

Identification of a Potent and Selective Noncovalent Cathepsin S Inhibitor

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Abbreviations used:

MHC II, major histocompatibility complex class II; CLIP, class II-associated invariant chain; p10Ii, p10 fragment of the invariant chain; LHVS, leucine homophenylalanine vinyl sulfone; PBMC, peripheral blood mononuclear cells; APC, antigen presenting cell; ECE, endothelin converting enzyme; ACE, angiotensin converting enzyme; MMP-13, matrix metalloproteinase 13; ADAM-28, a disintegrin and metalloproteases 28.

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Abstract

Cathepsin S is considered crucial for normal presentation of MHC class II-restricted antigens by antigen presenting cells to CD4⁺ T cells. It is a key enzyme for the degradation of the class II-associated invariant chain, a process that is required for effective antigen loading of class II molecules. Here we report a selective, orally available, high affinity cathepsin S inhibitor, JNJ 10329670, that represents a novel class of immunosuppressive compounds. JNJ 10329670 is a highly potent ($K_i \sim 30$ nM), nonpeptidic, noncovalent inhibitor of human cathepsin S, but is much less active against the mouse, dog, monkey, and bovine enzymes. The compound is inactive against other proteases including the closely related cathepsins L, F and K. This selectivity makes JNJ 10329670 an excellent tool for exploring the role of cathepsin S in human systems. Treatment of human B cell lines and primary human dendritic cells with JNJ 10329670 resulted in the accumulation of the p10 fragment of the invariant chain ($IC_{50} \sim 1$ μ M). In contrast, inhibition of invariant chain proteolysis was much less effective in a human monocytic cell line, suggesting that other enzymes may degrade the invariant chain in this cell type. JNJ 10329670 also blocked the proteolysis of the invariant chain in vivo using immunocompromised mice injected with human peripheral blood mononuclear cells (PBMC). Furthermore, this inhibitor blocks the presentation of tetanus toxoid and giant ragweed by human PBMC. The properties of JNJ 10329670 make it a candidate for immunosuppressive therapy of allergies and autoimmune diseases.

The presentation of antigens by MHC class II (MHC II)¹ molecules is the crucial initiating step in a CD4⁺ T cell mediated immune response. Antigen presenting cells, mainly dendritic cells, B cells and macrophages, take up and present antigens from the extracellular environment. The internalized protein antigens are processed by endosomal or lysosomal proteases to generate peptides that become associated with MHC II molecules. Peptide-loaded MHC II molecules are subsequently transported to the cell surface for display to CD4⁺ T cells. Recognition of the MHC II/peptide complexes triggers the activation of antigen-specific CD4⁺ T cells, which in turn activate other components of the immune system such as B cells, macrophages and CD8⁺ T cells. These cellular responses are crucial for the body's response to pathogens, but they are also responsible for the development and symptoms of allergy and autoimmune disease.

Cathepsin S is a cysteine protease found in the lysosome of hematopoietic cells. It is a member of the papain superfamily and has 57% identity to cathepsins L and K. Unlike most other lysosomal proteases that are only active under acidic conditions, the activity of cathepsin S exhibits a broad pH optimum that extends to alkaline pH. In contrast to the housekeeping enzymes cathepsins B, D and L, which are expressed ubiquitously, cathepsin S is expressed mainly in dendritic cells, B cells and macrophages that act as the key antigen presenting cells of the immune system. The main substrate of cathepsin S in vivo is the invariant chain. The invariant chain binds to the MHC II $\alpha\beta$ heterodimers in the endoplasmic reticulum and is crucial for the stability and trafficking of MHC II molecules into the endosomal system. The invariant chain also prevents premature peptide loading by blocking the peptide binding groove of the MHC II molecules. When the MHC II-invariant chain complex reaches the endosomes, the invariant chain is degraded in a series of proteolytic steps. One of the key proteases involved in

this process is cathepsin S (Chapman, 1998; Nakagawa and Rudensky, 1999; Villadangos et al., 1999; Villadangos and Ploegh, 2000). Cleavage by cathepsin S leaves only a short fragment of the invariant chain, CLIP (class II-associated invariant chain peptide), bound to MHC II. This fragment is removed through an interaction with the endosomal/lysosomal resident protein HLA-DM, which allows for the binding of antigenic peptides.

Mice deficient in cathepsin S are healthy and normal in most respects, but exhibit defects in immune functions (Nakagawa et al., 1999; Shi et al., 1999). The cathepsin S deficient mice show a decrease in invariant chain degradation in dendritic cells and B cells, but only a moderate effect in macrophages. These mice also display a diminished capacity to present antigens and show a reduced susceptibility to collagen-induced arthritis.

There have only been a few reports of cathepsin S inhibitors and most of these are covalent modifiers of the enzyme. Inhibitors have been shown to block both invariant chain processing and antigen presentation in cells and in vivo (Riese et al., 1996; Villadangos et al., 1997; Allen et al., 2001; Fiebiger et al., 2001; Podolin et al., 2001). Furthermore, in vivo treatment with cathepsin S inhibitors leads to the attenuation of the antibody responses in mice when immunized with ovalbumin (Riese et al., 1998), as well as smaller increases in IgE titer and less lung eosinophil infiltration in a mouse model of pulmonary hypersensitivity (Riese et al., 1998). The degree of inflammation was also decreased in both the rat adjuvant-induced arthritis model (Biroc et al., 2001) and the collagen-induced arthritis model in mice (Podolin et al., 2001). Cathepsin S inhibitors have also been shown to be effective in treating a murine model for Sjögren syndrome (Saegusa et al., 2002).

The published data suggest that inhibition of cathepsin S will block the degradation of the invariant chain and disrupt the presentation of antigens, thereby acting as an immunosuppressive agent. However, unlike most commonly used immunosuppressives, cathepsin S inhibitors will not directly target T cells and will be specific for CD4⁺ T cells. Such an approach should reduce the side effects commonly associated with immunosuppression. Here we describe the first nonpeptidic and noncovalent inhibitor of cathepsin S that has high affinity and selectivity. The high degree of selectivity versus other cathepsins makes it an excellent tool for exploring the role of cathepsin S in humans. In addition, the high affinity for cathepsin S without relying upon covalent attachment to the active site thiol represents a major advance towards the development of small-molecule therapeutics targeting this protease.

Methods

Materials. Anti-human invariant chain antisera was raised by immunization of rabbits using an invariant chain peptide-Keyhole Limpet Hemocyanin (KLH) conjugate emulsified in CFA. The sequence of the invariant chain peptide was CVSKMRMATPLLMQA. KLH conjugation was carried out as suggested by the manufacturer (Pierce Biotechnology, Rockford, IL) using Inject Maleimide Activated Mariculture KLH. Bovine cathepsin S, human cathepsin B, human cathepsin D, and human cathepsin L were obtained from Calbiochem (La Jolla, CA). Human matrix metalloproteinase 13 (MMP-13) was purchased from Chemicon (Temecula, CA). E-64 was purchased from Sigma (St. Louis, MO). The substrate for cathepsin Z was purchased from Enzyme System Products (Livermore, CA). Tetanus toxoid (TT) was purchased from the Massachusetts Biological Laboratories (Jamaica Plain, MA). Giant ragweed extract was purchased from Hollister-Stier Laboratories, LLC. (Minneapolis, MN). Phytohemagglutinin-M *Phaseolus vulgaris* was purchased from CalBiochem (La Jolla, CA). SCID (C57Bl/6J) mice were purchased from The Jackson Laboratories (Bar Harbor, Maine). Blood donors were obtained in collaboration with The Scripps Research Institute (San Diego, CA) and the procedures were approved by the Scripps Institutional Review Board.

JNJ 10329670 was prepared following the procedure recently disclosed (Butler et al., 2002). The free base of JNJ 10329670 has a molecular weight of 651.16. The monohydrochloride (MW 687.61) was used for most biological assays. Both forms are white, free-flowing powders with limited (<0.002 mg/ml) aqueous solubility in pH 2 or pH 7 buffers.

Cloning, expression and purification of cathepsin S. Recombinant human cathepsin S, cathepsin L2, cathepsin K, cathepsin F, cathepsin Z, cathepsin C, legumain, dog cathepsin S, monkey cathepsin S and mouse cathepsin S were expressed using the baculovirus expression system. The cathepsin S proteins were purified using thio-propyl Sepharose chromatography as previously described (Baker et al., 2003). The other proteases were purified using cation exchange chromatography. Human cathepsin E was expressed in Drosophila cells and purified using pepstatin A affinity chromatography. Napsin was expressed as a GST fusion protein and purified via a glutathione affinity column. ADAM-28 was expressed in the baculovirus expression system with a His-Tag, and purified using nickel-chelating columns.

Enzymatic Assays. In general, the assays were run using FRET-based substrates. For example, the cathepsin S, L, and L2 assays used the substrate (Aedens)EKARVLAEEA(Dabcyl)K-amide and cathepsin S cleaves between amino acids Leu-6 and Ala-7. The fluorescence of the aedens group is quenched by the dabcyI moiety in the intact peptide. Upon cleavage by cathepsin S, the quenching is released and the fluorescence of the aedens group can be measured.

The cysteine (cathepsins S, Z, F, C, L, L2, B, and legumain) and aspartic protease (cathepsins E, D, and napsin) assays were run in 100 µl volume with a buffer consisting of 100 mM sodium acetate pH 5.0 containing 100 mM NaCl and 1 mM dithiothreitol, except for cathepsin Z which used 10 mM dithiothreitol and cathepsins E, D, and napsin where no dithiothreitol was present. The MMP-13 assay was run in 50 mM Tris-HCl pH 7.5 containing 5 mM MgCl₂, 200 mM NaCl, and 0.02% Brij35. The ADAM-28 assay was run in 10 mM HCl pH 7.5 containing 0.02%

Brij35. The enzymes were mixed with 7.5 ml buffer and then 75 μ l was added to a Dynax black Microfluor 2 plate. To this 5 μ l of a 20x solution of compound in 30% DMSO was added. This was followed by the addition of 20 μ l 5x substrate to initiate the reaction. In all cases an 11 point 1:2 dilution of the compound was used at seven substrate concentrations (also 1:2). The increase in fluorescence was measured on a Cytofluor II (PerSeptive Biosystems, Framingham, MA) with an excitation filter of 360/40 nm and an emission filter of 460/40 nm. A reading was made every minute for 20 to 60 minutes depending on the assay, and the slope obtained from a linear regression of this time course was used as the reaction rate. The elastase assay was run according to the method of Adeyemi et al. (1990). Endothelin converting enzyme (ECE) was run according to the method of Ahn et al. (1992). Cathepsin G was run according to the method of Nakajima et al. (1979). Angiotensin converting enzyme (ACE) was run according to the method of Hoorn and Roth (1993).

The inhibition constant, K_i , was calculated by fitting plots of the reaction rate (v) as a function of both the substrate concentration ($[S]$) and the inhibitor concentration ($[I]$) to the equation

$$v = \frac{V_{\max}}{1 + \frac{K_m}{[S]} \left(1 + \frac{[I]}{K_i} \right)}$$

Eq. 1.

Cathepsin S from all of the species exhibited substrate inhibition at high substrate concentrations.

This can be taken into account by the equation

$$v = \frac{V_{\max}}{1 + \frac{K_m}{[S]} \left(1 + \frac{[I]}{K_i} \right) + \frac{[S]}{K_s}}$$

Eq. 2.

These equations assume competitive inhibition and similar results were obtained when equations for mixed inhibition were used. The program GraFit (Erithacus Software Lt., Surrey, UK) was used to carry out the nonlinear fits.

Reversibility of the compound was tested by incubating cathepsin S with 100 μ M of test compound for 15 min. The mixture was then dialyzed for 20 h at 4 °C using a mini Slide-a-lyzer (Pierce Biotechnology, Rockford, IL) with a 10,000 MWCO against 150 mM NaAc pH 5.0 containing 1 mM dithiothreitol. The mixture was then assayed for cathepsin S activity as given above.

The pH dependence was determined using the same assay as given above except that different buffers were used for the different pH. The buffers used for the pH titrations were 100 mM sodium acetate for pH 3.5-5.5, 100 mM MES for pH 6 and 6.5, 100 mM HEPES for pH 7.0, and 100 mM Tris-HCl for pH 7.4-9.5. The reaction rates were determined as given above for 11 different inhibitor concentrations at each pH and the percentage of inhibition was determined by comparison to the reaction rate in the absence of inhibitor. IC₅₀ values were determined by four parameter fits of plots of the percentage of inhibition versus inhibitor concentration using the program GraFit (Erithacus Software Lt., Surrey, UK).

Invariant chain degradation assays. Human JY B cell line, human THP-1 monocyte cell line, and monkey SML B cell line were purchased from ATCC. Human PBMC were purified from heparinized blood of healthy donors by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density

gradient centrifugation. Purification of human dendritic cells (DC) was done using a MACS blood dendritic cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

For invariant chain degradation assays, cells were cultured in 24-well plates (2.5×10^5 JY cells/well; 1×10^6 THP-1 cells/well; 5×10^5 SML cells/well; 5×10^6 hPBMC/well; 5×10^5 DC/well) in the presence of various concentrations of test compound for 24 h. Compound stock solutions (1000x) were prepared in DMSO and the final DMSO concentration in cell culture was 0.1%. Cells were harvested and then lysed in 1% Triton X-100/1x PBS with Complete® protease inhibitor cocktail (Roche, Indianapolis, IN). The accumulation of the invariant chain p10 fragment (p10Ii), which is the substrate for cathepsin S, was quantitated by Western blot using an invariant chain specific antibody. The chemiluminescence intensity of the p10Ii bands was quantified on a Typhoon 8600 Imager (Molecular Dynamics, Amersham Pharmacia Biotech, Piscataway, NJ). The relative amount of p10Ii was determined by comparison to the maximum amount seen with 50 nM of a standard cathepsin S inhibitor, LHVS. IC₅₀ values were determined by four parameter fits of plots of the relative amount of p10Ii observed versus inhibitor concentration using the program GraFit (Erithacus Software Lt., Surrey, UK). For titrations of LHVS, the values are given relative to the maximum accumulation of p10Ii.

Antigen induced PBMC proliferation. Blood donors were screened for reactivity to ragweed by RAST test. DR typing of donors was done using the Olerup SSP DR low-resolution kit from Genovision (West Chester, PA). Human PBMC were purified from heparinized blood of healthy donors by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Purified PBMC from either normal donors (n = 11) or allergic donors (n = 3) were cultured in

duplicate or triplicate cultures in 96-well plates in the presence of antigen (1-10 µg/ml TT or 1:30000 dilution of ragweed extract) for seven days, or 5 µg/ml phytohemagglutinin for four days. ³H-thymidine (TdR) was added for the last 18 hour of the culture. Cells were harvested onto filter plates and ³H-TdR incorporation was then determined using a TopCount (PerkinElmer Life Sciences Inc., Boston, MA). Inhibitors were first titrated in DMSO, then a 1:15 dilution of DMSO stocks was made into 40% cyclodextrin in H₂O, which was then added into cell cultures (3 µl into 200 µl/well). Inhibitors were added at the same time as the antigens or phytohemagglutinin. The results given are the average of 3 to 4 determinations and the error is given as the SEM.

In vivo invariant chain assay. Purified human PBMC (10⁸ cells/0.5ml/mouse) were injected intraperitoneally into SCID (C57Bl/6J) mice that received whole body irradiation (4 Gy) one day earlier. JNJ 10329670 administration (by subcutaneous injection) started two hours prior to adoptive transfer of hPBMC and was repeated every 6 h. Mice were euthanized 20 h after the first dose of compound. Peritoneal lavage was immediately performed using 5 ml PBS plus 3 mM EDTA. The level of p10Ii was determined by Western blot assay as described above.

Pharmacokinetic analysis Female Sprague-Dawley rats, mature male beagle dogs and mature female Cynomolgus monkeys were used in pharmacokinetics studies. Briefly, animals were fasted for 18 h before oral dosing. Oral dosing was carried out either by oral gavage for rats or via a stomach tube for dogs and monkeys. Intravenous (i.v.) dosing was done via tail vein (rats), or jugular or saphenous veins for dogs and monkeys. For oral dosing, the compound was prepared in 0.5% methylcellulose as a suspension; for i.v. dosing, the compound was dissolved

in 10% solutol and the pH value was adjusted to 6. After dosing, blood samples were collected via tail vein (rats), or jugular or saphenous veins for dogs and monkeys into tubes containing heparin at the specific time points, which normally include 0.5, 1, 2, 4, 7, and 24 h for oral dosing and dosing 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, and 7 h for i.v. dosing. Plasma samples were then immediately prepared and compound concentrations were determined by LC-MS following acetonitrile extraction. Pharmacokinetic parameters were estimated using the program Winnonlin (Pharsight Corp., Mountain View, CA).

Results

A model of cathepsin S was generated using the coordinates from the X-ray crystal structure of the related cysteine protease cathepsin K (McGrath et al., 1997). A virtual screen of the J&J PRD compound database against the pre-defined active site of cathepsin S was carried out using DOCK software (Kuntz et al., 1982). Selected compounds were screened for cathepsin S inhibitory activity using recombinant human cathepsin S. This screen yielded two related compounds containing a tetrahydropyridyl[4,3-*a*]pyrazole pharmacophore that became the basis of a medicinal chemistry effort. Structure activity relationship (SAR) analysis indicated that three elements were optimal for cathepsin S inhibition: an aryl-bearing piperazine or piperidine, a 3-4 carbon linker, and a 3-aryl-4,5,6,7-tetrahydropyridyl[4,3-*a*]pyrazole (H. Cai, S.P. Meduna, D. L. Gustin and J.P. Edwards, unpublished results). Further refinement showed that replacement of the arylpiperazine with a ketobenzimidazole piperidine resulted in a new series of potent non-covalent cathepsin S inhibitors (C.A. Sehon and J.P. Edwards, unpublished results). This led to the development of JNJ 10329670 (Figure 1). Here we describe the pharmacological characterization of JNJ 10329670.

The inhibition of recombinant human cathepsin S by JNJ 10329670 was determined by measuring cleavage of a synthetic 11 amino acid peptide substrate. Figure 2A shows the reaction rate as a function of the substrate concentration for different concentrations of the inhibitor for one representative determination. It is evident from the curves that at high substrate concentrations either substrate inhibition or quenching was occurring. This can be accounted for by Eq. 2 and an inhibition constant, K_i , of 40 ± 5 nM can be calculated. If the substrate concentration range is limited (Figure 2B) then the data can also be fit using the standard

equation for competitive inhibition (Eq. 1). This yields a K_i value of 37 ± 5 nM. Since the two methods yielded similar results, only the values from fits using Eq. 1 were used. The average values from a number of different determinations are given in Table 1. Thus, JNJ 10329670 had a high affinity for human cathepsin S with a K_i of 34 ± 6 nM ($n = 11$). Eqs. 1 and 2 assume competitive inhibition and no improvement in the fits were obtained using equations for mixed or noncompetitive inhibition. The double-reciprocal plot in Figure 2C emphasizes that the inhibitor was competitive versus the peptide substrate.

The reversibility of the inhibition of human cathepsin S was studied by incubating the enzyme with the inhibitor for 15 min followed by dialysis to remove the compound. Figure 3 shows the recovery of cathepsin S activity under these conditions. Before the dialysis there was complete inhibition of cathepsin S activity by JNJ 10329670. After dialysis the activity was restored to that of the DMSO control. In contrast the activity was not recovered after dialysis of two irreversible inhibitors, N-morpholinurea-homo-phenylalanyl-leucyl-vinylsulfonemethyl (LHVS) (Riese et al., 1996) and E-64, even though the estimated final concentration does not yield any inhibition. These results indicate that the inhibition of cathepsin S by JNJ 10329670 was completely reversible.

A kinetic analysis for JNJ 10329670 inhibition was carried out with cathepsin S from different species. As for the human isoform, all of the enzymes from the other species exhibited substrate inhibition at high substrate concentrations. However, fits with either Eq. 1 or 2 yielded similar K_i values and only the ones from fits to Eq. 1 are given in Table 2. The K_i values for mouse, dog, monkey and bovine cathepsin S were dramatically higher than that for the human enzyme, with

the dog homologue showing the closest activity. In all cases the inhibition was competitive for the peptide substrate.

Selectivity of synthetic cathepsin inhibitors versus other proteases has been an issue. To address this, the inhibition of a number of other closely related proteases was investigated (Table 2). The compound did not inhibit the cysteine proteases cathepsin B, F, L, L2, K, Z, C, and legumain nor the aspartic proteases cathepsin E, D, or napsin at concentrations up to 50 μ M. It also did not inhibit the metalloproteases MMP-13, ADAM-28, endothelin-converting enzyme (ECE) or angiotensin-converting enzyme (ACE). There was no inhibition observed for the serine proteases elastase or cathepsin G. Therefore JNJ 10329670 is highly selective for human cathepsin S with at least 7-fold selectivity over cathepsin S from other species and greater than 50-fold selectivity over a number of other proteases. As part of a general safety profiling, JNJ 10329670 has been assayed against 68 other targets representing major classes of biogenic amine receptors, neuropeptide receptors, ion channel binding sites, neurotransmitter transporters, kinases, and phosphatases. No inhibition greater than 40% was seen at 1 μ M for the receptors, ion channels and transporters and at 10 μ M for the other enzymes (data not shown).

In contrast to other lysosomal proteases, cathepsin S is active over a fairly broad pH range (Figure 4A). This may be important for the biological role of cathepsin S in invariant chain processing since a pH gradient exists in the endosomal/lysosomal compartment where the cleavage occurs. All of the inhibition studies above were carried out at pH 5.0. To explore the possibility that the inhibition could vary with pH, the IC_{50} for JNJ 10329670 inhibition of cathepsin S activity was measured over the pH range of 4.5-7.5 (Figure 4B). There are minor

changes in the potency of the compound with it being the most potent over the range where the enzyme has the greatest activity (pH 5-6). On either extreme the IC₅₀ is increased 2-3 fold. This difference is not expected to have a great impact on the biological activity.

An in vivo substrate of cathepsin S is the invariant chain. The proteolysis of this protein can be followed in cells by Western blot analysis. When a human B cell line (JY) was incubated with JNJ 10329670, the proteolysis of the invariant chain was disrupted and there was an accumulation of an intermediate product known as the p10 fragment that was detected by Western blot analysis using an antibody specific for invariant chain. A representative Western blot is shown in Figure 5A with IC₅₀ plots shown in Figure 5B. The irreversible cathepsin S inhibitor LHVS was very potent in this assay with an IC₅₀ of around 14 nM (Figure 5B). Incubation of JY cells with JNJ 10329670 also led to the accumulation of the p10li fragment with an IC₅₀ of approximately 1 μ M (Figure 5B). We have also carried out the experiments with the human Raji B cell line and found similar results (results not shown). In contrast JNJ 10329670 was not very potent in a monkey B cell line (SML), which is consistent with its lack of potency versus the purified monkey enzyme (Table 1). This data indicates that JNJ 10329670 can inhibit cathepsin S and thereby block the proteolysis of the invariant chain in human B cell lines.

Inhibition of cathepsin S can also block invariant chain degradation in primary cells. Dendritic cells are considered to play a major role in the presentation of antigens to CD4⁺ T cells. Both LHVS and JNJ 10329670 had potency in dendritic cells that matched their potency in the JY cells (Figure 5B). Therefore, inhibition of cathepsin S can block invariant chain degradation in

primary human dendritic cells. This assay was also run using purified human PBMC. Here, LHVS had the same potency as it does for JY cells or dendritic cells, but the potency of JNJ 10329670 was shifted by at least one log. The major cell type in PBMC is B cells and so the reason for the differences seen with the compound is unclear.

It has been reported that in the mouse, the processing of the invariant chain in macrophages is less dependent on cathepsin S than it is in B cells and dendritic cells (Nakagawa et al., 1999; Shi et al., 1999). To address this issue in humans we have studied the invariant chain processing in a human monocytic cell line, THP-1, which was treated overnight with IFN γ to induce differentiation to a macrophage-like phenotype. Figure 6A shows that LHVS inhibited the proteolysis of the invariant chain and led to the accumulation of the p10Ii fragment in these cells. However, the IC₅₀ was higher than in dendritic cells or B cells, which implies that another enzyme was responsible for this degradation (Figure 6B). The requirement for a different protease was clearly shown with the cathepsin S-specific compound JNJ 10329670 (Figure 6A) where no accumulation of the invariant chain p10 fragment was observed. Therefore, as seen in mice, it appears that human macrophages use another enzyme in addition to cathepsin S for proteolysis of the p10Ii fragment.

Inhibition of invariant chain degradation should block antigen presentation to CD4⁺ T cells. To test this we have used a human ex vivo system. When PBMC derived from blood of allergic human donors are incubated with allergen extracts there is a proliferation of antigen specific memory CD4⁺ T cells. This proliferation is dependent on the uptake of the allergen by antigen presenting cells (APC) and the presentation to T cells. This is similar to the process that occurs in

vivo. Blockage of cathepsin S should block the invariant chain degradation and reduce the amount of allergen that is presented. Figure 7 shows this for two different antigens, tetanus toxoid and giant ragweed. JNJ 10329670 was able to block proliferation of T cells in response to both antigens, but less so to the nonspecific T cell mitogen, phytohemagglutinin. This indicates that inhibition of cathepsin S blocks the presentation of these antigens to T cells. We have carried this assay using PBMC purified from donors representing many different MHC II haplotypes. Eleven donors with reactivity to tetanus toxoid and three donors with reactivity to both tetanus toxoid and giant ragweed were used. These results are given in Table 3 for both antigens. Regardless of the haplotype, inhibition of cathepsin S was able to block the presentation of these antigens.

Due to the species difference in the cathepsin S inhibition by JNJ 10329670, it was not possible to carry out in vivo studies in mice. This issue has been circumvented by using SCID mice (C57Bl/6) that were injected intraperitoneally with PBMC isolated from human blood. The mice were dosed subcutaneously at 60 mg/kg of JNJ 10329670 every six hours. Even though JNJ 10329670 was orally available in several species (cf. Table 4) the compound was dosed subcutaneously due to ease of administration and the ability to achieve higher peak plasma levels with lower doses. After 20 h the animals were sacrificed and the human PBMC were harvested by peritoneal lavage. As for the cell-based studies, the accumulation of the p10 fragment of the invariant chain was quantitated by Western blot analysis. The results (Figure 8) show that there was significant accumulation of the p10li fragment when the animals were treated with JNJ 10329670. The plasma concentration of JNJ 10329670 was predicted to be between 2-7 μ M throughout the experiment. At the time of euthanization (4 hours after the last dose), the average

plasma concentration of the compound was $5.7 \pm 0.8 \mu\text{M}$. These results show that JNJ 10329670 can block the proteolysis of the invariant chain in vivo.

An ideal therapeutic cathepsin S inhibitor should be orally bioavailable. To address this single dose pharmacokinetics of JNJ 10329670 have been evaluated in rats ($n = 4$), dogs ($n = 3$) and monkeys ($n = 3$). The compound was administered orally at 30 mg/kg for rats and 10 mg/kg for dogs and monkeys. To determine the bioavailability (%F) the compound was also dosed intravenously at 3 mg/kg for rats and 1 mg/kg for dogs and monkeys. LC/MS analysis was used to quantitate the plasma levels. After oral administration, JNJ 10329670 was orally available in all species with an absolute oral availability of 40-75% and a half-life of 4-14 h (Table 4).

Discussion

Many of the current treatments for autoimmune diseases and allergies are centered on controlling the inflammatory response and have a fairly broad specificity. It would be of benefit to have more specific treatments that target the immune response that is thought to initiate these diseases. One point of intervention is the antigen presentation pathway. Here we describe a novel compound that targets cathepsin S, one of the key enzymes in this pathway.

JNJ 10329670 is a potent inhibitor of cathepsin S that represents a new class of cysteine protease inhibitors. To date most of the cathepsin S inhibitors described in the literature or in patents are covalent modifiers of the active site cysteine. JNJ 10329670 does not have the capacity for covalent modification, yet it has an affinity for the enzyme in the low nanomolar range. This is the first potent noncovalent cathepsin S inhibitor described.

Although JNJ 10329670 is very potent versus the human enzyme, the potency drops dramatically when cathepsin S from other species are analyzed. The compound is the most potent versus dog cathepsin S where the K_i is 7-fold higher than for the human isoform. Versus the mouse, monkey and bovine enzyme the K_i values are 10-1000 fold higher than for the human enzyme. This is somewhat surprising since the enzyme is fairly well conserved among the different species with the homology ranging from 88-96% (Baker et al., 2003). However, minor differences could account for the changes in potency. As expected, the catalytic triad of C139, H279 and N299 is conserved among all of the species as it is for all thiol cathepsins. Also, the key oxyanion hole residues of Q133 and W301 are conserved. There are, however, some differences in the active sites. The S2 pocket is thought to be the primary determinant of specificity and only the human

form has a tyrosine at residue 326 that forms the bottom of the pocket whereas all of the other species have a phenylalanine at this position. Another potentially important change is in the S1' pocket where the human form has an arginine at position 256, where it is serine in all other species. The charged residue at this position is unusual since both cathepsin K and L have a glycine at this position. Another interesting difference is at position 188, where both the human and dog enzyme contain a threonine, but the mouse and monkey have glutamic acid at this position. This residue is adjacent to the activity site, but not a part of the traditional substrate-binding pocket.

In antigen presenting cells like B cells, dendritic cells and macrophages, the invariant chain must be released from the MHC II molecules before peptides can be loaded. This occurs by a series of proteolytic events that cumulates in only a small fragment, CLIP, remaining in the peptide-binding groove. This fragment is then removed by interaction with HLA-DM by mechanisms that are still unclear. The exact identity and number of proteases involved in the invariant chain degradation are unknown but it is clear that in some cell types cathepsin S is responsible for the final proteolytic step that generates CLIP. We have used the accumulation of the precursor for CLIP, the invariant chain p10 fragment (p10Ii), as a readout of cathepsin S activity in cells. Both LHVS and JNJ 10329670 are able to block the cleavage of p10Ii in B cell lines and in primary human dendritic cells. Since LHVS is not completely specific for cathepsin S, the use of the cathepsin S specific compound, JNJ 10329670, convincingly demonstrates that cathepsin S is the major p10Ii degrading enzyme in these cells.

In mice it has been reported that cathepsin S is not essential for the degradation of the invariant chain in macrophages. The human monocytic cell line THP-1 differentiates to a macrophage-like phenotype after IFN γ treatment, and we used these differentiated cells to investigate whether human macrophages are dependent on cathepsin S for invariant degradation. Without treatment with IFN γ there was no expression of invariant chain, but cathepsin S was still expressed (data not shown). Upon differentiation the levels of both the invariant chain and cathepsin S increased (data not shown). LHVS could block the degradation of the invariant chain, albeit with a shift in potency compared to B cells or dendritic cells, which suggest that a protease different from cathepsin S can perform this task. More definitive evidence for this can be seen with our selective cathepsin S inhibitor where there was no accumulation of p10Ii. This shows that in these cells cathepsin S is not essential for the degradation of the invariant chain. The shift in potency with LHVS, along with the lack of activity of JNJ 10329670, suggests that other proteases such as cathepsin L may be the responsible for the invariant chain degradation in these cells. Cathepsin F is also a candidate, but the affinity of LHVS for cathepsin F is similar to that of cathepsin S. Therefore if cathepsin F is involved, one would expect LHVS to be as potent in THP-1 cells as it is in JY cells, which was not observed.

One interesting finding is that JNJ 10329670 had a reduced potency in human PBMC when compared to B cell lines or dendritic cells. This shift was not seen with LHVS. B cells make up ~15% of PBMC and one would expect the potency of a cathepsin S inhibitor to be similar to that in JY or Raji cells. One possible explanation is that another protease exists in these primary B cells which can substitute for cathepsin S. This explanation would require that the unknown protease and cathepsin S are equally sensitive to LHVS, which suggests a role for cathepsin F.

As mentioned, LHVS has a similar potency for cathepsins S and F, whereas our compound does not inhibit cathepsin F.

Since JNJ 10329670 has very little activity versus the mouse cathepsin S, we resorted to a human/mouse chimera model for in vivo studies. In this model, human PBMC were injected intraperitoneally into SCID mice and the compound was administered subcutaneously. PBMC were subsequently harvested and the p10Ii fragment quantitated. Although this system is obviously highly artificial, it nonetheless demonstrates that the compound is able to block cathepsin S activity in vivo.

Blocking cleavage of the invariant chain should inhibit the loading of peptides onto MHC II molecules and block CD4⁺ T cell activation. This can be mimicked in vitro by treating PBMC from sensitized patients with antigen and measuring CD4⁺ T cell proliferation. We show that JNJ 10329670 blocked the proliferation in response to both tetanus toxoid and giant ragweed. These results are somewhat difficult to analyze quantitatively since the inhibition will depend on many factors such as antigen dose and the initial number of specific T cells. Therefore, we have not attempted to calculate IC₅₀ values from these results. However a qualitative assessment for both antigens indicate that the potency of the compound is consistent with the potency for inhibiting invariant chain degradation in dendritic cells. It is also noted that the compound has effects on the phytohemagglutinin-induced proliferation. PHA induced proliferation should not depend on cathepsin S activity since it is antigen independent and works by crosslinking receptors on the T cells. This is complicated, however, by the fact that the presence of antigen presenting cells actually amplifies the phytohemagglutinin response, perhaps by providing other costimulatory

molecules. Nevertheless, even when JNJ 10329670 is incubated with phytohemagglutinin and T cells alone there is inhibition of proliferation at concentrations above 10 μ M (data not shown). This indicates that the compound may have another target or some nonspecific activity (for example, it accumulates readily in membranes due to its hydrophobic nature). However, there is a separation between the concentration needed to inhibit antigen presentation and that which inhibits phytohemagglutinin-induced proliferation. While it is difficult to prove complete selectivity, JNJ 10329670 has no activity in vitro versus many other targets. This data points very strongly to the role of cathepsin S in the presentation of these antigens.

In mice it is known that the affinity of different MHC II molecules for the invariant chain varies. This means that in some haplotypes invariant chain degradation is not essential for peptide loading to MHC II. Thus, cathepsin S deficient B cells and dendritic cells from H-2^b mice (where invariant chain has high affinity for H2-A^b) are not able to present antigens, whereas cells from H-2^q mice are, in line with the fact that H2-A^q binds invariant chain less well (Nakagawa et al., 1999). This finding led us to evaluate antigen-induced proliferation assays using PBMC from donors with a variety of haplotypes (Table 3). Somewhat surprisingly we found that regardless of the haplotype the antigen presentation was blocked by inhibition of cathepsin S.

Taken together, the data presented here and elsewhere suggests that inhibitors of cathepsin S may be useful in treating T cell-mediated diseases in a large population of patients. Indeed, the fact that cathepsin S inhibitors can block the T cell proliferation induced by giant ragweed suggests that allergic conditions may be possible clinical indications. Other obvious indications are autoimmune diseases like rheumatoid arthritis or multiple sclerosis where autoreactive T

cells are considered the root cause of the pathogenic conditions. The limited knowledge of relevant antigens in these diseases makes target validation difficult, and it is likely that the usefulness of cathepsin S inhibitors for the treatment of autoimmune diseases will have to be demonstrated in the clinic.

In conclusion, we have identified a novel potent and noncovalent cathepsin S inhibitor that displays high selectivity for human cathepsin S over other proteases. In vitro the compound blocks invariant chain proteolysis in B cells and dendritic cells, as well as antigen-induced T cell proliferation. The noncovalent nature of JNJ 10329670 as well as its selectivity versus other cathepsins makes it an ideal tool for exploring cathepsin S biology. Furthermore, since this compound is bioavailable in several species and shows activity in an in vivo model system, we propose that it may be useful for the treatment of allergies or autoimmune diseases.

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Figures Legends

Figure 1. The structure of JNJ 10329670.

Figure 2. JNJ 10329670 inhibition of human cathepsin S. Representative non-linear fits to Eq. 2 (A) and Eq. 1 (B) of the reaction rate versus the substrate concentration for different concentrations of JNJ 10329670. The concentrations of inhibitor used where: 5 μM (●), 2.5 μM (○), 1.25 μM (□), 0.62 μM (■), 0.31 μM (△), 0.16 μM (▲), 0.08 μM (▽), 0.04 μM (▼), 0.02 μM (◇), 0.01 μM (◆), 0.005 μM (⊕), 0 μM (●). (C) Double-reciprocal plot of 1 / rate (min/RFU) versus 1 / [substrate] (μM^{-1}) at six concentrations of JNJ 10329670. The concentrations of inhibitor used where: 2.5 μM (●), 1.25 μM (○), 0.62 μM (▼), 0.31 μM (▽), 0.16 μM (■), 0 μM (□). A K_i of 34 ± 6 nM was determined from 11 different replicates.

Figure 3. Reversibility of cathepsin S inhibition by JNJ 10329670. Cathepsin S was incubated for 15 min with DMSO, JNJ 10329670, LHSV or E-64 each at 100 μM . Afterwards the compounds were dialyzed overnight at 4 °C. Enzyme activity was determined before dialysis (gray bar), after dialysis (open bar) and after addition of compounds diluted as they would be during dialysis (black bar). The results shown are the average of two experiments and the error bars represent the SEM.

Figure 4. pH profile of cathepsin S inhibition by JNJ 10329670. (A) pH dependence of cathepsin S activity over the pH range of 3.5 to 8.5. The rate of substrate hydrolysis (RFU / min) is plotted as a function of pH. (B) The IC_{50} of JNJ 10329670 inhibition of cathepsin S at different pH values. At each pH an 11-point titration of compound was run and the percent inhibition

calculated from the reaction rate relative to the no compound control. The IC_{50} was calculated by a four-point fit to the plot of percent inhibition versus compound concentration. The results shown are the average of two experiments and the error bars represent the SEM.

Figure 5. Inhibition of invariant chain proteolysis in cells. (A) Representative Western blot of JY cells treated with JNJ 10329670. The blot was probed with an antibody specific for invariant chain. In lane 1 cells were treated with 50 nM LHVS. Lanes 2-11 were treated with JNJ 10329670 starting at 10 μ M (lane 2) and continuing with a 2-fold dilution. Lane 12 is DMSO treatment only. (B) IC_{50} plots for the accumulation of the invariant chain p10 fragment was measured in various cells after incubation with either LHVS or JNJ 10329670. The amount of p10Ii fragment was normalized to the maximum amount seen with 50 nM LHVS treatment for the JNJ 10329670 titrations and to the maximum level of p10Ii accumulation for the LHVS titrations. Shown are the average of several experiments and the error bars represent the SEM. The different cells are JY cells with JNJ 10329670 (●, n = 10), human dendritic cells with JNJ 10329670 (○, n = 2), SML cells with JNJ 10329670 (▼, n = 2), human PBMC treated with JNJ 10329670 (◇, n = 3), human dendritic cells with LHVS (▽, n = 1), JY cells with LHVS (■, n = 2), and human PBMC treated with LHVS (□, n = 1). Shown are the average of several experiments and the error bars represent the SEM.

Figure 6. Inhibition of invariant chain proteolysis in human macrophages. (A) Representative Western blot of THP-1 cells treated with JNJ 10329670 or LHVS and probed with invariant chain antibody. In lanes 1-10 cells were treated with LHVS starting at 10 μ M (lane 1) and continuing with a 1/2-log dilution. Lane 11 is DMSO treatment only. In lane 13 cells were

treated with 50 nM LHVS. Lanes 14-23 were treated with JNJ 10329670 starting at 10 μ M (lane 14) and continuing with a 2-fold dilution. Lane 24 is DMSO treatment only. The accumulation of the p10 fragment of the invariant chain (the band at approximately 10 kDa) can be seen for the LHVS treated cells but not with the cells treated with JNJ 10329670. (B) IC₅₀ plots for the accumulation of the invariant chain in LHVS treated THP-1 cells (●, n = 6) compared to JY cells (○, n = 2). Shown are the average of several experiments and the error bars represent the SEM.

Figure 7. The effect of cathepsin S inhibition on antigen presentation. The effects of JNJ 10329670 on the PBMC proliferation induced by tetanus toxoid (A), giant ragweed (B) both in black bars or a nonspecific T cell mitogen, phytohemagglutinin, in gray bars. The bars represent the average from different replicates (n = 4 for tetanus toxoid and n = 3 for giant ragweed) and the error bars represent the SEM.

Figure 8. In vivo accumulation of the p10 fragment of the invariant chain in mice. Humanized SCID mice dosed with JNJ 10329670. The accumulation of human p10Ii from recovered human PBMC is shown. The results were judged to be significant (p value < 0.05 using a Student's t-test). Data for each individual mouse is shown (●) along with the average (—).

Tables

Table 1: *In vitro* inhibition of cathepsin S

Enzyme	$K_i \pm \text{SEM}$ (nM)
Human cathepsin S	34 ± 6 (n = 11)
Monkey cathepsin S	266 ± 78 (n = 2)
Dog cathepsin S	124 ± 23 (n = 3)
Bovine cathepsin S	411 ± 22 (n = 2)
Mouse cathepsin S	2364 ± 415 (n = 4)

Table 2: Selectivity of JNJ 10329670 versus other proteases (n = 3)

Protease Class	Enzyme	K _i (μM)
Cysteine	Cathepsin L	>50
	Cathepsin B	>50
	Cathepsin F	>50
	Cathepsin K	>50
	Cathepsin Z	>50
	Cathepsin C	>50
	Cathepsin L2	>50
	Legumain	>50
Aspartic	Cathepsin E	>50
	Cathepsin D	>50
	Napsin	>50
Metallo	ECE	>1
	ACE	>1
	MMP-13	>1
	ADAM-28	>1
Serine	Elastase	>1
	Cathepsin G	>1

Table 3: Inhibition of antigen-induced proliferation of human PBMC

Donor DR Haplotype ^a	Tetanus Toxoid ^b		Giant Ragweed ^b	
	LHVS	JNJ 10329670	LHVS	JNJ 10329670
4, 4	+			
13, 16	+	+		
11, 15	+			
7, 13	+			
4, 7	+	+		
13, 13	+	+		
1, 7	+			
11, 15	+			
8, 15	+	+		
8, 14	+	+		
11, 14	+	+		
7, 15	+		+	+
4, 11	+	+	+	+

^aEach line represents the results from an individual donor.

^bA “+” indicates that there was greater than 50% inhibition at 5 μ M or less. In all cases n = 3-4 determinations. A blank cell indicates that it was not run.

Table 4: Pharmacokinetic data for JNJ 10329670

Species	Route	C _{max} (μM)	t _{1/2} (h)	%F
Rat (n = 4)	i.v.		4 ± 3	
	p.o.	1.8 ± 0.5	14 ± 9	75
Dog (n = 3)	i.v.		3.1 ± 0.3	
	p.o.	2.2 ± 0.3	4.6 ± 0.7	78
Monkey (n = 3)	i.v.		0.7 ± 0.1	
	p.o.	1.5 ± 0.6	4 ± 1	40

Figure 1

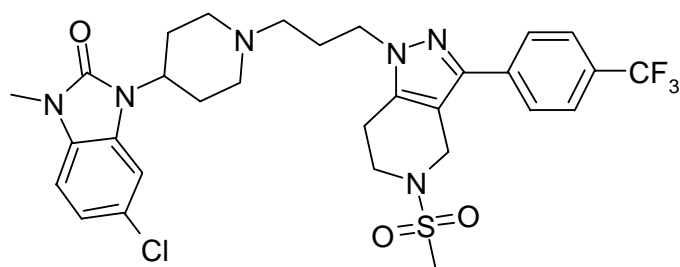


Figure 2

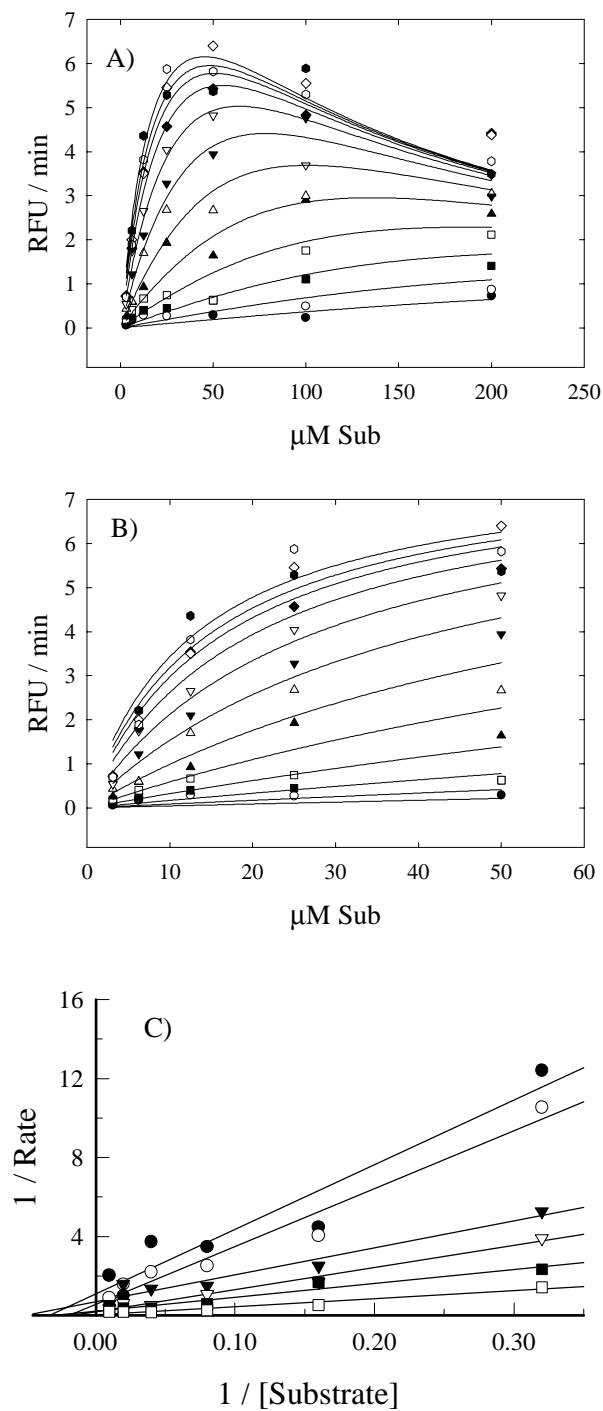


Figure 3

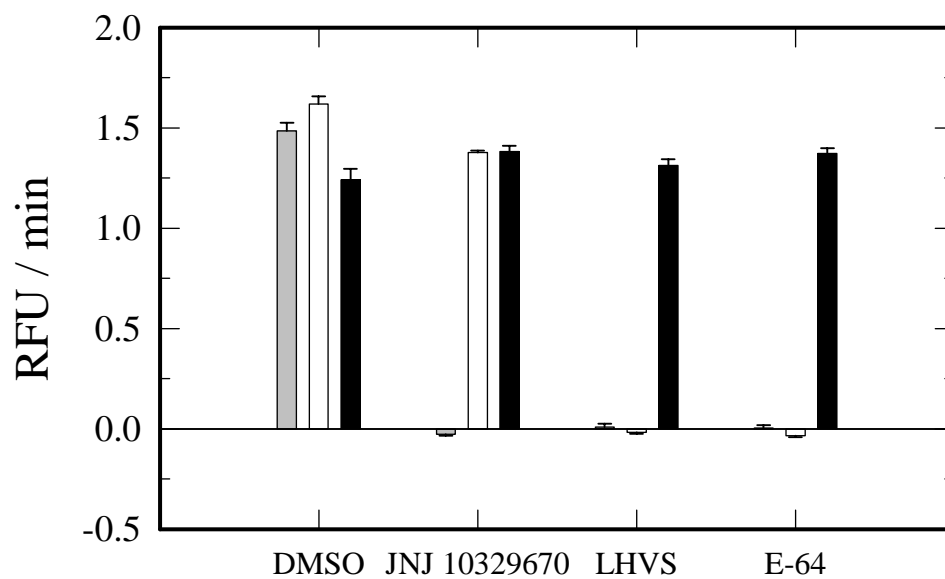


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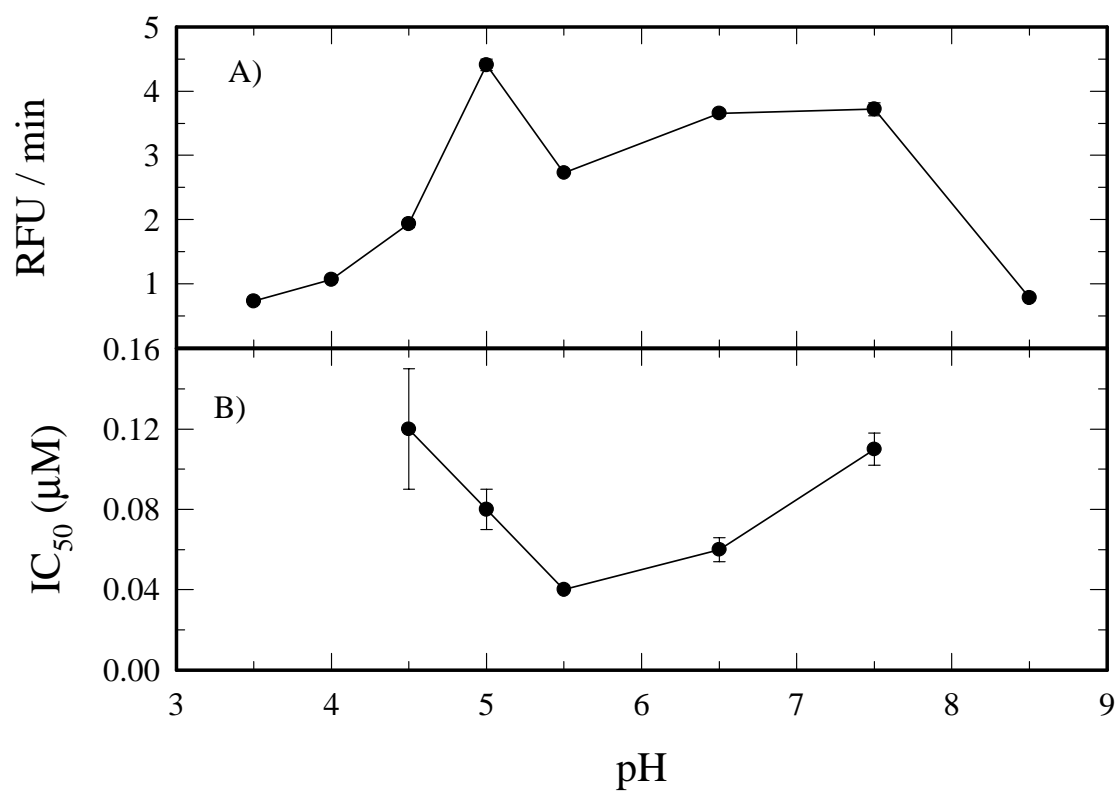


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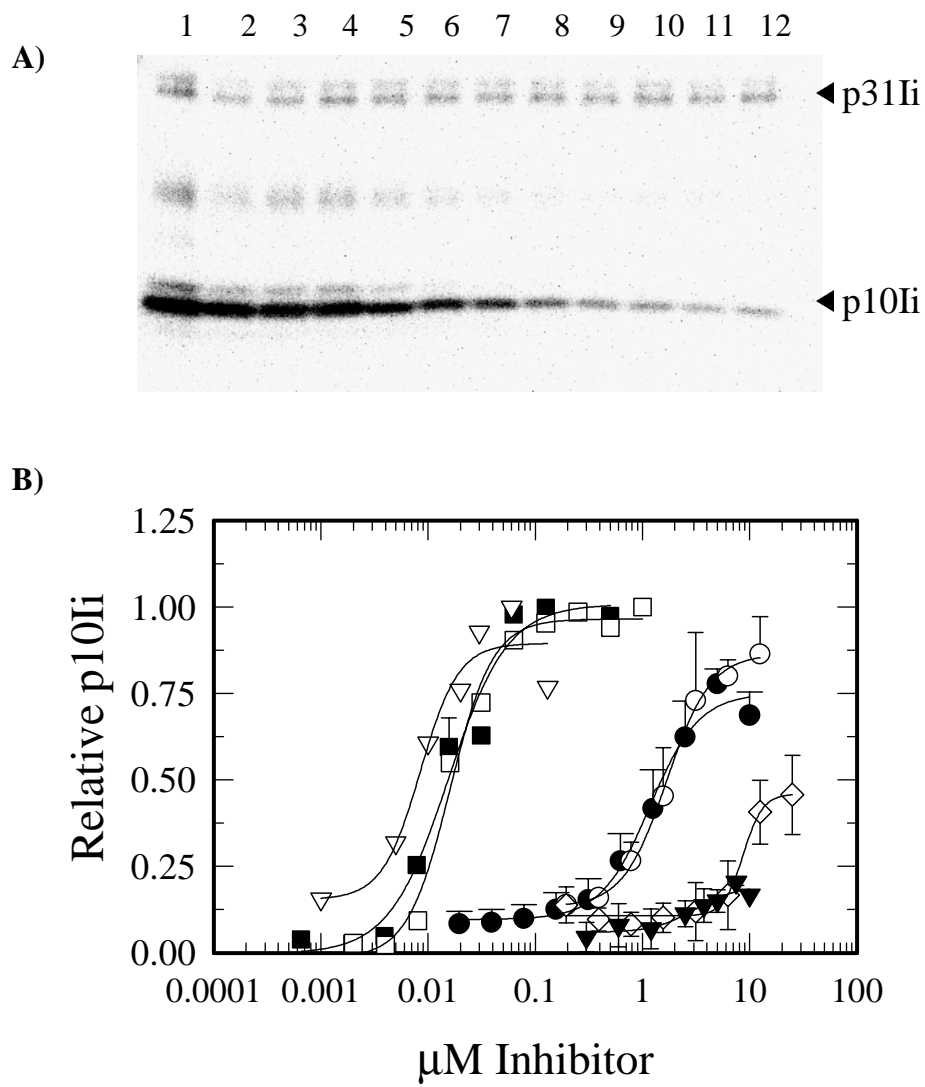


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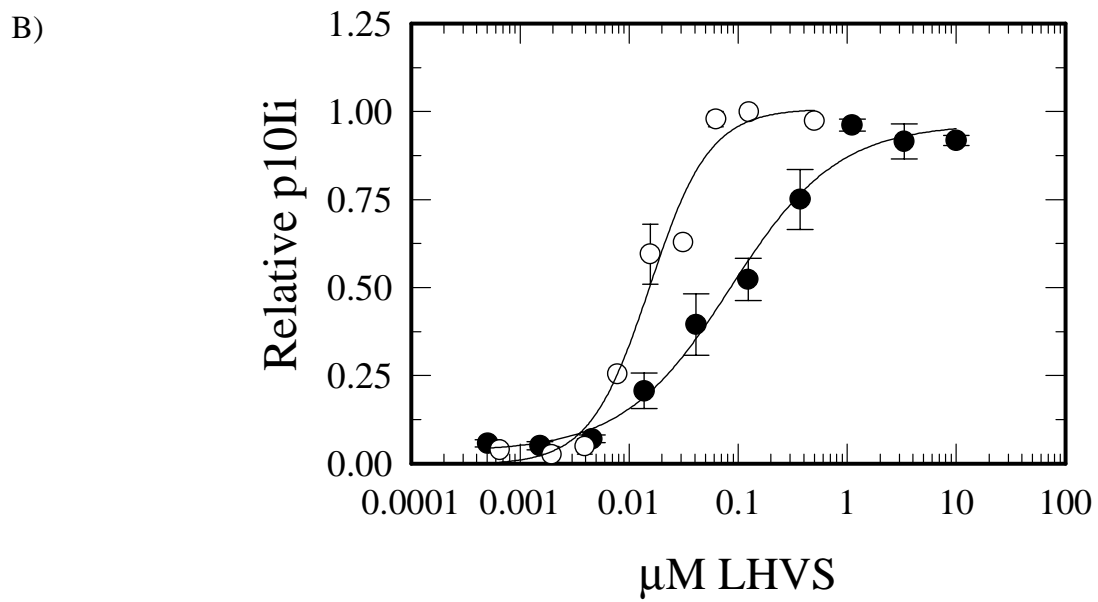
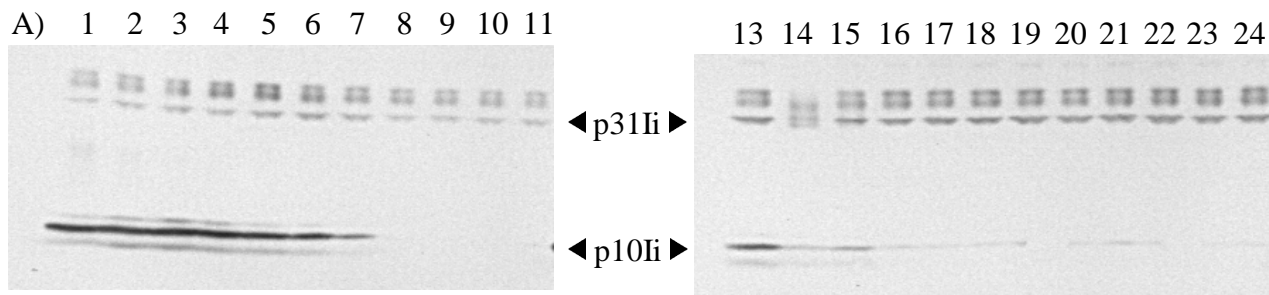


Figure 7

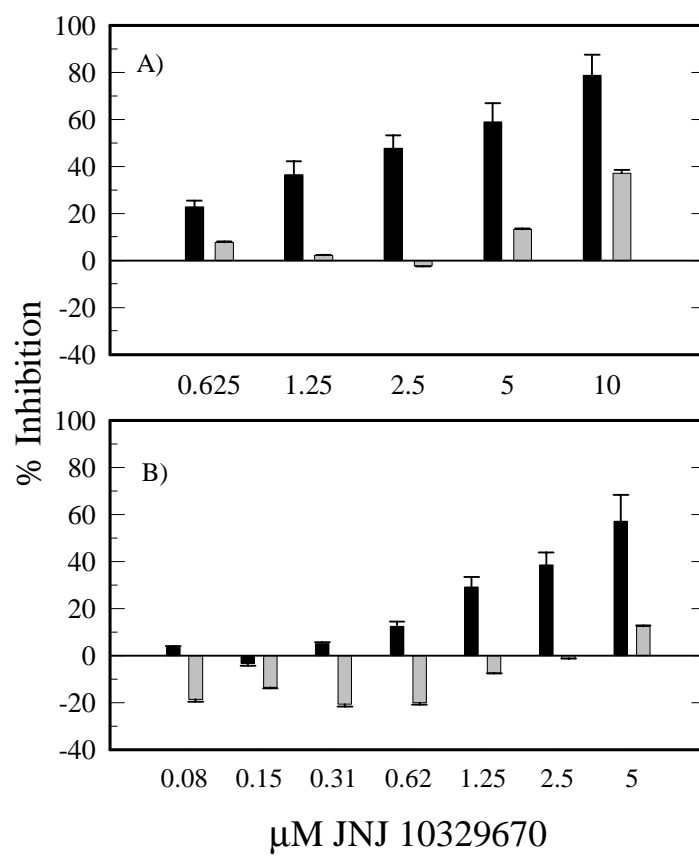


Figure 8

