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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and anti-endoplasmic reticulum stress activity of *N*-substituted-2-arylcarbonylhydrazinecarbothioamides

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Abstract

Misfolded or unfolded proteins are accumulated in lumen of endoplasmic reticulum (ER) in ER stress condition. It has been implicated in many pathological conditions such as Alzheimer's disease, diabetic retinopathy, atherosclerosis, β-cell apoptosis and lung inflammation. We found a series of *N*-substituted-2-arylcarbonylhydrazinecarbothioamides to potently decrease ER stress signal, showing up to almost 300-fold better activity than 1-hydroxynaphthoic acid and tauro-ursodesoxycholic acid, positive controls, respectively. Structure−activity relationship (SAR) study showed that 2-arylcarbonyl moiety is critical for the activity of the hydrazinecarbothioamide analogues and side chains tethering on thioamide moiety were relatively insensitive to the activity. Some analogues were found to consistently exert the potency under more physiologically relevant condition where ER stress was induced by palmitic acid. ER stress markers such as CHOP and phosphorylated eIF2α and PERK were accordingly decreased in western blotting upon treatment of compound **4h**. Potential ER stress inhibitory activity and novel structures could provide a novel platform for new chemical chaperone and therapy for protein misfolding diseases.

Keywords Endoplasmic reticulum stress · 2-Arylcarbonylhydrazinecarbothioamides · Chemical chaperone · Misfolded protein

Introduction

Folding of newly synthesized proteins primarily takes place in endoplasmic reticulum (ER). In a condition where unfolded or misfolded peptides are accumulated inside ER (ER stress), a series of cellular events, known as unfolded protein responses (UPRs) or ER stress responses, are triggered in order to adapt the cellular damages caused by ER stress condition (Walter and Ron 2011). They consist of the following canonical cellular processes: (i) translational attenuation, (ii) transcriptional activation of chaperones and degradation factors, (iii) ER-associated degradation (ERAD), and (iv) apoptosis. For example, expression of chaperone molecules, such as GRP78, is upregulated to assist the protein folding process and to reduce the load of the defect peptides.

ER stress can be monitored by three representative and evolutionarily conserved ER-resident sensor molecules: protein kinase RNA-activated (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1α (IRE1α), which relay complex and interrelated downstream signal cascade deciding

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'survival' (adaptive signalling) or 'suicide' (apoptotic signalling) (Xu et al. 2005). The activated PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α) which attenuates protein synthesis to reduce ER protein overloading (Harding et al. 1999) and also increases the expression of activating transcription factor 4 (ATF4) to augment the expression of many genes including apoptosis inducing factors i.e. C/EBP homology protein (CHOP) (Harding et al. 2003). ATF6 is also involved in activating the transcription of many UPR mediators including an ER chaperone protein, glucose-regulated protein (GRP78) (Haze et al. 1999). IRE1α, a serine/threonine kinase, processes X-box binding protein 1 (XBP1) mRNA through its intrinsic endoribonuclease activity to produce active spliced form which becomes a competent transcription factor for UPR-related genes.

ER stress condition has been implicated in various diseases and pathologies such as Alzheimer's disease, diabetic retinopathy, atherosclerosis, type 2 diabetes, β-cell apoptosis, and lung inflammation (Hetz et al. 2013). Recent studies suggested intriguing role of ER stress in the initiation and maintenance of diabetes to induce β-cell apoptosis (Papa 2012). Several chemical inducers of ER stress, such as tunicamycin, thapsigargin, and dithiothreotol, are known.

Reversely, chemical version of chaperone molecules, referred to as chemical chaperones (Fig. 1), are also identified and implicated for the potential treatment against the ER stress-related pathologies. For example, 4-phenyburytic acid (4-PBA) can exert therapeutic values against several ER stress-related conditions (Iannitti and Palmieri 2011). Salubrinal was also found to protect cells from ER stress by hampering eIF2α from dephosphorylation which is one of the hallmark events of UPR (Boyce et al. 2005). tUDCA (tauro-ursodeoxycholic acid) is considered as one of the best examples of chemical chaperones (Ozcan et al. 2006).

In search of novel chemical chaperones, we have developed a cell-based assay where *renilla* luciferase activity is driven by GRP78 promoter, which is one of the most important chaperone molecules and plays a crucial role in various UPR mechanisms such as PERK, ATF6, and IRE1 pathways. We have reported that hydroxynaphthoic acids (HNAs) have $EC_{50} \cong 0.2$ mM, about an order of magnitude lower EC_{50} than salicylate and tDUCA. They downregulate UPR markers such as GRP78, CHOP and phosphorylated PERK as a potent chemical chaperone (Jeong et al. 2013; Park et al. 2016). For the discovery of new chemical scaffolds with more drug-likeness than HNAs, we in this study identified *N*-substituted-2-arylcarbonylhydrazinecarbothioamides (**4**) as potent ER stress-reducing compounds. The new scaffold is much more potent than HNA in reducing ER stress signal and is much more diverse in heteroatom compositions by which drug-likeness might be improved. Also, it is possible that the open chain compound (**4**) can be cyclized to afford 1,3,4-thiadiazoles (**5**) and 1,2,4-triazoles (**6**). The cyclic structure can provide some rigidity necessary for better binding.

We report here the synthesis and structure−activity relationship of various *N*-substituted-2-arylcarbonylhydrazine-carbothioamides and their cyclic analogues based on tunicamycin- and palmitate (PA)-induced ER stress conditions. *N*-substituted-2-arylcarbonylhydrazinecarbothioamides (**4**), highly heteroatom-containing scaffold, were identified as strong hits with $EC_{50} < 10$ μM, which is about three orders of magnitude better EC_{50} than tUDCA and salicylate (Jeong et al. 2013), known chemical chaperones. It is noteworthy that EC_{50} of 1-hydroxy-2-naphthoic acid (1-HNA), our initial hit series, was 183 μM (Table 1) while best examples shown here have EC_{50} in μM range.

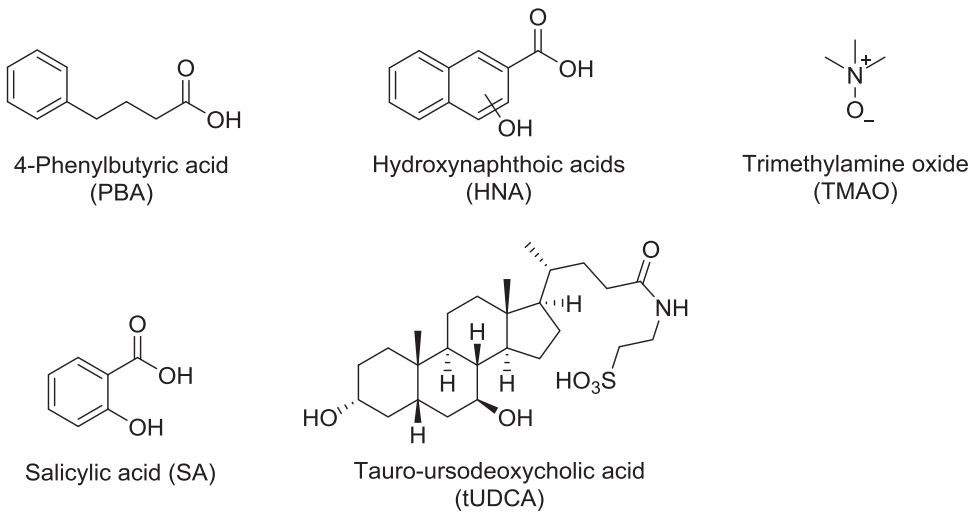


Fig. 1 Examples of chemical chaperones

Table 1 Activity of (**4**), (**5**) and (**6**) against tunicamycin-induced ER stress (data represent mean of three experiments in duplicate)

Compounds	EC_{50} (μM)	CC_{50} (μM)	SI[a]
4a	1.52 ± 0.09	>100	83.3
4b	2.77 ± 0.24	>100	53.0
4c	1.13 ± 0.12	>100	88.5
4d	4.53 ± 0.37	>100	24.0
4e	1.61 ± 0.24	>100	62.1
4f	6.96 ± 1.39	>100	14.4
4g	3.19 ± 0.25	>100	31.4
4h	0.62 ± 0.11	>100	160
4i	0.87 ± 0.11	—	—
4j	>100	>100	1.0
4k	94.9 ± 9.49	>100	1.05
4l	47.6 ± 9.50	>100	2.10
4m	31.0 ± 7.47	—	—
4n	80.0 ± 12.0	>100	1.25
4o	82.1 ± 9.03	>100	1.22
4p	>100	>100	1.0
4q	3.90 ± 0.78	—	—
4r	>100	>100	1.0
4s	3.38 ± 0.37	—	—
4t	16.9 ± 0.21	—	—
5b	19.1 ± 3.05	>100	5.23
5e	>100	>100	1.0
5h	>100	>100	1.0
5i	41.8 ± 6.27	>100	2.39
6b	>100	>100	1.0
6e	>100	>100	1.0
6h	>100	>100	1.0
6i	>100	>100	1.0
1-HNA	183		
tUDCA	5.2 mM		

[a]Safety index is expressed as a ratio of CC_{50}/EC_{50}

Materials and methods

Chemistry

Unless noted otherwise, materials were purchased from commercial suppliers and used without further purification. Air- or moisture-sensitive reactions were carried out under an inert gas atmosphere. Progress of reaction was monitored by thin layer chromatography (TLC) using silica gel F_{254} plates. Purification of the products was performed by flash column chromatography using silica gel 60 (70–230 mesh) or by Biotage 'Isolera One' system with indicated solvents. Melting points were determined using a Kruss melting pointer metre and were not corrected. NMR spectra were obtained using a Bruker spectrometer 400 and 700 MHz for ^{1}H-NMR, 100 and 175 MHz for ^{13}C-NMR, respectively. Chemical shifts (δ) were expressed in ppm using solvent as an internal standard and coupling constant (J) in hertz. Low-resolution mass spectra (LRMS) were obtained using an Advion Expression CMS, and recorded in a positive ion mode with an electrospray (ESI) source. High-resolution mass spectra (HRMS) were obtained using a Thermo Scientific LTQ Orbitrap XL mass spectrometer, and recorded in positive ion mode with an electrospray (ESI) source.

General procedure for the synthesis of 4a–4t

To a solution of hydrazide (**2**) (1.0 mmol) in EtOH was added isothiocyanate (1.1 mmol). The reaction mixture was refluxed overnight and cooled to room temperature. Half of the solvent was removed under reduced pressure and the residue was poured into ice. The precipitate was filtered and washed with ice-cold EtOH to give a product.

N-(3,4-Dichlorophenyl)-2-picolinoylhydrazinecarbothioamide (4a)

Yield: 91%; m.p.: 208–210 °C; ^{1}H-NMR (700 MHz, DMSO-d_6) δ 10.80 (s, 1H, C(=S)N<u>H</u>), 10.01 (s, 1H, N<u>H</u>(C=S)), 9.84 (s, 1H, (C=O)N<u>H</u>), 8.70 (d, $J = 4.1$ Hz, 1H, PyrC$_6$<u>H</u>), 8.08 (d, $J = 7.7$ Hz, 1H, PyrC$_3$<u>H</u>), 8.04 (t, $J = 7.1$ Hz, 1H, PyrC$_5$<u>H</u>), 7.85 (s, 1H, Ph), 7.66 (t, $J = 7.1$ Hz, 1H, PyrC$_4$<u>H</u>), 7.57–7.55 (m, 2H, Ph); ^{13}C-NMR (175 MHz, DMSO-d_6) δ 181.11 (C=S), 164.30 (C=O), 149.76 (C_{Pyr}), 148.99 (C_{Pyr}), 139.97 (C_{Pyr}), 138.19 (C_{Pyr}), 130.46 (C_{Pyr}), 130.21 (C_{Ph}), 127.49 (C_{Ph}), 127.01 (C_{Ph}), 125.78 (C_{Ph}), 125.66 (C_{Ph}), 123.00 (C_{Ph}); MS (ESI) *m/z*: 341.1 $[M+H]^+$; HRMS (ESI) *m/z* calcd for $C_{13}H_{10}Cl_2N_4OS$ $[M+H]^+$ 341.0025, found 341.0022.

2-Picolinoyl-*N*-(2-(trifluoromethyl)phenyl) hydrazinecarbothioamide (4b)

White solid. Yield: 92%; m.p.: 192.9–195.0 °C; ^{1}H-NMR (700 MHz, DMSO-d_6) δ 10.79 (s, 1H, C(=S)N<u>H</u>), 9.90 (s, 1H, N<u>H</u>(C=S)), 9.49 (s, 1H, (C=O)N<u>H</u>), 8.70 (d, $J = 4.3$ Hz, 1H, PyrC$_6$<u>H</u>), 8.06–8.00 (m, 3H, Pyr<u>H</u>), 7.68–7.65 (m, 3H), 7.47 (t, $J = 7.4$ Hz, 1H); ^{13}C-NMR (175 MHz, DMSO-d_6) δ 183.17 (C=S), 164.31 (C=O), 149.86 (C_{Pyr}), 149.01 (C_{Pyr}), 138.19 (C_{Pyr}), 132.89 (C_{Ph}), 127.56 (C_{Ph}), 127.47 (C_{Ph}), 126.54 (C_{Pyr}), 126.51 (CF_3), 124.74 (C_{Ph}), 123.18 (C_{Ph}), 122.95 (C_{Pyr}), 121.63 (C_{Ph}); MS (ESI) *m/z*: 341.1 $[M+H]^+$; HRMS (ESI) *m/z*: Calcd. for $[M+H]^+$ $C_{14}H_{11}F_3N_4O_S$: 341.0678; found: 341.0676.

N-Phenyl-2-picolinoylhydrazinecarbothioamide (4c)

White solid. Yield: 85%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.75 (s, 1H), 9.74 (br s, 2H), 8.69 (ddd, $J = 4.7$, 1.5,

1.0 Hz, 1H), 8.11–7.95 (m, 2H), 7.64 (ddd, $J = 7.3$, 4.8, 1.5 Hz, 1H), 7.47 (d, $J = 6.2$ Hz, 2H), 7.32 (t, $J = 7.8$ Hz, 2H), 7.17–7.10 (m, 1H); MS (ESI) *m/z* 273.1 $[M+H]^+$.

N-(4-Chlorobenzyl)-2-picolinoylhydrazinecarbothioamide (4d)

Yield: 93%; m.p.: 192.2–194.1 °C; ^{1}H-NMR (700 MHz, DMSO-d_6) δ 10.68 (s, 1H, HNC(=S)), 9.53 (s, 1H, C(=O)NH), 8.68 (d, $J = 5.2$ Hz, 1H, PyrC$_6$H)), 8.58 (s, 1H, C(=S)NH), 8.06 (d, $J = 7.8$ Hz, 1H, PyrC$_3$H), 8.02 (td, $J = 7.7$, 1.7 Hz, 1H, PyrC$_5$H)), 7.64 (ddd, $J = 7.5$, 4.7, 1.2 Hz, 1H, PyrC$_4$H)), 7.35 (q, $J = 8.6$ Hz, 4H, Ph), 4.70 (d, $J = 6.0$ Hz, 2H, CH$_2$); ^{13}C-NMR (175 MHz, DMSO-d_6) δ 182.48 (C=S), 164.14 (C=O), 149.86 (C_{Pyr}), 148.92 (C_{Pyr}), 138.97 (C_{Pyr}), 138.12 (C_{Ph}), 131.55 (C_{Ph}), 129.44 (C_{Ph}), 128.36 (C_{Ph}), 127.39 (C_{Pyr}), 123.00 (C_{Pyr}), 46.58 (CH_2); MS (ESI) *m/z*: 321.1 $[M+H]^+$; HRMS (ESI) *m/z*: Calcd. for $[M+H]^+$ $C_{14}H_{13}ClN_4OS$: 321.0571; found: 321.0575.

N-Butyl-2-picolinoylhydrazinecarbothioamide (4e)

White solid. Yield: 76%; m.p.: 141.5–143.2 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.51 (s, 1H), 9.28 (s, 1H), 8.69 (d, $J = 4.7$ Hz, 1H), 8.08–7.98 (m, 2H), 7.97 (t, $J = 5.5$ Hz, 1H), 7.64 (ddd, $J = 6.8$, 4.8, 2.2 Hz, 1H), 3.41 (dd, $J =$ 13.3, 6.7 Hz, 2H), 1.52–1.39 (m, 2H), 1.32–1.19 (m, 2H), 0.86 (t, $J = 7.3$ Hz, 3H); ^{13}C-NMR(100 MHz, DMSO-d_6) δ 181.2 (C=S), 163.4 (C=O), 149.4 (C_{Pyr}), 148.5 (C_{Pyr}), 137.7 (C_{Pyr}), 126.9 (C_{Pyr}), 122.5 (C_{Pyr}), 43.4 (C^1butyl), 30.9 (C^2butyl), 19.4 (C^3butyl), 13.8 (CH_2); MS (ESI) *m/z* 253 $[M+H]^+$.

N-Benzyl-2-picolinoylhydrazinecarbothioamide (4f)

White solid. Yield: 42 %; m.p.: 175–178 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.64 (s, 1H, HNC(=S)), 9.48 (s, 1H, C(=O)NH)), 8.66 (ddd, $J = 4.7$, 1.4, 0.9 Hz, 1H, PyrC$_6$H), 8.54 (s, 1H, C(=S)NH)), 8.07–8.03 (m, 1H, PyrC$_3$H), 8.01 (td, $J = 7.6$, 1.7 Hz, 1H, PyrC$_5$H), 7.63 (ddd, $J = 7.3$, 4.8, 1.5 Hz, 1H, PyrC$_4$H), 7.33–7.26 (m, 4H, Ph), 7.24–7.18 (m, 1H, Ph), 4.71 (d, $J = 6.0$ Hz, 2H, CH$_2$); ^{13}C-NMR(100 MHz, DMSO-d_6) δ 181.88, 163.64, 149.39, 148.42, 139.37, 137.62, 127.95, 127.05, 126.88, 126.50, 122.50, 46.72; MS (ESI) *m/z* 287.1 $[M+H]^+$; CAS registry 211572-58-6.

N-(4-Methoxyphenyl)-2-picolinoylhydrazinecarbothioamide (4g)

White solid. Yield: 81%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.71 (s, 1H), 9.63 (s, 2H), 8.68 (ddd, $J = 4.7$, 1.5, 1.0 Hz, 1H), 8.10–7.95 (m, 2H), 7.64 (ddd, $J = 7.3$, 4.8, 1.5 Hz, 1H), 7.30 (d, $J = 8.3$ Hz, 2H), 6.88 (d, $J = 8.9$ Hz, 2H), 3.74 (s, 3H); MS (ESI) *m/z* 303.1 $[M+H]^+$.

N-(4-Chlorophenyl)-2-picolinoylhydrazinecarbothioamide (4h)

White solid. Yield: 91%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.77 (s, 1H), 9.86 (br s, 1H), 9.78 (br s, 1H), 8.69 (d, $J =$ 4.3 Hz, 1H), 8.10–7.97 (m, 2H), 7.65 (ddd, $J = 7.2$, 4.8, 1.5 Hz, 1H), 7.50 (d, $J = 6.6$ Hz, 2H), 7.37 (d, $J = 8.8$ Hz, 2H); ^{13}C-NMR (100 MHz, DMSO) δ 180.72 (C=S), 163.79 (C=O), 149.39 (C_{Pyr}), 148.50 (C_{Pyr}), 138.28 (C_{Pyr}), 137.71 (C_{Ph}), 128.82 (C_{Ph}), 127.87 (C_{Ph}), 127.32(C_{Ph}), 126.98 (C_{Pyr}), 122.52(C_{Pyr}).; MS (ESI) *m/z*: 329.0 $[M+Na]^+$.

N-Cyclohexyl-2-picolinoylhydrazinecarbothioamide (4i)

White solid. Yield: 82%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.49 (s, 1H), 9.31 (s, 1H), 8.70–8.63 (m, 1H), 8.07–7.96 (m, 2H), 7.85–7.47 (m, 2H), 4.30–4.02 (m, 1H), 1.80–1.78 (m, 2H), 1.67–1.66 (m, 2H), 1.57–1.53 (m, 1H), 1.32–1.15 (m, 4H), 1.08–1.03 (m, 1H); MS (ESI) *m/z*: 279.3 $[M+H]^+$.

N-(4-Chlorophenyl)-2-nicotinoylhydrazinecarbothioamide (4j)

White solid. Yield: 85%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.79 (s, 1H), 9.91 (s, 2H), 9.10 (d, $J = 1.7$ Hz, 1H), 8.76 (dd, $J = 4.8$, 1.7 Hz, 1H), 8.27 (dt, $J = 8.0$, 1.9 Hz, 1H), 7.56 (ddd, $J = 8.0$, 4.8, 0.8 Hz, 1H), 7.46 (s, 2H), 7.42–7.34 (m, 2H); MS (ESI) *m/z*: 307.7 $[M+H]^+$.

N-(4-Chlorophenyl)-2-isonicotinoylhydrazinecarbothioamide (4k)

White solid. Yield: 90%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.88 (s, 1H), 9.93 (s, 2H), 8.78 (dd, $J = 4.4$, 1.6 Hz, 2H), 7.85 (d, $J = 6.0$ Hz, 2H), 7.46 (s, 2H), 7.43–7.31 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 181.10 (C=S), 164.48 (C=O), 150.25 (C_{Pyr}), 139.52 (C_{Pyr}), 138.14 (C_{Ph}), 129.27 (C_{Ph}), 127.99 (C_{Ph}), 127.68 (C_{Ph}), 121.69 (C_{Pyr}); MS (ESI) *m/z*: 307.7 $[M+H]^+$.

2-Benzoyl-*N*-(4-chlorophenyl)hydrazine-1-carbothioamide (4l)

White solid. Yield: 95%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.57 (s, 1H), 9.84 (s, 2H), 7.97 (d, $J = 7.3$ Hz, 2H), 7.61–7.57 (m, 1H), 7.52–7.49 (m, 4H), 7.42–7.33 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 181.09 (C=S), 165.93 (C=O), 138.27 (C_{Ph}), 132.43 (C_{Ph}), 131.91 ($C_{Benzoyl}$), 128.94 ($C_{Benzoyl}$), 128.45 (C_{Ph}), 128.27 (C_{Ph}), 127.90 ($C_{Benzoyl}$), 127.66 ($C_{Benzoyl}$); MS (ESI) *m/z*: 328.0 $[M+Na]^+$.

2-(2-chlorobenzoyl)-*N*-(5-methoxy-2-methylphenyl) hydrazine-1-carbothioamide (4m)

Yield: 82%; m.p.: 155.5–157.8 °C; ^{1}H-NMR (700 MHz, $CDCl_3$) δ 10.10 (br s, 2H, NHC(=S)NH), 8.73 (s, 1H, C(=O)NH), 7.90–7.80 (m, 1H, Cl-PhH), 7.81 (d, J = 7.5 Hz, 1H, Cl-PhH), 7.46–7.43 (m, 2H, Cl-PhH), 7.35 (t, J = 7.3 Hz, 1H, Ph), 6.98 (dd, J = 8.3, 1.1 Hz, 1H, Ph), 6.79 (d, J = 8.4 Hz, 1H, Ph), 3.70 (s, 3H, OCH_3), 2.31 (s, 3H, CH_3); ^{13}C-NMR (175 MHz, $CDCl_3$) δ 181.51 (C=S), 161.43 (C=O), 149.31 (C_{Ph}), 132.39 (C_{Ph}), 131.67 (C_{Ph}), 131.22 (C_{Ph}), 130.72 (C_{Ph}), 130.61 (C_{Ph}), 129.54 (C_{Ph}), 128.53 (C_{Ph}), 127.19 (C_{Ph}), 125.85 (C_{Ph}), 124.89 (C_{Ph}), 111.14 (C_{Ph}), 55.76 (OCH_3), 20.71 (CH_3); MS (ESI) *m/z*: 350.1 $[M+H]^+$; HRMS (ESI) *m/z*: Calcd. for $[M+H]^+$ $C_{16}H_{16}ClN_3O_2S$: 350.0725; found: 350.0724.

N-(4-Chlorophenyl)-2-(thiophene-2-carbonyl)hydrazine-1-carbothioamide (4n)

White solid. Yield: 94%; m.p.: 193.3–193.6 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.59 (s, 1H), 9.93 (s, 1H), 9.86 (s, 1H), 7.86 (d, J = 4.9 Hz, 2H), 7.48 (s, 2H), 7.38 (d, J = 8.7 Hz, 2H), 7.21 (dd, J = 4.8, 4.0 Hz, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 181.2 (C=S), 161.1 (C=O), 138.2 ($C_{thiophene}$), 137.4 (C_{ph}), 131.8 (C_{ph}), 129.6 ($C_{thiophene}$), 129.1 ($C_{thiophene}$), 128.1 (C_{ph}), 127.9 (C_{ph}), 127.6 ($C_{thiophene}$); MS (ESI) *m/z*: 312.8 $[M+H]^+$.

N-Phenyl-2-(thiophene-2-carbonyl) hydrazinecarbothioamide (4o)

White solid. Yield: 79%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.56 (s, 1H), 9.88 (s, 1H), 9.74 (s, 1H), 7.86 (d, J = 4.8 Hz, 2H), 7.43 (s, 2H), 7.32 (t, J = 7.8 Hz, 2H), 7.25–7.07 (m, 2H); MS (ESI) *m/z* 278.4 $[M+H]^+$.

N-(4-Methoxyphenyl)-2-(thiophene-2-carbonyl) hydrazinecarbothioamide (4p)

White solid. Yield: 87%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.53 (s, 1H), 9.76 (s, 1H), 9.64 (s, 1H), 7.89–7.82 (m, 2H), 7.27 (d, J = 8.2 Hz, 2H), 7.20 (dd, J = 4.9, 3.8 Hz, 1H), 6.89 (d, J = 8.9 Hz, 2H), 3.74 (s, 3H); MS (ESI) *m/z* 308.1 $[M+H]^+$.

N-(4-Isopropylphenyl)-2-(thiophene-2-carbonyl)hydrazine-1-carbothioamide (4q)

Yield: 89%; m.p.: 192.5–194.1 °C; ^{1}H-NMR (700 MHz, DMSO-d_6) δ 10.54 (s, 1H, C=(S)NH)), 9.80 (s, 1H, NHC(=S)), 9.68 (s, 1H, C(=O)NH), 7.88 (s, 1H, ThiopheneC_3H), 7.86 (d, J = 4.9 Hz, 1H, ThiopheneC_5H), 7.35 (s, 2H, PhH), 7.20−7.21 (m, 3H, PhH + ThiopheneC_4H), 2.88 (dt, J = 13.8, 6.9 Hz, 1H, CH), 1.21 (d, J = 6.9 Hz, 6H, CH_3); ^{13}C-NMR (175 MHz, DMSO-d_6) δ 181.75 (C=S), 161.54 (C=O), 145.62 (C_{ph}), 137.97 (C_{ph}), 137.37 ($C_{thiophene}$), 132.25 ($C_{thiophene}$), 132.09 ($C_{thiophene}$), 130.26 ($C_{thiophene}$), 130.04 (C_{ph}), 128.45 (C_{ph}), 126.63 (C_{ph}), 126.25 (C_{ph}), 33.43 (CH), 24.40 (CH_3), 24.29 (CH_3); MS (ESI) *m/z*: 320.1 $[M+H]^+$; HRMS (ESI) *m/z*: Calcd. for $[M+H]^+$ $C_{15}H_{17}N_3OS_2$: 320.0886; found: 320.0889.

N-Cyclohexyl-2-(thiophene-2-carbonyl) hydrazinecarbothioamide (4r)

White solid. Yield: 81%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.29 (s, 1H), 9.23 (s, 1H), 7.84 (d, J = 4.6 Hz, 2H), 7.80 (d, J = 8.2 Hz, 1H), 7.18 (t, J = 4.4 Hz, 1H), 4.16–4.13 (m, 1H), 1.78–1.76 (m, 2H), 1.69–1.68 (m, 2H), 1.58–1.57 (m, 1H), 1.35–1.13 (m, 4H), 1.06–1.05 (m, 1H); MS (ESI) *m/z*: 284.2 $[M+H]^+$.

2-(5-Chlorothiophene-2-carbonyl)-*N*-cyclohexylhydrazinecarbothioamide (4s)

Yield: 92%; m.p.: 182.2–184.8 °C; ^{1}H-NMR (700 MHz, DMSO-d_6) δ 10.37 (s, 1H, NHC(=S)), 9.23 (s, 1H, C(=O)NH), 7.82 (d, J = 7.2 Hz, 1H, thiopheneC_3H), 7.70 (s, 1H, C(=S)NH), 7.24 (d, J = 4.0 Hz, 1H, thiopheneC_4H), 4.30–4.10 (m, 1H), 1.79–1.78 (m, 2H), 1.71–1.70 (m, 2H), 1.59–1.58 (m, 1H), 1.34–1.21 (m, 4H), 1.07–1.06 (m, 1H); ^{13}C-NMR (175 MHz, DMSO-d_6) δ 181.14 (C=S), 160.47 (C=O), 137.26 ($C_{thiophene}$), 134.20 ($C_{thiophene}$), 129.83 ($C_{thiophene}$), 128.60 ($C_{thiophene}$), 53.59 (CH), 32.29 (CH_2), 25.64 (CH_2), 25.42 (CH_2); MS (ESI) *m/z*: 318.1 $[M+H]^+$; HRMS (ESI) *m/z*: Calcd. for $[M+H]^+$ $C_{12}H_{16}ClN_3OS_2$: 318.0496; found: 318.0499.

N-Cyclohexyl-2-(4-ethyl-5-methylthiophene-3-carbonyl) hydrazinecarbothioamide (4t)

Yield: 89%; m.p.: 177.3–179.3 °C; ^{1}H-NMR (700 MHz, DMSO-d_6) δ 9.90 (s, 1H, NHC(=S)), 9.15 (s, 1H, thiopheneC_2H), 7.84 (s, 1H, C(=O)NH), 7.55 (s, 1H, C(=S)NH), 4.11 (s, 1H, CH), 2.71 (q, J = 7.4 Hz, 2H, CH_2CH_3), 2.35 (s, 3H, CH_3), 1.80–1.79 (m, 2H), 1.73–1.64 (m, 2H), 1.57 (d, J = 12.5 Hz, 1H), 1.34–1.19 (m, 4H), 1.03 (t, J = 7.4 Hz, 3H, CH_3+CH_2); ^{13}C-NMR (175 MHz, DMSO-d_6) δ 184.22 (C=S), 164.03 (C=O), 140.07 ($C_{thiophene}$), 137.87 ($C_{thiophene}$), 133.87 ($C_{thiophene}$), 126.10 ($C_{thiophene}$), 53.20 (CH), 32.28 (CH_2), 25.62 (CH_2), 25.26 (CH_2), 20.16 (CH_2), 15.42 (CH_3), 12.89 (CH_3); MS (ESI) *m/z*: 326.1 $[M+H]^+$; HRMS (ESI) *m/z*: Calcd. for $[M+H]^+$ $C_{15}H_{24}N_3OS_2$: 326.1355; found: 326.1358.

General procedure for the synthesis of 2a–2i, 2j–2l, 2n–2r

To a solution of 100 mmol of ethyl picolinate (for **2a**–**2i**), ethyl nicotinate (for **2j**), ethyl isonicotinate (for **2k**), ethyl benzoate (for **2l**) or thiophene-2-carboxylate (for **2n**–**2r**) in EtOH was added hydrazine monohydrate (400 mmol). The reaction mixture was refluxed overnight and cooled to room temperature. Half of the solvent was removed under reduced pressure and the residue was poured into ice. The precipitate was filtered and washed with ice-cold EtOH to give a product.

Picolinohydrazide (2a–2i)

Yield: 79%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.88 (s, 1H), 8.64–8.57 (m, 1H), 8.03–7.94 (m, 2H), 7.56 (ddd, $J = 6.1$, 4.8, 2.9 Hz, 1H), 4.56 (s, 2H); MS (ESI) m/z 138.1 $[M+H]^+$.

Nicotinohydrazide (2j)

Yield: 81%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.96 (s, 1H), 8.96 (dd, $J = 2.3$, 0.9 Hz, 1H), 8.69 (dd, $J = 4.8$, 1.7 Hz, 1H), 8.15 (ddd, $J = 7.9$, 2.3, 1.7 Hz, 1H), 7.49 (ddd, $J = 7.9$, 4.8, 0.9 Hz, 1H), 4.58 (s, 2H); MS (ESI) m/z 138.1 $[M+H]^+$.

Isonicotinohydrazide (2k)

Yield: 88%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.88 (s, 1H), 8.64–8.57 (m, 1H), 8.01–7.93 (m, 2H), 7.56 (ddd, $J = 6.1$, 4.8, 2.9 Hz, 1H), 4.56 (s, 2H); MS (ESI) m/z 138.1 $[M+H]^+$.

Benzohydrazide (2l)

Yield: 94%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.77 (s, 1H), 7.86–7.78 (m, 2H), 7.54–7.40 (m, 3H), 4.49 (s, 2H); MS (ESI) m/z 137.2 $[M+H]^+$.

Thiophene-2-carbohydrazide (2n–2r)

Yield: 81%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.75 (s, 1H), 7.74 (dd, $J = 5.0$, 1.1 Hz, 1H), 7.70 (dd, $J = 3.7$, 1.1 Hz, 1H), 7.12 (dd, $J = 5.0$, 3.7 Hz, 1H), 4.45 (s, 2H); MS (ESI) m/z 143.2 $[M+H]^+$.

4-Ethyl-5-methylthiophene-3-carbohydrazide (2t)

^{1}H-NMR (700 MHz, DMSO-d_6) δ 9.38 (s, 1H), 7.54 (s, 1H), 4.46 (s, 2H), 2.70 (q, $J = 7.5$ Hz, 2H), 2.33 (s, 3H), 1.02 (t, $J = 7.5$ Hz, 3H); ^{13}C-NMR (175 MHz, DMSO) δ 164.81 (C=O), 139.58 (C4-$_{\text{Thiophene}}$), 135.90 (C5-$_{\text{Thiophene}}$), 133.70 (C2-$_{\text{Thiophene}}$), 123.76 (C2-$_{\text{Thiophene}}$), 20.10 (CH_2), 15.50 (CH_3), 12.94 (CH_3); MS (ESI) *m/z*: 185.1 $[M+H]^+$.

General procedure for the synthesis of 3d (y. 71%), 3m (y.75%) and 3q (74%), which are all known compounds

To a solution of aniline analogues (2.0 mmol) in EtOH (10 mL) were added CS_2 (2.4 mmol), Boc_2O (2.4 mmol), Et_3N (2.4 mmol) and 4-dimethylaminopyridine (0.2 mmol) at 0 °C. The reaction mixture was stirred for 1 h at room temperature. The reaction mixture was concentrated under reduced pressure, then diluted with CH_2Cl_2. Organic layer was washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated. The residue was purified on silica gel column chromatography to give isothiocyanates.

5-(Pyridin-2-yl)-*N*-(2-(trifluoromethyl)phenyl)-1,3,4-thiadiazol-2-amine (5b)

To a solution of hydrazinecarbothioamide (1 mmol) in EtOH (3.5 mL) was added H_2SO_4 (553 µL, 0.01 mmol). The reaction mixture was refluxed for 1 h and poured to ice-water. The resulting slurry was filtered and the filter cake was recrystallized with 33% AcOH in D.W. to give **5b** as white solid (y. 60%); m.p.: 249.6–250.6 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.98 (s, 1H), 8.66 (ddd, $J = 4.9$, 1.6, 1.0 Hz, 1H), 8.28 (s, 1H), 8.17 (dt, $J = 8.0$, 1.0 Hz, 1H), 8.00 (td, $J = 7.7$, 1.7 Hz, 1H), 7.80 (dd, $J = 7.9$, 1.5 Hz, 1H), 7.61 (t, $J = 8.0$ Hz, 1H), 7.52 (ddd, $J = 7.5$, 4.9, 1.2 Hz, 1H), 7.38 (d, $J = 7.7$ Hz, 1H); MS (ESI) *m/z* 323 $[M+H]^+$.

General procedure for the synthesis of 5e, 5h and 5i

Hydrazinecarbothioamide (0.3 mmol) was added to H_2SO_4 (10 mL) at 0 °C and stirred for 3 h at room temperature. The reaction mixture was neutralized with 2 N NaOH and filtered. Filter cake was recrystallized with EtOH to give thiadiazoles.

N-Butyl-5-(pyridin-2-yl)-1,3,4-thiadiazol-2-amine (5e)

White solid. Yield: 29%; m.p.: 110.9–112.8 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.58 (ddd, $J = 4.9$, 1.5, 1.0 Hz, 1H), 8.08–8.04 (m, 1H, N<u>H</u>), 8.03 (t, $J = 1.0$ Hz, 1H, PyrC$_3$<u>H</u>), 7.91 (td, $J = 7.7$, 1.7 Hz, 1H, PyrC$_4$<u>H</u>), 7.41 (ddd, $J = 7.5$, 4.9, 1.1 Hz, 1H, PyrC$_5$<u>H</u>), 3.33–3.29 (m, 2H, NHC<u>H$_2$</u>), 1.62–1.53 (m, 2H, C<u>H$_2$</u>), 1.36 (dq, $J = 14.4$, 7.3 Hz, 2H, C<u>H$_2$</u>), 0.91 (t, $J = 7.4$ Hz, 3H, C<u>H$_3$</u>); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 169.9 ($C_{\text{Thiadiazole}}$), 157.7 ($C_{\text{Thiadiazole}}$), 149.6 (C_{Pyr}), 149.5 (C_{Pyr}), 137.3 (C_{Pyr}), 124.2

(C_{Pyr}), 119 (C_{Pyr}), 44.5 (CH_2), 30.6 (CH_2), 19.5 (CH_2), 13.6 (CH_3); MS (ESI) *m/z* 235 $[M+H]^+$.

N-(4-Chlorophenyl)-5-(pyridin-2-yl)-1,3,4-thiadiazol-2-amine (5h)

Yield: 41%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.75 (s, 1H), 8.64 (d, $J = 4.7$ Hz, 1H), 8.14 (d, $J = 7.9$ Hz, 1H), 7.97 (td, $J = 7.8$, 1.6 Hz, 1H), 7.76–7.66 (m, 2H), 7.49 (ddd, $J =$ 7.4, 4.9, 0.9 Hz, 1H), 7.47–7.37 (m, 2H); MS (ESI) *m/z* 289 $[M+H]^+$.

N-Cyclohexyl-5-(pyridin-2-yl)-1,3,4-thiadiazol-2-amine (5i)

White solid. Yield: 71%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.57 (ddd, $J = 4.9$, 1.6, 1.0 Hz, 1H), 8.07–7.98 (m, 2H), 7.90 (td, $J = 7.7$, 1.7 Hz, 1H), 7.41 (ddd, $J = 7.5$, 4.9, 1.2 Hz, 1H), 3.58 (tt, $J = 13.3$, 6.8 Hz, 1H), 2.06 – 1.93 (m, 2H), 1.78 – 1.66 (m, 2H), 1.62 – 1.52 (m, 1H), 1.39 – 1.12 (m, 5H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 168.94 (C4′-thiophene), 157.55 (C2′-thiophene), 149.55 (C2′-pyridine), 137.29 (C6′-pyridine), 124.15 (C4′-pyridiine), 118.94 (C3′- and C5′-pyridine), 53.74 (CH-cyclohexyl), 32.02 (C2′-cyclohexyl), 25.20 (C4′-cyclohexyl), 24.26 (C3′-cyclohexyl); MS (ESI) *m/z* 261 $[M+H]^+$.

General procedure for the synthesis of 6b, 6e, 6h and 6i

Hydrazinecarbothioamide (1 mmol) was added to 2 N NaOH (6.6 mL) and refluxed for 3 h. The reaction mixture was cooled to room temperature and acidified with 2 N HCl to pH = 3. Resulting solid was filtered and recrystallized with EtOH to give triazoles.

5-(Pyridin-2-yl)-4-(2-(trifluoromethyl)phenyl)-4*H*-1,2,4-triazole-3-thiol (6b)

White solid. Yield: 71%; m.p.: 223.2–224.3 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 14.33 (s, 1H), 8.26 (dt, $J = 4.8$, 1.4 Hz, 1H), 7.97–7.89 (m, 2H), 7.84–7.76 (m, 2H), 7.74–7.63 (m, 2H), 7.43–7.37 (m, 1H); MS (ESI) *m/z* 323 $[M+H]^+$.

4-Butyl-5-(pyridin-2-yl)-4*H*-1,2,4-triazole-3-thiol (6e)

White solid. Yield: 72%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 14.08 (s, 1H), 8.73 (dt, $J = 4.8$, 1.4 Hz, 1H), 8.04−7.98 (m, 2H), 7.60–7.53 (m, 1H), 4.49 (t, J = 7.6 Hz, 2H), 1.67−1.58 (m, 2H), 1.30−1.20 (m, 2H), 0.84 (t, $J = 7.4$ Hz, 3H); MS (ESI) *m/z* 235 $[M+H]^+$.

4-(4-Chlorophenyl)-5-(pyridin-2-yl)-4*H*-1,2,4-triazole-3-thiol (6h)

White solid. Yield: 96%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 14.29 (s, 1H), 8.35 (ddd, $J = 4.8$, 1.7, 1.0 Hz, 1H), 7.96 −7.85 (m, 2H), 7.56–7.47 (m, 2H), 7.41 (ddd, $J = 7.4$, 4.8, 1.4 Hz, 1H), 7.38−7.29 (m, 2H); MS (ESI) *m/z* 289 $[M+H]^+$.

4-Cyclohexyl-5-(pyridin-2-yl)-4*H*-1,2,4-triazole-3-thiol (6i)

White solid. Yield: 81%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 14.02 (s, 1H), 8.76 (ddd, $J = 4.8$, 1.7, 0.9 Hz, 1H), 8.01 (td, $J = 7.8$, 1.8 Hz, 1H), 7.83 (dt, $J = 7.9$, 1.0 Hz, 1H), 7.60 (ddd, $J = 7.7$, 4.8, 1.2 Hz, 1H), 4.80 (tt, $J = 12.3$, 3.8 Hz, 1H), 2.29 (s, 2H), 1.70 (dd, $J = 25.5$, 12.6 Hz, 4H), 1.57 (d, $J = 12.6$ Hz, 1H), 1.31–1.15 (m, 2H), 1.12–0.95 (m, 1H); MS (ESI) *m/z* 261 $[M+H]^+$.

Cell culture

HEK 293 cells (human embryonic kidney cell) and HepG2 cells (human hepatocarcinoma cell) were purchased from ATCC. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin and streptomycin in a humidified incubator with 5% CO_2 atmosphere at 37 °C.

Palmitate preparation

A 20 mM solution of palmitate in 0.01 N NaOH was incubated at 80 °C for 30 min and 1 N NaOH was added up to 0.2 N final concentration for clearing up the solution. The fatty acid soaps were then combined with 5% fatty acid-free BSA in phosphate-buffered saline (PBS) in 1:3 volume ratio. PA/BSA conjugates consisted of 5 mM PA and 3.75% BSA. PA/BSA conjugates were diluted in DMEM (4.5 g/L glucose) supplemented with 10% FBS. Concentration of BSA in 0.3 mM PA containing DMEM was approximately 0.5% and the molar ratio of PA to BSA in this solution was approximately 5:1.

Multiplex luciferase assay

The stable human embryonic kidney (HEK) 293 cell line harbouring a reporter gene expressed by human grp78 promoter was used as previously described. Synthesized human grp78 promoter (−137 ~ +25) containing three ER stress response elements (ERSE) in tandem was cloned into pGL4.79 vector (Promega, Madison, USA). A stable human embryonic kidney (HEK) 293 cell line harbouring the

reporter gene was selected under 400 µg/mL of G418 selection pressure. Five thousand selected stable cells per well were seeded in white 384-well plate (Greiner, Solingen, Germany). After 24 h, media were changed with BSA or PA-conjugated BSA media, and serially diluted compounds were added. Following 22 h incubation, 60 µM of EnduRen™ live cell substrate (Promega, Madison, USA) was added to medium and, after 2 h incubation, *Renilla* luciferase (rLuc) activity was measured using EnVision® (Perkin-Elmer). To normalize the rLuc activity, total viable cells were counted using Celltiter-glo™ reagent (Promega). The 50% inhibitory concentration (IC_{50}) values for tested compounds were determined by non-linear regression analysis of log-dose/response curves using Prism® 5 software (Graphpad software Inc., CA, USA). Data from three independent experiments were expressed as the geometric mean IC_{50} and 95% confidence intervals (95% CI) were calculated.

Immunoblot analysis

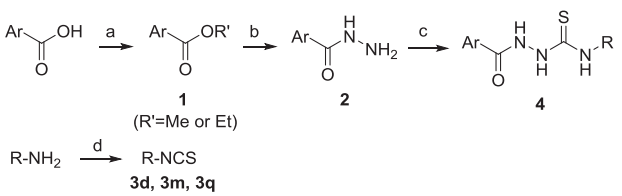
HepG2 cells in a six-well plate at a density of 500,000 cells/well were treated with tunicamycin (Tm) and test compound and incubated for 22 h. Cells were lysed in buffer A (50 mM Hepes pH 7.4, 100 mM NaF, 50 mM NaCl, 10 mM sodium pyrophosphate, 10 mM glycerol-2-phosphate, 5 mM EDTA, 5 mM EGTA and 1% TritonX-100) supplemented with protease inhibitor cocktail (Roche, Germany) and phosphatase inhibitor cocktail (Sigma-Aldrich). Following incubation on ice for 20 min, cleared lysates were boiled with 6× SDS sample buffer for 5 min and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Bio-Rad, CA, USA) and analysed by immunoblot. Anti-phospho PERK (Thr980), anti-phospho eIF2α (S51), anti-CHOP were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA) and anti-beta-action was from Cell Signaling (Beverly, MA, USA). AlexaFluor 488-conjugated goat anti-rabbit secondary antibody was from Thermo Fisher Scientific (MA, USA).

Results and discussion

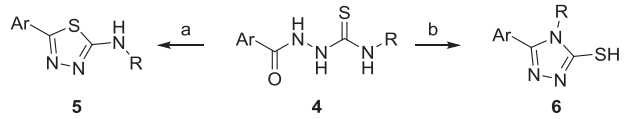
Previously, we have reported a cell-based reporter assay (Jeong et al. 2013) in which *renilla* luciferase (rLuc) activity is driven by human GRP78 promoter to search ER stress-reducing compounds. Since GRP78 is one of the most important chaperones and plays a pivotal role in various UPR mechanisms, monitoring the GRP78 signal in the presence of test compounds can be a useful way to represent ER stress-reducing activity of the compounds. Briefly, a library of ~20,000 heteroatom-containing compounds, including pyridines, pyrimidines, pyrroles, imidazoles, thiophenes, was screened in 384-well format at 20 mM concentration to identify ER stress-reducing compounds. Tunicamycin (1.2 µM final concentration) was treated to induce ER stress and test compounds were treated 24 h after Tm-induction. Compounds which decreased the luciferase signal that was induced by Tm by more than two-fold of the plate mean were selected as primary hits. Hydroxynaphthoic acids (HNAs) were identified as the initial hit showing about tenfold better activity than salicylate, a positive control. HNAs significantly decreased ER stress markers such as CHOP and phosphorylated PERK and eIF2α in western blot. Although HNAs are highly potent in modulating ER stress markers, they still need to be improved in drug-likeness and activity. *N*-Substituted-2-arylcarbonylhydrazinecarbothioamides (**4**) were identified in screening to show much better activity and to be more drug-like due to higher heteroatom content than HNA.

Scheme 1 shows the structures and synthesis of *N*-substituted-2-arylcarbonylhydrazinecarbothioamides (**4a**–**4t**). Except for the synthesis of **4s**, various aryl ethyl esters (**1**) were treated with hydrazine in refluxing ethanol to give the corresponding acylhydrazides (**2**) in 76–94% yields. For the synthesis of **4s**, methyl 5-chlorothiophene-2-carboxylate was prepared in refluxing MeOH under Fisher esterification condition, then it was subsequently reacted with hydrazine to afford the acylhydrazides (**2s**). All the prepared acylhydrazides (**2a – 2t**) were reacted with various isothiocyanates (**3**) to afford hydrazinecarbothioamides (**4a – 4t**) in 79–94% yields. Commercially unavailable isothiocyanates (**3d**, **3m** and **3q**) were prepared through a reaction of the corresponding amines with CS_2 and Boc_2O (Munch et al. 2008). Ar group includes phenyl, substituted phenyl, 2- and 3-thiophenyl, and 2-, 3-, and 4-pyridyl while R group ranges from alkyl, cycloalkyl and substituted phenyl.

Next, we investigated the feasibility of the cyclization of hydrazinecarbothioamides **4**. **4b, 4e, 4h** and **4i** were chosen as representative examples with high activities and underwent further cyclization (Scheme 2). They were refluxed in EtOH in the presence of 2 N NaOH to give corresponding 1,2,4-triazoles **6b**, **6e**, **6h**, and **6i**, respectively, in 71–96% yields (Ainsworth 1960). On the other hand, 1,3,4-thiadiazoles **5** (Oruç et al. 2004) were generally obtained through a



Scheme 1 Synthetic scheme for compounds **4**. Reagents and conditions: (a) MeOH, H_2SO_4, reflux, 4 h, 94%; (b) $H_2NNH_2{\cdot}H_2O$ (4 eq.), EtOH, reflux, 4 h, 76–94%; (c) R-NCS (**3**), EtOH, reflux, 5 h, 79–94%; (d) CS_2, Boc_2O, DMAP, Et_3N, EtOH, 0 °C → r.t., 1 h, 71–75%



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Scheme 2 Synthetic scheme for 1,3,4-thiadiazoles **5** and 1,2,4-triazoles **6**. Reagents and conditions: (a) (i) H_2SO_4 or HCl, EtOH, 0 °C → r.t., 2 h, 60% for **5b**, 29% for **5e**, 41% for **5h**, 71% for **5i**; (**b**) 2 N NaOH, reflux, 2 h, 71% for **6b**, 72% for **6e**, 96% for **6h**, 81% for **6i**

reaction with hydrazinecarbothioamides **4** with 1 mol% H_2SO_4 in refluxing EtOH. **5b** was obtained in 60% yield accordingly. However, other hydrazinecarbothioamides (**4e, 4h** and **4i**) afforded complex mixture of products under the same condition. The corresponding 1,3,4-thiadiazoles (**5e, 5h** and **5i**) were obtained by addition of **4e, 4h**, and **4i** into ice-cold H_2SO_4, followed by stirring at room temperature.

Compounds **4**, **5**, and **6** were evaluated using our cell-based reporter assay where ER stress is induced by Tm. As shown in Table 1, a number of compounds showed very potent activities to reduce Tm-induced ER stress signal. There are several features in the SAR. First of all, analogues with pyridine and thiophene moiety on Ar position were more active than those with phenyl and halogenated phenyl groups (**4l**, **4m**) and, 2-pyridyl analogues (**4a**–**4i**) showed much superior activities over the other regioisomers (**4j** and **4k**). Secondly, all the 2-pyridyl analogues with either alkyl or aryl groups on R position showed significant activities. Especially, when R = alkyl (**4d**–**4f** and **4i**), cyclohexyl analogue (**4i**) is the most active showing more than 200-fold better than our initial hit compound, 1-HNA. Of aromatic R groups, 4-chlorophenyl group showed the best activity (**4h**) that is almost 300-fold better than 1-HNA. Moreover, **4h** showed considerably low cytotoxicity (CC_{50}) suggesting a significant safety profile (SI) of the compound. Lastly, aromatic R groups should be attached directly to thiourea moiety. A methylene spacer between aromatic R group and thiourea moiety decreases the activity (**4c** vs. **4f**; **4d** vs. **4h**) by at least several folds.

With the identical R group of 4-chlorophenyl, **4j** and **4k** showed much less activity than their 2-pyridyl analogue, **4h**, demonstrating a high level of regioselectivity in pyridyl substitution on Ar position. 2-Pyridyl analogues consistently showed strong activities regardless of R group. It does not seem that R group is a strong determinant for the activity and shows significant electronic or steric effect on the activity. For example, although 4-chlorophenyl R group is commonly found in 2-pyridyl (**4h**), 3-pyridyl (**4j**), 4-pyridyl (**4k**), and phenyl (**4l**) analogues, only 2-pyridyl analogue (**4e**) showed the high level of activity while the others showed much weaker activities. Dependency of activity on Ar group was reproduced in thiophene series (Table 1, entries **4n**–**4t**) as well. Thiophene analogues with R group of 4-methoxyphenyl (**4g**), 4-chlorophenyl, (**4h**) and cyclohexyl R group (**4i**) showed almost 100-fold less activity than 2-pyridyl analogues with identical R groups (**4p**, **4n**, and **4r**, respectively). Albeit not significant, electron withdrawing R groups and/or substitution on thiophene ring are somewhat favoured for the activity. With the same cyclohexyl R group attached, **4s** which has a chloro substituent on thiophene ring showed the best activity among **4r** – **4t**.

Transformation of **4** to the more rigid structures (**5** and **6**) generally resulted in detrimental effects to the activity. Although they are all derived from active hydrazinecarbothioamide compounds, cyclized analogues **5** and **6** did not show comparable activity to their acyclic analogues (**4b**, **4e**, **4h**, and **4i**). Interestingly, most compounds in this study showed decent cytotoxicity (CC_{50}) profile (Table 1), thereby rendering maximized safety windows (SI).

Tunicamycin (Tm) induced ER stress in our cell-based assay for the sake of facile HTS campaign. It causes ER stress by perturbing *N*-linked glycosylation which is important in folding events of eukaryotic proteins (Heifetz et al. 1979). It is well established that high level of free fatty acids such as palmitic acid alone (lipotoxicity) or in combination with high level of glucose is one of the most important factors to trigger ER stress. The activity of the analogues were tested in PA-induced ER stress assay because high level of free fatty acids such as palmitic acid (lipotoxicity) is considered to be more physiologically important factors to trigger ER stress. At the same time, it would be interesting to investigate the correlation of the activity in between Tm- and PA-induced assays. As shown in Table 2, some pyridinyl compounds slightly lost their activities (**4a**, **4b, 4g–4i**) while others gained activity (**4c, 4d** and **4f**). However, level of changes remained within only a few folds (1.1–3.9-fold decrease and 1.2–2.6-fold increase) except for **4h** and **4f** which showed 6-fold decrease and 5.4-fold increase, respectively, suggesting a rough correlation between Tm-induced assay and PA-induced assay. Thiophenyl analogues, 1,3,4-Thiadiazoles (**5**) and 1,2,4-triazoles (**6**) which showed weak activity under Tm-induced condition still showed trivial activities (some data not shown).

Next, we investigated the effects of the active compounds identified from our reporter assay on cellular ER stress markers. Compound **4h** was treated at various concentrations along with Tm to HepG2 cells and the changes of some UPR markers were analysed by western blot (Fig. 2). When sensing ER stress, GRP78 which normally binds to PERK is released from the complex, which is followed by the oligomerization and transphosphorylation of PERK. Compound **4h** successfully diminished the phosphorylated PERK and phosphorylated eIF2α both of which were induced by Tm treatment. Level of CHOP were also attenuated in line with well-documented PERK-eIF2α-AFT4

Table 2 Activity of (**4**) against palmitate-induced ER stress

Compounds	EC_{50} (μM)[a]
4a	3.13 ± 0.68
4b	4.58 ± 0.73
4c	0.96 ± 0.19
4d	4.20 ± 1.26
4f	1.29 ± 0.16
4g	3.39 ± 0.51
4h	3.72 ± 0.30
4i	3.41 ± 0.46
4j	>100
4k	>100
4l	25.6 ± 2.8
4m	61.6
4n	24.1 ± 3.6
4o	>100
4p	>100
4q	45.5 ± 5.9
4r	>100
4s	37.6
4t	16.6 ± 3.3

[a]Data represent mean of three experiments

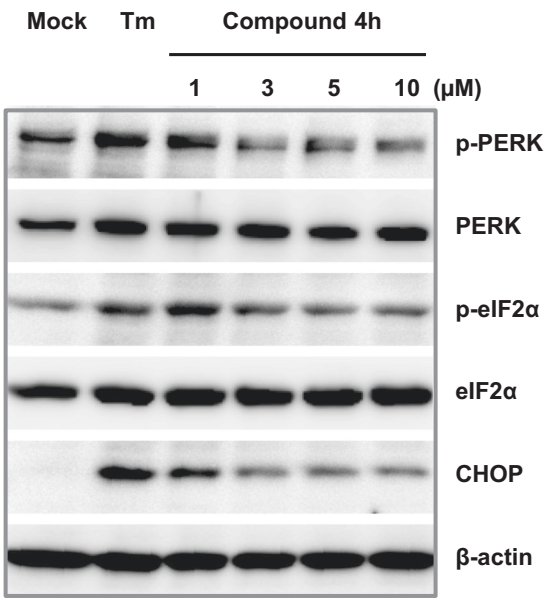

Fig. 2 Western blot of ER stress markers upon treatment of **4h**

pathway which is usually implicated in apoptosis. The degree of those changes was in concert with the results of our reporter assay, which demonstrated the robustness of our reporter assay.

Although we have shown here that representative ER stress markers are modulated upon treatment of our compounds, MOA by which our compounds decrease ER stress or misfolding load is still unclear and requires a lots of investigations. Nevertheless, we can speculate the MOA based on literature. Chemical chaperones can usually be classified into the following groups: (Papp and Csermely 2006) osmolytes, hydrophobic compounds or detergents, and pharmacological chaperones. The most common types are osmolytes which include glycerol, trimethylamine N-oxide (TMAO) and proline and so on. MOA of osmolytes is believed to limit free movement of proteins by elevating the density of the solvent, thus preventing aggregation of unfolded proteins. Therefore, this type of chemical chaperones typically require high enough concentration to exert the activity and are usually charged or uncharged polar compounds to be perfectly soluble in aqueous environment. Based on polarity and water solubility of our compounds, it is hard to suppose our compounds fit into the category. The second group consists of hydrophobic compounds, e.g., sodium salt of PBA, lipids, and detergents. MOA was proposed that hydrophobic moiety of these compounds binds to the hydrophobic surface of unfolded proteins to protect them from aggregation. Detergent-like structural feature of this category could make our compounds distinct from them. The last category is so-called pharmacological chaperones such as enzyme inhibitors and receptor ligands. Detailed MOA of this type of chemical chaperones is not clearly understood. However, it is proposed that binding of selective compound to a target protein can promote proper folding and transport of the mutant or misfolded protein. Pharmacological chaperones are to bind the native conformation of the target protein, stabilizing its conformation and pushing the balance toward the native state. In this regard, our compounds should bind to a target protein in a specific manner to be pharmacological chaperones. Unfortunately, we lack experimental data to identify their binding partner, or, to the best of our knowledge, any binding partners of similar compounds are not known at this point. Investigation of MOA will be conducted as a future direction.

In summary, we have identified and synthesized hydrazinecarbothioamides and their cyclized forms, thiadiazoles and triazoles which reduce ER stress induced by tunicamycin. Open chain forms (**4**) showed up to about 300-fold better activity than 1-hydroxynaphthoic acid (1-HNA) which is, in turn, 10-fold better than salicylate, a positive control. Some hydrazinecarbothioamides (**4**) also showed strong activity under more physiologically relevant PA-induced ER stress model. The cyclized analogues, 1,3,4-thiadiazoles and 1,2,4-triazoles are in general less active than **4**. Indeed, a selected compound (**4h**) reduced representative ER stress markers in western blot and we believe this scaffold could be a platform for further investigation to identify novel chemical chaperones.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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