Benzodiazepinone Derivatives Protect against Endoplasmic Reticulum Stress-Mediated Cell Death in Human Neuronal Cell Lines

Haixia Zou, Allison S. Limpert, Jiwen Zou, Anna Dembo, Pooi-San Lee, Daniel Grant, Robert Ardecky, Anthony B. Pinkerton, Gavin K. Magnuson, Mark E. Goldman, Juan Rong, Peter Teriête, Douglas J. Sheffler, John C. Reed, and Nicholas D. P. Cosford

**ABSTRACT:** Endoplasmic reticulum (ER) stress causes neuronal dysfunction followed by cell death and is recognized as a feature of many neurodegenerative diseases. Using a phenotypic screen, we recently identified benzodiazepinone derivatives that reduce ER stress-mediated apoptosis in a rat neuronal progenitor cell line (CSM14.1). Herein we describe how structure–activity relationship (SAR) studies around these screening hits led to compounds that display robust cytoprotective activity against thapsigargin-induced ER stress in SH-SY5Y and H4 human neuronal cell lines. We demonstrate that the most potent of these derivatives, compound 4hh, inhibits the activation of p38 MAP kinase (p38) and c-Jun N-terminal kinase (JNK), protein kinases that are downstream signal effectors of the unfolded protein response (UPR). Compound 4hh specifically protects against thapsigargin-induced cell death and displays no protection against other insults known to induce cellular stress or activate p38. However, compound 4hh provides moderate inhibition of p38 activity stimulated by compounds that disrupt calcium homeostasis. Our data indicate that probe compound 4hh is a valuable small molecule tool that can be used to investigate the effects of ER stress on human neurons. This approach may provide the basis for the future development of therapeutics for the treatment of neurodegenerative diseases.

**KEYWORDS:** Benzodiazepinone, ER stress, p38 MAPK, calcium homeostasis, neurodegeneration, thapsigargin

Cell death induced by endoplasmic reticulum (ER) stress is a critical component of many disorders including diabetes, cardiovascular disease, ischemic insult, and prion disease. Additionally, many devastating neuronal disorders are characterized by proteinopathies that upregulate the unfolded protein response (UPR). The UPR cell signaling mechanism activates ER stress pathways that eventually lead to neuronal death. Thus, ER stress is implicated in several neurodegenerative disorders that have limited treatment options, including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and amyotrophic lateral sclerosis (ALS). Consequently, the identification of novel compounds that ameliorate ER stress and reduce cellular apoptosis may have clinical relevance for the treatment of numerous disease states.

The ER is the organelle responsible for accurate protein folding, which is achieved through post-translational modifications of the amino acid chain. The ER can undergo stress if subjected to a variety of physiological insults, including accumulation of misfolded proteins, misglycosylation of proteins, dysregulation of calcium, and oxidative stress. These pathological conditions stimulate the UPR, an adaptive process that leads to the reduction of protein synthesis and the upregulation of molecular chaperones. In the event of the failure of these processes to alleviate ER stress, apoptotic pathways are initiated.

ER stress stimulates the activation of three distinct signaling pathways to elicit cellular adaptation or cell death (Figure 1). These separate pathways are initiated by three transmembrane proteins: protein kinase R (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). When cell stress remains unresolved, IRE1 complexes with the adaptor molecule tumor necrosis factor receptor-associated factor 2 (TRAF2), which then binds apoptosis signal regulating kinase 1 (ASK1), a mitogen-activated protein (MAP) kinase kinase kinase. This IRE1-TRAF2-ASK1 complex promotes activation of the proapoptotic signaling proteins p38 MAP kinase (p38) and c-Jun N-terminal kinase (JNK). p38 is able to induce cell death through the phosphorylation of two serine residues (78 and 81) on the transactivation domain of the transcription factor C/EBP homologous protein (CHOP). This phosphorylation event increases CHOP transcriptional activity, which upregulates the...
expression of apoptotic genes.\textsuperscript{3,9} Activation of JNK results in the phosphorylation of transcription activator protein 1 (AP1) which also induces the expression of inflammatory and proapoptotic genes (Figure 1).\textsuperscript{10}

The natural product thapsigargin (Figure 2) is one of a number of chemical agents that induce ER stress and activate the UPR in cells.\textsuperscript{11} Thapsigargin initiates ER stress through inhibition of sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) calcium transporters that facilitate calcium entry into the ER. This causes a depletion of ER calcium stores and an elevation of cytosolic calcium levels. As many ER chaperones depend on ER Ca\textsuperscript{2+} ions for proper functioning, disruption of ER Ca\textsuperscript{2+} concentrations results in ER stress.\textsuperscript{12} Disruptions in calcium regulation are also observed in many neuronal disorders. Calcium is an essential second messenger involved in cellular signaling, and tight control over the concentration of Ca\textsuperscript{2+} ions is especially important in neuronal cells to maintain membrane excitability and to allow depolarization. Importantly, the dysregulation of calcium homeostasis has been identified as a component of neurodegenerative diseases such as Alzheimer’s disease.\textsuperscript{13–15}

The upregulation of ER stress pathways is a common characteristic of many neurodegenerative diseases. ER stress and activation of the UPR have been observed in Alzheimer’s disease patients\textsuperscript{16} where the accumulation of amyloid beta protein contributes to cellular dysfunction.\textsuperscript{12} In addition, the E3 ubiquitin ligase Parkin has been demonstrated to alleviate ER stress in neurons, and mutations in this protein have been characterized as the predominant cause of Parkinson’s disease.\textsuperscript{17} Furthermore, familial ALS is frequently caused by mutations in superoxide dismutase 1 (SOD1) that cause this protein to form toxic aggregates in the ER.\textsuperscript{18,19} Treatments for Alzheimer’s disease, Parkinson’s disease, and ALS are extremely limited, and therefore, the discovery and characterization of compounds that modulate ER stress mechanisms would represent an important step toward the development of therapeutics for these disorders.

We recently reported a high-throughput screen (HTS) performed through the Molecular Libraries Probe Production Center Network (MLPCN) using a phenotypic cell-based assay to identify small molecule compounds that rescue a rat neuronal cell line (CSM14.1) from cell death induced by thapsigargin.\textsuperscript{20} In these studies, we demonstrated that certain benzodiazepinone screening hits (Figure 3) reduce ER stress-induced cell death in rodent neuronal cell lines and cultured neurons. These active benzodiazepinone derivatives specifically inhibited stress signals propagated through ASK1 kinase by enhancing the association of ASK1 with 14–3–3 proteins, thereby reducing its ability to bind to and activate downstream effectors of cell stress, such as p38 and JNK.\textsuperscript{20} However, these compounds appear to have no direct effect on ASK1 kinase activity or the activity of over 400 kinases tested.\textsuperscript{20} In addition, the benzodiazepinone hits were shown to exhibit cytoprotective activity against ER stress mediated by thapsigargin, but not staurosporine, VP16, or TNF, in human cervical (HeLa),
Herein we describe the expansion of the structure–activity relationship (SAR) around the benzodiazepinone derivatives and evaluate their activity as inhibitors of thapsigargin-induced p38 activity and apoptosis in human neuronal cell lines. We also describe experiments that provide insight into the cellular mode of action (MOA) of these ER stress inhibitors and evaluate their druglike properties in readiness for in vivo proof-of-concept (POC) studies.

### RESULTS AND DISCUSSION

Our preliminary analysis of the benzodiazepinone hits from phenotypic screening in rat neuronal cells led to the identification of several compounds that inhibit ER stress-mediated cell death through suppression of the ASK1 pathway. Among the hits from screening, the 3-phenyl derivative ML037 (Figure 3) was identified and characterized as an MLPCN probe compound for this project. To determine the structural characteristics that could impart cytoprotective potency and efficacy in human neuronal cells, we synthesized a library of new analogues (Table 1) to diversify the SAR around the benzodiazepinone core scaffold present in the screening hits. The analogues were designed to probe the structural requirements for cytoprotective potency and efficacy in human neuronal cells. We therefore synthesized a series of benzodiazepinone derivatives (Figure 3) in which substituents were varied at the 3-position (R2 = H or Me), the 7-position (R1 = Cl, Br, Ph, CF3, or COPh), or the 4′-position of the 11-phenyl moiety (R3). Our rationale for making these specific

<table>
<thead>
<tr>
<th>CPD #</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>SH-SY5Y MAX ± SEM (% Viability)</th>
<th>H4 MAX ± SEM (% Viability)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% DMSO</td>
<td></td>
<td></td>
<td></td>
<td>100.0 ± 1.8</td>
<td>100.0 ± 5.6</td>
</tr>
<tr>
<td>7.5 μM Tg</td>
<td></td>
<td></td>
<td></td>
<td>55.5 ± 0.4</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Salubrinal</td>
<td></td>
<td></td>
<td></td>
<td>34.0 ± 0.9</td>
<td>14.0 ± 1.2</td>
</tr>
<tr>
<td>4a</td>
<td>Cl</td>
<td>H</td>
<td></td>
<td>3.7 ± 0.5</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>4b</td>
<td>Cl</td>
<td>H</td>
<td></td>
<td>2.8 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>4c</td>
<td>Cl</td>
<td>H</td>
<td></td>
<td>2.4 ± 0.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>4d</td>
<td>Cl</td>
<td>H</td>
<td></td>
<td>2.5 ± 0.2</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>4e</td>
<td>Cl</td>
<td>H</td>
<td></td>
<td>4.4 ± 0.3</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>4f</td>
<td>Br</td>
<td>H</td>
<td></td>
<td>2.1 ± 0.0</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>4g</td>
<td>Br</td>
<td>H</td>
<td></td>
<td>2.2 ± 0.4</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>4h</td>
<td>Br</td>
<td>H</td>
<td></td>
<td>1.9 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>4i</td>
<td>Br</td>
<td>H</td>
<td></td>
<td>3.8 ± 0.3</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>4j</td>
<td>CF3</td>
<td>H</td>
<td></td>
<td>18.6 ± 0.9</td>
<td>1.8 ± 0.9</td>
</tr>
<tr>
<td>4k</td>
<td>CF3</td>
<td>H</td>
<td></td>
<td>19.8 ± 1.0</td>
<td>11.5 ± 3.3</td>
</tr>
<tr>
<td>4l</td>
<td>CF3</td>
<td>H</td>
<td></td>
<td>7.1 ± 0.1</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>4m</td>
<td>Cl</td>
<td>CH3</td>
<td></td>
<td>0.0 ± 1.1</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>4n</td>
<td>Cl</td>
<td>CH3</td>
<td></td>
<td>6.8 ± 0.7</td>
<td>8.1 ± 1.4</td>
</tr>
<tr>
<td>4o</td>
<td>Cl</td>
<td>CH3</td>
<td></td>
<td>17.4 ± 0.0</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>4p</td>
<td>Cl</td>
<td>CH3</td>
<td></td>
<td>3.8 ± 0.0</td>
<td>6.1 ± 0.8</td>
</tr>
<tr>
<td>4q</td>
<td>Cl</td>
<td>CH3</td>
<td></td>
<td>4.4 ± 0.4</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>4r</td>
<td>Cl</td>
<td>CH3</td>
<td></td>
<td>7.5 ± 0.2</td>
<td>7.4 ± 0.1</td>
</tr>
</tbody>
</table>

**SH-SY5Y or H4 cells were pretreated with DMSO or compounds (50 μM salubrinal; 25 μM benzodiazepinones) for 2 h and then treated with DMSO or 7.5 μM thapsigargin for an additional 18 h. Cell viability was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay. Wells containing 100 μL of DMEM but no cells were used as background, whereas wells treated with DMSO but no thapsigargin were used as positive controls. The viability (% of control) = 100 × (well value − average of background)/(average of positive control − average of background). The data were analyzed using MS Excel software. The experiments were repeated at least three times, and the data are represented as the average ± SEM.**
Research Article

Scheme 1

Reagents and conditions: (a) 2, PTSA, toluene, Dean–Stark trap. (b) Zn, FeSO₄, NH₄Cl, ethanol, water. (c) R’CHO, MgSO₄, AcOH, ethanol.

modifications included removal of the phenyl group at position 3 (R²) to eliminate a chiral center and simplify the structures. We also sought to investigate the effects of varying R¹ to determine the importance of the N-ethyl substituents present in ML037 (Figure 3). The analogues were synthesized using the chemistry shown in Scheme 1. Accordingly, treatment of 2-nitroaniline derivative 1 with 1,3-dione derivative 2 in benzene containing a catalytic amount of p-toluensulfonic acid produces the homologous amide 3a. Reduction of the nitro group in 3a with zinc, ferrous sulfate and ammonium chloride produces the amine derivative 3b. Treatment of intermediate 3b with an aldehyde (R’CHO = R’C₆H₄CHO) in a hot solution of acetic acid in ethanol produces the desired benzodiazepinone analogues 4 in high overall yield.

The newly synthesized compounds were then tested in viability assays to determine their ability to protect against thapsigargin-induced cell death. Since ER stress is a critical component of many human neurodegenerative diseases, the benzodiazepinone derivatives were tested in two human neuronal cell lines, SH-SYSY neuroblastoma cells and H4 glioma cells. Testing was accomplished by incubating cells for 2 h with the benzodiazepinone derivatives followed by addition of thapsigargin. Cell viability measurements were taken 18 h after thapsigargin treatment. As shown in Table 1, several compounds were found to exhibit cytoprotective effects against thapsigargin (7.5 μM) treatment. Dose–response experiments were then performed to determine the potencies (EC50 values, Table 2) for cytoprotective effects of those compounds initially determined to protect against ER stress in the single concentration assays. Analysis of the SAR data shown in Tables 1 and 2 revealed some interesting trends. For example, it became apparent that substitution at the 3-position (R²) is necessary for activity since all of the analogues lacking substituents at this position (4a−4i) were inactive. In the series with R² = Me, all of the compounds with R¹ = Cl (4m−4r) displayed EC50 values for cytoprotection of >20 μM (Table 2). Interestingly, however, in the R¹ = Br series, compound 4s was cytotoxic in H4 cells (EC50 ≈ 13 μM) while compound 4t was cytotoxic in SH-SYSY cells (EC50 ≈ 15 μM). In the same vein, compound 4w in the Br series showed protective activity in H4 cells (EC50 ≈ 13 μM) but not in SH-SYSY cells. On the other hand, in the series of analogues where R¹ = Ph (4x−4bb), all but one analogue (4bb) exhibited robust cytoprotective activity in both cell lines. Compounds 4y and 4z were the most potent in the R¹ = Ph series, with EC50 values of 9.60 and 6.85 μM, respectively, in H4 cells. Potency values declined somewhat in the R¹ = CF₃ series (4cc−4ff), although compounds 4ee and 4ff displayed EC50 values of <20 μM in both cell lines. In contrast, five compounds in the R¹ = COPh (benzophenone) series (4gg−4kk) all showed robust cytoprotective activity in both cell lines. It is notable, however, that compounds 4ll (R¹ = OH) and 4mm (R¹ = 3-thiophenyl) were devoid of cytoprotective activity and were therefore used as inactive controls in further experiments. Based on its overall profile in the cell viability assays, compound 4hh (EC50 = 7.00 μM in SH-SYSY cells, EC50 = 11.4 μM in H4 cells) was selected for additional characterization. The robust, dose-dependent cytoprotective activity of compound 4hh is shown graphically in Figure 4. A subset of the most potent compounds was then evaluated via in vitro absorption, distribution, metabolism, and excretion (ADME) assays to determine their plasma stability.

Table 2. Potency and Efficacy of Cytoprotective Benzodiazepinone Analogues

<table>
<thead>
<tr>
<th>compd</th>
<th>SH-SYSY EC50 ± SEM (μM)</th>
<th>MAX ± SEM (% viability)</th>
<th>H4 EC50 ± SEM (μM)</th>
<th>MAX ± SEM (% viability)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4j</td>
<td>&gt;50 ± na</td>
<td>41.1 ± 4.6</td>
<td>&gt;50 ± na</td>
<td>23.3 ± 7.7</td>
</tr>
<tr>
<td>4k</td>
<td>&gt;50 ± na</td>
<td>22.5 ± 2.7</td>
<td>&gt;50 ± na</td>
<td>12.1 ± 2.8</td>
</tr>
<tr>
<td>4n</td>
<td>&gt;50 ± na</td>
<td>60.4 ± 3.9</td>
<td>&gt;50 ± na</td>
<td>22.0 ± 3.9</td>
</tr>
<tr>
<td>4o</td>
<td>35.62 ± 2.74</td>
<td>62.9 ± 7.3</td>
<td>20.61 ± 0.66</td>
<td>22.5 ± 2.3</td>
</tr>
<tr>
<td>4p</td>
<td>40.44 ± 2.06</td>
<td>69.0 ± 1.1</td>
<td>27.50 ± 3.32</td>
<td>26.0 ± 1.9</td>
</tr>
<tr>
<td>4r</td>
<td>&gt;50 ± na</td>
<td>61.1 ± 5.0</td>
<td>22.25 ± 1.55</td>
<td>24.7 ± 2.5</td>
</tr>
<tr>
<td>4s</td>
<td>&gt;50 ± na</td>
<td>22.3 ± 4.6</td>
<td>12.86 ± 0.92</td>
<td>60.9 ± 4.4</td>
</tr>
<tr>
<td>4t</td>
<td>14.57 ± 1.61</td>
<td>59.3 ± 6.7</td>
<td>&gt;50 ± na</td>
<td>28.8 ± 1.8</td>
</tr>
<tr>
<td>4u</td>
<td>31.39 ± 0.99</td>
<td>69.8 ± 0.8</td>
<td>26.30 ± 1.34</td>
<td>29.9 ± 1.7</td>
</tr>
<tr>
<td>4w</td>
<td>&gt;50 ± na</td>
<td>52.2 ± 2.4</td>
<td>13.11 ± 2.53</td>
<td>25.7 ± 3.4</td>
</tr>
<tr>
<td>4x</td>
<td>21.04 ± 0.26</td>
<td>63.4 ± 6.5</td>
<td>14.32 ± 0.31</td>
<td>44.9 ± 1.0</td>
</tr>
<tr>
<td>4y</td>
<td>11.42 ± 1.03</td>
<td>66.2 ± 5.0</td>
<td>9.60 ± 1.06</td>
<td>52.0 ± 2.2</td>
</tr>
<tr>
<td>4z</td>
<td>13.78 ± 3.62</td>
<td>64.3 ± 5.2</td>
<td>6.85 ± 0.98</td>
<td>45.6 ± 0.6</td>
</tr>
<tr>
<td>4aa</td>
<td>21.69 ± 2.27</td>
<td>64.4 ± 10.2</td>
<td>16.99 ± 1.41</td>
<td>46.9 ± 1.5</td>
</tr>
<tr>
<td>4bb</td>
<td>&gt;50 ± na</td>
<td>67.7 ± 6.2</td>
<td>&gt;50 ± na</td>
<td>32.9 ± 3.8</td>
</tr>
<tr>
<td>4ee</td>
<td>14.02 ± 0.18</td>
<td>71.2 ± 1.6</td>
<td>18.81 ± 2.77</td>
<td>31.3 ± 2.5</td>
</tr>
<tr>
<td>4ff</td>
<td>16.02 ± 0.82</td>
<td>78.3 ± 8.1</td>
<td>19.96 ± 2.15</td>
<td>31.1 ± 4.1</td>
</tr>
<tr>
<td>4gg</td>
<td>15.30 ± 1.15</td>
<td>87.4 ± 0.6</td>
<td>15.10 ± 0.67</td>
<td>69.9 ± 3.2</td>
</tr>
<tr>
<td>4hh</td>
<td>7.00 ± 0.16</td>
<td>84.8 ± 3.3</td>
<td>11.40 ± 2.26</td>
<td>59.5 ± 0.2</td>
</tr>
<tr>
<td>4ii</td>
<td>12.33 ± 1.59</td>
<td>70.5 ± 4.7</td>
<td>19.02 ± 1.28</td>
<td>60.9 ± 1.4</td>
</tr>
<tr>
<td>4jj</td>
<td>14.98 ± 0.47</td>
<td>71.7 ± 5.7</td>
<td>14.73 ± 3.52</td>
<td>65.6 ± 5.1</td>
</tr>
<tr>
<td>4kk</td>
<td>19.81 ± 2.38</td>
<td>71.3 ± 2.7</td>
<td>18.87 ± 1.30</td>
<td>59.6 ± 3.1</td>
</tr>
</tbody>
</table>

"EC50" values and percent maximal viability for analogues were obtained by treating SH-SYSY or H4 cells with 7.5 μM thapsigargin and various concentrations of hit compounds. Cell viability was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay. The results were analyzed using GraphPad Prism 5. Experiments were repeated three times, and the data are represented as the average ± SEM.
Research Article

Figure 4. Compound 4hh dose-dependently increases viability in SH-SYSY and H4 cells. SH-SYSY or H4 cells were pretreated with 4hh for 2 h and then treated with 7.5 μM thapsigargin for an additional 18 h. Cell viability was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay. Wells containing 100 μL of DMEM but no cells were used as background, whereas wells treated with DMSO but no thapsigargin were used as positive controls. The viability (% of control) = 100 × (well value − average of background)/(average of positive control − average of background). Experiments were repeated at least three times, and the data are represented as the average ± SEM.

Figure 5. Benzodiazepinones inhibit thapsigargin-induced p38 MAPK and JNK activation. H4 cells were pretreated with DMSO or benzodiazepinones for 2 h and then treated with 20 μM thapsigargin for one more hour. Cell lysates were collected and analyzed by SDS-PAGE/immunoblotting. Specific antibodies for phospho-p38 MAPK, p38 MAPK, phospho-JNK, and JNK were used. 4hh is an active benzodiazepinone, while 4mm is inactive.

with the inactive analogue 4mm had no effect. These data suggest that, as with the previously characterized screening hits, 4hh inhibits thapsigargin-induced activation of the proapoptotic kinases p38 and JNK that are activated downstream of ASK1.

We next investigated whether 4hh possessed cytoprotective activity against cell death initiated in response to activators of cellular stress other than thapsigargin. The compounds tested covered a range of cell stress events, including ER stress [tunicamycin, carboxenzoxy-Leu-Leu-leucinal (MG132), or dithiothreitol (DTT)], broad kinase inhibition (staurosporine), oxidative stress [6-hydroxydopamine (6-OHDA), paragrot, or H2O2], and activation of the ASK1 kinase pathway [3’,4’-dichloro-3-(3,4-dichlorophenylacetyl)-2,4,6-trihydroxymethylbenzo (DDTD)]. Thus, SH-SYSY cells were pretreated with 4hh or the inactive analogue 4mm, which was utilized as a negative control, and then stimulated with the various cell stress inducers. Cellular viability was quantified using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) or ATPlite (PerkinElmer). Interestingly, we found that 4hh specifically protected against thapsigargin-induced cell death and offered no protection against other activators of cell stress (Figure 6). This specificity for thapsigargin-induced cell stress suggests that 4hh may act upon pathways uniquely initiated through thapsigargin treatment.

Thapsigargin is a noncompetitive SERCA inhibitor that increases the cytosolic calcium concentration in cells by preventing uptake of calcium by the ER. Therefore, we next examined whether 4hh inhibited p38 activation by other compounds that disrupt calcium homeostasis. To analyze p38 activity, we utilized H4 cells expressing a CHOP-luciferase reporter system. When active, p38 MAPK upregulates the transcriptional activity of CHOP through the phosphorylation of two serine residues in the CHOP transactivation domain. H4 cells pretreated with 4hh showed reduced p38 activation initiated by thapsigargin, CDDO-IM, or ionomycin, but had no effect on p38 activation induced by oxidative stress [6-hydroxydopamine (6-OHDA), paraquat, or H2O2], and activation of the ASK1 kinase pathway [3’,4’-dichloro-3-(3,4-dichlorophenylacetyl)-2,4,6-trihydroxymethylbenzo (DDTD)]. Thus, SH-SYSY cells were pretreated with 4hh or the inactive analogue 4mm, which was utilized as a negative control, and then stimulated with the various cell stress inducers. Cellular viability was quantified using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) or ATPlite (PerkinElmer). Interestingly, we found that 4hh specifically protected against thapsigargin-induced cell death and offered no protection against other activators of cell stress (Figure 6). This specificity for thapsigargin-induced cell stress suggests that 4hh may act upon pathways uniquely initiated through thapsigargin treatment.

Thapsigargin is a noncompetitive SERCA inhibitor that increases the cytosolic calcium concentration in cells by preventing uptake of calcium by the ER. Therefore, we next examined whether 4hh inhibited p38 activation by other compounds that disrupt calcium homeostasis. To analyze p38 activity, we utilized H4 cells expressing a CHOP-luciferase reporter system. When active, p38 MAPK upregulates the transcriptional activity of CHOP through the phosphorylation of two serine residues in the CHOP transactivation domain. H4 cells stably expressing pFR-Luc and pFA-CHOP plasmids were pretreated with DMSO, 4hh, or the inactive analogue 4mm. Additionally, a p38 inhibitor was used as a positive control. p38 activation was induced using the synthetic triterpenoid, 25 We found that treatment with 4hh reduced p38 activation initiated by thapsigargin, CDDO-IM, or ionomycin, but had no effect on p38 activity stimulated by MG132 (Figure 7). These data

stably in plasma, but have low stability in liver microsomes and may have poor blood-brain barrier (BBB) permeability as predicted by a parallel artificial membrane permeability assay (PAMPA). In addition, compound 4hh was tested against a panel of 46 receptors, ion channels, and transporters through the NIMH Psychoactive Drug Screening Program (PDSP), and the data are included in Table S1 in the Supporting Information. No significant off-target interactions were detected.

We previously performed experiments suggesting that this series of benzodiazepinone derivatives specifically target the ASK1 signaling pathway, resulting in reduced signaling from this stress kinase. Consequently, we evaluated the activity of whether it inhibited the activation of signaling effectors downstream of ASK1. As ASK1 activity results in the phosphorylation and subsequent activation of p38 MAPK and JNK, immunoblots using phospho-specific antibodies were performed on H4 cellular lysates. H4 cells pretreated with DMSO alone displayed robust phosphorylation of both p38 and JNK following 1 h of thapsigargin treatment. However, in cells pretreated with various concentrations of 4hh, p38 and JNK activity in response to thapsigargin treatment was significantly reduced (Figure 5). Conversely, pretreatment

Table 3. ADME Values for Selected Compounds

<table>
<thead>
<tr>
<th>Compd</th>
<th>Microsomal Stability</th>
<th>Plasma Stability</th>
<th>PAMPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4hh</td>
<td>7.9 ± 6.3</td>
<td>100</td>
<td>−7.33</td>
</tr>
<tr>
<td>4t</td>
<td>0.1 ± 0.1</td>
<td>100</td>
<td>N/A</td>
</tr>
<tr>
<td>4y</td>
<td>1.8 ± 2.1</td>
<td>97.9 ± 5.7</td>
<td>−7.12</td>
</tr>
<tr>
<td>4ee</td>
<td>1.8 ± 0.4</td>
<td>98.8 ± 3.7</td>
<td>−7.54</td>
</tr>
<tr>
<td>4ff</td>
<td>4.7 ± 1.7</td>
<td>100</td>
<td>−7.46</td>
</tr>
<tr>
<td>4jj</td>
<td>12.9 ± 1.6</td>
<td>100</td>
<td>−10.20</td>
</tr>
</tbody>
</table>

*Compounds with the highest relative potency were analyzed for stability in mouse microsomes and plasma, as well as for BBB permeability.

stable in plasma, but have low stability in liver microsomes and may have poor blood-brain barrier (BBB) permeability as predicted by a parallel artificial membrane permeability assay (PAMPA). In addition, compound 4hh was tested against a panel of 46 receptors, ion channels, and transporters through the NIMH Psychoactive Drug Screening Program (PDSP), and the data are included in Table S1 in the Supporting Information. No significant off-target interactions were detected.

We previously performed experiments suggesting that this series of benzodiazepinone derivatives specifically target the ASK1 signaling pathway, resulting in reduced signaling from this stress kinase. Consequently, we evaluated the activity of whether it inhibited the activation of signaling effectors downstream of ASK1. As ASK1 activity results in the phosphorylation and subsequent activation of p38 MAPK and JNK, immunoblots using phospho-specific antibodies were performed on H4 cellular lysates. H4 cells pretreated with DMSO alone displayed robust phosphorylation of both p38 and JNK following 1 h of thapsigargin treatment. However, in cells pretreated with various concentrations of 4hh, p38 and JNK activity in response to thapsigargin treatment was significantly reduced (Figure 5). Conversely, pretreatment

Table 3. ADME Values for Selected Compounds

<table>
<thead>
<tr>
<th>Compd</th>
<th>Microsomal Stability</th>
<th>Plasma Stability</th>
<th>PAMPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4hh</td>
<td>7.9 ± 6.3</td>
<td>100</td>
<td>−7.33</td>
</tr>
<tr>
<td>4t</td>
<td>0.1 ± 0.1</td>
<td>100</td>
<td>N/A</td>
</tr>
<tr>
<td>4y</td>
<td>1.8 ± 2.1</td>
<td>97.9 ± 5.7</td>
<td>−7.12</td>
</tr>
<tr>
<td>4ee</td>
<td>1.8 ± 0.4</td>
<td>98.8 ± 3.7</td>
<td>−7.54</td>
</tr>
<tr>
<td>4ff</td>
<td>4.7 ± 1.7</td>
<td>100</td>
<td>−7.46</td>
</tr>
<tr>
<td>4jj</td>
<td>12.9 ± 1.6</td>
<td>100</td>
<td>−10.20</td>
</tr>
</tbody>
</table>

*Compounds with the highest relative potency were analyzed for stability in mouse microsomes and plasma, as well as for BBB permeability.
Figure 6. Compound 4hh exhibits strong cytoprotection only with thapsigargin treatment among different p38 activating cell death inducers. SH-SY5Y cells were pretreated with DMSO or 25 μM compounds for 2 h and then treated with different inducers, including thapsigargin (7.5 μM), tunicamycin (15 μM), staurosporine (2 μM), 6-OHDA (200 μM), DTT (5 mM), and H2O2 (200 μM) for 18 h, and MG132 (5 μM), paraquat (0.5 mM), and DDTD (10 μM) for 48 h. Cell viability was assessed using the CellTiter 96 AQ One Solution Cell Proliferation Assay or ATPiLight. Wells with DMSO but no inducer and no compound were used as 100% controls, and wells with DMSO and inducer but no compound were used as negative controls. The experiments were repeated at least three times, and the data are represented as the average ± SEM. Asterisk (*) shows that 4hh exhibits very strong cytoprotective activity against thapsigargin-induced cell death whereas the inactive compound 4mm does not.

Figure 7. Active benzodiazepinone 4hh, but not inactive compound 4ll, inhibits calcium regulator-induced p38 MAPK activation. H4-CHOP-luciferase reporter cells were pretreated with DMSO or compounds for 1 h and then treated with thapsigargin (20 nM), CDDO-IM (600 nM), ionomycin (120 nM), or MG132 (740 nM) for an additional 18 h. The luciferase expression was assessed using the Steady-Glo luciferase assay reagent. The p38 inhibitor was used at 20 μM. The benzodiazepinones 4hh or 4ll (10 μM) were used with thapsigargin or CDDO-IM treatment, while concentrations of 20 and 50 μM were tested with MG132 and ionomycin treatment, respectively. Wells treated with compounds only but no inducers were used as background control correspondingly, and wells treated with inducers but no compounds were used as 100% controls. The RLU (% of control) = 100 × (well value − average of background)/ (average of 100% control − average of background). The data were analyzed using MS Excel software. The experiments were repeated at least three times, and the data are represented as the average ± SEM. Statistical significance (P < 0.05) was determined using one-way analysis of variance. *Significance was compared with inducer treatment groups. This compound is also very strong cytoprotective activity against thapsigargin-induced cell death whereas the inactive compound 4mm does not.

suggest that a potential mechanism by which 4hh alleviates stress-induced apoptosis is through reducing the activation of stress kinases in response to calcium dysregulation.

■ CONCLUSIONS

Compounds that protect against ER stress have great potential to ameliorate neuronal death and dysfunction associated with neurodegenerative disorders. We previously demonstrated that benzodiazepinone derivatives have cytoprotective properties in rodent neurons treated with thapsigargin.20 We have extended these studies by expanding the SAR in this series. Thirty nine new benzodiazepinone analogues were synthesized and tested for their ability to inhibit ER stress-mediated cell death. Of these, the most potent compound was 4hh, which protects against thapsigargin-induced ER stress in SH-SY5Y cells with a potency of approximately 7 μM. This compound is also cytoprotective in H4 human neuronal cells and reduces the activity of the stress kinases JNK and p38 MAPK in response to thapsigargin. Additionally, 4hh appears to specifically reduce the activation of stress kinases in response to the dysregulation of calcium homeostasis. Calcium plays a critical role in neuronal signaling and disruptions in calcium homeostasis in both the ER and mitochondria have been identified in ALS.26 Specifically, motor neurons expressing G93A hSOD1 mutations, which have been linked to familial ALS, displayed increased calcium uptake by SERCA receptors coupled with alterations in mitochondrial calcium efflux.27,28 In addition, some motor neurons have been demonstrated to be particularly vulnerable to alterations in calcium.29,30 The biological characteristics of the benzodiazepinone derivatives in our study suggest that these compounds may provide the basis of a treatment for disease states where ER stress leads to neuronal death and dysfunction. Taken together, our data support the continued investigation of benzodiazepinone analogues as potential therapeutic agents for neuronal disorders that feature neuron loss in response to ER stress. It is notable that these compounds specifically inhibit stress kinase activation in response to the disruption of calcium homeostasis, an initiator of both ER stress and neuronal dysfunction.

■ METHODS

General Methods for the Synthesis of Benzodiazepinone Derivatives. General Method A. A stirred solution of the substituted 2-nitroaniline 1 (1 mmol, 1 equiv), 1,3-cyclohexanedione 2 (1.5 mmol, 1.5 equiv), 50 mL of toluene, and PTSA (0.1 mmol, 0.1 equiv) was heated at reflux using a Dean–Stark trap for 12–24 h. When the reaction was completed, as determined by HPLC-MS analysis, the reaction was cooled to room temperature. The crude reaction mixture was diluted with 100 mL of ethyl acetate and washed twice with saturated NaHCO3 solution (50 mL). The organic layers were collected and dried over Na2SO4. The solvents were removed by rotary evaporation, and the products were isolated by flash chromatography (hexane/ethyl acetate 90:10 to 50:50 gradient) and concentrated in vacuo to provide the compound 3a (yield 50–85%) which was determined to be >95% pure by HPLC-MS and 1H NMR.

General Method B. To solution of compound 3a (1 mmol, 1 equiv), ethanol (50 mL), iron sulfate heptahydrate (3 mmol, 3 equiv), water (9 mL), and ammonium chloride (8 mmol, 8 equiv) was added with efficient stirring zinc powder (3 mmol, 3 equiv). The reaction mixture was then heated for 3–12 h at 50 °C. When the reaction was completed, as determined by HPLC-MS analysis, the reaction mixture was cooled to room temperature and filtered over a pad of Celite (5g) with suction. The filter cake was washed with ethanol, and the filtrate was concentrated under reduced pressure to a residue. The residue was dissolved in CH2Cl2 (50 mL), and 50 mL of water was added. The

DOI: 10.1021/cn500297v
ACS Chem. Neurosci. 2015, 6, 464–475

ACS Chemical Neuroscience

Research Article
organic layer was separated and dried over Na2SO4. The solvents were removed by rotary evaporation and the products were isolated by flash chromatography (hexane/ethyl acetate 90:10 to 50:50 gradient) to provide the compound 3b (yield 90–95%) which was determined to be >95% pure by HPLC-MS and 1H NMR.

**General Method C.** A stirred solution of compound 3b (1 mmol, 1 equiv) in ethanol (50 mL), RCHO (1.1 mmol, 1 equiv), and MgSO4 (2 mmol, 2 equiv) was heated for 12–24 h at 70 °C. When the reaction was completed, as determined by HPLC-MS analysis, the reaction was cooled to room temperature and filtered, the filtrate was removed by rotary evaporation, and the products were isolated by preparative HPLC (C-18 column eluted with MeOH containing 0.05% formic acid and water containing 0.5% formic acid, 10:90 to 100:0 gradient) to provide the final compound 4 (yield 55–78%) which was determined to be >95% pure by HPLC-MS and 1H NMR.

**7-Chloro-11-(4-dimethylamino)phenyl)-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one (4a).** 1H NMR (400 MHz, CDCl3): δ 6.87 (m, 2H), 6.70 (m, 1H), 6.44 (m, 3H), 6.21 (m, 1H), 5.81 (s, 1H), 3.23 (m, 4H), 2.70–2.60 (m, 2H), 2.40 (m, 2H), 2.08 (m, 2H), 1.07 (m, 3H). 13C NMR (100 MHz, CDCl3): δ 192.5, 156.0, 145.7, 138.1, 132.1, 130.4, 128.0, 122.2, 116.7, 118.7, 112.9, 54.5, 43.5, 36.0, 31.0, 21.5. ESI-MS m/z 396 [M + H]+. HRMS m/z calculated for C25H27ClN3O [M + H]+: 396.1837. Found: 396.1838.

**7-Chloro-11-(4-pyrrolidin-1-yl)phenyl)-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one (4b).** 1H NMR (400 MHz, CDCl3): δ 7.83 (s, 1H), 6.90–6.96 (m, 2H), 6.60–6.65 (m, 2H), 6.29–6.27 (m, 2H), 6.59 (s, 1H), 3.09 (m, 4H), 2.65 (m, 2H), 2.23 (m, 2H), 1.93–1.87 (m, 6H). 13C NMR (100 MHz, CDCl3): δ 192.5, 156.1, 145.9, 127.9, 121.6, 118.7, 113.8, 54.7, 47.2, 35.9, 30.7, 24.8, 21.4. ESI-MS m/z 408 [M + H]+. HRMS m/z calculated for C24H27ClN3O [M + H]+: 408.1933. Found: 408.1924.

**1H NMR (400 MHz, CDCl3): δ 6.91 (m, 2H), 6.71 (m, 3H). 6.5–6.20 (m, 2H), 5.84 (s, 1H), 3.04 (m, 4H), 2.70–2.60 (m, 2H), 2.40 (m, 2H), 2.11 (m, 2H), 1.52 (m, 6H). 13C NMR (100 MHz, CDCl3): δ 194.1, 153.8, 151.7, 138.7, 136.2, 131.9, 125.8, 123.4, 122.6, 120.9, 120.5, 119.1, 116.3, 113.9, 113.5, 57.2, 57.0, 50.6, 36.2, 32.8, 25.8, 24.1, 21.6. ESI-MS m/z 408 [M + H]+. HRMS m/z calculated for C25H27ClN3O [M + H]+: 408.1933. Found: 408.1924.

**7-Chloro-11-(4-(morpholin-4-yl)phenyl)-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one (4d).** 1H NMR (400 MHz, CDCl3): δ 6.94 (m, 2H), 6.68 (m, 4H), 6.50–6.30 (m, 2H), 5.85 (s, 1H), 3.18 (m, 4H), 3.04 (m, 4H), 2.75–2.60 (m, 2H), 2.41 (m, 2H), 2.06 (m, 2H). 13C NMR (100 MHz, CDCl3): δ 192.6, 156.1, 149.0, 138.0, 134.6, 132.2, 127.5, 122.4, 121.0, 118.9, 111.4, 103.2, 56.6, 50.7, 50.6, 36.2, 24.9, 21.6, 20.3, 13.9. ESI-MS m/z 452 [M + H]+. HRMS m/z calculated for C25H27ClN3O [M + H]+: 452.2463. Found: 452.2453.

**7-Chloro-11-(4-(pyridin-1-yl)phenyl)-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one (4e).** 1H NMR (400 MHz, CDCl3): δ 6.88–6.75 (m, 3H), 6.65 (m, 1H), 6.37 (m, 3H), 5.81 (s, 1H), 3.15 (m, 4H), 2.55 (m, 2H), 2.37 (m, 2H), 2.00 (m, 2H), 1.46 (m, 2H), 1.27 (m, 2H), 0.90 (m, 6H). 13C NMR (100 MHz, CDCl3): δ 194.4, 155.0, 146.9, 138.8, 136.4, 132.0, 130.0, 128.0, 125.3, 123.2, 122.2, 120.7, 119.5, 113.9, 111.4, 110.6, 57.0, 50.7, 57.0, 50.6, 36.2, 24.9, 21.6, 20.3, 13.9. ESI-MS m/z 452 [M + H]+. HRMS m/z calculated for C25H27ClN3O [M + H]+: 452.2463. Found: 452.2453.
**Research Article**

ACS Chemical Neuroscience

---

'\(^3\)H NMR (400 MHz, CDCl\(_3\)): \(d = 6.91 (m, 2H), 6.70 (m, 2H), 6.35 (m, 3H), 6.10 (s, 1H), 5.80 (s, 1H), 3.17 (m, 4H), 2.60 (m, 1H), 2.29 (m, 3H), 1.92 (m, 4H), 1.15 (s, 3H), 1.08 (s, 3H).'\(^1\)C NMR (100 MHz, CDCl\(_3\)): \(\delta 193.8, 151.5, 146.7, 156.3, 130.0, 130.3, 127.8, 123.4, 122.6, 119.0, 113.1, 111.4, 57.4, 49.7, 47.4, 46.5, 32.4, 29.0, 27.7, 25.4. ESI-MS m/z 422 [M + H\(^+\)]. HRMS m/z calc for C\(_{29}\)H\(_{39}\)BrN\(_3\)O \([M + H]\): 484.1421. Found: 484.1410.

7-Chloro-3,3-dimethyl-11-(4-piperidin-1-ylphenyl)-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one (4p).

'\(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 7.00 (m, 2H), 7.00 (m, 1H), 7.00 (m, 1H), 6.98 (m, 5H), 6.55 (m, 1H), 6.15 (m, 4H), 2.75 (m, 1H), 1.08 (s, 3H), 0.91 (s, 3H).'\(^1\)C NMR (100 MHz, CDCl\(_3\)): \(\delta 193.8, 151.2, 146.7, 156.3, 130.0, 130.3, 127.8, 123.4, 122.6, 119.0, 113.1, 111.4, 57.4, 49.7, 47.4, 46.5, 32.4, 29.0, 27.7, 25.4. ESI-MS m/z 422 [M + H\(^+\)]. HRMS m/z calc for C\(_{29}\)H\(_{39}\)BrN\(_3\)O \([M + H]\): 484.1421. Found: 484.1410.

---
112.3, 57.0, 49.7, 46.2, 40.5, 32.4, 29.0, 27.4. ESI-MS m/z 430 [M + H]−. HRMS m/z calcd for C32H33F6N4O [M + H]−: 430.2101. Found: 430.2083.

11-(4-Diethylaminophenyl)-3,3-dimethyl-7-(trifluoromethyl)-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one (193.9, 152.3, 142.6, 138.4, 134.3, 131.7, 130.0, 129.5, 128.2, 127.5, 126.8, 121.8, 120.0, 116.3, 112.6, 56.7, 49.6, 46.2, 32.2, 29.1, 27.5, 25.7, 24.1. ESI-MS m/z 506 [M + H]−. HRMS m/z calcd for C36H33F6N4O [M + H]−: 506.2802. Found: 506.2793.

7-Benzoyl-3,3-dimethyl-11-(4-morpholinophenyl)-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one (4R). 1H NMR (400 MHz, CDCl3): δ 7.89 (dl, J = 7.3 Hz, 2H), 7.52 (m, 1H), 7.46 (m, 2H), 7.15 (m, 1H), 7.04 (dl, J = 6.8 Hz, 2H), 6.76 (s, 1H), 6.71 (dl, J = 6.8 Hz, 2H), 6.59 (m, 1H), 5.93 (s, 1H), 3.78 (m, 4H), 3.05 (m, 4H), 2.59 (m, 1H), 2.31 (m, 3H), 1.13 (s, 3H), 1.04 (s, 3H). 13C NMR (100 MHz, CDCl3): δ 195.4, 153.9, 149.7, 147.3, 143.2, 138.2, 133.5, 131.8, 130.1, 129.5, 128.2, 127.8, 127.1, 122.0, 119.9, 115.5, 112.3, 116.9, 56.6, 49.7, 49.2, 46.2, 32.2, 29.1, 27.4. ESI-MS m/z 508 [M + H]−. HRMS m/z calcd for C36H33F6N4O [M + H]−: 508.2595. Found: 508.2579.

7-Benzoyl-11-(4-hydroxyphenyl)-3,3-dimethyl-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one (21). 1H NMR (400 MHz, DMSO-d6): δ 8.86 (s, 1H), 7.54–7.46 (m, 5H), 7.08 (m, 1H), 6.92–6.86 (m, 3H), 6.62 (m, 1H), 6.48 (m, 2H), 5.62 (s, 2H), 2.49 (s, 2H), 2.18–2.00 (m, 2H), 1.00 (s, 3H), 0.94 (s, 3H). 13C NMR (100 MHz, DMSO-d6): δ 193.7, 192.2, 155.5, 154.6, 143.7, 138.3, 134.9, 131.5, 129.8, 129.0, 128.3, 127.9, 127.3, 125.2, 122.3, 119.4, 114.7, 111.1, 54.5, 49.3, 43.8, 31.7, 28.7, 27.2. ESI-MS m/z 439 [M + H]+. HRMS m/z calcd for C36H33F6N4O [M + H]−: 439.1943. Found: 439.1942.

11-(4-Benzoyl-3,3-dimethyl-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one (4M)). 1H NMR (400 MHz, DMSO-d6): δ 8.94 (s, 1H), 7.54–7.42 (m, 7H), 7.21 (m, 1H), 7.06–6.84 (m, 4H), 6.66 (m, 1H), 6.40 (m, 6H), 6.50 (s, 2H), 2.39 (s, 2H), 2.18–2.00 (m, 2H), 0.98 (s, 3H), 0.93 (s, 3H). 13C NMR (100 MHz, DMSO-d6): δ 194.4, 192.5, 154.0, 145.5, 138.2, 130.0, 130.4, 129.1, 128.2, 127.4, 125.8, 123.0, 119.2, 116.9, 56.7, 49.7, 46.4, 32.4, 28.9, 27.5, 25.8, 24.1. ESI-MS m/z 470 [M + H]+. HRMS m/z calcd for C36H33F6N4O [M + H]−: 470.2414. Found: 470.2403.

Cell Culture. SH-SYSY cells (ATCC, CRL-2266) were maintained in DMEM/F12 (1:1, Life Technologies, 11330-032) supplemented with 10% FBS (HyClone, SH30396.03), 100 μg/mL streptomycin, 100 IU penicillin (Life Technologies, 15140-122), and 2 mM l-glutamine (Omega, GS-60). H4 cells (ATCC, HTB-148) were maintained in DMEM (Cellgro, 15–013-CV) supplemented with 10% FBS, 100 μg/mL streptomycin, 100 IU penicillin, and 2 mM l-glutamine. The H4-CHOP-luciferase reporter cell line was maintained in DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, 100 IU penicillin, 2 mM l-glutamine, 250 μg/mL Gentamicin (Gibco, 10131), and 1 μg/mL Purimycin (Sigma-Aldrich, P8833). All cell lines were incubated at 37 °C with 5% CO2.

Cell Viability Assay. Thapsigargin as Stressor. SH-SYSY or H4 cells were plated in 96-well plates (Costar, 3596) with a density of 4 × 104 or 2.5 × 105 cells per well in 90 μL of phenol red-free DMEM (Cellgro, 17-205-CV) containing 2% FBS, 100 μg/mL streptomycin, 100 IU penicillin, and 2 mM l-glutamine. The plates were incubated overnight at 37 °C with 5% CO2. The test compounds (5 μL in 10% DMSO) were added to wells to achieve a final concentration of 25 μM for screening or concentrations in dose-response experiments. Subalural (EMD Millipore, in 5 μL in 10% DMSO) was added to obtain a final concentration of 50 μM, whereas 5 μL of 10% DMSO was used as control. After a 2 h incubation, thapsigargin (Calbiochem, 5 μL in 10% DMSO) was added to each well to give a final concentration of 7.5 μM and, 5 μL of 10% DMSO was added to no-compound and no-thapsigargin wells as controls. The plates were incubated at 37 °C for an additional 18 h. Cell viability was assessed by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, G3880). The plates were recorded using a BMG POLARStar Omega (BMG Labtech) multimode plate reader in absorbance mode. The absorbance was measured at 570 nm. Wells containing 100 μL of DMEM but no cells were used as background, whereas wells treated with DMSO but no thapsigargin were used as positive controls. The viability (% of control) = 100 × (well value – average of background)/(average of positive control – average of background). The data were analyzed by using EXCEL software for screening and GraphPad Prism 5 for dose–response results. The
Used detection reagents (GE Healthcare Life Sciences, RPN2106) were HRP-linked anti-mouse IgG (Cell Signaling Technology, 7076) were HRP-conjugated goat anti-rabbit IgG (Thermo Scientific, H32323, 200 μM). The protocol was the same as that for thapsigargin treatment.

SH-SYSY cells and 96-well plates were also used when treating cells with MG132 (EMD Millipore, 474790, 5 μM), pararuech dichloride (Sigma-Aldrich, 36541, 0.5 μM), or 3',4'-dicloro-3-(3,4-dichloro-phenylactetyl)-2,4,6-trihydroxyxoe benzoin (DDTD, 2410 μM). The protocol was the same as that for thapsigargin treatment except for exposing cells to the cell death inducers for 48 h.

However, for dithiothreitol (Sigma-Aldrich, D0632) treatment, SH-SYSY cells (10^4 cells/well) and solid white 384-well plates (Greiner Bio-one, 781080) were used by adjusting all the volumes used in the thapsigargin protocol to 1/4. The plates were assessed by ATPPlite (PerkinElmer Life Sciences, 6016941) and read on a BMG POLARstar Omega plate reader in luminescence mode. The experiments were repeated at least three times, and the data are presented as the average ± SEM.

Immunoblotting. H4 cells in phenol red-free DMEM containing 2% FBS, 100 μg/mL streptomycin, 100 μM penicillin, and 2 mM l-glutamine were seeded at a density of 2.5 × 10^5 in 10 cm dishes. After overnight incubation, the culture media were changed to serum-free DMEM. Following 2 h starvation, cells were pretreated with DMSO or compounds (25 μM and 5 μM) for 2 h and then exposed to 20 μM thapsigargin for an additional hour. Cells were collected in ice-cold PBS and lysed with RIPA buffer (Sigma-Aldrich, R0278) supplemented with a protease inhibitor (Roche Applied Science, 04693124001) and a phosphatase inhibitor (Roche Applied Science, 04906848001). The cell lysates were centrifuged, and supernatants were recovered. The protein concentrations were determined by using the BCA Protein Assay Kit (Pierce, 23225). Subsequently, 50 μg of cell extract was analyzed by SDS PAGE immunoblotting. Here, NuPAGE 4–12% Bis-Tris Gels (Life Technologies, NP0321) and iBlot Transfer Stack, PVDF Regular (Life Technologies, IB401001) were used. The primary antibodies included anti-phospho-p38 MAPK antibody (Cell Signaling Technology, 9211), anti-phospho MAPK (Cell Signaling Technology, 9212), anti-phospho-JNK antibody (Promega, V7931), and anti-JNK antibody (Santa Cruz Biotechnology, sc-7345). HRP conjugated goat anti-rabbit IgG (Thermo Scientific, 31466) and HRP-linked anti-mouse IgG (Cell Signaling Technology, 7076) were used as secondary antibodies. To develop the Western blots, ECL Detection Reagents (GE Healthcare Life Sciences, RP2106) were used.

Activity of p38 MAPK Pathway. H4-CHOP-luciferase reporter cells (stable expressing pFR-Luc and pFA-CHOP plasmids (Agilent)) were plated in 384-well white LIA-plate (Greiner # 781080) at a cell density of 1000 cells/well in DMEM containing 10% FBS, 100 μg/mL streptomycin, 100 μIU penicillin, and 2 mM l-glutamine. After 24 h incubation at 37°C, 5% CO2, the media in each well was replaced with 45 μL of assay media (phenol red-free DMEM containing 2% FBS, 100 μg/mL streptomycin, 100 μIU penicillin, and 2 mM l-glutamine). Either 2.5 μL of test compounds or p38 MAPK inhibitor (EMD Millipore, 506148) in 4% DMSO was added to achieve designated concentrations or 2.5 μL of DMSO was added as control. After 1 h incubation at 37°C, 2.5 μL of inducers including thapsigargin, 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazole (CDDO-IM) and thapsigargin protocol for 1/4. The plates were assessed by using the BCA Protein Assay Kit (Pierce, 23225). Subsequently, 50 μg of cell extract was analyzed by SDS PAGE immunoblotting. Here, NuPAGE 4–12% Bis-Tris Gels (Life Technologies, NP0321) and iBlot Transfer Stack, PVDF Regular (Life Technologies, IB401001) were used. The primary antibodies included anti-phospho-p38 MAPK antibody (Cell Signaling Technology, 9211), anti-phospho MAPK (Cell Signaling Technology, 9212), anti-phospho-JNK antibody (Promega, V7931), and anti-JNK antibody (Santa Cruz Biotechnology, sc-7345). HRP conjugated goat anti-rabbit IgG (Thermo Scientific, 31466) and HRP-linked anti-mouse IgG (Cell Signaling Technology, 7076) were used as secondary antibodies. To develop the Western blots, ECL Detection Reagents (GE Healthcare Life Sciences, RP2106) were used.

Activity of p38 MAPK Pathway. H4-CHOP-luciferase reporter cells (stable expressing pFR-Luc and pFA-CHOP plasmids (Agilent)) were plated in 384-well white LIA-plate (Greiner # 781080) at a cell density of 1000 cells/well in DMEM containing 10% FBS, 100 μg/mL streptomycin, 100 μIU penicillin, and 2 mM l-glutamine. After 24 h incubation at 37°C, 5% CO2, the media in each well was replaced with 45 μL of assay media (phenol red-free DMEM containing 2% FBS, 100 μg/mL streptomycin, 100 μIU penicillin, and 2 mM l-glutamine). Either 2.5 μL of test compounds or p38 MAPK inhibitor (EMD Millipore, 506148) in 4% DMSO was added to achieve designated concentrations or 2.5 μL of DMSO was added as control. After 1 h incubation at 37°C, 2.5 μL of inducers including thapsigargin, 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazole (CDDO-IM) and thapsigargin protocol for 1/4. The plates were assessed by using the BCA Protein Assay Kit (Pierce, 23225). Subsequently, 50 μg of cell extract was analyzed by SDS PAGE immunoblotting. Here, NuPAGE 4–12% Bis-Tris Gels (Life Technologies, NP0321) and iBlot Transfer Stack, PVDF Regular (Life Technologies, IB401001) were used. The primary antibodies included anti-phospho-p38 MAPK antibody (Cell Signaling Technology, 9211), anti-phospho MAPK (Cell Signaling Technology, 9212), anti-phospho-JNK antibody (Promega, V7931), and anti-JNK antibody (Santa Cruz Biotechnology, sc-7345). HRP conjugated goat anti-rabbit IgG (Thermo Scientific, 31466) and HRP-linked anti-mouse IgG (Cell Signaling Technology, 7076) were used as secondary antibodies. To develop the Western blots, ECL Detection Reagents (GE Healthcare Life Sciences, RP2106) were used.

Activity of p38 MAPK Pathway. H4-CHOP-luciferase reporter cells (stable expressing pFR-Luc and pFA-CHOP plasmids (Agilent)) were plated in 384-well white LIA-plate (Greiner # 781080) at a cell density of 1000 cells/well in DMEM containing 10% FBS, 100 μg/mL streptomycin, 100 μIU penicillin, and 2 mM l-glutamine. After 24 h incubation at 37°C, 5% CO2, the media in each well was replaced with 45 μL of assay media (phenol red-free DMEM containing 2% FBS, 100 μg/mL streptomycin, 100 μIU penicillin, and 2 mM l-glutamine). Either 2.5 μL of test compounds or p38 MAPK inhibitor (EMD Millipore, 506148) in 4% DMSO was added to achieve designated concentrations or 2.5 μL of DMSO was added as control. After 1 h incubation at 37°C, 2.5 μL of inducers including thapsigargin, 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazole (CDDO-IM) and thapsigargin protocol for 1/4. The plates were assessed by using the BCA Protein Assay Kit (Pierce, 23225). Subsequently, 50 μg of cell extract was analyzed by SDS PAGE immunoblotting. Here, NuPAGE 4–12% Bis-Tris Gels (Life Technologies, NP0321) and iBlot Transfer Stack, PVDF Regular (Life Technologies, IB401001) were used. The primary antibodies included anti-phospho-p38 MAPK antibody (Cell Signaling Technology, 9211), anti-phospho MAPK (Cell Signaling Technology, 9212), anti-phospho-JNK antibody (Promega, V7931), and anti-JNK antibody (Santa Cruz Biotechnology, sc-7345). HRP conjugated goat anti-rabbit IgG (Thermo Scientific, 31466) and HRP-linked anti-mouse IgG (Cell Signaling Technology, 7076) were used as secondary antibodies. To develop the Western blots, ECL Detection Reagents (GE Healthcare Life Sciences, RP2106) were used.
Funding
This work was supported by NIH Grant HG005033 (NIGMS/NIMH), the Department of Defense Amyotrophic Lateral Sclerosis Research Program under Award Number W81XWH-12-1-0373 (to N.D.P.C.), and Janssen Research and Development LLC.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
We would like to thank Dr. Nicholas Carruthers (Janssen Research and Development LLC) for helpful comments. The binding profile for compound 4hh was generously provided by the National Institute of Mental Health’s Psychoactive Drug Screening Program, Contract HHSN-271-2008-00025-C (NIMH PDSP).

DEDICATION
This paper is dedicated to our departed colleague Professor Gregory P. Roth.

ABBREVIATIONS
6-OHDA, 6-hydroxydopamine; ADME, absorption, distribution, metabolism and excretion; ALS, amyotrophic lateral sclerosis; API, transcription activator protein 1; ASK1, apoptosis signal regulating kinase 1; ATF6, activating transcription, metabolism and excretion; ALS, amyotrophic lateral sclerosis; AP1, transcription activator protein 1; ASK1, apoptosis signal regulating kinase 1 pathway in human osteosarcoma cells.

REFERENCES


