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Effect of Cathepsin K Inhibitor Basicity on in Vivo Off-Target Activities[S]

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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 based on their potencies against these isolated enzymes. Long-term administration of the basic cathepsin K inhibitors *N*-(1-(((cyanomethyl)amino)carbonyl)cyclohexyl)-4-(2-(4-methylpiperazin-1-yl)-1,3-thiazol-4-yl)benzamide (L-006235) and balicatib to rats at a supratherapeutic dose of 500 mg/kg/day for 4 weeks resulted in increased tissue protein levels of cathepsin B and L but had no effect on cathepsin B and L message. This is attributed to the inhibitor engagement of these off-target enzymes and their stabilization to proteolytic degradation. No such increase in these tissue cathepsins was detected at the same dose of *N*-(cyanomethyl)-N^2-{(1*S*)-2,2,2-trifluoro-1-[4'-methylsulfonyl)biphenyl-4-yl]ethyl}-L-leucinamide (L-873724), a potent nonbasic 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 nonbasic analogs and potentially have a greater risk of adverse effects associated with inhibition of these cathepsins.

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 synthesized 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 characterized by an imbalance of bone resorption over bone formation (performed by osteoblasts) (Deaton and Tavares, 2005; Grabowskal et al., 2005; Yasuda et al., 2005; Boyce et al., 2006; Close 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

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ABBREVIATIONS: Cat, cathepsin; BIL-DMK, biphenyl-leucine-diazomethylketone; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; Ca-074, *N*-(3-propylcarbamoyloxirane-2-carbonyl)-isoleucyl-proline; CHO, aldehyde; Z-, *N*-benzyloxycarbonyl; AMC, amino-4-methylcoumarin; Ac-, *N*-acetyl; EDANS, 5-[(2-aminoethyl)amino]naphthalene-1-sulfonyl; DABCYL, 4-dimethylaminoazobenzene-4′-sulfonyl; MES, 2-(*N*-morpholino)-ethanesulfonic acid; NAGA, β-*N*-acetylglucosaminidase; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; PCR, polymerase chain reaction; TBST, Tris-buffered saline/0.1% Tween 20; AUC, area under the curve; ABP, activity-based probe; L-873724, *N*-(cyanomethyl)-N^2-{(1*S*)-2,2,2-trifluoro-1-[4'-methylsulfonyl)biphenyl-4-yl]ethyl}-L-leucinamide.

believed 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.

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 behavior (Falgueyret et al., 2005; Black and Percival, 2006). 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 with potencies against isolated enzymes (Falgueyret et al., 2004, 2005; Black and Percival, 2006). In contrast, nonbasic α-aminoacetonitrile Cat K inhibitors do not accumulate in lysosomes, and their potencies are generally similar, or weaker, in whole cells compared with 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 localized (Falgueyret et al., 2005; Black and Percival, 2006). For unexplained reasons, examples of basic and nonbasic Cat K inhibitors show similar Cat K activities in both a functional cell-based bone resorption assay and 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 nonbasic analogs, 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 nonbasic Cat K inhibitor, L-873724 (Fig. 1) (Li et al., 2006). We examined whether chronic treatment of rats with a high dose of these Cat K inhibitors caused a perturbation of tissue enzyme levels compared with vehicle-treated animals. Because any effect of the basic inhibitors could be due to lysosomal alkalinization, rather than direct inhibition of lysosomal protease activities, we also profiled an inactive analog of L-006235, Cmpd A (Fig. 1). To directly examine the off-target cathepsin activities of these inhibitors in vivo, we developed an enzyme occupancy assay in which the radiolabeled, nonselective, 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 the nonbasic analog L-873724, which has a similar profile against these isolated enzymes.

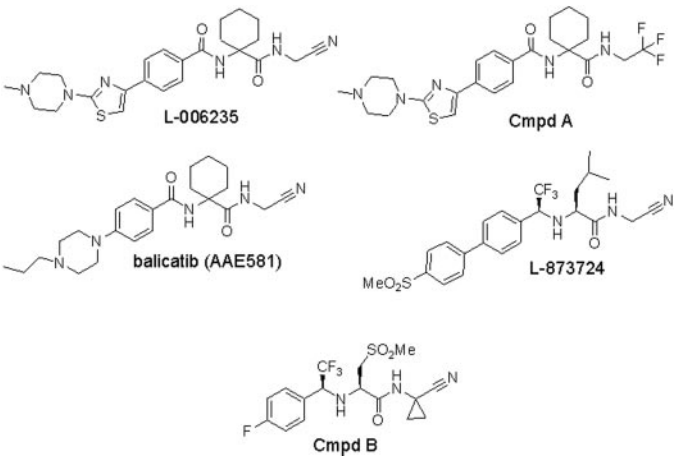

Fig. 1. Cathepsin inhibitors used in this study.

Methods and Materials

Cysteine Cathepsins, Substrates, Inhibitors and Antibodies. Recombinant humanized rabbit, rat, and mouse Cat K and human Cat L and S were provided by Celera (Rockville, MD) (Robichaud et al., 2003). Human liver Cat B was from Sigma (St. Louis, MO). Rat Cat B and L were purified from isolated liver lysosomes, whereas rat Cat S was isolated from spleen (Barrett and Kirschke, 1981; Kirschke and Wiederanders, 1994). Recombinant mouse pro-Cat B and L were from R and D Systems (Minneapolis, MN). Recombinant mouse Cat S was prepared by Merck Frosst Canada (Kirkland, QC, Canada) (McGrath et al., 1998). Human Cat D was from BIOMOL Research Laboratories (Plymouth Meeting, PA). The protease substrates were as follows: Z-Arg-Arg-AMC (Calbiochem, San Diego, CA), Z-Leu-Arg-AMC (Novabiochem, Laufelfingen, Switzerland), 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), and *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 (Torrance, CA), respectively. Z-Phe-Tyr-CHO was from Santa Cruz Biotechnology (Santa Cruz, CA). Complete protease inhibitor tablets were purchased from Roche Diagnostics (Indianapolis, IN). The conditions used to assess inhibitor potencies against human, rat, and mouse Cat K, B, L, and S were as described previously (Falgueyret et al., 2004).

Rat Long-Term Inhibitor Dosing and Evaluation of Tissue Cathepsin Activities. Adult female Sprague-Dawley rats were orally dosed at 500 mg/kg/day (dose volume, 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). After 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 two animals was dosed with each test compound individually for 14 or 28 days. On the final day, 2 to 4 h after dosing, plasma was collected and inhibitor levels were measured as described previously (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 volumes of ice-cold homogenization buffer (50 mM MES, pH 5.5, 2.5 mM dithiothreitol, and 2.5 mM EDTA) using a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland). The total protein concentration in tissue lysates was determined by detergent-compatible–Lowry protein assay (Bio-Rad Laboratories, Hercules, CA) according to 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 to 10 μg of protein. Assays were performed in the 96-well plate format, and the plates were 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 dithiothreitol, 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 Z-Leu-Arg-AMC as substrate. To eliminate any contribution from other cysteine pro-

teases, the assays 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-Asp(EDANS)-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Gly-Lys(DABCYL)-Glu-NH_2 in presence of 10 μM E-64 (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 (Sellinger et al., 1960; Arai et al., 1991). The acid phosphatase assay was carried out at 37°C in 90 mM acetate buffer, pH 5.0, using 8 mM *p*-nitrophenyl phosphate as substrate. After 20-min incubation, the activity was stopped by adding NaOH, and the absorbance was measured at 410 nm. Succinate dehydrogenase activity was assayed as described previously (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 95°C and 25 μl was loaded on Tris-glycine 10 to 20% PAGE gels (Invitrogen, Carlsbad, CA). Western blotting was performed with the Novex transfer system and buffer (Invitrogen) onto nitrocellulose using enhanced chemiluminescence detection (GE Healthcare, Baie d'Urfé, QC, Canada). Primary antibodies employed were the following: anti-rat Cat B (1/1000 dilution; US Biological, Swampscott, MA), goat anti-human pro-Cat L (1/200 dilution; Santa Cruz Biotechnology), goat anti-human Cat L (1/100 dilution; Santa Cruz Biotechnology). Secondary antibodies were from Santa Cruz Biotechnology and were diluted 1/1000: donkey anti-goat horseradish peroxidase and donkey anti-rabbit horseradish peroxidase.

Reverse Transcription. Reverse transcription (RT) reactions were performed for each RNA sample in MicroAmp reaction tubes using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Each reaction tube contained 250 ng of total RNA in a volume of 50 μl containing 1× TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM concentrations of each dNTP, 2.5 μM 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, Cat L, 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-tetramethylrhodamine) 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 and 100 bp and designed to span exon junctions. Glyceraldehyde-3-phosphate dehydrogenase 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 1× Universal Master Mix (Applied Biosystems) were combined. Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min. All reactions were performed in ABI Prism 7700 Sequence Detection System in duplicate using Sequence Detector ver. 1.6 program.

Cathepsin S Assay in THP-1 cells. THP-1 cells were grown in RPMI 1640 medium, 10% fetal bovine serum, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/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% bovine serum albumin. After 24 h, the cells were plated at 1.5×10^6 cells/ml in 200 μl of 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 nonradioactive BIL-DMK for 5 min, and cells were centrifuged at 300*g* for 4 min. The medium was removed, and the cells washed with phosphate-buffered saline 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 Tris-buffered saline/0.1% Tween 20 (TBST) for 1 h then probed with goat anti-Cat S (diluted 1:200 in 3% milk TBST; Santa Cruz Biotechnology) for 1 h. Blots were washed 3 × 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. Detection was performed using ECL Plus reagent (GE Healthcare) and chemiluminescence captured using a Fuji charge-coupled device camera. Blots were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and exposed to BioMax MS film (PerkinElmer Life and Analytical Sciences) 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 volume, 10 ml/kg using 1% methocel suspensions) or vehicle. After 1 or 2 h, the mice were dosed intravenously with ^{125}I-BIL-DMK (0.03 mCi, 0.01 nmol; dose volume, 5 ml/kg using 60% polyethylene glycol as vehicle) and after 60 min were euthanized by CO_2; tissues were 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 E-64. The tissue lysates were diluted to 2.5 mg/ml protein in Laemmli sample buffer. Labeled proteins were separated on Tris-glycine 12% PAGE (Invitrogen) and quantified as described previously (Falgueyret et al., 2004). A satellite arm of 2 animals was dosed with each test compound individually, followed at 1 h by an intravenous dose (5 ml/kg) of 60% polyethylene glycol. After an additional hour, plasma and tissues were collected and inhibitor levels measured as described previously (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 analog of L-006235 in which the electrophilic nitrile warhead was replaced by a nonreactive trifluoromethyl group (Fig. 1, Cmpd A) 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 clan 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 Long-Term 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/day. Peak plasma levels (2–4 h after 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). After 4 weeks of daily dosing and 24 h after the last dose, the brains, livers, kidneys, and spleens of vehicle- and compound-treated animals were harvested. All four compounds were well tolerated; no clinical signs of toxicity were 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 after 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 with 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 generally to a lower degree than that found for Cat B and L. Only small and often nonsignificant increases in the lysosomal enzyme activities of NAGA and acid phosphatase 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 nonbasic 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 the increases were not observed in other tissues (Table 3). Only very minor changes in the activity of the mitochondrial enzyme succinate dehydrogenase 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 compared with vehicle-treated animals 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 with 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. 2A), as well as the spleen, liver, and brain (data not shown). Processing of the single to the two-chain form of Cat B, which are both catalytically active, is blocked in mice deficient for the lysosomal cysteine protease legumain (Shirahama-Noda et al., 2003) and in cells by the nonselective cysteine protease inhibitors E-64d and leupeptin (Hara et al., 1988). In this study, the ratio of these two species was not affected by cathepsin inhibitor treatment (Fig. 2A). Only a single form of mature Cat L was detected in the kidney (Fig. 2B) 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 with vehicle-treated animals.

mRNA 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 with the relatively highly expressed Cat B and Cat L.

Correlation between Cathepsin S Inhibition and Protein Stabilization in THP-1 Cells. To gain some understand-

TABLE 1

Potencies of cathepsin inhibitors across species

All data are the average of at least 3 independent titrations.

Compounds & Species	IC_{50}			
	Cat K	Cat B	Cat L	Cat S
	nM			
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	>10,000	>10,000	>10,000
Rat		>10,000	>10,000	>10,000
Mouse				>10,000
Cmpd B				
Human	410	1100	1010	1.8
Mouse	1650	230	3040	0.6

TABLE 2

Rat pharmacokinetic parameters for cathepsin inhibitors when dosed at 500 mg/kg

Plasma C_{max} values were obtained 2 to 4 h after oral dosing. Plasma exposure is over 24 h.

	Plasma C_{max}	Plasma Exposure
	μM	$AUC_{0-24\ h}$, *μM/h*
L-006235	50	690
Balicatib	67	1080
Cmpd A	25	
L-873724	22	460

TABLE 3

Effect of long-term 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.

Compounds & Tissues	Cat B	Cat L	Cat D	NAGA	Acid Phosphatase	Succinate Dehydrogenase
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*

* $P < 0.05$.

ing 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 because a potent and selective nonbasic 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 labeled with 1 nM ^{125}I-BIL-DMK, a nonselective, irreversible cysteine cathepsin activity-based probe (ABP) 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). After separation of cell lysate proteins by SDS-PAGE and transfer to nitrocellulose, Cat S protein was detected by Western blotting and ^{125}I-labeled 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-labeling by the ABP (Fig. 4, A and C), and the increase in Cat S protein in the THP-1 cells (Fig. 4, B and C), with EC_{50} values of 5 to 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-labeled 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 labeled ABP has been used to identify the targets of active site-directed inhibitors toward proteases, as well as kinases and phosphatases (Fonović and Bogyo, 2007). In general, 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). One hour after intravenous administration of ^{125}I-BIL-DMK, mice were euthanized, and spleen, liver, kidney and lung tissue lysates rapidly prepared in the presence of 100 μM E-64. Radioactivity levels in each organ corresponded to tissue levels of approximately 0.1 nM parent or its metabolite(s). Separation of the radiolabeled proteins by two-dimensional gels followed by autoradiography (data not shown) demonstrated that, as was the case with intact cells, relatively few labeled proteins were obtained (Falgueyret et al., 2004). These labeled 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-labeled Cat B and L, whereas lung and spleen contain an additional radiolabeled band corresponding to Cat S. The cathepsin labeling obtained in each tissue is consistent with known tissue localizations, because 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

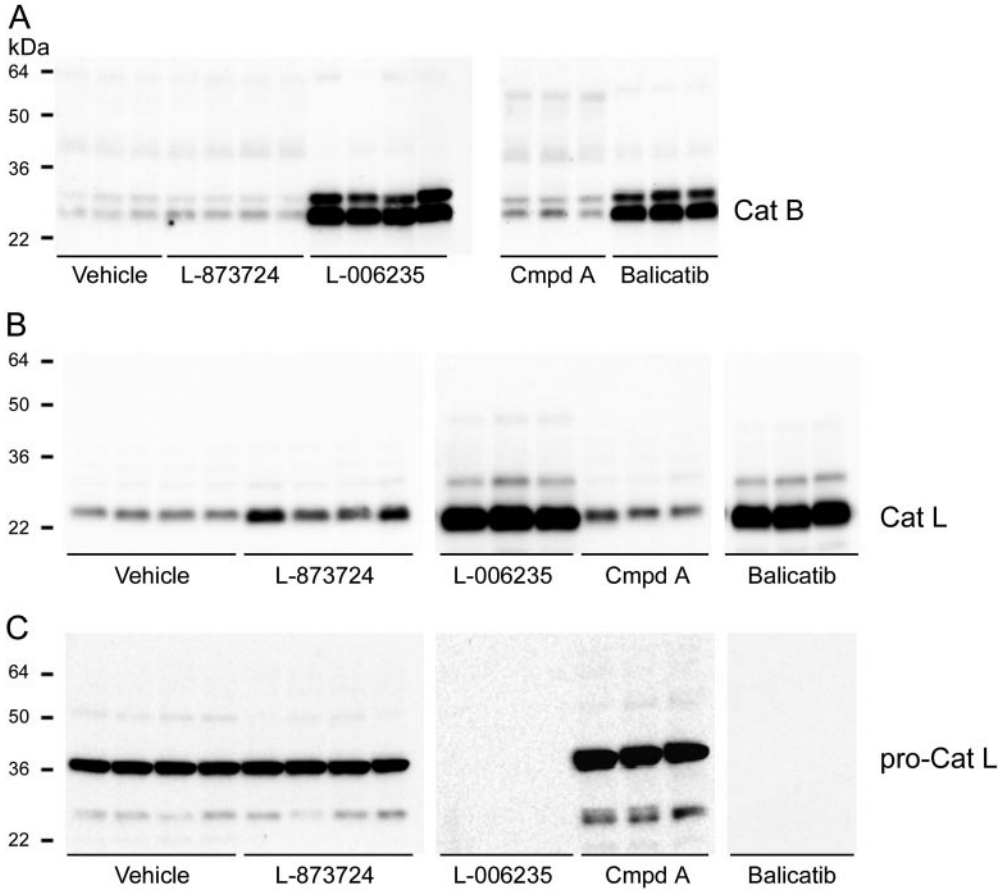

Fig. 2. Western blot analysis of rat kidney extracts of animals dosed with vehicle or 500 mg/kg/day L-873724, L-006235, Cmpd A, or balicatib for 4 weeks. The same extracts (50 μg of protein per lane) were run on three different gels and each was blotted against antimature Cat B (A), mature Cat L (B), and pro-Cat L (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.

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 nonbasic selective Cat S inhibitor (Fig. 1 and Table 1) was then tested for its ability to compete for ^{125}I-BIL-DMK cathepsin labeling 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 before the intravenous administration of ^{125}I-BIL-DMK. After an additional hour, the organs were harvested as described under *Materials and Methods*. 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 labeling 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 after oral dosing of the prodrug to satellite animals are shown in Supplemental Data.

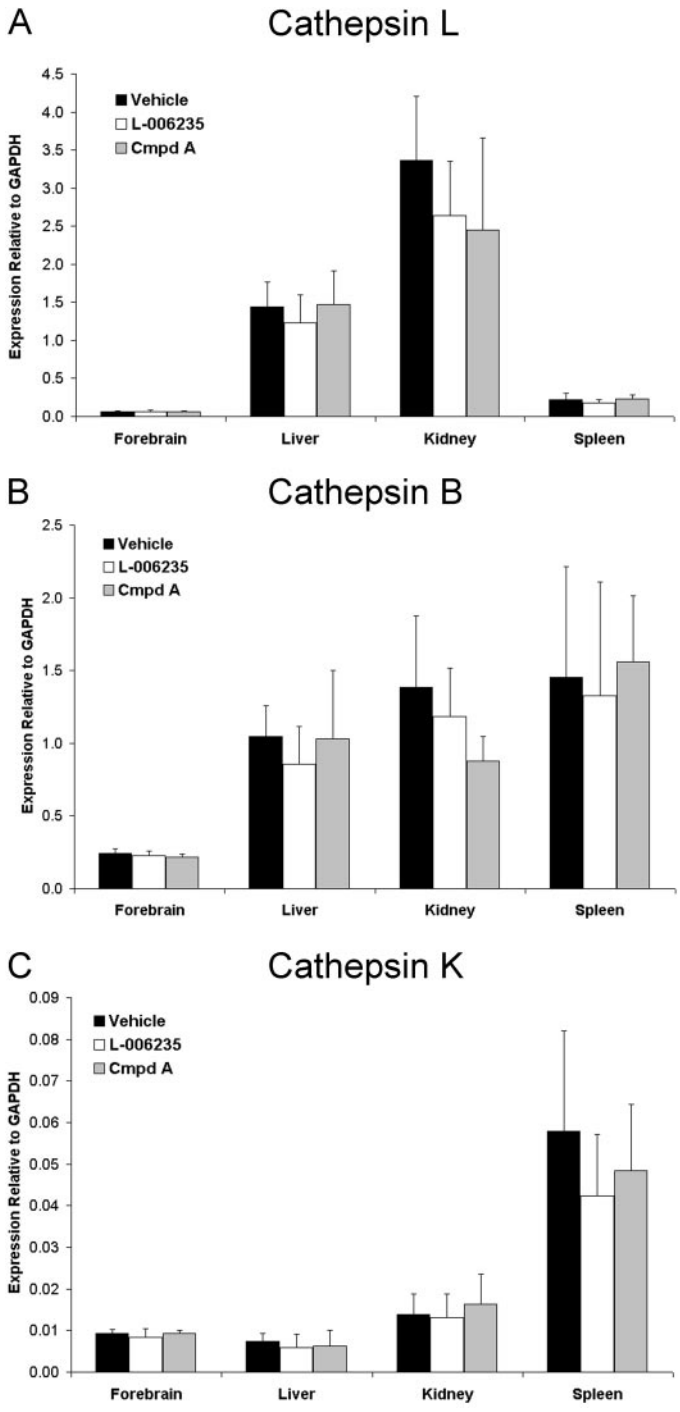

Fig. 3. Messenger RNA analysis of rat tissues of animals dosed with vehicle or 500 mg/kg/day L-006235 or Cmpd A for 4 weeks. The expression levels shown are relative to that of glyceraldehyde-3-phosphate dehydrogenase and represent averages ± S.D. of analyses of tissue from three to four animals. A, Cat L. B, Cat B. C, Cat K.

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 because of 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 after an additional 1 h, the animals were euthanized and the tissues removed and rapidly processed. 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 approximately 5- to 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 nonbasic 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 labeling 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 nonbasic 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 days' treatment. The efficacy in this model was comparable with that of a high dose of the effective bisphos-

phonate 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/days (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 because 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 long-term treatment rat studies described here were therefore performed at supratherapeutic doses with C_{max} values and exposures of L-006235 and L-873724 on the order of 25 to 300 times that required for efficacy in rabbits (Table 2).

These long-term treatment 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 long-term treatment with a supratherapeutic dose of the nonbasic 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 L levels occurred in the absence of increased message (Fig. 3). Previous reports have shown that both nonselective, nonbasic cysteine cathepsin inhibitors (Kominami et al., 1987; Montenez et al., 1994) and lysosomotropic compounds, such as chloroquine (Gerard et al., 1988; Gerbaux et al., 1996), 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 stabilization toward 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 nonbasic 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

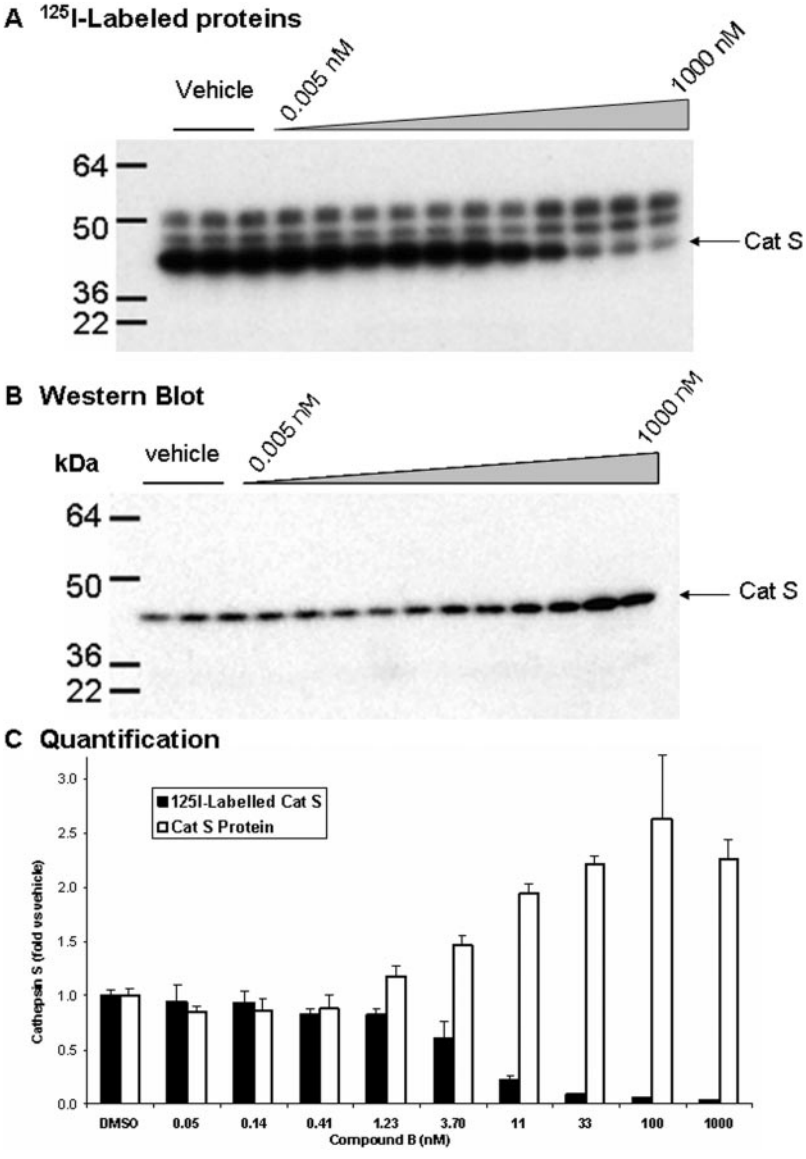

Fig. 4. Effect of Cmpd B on Cat S activity and protein levels in THP-1 cells. THP-1 cells were cultured in the presence of Cmpd B for 24 h, and the cells were then labeled with 1 nM ^{125}I-BIL-DMK for 25 min. The cell lysate proteins were separated by SDS-PAGE and transfer to nitrocellulose, Cat S protein being detected by Western blotting. ^{125}I-labeled proteins were detected by autoradiography. A, representative whole-cell enzyme occupancy titration of Cat S activity (^{125}I-labeled proteins) with Cmpd B (left to right; 0.005, 0.015, 0.05, 0.14, 0.41, 1.2, 3.7, 11, 33, 100, 1000 nM). B, representative Western blot titration of cell lysate Cat S with Cmpd B. 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.

L-006235 result in the lysosomal accumulation of these cathepsins because of their stabilization to proteolysis. Only balicatib and L-006235 cause this phenomenon, because their lysosomotropic properties cause them to concentrate within the lysosomes to attain levels that inhibit the Cat B and L, which are localized within these organelles. Indeed, the activities of these two basic inhibitors, but not the nonbasic L-873724, against intracellular Cat B and L are increased up to 100-fold in cell-based assays compared with activities against the purified enzymes (Falgueyret et al., 2005). The lack of any off-target effect of the nonbasic 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 probably similar to their intrinsic enzyme potencies (Table 1), because little serum protein binding has been observed for these compounds (S. Desmarais, unpublished data). 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, these compounds target both intra- and extracellular cathepsins, but the basic inhibitors suffer increased off-target activity (against the lysosomal cathepsins) because of their lysosomotropic properties.

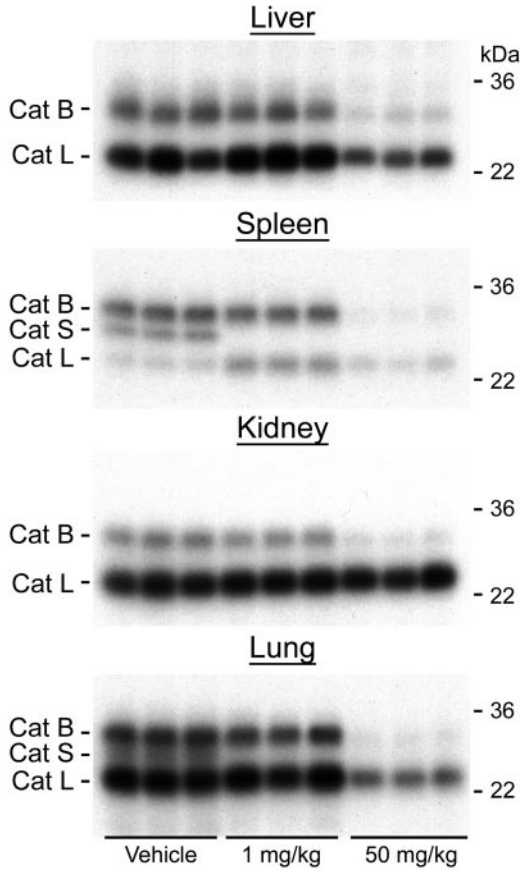

Fig. 5. Effect of Cmpd B on tissue cathepsin B, L, and S active site occupancy. Mice were dosed orally with Cmpd B (1 and 50 mg/kg) or vehicle. After 1 h, the mice were dosed intravenously with ^{125}I-BIL-DMK and after a further 60 min were euthanized. The tissues (liver, spleen, kidney, and lung) were rapidly collected and homogenized in the presence of E-64. Tissue lysate proteins were separated on SDS-PAGE gels that were subjected to autoradiography. Each lane represents a sample of a tissue lysate from a single mouse.

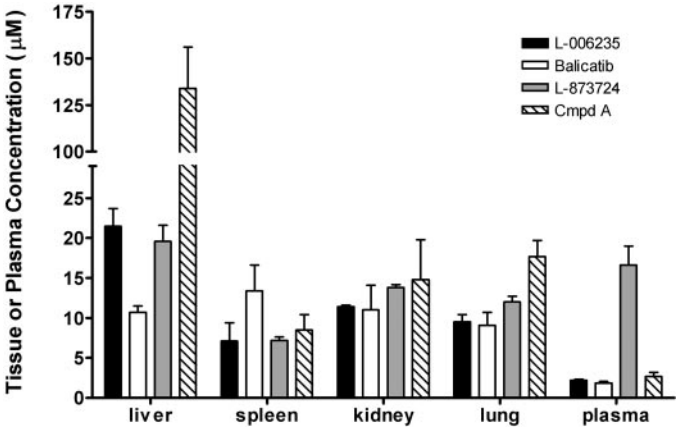

Fig. 6. Tissue and plasma concentrations of mice dosed with L-006235, balicatib, L-873724, and Cmpd A. Satellite animals to the study described in Fig. 7 were each dosed orally with 10 mg/kg L-006235, 25 mg/kg balicatib, 10 mg/kg L-873724, and 60 mg/kg Cmpd A and were euthanized after 2 h. Drug tissue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

The majority of the effects of L-006235 and balicatib on tissue enzyme activities are not a consequence of lysosomal alkalinization resulting in inhibition of lysosomal protease activities, because a only a minor modulation of lysosomal enzymes was caused by treatment with the basic, inactive analog 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 is 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). It is noteworthy that 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 analog (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 μM (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 labeling 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 equiv-

alent (Fig. 6), no competition of Cat B, L, and S labeling 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 severalfold higher than that predicted to provide efficacy also showed off-target activity. The nonbasic Cat K inhibitor L-873724 has similar, and in many cases greater, activities against isolated Cat B, L, and S compared with 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

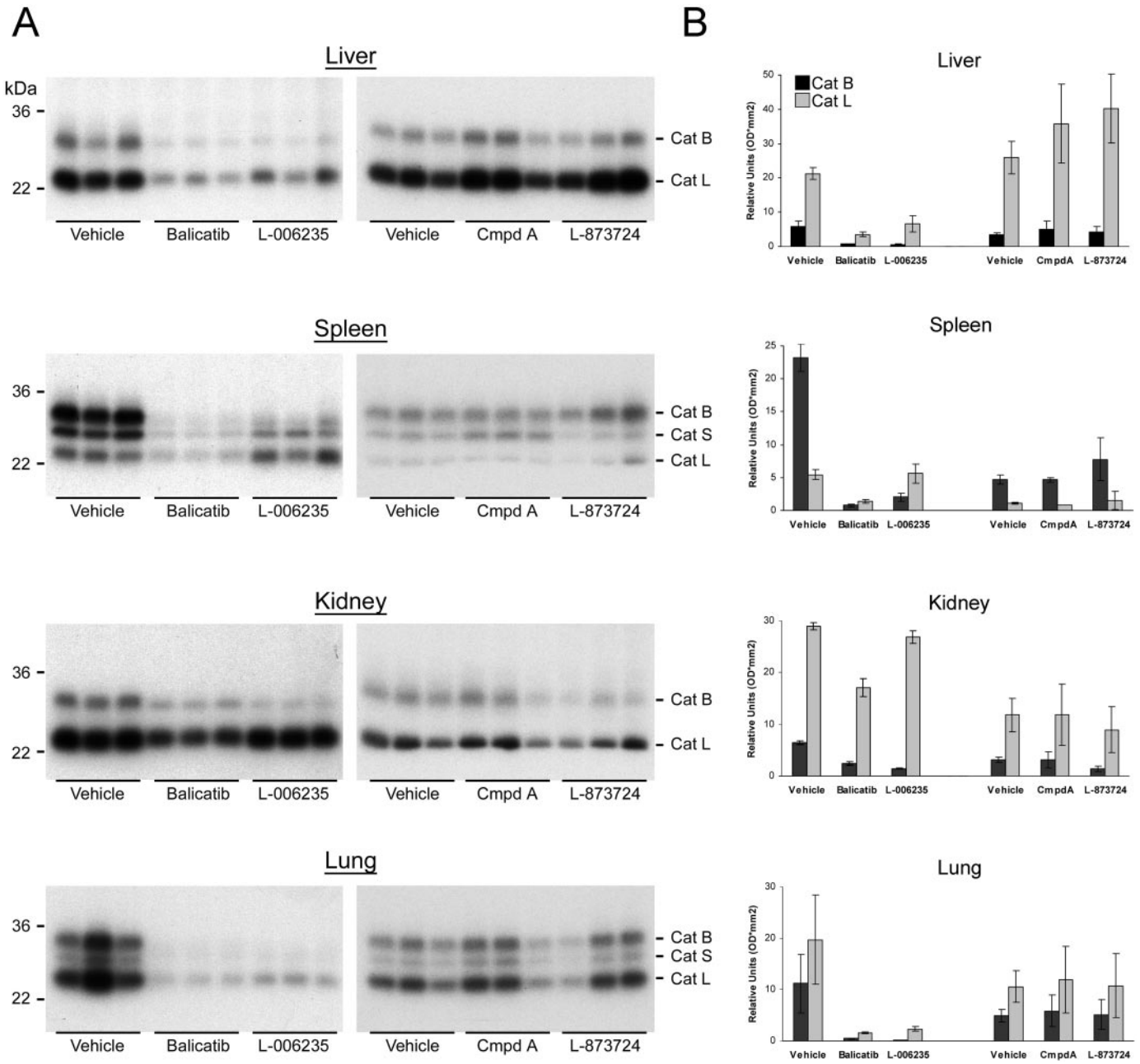

Fig. 7. Effect of L-006235, balicatib, L-873724, and Cmpd A on tissue cathepsin B, L, and S active site occupancy. Mice were dosed orally with 10 mg/kg L-006235, 25 mg/kg balicatib, 10 mg/kg L-873724, and 60 mg/kg Cmpd A or vehicle. After 1 h, the mice were dosed intravenously with ^{125}I-BIL-DMK and after a further 60 min were euthanized. The tissues (liver, spleen, kidney, and lung) were rapidly collected and homogenized in the presence of E-64. A, tissue lysate proteins were separated on SDS-PAGE gels that 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. B, the relative intensities of the cathepsin bands were quantified by densitometry for each group (mean ± S.D.).

are localized. Based on this study, inhibition of off-target lysosomal cysteine cathepsins by L-873724 or structurally related nonbasic Cat K inhibitors may not be expected, even at doses manyfold higher than that required for efficacy.

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