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Pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives as novel small molecule chaperone amplifiers

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ABSTRACT

Pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives were investigated as novel small molecule amplifiers of heat shock factor 1 transcriptional activity. Lead optimization led to the discovery of compound 4A-13, which displayed potent HSF1 activity under mild heat stress (EC_{50} = 2.5 μM) and significant cytoprotection in both rotenone (EC_{50} = 0.23 μM) and oxygen-glucose deprivation cell toxicity models (80% protection at 2.5 μM).

Protein misfolding has recently been implicated as one of the fundamental mechanisms that link the pathogenesis of disparate illnesses such as neurodegenerative diseases and prion-related infectious diseases. Indeed, there is increasing experimental evidence that links protein misfolding to sporadic and familial amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease (PD), Huntington's disease and related polyglutamine expansion diseases as well as Transmissible Spongiform Encephalopathy diseases.[1–5] Molecular chaperones, many of which are also commonly known as heat shock proteins (HSPs), play a major role in protein folding and maintenance of proteome homeostasis against stress. This cytoprotective mechanism involves stress-induced expression of heat shock proteins such as HSP70, HSP90, HSP27 among others.

We are interested in identifying compounds similar to the current clinical candidate arimoclomol,[9] that do not induce chaperone proteins de novo in non-stressed cells, but amplify the endogenous induced chaperone expression in stressed or diseased cells. We hypothesized that such a unique molecular mechanism with high selectivity for diseased (stressed) cells may offer therapeutic advantage with reduced side effects compared to general HSP inducers that indiscriminately activate chaperone expression even in non-stressed cells.

The pivotal transcription factor that regulates the expression of these heat shock proteins is the heat shock transcription factor 1 (HSF1). It has been reported that the kinetics of HSF1 granule formation correlates well with its transcriptional activity and is generally believed to be a visual indicator of the active transcriptional complex.[6,7] Based on these observations, we have developed a quantitative high content image-based assay to measure the HSF1 granule formation.[8] We applied this assay as the primary screen to identify and rank novel small molecule chaperone amplifiers from chemical libraries. In this communication, we report the discovery, synthesis, and initial structure–activity relationship (SAR) of a series of pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives as novel small molecule chaperone amplifiers that are structurally distinct from arimoclomol. The biological profile of this series of compounds suggests its potential therapeutic application in a number of indications, including stroke and neurodegenerative diseases such as PD.

During our in-house library screening using high content image-based HSF1 granule assay, we discovered that 1-ethyl-6-methyl-3-(thiophen-2-yl)pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-

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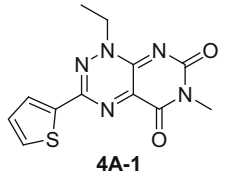


Figure 1. HSF1 amplifier.

dione (**4A-1** in Fig. 1) exhibited potent HSF1 amplifying activity (Table 1). Therefore we focused on the lead optimization of this chemical series aiming to discover more potent compounds that amplify the HSF1-dependent stress response for cytoprotection in neurodegenerative disease cell models for potential use as targeted chaperone therapies.

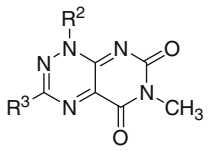
The synthesis of the pyrimidotriazinedione derivatives was fairly straightforward. It involved a 3-step synthesis. As shown in Scheme 1, first 6-chloro-pyrimidine-2,4(1*H*,3*H*)-dione (**1**), either commercially available or synthesized according to literature methods,[10] was converted to 6-hydrazinyl-pyrimidine-2,4(1*H*,3*H*)-dione (**2**) by the treatment of hydrazine under heating.[11] Compound **2** was then reacted with an aldehyde to form an imine intermediate **3** under heating which was further cyclized by the treatment of $NaNO_2$ in the presence of acetic acid to form a mixture of 3-(2-yl)pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione (**4A**) and 5,7-dioxo-3-(2-yl)-1,5,6,7-tetrahydropyrimido[5,4-*e*][1,2,4]triazine 4-oxide (**4B**).[12] Desired products of **4A** and **4B** were separated by HPLC purification. Alternatively compounds **4B** were reduced to form **4A** by the treatment of dithioerythritol (DTE) or $Na_2S_2O_4$.

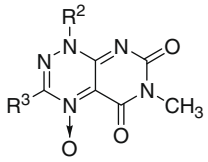
Our lead optimization initially focused on the modification of R^2 and R^3 substituents (where R^1 = Me) to study the structure and activity relationship (SAR). The high content image-based HSF1 granule assay was used to measure the compound-induced HSF1 amplification activity.[13] Rotenone is a broad-spectrum insecticide and pesticide that is known to cause PD-like symptoms in rats.[14] Therefore we developed a rotenone toxicity model in SK-N-SH neuroblastoma cells to measure the cytoprotection from rotenone induced cellular damage.[15] The results are summarized in Table 1.

As shown in Table 1, the N-oxide analogs of **4B** were generally slightly more potent than the corresponding **4A** analogs in the HSF1 granule assay (e.g., **4A-2** vs **4B-2**, **4A-3** vs **4B-3**, **4A-5** vs **4B-5**, **4A-7** vs **4B-7**) but with the drawback of increased cell toxicity. Therefore we subsequently focused our optimization on **4A** analogs.

The R^2 moiety had noticeable impact on HSF1 activity. Analogs with *n*-butyl R^2 group were generally more active than the analogs

Table 1
SAR in HSF1 granule and Rotenone assays





R^2	R^3	Product[a]	HSF1 EC_{50}[b] (μM)	Rotenone EC_{50}[c] (μM)	Increase in Rotenone viability[c] (%)	HeLa CC_{50}[d] (μM)	SK CC_{50}[d] (μM)
Et	2-Thiophene	**4A-1**	5.1	0.41	53	10.5	9.9
Et	5-Me-2-thiophene	**4A-2**	17.3	1.33	50	22.2	63.5
Et	5-Me-2-thiophene	**4B-2**	11.6	1.10	57	15	27.2
n-Bu	5-Me-2-thiophene	**4A-3**	16.8	44%@1.25	44	15.3	18.2
n-Bu	5-Me-2-thiophene	**4B-3**	8.6	0.19	27	6.6	7.2
n-Bu	5-Me-2-Furan	**4A-4**	9.9	0.33	23	7.8	5.3
n-Bu	5-Me-2-Furan	**4B-4**	10	49%@1.25	49	9	6.6
Et	5-Me-2-Furan	**4A-5**	46.3	1.84	50	22.3	8.2
Et	5-Me-2-Furan	**4B-5**	20.6	56%@1.25	56	9.8	3.3
Et	2-Furan	**4A-6**	10.1	64%@1.25	64	5.6	2.8
Et	2-Pyrrol	**4A-7**	35	0.68	44	10.7	4.3
Et	2-Pyrrol	**4B-7**	24.7	0.17	35	5.2	1.9
n-Bu	2-Pyrrol	**4B-8**	14.9	0.64	49	6.3	1.5
Et	2-Imidazole	**4A-8**	39.8	2.71	62	11.9	3.6
n-Bu	2-Imidazole	**4A-9**	7.7	0.30	56	3.6	1.3
n-Bu	2-Thiazole	**4A-10**	2.1	55%@0.63	55	2.5	1.4
n-Bu	2-Benzimidazole	**4A-11**	40.8	1.9	59	76%@80	13.1
n-Bu	2-Quinoline	**4A-12**	2.5	0.29	71	9.5	4.0
n-Bu	2-Benzothiazole	**4A-13**	2.5	0.23	53	8.7	8.4
isoamyl[e]	2-Benzothiazole	**4A-14**	2.5	0.24	44	16.9	10.7
i-Bu	2-Benzothiazole	**4A-15**	1.3	0.27	67	7.9	3.7
n-Pr	2-Benzothiazole	**4A-16**	25%@80	0.55	55	15.6	5.4
Bn	2-Benzothiazole	**4A-17**	4.1	2.34	27	7.3	5.6
p-F-Bn	2-Benzothiazole	**4A-18**	14.0	No activity	NA	23.4	21.3
n-Bu	Phenyl	**4A-19**	14.8	0.49	58	18.5	9.8
H	2-Thiophene	**4A-20**	No activity	No activity	NA	>80	>80
NA	NA	Celastrol	1.1[f]	24%@0.16	24	3.0	1.6

[a] The desired mass was found for each compound in LC–MS analysis and structure was confirmed by ^{1}H NMR. All the compounds of **4A** and **4B** had no activity in HSF1 granule assay without heat shock stress (no de novo activation activity, data not shown). The data are the average of multiple testing.

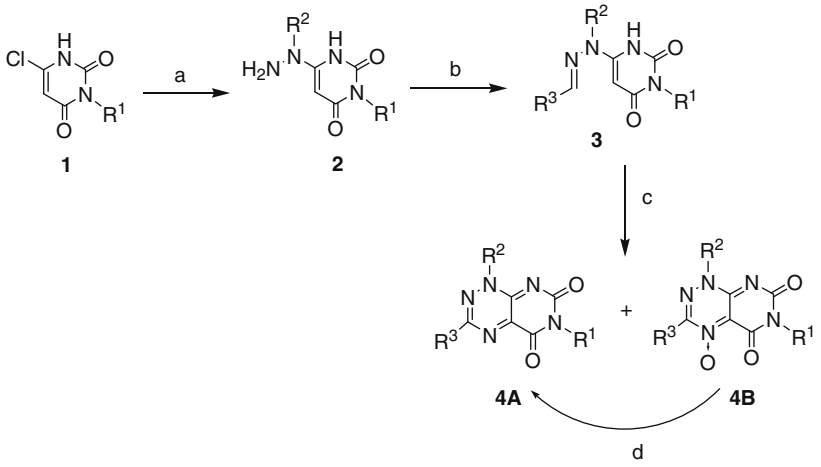
[b] See Ref. 13 for the HSF1 granule assay condition. EC_{50} is the concentration at which the percentage of cells that has positive HSF1 granules is half of the maximum.

[c] See Ref. 15 for rotenone stress assay condition and the calculation of the maximum cytoprotection. EC_{50} is the concentration at which cytoprotection from rotenone is half of maximal protection.

[d] Viability was determined by the MTS assay described in Ref. 16. CC_{50} is the concentration at which cytotoxicity is half of maximum.

[e] Isoamyl = 3,3-dimethylpropyl.

[f] Celastrol has EC_{50} of 2.6 μM in HSF1 granule assay without heat shock stress (de novo activation).



Scheme 1. Reagents and conditions: (a) $R^2NHNH_2{\cdot}2HCl$, $^iPrNEt_2$, EtOH, 100 °C, overnight; (b) R^3CHO, EtOH, 80 °C, 1 h–overnight; (c) aq $NaNO_2$, CH_3CO_2H, rt, overnight; (d) DTE, MeOH, rt, 1 h–3 days.

with ethyl R^2 group (e.g., **4A-4** vs **4A-5**, **4B-4** vs **4B-5**, **4B-7** vs **4B-8**, **4A-8** vs **4A-9**) without any effect on cell toxicity. *n*-Propyl R^2 groups led to less active compounds than *n*-butyl R^2 moiety (compare **4A-16** with **4A-13**). In addition, the R^2 group with same size in chain length as *n*-Bu normally led to compounds with similar HSF1 activity. For example, isoamyl R^2 groups resulted in similar HSF1 activity to that of *n*-butyl moiety (**4A-14** vs **4A-13**). The compound with *iso*-butyl R^2 group (**4A-15**) was equipotent as celastrol, a reference compound that entered into clinical trial for treating rheumatoid arthritis.[17] Interestingly, when R^2 = H, HSF1 activity diminished completely (**4A-20**) (as compared with **4A-1**) suggesting that the N-1 position needs to be substituted in order to gain HSF1 amplifying activity.

R^3 groups also had significant impact on HSF1 activity. The addition of a methyl group at the *ortho*-position of the thiophene ring resulted in significant activity loss (compare **4A-2** or **4A-3** with **4A-1**). A similar SAR trend was observed when 2-furan was the R^3 group (**4A-5** vs **4A-6**). Replacement of 2-thiophene with either 2-pyrrol or 2-imidazole ring led to much less potent compounds (compare **4A-7** or **4A-8** with **4A-1**). In addition, both 2-thiazole and 2-benzothiazole R^3 groups led to more potent compounds (**4A-10** and **4A-13**) with the later being less toxic in both HeLa and SK-N-SH cells. However, replacement of the 5-membered thiophene ring of **4A-1** with a 6-membered phenyl ring as R^3 (**4A-19**) resulted in a significant loss of HSF1 activity.

It is important to note that none of the compounds tested in this series of pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives showed activity in HSF1 granule assay in the absence of heat shock stress while celastrol displayed potent HSF1 activity (EC_{50} = 2.6 μM) without heat shock stress. Therefore, this class of novel small molecules, with a selective chaperone amplifying effect only to those cells under stress, may thus provide therapeutic advantages with potentially fewer side effects compared to chaperone inducers like celastrol.

Next, in order to further evaluate the potential applications of this series of pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives, the cytoprotective effects of these compounds in biologically relevant cell models were also investigated. The rotenone model of PD is a cell-based system for study of protein misfolding induced cytotoxicity.[18]

In the control experiment, when 200 nM of rotenone was applied with DMSO for 48 h, about 40% of SK-N-SH cells were killed. However, treatment with compounds **4A** or **4B** in the presence of rotenone stress resulted in significant cytoprotection (27–71% maximum percent cell viability increase compared with the DMSO control with rotenone treatment) with 76–103% cell survival compared with that of the DMSO control without rotenone treatment (e.g., **4A-12** has cell viability of 103%, Fig. 2). Both the EC_{50} value and the maximum percent cell viability increase (compared with the DMSO control with rotenone treatment, for example, **4A-12** has a maximum cytoprotection of 71%) are listed in Table 1.

The majority of the compounds tested were very potent in the rotenone stress assay with EC_{50} < 1 μM. However, addition of a methyl group at the *ortho*-position of a thiophene ring as R^3 group resulted in less potent compounds with EC_{50} > 1 μM (compared **4A-2** or **4A-3** with **4A-1**). The R^3 moieties such as 2-benzimidazole, 2-imidazole (R^2 = Et) and 5-methyl-2-furan (R^2 = Et) also led to less potent compounds with EC_{50} > 1 μM (**4A-11**, **4A-8** and **4A-5**). Furthermore, benzyl and *para*-F-benzyl R^2 moieties led to either a less potent compound with EC_{50} > 1 μM (**4A-17**) or complete loss of cytoprotection from rotenone toxicity (**4A-18**). Without substitution (R^2 = H), the cytoprotection diminished completely suggesting that the R^2 group is critical to the cytoprotection effect which was consistent with the diminished HSF1 amplifying activity of this compound (**4A-20**). Overall both R^2 and R^3 groups have effects on the level of cytoprotection from rotenone-induced toxicity.

In general, the therapeutic window (CC_{50}/EC_{50}) of the compounds in SK cells in this series is significant, up to 48-fold (**4A-2**) suggesting significant therapeutic potential of this series of compounds for PD. The HSF1 EC_{50}/CC_{50} ratio in HeLa cells is generally smaller than the corresponding ratio in the rotenone protection assay in SK cells (sevenfold or less), mostly because the EC_{50} for cytoprotection is substantially lower than the EC_{50} for HSF1 activation. This suggests that relatively small increases in HSF1 activation are sufficient to protect against rotenone cytotoxicity. Since the

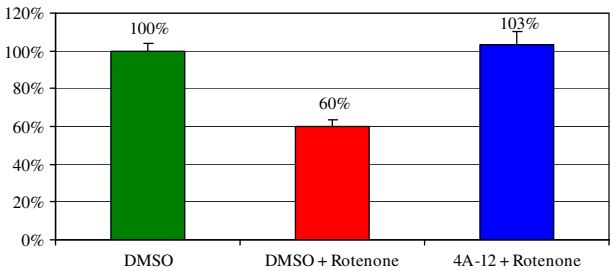


Figure 2. Percent cell survival with rotenone treatment in the presence or absence of the compound (0.625 μM) as compared with the DMSO control with or without rotenone treatment.

Table 2
Cytoprotective effect in OGD cell model in SH-SY-5Y cells

Compound	Cytoprotection (%)		
	0.5 μM	1.0 μM	2.5 μM
Celastrol	8	18	−22
4A-1	18	48	14
4A-10	7	23	39
4A-13	14	38	80

rotenone assay is a functional assay and a disease relevant cell model system, we consider the therapeutic window related to cytoprotection in SK cells to be more important and relevant than the corresponding pharmacodynamic effects in the HeLa cells used for screening.

In addition, the correlation between HSF1 activity and rotenone cytoprotection is not linear. This lack of direct correlation may suggest that HSF1 is not the direct molecular target for this series of compounds but is likely downstream from the direct target in this pathway. We believe that the initial compound modulates at least two separate mechanistic pathways, one that amplifies the HSF1 response and one that is toxic. When structural changes are made in the molecule, some presumably allow for improved binding to one of the targets versus the other.

Oxygen and glucose deprivation (OGD) is an in vitro cell-based model system of ischemia and stroke. Cytotoxicity induced by OGD is at least partly due to protein misfolding and aggregation.[19] A few selected compounds along with the reference compound celastrol were tested in an OGD cell model in SH-SY-5Y cells and the results are summarized in Table 2.[20] Compounds **4A-1**, **4A-10** and **4A-13** started to show some cytoprotective effect at concentration as low as 0.5 μM with the maximum cytoprotection at 1 or 2.5 μM. Among them compound **4A-13** displayed the maximum cytoprotection of 80% at 2.5 μM compared with the OGD DMSO control and it provided much higher cytoprotection than celastrol at 1 μM (38% vs 18%) and at 2.5 μM (80% vs −22%).

In summary, we synthesized a number of pyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione derivatives as novel small molecule chaperone amplifiers. Our SAR studies revealed that both R^2 and R^3 substituents had effects on HSF1 amplifying activity. Most compounds under study showed potent cytoprotection in the rotenone stress cell model. One of the compounds (**4A-13**) exhibited potent HSF1 amplifying activity and excellent cytoprotection effect in both rotenone and OGD cell models. As the rotenone and OGD cell models are directly relevant to PD and stroke respectively, these compounds could potentially be used in treating these indications.

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11. *General procedure for synthesis of* **2** *where* R^1 *= Me*: diisopropylethylamine (5.2 mL, 30 mmol) and dry ethanol (30 mL) were mixed with 6-chloro-3-methyluracil (10 mmol) and a hydrazine salt (15 mmol) in a sealed tube. The mixture was stirred at 100 °C overnight. After cooling to the room temperature, the product was extracted with dichloromethane (3 × 50 mL). The organic phase was dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by automated flash chromatography (ISCO, CombiFlash Companion purchased from Teledyne ISCO) on silica gel (0–10% of MeOH in DCM) to give the desired products (**2**). The yields ranged from 44% to 95%. The synthesis was referred to the procedure as described in WO 2004/007499 A1 and WO04/065387.
12. *General procedure for synthesis of* **4A** and **4B** *where* R^1 *= Me*: an aldehyde (0.75 mmol) was added to a solution of 6-(1-hydrazinyl)-3-methyluracil (0.75 mmol) in ethanol (6 mL) and the mixture was stirred at 80 °C for 1 h–overnight. The solid precipitate was collected by filtration, washed with hexanes, and dried in vacuo. The imine intermediate thus obtained was of sufficient purity to be carried to the next step without further purification. This imine intermediate was then treated with glacial acetic acid (2 mL). An aqueous solution of sodium nitrite (37 mg in 93 μL of H_2O) was added and the resulting mixture was stirred at room temperature overnight, neutralized by aqueous Na_2CO_3 and extracted by 5% MeOH in DCM. The residue was purified by preparative thin layer chromatography on silica gel (40% toluene in ethyl acetate) or by reverse-phase HPLC (Gilson 215 liquid handler) (5–95% ACN in water with 0.05% TFA) to afford pure desired products **4A** and **4B**. Alternatively, the mixture after work-up was first dissolved in methanol (10 mL), then dithioerythritol (2.25 mmol) was added and the mixture was stirred at room temperature for 72 h. The crude mixture was purified by reversed-phase HPLC (5–95% ACN in water with 0.05% TFA) to afford the desired product **4A** as a solid after evaporation. The isolated yields ranged from 6–75%. The desired mass was found by LC–MS analysis for each product listed in Table 1. Example (**4A-1**): 1-ethyl-6-methyl-3-thiophen-2-yl-1*H*-pyrimido[5,4-*e*][1,2,4]triazine-5,7-dione: yield: 75%; LC–MS (ES^+, *m/z*) = 290 $[M+1]^+$; ^1H NMR (DMSO-d_6, 400 MHz): δ 1.43 ppm (t, 3H, *J* = 5.72 Hz), 3.28 (s, 3H), 4.43 (q, 2H, *J* = 5.79), 7.26 (t, 1H, *J* = 3.06 Hz), 7.87 (d, 1H, *J* = 4.25 Hz), 7.88 (d, 1H, *J* = 2.47 Hz). The synthesis was referred to the procedure described in Lacrampe, J. F. A., et al. WO 2004/007498 A2, **2004**.
13. *HSF1 granule assay*: The assay was performed according to the previous publications (Ref. 8 and Zhou, Y. et al. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3128). Briefly, HeLa cells were pretreated with compounds 1 h before mild heat shock at 41 °C for 2 h with no recovery time. As a control, HeLa cells were pretreated with compounds at 37 °C for 3 h in order to eliminate compounds that induce heat shock response in non-stressed cells. The overall dilution of compound was 200-fold, with a concentration ranging from 0.3 μM to 80 μM. Immunocytochemical staining for HSF1 in HeLa cells was performed as described in the Ref. 8 with some modifications. Image acquisition was performed using an INcell 1000 analyzer (GE Healthcare, Piscataway, NJ) with a 10× object. Image analysis was carried out using Multi Target Analysis module from Workstation 3.6. Algorithms for the HSF1 total granule counts were established according to assay conditions and manufacture instructions. EC_{50} values and curve fitting were calculated using Prism 4.0 (GraphPad Software, San Diego, CA) with nonlinear regression analysis. A maximum percentage <20% HSF1 granule positive cells (compared to 7% in the DMSO negative control) was considered inactive.
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15. *Rotenone stress assay condition*: SK-N-SH neuroblastoma cells (within passage 6, purchased from ATCC, Manassas, VA) were seeded in a 96 well plate with 8000 cells/well in DMEM containing 0.1% FBS for 18 h before Rotenone treatment. Using Sciclone liquid handling workstation (Caliper Life Science, Hopkinton, MA), fresh media containing rotenone with or without testing compounds were added to the assay plates with a final concentration of rotenone at 200 nM and compound concentration ranging from 0.019 μM to 10 μM. Cells were incubated for 48 h followed by MTS viability assay. Percent cytoprotection was determined using the following formula: $(MTS_{compound} - MTS_{rotenone\ \ alone})/MTS_{rotenone\ \ alone} \times 100\%$. EC_{50} values and curve fitting were calculated using Prism 4.0 (GraphPad Software, San Diego, CA) with nonlinear regression analysis. Maximum percent cytoprotection <20% was considered inactive.
16. *MTS viability assay*: HeLa (8000 cells/well) and SK-N-SH (14,000 cells/well) cells were seeded in DMEM containing 10% FBS in 96-well plates (Catalog # Costar 3598, Cornings, MA) for 18 h before experiment. Compounds or DMSO control were added to the culture at 1–200 dilutions with a final concentration ranging from 0.3 μM and 80 μM (final DMSO concentration is 0.5% v/v). Cells were incubated with compounds for 72 h followed by MTS/PMS addition into the plates and incubated for four additional hours. SDS was added to a final concentration of 1.4% (w/v%). Plates were then measured for absorbance at 492 nm using envision excite (Perkin Elmer, Wellesley, MA). The absorbance at 492 nm is directly proportional to the living cells in the culture. Percent inhibition was determined using the following formula: $(MTS_{DMSO} - MTS_{compound})/MTS_{DMSO} \times 100\%$. IC_{50} values and curve fitting were calculated using Prism 4.0 (GraphPad Software, San Diego, CA) with nonlinear regression analysis.
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20. *Oxygen glucose deprivation (OGD) assay*: The assay was performed according to a previous publication (Ref. 8). Briefly, SHSY5Y cells (purchased from ATCC,

Manassas, VA) were plated at a density of 25,000 cells/well in 96-well plates pre-coated with collagen I (BD Biosciences, San Diego, CA) and grown for 16–24 h in complete medium. For the induction of oxygen glucose deprivation (OGD), cells were washed twice in pre-deoxygenated medium with no glucose or serum. Selected compounds were added to the cells after medium change within 1 h before OGD stress, and the plates were placed in modular incubator chambers (Billups-Rothenberg, Del Mar, CA). The chambers were flushed with a gas mixture of 95% N_2/5% CO_2 at a flow rate of 10 L/min for 30 min at room temperature. The residual O_2 concentration was monitored by a PO_2 meter which was connected to the deoxygen chamber with a final concentration less than 1%. After flushing, the chambers were sealed and maintained in a 37 °C incubator for 28 h. MTS viability assay was performed. Percent cytoprotection (the percent of the cell viability increase) compared with the OGD DMSO control was determined using the following formula: $(MTS_{compound} - MTS_{DMSO})/MTS_{DMSO} \times 100\%$.