Inhibiting dipeptidyl peptidase activity partially ameliorates colitis in mice

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1. ABSTRACT

New treatment strategies are required for the debilitating inflammatory bowel diseases (IBD), Crohn’s Disease and Ulcerative Colitis. DP inhibitors can prolong the bioactivity of the potent intestinal trophic growth factor glucagon-like peptide-2 (GLP-21-33). We investigated whether novel inhibitors of DP activity could modify the course of disease activity in the dextran sulfate sodium (DSS) model of colitis. C57BL/6 mice consumed 2% DSS in drinking water for 6 days. Mice were orally gavaged twice daily with 0.9% saline, 10mg/kg isoleucyl-cyanopyrrolidine (P59/99) or isoleucyl-thiazolidine (P32/98). Assessment of disease severity incorporated a disease activity index (DAI), together with histological assessment of crypt area and depth in the distal colon. DP activity was significantly inhibited at all time points. The DAI was significantly lower in the P59/99 and P32/98 treatment groups compared to saline treatment in all three time courses. Crypt hyperplasia (p<0.05) was observed in the saline group compared to P32/98 treatment at day 9. This preliminary study shows that novel inhibitors of DP activity may provide a new treatment strategy for IBD.

2. INTRODUCTION

Inflammatory bowel disease (IBD) encompasses a group of inflammatory disorders, including Crohn’s disease and ulcerative colitis, which affect the gastrointestinal tract (1). Although the aetiology of these diseases is not well understood, there is substantial evidence that genetic, environmental and immunological factors can contribute to disease pathogenesis (2, 3). There is a clear need for the development of novel treatment strategies. Recent reports suggest that glucagon-like peptide-2 (GLP-2) represents one such treatment modality.

GLP-21-33, a member of a family of glucagon-like peptides, is a 33 amino acid peptide (4, 5). It is synthesised in the enteroendocrine L-cells of the small intestine and colon, and signals through a G-protein coupled receptor (GLP-2R), expressed predominantly on the subepithelial myofibroblasts of the small and large intestine (6). GLP-21-33 has been identified as a potent gastrointestinal growth factor by stimulating crypt cell proliferation and inhibiting crypt cell apoptosis (7, 8). Previous studies have demonstrated the capacity for exogenous GLP-21-33 to
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reduce the severity of intestinal damage in a range of animal models of small intestinal and colonic injury (9-11). However, the potential for GLP-2 analogues to act as a treatment strategy for IBD is limited by activity of the serine protease, dipeptidyl peptidase (DP) IV (12).

DP IV is a 110kDa membrane associated peptidase expressed on epithelial cells of the intestine, kidney, liver, lung and prostate as well as on fibroblasts, melanocytes and certain leucocyte subsets (13). There is also a basal level of circulating soluble DP IV (14, 15). Several studies have found reduced serum DP IV activity in various inflammatory conditions, including IBD (16, 17), suggesting an innate compensatory down regulation of DP IV activity during IBD. DP IV plays a key role in the inactivation of a number of regulatory peptides, cleaving N-terminal dipeptides from polypeptides with either Xaa-Pro or Xaa-Ala at the penultimate position (13, 18), including GLP-2. DP IV cleaves bioactive GLP-2 both in vitro and in vivo to the inactive form, GLP-2 (19). Furthermore, GLP-2 acts as a GLP-2R antagonist, thus inhibiting the action of GLP-2 (20). DP IV is structurally homologous to other proteins of the DP IV gene family, including DP8, DP9 and fibroblast activation protein (FAP), which are each known to possess DP activity (21, 22). Recently DP8 and DP9 were shown to cleave a number of DP IV substrates, including GLP-2, in vitro (23). Therefore, in order to overcome the enzymatic degradation of GLP-2, strategies to limit DP IV activity are required.

One such strategy is to engineer a DP IV-resistant GLP-2 analogue. Drucker et al have demonstrated that administration of supra-physiological levels of a DP IV-resistant GLP-2 analogue enhanced small and large bowel growth in mice and reduced the severity of DSS-colitis (24). More recent clinical studies in settings of short bowel syndrome and Crohn’s disease have demonstrated that the DP IV-resistant analogue, Teduglutide, improves intestinal function and increases the likelihood of remission in Crohn’s disease patients (25, 26).

Supra-physiological doses of GLP-2 and DP IV resistant GLP-2 analogues have been shown to increase intestinal growth (10, 12, 24, 27). However, supra-physiological levels of the DP IV resistant GLP-2 analogue can accelerate the growth of colonic neoplasms in mice, potentially increasing the risk for gastrointestinal cancer (28). Inhibitors of DP activity have been shown to prolong the GLP-2 bioactivity (12). DP inhibition may be a safer treatment alternative as it aims to raise only endogenous levels of native GLP-2 as opposed to daily injections of pharmaceutical doses of the peptide hormone. Furthermore, DP inhibitors are orally available, so that patient compliance for such a treatment option is significantly increased (29). DP IV also inactivates the incretin hormone, GLP-1, which acts to lower blood glucose (30). In late 2006, MK-0341 became the first DP IV inhibitor licensed for sale as a type II diabetes therapy, under the brand name Januvia (31). Thus there is a precedent for use of DP inhibitors as a therapy in humans.

In addition DP IV inhibitors have shown potent anti-inflammatory effects in a number of animal models of inflammation, including rheumatoid arthritis (32, 33) multiple sclerosis (34, 35) and acne (36). Thus there is an accumulation of evidence suggesting that inhibition of DP IV enzymatic activity could be a useful clinical tool for managing inflammatory disorders, including IBD.

In previous studies utilising DPIV knockout (DPIV/−/) mice in a DSS-colitis model, we have demonstrated that DPIV may not be the sole protease involved in GLP-2 degradation (37). The primary aim of the current study was to investigate the role that proteases within the DPIV gene family play in the development and resolution of experimentally-induced colitis in mice, utilizing inhibitors of DP activity. We hypothesised that mice treated with DPIV inhibitors would have greater resistance to the development of DSS-induced colitis and would display an enhanced rate of intestinal repair.

3. MATERIALS AND METHODS

3.1 Animal studies

Male C57BL/6 mice (age 6-7 weeks) were group-housed in a temperature-controlled room with a 12hr light-dark cycle. All animals were acclimatized for a minimum of one week before the commencement of the trial. All animal studies were conducted in compliance with the Australian Code of Practice for the Care and Use of Animals and were approved by the Children, Youth and Women’s Health Service Animal Ethics Committee.

Mice consumed 2% DSS (ICN Biomedical Inc., Aurora, Ohio, GA) ad libitum in drinking water for 6 days to induce experimental colitis. DSS was then substituted with drinking water on day 6 and mice continued to drink regular water for a further 3 or 8 days for the 9 and 14-day time points, respectively. Mice were randomly assigned to 3 groups. Groups 1 and 2 and received either 100 µg P95/99 or P32/98 (Probiodrug AG, Halle, Germany) at a dose of 10mg/kg (30, 38) twice daily at 8am and 5pm by oral gavage. Group 3, mice received 100 µg/kg of vehicle (0.9% saline) twice daily. Animals were treated with either inhibitor or vehicle beginning at day 0 and continuing through to day 6 (n=10), 9 (n=6) or 14 (n=6), at which point mice were killed.

Body weight was monitored daily in addition to colitic disease activity. This was scored daily using a disease activity index (DAI) based on weight loss, stool consistency, rectal bleeding and overall condition of the animal. Mice received a score ranging from 0 (unaffected) to 3 (severe). Scores were then totalled to achieve an overall DAI (39, 40).

On days 0, 6, 9 and 14, mice were killed by CO2 overdose and cervical dislocation. Weights and lengths of all visceral organs were recorded. Tissue from jejunum, ileum and colon was collected and fixed in 10% buffered formalin for 24hrs. Blood was collected at the time of kill by cardiac bleed and stored in centrifuge tubes containing...
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3.2. Plasma DP activity

Plasma DP activity was measured by a fluorogenic assay described previously (21). Fluorescence was determined using a spectrofluorometer (excitation at 405nm and emission at 510nm; Fluoroskan Ascent 1.8, Labsystems, Finland and Ascent Research Edition Version 2.2 software). DP activity in plasma was expressed as change in fluorescence units per minute (dF/min). * denotes p<0.05 for inhibitor treatment compared to saline treatment.

3.3. Histological analyses and cell kinetics

Tissue samples were processed and embedded in paraffin wax. Sections (4µm) were stained with haematoxylin and eosin (H&E) for histological analyses including overall severity of damage, crypt depth, and crypt area. Overall damage severity is a blinded, subjective, score based assessment of various parameters, including crypt loss, thickening of the submucosa and muscularis externa, immune cell infiltration, goblet cell loss and surface enterocyte disruption. Scores for each parameter ranged from 0 (unaffected) to 3 (severe). Scores were then totaled to achieve a maximum overall damage severity score out of 18. Overall damage severity and crypt area were calculated using 16 fields of view from 4 cross sections per tissue segment. For crypt depth analyses 40 well-oriented crypts from four cross sections per tissue segment were selected for measurements of crypt depth and a mean value was then obtained. All microscopic analysis was carried out using an Olympus BH-2 light microscope (Tokyo, Japan) with a Sony digital camera (Tokyo, Japan) and the Image Pro Plus software package V4.5.1.27 (Media Cybernetics, Maryland USA).

3.4. Statistical analyses

Overall damage severity data was expressed as a median score (range), and DAI was expressed as a mean score ± standard error of the mean (SEM). Pair-wise comparisons with a Holmes post-hoc test between saline and treatment groups were made at each day with a family-wise significance level of 0.05. For all other analyses, comparisons between groups were made using a one-way ANOVA, with a Tukey’s post-hoc test. For all analyses, p<0.05 was considered to be significant. Statistical comparisons were made using SPSS for Windows software package V11.5.0 (SPSS Inc. Chicago, Illinois, USA).

RESULTS

4.1. Plasma DP activity

To confirm inhibition of DP activity, plasma was collected at the time of kill and DP activity was measured using a fluorogenic assay. DP activity was significantly inhibited at all time points following inhibitor treatment. At day 6 of DSS colitis, DP activity was significantly inhibited in mice treated with P32/98 (2.23 ± 0.2dF/min) compared to saline treated mouse (3.3 ± 0.14dF/min; p<0.05; analysis not performed for P59/99 treatment group). Following both 9- and 14-day treatment with inhibitor, plasma DP activity was found to be significantly reduced by at least 30% in both inhibitor groups compared to mice treated with saline (Figure 1).

4.2. Disease activity index

In order to determine effects of DSS on animal health, mice were scored daily using a DAI. Data from each group at the 9 and 14-day time points were pooled to achieve an overall 9-day disease activity score. During each time course all treatment groups were observed to have developed colitis, as indicated by DAI between day 5 and day 9 being significantly greater compared to day 0 (Figure 2a).

By inhibiting DP activity, DAI scores were observed to be significantly lower in the P59/99 and P32/98 treatment groups compared to saline treatment in all three time courses (Figure 2a and b). In the 6-day experiment, saline treated animals had a significantly higher DAI (4.0 ± 0.4) compared to P59/99 (2.3 ± 0.4) and P32/98 (1.9 ± 0.3; data not shown). One of the key determinants of the DAI is body weight loss. Throughout each time course, mice treated with P32/98 had a significant and consistently higher percentage body weight when compared to mice treated with saline and P59/99 (Figure 2c, data not shown for 6 and 14 day trials).

4.3. Colon weight and length

The DSS model of colitis is known to significantly decrease colon length (41). In the current study, colon length was decreased in all treatment groups at 6, 9 and 14 days compared to day 0 (Table 1). Colon

![Figure 1](image-url)
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Figure 2. Reduced disease activity and improved percentage bodyweight in inhibitor treated mice compared to saline treated mice. Disease activity was monitored over a 9 (n=12, a) and 14-day (n=6, b) period. Bodyweight was monitored daily, and expressed as a percentage bodyweight (n=12, c) DAI and bodyweight data was pooled from 9 and 14-day trials so that n=12 at 9-days. Data are expressed as mean ± SEM. * denotes p<0.05 for DAI scores in P59/99 and P32/98 treatment groups compared to saline treatment. # denotes p<0.05 for DAI scores with P32/98 treatment compared to the P32/98 administration.

weight was significantly greater in all treatment groups at day 9 compared to day 0 following adjustment for body weight. In the 6-day trial colon weight was 16% (p<0.05) and 20% (p<0.05) greater in the P32/98 and P59/99 treated mice respectively when compared to saline treated mice. At day 14, colon weight corrected for body weight was significantly greater by 39% only in the P32/98 treatment group compared to day 0 (p<0.05). When expressed relative to body weight, colon weight in the inhibitor treated groups was still higher; however, there was no statistical significance when compared to saline administration (Table 1).

4.4. Histological analyses

Damage in the DSS-induced colitis model manifests predominantly in the distal colon and is characterized histologically by an increased overall damage severity, increase in crypt depth and a decrease in crypt area (41) (Figure 3). Although no statistically significant differences were observed for overall damage severity, at day 9 mice treated with P59/99 and P32/98 had lower scores of 7.75 (0.5-18) and 2 (1.5-15) respectively compared to saline treatment 12.5 (3.5-18).

Crypt hyperplasia was observed in all three treatment groups at day 9 compared to mice at day 0 (Figure 4a), further confirming that DSS treatment had induced significant intestinal damage. Crypt depth was 17% greater for mice treated with saline compared to P32/98 treatment at day 9 (p<0.0001). By day 14, there was a 35% recovery in crypt depth in the P59/99 treatment group compared to that observed at day 9 (Figure 4a).

At day 9, recovery of crypt area was seen only in the P32/98 treatment group, whereas crypt area was 59% lower (p<0.01) in the saline treated mice compared to day 0 (Figure 4b). Following treatment with P32/98, crypt area was greater by 64% compared to P59/99 at day 9 (p<0.001). This again suggests a greater protective effect of P32/98 in this acute model of colitis. At day 14, crypt area in inhibitor treated mice had almost returned to normal; however, crypt area in the saline treatment group at day 14 was still 35% lower than day 0, (p<0.05, Figure 4b).

As GLP-2 is known to promote crypt cell proliferation (42), PCNA was used to label proliferating crypt cells. No differences were found between groups at day 6. The LI was significantly higher at day 14 in both P59/99 and P32/98 treatment groups compared to day 0. At day 14 mice treated with P59/99 had a significantly higher LI (44.1 ± 6.0%) compared to saline treatment (16.0 ± 4.1%) (Figure 5; p<0.0001).

3. DISCUSSION

This study follows on from preliminary data presented by our research group (43) and further demonstrates that inhibition of DP activity alone, using either P59/99 or P32/98, partially attenuates DSS-induced colitis in mice. This compares to a recent study by Bank et al, which found inhibition of DP activity alongside inhibition of aminopeptidase N was able to partially reduce the severity of DSS-induced colitis in mice (44). In the current study, we observed a significant decrease in the physical symptoms of experimental colitis such as diarrhea, rectal bleeding and body weight loss following treatment using a DP inhibitor only.
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Table 1. Colon length, colon weight and colon weight relative to body weight of mice treated with saline, P59/99 and P32/98 during development and resolution of DSS-induced colitis

<table>
<thead>
<tr>
<th>Day</th>
<th>Length (cm)</th>
<th>Weight (g)</th>
<th>Weight/Body weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.73 ± 0.73</td>
<td>0.20 ± 0.02</td>
<td>0.73 ± 0.08</td>
</tr>
<tr>
<td>6</td>
<td>4.76 ± 0.30</td>
<td>0.16 ± 0.01</td>
<td>0.78 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>P59/99</td>
<td>4.44 ± 0.18</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>P32/98</td>
<td>5.18 ± 0.19</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>9</td>
<td>5.55 ± 0.47</td>
<td>0.24 ± 0.02</td>
<td>1.27 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>P59/99</td>
<td>4.92 ± 0.36</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>P32/98</td>
<td>4.60 ± 0.37</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>14</td>
<td>5.60 ± 0.83</td>
<td>0.20 ± 0.01</td>
<td>0.93 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>P59/99</td>
<td>5.57 ± 0.23</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>P32/98</td>
<td>4.92 ± 0.36</td>
<td>0.18 ± 0.01</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM (n=6, except at day 6, n=10). Statistical comparisons were made between the three groups using one-way ANOVA with a Tukey’s post hoc test. *p<0.05 for day 0 colon length compared to day 9 and day 14 mice; **p<0.05 for colon length in P32/98 group compared to P59/99 treatment at day 6; †p<0.05 for colon weight in P59/99 and P32/98 group compared to saline treatment at day 6 and 14; ‡p<0.05 for colon weight corrected for body weight at day 9 and 14 compared to day 0; ‡p<0.05 for colon weight relative to body weight in P59/99 and P32/98 group compared to saline treatment at day 14.

While DP activity was significantly reduced in plasma from P59/99 and P32/98 treatment groups a residual DP activity was still observed. Previous studies in both animal models and humans have demonstrated that DP plasma activity is only totally inhibited in vivo up to three to five hours after initial administration of a 10mg/kg dose of DP inhibitor, after which activity slowly returns to basal levels (29, 30, 45). In this study plasma was collected approximately three hours after treatment with inhibitors; therefore, at this time point the inhibitors may no longer be exerting their maximal effect. While DPIV is thought to be the major DP present in plasma an alternative hypothesis is that residual DP activity resulted from another enzyme possessing DP activity that is not targeted by the inhibitors used.

P59/99 and P32/98 are small molecule inhibitors, exerting their actions by competitively binding the active site; however, they possess differing inhibitory properties and specificities in vitro. Both inhibit DPIV in the nM-range; P59/99 is a slow-binding DPIV specific inhibitor, with a Kᵢ=2nM for DPIV, compared to P32/98, which has a Kᵢ=80nM (29). More recently P32/98 has been shown to inhibit DP8 and DP9 as well as DPIV (46, 47). The inhibitors reduced plasma DP activity equally therefore it is unlikely that the residual plasma activity was due to either DP8 or DP9. In addition DP8 and DP9 are cytoplasmic enzymes and so are most probably not present in plasma.

Despite the differing in vitro properties of the two inhibitors, in vivo glucose tolerance tests have shown that both inhibitors are equally capable of lowering blood glucose following glucose challenge (29). In our study, there were clear differences between their abilities to ameliorate disease activity in DSS colitis, despite both inhibiting DP plasma activity equally by approximately 30%. The protective effect of P32/98 was rapid and observed throughout the experimental period. At the site of tissue injury in the gut, P32/98 may have had more physiological effects due to off target inhibition and its ability to inhibit DP8 and DP9 may have led to the better protection observed. Furthermore, in vitro experiments have demonstrated P32/98 to rapidly bind the mucosal peptide transporter, PepT1, suggesting a rapid uptake of GLP-21-33 at day 6, potentially due to the greater selectivity of P32/98 for DPIV compared to P32/98 (49). This may explain why P59/99 had significant growth effects during the repair phase.

Several studies have shown GLP-21-33 to increase intestinal growth by stimulating crypt cell proliferation. In our study, there was a significantly greater percentage of PCNA-positive cells observed at day 14 in P59/99 and P32/98 treatment groups when compared to saline, suggesting there were higher concentrations of GLP-21-33 in these groups to promote greater crypt cell proliferation during the recovery period. Previous animal studies have reported significant increases in various histological parameters, including villus height and crypt depth following exogenous administration of either native or DPIV-resistant GLP-21-33 (12, 24, 48). Significantly greater improvements in crypt area, crypt length and LI were seen in the P59/99 treatment group at day 14 compared to both saline and P32/98. Preliminary studies in our lab have suggested that treatment with P59/99 in mice with DSS colitis significantly increases circulating concentrations of GLP-21-33 at day 6, potentially due to the greater selectivity of P59/99 for DPIV compared to P32/98 (49). This may explain why P59/99 had significant growth effects during the repair phase.

A secondary hypothesis as to why a decrease in disease severity was observed in inhibitor treated mice is that the DP inhibitors may have been preventing production of pro-inflammatory cytokines, as well as increasing levels of TGF-beta1. In vitro studies have shown that DP inhibitors can suppress T-cell proliferation by indirectly inhibiting the production of various pro- and anti-inflammatory cytokines including interleukin (IL)-2, IL-10, IL-12, TNF-alpha and IFN-gamma (34, 50-52) as well as enhancing production of the immunosuppressive cytokine TGF-beta (34). In the DSS model of colitis there is an up-regulation of the pro-inflammatory cytokines IL-12, IFN-gamma and TNF-alpha (53), reflective of the Th1 cytokine profile seen in patients with Crohn’s disease. This suggests that in this DSS animal model, DP inhibitors may result in a down-regulation of pro-inflammatory cytokines as well as an increase in anti-inflammatory cytokines such as TGF-beta1.
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Figure 3. Reduced crypt damage in inhibitor treated mice compared to saline treated mice. Photomicrographs (200x) of 4µm sections of distal colon stained with H&E. (a) wild-type mice at day 0, (b) saline treatment at day 6, (c) P59/99 treatment at day 6, (d) P32/98 treatment at day 6, (e) saline treatment at day 9 (f) P59/99 treatment at day 9 (g) P32/98 treatment at day 9 (h) saline treatment at day 14 (i) P59/99 treatment at day 14 (j) P32/98 treatment at day 14

Recently, in vivo studies demonstrated that during DSS colitis, mice treated with the DP inhibitor Lys (Z (NO₂))-pyrrolidide had lower DAI compared to saline treated animals as well as increased levels of TGF-beta1. This effect was marginally improved upon co-administration of an aminopeptidase N inhibitor (44). Lys (Z (NO₂))-pyrrolidide has since been shown to have a greater specificity for DP8 and DP9 compared to DPIV (46). This suggests a potential immuno-regulatory role of DP8 and DP9 during DSS colitis and may in part explain the observed protective effects of P59/99 and P32/98 in this study. This is further supported by our previous report which demonstrated that DPIV deficient mice were not protected from DSS-colitis (37). We hypothesize that more than one DP may need to be targeted in order to gain the best protection. However, further work is required in order to characterize the specific target of the DP inhibitors and whether they regulate cytokine expression in the DSS colitis model.
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Chronic inhibition of DP activity in mice and rats has been reported to result in a decreased food intake, reduced body weight and varying behavioral changes (46, 54). DPIV-/- mice are known to have a decreased food and water intake as well as a decreased body weight and increased activity levels (54). It is important that all potential effects of inhibition of DP activity be investigated before DP inhibition is offered as a long-term treatment option for IBD patients.

In summary, this study provides further evidence that inhibition of DP activity alone using P59/99 or P32/98 can have significant effects in reducing disease activity in the DSS colitis model. These findings may lead to the development of new treatment strategies for IBD centered on inhibition of DP activity.

4. ACKNOWLEDGEMENTS

Gordon S. Howarth and Catherine A. Abbott contributed equally to this manuscript. This project was supported by funding from the Channel 7 Children's Research Foundation of South Australia. The authors would like to thank Kerry Lynn for assistance with animal experimentation and thank Kylie Lange and Professor Alan Branford of Flinders University, for assistance with statistical analyses. Fig 2 (a), 2 (b) and 3 (a) have been reproduced with the kind permission of Springer Science and Business Media (43).

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**Abbreviations:** Inflammatory bowel disease (IBD), Glucagon like-peptide-2 (GLP-2), Dipeptidyl peptidase (DP), Dextran sulphate sodium (DSS), Proliferating cell nuclear antigen (PCNA), epithelial cell proliferative labeling index (LI), Interleukin (IL), Interferon (IFN), Tumour necrosis factor (TNF), Transforming growth factor (TGF)

**Key Words:** Dextran Sulfate Sodium, Dipeptidyl Peptidase Inhibitor, Glucagon-Like Peptide-2, Inflammatory Bowel Disease, Mouse Model

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