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Title: VX-765, an orally available selective interleukin converting enzyme (ICE)/caspase-1 inhibitor exhibits potent anti-inflammatory activities by inhibiting the release of IL-1β and IL-18

Authors: Woods Wannamaker, Robert Davies, Mark Namchuk, John Pollard, Pamella Ford, George Ku, Caroline Decker, Paul Charifson, Peter Weber, Ursula A Germann, Keisuke Kuida, and John C.R. Randle

Department of Chemistry (W.W., R.D.), Department of Protein Sciences (M.N.), Department of Biology (P.F., G.K., UAG, K.K.), Drug Discovery Support Unit (C.D.), Department of Modeling (P.C.), Strategic Development (J.C.R.R.) Vertex Pharmaceuticals, Inc.

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Corresponding author:

John C.R. Randle

Vertex Pharmaceuticals, Inc.

130 Waverly Street, Cambridge, MA 02139

TEL: (617) 444-6478

FAX: (617) 444-6680

e-mail: john_randle@vrtx.com

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List of abbreviations:

$AUC_{0\text{-last}}$	area under the curve calculated between time 0 and the last time point
BID	twice daily
C_{max}	maximum plasma concentration
ICE	interleukin converting enzyme
IFNγ	interferon gamma
IL-1β	interleukin-1β
IL-4	interleukin-4
IL-18	interleukin-18
MCP-1	monocyte chemotactic protein-1
MIP-1α	monocyte inhibitory protein-1α
MIP-2	monocyte inhibitory protein-2
SAC	*Staphylococcus aureus*-Cowan strain 1
T_{max}	time to maximum plasma concentration
TNF-α	tumor necrosis factor-α

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Abstract

VX-765 is an orally-absorbed pro-drug of VRT-043198, a potent and selective inhibitor of ICE/caspase-1 sub-family caspases. VRT-043198 exhibits 100-10,000-fold selectivity against other caspase-3 and -6-9. The therapeutic potential of VX-765 was assessed by determining the effects of VRT-043198 on cytokine release by monocytes *in vitro* and of orally-administered VX-765 in several animal models *in vivo*. In cultures of peripheral blood mononuclear cells and whole blood from healthy subjects stimulated with bacterial products, VRT-043198 inhibited the release of Interleukin (IL)-1β and IL-18, but had little effect on the release of several other cytokines, including IL-1α, tumor necrosis factor-α, IL-6 and IL-8. In contrast, VRT-043198 had little or no demonstrable activity in cellular models of apoptosis and did not affect the proliferation of activated primary T-cells or T-cell lines. VX-765 was efficiently converted to VRT-043198 when administered orally to mice and inhibited LPS-induced cytokine secretion. In addition, VX-765 reduced disease severity and the expression of inflammatory mediators in models of rheumatoid arthritis and skin inflammation. These data suggest that VX-765 is a novel cytokine inhibitor useful for treatment of inflammatory diseases.

Introduction

The interleukin converting enzyme (ICE), also known as caspase-1, is the cysteine protease that cleaves pro-interleukin-1β (IL-1β) and pro-interleukin-18 (IL-18) to form the mature, active cytokines, IL-1β and IL-18. IL-1β and IL-18 have important roles in the acute and chronic stages of inflammatory immune responses (reviewed in Braddock and Quinn, 2004). IL-1β induces the expression of several mediators of immune cell response, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), cyclooxygenase-2, chemokines, and cell-surface adhesion molecules that target cells to a site of infection or injury. IL-18, originally identified as interferon γ (IFNγ)-inducing factor, has structural similarity to IL-1 and belongs to the IL-1 superfamily (reviewed in Dinarello, 2002). It also induces chemokine and adhesion molecule expression, synergizes with IL-12 to induce the production of IFNγ by T helper cell type 1 (T_H1) cells, and activates natural killer cells (Yoshimoto et al., 1998). ICE/caspase-1 is not directly involved in the processing or activation of the other IL-1 cytokine such as IL-1α or the naturally occurring IL-1 receptor antagonist (IL-1Ra).

ICE/caspase-1 is the first member identified in the caspase family of cysteine proteases that now has 14 known members, 11 of which are expressed in humans (reviewed in Earnshaw et al., 1999). The caspases are typically divided into three sub-families on the basis of sequence homology and function. The caspase-1 sub-family includes ICE/caspase-1 along with caspase-4 and caspase-5 in humans. This subfamily appears to be involved primarily in inflammatory response and the production of IL-1β and IL-18

(reviewed in Martinon and Tschopp, 2004). While ICE/caspase-1 directly cleaves and activates the cytokines, the other family members may participate in the proteolytic activation of ICE/caspase-1 in response to signaling from membrane receptors. The remaining caspases are divided into two subfamilies based on their key roles in the initiation and execution of programmed cell death, or apoptosis, in a wide variety of cell types. ICE/caspase-1 is constitutively expressed and highly inducible in macrophages, T cells, and neutrophils. ICE/caspase-1 expression is also induced under certain conditions in other cell types such as keratinocytes (Zepter et al., 1997). The ICE/caspase-1 subfamily caspases do not appear to play a prominent direct role in apoptosis (Li et al., 1995; Smith et al., 1997), although they may play indirect roles through their influence on cytokine-mediated inflammatory responses that ultimately lead to apoptosis. In addition, Thalappilly et al. recently reported that activation of ICE/caspase-1 induces changes in the mitochondria leading to caspase-9 activation in apoptosis mediated by a phosphatase, thus suggesting a substantial role of ICE/caspase-1 in limited circumstances (Thalappilly et al., 2006). ICE/caspase-1-deficient mice develop and reproduce normally and have normal T-cell development (Kuida et al., 1995; Li et al., 1995). Apoptotic pathways in mature T cells are unimpaired, and the only discernible defect in apoptosis is in Fas-mediated apoptosis in thymocytes (Kuida et al., 1995). Under normal rearing conditions up to one year of age, ICE/caspase-1-deficient mice exhibit no obvious increase in the incidence of infection or malignancy (Kuida et al., 1995; D. Boucher, KK, JR, unpublished data).

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VX-765 is an orally-absorbed pro-drug of VRT-043198 (Figure 1), a potent and selective inhibitor of caspases belonging to the ICE/caspase-1 subfamily. VX-765 is converted rapidly to VRT-043198 under the action of plasma and liver esterases and also much more slowly in aqueous solution. This paper describes studies performed to evaluate the therapeutic potential of VX-765 based on its inhibition of cytokine release by monocytes *in vitro* and clinical and biomarker responses in animal models of inflammatory, autoimmune joint and skin disease *in vivo*.

Methods

VX-765 and VRT-043198

VX-765, (S)-1-((S)-2-{[1-(4-Amino-3-chloro-phenyl)-methanoyl]-amino}-3,3-dimethyl-butanoyl)-pyrrolidine-2-carboxylic acid ((2R,3S)-2-ethoxy-5-oxo-tetrahydro-furan-3-yl)-amide, and VRT-043198, (S)-3-({1-[(S)-1-((S)-2-{[1-(4-amino-3-chloro-phenyl)-methanoyl]-amino}-3,3-dimethyl-butanoyl)-pyrrolidin-2-yl]-methanoyl}-amino)-4-oxo-butyric acid, were synthesized at Vertex Pharmaceuticals, Inc. as described in International Patent application WO 01/90063 (Figure 1).

Protease enzyme assays

Caspase-1, -3, -7, and -8 were produced at Vertex Pharmaceuticals. Caspase-6 and –9 were purchased from Pharmingen (San Diego, CA) and Chemicon (Hampshire, UK), respectively. Caspase-4 was purchased from Eurogenetics. Granzyme B was purchased from Alexis Biochemical (Carlsbad, CA). Cathepsin B from bovine spleen and trypsin

from bovine pancreas were purchased from Sigma (St Louis, MO). Enzyme inhibition was assayed by tracking of the rate of hydrolysis of an appropriate substrate labeled with either p-nitroanaline (pNA) or aminomethyl coumarin (AMC) as follows: ICE/caspase-1, suc-YVAD-pNA; caspase-4, Ac-WEHD-AMC; caspase-6, Ac-VEID-AMC; caspase-3, -7, -8, and –9, Ac-DEVD-AMC; granzyme B, Ac-IEPD-AMC (Bachem, King of Prussia, PA). Enzymes and substrates were incubated in a reaction buffer (10 mM TRIS, pH 7.5, 0.1% (w/v) CHAPS, 1 mM DTT, 5% (v/v) DMSO) for 10 minutes (min) at 37°C. Glycerol was added to the buffer at 8% (v/v) for caspase-3, -6, -9 and granzyme B to improve stability of enzymes. The rate of substrate hydrolysis was monitored using a fluorometer. Assays for cathepsin B and trypsin were performed as described previously (Fox et al., 1992).

PBMC and whole blood assays

Buffy coat fractions from healthy volunteer donors were purchased from the Massachusetts General Hospital. PBMCs were prepared from the buffy coat fractions by a Ficoll gradient using Ficoll-Hypaque (Amersham Bioscience, Uppsala, Sweden) and washed twice in RPMI1640 (JRH Biosciences, Lenexa, KS). Cells were then transferred to 96-well microtiter plates at 4.8 x 10^5cells/well and stimulated with either 1 μg/ml of *E. coli* LPS (O111:B4, Sigma) or 1:1000 dilution of *Staphylococcus aureus*-Cowan strain 1 (SAC). *aureus Cowan* (SAC) strain 1 (2 mg/ml solution, Calbiochem, San Diego, CA). Plates were incubated overnight (16-20 hours (h)) at 37^{o}C in 5% CO_2. For the whole blood assay, blood was drawn from healthy volunteers at Vertex Pharmaceuticals using

Becton-Dickenson (Franklin Lakes, NJ) vacutainers. The blood was diluted with equal volume of RPMI1640 and 0.2 ml of the diluted blood was added to each of a 96-well costar plate. Cells were stimulated with 5 ng/ml of *E. coli* LPS (O111:B4). At the end of an 18-h incubation period, the plates were shaken, centrifuged at 200 x g for 5 min and supernatant was removed for cytokine evaluation. IL-1β, IL-18 and other cytokines in culture media were measured using specific ELISA kits (R&D Systems, Minneapolis, MN).

Phamacokinetic analysis of VX-765 and VRT-043198 in mice

Single doses of VX-765 (10, 21, 43 and 84 mg/kg) in vehicle (25% Cremophor EL, Sigma, in water) were administered via oral gavage. Blood samples (approximately 0.25-0.3 ml) were collected prior to dose administration and 0.167, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 24 h after dosing via the retroorbital sinus and processed for plasma. A high-performance liquid chromatography/mass spectrometry methodology was used to determine the concentration of VX-765 and VRT-043198 in plasma samples. Non-compartmental analysis was carried out using WinNonlin Pro Version 4.0.1 (Pharsight, Moutain View, CA).

Induction of IL-1β by intravenous injection of LPS

Naïve male CD-1 mice (Charles River Laboratory, Wilmington, MA) 6-7 weeks of age (30-32 gram body weight) were randomized and dosed by oral gavage with VX-765 1 h before intravenous (i.v.) injection of 2 mg/kg E. coli LPS (strain 0111:B4, lot 32K4092,

Sigma). Vehicle (25% Cremophor EL in water) was dosed at 1 h prior to the LPS challenge as a control. Peripheral blood samples were harvested 2.5 h following the LPS challenge. The blood was allowed to clot overnight at 4°C and then centrifuged to obtain sera for analysis on levels of IL-1β by a specific ELISA system (R&D Systems). All studies were performed with 6 mice per group.

Oxazolone-induced delayed-type hypersensitivity responses (DTH) in CD-1 mice

Naïve male CD-1 mice (Charles River Laboratory) 6-7 weeks of age (30-32 gram body weight, n = 9/group) were used in this study. The abdomen was shaved and 150 μL of a 5% (w:v) solution of oxazolone (Sigma) in a solvent composed of ethanol and acetone (4:1, v:v) was applied. Three days later the mice were challenged with 10 μl of 1-3% oxazolone applied to each side of the right ear. The left ears were treated with the same volume of the solvent as control. Mice were then treated twice by oral gavage with either the vehicle (25% Cremophor EL in water) or VX-765 (10-100 mg/kg in a dosing volume of 10 ml/kg body weight) 24 and 36 h after the ear challenge. Prednisolone (5 mg/kg) was used as positive control and dose orally. At 48 h after the ear challenge, 9 mm diameter biopsy samples were collected from both right and left ears and weighed. Edema in the oxazolone-challenged right ear was determined as the difference in weight between the right and control left ear biopsy samples. The biopsy samples were then homogenized individually in 1 ml of PBS pH 7 with a standardized cocktail of protease inhibitors (Roche Biochemicals, Indianapolis, IN). The homogenates were spun at 15,000 rpm for 15 min and the resulting supernatants were analyzed by specific ELISA

(R&D Systems) for IL-1β, IL-18, IFNγ, IL-4, monocyte chemotactic protein-1 (MCP-1), monocyte inhibitory protein–1 alpha (MIP-1α), monocyte inhibitory protein-2 (MIP-2), myeloperoxidase and nitric oxide.

Collagen-induced arthritis (CIA) model in mouse

Naïve male DBA/1 mice (Jackson Laboratory, Bar Harbor, ME) at 8-10 weeks of age were immunized, intradermally at the base of the tail, 0.1 ml aliquots of a 1:1 (vol:vol) emulsion of complete Freund's adjuvant (CFA, Sigma) and chick type II collagen (4 mg/ml 100 mM acetic acid, Elastin Products, Owensville, MO). The mice were immunized again with the same material three weeks later (Ku et al., 1996). Forepaw inflammation was monitored every other day and graded in a blinded manner semi-quantitatively on a scale from 0-5: level 0 = no evidence for inflammation; level 1 = erythema around the wrist joint; level 2 = erythema plus partial swelling of tissue around the wrist joint; level 3 = erythema plus pronounced swelling of tissue around the wrist joint; level 4 = erythema plus pronounced swelling of the wrist and palm; level 5 = erythema plus pronounced swelling of the wrist, palm and fingers of each forepaw. In the prophylactic regimen, immediately following the second immunization mice were assigned to groups and treated by oral gavage twice-daily (BID) with vehicle (25% Cremophor EL), VX-765 (10-100 mg/kg), or prednisolone (5 mg/kg). In the therapeutic regimen, the disease was allowed to progress until a portion of the mice (usually 60-70%) exhibited level 2 paw inflammation score in each front paw. Then these mice were assigned to different treatment groups. Dosing materials were provided blinded to the investigator performing the drug treatment and disease scoring. The sum of the forepaw

inflammation scores was recorded every second day. Area-under-the-curve (AUC) of forepaw inflammation scores for each mouse was calculated using the trapezoidal rule. Statistical significance of treatment was analyzed based on AUC using the Wilcoxon rank sum test. At the end of studies, forepaws were collected for histological examination. The incidence of cartilage and bone lesions was noted and a histological score was assigned to each joint as follows: level 1 = infiltration in the synovium; level 2 = level 1 plus erosion in the cartilage; level 3 = level 2 plus erosion in the bone; level 4 = level 3 plus pannus formation.

Results

Selectivity of VRT-43198 against caspases and other proteases

We evaluated *in vitro* the potency of VRT-043198 against ICE/caspase-1 and caspase-4 and its selectivity against representatives of the three subfamilies of caspases, and other proteases, including granzyme B and trypsin (serine proteases), and cathepsin B (cysteine protease). As shown in Table 1, VRT-043198 exhibited potent inhibition of ICE/caspase-1 (Ki = 0.8 nM) and caspase-4 (Ki < 0.6 nM) and at least 100-fold lower potency against other non-ICE subfamily caspases. VRT-043198 exhibited no significant inhibition of trypsin or cathepsin B, and only weak inhibition of granzyme B (Ki = 9 μM).

Inhibition of cytokine release from human PBMCs and whole blood

We evaluated the ability of VRT-043198 to inhibit the LPS-stimulated release of cytokines from human PBMCs and whole blood obtained from healthy volunteers. VRT-

043198 inhibited IL-1β release from both PBMCs (n = 8) and whole blood (n = 4) with IC_{50} values of 0.67 ± 0.55 μM and 1.9 ± 0.80 μM (mean ± SD), respectively. Additional experiments were conducted using PBMCs stimulated with SAC, which induces the release of large amounts of IL-1β, as well as sufficient IL-18, IFNγ and TNF-α to determine the inhibitory effects of VRT-043198. VRT-043198 dose-dependently inhibited production of IL-1β, IL-18 and IFNγ, but did not affect TNF-α release (Table 2).

VRT-043198 lacks potent anti-apoptotic activity

VRT-043198 was evaluated in a hypoxia-induced apoptosis assay using the human neuroblastoma cell line NT2 where caspase-9 may play a role in the initiation of hypoxia- and ischemia-induced apoptosis. VRT-043198 did not alter ischemia-induced apoptosis at concentrations up to 100 μM (see Supplemental Figure 1), consistent with its low potency against caspase-9 (Table 1). In addition, the effects of VRT-043198 on Fas-induced apoptosis were evaluated in the Jurkat human T-cell line, where initiation of the apoptosis cascade is mediated by caspase-8 activity. VRT-043198 only affected cell death at concentrations of 200 μM (about 50% inhibition) (see Supplemental Figure 2). Comparison of these apoptosis studies with the cytokine inhibition studies suggests that approximately 100-fold lower concentrations of VRT-043198 are required for anti-inflammatory activity than for caspase-8 mediated anti-apoptotic activity, consistent with the 100-fold difference in potency on isolated ICE/caspase-1 and caspase-8 enzymes (Table 1).

Oral administration of VX-765 resulted in high plasma concentrations of VRT-043198

To evaluate the pharmacokinetic properties of VX-765, plasma concentrations of VX-765 and VRT-043198 were monitored after oral dosing of VX-765 for 24 h in mice. Exposure of VRT-043198 was greater than that of VX-765 following oral administration of VX-765 at all dose levels tested (Table 3). The maximum plasma concentration (C_{max}) values of VRT-043198 observed in the mouse were higher than or close to *in vitro* IC_{50} values for inhibition of IL-1β and IL-18 in human PBMCs and whole blood (Table 2 and see above).

VX-765 inhibits LPS-induced IL-1β production *in vivo*

Intravenous injection of *E. coli* LPS in mice provokes a spike of serum IL-1β levels within 2-2.5 h. To evaluate the inhibition of ICE/caspase-1-mediated IL-1β production *in vivo*, a single oral dose of VX-765 (25, 50, 100 or 200 mg/kg) was administered 1 h before i.v. LPS injection and peripheral blood samples were harvested at 2.5 h and assayed for IL-1β concentrations. VX-765 doses 50, 100 and 200 mg/kg significantly ($p < 0.05$; Dunnett's ANOVA test) reduced serum IL-1β levels by as much as 60%, while 25 mg/kg had a smaller effect (~35% inhibition) that was not statistically significant (Figure 2). Of note, the effect of VX-765 on the release of IL-1β induced by LPS reached a plateau at 100 mg/kg. Although the reason for this ceiling effect is not clear, we speculate that intravenous injection of LPS may cause substantial release of pro-IL-

1β, which was detected by the ELISA system used in this study. Alternatively, IL-1β immunoreactivity may be produced by a processing mechanism other than ICE/caspase-1. Finally, the release of IL-1β may be regulated by two phases, i.e., acute induction and processing of IL-1β followed by a sub-acute process of IL-1β production mediated through production of other cytokines and/or mediators such as TNFα which is known to induce IL-1β production (Fong et al., 1989; Covert et al., 2005). It is quite possible that drug concentrations were not high enough to suppress the second process by the time it began.

VX-765 alleviates oxazolone-induced DTH dermatitis

DTH is an immune response characterized by large influx of inflammatory cells, of which macrophages are a major participant. DTH is considered to be a useful model to evaluate the efficacy of compounds on T cell function, cytokine production and signaling and inflammatory cell function. The role of ICE/caspase-1 and IL-1β has been studied in these models and ICE-deficient mice were shown to be resistant to contact sensitization induced by oxazolone and 2,4-dinitrofluorobenzene (Antonopoulos et al., 2001). VX-765 was evaluated therapeutically in the oxazolone-induced DTH model. Mice were sensitized with oxazolone applied to the abdominal skin and then challenged three days later with oxazolone applied to one ear. Mice were dosed orally with VX-765, 24 and 36 h after their ears were challenged. Prednisolone (5 mg/kg x 2) was used as a positive control (Murray et al., 1994). Biopsy samples were harvested from treated and control ears 48 h after the ear challenge. VX-765 (25, 50 and 100 mg/kg x 2) significantly

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reduced ear edema, as did prednisolone ($p < 0.05$, Dunnett's ANOVA test) (Figure 3). VX-765 also dose-dependently reduced the concentrations of cytokines, chemokines, and inflammatory mediators in the ear biopsy samples (Figure 4). The maximal effects of VX-765 (at 50 or 100 mg/kg x 2) were similar to those of prednisolone.

VX-765 attenuates CIA responses in mice

Cytokines appear to play a pivotal role in the pathogenesis of rheumatoid arthritis (RA). IL-1β has been detected in arthritic joints of patients with rheumatoid arthritis and administration of IL-1β is sufficient to induce arthritis in experimental animals (reviewed in van den Berg, 2002). The efficacy of VX-765 administered orally twice daily was evaluated in CIA, a mouse model of rheumatoid arthritis, using both prophylactic and therapeutic treatment regimens. VX-765 was well tolerated even at 100 mg/kg twice daily for 28 days and did not show substantial changes in body weight (data not shown). In the prophylactic study, compounds and vehicle were dosed beginning after booster immunization. In the therapeutic study, treatment was started when erythema and partial swelling of the tissue around the wrist joint were apparent (level 2) in both forepaws. Figure 5A & B shows the mean forepaw inflammation scores, evaluated every other day. In both studies, VX-765 induced a dose-dependent, statistically significant reduction in the inflammation scores ($p < 0.05$; Wilcoxon rank-sum test), and VX-765 (100 mg/kg) was as efficacious as prednisolone (5 mg/kg) (Ku et al., 1996; Rioja et al., 2004). Histological analysis of forepaws at the end of both studies revealed good correlation between the degree of joint structural damage and the inflammation scores (Table 4 and Figure 6). In the therapeutic study, a majority of mice in the vehicle-treated group

exhibited severe inflammation and extensive damage to bone and cartilage (Table 4 and Figure 6C). In contrast, mice treated with VX-765 showed pronounced protection from joint changes, with maximum benefit being similar to that observed with prednisolone (Table 4 and Figure 6D).

Discussion

Cytokines are implicated in a number of human immune and inflammatory diseases, and modulation of production of key cytokines has emerged as an important therapeutic approach. Increased production of IL-1β and IL-18 has been demonstrated in patients with inflammatory diseases such as psoriasis, RA and Crohn's disease. A recombinant form of human IL-1Ra (anakinra) has been shown to be effective in clinical trials of RA and is approved for the treatment of RA (reviewed in Braddock and Quinn, 2004). In addition, anakinra markedly suppressed inflammatory manifestations of autoinflammatory syndromes, including Muckle-Wells syndrome, neonatal onset multi-system inflammatory disease, familial cold autoinflammatory syndrome (FCAS) (reviewed in Ting et al., 2006) and systemic onset juvenile idiopathic arthritis (Pascual et al., 2005) and adult Still's disease (Fitzgerald et al., 2005).

In this report we characterized another approach to suppress excessive inflammation in diseases by inhibiting the ICE/caspase-1 subfamily of caspases essential for generation of biologically active IL-1β and IL-18. Previously we developed an ICE/caspase-1 inhibitor, pralnacasan, and found it to be effective in a number of pre-clinical models and

in RA patients (Ku et al., 2001; Rudolphi et al., 2003; Loher et al., 2004). VX-765 was developed from a structurally distinct class of caspase-1 inhibitors that share with pralnacasan the aspartyl-hemiacetal ester moiety that serves as a prodrug. VRT-043198, the active metabolite of VX-765, is a potent, specific inhibitor of the caspase-1 subfamily. VRT-043198 inhibits the release of IL-1β and IL-18 from human monocytes *in vitro* and their production *in vivo* in models of inflammation. Note that we recently studied PBMCs from FCAS patients, which produce high levels of IL-1β and IL-18 upon LPS stimulation. Consistent with the current report, VX-765 inhibited the release of IL-1β and IL-18 in FCAS patient PBMCs and equally potent at inhibiting the release of IL-1β in both normal and FACS patient PBMCs (Stack *et al*. 2005). VRT-043198 exhibited little or no effect on cell apoptosis and proliferation, as expected based on observations in caspase-1-deficient mice. This activity profile endows VX-765 with beneficial activity in mouse models of dermatological and bone and joint inflammation and autoimmunity.

We used the mouse DTH model to evaluate the effect of VX-765 on skin inflammation and to provide support for its use in inflammatory skin disorders such as psoriasis and atopic dermatitis. DTH responses are typically measured as skin reactions to haptens and mediated by inflammatory cytokines (Askenase, 2001). In particular, IL-18 is constitutively expressed at high levels in macrophages, keratinocytes and Langerhans cells (LCs) (Stoll et al., 1998; Naik et al., 1999). LCs are a key component of DTH responses in the skin against reactive molecules such as oxazolone and picryl chloride. IL-18 has been shown to play an essential role in migration of LCs into draining lymph nodes by an IL-1β– and TNFα-dependent mechanism (Cumberbatch et al., 2001). IL-18

has also been implicated in IL-12-driven IFN-γ production by T_H1 cells, thus contributing to DTH responses in the skin (Wang et al., 2002). As shown in Figure 4, VX-765 inhibited the release of IL-1β and IL-18 in a dose-dependent manner resulting in reduced production of IFNγ and chemokines. Reduction of theses inflammatory mediators presumably contributed to diminished T_H1 responses and recruitment of neutrophils and macrophages, the latter reflected in decreased levels of myeloperoxidase and nitric oxide in the ear biopsy samples from mice treated with VX-765. Moreover, the highest dose of VX-765 suppressed production of IL-4, whereas prednisolone did not. In DTH responses, IL-4 is produced by NK T cells and promotes activation of a subset of B cells. Although IL-18 is an inducer of the T_H1 cytokine IFN-γ, IL-18 is known to induce the expression of the T_H2 cytokines, IL-4 and IL-13, in T cells, NK cells, mast cells and basophils (Hoshino et al., 2001). Thus, it is most likely that decreased IL-18 production by VX-765 resulted in reduction of IL-4 in the inflamed ear discs. Taken together, our results in the DTH model suggest that VX-765 may be useful in the treatment of inflammatory skin disorders such as psoriasis and atopic dermatitis.

RA is a chronic syndrome characterized by systematic inflammation of peripheral joints, potentially resulting in destruction of articular and periarticular structures. Although no animal model fully represents the patho-physiological changes in human RA patients, mouse CIA is widely used as a surrogate for analyzing pathogenic mechanisms of joint inflammation and evaluating therapeutic agents (Iwakura, 2002). Mouse CIA is induced by immunization with type II collagen to a susceptible strain of mouse and is mediated by both humoral and cellular immunity. Similarities between mouse CIA responses and

human RA include linkage of disease to genes located in the histocompatibility locus, mononuclear cell infiltration, pannus development, fibrin deposition, erosion of cartilage and bone destruction. In addition, inflammatory cytokines such as IL-1β, TNFα and IL-6 are also produced in CIA joints (Rioja et al., 2004). Although high levels of TNF-α are produced in the synovial fluid of RA patients (Neidel et al., 1995), analyses of paw tissues from CIA studies in animals indicate that arthritic joints of CIA produce low levels of TNF-α compared with IL-1β and IL-6 (Rioja et al., 2004). Consistent with these data, blockade of TNF-α is partially effective at an early stage of CIA, whereas blocking of IL-1, either right after the onset or during established CIA, effectively suppresses progression of arthritis (Joosten et al., 1996). In mice deficient in TNF-α or TNF receptor, the incidence of CIA is reduced. However, once initial signs of arthritis develop, the condition tends to progress to full-blown, destructive arthritis in the mice (Mori et al., 1996; Campbell et al., 2001). In contrast, IL-1α/β-deficient mice are markedly resistant to development of CIA and mice deficient in either IL-1α or IL-1β exhibit reduced progression of CIA (Saijo et al., 2002). Thus, TNF-α plays a role mainly in the early stage of CIA whereas IL-1 plays a more pivotal role both in the onset and progression of the disease (van den Berg, 2002). Here we show that caspase-1 inhibition with VX-765 delayed the onset of arthritis and suppressed progression of the disease when dosed prophylactically and reversed paw inflammation and prevents joint damage in established disease when dosed therapeutically. This suggests the potential of VX-765 for therapeutic benefit against established arthritis in RA patients. It is noteworthy that IL-1 is linked to osteoclast activation by inducing expression of RANKL (receptor activator of the NF-κB ligand) resulting in bone resorption and strongly suppresses

aggrecan synthesis causing cartilage erosion (Goldring and Gravallese, 2000). This is highlighted by the fact that IL-1 deficient mice or mice treated with IL-1Ra do not develop joint erosion whereas treatment with anti-TNFα antibodies or soluble TNF receptor protein in CIA models does not result in any measurable effect on cartilage or bone destruction (Wooley et al., 1993; Joosten et al., 1996; Saijo et al., 2002). Given that VX-765 treatment reduced histological changes in the prophylactic regimen and resulted in significant improvement of joint histology in the therapeutic regimen comparable to treatment with prednisolone (Table 4 and 5), VX-765 may act as a disease-modifying agent in the treatment of RA.

Several therapeutic agents targeting IL-1 and IL-18 have been tested in clinic for inflammation (reviewed in Braddock and Quinn, 2004). The data described in this report confirm the therapeutic promise of a novel approach, selective ICE/caspase-1 inhibition. VX-765 is a potent and selective ICE/caspase-1 inhibitor that reduces the production of IL-1β and IL-18 both *in vitro* and *in vivo* in correlation with tissue protective effects in animal models of inflammatory disease. VX-765 is currently in clinical trials in inflammatory and autoimmune indications, the results of which will be reported separately.

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Legends for Figures

Figure 1 Structure of VX-765 and its active metabolite, VRT-043198.

Figure 2 Dose-response of VX-765 in LPS-induced IL-1β production *in vivo*. VX-765 (25, 50, 100 or 200 mg/kg) was administered by oral gavage 1h prior to the intravenous LPS challenge. Blood samples were collected 2.5 h after the LPS challenge serum IL-1β was assayed by specific ELISA. Data are means ± SD. Dunnett's ANOVA test was performed for statistical analysis (*, $p < 0.05$ when compared with the LPS-control group).

Figure 3 Effects of VX-765 on ear swelling induced by oxazolone. Forty-eight hours after the challenge on the right ear, biopsy samples 9 mm in diameter were taken from both ears of each animal and weighed. Data are expressed as the difference in weight between the right and left ear discs of animal (n = 9/group, means ± SD). Dunnett's ANOVA test was performed for statistical analysis (*, $p < 0.05$ when compared with the vehicle-treated group). Similar results were obtained from at least two independent experiments.

Figure 4 Effects of VX-765 on production of inflammatory mediators in biopsy samples from oxazolone-challenged mouse ears (n = 6/group). Cytokines (A-C), nitric oxide (D), myeloperoxidase (E) and chemokines (G-I) were quantified by specific ELISA

kits. Data are means ± SD. Dunnett's ANOVA test was performed for statistical analysis (*, $p < 0.05$ when compared with control). Similar results were obtained from at least two independent experiments.

Figure 5 Effects of VX-765 on forepaw inflammation in the mouse CIA model administered in either the prophylactic (A) or therapeutic (B) regimen. In the prophylactic study, mice (n = 4-6/group) were treated orally twice daily with vehicle, prednisolone (5 mg/kg) or VX-765 (25, 50 or 100 mg/kg) after the second immunization of the type II collagen for 28 days. In the therapeutic regimen, mice (9-10/group) were allowed to develop level 2 inflammation in both forepaws before assignment to treatment groups (n = 10/group) and then treated with either vehicle, prednisolone (5 mg/kg BID) or VX-765 (10, 25, 50 or 100 mg/kg BID) for 24 days. Paw inflammation was scored every other day. Scores are reported as the average of the sum of inflammation scores from both forepaws of each mouse in a given treatment group with standard deviation. Similar results were obtained from two independent experiments both in the prophylactic and therapeutic dosing regimens.

Figure 6 Histological examination of representative forepaw wrist joints from the prophylactic and therapeutic studies. Joint issues were harvested at the end of studies and stained with hematoxyline and eosin. In the prophylactic study, a representative forepaw joint from a mouse treated with the vehicle (A) shows substantial cartilage erosion and synovial infiltration in the joint space while in a forepaw from an animal treated with VX-765 (100 mg/kg) exhibits minimal signs of such changes (B). Bone erosion in addition to synovial infiltration and cartilage erosion is obvious in a representative joint

from a mouse treated with the vehicle in the therapeutic study (C). These changes were not observed in a mouse treated therapeutically with VX-765 (100 mg/kg) (D).

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Table 1 Ki values for VRT-043198 against caspases and non-caspase proteases.

Caspases		**Non-Caspase Proteases**	
Enzyme	**Ki (nM)**	**Enzyme**	**Ki (nM)**
Caspase-1 (ICE)	0.8	Granzyme B	9000
Caspase-4	<0.6	Cathepsin B	48% inhibition at 100 μM
Caspase-8	100	Trypsin	4% inhibition at 100 μM
Caspase-6	560		
Caspase-9	1030		
Caspase-7	16000		
Caspase-3	21500		

Table 2 IC_{50} values (mean ± SD) for VRT-043198 inhibition of SAC-stimulated cytokine release from human PBMCs *in vitro*.

Cytokine	**IL-1β**	**IL-18**	**IFNγ**	**TNFα**
N	9	6	5	2
IC_{50} (μM)	0.87 ± 0.77	2.8 ± 3.5	5.6 ± 1.3	>50

N = Number of determinations in separate experiments

Table 3 Plasma pharamacokinetic parameters of mice dosed orally with VX-765.

Dose of VX-765 (mg/kg)	**VX-765 Parameters**			**VRT-043198 Parameters**		
	C_{max} μg/mL	**T_{max} h**	**AUC_{last} μg.h/mL**	**Cmax μg/mL**	**T_{max} h**	**AUC_{last} μg.h/mL**
10	0.18	0.25	0.27	0.66	0.25	0.40
21	0.23	3.0	0.91	2.14	0.17	1.90
43	0.49	2.0	1.37	2.12	0.17	2.63
84	0.78	1.0	2.06	3.26	0.17	8.66

Table 4 Histological assessment of forepaws in the collagen-induced arthritis experiments with prophylactic or therapeutic dosing of VX-765

Group	# of joints	Histological scores[§]	# of joints with cartilage erosion	# of joints with bone changes
Prophylactic dosing experiment				
Vehicle	9	1.83 ± 0.50	9	0
VX-765 25 mg/kg	10	1.50 ± 0.58	4	0
VX-765 50 mg/kg	8	1.25 ± 0.42*	2	0
VX-765 100 mg/kg	9	1.13 ± 0.35*	1	0
Therapeutic dosing experiment				
Vehicle	7	2.93 ± 1.02	7	4
VX-765 10 mg/kg	10	1.70 ± 0.54*	7	1
VX-765 25 mg/kg	9	1.22 ± 0.44*	2	0
VX-765 50 mg/kg	10	1.15 ± 0.34*	2	0
VX-765 100 mg/kg	9	1.11 ± 0.22*	2	0
Prednisolone	10	1.20 ± 0.35*	3	0

[§] Values are shown as the mean ± SD

* $p < 0.05$ by Dunnett's ANOVA test compared with the vehicle control

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Figure 1

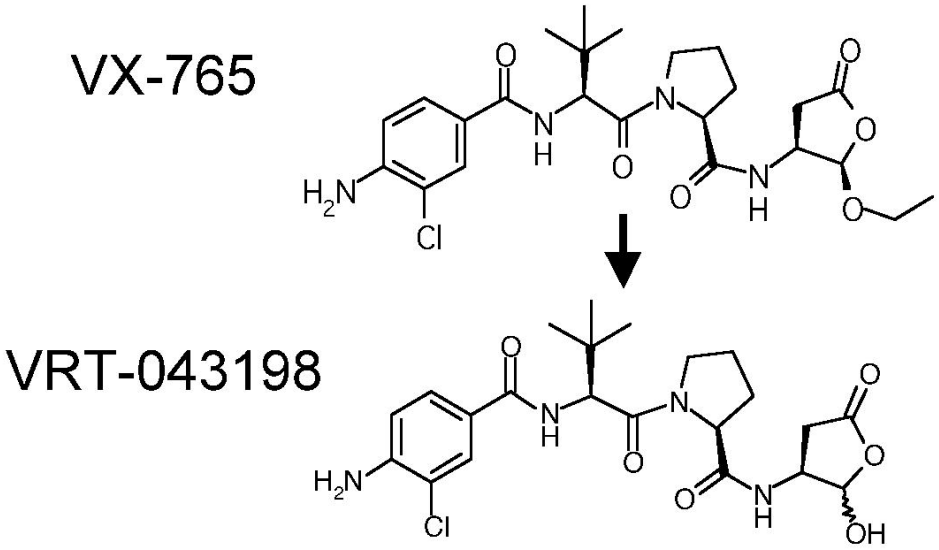

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Figure 2

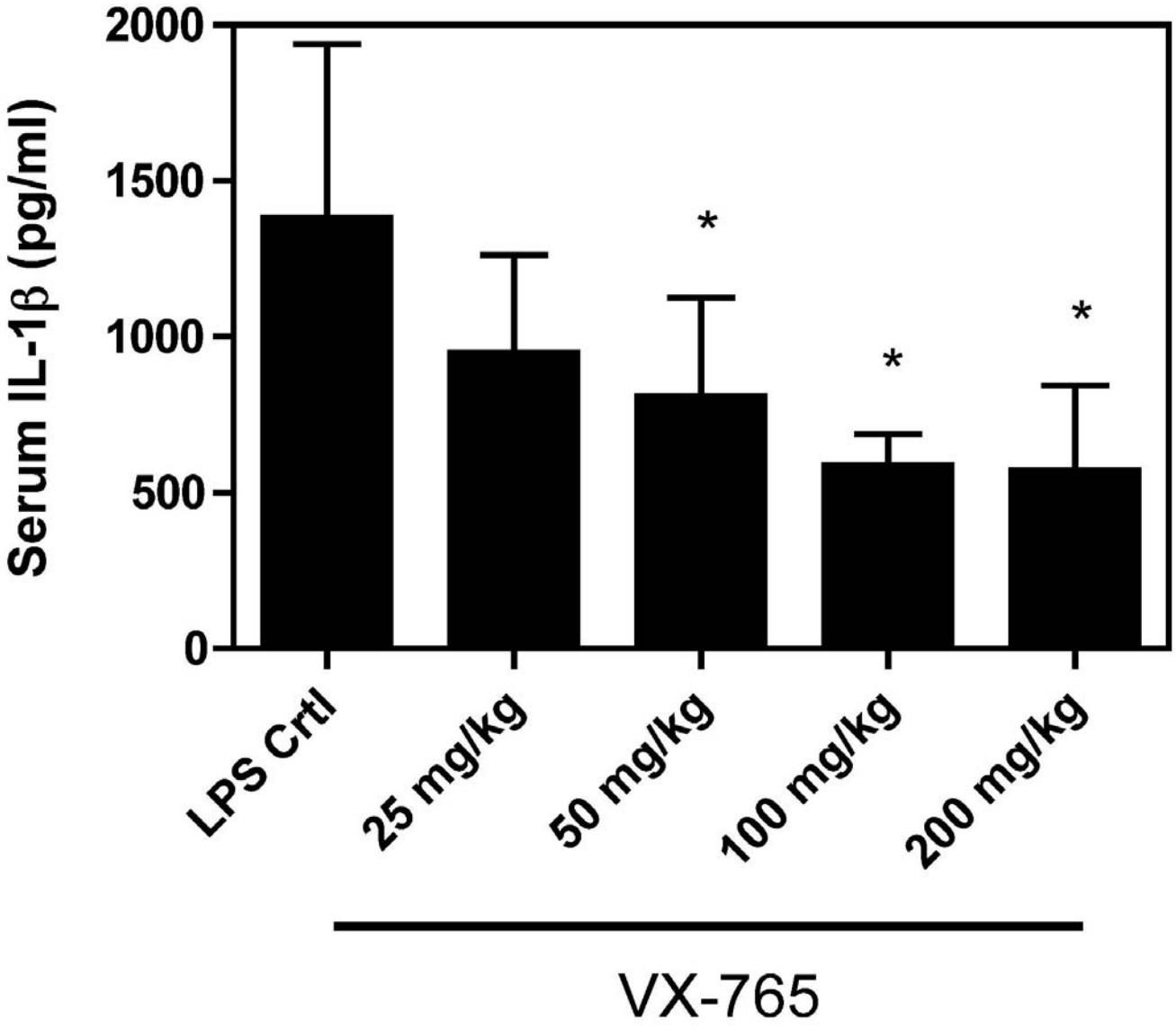

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Figure 3

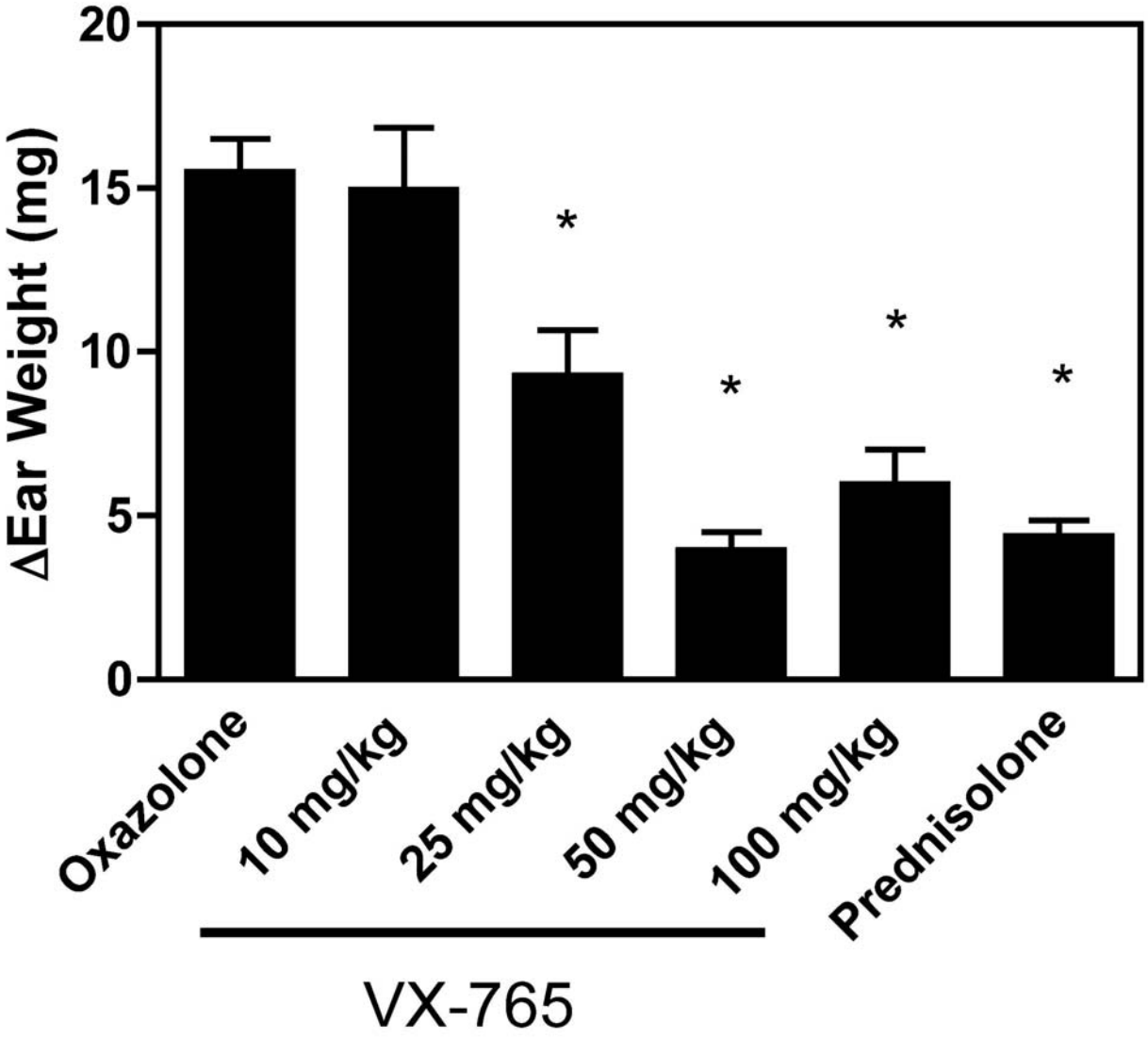

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Figure 4

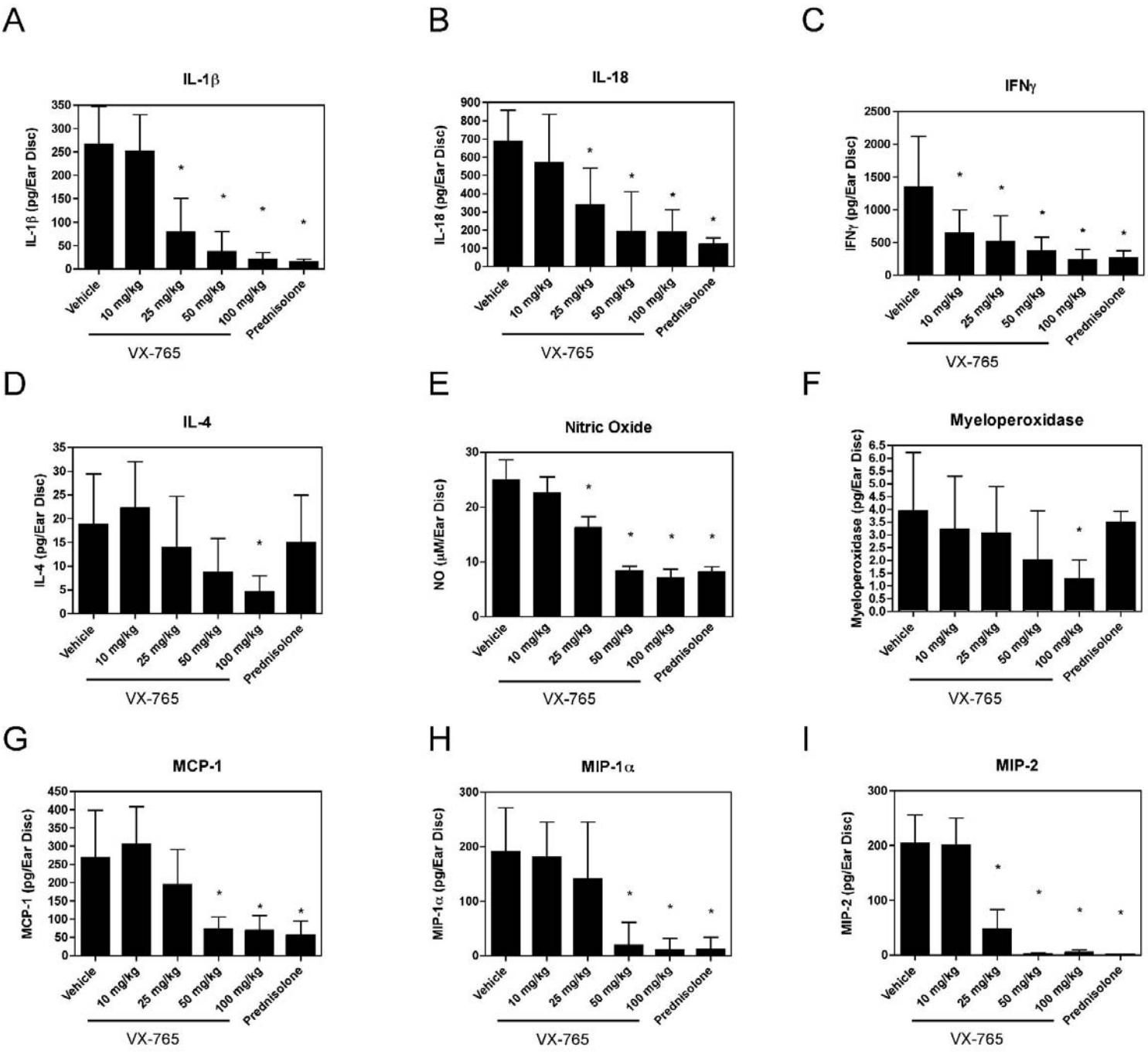

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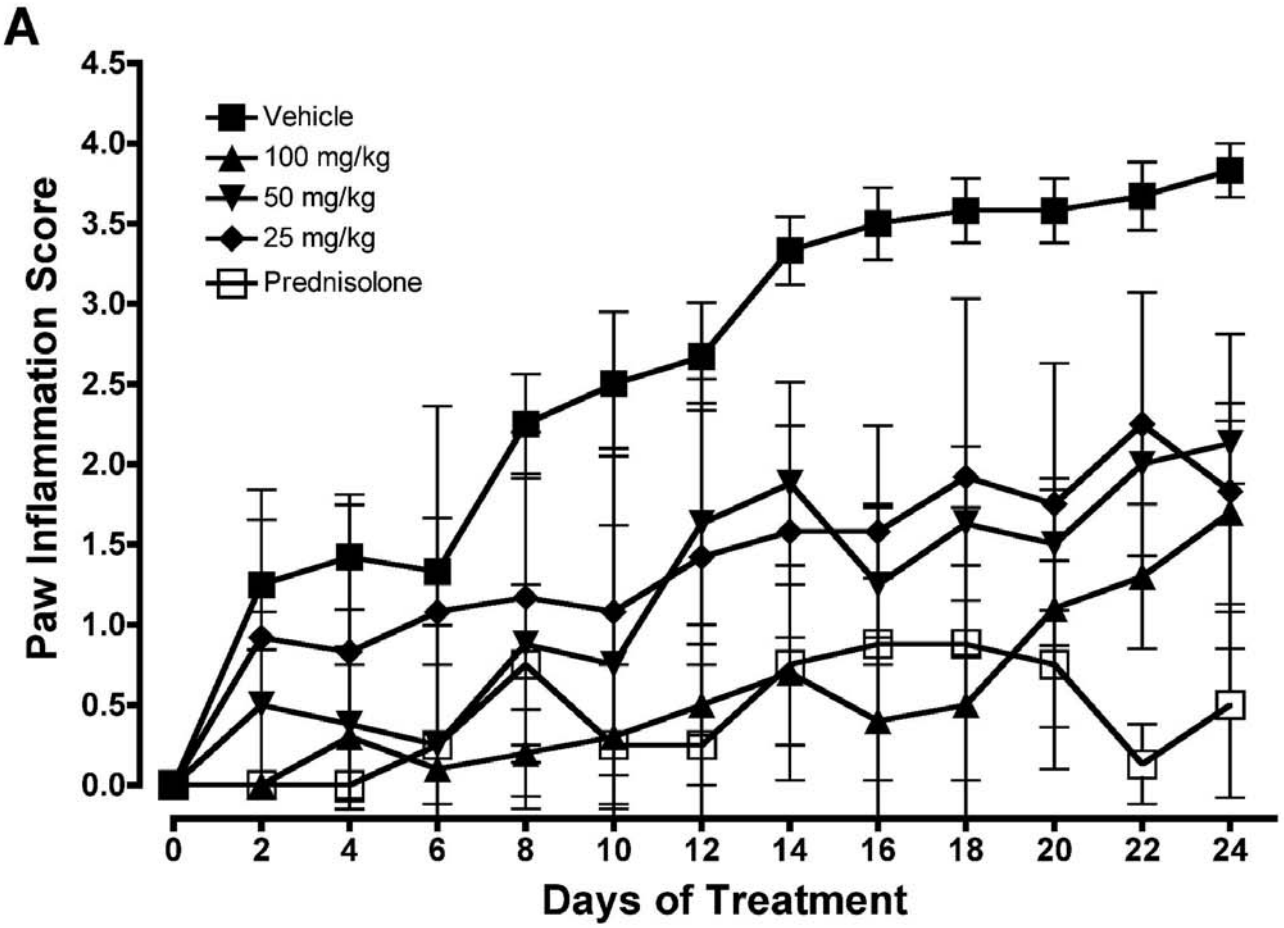
Figure 5



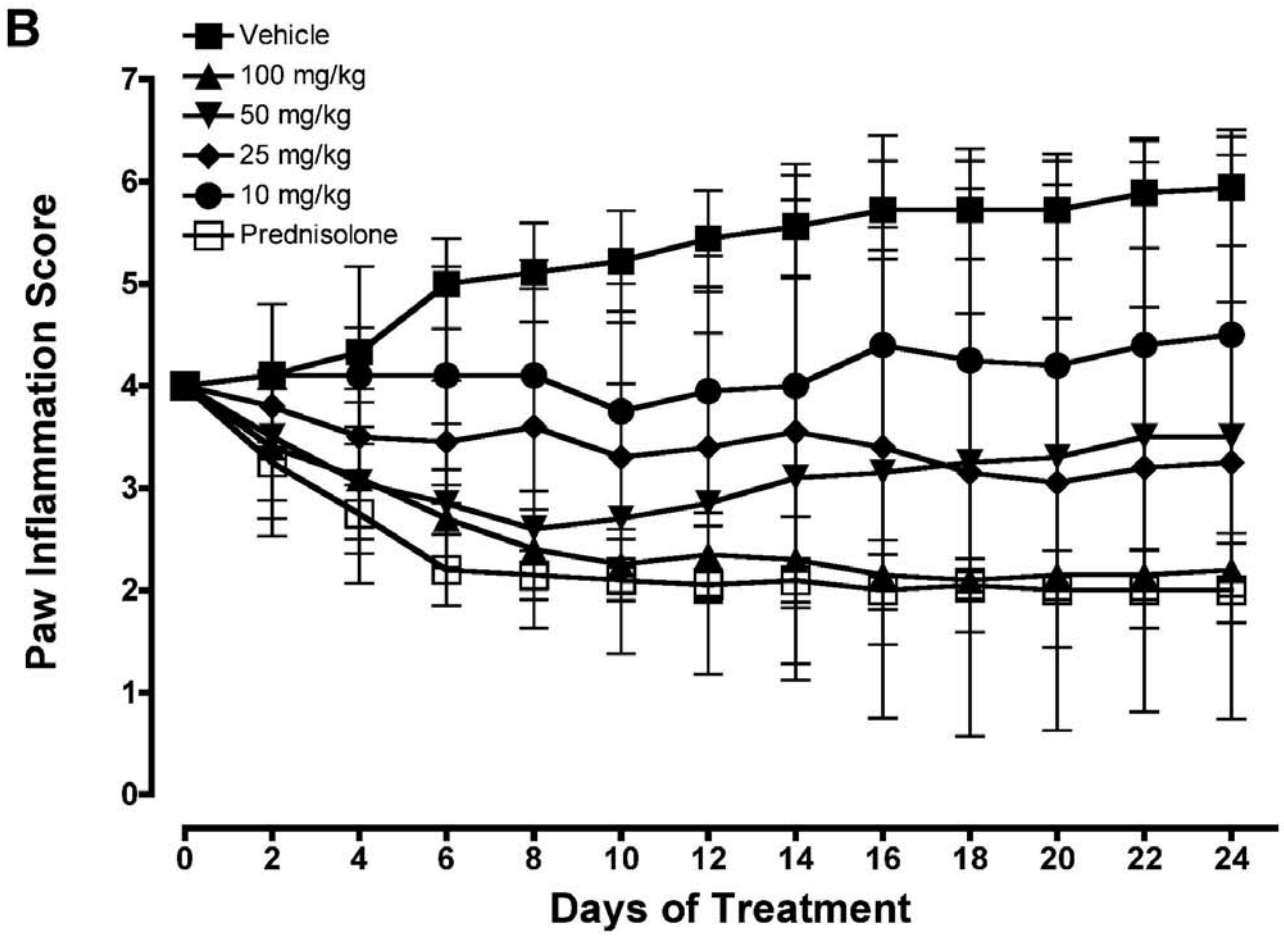


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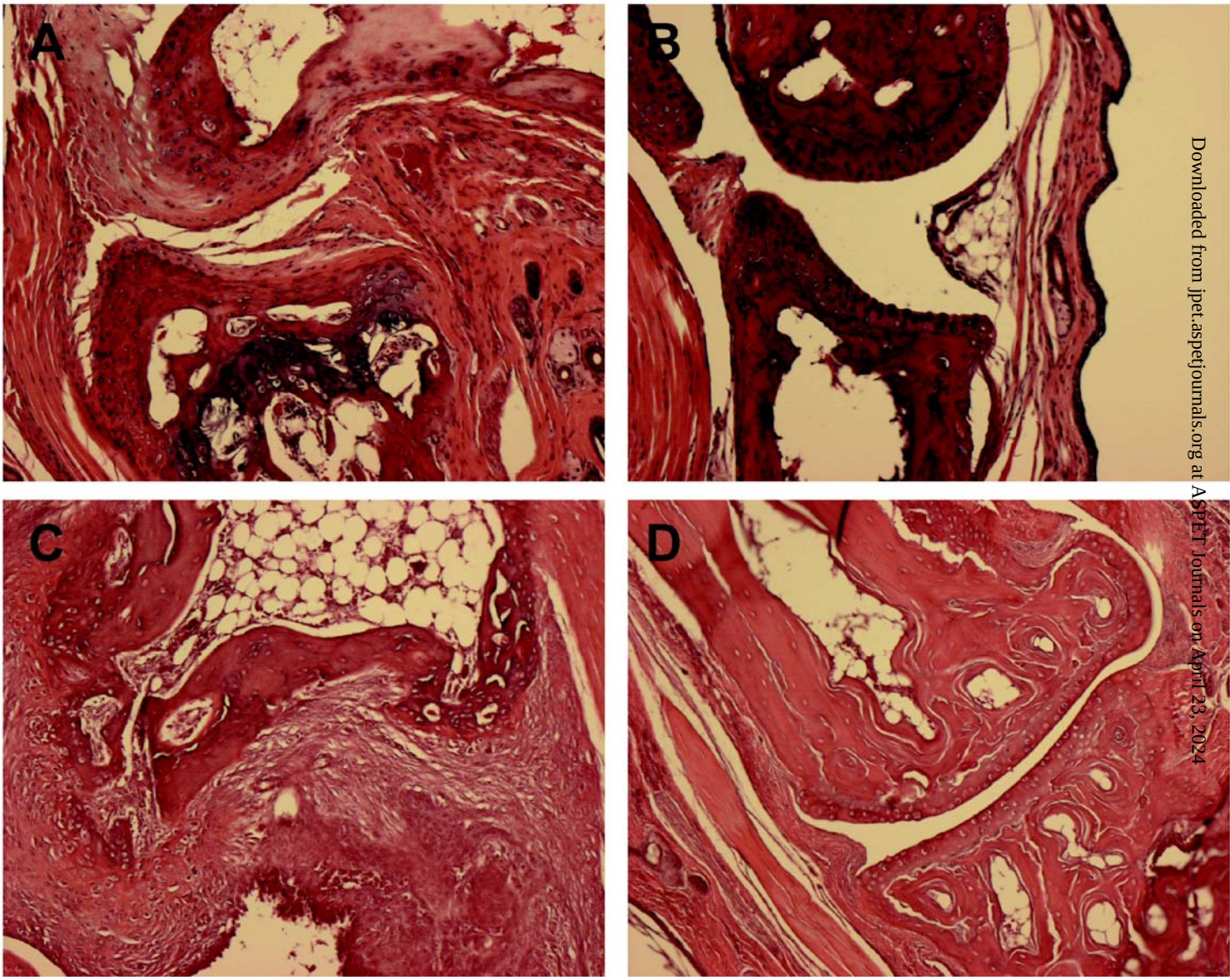
Figure 6



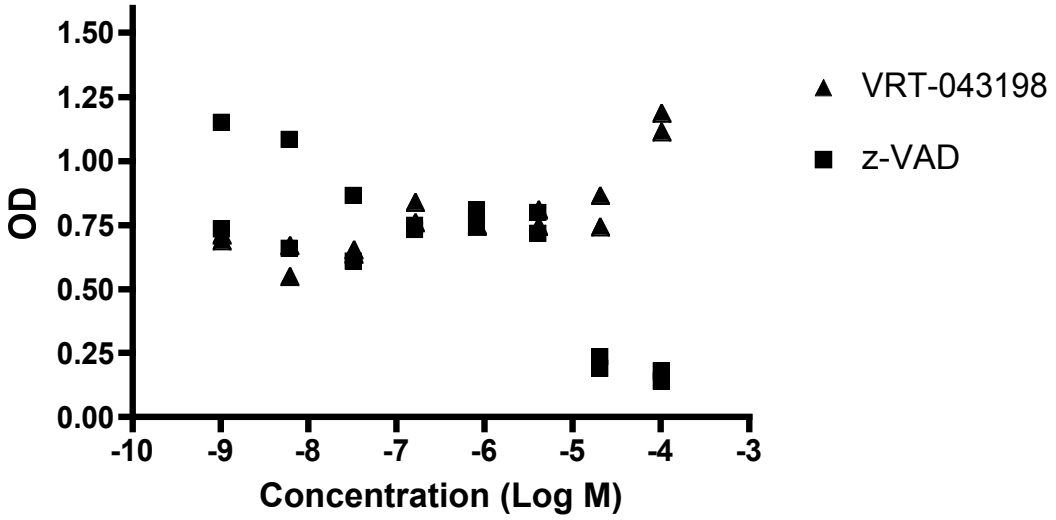


Figure 1 Dose response curve for VRT-043198 (triangles) in hypoxia-induced apoptosis in NT2 cells. NT2 cells were incubated with 100% N_2 at 37°C for 18 h in the presence or absence of compound. Apoptosis was measured by determination of the OD_{405} using a Cell Death Detection ELISA kit (Roche Diagnostics). Individual data are shown in this graph. z-VAD-fmk (squares) was used as positive control. An average of OD_{405} values in hypoxic NT2 cells treated only with DMSO was 1.40.

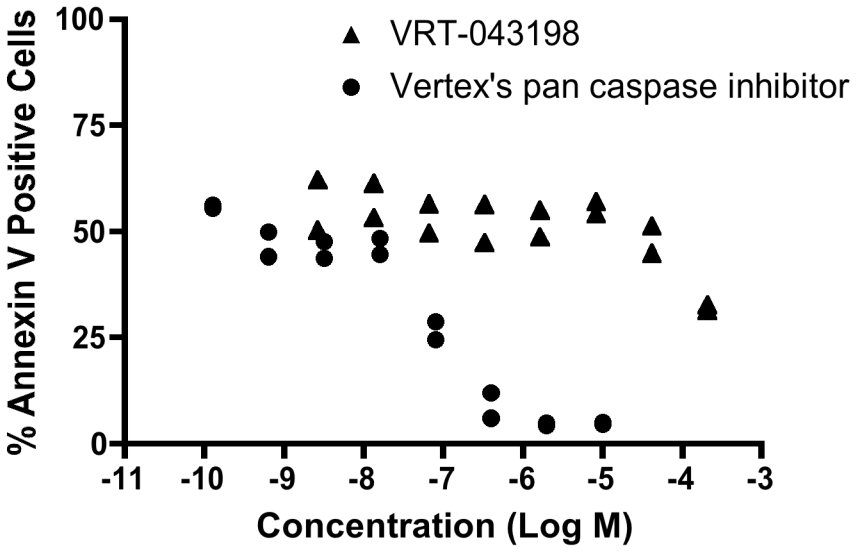


Figure 2 Dose response curve for VRT-043198 (triangles) and Vertex's pan-caspase inhibitor (circles) in anti-Fas antibody-induced apoptosis in Jurkat cells. Cells were incubated at 37°C for 18 h in the presence of anti-Fas antibody at 10 ng/ml (clone CH-11, Upstate) and then stained with Cy5-conjugated Annexin V. Apoptotic cells were measured by flow cytometry. Individual data are indicated as the percentage of AnnexinV positive cells. Anti-Fas antibody induced 60.3 ± 4.53% of Annexin V positive cells whereas naïve Jurkat cells only treated with 0.2% DMSO showed 6.26 ± 0.93% of apoptotic cells. The assay for those controls was done in quadruplicate. Two other experiments exhibited similar results.