Brief Articles

Identification of 3-Acetyl-2-aminoquinolin-4-one as a Novel, Nonpeptidic Scaffold for Specific Calpain Inhibitory Activity

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Received November 24, 2008

A series of 3-acetyl-2-aminoquinolin-4-one derivatives selected from the Korean Chemical Bank were screened for calpain inhibitory activity by using a high-throughput fluorimetric calpain assay. We identified a potent and selective μ-calpain inhibitor, compound **17**, whose specificity and efficacy for μ-calpain inhibition was better than MDL28170. Docking studies revealed that the efficacy of its inhibitory effect on calpain depended on the size and charge properties of the substitutions on the phenylamino ring.

Introduction

Calpains are calcium-dependent cytosolic proteases that play key roles in intracellular signaling cascades and calcium-induced neuronal degeneration.[1] Calpains perform limited proteolytic cleavage of a diverse range of cellular substrates in all eukaryotic cells.[2] The two most widely distributed isoforms, μ-calpain and m-calpain, have almost the same substrate specificities but exhibit differences in their calcium concentration requirements for activation in vitro as well as their tissue-distribution profiles. The m-calpain necessitates calcium levels in the mM range for full activity in vitro, although intracellular factors increase its sensitivity to calcium in vivo. The ubiquitous μ-calpain, which is the major form expressed in neuronal cells and many blood elements, requires low calcium concentrations within the μM range for optimal activation.[3] The overactivation of calpains is linked to cellular aging, and this is demonstrated by the strong inverse correlation of the levels of soluble calpain activity in the brain and the lifespan of mammals.[4] During normal aging, increased activity of calpains promotes the development of neurological disorders involving impaired calcium homeostasis. In addition, overactivation of calpain is a prominent factor in cellular degeneration during acute cell injury triggered by abnormal calcium influx in the nervous system.[5] This ability of calpains to promote cellular degeneration and cell death indicates that calpains may play a role as synergistic factors in between causing and progressing neurodegenerative disorders in the elderly.

Alzheimer's disease (AD[a]) is a typical neurodegenerative disorder. Persistent calpain activation develops at an early stage in the AD process, well before the neurons die. This level of calpain activation exceeds aging-related changes and may contribute to multiple aspects of AD development, including the amyloidogenic process of the amyloid precursor protein (APP) to short hydrophobic amyloid β peptides (Aβ 1−40, Aβ 1−42)[1a,6] and the abnormal development of neuronal tau aggregates (neuropil threads and neurofibrillary tangles).[7] In recent years, cyclin-dependent kinase 5 (cdk5) localized in the brain has generated much interest since increased levels of cdk5/p25 complex were observed in the brains of AD patients. The cdk5/p25 complex is produced by calpain-mediated cleavage of p35 to p25. The breakdown of cdk5/p35 into cdk5/p25 prolongs cdk5 kinase activity, induces mislocalization of cdk5, and increases neurotoxicity in neuronal cells via hyperphosphorylation of tau, leading to the formation of neurofibrillary tangles.[8] In addition, development of the drug, R-flurbiprofen, a specific inhibitor of Aβ production through the modulation of γ-secretase, was discontinued in phase 3 clinical trials last year since no significant efficacy was achieved (http://www.myriad.com). Therefore, the inhibition of neurofibrillary tangle formation through reduction of calpain activity might be a more promising route to discovering potent drug candidates for the treatment of AD. For this reason, many researchers have focused their attention on the discovery of calpain inhibitors such as peptidyl aldehyde derivatives,[9] diketopiperazine derivatives,[10] and α-ketoamide derivatives.[11]

However, the development of calpain inhibitors with the aim of lessening the damage done in long-term neurodegenerative disease such as AD has yielded inadequate selectivity for calpain over other cysteine proteases, poor metabolic stability, and/or deficient cell permeability in the past. The development of adequately selective and efficient calpain inhibitors is challenging due to the ability of calpain's active site to accommodate a wide variety of residues where its specificity is similar to other related cysteine proteases.[12] In addition, most of the known inhibitors possess peptide character and highly reactive aldehyde moiety that affect nonselectivity, instability during storage, and

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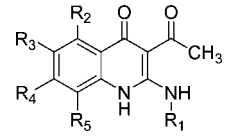
[a] Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; ANOVA, analysis of variance; CRD, completely randomized design; cdk5, cyclin-dependent kinase 5; DMRT, Duncan's multiple range test; DMSO, dimethyl sulfoxide; PDB, Protein Data Bank; RFU, Relative fluorescence unit; rt, room temperature; SAS, statistical analysis system.

10.1021/jm8014734 CCC: $40.75
Published on Web 04/20/2009

Table 1. Structure and μ-Calpain[a] Inhibitory Activity of Compounds **1**−**22**



						μ-calpain inhibition IC_{50} (μM)[b]	
						substrate	
compd	R_1	R_2	R_3	R_4	R_5	pep1[c]	pep2[d]
1	4-chlorophenyl	H	NO_2	H	Cl	4.56 ± 0.39	8.08 ± 0.30
2	4-*tert*-butylphenyl	H	NO_2	H	Cl	3.69 ± 0.23	7.43 ± 0.18
3	3-trifluoromethyl-phenyl	H	H	Cl	Cl	>30	NT[f]
4	4-trifluoromethyl-phenyl	H	Cl	H	CF_3	>30	NT
5	2-chloro-4-methylphenyl	H	Cl	H	NO_2	3.73 ± 0.36	2.39 ± 0.34
6	2,4-dichlorophenyl	H	Cl	H	NO_2	4.64 ± 0.11	3.16 ± 0.11
7	2,4-dichlorophenyl	H	H	H	CF_3	19.20 ± 0.30	NT
8	3-fluorophenyl	H	H	H	phenyl	>30	NT
9	4-bromophenyl	Br	H	H	Br	3.59 ± 0.19	1.69 ± 0.019
10	4-chlorophenyl	Cl	H	H	Br	3.53 ± 0.19	9.94 ± 0.14
11	2,4-dichlorophenyl	Cl	H	H	Cl	3.99 ± 0.24	3.17 ± 0.28
12	3-methylphenyl	NO_2	H	H	Cl	>30	NT
13	phenyl	F	H	H	Cl	>30	NT
14	4-*tert*-butylphenyl	F	H	H	F	>30	NT
15	4-chlorophenyl	F	H	H	F	>30	NT
16	3,5-ditrifluoromethyl-phenyl	F	H	H	F	>30	NT
17	2,4-dibromophenyl	CH_3	H	H	Cl	0.277 ± 0.006	3.06 ± 0.28
18	2-fluoro-4-chlorophenyl	CH_3	H	H	Cl	>30	NT
19	2,4,5-trifluorophenyl	CH_3	H	H	Cl	>30	NT
20	2,3,4-trifluorophenyl	CH_3	H	H	Cl	>30	NT
21	2-fluoro-5-methylphenyl	CF_3	H	H	Cl	>30	NT
22	2,4,5-trifluorophenyl	H	F	H	F	>30	NT
	MDL28170					0.200 ± 0.03	0.199 ± 0.002

[a] Human erythrocyte μ-calpain (Calbiochem). [b] Each data point represents mean ± SD from three different experiments performed in triplicate. [c] Calpain inhibitory activity of chemicals on pep1 substrate was evaluated in reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 7.5) with 100 μM pep1, 2.5 mM $CaCl_2$, and 5.25 units/mL μ-calpain. Thereafter, reaction mixture was incubated with shaking at rt for 30 min. Fluorescence intensities were measured using 360 nm excitation and 420 nm emission wavelengths. The substrate of pep1 possess the calpain-cleavage site in p35 which is [2-Abz]-Ser-Thr-Phe-Ala-Gln-Pro-[3-nitrotyrosine]-NH_2. [d] Calpain inhibitory activity of chemicals on pep2 substrate was assayed with IQ protease assay trial pack-calpain-1 kit (Pierce) as following the protocol provided by manufacture. Fluorescence intensities were determined using 535 nm excitation and 595 nm emission wavelengths. The substrate pep2 was originated from α-spectrin. [e] MDL28170 (Sigma Aldrich). [f] Not tested.

excessive metabolism.[9−12] Therefore, we tried to identify a new core for selective calpain inhibitors that possessed less or no peptidic character. Here, we report a series of 3-acetyl-2-aminoquinolin-4-one derivatives with calpain inhibitory activity, obtained from high-throughput fluorimetric calpain assays using the chemical library provided from the Korean Chemical Bank. Compounds with the quinolin-4-one core were previously reported by Graybill et al. as a nonpeptide calpain inhibitors.[13]

Results and Discussion

To develop novel nonpeptidic calpain inhibitors possessing good potency and selectivity as potent drug-like compounds for the treatment of AD, we modified a previously reported method that uses the substrate derived from α-spectrin.[14] By using a fluorescence-based probe pep1, the specific μ-calpain activity of proteolysis of a peptide based on the cleavage site in cdk5/p35, [2-Abz]-Ser-Thr-Phe-Ala-Gln-Pro-[3-nitrotyrosine]-NH_2, was detected.[7] This method was easy to carry out and was capable of detecting enzymatic activity in the nanomolar range at room temperature (rt). Using pep1 as the substrate had the additional advantage that it was specific to μ-calpain over other cysteine proteases such as cathepsins B, L, and H. This was confirmed by attempting fluorimetric cathepsin assays using pep1 instead of their substrates (Results not shown). The current method using pep1 was very useful and more straightforward than the conventional method that adopted the cleavage site based on α-spectrin as the substrate because no interference by proteolysis of cathepsins was observed and the CDK/p25 complex is implicated in AD patients.[8,15] Approaches to discovering novel calpain inhibitors using this method have provided opportunities to screen large chemical libraries containing 5000 compounds provided by the Korean Chemical Bank for their calpain inhibitory capabilities.

A fluorimetric calpain assay 2, which uses pep2 possessing the peptide sequence that originates from the μ-calpain cleavage site in α-spectrin as the substrate, was performed to covalidate results from fluorimetric calpain assay 1 using pep1 as the substrate (Table 1). Compounds **1** and **10** showed μ-calpain inhibitory activity during the first screening. Subsequently, the assay was extended to all the compounds possessing the core of 3-acetyl-2-aminoquinolin-4-one in the Korean Chemical Bank for the purpose of identifying a novel μ-calpain inhibitor.[16] Compound **17** was identified as showing good potency for μ-calpain inhibitory activity. Its activity was comparable to that of MDL28170, a known peptidyl aldehyde derivative of the calpain inhibitor.

Docking studies were carried out for compound **17** in μ-calpain containing the domains I−II from previously published X-ray crystal structure analyses (Protein Data Bank (PDB) code 2G8J) to further explain our experimental data using the Autodock4.0/ADT.[17] The overall bound structure was consistent with other known structures in that the ligand fitted into the active site of calpain.[11b,12,18] With other peptidomimetic calpain inhibitors such as leupeptin, there are extensive hydrogen

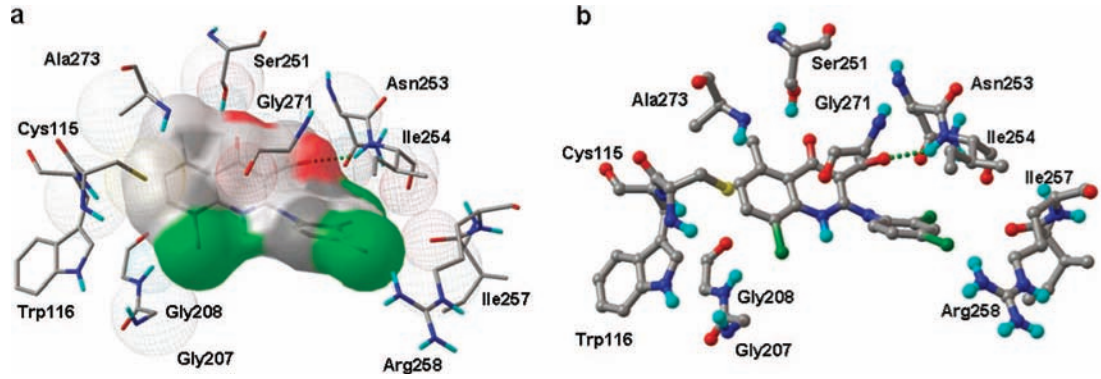


Figure 1. The active site view of the docked complex. The residues of the active site of μ-calpain that have van der Waals contact with compound **17** are shown. (a) The stick and surface representation of the complex colored by atom type (carbon, gray; oxygen, red; nitrogen, blue; hydrogen, cyan; halides, green), and the contacts are represented by wireframe spheres. (b) A ball and stick representation. Dotted green lines indicate hydrogen bonding interactions.

Table 2. The Activities of Selected Compounds in Inhibition of Cathepsins B, L, H, and D

	inhibitory activity as IC_{50} (μM)[a] of			
compd	cathepsin B	cathepsin L	cathepsin H	cathepsin D
MDL28170	0.0142 ± 0.0017	0.000437 ± 0.000053	0.145 ± 0.0025	>100
CA-074	0.00311 ± 0.00012	>100	>100	>100
Z-FF-FMK	0.3968 ± 0.0273	0.00166 ± 0.00017	>100	>100
pepstatin A	12.69 ± 0.31	19.82 ± 1.12	32.57 ± 0.74	0.042 ± 0.00762
1	1.64 ± 0.12	1.06 ± 0.01	91.76 ± 3.86	>100
2	1.53 ± 0.14	1.00 ± 0.14	>100	>100
5	3.89 ± 0.34	1.24 ± 0.10	>100	>100
6	1.75 ± 0.08	1.37 ± 0.10	66.52 ± 1.49	>100
9	1.36 ± 0.11	1.27 ± 0.07	>100	>100
10	7.56 ± 0.48	3.09 ± 0.18	>100	>100
11	0.68 ± 0.042	0.51 ± 0.07	>100	>100
17	4.26 ± 0.26	0.80 ± 0.04	>100	>100

[a] Each data point represents mean ± SD from three different experiments performed in triplicate.

bonding interactions. Because of the small number of hydrogen bonding donors and acceptors in compound **17**, such hydrogen bonding interactions were not prominent. However it showed 11 van der Waals contacts from residues Cys115, Trp116, Gly207, Gly208, Ser251, Asn253, Ile254, Ile257, Arg258, Gly271, and Ala273 (Figure 1). Compared to the leupeptin complex (PDB code 1TLO), compound **17** showed more interaction with residues from domain II because the phenylamino group is juxtaposed to the 251−261 loop in domain II.[18a] The amide group of the residue Ile243 showed hydrogen bonding interactions with the acetyl group of compound **17**. The quinoline moiety showed hydrophobic interaction with Cys115, Trp116, Gly207, and Gly208, which was matched to the P2 residue in leupeptin and E64.[18a] The quinoline ring was shown to be surrounded by these hydrophobic residues and therefore blocked the substrates from the catalytic triad comprising Cys115, His272, and Asn296. From comparisons of the IC_{50} between compound **17** and **9**, the methyl group on the R_2 position was shown to be essential. The acetyl and ketone moiety on the quinoline ring interacted with polar residues such as Ser251 and Asn253. The 2,4-dibromophenylamino group fitted well into the active site pocket of calpain. The efficacy of the inhibitory effect of the calpain was shown to be dependent on the size and charge properties of the substitutions on the phenylamino ring. When the substitutions on the phenylamino group were smaller, such as the chloro or fluoro group (compounds **18**−**20**), the ligand was shown to be loosely fitted into the pocket. This explained its inactivity as a calpain inhibitor. Also the 4-bromo side chain on the phenyl ring interacted with the bulky, positively charged Arg258 residue that had a complementary charge with a partially negatively charged bromo group. Compounds **1** and **2** showed some calpain inhibitory activity and possessed a negatively charged nitro group at R_3 position. Because it was favorable to have a nonpolar, hydrophobic group at this position, it was suggested that the compound flipped over to have nitro group to face the Arg258 residue. To test this hypothesis, synthesis of a new 3-acetyl-2-aminoquinolin-4-one derivative having more nucleophilic substitutions (such as a nitro group) on the phenylamino ring and hydrophobic group on the R_2 position is in process.

Many of the reported calpain inhibitors including MDL28170 were found to inhibit closely related cysteine proteases owing to poor selectivity. Thus a total of eight compounds were further assayed quantitatively to test the potency of cathepsins B, L, H, and D inhibition, respectively, following the method of fluorimetric end-point assay (Table 2). Cathepsins B and L were shown to be active-site related and physiologically relevant to μ-calpain.[12] CA-074 as a cathepsin B inhibitor,[19a] Z-FF-FMK as a cathepsin L inhibitor,[19b] and pepstatin A as cathepsin D inhibitor[19c] were used as positive controls. Cathepsins B and L inhibition potencies in terms of IC_{50} values of tested compounds showed broad ranges from 0.68 to 7.56 μM for cathepsin B and 0.51 to 3.09 μM for cathepsin L. However, the compounds showed weak inhibition potency against cathepsins H and D. Upon comparison of the IC_{50} values, compound **17** showed greater specificity for μ-calpain over cathepsin enzymes. Notably, compound **17** demonstrated 15 times greater efficacy for calpain over cathepsin B. Among the compounds, some showed good inhibition potency against cathepsin. Compound

11 demonstrated almost an 8-fold difference in specificity for cathepsins B and L over calpain.

Conclusion

In conclusion, compound **17** may pave the way for the development of efficient calpain-specific inhibitors that can provide insight in defining the normal physiological roles of calpains. In fact, a complete understanding on the physiological role of calpain remains to be defined because known calpain inhibitors currently in use such as calpeptin, MDL28170, and ALLN lack specificity. Thus, these compounds can cross-react with cathepsins, papain, and even, to some degree, with the proteasome.[12,15,19] Indeed, our data and screening method utilizing the fluorescence-probed substrate of μ-calpain originating from p35 cleavage site will be a valuable tool to attempt cdk5 regulation with modulating p35 and this may further lead to the development of AD therapeutic agents.

Experimental Section

Materials. μ-Calpain (human erythrocyte) as well as cathepsins B, L, H, and D (human liver) were purchased from Calbiochem (Darmstadt, Germany). Pep1, a substrate of μ-calpain, was synthesized by the Peptron Corporation (Daejeon, Korea). The IQ protease assay trial pack-μ-calpain kit and BCA protein assay reagent kit were purchased from Pierce (Rockford, IL). The cathepsin substrates of Z-FR-AMC and R-AMC and hemoglobin were purchased from Sigma Chemical Co. (St. Louis, MO). MDL28170 (μ-calpain inhibitor), CA-074 (cathepsin B inhibitor), Z-FF-FMK (cathepsin L inhibitor), and pepstatin A (cathepsin D inhibitor) were purchased from Sigma Chemical Co. Compounds possessing the core of 3-acetyl-2-aminoquinolin-4-one tested as novel calpain inhibitors were provided by the Korean Chemical Bank. The syntheses and characterization of these compounds are described elsewhere.[16] The purity of the compounds was analyzed by LC/MS, which showed ≥95% purity for most of the compounds. The purity of the compounds used in this study is summarized in Table S1 in the Supporting Information.

Optimization of Fluorimetric Calpain Assay 1. In the fluorimetric calpain assay 1, the composition of reaction buffer and the concentration of pep1 were determined by using the method of Stifun Mittoo et al.[14] The concentration of $CaCl_2$ and the unit of μ-calpain were determined through an optimization process. The typical standard curve obtained with 0.001−10 mM of $CaCl_2$ and 0.1−100 units/mL of μ-calpain are shown in Figure S1 in the Supporting Information, where the effects of $CaCl_2$ and μ-calpain concentration on the activities of μ-calpain are also shown. Calpain activity appeared to be fully saturated when more than 2.5 mM of $CaCl_2$ and 10 units/mL of μ-calpain were present in the reaction buffer. A linear relationship was observed with 1−2.5 mM of $CaCl_2$ and 1−10 units/mL of μ-calpain. Therefore, constant concentrations of 2.5 mM of $CaCl_2$ and 5.25 units/mL of μ-calpain were maintained throughout fluorimetric calpain assay 1.

Fluorimetric μ-Calpain Inhibition Assay. The fluorimetric assay was performed in 96-well plates. The end-point fluorescence intensity in each well was measured by using a microplate fluorescence reader (FL600, Bio Tek), and the IC_{50} values were obtained by using the data graphing software TableCurve 2D (Systat Software Inc.). Fluorescence intensity was indicated by relative fluorescence units (RFU). RFU was calculated by subtracting the RFU of the control from all other values. To determine percent inhibition, the percent change in RFU between the activity of the enzyme in the presence and absence of the inhibitor was calculated. The resulting RFU in the absence of inhibitor represented 100% enzyme activity.

Fluorimetric Calpain Assay 1. Calpain activity was quantified by using a modified method by Mittoo et al.[14] The substrate used was a fluorescence-based probe and designed to possess the calpain-cleavage site in p35, which was [2-Abz]-Ser-Thr-Phe-Ala-Gln-Pro-[3-nitrotyrosine]-NH_2, named pep1. The specific cleavage in pep1 by μ-calpain occurs between phenylalanine and alanine.[7] It consists of a donor fluorescence group and an acceptor moiety that is capable of intramolecularly quenching the donor's fluorescence. The donor and acceptor are separated by six amino acid residues corresponding to three amino acid residues on either side of the cleavage site. This separation by six amino acid residues is known to provide maximal efficacy of internal quenching. The cleavage between phenylalanine and alanine results in the increase in fluorescence intensity and thus an indication of calpain activity. The assay was performed in a final volume of 100 μL. Stock solutions of pep1 and inhibitors were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. Subsequent dilutions were performed by using reaction buffer such that the final concentration of DMSO was less than 5%. Calpain inhibition was assayed in reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol, pH 7.5) with 100 μM pep1, 2.5 mM $CaCl_2$, and 5.25 units/mL μ-calpain. The concentration of $CaCl_2$ and the unit of μ-calpain used in the present study were determined through the optimization process in the laboratory. The reaction was initiated by the addition of substrate, μ-calpain, inhibitor, and $CaCl_2$ solution. Thereafter, it was incubated with shaking at rt for 30 min. Fluorescence intensities were measured by using 360 nm excitation and 420 nm emission wavelengths.

Fluorimetric Calpain Assay 2. Fluorimetric calpain assay 2 was performed by using the IQ μ-calpain protease assay kit (Pierce) according to the manufacturer's instructions. This method was similar to the fluorimetric calpain assay 1, but it used a different substrate, pep2, which originated from the calpain cleavage site in α-spectrin. The fluorophore-labeled peptide substrate provided by the manufacture and named pep2 was dye-Gln-Gln-Gln-Glu-Val-Tyr-Gly-Met-Met-Pro-Arg-Asp-pSer-Ala. μ-Calpain cleaved between tyrosine and glycine. Fluorescence intensities were determined by using 535 nm excitation and 595 nm emission wavelengths.

Fluorimetric Cathepsins B, L, and H Assay. The method for cathepsins B, L, and H activity measurement was modified from the method published by Dominic Cuerrier et al.[12] This method was performed in a final volume of 100 μL. Cathepsins B, L, and H inhibitory activities were assayed in the reaction buffer (0.1 M NaOAc-HCl, pH 5.5 for cathepsins B and L, pH 6.8 for cathepsin H with 1 mM EDTA, and 0.1% β-mercaptoethanol) with 20 μM substrate, 1.5 nM cathepsin B, 4 nM cathepsin L, and 30 nM cathepsin H, respectively. The substrates used were Z-FR-AMC for cathepsins B and L and R-AMC for cathepsin H. The cathepsins were reductively activated by preincubation in assay buffer for 30 min prior to initiating the reaction by addition of substrate and inhibitor. Afterward, shaking incubation was performed at rt for 30 min. Fluorescence intensities were determined by using 360 nm excitation and 460 nm emission wavelengths.

Cathepsin D Inhibitory Activity Measurement. The reaction mixture contained 7.5 μL of 100 mM formic acid buffer (pH 3.3), 0.5 μL of inhibitor dissolved in DMSO, and 1.5 μL of 2.5% hemoglobin substrate. After incubating at 37 °C for 10 min, 0.5 μL of 14.4 units/mL of cathepsin D was added. The cathepsin D was activated by incubating at 37 °C for 30 min in the reaction mixture. Following this, 12.5 μL of 10% trichloroacetic acid (stop solution) was added.[20] The reaction mixture with trichloroacetic acid was centrifuged at 2000*g* for 3 min, and the supernatant was taken. The absorbance of the supernatant at 280 nm was measured by using a Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies, USA).

Statistical Analysis. In all experiments, data were expressed as mean ± standard deviation, with at least three repeats in each experimental group. All data were subjected to one-way analysis of variance (ANOVA) in a completely randomized design (CRD) and Duncan's multiple range test (DMRT) by using general linear models in a statistical analysis system (SAS) program in order to determine differences between the experimental groups. *P* values less than 0.05 were considered to be statistically significant.

Docking Studies. The coordinates for the calpain domains I−II (2G8J) were retrieved from the PDB. The water molecules were removed. The structure of compound **17** was constructed by Sybyl

8.0 and energetically minimized by using a Tripos force field with Gasteiger−Huckel charges. The receptor and the ligand file were prepared as the original publication protocols.[17] Docking was carried out with Autodock4.0/ADT by using the Lamarckian genetic algorithm search parameters.

Acknowledgment. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-531-E00109). D.-H. Kang and K.-Y. Jun were partially supported by the Brain Korea 21 Project.

Supporting Information Available: Figure depicting the optimization of the effect of $CaCl_2$ concentration and μ-calpain amount in the proteolytic activity of μ-calpain and LC/MS analyses data for compounds **1**−**22**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM8014734