Hydroxynaphthoic Acids Identified in a High Throughput Screening Potently Ameliorate Endoplasmic Reticulum Stress as Novel Chemical Chaperones

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Folding of newly synthesized protein occurs in endoplasmic reticulum (ER) and is assisted by chaperone molecules. In ER stress conditions, misfolded proteins are enriched in a lumen of ER perturbing its normal function, which triggers cellular self-defense mechanism, the unfolded protein response (UPR). It was reported that tunicamycin-induced ER stress can be modulated with high concentration of chemicals such as 4-phenylbutyric acid and salicylate. In search of assay systems to identify such compounds, we have developed a cell-based reporter assay where *renilla* luciferase activity is driven by glucose-regulated protein 78 (GRP78) promoter. Using our reporter assay, we have screened chemical libraries and found that hydroxynaphthoic acids, especially 1-, 3-, and 6-hydroxy-2-naphthoic acids, potently decrease the ER stress signal, showing an order of magnitude better activity than salicylate. UPR markers such as GRP78, C/EBP homology protein (CHOP) and phosphorylated protein kinase RNA-activated (PKR)-like ER kinase (PERK) were significantly down-regulated with hydroxynaphthoic acids in Western blot. Among the analogues, 1-hydroxy-2-naphthoic acid was the most potent in down-regulating those UPR markers. Further, both phosphorylated inositol-requiring enzyme 1α (IRE1α) and spliced form of X-box binding protein 1 (XBP1) were decreased in the protein and the mRNA level, implying both PERK and IRE1α branches in UPR mechanism are controlled with hydroxynaphthoic acids. Taken together, it was suggested that hydroxynaphthoic acids exert their ER stress-reducing activity prior to the UPR activation as chemical chaperones do. In summary, we report a cell-based assay system for the screening of ER stress-reducing compounds and hydroxynaphthoic acids as novel series of chemical chaperones.

Key words endoplasmic reticulum stress; hydroxynaphthoic acid; chemical chaperone; unfolded protein response

Endoplasmic reticulum (ER) is the essential location of synthesis and folding of proteins destined for either secretion or intracellular uses. Various chemical agents, including tunicamycin (Tm), thapsigargin (Tg) and dithiothreotol (DTT), cause detrimental effects, called 'ER stress,' on the normal function of ER by perturbing *N*-linked glycosylation,[1] cellular calcium homeostasis[2] and lumenal oxidizing environment,[3] respectively. As a result, misfolded proteins are accumulated in the lumen of ER, which triggers a self-defense mechanism termed 'unfolded protein response (UPR)' which consists of the following canonical steps: i) translational attenuation,[4] ii) transcriptional activation of chaperones and degradation factors,[5,6] iii) ER-associated degradation (ERAD),[7] and iv) apoptosis.[8,9] 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 'survival' (adaptive signaling) or 'suicide' (apoptotic signaling).[10] The activated PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α) which attenuates protein synthesis to reduce ER protein overloading[11] 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).[12] ATF6 is also involved in activating the transcription of many UPR mediators including an ER chaperone protein, glucose-regulated protein (GRP78).[13] 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.[14]

ER stress has been implicated in a number of diseases such as neurodegeneration,[15] atherosclerosis,[16] diabetes[17] and its complications.[18–20] Recently, some ER stress reducing chemicals, referred to as chemical chaperones, have been identified for their potential activity against the related pathologies.[21] For example, evidences have suggested that 4-phenylbutyric acid (4-PBA) can exert therapeutic values against several ER stress-related conditions such as type 2 diabetes,[22] diabetic retinopathy[23] and nephropathy,[24] autophagy[25] and Alzheimer's disease.[26] Cycloheximide, a translation elongation inhibitor, reduced cell death caused by various ER stress inducers.[27] Salubrinal also protected against Tm-induced cell death by specifically inhibiting eIF2α dephosphorylation.[28] Interestingly, aspirin and its metabolite, salicylate, have been reported to reduce Tm-induced neuronal cell death.[29] In primary human adipocytes, salicylate clearly alleviated ER stress induced by lipopolysaccharide (LPS), high glucose, saturated fatty acid or Tm.[30] Salicylate was also reported to inhibit protein synthesis by inducing PERK-dependent eIF2α phosphorylation.[31] The effective concentrations of aspirin and salicylate in those

The authors declare no conflict of interest.

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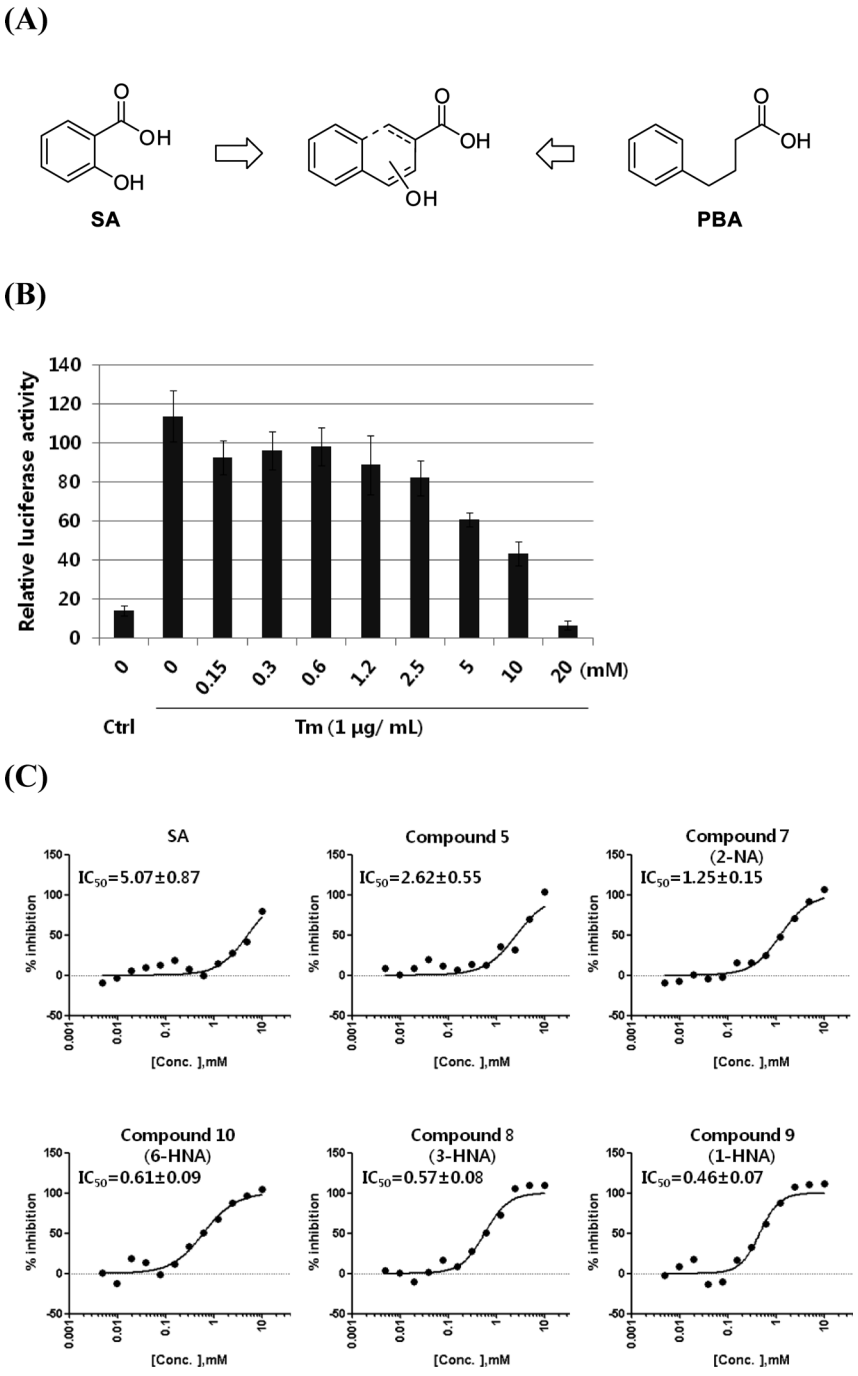


Fig. 1. (A) Chemical Structures of Salicylate (SA) and PBA

(B) Salicylate reduces Tm-induced ER stress in dose-dependent manner. 293 stable cells carrying GRP78 promoter-driven reporter were treated with indicated amounts of salicylate and Tm for 22 h. Following rLuc activity and cell viability were measured, the relative luciferase activity was calculated and plotted on the graph. Error bars were standard deviations of three independent experiments. (C) Dose–response curves of selected compounds and their IC_{50} values in our reporter assay. Percent inhibition was expressed as 100%−percent of control where ER stress was induced but no test compounds were added.

studies were, however, significantly higher than its therapeutic concentration as a non-steroidal anti-inflammatory drug (NSAID) (10–20, 0.15–0.5 mM, respectively) (Fig. 1B).

In the present study, we report the identification and preliminary characterization of novel class of ER stress-reducing agent, hydroxynaphthoic acids (HNAs). We have developed a cell-based reporter assay based on *renilla* luciferase (rLuc) activity driven by GRP78 gene by which we conducted a high throughput screening. We have found that hydroxynaphthoic acids (HNAs) exhibited better activities than salicylate in the reporter assay and that they also reduced various UPR markers in protein and mRNA level in HepG2 cell. We propose this study will provide basis for a scaffold of novel chemical chaperones and a potential perturbation against ER stress related pathologies.

Results and Discussion

Cell-Based Reporter Assay and High Throughput Screening GRP78 is one of the most important chaperones and plays a crucial role in various UPR mechanisms such as PERK, ATF6, and IRE1 pathways. Based on the notion, we established a cell-based reporter assay. 293 cells were stably

transfected with rLuc reporter driven by human GRP78 promoter (−137~+25) harboring three ER stress response elements (ERSEs) in tandem. Using this assay system, a library of *ca*. 20000 compounds was screened in 384-well format at 20 mM concentration to identify ER stress-reducing compounds. Compounds that decreased luciferase signal induced with tunicamycin by more than two-fold of the plate mean were selected for further testing. Compounds were tested in dose–response experiments, and strong hits with IC_{50} values smaller than 2 mM were identified (IC_{50} of salicylate=5.1 mM). Toxic compounds that had a CC_{50} value less than three-fold the EC_{50} value were excluded. Of the compounds screened, hydroxynaphthoic acid analogues met the efficacy and toxicity criteria and were chosen for further analysis.

Salicylate Reduces ER Stress in HepG2 Cells We wanted to confirm the results of reporter assay in protein level in human liver carcinoma HepG2 cells. First, we have tested salicylate to check if it reduces the Tm-induced ER stress as previously reported in other cell types.[29,30] As shown in Fig. 2, ER stress was induced by Tm, which was characterized by the phosphorylation of PERK, an adaptive signal transducer, and up-regulated CHOP, a pro-apoptotic factor. GRP78 was also induced by Tm treatment. PERK phosphorylation occurred at early time points whereas CHOP and other markers have changed in a prolonged period after Tm treatment when ER stress began to damage the cells (data not shown). In this study, PERK phosphorylation was analyzed at 4 h post-treatment while the other markers and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a loading control, at 22 h post-treatment. High concentrations of salicylate clearly reduced the phosphorylation of PERK and the level of GRP78 and CHOP, indicating that it decreases Tm-induced ER stress in HepG2 cells (Fig. 2).

Structure–Activity Relationship Study We envisioned that hydroxynaphthoic acids can be considered as a hybrid structure between 4-PBA and salicylate (Fig. 1A) and decided to elucidate the structural requirement for the ER stress reducing activity using the reporter assay. Salicylate supressed the ER stress signal in a dose-dependent manner (Figs. 1B, C) which, in line with Fig. 2, strongly supports the robustness of our reporter assay and its feasibility as a screening tool for ER stress-reducing compounds.

About twenty analogues of salicylate which are varied in aromatic ring, aliphatic chain, and the position of hydroxy group were tested in our reporter assay and their ER stress-reducing activities are listed as their IC_{50}'s in Table 1. Dose–response curves of the representative compounds are shown in Fig. 1C. A substitution of phenol moiety of SA with a pyridinol significantly decreased the activity (Table 1, compound **1**). A methylene spacer between the phenyl ring and carboxylic acid of SA resulted in a marginal decrease in activity (compound **2**), and its *para*-hydroxy analogue completely lost the activity (compound **3**). Addition of another methylene group slightly increased the activity (compound **4**), and the introduction of an α,β-unsaturated system further increased the activity (compound **5**). Interestingly, a *para*-hydroxy analogue of compound **5** again completely lost the activity (compound **6**), suggesting the consequence of the proximity between the hydroxy group and the carboxylic acid group. Compound **5** lead us to a combination of salicylic acid and PBA toward a bicyclic system (Fig. 1A). Indeed, 2-naphthoic acid (2-NA, compound **7**) further decreased IC_{50}, showing about 4-times better activity than SA. Installation of a hydroxy group at *ortho*-position to the acid group of 2-NA afforded compound **8** (3-HNA), a direct bicyclic analogue of SA, which showed almost nine-fold better activity than SA. Its regioisomer, compound **9** (1-HNA), showed even better activity; more than an order of magnitude better activity than SA, confirming the significance of hydroxy group for the activity. 6-HNA (compound **10**) which does not have *ortho*-hydroxy acid functionality also showed a significant activity, which suggested more generous regioisomeric requirement for hydroxy-acid functional group in the bicyclic 2-NA system than monocyclic SA. However, the position of the acid group is critical; 1-naphthoic acid (compound **11**) showed several times weaker activity than 2-NA (compound **7**). The addition of a hydroxy group at *ortho*-position to the carboxylic acid once again increased the activity (compound **12**), which, however, is still about 2.5-times weaker than its regioisomer, 3-HNA. Capping the hydroxy group as a methoxy group (compounds **13**, **14**) dramatically decreased the activity, suggesting a requirement for a hydrogen bonding donor. However, compound **15** which has an amino group afforded only 25% of the activity of the hydroxy analogue (3-HNA), indicating a hydroxy group is a specific prerequisite for the activity. Putting an additional hydroxy group on 3-HNA system more or less decreased the activity depending on the position of the two hydroxy groups (compounds **16**, **17**).

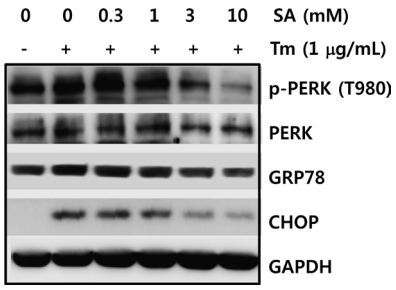
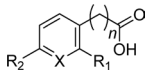
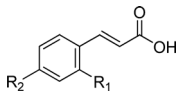


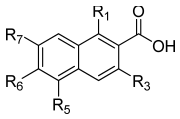
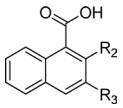
Fig. 2. Salicylate Inhibits Tm-Induced UPR Activation in HepG2 Cells

Salicylate was treated to HepG2 cells in the media containing Tm (1 μg/mL) and cells were harvested at 4 h for PERK and at 22 h for other markers after salicylate treatment. Cell lysates were analyzed by Western blot using the indicated antibodies.

HNA Decrease ER Stress Markers Next, we investigated the effects of the active compounds identified from our reporter assay on cellular ER stress markers. The compounds were treated (0.3 mM final concentration) along with Tm (1 μg/mL) to HepG2 cells and the changes of UPR markers were analyzed by Western blot (Fig. 3A). When sensing ER stress, GRP78 which normally binds to PERK is released from the complex, which is followed by the oligomerization and trans-phosphorylation of PERK. Our compounds successfully diminished the phosphorylated PERK and GRP78 both of which were induced by Tm (Fig. 3A). CHOP were also attenuated in line with well-documented PERK-eIF2α-AFT4 pathway which is usually implicated in apoptosis. In particular, 3-HNA (compound **8**) and 1-HNA (compound **9**) showed very potent activities in decreasing the levels of CHOP, GRP78, and phosphorylated PERK. The degree of those changes was in concert with the results of our reporter assay, which demonstrated the robustness of our reporter assay. On the other hand, the activities of 2-NA (compound **7**) and 6-HNA (compound **10**) seem

Table 1. Chemical Structures and IC_{50} Values of HNA Analogues Tested in Our Reporter Assay Are Shown

Structure	Compd. No.	*n*	R_1	R_2	X	IC_{50} (mM)	Fold increase of activity (IC_{50SA}/IC_{50})
	SA	0	OH	H	CH	5.07	1
	1	0	OH	H	N	19.4	0.3
	2	1	OH	H	CH	5.66	0.9
	3	1	H	OH	CH	>20	<0.3
	4	2	OH	H	CH	3.45	1.5
	**5**	—	OH	H	—	2.62	1.9
	6	—	H	OH	—	>20	<0.3

Structure	Compd. No.	R_1	R_2	R_3	R_5	R_6	R_7	IC_{50} (mM)	Fold increase of activity (IC_{50SA}/IC_{50})
	**7**	H	—	H	H	H	H	1.25	4.1
	8	H	—	OH	H	H	H	0.57	8.9
	9	OH	—	H	H	H	H	0.46	11.0
	10	H	—	H	H	OH	H	0.61	8.3
	13	H	—	OMe	H	H	H	5.65	0.9
	15	H	—	NH_2	H	H	H	2.38	2.1
	16	H	—	OH	H	H	OH	1.00	5.1
	17	H	—	OH	OH	H	H	2.30	2.2
	**11**	—	H	H	—	—	—	4.03	1.3
	12	—	OH	H	—	—	—	1.44	3.5
	14	—	OMe	H	—	—	—	5.65	0.9

Cytotoxicity expressed as CC_{50} values were more than 30 mM for all compounds except for salicylate (*ca.* 10 mM).

to be rather focused on GRP78. The multiple activities of compounds **8** and **9** might be caused by their *ortho*-hydroxy-acid structures which is absent in compounds **7** and **10**. Albeit an *ortho*-hydroxy-acid structure, salicylate was virtually inactive in regulating the level of GRP78, CHOP, and phosphorylated PERK at 0.3 mM concentration (Fig. 3A), indicating the intrinsic limitation of the monocyclic scaffolds. Densitometry analysis (Fig. 3B) confirmed that 1-HNA and 3-HNA decreased phosphorylated PERK to about 50% of control level while 2-NA and 6-HNA afforded only comparable level to the control. Effects on GRP78 and CHOP showed the similar pattern in that both 1-HNA and 3-HNA were the most effective, showing more than double the activity than SA, while 6-HNA seemed less effective than 1-HNA and 3-HNA.

1-HNA Down-Regulates the Level of IRE1*α* and XBP1 and Splicing of mRNA of XBP1 1-HNA (compound **9**), as a representative, was further characterized in Western blot (Fig. 4). It turned out that, in addition to PERK pathway, it also down-regulated IRE1*α* pathway in a dose dependent manner (Fig. 4A). Upon sensing ER stress, IRE1*α* is activated by autophosphorylation, which results in splicing and activation of a transcription factor, XBP1. Treatment of 1-HNA dramatically decreased the levels of XBP1 and phosphorylation of IRE1*α* (Fig. 4A). It is noticeable that the levels of CHOP, XBP1, phosphorylated IRE1*α*, and phosphorylated PERK were all decreased even at 0.1 mM, lower concentration than previous experiments. The splicing of XBP1 pre-mRNA to mature mRNA by phosphorylated IRE1*α* is another crucial event in UPR, which results in the transcriptional activation of ERAD components. It was also found that 1-HNA indeed suppress the splicing of pre-mRNA of XBP1 gene (Fig. 4B).

A few chemical versions of chaperone molecules have been identified and the mechanisms by which they work have been suggested. However, little is known for molecular targets responsible for their activities. For example, studies on the target or binding partner of 4-PBA and salicylate are hard to encounter in literatures. A typical chemical biology approach to identify the target of a compound is a pull-down assay using affinity chromatography. To this end, insoluble resin is usually attached to a compound through a linker moiety which is linked to a compound at the position tolerable to chemical modification. This study suggested that, in HNA structure, with a carboxylic acid group at *C*(2)-position, *C*(6)-position (Table 1, compound **10**) can be quite tolerable to chemical modification without a significant loss of activity and, therefore, can be a good candidate position to attach a linker moieties such as ethylene glycol unit. Since HNAs seem to decrease ER stress markers prior to UPR activation, it might be possible that the target molecule with which HNAs interact is a master controller of these pathways.

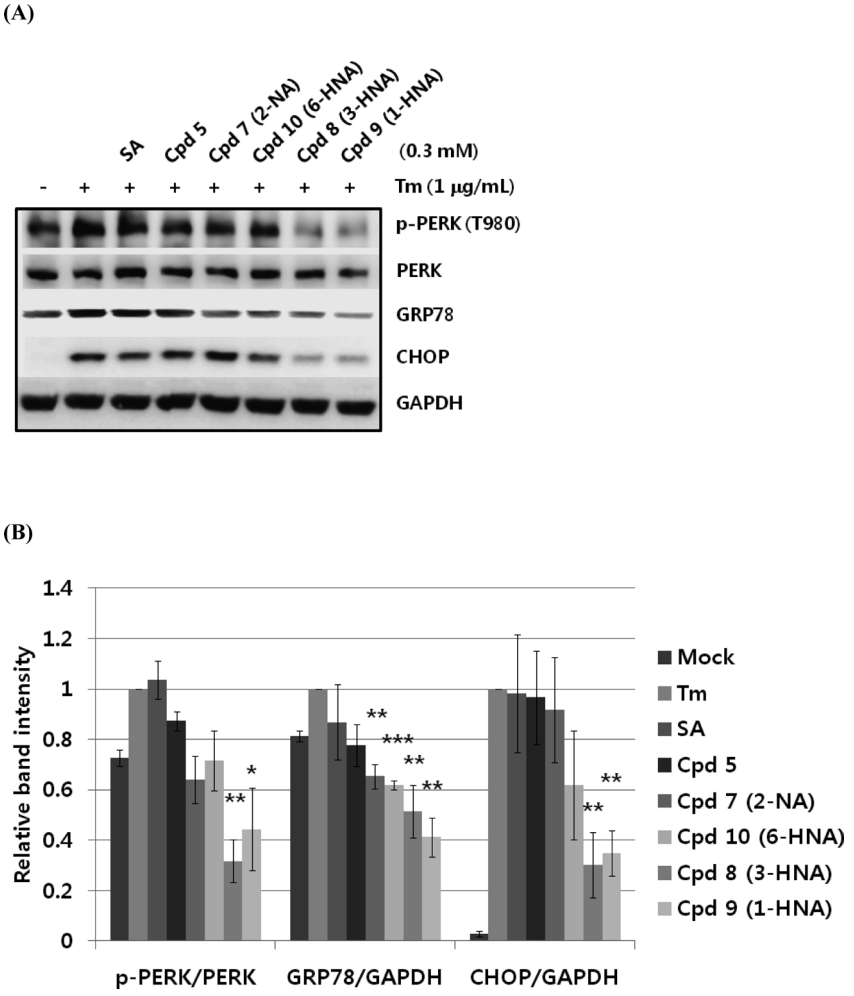


Fig. 3. (A) Six Selected Compounds Were Analyzed in Western Blot for the Changes in UPR Markers in HepG2 Cells

(A) Experimental procedure was identical to that of Fig. 1B. The representative data set out of three independent experiments was shown. (B) Densitometry analysis of Western blot data of Fig. 3A. Data are mean±S.D. (n=3) from three individual experiments. *p<0.05, **p<0.01, ***p<0.001.

Conclusion

In this study, we have developed a cell-based reporter assay to identify ER stress-reducing compounds and found that hydroxy-2-naphthoic acids (HNAs) possess potent activities. HNAs showed IC_{50}'s≅0.5 mM in our reporter assay and alleviated UPR markers including PERK, GRP78, and CHOP in Western blot as well. 1-Hydroxy-2-naphthoic acid (1-HNA), the most potent analogue, down-regulated XBP1 and IRE1α phosphorylation, and protected XBP1 mRNA from being spliced even at 0.1 mM concentration. These data suggest that HNAs, in particular 1-HNA, have activities as chemical chaperone which block the upstream of UPR pathway. Because some chemical chaperones have been implicated in the treatment of ER stress-mediated diseases, future works will warrant the therapeutic values of hydroxy-2-naphthoic acids in more physiologically relevant conditions.

Experimentals

Chemicals Tunicamycin and the compounds used in screening were purchased from commercial vendors and used without further purification unless mentioned otherwise.

Cell Culture 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.

Dual Reporter Assay System Synthesized human grp78 promoter (−137~+25) containing three ER stress response elements (ERSE) in tandem was cloned into pGL4.79 vector (Promega). The plasmid was transfected in 293 cells using Superfect™ reagent (Qiagen). To select stable cells, G418 was applied to the culture medium at a concentration of 400 μg/mL. Selected clones were screened to confirm whether they carried the GRP78 promoter driven rLuc expression cassette as following. Five thousand selected cells per well were seeded in white 384-well plate (Greiner). After 24 h, Tm or DMSO, a negative control, was added (1 μg/mL final concentration). Following 22 h incubation, 60 μM of EnduRen™ live cell substrate (Promega) was added to medium and, after 2 h incubation, rLuc activity was measured using EnVision® (Perkin-Elmer). To normalize the rLuc activity, total viable cells were counted using Celltiter-glo™ reagent (Promega). Clones showing high level of Tm-induced promoter induction were expanded and freeze-stored. ER stress-reducing activity was expressed as percent of control where ER stress was induced but no test compounds were added. For IC_{50} values, serially diluted compounds were added along with Tm and measured

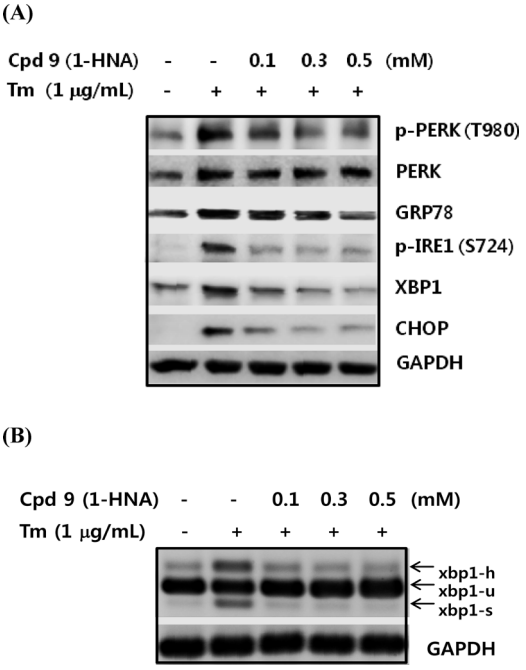


Fig. 4. 1-HNA (Compound **9**) Reduces Tm-Induced ER Stress

(A) Increasing doses of 1-HNA was added to Tm-treated HepG2 cells. Cell lysates were analyzed by Western blot by using indicated antibodies. (B) Following the same treatment as Fig. 4A, spliced xbp-1 mRNA was detected by RT-PCR. The PCR products of xbp1 mRNA were shown as three bands: xbp1-h (hybrid), xbp1-u (unspliced) and xbp1-s (spliced). xbp1-h is considered as a hybridized product of xbp1-u and xbp1-s which is formed during annealing in the final PCR step.[33] GAPDH is a loading control.

likewise. IC_{50}'s were calculated using Prism® 5 software (Graphpad Software Inc.).

High Throughput Screening 293 cells described above were suspended in IMDM media containing 10% FBS, penicillin (50 U/mL), and streptomycin (50 μg/mL) and plated in 384 well plates (5000 cells/well). After 24 h, Tm (1 μg/mL final concentration) and test compounds (20 μM final concentration, chemical library of *ca.* 2×10^4 commercial and in-house compounds) were added to plates. After incubation for 2 d, 60 μM of EnduRen™ live cell substrate (Promega) was added to medium and, after 2 h incubation, rLuc activity was measured using EnVision® (Perkin-Elmer). HTS assay quality was confirmed by measuring the coefficient of variation (CV, 10%), signal to noise ratio (>90), and *z*-factor (0.56) with DMSO as positive and negative control, respectively.

Western Blot Analysis HepG2 cells were seeded in a 6-well plate at a density of 500000 cells/well. After 24 h, Tm and compounds were added and incubated for 22 h. Cells were harvested and lysed in cell lysis buffer (50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) pH 7.4, 100 mM NaF, 50 mM NaCl, 10 mM sodium pyrophosphate, 10 mM glycerol-2-phosphate, 5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 1% TritonX-100) supplemented with protease inhibitor cocktail (Roche). Following incubation on ice for 20 min, cleared lysates were boiled for 5 min with 6× sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose membrane (Bio-rad) and analyzed by Western blot. Anti-phospho PERK (Thr980), anti-PERK, anti-GRP78, anti-CHOP and anti-XBP1 were purchased from Santa Cruz Biotech. Anti-GAPDH was from Cell Signaling and an anti-phopho IRE1α (S724) was from Abcam. The band intensity of three independent experiments was quantitated using UN-SCAN-IT-Gel 6.1™ software (Silk Scientific Software) and analyzed by Student's *t*-test.

XBP1 mRNA Splicing Assay Following treatment of the compound on HepG2 cells for 22 h, total RNA was purified using Hibrid-R™ kit (Geneall) and quantified. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using Topscript one step RT-PCR kit (Enzynomics). The primer sequence for XBP1 and GAPDH and reaction condition was previously described.[32] PCR products were separated in 2.5% TBE agarose gel and ETBR-stained gels were visualized using LAS-3000 mini (FUJIFILM).

Cytotoxicity Cells were plated in new plates and treated with serially diluted compounds in a parallel manner to the reporter assay. After incubation, cells were treated with CellTiter-Glo reagent (Promega), incubated for 30 min at room temperature. Luminescence was measured using Envision (Perkin Elmer) reader. Cytotoxicity was expressed as CC_{50} values.

Protein Determination Protein concentration of the cell lysates was determined using the standard curve obtained using bovine serum albumin (BSA) as a protein standard.

Statistical Analysis Data are presented as mean S.D. and were analyzed using student *t*-test. *p* values of <0.05 were considered statistically significant. IC_{50}'s and CC_{50}'s were calculated using Prism® 5 software (Graphpad Software Inc.).

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