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Protective effect of 7-*O*-succinyl macrolactin A against intestinal inflammation is mediated through PI3-kinase/Akt/mTOR and NF-κB signaling pathways

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ABSTRACT

Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, are pivotal for the development of inflammatory bowel disease (IBD), and down-regulation of the cytokines and cytokine-induced inflammatory responses therefore constitute pharmacological targets for the development of therapeutic strategies in IBD. In the current study, we found that 7-*O*-succinyl macrolactin A (SMA), a macrolide, potently inhibited TNF-α-induced adhesion of monocytes to colonic epithelial cells in a concentration-dependent manner, similar to rapamycin, a mTOR inhibitor. In addition, oral administration of SMA resulted in a significant suppression of clinical signs of TNBS-induced rat colitis, including weight loss, colon tissue edema, and myeloperoxidase activity, a marker for inflammatory cell infiltration, as well as microscopic damage score in a histomorphological examination of HE-stained colon tissue. More importantly, SMA was more efficacious in inhibition of intestinal inflammation than 5-aminosalicylic acid (5-ASA), an active metabolite of sulfasalazine, the most commonly prescribed agent for the treatment of IBD. Such anti-inflammatory activity showed correlation with significant suppression of adhesion molecules (ICAM-1 and VCAM-1), T-helper 1-type cytokines (TNF-α, IL-6), and chemokines (MCP-1, IL-8). In addition to inhibition of NF-κB nuclear translocation, SMA also caused significant suppression of TNF-α-induced phosphorylation of PI3K, Akt, mTOR and p70S6 kinase, similar to the effect of rapamycin, an immunosuppressant macrolide. Taken together, the current results suggest that managing both mTOR and NF-κB activation pathways using SMA may be a good therapeutic intervention for the treatment of IBD.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract, encompassing two major types, Crohn's disease (CD) and ulcerative colitis (UC) (Elson and Weaver, 2005; Podolsky, 2002). Although its etiology is largely unknown, it is widely accepted that genetic, environmental, and intestinal immune factors interact (Khor et al., 2011). For the majority of IBD patients, nonspecific immunosuppression with aminosalicylates and corticosteroids is considered standard therapy. In patients with severe flare-up who are refractory to steroid therapy and facing the risk of life-threatening complications, fast working immunosuppressants such as cyclosporine have been considered as a choice of therapy (D'Haens et al., 2001; Lichtiger et al., 1994; Loftus et al., 2003). However, instead of cyclosporine A, which has severe side effects, recent studies have suggested rapamycin, one of the macrolide compounds which has antifungal and immunosuppressant activities, as an effective agent for treatment of refractory IBD (Farkas et al., 2006; Massey et al., 2008; Ogino et al., 2012). Rapamycin inhibits mammalian target of rapamycin (mTOR) and prevents PI3K and Akt signaling in

Abbreviations: 5-ASA, 5-aminosalycilic acid; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester; CD, Crohn's disease; DMSO, dimethyl sulfoxide; ELISA, enzyme linked immunosorbent assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IBD, inflammatory bowel disease; IKK, IκB kinase; MA, macrolactin A; MMA, 7-*O*-malonyl macrolactin A; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative real-time PCR; SMA, 7-*O*-succinyl macrolactin A; TBST, Tris-buffered saline TBS-Tween 20; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; UC, ulcerative colitis

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response to growth factors, hormones, and nutrients (Tokunaga et al., 2004).

During the recurrent inflammatory process, attachment and infiltration of leukocytes into the damaged gut epithelium by pro-inflammatory cytokines such as TNF-α is one of the critical steps of inflammation and tissue injury in IBD-inflicted mucosa (Spiik et al., 2002; van Assche and Rutgeerts, 2002), which was also demonstrated in colonic biopsy samples from patients with Crohn's disease (Ellis et al., 1998) (Nikolaus et al., 1998; Pallone and Monteleone, 1996; Van Deventer, 1997). TNF-α induces expression of other pro-inflammatory cytokines and adhesion molecules which are required for leukocyte attachment and infiltration through activation of transcription factor NF-κB (Baldwin, 1996; Barnes and Karin, 1997; Chen and Manning, 1995). Activation of NF-κB by TNF-α stimulus relies on phosphorylation-dependent ubiquitination and degradation of inhibitor of κB (IκB) proteins mediated by IκB kinase (IKK) complex (Ghosh and Karin, 2002). In addition, TNF-α has been reported to stimulate activation of the phosphoinositide-3-kinase (PI3K)/Akt pathway which, in turn, phosphorylates the IKK complex (Ozes et al., 1999).

7-*O*-succinyl macrolactin A (SMA) is a derivative of macrolactin A (MA). The activity of MA is derived from the presence of a large macrocyclic lactone ring and it is known to have anti-biotic and immunosuppressive activities (Gustafson et al., 1989). In addition to its antibacterial activity against vancomycin-resistant enterococci and methicillin-resistant *staphylococcus aureus* (Kim et al., 2011), MA protects not only T-lymphoblast cells against human HIV viral replication (Gustafson et al., 1989), but also neuronal cells against glutamate toxicity (Kim et al., 1997). SMA has better antibacterial activity and less cytotoxicity than MA (Romero-Tabarez et al., 2006). However, despite its biological activities, the potential of MA and SMA for treatment of IBD has not been fully investigated. In present study, we attempted to determine whether SMA exerts inhibitory effects on intestinal inflammation *in vitro* and *in vivo*, and the signaling pathway of SMA action.

2. Materials and methods

2.1. Materials

The HT-29 human colon cancer cell line and U937 human monocytic cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.). RPMI-1640, fetal bovine serum (FBS), and penicillin-streptomycin solution were purchased from Thermo Scientific HyClone (Logan, UT, U.S.). M199 and Trizol reagent were purchased from Invitrogen (Carlsbad, California, U.S.). TNF-α was purchased from Biosource-Invitrogen (Carlsbad, California, U.S.). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM), trinitrobenzene sulfonic acid (TNBS) solution, 5-aminosalicylic acid (5-ASA), prednisolone, and rapamycin were supplied by Sigma-Aldrich (St. Louis, MO, U.S.). SMA (Fig. 1) was provided by Deawoo pharmaceutical company (Busan, Korea).

2.2. Cell culture

HT-29 and U937 cells were maintained in RPMI-1640 media supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were subcultured every two or three days at 80–90% confluence.

2.3. Cell viability assay

Cell viability was determined by MTT assay. HT-29 colon epithelial cells were seeded in a 96-well plate at a density of 1×10^5 cell/cm^2. After incubation overnight, the media was changed to RPMI media supplemented with 1% FBS. SMA stock solution was dissolved in ethanol and diluted with phosphate-buffered saline (PBS). SMA was then diluted subsequently in RPMI media just before use. Cells were treated with various concentrations of SMA (0.1 to 30 μM) for 24 h, 48 h, and 72 h, respectively. Following incubation, 20 μl of MTT solution (5 mg/ml in Hanks' balanced salt solution) was added and incubated for an additional 4 h. Then, the media was removed and the insoluble MTT formazan was dissolved in 200 μl of dimethyl sulfoxide (DMSO) for 30 min at 37 °C. Absorbance was measured at 540 nm using a microplate reader (VERSAmax, Molecular Devices).

2.4. Adhesion assay

Adhesion of monocytic U937 cells to HT-29 colon epithelial cells was measured as previously reported (Thapa et al., 2008, 2009). HT-29 cells were seeded at a density of 2×10^5 cells/cm^2 in a 48-well plate. After 1 h pretreatment with drugs, cells were stimulated with TNF-α (10 ng/ml) for 3 h. U937 cells were pre-labeled with 10 μg/ml of BCECF-AM for 60 min at 37 °C in serum-free RPMI 1640 media, centrifuged and resuspended in M199 media. After 3 h, the media from HT-29 cells was removed and co-incubated with fluorescence labeled U937 cells for 1 h at 37 °C. Cells were gently washed twice with warm PBS to remove non-adhering U937 cells. Cells were lysed with 0.1% Triton X-100 in 0.1 M Tris (pH 8.0), and BCECF fluorescence was measured using

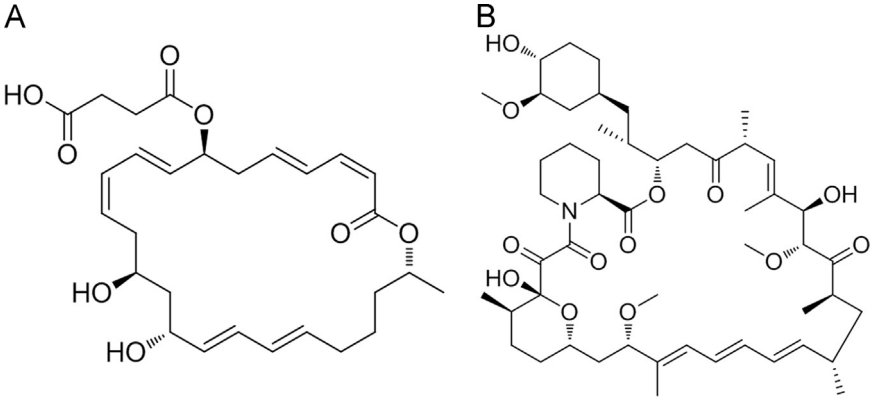

Fig. 1. Chemical structure of SMA and rapamycin.