Neutrophil elastase inhibition improves intestinal mucosal damage and gut microbiota in a mouse model of 5-fluorouracil–induced intestinal mucositis

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Abstract

Background and Purpose: 5-Fluorouracil (5-FU)-based chemotherapy is the first-line chemotherapeutic agent for colorectal cancer. However, 5-FU–induced intestinal mucositis (FUIIM) is a common adverse effect that severely impairs drug tolerance and results in poor patient health.

Experimental Approach: Neutrophil elastase (NE) overexpression contributes to FUIIM via abnormal inflammatory responses, microbiota imbalance, and tissue damage. Therefore, restoring NE homeostasis could prevent or improve FUIIM.

Key Results: This study shows that treatment with the specific NE inhibitor MPH966 (7.5 mg/kg; p.o.) significantly reversed 5-FU–induced losses in body weight; reversed villus atrophy; significantly suppressed myeloperoxidase, NE, and proteinase 3 activity; and reduced pro-inflammatory cytokine levels in a mouse model of FUIIM. In addition, MPH966 prevented 5-FU–induced intestinal barrier dysfunction, as was indicated by modulated expression of the tight junction proteins zonula occludin-1 and occludin. MPH966 also reversed 5-FU–induced changes in gut microbiota diversity and abundances, specifically the Firmicutes-to-Bacteroidetes ratio; *Muribaculaceae*, *Ruminococcaceae*, and *Eggerthellaceae* abundances at the family level; and *Candidatus Arthromitus* abundance at the genus level.

Conclusion and Implications: These data indicate that NE inhibitors are a potential treatment candidate to alleviate FUIIM by regulating abnormal inflammatory responses, intestinal barrier dysfunction, and gut microbiota imbalance.

Keywords：5-Fluorouracil, intestinal mucositis, microbiota, and neutrophil elastase

What is already known

- The clinical drug is absent that against 5-FU induced intestinal mucositis.
- The Phase II clinical trial for alpha-1 antitrypsin deficiency of MPH-966 is ongoing.

What this study adds

- MPH-966 alleviated 5-FU induced body weight loss and tissue damage in mice.
- MPH-966 attenuated inflammation, intestinal barrier dysfunction and the gut microbiota in FUIIM in mice.

What is the clinical significance

- MPH-966 could be a candidate to alleviate chemotherapy-induced intestinal mucositis.

Abbreviations

5-FU: 5-Fluorouracil

5-FU–induced intestinal mucositis: FUIIM

Neutrophil elastase: NE

Proteinase 3: Pr3

Reactive oxygen species: ROS

Myeloperoxidase: MPO

Interleukin-1β: IL-1β

Tumor necrosis factor-α: TNF-α

Zona occludin-1: ZO-1

Principal coordinates analysis: PCoA

Linear discriminant analysis: LDA

Introduction

5-Fluorouracil (5-FU) is a critical chemotherapy drug for patients with cancer. The drug works via inhibiting thymidylate synthase and incorporating 5-FU metabolites, which block DNA and RNA synthesis in normal and tumor cells (Vodenkova et al., 2020). 5-FU has been the primary chemotherapeutic agent for colorectal cancer since the 1990s. However, 5-FU treatment is accompanied by severe adverse effects. Intestinal mucositis associated with severe diarrhea, nausea, vomiting, and anorexia has been reported in 50–80% of patients (Hamouda et al., 2017; Wardill, Bowen, & Gibson, 2014). Moreover, 5-FU–induced intestinal mucositis (FUIIM) could severely impact drug tolerance and promote high mortality in patients with cancer (Sangild, Shen, Pontoppidan, & Rathe, 2018). Thus, it is necessary to develop new agents to treat or prevent FUIIM.

Multiple mechanisms contribute to the complicated pathogenesis of FUIIM, including the production of reactive oxygen species (ROS), release of proinflammatory cytokines from epithelial cells, and neutrophil infiltration. These processes promote inflammatory responses, thereby mediating intestinal barrier dysfunction and gut imbalance of microbiota (de Barros et al., 2018; Sonis, 2004; Tang et al., 2017; Yan et al., 2019). Neutrophils provide a key first-line immune cell response against pathogens via phagocytosis, degranulation, and formation of

neutrophil extracellular traps (Teng, Ji, Ji, & Li, 2017). Myeloperoxidase (MPO) and neutrophil elastase (NE) are good neutrophil biomarkers because they are abundantly released from neutrophils during degranulation (Korkmaz, Horwitz, Jenne, & Gauthier, 2010; Muthas et al., 2017). In mice, abnormal inflammation in IM is characterized by increased MPO expression in the inflamed small intestine (Oh, Lee, Lee, Lee, & Kim, 2017; J. Wu et al., 2020). Neutrophil transmigration and release of NE-related ser ine proteases degrade intestinal epithelial junction proteins, resulting in intestinal barrier permeability and gut microbiota imbalance (Ginzberg et al., 2001; Matthews, Weight, & Parkos, 2014). Gut microbiota alters the development of chemotherapy-induced intestinal mucositis by alleviating abnormal inflammatory responses and intestinal barrier dysfunction (Touchefeu et al., 2014). Many studies have indicated that regulating gut microbiota is a potential target for treating FUIIM (Chang et al., 2020; Yeung et al., 2019).

MPH966 (AZD9668) is a specific NE inhibitor that is safe and well-tolerated in patients with respiratory diseases (Stockley et al., 2013; Vogelmeier, Aquino, O'Brien, Perrett, & Gunawardena, 2012). This drug may serve as a potential pharmacotherapy to normalize FUIIM-associated inflammatory states and gut microbiota imbalance. However, it is still unclear how MPH966 works to restore homeostasis in FUIIM. Therefore, in this study, we investigated the effects of MPH966 on FUIIM in mice.

Results

Effects of MPH966 on body weight loss and histological changes in FUIIM. Loss of body weight is a common phenomenon in FUIIM (Mashtoub, Tran, & Howarth, 2013; Yasuda et al., 2013). Therefore, mouse body weight was recorded each day during MPH966 treatment. In addition, intestinal tissues were collected and morphological changes were determined. The results indicated significant body weight loss on days 2 through 6 in the 5-FU–treated group compared to the vehicle group ($p < 0.001$). Following treatment, body weight in the 5-FU group reduced to 79% of the vehicle group (Fig. 1B). By contrast, MPH966 treatment significantly rescued body weight loss compared to the 5-FU group mice (all $p < 0.001$) in a dose-dependent manner. The MPH966 treatment groups lost 15% (5 mg/kg) and 9% (7.5 mg/kg) of their total body weight (Fig. 1B).

Histological analysis of the jejunum of the small intestine was completed using hematoxylin and eosin (H&E) staining (Fig. 1C). 5-FU induced villus length atrophy and epithelial cell necrosis compared to vehicle treatment, whereas MPH966 treatment ameliorated these 5-FU–induced changes in a dose-dependent manner (all $p < 0.001$) (Fig. 1D). Notably, 5-FU and MPH966 administration did not alter crypt depth (Fig. 1E). These findings indicate that MPH966 was able to restore 5-FU–induced body weight loss and histological changes.

MPH966 suppresses MPO, NE, and proteinase 3 activity in FUIIM. MPO is widely used as an inflammatory marker in FUIIM models (de Miranda et al., 2020; Justino et al., 2014). Additionally, neutrophil infiltration and degranulation is mediated by NE and proteinase 3 (Pr3) release and causes tissue damage (Muthas et al., 2017). To study the effects of MPH966 treatment on FUIIM, mice jejunums were collected and homogenized. Whether MPH966 confers protective effects was determined by analyzing MPO, NE, and Pr3 activity. The results showed that MPH966 significantly attenuated 5-FU–induced MPO, NE, and Pr3 activity in a dose-dependent manner (Fig. 2A-C). MPH966's ability to decrease NE expression in mice jejunums was further confirmed using IHC staining (Fig. 2D). This is the first report that indicates the involvement of NE and Pr3 in FUIIM pathogenesis. Further, it supports a role for MPH966 in modulating NE homeostasis and preventing FUIIM.

MPH966 attenuates pro-inflammatory cytokine expression in FUIIM. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are critical pro-inflammatory cytokines of intestinal inflammation. IL-β and TNF-α contribute to abnormal immune cell responses, crypt cell apoptosis, and the severity of intestine injury in FUIIM (Kato et al., 2015; Soares et al., 2013). In this study, mouse jejunums

were analyzed for IL-1β and TNF-α levels using qPCR technology. The results suggested 5-FU significantly augmented IL-1β and TNF-α levels compared to vehicle, whereas treating MPH966 significantly attenuated 5-FU–induced IL-1β and TNF-α levels (Fig. 3A-B).

MPH966 enhances zona occludin-1 and occludin expression in FUIIM mice. Zona occludin-1 (ZO-1) and occludin are important proteins that maintain mucosal barrier function and tight junction stability (Boeing et al., 2020). These proteins are markedly downregulated in FUIIM (de Barros et al., 2018; Gan et al., 2020). To investigate whether MPH966 has protective effects on tight junction proteins, IHC staining and integrated optical density analysis were used to determine ZO-1 and occludin expression. The results obversed 5-FU decreased ZO-1 and occludin expression compared to vehicle, but MPH966 improved 5-FU–induced degradation of ZO-1 and occludin (all $p < 0.05$) (Fig. 4A-C). These data suggest that MPH966 confers protective effects on intestinal barrier dysfunction.

MPH966 modulates the gut microbiota in FUIIM mice. These results indicate that MPH966 may protect intestinal barrier damage by inhibiting neutrophil infiltration, inhibiting MPO and NE expression, and modulating cytokines released from intestinal

epithelial cells. Modulating chemotherapy-induced intestinal microbial imbalances is a target to ameliorate abnormal inflammatory responses and intestinal barrier dysfunction (Tang et al., 2017; Yeung et al., 2019). Therefore, the gut microbiota was also analyzed in the current samples.

Alpha diversity was quantified using the Chao1 and Shannon indices, which indicate the community richness and diversity of the microbiota, respectively (Fig. 5A and 5B). Although the Chao1 index indicated no differences in community richness (Fig. 5A), the Shannon index indicated a trend toward a significant change in community diversity in the 5-FU group ($p < 0.01$), which was restored by MPH966 administration (Fig. 5B).

Similarities in the community structure of the microbiota among the five groups was plotted by a weighted UniFrac metric PCoA (Fig. 5C). Clear segregation was observed between the vehicle group and the 5-FU and low-dose MPH966 + 5-FU groups. However, the clusters from the high-dose MPH966 + 5-FU group overlapped with the vehicle group clusters. This evidence suggests that high-dose MPH966 could improve microbial community diversity and structure.

The relative abundances of the top 10 species were drawn as distribution histograms. A statistical table indicates the phylum (Fig. 5D, Table 2) and genus (Fig. 5E, Table 3). The major microbiota composition was dominated by two bacterial phyla: Firmicutes

and Bacteroidetes. 5-FU treatment increased the relative abundance of Firmicutes, but decreased the relative abundance of Bacteroidetes. Abundances were restored by high-dose MPH966 treatment. The Firmicutes-to-Bacteroidetes ratio is correlated with inflammation in FUIIM (H. L. Li et al., 2017). Our data indicated that high-dose MPH966 administration restores this ratio.

The major microbiota composition was dominated by five bacterial genera in the jejunm of small intestine: *Candidatus Arthromitus*, *Lactobacillus*, *Lachnospiraceae_NK4A136_group*, *Ruminococcaceae_UCG_014*, and *Bacteroides*. Compared to vehicle, 5-FU treatment reduced the relative abundances of *Lachnospiraceae_NK4A136_group*, *Ruminococcaceae_UCG_014*, and *Bacteroides*, but elevated the relative abundances of *Candidatus Arthromitus*. High-dose MPH966 significantly attenuated the relative abundance of *Candidatus Arthromitus* and slightly augmented the relative abundances of *Lachnospiraceae_NK4A136_group*, *Ruminococcaceae_UCG_014*, and *Bacteroides* compared to 5-FU. These results suggest that MPH966 may be useful to shape the gut microbiota.

Linear discriminant analysis (LDA) scores indicated differences in gut microbiota biomarkers between the five groups (Fig. 6A). Accordingly, several predominant biomarkers were selected at the genus level (Fig. 6B-C) and the family level (Fig. 6D-F). In the 5-FU group, *Candidatus Arthromitus* was a predominant biomarker at

the genus level, which was consistent with the relative abundance of top 10 gut microbiota at the genus level (Fig. 6B). In the vehicle group, three families (*Muribaculaceae*, *Ruminococcaceae*, and *Eggerthellaceae*) and a genus (*Lachnospiraceae_NK4A136_group*) were the major expressed bacteria. These bacteria were significantly suppressed by 5-FU treatment and were restored by MPH966 treatment (Fig. 6D-F).

Finally, the PICRUSt software and level 3 KEGG database pathways were used to predict the microbial genes involved in cellular processes and organismal systems. The results suggested that 5-FU modified cellular processes (peroxisome, lysosome, and bacterial chemotaxis) and organismal systems (plant-pathogen interaction and adipocytokine signaling pathway). MPH966 treatment restored the peroxisome and plant–pathogen interaction pathway components (Fig. 7A and 7B).

Discussion

Ulceration of the intestinal mucosal is an initial feature of FUIIM pathogenesis that can further promote intestinal permeability and block nutrient absorption in the small intestine, thereby causing a loss in body weight (Generoso Sde et al., 2015). Previous studies have demonstrated that 5-FU treatment increased intestinal epithelial cell apoptosis and contributed to loss of crypt cells, villus atrophy, and immune cells

infiltration (Ali et al., 2019; Trindade et al., 2018). Sivelestat, a commercial NE inhibitor, can ameliorate 5-FU–induced diarrhea by inhibiting neutrophil recruitment and modulating aquaporin 4 and 8 levels in mice (Sakai et al., 2014). Notably, MPH966 has more potent NE inhibitory effects (von Nussbaum & Li, 2015). Our study demonstrated that MPH966 can attenuate 5-FU–induced body weight loss and prevent 5-FU–induced villus atrophy and epithelial cell necrosis.

Intestinal homeostasis is maintained by cytokines that are released from immune and epithelial cells (Z. P. Hu et al., 2006; Winsor, Krustev, Bruce, Philpott, & Girardin, 2019). IL-β and TNF-α are critical pro-inflammatory cytokines that synergistically enhance inflammation by promoting leukocyte migration into inflamed tissues (Dinarello, 2000). Furthermore, IL-β and TNF-α are involved in 5-FU–induced crypt cell apoptosis, which was associated with jejunum damage in our study. Therefore, inhibiting pro-inflammatory cytokine release could suppress small intestine damage (Kato et al., 2015). We found that MPH966 treatment significantly reduced 5-FU–induced pro-inflammatory cytokine levels, which is similar to reductions noted in the absence of NE (G. Li et al., 2016). As such, MPH966 might suppress small intestine damage via modulating pro-inflammatory cytokine expression.

Tight junction function is another important marker of FUIIM. Several proteins comprise tight junctions, including claudins, ZOs, occludin, and junctional adhesion molecules. These proteins can manipulate intestinal barrier function and prevent intestinal bacterial translocation into the lamina propria (Oshima & Miwa, 2016; Sanchez de Medina, Romero-Calvo, Mascaraque, & Martinez-Augustin, 2014). 5-FU decreased ZO-1 and occludin expression, suggesting enhanced intestinal permeability and intestinal barrier dysfunction. However, MPH966 treatment significantly increased ZO-1 and occludin expression. These results suggest that NE inhibition could restore intestinal permeability and the intestinal barrier dysfunction (de Barros et al., 2018).

Gut microbiota plays an indispensable role in gut homeostasis. Crosstalk between immune cells and microbiota or microbiota metabolites (e.g., short-chain fatty acids) has been associated with intestinal inflammation (Hiippala et al., 2018). Previous studies demonstrated that microbiota dysbiosis occurred with intestinal barrier dysfunction in FUIIM (Hamouda et al., 2017; J. Wu et al., 2020) and was attenuated by probiotic treatment or FMT from healthy mice (Chang et al., 2020; Justino et al., 2014; Yuan et al., 2015). In our study, gut microbiota distribution in the small intestine was analyzed using 16S rRNA amplifying sequencing. The results demonstrated that

microbial diversity and microbiota community structure were clearly segregated between 5-FU–treated and control mice. MPH966 treatment modulated microbial diversity and microbiota community structure, indicating its use as a potential therapeutic agent to restore microbiota homeostasis.

The composition and abundance of gut microbiota found in each treatment group confer different functions in the small intestine. At the phylum level, our data revealed that Firmicutes, Bacteroidetes, and Proteobacteria were the dominant bacteria in the small intestine of the vehicle group. The Firmicutes-to-Bacteroidetes ratio is commonly used to evaluate the pathogenic potential of gut diseases (Benabid et al., 2012; Nourrisson et al., 2014; Rojas-Feria et al., 2017) and has been shown to be significantly decreased in the feces and cecum after 5-FU administration (J. Wang et al., 2019). Interestingly, our data demonstrated that the Firmicutes-to-Bacteroidetes ratio was significantly increased in the small intestine following 5-FU treatment, and this increase was suppressed by MPH966.

At the family level, our study revealed that the abundances of *Muribaculaceae*, *Ruminococcaceae*, and *Eggerthellaceae* observed in the vehicle group were significantly diminished by 5-FU treatment and were recovered by MPH966 treatment.

The families *Muribaculaceae* and *Eggerthellaceae* play an important role in metabolizing carbohydrates and polyphenols (Chung, Gwak, Moon, Rho, & Ryu, 2020; Selma et al., 2017), but their roles in FUIIM remain unclear. Additionally, the family *Ruminococcaceae* contributes to polysaccharide degradation and reverses dextran sodium sulfate-induced colitis (Y. Wang et al., 2019). Because the abundances of these families are highly associated with intestinal barrier integrity, these families might be considered as FUIIM markers.

At the genus level, this study demonstrated that *Candidatus Arthromitus*, *Lactobacillus*, *Lachnospiraceae_NK4A136_group*, *Ruminococcaceae_UCG_014*, and *Bacteroides* were dominant bacteria in the vehicle group. Of these, the genus *Candidatus Arthromitus* is a kind of segmented filamentous bacterium that regulates proinflammatory cytokine expression in the immune system (Santiago et al., 2019). Indeed, the genera *Lactobacillus, Lachnospiraceae_NK4A136_group*, *Ruminococcaceae_UCG_014*, and *Bacteroides* are short-chain fatty acid-producing–related bacteria that modulate inflammation (S. Hu et al., 2019; C. Wang et al., 2019). Increasing the abundance of genera *Lactobacillus* and *Bacteroides* significantly attenuated FUIIM (H. Chen et al., 2020; Yeung et al., 2019). Similarly, augmenting the abundance of *Lachnospiraceae_NK4A136_group* and genera

Ruminococcaceae_UCG_014 could reverse experimentally induced colitis (A. L. Li et al., 2020; C. Wang et al., 2019). Our study confirmed that 5-FU administration could significantly decrease the abundance of *Lachnospiraceae_NK4A136_group*, *Ruminococcaceae_UCG_014*, and *Bacteroides,* which was non-significantly reversed by MPH966 treatment. Our results also demonstrated that MPH966 suppressed 5-FU–induced abundance of *Candidatus Arthromitus*.

This study used the PICRUSt software to reveal the small intestine microbiota pathways related to cellular processes and organismal systems at KEGG level 3. MPH966 treatment normalized 5-FU–induced changes in the peroxisome and plant–pathogen interaction pathways. The peroxisome pathway regulates inflammation produced by oxidative metabolism and the utilization of short-chain fatty acids (Ganguli, Mukherjee, & Sonawane, 2019). Therefore, these results suggest that MPH966 modified the gut microbiota and modulated inflammatory processes in FUIIM via inhibition of 5-FU–induced NE overexpression.

In conclusion, this study presents evidence of a pathogenic role of NE in FUIIM. Treatment with MPH966, a highly specific NE inhibitor, alleviated FUIIM-associated symptoms, including dysregulated inflammatory responses, intestinal barrier

dysfunction, and gut microbiota imbalances. Thus, NE inhibitors may be candidates to alleviate chemotherapy-induced intestinal mucositis.

Materials and methods

Materials 5-FU (F6627), dimethyl sulfoxide (DMSO, D2650), hexadecyltrimethylammonium bromide (HETAB, H-9151), protease inhibitor cocktail (P8340), bovine serum albumin (BSA, A7906), o-dianisidine dihydrochloride (D3252), hydrogen peroxide (18304), calcium chloride ($CaCl_2$, C5670), sodium azide (NaN_3, S8032), and Triton X100 (T9284) were obtained from Sigma-Aldrich (St. Louis, MO, USA). MPH966 (HY-15651) was obtained from MedChemExpress (NJ, USA). Tween 80 (T2533) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Phosphate buffered saline (PBS, UR-PBS001-5L) was obtained from UniRegion Biotech (New Taipei, Taiwan). Neutralized formalin solution (10%) (3933.9020), dipotassium hydrogen phosphate (3252-01), potassium dihydrogen phosphate (3246-01), TRIS hydrochloride (Tris-HCl, 4103-02), and HEPES (4153-01) were obtained from J. T. Baker (Gliwice, Poland). Bio-Rad protein assay dye reagent concentrate (#5000006) and iQ™ SYBR® Green Supermix (170-8882) were obtained from Bio-Rad (CA, USA). MPO (M9760-07) was obtained from USBiological (MA, USA). Human NE (HNE, BML-SE284) and Pr3 (BML-SE498) were obtained from Enzo Life Sciences (NY, USA). NE substrate

(methoxysuccinyl-Ala-Ala-Pro-Val-pNA, 454454) was obtained from Merck (NJ, USA). Pr3 substrate (Abz-VAD-norV-ADRQ-EDDnp) was obtained from Alta Biosciences (Redditch, UK). Sodium chloride (NaCl, AS0480-1000) was obtained from Bionovas (Ontario, Canada). Dewax solution (AR9222), citrate buffer (AR9961), EDTA buffer (AR9640), and BOND Polymer Refine Detection kit (DS9800) were obtained from Leica Biosystems Inc. (IL, USA). NE antibody (ab68672, RRID: AB_1658868) was obtained from Abcam (Cambridge, UK). ZO-1 antibody (21773-1-AP, RRID: AB_10733242) and occludin antibody (13409-1-AP, RRID: AB_2156308) were obtained from Proteintech (IL, USA). TOOLSmart RNA Extractor (DPT-BD24) and ToolsQuant II Fast RT kit (KRT-BA06) were obtained from BIOTOOLS (New Taipei, Taiwan). Primers were synthesized by Sigma-Aldrich (St. Louis, MO, USA). Stool DNA Isolation Kit (27600) was obtained from Norgen Biotek Corp. (ON, Canada).

Animals. Male C57BL/6 mice (age, 6–9 weeks), each weighing 22–28 g, were purchased from BioLasco (Ilan, Taiwan). The experimental procedures were approved by the Institutional Animal Care and Use Committee of Chang Gung University (IACUC Approval no.: CGU107-081) and complied with the ARRIVE guidelines (Kilkenny et al., 2010) and with the recommendations made by the *British Journal of*

Pharmacology. All mice were housed in a room maintained with a 12:12 h light/dark cycle with water and food supplied ad libitum.

FUIIM. All mice were acclimatized for 7 d, then were randomly allocated into five groups (n=5 for vehicle group, n=8 for other each groups): 1) Vehicle, 2) 5-FU (50 mg/kg), 3) MPH966 (5 mg/kg) + 5-FU (50 mg/kg), 4) MPH966 (7.5 mg/kg) + 5-FU (50 mg/kg), and 5) MPH966 (7.5 mg/kg). 5-FU was dissolved in normal saline. MPH966 was dissolved in its excipient (60% normal saline, 20% DMSO, and 20% Tween 80). According to previous protocols, the mice treated with 5-FU (50 mg/kg) received intraperitoneal (i.p.) injections for 5 consecutive days to induce IM. Control mice were i.p. injected with normal saline (Yan et al., 2019). MPH966 (0, 5, or 7.5 mg/kg) was gavaged 30 min prior to 5-FU treatment over the 5 consecutive days. The experimental design is presented in Figure 1A. Daily body weight and food intake recordings were conducted until mice were sacrificed under deep anesthesia with isoflurane on day 6 (24 h after the last injection). The small intestine (jejunum) was removed and washed with cold PBS. Several 1-cm jejunum sections were collected in 10% neutralized formalin solution. The other part of the jejunum was collected and frozen immediately at −80°C until used.

Histological analysis. Jejunum tissue was fixed with 10% neutralized formalin overnight. Tissues were then embedded in paraffin wax and stained with H&E for histological analysis. The average villus length and crypt depth were obtained by evaluating three villi (from the top of the villus to the villus-crypt junction) and three crypts (defined as invagination depth between adjacent villus) per sample, for details see (Fu et al., 2017). Jejunum images were captured using an Olympus microscope IX81 (Tokyo, Japan).

Neutrophil-related enzymes sample preparation. Jejunum tissue was collected and homogenized in 50-nM potassium phosphate buffer (1 mL, Kpi buffer, pH 6.0) with 0.5% HETAB and 0.1% protease inhibitor cocktail. After homogenization, the samples were centrifuged at 12,000 rpm for 15 min and the supernatants were collected. The supernatant protein concentration was measured using the Bio-Rad protein assay dye reagent and calculated based on a standard curve derived from commercial standard, BSA.

Measurement of MPO activity. Neutrophil infiltration was determined by MPO activity (Y. L. Chen et al., 2017). The supernatant (10 μL) was transferred into a 96-well plate including a reaction solution [300 μL; 0.0005% hydrogen peroxide and

0.167 mg/mL *o*-dianisidine dihydrochloride in Kpi buffer (pH 6.0)] and incubated for 15 min. The mixture absorbance was measured at 450 nm (Multiskan Ascent 354 Plate Reader, Thermo Labsystems). MPO activity was calculated based on a standard curve derived from commercial MPO and is shown as units/protein concentration (mg).

Measurement of NE activity. The supernatant (20 μL) was transferred into a 96-well plate with the substrate solution (500 μM, 80 μL) in a working solution [20 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$, and 0.1% NaN_3] and incubated for 180 min. The mixture absorbance was recorded at 405 nm (Multiskan Ascent 354 Plate Reader, Thermo Labsystems) (Y. L. Chen et al., 2017). The NE activity was calculated based on a standard curve derived from commercial NE and shown as NE (nmol)/protein concentration (g).

Measurement of Pr3 activity. The supernatant (100 μL) was transferred into a 96-well black polypropylene low-binding plate with the Pr3 substrate solution (40 μM, 50 μL) in a working solution [50 mM HEPES (pH 7.4), 150 mM NaCl, and 0.05% Triton X100) and incubated for 10 min. Fluorescence of the mixture was measured at excitation 320 nm and emission 420 nm (Infinite 200 pro, TECAN) (Sinden &

Stockley, 2013). Pr3 activity was calculated based on a standard curve derived from commercial Pr3 and shown as Pr3 (nmol)/protein concentration (g).

Immunohistochemical (IHC) evaluation. IHC was used to detect NE and tight junction protein (ZO-1 and occludin) expression. The experimental procedures followed those of the Molecular Medicine Research Center of Chang Gung University (Taoyuan, Taiwan) and comply with the recommendations made by the *British Journal of Pharmacology*. Tissue sections were dewaxed with dewax solution, and epitope retrieval was performed by citrate buffer (for NE and ZO-1) or EDTA buffer (for occludin) for 20 mins at 100°C. Using anti-NE (1:2000 dilution), anti-ZO-1 (1:300 dilution), and anti-occludin (1:1000 dilution) antibodies were detected the target protein, and IHC staining was performed by an automatic IHC staining device (Vision BioSystems, Australia) with BOND Polymer Refine Detection kit. The images were observed and recorded using an Olympus microscope (BX51; Tokyo, Japan). The integrated optical density showed positive staining and was measured using the ImageJ software (RRID: SCR_003070), version 1.8.0 (Gan et al., 2020).

qPCR analysis of pro-inflammatory cytokines, IL-1β and TNF-α. Total RNA was extracted from the jejunum using TOOLSmart RNA Extractor. After confirming the

RNA quality, the total RNA was reverse transcribed into cDNA using ToolsQuant II Fast RT kit following the manufacturer's instructions. Pro-inflammatory cytokine mRNA expression was observed using iQ™ SYBR® Green Supermix (Bio-Rad) with primers in the CFX Connect Real-Time PCR Detection System (Bio-Rad). The primer sequences are listed in Table 1. Pro-inflammatory cytokine expression was quantified as the expression relative to the housekeeping gene *GAPDH* using the $2^{-\Delta\Delta Ct}$ method in the same sample (Dos Santos Filho et al., 2016). Differentiation samples treated with vehicle were used as controls for mRNA levels for each gene of interest.

Gut microbiota analysis. The microbial genomic DNA from the jejunum was extracted using the Stool DNA Isolation Kit according to the manufacturer's instructions. As previously reported, the V3–V4 regions of 16S rRNA were amplified, quality control was performed, and QIIME-based microbiota analysis was completed with the Illumina MiSeq platform (CA, USA) (T. R. Wu et al., 2019). The detail protocols/ methods see Supplementary information.

Statistical analysis The data and statistical analysis in this study comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology. All data are expressed as mean ± S.E.M. and were analyzed

with two-tailed independent-sample Student *t*-tests or one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 5.01 (RRID: SCR_002798, GraphPad Software, Inc., USA).

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Author contributions

K.-R.C. Y.-L.C. and P.-W.H. participated in the study designs. S.-H.U. carried out histology and pathology analysis in the study. K.-R.C. wrote the manuscript. P.-W.H. and L,-M.K. were in charge of the whole experimental conduction and proofread the manuscript.

Competing financial interests: The authors declare no competing financial interests.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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Legends

Figure 1. MPH966 alleviated 5-fluorouracil (FU)–induced body weight loss and histological changes in mice. (A) Experiment protocol. (B) Loss of body weight after 5-FU administration was significantly alleviated by MPH966 treatment. (C) Representative H&E staining for villus length and epithelial cell necrosis (scale bar = 200 μm in 160X magnification). (D) 5-FU administration reduced villus length, which was alleviated by MPH966 treatment. (E) 5-FU and MPH966 did not affect crypt depth. Each point represents the mean ± S.E.M. (n=5 for vehicle group, n=8 for other each groups). Compared with the vehicle group: *** $p < 0.001$; compared with the 5-FU group: ### $p < 0.001$.

Figure 2. MPH966 alleviated 5-fluorouracil (FU)–induced inflammation and neutrophil degranulation in mice. MPH966 inhibited 5-FU–induced (A) MPO, (B) NE, and (C) Pr3 activity in the small intestine. (D) IHC confirmed decreased NE expression (red arrow; scale bar = 100 μm in 200X magnification). Each point represents the mean ± S.E.M. (n=5 for vehicle group, n=8 for other each groups). Compared with the vehicle group: * $p < 0.05$ and ** $p < 0.01$; compared with the 5-FU group: # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$.

Figure 3. MPH966 alleviated 5-fluorouracil (FU)–induced pro-inflammatory cytokine expression in mice. MPH966 alleviated 5-FU-induced (A) IL-1β and (B) TNF-α expression. Each point represents the mean ± S.E.M. (n=5). Compared with the vehicle group: * $p < 0.05$ and ** $p < 0.01$; compared with the 5-FU group: # $p < 0.05$ and ## $p < 0.01$.

Figure 4. MPH966 alleviated 5-fluorouracil (FU)–induced intestinal mucosal barrier dysfunction in mice. MPH966 alleviated 5-FU–induced expression of the tight junction proteins (A–B) ZO-1 and (C–D) occludin. IHC staining (scale bar = 100 μm in 200X magnification) indicated that ZO-1 presented around the villus and occludin presented in the villus and crypt (red arrow). Each point represents the mean ± S.E.M. (n=5 for vehicle group, n=6 for other each groups). Compared with the vehicle group: ** $p < 0.01$ and *** $p < 0.001$; compared with the 5-FU group: # $p < 0.05$.

Figure 5. Effects of MPH966 on 5-fluorouracil (FU)–induced changes in gut microbiota composition in the small intestine. Alpha diversity demonstrated by (A) Chao1 and (B) Shannon indices indicated that the gut microbiota diversities differed

between the treatment groups. Beta diversity demonstrated by (C) PCoA indicated two major gut microbiota compositions. PCoA was based on weighted UniFrac distance, plotted by the first principal component on the x-axis and second principal component on the y-axis. The top 10 relative abundances of gut microbiota distributed at the (D) phylum and (E) genus levels. Each group represents the mean ± S.E.M. (n=5 for vehicle group, n=8 for other each groups). Compared with the vehicle group: ** $p < 0.01$. VEH: Vehicle; Control: 5-FU (50 mg/kg); LD: MPH966 (5 mg/kg) + 5-FU (50 mg/kg); HD: MPH966 (7.5 mg/kg) + 5-FU (50 mg/kg); Alone: MPH966 (7.5 mg/kg).

Figure 6. Effects of MPH966 on 5-fluorouracil (FU)–induced changes in various microbial in the small intestine. (A) LDA indicated differential abundance of gut microbiota biomarkers between the treatment groups, as evaluated by metagenomeSeq. At the genus level, MPH966 significantly decreased (B) the abundance of *Candidatus Arthromitu* and partly increased (C) the abundance of *Lachnospiraceae NK4A136* compared with 5-FU treatment. At the family level, MPH966 significantly increased the abundance of (D) *Muribaculaceae*, (E) *Ruminococcaceae*, and (F) *Eggerthellaceae* compared with 5-FU treatment. Each group represents the mean ± S.E.M. (n=5 for vehicle group, n=8 for other each

groups). Comparisons between two different groups: * $p < 0.05$ and ** $p < 0.01$. PCoA: principal coordinates analysis. VEH: Vehicle; Control: 5-FU (50 mg/kg); LD: MPH966 (5 mg/kg) + 5-FU (50 mg/kg); HD: MPH966 (7.5 mg/kg) + 5-FU (50 mg/kg); Alone: MPH966 (7.5 mg/kg).

Figure 7. Effects of MPH966 on 5-fluorouracil (FU)–induced changes in the microbial gene pathway of the small intestine. The relative abundance of predicted microbial genes related to (A) cellular processes and (B) organismal systems were analyzed using the KEGG database. Each group represents the mean ± S.E.M. (n=5 for vehicle group, n=8 for other each groups). VEH: Vehicle; Control: 5-FU (50 mg/kg); HD: MPH966 (7.5 mg/kg) + 5-FU (50 mg/kg).