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Title: Effect of cathepsin K inhibitor basicity on *in vivo* off-target activities

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Running Title: Off-target inhibition of basic cathepsin K inhibitors

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5-[(2-Aminoethyl)amino]naphthalene-1-sulfonyl;DABSYL,4-dimethylaminoazobenzene-

4'-sulphonyl;Mes, 2-(N-morpholino)ethanesulfonic acid; NAGA, β-N-

acetylglucosaminidase; Acid P, acid phosphatase; Succ Dehyd, succinate dehydrogenase;

TBST, TBS-0.1% Tween 20; RT, Reverse transcription

Abstract

Cathepsin K is a lysosomal cysteine protease that is a pharmacological target for the treatment of osteoporosis. Previous studies showed that basic, lipophilic cathepsin K inhibitors are lysosomotropic and have greater activities in cell-based assays against cathepsin K, as well as the physiologically important lysosomal cysteine cathepsins B, L and S than expected on the basis of their potencies against these isolated enzymes. Chronic administration of the basic cathepsin K inhibitors L-006235 and balicatib to rats at a supratherapeutic dose of 500 mg/kg/d for four weeks resulted in increased tissue protein levels of cathepsin B and L, but no effect on cathepsin B and L message. This is attributed to the inhibitor engagement of these off-target enzymes and their stabilisation to proteolytic degradation. No such increase in these tissue cathepsins was detected at the same dose of L-873724, a potent non-basic cathepsin K inhibitor with a similar off-target profile, although all three inhibitors provided similar plasma exposures. Using an activity-based probe [^{125}I]-BIL-DMK, *in vivo* inhibition of cathepsins B, L and S was detected in tissues of mice given a single oral dose of L-006235 and balicatib, but not in mice given L-873724. In each case, similar tissue levels were achieved by all three compounds, thereby demonstrating the *in vivo* cathepsin selectivity of L-873724. In conclusion, basic cathepsin K inhibitors demonstrate increased off-target cysteine cathepsin activities than their non-basic analogues and potentially have a greater risk of adverse effects associated with inhibition of these cathepsins.

Introduction

The CA1 family of human papain-like cysteine proteases comprises 11 members. These enzymes are collectively known as cathepsins, a name which is derived from the Greek, *kathepsein*, to digest. Being largely lysosomal enzymes, cathepsins have acidic pH activity and stability optima. These enzymes are synthesised as preproenzymes, the mature proteins sharing between 25 and 80% sequence identity (Lecaille *et al.*, 2002;Turk *et al.*, 2003). Cathepsin K (Cat K) is highly and somewhat specifically expressed in osteoclasts, the multinucleated giant cells of hematopoietic origin that are responsible for the normal physiological process of bone resorption. Cat K destroys the organic fraction of bone, through its potent collagenase activity, this process taking place in the acidic pit between the osteoclast and the bone surface and also intracellularly within lysosomes of the osteoclast (Saftig *et al.*, 1998). A large volume of genetic and pharmacological data points to a pivotal role for Cat K in bone resorption and Cat K inhibitors are presently being evaluated in clinical trials as a treatment of osteoporosis, a disease characterised by an imbalance of bone resorption over bone formation (performed by osteoblasts) (Deaton and Tavares, 2005;Grabowskal *et al.*, 2005;Yasuda *et al.*, 2005;Close *et al.*, 2006;Boyce *et al.*, 2006). Both physiological and pathological roles have been identified for the remaining 10 human papain-like cysteine proteases including apoptosis, antigen presentation, epidermal homeostasis, proenzyme activation, atherosclerosis and cancer growth (Lecaille *et al.*, 2002). Because of the multitude of roles identified for these cysteine cathepsins, it is considered that a high degree of selectivity is required for a Cat K inhibitor for use as a long-term therapy for osteoporosis to avoid potential adverse events caused by off-target activities.

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We have previously shown that members of a basic nitrogen-containing class of reversible α-aminoacetonitrile Cat K inhibitors, such as L-006235 and balicatib (Fig. 1) demonstrate lysosomotropic behaviour (Black and Percival, 2006;Falgueyret *et al.*, 2005). These basic compounds concentrate in acidic subcellular organelles of isolated cells and in rat tissues with high lysosome content, such as the lung, liver, kidney and spleen. Members of this class of Cat K inhibitors show significantly increased potencies in cell-based assays against both Cat K and the off-target family members Cat B, L and S, compared to potencies against isolated enzymes (Falgueyret *et al.*, 2004;Falgueyret *et al.*, 2005;Black and Percival, 2006). In contrast, non-basic α-aminoacetonitrile Cat K inhibitors do not accumulate in lysosomes and their potencies are generally similar, or weaker, in whole cells compared to purified enzyme assays. The increased cellular activity of basic inhibitors was therefore attributed to their ability to concentrate within lysosomes, where both target and anti-target cathepsins are localised (Black and Percival, 2006;Falgueyret *et al.*, 2005). For unexplained reasons, examples of basic and non-basic Cat K inhibitors show similar Cat K activities in both a functional cell-based bone resorption assay and in an *in vivo* model of bone resorption, suggesting that the basic inhibitors suffer from a loss of functional selectivity in vivo (Black and Percival, 2006).

The studies described herein were performed to determine whether the basic nitrogen-containing Cat K inhibitors L-006235 and balicatib (Fig.1) show evidence of greater off-target activities in vivo than non-basic analogues, as was suggested by previous in vitro studies (Falgueyret *et al.*, 2005;Black and Percival, 2006). The profiles of these inhibitors were compared with that of a non-basic Cat K inhibitor, L-873724 (Fig.1) (Li *et al.*, 2006). We examined whether chronic treatment of rats with a high dose

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of these Cat K inhibitors caused a perturbation of tissue enzyme levels compared to vehicle-treated animals. Since any effect of the basic inhibitors could be due to lysosomal alkalinisation, rather than direct inhibition of lysosomal protease activities, we also profiled an inactive analogue of L-006235, Cmpd A (Fig. 1). In order to directly examine the off-target cathepsin activities of these inhibitors in vivo, we developed an enzyme occupancy assay in which the radiolabelled, non-selective, cysteine cathepsin activity-based probe (ABP) [^{125}I]-BIL-DMK was infused in mice previously orally dosed with each of the reversible Cat K inhibitors. The results of both studies provide consistent evidence that the basic Cat K inhibitors L-006235 and balicatib provide greater off-target cathepsin activity in vivo than a non-basic analogue L-873724, which has a similar profile against these isolated enzymes.

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Methods and Materials

Cysteine cathepsins, substrates, inhibitors and antibodies. Recombinant humanized rabbit, rat and mouse Cat K, human Cat L and S were provided by Celera Inc (Robichaud *et al.*, 2003)*.* Human liver Cat B was from Sigma. Rat Cat B and L were purified from isolated liver lysosomes, while rat Cat S was isolated from spleen (Kirschke and Wiederanders, 1994;Barrett and Kirschke, 1981). Recombinant mouse pro-Cat B and L were from R & D Systems. Recombinant mouse Cat S was prepared by Merck Frosst Canada (McGrath *et al.*, 1998). Human Cat D was from Biomol. The protease substrates were as follows: Z-Arg-Arg-AMC (Calbiochem), Z-Leu-Arg-AMC (Nova Biochem), Ac-Glu-Asp(EDANS)-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Gly-Lys(DABCYL)-Glu-NH$_2$ (Bachem), N-methyl umbelliferyl-N-acetyl-β-D-glucosamidine (Sigma-Aldrich), p-nitrophenyl phosphate (Sigma-Aldrich), p-iodonitrotetrazolium violet (Sigma-Aldrich). Cathepsin inhibitors and the activity based probe [^{125}I]-BIL-DMK were prepared by the Medicinal Chemistry Department at Merck Frosst Canada (Falgueyret *et al.*, 2004). Protease inhibitors E-64 and Ca-074 were from Sigma-Aldrich and Bachem, respectively. Z-Phe-Tyr-CHO was from Santa Cruz Biotechnology. Complete protease inhibitor tablets were purchased from Roche Diagnostics. The conditions used to assess inhibitor potencies against human, rat and mouse Cat K, B, L and S were as previously described (Falgueyret *et al.*, 2004).

Rat chronic inhibitor dosing and evaluation of tissue cathepsin activities

Adult female Sprague Dawley rats were orally dosed at 500 mg/kg/day (dose vol. 5 mL/kg using vehicle 0.5% methocel) for 4 weeks with either L-873724, balicatib, L-006235, Cmpd A or vehicle (3-4 animals per group). Following euthanization by CO$_2$,

24 h after the final dose, brain cortex, liver, kidney and spleen tissues were collected, rinsed with phosphate-buffered saline and frozen in liquid nitrogen. Samples were kept at -80° C until analysis. A satellite arm of 2 animals was dosed with each test compound individually for 14 or 28 d. On the final day, 2-4 h post dosing, plasma was collected and inhibitor levels measured as previously described (Falgueyret *et al.*, 2005). Compound exposure ($AUC_{0-24 h}$) was determined in 2 animals in single dose studies. Frozen tissues were weighed and homogenized in 10 vol. of ice-cold homogenization buffer (50 mM Mes, pH 5.5, 2.5 mM DTT and 2.5 mM EDTA) using a Brinkmann Polytron homogenizer. The total protein concentration in tissue lysates was determined by DC-Lowry protein assay (BioRad Laboratories) as per the manufacturer's instructions. The tissue lysates were divided in two and protease inhibitors were added to that used for western blot analysis. Tissues lysates were diluted to 1 mg/mL with homogenization buffer and enzyme activities were carried out using 5-10 µg of protein. Assays were performed in the 96-well plate format and the plates read using a Gemini EM (Molecular Devices) plate reader (λ_{ex} = 355 nm, λ_{em} = 460 nm). Cat B activity was measured using 50 mM Mes, pH 5.5, 2.5 mM DTT and 2.5 mM EDTA and 50 µM Z-Arg-Arg-AMC as a selective substrate. Determination of Cat L activity was made in the same buffer as Cat B, using 10 µM of Z-Leu-Arg-AMC as substrate. To eliminate any contribution from other cysteine proteases, the assay were performed in two steps; first in presence of 5 µM Ca-O74 (selective Cat B inhibitor), then in presence of 5 µM Ca-074 plus 20 nM Z-Phe-Tyr-CHO (selective Cat L inhibitor). Cat L activity was obtained by subtracting the rate obtained in presence of Ca-074 + Z-Phe-Tyr-CHO from the rate obtained with Ca-074. Cat D activity was measured at 37° C in 50 mM Gly-HCl, pH 3.5 using 10 µM Ac-Glu-

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Asp(EDANS)-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Gly-Lys(DABCYL)-Glu-NH$_2$ in presence of 10 µM E64 (λ_{ex} = 349 nm, λ_{ex} =487 nm). β-N-Acetylglucosaminidase (NAGA) activity was measured using N-methyl umbelliferyl-N-acetyl-β-D-glucosamidine as substrate (Arai *et al.*, 1991;SELLINGER *et al.*, 1960). The acid phosphatase assay was carried out at 37º C in 90 mM acetate buffer pH 5.0 using 8 mM p-nitrophenyl phosphate as substrate. Following 20 min incubation, the activity was stopped by adding NaOH and the absorbance measured at 410 nm. Succinate dehydrogenase activity was assayed as described (Davis and Bloom, 1973).

Western blot analysis of rat tissue lysates. Tissue lysates were diluted to 3 mg/mL in Laemmli sample buffer. Samples were heated at 95º C and 25 µL was loaded on Tris-glycine 10-20% PAGE gels (Invitrogen). Western blotting was performed with the Novex transfer system and buffer (Invitrogen) onto nitrocellulose using enhanced chemiluminescence detection (Amersham). Primary antibodies employed were the following: anti-rat Cat B, US Biological, C-2097-05, 1/1000 dilution; goat anti-human pro-Cat L, Santa Cruz Biotechnology, 10778, 1/200 dilution; goat anti-human Cat L, Santa Cruz Biotechnology, 6501, 1/100 dilution. Secondary antibodies were from Santa Cruz Biotechnology and were diluted 1/1000: donkey anti-goat HRP, 2020 and donkey anti-rabbit HRP, 2004.

Reverse transcription. Reverse transcription (RT) reactions were performed for each RNA sample in MicroAmp reaction tubes using TaqMan reverse transcription reagents (Applied Biosystems) . Each reaction tube contained 250 ng of total RNA in a volume of 50 µL containing 1X TaqMan RT buffer, 5.5 mM MgCl$_2$, 500 µM of each dNTP, 2.5 µM of oligo-d(T)16 primers, random hexamers, 0.4 U/µL RNase inhibitor, and 1.25 U/µL

MultiScribe Reverse Transcriptase. RT reaction was carried out at 25°C for 10 min, 48°C

for 30 min and 95°C for 5 min. The RT reaction mixture was then placed at 4°C for use

in PCR amplification.

TaqMan real-time quantitative PCR. The relative abundance of Cat K and Cat B mRNA

was evaluated using the 5' fluorogenic nuclease assay to perform real-time quantitative

PCR with Taqman chemistry. All probes were synthesized by Applied Biosystems with

the fluorescent reporter dye FAM (6-carboxy-fluorescein) attached to the 5'-end and the

quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) attached to the 3'-end.

Primers and fluorogenic probes were designed using Primer Express v. 1.0 (Applied

Biosystems) and are listed in the supplemental data. Amplified products were between

70-100 bp and designed to span exon junctions. GAPDH primers and probe were used as

the endogenous control (Applied Biosystems). Real-time PCR was performed in a

MicroAmp Optical 96-well reaction plate. For each 50 µL reaction, 10 µL of RT product

(50 ng total RNA), 0.1 µM forward primer, 0.1 µM reverse primer, 0.1 µM probe and 1X

Universal Master Mix (Applied Biosystems) were combined. Amplification conditions

were 2 min at 50°C, 10 min at 95°C followed by 40 cycles at 95°C for 15 sec, 60°C for 1

min. All reactions were performed in ABI Prism 7700 Sequence Detection System in

duplicate using Sequence Detector v 1.6 program.

Cathepsin S Assay in THP-1 cells. THP-1 cells were grown in RPMI 1640, 10% FBS,

10 mM Hepes, 2 mM Glutamine, 1 mM sodium pyruvate, 100U/mL penicillin–

streptomycin at 37 °C in the presence of 5% CO_2. The cells were centrifuged at 300 g for

4 min, washed and resuspended in serum-free medium containing 0.2% BSA. After 24 h,

the cells were plated at 1.5 x 10^6 cells/mL in 200 uL medium in a 96-well plate. Cells

were incubated for 24 h with test compound (titration from 1000 nM final concentration

with a 3-fold serial dilution) in 1% DMSO final concentration. [^{125}I]-BIL-DMK (1 nM)

was then added for 25 min, the reaction was stopped with 1 μM of cold BIL-DMK for 5

min, and cells were centrifuged at 300 g for 4 min. The medium was removed and the

cells washed with PBS and finally resuspended in sample buffer. Labeled proteins were

separated by 12% Tris-Glycine PAGE and transferred onto nitrocellulose membranes.

Ponceau red staining of the membranes showed that total THP-1 cellular protein levels

were not affected by Cmpd B treatment. The membranes were blocked with 5% milk

TBS-0.1% Tween 20 (TBST) for 1 h then probed with goat anti-Cat S (Santa Cruz, sc-

6505, diluted 1:200 in 3% milk TBST) for 1 h. Blots were washed 3 x 10 min with

TBST, followed by incubation with anti-goat IgG Horseradish Peroxidase-conjugated

antibody diluted 1:3000 in 3% milk TBST for 1 h and washed again as described.

Detection was performed using ECL plus reagent (Amersham) and chemiluminescence

captured using a Fuji CCD camera. Blots were stripped with Restore Western Blot

Stripping Buffer (Pierce) and exposed to Biomax MS film for quantification (Falgueyret

et al., 2004).

Mouse in vivo enzyme occupancy assay. C57BL/6 mice (male, 4 weeks, ~22 g,

fasted, 3-4 animals per group) were dosed orally with test compounds (10-60 mg/kg, dose

vol. 10 mL/kg using 1% methocel suspensions) or vehicle. After 1 or 2 h, the mice were

dosed i.v. with [^{125}I]-BIL-DMK (0.03 mCi, 0.01 nmol, dose vol. 5 mL/kg using 60%

PEG as vehicle) and after 60 min were euthanized by CO_2 and tissues collected and kept

on ice. Fresh tissues were rapidly homogenized, as described above, in ice-cold

homogenization buffer containing protease inhibitor cocktail and 100 μM E64. The tissue

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lysates were diluted to 2.5 mg/mL protein in Laemmli sample buffer. Labelled proteins were separated on Tris-glycine 12 % PAGE (Invitrogen) and quantified as described (Falgueyret *et al.*, 2004). A satellite arm of 2 animals was dosed with each test compound individually, followed at 1 h by an i.v. dose (5 mL/kg) of 60% PEG. After an additional h, plasma and tissues were collected and inhibitor levels measured as previously described (Falgueyret *et al.*, 2005).

Results

Potencies of human cathepsin K inhibitors against rat and mouse enzymes. The potent and selective human Cat K inhibitors L-006235, balicatib and L-873724 (Fig. 1) are relatively weak inhibitors of rodent Cat K enzymes, but generally maintain their activities against off-target rodent Cat B, L and S (Table 1). An analogue of L-006235, in which the electrophilic nitrile warhead was replaced by a non-reactive trifluoromethyl group (Cmpd A, Fig. 1) was essentially inactive against all cysteine cathepsins (Table 1). All compounds were inactive ($IC_{50} > 10$ μM) against the aspartyl lysosomal protease Cat D, the CD family cysteine protease legumain, and rat liver lysosomal acid phosphatase. L-006235 and L-873724 were also inactive versus a panel of 8 matrix metalloproteases, 19 serine proteases and 5 aspartyl proteases.

Effect of chronic inhibitor dosing on rat tissue enzyme activities. L-006235, balicatib, L-873724 and Cmpd A was orally dosed to adult female rats at 500 mg/kg/d. Peak plasma levels (2-4 h post dosing) for each compound were similar, as were 24 h plasma exposures ($AUC_{0-24\ h}$) obtained from single dose rat pharmacokinetic studies (Table 2). Following 4 weeks of daily dosing and 24 h following the last dose, the brain, liver, kidney and spleen of vehicle and compound-treated animals were harvested. All four compounds were well tolerated with no clinical signs of toxicity detected. Tissue lysates were prepared and cathepsin and other enzyme activities were determined. The concentrations of each cathepsin inhibitor remaining in these lysates were such that following dilution in the assay mixtures, little or no inhibition would be anticipated (data not shown). Enzyme activities of the cysteine Cat B and L in the brain cortex, liver,

spleen and kidney lysates of L-006235 and balicatib-treated animals were significantly

increased compared to those animals treated with vehicle. Cat L activities in liver, spleen

and kidney were most affected, with increases in activities ranging from 22 to 80-fold

over vehicle-treated animals (Table 3). The activity of the lysosomal aspartyl protease

Cat D in these tissues was also significantly increased by both L-006235 and by balicatib,

but the magnitude was generally to a lower degree than that found for Cat B and L. Only

small and often non-significant increases in the lysosomal enzyme activities of N-

acetylgalactosaminidase (NAGA) and acid phosphatase (Acid P) were observed with

animals treated with L-006235 and balicatib. By contrast, no significant changes in

enzyme activities across all tissues were noted for the non-basic L-873724-treated

animals, with the exceptions of a 1.5-fold increase in liver Cat B activity and minor

changes in NAGA activities in liver and kidney (Table 3). The basic, inactive cathepsin

inhibitor Cmpd A caused small, but significant increases in liver Cat B and NAGA and

brain cortex Cat D, but increases were not observed in other tissues (Table 3). Only very

minor changes in the activity of the mitochondrial enzyme succinate dehydrogenase

(Succ Dehyd) were detected for all four compounds tested.

To further investigate the apparently large increases in Cat B and L activities in L-

006235 and balicatib-treated animals, western blots were performed with antibodies

against mature Cat B and L using tissue lysates from inhibitor and vehicle-treated

animals. Consistent with the enzyme activity increases, Cat B and L protein levels in the

kidney (Fig. 2, A and B) were increased in L-006235 and balicatib-treated animals

compared to vehicle, but were not consistently increased in animals treated with L-

873724 or Cmpd A. Cat B protein was also increased in the spleen, liver and brain of L-

006235-treated rats compared to vehicle (data not shown), as was liver Cat L (data not shown). Both the single chain mature Cat B (predicted mass 27.8 kDa) and the heavy chain (predicted mass 22.4 kDa) of the two chain form of Cat B were detected in kidney (Fig. 2 A), as well as the spleen, liver, brain (data not shown). Processing of the single to the two chain form of Cat B, both of which are catalytically active, is blocked in mice deficient for the lysosomal cysteine protease legumain (Shirahama-Noda *et al.*, 2003) and in cells by the non-selective cysteine protease inhibitors E64d and leupeptin (Hara *et al.*, 1988). In this study, the ratio of these two species was not affected by cathepsin inhibitor treatment (Fig. 2 A). Only a single form of mature Cat L was detected in the kidney (Fig. 2 B) and liver (data not shown) of both vehicle and compound-treated rats.

Kidney tissue lysates were also probed with an antibody directed against the pro-domain of Cat L. For animals dosed with L-873724 and Cmpd A, no changes in the levels of pro-Cat L were apparent. Animals treated with L-006235 and balicatib showed a clear reduction of pro-Cat L levels in the kidney (Fig. 2 C) and liver (data not shown) compared to vehicle-treated animals.

Messenger RNA analysis of rat tissues showed that treatment for 4 weeks with L-006235 and Cmpd A had no significant effect on Cat K, B and L gene expression in the kidney, forebrain, liver and spleen (Fig. 3). These results also demonstrate the low degree of Cat K expression in these tissues, compared to the relatively highly expressed Cat B and Cat L.

Correlation between cathepsin S inhibition and protein stabilisation in THP-1 cells.

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In order to gain some understanding of the mechanism underlying the increase in Cat B and L protein in the tissues of L-006235 and balicatib-treated rats, further studies were performed using the human monocytic THP-1 cell line and the highly selective cell-permeable Cat S inhibitor Cmpd B (Gauthier *et al.*, 2007). This inhibitor was chosen for these in vitro experiments since a potent and selective, non-basic, Cat B or L inhibitor was not available. THP-1 cells were cultured in the presence of varying concentrations of Cmpd B for 24 h and the cells were then labelled with 1 nM of the non-selective, irreversible cysteine cathepsin activity-based probe (ABP) [^{125}I]-BIL-DMK for 25 min. This cell-permeable ABP was previously shown to specifically label Cat B, K, L and S in intact human and rabbit cells (Falgueyret *et al.*, 2004). Following separation of cell lysate proteins by SDS-PAGE and transfer to nitrocellulose, Cat S protein was detected by western blotting and ^{125}I-labelled proteins were detected by autoradiography (Fig 4). The radioactive band and immunoreactive Cat S comigrate, confirming the expression of Cat S in this cell line. A close correlation was observed between the degree of Cat S inhibition, as assessed by competition of Cat S-labelling by the ABP (Fig. 4 A, C), and the increase in Cat S protein in the THP-1 cells (Fig. 4 B, C), with EC_{50} values of 5-10 nM for both processes. This value is close to the IC_{50} value for Cmpd B against isolated Cat S (2 nM) and is several orders of magnitude lower than the IC_{50} values for Cat B or L (Table 1). Furthermore, no competition of the other [^{125}I]-BIL-DMK-labelled proteins (presumably Cat B and L (Falgueyret *et al.*, 2004)) were detected at doses of Cmpd B in THP-1 cells up to 1000 nM. Cellular levels of Cat S protein therefore correlate closely with Cat S inhibition in THP-1 cells.

Evaluation of in vivo cathepsin inhibition profile using an Activity-Based Probe.
Competition with labelled ABP has been used to identify the targets of active site-directed inhibitors towards proteases, as well as kinases and phosphatases (Fonovic and Bogyo, 2007). Generally, these studies have been performed using cell and tissue lysates, although several ABP have shown utility in vivo. Pharmacokinetic studies with the ABP BIL-DMK showed that when dosed intravenously to mice at a dose of 1 mg/kg, a peak plasma concentration of 1.0 μM was achieved, which then declined with α and β half lives of 12 and 60 min respectively (data not shown). Following intravenous administration of [^{125}I]-BIL-DMK, mice were euthanised after 1 h and spleen, liver, kidney and lung tissue lysates rapidly prepared in the presence of 100 μM E64. Radioactivity levels in each organ corresponded to tissue levels of approximately 0.1 nM parent or its metabolite(s). Separation of the radiolabelled proteins by 2D gels followed by autoradiography (data not shown) demonstrated that, as was the case with intact cells, relatively few labelled proteins were obtained (Falgueyret *et al.*, 2004). These labelled proteins were identified as Cat B, L and S from their predicted mass and pI values. As shown by single dimension SDS-PAGE (Fig. 5, vehicle), liver and kidney lysates contain ^{125}I-labelled Cat B and L, whereas lung and spleen contain an additional radiolabelled band corresponding to Cat S. The cathepsin labelling obtained in each tissue is consistent with known tissue localisations, as both Cat B and L are widely distributed (Qian *et al.*, 1991), whereas Cat S distribution is restricted, with highest levels in spleen, lung and heart (Shi *et al.*, 1994). No signal was obtained that corresponded with Cat K, consistent with the relatively low degree of expression of this gene in these tissues, as demonstrated by the results of Fig. 3 (Rantakokko *et al.*, 1996). Cmpd B, the non-basic selective Cat S

inhibitor (Fig. 1 and Table 1) was then tested for its ability to compete for [^{125}I]-BIL-DMK cathepsin labelling *in vivo*. Improved plasma exposure dose proportionality in mice was achieved by orally dosing the sulfoxide Cmpd B prodrug, which is rapidly converted to the active sulfone *in vivo* (data not shown). Cmpd B sulfoxide prodrug or vehicle was dosed orally to mice 2 h prior to the intravenous administration of [^{125}I]-BIL-DMK. After an additional hour, the organs were harvested as described above. The autoradiograms of the separated tissue lysates show that Cmpd B caused a selective blockade of the Cat S signal at a dose of 1 mg/kg and inhibition of both Cat S and B at a dose of 50 mg/kg (Fig.5). The relative degree of Cat B and L competition of ABP labelling by Cmpd B in vivo (50 mg/kg, Fig. 5) is consistent with its approximately 13-fold selectivity for mouse Cat B over mouse Cat L (Table 1). The plasma and tissue concentrations of Cmpd B at a time 3 h following oral dosing of the prodrug to satellite animals are shown in Supplemental Data.

Having established that [^{125}I]-BIL-DMK can be used as an ABP to identify the targets of a cysteine cathepsin inhibitor in vivo, the experiments were then repeated to identify the targets of L-006235, balicatib, L-873724 and Cmpd A. These experiments were performed in mice, rather than rats, due to constraints on the availability of the radioiodinated ABP.   Mice were dosed orally with vehicle, 10 mg/kg L-006235, 25 mg/kg balicatib, 10 mg/kg L-873724 and 60 mg/kg Cmpd A. After 1 h, [^{125}I]-BIL-DMK was dosed intravenously and following an additional 1 h, the animals were euthanised and the tissues removed and rapidly processed as described above. Analysis of tissues from satellite animals dosed with L-006235, balicatib, L-873724 and Cmpd A showed that at each of these doses, roughly equivalent kidney, spleen, lung and liver inhibitor

concentrations were achieved (Fig. 6). The tissue levels of mice dosed with L-006235, balicatib and Cmpd A were on the order of 5-10-fold higher than those attained in plasma, consistent with their lysosomotropic properties and previous tissue level studies in rats (Falgueyret *et al.*, 2005). In contrast, plasma and tissue levels of L-873724 were similar, consistent with its volume of distribution of around unity and non-basic nature (Li *et al.*, 2006). Comparison of SDS-PAGE autoradiograms of tissue lysates from vehicle, L-006235 and balicatib-treated animals show that Cat B, L and S labelling was consistently competed in each organ by the basic inhibitors. In contrast, competition of Cat B, L and S was not consistently observed in organs of mice treated with L-873724 or Cmpd A (Fig. 7).

Discussion

These studies were performed to investigate whether the basic lysosomotropic Cat K inhibitors L-006235 and balicatib, which show promising preclinical and clinical activity as inhibitors of bone resorption, have greater potential for off-target cathepsin activity than a non-basic inhibitor L-873724. The weak activities of these Cat K inhibitors against the rodent Cat K enzymes (Table 1) precludes the use of rat or mouse models of bone resorption to compare efficacy with off-target activity in the same species. However, this comparison can be made on the basis of doses and exposures in the species used for efficacy (rabbit and rhesus monkey) and off-target activity (rat and mouse). In the rabbit Schenk model of bone resorption, L-006235 (10 mg/kg) and L-873724 (10 mg/kg) both caused a 15% increase in bone mineral density after 10 d treatment. The efficacy in this model was comparable to that of a high dose of the effective bisphosphonate bone resorption inhibitor alendronate. The C_{max} and 24 h AUC exposures for L-006235 and L-873724 in these rabbit studies were 1.0 μM, 1.7 μM.h and 0.75 μM, 1.1 μM.h respectively (Pennypacker *et al.*, 2006). Equivalent efficacies of L-006235 and L-873724 (68% inhibition of the collagen degradation marker urinary NTx) were also observed in an ovariectomized rhesus monkey model of bone resorption at doses of 3 mg/kg/d (Black and Percival, 2006). Data for balicatib in a preclinical model of bone resorption has not been published, although higher exposures may be required for efficacy as it is 5 to 20-fold weaker than L-006235 against rabbit Cat K in enzyme and cell-based bone resorption assays (Falgueyret *et al.*, 2005). The chronic rat studies described here were therefore performed at supratherapeutic doses with Cmax values and

exposures of L-006235 and L-873724 on the order of 25-300 times that required for efficacy in rabbits (Table 2).

These chronic rat studies revealed that treatment with the basic Cat K inhibitors L-006235 and balicatib caused large increases in Cat L activity, and to a lesser extent Cat B activity and protein levels in a number of tissues. On the other hand, no consistent perturbation of these tissue enzymes was caused by chronic treatment with a supratherapeutic dose of the non-basic L-873724 (Table 3, Fig. 2), despite similar compound exposures and potencies against these purified enzymes (Table 1). The increases in tissue Cat B and Cat L levels occurred in the absence of increased message (Fig. 3). Previous reports have shown that both non-selective, non-basic cysteine cathepsin inhibitors (Montenez *et al.*, 1994;Kominami *et al.*, 1987) and lysosomotropic compounds, such as chloroquine (Gerbaux *et al.*, 1996;Gerard *et al.*, 1988), induce an increase of cathepsin and other lysosomal protein levels in both isolated cells and in vivo. The formation of a protein-ligand complex often results in protein stabilisation towards thermal and chaotropic agent denaturation (Kleanthous *et al.*, 1991), as well as protease susceptibility (Tawa *et al.*, 2004). That this can occur in the cases of cysteine cathepsins is supported by the coincidence of EC_{50} values for inhibition and protein increase of Cat S in THP-1 cells treated with the non-basic selective Cat S inhibitor Cmpd B (Fig. 4). Thus, the results described here imply that off-target inhibition of Cat B and L by balicatib and L-006235 result in the lysosomal accumulation of these cathepsins due to their stabilisation to proteolysis. Only balicatib and L-006235 cause this phenomenon because their lysosomotropic properties cause them to concentrate within the lysosomes to attain levels which inhibit the Cat B and L which are localised within these organelles.

Indeed, the activities of these two basic inhibitors, but not the non-basic L-873724, against intracellular Cat B and L are increased up to 100-fold in cell-based assays compared to activities against the purified enzymes (Falgueyret *et al.*, 2005). The lack of any off-target effect of the non-basic inhibitor L-873724 is not simply due to low cell permeability. The IC_{50} values of L-873724 against intracellular Cat K, L, B and S are all within 1 to 5-fold of the intrinsic enzyme potencies, implying a high degree of cell permeability (Black and Percival, 2006). The potencies of L-873724, as well as the basic inhibitors balicatib and L-006235 against cathepsins in an extracellular milieu, are likely similar to their intrinsic enzyme potencies (Table 1), as little serum protein binding has been observed for these compounds (unpublished data, S. Desmarais). This is exemplified by the similarity of the in vivo activities of L-873724 and balicatib in models of bone resorption, where the activity of Cat K is predominantly an extracellular process. Thus, both intra- and extracellular cathepsins are targeted by these compounds, but the basic inhibitors suffer increased off-target activity (against the lysosomal cathepsins) due to their lysosomotropic properties.

The majority of the effects of L-006235 and balicatib on tissue enzyme activities are not a consequence of lysosomal alkalinisation resulting in inhibition of lysosomal protease activities, since a only a minor modulation of lysosomal enzymes was caused by treatment with the basic, inactive analogue, Cmpd A (Table 3, Fig. 2). Activities of Cat D and the other lysosomal enzymes acid phosphatase and NAGA were also increased to a small degree by treatment with L-006235 and balicatib (with the exception of a 9.5-fold increase in kidney Cat D activity caused by L-006235). Neither the aspartyl protease Cat D, acid phosphatase, nor NAGA are significantly inhibited by L-006235 and balicatib

(Table 1). These results suggest that inhibitor binding to Cat B and L blocks their autolysis and that the increased level of the other enzymes reflects a lowering of lysosomal protease activities. The targeting of the lysosomes by these inhibitors is demonstrated by the small effect on the activity of the cytosolic enzyme succinate dehydrogenase (Table 3). Interestingly, a reduction in tissue levels of pro-Cat L was detected in L-006235 and balicatib-treated animals (Fig. 2 C) which may reflect an increased activation of this zymogen in response to inhibition of mature Cat L activity by these compounds.

Off-target activity of Cat K inhibitors was assessed in mice directly by competition with the cysteine cathepsin ABP [^{125}I]-BIL-DMK. The drug doses used in these studies were designed to provide approximately equal tissue levels for each of the three Cat K inhibitors and the inactive analogue (Fig. 6) to enable a direct comparison of their off-target activities. Plasma levels of L-006235 in mice were approximately 2-fold higher than that providing efficacy in the rabbit Schenk model of bone resorption (~ 1 uM (Pennypacker *et al.*, 2006)), whereas plasma levels of L-873724 exceeded that providing efficacy by approximately 22-fold (0.75 uM (Pennypacker *et al.*, 2006)). Competition of [^{125}I]-BIL-DMK labelling of Cat B, L and S by L-006235 and balicatib was observed in each tissue tested (Fig. 7). However, although the plasma level of L-873724 in mice was 8-fold greater than that of L-006235 and balicatib, and bulk tissue levels were equivalent (Fig. 6), no competition of Cat B, L and S labelling in tissues by L-873724 was detected (Fig. 7).

In summary, evidence of in vivo off-target Cat B, L and S inhibition of the basic Cat K inhibitors L-006235 and balicatib was obtained in rats and mice. This off-target

activity in rats was found at the equivalent of a supratherapeutic dose, whereas in mice, inhibitor plasma levels only several fold higher than that predicted to provide efficacy also showed off-target activity. The non-basic Cat K inhibitor L-873724 has similar, and in many cases greater activities against isolated Cat B, L and S compared to L-006235 and balicatib (Table 1). However, in neither in vivo study did L-873724 show evidence of off-target activity, although plasma and tissue levels and exposures were similar or greater than those of the basic inhibitors in both studies.

Balicatib has been reported to cause incidences of skin rashes and skin scleroderma, a form of fibrosis and has been withdrawn from human osteoporosis clinical trials (Adami *et al.*, 2006). Cat K, B and L are each expressed in human skin fibroblasts and it is possible that these adverse events result from the concurrent inhibition of these three collagenolytic enzymes, resulting in a pathological increase in matrix proteins. This phenomenon may occur because of the tendency of balicatib to concentrate in lysosomes where these enzymes are localised. On the basis of this study, inhibition of off-target lysosomal cysteine cathepsins by L-873724, or structurally-related non-basic Cat K inhibitors may not be expected, even at doses many fold higher than that required for efficacy.

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Figure Legends

Fig. 1. Cathepsin inhibitors used in this study.

Fig. 2. Western blot analysis of rat kidney extracts of animals dosed with vehicle or 500 mg/kg/d L-873724, L-006235, Cmpd A or balicatib for 4 weeks. The same extracts (50 µg protein per lane) were run on 3 different gels and each was blotted against anti mature Cat B (panel A), mature Cat L (panel B) and pro-Cat L (panel C). Each lane represents an extract from a single animal. For each separate blot showing drug-treated animals, lanes were run containing extracts from vehicle-treated animals. In these cases, vehicle band intensities were similar to those shown.

Fig. 3. Messenger RNA analysis of rat tissues of animals dosed with vehicle or 500 mg/kg/d L-006235 or Cmpd A for 4 weeks. The expression levels shown are relative to that of GAPDH and represent averages $\pm$ SD of analyses of tissue from 3-4 animals. Panel A, Cat L. Panel B, Cat B. Panel C, Cat K.

Fig. 4. Effect of Cmpd B on Cathepsin S activity and protein levels in THP-1 cells. THP-1 cells were cultured in the presence of Cmpd B for 24 h and the cells were then labelled with 1 nM [^{125}I]-BIL-DMK for 25 min. The cell lysate proteins were separated by SDS-PAGE and transfer to nitrocellulose, Cat S protein being detected by western blotting. ^{125}I-labelled proteins were detected by autoradiography Panel A. Representative whole cell-enzyme occupancy titration of Cat S activity (^{125}I-labelled proteins) with Cmpd B

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(left to right; 0.005, 0.015, 0.05, 0.14, 0.41, 1.2, 3.7, 11, 33, 100, 1000 nM). Panel B. Representative western blot titration of cell lysate Cat S with Cmpd B. Panel C, Quantification of enzyme occupancy and western blot titrations of Cat S activity and protein with Cmpd B. The data are plotted as ratios of Cat S activity or protein expression versus vehicle control. The data are average ± range of duplicate experiments. The arrows correspond to the mobility of Cat S.

Fig. 5. Effect of Cmpd B on tissue cathepsin B, L and S active site occupancy. Mice were dosed orally with Cmpd B (1 and 50 mg/kg) or vehicle. After 1 h, the mice were dosed intravenously with [^{125}I]-BIL-DMK and after a further 60 min were euthanized. The tissues (liver, spleen, kidney and lung) were rapidly collected and homogenized in the presence of E64. Tissue lysate proteins were separated on SDS-PAGE gels which were subjected to autoradiography. Each lane represents a sample of a tissue lysate from a single mouse.

Fig.6. Tissue and plasma concentrations of mice dosed with L-006235, balicatib, L-873724 and Cmpd A. Satellite animals to the study described in Fig. 7 were each dosed orally with 10 mg/kg L-006235, 25 mg/kg balicatib, 10 mg/kg L-873724 and 60 mg/kg Cmpd A and were euthanized after 2 h. Drug tissue and plasma concentrations were determined by LC-MS and represent the average ± range for 3 animals.

Fig. 7. Effect of L-006235, balicatib, L-873724 and Cmpd A on tissue cathepsin B, L and S active site occupancy. Mice were dosed orally with 10 mg/kg L-006235, 25 mg/kg

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balicatib, 10 mg/kg L-873724 and 60 mg/kg Cmpd A or vehicle. After 1 h, the mice

were dosed intravenously with [^{125}I]-BIL-DMK and after a further 60 min were

euthanized. The tissues (liver, spleen, kidney and lung) were rapidly collected and

homogenized in the presence of E64. Panel A) Tissue lysate proteins were separated on

SDS-PAGE gels which were subjected to autoradiography. Each lane represents a sample

of a tissue lysate from a single mouse. The data shown for vehicle, L-006235 and L-

873724 are representative of two distinct experiments. Panel B) The relative intensities of

the cathepsin bands were quantified by densitometry for each group (mean +/- SD).

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Table 1. Potencies of cathepsin inhibitors across species[a]

Compound	Species	IC$_{50}$ (nM)			
		Cat K	Cat B	Cat L	Cat S
L-006235	Human	0.2	1100	6300	47000
	Rat	12	350	3690	5650
	Mouse	20	130	2300	5400
Balicatib	Human	1.4	4800	500	65000
	Rat	56	2880	5800	7320
	Mouse	480	1170	6750	6350
L-873724	Human	0.2	5239	264	180
	Rat	20	925	90	2440
	Mouse	43	540	250	250
Cmpd A	Human	3878	>10000	>10000	>10000
	Rat	_	>10000	>10000	>10000
	Mouse	_	_	_	>10000
Cmpd B	Human	410	1100	1010	1.8
	Mouse	1650	230	3040	0.6

[a] all data are the average of at least 3 independent titrations.

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Table 2. Rat pharmacokinetic parameters for cathepsin inhibitors when dosed at 500 mg/kg. Plasma C_{max} values were obtained 2-4 h post oral dosing. Plasma exposure is over 24 h

	Plasma C_{max} (µM)	Plasma exposure (AUC$_{0-24\ h}$, µM.h)
L-006235	50	690
Balicatib	67	1080
Cmpd A	25	–
L-873724	22	460

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Table 3: Effect of Chronic Dosing of Cathepsin Inhibitors (500 mg/kg/d for 4 weeks) on Rat Tissue Enzyme Activities. Values represent the ratio of the activity compared to vehicle-treated animals. Each value represents the average of duplicate determinations from tissues from at least n=3 vehicle and compound-treated rats. *P<0.05.

	Tissue	Cat B	Cat L	Cat D	NAGA	Acid P	Succ Dehyd
L-873724	**Liver**	1.5*	3.6	1.4	1.2*	1.0	1.0
	Spleen	1.6	3.6	1.6	1.1	1.0	0.9
	Kidney	1.3	4.3	1.9	1.1*	1.0	1.1
L-006235	**Cortex**	2.2*	2.4	3.4*	0.9	1.3	1.0
	Liver	7.1*	80*	2.0*	3.2*	1.5	1.1
	Spleen	4.9*	50*	2.4*	2.2*	1.1	1.1
	Kidney	5.8*	49*	9.5*	2.1*	0.9	1.2*
Cmpd A	**Cortex**	0.8	2.0	3.4*	0.9	1.0	1.1
	Liver	1.8*	1.0	1.4	1.6*	1.0	1.0
	Spleen	1.1	1.0	1.2	1.0	0.8	1.0
	Kidney	1.3	1.0	1.4	0.9	1.0	1.0
Balicatib	**Cortex**	1.8	3.0	1.5	0.8	0.9	1.0
	Liver	8.3*	42*	3.2*	2.7*	1.4*	1.0
	Spleen	5.7*	40*	1.6*	2.6*	0.9	1.1*
	Kidney	8.0*	22*	1.7	2.2*	1.1	1.1*

Figure 1   MOL #39511

L-006235

Cmpd A

balicatib (AAE581)

L-873724

Cmpd B

Figure 2   MOL #39511

Figure 3   MOL #39511

A) Cathepsin L

B) Cathepsin B

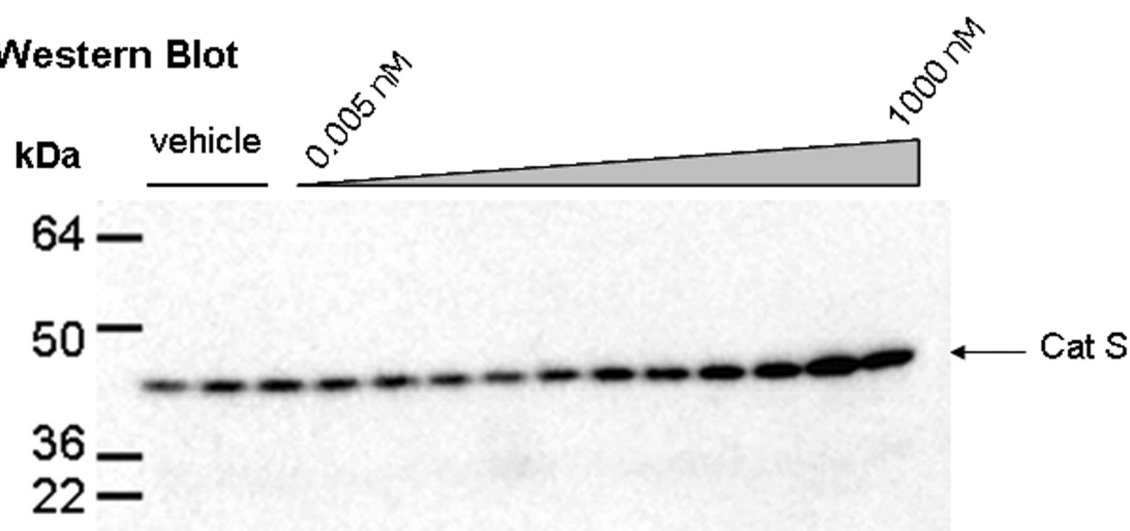C) Cathepsin K
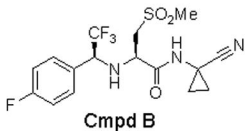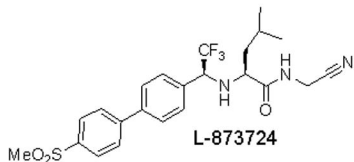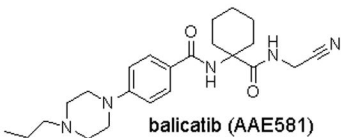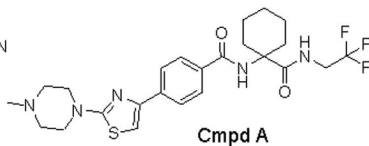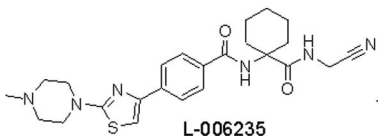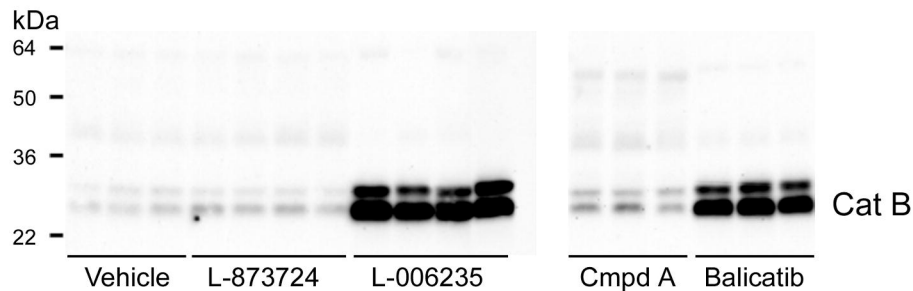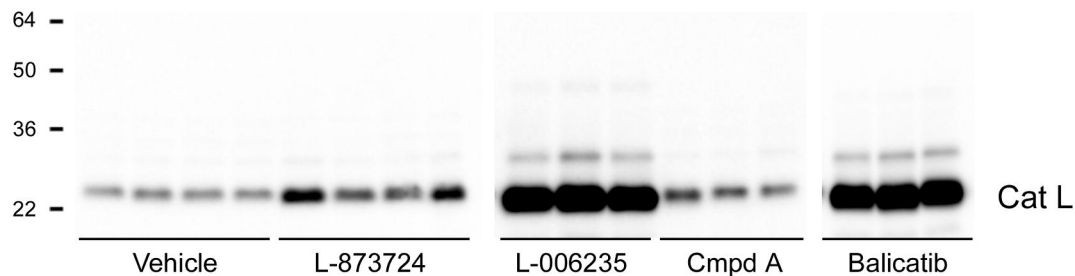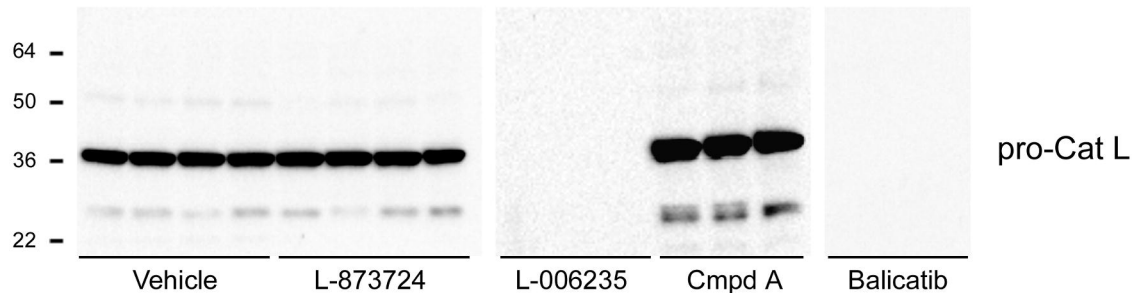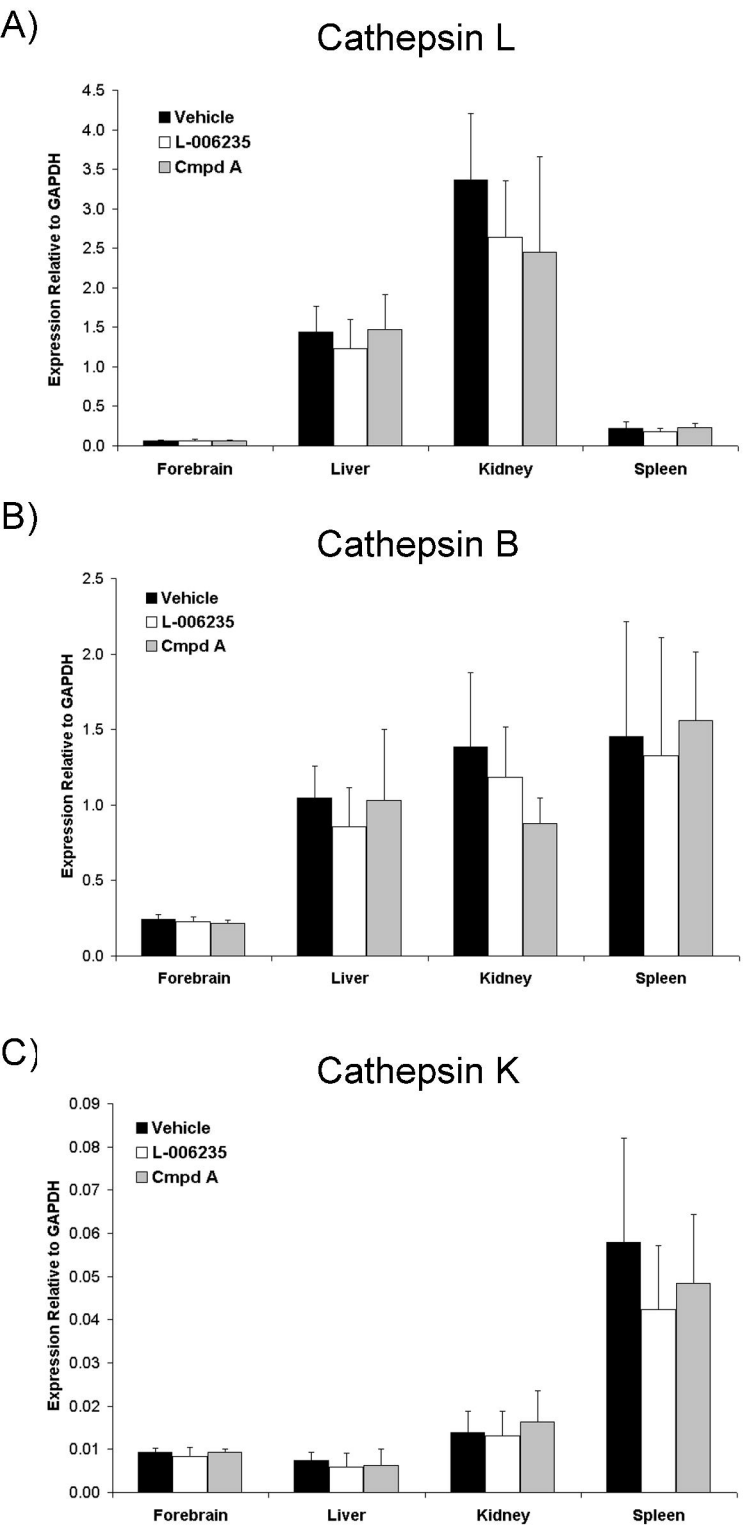
Figure 4   MOL #39511

A) ^{125}I-Labeled proteins
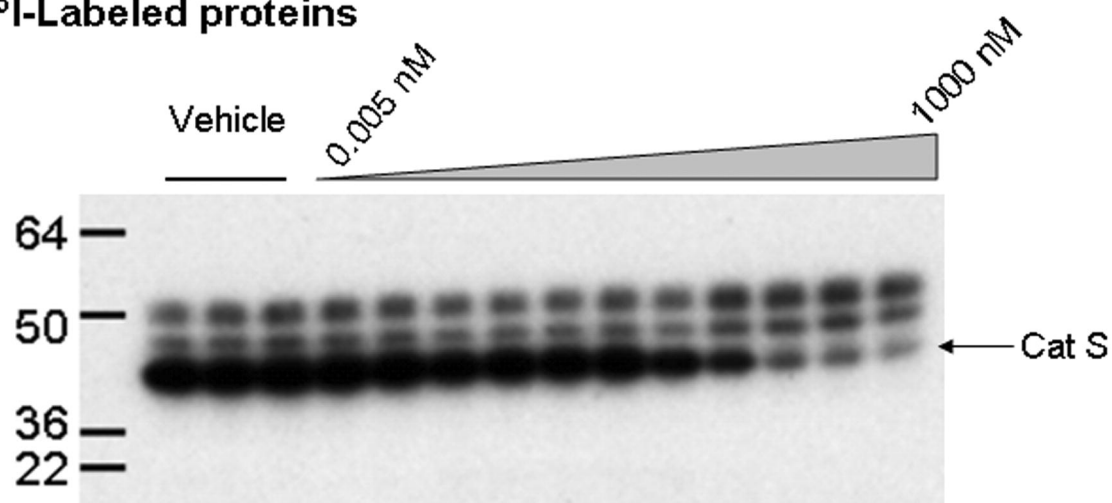
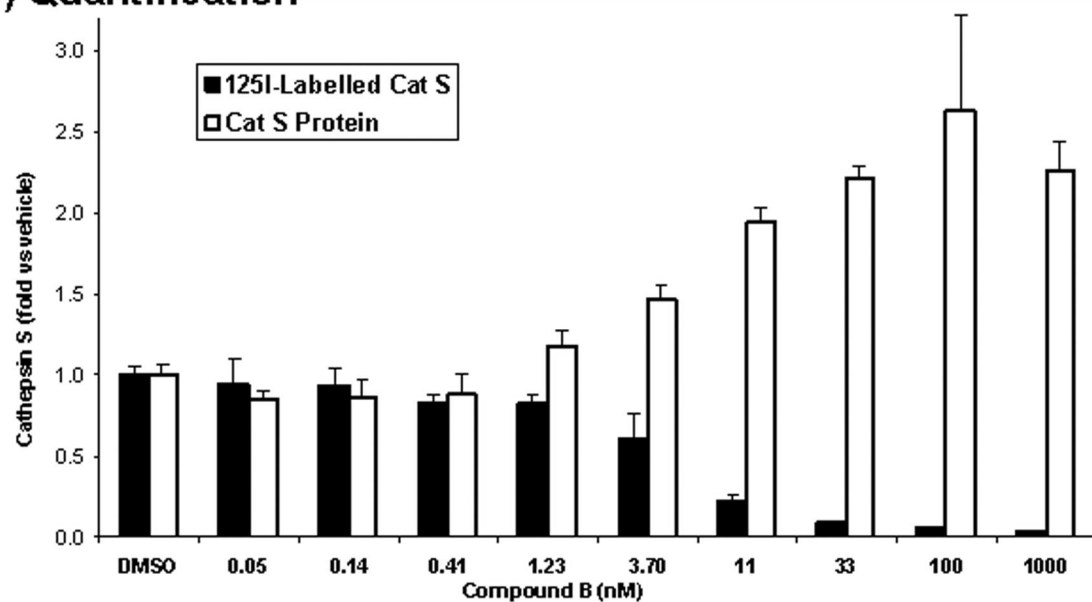**B) Western Blot**

C) Quantification

Figure 5 MOL #39511
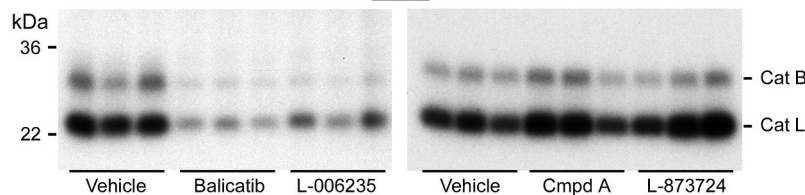

Liver

Cat B -
Cat L -

kDa
- 36
- 22

Spleen

Cat B -
Cat S -
Cat L -

- 36
- 22

Kidney

Cat B -
Cat L -

- 36
- 22

Lung

Cat B -
Cat S -
Cat L -

- 36
- 22

Vehicle   1 mg/kg   50 mg/kg

Figure 6   MOL #39511

Figure 7

A)

B)