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Inhibition of the Activation of Multiple Serine Proteases with a Cathepsin C Inhibitor Requires Sustained Exposure to Prevent Pro-enzyme Processing*[S]

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Cathepsin C is a cysteine protease required for the activation of several pro-inflammatory serine proteases and, as such, is of interest as a therapeutic target. In cathepsin C-deficient mice and humans, the N-terminal processing and activation of neutrophil elastase, cathepsin G, and proteinase-3 is abolished and is accompanied by a reduction of protein levels. Pharmacologically, the consequence of cathepsin C inhibition on the activation of these serine proteases has not been described, due to the lack of stable and non-toxic inhibitors and the absence of appropriate experimental cell systems. Using novel reversible peptide nitrile inhibitors of cathepsin C, and cell-based assays with U937 and EcoM-G cells, we determined the effects of pharmacological inhibition of cathepsin C on serine protease activity. We show that indirect and complete inhibition of neutrophil elastase, cathepsin G, and proteinase-3 is achievable in intact cells with selective and non-cytotoxic cathepsin C inhibitors, at concentrations ~10-fold higher than those required to inhibit purified cathepsin C. The concentration of inhibitor needed to block processing of these three serine proteases was similar, regardless of the cell system used. Importantly, cathepsin C inhibition must be sustained to maintain serine protease inhibition, because removal of the reversible inhibitors resulted in the activation of pro-enzymes in intact cells. These findings demonstrate that near complete inhibition of multiple serine proteases can be achieved with cathepsin C inhibitors and that cathepsin C inhibition represents a viable but challenging approach for the treatment of neutrophil-based inflammatory diseases.

Cathepsin C (also known as dipeptidyl peptidase-1, EC 3.4.14.1) is a cysteine dipeptidyl aminopeptidase expressed in the lysosomes of several tissues, with levels highest in lung, macrophages, neutrophils, CD8+ T cells, and mast cells (1–5). The enzyme cleaves two-residue units from the N termini of proteins until it reaches a stop sequence, typically an arginine or lysine in P2 (6), a proline residue in P1 or P1′ (7), or an isoleucine residue in P1 (8).

Cathepsin C (CG)[3] is implicated in processing of certain lysosomal cathepsins (9) and in the degradation of intracellular proteins (7, 10, 11). More recently, cathepsin C was shown to activate several chymotrypsin-like serine proteases by removing an inhibitory N-terminal dipeptide. CG, proteinase-3 (Pr-3), neutrophil elastase (NE), granzymes A, B, and C, and mast cell chymase and tryptase all require cathepsin C-mediated cleavage for their full activation (12–18).

Serine proteases, particularly NE, are believed to play important roles in human pathologies. This is clearly evident from early onset emphysema in patients deficient in plasma α1AT, the main NE inhibitor in the lung (19; reviewed in Ref. 20). Cystic fibrosis is another disease in which excess NE leads to inflammation and tissue destruction (21). *In vitro*, NE affects the levels of tumor necrosis factor α (22, 23) and transforming growth factor α and stimulates mucus secretion (22). NE may potentiate the oncogenicity of PML-RARα in acute promyelocytic leukemia and in various other cancers (24; reviewed in Ref. 25). Another serine protease, mast cell tryptase, is thought to participate to asthma pathophysiology through its effect on bronchodilating peptides and protease-activated receptor-2 (PAR-2) (26, 27). CG and Pr-3 have distinct *in vitro* effects on cell signaling and cytokine and chemokine processing. CG modulates chemokine and reactive oxygen species release in murine polymorphonuclear leukocytes (28) and activates PAR-4 (29). Pr-3 mediates interleukin-18 release (30) and stimulates cell proliferation (31, 32).

Non-overlapping roles for CG, NE, and Pr-3 are less clear *in vivo*, but some differences have emerged, particularly with respect to host responses to bacteria (33–35) and to inflammatory diseases (reviewed in Ref. 36). In a murine model of collagen-antibody induced arthritis, the clinical score of arthritis was lower in $NE^{-/-}/CG^{-/-}$ double mutant mice compared with single deletions of either NE or CG. Deletion of cathepsin C provided equivalent protection as NE/CG double mutants in

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Tables S1–S3.

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[3] The abbreviations used are: CG, cathepsin G; AMC, aminomethyl coumarin; DMK, diazomethylketone; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, *N*-3-(2-4-dinitrophenyl)-1-2-3-diaminopropyonyl; L2p, lysyl-2-(picolinoyl); NE, neutrophil elastase; Pr-3, proteinase-3; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid; DTT, dithiothreitol; PAR, protease-activated receptor.

this model (14) and significant protection in the collagen-induced arthritis model (37).

Based on this genetic data, cathepsin C inhibition should result in a reduction in activity of several clinically important serine protease targets and could potentially be of superior efficacy to blocking individual serine proteases, especially in complex inflammatory diseases such as chronic obstructive pulmonary disease. Several potent and selective cathepsin C inhibitors have been described, but most suffer from poor metabolic stability and cytotoxicity (38–41). Partial inhibition of serine proteases by a cathepsin C inhibitor has been shown with Gly-Phe-DMK, a metabolically unstable and cytotoxic compound (12, 18, 42).

We characterize here novel non-cytotoxic, cell-permeable, and reversible cathepsin C inhibitors. We used two neutrophil-like cell lines to demonstrate for the first time that near complete inhibition of NE, CG, and Pr-3 is achievable with selective cathepsin C inhibitors but not by inhibitors of other cysteine cathepsins. Inhibition of CG and NE was maintained for several days, but removal of the reversible inhibitor resulted in a gradual recovery of serine protease activities. These data validate the development of small molecule inhibitors of cathepsin C as a strategy to block the processing and activity of multiple pro-inflammatory proteases but also highlight a potential difficulty, in that a high and sustained level of cathepsin C inhibition may be required for efficacy.

EXPERIMENTAL PROCEDURES

Reagents—Gly-Phe-DMK was purchased from ICN Pharmaceuticals (Aurora, OH). Ala-4-(I)Phe-DMK (where "I" represents iodine), Ala-4-[^{125}I]Phe-DMK, leucine-homophenylaline-vinylsulfone, and dipeptide nitriles were synthesized at Merck Frosst. A complete description of the synthesis of novel compounds will be published elsewhere. BIL-DMK (43), compound 6,[4] and compound 7 (gift from Renata Oballa) were obtained from Merck Frosst. L-694,458 (44) was obtained from Merck & Co. Rabbit anti-human cathepsin G antibody and cathepsin G inhibitor I were obtained from EMD Biosciences (San Diego, CA). Aprotinin-agarose, cycloheximide, β-estradiol, E64, and E64d were purchased from Sigma-Aldrich. Z-Phe-Arg-AMC, Z-Val-Val-Arg-AMC, *t*-butoxycarbonyl-Leu-Lys-Arg-AMC, methoxysuccinyl-Ala-Ala-Pro-Val-AMC, and Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide were obtained from Bachem AG, and NH_2-Gly-Arg-AMC was from Nova Biochem. Mca-L2p-Tyr-Asp-Ala-Lys-Gly-Asp-DpaNH_2 was custom-synthesized at AnaSpec Inc. (San Jose, CA). Human sputum NE was purchased from Elastin Products Co., Inc., whereas CG was obtained from Biodesign Int. (Saco, ME). Recombinant human cathepsins C and L were purchased from R&D Systems, recombinant human cathepsin S from Calbiochem, human liver cathepsin H from Biomol, and human liver cathepsin B from Sigma-Aldrich. Methionine and glutamine-free RPMI 1640 and mouse recombinant granulocyte macrophage-colony stimulating factor were obtained from BIOSOURCE Int. RPMI 1640, sodium pyruvate, penicillin-streptomycin, PBS solutions were from Mediatech, Inc. Fetal bovine serum was purchased from HyClone. [^{35}S]Methionine (1000 Ci/mmol) was obtained from Amersham Biosciences.

Cell-based Assays—U937 cells (ATCC 1593.2) were propagated in U937 culture media (RPMI 1640 supplemented with 10% fetal bovine serum, 10 mM Hepes, 1 mM sodium pyruvate, 100 units/ml each of penicillin and streptomycin). EcoM-G cells (clone EPSΔ1 ER 3.3 (45) from Dr. Mark Kamps, University of California-San Diego) were grown in RPMI 1640, 10% fetal bovine serum, 100 units/ml each of penicillin and streptomycin, 10 ng/ml recombinant murine granulocyte macrophage-colony stimulating factor, and 1 μM β-estradiol. The U937 cathepsin G aprotinin-agarose assay is described in greater details below. The U937 NE 7-day incubation assay is described in the legend of Fig. 3. All experiments using EcoM-G cells were performed with cultures <3 months of age. Exponentially growing EcoM-G cells were washed once with PBS and seeded in 96-well plates at 0.5×10^6 cells/ml in EcoM-G culture media without β-estradiol. The cells were incubated for 24 h, at which time compound diluted in Me_2SO (final Me_2SO, 0.5%) was added. EcoM-G cells were differentiated for another 24 h, harvested by centrifugation at 400 × *g*, and processed for serine protease activity determination. Data were plotted using SigmaPlot 9.0, and sigmoidal curve fitting was performed using the Hill four-parameter equation.

Cytotoxicity Assays—U937 cells were plated at a density 0.4×10^6 cells/ml in U937 culture media, with 0.2 ml of cell suspension per well, in 96-well dishes. Vehicle (Me_2SO) or compound diluted in Me_2SO, were added. In all wells the final Me_2SO concentration was 0.5%. After 24 h, some of the cells were harvested, washed with PBS, and processed to measure either cathepsin C activity (see below) or cell viability. For incubations exceeding 24 h, the cells were harvested every 24 h and re-seeded in U937 culture media containing freshly added inhibitors. Viability was determined using the TACS Annexin V-FITC kit (R&D Systems) according to the manufacturer's specifications and two-color flow cytometric analysis on a FACSCalibur system (BD Biosciences). Cells were considered viable if they failed to stain for annexin V or propidium iodide.

Cathepsin C Active Site Labeling—Ala-4-[^{125}I]Phe-DMK (2000 Ci/mmol) was added directly to a final concentration of 0.5 nM to U937 cells (200,000 cells) seeded in culture media and that had been preincubated for 30 min with either vehicle or cathepsin inhibitors. Labeling proceeded for 15 min before quenching by addition of unlabeled Ala-4-(I)Phe-DMK (1 μM). The cells were pelleted by centrifugation at 400 × *g* for 5 min and washed with PBS containing 1 μM of Ala-4-(I)Phe-DMK. The cells were lysed 15 min on ice with 20 mM MES, pH 6.0, 50 mM NaCl, 0.5% Nonidet P-40, and debris was removed by centrifugation at 15,000 × *g* for 10 min. Proteins were denatured by addition of reducing Laemmli buffer, heated at 95 °C for 5 min, and resolved on 10–20% SDS-PAGE gels. The gels were fixed as described below and exposed to film for 6–24 h.

[^{35}S]Methionine Labeling and Aprotinin-Agarose Binding—Exponentially growing U937 cells were washed in PBS and plated at 2×10^6 cells/ml in methionine glutamine-free RPMI

[4] Gauthier, J.-Y., Black, C. W., Courchesne, I., Cromlish, W., Desmarais, S., Houle, R., Lamontagne, S., Li, C. S., McKay, D. J., Ouellet, M., Robichaud, J., Truchon, J.-F., Truong, V.-L., Wang, Q., and Percival, M. D. (2007) *Bioorg. Med. Chem. Lett.*, in press.

1640/10% dialyzed fetal bovine serum. The cells were methionine-starved for 30 min before the addition of [^{35}S]methionine (10 μCi/ml). Me_2SO (0.5%) or cathepsin inhibitors were added during the methionine starvation. After 30 min, the cells were harvested, washed twice with PBS, re-seeded in complete U937 culture medium in the presence of vehicle or cathepsin C inhibitors, and incubated for an additional 3 h. At the end of the chase period, the cells were washed two times with PBS and lysed on ice for 15 min in lysis buffer A (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% Nonidet P-40). Cell debris was removed by centrifugation at 15,000 × *g* for 10 min. The supernatant was mixed with 4 volumes of binding buffer A (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 1% Nonidet P-40). Where indicated, L-694,458 or cathepsin G inhibitor I was mixed with the lysate 30 min before addition of aprotinin-agarose. The beads were incubated with lysate (or purified serine proteases) for 1 h at room temperature with gentle rotation and pelleted by brief centrifugation at 750 × *g*. The beads were washed three times with binding buffer and once with lysis buffer A. Bound proteins were eluted by addition of Laemmli buffer and heating at 95 °C for 5 min. The polypeptides were resolved on 10–20% SDS-PAGE gels (Invitrogen), fixed in 40% methanol/7.5% acetic acid before soaking in Enlightning™ (PerkinElmer Life Sciences) and drying. The gels were exposed to Kodak BioMax MR film with intensifying screens for 24–48 h. Densitometry on ^{35}S-labeled CG was performed using a Bio-Rad GS-800 calibrated densitometer and QuantityOne software. Curve fitting was performed with Sigma Plot 9.0 using the Hill four-parameter equation.

Western Blotting, Immunoprecipitation, and Silver Stains—New Zealand White rabbits were immunized with keyhole limpet hemocyanin-conjugated peptide (H-SEIVGGRRARPHC) corresponding to the unprocessed N terminus of pro-human NE using a protocol approved by the Merck & Co. animal care committee. The immunization was followed by successive boosts in complete Freund's adjuvant. Antibodies were purified from crude antisera on an H-SEIVGGRRARPHC column. The affinity-purified antibody was diluted to 5 μg/ml in TBS supplemented with 0.1% Tween 20 (TBS-T) and 5% powdered milk and was used to probe nitrocellulose membranes onto which 50 μg of extracts had been transferred. Antigen-antibody complexes were revealed by incubation with anti-rabbit-IgG-horseradish peroxidase (Amersham Biosciences) diluted 5000-fold in TBS-T plus 5% milk and development with West-femto chemiluminescence reagents (Pierce) on Hyperfilm ECL (Amersham Biosciences). The limit of detection was 20 ng of purified human sputum NE. Immunoprecipitations for each experimental conditions were carried out with extracts from 2×10^6 U937 cells that had been metabolically labeled with [^{35}S]methionine as described above. Rabbit anti-cathepsin G antibodies (10 μg) were added to the extract in a buffer that contained 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, and 0.5 M NaCl, and incubated for 1 h at room temperature. Protein A-Sepharose beads were added for another hour. The beads were pelleted by centrifugation at 300 × *g* and washed 3× in binding buffer A and 1× in lysis buffer A. Bound proteins were eluted from the beads with 1× Laemmli buffer and resolved by 10–20% SDS-PAGE. Fluorography was performed as described above. Silver staining was performed using a silver nitrate method described previously (46).

Protease Activity Assays—Neutrophil elastase, cathepsin G, or proteinase-3 enzymatic activities were measured from U937 and EcoM-G cytosolic lysates, in a final assay volume of 110 μl. Briefly, cells were washed twice with PBS and lysed with either NE lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.2% Nonidet P-40) for serine protease assays, or with cathepsin C lysis buffer (25 mM MES, pH 6.0, 50 mM NaCl, 5 mM DTT, 0.2% Nonidet P-40) for cathepsin C assays. Debris was removed by centrifugation at 15,000 × *g* for 10 min, and supernatants were retained. The extracts were mixed with assay buffer (100 mM Tris-HCl, pH 7.5, and 1 M NaCl) supplemented with a peptide substrate specific for the serine protease assessed: for NE, 400 μM methoxysuccinyl-Ala-Ala-Pro-Val-AMC; for CG, 1 mM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide; and for Pr-3, 10 μM L2p-Tyr-Asp-Ala-Lys-Gly-Asp-Dpa-NH_2 (47). Cleavage of the substrate was monitored spectrophotometrically using Spectramax plate readers (Molecular Devices), and kinetic rates were calculated from the linear portion of the reaction. Release of AMC was measured with excitation at 370 nm and emission of 450 nm for 10 min. For *p*-nitroanilide, substrate cleavage was measured by absorbance at 405 nm for 5 min. Pr-3 substrate cleavage was monitored by loss of intramolecular substrate quenching (excitation, 328 nm; emission, 393 nm) for 5 min. The specificity of each reaction was ascertained by using protease inhibitors (L-694,458 for NE, and cathepsin G inhibitor I for CG). NE activity in U937 and EcoM-G was fully inhibited by L-694,458, and similarly, the CG activity detected in crude lysates was completely inhibited by cathepsin G inhibitor I. To demonstrate that cleavage of L2p-Tyr-Asp-Ala-Lys-Gly-Asp-Dpa-NH_2 was due to Pr-3 and not NE in crude EcoM-G lysates, the serpin SLPI was used. SLPI is active against NE but not against Pr-3. Cleavage of L2p-Tyr-Asp-Ala-Lys-Gly-Asp-Dpa-NH_2 was not inhibited by SLPI, whereas cleavage of methoxysuccinyl-Ala-Ala-Pro-Val-AMC was abolished in the presence of SLPI.[5] Cathepsin C activity was determined in a similar manner on cytosolic extracts normalized for protein content and on purified recombinant protein (R&D Systems). The assay was performed by mixing the lysate (diluted 1:10 in cathepsin C lysis buffer) to 25 mM MES, pH 6.0, 50 mM NaCl, 5 mM DTT, supplemented with 50 μM of NH_2-Gly-Arg-AMC. The concentration of substrate used for the cathepsin C assay is equivalent to twice the K_m value, under the assay conditions used. When using purified enzyme, 1 nM cathepsin C was used. Cathepsin L enzymatic assays were performed with purified recombinant cathepsin L (R&D Systems) with 0.35 nM enzyme, 2 μM substrate (Z-Phe-Arg-AMC) in 50 mM MES, pH 5.5, 2.5 mM EDTA, 2.5 mM DTT, and 10% Me_2SO. Human recombinant cathepsin S (Calbiochem) was used at 1 nM with 40 μM Z-Val-Val-Arg-AMC in 50 mM MES, pH 6.5, 2.5 mM EDTA, 2.5 mM DTT, 100 mM NaCl, 0.001% bovine serum albumin, and 10% Me_2SO. Cathepsin H activity was assayed with 2.1 μg/ml enzyme in 50 mM Tris-HCl, pH 6.8, 1 mM EDTA, 2.5 mM DTT, and 20 μM Z-Arg-AMC. Cathepsin B assays were performed with 1 nM

[5] N. Méthot, data not shown.

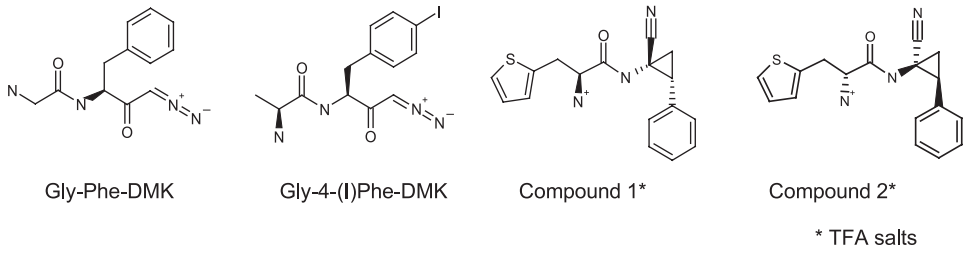

FIGURE 1. **Structure of DMKs and novel dipeptide nitrile inhibitors used in this study.** Compounds with an *asterisk* were synthesized as trifluoroacetic acids salts. Details on the synthesis of Gly-4-(I)Phe-DMK, compounds 1, 2, and 3 will be published elsewhere (D. Guay, C. Beaulieu, T. J. Reddy, N. Methot, D. Ethier, and J. Rubin, manuscript in preparation). Structures and potencies for additional cathepsin C and other poly-cathepsin inhibitors can be found in the supplemental information.

enzyme in 50 mM MES, pH 6.0, 1 mM EDTA, 2.5 mM DTT, 0.001% Triton X-100, 10% Me_2SO, and 83 μM *t*-butoxycarbonyl-Leu-Lys-Arg-AMC. In all cases, the potencies of the compounds on the various cathepsins were determined by pre-incubating the diluted enzyme with the inhibitor for 10 min, and then adding the protease substrate. All reactions were carried out at room temperature.

RESULTS

Cell Permeability and Cytotoxicity of Selective Nitrile Cathepsin C Inhibitors—Gly-Phe-DMK is the only cathepsin C inhibitor described to date that was shown to block NE and CG activities in U937 cells, albeit incompletely, and only after 3 days or more of incubation (12, 18, 42). We prepared a 50-fold more potent inhibitor of cathepsin C, Ala-4-(I)Phe-DMK, by substituting the glycine in P2 for an alanine, and by adding an iodine in the para position of the phenylalanine in P1. The structures and potencies of this inhibitor and of other novel inhibitors used in these studies are shown in Fig. 1, Table 1, and in the online supplemental information section.

To test whether complete NE inhibition could be achieved with Ala-4-(I)Phe-DMK, U937 cells were incubated with either Gly-Phe-DMK or Ala-4-(I)Phe-DMK. Both inhibitors fully blocked cathepsin C activity but showed significant cytotoxicity, with only 32% viable cells remaining after 72 h of incubation with 1 μM inhibitor (Table 2). New cathepsin C inhibitors from the dipeptide α-aminoacetonitrile class (supplemental Table S1) were therefore prepared and tested for cytotoxicity and cathepsin C inhibition. Nitrile inhibitors reversibly block cysteine protease activity (48), unlike diazomethylketones (DMKs), which bind irreversibly to the active site cysteine (reviewed in Ref. 49). No cytotoxicity on U937 cells was detected with the nitrile inhibitors at 30 μM, even after 72 h of incubation (Table 2).

To determine whether these nitrile inhibitors were cell-permeable, we radiolabeled Ala-4-(I)Phe-DMK with ^{125}I and used it as an active site probe in competition experiments. When added to U937 cells, Ala-4-[^{125}I]Phe-DMK labeled 23-, 27-, and 43-kDa polypeptides (Fig. 2, *lane 1*). The 23-kDa protein migrates at the size expected for the cathepsin C large subunit bearing the active site cysteine. When the cathepsin C-selective inhibitor compound 1 was added prior to Ala-4-[^{125}I]Phe-DMK, labeling of the 23-kDa, but not the of 27 and 43-kDa proteins, was abolished (*lane 2*). Labeling of the latter two proteins was prevented by the non-selective cathepsin inhibitor BIL-DMK (43) and leucine-homophenylalanine-vinylsulfone (*lanes 3* and *4*). The 23-kDa protein was absent in cathepsin $C^{-/-}$ bone marrow cells labeled with Ala-4-[^{125}I]Phe-DMK, whereas labeling of the 27- and 43-kDa proteins was unaltered.[6] These results confirm that the 23-kDa protein labeled by Ala-4-[^{125}I]Phe-DMK is the large subunit of cathepsin C. To verify that labeling occurred intracellularly, the cells were incubated with the cell-impermeable cysteine protease inhibitor E64, or the cell-permeable pro-drug analog, E64d (49). E64 did not block labeling of cathepsin C, in contrast to E64d. Taken together, these data demonstrate that the dipeptide nitriles are non-toxic cell-permeable active site inhibitors of cathepsin C.

Activation of Cathepsin G U937 Cells—Next, we investigated the effect of cathepsin C inhibitors on downstream serine protease activation. Importantly, neither dipeptide nitriles nor DMK inhibitors directly blocked the activities of purified NE, CG, or Pr-3 ($IC_{50} > 50$ μM, see supplemental information).

The processing of NE and CG has been studied by metabolically labeling cells with [^{35}S]methionine and chasing with unlabeled methionine. Active NE and CG can be separated from their inactive pro-enzymes by binding to aprotinin-agarose and resolved by SDS-PAGE (50). We performed pulse-chase experiments in U937 cells and recovered a ^{35}S-labeled 29-kDa protein from aprotinin-agarose beads after 3 h of chase (Fig. 3*A*, *lane* 3) but not after a shorter 30-min chase (*lane 2*). This labeled protein was not seen in cells treated with 5 μM Ala-4-(I)Phe-DMK (*lane 4*), consistent with the inhibition of cathepsin C-mediated serine protease processing that would have led to the production of mature enzyme. To identify the 29-kDa labeled protein, the NE inhibitor L-694,458 (44) and the CG inhibitor I (Calbiochem) were used but first characterized for their specificity with purified human sputum NE, CG, and unlabeled U937 extracts. A silver-stained SDS-PAGE gel of aprotinin-agarose-bound proteins shows that 1 μM L-694,458 prevented binding of purified NE to aprotinin without significantly affecting CG binding (Fig. 3*B*, *lanes 3* and *5*). CG inhibitor I (5 μM) partially blocked binding of purified CG to aprotinin (*lane 6*). The heterogeneity of the purified proteins may be due to differential N- and C-terminal processing of NE and CG, and glycosylation (51). With U937 extracts, three polypeptides of 30, 29, and 27 kDa associated with aprotinin-agarose (*lane 7*). L-694,458 blocked the association of the 27-kDa protein with aprotinin, whereas CG inhibitor I partially prevented binding of the 29- and 30-kDa proteins, without affecting recovery of the 27-kDa polypeptide (*lanes 9* and *10*). Identification of the ^{35}S-labeled 29-kDa protein was performed with U937 cells pulse-chased as described above. Extracts were prepared and treated

[6] N. Méthot, unpublished data.

TABLE 1

Potency and selectivity of inhibitors on human cysteine cathepsins

The inhibitor potencies were determined on recombinant or purified human enzymes under conditions described under "Experimental Procedures." Information on more nitrile inhibitors (compounds 3–7) is found in the supplemental information. A complete description of the synthesis of compounds Gly-4(I)Phe-DMK, and compounds 1–6 will be published elsewhere (D. Guay, C. Beaulieu, T. J. Reddy, N. Methot, D. Ethier, and J. Rubin, manuscript in preparation).[4] BIL-DMK is described in Ref. 43.

Compound	Inhibitor class	IC_{50}				
		Cat C	Cat B	Cat L	Cat S	Cat H
				μM		
Gly-Phe-DMK	DMK	0.051	>30	25.5	ND[a]	29
Gly-4-(I)Phe-DMK	DMK	0.001	2.94	29.5	>50	9.8
BIL-DMK	DMK	0.45	0.065	0.01	0.009	ND
E64	Epoxysuccinyl	0.77	0.01	0.013	0.007	0.19
LHVS	Vinyl sulfone	>10	1.36	0.007	0.001	ND
Compound 1	Nitrile	0.014	>50	5.40	5.90	>50
Compound 2	Nitrile	>50	>50	>50	>50	>50

[a] ND, not determined.

TABLE 2

Potency and cytotoxicity of cathepsin C inhibitors on U937 cells

Numbers in parenthesis indicate the compound concentration at the beginning of the incubation. The percentage of residual cathepsin C activity in cell lysates was determined after 24 h, at which time cytotoxicity of DMKs was minimal. Residual cathepsin C activity was not measured for dipeptide nitriles, because they reversibly inhibit cathepsin C.

Compound	Reversibility	Cathepsin C inhibition, 24 h	Viable cells, 72 h
		%	
Gly-Phe-DMK (1 μM)	No	100	32
Ala-4-(I)Phe-DMK (1 μM)	No	99.6	32
Compound 1 (30 μM)	Yes	ND[a]	100
Compound 2 (30 μM)	Yes	ND	98

[a] ND, not determined.

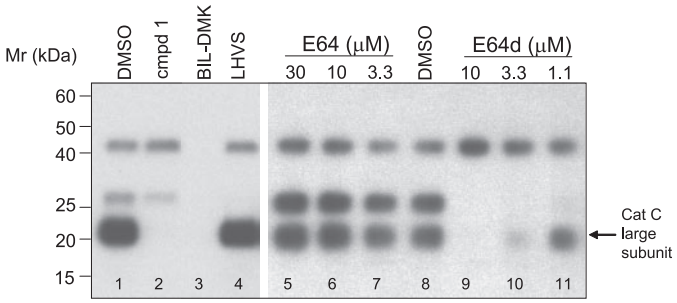

FIGURE 2. **Dipeptide nitrile and DMK inhibitors of cathepsin C block intracellular cathepsin C active site.** Autoradiography of polypeptides extracted from U937 cells incubated with Ala-4-[^{125}I]Phe-DMK and resolved on SDS-PAGE. All cells were treated with vehicle (0.5% Me_2SO (*DMSO*)) or 10 μM competitor inhibitor (compound 1, BIL-DMK, or LHVS) for 30 min before the addition of Ala-4-[^{125}I]Phe-DMK to the cell culture media. U937 cytosolic extracts were used for *lanes 1–4*, and total extracts were used in *lanes 5–11*. The large subunit of cathepsin C labeled by the probe is marked by an *arrow*. The broad cysteine protease inhibitors E64 (cell-impermeable) and E64d (cell-permeable) were added to the culture media before probe addition to distinguish between intra- and extracellular cathepsin C labeling. In this figure and all subsequent figures, compound is abbreviated as *cmpd*.

with NE or CG inhibitors before addition of aprotinin-agarose. Binding of the ^{35}S-labeled 29-kDa protein was not affected by L-694,458 but was significantly reduced by CG inhibitor I (Fig. 3*C*), suggesting that this protein is CG. To determine whether Ala-4-(I)Phe-DMK blocked binding of the 29-kDa protein by affecting its activity or its synthesis, an immunoprecipitation with anti-CG antibodies was performed. U937 cells were pulsed with [^{35}S]methionine and chased in the presence of Ala-4-(I)Phe-DMK. Extracts were prepared and affinity precipitations were performed with aprotinin-agarose or anti-CG antibodies. As before, Ala-4-(I)Phe-DMK blocked binding of CG to aprotinin-agarose (Fig. 3*D*, *lanes 1* and *4*). Immunoprecipitation with CG antibodies recovered a polypeptide that comigrated with aprotinin-bound CG (*lanes 2* and *3*), in quantities similar to when Ala-4-(I)Phe-DMK was present (*lanes 5* and *6*). These data indicate that Ala-4-(I)Phe-DMK does not affect the synthesis of CG but, rather, prevents cathepsin C-mediated processing and activation of newly formed pro-CG.

Reversible Cathepsin C Inhibitors Fully Block CG and NE Processing in U937 Cells—Having established that CG processing was completely inhibited by Ala-4-(I)Phe-DMK, we tested the reversible nitrile compounds in the pulse-chase cellular assay, and monitored maximum achievable inhibition and concentration dependence.

Compound 1 is one of the most potent dipeptide nitrile cathepsin C inhibitors (Table 1, IC_{50} 14 nM). As shown in Fig. 4*A*, compound 1 decreased the amount of activated, aprotinin-bound CG. The inhibition of CG activation was dose-dependent and, with 10 μM, nearly complete (Fig. 4*B*). The cell-based potency of nitrile inhibitors to reduce CG processing was proportional to their intrinsic potency on purified cathepsin C, but consistently 6- to 10-fold weaker (Table 3, and on-line supplemental information). Importantly, compound 2, an inactive enantiomer of compound 1 did not block CG processing. A potent cathepsin S inhibitor[4] was ineffective as well (supplemental information). Thus, processing of CG in U937 cells is fully dependent on cathepsin C activity.

U937 cells also express NE, but no ^{35}S-labeled NE bound to aprotinin-agarose was detected under our pulse-chase assay conditions. Because the dipeptide nitriles are non-cytotoxic, U937 cells were incubated with inhibitors for up to 7 days to allow turnover of stored active NE and *de novo* NE synthesis. After 7 days, compound 1 inhibited >90% of NE processing, with an IC_{50} of 0.22 μM (Fig. 4*C*). A shorter incubation time led to incomplete inhibition, presumably due to the presence of previously stored and active NE (see supplemental information). Other cathepsin C inhibitors were tested, and the IC_{50} values obtained were very similar to those measured for CG activation (see supplemental information). Taken together, these data demonstrate that reversible cathepsin C inhibitors can achieve nearly complete blockade of CG and NE activation.

Cathepsin C Inhibitors Block the Activation of NE, CG, and Pr-3 in the Neutrophil Cell Line EcoM-G—U937 cells express NE and CG constitutively. On the other hand, neutrophils, which are the clinically relevant cells for cathepsin C inhibitors,

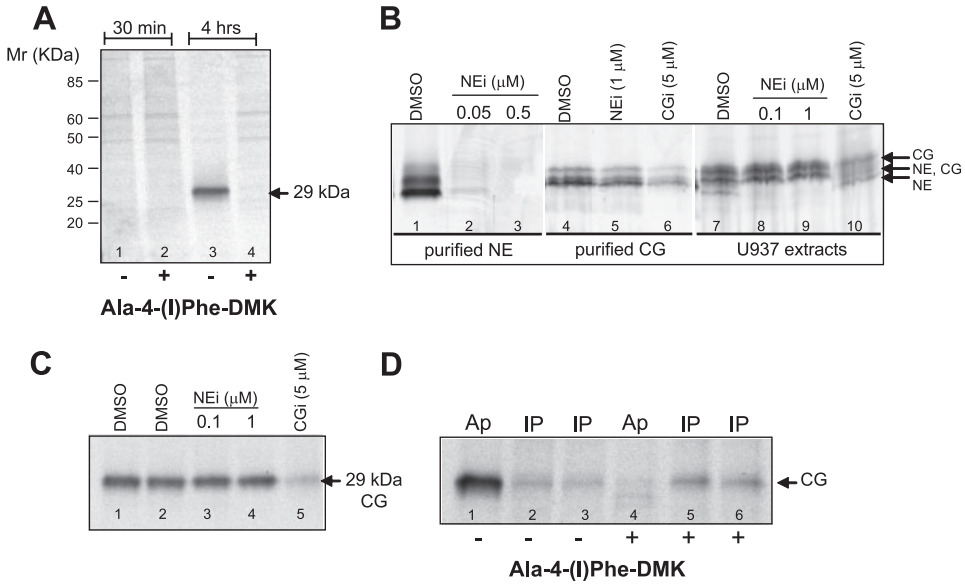

FIGURE 3. **Cathepsin G processing is blocked by Ala-4-(I)Phe-DMK in U937 cells.** *A*, fluorograph of aprotinin-agarose-bound proteins after metabolic labeling. U937 cells were pulsed with [^{35}S]methionine and chased for 30 min (*lanes 1* and *2*) or 4 h (*lanes 3* and *4*) in the presence of a Ala-4-(I)Phe-DMK (*lanes 2* and *4*) or with vehicle (*lanes 1* and *3*). A 29-kDa polypeptide was the most abundant species recovered. *B*, SDS-PAGE and silver stain of purified human sputum NE (300 ng; *lanes 1–3*) or CG (300 ng; *lanes 4–6*) or U937 extracts (*lanes 7–10*) bound to aprotinin-agarose, in the presence of NE inhibitor (*NEi* = L-694,458, *lanes 2*, *3*, *5*, *8*, and *9*) or CG inhibitor (*CGi* = Calbiochem cathepsin G inhibitor I, *lanes 6* and *10*). The inhibitors were mixed with the protein or U937 extracts 30 min before addition of aprotinin-agarose and processed as described under "Experimental Procedures." NEi and CGi specifically blocked binding of their respective targets to aprotinin-agarose. Both NE and CG from U937 lysates bound to the aprotinin-agarose. *C*, fluorograph of aprotinin-agarose-bound 29-kDa protein recovered from [^{35}S]methionine-labeled U937 cells. U937 extracts were prepared as in *A* and incubated with aprotinin-agarose in the presence of NEi (*lanes 3* and *4*) of CGi (*lane 5*). Only the CGi blocked binding of the ^{35}S-labeled protein to aprotinin-agarose. *D*, fluorograph of [^{35}S]methionine-labeled proteins in U937 cells, isolated with aprotinin-agarose (*Ap*; *lanes 1* and *4*) or CG immunoprecipitated (*IP*; *lanes 2*, *3*, *5*, and *6*), in the absence (*lanes 1–3*) or presence (*lanes 4–6*) or of Ala-4-(I)Phe-DMK.

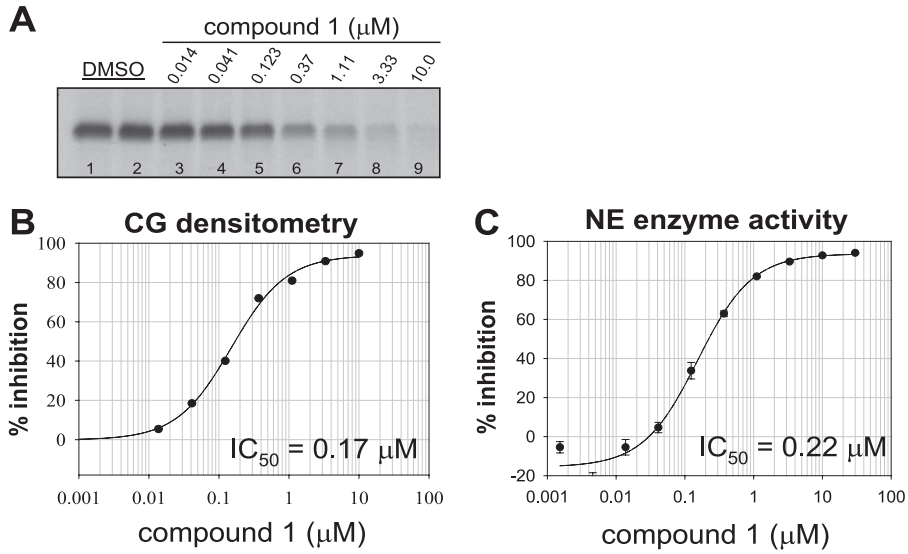

FIGURE 4. **Complete and dose-dependent inhibition of CG and NE activation by reversible cathepsin C inhibitors in U937 cells.** *A*, fluorograph of aprotinin-agarose bound ^{35}S-labeled CG after metabolic labeling of U937 cells and 3-h chase, in the presence of cathepsin C inhibitors. Cells were treated with either 0.5% Me_2SO (*DMSO*) or increasing concentrations of compound 1, as indicated. *B*, results from *A* were quantitated by densitometry and are expressed as percent inhibition of CG binding compared with vehicle-treated cells. *C*, NE activity expressed as percent remaining activity relative to vehicle-treated cells, after 7 days of incubation with compound 1. U937 cells were seeded at a density of 1×10^5 cells/ml with either vehicle or compound 1. The cells were passaged and diluted 10-fold into fresh media with either vehicle or compound on days 3 and 5, and harvested on day 7. After washing twice with PBS, the cells were lysed and the protein extracts tested for NE activity using a fluorogenic substrate (see "Experimental Procedures"). Each point represents the average percent inhibition of NE activity ± S.E. measured in four individual wells and calculated using vehicle-treated wells as 0% inhibition. The IC_{50} for NE and CG activation is indicated in the *right-hand corner*. Nearly 100% inhibition of processing was achieved with 10 μM cathepsin C inhibitor, for both NE and CG.

express NE, CG, and Pr-3 only during their pro-myelocyte developmental stage (52, 53).

EcoM-G is a murine pro-myoelocytic cell line immortalized with an estrogen-regulated E2a/Pbx-1 fusion protein. Upon β-estradiol withdrawal, the cells differentiate into mature granulocytes in 7 days (45, 54). We characterized the activities of CG, Pr-3, and NE in EcoM-G during early differentiation. No detectable activities were measured in cells grown in the presence of β-estradiol. Removal of β-estradiol triggered an increase in all three enzymatic activities, starting ~24 h post-estrogen removal and peaking at 48 h. NE, Pr-3, and CG activities were maintained until 80-h post-estrogen removal, after which they started to decline (Fig. 5). Addition of compound 1 (10 μM) 24 h after estrogen removal prevented the rise of enzymatic activities for NE, CG, and Pr-3, and the inhibition was maintained for up to 3 days (Fig. 5).

To further characterize the role of cathepsin C on the activation of NE, CG, and Pr-3, we differentiated EcoM-G cells for 24 h and added cathepsin C inhibitors. The cells were harvested 24 h later, and serine protease activities were measured. At high concentrations, cathepsin C inhibitors blocked the activation of Pr-3, NE, and CG by >90% (Fig. 6). Compound 1 blocked NE processing in EcoM-G cells with an IC_{50} of 0.16 μM (Table 4). For other inhibitors, the whole cell potencies for NE and Pr-3 activation correlated well with the purified enzyme potency but were consistently decreased by a factor of ten. Interestingly, for CG activation, the shift was 30- to 50-fold. Inhibitors potent against cathepsin S[2], cathepsin B, cathepsins S and L, and cathepsins S, L, and B, were inactive or poor in the EcoM-G cell-based assay (see supplemental information). These results demonstrate that cathepsin C inhibitors block the activation of multiple serine proteases in cells that closely resemble maturing granulocytes and that NE, CG, and

TABLE 3
Potency of cathepsin C inhibitors for blockade of NE and CG processing in U937 cells
Data for compounds 3–6 are found in the supplemental information.

Compound	IC_{50}		
	Cathepsin C intrinsic potency[a]	U937 whole cell potency	
		CG activation[b]	NE activation[c]
		μM	
Compound 1	0.014	0.15	0.22
Compound 2	>30	>50	30

[a] Determined with recombinant human cathepsin C and NH_2-G-R-AMC fluorogenic substrate.
[b] Measured by densitometry of CG labeled with [^{35}S]methionine and bound to aprotinin-agarose.
[c] Measured by NE enzymatic activity on a fluorogenic substrate MeOSuc-A-A-P-V-AMC in crude extracts of U937 cells exposed to inhibitors for 7 days.

Pr-3 processing is highly dependent on cathepsin C and no other cysteine proteases.

Activation of Serine Protease Pro-enzymes upon Withdrawal of Cathepsin C Inhibitors—Western blotting was used to determine whether NE protein levels were affected by cathepsin C inhibition. NE protein expression was undetectable in the presence of β-estradiol (Fig. 7*A*, *lane 1*) but was clearly seen 48 h post-estrogen removal. Greater levels of NE protein were measured when EcoM-G cells were treated with compound 1 (compare *lanes 2* and *3*). Thus, NE activation, and not NE protein synthesis, is blocked by the cathepsin C inhibitor. In U937 cells, similar levels of NE protein were obtained after 7 days of treatment with compound 1 (*lanes 4* and *5*). Based on these results, differentiating EcoM-G and U937 cells exposed to compound 1 contain significant amounts of unprocessed NE, presumably its zymogen.

We asked whether the NE zymogen could be activated following removal of cathepsin C inhibition. We differentiated EcoM-G cells for 24 h, added 1 μM compound 1, and measured NE activity 48 h post-estrogen withdrawal. As expected, NE activation was strongly inhibited (>90%, Fig. 7*B*, $t = 0$). An aliquot of the compound 1-treated cells was then washed twice with PBS and re-seeded under identical conditions but in the absence of compound 1. With rapidly reversible dipeptide nitrile cathepsin C inhibitors, cellular cathepsin C inhibition is lost when cells are incubated in compound-free culture media.[7] As early as 2 h after re-seeding, NE activity had increased 2.5-fold. By 6 h, NE activity had further increased, to levels 5-fold greater than originally present before the PBS wash (Fig. 7*B*). This is in contrast to the total NE activity measured in cells that had been exposed only to vehicle throughout the experiment, which had slightly decreased at the 6-h time point.

To assess the contribution of *de novo* NE protein synthesis to the observed increase in NE activity, the experiment was repeated in the presence of 100 μM cycloheximide, a protein synthesis inhibitor. Differentiated EcoM-G cells were incubated for 24 h with compound 1 (1 μM) and matured for an additional 24 h. The cells were washed twice in PBS and re-seeded with either compound 1, vehicle, or vehicle plus 100 μM cycloheximide. NE activity was measured 6 h later and was found to be blocked by 80% when the cells were re-exposed to compound 1. The absence of compound 1 for the last 6 h of incubation resulted in a 2.5-fold increased in NE activity. In the presence of cycloheximide, NE activity increased 2-fold (Fig. 7*C*). These data indicate that the majority of the NE activity recovered following the removal of cathepsin C inhibition resulted from the subsequent conversion of the inactive zymogen and not from *de novo* protein synthesis. Similar results were obtained with the recovery of Pr-3 activity. CG activity was reduced by cycloheximide (data not shown).

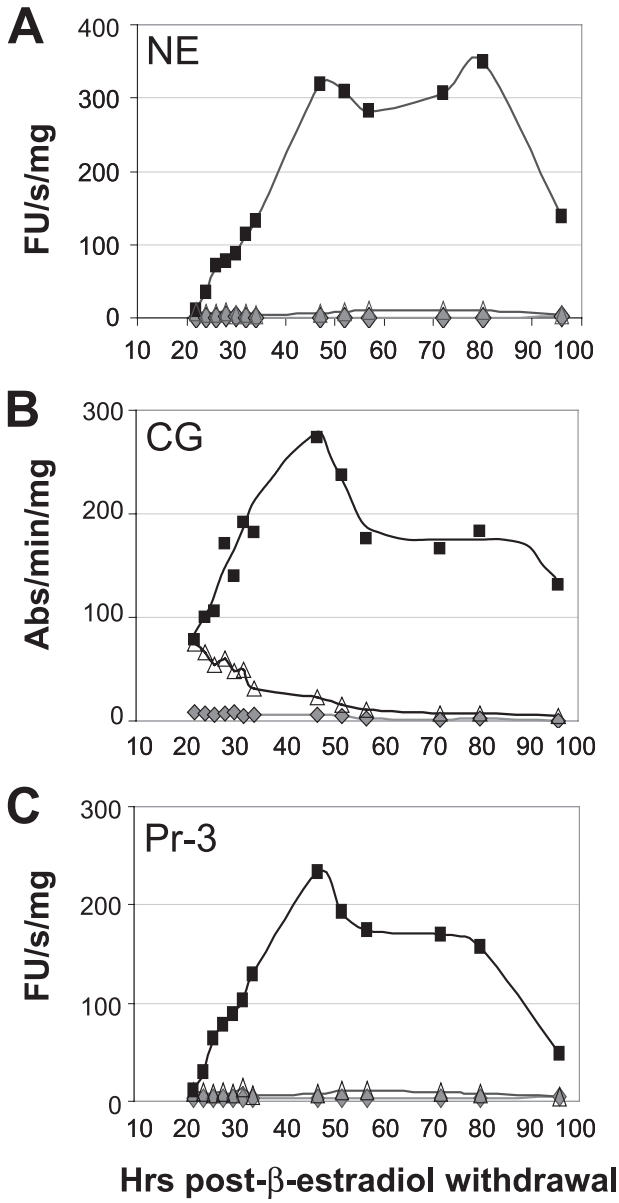

FIGURE 5. **Time-dependent increase of serine protease activities in differentiating EcoM-G cells.** EcoM-G cells were seeded in the presence, or absence of β-estradiol to induce differentiation into granulocytes. After 24 h, compound 1 (10 μM) or vehicle were added to the culture, aliquots were removed at the indicated time points. Protease activities were measured in the protein extracts using fluorogenic substrates, as described under "Experimental Procedures." *A*, NE activity; *B*, CG activity; *C*, Pr-3 activity. *Squares*, no β-estradiol; *lozenge*, with β-estradiol; *open triangles*, no β-estradiol, with compound 1. No serine protease activity was detectable when β-estradiol was present throughout the test period. Addition of compound 1 at 24 h post-β-estradiol removal abolished the induction of the three serine proteases for up to 3 days.

[7] N. Méthot, J. Rubin, D. Guay, C. Beaulieu, D. Ethier, T. J. Reddy, D. Riendeau, and M. D. Percival, unpublished data.

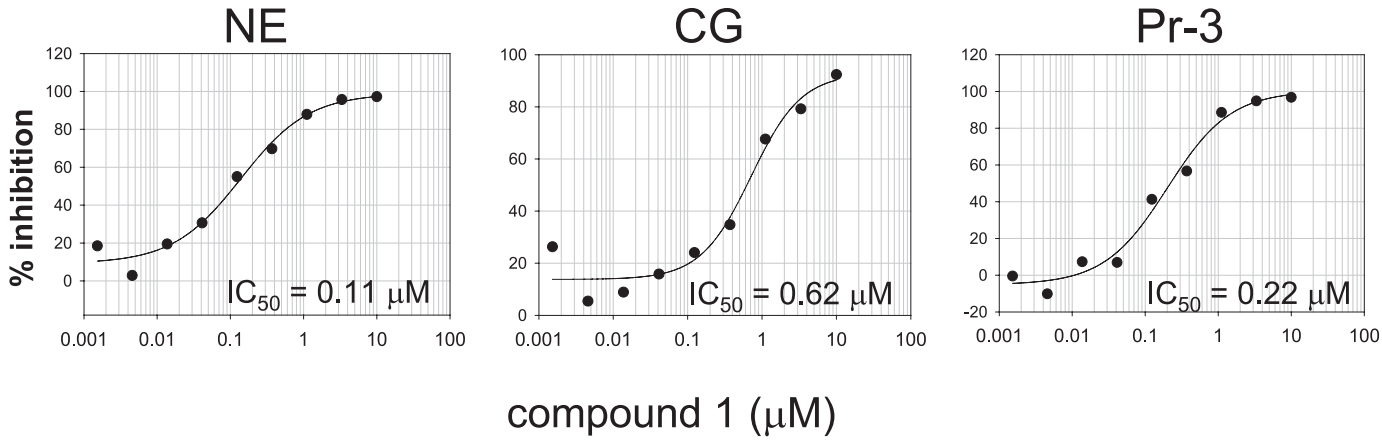

FIGURE 6. **Complete and dose-dependent inhibition of the activities of Pr-3, NE, and CG in differentiating EcoM-G cells by a reversible cathepsin C inhibitor.** Representative graphs for dose-dependent inhibition of NE, CG, and Pr-3 in differentiating EcoM-G cells treated with compound 1. EcoM-G cells were seeded in the presence or absence of β-estradiol to induce differentiation into granulocytes. After 24 h, compound 1 at increasing concentrations, or vehicle was added to the culture. The cells were harvested 24 h later, washed, and lysed. Protease activities were measured in the protein extracts using fluorogenic substrates, as described under "Experimental Procedures." Enzymatic activities for all three serine proteases were measured from the same lysates. IC_{50} values obtained for the representative experiment, for each serine protease, is indicated on the *right-hand side* of the graph. The average IC_{50} values and their standard mean error are shown in Table 4.

TABLE 4

Cathepsin C inhibitor potency at blocking activation of NE, CG, and Pr-3 in differentiating EcoM-G cells

EcoM-G cells were differentiated for 24 h before addition of inhibitors in the cell culture media. The cells were harvested 24 h later, washed, and lysed. Enzymatic activities for NE, CG, and Pr-3 were determined with peptide substrates as described under "Experimental Procedures." Data for compounds 3–7 are found in the supplemental information.

Compound	Downstream protease inhibition, IC_{50} ± S.E.		
	NE	CG	Pr-3
		μM	
Compound 1	0.16 ± 0.03	0.48 ± 0.06	0.14 ± 0.02
Compound 2	>50	>50	>50
E64d	>10	>10	>10
LHVS	25.5	39.8	21.2

The recovery of NE activity was evaluated in U937 cells. After 7 days of culture with compound 1, the cells were washed twice with PBS and re-seeded with or without compound 1 for an additional 6 h. Compound 1 at 10 μM blocked >85% of NE activity. When the culture media was replaced with inhibitor-free media, total NE activity had tripled by 6 h, compared with inhibitor-treated cells (Fig. 8*A*). To control for *de novo* NE synthesis, we measured new NE protein activation using the slowly reversible inhibitor L-694,458 ($t_{1/2}$ 15 h 44). NE activity in cells treated for 15 min with L-694,458 was ablated. When the cells were washed to remove L-694,458, re-seeded, and assayed after 6 h for new NE activity, a small increase was measured, accounting for 2% of the total NE activity (Fig. 8*A*). *De novo* NE protein synthesis is not significant under these conditions, and thus, we conclude that the majority of the new NE activity generated during the 6 h spent without cathepsin C inhibitor is due to activation of pro-NE. CG activity recovery could not be assessed under these conditions due to the lack of selectivity of the peptide substrate when using crude U937 cell lysates (Ref. 12 and data not shown). Instead, the recovery of active CG was tested in U937 cells using pulse-chase and aprotinin-agarose binding. The cells were labeled with [^{35}S]methionine for 30 min and chased with unlabeled methionine for 3 h. Cathepsin C inhibition reduced by 62% the amount of [^{35}S]CG that bound to aprotinin-agarose, compared with the vehicle control (Fig. 8*B*, *lanes 1* and *2*). When cells were washed with PBS, re-seeded with the same inhibitor and chased for an additional 3 h (*lanes 3* and *4*), the amount of bound [^{35}S]CG remained reduced by 61% (*lanes 5* and *6*). However, if the cells were re-seeded without cathepsin C inhibitor, the reduction was not as large (38%; *lanes 7* and *8*). Thus, as with NE, pro-CG processing occurs when cathepsin C inhibition is removed.

DISCUSSION

In this report, we describe the properties of cell-permeable and non-cytotoxic cathepsin C inhibitors and the cell-based assays used to evaluate downstream serine protease activation. The feasibility of fully blocking multiple inflammatory neutrophil serine proteases in intact cells, using a single selective and reversible cathepsin C inhibitor, is shown for the first time. The most potent cathepsin C inhibitor tested, compound 1 (IC_{50} = 14 nM), blocked processing and activation of cellular NE, CG, and Pr-3 but was inactive against the purified serine protease themselves. The inhibition of these serine proteases is therefore indirect and attributable to blockade of cathepsin C activity. In contrast, potent inhibitors of cathepsins B, L, H, or S, but weak against C, were ineffective inhibitors of cellular serine protease activation. These results validate cathepsin C as an attractive target for inflammatory diseases in which multiple serine proteases play a role and demonstrate its amenability to pharmacological inhibition.

Despite the different methodologies and cell systems used to follow the activation of the various serine proteases, the cathepsin C inhibitors exhibited remarkably similar potencies at blocking NE, CG, and Pr-3 processing. In U937 cells, the IC_{50} values obtained for CG and NE were virtually identical and similar to those measured in EcoM-G cells for NE and Pr-3 (Tables 3 and 4). The only exception was CG in EcoM-G, the inhibition of which required significantly (3- to 5-fold) higher concentration of cathepsin C inhibitor compared with NE or Pr-3. The reason for this is unknown. Addition of compound 1 at earlier time points after initiation of differentiation did not

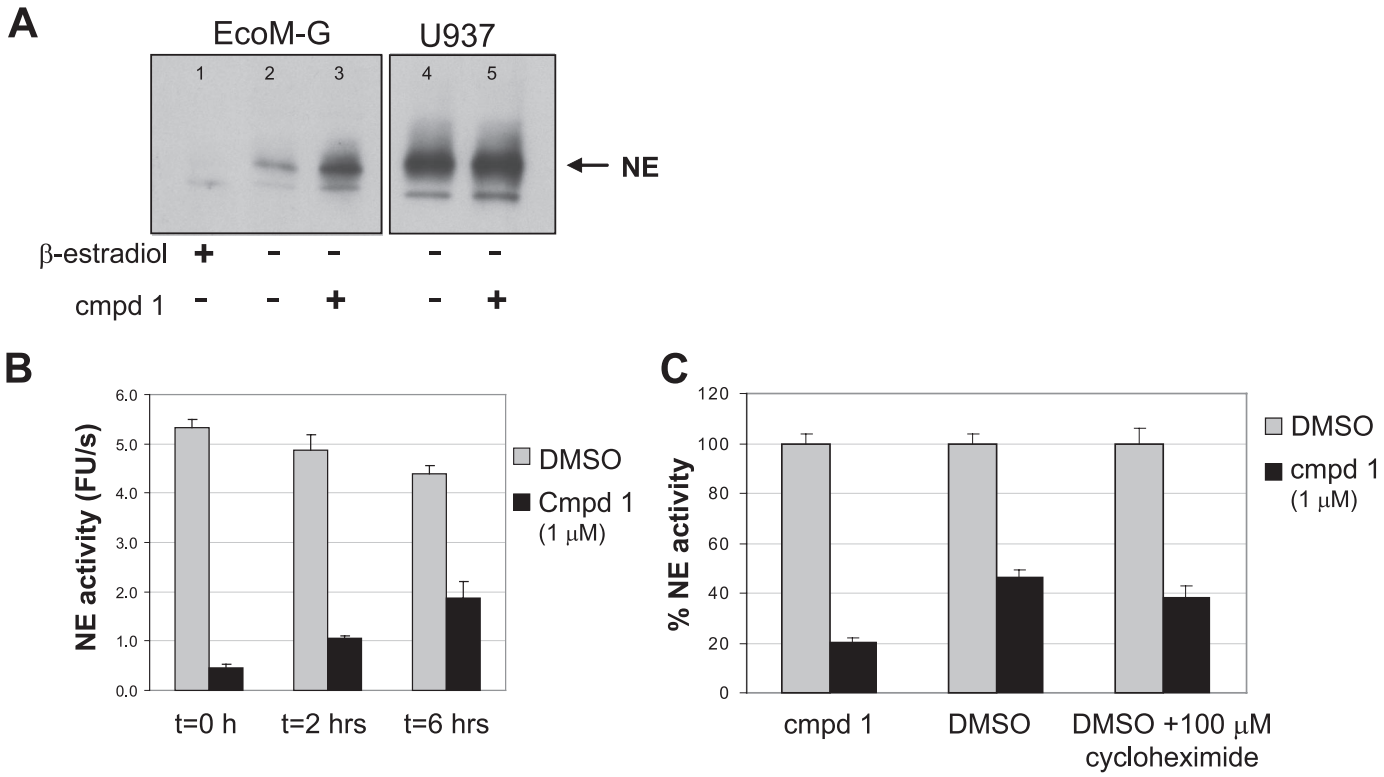

FIGURE 7. **Slow recovery of NE activity following removal of cathepsin C inhibition.** *A*, anti-NE Western blot showing NE protein levels in non-differentiated (*lane 1*) or differentiated (*lanes 2* and *3*) EcoM-G cells, and in U937 cells grown for 7 days with vehicle or 30 μM of compound 1 (*lanes 4* and *5*). The cathepsin C inhibitor increased the amount of NE protein in EcoM-G but not U937 cells. *B*, NE enzymatic activity measured in lysates of cells discontinuously exposed to compound 1. The cells were differentiated for 24 h before addition of inhibitor and matured for a further 24 h, then harvested and washed with PBS. Some cells were frozen immediately ($t = 0$), or re-seeded in growth media, without compound 1 for an additional 2–6 h. NE activity was measured in cell lysates. *C*, effect of protein synthesis inhibition on NE activity recovery during the inhibitor-free growth period. EcoM-G cells were differentiated for 24 h before addition of compound 1 and matured for a further 24 h, then harvested and washed with PBS. The cells reseeded with either 1 μM compound 1, vehicle, or vehicle plus 100 μM cycloheximide and incubated for a further 6 h. Cell lysates were prepared to measure NE activity.

significantly change the respective potencies on NE, CG, and Pr-3.[8]

At least 10-fold greater concentrations of inhibitor were consistently needed for effectiveness in cellular assays, compared with purified cathepsin C. Reduced cell permeability, high intracellular concentrations of the endogenous substrates, or an excess of cathepsin C activity for zymogen processing may account for the cellular potency shift. The latter possibility may translate into a requirement for high degree of cathepsin C inhibition. It is also known that NE, CG, and Pr-3 are stored at very high concentrations (>3 mM 55) in granules, and in theory, these substrates could compete with the inhibitor for the cathepsin C active site. The enzymatic activities of NE and CG in cathepsin $C^{+/-}$ mice are very similar to those measured in +/+ animals, despite the 50% reduction in cathepsin C enzymatic activity.[9] These data would indicate that *in vivo*, >50% of cathepsin C inhibition would be required to affect downstream serine protease activities. High fractional inhibition has been shown for biological processes far downstream of the primary site of inhibition. High caspase-3 fractional inhibition is required to block DNA fragmentation in apoptosis but not to block direct caspase-mediated αII-spectrin cleavage (56).

Important aspects to consider when designing a therapeutic strategy are the *in vivo* site of inhibition and the duration of treatment for efficacy. For cathepsin C and neutrophil serine proteases, the site of action is expected to be bone marrow, where granulopoiesis and activation of neutrophil serine proteases take place (53). Cathepsin C inhibitors will not be effective in granulocytes that have passed the myelocyte stage. Based on the maturation kinetics of normal human neutrophils (57) treatment with a cathepsin C inhibitor will require exposure for 11–14 days to be efficacious. In addition to these considerations, the present data show that, with a reversible inhibitor, a brief cessation of treatment may result in reactivation of stored neutrophil serine proteases. Whether this will be the case *in vivo* with normal human neutrophils is not known, but some human and mouse genetic data suggest that the proposed activation of stored, inactive serine proteases may not occur for all serine proteases. Bone marrow-derived neutrophils from cathepsin C-null mice lacked CG protein but had normal NE protein levels (14). Neutrophils from Papillon-Lefèvre Syndrome patients, which are cathepsin C-deficient, contain no CG and very low levels of NE and Pr-3 proteins (16). If the absence of cathepsin C activity results in the degradation of its target serine proteases, then an interruption in cathepsin C inhibition will not result in a body-wide activation of these targets. In the cellular assays presented here, however, NE protein

[8] J. Rubin, unpublished data.
[9] D. Ethier, unpublished data.

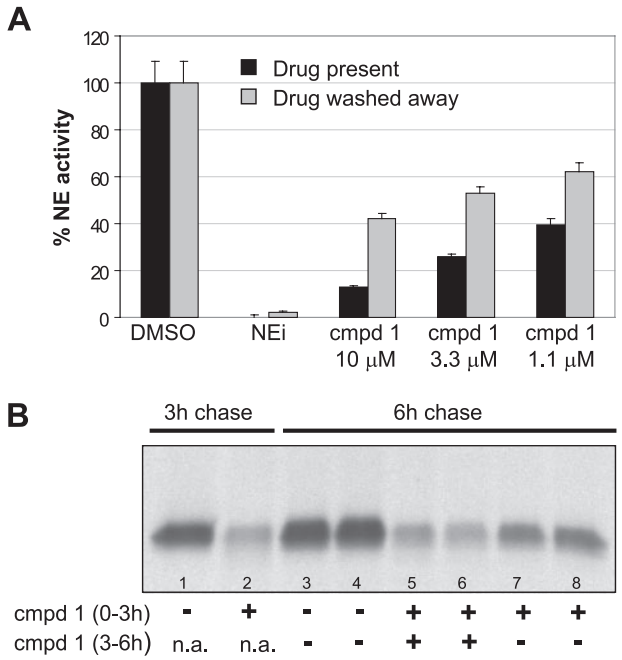

FIGURE 8. **Recovery of NE and CG activities in U937 cells after removal of cathepsin C inhibition.** *A*, NE enzymatic activity. U937 cells were cultured for two passages with either vehicle or 10 μM compound 1. After 7 days, the cells were washed with PBS and re-seeded with inhibitor (*black bars*) or Me_2SO (*gray bars*) for an additional 6 h before NE activity determination. In some cases, the cells were treated with 5 μM NE inhibitor (*NEi*) for 30 min, washed twice, and either re-seeded with Me_2SO for 6 h (*gray bar*), or frozen. *Error bars* represent $\pm$ S.E. and were calculated from at least three individual values for each condition tested. *B*, recovery of active CG in U937 cells. Cells were labeled with [^{35}S]methionine and chased in the presence of vehicle (*lanes 1*, *3*, and *4*) or 1 μM cathepsin C inhibitor (*lanes 2* and *6–8*) for 3 h. The cells were then washed with PBS, and either frozen (*lanes 1* and *2*) or re-seeded with 1 μM of inhibitor (*lanes 5* and *6*) or vehicle (*lanes 3*, *4*, *7*, and *8*) for an additional 3 h. Lysates were prepared, and active ^{35}S-labeled CG was recovered from aprotinin-agarose (see "Experimental Procedures" for details). *n.a.*, not applicable.

levels were not decreased by cathepsin C inhibition (Fig. 7). Perhaps the NE or CG degradation requires longer duration of cathepsin C inhibition or is uniquely observed when activity is genetically ablated. It will be interesting to follow serine protease activities and protein levels in animals treated with a cathepsin C inhibitor for several weeks and to determine if there is serine protease activity recovery after treatment cessation.

In conclusion, near complete inhibition of multiple inflammatory serine proteases was demonstrated with potent, selective, and reversible cathepsin C inhibitors, using novel cell-based assays that mimic neutrophil differentiation. Inhibition must be continuous to prevent the activation of stored proenzymes, at least in cultured cells. The next challenge will be to identify cathepsin C inhibitors with the proper pharmacokinetic profile for *in vivo* testing.

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