

Inhibition of HDAC Activity by ITF2357 Ameliorates Joint Inflammation and Prevents Cartilage and Bone Destruction in Experimental Arthritis

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Inhibition of histone deacetylases (HDAC) has been shown to modulate gene expression and cytokine production after stimulation with several stimuli. In the present study, the antiinflammatory effect of a potent HDACi, ITF2357, was explored in different experimental models of arthritis. In addition, the bone protective effect of ITF2357 was investigated *in vitro*. Treatment of acute arthritis (*Streptococcus pyogenes* cell wall (SCW) arthritis) with ITF2357 showed that joint swelling and cell influx into the joint cavity were reduced. Furthermore, the chondrocyte metabolic function was improved by treatment of ITF2357. The production of proinflammatory cytokines by synovial tissue was reduced after ITF2357 treatment. To examine the effect of HDAC inhibition on joint destruction, ITF2357 was applied to both rat adjuvant arthritis and mouse collagen type II arthritis. ITF2357 treatment both ameliorates the severity scores in arthritis models and prevents bone destruction. In an *in vitro* bone destruction assay, ITF2357 was highly effective at a dose of 100 nmol/L. In conclusion, inhibition of HDAC prevents joint inflammation and cartilage and bone destruction in experimental arthritis.

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INTRODUCTION

Although synthetic histone deacetylase inhibitors (HDACi) hyperacetylate histones via the conserved N-terminal lysines present on histones, they also hyperacetylate cytoplasmic proteins, including transcription factors. Therefore, unraveling chromatin and thus permitting transcription factors to bind to DNA is a prominent property of HDACi, but not their only mechanism of action. Thus, the ability of HDACi to acetylate cytoplasmic proteins may affect cellular functions independent of their role on gene expression. In humans, there are 18 distinct HDACs (1) and their specific

targeting with synthetic HDACi may have a role in treating diseases, particularly in chronic diseases such as degenerative joint diseases. Also, in order to be used during chronic disease states, such as occurs with inflamed joints, HDACi need to be safe for long-term use.

Inhibitors of HDAC are used widely in medicine. For example, valproic acid is used chronically to treat epilepsy and obsessive disorders (2,3). Whereas several synthetic inhibitors of HDACs have been developed to bring about terminal differentiation of cancer cells and to increase proapoptotic genes (4), to our knowledge

the only HDACi used in humans as an antiinflammatory agent is ITF2357 (givinostat) (14). ITF2357, a Class I and II HDACs inhibitor (14), was given to children with systemic onset juvenile idiopathic arthritis (SOJIA) at a dose of 1.5 mg/kg for 12 weeks. The study exhibited no organ toxicity and achieved significant reduction in parameters of systemic disease but particularly the number of painful joints (5).

In general, micromolar concentrations of synthetic HDAC inhibitors are required to increase the expression of several proapoptotic genes in malignant cells (4,6–8). However, HDACi also exhibit immunosuppressive and anti-inflammatory properties at lower concentrations (9). For instance, nanomolar concentrations of ITF2357 are sufficient to reduce the production and/or activity of proinflammatory cytokines *in vitro* or to exert a potent effect in animal models of inflammatory and autoimmune diseases (9–17). Other HDACi, such as SAHA, share with ITF2357 many of the

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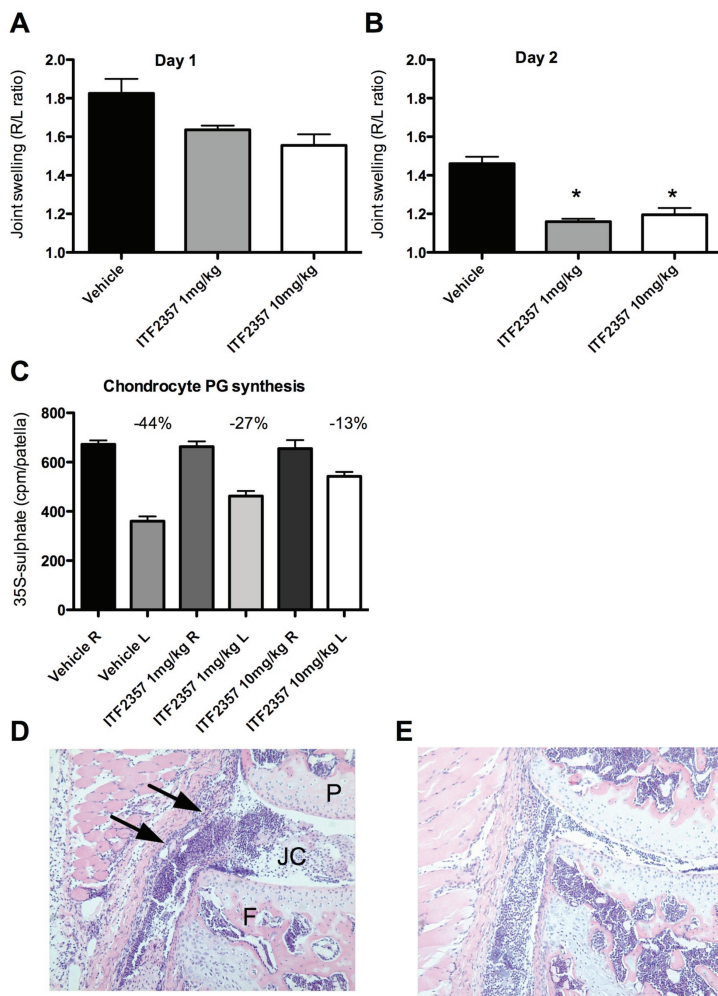


Figure 1. Effect of HDACi ITF2357 on SWC-induced arthritis. (A,B) Joint inflammation was induced by injection of 25 μ g SCW fragments into the right knee joint of C57/Bl6 mice 2 h after ITF2357 was orally supplied ($n = 5$ mice per group). Joint swelling was determined by 99m Tc-uptake method (30) at day 1 (A) and day 2 (B). Mice were treated with vehicle, 1 and 10 mg/kg ITF2357. Note the suppression of joint swelling at day 2 by both 1 and 10 mg/kg ITF2357. (C) At day 2 chondrocyte proteoglycan (PG)-synthesis was analyzed by using 35 S-sulphate incorporation method (31). Treatment with ITF2357 (10 mg/kg/day) almost restored (-13%) the chondrocyte PG-synthesis when compared with vehicle (-44%). (D) Vehicle treatment showed clear cell influx (predominantly PMNs) in the joint cavity and synovial lining layer (see arrows). After treatment with 10 mg/kg ITF2357, reduced cell influx in the joint cavity was seen. P = patella, F = femur, JC joint cavity. Mann-Whitney U test was used for statistical analysis. * $P < 0.05$.

antiinflammatory properties in models of disease in experimental animals (12,14).

In the present study, we have evaluated the effect of ITF2357 in several models of arthritis, ranging from acute to chronic models of arthritis. In addition, the effect of ITF2357 was studied on both TNF α - and IL-1 β -induced bone resorption.

HDAC INHIBITORS DECREASE CYTOKINE PRODUCTION BY RA SYNOVIAL CELLS AND RA TISSUE EXPLANTS

Recently, it was demonstrated that HDAC expression and activity in synovial tissue of RA patients correlates positively with the concentration of TNF α (18). In addition, it was shown that HDAC in-

hibitors suppress cytokine production by RA synovial tissue explants and that both TNF α - and LPS-induced cytokine and chemokine production were decreased by inhibition of HDAC activity (19). However, HDAC inhibition did not influence the spontaneous cytokine production of RA synovial macrophages or intact synovial tissue explants. Interestingly, HDACi induced apoptosis in RA synovial macrophages by suppressing antiapoptotic Bcl-1 protein (19). It has been reported that HDACi activate either the extrinsic or the intrinsic death pathway or both of these pathways (20). In addition, it was demonstrated that HDACi induce growth arrest in RA synovial fibroblasts through induction of p21 and suppression of NF- κ B nuclear accumulation (21). HDACi suppress cytokine production without an effect on cell death, which is of high interest. This indicates that it might be possible to uncouple inhibition of cytokine production and induction of apoptosis (14).

INHIBITION OF HDAC ACTIVITY SUPPRESSES EXPERIMENTAL ARTHRITIS

Since it was demonstrated that HDACi suppress the production of proinflammatory cytokines, several studies have been performed to investigate their anti-inflammatory properties in models of arthritis. Anti-type II collagen antibody-induced arthritis (CAIA) is based on the formation of immune complexes in the joints of susceptible mice. The onset of this experimental model of arthritis is highly dependent on cytokines, such as TNF α and IL-1. Both prophylactic and therapeutic treatment of CAIA with HDACi (TSA or FK228) suppressed arthritis severity (22,23) and reduced joint pathology. Of interest, enzymes that promote joint destruction such as matrix metalloproteinases (MMP)-3 and MMP-13 were reduced compared with vehicle. It is known that these MMPs are induced by TNF α but predominantly IL-1 β . HDAC inhibition will lead to suppression of these cytokines and the resultant reduction of MMP expression or activity can account for the benefit of HDACi in models of rheumatoid arthritis.

Table 1. Protective effect of ITF2357 on acute joint inflammation and cartilage catabolism.

mg/kg	Inhibition (%) of synovial cytokine production after ITF2357 treatment							
	TNF α	IL-1 β	IL-1 α	IL-6	IFN γ	IL-12	MIP-1 α	KC
1	-11.8	16.7	36.7	-9.3	4.8	0.5	0.7	17.4
5	17.5	30.1	35.2	14.3	42.1	23.8	17.2	32.0
10	45.3	61.7	66.3	39.3	59.1	64.2	51.9	25.8

Joint inflammation was induced by injection of 25 μ g SCW fragments into the right knee joint of C57/Bl6 mice 2 h after ITF2357 had been supplied orally ($n = 5$ mice per group). After 4 h, patellae with surrounding synovial tissue were isolated and cultured for 1 h at RT in RPMI 1640 medium. Cytokines were determined by Luminex bead array system. Values represent the reduction in cytokine production compared with vehicle control. A negative value indicates induction of cytokines.

Inhibition of HDAC activity by ITF2357 modulates several *in vivo* models of inflammation, such as LPS-induced shock, Con A-induced hepatitis, DSS colitis and even traumatic brain injury (24,14,25). In these models, it was demonstrated that IL-1 production is reduced after treatment with ITF2357. IL-1 is the classical proinflammatory cytokine that drives cartilage destruction during chronic joint inflammation. Of interest, in the beginning of the cytokine era, IL-1 was also named by Jeremy Saklatvala "catabolin," referring to its potent cartilage destructive properties (26). Thereafter, it was demonstrated that IL-1 contributes to joint inflammation and severe cartilage destruction (27). IL-1 exerts potent arthritogenic activity when injected directly into murine knee joints, whereas TNF α induces joint swelling and influx of cells into the joint space (28). Cartilage exposure to IL-1, both *in vitro* and *in vivo*, inhibits matrix proteoglycan synthesis and promotes cartilage destruction by upregulation of matrix MMPs. Irreversible cartilage damage elicited by MMPs is one of the hallmarks of joint destruction in inflammatory arthritis. The role of IL-1 was underlined further by elegant studies using IL-1 deficient mice. They revealed that IL-1 β is the pivotal mediator that directs chronic joint inflammation, but both IL-1 α and IL-1 β promote cartilage damage (29,30).

PROTECTIVE EFFECT OF ITF2357 ON ACUTE JOINT INFLAMMATION AND CARTILAGE CATABOLISM

The effects of ITF2357 on cartilage catabolism were studied in a mouse model

of *Streptococcus pyogenes* cell wall (SCW)-induced arthritis (31). This is a predominantly macrophage-driven arthritis model, initiated by TLR2 and NOD2 triggering (32). The study was performed in a prophylactic fashion in which the compound was administered orally before an intraarticular injection of 25 μ g SCW fragments (30–32). ITF2357 was given orally at 1 and 10 mg/kg at -2 hours, 6 hours, day 1 and day 2. Treatment with 1 and 10 mg/kg ITF2357 mildly suppressed joint swelling as determined by 99m Tc-uptake at days 1 and 2 (Figure 1A, B). Since inhibition of HDAC suppressed TNF α production *in vitro* as well as *in vivo*, it is likely that the reduced joint swelling seen after ITF2357 treatment is due to lower TNF α levels. Indeed, when the local cytokine production was determined, a 45% reduction of TNF production was seen in mice treated with 10 mg/kg ITF2357 (Table 1). In line with these findings, SCW-induced joint swelling is completely absent in TNF α gene deficient mice or mice treated with neutralizing anti-TNF α antibodies (28,30).

Induction of SCW arthritis results in a dramatic suppression of chondrocyte metabolic function as measured by the incorporation of radiolabeled 35 S-sulphate into the patellar cartilage. As an example, an inhibition of chondrocyte proteoglycan (PG)-synthesis of 44% is seen in Figure 1C. Oral treatment with 1 and 10 mg/kg ITF2357 dose dependently reduced the suppression of chondrocyte PG synthesis compared with the control group (27% and 13%, respectively, versus 44% in the

control group). It is known that inhibition of chondrocyte PG synthesis due to joint inflammation, progressively leads to severe cartilage damage. IL-1 β appears to be the most potent cytokine studied to date with respect to chondrocyte PG-synthesis. Overexpression of IL-1 β results in irreversible cartilage destruction (27). It is likely that the same modulation of IL-1 by HDACi as seen in other models of inflammation accounts for the reduced inhibition of chondrocyte PG-synthesis seen here, and hence in a protective effect against cartilage catabolism during arthritis. This was confirmed by the finding that treatment with 1 mg/kg or 10 mg/kg ITF2357 produced less IL-1 β , namely 17% and 62%, respectively, compared with SCW-control animals (Table 1). Interestingly, other proinflammatory cytokines (for example, IL-1 α and IFN γ) including arthritogenic ones such as IL-6 and IL-12 or chemokines MIP-1 α KC were dose dependently inhibited by ITF2357, suggesting a broad antiinflammatory effect for the drug (Table 1).

In SCW-induced arthritis, influx of predominantly PMNs can be noted in the joint cavity of inflamed joints (Figure 1D). Figure 1E shows the cellular influx at day 2 of SCW-induced arthritis and demonstrates that treatment with ITF2357 (10 mg/kg) reduces the number of inflammatory cells in the joint cavity. In contrast, no protective effect was noted on PG loss from the cartilage layers with either 1mg/kg or 10mg/kg ITF 2357 as measured by Safranin O staining. However, since inhibition of HDAC activity results in protection against inhibition of chondrocyte PG synthesis, the overall effect on cartilage of ITF2357 is beneficial to the cartilage structure.

ITF2357 INHIBITS SEVERE BONE DESTRUCTION IN COLLAGEN-INDUCED ARTHRITIS AND ADJUVANT-INDUCED ARTHRITIS

Since inhibition of HDAC activity by ITF2357 resulted in amelioration of acute joint inflammation and cartilage damage (improved chondrocyte proteoglycan synthesis) the effect of the drug was stud-

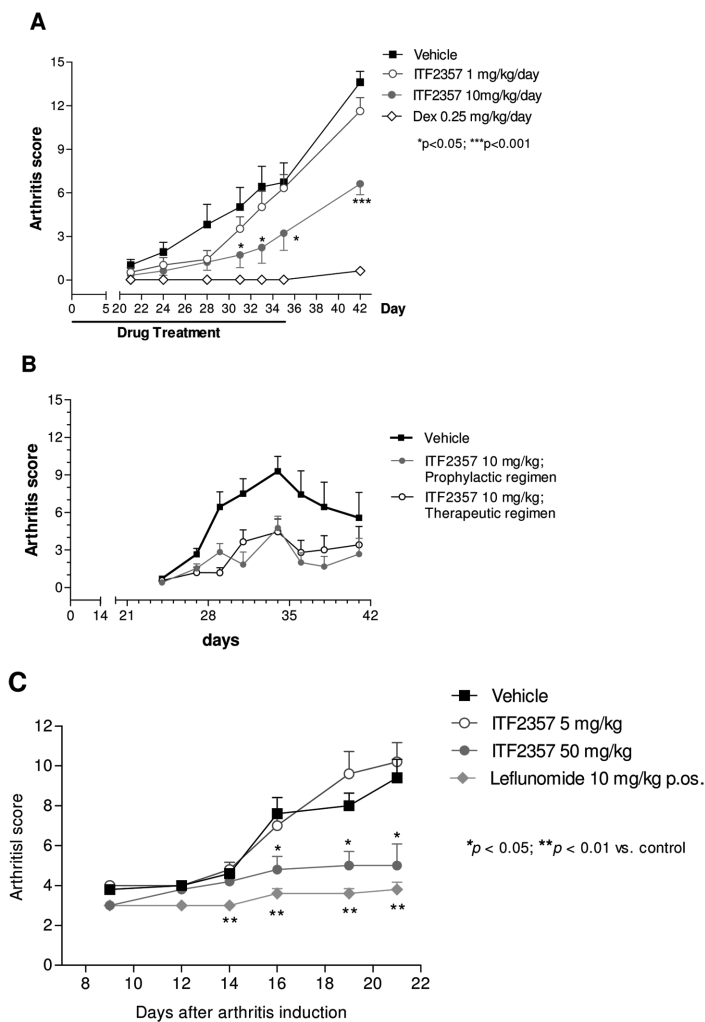


Figure 2. ITF2357 ameliorates both murine CIA and rat AA. (A) Murine type II collagen-induced arthritis was induced according to standard procedures. DBA-1 mice were treated orally with vehicle or ITF2357 at 1 and 10 mg/kg/day. Dexamethasone (0.25mg/kg) was used as positive control (n = 10 mice per group). Treatment was started just after the mice were immunized with CII/FCA (prophylactic). Mice were examined visually for arthritis expression. Noted that ITF2357 showed a potent antiinflammatory effect at a dose of 10 mg/kg/day. (B) ITF2357 was administered orally at a dose of 10 mg/kg in a prophylactic regimen (as in panel A) or starting on day 22 after arthritis induction (therapeutic regimen). (C) Rat adjuvant arthritis (AA) treated with ITF2357 (5 and 50 mg/kg). AA was induced using a standard protocol. Rats (n = 8 per group) were treated for 21 d, starting directly after immunization with FCA/MTB. Leflunomide (10 mg/kg/day) was used as positive control. Potent suppression of AA with both ITF2357 (50 mg/kg) and Leflunomide (10 mg/kg). Mann-Whitney U test was used for statistical analysis.

ied in arthritis models expressing severe joint destruction (bone and cartilage), namely murine type II collagen-induced arthritis (CIA) and rat adjuvant-induced arthritis (AA). Joint destruction is the hallmark of RA and one of the main targets for targeted therapies in RA.

In the CIA model, ITF2357 was administered either immediately after immunization with CII/FCA (prophylactic treatment) or 22 days after the induction of arthritis (therapeutic treatment). As shown in Figure 2A, 10 mg/kg ITF2357 given prophylactically had a suppressive effect

on arthritis development (macroscopic disease activity), when compared with vehicle. Interestingly, a virtually identical effect was seen when ITF2357 (10 mg/kg) was administered in the therapeutic regimen (Figure 2B). The beneficial effects of ITF2357 treatments were demonstrated further by histopathology analysis of the joint carried out on day 35. As seen in Table 2, both inflammatory cell influx and joint destruction were decreased dramatically after exposure to 10 mg/kg/day of ITF2357, irrespective of the treatment regimen.

Since rat AA displays more severe prominent bone destruction compared with murine CIA, rats with AA were treated with 5–50 mg/kg ITF2357. Prophylactic treatment with 50 mg/kg/day showed an optimal antiinflammatory effect when compared with vehicle control (Figure 2C). This suppressive effect was almost similar to leflunomide treatment (positive control) and resulted in strong reduction of bone pathology (data not shown).

Several reports confirmed the above findings that inhibition of HDAC strongly reduced disease severity in models of experimental arthritis (25). In line with our results, prominent protection against bone destruction was seen after treatment with HDACi (33).

BONE PROTECTIVE EFFECT OF ITF2357

Several preclinical studies showed that HDACi, including ITF2357, protect against severe bone destruction. TNF α and IL-1 are important cytokines that drive bone erosion in arthritis as shown in transgenic mice overexpressing human TNF α , where bone destruction was a prominent feature, and by blocking IL-1 receptors with antibodies early in the disease, which prevented bone loss. Furthermore, massive bone destruction was strongly reduced in mice transgenic for human TNF α but deficient in IL-1 (34,35). The effect of ITF2357 on bone turnover was, therefore, studied in a bone assay where bone destruction is induced by either TNF α or IL-1 β (36). Calcium release from 5-day-old mouse calvaria was measured 48 hours after addi-

Table 2. Histopathology evaluation of the effect of givinostat administration (10 mg/kg) on joint inflammation and tissue damages in the CIA model at day 28.

Treatment	Periarticular score	Bone and cartilage damage	Dilatation of joint cavity	Necrotic and inflammatory deposits in the joint cavity
Vehicle	2.08	1.50	1.25	1.75
Prophylactic	0.75	0.67	0.58	0.58
Therapeutic	0.83	0.42	0.33	0.50

tion of the cytokines in the absence or the presence of increasing amounts of the drug. As expected, a much larger concentration of TNF α was needed to induce the same degree of bone resorption as that of IL-1 β (1 μ g/mL versus 5 ng/mL) consistent with the prominent role of the latter on bone destruction as compared with the former. ITF2357 strongly inhibited bone resorption induced by either TNF α or IL-1 β (Figure 3A, B). The dose of ITF2357 needed for optimal inhibition was approximately 100 nmol/L. Bone resorption induced by the classic activators PGE2, 1,25 OH-Vit D3, parathyroid hormone or LPS also was blocked by ITF2357 although concentrations of about 1 μ mol/L were needed for a 50% reduction (data not shown).

Recently, it was shown that HDACi inhibit osteoclastogenesis and bone resorption by suppressing the induction of c-Fos by RANKL (37). In addition, HDACi induce IFN- β and thereby suppress osteoclastogenesis and bone destruction (38). In addition, HDACi are potent MMP inhibitors which are involved in both cartilage and bone destruction (39–41). Taken together, these data indicate that HDACi may be a future therapy for chronic joint diseases, in which bone destruction is difficult to treat.

FINAL REMARKS

Collectively the data from the literature and the results on ITF2357 presented here demonstrate that HDACi have a strong antiinflammatory and antidestructive effect in several models of arthritis. ITF2357, in particular, suppressed both acute and chronic arthritis. In the SCW model, joint swelling, the inhibition of chondrocyte proteoglycan synthesis and the influx of inflammatory cells to the inflamed joints

were suppressed dose dependently by oral treatment with the drug. TNF α and IL-1 β in the joint cavity also were reduced by ITF2357. This is consistent with the role played by the two cytokines in this model where joint swelling and cell influx are driven by TNF α , and IL-1 β is crucial in the inhibition of chondrocyte PG synthesis. Inhibition of TNF α and IL-1 β is not the only mechanism by which ITF2357 exerts its protective effect in the SCW-model, since other proinflammatory cytokines including arthritogenic IL6 and IL12 and chemokines were downmodulated equally by the drug (Table 1).

In addition to the suppressive effect in the acute model of arthritis, ITF2357 and other HDACi are protective in models of chronic arthritis. Remarkably, the efficacy seen upon administration of the drug before arthritis induction is comparable to that obtained when the treatment begins after the onset of the disease (25,33 and Figure 2). Furthermore, as with the SCW model, HDAC inhibition reduces bone destruction in these models of chronic arthritis (33 and Table 2; see Figure 2).

Inhibition of bone resorption also was observed *in vitro* where ITF2357 suppressed calcium release induced in mouse calvaria by IL-1 β or TNF α or other stimuli (Figure 3). Thus, not only is HDAC inhibition able to downregulate the expression of proinflammatory cytokines, it also is repressing their destructive effects on the bone. This is consistent with the results from LPS-induced activation of human PBMC where ITF2357, vorinostat and other HDACi suppress monocyte/macrophage production of TNF α , IL12, IL-1 β , etc. and their activity on lymphocytes (9).

One of the critical questions for the treatment of human arthritic conditions

with HDACi is whether or not sufficient circulating concentrations can be achieved by safe doses of these drugs. Approximately 500 patients with different pathologies have been treated to date with ITF2357. Safe doses range from 1.4 to 2.1 mg/kg/day. Owing to differences in the metabolic and adsorption processes, the C_{\max} and AUC values of a dose of 1.0 mg/kg/day ITF2357 in humans (including children) corresponds to 25–30 and 40 mg/kg/day, respectively, in rodents. Therefore the efficacious doses of 10 or 50 mg/kg/day seen here in the var-

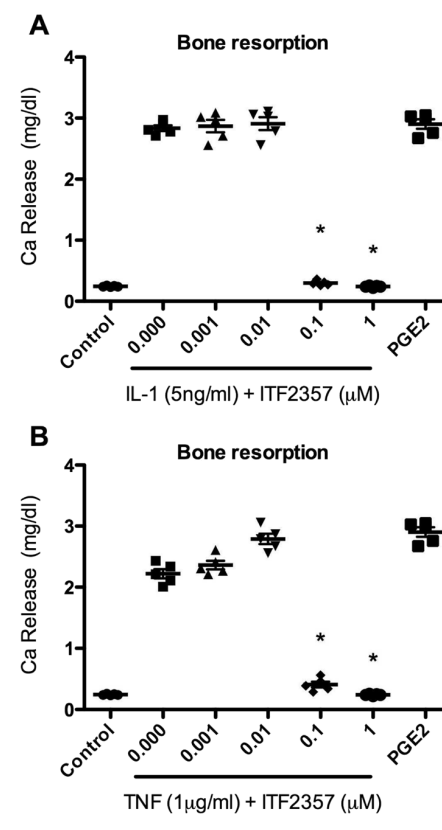


Figure 3. IL-1 β - or TNF α -induced bone resorption is inhibited by ITF2357. (A) IL-1 β -induced Ca-release using the standard mouse calvaria assay. Bone resorption assay (calcium release in 48 h) from 5-day-old mouse calvaria (n = 5 per group). ITF2357 was added in a dose-range from 0 up to 1 μ M. PGE2 served as positive control. IL-1 β at 5ng/mL. (B) Identical for TNF α -induced bone resorption. TNF α at 1 μ g/mL. Mann-Whitney *U* test was used for statistical analysis. **P* < 0.001.

ious models of experimental arthritis are consistent with a human use. Indeed, in a clinical trial in juvenile idiopathic arthritis (JIA), givinostat (ITF2357) was given to children at 1.5 mg/kg (in two divided doses) for 12 weeks (5). The drug was well tolerated and the overall arthritis score was suppressed in these children.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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