Identification of Cathepsin B as a Mediator of Neuronal Death Induced by Aβ-activated Microglial Cells Using a Functional Genomics Approach*

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Alzheimer’s disease is a progressive neurodegenerative disease characterized by senile plaques, neurofibrillary tangles, dysstrophic neurites, and reactive glial cells. Activated microglia are found to be intimately associated with senile plaques and may play a central role in mediating chronic inflammatory conditions in Alzheimer’s disease. Activation of cultured murine microglial BV2 cells by freshly sonicated Aβ42 results in the secretion of neurotoxic factors that kill primary cultured neurons. To understand molecular pathways underlying Aβ-induced microglial activation, we analyzed the expression levels of transcripts isolated from Aβ42-activated BV2 cells using high density filter arrays. The analysis of these arrays identified 554 genes that are transcriptionally up-regulated by Aβ42 in a statistically significant manner. Quantitative reverse transcription-PCR was used to confirm the regulation of a subset of genes, including cysteine proteases cathepsin B and cathepsin L, tissue inhibitor of matrix metalloproteinase 2, cytochrome c oxidase, and allograft inflammatory factor 1. Small interfering RNA-mediated silencing of the cathepsin B gene in Aβ-activated BV2 cells diminished the microglial activation-mediated neurotoxicity. Moreover, CA-074, a specific cathepsin B inhibitor, also abolished the neurotoxic effects caused by Aβ42-activated BV2 cells. Our results suggest an essential role for secreted cathepsin B in neuronal death mediated by Aβ-activated inflammatory response.

Important role in AD pathogenesis because numerous epidemiological studies have revealed significant protective effects of nonsteroidal anti-inflammatory drugs (5–10).

Microglia, the resident macrophages in the brain, play a central role in mediating chronic inflammatory conditions in AD. They are closely associated with amyloid plaques and exhibit a reactive phenotype with elevated expression of cell surface markers, including CD45, Mac1, and major histocompatibility complex class II antigens (11). They are also capable of releasing numerous acute phase proteins, such as α-antichymotrypsin, α-antitrypsin, complement proteins, and proinflammatory cytokines, such as interleukins 1α, 1β, and 6, tumor necrosis factor (TNF-α), and others (12–16).

There is compelling evidence that Aβ peptides serve as inflammatory stimuli to provoke a microglial-mediated inflammatory response that contributes significantly to neuronal loss and cognitive decline (3). In vitro studies have shown that stimulation of microglia by high concentration of fibrillar Aβ results in TNF-α-dependent expression of inducible nitric oxide synthase and neuronal apoptosis (16, 17). Other studies have shown that Aβ can amplify microglial activation by other coexisting inflammatory stimuli, such as lipopolysaccharide (LPS), interferon γ (IFNγ), and advanced glycation end products (18).

Besides fibrillar Aβ42, soluble Aβ42 also appeared to be a potent activator of microglia. Purified dimeric and trimeric components of Aβ42 peptides from neuritic and vascular amyloid deposits elicit a profound neurotoxicity in hippocampal neurons in the presence of microglia (19). In addition, although the identity of the neurotoxic agent(s) has not been determined, studies indicate that activation of cultured microglia by senile/neuritic plaque fragments leads to the release of toxic factors that kill cultured hippocampal neurons (20, 21). Therefore, identification of molecular targets involved in the initiation and maintenance of microglial activation caused by Aβ42 peptides may lead to a better understanding of inflammatory processes leading to AD.

In this study, we investigated molecular mechanisms underlying the Aβ42-mediated inflammatory response in microglial cells using large scale profiling of transcriptional induction by Aβ42 peptides in murine microglial BV2 cells. Cathepsin B was identified to be one of the 554 genes transcriptionally induced by Aβ42. Specific inhibition of cathepsin B in BV2 cells using either small interference RNA (siRNA)-mediated gene silencing or a specific cathepsin B inhibitor leads to diminished toxic effects on primary neurons. Our studies indicate that cathepsin B is a key player in microglial-mediated neuronal death. In addition, by combining large scale array analyses with siRNA-mediated gene silencing, our approach provides a useful plat-
form to systematically probe molecular pathways involved in inflammation-mediated neuronal degeneration in AD.

**EXPERIMENTAL PROCEDURES**

**Aβ Preparation and Treatment of BV2 Cells with Aβ Peptides—** Freshly sonicated Aβ42 was prepared by adding 1 ml of DMEM/F12/N2 medium to 1 mg of Aβ42 lyophilized powder (California Peptide, Napa, CA) and sonicating for 5 min. The resulting Aβ42 solution was then diluted with DMEM/F12/N2 to a final concentration of 5 or 11 μM and added to the BV2 cells for 15–16 h.

Aged Aβ was prepared using a modified protocol from Dr. LaDu’s laboratory (24). Briefly, Aβ42 powder lyophilized with hydroxy-furoisopropanol (California Peptide, Napa, CA) was dissolved in Me2SO and resuspended in DMEM/F12 at 44 μM before each use. The resulting solution was aged for 48 h at 4 °C. On the day of the treatment, the Aβ42 solution was diluted with DMEM/F12/N2 to a final concentration of 22 μM and added to the BV2 cells for 15–16 h.

**RNA Preparation and Library Construction—** Total RNA from cells was isolated using TRI Star reagent according to the manufacturer’s instructions (Invitrogen). The resulting RNA was treated with RNase-free DNase (Ambion, Austin, TX) for 40 min at 37 °C. Isolation of mRNA was carried out using Oligotex (Qiagen). To ensure the quality of mRNA preparations, denaturing agarose gel electrophoresis was used to assess the quality of each preparation. To decrease the redundancy and increase the relative abundance of rare transcripts, subtractive and normalized libraries were made. Reverse transcription and second strand synthesis were performed using the Superscript II reverse transcription kit (Invitrogen) with an anchored oligo(dT) (30) primer. Subtractive and normalized cDNA libraries were generated using suppression PCR as described previously (26).

**Array Production, Probe Generation, and Hybridization—** Subtracted and normalized cDNA libraries were cloned into the pCR2.1 vector (Invitrogen) and plated. Single colonies were picked into 384-well plates using an automated colony picker (Q-Pix, Genetix, Cambridge, UK) to grow overnight to serve as templates for PCR. PCR products were spotted in 12 replicate arrays using a 96-pin head arrayer (Genetix, Genetix, Cambridge, UK). More than 50,000 individual clones along with standards and empty wells were spotted onto three filters in each array. The probes were generated from three independent BV2 cultures treated with freshly sonicated Aβ42 peptide (California Peptide) along with controls. Briefly, mRNA was isolated from those cells as described previously. Double-stranded cDNA was generated by reverse transcription and second strand synthesis and digested with RsaI (New England Biolabs, Beverly, MA). The probes were generated through labeling with [32P]dCTP (PerkinElmer Life Sciences) using a Decamer labeling kit (Ambion). Hybridization was performed for 42 °C for 24 h in a hybridization solution containing 50% formamide.

**Array Analysis and Gene Annotation—** Cyclone phosphorimaging system (Packard Biosciences, Meriden, CT) and GenePix software analysis (Axon Instruments, Foster City, CA) were used to obtain densitometry of the [32P] signal. After normalization with the global background calculated from 1100 empty array spots, the mean and standard deviation from triplicate measurements for each clone was determined to calculate the confidence level (Student’s t test) and ratio of expression between control and Aβ-activated BV2 arrays. Arrays experiments were performed twice with the same probes, and the clones confirmed to be up-regulated in either experiments were submitted for sequencing. Sequence analysis was performed using Phrap (CodonCode, Dedham, MA), and a comprehensive homology analysis was conducted using a Blast algorithm against the RefSeq (NCBI) data base on accelerated computer resources (27). A comprehensive homology analysis was conducted using a Blast algorithm against the RefSeq (NCBI) data base on accelerated computer resources (27). A comprehensive homology analysis was conducted using a Blast algorithm against the RefSeq (NCBI) data base on accelerated computer resources (27).

**RESULTS**

**Activation of BV2 Cells by Freshly Sonicated Aβ, Not Aged Aβ, Induced Neuronal Death—** Numerous studies have described how microglial lineage cells secrete neurotoxic factors upon stimulation with synthetic Aβ peptides or Aβ isolated from human plaques. Most of the studies focused on stimulation of microglial lineage cells with fibrillar Aβ at very high concentrations or in combination with IFNγ (12, 16–18, 29). More recent studies demonstrate that soluble Aβ peptides, including oligomers and amyloid-derived diffusible ligands, are also neurotoxic. We reasoned that soluble Aβ42 might play an important role in microglial-mediated neurodegeneration. In our *in vitro* system that mimics microglial-dependent neuronal death in AD, we compared neuronal damage in primary rat cortical neurons upon treatment with the conditioned medium from murine microglial BV2 cells stimulated with either freshly sonicated soluble Aβ42 or aged Aβ42 peptides.

Consistent with prior studies, aged Aβ42 peptide alone caused no significant direct neuronal death in primary cortical neurons (Refs. 30–32 and Fig. 1). However, when aged Aβ42 peptide was incubated with BV2 cells, the conditioned medium caused no significant neurotoxicity. The loss of neurotoxicity is
pendent experiments using different A\textsubscript{42} treatment with 100 ng/ml LPS and 100 ng/ml IFN\textgamma. A profiling experiment was performed using BV2-specific arrays containing 50,000 clones, 9248 clones up-regulated by A\textsubscript{42} by a ratio of \( \geq 1.2 \) in at least one of the A\textsubscript{42}-treated BV2 cell samples and sequenced. Of the 9248 clones sequenced, 6929 clones yielded annotable sequence, based on the base call quality. These 6929 clones were contiged to 2176 unique transcripts to estimate the redundancy of the up-regulated clone selection. From the 6929 annotable clones, we selected 2851 clones that were up-regulated by A\textsubscript{42} by a ratio \( \geq 1.2 \) in at least two BV2 cell samples (Fig. 2A). Following gene annotation of these 2851 clones, we identified 554 nonredundant genes whose expression was induced by more than 1.2-fold. Clones lacking annotation to a gene in NCBI RefSeq were not analyzed further. The 554 up-regulated genes were mapped using GO molecular function terms (27). We found a high percentage of functional mappings to enzymes (40%), especially hydrolases (14%), such as cathepsin B and cathepsin L, and oxidoreductases (7%) among other enzymes. In addition, of all functional mappings for the 554 genes, 29% of them map to the binding factors, including protein-binding (5%), nucleotide-binding (9%), nucleic acid-binding (10%), metal ion-binding (2%), and other binding factors (3%) (Fig. 2B). Finally, 32 of the 554 genes mapped to the GO molecular function unknown category, representing 3% of all functional mappings and 5% of all genes. The NCBI RefSeq accession numbers for all 554 up-regulated genes, along with corresponding array data, has been deposited in the Gene Expression Omnibus hosted by NCBI (www.ncbi.nlm.nih.gov/geo/, series submission number GSE772 and platform accession numbers GPL561 and GPL562).

**Expression Analysis of Cathepsins B and L, TIMP2, AIF1, and Cytochrome c**—From the 554 genes, we selected a subgroup of genes from the two major functional mapping groups, namely enzymes and binding factors for further analysis. From the enzyme group, cathepsin B, cathepsin L, TIMP2, and cytochrome c oxidase were further analyzed for their roles in mediating inflammatory responses in microglia. AIF1, an IFN\textgamma-inducible Ca\textsuperscript{2+}-binding EF-hand protein, was selected from the binding factor group. Cathepsin B and L are lysosomal proteinases that are found extracellularly at high levels in the senile plaques of AD brain, which are known to contain activated microglia (36). Moreover, the activation and differentiation of mononuclear phagocytes is known to be accompanied by increased cathepsin B/L enzymatic activities (37). TIMP2, a tissue inhibitor of matrix metalloproteinases, is one of the major endogenous counter-regulators of matrix metalloproteinases. Both matrix metalloproteinases and TIMPs are implicated in the pathogenesis of inflammatory disorders of the central nervous system (38). Mitochondrial cytochrome c oxidase is known to be involved in apoptosis induced by various stressful stimuli, including inflammation (39). AIF1 has been associated with microglial activation in experimental models and in human cerebral infarctions (40).

We first used quantitative RT-PCR to confirm the gene regulation in sonicated A\textsubscript{42}-treated BV2 cells and compare gene regulation induced by sonicated and aged A\textsubscript{42}. In addition, regulation by LPS/IFN\textgamma, strong inflammatory stimuli, was used as a positive control for inflammatory responses. Of the five selected genes, we confirmed that the mRNA levels of all of them, except that of TIMP2, were significantly up-regulated by freshly sonicated A\textsubscript{42} in three or four independent experiments using quantitative RT-PCR (Table I, \( p < 0.05 \)). Interestingly, three of the five selected genes, AIF1 and cathepsins B and L, were up-regulated by both LPS/IFN\textgamma and freshly soni-
cated Aβ42, suggesting overlapping pathways shared by LPS/IFN-γ and freshly sonicated Aβ42 stimulation. However, none of the three genes were up-regulated by aged Aβ42, supporting our previous finding that stimulation of BV2 cells by freshly sonicated Aβ42 and aged Aβ42 involves divergent pathways (Fig. 1). Although in the array analyses, mRNA of TIMP2 was shown to be up-regulated by freshly sonicated Aβ42 in a statistically significant manner (Average ratio = 1.35 ± 0.09, p < 0.01), we were not able to confirm the finding in three independent quantitative RT-PCR analyses. This discrepancy is likely due to nonspecific hybridization with other isoforms of TIMPs in the large scale array analysis because large cDNA fragments were used as probes. It is therefore very important to confirm the results from large scale profiling studies in more detailed studies, such as quantitative RT-PCR using isoform-specific primers.

Knock-down Analysis of TIMP2, and AIF1—To identify whether any of the five selected genes play a key role in mediating the neurotoxic effects of Aβ-activated microglia, we used a strategy outlined in Fig. 3A. Expression of specific genes in BV2 cells was inhibited using siRNA (41) by transient transfection, followed by activation with freshly sonicated Aβ42. The rat primary cortical neurons were then exposed to the conditioned medium from activated BV2 cells transfected with siRNAs. We reasoned that specific inhibition of a key mediator in BV2 cells would abolish the neuron-killing effects of the toxic factors released by BV2 cells.

Gene-specific siRNAs induced highly efficient knock-down of mRNA levels for both TIMP2 and AIF1 (Fig. 3B). The efficiency of knock-down was similar in both control cells and cells treated Aβ42 (data not shown). However, the inhibition of TIMP2 and AIF1 expression did not affect the toxicity exerted...
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We next specifically inhibited the expression of cathepsin B and L by siRNAs (Fig. 4A). Inhibition of cathepsin B expression in BV2 cells completely abolished the neurotoxic effects exerted by conditioned medium from Aβ42-activated BV2 cells. However, inhibition of cathepsin L expression in BV2 cells did not affect the neurotoxic effects mediated by Aβ42-activated BV2 cells, indicating that cathepsin B, not cathepsin L, plays a crucial role in this process (Fig. 4B).

We then addressed the question of whether intracellular or extracellular cathepsin B is essential in mediating neurotoxicity in primary cortical neurons. CA-074, a nonpermeable cathepsin B inhibitor, was used to selectively inhibit extracellular cathepsin B activity (42). Indeed, inhibition of the enzymatic activity of cathepsin B in the conditioned medium from Aβ42-activated BV2 cells abolished its neurotoxic effects on primary neurons (Fig. 4C). Significant rescue effects were observed at both the 0.5 and 1 μM concentrations (Fig. 4C). Our data suggest that cathepsin B, especially extracellular cathepsin B, is probably a crucial factor released by Aβ42-activated microglial cells to mediate the neurotoxicity in cortical neurons or BV2 cells. Interestingly, we found that activation of BV2 cells by different forms of Aβ42 resulted in completely different neurotoxic effects in primary cortical neurons (Fig. 1). Freshly sonicated Aβ42 did not cause neuronal death when added directly to neurons, but activated BV2 cells to release toxic factors that caused significant neuronal death (Fig. 1). On the contrary, aged Aβ42 elicited significant direct neuronal toxicity, but its toxic effect on neurons was abolished after incubating with BV2 cells. Our current observation that aged Aβ42 peptide does not induce microglial-mediated neurotoxicity is different from some previous studies in which aged fibrillar Aβ42 was found to be effective. This difference could be explained by the fact that those studies used much higher concentrations (e.g., 60 μM) of aged fibrillar Aβ or a combination of aged Aβ42 and IFNγ to stimulate microglial cells to release toxic factors (16–18, 29). More importantly, we found that a relatively low concentration (11 μM) of freshly sonicated Aβ42 alone can induce microglia-mediated neurotoxicity. Although TNF-α and nitric oxide appear to be intimately involved in microglial-mediated neurotoxicity with a high dose of fibrillar Aβ40 (16, 17), no TNF-α or nitric oxide was detected in the conditioned medium from BV2 cells activated by freshly sonicated Aβ42,2 indicating different pathways involved. More detailed studies will be needed to understand the roles of different species of Aβ42 peptides in microglial activation as well as their pathophysiological relevance in the AD brain.

To understand the molecular pathways activated by freshly sonicated Aβ42, we conducted a large scale expression profiling analysis using filter-based cDNA arrays made from BV2 cDNA libraries enriched for Aβ42-activated microglial genes. The distinct advantage of this approach over presynthesized oligonucleotide arrays is that the clone collections are cell type- and disease-specific and are enriched with transcripts that are induced in specific disease conditions. Using this approach, we identified a total of 554 genes that are up-regulated by freshly sonicated Aβ42, of which 40% are enzymes. The unusual enrichment of enzymes, especially proteases, suggests that proteases activated in the chronic inflammatory conditions might play important roles in neuronal degeneration in AD (48).

To identify crucial players in Aβ-mediated inflammatory responses for therapeutic intervention, we used siRNA-mediated gene knock-out to establish a causative functional link between candidate genes with microglial-mediated neurotoxicity. Indeed, transfection of siRNAs corresponding to the candidate genes in BV2 cells resulted in 80–90% inhibition of expression of all four selected genes, indicating that siRNA-mediated gene silencing is a very powerful tool in analyzing gene function and identifying therapeutic targets in microglial BV2 cells. Our study found that inhibition of cathepsin B expression using siRNA completely abolished the neurotoxicity mediated by Aβ-activated BV2 microglial cells, suggesting that cathepsin

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**TABLE I**

| Gene name LPS/IFNγ | Sonicated Aβ42 | Aged Aβ42 |
|--------------------|----------------|-----------|
| Allograft inflammatory factor 1 | 6.74 ± 2.28 | 2.49 ± 0.91 | 0.65 ± 0.12 |
| Tissue inhibitor of metalloproteinase 2 | 0.64 ± 0.12 | 1.04 ± 0.18 | 0.85 ± 0.05 |
| Cathepsin B | 2.02 ± 0.45 | 1.82 ± 0.45 | 0.85 ± 0.15 |
| Cathepsin L | 1.92 ± 0.40 | 1.33 ± 0.12 | 0.74 ± 0.23 |
| Cytochrome c oxidase | 1.22 ± 0.19 | 1.45 ± 0.30 | ND³ |

³ ND stands for not determined.

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**DISCUSSION**

In the present study, we used a functional genomics approach to identify crucial molecular mediators of the neurotoxic effects caused by Aβ42-activated microglial BV2 cells. Our approach integrated large scale expression profiling with siRNA-mediated gene-specific silencing to identify cathepsin B as one of the key players in this process. Its crucial role was further confirmed using a highly specific inhibitor, CA-074, which completely abolished the neurotoxic effects mediated by Aβ42-activated microglial BV2 cells.

One important aspect of the pathophysiology of AD is its chronic inflammation associated with microglial activation, which is known to play multiple-functional roles in the neurodegeneration process (49). Proinflammatory factors and acute phase proteins released by chronically activated microglia are known to play important roles in promoting neurodegeneration in AD brain (3, 4). However, there is also mounting evidence demonstrating beneficial roles of microglial activation and inflammatory responses in plaque clearance (44–46) and neurodegeneration (47).

Interestingly, we found that activation of BV2 cells by different forms of Aβ42 resulted in completely different neurotoxic effects in primary cortical neurons (Fig. 1). Freshly sonicated Aβ42 did not cause neuronal death when added directly to neurons, but activated BV2 cells to release toxic factors that caused significant neuronal death (Fig. 1). On the contrary, aged Aβ42 elicited significant direct neuronal toxicity, but its toxic effect on neurons was abolished after incubating with BV2 cells. Our current observation that aged Aβ42 peptide does not induce microglial-mediated neurotoxicity is different from some previous studies in which aged fibrillar Aβ42 was found to be effective. This difference could be explained by the fact that those studies used much higher concentrations (e.g., 60 μM) of aged fibrillar Aβ or a combination of aged Aβ42 and IFNγ to stimulate microglial cells to release toxic factors (16–18, 29). More importantly, we found that a relatively low concentration (11 μM) of freshly sonicated Aβ42 alone can induce microglia-mediated neurotoxicity. Although TNF-α and nitric oxide appear to be intimately involved in microglial-mediated neurotoxicity with a high dose of fibrillar Aβ40 (16, 17), no TNF-α or nitric oxide was detected in the conditioned medium from BV2 cells activated by freshly sonicated Aβ42, indicating different pathways involved. More detailed studies will be needed to understand the roles of different species of Aβ42 peptides in microglial activation as well as their pathophysiologically relevant in the AD brain.

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² S. Yi and L. Gan, unpublished observations.
B plays a crucial role in this process. Cathepsin B is an abundant and ubiquitously expressed cysteine peptidase. Intracellular cathepsin B is localized in the lysosome and is partly responsible for terminal degradation of intracellular proteins. It has been implicated in a variety of diseases involving tissue-remodeling states, such as inflammation (49), parasite infection (50), and tumor metastasis (51). In microglial BV2 cells, cathepsin B consists of two major single-chain species of 32 and 34 kDa, which are processed from pro-cathepsin B in acidic pH. Stimulation of BV2 cells with LPS results in secretion of both pro-cathepsin B and 32-kDa single-chain cathepsin B species (52). Release of cathepsin B in the conditioned medium from microglial cells activated by LPS or peptide chromogranin induces neuronal apoptosis and activation of caspase 3 (53, 54).

More over, in slice cultures from rodents as well as in in vivo studies from primates, selective cathepsin B inhibitors were shown to protect against ischemia-induced neuronal damage (55), which is at least in part due to inflammatory and immunological reactions (56, 57).

In the present study, CA-074, an irreversible cathepsin B-specific inhibitor with very low membrane permeability, completely abolished the toxicity in the conditioned medium released by activated BV2 cells. Our study indicates that cathepsin B, most likely extracellular cathepsin B released from Aβ42-activated BV2 cells, is a key mediator in causing neuronal death in primary neurons. To the best of our knowledge, our study is the first to establish the functional link of cathepsin B with Aβ-mediated microglia activation and highlight the potentially crucial role of cathepsin B in AD pathogenesis.

In summary, we identified cathepsin B as a key player in microglial-mediated neuronal death using a strategy that inte-
Fig. 4. Cathepsin B plays a key function in mediating the toxic effects caused by conditioned medium from Aβ42-activated BV2 cells. A, expression of cathepsin B (n = 8) and cathepsin L (n = 8) was strongly inhibited by siRNAs with corresponding sequences but not by siRNA with a scrambled sequence (si-Control). The graph represents the means ± S.E. from triplicate wells. Similar results were obtained in three independent experiments with different Aβ preparations. B, inhibition of cathepsin B activity leads to increased neuronal viability after treatment with Aβ42-activated BV2 supernatants. BV2 cells were pretreated with 0.5 or 1 μM CA-074 for 30 min followed by treatment with 11 μM freshly sonicated Aβ42 for 15–17 h in the presence of CA-074. Neuronal viability was quantified using CellTiter-Glo Luminescent cell viability assay and expressed as relative luminescent signal units (RLU). Significant neuronal toxicity was observed in control (−), expression of cathepsin B (siCatB) abolished the CA-074-mediated neurotoxicity (p = 0.29). The graph represents the means ± S.E. in triplicate wells. Similar results were obtained in three independent experiments with different Aβ preparations. C, inhibition of cathepsin B activity leads to increased neuronal viability after treatment with Aβ42-activated BV2 supernatants. BV2 cells were pretreated with 0.5 or 1 μM CA-074 for 30 min followed by treatment with 11 μM freshly sonicated Aβ42 for 15–17 h in the presence of CA-074. Significant neuronal viability was quantified using CellTiter-Glo Luminescent cell viability assay and expressed as relative luminescent signal units (RLU). Significant neuronal toxicity was observed in control (−), expression of cathepsin B (siCatB) abolished the CA-074-mediated neurotoxicity (p = 0.29). The graph represents the means ± S.E. from triplicate wells. Similar results were obtained in three independent experiments with different Aβ preparations.
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