A Novel mTOR Inhibitor is Efficacious in a Murine Model of Colitis


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Running Title: P2281 inhibition of DSS-induced colitis

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Abstract

Ulcerative colitis is an autoimmune-inflammatory disease characterized by increased proliferation of colonic epithelial cells, dysregulation of signal transduction pathways, elevated mucosal T-cell activation, increased production of pro-inflammatory cytokines, and enhanced leukocyte infiltration into colonic interstitium. Several compounds that possess anti-proliferative properties and/or inhibit cytokine production exhibit a therapeutic effect in murine models of colitis. Mammalian target of rapamycin (mTOR), a protein kinase regulating cell proliferation, is implicated in colon carcinogenesis. In this study, we report that a novel haloacyl aminopyridine based molecule (P2281) is a mTOR inhibitor and is efficacious in a murine model of human colitis. In vitro studies using western blot analysis and cell-based ELISA assays showed that P2281 inhibits mTOR activity in colon cancer cells. In vitro and in vivo assays of pro-inflammatory cytokine production revealed that P2281 diminishes induced IFN-γ production but not TNF-α production indicating preferential inhibitory effects of P2281 on T-cell function. In the DSS model of colitis (i) macroscopic colon observations demonstrated that P2281 significantly inhibited DSS-induced weight loss, improved rectal bleeding index, decreased disease activity index and reversed DSS-induced shortening of the colon; (ii) histological analyses of colonic tissues revealed that P2281 distinctly attenuated DSS-induced edema, prominently diminished the leukocyte infiltration in the colonic mucosa and resulted in protection against DSS-induced crypt damage and (iii) western blot analysis showed that P2281 blocks DSS-induced activation of mTOR. Collectively, these results provide direct evidence that P2281, a novel mTOR inhibitor, suppresses DSS-induced colitis by inhibiting T-cell function, and is a potential therapeutic for colitis. Given that compounds with anti-cancer activity show
promising anti-inflammatory efficacy, our findings reinforce the cross-therapeutic functionality of potential drugs.

**Keywords:** colon, inflammation, T-cells, IFN-\(\gamma\), mTOR
Introduction

Ulcerative colitis (UC)\(^1\) is an auto-immune/inflammatory disease affecting millions of people worldwide. Neither the initiating event nor the sequence of propagating events that lead to and sustain colitis have been fully elucidated. Nevertheless, it is increasingly clear that a dysfunctional immune-response, involving Toll-like receptor-4 (TLR-4) (17) and components of normal gastrointestinal enteric bacteria (24, 49), plays a key role in the pathogenesis of colitis. Thus, an early step is macrophage antigen presentation to activated mucosal T-cells (53) which leads to interferon (IFN) production and release (6). Bacterial components (e.g., LPS) and IFNs trigger signal transduction cascades (e.g., NF-κB pathway; mTOR-STAT1 pathway) (4, 13) leading to increased proliferation of colonic epithelial cells (56) and elevated expression of pro-inflammatory genes (e.g. cytokines such as TNF-α) (8). Pro-inflammatory cytokines stimulate leukocytes and endothelium leading to aberrant leukocyte recruitment and enhanced infiltration into damaged colonic interstitium (29, 38).

The above observations have led to therapeutic approaches that seek to diminish colitis (and related diseases) by attenuating the immune/inflammatory response. Indeed, in various experimental models of acute and/or chronic colitis (i) suppression of T-cell function (e.g., by cyclosporin A) (37), (ii) blockade of signal transduction pathways (e.g., mTOR pathway, NF-κB pathway) (15, 34, 52), (iii) inhibition of pro-inflammatory cytokine expression (3, 46), and (iv) attenuation of leukocyte-endothelial interactions (1) separately provides a beneficial effect.
Mammalian target of rapamycin (mTOR) is a serine-threonine protein kinase that regulates protein synthesis, cell growth and cell proliferation in response to growth factors and nutrients (18, 40). It is well-established that mTOR plays a crucial role in tumorogenesis (40). More recently, accumulating evidence causally links increased mTOR activity to heightened inflammatory responses. Indeed, LPS stimulation of macrophages leads to the phosphorylation and activation of p70S6K1 as well as that of 4EBP1/PHAS-1 (13); both proteins are bonafide targets of mTOR. Moreover, the mTOR pathway regulates the production of nitric oxide (58) and activates STAT1-dependent transcription in macrophages in response to LPS (27). Interestingly, a recent study showed that rapamycin, a mTOR inhibitor, blunts leukocyte adhesion and extravasation in the gut mucosa leading to suppression of experimental chronic colitis (15). In a complementary study, treatment with everolimus (another mTOR inhibitor) reduced the number of T-cells in lamina propria and blocked lymphocytic IFN-γ release thereby ameliorating established murine colitis (34). These findings suggest that mTOR inhibitors may be useful for treatment of UC.

The pyridine scaffold is a very common structural motif that can be found in many natural products and in several pharmacologically interesting compounds (20, 22, 30). Therefore, the synthesis of pyridine derivatives, with the objective of developing new drugs, is an active area of research. Indeed, (i) it has been claimed that 2-cyanopyridylureas derivatives can treat hyperproliferative and angiogenesis disorders (50) (ii) 3-cyano-2,6-dihydropyridine inhibits dihydouracil dehydrogenase and its coadministration with 1-ethoxymethyl-5-fluorouracil enhances the antitumor effect (54), (iii) pyridothienopyrimidines exhibit cytotoxic activity (41). In our general search for novel anti-cancer agents, we found that 2-chloro-N-(6-cyanopyridin-3-
(2E)-1-(2,2-dimethylpropanamide (P2281; Fig. 1) markedly inhibits mTOR activity in colon cancer cells. Given these observations, and the fact that mTOR inhibitors may be useful for treatment of UC, we probed the use of P2281 as a therapeutic for colitis. For this, we used the DSS-induced murine model of acute colitis, a model which is well-recognized and known to mimic the pathologic features of human colitis (28, 57). This model is characterized by dysregulated inflammatory response indicated by presence of edema, infiltration of inflammatory cells, and extensive mucosal damage (28, 57). We established this model in our center and used it to assess the efficacy of P2281 on the gross pathology of colitis.
Materials and Methods

Synthesis of 2-chloro-N-(6-cyanopyridin-3-yl)propanamide (P2281): To a stirring suspension of 5-amino-2-cyano pyridine (2.0 g, 16.8 mmole) in chloroform (50 ml), triethyl amine (2.54 g, 25.2 mmole) and 2-chloropropanoyl chloride (2.35 g, 18.48 mmole) were added drop wise at 0 °C. After complete addition of chloroacetyl chloride, the reaction mixture was allowed to come to room temperature (RT) and stirred overnight. The reaction mixture was diluted with chloroform (100 ml) and washed with water (2 x 50 ml). Subsequently, the organic layer was dried over sodium sulfate, concentrated on vaccuo and resulting product was crystallized from chloroform: petroleum ether (1:2) to obtain the desired compound (Fig. 1) in 95% yield (3.3 g).

$^1$H NMR (DMSO-d$_6$ 300 MHz) $\delta$: 10.99 (s, 1H), 8.87-8.86 (d, 1H, J=2.7Hz), 8.29-8.25 (dd, 1H, J= 2.7 & 8.7), 8.01-7.98 (d, 1H, J=8.7Hz), 4.66-4.73 (m, 1H), 1.60-1.62 (d, 3H, MS m/z 210 (M+1) calcd for C$_9$H$_8$N$_3$ClO 209.05. HRMS hplc 99.42% (acetonitrile : ammonium acetate : triethyl amine pH 5.0). The discovery and structure-activity-relationship leading to P2281 will be presented elsewhere (S. Kumar, manuscript in preparation).

Cell lines: H460 human non-small cell lung cancer cells and HCT-116 human colon carcinoma cells were purchased from ATCC (Manassas, VA). Both the cell lines were cultured in RPMI 1640 (Gibco BRL; Pasley, UK) supplemented with 10% heat inactivated fetal calf serum (FCS; JRH; Australia), 100 units/ml penicillin (Sigma Aldrich; St Louis, MO) and 100 µg/ml streptomycin (Sigma Aldrich).
**Assays for analyzing mTOR activity:** Cell based ELISA (11) detecting the phosphorylation of p70S6 kinase at Thr 389, which is a bonafide target for mTOR kinase (18), was used to characterize the mTOR activity. Briefly, H460 and/or HCT-116 cells were plated on 96-well tissue culture plates and allowed to adhere for 24 hr. Subsequently, the cells were serum starved for 18-24 hr. The cells were then pre-treated with P2281 or 0.5% DMSO (carrier control for P2281) for 1 hr, following which the cells were stimulated with 20% FCS to induce the signaling cascade via mTOR. After 30 min incubation at 37 °C, the cells were washed, fixed in 3.7% formaldehyde at ambient temperature for 15 min, washed with PBS, 0.1% Triton-X (PBS-Triton), and incubated in PBS-Triton containing 10% FCS. Unless otherwise noted, from this point on all antibody dilutions and washes were carried out in PBS-Triton. Rabbit polyclonal antibody to phospho-p70S6K1-Thr39 (Cell Signaling; Danvers, MA) was added (diluted 1:500), and the cells were incubated at ambient temperature for 1 hr. After the incubation, the wells were washed and a peroxidase-conjugated polyclonal (secondary) antibody to rabbit IgG (Santa Cruz, CA) was added (diluted 1:500). Following 1 hr incubation at room temperature, the wells were washed and treated with o-phenylene diamine dihydrochloride (OPD; Sigma Aldrich; St. Louis, MO). After a 5 min-incubation at room temperature, the reaction was stopped using H$_2$SO$_4$. The absorbance of the fluid in each well was determined at 490 nm using a microwell plate spectrophotometer (Molecular Devices, Sunnyvale, CA). In each experiment, rapamycin (0.2 µM; Sigma Aldrich) was used as a positive control for mTOR inhibition. In every experiment, each condition was run in triplicate wells.

Western blot analysis (45) was used to confirm the mTOR activity. H460 and/or HCT-116 cells were serum starved for 24 hr. Subsequently, the cells were pre-treated with P2281 or DMSO for
1 hr, following which the cells were stimulated with 20% FCS. After 30 min incubation at 37°C, the cells were washed extensively and lysed using mammalian cell lytic reagent (Sigma Aldrich) supplemented with protease inhibitor cocktail (Sigma Aldrich). In experimental colitis (described below), colon samples from various groups of mice were homogenized and lysed using mammalian cell lytic reagent supplemented with protease inhibitor cocktail. Lysates were used immediately or stored at –20°C for later use. The protein levels in lysates were quantified using Bradford Reagent (Bio-Rad Laboratories; Hercules, CA). For SDS-PAGE and Western blotting, equal amounts of lysates (40 µg) were diluted in reducing sample buffer, boiled and then separated on 10% Tris-HCl SDS-PAGE gels. Subsequently, the resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore) and membrane blocked with 5% non-fat milk. Membranes were washed and incubated with primary antibodies (anti-phospho-p70S6K1 and anti-phospho-4E-BP1; Cell Signaling) overnight at 4°C. After extensively washing the membranes with PBS, 0.1% Tween 20, the membranes were incubated with appropriate horse-radish peroxidase (HRP) conjugated secondary antibody (Santa Cruz, CA). Following 1 hr. incubation, membranes were washed and proteins of interest detected using the chemiluminescent substrate (SuperSignal West Femto Substrate; Pierce Biotechnology; Rockford, IL). The images were captured using a Kodak FX documentation system. In each in vitro experiment, rapamycin (1 µM) was used as a positive control for mTOR inhibition. To quantify the relative differences in phosphorylation, densitometry analysis was performed using the Quantity One software (Bio-Rad Laboratories) with high resolution “tiff” images. The optical density of each band was determined keeping the area and the pixel number constant. Following background subtraction and normalization to the β-actin band, the % phosphorylation of the relevant protein (p4E-BP1 or p70S6K1) was calculated.
**Pro-inflammatory cytokine production assay using hPBMCs:** Regular blood was collected from normal healthy volunteers after informed consent. Peripheral blood mononuclear cells (hPBMC) were harvested using Ficoll-Hypaque density gradient centrifugation (1.077 g/ml; Sigma Aldrich) (10). hPBMCs were resuspended in RPMI 1640 culture medium (Gibco BRL, Pasley, UK) containing 10% FCS, 100 U/ml penicillin (Sigma Chemical Co. St Louis, MO) and 100 µg/ml streptomycin (Sigma Chemical Co. St Louis, MO) at $1 \times 10^6$ cells/ml. $1 \times 10^5$ hPBMCs/well were pre-treated with P2281 or 0.5% DMSO (carrier control for P2281) for 30 min at 37°C. Subsequently, these cells were stimulated with 1 µg/ml LPS (Escherichia coli 0111:B4, Sigma Chemical Co., St. Louis, MO) or concanavalin A (Sigma Chemical Co., St. Louis, MO). Following 5 hr (for LPS experiments) or 18 hr (for concanavalin A experiments) incubation at 37°C, supernatants were collected and stored at −70°C until assayed for human TNF-α, IL-1, IL-6, IL-8 or IFN-γ by ELISA as described by the manufacturer (OptiEIA ELISA sets, BD BioSciences). In every experiment, positive controls were used for inhibiting induced pro-inflammatory cytokine production: rolipram (300 µM; Sigma Aldrich) for TNF-α and cyclosporine (1 µM; Sigma Aldrich) for IFN-γ. In every experiment, each condition was run in triplicate wells.

**In vivo pro-inflammatory cytokine production assay:** BALB/c mice (6 weeks of age, weighing 18 – 22 gms) were obtained from Jackson Laboratories (Bar Harbor, Maine) and housed in individually ventilated cages. All animal experiments were carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and “Guide for Care and Use of Laboratory Animals” (NIH Publication No. 85-23,
revised 1985). All animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of Piramal Life Sciences Limited. In preliminary experiments, we ascertained the pharmacokinetic profile of P2281 administered intraperitoneally (i.p.) to mice. These studies revealed that a dose of 100 mg/kg of P2281 results in a maximal concentration ($C_{\text{max}}$) of 180 µM in the plasma of mice (data not shown). A rough extrapolation of these findings suggests that a dose of 15 mg/kg P2281 (i.p.) would result in a $C_{\text{max}}$ of ~30 µM (concentration at which mTOR inhibition is observed in HCT116 colon cancer cells; see Results section). Accordingly, the effect of P2281 on experimental colitis was studied at a dose of 15 mg/kg (i.p.) (described below). Furthermore, given that P2281 did not inhibit induced TNF-α production in vitro (essentially negative data), we investigated the effect of P2281 on induced TNF-α production in vivo at 30 mg/kg (i.e., 2X the dose used in efficacy studies) to rule out any dose-related issues. 30 mg/kg P2281, suspended in 0.5% Tween80-carboxymethylcellulose sodium salt (CMC; Sigma Aldrich) was administered i.p. to mice. One hour later, LPS (E. coli serotype 0127:B8; 1 mg/kg; Sigma Aldrich) dissolved in sterile pyrogen-free normal saline was administered i.p. The negative control group received normal saline as an i.p. injection, while all other groups received LPS. Rolipram (30 mg/kg; p.o.) was used as a positive control for inhibiting induced TNF-α production. After 1.5 hr, blood was collected from the abdominal artery using a 1 ml syringe flushed with heparinized saline (50 IU/ml). Plasma was separated by centrifugation at 2000g at room temperature, aliquoted and stored at −70°C until assayed for mouse TNF-α levels by ELISA as per manufacturer’s instructions (OptiEIA ELISA sets, BD BioSciences). In each experiment, every group consisted of 6 mice.
**Induction of colitis and P2281 treatment:** C57BL/6J mice (6 weeks of age, weighing 18 – 22 gms) were obtained from Jackson Laboratories and housed in individually ventilated cages. Colitis was induced in mice by giving 3% (wt/vol) DSS (MW 30-40 kDa; ICN Biomedicals, Aurora, OH) in drinking water *ad libitum* as reported by others (26, 35, 48). Importantly, 30-40kDa DSS was utilized in this study for it is known to induce more severe colitis than 5kDa DSS and 500kDa DSS (25). For each mouse, weight, and rectal bleeding were determined every day following the introduction of DSS. DSS-induced colitis was assessed by macroscopic and histological analyses of the colon (described below). To probe the efficacy of P2281, a group of 6 mice were given daily i.p. injections of 15 mg/kg P2281 suspension in CMC. On the day of sacrifice, various parameters indicative of clinical disease were graded as mentioned in Table 1. All these parameters were lumped to obtain an overall clinical disease activity index.

**Macroscopic Colon Assessment:** At the end of DSS treatment period, mice were anesthetized with urethane (1.5 g/kg, i.p.). Maximum possible quantity of blood was collected through abdominal aorta in a heparinized tube. Subsequently, the whole colon was excised. The colon was macroscopically assessed by determining (a) the presence or absence of blood and (b) the longitudinal length. The whole colon was utilized for histological analyses and/or western blot analysis.

**Histological Analysis of Colon:** Colon biopsies from proximal colon, medial colon and distal colon were collected and fixed in neutral buffered formalin. Paraffin embedded sections (5µm thickness) of the colon specimens were stained with Mayer’s hematoxyllin (Sigma Aldrich) and Eosin (Loba Chemie; Mumbai, India) and graded by an investigator blinded to the treatment
groups. Histopathological scoring was performed based on presence of inflammatory cells, extent of crypt damage, erosions and overall architectural damage, each scored on a scale of 0 to 3 as described elsewhere (12). Sections were scored for each feature separately and the scores added to arrive at the final histopathological scoring for individual colon specimen. Histological assessment of disease activity was carried out by analyzing the average histopathological score of a group.

**Statistical Analysis:** For analyzing differences between two groups, Student’s T-test was used. For analyzing differences among multiple (more than two) groups, a single factor ANOVA followed by Bonferroni’s multiple pair-wise comparison tests was used. P values < 0.05 were considered statistically significant. Unless stated otherwise, all error bars represent standard error of mean.
Results

**P2281, a haloacyl aminopyridine based molecule, inhibits mTOR activity in cancer cells**

Prior studies have shown that pyridine based molecules are known to possess anti-proliferative properties (20, 22, 30). mTOR is a protein kinase that regulates cell proliferation (18, 40). Thus, in our attempt to identify novel anti-cancer agents we ascertained the effect of library compounds on mTOR activity. As part of this general screening process, we investigated the effect of P2281, a haloacyl aminopyridine based molecule (Fig. 1), on mTOR activity. Initially, we performed cell-based ELISA and used the phosphorylation of p70S6 kinase at Thr 389 as a measure of mTOR activity. Consistent with results of prior studies (36), naïve H460 cells exhibited little, if any, mTOR activity (Fig. 2A). Upon stimulation with 20% FCS the phosphorylation of p70S6K1 at Thr 389 (18), was markedly increased indicating elevated mTOR activation (Fig. 2A). Pre-treatment of cancer cells with P2281 or with positive control rapamycin each led to a significant decrease in the phosphorylation of p70S6K1 at Thr 389 (Fig. 2A). To further characterize the mTOR inhibitory activity of P2281, we examined its effect on phosphorylation of 4E-BP1 protein. Western blot analysis of lysates from FCS-stimulated H460 cells showed prominent up-regulation in phosphorylation status of 4E-BP1 in comparison to lysates from naïve cells. In line with observations from cell based ELISA, pre-treatment with P2281 or with positive controls LY294002 and Rapamycin, each led to a distinct decrease of 4E-BP1 phosphorylation (Fig. 2B). Of note, P2281 inhibited the FCS-induced phosphorylation of p70S6K1 (Thr 389) in HCT-116 colon cancer cells (Fig. 2C). The relative differences in phosphorylation were quantified using densitometry. For this, the phosphorylation of the protein
in the serum-stimulated, DMSO-treated samples (Figs. 2B and 2C; Lane 2) was considered to be 100%. The phosphoprotein levels in the remaining samples of the respective experiment were calculated with reference to the serum-stimulated, DMSO-treated samples. Thus, following treatment of H460 cells with P2281 (Fig. 2B; Lanes 3 and 4), the residual phosphorylation of 4E-BP1 was 47% whereas the residual phosphorylation of 4E-BP1 following treatment with LY294002 (Fig. 2B; Lane 5) and rapamycin (Fig. 2B; Lane 6) was 33% and 48%, respectively. Similarly, the residual phosphorylation of p70S6K1 in HCT-116 cells following P2281 treatment (Fig. 2C; Lane 3) was 55% as compared to 17% following LY294002 treatment (Fig. 2C; Lane 4). It is well-established that, in addition to being phosphorylated by mTOR, p70S6K1 can also be phosphorylated via the phosphotidyl-inositol 3’ kinase (PI3K) pathway by PDK1 at Thr 389 (47, 55). Given these findings of earlier studies, it is not entirely surprising that treatment of HCT-116 cells with LY294002 (which inhibits both PI3K and mTOR) results in more potent abrogation of p70S6K1 phosphorylation. Interestingly, P2281 did not significantly inhibit the PI3K activity as evidenced by lack of effect on phosphorylation of Akt (18% inhibition; data not shown) suggesting that is a selective/preferential mTOR inhibitor.

**P2281 inhibits induced IFN-γproduction but not TNF-αproduction**

It is increasingly being recognized that anti-cancer therapeutics can possess anti-inflammatory properties (23, 31, 39). More importantly, a growing body of evidence associates increased mTOR activation to inflammatory complications (27, 58). Accordingly, we sought to investigate the effects of P2281 in inflammation assays. LPS stimulation of macrophages leads to not only activation of mTOR but also secretion of pro-inflammatory cytokines including TNF-α. This fact, combined with the documented role of bacterial endotoxin and TNF-α in the pathogenesis
of inflammatory disorders including UC (5, 9), led us to initially probe the effect of P2281 on the LPS-induced expression of TNF-α. Freshly isolated human peripheral blood mononuclear cells (hPBMCs) were pre-treated with P2281 or 0.5% DMSO (carrier control) and subsequently challenged with LPS for 5 hr. ELISA of supernatants revealed that LPS stimulation induced TNF-α production from hPBMCs (Fig. 3A). Rolipram, the positive control, significantly inhibited LPS-induced production of TNF-α (Fig. 3A). However, P2281 had little, if any, effect on LPS-induced production of TNF-α (Fig. 3A). Similar results were obtained with rapamycin (Fig. 3A). These in vitro findings were corroborated by in vivo studies wherein P2281 did not inhibit LPS-induced TNF-α production (Fig. 3B).

Given that everolimus, a mTOR inhibitor, inhibits IFN-γ production (34), we next investigated the effect of P2281 on the induced production of IFN-γ. Freshly isolated hPBMCs were pre-treated with P2281 or 0.5% DMSO and subsequently stimulated with concanavalin A for 18 hrs. ELISA of supernatants revealed that concanavalin stimulation induced IFN-γ production from hPBMCs (Fig. 4A). Cyclosporine A, the positive control, significantly inhibited concanavalin-induced production of IFN-γ (Fig. 4A). More importantly, P2281 also significantly inhibited concanavalin-induced production of IFN-γ (Fig. 4A). Accordingly, dose-response studies were carried out. P2281 inhibited induced IFN-γ production in a dose dependent manner with significant inhibition being observed at P2281 concentration ≥ 30 µM (Fig. 4B). Rapamycin also inhibited concanavalin-induced IFN-γ production in a dose-dependent manner (data not shown). Collectively, these results clearly demonstrate that P2281 inhibits induced IFN-γ production but not induced TNF-α production.
**P2281 suppresses DSS-induced colitis**

The observations that rapamycin and evorilimus (both mTOR inhibitors) are efficacious in animal models of colitis (15, 34), combined with the findings that blocking IFN-γ production elicits a therapeutic effect in experimental colitis (16), led us hypothesize that P2281 (that inhibits mTOR activation as well as IFN-γ production) would be efficacious in a murine model of colitis. Accordingly, we investigated the effect of P2281 in an experimental model of colitis. A group of 6 mice was given DSS solution from day 1 to 10. As a control for DSS treatment, a group of 6 mice was given regular drinking water from day 1 to 10 (normal mice; naïve). Separate groups of mice (6 mice per group) were given DSS solution from day 1 to 10 and received daily injections of P2281 (15 mg/kg; i.p.) or 0.5% CMC (vehicle control for P2281). The P2281 and CMC administration were started on the same day as DSS was added to the water. All mice were sacrificed after day 10 and macroscopic and histological analysis of the colon was performed.

As reported by others (48), DSS-induction of colitis was manifested with significant increase in clinical disease activity index associated with significant weight loss, presence of rectal bleeding, diarrhea and distinct occurrence of occult blood in feces (Fig. 5). Consistent with these observations, DSS treatment significantly reduced the colon length (Fig. 5F). CMC (vehicle control for P2281), given coincident with DSS treatment, had no effect on DSS-induced disease (data not shown). Importantly, P2281, given coincident with DSS treatment, significantly inhibited DSS-induced weight loss (Fig. 5A) and significantly inhibited DSS-induced disease.
activity index (Fig. 5E). In line with these observations, P2281 treatment significantly inhibited the DSS-induced shortening of the colon (Fig. 5F). P2281 treatment diminished (albeit at not statistically significant levels) DSS-induced rectal bleeding (Fig. 5B) and attenuated (albeit at not statistically significant levels) DSS-induced decreases in hemoglobin levels (Fig. 5D).

Histological analysis confirmed the DSS-induction of colitis. As reported by others (25), colonic tissue sections from DSS mice, but not from normal mice, revealed severe inflammation, characterized by presence of edema, distinct inflammatory cellular infiltrate, extensive damage to mucosa and epithelium along with crypt destruction (Fig. 6A). Remarkably, tissue sections from P2281-treated DSS mice, but not CMC-treated DSS mice, revealed attenuation in inflammation, characterized by suppression of edema, reduction in inflammatory cellular infiltrate, and protection against epithelium and crypt damage (Fig. 6A and data not shown). Accordingly, the histopathological score of P2281-treated DSS mice was significantly lower than that of CMC-treated DSS mice (Fig. 6B).

Western blot analysis revealed that DSS induced phosphorylation of 4E-BP1 in the colon (Fig. 7). Consistent with in-vitro observations, P2281, at concentration efficacious in suppressing colitis, prominently suppressed DSS-induced phosphorylation of 4E-BP1 in colon (Fig. 7). The relative differences in phosphorylation were quantified using densitometry. For this, the phosphorylation of the protein in the lysates from colon of CMC-treated DSS mice (Fig. 7; Lane 2) was considered to be 100%. The phosphoprotein levels in the remaining samples were calculated with reference to the CMC-treated DSS mice. In P2281-treated DSS mice (Fig. 7; Lane 3), the residual phosphorylation of 4E-BP1 was found to be 47% compared to 35% in naïve
mice (Fig. 7; Lane 1). Thus, P2281 treatment causes a marked reduction in DSS-induced mTOR activity.

Taken together, the above results clearly demonstrate that P2281 significantly inhibits DSS-induced macroscopic and histological abnormalities in the colon by inhibiting mTOR activation.
**Discussion**

A critical component of ulcerative colitis is increased proliferation of colonic epithelial cells (56), dysregulation of signal transduction pathways (4), elevated mucosal T-cell activation (53), increased production of pro-inflammatory cytokines (6, 8), and enhanced leukocyte infiltration into colonic interstitium (29, 38). A growing body of evidence supports the notion that kinase inhibitors, which possess anti-proliferative activities, are potential therapeutics for ulcerative colitis (15, 34, 39). In our search for anti-cancer therapeutics, we have found that P2281 inhibits mTOR activity in colon cancer cells and suppresses DSS-induced colitis. Given that prior studies have demonstrated that compounds with anti-cancer activity show promising anti-inflammatory efficacy (39, 43, 51, 59), the findings from this study, thus, reinforce the cross-therapeutic functionality of potential drugs.

Our results clearly demonstrate that P2281 can markedly suppress DSS-induced colitis. Specifically, P2281 significantly inhibits DSS-induced weight loss and significantly reverses DSS-induced shortening of the colon (Figs. 5A and 5F). P2281 also diminished rectal bleeding index and attenuated DSS-induced reduction in hemoglobin levels (Figs. 5B and 5D); however the effect of P2281 on these DSS-induced disease parameters did not reach statistical significance. The reason for preferential efficacy of P2281 on certain parameters of DSS-induced disease is currently unknown. However, microscopic histological analyses corroborate the macroscopic observations of P2281 efficacy in protecting mice against DSS-induced colitis. In particular, P2281 dramatically suppresses edema, reduces leukocyte infiltration and maintains mucosal integrity in DSS-treated mice (Fig. 6A). The results of our study are in line with
observations from other studies (15, 34) and suggest that marked mTOR inhibitory activity (Fig. 7) can elicit a meaningful physiological effect. Given that the macroscopic and microscopic manifestations of colitis observed in our system have been reported in other animal models of colitis and in humans (28), our data are likely broadly applicable to other experimental colitis models, and importantly to human colitis. Further experiments are warranted to confirm this hypothesis.

The current therapies for inflammatory bowel disease include immunomodulating agents such as mesalazine, corticosteroids, and cyclosporine A (5). The latter is believed to act primarily through effects on T-cell function. P2281 inhibits mTOR activity and also suppresses induced production of IFN-γ (cytokine released by T-cells) (Figs. 2 and 4). Interestingly, we found that P2281 failed to block in-vitro and in-vivo LPS-induced production of TNF-α (cytokine released by macrophages). The inability of P2281 to inhibit in-vivo LPS-induced TNF-α production was not because of pharmacokinetic issues for sufficient levels ($C_{\text{max}}$: 38 µg/ml i.e., 180 µM) of P2281 were seen in the plasma of mice after administration of 100 mg/kg P2281 (data not shown). These observations, combined with the fact that LPS stimulates mTOR activity (13, 27), indicate that mTOR activation plays little, if any, role in induced TNF-α production. Furthermore, P2281 had little if any effect on LPS-induced IL-6, IL-8 and IL-1 production from hPBMCs (data not shown). Taken together, these results suggest that P2281 may have preferential effects on T-cells in comparison to macrophages. Our findings are not entirely surprising particularly since mTOR inhibitors (such as rapamycin) are known to be potent inhibitors of T-cell function (7). In view of our observations, it will, thus, be of interest to determine the efficacy of P2281 in other T-cell mediated disorders (e.g., psoriasis (33)).
The findings from this study have important implications for oncology too. It is well established that mTOR is a critical integrator of various signals emanating from growth factors, cytokines, hormones and nutrients (18). Activation of mTOR leads to phosphorylation of two major effector molecules – p70S6 kinase and 4E-BP1 which in turn enhances protein translation and cell growth. Several studies have implicated mTOR in tumorigenesis (40) and the fact that mTOR inhibitors such as rapamycin, temserolimus, CCI-779 are showing promising results in clinical trials (18) is in itself a fair indication of the importance of mTOR in oncology. Moreover, due to their anti-proliferative effects on T and B cells, rapamycin (7) and its analogs have also been successfully used as immunosuppressants indicating that mTOR also plays a crucial role in functioning of the immune system. The activation of mTOR is governed by Akt (protein kinase B) which in turn is activated by the PI3K pathway (19, 32). Thus, triggering of the PI3K-Akt-mTOR arm of the cellular signaling machinery in many cases is necessary and sufficient for tumorigenesis. Of note, the PI3K-Akt pathway has been implicated in colon carcinogenesis (44). Interestingly, P2281 did not significantly inhibit the phosphorylation of Akt (18% inhibition; data not shown) indicating that it is a preferential mTOR inhibitor. Separately, overexpression of the protein synthesis initiation factor, eIF4E has been documented in colon cancers (42). Given that mTOR positively regulates the function of eIF4E, it follows that mTOR is a critical target for colon cancer. A connection between Wnt and mTOR has also been reported. Wnt pathway can inactivate TSC1/2 proteins which are the negative regulators of mTOR, thus relieving the inhibition on mTOR (21). The enhanced activation of mTOR would then drive colon carcinogenesis. mTOR activation was also shown to lead to chromosomal instability and the formation of colonic polyposis (2). Taken together, these findings
demonstrate the importance of targeting mTOR in colorectal cancer. Moreover, inflammatory disorders such as Crohn’s disease and ulcerative colitis may also lead to colon cancer in the long run (14). Our findings, thus, suggest that targeting mTOR may not only prevent colitis but also exert a further inhibitory effect on colon cancer development in inflammatory bowel disease patients.

In conclusion, we have demonstrated that P2281 is a novel mTOR inhibitor and that systemic application (i.p.) of P2281 significantly suppresses chemically-induced murine colitis. Thus, P2281 may be a potential therapeutic for ulcerative colitis with important implications for colon cancer too.
Footnotes:

1. Abbreviations used in this paper: $C_{\text{max}}$, maximal concentration; CMC, carboxymethylcellulose sodium salt; DSS, dextran sulfate sodium; FCS, fetal calf serum; hPBMCs, human peripheral blood mononuclear cells; HRP, horse-radish peroxidase; IFN-$\gamma$, interferon-$\gamma$; i.p., intraperitoneal; LPS, lipopolysaccharide; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; OPD, o-phenylenediamine hydrochloride; PBS, phosphate buffer saline; PI3K, phosphotidyl-inositol 3’ kinase; PVDF, polyvinylidene difluoride; STAT1; Signal Transducer and Activator-1; TLR-4, toll like receptor-4; TNF-$\alpha$, tumor necrosis factor-$\alpha$; UC, ulcerative colitis.
Acknowledgements

We would like to thank Rai Ajit Srivastava for critical review of the manuscript, Kumar Nemmani for useful discussions and Lyle Fonseca, Divya Kamath and Merlene Ann Babu for expert technical assistance.
Figure 1. Structure of P2281.

Figure 2. P2281 inhibits mTOR activity. (A) The phosphorylation status of p70S6K1 in unstimulated and 20% FCS stimulated serum-starved adhered H460 cells, pre-treated with or without P2281 or rapamycin or 0.5% DMSO, was determined using ELISA. All values are means of triplicate wells. Results presented are representative of n = 3 separate experiments. The values in the treated samples are expressed in terms of percentage decrease in 4E-BP1 phosphorylation with respect to the 20% FCS stimulated, DMSO-treated sample. (B) Serum starved H460 cells were pre-treated with P2281, LY2942002, Rapamycin or DMSO followed by stimulation (+) or no stimulation (-) with 20% FCS. Equal amounts of lysates from various treatment groups were separated by SDS PAGE, transferred onto PVDF membrane and blotted with antibody to p4E-BP1 or β-actin. Blots presented are representative of n = 2 experiments. (Lanes 1 and 2: pre-treatment with 0.5% DMSO; Lane 3: pre-treatment with 3 μM P2281; Lane 4: pre-treatment with 10 μM P2281; Lane 5: pre-treatment with 30 μM LY294002 and Lane 6: pre-treatment with 1 μM rapamycin.) The numerical values on top of the blot indicate relative phosphorylation with reference to the serum-stimulated, DMSO-treated samples. (C) Serum starved HCT-116 cells were pre-treated with P2281, LY2942002, or DMSO followed by stimulation (+) or no stimulation (-) with 20% FCS. Equal amounts of lysates from various treatment groups were separated by SDS PAGE, transferred onto PVDF membrane and blotted with antibody to p70S6K1 or β-actin. Blots presented are representative of n = 2 experiments. (Lanes 1 and 2: pre-treatment with 0.5% DMSO; Lane 3: pre-treatment with 30 μM P2281 and
Lane 4: pre-treatment with 30 µM LY294002.) The numerical values on top of the blot indicate relative phosphorylation with reference to the serum-stimulated, DMSO-treated samples.

**Figure 3. P2281 does not inhibit induced TNF-α production.** (A) Freshly isolated human peripheral blood mononuclear cells (hPBMCs) were pre-treated with 30 µM P2281 or 0.5% DMSO and subsequently challenged with LPS. Following 5 hr. incubation, supernatants were collected and TNF-α levels estimated by ELISA. Results presented are representative of n = 3 separate experiments. (B) Female BALB/c mice were pre-treated with 30 mg/kg P2281 for 1 hr. and subsequently challenged with LPS. Following 1 hr. stimulation, plasma was collected from each mice and TNF-α levels estimated by ELISA. Values presented are average of n = 6 mice. For both in-vitro and in-vivo experiments, rolipram (Roli) served as a positive control for inhibiting induced TNF-α expression. * indicates p < 0.05 compared to DMSO control.

**Figure 4. P2281 inhibits induced IFN-γ production.** Freshly isolated human peripheral blood mononuclear cells (hPBMCs) were pre-treated with P2281 or 0.5% DMSO and subsequently stimulated with concanavalin A. Following 18 hr. incubation, supernatants were collected and IFN-γ levels estimated by ELISA. Cyclosporine A (CS) served as a positive control for inhibiting induced IFN-γ expression. (A) Inhibition of induced IFN-γ production using a single dose of P2281 (100 µM) (B) P2281 inhibits induced IFN-γ production in a dose-dependent manner. Results presented are representative of n = 3 separate experiments. * indicates p < 0.05 compared to DMSO control.
Figure 5. P2281 significantly inhibits DSS-induced weight loss, diminishes DSS-induced rectal bleeding, attenuates DSS-induced decrease in hemoglobin levels and significantly inhibits DSS-induced shortening of the colon. Various groups of mice received DSS daily with some groups receiving daily injections of 15 mg/kg P2281 or 0.5% CMC. (A) The percentage weight loss during the study. (B) The presence or absence of rectal bleeding depicted in the form of rectal bleeding index. (C) Stool consistency (D) Hemoglobin levels (E) Disease activity index and (F) The longitudinal length of the colon. All values are averages of 6 mice. Results presented are representative of n = 3 separate experiments. * indicates p < 0.05 compared to DSS-treated, CMC administered control group. (Legend: Naive indicates mice were given regular drinking water from day 0 to day 10. DSS indicates mice were given DSS in drinking water from day 0 to day 10 and administered 0.5% CMC daily from day 0 to day 10, P2281 indicates mice were given DSS in drinking water from day 0 to day 10 and administered 15 mg/kg P2281, i.p., daily from day 0 to day 10).

Figure 6. P2281 attenuates DSS-induced histological abnormalities. Various groups of mice received DSS daily with some groups receiving daily injections of 15 mg/kg P2281 or 0.5% CMC. Histological analyses of the colons from various treatment groups were performed. Approximately 5 stained sections of each colon segment were observed from all mice in each treatment group. (A) Representative images from a single experiment are presented. Tissue sections from DSS mice are characterized by the prominent presence of edema, distinct infiltration of inflammatory cells (stealth arrow) and crypt destruction (oval arrow). In contrast, tissue sections from P2281-treated DSS mice reveal attenuation in inflammation, characterized by suppression of edema, reduction in inflammatory cellular infiltrate (stealth arrow), and
protection against epithelium and crypt damage (oval arrow). Results presented are representative of n = 2 separate experiments. Top panel: Images taken at 40X magnification. Bottom panel: Images taken at 100X magnification. (B) Histopathological scoring. * indicates p < 0.05 compared to DSS-treated, CMC administered control group.

Figure 7. P2281 blocks DSS-induced mTOR activation. Various groups of mice received DSS daily with some groups receiving daily injections of 15 mg/kg P2281 or 0.5% carboxymethylcellulose sodium salt (CMC). On the day of sacrifice, colon specimen from various groups were homogenized and lysed. Equal amounts of lysates from various treatment groups were separated by SDS PAGE, transferred onto PVDF membrane and blotted with antibody to p4E-BP1 or β-actin. (Lane 1: naïve mice; lane 2: DSS-treated, CMC administered mice; lane 3: DSS-treated, P2281 administered mice.) The numerical values on top of the blot indicate relative phosphorylation with reference to the DSS-fed mice treated with CMC.
References


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* Presence of blood: +1, slightly present; +2 present; +3 heavy

Disease activity index is the sum of all scores
Figure 1 (Bhonde et. al )

\[
\text{P2281}
\]
Figure 2 (Bhonde et. al)

A

% of phosphorylated protein

FCS  DMSO  P2281  Rapamycin

B

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Figure 3 (Bhonde et. al)

A

LPS
- + + + +
Pre-treat - DMSO Roli P2281

B

LPS
- + + + +
Pre-treat - DMSO Roli P2281

TNF-α (pg/ml)
Figure 4 (Bhonde et. al)

A

B

Con A

Pre-treat

DMSO

CS

P2281

Conc. (μM)

0.3

1

3

10

30

100

IFN-γ (pg/ml)

0

200

400

600

800

1000

1200

*
Figure 5 (Bhonde et al.)

A. % Weight Loss

B. Rectal Bleeding Index

C. Stool Consistency Index

D. Hemoglobin Levels

E. Disease Activity Index

F. Colon Length (cm)
**Figure 6 (Bhonde et. al)**

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Figure 6 (Bhonde et. al)

Histopathological score

DSS Treat
- - + P2281
Figure 7 (Bhonde et. al)

Relative phosphorylation

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