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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.

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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

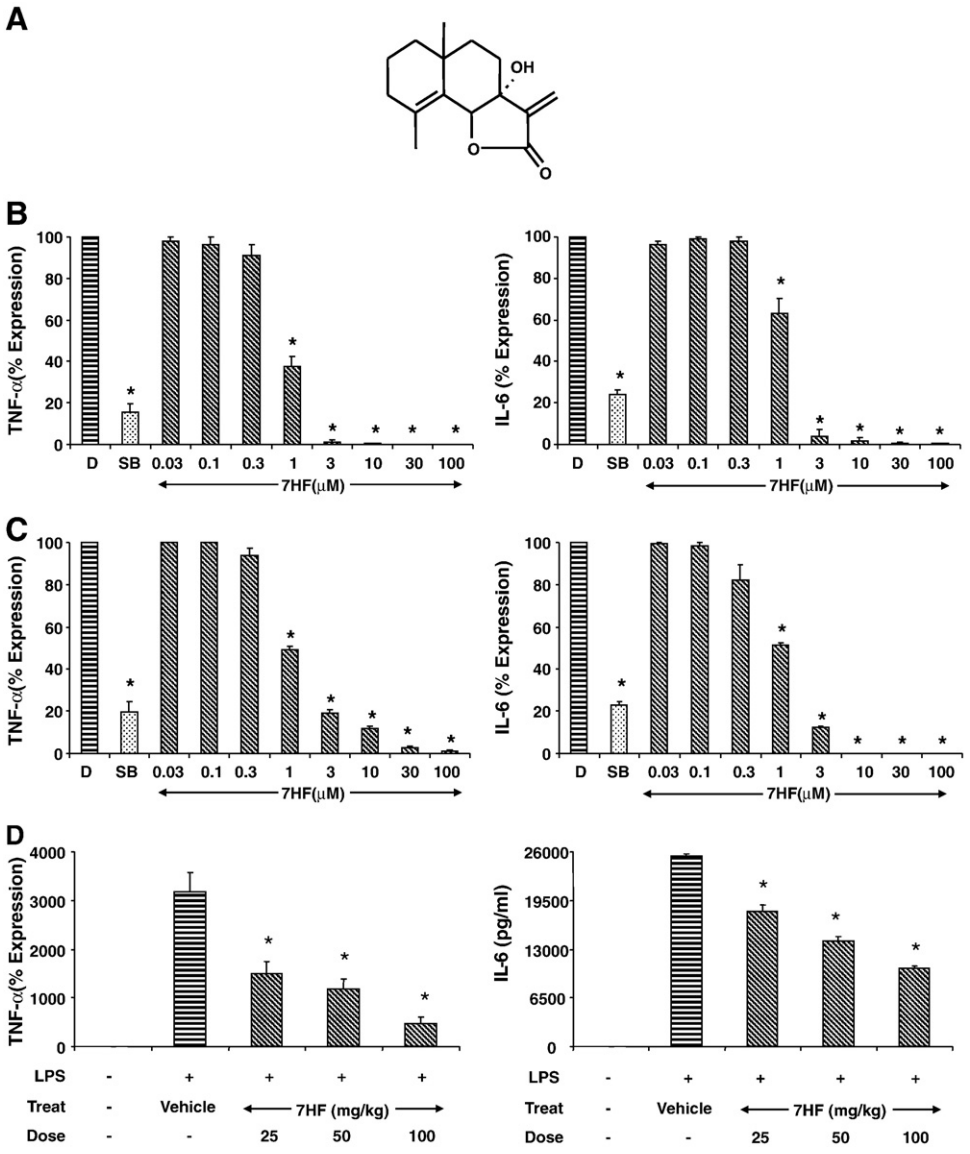


Fig. 1. 7HF potently suppresses *in vitro* and *in vivo* production of TNF-α and IL-6. (A) Structure of 7HF. (B) TNF-α and IL-6 production from LPS-stimulated human peripheral blood mononuclear cells. The cytokine level in DMSO pre-treated, LPS-stimulated human peripheral blood mononuclear cells was set at 100% in every experiment and used to normalize the other data. All values are average ± S.E.M. of 3 separate experiments. * indicates $P<0.05$ compared to DMSO pre-treated control cells. (C) TNF-α and IL-6 production from human synovial tissue cells. The cytokine level in DMSO pre-treated control group was set at 100% in every experiment and used to normalize the other data. All values are average ± S.E.M. from a single experiment. Results presented are representative of 3 separate experiments. * indicates $P<0.05$ compared to DMSO pre-treated control cells. (D) TNF-α and IL-6 production from LPS-stimulated BALB/c mice. All values are average ± S.E.M. of 8 mice from a single experiment. Results presented are representative of 3 separate experiments. * indicates $P<0.05$ compared to vehicle control.

for the Purpose of Control and Supervision of Experiments on Animals". All animal experiments were approved by Institutional Animal Ethics Committee of Piramal Life Sciences Limited. Prior to conducting *in vivo* efficacy studies, we ascertained the pharmacokinetic profile of 7HF administered orally to mice. These studies revealed that a dose of 50 mg/kg of 7HF results in a maximal concentration (C_{max}) of 1.6 μM and half-life ($t_{1/2}$) of 8.89 h in the plasma of mice (data not shown). The *in vitro* IC_{50} values of 7HF-mediated inhibition of TNF-α and IL-6 production were all in the range of 0.8–1.4 μM (see Results section). Accordingly, the efficacy of 7HF in experimental models of chronic inflammation (e.g., DSS-induced colitis, collagen-induced arthritis) was studied at this dose (i.e., 50 mg/kg) or one dose above (75 mg/kg) or below (25 mg/kg) this dose. Furthermore, given that $t_{1/2}$ of 7HF (dosed orally at 50 mg/kg) was 8.89 h, we chose to administer this compound twice daily in these chronic models of inflammation.

2.5. In vivo LPS assay

The procedure followed to ascertain the effects of 7HF on *in vivo* LPS-induced production of TNF-α and IL-6 was as reported elsewhere (Bhonde et al., 2008). 7HF was orally administered to BALB/c mice ($n=8$) at doses of 25, 50 and 100 mg/kg in the form of a suspension in carboxymethylcellulose (CMC; Sigma Aldrich). One hour later, LPS (*E. coli* serotype 0127:B8; 1 mg/kg; Sigma Aldrich) dissolved in sterile pyrogen-free normal saline was administered i.p. The negative control group received normal saline as an i.p. injection, while all other groups received LPS. Dexamethasone (10 mg/kg) known to inhibit LPS-induced production of TNF-α and IL-6 was used as a positive control. After 2 h, blood was collected and plasma separated by centrifugation at 2000 × *g* at room temperature. The aliquoted plasma was stored at −70 °C until assayed for mouse TNF-α and IL-6 levels by ELISA.

2.6. LPS-induced septic shock

The procedure followed was as described by others (Kotanidou et al., 2002). Male C57BL/6J mice were challenged with increasing doses of LPS (*E. coli* serotype 0111:B4; Sigma Aldrich) by i.p. administration. Mice were then observed for survival over a period of 7 days. Preliminary experiments revealed that 7 mg/kg of LPS consistently elicited lethality in 70% of mice by day 5 (data not shown). Accordingly, this dose of LPS was utilized to assess the effect of 7HF on survivability of mice. 7HF (50 mg/kg) or 0.5% CMC (vehicle control) were orally administered to a group of mice (n = 10) 1 h prior to LPS challenge. Dexamethasone (10 mg/kg) was used as positive control.

2.7. Colitis

2.7.1. Disease induction

Colitis was induced in C57BL/6J mice by giving 3% (weigh/volume) dextran sulfate sodium (DSS; MW30–40 kDa; ICN Biomedicals, Aurora, OH) in drinking water *ad libitum* as described elsewhere (Bhonde et al., 2008). For each mouse, body weight, and rectal bleeding were determined every day following introduction of DSS. Colitis was assessed by macroscopic and histological analyses of the colon (described below). To probe the efficacy of 7HF, a group of mice (n = 6) were given twice daily, oral administration of 75 mg/kg 7HF suspension in CMC. Sulfasalazine (100 mg/kg, p.o., b.i.d.; Sigma Aldrich) was used as a positive control. On day 10, animals were euthanized and multiple parameters indicative of clinical disease of colitis were graded as described elsewhere (Bhonde et al., 2008) and used to evaluate overall clinical disease activity index (Bhonde et al., 2008).

2.7.2. Macroscopic colon assessment

At the end of DSS treatment, mice were euthanized with 15% urethane (i.p). The whole colon was excised. The colon was macroscopically assessed by determining (a) the presence or absence of blood and (b) the length. Subsequently, the whole colon was cleaned off fecal contents, washed in saline and divided for myeloperoxidase (MPO) activity, RTQ-PCR and histological analyses.

2.7.3. MPO activity

MPO activity was assessed to indicate the extent of neutrophil infiltration. The procedure followed was as described by others (Sanchez-Fidalgo et al., 2007). Colonic tissue was immediately rinsed with ice-cold PBS, blotted dry, and snap frozen at − 70 °C until further analysis. For MPO assay the tissues were first weighed and homogenized in 50 mM PBS, (pH = 7.4) to obtain 100 mg/ml homogenate. After centrifuging at 20,000 × *g* for 20 min at 4 °C the pellets were again homogenized in a similar volume of 50 mM PBS (pH = 6.0) containing 0.5% hexadecyl-trimethylammonium bromide (Sigma Aldrich) and 10 mM ethylenediaminetetraacetic acid (Sigma Aldrich). The homogenates were then subjected to one freeze/thaw cycle and sonicated for 5 min The homogenates were centrifuged at 9600 × *g* for 10 min at 4 °C and 20 µl of supernatents was added to 50 µl 3,3′,5,5′-tetramethylbenzidine (BD Biosciences) in a 96 well plate. The reaction mixture was incubated at 37 °C for 5 min followed by addition of 50 µl H_2O_2 (BD Biosciences). The plate was incubated for 3 min at 37 °C. Reaction was terminated by 50 µl 2 N H_2SO_4. The absorbance at 460 nm was measured by microplate reader Spectramax 384 Plus (Molecular devices; Sunnyvale, CA). MPO levels were determined by plotting standard curve using serial dilutions of MPO standard (Sigma Aldrich). Results were quantified as U/100 mg of sample.

2.7.4. Histological analysis of colon

Colon biopsies were fixed in 10% neutral buffered formalin. Paraffin embedded sections (5 µm thickness) of the colon specimens were stained with Hematoxylin (Sigma Aldrich) and Eosin (Loba Chemie; Mumbai, India) and graded by an investigator blinded to the treatment groups. Histological scoring was performed based on presence of inflammatory cells, extent of crypt damage, mucosal erosions, edema and over all architectural damage, each scored on a scale of 0 to 3 as described elsewhere (Bhonde et al., 2008). Sections were scored for each of the above parameters. Cumulative score for all the parameters of each mouse was used for assessment of microscopic disease activity by comparing the average for each group.

2.7.5. Quantitative real time PCR analysis

The mRNA levels of pro-inflammatory mediators in colonic tissue were ascertained in a manner similar to that described previously (Dagia et al., 2009). The following primers were used: TNF-α, forward: AACCTCCTCTCTGCCATCAA, backward: GGAAGACCCCTCCCAGATAG; IL-6, forward: ATGCAATAACCACCCCTGAC, backward: GCGCAGAATGAGATGAGTTG.

2.8. Carrageenan induced paw edema

The procedure followed was as described in literature (Winter et al., 1962). Eighteen female Wistar rats were randomly divided into 3 groups and fasted overnight before the experiment with free access to water. Animal paw was marked with indelible ink for reference at subsequent measurements. 7HF (100 mg/kg) was administered orally to rats 1 h prior to s.c. injection of 1% carrageenan (λ-carrageenan, type IV; Sigma Aldrich) into the plantar surface of the right hind paw. The control group received an equal volume of 0.5% CMC (vehicle) and the positive control group received dexamethasone (10 mg/kg). The paw volumes before and after carrageenan injection (at various time points up to 8 h) were measured using a plethysmometer (LE-7500, Lectica Scientific Instruments; Barcelona, Spain). Percentage change in paw volume at each time point was calculated as difference of pre- and post-carrageenan injection paw volume.

2.9. Collagen induced arthritis

2.9.1. Disease induction

The procedure followed for collagen-induced arthritis was as described by others (Terato et al., 1985). Male DBA/1J mice (8–10 weeks of age) were immunized intradermally at the base of the tail with 200 µg type II collagen (Elastin products; Owensville, MI) emulsified in Complete Freund's Adjuvant (CFA; Sigma Aldrich). On day 21, mice were boosted with 200 µg type II collagen emulsified in CFA. Gradual disease onset occurs within 1 week after the second collagen injection. Following the day 21-booster injection, the mice (8 per treatment group) were monitored for the development and severity of arthritis using articular index and paw thickness as parameters. Articular index scoring was performed employing the following criteria—*Fore limbs* (*Scale 0–3*): 0, no redness or swelling; 1, redness but no swelling; 2, redness and swelling of the paw; 3, redness and severe swelling of the paw. *Hind limbs* (*Scale 0–4*): 0, no redness or swelling; 1, redness and mild swelling of paw; 2, redness and moderate swelling of paw and/or swelling of at least one of the digits; 3, redness and moderate/severe swelling of paw, swelling of ankle joint and/or swelling of one or more digits; 4, redness and severe swelling of paw, digits and ankle joint, with joint stiffness and altered angle of digits. The total articular index for a mouse is sum of individual articular index scores of fore limbs and hind limbs. Swelling of each of the paws of mice was measured with constant-tension, spring-loaded calipers (POCO 2 T; Krœplin Längenmesstechnik, Schlüchtern, Germany). Since the disease is more pronounced in the hind limbs, the paw thickness depicted in the figures is sum of paw thickness of both hind limbs. All measurements and scoring were performed by an operator blinded to the treatment groups. In the therapeutic dosing regimen, administration of 7HF (25 mg/kg, 50 mg/kg and 75 mg/kg, p.o., b.i.d.) was initiated only after hind limb of an

animal achieved a score of 2 (typically within 1 week after the second collagen immunization), at which time the animal was randomly assigned to a treatment group. Treatment continued daily for 10 days thereafter, and the body weight of the animal along with the severity of inflammation for all 4 paws was monitored daily (as described above). In every experiment, separate groups of inducted mice were treated with either 0.5% CMC (vehicle control) or Enbrel (2 mg/kg, s.c., q.d.; positive control; Wyeth Ltd.; Hampshire, UK). On the last day of experiment, 1 h after the compound administration, the animals were euthanized. Hind limbs of all the animals were excised and stored in PBS for immediate radiological analyses following which they were fixed in 10% neutral buffered formalin for 7 days for histological evaluations. The radiological analyses and histological evaluations were performed by a pathologist blinded to the treatment groups.

2.9.2. Radiological analyses

The hind limbs were removed from PBS and blotted dry. Dorso-ventral view was captured using Kodax Image Station (In-Vivo-FX, Kodak Molecular Imaging Systems; CT, USA). Radiological scoring was performed using the following criteria (Fletcher et al., 1998): *Soft tissue swelling* (*Scale 0–3*): 0, absence of soft tissue swelling; 1, soft tissue swelling only in any one digit; 2, soft tissue swelling in more than one but not all digits; 3, soft tissue swelling in all digits. *Reduction in joint space* (*Scale 0–3*)**:** 0, overall maintenance of joint space; 1, reduction of joint space only in one digit; 2, reduction of joint space in more than one but not all digits; 3, reduction of joint space in more than one but not all digits. *Osteolysis* (*Scale 0–3*): 0, absence of osteolysis; 1, bone thinning only in any one digit; 2, osteolysis in more than one but not all digits; 3, osteolysis in all digits. *Periosteal reaction* (*Scale 0–3*)*:* 0, absence of periosteal reaction; 1, presence of periosteal reaction in only any one digit; 2, presence of periosteal reaction in one but not all digits; 3, presence of periosteal reaction all digits. *Degenerative joint disease* (*Scale 0–3*)**:** 0, joint architecture maintained; 1, changes in joint architecture in only any one digit; 2, changes in joint architecture in more than one digit but not all; 3, changes in joint architecture in all digits. The mean of the total score was compared to vehicle treated group.

2.9.3. Histological evaluations

The procedure followed was as described by others (Williams et al., 1992). 10% neutral buffered formalin-fixed paws were decalcified in Gooding and Stewart's fluid for 4 days, processed and finally embedded in paraffin. Sections (6 μm) were stained with either hematoxylin and eosin or safranin O (Sigma Aldrich) and evaluated microscopically. Histological changes in sections were scored employing the following criteria—*Synovitis* (*Scale 0–3*): 0, absence of inflammatory cell infiltration; 1, minimum cell infiltration such as to maintain joint space; 2, moderate cellular infiltration with pannus formation; 3, pannus formation obstructing most of the joint space. *Bone damage* (*Scale 0–3*): 0, absence of bone damage; 1, bone erosions limited to discrete foci; 2, multiple foci of bone damage but joint architecture intact; 3, complete disruption of joint architecture. *Cartilage erosions* (*Scale 0–3*)*:* 0, cartilage integrity maintained; 1, cartilage erosions limited to discrete foci; 2, multiple foci of cartilage erosions; 3, extensive erosions or complete loss. The mean total score was compared to that of vehicle treated group. Cartilage depletion was indicated visually by diminished safranin O staining of proteoglycan matrix.

2.10. Statistical analysis

For analyzing differences between two groups, Student's T-test was used. For analyzing differences among multiple (more than two) groups, a single factor ANOVA followed by Dunnett's multiple comparison tests or Bonferroni's multiple pair-wise comparison tests were used (as appropriate). For septic shock experiments, significance of differences between survival rates (Kaplan–Meier survival plots) was ascertained by the log-rank test. In case of radiological and histolopathological analyses, Kruskal–Wallis test followed by Dunnett's multiple comparison tests was used to evaluate the statistical difference between two groups. P values <0.05 were considered statistically significant. Unless stated otherwise, all error bars represent standard error of mean.

3. Results

3.1. 7HF inhibits in vitro and in vivo production of pro-inflammatory cytokines

As a first step towards ascertaining the anti-inflammatory potential of 7HF, we investigated its effect on induced production of pro-inflammatory cytokines. LPS stimulation induced TNF-α and IL-6 production from human peripheral blood mononuclear cells (Fig. 1B). 7HF potently and dose-dependently inhibited the LPS-induced production of TNF-α and IL-6 from human peripheral blood mononuclear cells (IC_{50}: 0.8 μM and 1.4 μM for inhibition of TNF-α and IL-6 production, respectively; $IC_{50}>100$ μM for cytotoxicity; Fig. 1B and data not shown). We next sought to determine whether 7HF could attenuate the production of pro-inflammatory cytokines from pathologically relevant cells. Accordingly, we probed its effect on freshly isolated synovial tissue cells obtained from patients with active rheumatoid arthritis. We observed a robust inhibition of spontaneous production of TNF-α and IL-6 from synovial tissue cells by 7HF (IC_{50}: 1.0 μM and 1.0 μM for inhibition of TNF-α and IL-6 production, respectively; IC_{50}: 15.0 μM for cytotoxicity; Fig. 1C and data not shown). To assess whether the 7HF-mediated inhibition of pro-inflammatory cytokine production observed *in vitro* could be translated into a meaningful pharmacological effect *in vivo*, we used an acute model of inflammation. In these studies, 7HF inhibited LPS-induced production of TNF-α (53% inhibition at 25 mg/kg, 63% inhibition at 50 mg/kg, and 86% inhibition at 100 mg/kg) and IL-6 (29% inhibition at 25 mg/kg, 45% inhibition at 50 mg/kg, and 59% inhibition at 100 mg/kg) from BALB/c mice in a dose-dependent manner (Fig. 1D) corroborating the *in vitro* findings.

3.2. 7HF protects mice against endotoxin-mediated mortality

To determine the functional consequence of 7HF-mediated inhibition of pro-inflammatory cytokines, we used a mouse model of septic shock. In this model, mice pre-treated with 50 mg/kg 7HF showed significant protection against LPS-induced mortality (60% protection compared to vehicle control; Fig. 2). The protective effects of 7HF were found to be similar to that of dexamethasone (Fig. 2). No mortality was observed in mice given 50 mg/kg 7HF alone without LPS challenge (data not shown).

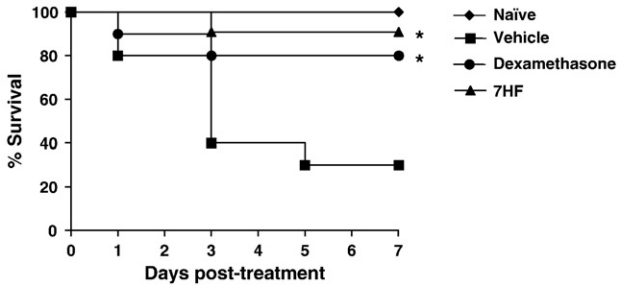


Fig. 2. 7HF shows significant protection against endotoxin-mediated mortality. Kaplan–Meier survival plot of C57BL/6 J mice treated with 50 mg/kg 7HF or 10 mg/kg Dexamethasone or 0.5% CMC (vehicle control) and subsequently challenged with 7 mg/kg LPS. All values presented are from a single experiment consisting of 10 mice per treatment group. Results presented are representative of two separate experiments. * indicates $P<0.05$ compared to vehicle control.

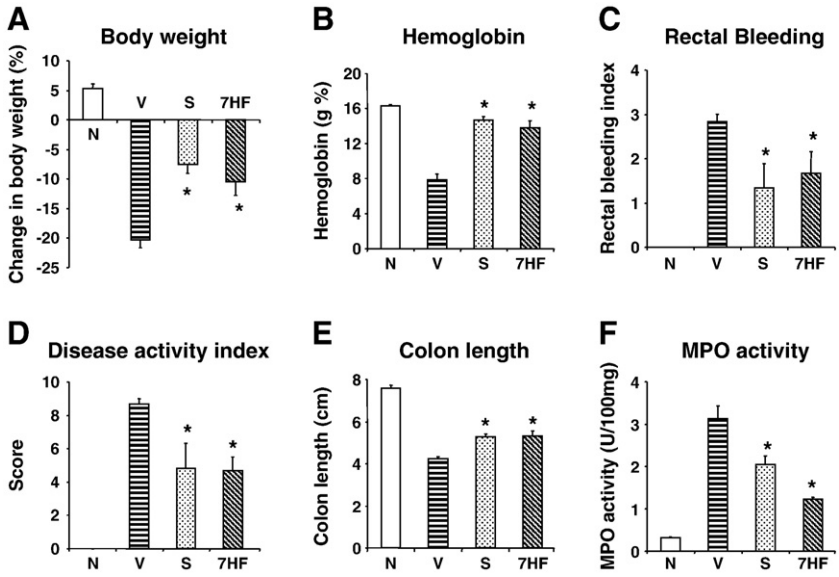


Fig. 3. 7HF suppresses DSS-induced colitis. Various groups of mice received DSS daily with some groups receiving twice daily, oral administration of 75 mg/kg 7HF (7HF) or 100 mg/kg sulfasalazine (S) or 0.5% CMC (vehicle control) (V) from day 1 onwards. Naïve mice (N) received regular drinking water throughout the study. (A) Change in body weight during the study. (B) Blood hemoglobin levels (C) The presence or absence of rectal bleeding depicted in the form of rectal bleeding index (D) Disease activity index (E) Colon length (F) MPO activity. All values are averages ± S.E.M. of 6 mice. * indicates $P<0.05$ compared to vehicle treated, DSS-fed mice.

3.3. 7HF suppresses DSS-induced mouse colitis

The observations that bacterial endotoxin (Khan et al., 2006) and pro-inflammatory cytokines (Baumgart and Sandborn, 2007) have been implicated in the pathogenesis of colitis, combined with the findings that therapies targeting pro-inflammatory cytokines have proved quite successful in reducing the severity of experimental colitis (Assi et al., 2006), led us to hypothesize that 7HF (which inhibits LPS-induced production of TNF-α and IL-6) might be efficacious in a murine model of colitis. Accordingly, we investigated the effect of 7HF in an experimental model of colitis.

As reported previously (Bhonde et al., 2008; Dagia et al., 2009), DSS-induction of colitis was manifested with significant increase in clinical disease activity index associated with marked weight loss, presence of rectal bleeding, diarrhea, distinct occurrence of occult blood in faeces, reduction in hematocrit, presence of colon bleeding and marked neutrophil infiltration as evidenced by increased levels of MPO (Fig. 3 and data not shown). Consistent with these observations, DSS treatment significantly reduced the colon length (Fig. 3). More importantly, 75 mg/kg 7HF, administered orally twice daily, showed significant inhibition of DSS-induced weight loss and reduction in blood hemoglobin levels, improved rectal bleeding index, markedly attenuated DSS-induced shortening of the colon and significantly reduced the neutrophil infiltration in colon as evidenced by decreased levels of MPO (Fig. 3). Histological analysis confirmed the DSS-induction of colitis. As reported previously (Bhonde et al., 2008; Dagia et al., 2009), colonic tissue sections from DSS mice, but not from normal mice, revealed severe inflammation, characterized by presence of edema, distinct inflammatory cellular infiltrate, extensive damage to mucosa and epithelium along with crypt destruction (Fig. 4). In contrast, tissue sections from 7HF-treated DSS mice revealed attenuation in inflammation, characterized by suppression of

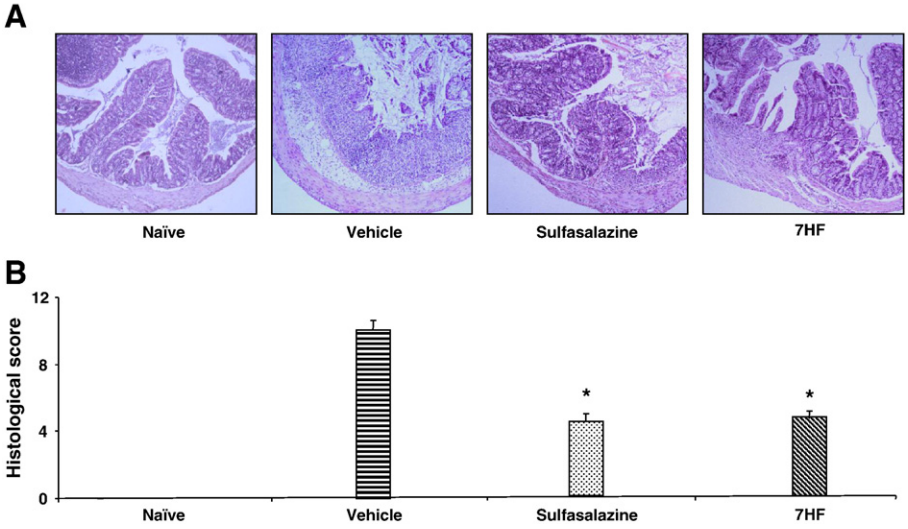


Fig. 4. 7HF markedly inhibits DSS-induced histological abnormalities. Histological analyses of the colons from various treatment groups (described in Fig. 3) were performed. Approximately 15 stained sections were observed from all 6 mice in each treatment group. (A) Representative images are presented. Compared to 0.5% CMC (vehicle control) treated DSS-fed mice, tissue sections from 7HF-treated DSS mice reveal attenuation of edema, reduction in infiltration of inflammatory cells and protection against DSS-induced crypt damage. (B) Histological scoring. All values are averages ± S.E.M. of 6 mice. * indicates $P<0.05$ compared to vehicle treated, DSS-fed mice.

edema, reduction in inflammatory cellular infiltrate, and protection against mucosal and crypt damage (Fig. 4). We next sought to determine if the efficacy of 7HF in attenuating DSS-induced colitis was associated with reduced production of TNF-α and IL-6. Accordingly, we performed RTQ-PCR analyses of colonic tissues from DSS-induced colitis experiment. Consistent with prior reports (Baumgart and Sandborn, 2007), DSS induced TNF-α and IL-6 mRNA expression in the colon (Fig. 5). More importantly, in line with our *in vitro* and *in vivo* cytokine inhibition studies, 7HF significantly suppressed DSS-induced increased TNF-α and IL-6 mRNA expression (Fig. 5).

3.4. 7HF abrogates carrageenan-induced paw edema

The observations that pro-inflammatory cytokines (Maini et al., 1995; Park and Pillinger, 2007) have been implicated in the pathophysiology of rheumatoid arthritis, combined with the findings that therapies uniquely targeting pro-inflammatory cytokines have proved quite successful in reducing the severity of rheumatoid arthritis in clinical trials (Koller, 2006), led us to hypothesize that 7HF (which inhibits spontaneous production of TNF-α and IL-6 from synovial tissue cells isolated from patients with active rheumatoid arthritis) might be efficacious in experimental models of arthritis. Initially, we evaluated its effects in an acute model of inflammatory arthritis i.e., the carrageenan-induced paw edema model. Of note, the development of paw edema in this model is dependent, at least in part, on production and release of pro-inflammatory cytokines including TNF-α (Rocha et al., 2006). As reported by others (Min et al., 2009), intraplantar injection of carrageenan induced an increase in paw volume as early as 2 h after injection (Fig. 6). This increase in paw volume was sustained at least till 8 h after injection (Fig. 6). Remarkably, this carrageenan-induced paw edema was significantly inhibited by pre-treatment with 100 mg/kg 7HF (Fig. 6). Furthermore, the anti-inflammatory efficacy of 7HF in this model was similar to that of dexamethasone at early (1–3 h) as well as late (8 h) time points (Fig. 6).

3.5. 7HF arrests collagen-induced arthritis

We next evaluated the effects of 7HF in a chronic model of arthritis; i.e., the well-established mouse collagen-induced arthritis model. As reported by others (Durie et al., 1994), the collagen-induced arthritis in DBA/1J mice was manifested with significant increases in paw thickness and articular index (Fig. 7) and these clinical signs of arthritic disease were markedly reduced in mice receiving 7HF (Fig. 7). Importantly, the effects of 7HF were dose-dependent and significant protection was observed with 50 mg/kg and 75 mg/kg doses (Fig. 7). The degree of macroscopic protection provided by 75 mg/kg 7HF showed a trend towards being statistically similar to the protection demonstrated by Enbrel (Fig. 7). Treatment with 7HF or Enbrel did not result in significant change in body weight of experimental mice (Fig. 7).

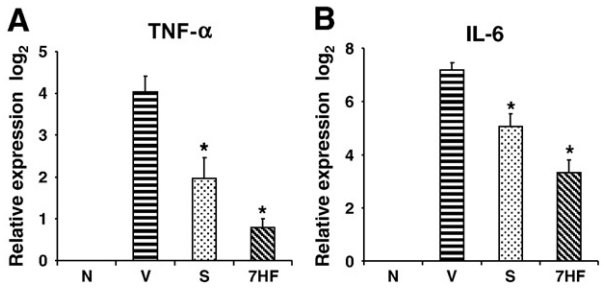


Fig. 5. 7HF inhibits DSS-induced expression of pro-inflammatory cytokines in colon. RNA isolated from tissue sections of colon of mice from various treatment groups (described in Fig. 3) was subjected to RTQ-PCR analyses using appropriate primers for (A) TNF-α and (B) IL-6. GAPDH was used as the loading control. Results presented are normalized to loading control. All values are averages ± S.E.M. of 6 mice. * indicates P<0.05 compared to vehicle treated, DSS-fed mice.

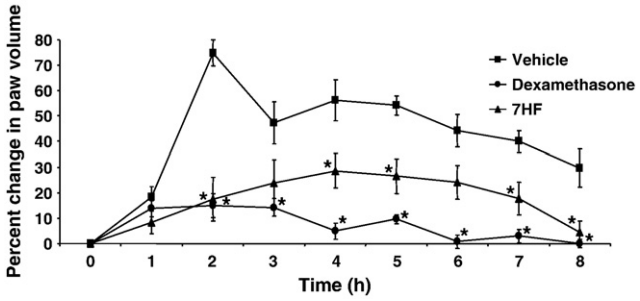


Fig. 6. 7HF inhibits carrageenan-induced paw edema. Wistar rats were pre-treated with 100 mg/kg 7HF or 10 mg/kg dexamethasone or 0.5% CMC (vehicle control) and, subsequently, challenged with carrageenan. The paw volume of each rat was measured every hour over a period of 8 h Data are expressed as percent change in paw volume with respect to paw volume of rats at time t = 0 (i.e. when carrageenan was injected). All values are averages ± S.E.M. of 6 rats. * indicates P<0.05 compared to vehicle treated group at the corresponding time point.

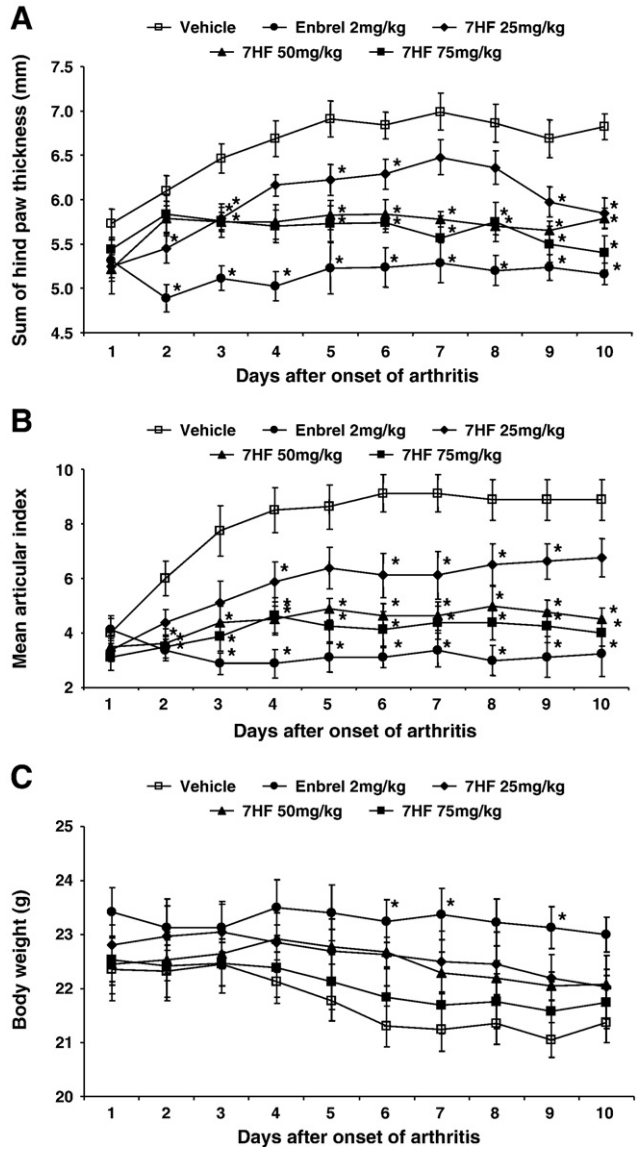


Fig. 7. 7HF arrests progression of collagen-induced arthritis. Immunized DBA/1J mice showing clinical symptoms of arthritis were treated (for 10 days) with twice daily oral administration of varying doses of 7HF or 0.5% CMC (vehicle control) or Enbrel (2 mg/kg; s.c.; q.d.). (A) Sum of hind paw thickness (B) Sum of articular index of all 4 paws (C) Body weight. All values are averages ± S.E.M. of 8 mice. * indicates P<0.05 compared to vehicle treated arthritic mice on the corresponding day.

Radiological analyses revealed extensive bone destruction and obvious joint deformities in hind paws of arthritic mice treated with vehicle control (Fig. 8). In accordance with the observed effects of reduced disease incidence and severity, hind paws of mice treated with 75 mg/kg 7HF showed significant protection against bone erosion and joint space narrowing (Fig. 8) whereas Enbrel showed complete protection against bone destruction and joint deformities (Fig. 8). Histological analyses of paw tissues of diseased mice treated with vehicle revealed severe destruction in the joints characterized by synovitis, pannus formation, articular cartilage erosion and pronounced infiltration of inflammatory cells invading bony cortex at multiple foci (Fig. 9). In contrast, hind paws of mice treated with 75 mg/kg 7HF or Enbrel showed maintenance of joint architecture with diminished pannus formation and reduced infiltration of inflammatory cells (Fig. 9). Furthermore, both, 75 mg/kg 7HF and Enbrel, preserved proteglycan matrix as seen after safranin O staining (Fig. 9).

4. Discussion

In this study, we report that 7HF inhibits *in vitro* and *in vivo* induced production of TNF-α and IL-6 (Fig. 1). These functional properties of 7HF translate in it affording protection to mice against septic shock induced mortality (Fig. 2). Our findings are in accordance with prior studies that have demonstrated that genetic or pharmacological modulation of TNF-α and/or IL-6 elicits a protective effect in the acute model of septic shock (Jin et al., 1994; Libert et al., 1992; Tracey et al., 1987). 7HF treatment also significantly suppresses experimental colitis. Our results complement earlier observations wherein (i) genetic deletion of TNF-α or TNF-α-receptor (Corazza et al., 1999; Mizoguchi et al., 2002) or IL-6 (Suzuki et al., 2001) protected mice against DSS-induced colitis and (ii) blockade of TNF-α production attenuated DSS-induced colitis (Zhang et al., 2009). Given that 7HF diminished leukocyte infiltration in colon, it would be of interest to assess its effect on the expression of endothelial cell adhesion molecules (E-selectin, ICAM-1, VCAM-1) which are known to play a critical role in the leukocyte-endothelial cell adhesion cascade (Panes et al., 2007). These studies are currently ongoing in our laboratories. Besides heightened production and biological activity of TNF-α and IL-6, ulcerative colitis is characterized by increased proliferation of colonic epithelial cells (Fiocchi, 1998). Thus, it would also be of interest to determine if 7HF inhibits the proliferation of colonic epithelial cells. Given that inflammatory disorders such as Crohn's disease and ulcerative colitis may also lead to colon cancer in the long run (Fantini and Pallone, 2008), our observations warrant further investigation probing the ability of 7HF to reduce colon cancer. In this regards, it is important to note that TNF-α has been shown to play a crucial role in an experimental model of colitis-associated colon cancer (Onizawa et al., 2009).

7HF is also efficacious in acute and chronic models of arthritis. Specifically, 7HF prevents edema and shows significant protection against paw swelling in the carrageenan-induced paw edema model (Fig. 6). Our results are in line with earlier observations wherein small molecule inhibitors targeting TNF-α and/or IL-6 have elicited efficacy in this acute model of arthritis (Min et al., 2009). Of note, the inflammatory response in the carrageenan-induced paw edema model is mediated by not only increased production of pro-inflammatory cytokines (Rocha et al., 2006) but also release of histamine, serotonin and prostaglandin (Vinegar et al., 1969). Whether 7HF inhibits the aforementioned mediators of inflammation is currently unknown and warrants further investigation. We observed that oral administration of 7HF also elicits significant

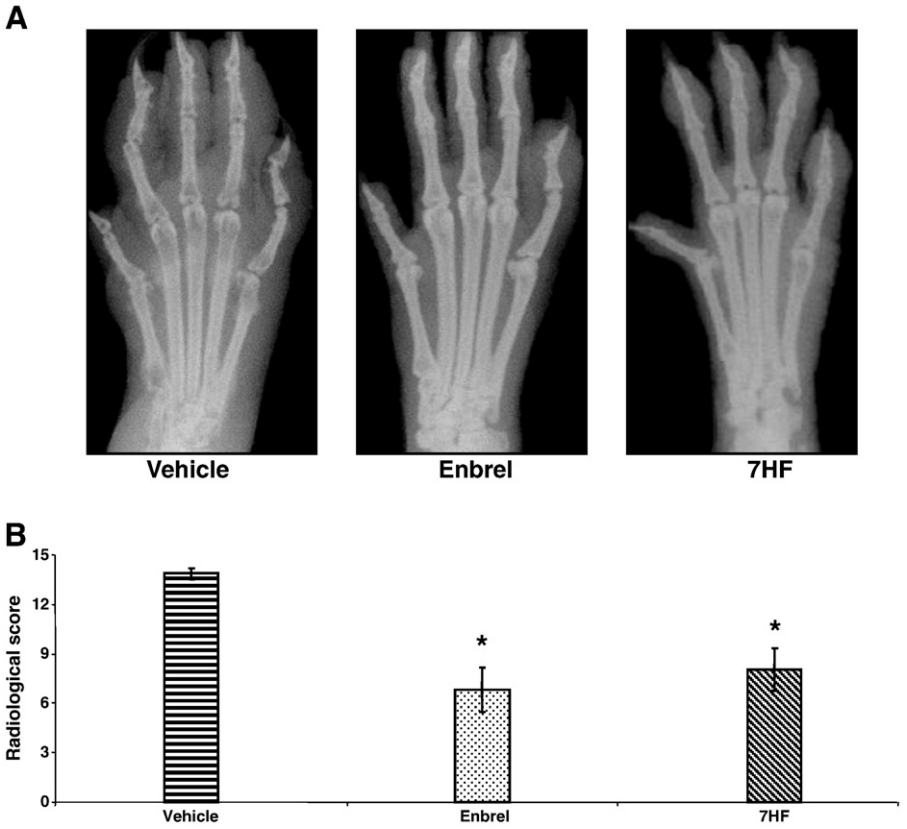


Fig. 8. 7HF provides protection against joint deformities and bone destruction associated with collagen-induced arthritis. Radiological analysis of hind limbs of mice from various treatment groups (described in Fig. 7) was carried out. (A) Representative X-ray images of the dorso-ventral view of the hind paw are presented. (B) X-ray images of the paws were scored for soft tissue swelling, reduction in joint space, osteolysis, periosteal reaction and degenerative joint disease. All values are averages ± S.E.M. of 8 mice. * indicates $P<0.05$ compared to vehicle treated mice.

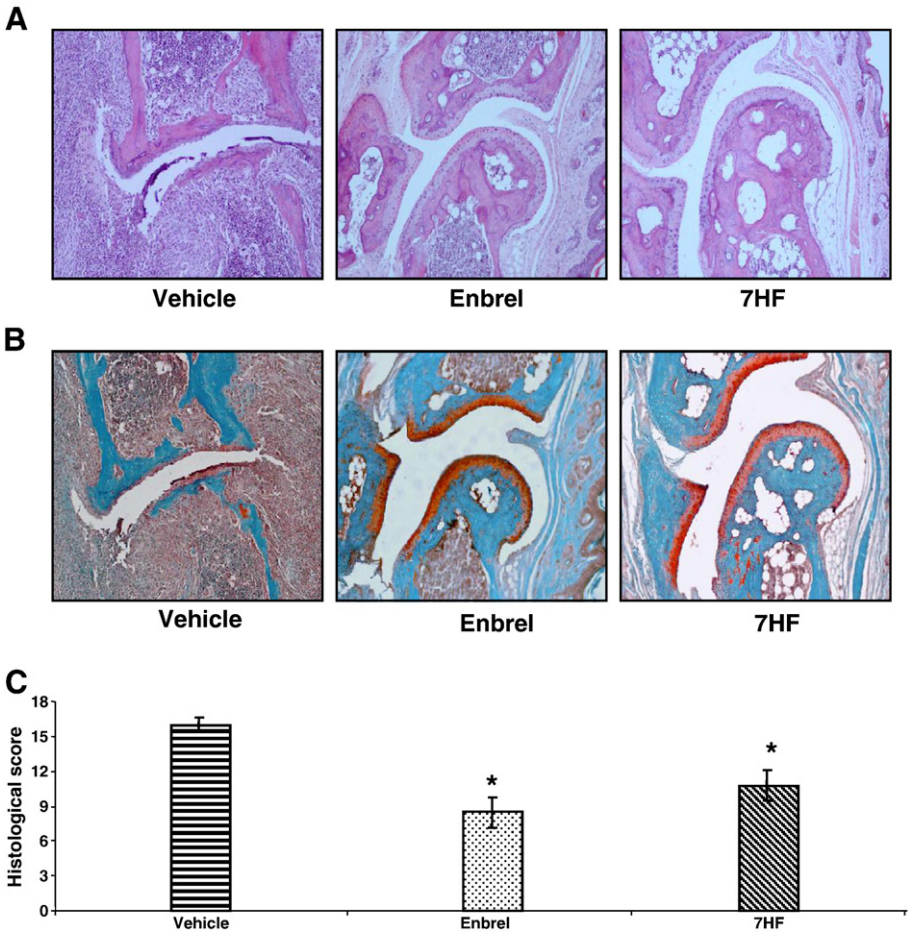


Fig. 9. 7HF prominently diminishes histological abnormalities in arthritic mice. Histological analysis of hind limbs of mice from various treatment groups (described in Fig. 7) was carried out. (A) Representative images of H&E stained sections of the hind paw are presented. (B) Representative images of safranin-O stained sections of the hind paw are presented. (C) Histological appearances of the H&E stained sections were scored for synovitis, bone damage and cartilage erosions. All values are averages ± S.E.M. of 8 mice. * indicates $P<0.05$ compared to vehicle treated mice.

protection against collagen-induced arthritis. Our findings support and extend earlier studies demonstrating TNF-α- or IL-6-deficient mice have reduced susceptibility for developing experimental arthritis (Hata et al., 2004), and neutralization of TNF-α and/or IL-6 decreases the incidence and severity of inflammatory collagen-induced arthritis (Liang et al., 2009; Williams et al., 1992). Besides increased production and activity of TNF-α and IL-6, rheumatoid arthritis is characterized by increased activation of T-cells and heightened production of other pro-inflammatory cytokines (e.g., IFN-γ, IL-1β). Indeed, (i) activated T-cells, by virtue of direct release of IFN-γ or initiating cell-contact-mediated stimulation of monocytes, play an important role in the pathogenesis of rheumatoid arthritis (McInnes et al., 2000) and (ii) the bone damage in rheumatoid arthritis is mediated, at least in part, by IL-1β (Zwerina et al., 2007). Of note, 7HF markedly inhibited induced production of IFN-γ from human peripheral blood mononuclear cells stimulated using a combination of anti-CD3 and anti-CD28 monoclonal antibodies (IC_{50}: 0.3 μM). Furthermore, 7HF blocked *in vitro* and *in vivo* induced production of IL-1β (*in vitro* IC_{50}: 0.5 μM; *in vivo* 58% inhibition at 100 mg/kg, p.o. dose). Since cartilage erosion in rheumatoid arthritis is mediated, at least in part, by matrix metalloproteinases (MMPs) (Goldbach-Mansky et al., 2000), it would be of interest to investigate the effect of 7HF on production of MMPs, particularly given that 7HF protected against cartilage destruction and bone deformities in collagen-induced arthritis.

The induced production of TNF-α and IL-6 is controlled at the gene level by the activity of transcription factors (Vallabhapurapu and Karin, 2009). Our observations that 7HF inhibits (both) TNF-α and IL-6 suggest that it is targeting transcription factor(s) which are common in the promoter regions for these pro-inflammatory cytokines. Clearly, future experiments are warranted to investigate the "target" and molecular basis of anti-inflammatory action of 7HF. These studies are currently ongoing in our laboratories. Preliminary investigations have revealed that 7HF does not inhibit p38 MAPK or PDE4D activity (data not shown).

In summary, the findings from our study provide direct evidence that 7HF, a sesquiterpene lactone present in a plant described in ancient Indian system of medicine, inhibits the production of pro-inflammatory cytokines, and is orally efficacious in experimental models of acute and chronic inflammation. Our findings reinforce the concept of targeting cytokines (Tincani et al., 2007) for treating various auto-immune/inflammatory disorders.

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