05 versus vehicle/TNBS for Vido/TNBS and Vido + Uri/TNBS treatment groups).

**Oral Vidofludimus + Uridine Administration to Rats Resulted in Similar Inhibitory Effects on Colonic STAT3 and IL-17.** Treatment of rats with Vido alone or in

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**Fig. 4.** Vido + Uri treatment improves the altered colonic histological pathology associated with TNBS administration to rats. A, the colon of a rat from the vehicle/PBS treatment group showed a relatively normal histological appearance. B, the intracolonic administration of TNBS to young rats resulted in colonic epithelial damage and ulceration (large arrow). There was also evidence of prominent leukocyte influx throughout the mucosa and submucosa, which extended to the muscularis propria (small arrow). C and D, histological pathology was less prominent in the colons of rats treated with Vido/TNBS (C) or Vido + Uri/TNBS (D). Magnification is 100-fold. E, the colonic histology scores (0–12 scale) were: 4.7 ± 0.7 (vehicle/PBS), 10.0 ± 0.4 (vehicle/TNBS), 7.6 ± 0.5 (Vido/TNBS), 8.9 ± 0.7 (Uri + TNBS), and 8.1 ± 0.7 (Vido + Uri/TNBS). *, p < 0.05 versus the vehicle/TNBS treatment group.
combination with Uri reduced the nuclear binding of STAT3 in the colon. The data obtained with the wild-type oligonucleotide control (Fig. 5A, brown bar) and the mutated oligonucleotide assay kit control (Fig. 5A, gray bar) demonstrates the specificity for measuring STAT3 binding from these colonic samples. In a similar fashion to the STAT3 data, rats treated with Vido + TNBS had lower levels of IL-17 in the colon (Fig. 5B). Specifically, normalized values (fold changes versus PBS) were: 1.00 ± 0.06 (vehicle/PBS), 1.53 ± 0.12 (vehicle/TNBS), 1.19 ± 0.11 (Vido/TNBS), 1.31 ± 0.98 ( Uri/TNBS), and 1.13 ± 0.14 (Vido + Uri/TNBS) (p < 0.05 versus vehicle/TNBS for Vido + Uri/TNBS treatment groups).

It is noteworthy that, as shown in Fig. 6A, individual rats that were treated with Vido alone or Vido plus Uri generally had lower levels of colonic NF-κB p65 expression (i.e., less dense bands on Western blots) than did vehicle/TNBS-treated animals. The overall quantification of the p65 data is shown in Fig. 6B. Normalized p65/actin values were: 1.0 ± 0.1 (vehicle/PBS), 2.0 ± 0.5 (vehicle/TNBS), 0.5 ± 0.2 (Vido/TNBS), 2.6 ± 0.9 ( Uri/TNBS), and 1.4 ± 0.4 (Vido + Uri/TNBS) (p < 0.05 versus vehicle/TNBS for Vido/TNBS treatment group). Therefore, treatment with Vido/TNBS (Fig. 6B, green bar) had the most profound effect on colonic p65 expression.

Oral Administration of Vidofludimus Alone Most Effectively Inhibits the Numbers of CD3+ Colonic T Cells. Figure 7A shows representative CD3 immunohistochemistry results from the rat colon. In Fig. 7B, there are relatively large numbers of CD3+ T cells (brown cellular staining, indicated by arrows) within the ulcerated area of the colon from a vehicle/TNBS-treated rat. In contrast, the numbers of CD3+ T cells were less prominent in the colons of rats treated with Vido + TNBS or concomitantly with Uri/TNBS (Fig. 7, C and D, respectively, arrows). These data were quantified as shown in Fig. 7E. Specifically, the mean numbers of colonic CD3+ T cells were: 5 ± 1 (vehicle/PBS), 34 ± 6 (vehicle/TNBS), 6 ± 2 (Vido/TNBS), 42 ± 10 ( Uri/TNBS), and 13 ± 7 (Vido + Uri/TNBS) (p < 0.05 versus vehicle/TNBS for Vido/TNBS and Vido + Uri/TNBS treatment groups). It is noteworthy that the number of positively stained CD3 T lymphocytes was also significantly (p < 0.05) less in the Vido + Uri/TNBS group of rats compared with the Uri cohort of animals.

Oral Vidofludimus Administration to Rats Modulates the Proliferation but Not Apoptosis of Colonic Leukocytes. Representative leukocyte proliferation results, as determined by PCNA immunohistochemistry, are shown in Fig. 8. Figure 8A shows only a few scattered proliferating leukocytes in the colonic lamina propria or submucosa (arrow) from a vehicle/PBS-treated rat. In contrast, large numbers of proliferating leukocytes, some with the morphological appearance of lymphocytes (Fig. 8B, left arrow), were evident within the ulcerated area of the colon from a vehicle/TNBS-treated rat. The presence of proliferating leukocytes was less prominent in the colon of a Vido + Uri/TNBS-treated rat (Fig. 8D) and very sparse in an animal treated with Vido + TNBS (Fig. 8C). The pattern of PCNA-positive cell staining...
in Uri/TNBS-treated rats was similar to that in vehicle/TNBS-treated animals (representative picture not shown). Figure 9 shows representative apoptosis results (TUNEL method) from the rat colon. Fig. 9A shows a few apoptotic leukocytes in the lamina propria (arrows) from a vehicle/PBS-treated rat. However, relatively large numbers of apoptotic leukocytes (Fig. 9B, black arrows) were evident within the ulcerated area of the colon from a vehicle/TNBS-treated rat. In contrast, only fewer scattered apoptotic leukocytes were present in the lamina propria and submucosa of rats treated with Vido/TNBS or in combination with Uri/TNBS (Fig. 9, C and D, respectively). The pattern of positive apoptotic cell staining in Uri/TNBS-treated rats was similar to that in vehicle/TNBS-treated animals (representative picture not shown).

**Discussion**

Previously published data showed that Vido potently inhibited rat DHODH, with an IC_{50} value of 1.3 μM (Kulkarni et al., 2010). Vido also attenuated lymphocyte proliferation, with an IC_{50} value of 12.9 μM, and uniquely blocked phytohemagglutinin-stimulated IL-17 production by PBMCs, with an IC_{50} value of 6 μM (Fitzpatrick et al., 2010a; Kulkarni et al., 2010). It is noteworthy that Vido also improved various parameters of TNBS-induced colitis in mice, including the up-regulation of IL-17 associated with this hapten-induced colitis model (Fitzpatrick et al., 2010a). More recently, we reported the activation of STAT3 and NF-κB pathways in the colons of mice with TNBS-induced colitis. Activation of these signal-transduction pathways was normalized in mice that were dosed orally with Vido in the range of 50 to 200 mg/kg (Fitzpatrick et al., 2011b).

To better understand the mechanisms responsible for the documented anticolitis actions of Vido, we tested this drug in our model of TNBS-induced colitis in young rats (Fitzpatrick et al., 2010b). Acute colitis can be induced in these animals by a single intracolonic administration of TNBS (8 mg) in 40% ethanol. This colitis is characterized by transmural colonic inflammation, submucosal collagen deposition, and enhanced production of various cytokines, including IL-17 (Fitzpatrick et al., 2010b). Therefore, this animal model of IBD was chosen to further evaluate the anticolitis profile of Vido in vivo.

Specifically, rats were dosed concomitantly with Vido and Uri. Other investigators have used this experimental approach to examine the in vivo mechanisms of action for leflunomide (another DHODH inhibitor) in rats (Chong et al., 1999). The pharmacological principle of this approach relates to the diminution of endogenous pyrimidine levels in the rat after DHODH inhibition and the restoration of pyrimidines in the tissue after the intraperitoneal administration of uridine at 500 mg/kg (Chong et al., 1999). Because DHODH and
pyrimidine biosynthesis play an integral role in lymphocyte proliferation, a significant reversal in the in vivo effects of DHODH inhibition (in the presence of Uri) was in fact observed by other investigators (Chong et al., 1999). However, because IL-17 inhibition by Vido seems to be independent from lymphocyte proliferation in vitro (Fitzpatrick et al., 2010a), we speculated that the inhibition of lymphocyte proliferation by Vido contributes only partly to its pharmacological effects in vivo.

The dose of Vido (60 mg/kg), which was used for this study, was within the effective dose range for attenuating TNBS-induced colitis in mice (Fitzpatrick et al., 2010a). Although we did not directly measure blood or tissue levels of Vido in this study, the relevant literature suggests $C_{max}$ plasma levels of Vido in the range of 80 to 147 μM after the oral administration of Vido (30–300 mg/kg) in rodents (Kulkarni et al., 2010). Therefore, the oral dose of Vido used in this study probably resulted in peak plasma levels of the drug well in excess of the IC50 value (12.9 μM) for inhibiting proliferation in PBMCs (Kulkarni et al., 2010), as well as the IC50 value (26.4 μM) for inhibiting cytokine-stimulated IL-17 secretion from the rat colon (Fig. 1).

In agreement with our previous results from a murine model of TNBS-induced colitis, treatment of young rats with Vido over a 6-day period resulted in significant reductions in the colonic ulcer score, macroscopic colonic score, and colonic histology score (Figs. 2–4). It is noteworthy that these parameters of colitis were also reduced in animals treated concomitantly with Vido + Uri, but not to the overall degree as with Vido treatment alone. With regard to the colonic weight data, significant attenuations in this parameter were not attained in either of the Vido treatment groups. Perhaps, this was caused by the lack of prominent inhibitory effects on edema and/or fibrosis in the colonic submucosa, which contributes to the increased colonic weight in this TNBS colitis model (Fitzpatrick et al., 2010b). In contrast, some other parameters of colitis (colonic MPO and IL-17) were generally comparable in Vido + Uri-treated rats. Specifically, a profound reversal in the pharmacological effects of Vido in the presence of Uri (i.e., to the levels found in vehicle/TNBS-treated rats) could not be observed for any parameters of colitis.

As a whole, these data imply that inhibition of T cell proliferation may only partially explain the anticolitis effects of Vido. In this regard, other mechanisms also seem to be operative (Fitzpatrick et al., 2010a, 2011b).

Intriguing information has been published regarding the dual activation of NF-κB and STAT3 pathways in pathological conditions such as hepatic inflammation and cancer.
(Danese and Mantovani, 2010; Grivennikov and Karin, 2010; He and Karin, 2011). It is noteworthy that Sutton et al. (2006, 2009) demonstrated that STAT3 and NF-κB pathways mediated IL-17 production from γ-δ T cells. Subsequently, they reported that both γ-δ and CD4+ T cells (via IL-17 production) promoted experimental autoimmune encephalomyelitis in mice (Sutton et al., 2009).

Interactions between the NF-κB and STAT3 pathways may also contribute to the pathogenesis of intestinal inflammation/IBD (Danese and Mantovani, 2010). Indeed, activation of these pathways has been described in conjunction with dextran sulfate sodium-induced colitis in mice and TNBS-induced colitis in rodents (Bai et al., 2007; Kretzmann et al., 2008; Youn et al., 2009; Fitzpatrick et al., 2011b). Moreover, we have reported that Vido inhibits activation of these intracellular signaling pathways in the mouse colon and murine splenocytes (Fitzpatrick et al., 2011b). Therefore, direct inhibition of these pathways may be another important mechanism by which Vido attenuated the macroscopic and histological parameters of TNBS-induced colitis in rats (Figs. 2–4).

As shown in Figs. 5 and 6, the nuclear binding of STAT3, the nuclear expression of p65, as well as IL-17, were in-

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**Fig. 8.** Representative PCNA immunohistochemistry photographs from the rat colon. Magnification is 400-fold. A, only a few proliferating leukocytes in the colonic lamina propria or submucosa (arrow) were present from a vehicle/PBS-treated rat. B, large numbers of proliferating leukocytes, some with the morphological appearance of lymphocytes (left arrow), were evident within the ulcerated area of the colon from a vehicle/TNBS-treated rat. C and D, evidence of proliferating leukocytes (arrows) was less in the colon of a Vido + Uridine/TNBS-treated rat (D) and very sparse in an animal treated with Vido/TNBS (C).

**Fig. 9.** Representative TUNEL histochemistry photographs from the rat colon. Magnification is 400-fold. Representative apoptosis results (TUNEL method) from the rat colon are shown. A, a few apoptotic leukocytes in the lamina propria (arrows) from a vehicle/PBS-treated rat are shown. B, relatively large numbers of apoptotic leukocytes (arrows) were evident within the ulcerated area of the colon from a vehicle/TNBS-treated rat. C and D, in contrast, fewer apoptotic leukocytes (arrows) were present in the lamina propria and submucosa of rats treated with Vido + TNBS (C) or Vido + Uridine/TNBS (D).
increased in the colons of vehicle/TNBS-treated rats. It is noteworthy that all of these parameters were down-regulated within a comparable range in the colons of rats treated with both Vido alone and in combination with Uri. IL-17 plays a potential integral role in the amplification of intestinal inflammation by stimulating various cell types (e.g., myeloblasts and epithelial cells) to produce proinflammatory mediators (IL-6 and IL-8) that amplify intestinal inflammation (Zhang et al., 2016; Strzepa and Szczepanik, 2011). Therefore, by attenuating IL-17 levels in the colon (Fig. 5) Vido also probably affected the amplification of intestinal inflammation that is controlled by IL-17. This statement is supported by the MPO data, which suggested that treatment with Vido alone or in combination with Uri significantly attenuated neutrophil influx into the colon.

To more directly evaluate its relevant pharmacological actions, Vido (25–100 μM) was also tested for inhibiting IL-17 secretion from rat colonic strips (Fig. 1). Dual cytokine (IL-1β + IL-23) stimulation of colonic explants resulted in the enhanced secretion of IL-17 (Fig. 1). Similar results have been reported by other investigators, who used CD161+ T cells from patients with IBD (Kleinschek et al., 2009). Our results (Fig. 1) clearly show that Vido could potently inhibit both basal and dual cytokine-stimulated IL-17 secretion from the colonic strips of rats with acute TNBS-induced colitis. In this ex vivo model, it is less likely that the observed inhibitory effect of Vido solely depends on the attenuation of leukocyte proliferation, at the time point (24 h) at which IL-17 was measured (Reiss and Williams, 1979; Morstyn et al., 1986). However, we did not directly measure cellular proliferation in conjunction with this colonic organ culture system.

Our unpublished results suggest that Vido (10–100 μM) can inhibit T-lymphocyte proliferation, as well as induce apoptosis in vitro, by a p53-dependent mechanism (Hamm et al., 2012). Similar results were reported previously by other investigators, who showed that leflunomide prevented the expansion of activated lymphocytes by interfering with progression through the G1 to S phase of the cell cycle, caused by mechanisms involving the accumulation of p53 (Fox et al., 1999). Intriguingly, the relevant literature also suggests that STAT3 and NF-κB play a role in the transcriptional control of proliferation and apoptosis (Bollrath and Greten, 2009). Therefore, it is possible that the effects on proliferation and apoptosis observed in Vido-treated T lymphocytes also involved inhibition of these two signal transduction pathways (Fitzpatrick et al., 2011b).

Other investigators have used CD3 immunohistochemistry to evaluate treatment effects on T-cell numbers in the colon after the administration of TNBS to rats (Gao et al., 2005). As shown in Fig. 7, the numbers of CD3+ T-lymphocytes were increased approximately 7-fold in the colons of vehicle/TNBS-treated rats. It is noteworthy that rats treated with Vido ± Uri had significantly (p < 0.05) fewer numbers of CD3+ cells in the colon than in rats treated with either vehicle or Uri. However, the inhibitory effect on CD3+ T-cells was most pronounced in rats treated with Vido alone. Our results (Fig. 8) suggest that Vido limited the proliferation of leukocytes in the lamina propria and submucosa of TNBS-treated rats. In contrast, Vido did not seem to induce apoptosis of infiltrating leukocytes in the colon (Fig. 9). However, it may be possible that Vido could selectively induce apoptosis of only T lymphocytes as described in a previous in vitro study (Hamm et al., 2012). This possibility could be further investigated in future studies. Based on the data presented in this article, we propose that the inhibition of CD3+ T-lymphocyte numbers in the Vido treatment groups most likely represents the effects of inhibiting cellular proliferation, as well as directly inhibiting IL-17 production, which would limit amplification of the colonic inflammatory response.

In summary, our data suggest that Vido improves TNBS-induced colonic inflammation by a unique dual mode of action: 1) inhibition of colonic T-cell expansion, and 2) direct suppression of colonic IL-17 production, by inhibition of STAT3 and NF-κB activation. This unique dual mode of action displayed by Vido makes it an attractive candidate as a new therapeutic entity for IBD. Currently, Vido is in clinical trials for IBD. Initial phase 2 data showed that Vido had a good efficacy and safety profile in patients with IBD (Herrlinger et al., 2011). Therefore, Vido may represent a future pharmacological alternative for patients with IBD currently being treated with other immunosuppressive drugs.

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Authorship Contributions
Participated in research design: Fitzpatrick, Dobhofer, and Ammendola.

Conducted experiments: Fitzpatrick and Small.

Performed data analysis: Fitzpatrick.

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