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Cellular and Molecular Impact of the Melanocortin Receptor Agonist PL8177 in Dextran Sulfate Sodium–Induced Colitis in Rats

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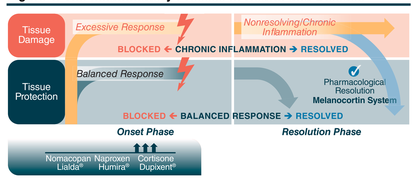
Introduction

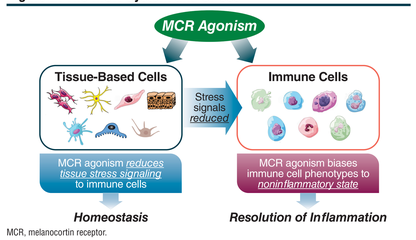
- The melanocortin system plays an important role in resolving inflammatory processes (**Figure 1**)[1,2]

Figure 1. The Inflammatory Process in Health and Disease[1]



- Melanocortin receptor (MCR) agonists simultaneously reduce tissue stress signaling and the stress response (**Figure 2**)

Figure 2. The MCR System and Stress[2-6]



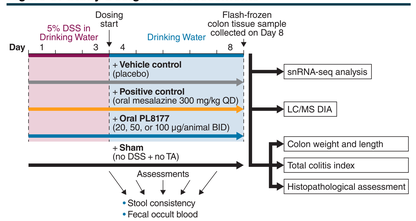
MCR, melanocortin receptor.

- The melanocortin 1 receptor (MC1R)–specific agonist PL8177 has demonstrated MC1R binding affinity and functional activity that mirrors that of α-melanocyte–stimulating hormone[4,7]
  - PL8177 has shown significant protective effects against dinitrobenzene sulfonic acid–induced colitis in rats after intracolonic administration via cannula of 1.5- and 5-µg doses[4]
- High potency and a lack of systemic absorption make PL8177 a promising new candidate for clinical development of an oral formulation for the treatment of inflammatory bowel disease
- Here, we report the results of a study that investigated the effects of orally delivered PL8177 on inflammation, cell population composition, and gene and protein expression in colons from a dextran sulfate sodium (DSS)-induced rat model of colitis

Methods

- PL8177 oral formulation was tested in a DSS-induced colitis rat model for potential curative effects
- Male Wistar rats (each group, n=6) received 5% DSS in drinking water for 3 days to induce colitis. Rats in the sham group had drinking water only (**Figure 3**)

Figure 3. Study Design and Assessments



BID, twice daily; DSS, dextran sulfate sodium, LC/MS DIA, data-independent acquisition tandem mass spectrometer; QD, once daily; snRNA-seq, single nuclei RNA sequencing; TA, test agent.

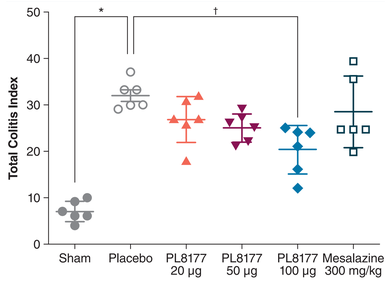
- At termination on day 8, 24 hours after the last dosing, colon tissues were harvested, dissected, and flash-frozen with liquid nitrogen
- Total colitis index scoring (score range, 0–60) to assess inflammatory damage was based on independent observers examining and summing the scores from 3 sections from each colon per animal
  - Sections were taken at 2.5 cm, 5 cm, and 7.5 cm distance from the anus
- Colon samples were analyzed with single nuclei RNA sequencing (snRNA-seq) and data-independent acquisition tandem mass spectrometry (LC/MS DIA)
- RNA sequences were plotted with Uniform Manifold Approximation and Projection (UMAP) (https://arxiv.org/abs/1802.03426) and annotated using gene signatures for known cell types
- LC/MS DIA–based phosphoproteomic profiling was performed on rat colon samples

Results

Total Colitis Index and Histologic Assessment

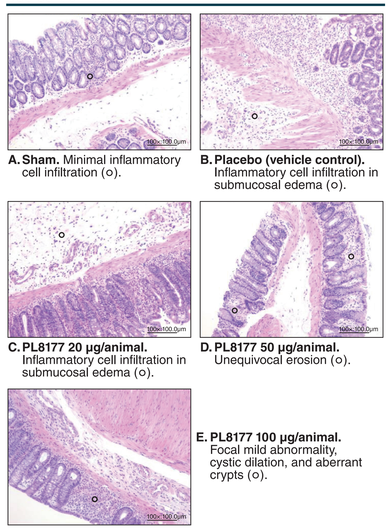
- There was a significant ($P<0.05$) improvement observed in the total colitis index for the PL8177 100-µg group compared to the vehicle control group, and all PL8177 cohorts showed greater improvement in the total colitis index compared to the mesalazine-treated cohort (**Figure 4**)

Figure 4. PL8177 Treatment Improves Total Colitis Index on Day 8



- Colon histopathologic examination showed injury and prominent ulcerations to the mucosa of the distal colon that extended for 2.5 to 7 cm in treated inflamed rats. Submucosal focal edema and pronounced transmural thickening of the colonic wall were also observed (**Figure 5**)

Figure 5. Representative Colon Histologic Sections of DSS Colitis-Induced Rats



A. Sham. Minimal inflammatory cell infiltration (○).

B. Placebo (vehicle control). Inflammatory cell infiltration in submucosal edema (○).

C. PL8177 20 µg/animal. Inflammatory cell infiltration in submucosal edema (○).

D. PL8177 50 µg/animal. Unequivocal erosion (○).

E. PL8177 100 µg/animal. Focal mild abnormality, cystic dilation, and aberrant crypts (○).

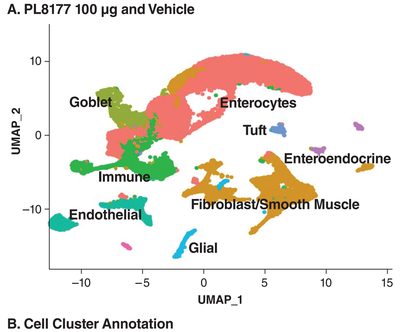
DSS, dextran sulfate sodium; snRNA-Seq. Note: Sham (A) is no challenge and no treatment. Vehicle control (placebo) (B) is no treatment but DSS challenge. C, D, and E are DSS challenge and treatment with PL8177.

snRNA-Seq

- UMAP was performed to visualize clustering of single nuclei from different colon samples (**Figure 6**)
- Unbiased clustering on the gene expression profiles of 33,337 colon nuclei from the PL8177 100-µg and vehicle groups identified 8 major colon cell types (**Figure 6A**). Cell clusters were annotated based on expression of marker, signature genes (**Figure 6B**)

Figure 6. UMAP Plot Displaying Cell Type Clusters of Nuclei Derived From Vehicle and PL8177-Treated Colon Samples

A. PL8177 100 µg and Vehicle



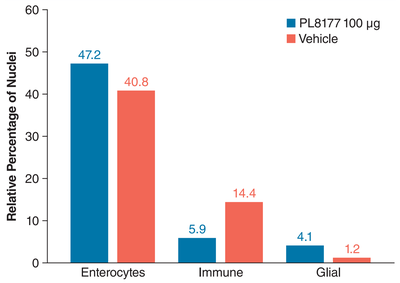
B. Cell Cluster Annotation

Cell Class	Signature Genes
Enterocytes	*Mep1a, Fgf15, Epcam, Fabp1, Cldn3, Krt8, Phgr1, Cldn7, Krt19*
Goblet	*Spink1, Tff3, Manf, Ccl9, Clca3, Cldn4, Fcgbp, Slc12a8, Spdef*
Fibroblast/ Smooth Muscle	*Col1a1, Col1a2,Col6a1,Col6a2, Myh11, Col4a1*
Immune	*Ptprc, Cd4, Skap1, Il7r, Cd79a, Cd19, Cd38, Cxcr4*
Endothelial	*Vwf, Kdr, Lyve1*
Enteroendocrine	*Chga, Chgb*
Tuft	*Lrmp, Dclk1, Cd24a, Trpm5, Ptgs1*
Glial	*Clu, S100b, Sox10*

UMAP, Uniform Manifold Approximation and Projection.

- PL8177 100-µg treatment causes a relative decrease in immune cells and an increase in enterocytes and enteric glial cells compared to vehicle control (**Figure 7**)
  - Enterocytes are the major epithelium cell type. A highly inflammatory milieu results in excessive intestinal epithelium shedding and compromised barrier integrity
  - Enteric glial cells promote barrier integrity and tissue repair. Studies have shown loss of enteric glial cells in inflamed tissue

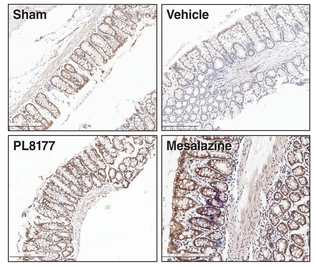
Figure 7. PL8177 Treatment Decreases Immune Cells and Increases Enterocytes and Enteric Glial Cells



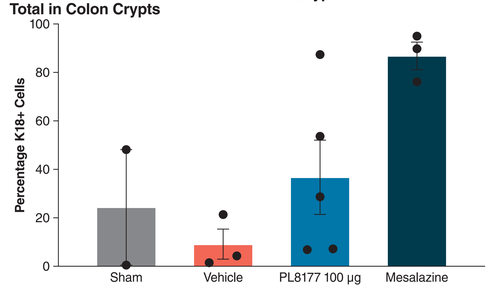
- Immunohistochemistry for cytokeratin 18 (K18), a marker for colon enterocytes, was performed in the DSS rat colons (**Figure 8A**)
  - K18+ enterocytes were decreased in vehicle colons vs sham. PL8177 treatment increased the percentage of K18+ enterocytes, similar to sham (**Figure 8B**)

Figure 8. Colon Histochemistry and Quantification of K18+ in Colon Crypts

A. Representative Colon Immunohistochemistry K18+ Staining



B. Quantification of K18+ Cells in Colon Crypts vs Total in Colon Crypts

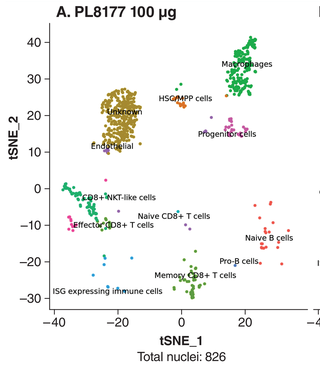


Sham is DSS, dextran sulfate sodium; K18, cytokeratin 18. Sham is no DSS challenge and no treatment. Vehicle is no treatment but DSS challenge. Mesalazine is positive control.

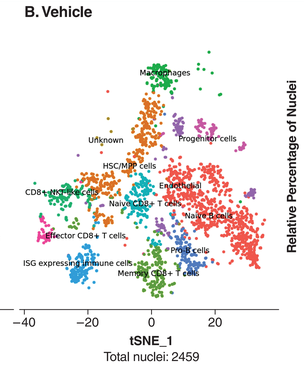
- PL8177-treated samples show a reduction in diverse immune cell subpopulations and relative increase in macrophages compared to vehicle (**Figure 9**)
  - Subclustering of immune cells reveals additional heterogeneity in PL8177 and vehicle (**Figure 9A** and **9B**)

Figure 9. PL8177-Treated Samples Show Reduction in Diverse Immune Cell Subpopulations and Relative Increase in Macrophages vs Vehicle

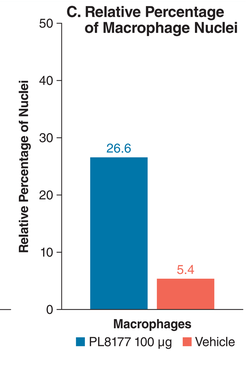
A. PL8177 100 µg



B. Vehicle



C. Relative Percentage of Macrophage Nuclei



tSNE, t-distributed stochastic neighbor embedding.

- PL8177 treatment repolarized macrophage cells to become pro-resolving of inflammation (**Figure 10**)
  - Macrophages are key cellular component of innate immunity
  - They have dual role as "killer" M1 (pro-inflammatory) and "builder" M2 (anti-inflammatory)
  - PL8177-treated immune cells have high expression of builder M2 marker genes compared to killer M1 markers in vehicle

Proteomic Data

- Differential phosphoproteome analysis identified 1200 unique proteins among all samples
- Supervised linear discriminant analysis (LDA) was performed on 34 principal components, which explain 95% of the variation
- Principal component LDA analysis showed clear separation between treatment groups (**Figure 11**)

Figure 10. PL8177 Treatment Biases Macrophages to the Anti-Inflammatory M2 State

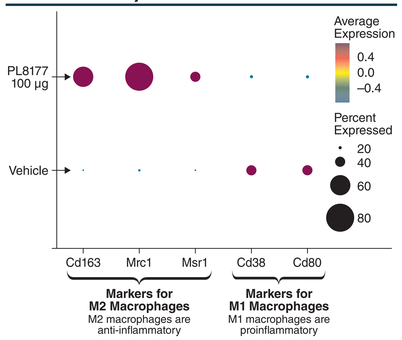
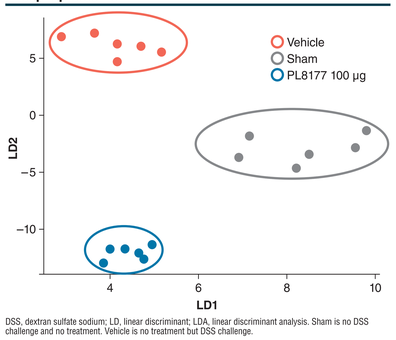


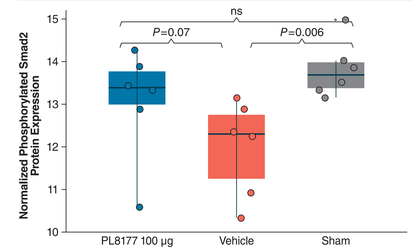
Figure 11. Supervised LDA of DSS Rat Colon Phosphoproteome



DSS, dextran sulfate sodium; LD, linear discriminant; LDA, linear discriminant analysis. Sham is no DSS challenge and no treatment. Vehicle is no treatment but DSS challenge.

- Among the proteins with the greatest differential phosphorylation was Smad2 (**Figure 12**)

Figure 12. Differential Phosphoproteome Analysis Demonstrates Increased Phosphorylation of Smad2 With PL8177 Treatment



DSS, dextran sulfate sodium; ns, not significant. Sham is no DSS challenge and no treatment. Vehicle is no treatment but DSS challenge. *P* values are from Wilcoxon test.

- Smad proteins are signal transducers and transcriptional modulators that mediate multiple signaling pathways. Smad2 and Smad3 play an essential role in inhibiting inflammation
  - Smad2 and Smad3 are phosphorylated by transforming growth factor ß receptor 1 (TGF-ßR1), which is activated by binding of TGF-ß1 to TGF-ß receptor II. Once phosphorylated, Smad2 and Smad3 interact with Smad4 and the complex moves to the nucleus, where it inhibits many inflammatory target genes[8]
  - Increased Smad2 phosphorylation with PL8177 treatment should lead to decreased inflammation

Conclusions

- Oral PL8177 treatment of inflamed colon showed significant improvement in markers of colitis in the rat model compared to the vehicle (placebo) and mesalazine control groups, which supports the aim of treating inflammatory bowel disease in humans
- There was significant ($P<0.05$) improvement in the total colitis index for the PL8177 100-µg group compared to the vehicle control group, and all PL8177 cohorts showed greater improvement compared to the mesalazine-treated cohort
- snRNA-seq analysis showed oral PL8177 100-µg treatment increased the relative proportion of enterocytes and enteric glial cells and decreased the proportion of immune cells compared to vehicle
- Subclustering analysis revealed that in vehicle-treated colons, the macrophages were primarily M1 macrophages, which are involved in inflammation, whereas in PL8177 100 µg–treated colons, macrophages were primarily M2, which are involved in resolution of inflammation
- Phosphoproteomic analysis showed that after treatment with PL8177 100 µg, the levels of phosphorylated Smad2 and Smad3 proteins, known to inhibit inflammation, were similar to the sham group and were markedly elevated compared to vehicle
- Oral PL8177 100-µg treatment causes diseased colons to move toward the healthy state and to resolve inflammation. Resolving inflammation—rather than blocking it—provides the possibility of efficacy coupled with safety in treating colitis and inflammatory bowel disease
- *PL8177 is currently under investigation in a phase 2a clinical trial in patients with ulcerative colitis (https://clinicaltrials.gov/study/NCT05466890)*

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Disclosures Paul S. Kayne, Priyanka Dhingra, Alison Obr, Carl Spana, and John H. Dodd are employees of Palatin Technologies, Inc.

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