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European Journal of Pharmacology 538 (2006) 168–174
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# An orally active reversible inhibitor of cathepsin S inhibits human trans vivo delayed-type hypersensitivity

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Received 19 December 2005; received in revised form 16 March 2006; accepted 22 March 2006
Available online 28 March 2006

**Abstract**

Cathepsin S is a major histocompatibility complex (MHC) class II associated invariant chain (Ii) degrading enzyme expressed in antigen presenting cells such as B cells and dendritic cells. This enzyme is essential for MHC class II associated antigen processing and presentation to $CD4^+$ T cells. Compound I, a selective, reversible and orally bioavailable, inhibitor of cathepsin S, with molecular $IC_{50}=9$ nM, has been recently described. We have tested the effects of compound I in a trans vivo model of delayed-type hypersensitivity. Human peripheral blood mononuclear cells ($7–10\times10^6$) from tetanus-sensitized donors were co-injected with tetanus toxoid (0.25 Lf) into C57Bl/6 mouse footpads. At 24 h, significant footpad swelling (+0.024±0.001 cm) characterized by an influx of mouse neutrophils and monocytes was observed. Injection of peripheral blood mononuclear cells alone caused negligible swelling (0.002±0.0002 cm). Anti-human MHC class II (HLA-DR, DP, DQ) antibody (5 mg/kg, i.p.) inhibited the swelling 91±7%, thus demonstrating a role of human antigen presenting cells in this model. Compound I (10, 30, and 100 mg/kg, p.o.) inhibited the response with an ED50 of ~18 mg/kg. Compound III, a less active analogue (molecular IC50>20 μM) had no effect. Furthermore, pretreatment of peripheral blood mononuclear cells with 10 nM compound II, an irreversible inhibitor (molecular IC50=11 nM) inhibited swelling 87±4%. These findings support the role of cathepsin S in human delayed-type hypersensitivity. Inhibition of cathepsin S with compound I may be useful in the treatment of human autoimmune diseases like rheumatoid arthritis and multiple sclerosis.
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*Keywords:* Cathepsin S; Delayed-type hypersensitivity; Antagonist

## 1. Introduction

The cysteine endoprotease cathepsin S mediates degradation of the major histocompatibility complex (MHC) class II associated chaperone invariant chain (Ii) in human and mouse antigen presenting cells. Cathepsin S is principally expressed in bone marrow derived antigen presenting cells, namely dendritic cells, B cells, and macrophages that act as the key antigen presenting cells of the immune system. Cathepsin S is highly expressed in the spleen, professional antigen presenting cells and other class II-positive cells (Shi et al., 1992; Shi et al., 1994). Cathepsin S, given its broad pH profile, is probably active throughout the endocytic pathway with capacity to mediate efficient invariant chain degradation in early and late endosomes as well as mature lysosomes (Driessen et al., 1999).

α, β-heterodimers of MHC class II are assembled in the endoplasmic reticulum with the assistance of the invariant chain (Ii) chaperone molecule. Upon entering the endosomes, Ii is degraded stepwise by lysosomal proteases. Cathepsin S is one of the key proteases involved (Chapman, 1998; Nakagawa and Rudensky, 1999; Villadangos et al., 1999; Villadangos and Ploegh, 2000). Internalized antigenic proteins are also degraded by lysosomal proteases in the endosomes. The MHC class II peptide-binding groove is protected from premature peptide loading by a fragment of Ii, known as the class II-associated invariant-chain peptide. HLA-DM in humans and H-2M in mice mediate the exchange of class II-associated invariant-chain

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doi:10.1016/j.ejphar.2006.03.051

peptide for the antigenic peptide. MHC class II molecules bind antigens within antigen presenting cells and then display these peptide antigens on the cell surface to interact with and activate $CD4^+$ T cells. Activation of antigen-specific $CD4^+$ T cells, results in immune responses involving B cells, macrophages, and $CD8^+$ T cells. These cellular responses defend against infectious agents but can also mediate autoimmune diseases. In cathepsin S deficient mice, B cells and dendritic cells do not convert invariant chain fragment p10 to class II-associated invariant-chain peptide and accumulate class-II-p10 complexes (Shi et al., 1999; Nakagawa et al., 1999).

Cathepsin S inhibitors have been shown to block antigen presentation in vitro and in vivo (Riese et al., 1996; Villadangos et al., 1997; Fiebiger et al., 2001). In vivo treatment with cathepsin S inhibitors results in reduced inflammation in rat adjuvant arthritis (Biroc et al., 2001), and also inhibits disease in a murine model of Sjögren syndrome (Saegusa et al., 2002). Inhibition of cathepsin S attenuates the ability of MHC class II to load and present antigenic peptide and thus provides a potential therapeutic mechanism for modulating autoimmunity. This approach involves indirect targeting of specifically MHC class II-restricted $CD4^+$ T cells. Since T-cells are not targeted directly, this approach may have the potential to minimize and/or avoid side effects associated with commonly prescribed immunosuppressants.

A novel, reversible and selective cathepsin S inhibitor, compound I, was recently identified. Compound I is a dipeptide nitrile (structure in Fig. 1A) with low nanomolar molecular potency. Accumulation of the invariant chain fragment p10, a substrate for cathepsin S in a cellular assay, demonstrates that the molecular target is inhibited with a minimal inhibitory concentration of 500 nM. Compound I selectively inhibits cathepsin S in an enzymatic and cellular assay as well as in vivo as evidenced by the accumulation of invariant chain fragment p10 in the splenocytes of $H\text{-}2A^b$ and $H\text{-}2A^d$ allele mice (Ward et al., 2002; Cywin et al., 2003).

Trans vivo human delayed-type hypersensitivity (Carrodeguas et al., 1999) is a humanized mouse model for testing compounds against human targets. The model is relatively rapid and simple, and an effective alternative to human skin testing for measuring human delayed-type hypersensitivity responses. The response requires presentation of tetanus toxoid antigen by human antigen presenting cells to human T cells injected into mouse foot pads. Use of this model allows for the testing of in vivo effects of compounds in a quick and efficient manner and alleviates the use of complex experiments in non-human primates that require more time and are resource intensive. In this model, we have shown that the delayed-type hypersensitivity response requires human MHC class II mediated antigen presentation and it was inhibited by treatment of the mice with anti-human MHC class II (HLA-DR, DP, DQ) antibody. The relevance of utilizing a delayed-type hypersensitivity protocol is that this reaction is regarded as a hallmark of T helper-1 mediated autoimmune diseases. The goal of the present study was to test the potency of the reversible cathepsin S inhibitor, compound I, and the irreversible inhibitor, compound II (structure in Fig. 1B), in the humanized mouse model of trans vivo delayed-type hypersensitivity. Compound III (structure in Fig. 1C), a less active analogue (molecular $IC_{50}>20$ μM) was also tested.

## 2. Materials and methods

### 2.1. Animals

Female C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 6–8 weeks of age. Cathepsin S knockout mice were generously supplied by Dr. Harold Chapman and are described (Shi et al., 1999). All mice were housed and experiments were conducted in conformity with the National Research Council's, "Guide for the Care and Use of Laboratory Animals." All animal experiments were reviewed by and approved by the institutional Animal Care and Use Committee at Boehringer Ingelheim Pharmaceuticals Inc.

### 2.2. Peripheral blood mononuclear cells

Blood was collected by venipuncture from normal human donors that were known to be good tetanus responders. One hundred milliliters of whole blood was drawn into Vacutainer® CPT™ tubes (Becton Dickinson, Franklin Lakes, NJ), spun at 1800×*g* for 30 min, and the buffy layer containing mononuclear cells and platelets was separated, washed three times, resuspended in phosphate-buffered saline (PBS) and counted. Platelet contamination was minimized by multiple washes in PBS. No more than 1:1 ratio of platelets to peripheral blood mononuclear cells was allowed. The cells were maintained on ice until injection into the mice. Aluminum phosphate-adsorbed tetanus toxoid (Tetguard, BI-Vetmedica, Inc. St. Joseph, MO) was used at a concentration of 0.25 Lf per injection site. (Lf unit is the flocculation value;

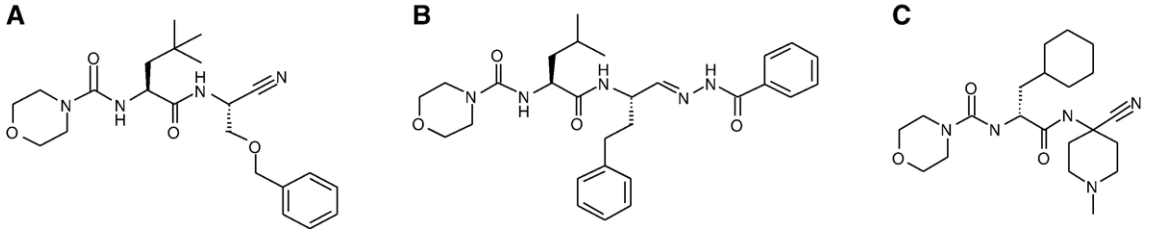


Fig. 1. (A) Structure of compound I, a reversible inhibitor of cathepsin S. (B) Structure of compound II, an irreversible inhibitor of cathepsin S. (C) Structure of compound III, an analogue with low activity against cathepsin S.

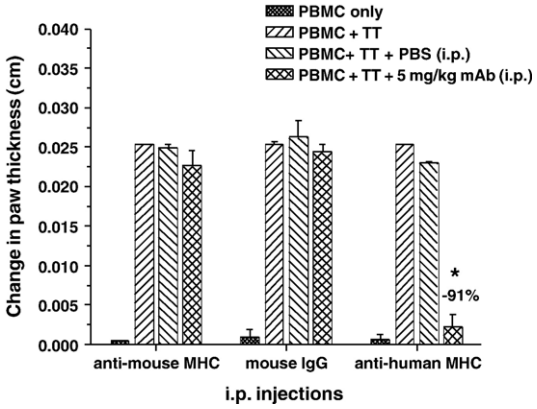


Fig. 2. Effect of anti-human MHC class II antibody on trans vivo delayed-type hypersensitivity. The anti-human MHC class II (HLA-DR, DP, DQ) antibody clone TU39, mouse IgG, its isotype control, or anti-mouse MHC class II antibody were injected i.p. at 5 mg/kg 1 h before footpad injections of peripheral blood mononuclear cells (PBMC) alone or with tetanus toxoid (TT). The mean± S.E.M. changes in paw thickness from 4 different donor peripheral blood mononuclear cell experiments are plotted. *$P<0.05$ versus vehicle control by analysis of variance (ANOVA).

the amount of toxoid which mixed with one International Unit of antitoxin produces an optimal flocculating mixture).

### 2.3. In vitro experiments

Antibodies and compounds were tested in an in vitro T-cell proliferation assay run in parallel with the in vivo assay. Peripheral blood mononuclear cells from the same donors were used in vivo and in vitro. Peripheral blood mononuclear cells were cultured in serum free media, AIM V (Invitrogen, Carlsbad, CA) substituted with HEPES, non-essential amino acids, sodium pyruvate and 2-mercaptoethanol, with or without tetanus toxoid and test agents in a humidified incubator (37 °C, 5% $CO_2$). On the fourth day culture wells were pulsed with 0.5 μCi tritiated thymidine and incubated overnight. The cells were harvested using a Skatron cell harvester on filter mats and counted in a beta counter. Proliferation was expressed as stimulation index (SI=mean cpm of stimulated wells divided by mean cpm of non-stimulated wells). Peripheral blood mononuclear cells from 4 donors per treatment were tested. The anti-mouse and anti-human MHC class II antibodies were diluted in PBS. Compounds I, II and III were dissolved in dimethyl sulfoxide and diluted 1000 times with culture media.

### 2.4. Trans vivo delayed-type hypersensitivity

$7–10\times10^6$ peripheral blood mononuclear cells mixed with 0.25 Lf units of tetanus toxoid in a total volume of 25–40 μl were injected into the hind footpads of mice. Footpad thickness was measured prior to injection and 24 h post-injection, using a dial thickness gauge (Mitutoyo, Aurora, IL). Pre-injection thickness was subtracted from post-injection thickness to obtain the change in paw thickness. All measurements were made in inches and converted to millimeters. Unless otherwise stated a minimum of four human donors and eight mice were used per dose or treatment.

### 2.5. Ex vivo pretreatment of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were treated with compound II at 10–1000 nM in vitro and incubated for 30 min at 37 °C, cells were spun down and the supernatant was removed. The pelleted cells were resuspended with or without tetanus toxoid in PBS and injected into the footpads of naïve mice. The footpad swelling was measured 24 h later.

### 2.6. In vivo testing

Anti-human and anti-mouse MHC class II antibodies and their isotype controls were tested at single doses of 5 mg/kg given i.p. 1 h before footpad injection. Antibodies were prepared in PBS. Compound I was dissolved in 30% Cremophor (Cremophor RH40; BASF, Ludwigshafen, Germany) and dosed orally at 10, 30 and 100 mg/kg at −0.5 and 4 h, post paw injection (peripheral blood mononuclear cells with or without tetanus toxoid). Compound I and a less active analogue, compound III, were tested with four and two different donors' peripheral blood mononuclear cells, respectively. Percent inhibition was calculated as:

$$100\times(\Delta\text{paw thickness}_{\text{vehicle}}-\Delta\text{paw thickness}_{\text{drug}})/(\Delta\text{paw thickness}_{\text{vehicle}}-\Delta\text{paw thickness}_{\text{cells only}}).$$

### 2.7. Statistics

All values are expressed as the mean±S.E.M. Statistical comparisons were done by an analysis of variance (ANOVA)

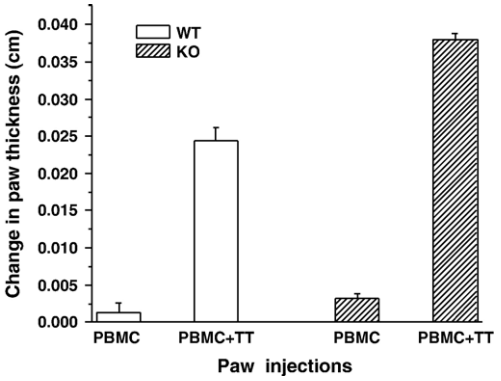


Fig. 3. Trans vivo delayed-type hypersensitivity response in cathepsin S knockout mice. The trans vivo delayed-type hypersensitivity response was tested in C57Bl/6 wild-type (WT) and cathepsin S knockout (KO) mice. Peripheral blood mononuclear cells, with and without tetanus toxoid, were injected into footpads of mice to determine the role of mouse antigen presenting cells in the trans vivo delayed-type hypersensitivity. The mean±S.E.M. responses from experiments using two donors' peripheral blood mononuclear cells are plotted. See text for additional discussion.

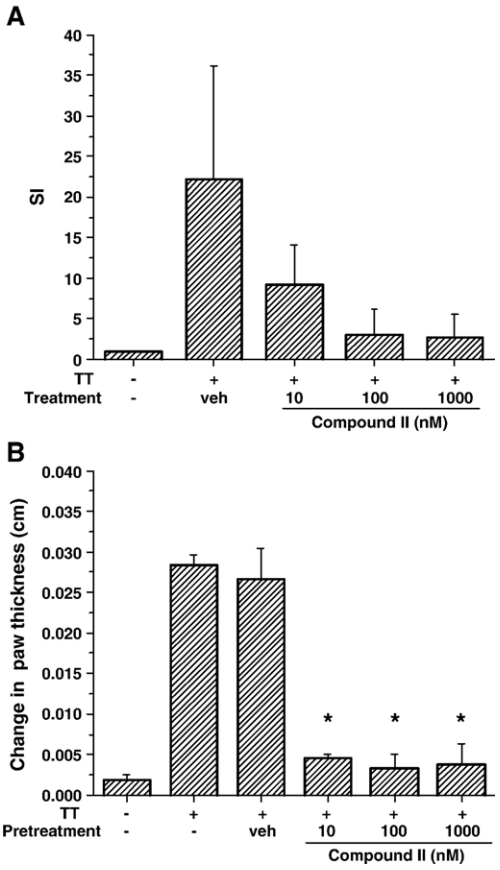


Fig. 4. Effect of treatment of peripheral blood mononuclear cells in vitro with an irreversible cathepsin S inhibitor, compound II, on proliferation and trans vivo delayed-type hypersensitivity. Peripheral blood mononuclear cells from 2 different donors were incubated with 10, 100, or 1000 nM compound II or DMSO vehicle and the cellular proliferation to tetanus toxoid (TT) was tested. The mean±S.E.M. stimulation indices (SI) are plotted (A). Peripheral blood mononuclear cells from 2 different donors were preincubated with 10, 100, or 1000 nM compound II or DMSO vehicle. Cells were subsequently injected with or without tetanus toxoid into footpads of mice. The mean±S.E.M. changes in footpad thickness (B) are plotted. *$P<0.05$ compared to vehicle (ANOVA).

followed by a Dunnett's test. A p value of less than 0.05 was considered statistically significant.

## 3. Results

To validate the human trans vivo delayed-type hypersensitivity model, it was critical to confirm that the human cells present the tetanus toxoid antigen to the human T cells in vivo. Anti-human MHC class II antibody, its isotype control mouse IgG and anti-mouse MHC class II antibody, were injected intraperitoneally 1 h before cell transfer. As seen in Fig. 2, anti-human MHC class II (HLA-DR, DP, DQ) antibody significantly inhibited the trans vivo delayed-type hypersensitivity whereas the isotype control and anti-mouse MHC class II antibody had no effect. Inhibition of trans vivo delayed-type hypersensitivity by anti-human MHC class II antibody and not anti-mouse MHC class II antibody, indicate that human and not mouse antigen presenting cells play a crucial role in this response.

Having established the requirement for human MHC class II molecules, cathepsin S knockout mice were tested to demonstrate the lack of requirement for mouse cathepsin S in the trans vivo delayed-type hypersensitivity response. As shown in Fig. 3, the response (paw swelling) was observed in both the wild-type C57Bl/6 and cathepsin S knockout mice, again indicating a lack of dependence on the mouse antigen presenting cells in this reaction (Fig. 3). Examination of Fig. 3 shows that paw swelling was actually somewhat increased in the knockout mice compared to wild-type mice. However, the knockout response was well within that observed in wild-type mice in other experiments. Comparing Figs. 4B and 6A and B illustrates this.

To explore further the role of cathepsin S in human antigen presentation, we tested the effect of an irreversible cathepsin S inhibitor (compound II) on in vitro proliferation of human peripheral blood mononuclear cells with tetanus toxoid antigen. Additionally, we investigated the effect of in vitro pretreatment of human peripheral blood mononuclear cells with compound II on the induction of trans vivo delayed-type hypersensitivity. Fig. 4A shows pooled proliferation results expressed as stimulation index (SI) in 2 donors' peripheral blood mononuclear cells treated with 10, 100, or 1000 nM compound II or dimethyl sulfoxide (DMSO) vehicle. Fig. 4B shows the footpad thickness changes in the trans vivo delayed-

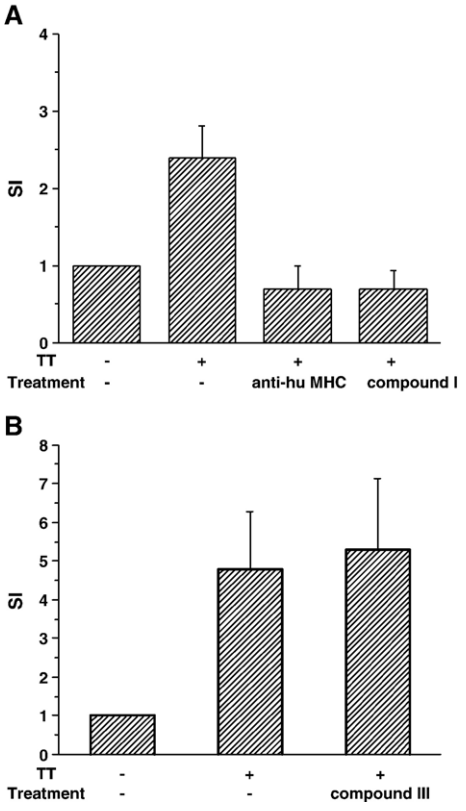


Fig. 5. Effects of compound I and the inactive analogue, compound III, on T-cell proliferation in vitro. Compound I at 30 μg/ml and anti-human MHC class II antibody (anti-hu MHC) (HLA-DR, DP, DQ) at 10 μg/ml were tested using peripheral blood mononuclear cells from 4 different donors (A). Compound III was similarly tested in 2 different donors (B). The mean±S.E.M. stimulation indices (SI) values are plotted.

type hypersensitivity response when peripheral blood mononuclear cells from 2 different donors were pretreated with 10, 100, or 1000 nM compound II or DMSO vehicle before footpad injection. A good correlation between in vitro and in vivo effects was observed. Treatment with compound II inhibited in vitro proliferation and the trans vivo delayed-type hypersensitivity response.

Compound I, a reversible inhibitor of cathepsin S, was also tested in vitro in the human peripheral blood mononuclear cell proliferation assay and by oral administration of the compound to mice in the trans vivo delayed-type hypersensitivity model. Fig. 5A shows the in vitro effect of compound I, on human T cell proliferation. Compound I at 30 μg/ml (72 μM) and anti-human MHC class II antibody at 10 μg/ml inhibited T cell proliferation to baseline levels. Compound III, a less active analogue, at 30 μg/ml (59 μM) had no effect on T cell proliferation (Fig. 5B).

Compound I, when administered orally in the trans vivo model, inhibited the delayed-type hypersensitivity response in all 4 donors in a dose-dependent manner (Fig. 6A). Pooled data from the 4 separate experiments using different donors showed 41±16%, 57±16% and 89±6% inhibition of the trans vivo delayed-type hypersensitivity response in mice administered 10, 30, and 100 mg/kg p.o., respectively (Fig. 6B). A less active analogue, compound III, dosed similarly had no effect on the trans vivo delayed-type hypersensitivity response.

## 4. Discussion

The trans vivo delayed-type hypersensitivity assay is a test of human T cell function whereby peripheral blood mononuclear cells from immunized individuals mediate an antigen-specific swelling response in the footpad of mice (Carrodeguas et al., 1999). The mouse footpad is used as a receptacle in which human delayed-type hypersensitivity responses can be induced. Human memory T cells respond to tetanus toxoid antigen presented by human antigen presenting cells resulting in a measurable swelling response in the footpad. This human-to-mouse model provides an effective tool to measure human cell-mediated immune responses in vivo. It is a relatively rapid and simple in vivo test for measuring human delayed-type hypersensitivity responses and an effective alternative to human skin testing. This humanized mouse model was modified to test compounds that affect human cell mediated immune responses. In the present study, in vivo activity of a selective, reversible, orally bioavailable, inhibitor of cathepsin S,

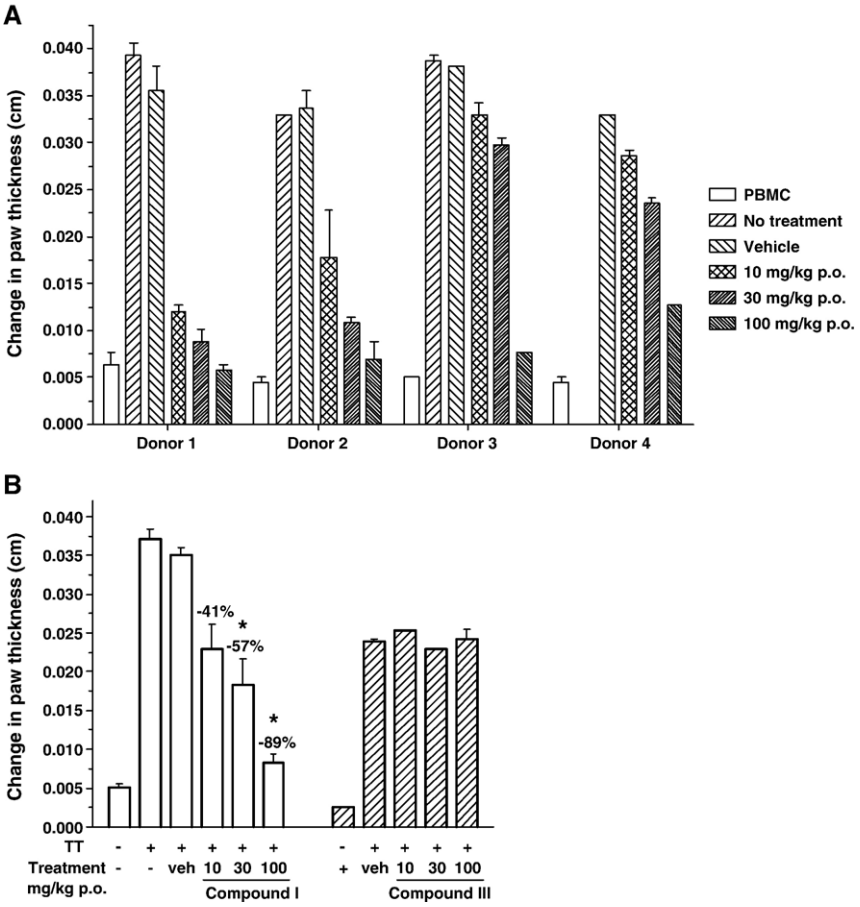


Fig. 6. Effect of the reversible cathepsin S inhibitor, compound I, and the inactive analogue, compound III, on trans vivo delayed-type hypersensitivity. Compound I and compound III were administered orally at 10, 30 or 100 mg/kg at −0.5 h and 4 h after paw injections with peripheral blood mononuclear cells (PBMC) with or without tetanus toxoid (TT). Mouse paw thickness responses using peripheral blood mononuclear cells from 4 different donors are shown individually in (A). The mean±S.E.M. responses to administration of compound I and compound III are shown in (B). *$P<0.05$ versus vehicle by ANOVA.

compound I, was tested in the trans vivo human delayed-type hypersensitivity model.

In the present study cathepsin S knockout mice responded in a manner similar to wild-type mice in the trans vivo delayed-type hypersensitivity model. This demonstrated that human antigen presenting cells play a major role in trans vivo delayed-type hypersensitivity. Also, anti-human MHC class II antibody inhibited trans vivo delayed-type hypersensitivity, whereas the same concentration of anti-mouse MHC class II antibody had no effect, again confirming that mouse antigen presenting cells are not required for the trans vivo delayed-type hypersensitivity response.

The design of compound I, a potent and reversible dipeptide nitrile inhibitor of the cathepsin S enzyme and the X-ray crystal structure of this compound co-crystallized with cathepsin S are published (Ward et al., 2002). The inhibitory concentrations of compounds against human recombinant cathepsin S have been determined by an in vitro enzyme inhibition assay. Selectivity of the compounds against elastase and human cathepsin B was also tested. Both compound I (molecular $IC_{50}=9$ nM) and compound II (molecular $IC_{50}=11$ nM) are selective for cathepsin S. The enantiomer of compound II shows an $IC_{50}=13{,}000$ nM (Cywin et al., 2003). Inhibition of cathepsin S in a human B cell line (HOM2) after treatment with compounds was measured via the accumulation of invariant chain fragment p10 and a decrease in class II-associated invariant chain peptide. In this cellular assay, the minimal inhibitory concentration of the drug required to detect an increase in invariant chain fragment p10 for compound I and compound II is 500 nM and 10 nM, respectively (Ward et al., 2002; Cywin et al., 2003). Western blot analysis for the detection of mouse invariant chain fragment p10 accumulation in the spleens of BALB/c male mice following a single oral dose of compound was conducted. Compound I and compound II show a significant accumulation of invariant chain fragment p10 at 100 mg/kg while its enantiomer shows no activity at the same dose (Cywin et al., 2003). Thus the in vivo activity of compounds I and II in the mouse is established. The in vivo activity of other cathepsin S inhibitors has been tested by injection of human peripheral blood mononuclear cells i.p. into irradiated scid mice (Thurmond et al., 2004). Peritoneal lavage cells collected 20 h after adoptive cell transfers were used to determine the effect of the compounds on accumulation of invariant chain fragment p10 as quantitated by Western blot analysis. In the present study, compounds I and II were tested in the trans vivo human delayed-type hypersensitivity model to determine their activity in a functional assay of human delayed-type hypersensitivity. Delayed-type hypersensitivity is regarded as a hallmark of Th1 mediated immune responses.

To determine the selective activity of the present compounds for cathepsin S inhibition, the activity of compound I was compared to a less active enantiomer (molecular $IC_{50}>20$ μM) of another potent inhibitor of cathepsin S. Compound I inhibited human T cell proliferation to baseline levels, whereas compound III was without effect despite extremely high concentrations of these compounds. Similarly, in vivo, compound I inhibited the trans vivo delayed-type hypersensitivity response ($ED_{50}\sim 18$ mg/kg), whereas compound III had no effect. Furthermore, there was a good correlation between the in vivo and in vitro effects of an irreversible inhibitor (compound II). Compound II inhibited in vitro human T cell proliferation in a dose-dependant manner whereas pretreatment of peripheral blood mononuclear cells with compound II inhibited trans vivo paw swelling. These findings support the role of cathepsin S in human delayed-type hypersensitivity.

Selective inhibition of cathepsin S has potential for modulating autoantigen driven immune responses in class II-restricted autoimmune diseases. Current therapies for autoimmune diseases, e.g. interferon beta, glatiramer acetate, mitoxantrone hydrochloride and corticosteroids (Noseworthy et al., 2000), are often only partially effective and are accompanied by undesirable side effects. Recently a promising therapy, anti-VLA-4 antibody, was temporarily suspended due to the activation of a potentially lethal viral infection in three individuals. Other immune-based therapies, e.g. corticosteroids, cyclosporine and cytokine inhibitors, target the effector molecules in inflammatory reactions. Thus targeting the antigen presentation pathway, responsible for triggering autoimmune diseases, may provide a novel immunotherapy.

Cathepsin S has been implicated in the pathogenesis of neurological disorders (Munger et al., 1995; Lemere et al., 1995), immune system regulation (Shi et al., 1999; Nakagawa et al., 1999), atherogenesis (Sukhova et al., 1998), chronic inflammatory states (Reddy et al., 1995), rheumatoid arthritis (Gay et al., 1993) and other diseases involving tissue destruction. Recent reports demonstrate a role of cathepsin S in human astrocytomas (Flannery et al., 2003), inflammatory myopathies (Wiendl et al., 2003), embryogenesis, atherosclerotic plaque growth, and angiogenesis, an important factor in tumor invasion (Shi et al., 2003; Sukhova et al., 2003). Thus inhibitors of cathepsin S may be of therapeutic value in treating various diseases in a large population of patients. Furthermore, it is believed that autoreactive CD4 T cells play a major role in the pathogenesis of rheumatoid arthritis and multiple sclerosis. Selective inhibition of the cysteine protease, cathepsin S, has the important therapeutic potential of treating such autoimmune diseases.

## Acknowledgments

This study was funded in full by Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT 06877. We thank Drs. Hans Reiser and Gerald Nabozny for critical review of this manuscript.

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