Center Probe Report

Project Title: Chemical Inhibitors of ER Stress

Grant Number: 1 R03 DA024887-01

Screening Center Name: San Diego Center for Chemical Genomics
Principal Investigator of Screening Center: John C. Reed, MD, Ph.D.

Assay Provider & Institution: John C. Reed, MD, Ph.D., Burnham Institute for Medical Research, La Jolla, CA

Assay or Pathway Target: ER Stress Pathway

Probe PubChem Compound Identifier (SID/CID): CID-2878746 (MLS-5962123; benzodiazepine series) and CID-2891837 (MLS-6239507)

Assay provider information

Specific Aim:
The stated goal is to identify compounds that selectively inhibit cell death induced by endoplasmic reticulum (ER) stress in mammalian cells. Myriad types of disturbances cause accumulation of unfolded proteins in the ER, triggering a cell signaling response, termed the unfolded protein response (UPR). Some changes of cell's environment such as (a) glucose deprivation (Ma et al. 2004), (b) aberrations of Ca^{2+} regulation in the ER (Rao et al. 2004), (c) viral infection (He et al. 2006), (d) disturbances in cellular redox regulation (Frand et al. 2000), and (e) situations that impair proteasome activity (Paschen et al. 2003) can induce UPR. The initial intent of the ER stress signaling (UPR) is to adapt to the changing environment, reestablish homeostasis and normal ER function. But, prolonged ER stress triggers cell suicide, usually in the form of apoptosis in animal cells to dispense of dysfunctional cells.

Because cell death linked to ER stress is a prominent feature of several neurological diseases (Yoshida et al. 2007; Lindholm et al. 2006; Xu et al. 2005), we focused on developing a primary chemical library screen utilizing neuronal cells. CSM14.1 is a rat striatal neuroprogenitor cell line that was established by immortalization using a temperature-sensitive variant of SV40 Large T antigen (Zhong et al. 1993; Haas et al. 2002). At permissive temperature (optimal at 32°C), the cells proliferate and can be easily expanded in standard culture media for high throughput screening (HTS) assays. When cultured at non-permissive temperature of 39°C, large T antigen is inactive and the cells cease proliferating and differentiate to produce neurons with characteristics of mature dopaminergic neurons

(Zhong et al. 1993; Haas et al. 2002). For convenience, and because transient reductions in temperature that might be associated with large screening experiments could restore Large T activity, we elected to develop our HTS using undifferentiated CSM14.1 cells, with the plan to then confirm hits using differentiated cells.

For monitoring cell death, we used a bioluminescence reagent that determines intracellular ATP content without requirement for complicated cell processing steps (ATPlite [Perkin Elmer]). Thus, ATP was used as a surrogate indicator of cell survival for the primary assay. To trigger cell death using a stimulus known to induce ER stress, we selected thapsigargin, a sesquiterpene lactone that irreversibly inhibits the Ca2+-ATPase of the ER membrane. After primary HTS, a variety of secondary assays were performed to determine if cytoprotection by the hit compounds is: (1) active for both undifferentiated and differentiated CMS14.1 cells; (2) active for additional cell lines stimulated with thapsigargin; (3) active for several inducers of ER stress, including thapsigargin and tunicamycin; and (4) selective for cell death induced by ER stress, but not cell death induced via other pathways, including the TNF/death receptor pathway and the mitochondrial pathway. Finally, the effects of active compounds on signaling events associated with ER stress were investigated.

This Probe report describes a potent single digit micromolar benzodiazepine compound (CID-2878746) that inhibits ER stress-induced cell death. The probe was identified and selected from a round of analog-by-catalog of the most potent cell active hit from screening (CID-2891837). Eleven additional representatives of this family of benzodiazepines inhibit ER stress-induced cell death, but not death induced by death receptor or mitochondrial pathways. Thus, they are pathway specific with respect to cell death pathways. Detailed analysis of CID-2891837 to narrow the locus of biological action was completed by the assay provider in advance of identification of the most potent Probe compound (CID-2878746). The screening hit (CID-2891837) selectively inhibits the UPR signaling pathway involving IRE1-ASK1-JNK/p38MAPK, without affecting the ATF6 proteolytic processing, sXBP1 mRNA splicing, ATF4 expression, or CHOP expression. Thus, CID-2891837 is also pathway specific in that it selectively suppresses one of the major ER stress signal transduction pathways that leads to activation of stress kinases. In this regard, CID-2891837 inhibits phosphorylation of c-Jun transcription factor and p38 MAPK without directly inhibiting JNK or p38MAPK, implying a site of action at or upstream of the kinase ASK1. The Probe compound's protective activity was prominent in CSM14.1 ($IC_{50} \simeq 10$ uM), several tumor cell lines (IC_{50} ranging from 10 to 20 uM), mouse neural stem cells, and primary cortical neuron cells, but not in Jurkat leukemia or undifferentiated PC12 pheochromocytoma cells. Altogether, 7 types of cells or cell lines were tested under 10 conditions (differentiated vs undifferentiated), with the compounds showing protective

activity against thapsigargin-induced death in 5 of 7 cell lines under 7 of 10 culture conditions. Thus, while exhibiting some cell-type specificity, the Probe compound shows broad-spectrum activity. Thus, compound (CID-2878746) represents a class of pathway-selective inhibitor of ER stress-induced cell death, which may be useful for identifying and analyzing targets that regulate UPR and ER stress-induced cell death signaling – mechanism of relevance to neurodegeneration and ischemic diseases. Moreover, because ER stress leading to JNK activation has been associated with insulin resistance in the context of high fat diet, the compound may also be useful for diabetes and obesity research.

Screening center information

PubChem Bioassay Name: High Throughput Screen for Inhibitors of ER stress-induced Cell Death.

List of PubChem bioassay identifiers generated for this screening project (AIDS): 1010

List of relevant AIDs that may be used as counter-screen information: 1006

Primary Assay Description as defined in PubChem

Final concentration of each reagent is:

(a)Compound control-Salubrinal: 100 uM

(b)Negative control: DMSO 1% with thapsigargin treatment

(c)Positive control: DMSO 1%, without thapsigargin treatment

(d)DMSO concentration during whole procedure: approximately 1%

(e)Thapsigargin: 15 uM

(f)Library compound: 15 ug/ml

(g)ATPlite solution: 40% of final culture volume (20uL/50uL)

1) Undifferentiated CSM14.1 cells were maintained in complete media at 32°C.

* Tissue culture complete media: DMEM with 10% FBS, 1% L-glutamine, 100 I.U. Penicillin/ml, 100 ug/ml streptomycin, 250 ng/ml Amphotericin.

2) CSM14.1 cells were recovered from cultures by trypsinization, and suspended at a density of 7.5 x 104 cells/mL in DMEM medium containing 2% FBS, and same concentration of antibiotics in step 1.

3) 40 uL per well of the cell suspension was seeded to each well of 96 well plastic microtiter plates by 'Well Mate' liquid dispenser, and cultured overnight at 32°C.

4) Library compounds were thawed at room temperature for 1 hr, and centrifuged to pellet liquid. Using a BIOMEK FX liquid handler (Beckman Coulter), 5 uL of test compounds in 10% DMSO were added to wells in columns 2-11 of the 96 well plates (leaving columns 1 and 12 intact) to achieve an approximate final concentration of 15 ug/mL of test compound and a final concentration of 1.1% DMSO at this point.

5) With step 4), 5 uL of 1 mM Salubrinal solution was added to wells of column 1/rows A-D of each plate, while 5 uL of DMSO solution was added to wells corresponding to column 1/rows E-H and to all wells in column 12. Multi channel pipettor was used to dispense 5uL solutions in column 1, and 12

* Salubrinal solution (1 mM) : 10% DMSO solution of 10mM Salubrinal + 90% DMEM.

* DMSO solution (10%): 10% DMSO + 90% DMEM

6) The plates were returned to the incubator for 2 hrs.

7) A WellMate bulk reagent dispenser (Thermo Fisher Scientific) was used to dispense 5 uL per well of thapsigargin solution in, thus achieving a final concentration of ~15 uM thapsigargin, to columns 1-11, leaving column 12 as a control for data comparison (only DMSO/no thapsigargin). Instead, column 12 received 5 uL of DMEM (2% FBS, and same antibiotics concentration) containing 0.0375uL of DMSO by multi channel pipettor.

* thapsigargin solution (150uM): 5uL uM thapsigargin solution is composed of 0.0375 ul of 20 mM TG plus 4.9625 ul of complete culture media with 2% FBS.

8) The plates were returned to the incubator for 24 hrs.

9) 30 minutes before performing the surrogate cell viability assay in which cellular ATP levels are measured, the components of the ATPliteTM (Perkin-Elmer) kit were prepared..

10) 20 uL of ATPlite solution was dispensed per well using a WellMate.

11) Within 30 minutes, ATP luminescence was evaluated using an Analyst HT multimode plate reader (Molecular Device Corporation) in the luminescence mode.

12) Data analysis was performed using CBIS software (ChemInnovation, Inc).

* For liquid dispensing in step 3), 7) and 10), small nozzle tubing (Thermo Fischer Scientific) was used. Before use, tubing was sterilized by 70% ethanol, and washed intensively by sterilized distilled water.

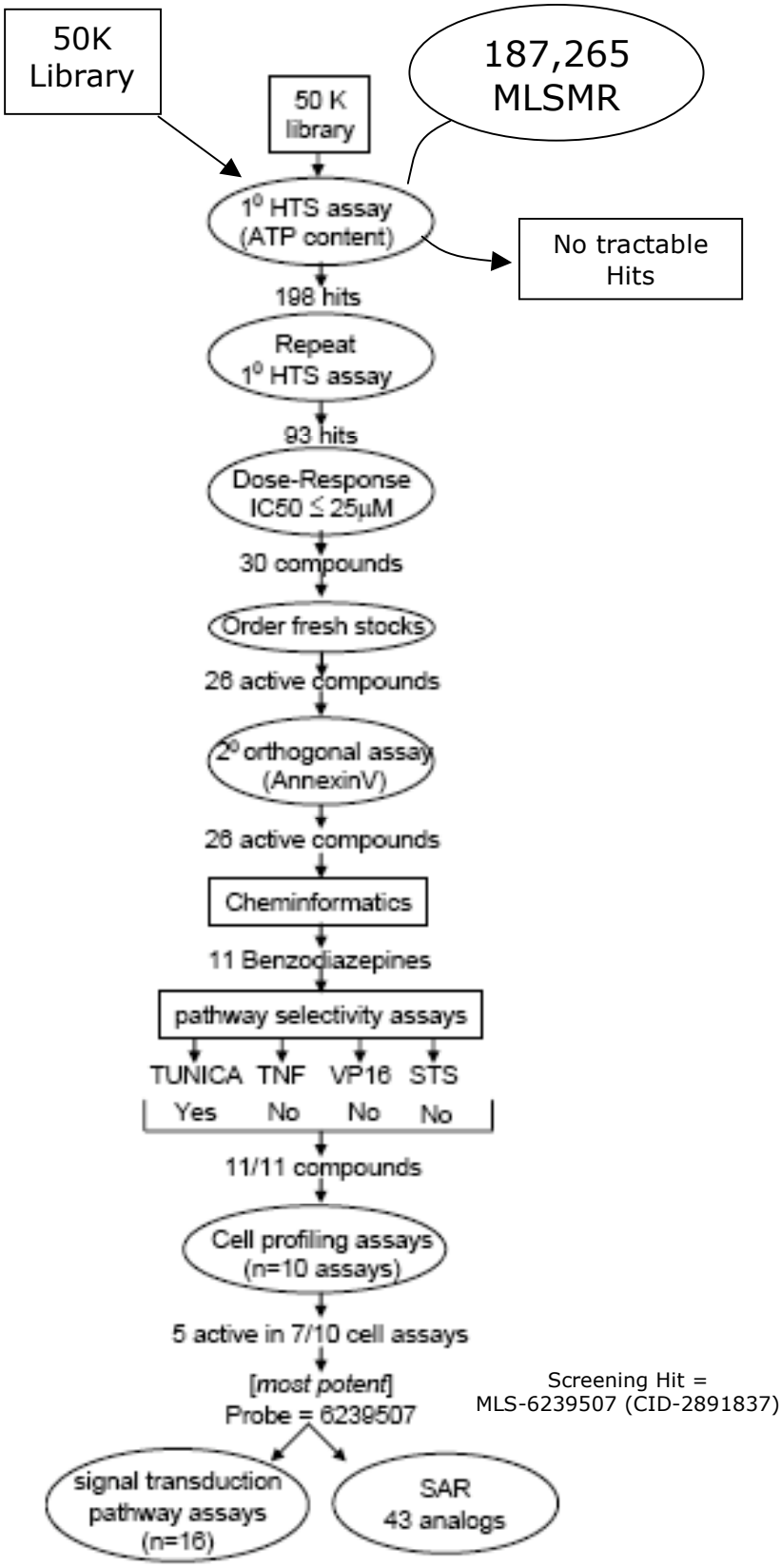
Activity Scoring

The maximum and minimum ATP content values for the assay were defined by the wells in that received DMSO only (max, assigned 100%) versus DMSO + thapsigargin (min = 0%), and all data were related to these maximum and minimum values on a 0-100% scale. Any screening values less than the minimum were assigned a score of 0%. Any screening values greater than the maximum were assigned a score of 100%, and were considered to be hits. Values between the minimum (0%) and maximum (100%) were considered to be hits if >50%.

Center Summary of the Primary Screen and confirmatory dose-response studies.

A library of 50,000 compounds (ChemBridge DiverSet) was screened. The primary screen resulted in 198 hits that maintained ATP content 50% of control levels (DMSO only) or greater, using undifferentiated CSM14.1 cells treated with thapsigargin, measuring ATP content. Repeat testing with the same primary assay resulted in 93 confirmed hits. Dose-response studies resulted in 30 compounds that displayed appropriate dose-dependent cytoprotection with $IC_{50} \leq$ 25 uM. Fresh stocks of the 30 compounds were purchased from the commercial source (ChemBridge), and 26 were found to be active, with similar potency to the originally tested material, whereas 4 showed only weak activity. All 26 active compounds were confirmed to be cytoprotective for undifferentiated CSM14.1 cells treated with thapsigargin by an orthogonal assay, measuring cell viability based on Annexin-V staining by flow-cytometry. Cheminformatics analysis revealed that 11 of the 26 confirmed hits constituted a series of 11 structurally related benzodiazepines (IC_{50} values ranging from 10 to 25 uM), which were evaluated extensively, using 31 secondary assays (described below). These secondary assays used for evaluating the 11 benzodiazepines included 15 cytotoxicity assays used to define pathway selectivity and cellular spectrum of activity, and 16 measures of signal transduction events associated with UPR.

Additionally, the MLSMR library of 187,265 compounds were screened in the primary assay, however, no hits confirmed that provided tractable leads. The screening flow is summarized in the following flowchart.

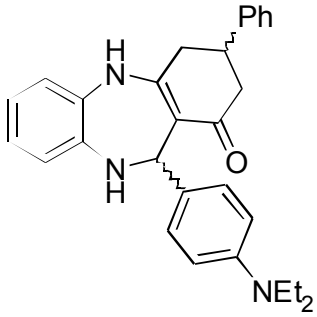


Flow Chart - From Screening to Hit Identification

Probe Chemical Structure

Chemical name of probe compound: 6-(4-diethylaminophenyl)-9-phenyl-5,6,8,9,10,11-hexahydrobenzo[c][1,5]benzodiazepin-7-one.

Draw probe chemical structure and show stereochemistry if known: (CID-2878746; see Figure).



CID-2878746

Description of Biological Activity of Probe Analog (CID-2891837)

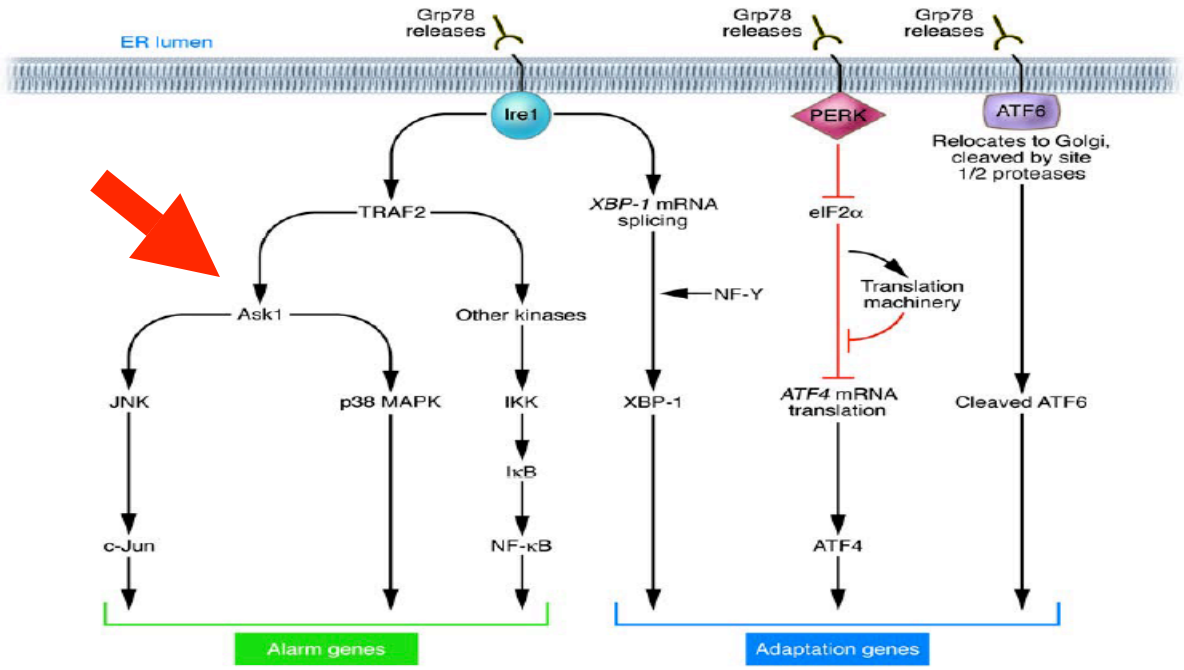
The most potent screening hit (CID-2891837) inhibits the thapsigargin (an inducer of ER stress)-induced death of both undifferentiated and differentiated rat neuronal cell line CSM14.1 with IC_{50} ~14 uM using two different indicators of cell viability—a) ATP content assay and b) Flow cytometry-based assay for Annexin V staining**.** This compound also inhibits cell death induced by Tunicamycin (another inducer of ER stress) in CSM14.1 cells, but does not inhibit CSM14.1 cell death induced by TNF-a (plus cycloheximide), an agonist of the death receptor (extrinsic) cell death pathway or by either VP-16 or staurosporine (agonists of the mitochondrial cell death pathway), suggesting it is a selective inhibitor of ER stress-induced cell death (i.e., pathway specific).

In addition to CSM14.1 cells, when tested at 25 uM, the Probe compound protected by >50% against thapsigargin-induced death of several tumor cell lines (HeLa human cervical cancer, SW1 melanoma cell, PPC1, human prostate cancer), mouse neural stem cell C17.2 (both differentiated [neuronal phenotype] and non-differentiated [stem cell phenotype]) as determined by ATP content assay, and primary rat cortical neurons as determined by microscopy assay measuring the percentage of NeuN-immunopositive cells with either normal or apoptotic nuclear morphology (Hoechst dye staining). However, this compound does not protect Jurkat human T-leukemia or either undifferentiated or differentiated (neuronal phenotype) PC12 rat pheochromocytoma cells from thapsigargin-induced cell death, as determined by an ATP content assay at 25 uM. In fact, CID-2891837 showed paradoxical cell death-promoting activity when tested on undifferentiated PC12 cells treated with thapsigargin. Overall, while showing some cell type-selectivity, the Probe compound is reasonably broad-spectrum in its cytoprotective activity, protecting 6 of 8 cell lines or cell types (primary neurons) tested.

Testing in thapsigargin-stimulated CSM 14.1 cells showed that this compound at 10 uM inhibits the UPR signaling pathway that results in phosphorylation of the JNK substrate c-Jun (measured by immunoblotting

with phospho-specific antibody) and phosphorylation of p38MAPK, as determined by immunoblotting using phospho-specific antibodies and by a quantitative ELISA-based assay (MSD assay), but not the UPR-pathways involving PERK-mediated phosphorylation of eIF2alpha (measured by immunoblotting with phosphospecific antibody), thapsigargin-induced expression of ATF4 (measured by immunoblotting), ATF6 proteolytic processing (assayed by immunoblotting to detect cleaved form), XBP1 mRNA splicing (assayed by RT-PCR to measure ratio of unspliced: spliced mRNA), Ire1 auto-phosphorylation (measured by *in vitro* kinase assay), ASK1 autophosphorylation (measured by *in vitro* kinase assay), thapsigargin-induced activation of ASK1 in cells (measured *in vitro* using a coupled kinase assay containing MKK6 and p38MAPK, to which ASK1 recovered from compound-treated cells by immunoprecipitation was added), or thapsigarin-induced expression of CHOP.

CID-2891837 inhibited thapsigargin-stimulated dephosphorylation of ASK1 at serine 967 at 50 uM, measured in ASK1-transfected/thapsigargin-stimulated HEK293T cells by immunoblotting using phospho-specific antibodies, and it also increased 14-3-3 binding to ASK1, as determined by co-immunoprecipitation assay using the same transfected HEK293T cells stimulated with thapsigargin. Thus, while not directly inhibiting ASK1 kinase activity, these events are predicted to reduce ASK1 *in vivo* kinase activity, and raise the possibility that the Probe compound inhibits a protein phosphatase that regulates phosphorylation of Ser967.



Compound distribution to MLSMR: The Probe has been submitted to the MLSMR.
Commercial vendor information if available for probe or analogs that have been purchased:
CID-2878746 and CID-2891837 are available from ChemBridge as vendor catalogue numbers 5962123 and 6239507, respectively. Recommended negative control compounds include ChemBridge 6075841 or 6048163. These benzodiazepines are inactive in the cell death assay used for primary screening and fail to suppress thapsigargin-induced phosphorylation of Jun.

Description of secondary screens used to optimize Probe structure
The secondary screens used to characterize the Probe compound (many of which were used to characterize all 11 benzodiazepines) are outlined above. Briefly, in total, 31 secondary screens have been used to date to characterize CID-2891837, in addition to the Probe compound, CID-2878746 This most potent screening compound (CID-2891837) is active with IC_{50} ~10 uM as an inhibitor of thapsigargin-induced cell death of undifferentiated CSM 14.1 cells as measured by ATP content (assay #1) and as an inhibitor of tunicamycin-induced cell death of undifferentiated CSM14.1 cells as measured by the ATP content assay (assay #2). The compound's activity against ER stress-induced cell death was confirmed by flow cytometric analysis, measuring annexin V staining of CSM14.1 cells treated with either thapsigargin (assay #3) or tunicamycin (assay #4). When tested at 25 uM against undifferentiated CSM14.1 cells by the ATP content assay, the Probe compound at 25 uM was not active against cell death induced by TNF-alpha plus cycloheximide (assay #5), VP-16 (assay #6), and staurosporine (assay#7). The compound's activity in neuronal cells was confirmed at 25 uM using differentiated rat neuronal CSM 14.1 cells treated with 10 uM thapsigargin using the ATP content assay (assay #8), differentiated mouse neuronal C17.2 cells treated with thapsigargin using the ATP content assay (assay #9), but not in differentiated rat pheochromocytoma PC12 cells treated with thapsigargin using the ATP content assay (assay #10). The compound showed paradoxical cell death-promoting activity against thapsigargin-treated undifferentiated PC12 cells (assay #11). Finally, CID-2891837 inhibits thapsigargin-induced cell death of rat primary cortical neurons (identified by staining with NeuN), as determined by counting apoptotic neurofilament (NeuN)–positive cells stained with the DNA-binding fluorochrome Hoechst dye to identify cells with condensed nuclear morphology indicative of apoptosis and evidence of neurite retraction (assay #12).

Cytoprotective activity of the compound was also demonstrated in several types of non-neuronal human tumor cell lines treated with thapsigargin using the ATP content assay, including cervical carcinoma HeLa

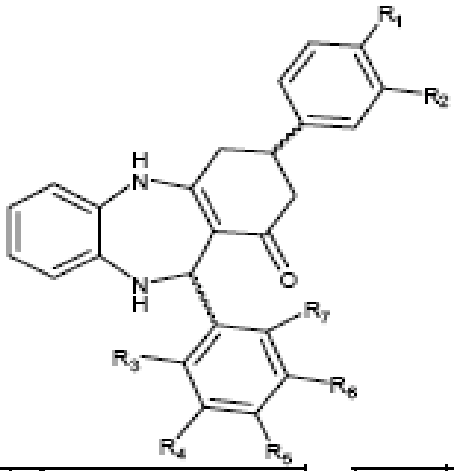
(assay #13), human prostate cancer PPC-1 (assay #14), and human melanoma SW1 (assay #15) cells. The compound, however, was inactive against thapsigargin-treated Jurkat T-leukemia cells (assay #16), as determined by the ATP content assay.

In terms of mechanism, the compound inhibits thapsigarin-induced phosphorylation of c-Jun and p38MAPK in CSM 14.1 cells, as determined by immunoblotting using phospho-specific antibodies (phospho-c-Jun Ser 63, and phosphor p38MAPK Thr180/Tyr182) (assays #17, #18). Suppression of thapsigargin-induced phosphorylation of p38MAPK was also measured by a quantitative ELISA-methods, with IC_{50} for p38MAPK phosphorylation estimated at <5 uM (assay #19). In contrast, thapsigargin-induced expression of CHOP (assay #20), expression of ATF4 (assay #21), proteolytic processing of ATF6 (assay #22), phosphorylation of eIF2a (Ser 51) (assay #23), auto-phosphorylation of Ire1a (assay #24) or auto-phosphorylation of ASK1 (assay #25) were not inhibited directly by the probe compound at concentrations up to 50 uM tested by *in vitro* kinase assay using p32-γ-ATP substrate. This compound also failed to inhibit cellular activation of ASK1, as determined by a coupled *in vitro* kinase assay containing purified MAPKK6 (MKK6/SKK3) and purified p38 MAPK, together with immunoprecipitated ASK1 derived from HEK293T cells that had been transfected with ASK1 plasmid and incubated with 100 uM compound plus 15 ug/mL Thapsigargin, prior to immunoprecipitating ASK1 and adding it to the couple assay (assay #26). Thapsigargin-induced reductions in phosphorylation of ASK1 at the serine 967 site in ASK1 transfected 293T cells (assay #27) are inhibited by this compound at concentrations of 50-100 uM, as determined by immunoblotting using anti-phospho-specific (ser 967) antibody, but thapsigargin-induced changes in phosphorylation of ASK1 at ser 83 and thr 845 are not modulated by this compound at concentrations as high as 100 uM (assays #28, 29) in ASK1-transfected HEK293T cells. This compound, at concentrations of 100 uM, also increases binding of ASK1 to 14-3-3 protein, as determined in a co-immunoprecipitation assay, using thapsigargin-stimulated, ASK1 transfected, HEK293T cells (assay #30). This compound, at a concentration of 100 uM, did not affect activity of protein phosphatase 2B (Calcineurin) tested by an *in vitro* phosphatase assay using immunoprecipitated ASK1 (ser 967 site) as the substrate (assay #31).

Chemistry strategy leading hit-to-probe identification

SAR analysis of the Probe compound (CID-2891837) was performed, addressing 3 functionalities by analyzing data on 41 purchased analogs, in addition to the SAR inherent in the primary screening data that demonstrated 11 active benzodiazepine hits. The assay used to compare the activity of compounds was the same as the primary HTS assay, in which undifferentiated CSM14.1 cells were challenged with thapsigargin and the cell viability was assessed using an ATP content assay. From the potency

data on the analog-by-catalog procedure shown in Table 1, the Probe compound, CID-2878746 (MLS-5962123) was selected based on potency and cellular activity profile. The 10 of 11 initial benzodiazapines identified from primary screening (Flowchart) are highlighted in Table 1 (1st 10 compounds)



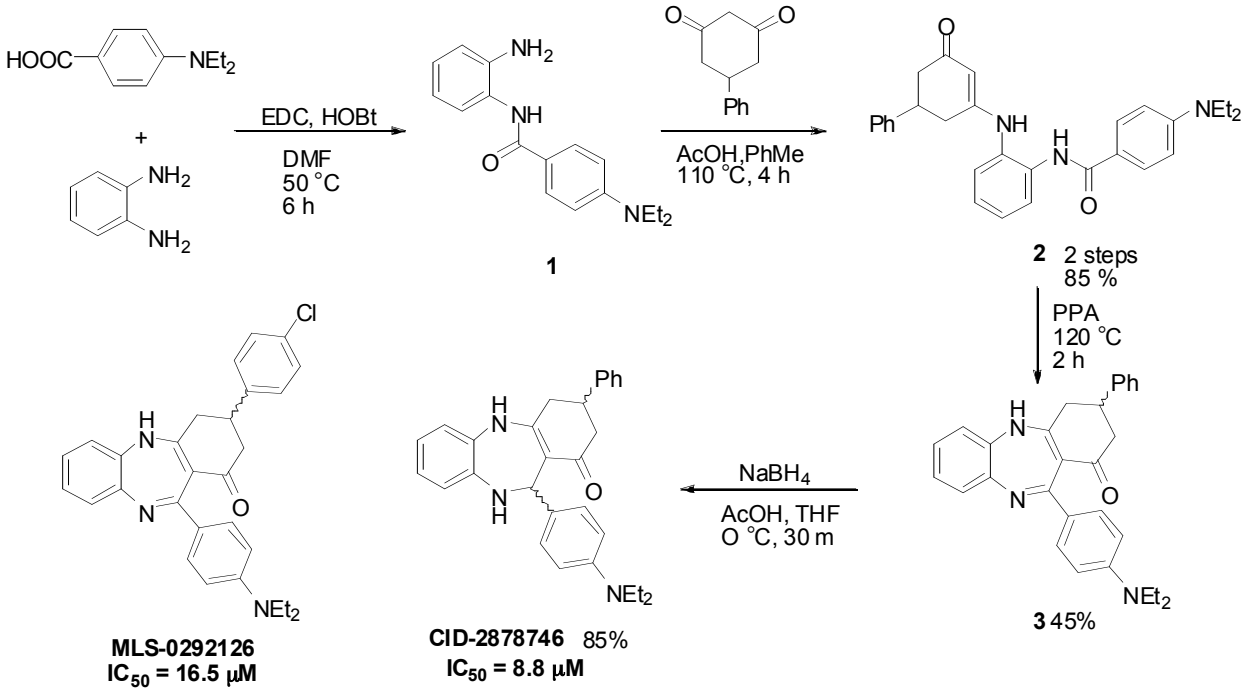
Cmpnd.	R1	R2	R3	R4	R5	R6	R7	IC_{50} (μM)
6239507	OMe	OMe				OPh		13.89 ±0.2596 : 10.17 ± 1.621
6237735	OMe	OMe	CF_3					17.16 ± 0.4705 : 12.5 ± 0.1859
6239475	OMe	OMe				CF3		18.81 ± 0.1966 : 12.5 ± 0.1695
6237877	OMe	OMe			O-n-Pr			16.26 ± 0.4383 : 12.5 ± 0.1695
6239538	OMe	OMe				Br		15.48 ± 0.2427 : 12.5 ± 0.1766
6239767	OMe		Me			Me		13.31 ± 1.1719 : 12.5 ± 0.1769
6049448	OMe			Cl	OH	OEt		24.83 ± 2.031 : 23.73 ± 0.642
5963958					SMe			18.55 ± 9.14 : 12.5 ± 0.1789
6237973			Cl				F	22.58 ± 0.6804 : 19.49 ± 0.1691
6044673	OMe					OEt		20.15 ± 0.7806 : 21.15 ± 0.3619
6047795	Cl				Me			10.95 ± 1.042
6049070	Cl				OMe			12.5 ± 0.1302
6047998	Cl		O-i-Pr					8.087 ± 0.8322
5962123					NEt_2			7.499 ± 0.9572
5968327	OMe					Cl		21.73 ± 1.070
6065973			Cl		Cl			> 200
6067345	OMe				NAc			> 100
6079090	OMe				F	F		21.67 ± 0.2472
6076307	OMe	OMe	Cl					41.7 ± 0.9159
5969590	OMe				NEt_2			11.43 ± 1.496

Cmpnd.	R1	R2	R3	R4	R5	R6	R7	IC_{50} (μM)
6049661	OMe				i-Pr			10.3 ± 1.284
6366991			1-Naphthyl					22.18 ± 2.797
6043558	OMe		O-i-Pr					24.02 ± 2.311
6048163	OMe					OH		> 200
6045744	OMe				OH			> 200
6240043	OMe	OMe	Nitro					39.61 ± 1.99
6069474					OMe			25 ± 0.1221
6045627	OMe		1-Naphthyl					13.36 ± 1.884
6066511			O-n-Bu					19.23 ± 2.16
6048306	OMe			Br	OH	OEt		18.22 ± 1.925
6044487	OMe		Cl				F	15.27 ± 1.505
6241186	OMe		F				F	25 ± 0.1749
6079349	OMe		Cl				Cl	31.48 ± 2.843
6239845	OMe	OMe			Ph			21.77 ± 2.974
6105705	OMe	OMe			Me			25 ± 0.1691
6075841	OMe	OMe	4-pyridyl replaces Ph					> 200
6369841	OMe	OMe	Me					36.45 ± 2.193
K813-0010	OMe	OMe				CH_3		24.91 ± 0.8326
5991-0620	OEt	OMe	Cl			F		> 200
3192-5300	OMe	OMe				OH		53.46 ± 3.454

Table 1. Potency data for analogs in the benzodiazepine series of ER Stress-active compounds

Reduced to practice detailed synthetic pathway for making probe:

Probe Compound CID-2878746 was resynthesized (Scheme 1), the analytical data and biological activity where identical to the purchased compound thus confirming its structure and potency. It is important to note that analytical data indicates the presence of all 4 possible stereoisomers in both the commercial and synthetic samples. In addition an analog of compound **3** (see below at Scheme 1), MLS-0292126 was synthesized via the same route and showed an IC_{50} of 16.5 μM.



Scheme 1. Route for resynthesis of CID-2878746 and synthesis of MLS-0292126

Known probe properties

A summary of the properties of CID-2891837 is provided in the table below**.**

Table 1. Cytoprotective activity against various cell death stimuli assessed using CSM 14.1 cells

Stimuli	Thapsigargin	Tunicamycin	VP- 16	TNFa + CHX.	Staurosporine
Readout	ATP & Annexin	Annexin	Annexin	ATP & Annexin	ATP & Annexin
Cell type	CSM 14.1	CSM 14.1	CSM 14.1	CSM 14.1	CSM 14.1
Result	+	+	-	-	-

(+ indicates > 50% rescue of cell viability relative to untreated cells not exposed to thasigargin at compound concentration of ≤ 25 uM)

(ATP = ATPlite assay measuring ATP content of cells)

(Annexin V staining = measuring % FITC-Annexin V-positive cells by flow-cytometry)

Table 2. Cytoprotective activity against other cell lines

Stimulus	thapsigargin								
Readout	ATP content								
Cell type	CSM14.1 diff	HeLa	PPC1	SW1	Jurkat	PC12	PC12 diff	C17.2	C17.2 diff
Result	+	+	+	+	-	E	-	+	+

(+ indicates ≥ 50% rescue of cell viability relative to untreated cells not exposed to thasigargin at compound concentration of ≤ 25 uM; thapsigargin concentration was 10 uM for C17.2 cells, 15 uM for all others)

E indicates compound enhanced thapsigargin-induced death

(diff.=differentiated; For PC12, cells were stimulated with 20 ng/ml NGF for 5 days; For C17.2, cells were stimulated with 'serum reduction, and 1% N2 incubation' for 3 days; For CSM14.1, cells were cultured at 39°C for 5-7 days.

Probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool

The solubility of the probe compound CID-2878746 in DMSO is excellent at concentrations of 25 mM. For adding compound to culture media, at least 0.2% (v/v/) final DMSO concentration was needed to avoid production of a visible cloudy precipitate. At concentrations exceeding 25 mM, the compound in DMSO shows yellow color.

Negative control compounds 6048163, and 6075841 show similar solubility as 6239507 in DMSO. At concentration of 25 mM, 6048163 showed light yellow color, while 6075841 has no color.

ER Stress Bibliography

Ma, Y. and L.M. Hendershot, ER chaperone functions during normal and stress conditions. J Chem Neuroanat, 2004. 28(1-2): p. 51-65.
Rao, R.V., H.M. Ellerby, and D.E. Bredesen, Coupling endoplasmic reticulum stress to the cell death program. Cell Death Differ, 2004. 11(4): p. 372-80.
He, B., Viruses, endoplasmic reticulum stress, and interferon responses. Cell death Differ, 2006.13(3): p.393-403.
Frand, A.R., J.W. Cuozzo, and C.A. Kaiser, Pathways for protein disulphide bond formation. Trends Cell Biol, 2000. 10(5): p. 203-10.
Paschen, W., Endoplasmic reticulum: a primary target in various acute disorders and degenerative diseases of the brain. Cell Calcium, 2003. 34(4-5): p. 365-83.
Yoshida, H., ER stress and diseases. Febs J, 2007. 274(3): p. 630-58.
Lindholm, D., H. Wootz, and L. Korhonen, ER stress and neurodegenerative diseases. Cell Death Differ, 2006. 13(3): p. 385-92.
Xu, C., B. Bailly-Maitre, and J.C. Reed, Endoplasmic reticulum stress: cell life and death decisions. J Clin Invest, 2005. 115(10): p. 2656-64.
Zhong, L. T., Sarafian, T., Kane, D. J., Charles, A. C., Mah, S. P., Edwards, R. H., and Bredesen, D. E. Bcl-2 inhibits death of central neural cells induced by multiple agents. Proc Natl Acad Sci USA, 1993. 90: p 4533-4537,.
Haas, S. J. and Wree, A. Dopaminergic differentiation of the Nurr1-expressing immortalized mesencephalic cell line CSM14.1 *in vitro*. J Anat, 2002. 201: p 61-69.