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M. M. B, F. A. Y. and A. A. Made equal contribution to this study.

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Abstract

EW-7197 is a TGF-β type I receptor kinase inhibitor with potential anti-inflammatory and anti-fibrotic properties. Here, we investigate the potential therapeutic effects of EW-7197 in a murine model of ulcerative colitis. EW-7197 attenuated the colitis disease activity index by improving rectal bleeding, body weight, and degree of stool consistency. EW-7197 also reduced colorectal tissue damage and the colon histopathological score by reducing crypt loss, mucosal damage, and tissue inflammation. Moreover, EW-7197 appeared to ameliorate the inflammatory and fibrotic responses by reducing oxidative stress, reducing sub-mucosal edema and inflammatory cell infiltration, down-regulating pro-inflammatory and pro-fibrotic genes, and inhibiting excessive collagen deposition in inflamed and fibrotic ulcerative colitis tissues. These results suggest that EW-7197 has potentially useful therapeutic properties against colitis, with clinically translational potential of inhibiting key pathological responses of inflammation and fibrosis in patients with colitis.

Keywords: EW-7197, TGF-β signaling, Colitis, Inflammation, Fibrosis,

Introduction

Ulcerative colitis (UC) is a chronic inflammatory disorder of the large intestine characterized by colicky abdominal pain, bloody diarrhea, fever and rectal urgency (Kornbluth and Sachar, 2010). Advanced age, gastrointestinal procedures, environmental and genetic factors, and abdominal surgery are risk factors for UC (Kappelman et al., 2013). Colitis-associated inflammation increases activation of oncogenic signaling pathways and enhances cell proliferation resulting in a higher risk for developing colorectal cancer (Lakatos and Lakatos, 2008). The treatment of UC depends mainly on its regional distribution, disease stage and frequency of relapses (Kornbluth and Sachar, 2010). Anti-inflammatory drugs like 5-aminosalicylic acid (5-ASA) are often the first-line treatment of patients with UC (Meier and Sturm, 2011), However, adverse effects and poor outcomes reduce treatment efficacy in long term therapy (Xu and Pan, 1999).

Transforming Growth Factor-β (TGF-β) pathway has a key role in the pathogenesis of pro-inflammatory and pro-fibrotic diseases including UC (Marek et al., 2002). EW-7197 is a TGF-β type I receptor kinase inhibitor, which targets TGF-β signaling by specifically inhibiting the activin receptor–like kinase (ALK)-induced activation of Smad2/3, the key signal transducers of TGF-β signaling. There are studies showing the anti-fibrotic properties of EW-7197 against hepatic, pulmonary, and renal fibrosis (Kim et al., 2016; Park et al., 2015). Moreover, the regulatory effects of this pivotal signaling pathway in several inflammatory diseases have been well established. For instance, TGF-β isoforms are increased in colonic mucosa of UC patients which is correlated with stage of disease (Babyatsky et al., 1996; Di Mola et al., 1999; Wiercińska-Drapało et al., 2001). Moreover, it has been shown that disruption of the Smad3 could reduce inflammation in skin wounds in mice model (Ashcroft et al., 1999; Flanders et al., 2002).

Consistent with the important regulatory role of TGF-β in pro-inflammatory and fibrotic diseases, and we have therefore investigated the therapeutic effects of EW-7197 as a TGF-β

type I receptor kinase inhibitor, against UC in a murine model. Our results showed that EW-7197 ameliorates the clinical symptoms of colitis, attenuates disease activity index, decreases histological damage to colon tissue, regulates oxidant/anti-oxidant balance, suppresses over-expression of pro-inflammatory and pro-fibrotic genes, and inhibits excessive collagen deposition and fibrosis in colitis tissues. Our results emphasize the therapeutic potential of this novel pharmacological inhibitor in attenuating colitis-associated pathological symptoms.

Material & Methods

Materials

EW-7197 and Dextran sulfate sodium (DSS-40kD) were obtained from Cayman Chemical Company (Ann Arbor, MI). Rabbit anti PI3K (p110α), anti-cyclin D1, and secondary antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA).

Animal

Eight-week-old C57BL/6 male mice were obtained from the Pasteur institute of Iran (Tehran, Iran) and kept according to the standard protocol of Institutional Animal Care Guidelines. The animals were housed under standard condition; room temperature (22-25°C), 12h light/dark cycle with free access to food and water ad libitum. All animal experiments were performed according to the guideline for Care and Use of Laboratory Animals from Mashhad University of Medical Sciences (MUMS).

Murine colitis model and experimental protocol

Mice were randomly divided into three groups (n= 6 for each group). 1. A control group received drinking water for 10 days. 2) A colitis group that received 1 % (w/v) DSS in drinking water for 7 days and then normal drinking water for the next 3 days. 3) The EW7197-treated mice received DSS 1% (w/v) from day 1 to 7, and EW7197 (5 mg/kg/day; oral gavage) from day 3 to 10 (Fig. 1). (Knod et al., 2016). It has been shown that this is an appropriate animal model for Inflammatory Bowel Disease (IBD) in man and is useful for investigating different aspects of the disease (Byrne and Viney, 2006; Egger et al., 2000; Miyazawa et al., 1967). A schematic representation of experimental protocol is presented in Fig. 1A. During the experiment, animals were monitored daily and evaluated for disease activity index (DAI) criteria consisting of the three parameters including weight loss, stool consistency, and rectal bleeding, as described previously (Table 1) (Cooper et al., 1993; Rijcken et al., 2004). After the experiment was carried

out, colon specimens were collected, washed, and weight and length of colons were recorded. Tissues were rapidly stored in 10% formalin or frozen in liquid nitrogen for further assessment.

Histopathological evaluation of colons

Colonic tissues were fixed in formalin, embedded in paraffin, sectioned using a microtome, and stained with Haematoxylin Eosin (H&E) as well as Masson's trichrome. Samples were analyzed individually using light microscopy and scored according to the standard histopathological scoring system presented in Table 2.

Evaluation of oxidative stress markers

The level of oxidative stress markers including MDA, total thiol and catalase activity were measured in the tissue homogenates as described previously (Aebi, 1984).

Real-time PCR

By using specific forward and reverse primers (Table 3) mRNA levels of proliferative, inflammatory and fibrotic genes were compared between groups as described (Hassanian et al., 2014). Briefly, RNA was extracted and converted to complementary cDNA (cDNA) by cDNA Reverse Transcription Kit according to the manufacturer's instruction (TaKaRa Bio, Shiga, Japan). The expression levels were normalized to a housekeeping control gene (GAPDH).

Western blotting

Total protein of colon tissues homogenate was extracted, separated by electrophoresis, and transferred to nitrocellulose membranes as described (Hassanian et al., 2016; Hassanian et al., 2015); tissues were blocked and incubated with primary and secondary antibodies. The signal intensity of blots were quantified by densitometric analysis using NIH ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

All data were expressed as means ± SEM and differences were considered to be statistically significant at $P < 0.05$. Statistical comparisons were determined using One-way analysis of variance (ANOVA) and the Wilcoxon Mann–Whitney tests. All data obtained from three independent experiments.

Results

EW-7197 ameliorated colitis clinical symptoms

The weight of animals was monitored daily. DSS treatment was associated with a reduction in weight of mice, and EW-7197 significantly decreased weight loss in the mice with induced colitis (Fig. 2A). Compared to the DSS-treated mice, administration of EW-7197 attenuated disease activity index (DAI) in colitis mice (Fig. 2B). To further investigate the protective effect of EW-7197 on colitis symptoms, we compared the colon length between different groups. EW-7197 suppressed DSS-induced colon shortening in colitis mice (Fig. 2C and D). EW-7197 treatment was also associated with a reduction in colon weight to colon length ratio, a marker of inflammation and tissue edema, in DSS-induced colitis mice, (Fig. 2E). These results clearly support the protective effects of EW-7197 against colitis pathological symptoms.

EW-7197 attenuated histological damage to colon tissue in colitis

To investigate the effect of EW-7197 on colon histological score, we compared colonic tissue damage between EW-7197-treated and -untreated mice. EW-7197 significantly reduced colon histological score in DSS-induced colitis mice (Fig. 3A), at least partially by decreasing tissue inflammation (Fig. 3B), mucosal damage (Fig. 3C) and crypt loss (Fig 3D). Further histological studies showed that DSS increased sub-mucosal edema and inflammatory cell infiltration which were decreased in the presence of EW-7197 in colitic mice (Fig. 3E), indicating the protective effect of EW-7197 on clinical and histological features of colon tissue in colitis.

Anti-oxidant and anti-inflammatory effects of EW-7197 in colitis

To further study the anti-inflammatory mechanism by which EW-7197 exerts its anti-colitis effect, we measured the oxidant and anti-oxidant balance in tissue homogenates in different mice groups. Our results revealed that compared to DSS-treated mice, administration of EW-7197 significantly increased total thiol concentrations and catalase activity, (Fig. 4A and

B) whereas decreased malonyl dialdehyde (MDA) levels (Fig. 4C) in colitis mice. In agreement with these results, we showed that EW-7197 significantly abrogated the stimulatory effect of DSS on the expression of inflammatory genes including interleukin-1β (IL-1β) and interferon gamma (IFN-γ) in colitis mice (Fig. 4D). These results suggest that EW-7197 elicits its anti-colitis properties partially by regulating inflammatory gene expression as well as oxidant/anti-oxidant balance in colitis mice.

EW-7197 inhibits fibrogenesis in colitis mice

Excessive collagen deposition and up-regulation of pro-fibrotic factors are key steps in the pathophysiology of colitis (Gordon et al., 2014). To evaluate the effect of EW-7197 on collagen deposition and fibrosis, colonic tissues were stained with Masson's trichrome. Results showed that EW-7197 significantly decreased DSS-induced collagen deposition in colitis mice (Fig. 5A). Consistent with these findings, EW-7197 decreased mRNA levels of pro-fibrotic genes including collagen type 1 alpha 1 (Col1a1), collagen type 1 alpha 2 (Col1a2), and alpha-actin-2 (Acta 2), in DSS-induced colitis mice (Fig. 5B), supporting the anti-fibrotic effects of EW-7197 in colitis. Taken together, these results showed that EW-7197 has anti-colitis properties by inhibiting key pathological of inflammation and fibrosis in ulcerative colitis**.**

Discussion

We have, for the first time, evaluated the therapeutic potential of EW-7197 in a murine model of ulcerative colitis and have demonstrated that this TGFβ signaling inhibitor can potently attenuate colitis disease activity index mainly by decreasing inflammation and fibrosis in ulcerative-colitis tissue. Decrease in sub-mucosal edema and inflammatory cell infiltration, down-regulation of pro-inflammatory and pro-fibrotic gene expression, regulation of oxidant/anti-oxidant balance, and reduction in excessive collagen deposition are some protective mechanisms of EW-7197 against ulcerative colitis. These results indicate that EW-7197 either alone or in combination with common therapy agents could attenuate colitis clinical symptoms.

Fibrosis is associated with excessive fibroblast proliferation and collagen deposition during wound healing process (Margadant and Sonnenberg, 2010). Recent studies revealed that TGF-β pathway has a key role in fibrogenesis through Smad dependent pathway by regulating several fibrogenic genes including Col1a1, Acta 2 and Col1a2 (Lan, 2011; Meng et al., 2016; Pohlers et al., 2009). In line with these findings, there are several studies supporting the therapeutic potency of EW-7197 in the pathogenesis of fibrotic diseases (Jun et al., 2017; Kim et al., 2016; Park et al., 2015). For instance, Kim et al. showed that oral administration of EW-7197 suppressed fibrosis and oxidative stress in liver fibrosis model (Kim et al., 2016). Moreover, Park et al. revealed that EW-7197 could significantly suppress fibrotic markers (collagen 1a1, Acta 2) and oxidative stress in hepatic, renal, and pulmonary fibrosis models through inhibiting TGF-β signaling pathway (Park et al., 2015). Consistently, our results showed that EW attenuates pro-fibrosis responses in DSS treated mice, supporting the therapeutic potency of this inhibitor in treatment of colitis induced fibrogenesis.

It should be noted that the exact regulatory function of TGFβ in IBD is diverse and to some extent, contradictory. There are studies supporting the anti-inflammatory signaling function of TGF-β pathway. For instance Boirivant et al. showed that suppression of Smad7 with a specific antisense oligonucleotide could improve TGF-β1-mediated inhibition of inflammation

in colitis mice model (Boirivant et al., 2006). Similarly, Di Sabatino et al. showed that activation of TGF-β signaling pathway down-regulate expression of inflammatory genes including IFN-γ, TNF-α, IL2, IL6, IL8 and IL17 as well as decreasing T cell-mediated tissue-damaging responses in the human gut (Di Sabatino et al., 2008). In contrast with these results, it has been shown that activation of TGF-β pathway exacerbate inflammatory responses in IBD by enhancing differentiation of T_H17 cells leading to over-production of cytokines and pro-inflammatory genes including IFN-γ, TNF-α and IL-6, (Liu et al., 2009; Mangan et al., 2006; Pohlers et al., 2009).

To further support of the complexity of TGFβ signaling on inflammation, Han et al. have reported that metallic stents covered with EW-7197 did not altered inflammatory cell infiltration after stent placement in a canine urethral model (Han et al., 2018). Moreover, Jun et al. indicated that although, EW-7197 could significantly decrease the level of fibrogenic markers but had no effect on inflammatory cell infiltration in stented esophagus of rats (Jun et al., 2017). Similarly, Tsauo et al. demonstrated that oral administration of EW-7197 only suppresses fibrogenesis in EW-7197 treated group with no effect on inflammatory response in mice model of peritoneal adhesion band (Tsauo et al., 2018).

In this study, we clearly show that EW-7197 reduces inflammation, possibly by decreasing oxidative stress, reducing sub-mucosal edema and inflammatory cell infiltration, and down-regulating pro-inflammatory genes in colitis mice model. The exact anti-inflammatory effect of TGF-β signaling in colitis is still unclear and further *in vivo* and *in vitro* studies are needed. Further investigation and preclinical studies are required to elucidate the exact molecular mechanisms and role of EW-7197 as an inhibitor of TGF-β signaling pathway in colitis patients. The information gained from all these studies will provide guidelines for clinical application of novel selective TGF-β receptor inhibitors in improve treatment of colitis patients.

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Figure Legends

Figure 1. Schematic representation of experimental procedure. Control group received drinking water for 10 days. Colitis group received 1 % (w/v) DSS in drinking water for 7 days and then normal drinking water for the next 3 days. The EW7197-treated mice received DSS 1% (w/v) from day 1 to 7, and EW7197 (5 mg/kg/day; oral gavage) from day 3 to 10.

Figure 2. EW-7197 ameliorates colitis clinical symptoms. Treatment of colitis mice with EW-7197 (5mg/Kg) declined DSS-induced body weight loss (Fig. 2A). Mice were treated with EW-7197 (5mg/Kg) and disease activity index score was measured in different groups (Fig. 2B). The effect of EW-7197 on DSS-induced colon shortening was measured (Fig. 2C,D). Compared to colitis mice, colon weight/length ratio was evaluated in EW-7197 treated groups (Fig. 2E). *p<0.05; **p<0.01; ***p<0.001.

Figure 3. The effect of EW-7197 on colorectal tissue damage in colitis. Colon histological score (Fig. 3A), inflammation score (Fig. 3B), mucosal damage score (Fig. 3C), and crypt loss (Fig. 3D), were measured in different groups. Histological studies to compare sub-mucosal edema and inflammatory cell infiltration between different groups (Fig. 3E) ***p<0.001.

Figure 4. EW-7197 modulates oxidative responses and inflammation in colitis. Mice were treated in the presence and absence of EW-7197 and serum thiol (Fig. 4A), catalase activity (Fig. 4B), and MDA (Fig. 4C) were compared between groups. Inhibitory effect of EW-7197 on DSS-induced over-expression of inflammatory genes is investigated in colon tissue homogenates (Fig. 4D). *p<0.05; **p<0.01; ***p<0.001.

Figure 5. EW-7197 inhibits fibrogenesis in colitis mice. EW-7197 decreased DSS-induced collagen deposition in colon tissues as visualized with Masson's trichrome staining (Fig.5A).

EW-7197 (5mg/kg) decreased mRNA levels of pro-fibrotic genes in DSS-induced colitis mice (Fig.5B). *p<0.05; **p<0.01; ***p<0.001.