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7-hydroxyfrullanolide, a sesquiterpene lactone, inhibits pro-inflammatory cytokine production from immune cells and is orally efficacious in animal models of inflammation

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ABSTRACT

A promising therapeutic approach to reduce pathological inflammation is to inhibit the increased production of pro-inflammatory cytokines (e.g., TNF-α, IL-6). In this study, we investigated the anti-inflammatory potential of 7-hydroxyfrullanolide (7HF). 7HF is an orally bioavailable, small molecule sesquiterpene lactone isolated from the fruit of *Sphaeranthus indicus*. 7HF significantly and dose-dependently diminished induced and spontaneous production of TNF-α and IL-6 from freshly isolated human mononuclear cells, synovial tissue cells isolated from patients with active rheumatoid arthritis and BALB/c mice. Oral administration of 7HF significantly protected C57BL/6J mice against endotoxin-mediated lethality. In the dextran sulfate sodium (DSS) model of murine colitis, oral administration of 7HF prevented DSS-induced weight loss, attenuated rectal bleeding, improved disease activity index and diminished shortening of the colon of C57BL/6J mice. Histological analyses of colonic tissues revealed that 7HF attenuated DSS-induced colonic edema, leukocyte infiltration in the colonic mucosa and afforded significant protection against DSS-induced crypt damage. 7HF was also significantly efficacious in attenuating carrageenan-induced paw edema in Wistar rats after oral administration. In the collagen-induced arthritis in DBA/1J mice, 7HF significantly reduced disease associated increases in articular index and paw thickness, protected against bone erosion and joint space narrowing and prominently diminished joint destruction, hyperproliferative pannus formation and infiltration of inflammatory cells. Collectively, these results provide evidence that 7HF-mediated inhibition of pro-inflammatory cytokines functionally results in marked protection in experimental models of acute and chronic inflammation.

1. Introduction

The standard therapy for auto-immune/inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease includes immunomodulating agents such as methotrexate, mesalazine, corticosteroids, and cyclosporine A (Baumgart and Sandborn, 2007). However, the use of these agents, particularly corticosteroids and cyclosporine A, is fraught with severe side effects. Evidence from *in vitro*, *in vivo* and, most importantly, clinically-relevant studies has established that pro-inflammatory cytokines (e.g., tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6)) play a critical role in the pathogenesis of rheumatoid arthritis and inflammatory bowel disease. Increased levels of TNF-α and IL-6 are observed in the diseased tissue of patients with active inflammatory disorder and elevated pro-inflammatory cytokine levels correlate with disease activity (Baumgart and Sandborn, 2007; Feldmann and Maini, 2008). Further, in animal models of experimental inflammation, administration of anti-TNF-α or anti-IL-6-receptor antibody leads to marked reduction in inflammatory responses and severity of disease (Fujimoto et al., 2008; Williams et al., 1992), and experimental inflammation is suppressed in TNF-receptor$^{-/-}$ and IL-6$^{-/-}$ mice (Alexopoulou et al., 1997; Naito et al., 2004). Therefore, a promising therapeutic approach to control the aberrant immune/inflammatory response is to inhibit the production of pro-inflammatory cytokines. Indeed, clinically approved or promising therapies for treating auto-immune/inflammatory disorders include TNF-α inhibitors (etanercept, infliximab and adalimumab) (Feldmann and Maini, 2008) and IL-6 inhibitor (tocilizumab) (Maini et al., 2006) thus validating the rationale for choosing pro-inflammatory cytokines as therapeutic targets.

Biologic response modifiers targeting TNF-α and/or IL-6 have revolutionized the clinical management of patients with inflammatory

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disorders including rheumatoid arthritis and inflammatory bowel disease (Kaser and Tilg, 2008). However, use of biological agents has certain limitations such as need for parenteral route of administration, high cost of therapy, risk of opportunistic infections, induction of allergic reactions, activation of latent tuberculosis, increased risk of cancer, and risk for worsening congestive heart disease (Feldmann and Maini, 2008). Various attempts have been made to develop orally active, pro-inflammatory cytokine inhibitors designed to target intracellular signal transduction pathways (e.g., PDE4D or p38 MAPK); to date, all have failed in clinical trials due to adverse affects (Cohen, 2009; Dyke and Montana, 2002). Of note, leflunomide is the only approved orally active drug in the market for rheumatoid arthritis; however its use also has several side effects (Osiri et al., 2003). Hence there is an unmet need for orally active inhibitors of pro-inflammatory mediators that can be used as an alternative to biological agents.

An attractive strategy to discover and develop orally efficacious small molecule cytokine inhibitors is to exploit the vast array of natural resources. A large number of compounds possessing anti-inflammatory properties have been derived from plants (Gautam and Jachak, 2009). The pharmacological activities of some of these medicinal plants, especially those from the family Asteraceae, are attributed to their contents of sesquiterpene lactones such as parthenolide from *Tanacetum parthenium*, budlein A from *Viguiera robusta*, mikanolide from *Mikania cordata*, helenalin from *Arnica montana*, and artemisinin from *Artemisia annua* (Ahmed et al., 2001; Berges et al., 2009; Jain and Kulkarni, 1999; Tawfik et al., 1990; Valerio et al., 2007).

In this study, we investigated the anti-inflammatory properties of 7-hydroxyfrullanolide (7HF), a sesquiterpene lactone isolated from the methanolic extract of *Sphaeranthus indicus* (Atta et al., 1989; Sohoni et al., 1988). ELISA assays were utilized to explore its potential to inhibit *in vitro* and *in vivo* production of TNF-α and IL-6. Subsequently, the efficacy of 7HF was probed in multiple *in vivo* models of acute and chronic inflammation.

2. Materials and methods

2.1. Isolation of 7-hydroxyfrullanolide

2.1.1. Plant material

Fresh flowering and fruiting heads of *S. indicus* were collected from Kelwe Road, Maharashtra, India and were authenticated in Piramal Life Sciences Limited, Mumbai, India. A voucher specimen (No. Herb-00230) was kept at the Piramal Life Sciences Limited herbarium for future reference.

2.1.2. Extraction and isolation

Dried flowering and fruiting heads of *S. indicus* (200 g) were pulverized. The powdered material was extracted using methanol (2.5 l) by stirring at 60 °C for 3 h and filtered under vacuum. This extraction process was repeated two more times. The extracts were combined and concentrated. Approximately 20 g of the methanolic extract were purified by column chromatography (silica gel, methanol in chloroform). Final purification was achieved by preparative HPLC (Kromasil 100-5-SIL 250×20 mm, 5 μm, Hexane: Isopropyl alcohol (95:5)) to obtain 7HF (Fig. 1A). ^{1}H Nuclear Magnetic Resonance ($CDCl_3$, 500 MHz): δ1.085 (3H, CH_3), 4.997 (1H, s), 5.801 (1H, s), 6.270 (1H, s); Mass Spectroscopy: m/e (ES) 248 (M^+). 7HF was characterized by comparing the obtained spectral data with the reported literature (Atta et al., 1989; Sohoni et al., 1988).

2.2. Human peripheral blood mononuclear cells assay

Peripheral blood was collected from healthy human donors after informed consent and Independent Ethics Committee approval. Human peripheral blood mononuclear cells were harvested using Ficoll-Hypaque density gradient centrifugation (1.077 g/ml; Sigma Aldrich; St. Louis, MO) (Bhonde et al., 2008; Dagia et al., 2006; Dagia et al., 2009) and suspended in assay medium [RPMI 1640 culture medium (Sigma Aldrich) containing 10% heat inactivated fetal bovine serum (FBS; JRH Biosciences; Lenexa, KA), 100 U/ml penicillin (Sigma Aldrich) and 100 μg/ml streptomycin (Sigma Aldrich)]. A cell suspension containing 2×10^5 human peripheral blood mononuclear cells per well was aliquoted into a 96-well plate. The cells were pre-treated with various concentrations of 7HF or 0.5% dimethyl sulfoxide (DMSO) or 10 μM 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole [SB203580; a p38 MAPK inhibitor which is known to suppress induced production of TNF-α and IL-6; Sigma Aldrich] for 1 h at 37 °C, 5% CO_2 and stimulated with 1 μg/ml lipopolysaccharide (LPS; *Escherichia coli* serotype 0127:B8; Sigma Aldrich). The cells were incubated for 6 h at 37 °C, 5% CO_2 following which supernatants were collected, stored at −70 °C and assayed later for TNF-α and IL-6 by Enzyme-Linked Immunosorbent Assay (ELISA; OptiEIA ELISA sets; BD Biosciences). The 50% inhibitory concentration (IC_{50}) values were calculated by a nonlinear regression method using GraphPad software (Prism 3.03). In all experiments, a parallel plate was run to ascertain the toxicity of 7HF. The toxicity was determined using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega; Madison, WI). In every experiment, each condition was run in triplicate wells.

2.3. Synovial tissue assay

Synovial tissue assay was conducted using a protocol reported by others (Brennan et al., 1989). After informed consent and Independent Ethics Committee approval, synovial tissue was obtained from rheumatoid arthritis patients undergoing knee replacement surgery. The tissue was minced into small pieces and digested in RPMI 1640 medium containing 100 U/ml penicillin-G, 100 μg/ml streptomycin, 50 ng/ml amphotericin B (Gibco BRL; Pasley, UK), 1.33 mg/ml collagenase Type I (Worthington Biochemical Corporation; NJ), 0.5 μg/ml deoxyribonuclease Type I (Sigma Aldrich) and 8.33 U/ml heparin (Biological E. Limited; India) for 3 h at 37 °C, 5% CO_2. The digested tissue was filtered through a cell strainer (mesh size 70 μm; BD Biosciences). Subsequently, the cells were washed three times and resuspended in complete medium (RPMI 1640 supplemented with 5% FBS and 5% human serum-AB+). Flow cytometric analyses of synovial tissue cells routinely revealed that majority of the cell population consisted of CD14+ monocytes, HLA-DR+activated macrophages and CD90+ fibroblasts (data not shown). Occasionally, CD3+ T-cells and CD19+ B-cells were also observed in synovial cell population (data not shown). For the experiment, 1×10^5 cells were added to the wells of a 96-well culture plate. 7HF or 0.5% DMSO were then added to the cells. SB203580 was used as a standard compound. The cells were incubated for 16 h at 37 °C, 5% CO_2 following which supernatants were collected, stored at −70 °C and assayed for TNF-α and IL-6 by ELISA. The 50% inhibitory concentration (IC_{50}) values were calculated by a nonlinear regression method using GraphPad software (Prism 3.03). In all experiments, a parallel plate was run to ascertain the toxicity of 7HF. The toxicity was determined using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). In every experiment, each condition was run in triplicate wells.

2.4. Animals

Male BALB/c mice (8–10 weeks of age, weighing 18–20 g), male DBA/1J mice (8–10 weeks of age, weighing 18–22 g), male C57BL/6J mice (8–10 weeks of age, weighing 18–22 g) and female Wistar rats (10–12 weeks of age, weighing 150–180 g) were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in individually ventilated cages in a temperature-controlled room, with access to water and food *ad libitum*. All animal experiments were double blinded and handled in accordance with the guidelines of "Committee