Regulation of IRE1α by the small molecule inhibitor 4µ8c in hepatoma cells

Claire Stewart, Andrea Estrada, Paul Kim, Dong Wang, Yuren Wei, Chris Gentile, Michael Pagliassotti*

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1 Introduction

The endoplasmic reticulum (ER) is critical for maintaining overall cellular homeostasis through its roles in calcium storage, lipid storage and trafficking, and protein folding [6-9]. Perturbations in the ER folding environment, commonly termed ER stress, elicit the unfolded protein response (UPR), an adaptive quality control system that aims to restore ER homeostasis via transcriptional and translational regulation [10-13]. In mammals, the UPR consists of three proximal transmembrane sensors: inositol-requiring ER-to-nucleus signaling protein (IRE1α), RNA-dependent protein kinase-like ER eIF-2α kinase (PERK), and activating transcription factor 6 (ATF6). Upon ER stress, activation of these three sensors elicits distinct but cooperative signaling cascades that ultimately reduce cellular stress or, in severe cases, eliminate stressed cells by inducing apoptosis [14, 15].

Although the three UPR sensors activate distinct signaling cascades, redundancies exist among the cascades in terms of the transcriptional and translational alterations they elicit (e.g. activation of IRE1α and ATF6α upregulate a common set of ER chaperones; ATF6α and XBP1 work cooperatively to regulate genes involved in quality control and degradation, as well as anterograde trafficking in the ER) [14, 15]. These redundancies have obscured our knowledge of the contribution of the individual proximal UPR sensors in maintaining cell health and affecting disease processes. Genetically modified animals and cells that lack particular UPR sensors have
provided some insight into the relative importance of each UPR branch, but evidence suggests that these approaches may be limited because of compensatory responses by non-targeted UPR components [14-16]. To overcome these obstacles, considerable attention has been given to the development of small-molecule inhibitors that directly and acutely target specific UPR sensors. Recent high-throughput screens identified 4μ8c, a small-molecule inhibitor of the endoribonuclease (RNase) activity of IRE1α, leaving the kinase activity unaffected [1, 3]. This inhibitor prevented the downstream splicing of the transcription factor X-box binding protein 1 (XBP1) and reduced or prevented IRE1α-mediated mRNA degradation (i.e. regulated IRE1α-dependent decay of mRNA or RIDD) [1, 3]. The IRE1α-XBP1 signaling pathway is involved in regulation of chaperone expression, ER-associated degradation, lipogenesis, and ER membrane biogenesis and expansion [17-19]. Although much less is known about the regulation and downstream consequences of RIDD, recent studies have suggested that RIDD can target mRNA encoding proteins that are localized in the cytosol, nucleus or ER and is involved in ER homeostasis under both basal and stressed conditions [20-22]. Recently, studies have demonstrated that 4μ8c attenuated the growth of multiple myeloma and pancreatic beta cells during ER stress, linking IRE1α RNase activity to cell proliferation [3, 4]. This inhibitor reduced IRE1α RNase activity in multiple cell culture models [1-5], but has not been examined in unstressed and stressed hepatoma cells, a cell type often used to identify mechanisms involved in chronic liver diseases and regulation of cell survival. Therefore, the aims of this study were to determine the effectiveness of this inhibitor under basal conditions (unstressed) as well as chemically-induced ER stress and to examine the role of IRE1α RNase activity in regulation of cell proliferation in H4IIE cells.

2 Materials and Methods

2.1 Cell Culture

H4IIE cells (American Type Culture Collection, Manassas, VA), a rat liver hepatoma cell line, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 8 mM glucose and supplemented with 10% fetal bovine serum, penicillin, and streptomycin sulfate. Each experiment was performed in triplicate at 80-100% confluency.

2.2 Experimental agents

Thapsigargin (780nM), a tumor promoting sesquiterpene lactone that inhibits the ER-associated calcium ATPase, was used to induce ER stress [23]. The IRE1α inhibitor, 7-Hydroxy-4-methyl-2-oxo-2H-1-benzopyran-8-carboxaldehyde (4μ8c) was purchased from TOCRIS Bioscience (Minneapolis, MN).

2.3 RNA isolation and analysis

Total RNA was extracted with TRIzol reagent using the manufacturer’s protocol (Invitrogen, Carlsbad, CA). For Real Time PCR, reverse transcription was performed using 0.5 µg of DNase-treated RNA, Superscript II RNase H- and random hexamers. PCR reactions were performed using the Bio-Rad CFX Connect Real Time analyzer and IQ-SYBR green master mix (Bio-Rad, Hercules, CA). PCR efficiency was between 90% and 105% for all primer and probe sets and linear over 5 orders of magnitude. The specificity of products generated for each set of primers was examined for each fragment using a melting curve and gel electrophoresis. Reactions were run in triplicate and data calculated as the change in cycle threshold (DCT) for the target gene relative to the DCT for β2-microglobulin (control gene) according to the procedures of Muller et al. [24]. Target genes (XBP1s, YAP1, MKI-67 , TOP2A, CHOP , GADD34, GRP-78) were normalized to β2-microglobulin. Primer sequences are listed in table 1.

2.4 Cell proliferation

The Cell Proliferation ELISA 5-bromo-2′-deoxyuridine (BrdU) colorimetric kit (Sigma-Aldrich, St. Louis, MO) was used to measure cell proliferation. This assay is based on BrdU incorporation into DNA in place of thymidine in replicating cells. Briefly, cells were grown in 96-well plates and were labeled with BrdU for 4 hours. The labeling medium was removed and cells were fixed using FixDenat. The anti-BrdU-POD antibody was added which formed a complex with newly synthesized cellular DNA that contained BrdU. The reaction complex was detected via absorbance at 450 nm using a multi-well spectrophotometer (Thermo Labsystems MultiskanEX model #355, Thermo Fisher Scientific, Waltham, MA).
2.5 Western blot analysis

Cells were washed with phosphate-buffered saline (PBS) and harvested using a lysis buffer containing 50 mM Tris-HCl pH 8.0, 1% SDS, and previously described protease and phosphatase inhibitors [25]. Equivalent amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes (EMD Millipore; Billerica, MA). The membranes were incubated with antibodies against total and phosphorylated eukaryotic initiation factor-2α (eIF2α sc-133132; Santa Cruz Biotechnology, Santa Cruz, CA; p-eIF2α NB110-56949; Novus Biologicals, Littleton, CO), or C/EBP homologous protein (CHOP #ab11419; Abcam, Cambridge, UK). The following day, membranes were washed in Tris-buffered saline and Tween 20 (TBST) pH 7.4, incubated for 1 hour with secondary detection antibodies ((IRDye 680 CS-conjugated anti-rabbit, IRDye 800 CS-conjugated anti-rabbit, IRDye 680 CS-conjugated anti-mouse, or IRDye 800 CW-conjugated anti-mouse (LI-COR Biosciences, Lincoln, NE)), washed in TBST, and scanned for infrared signal using the Odyssey Imaging System (LI-COR Biosciences). Density was quantified using Image Studio (LI-COR Biosciences).

2.6 Cell viability

Cell viability was measured using Trypan Blue Exclusion (Thermo Fisher Scientific). Briefly, 0.4% trypan blue solution (pH 7.2-7.3) was added to 1 mL of 90%-100% confluent cells and then examined under a microscope. Using a hemocytometer, at least 1 quadrant of 16 units containing on average 50-100 cells were counted manually and percent viable cells was calculated as:

Trypan blue positive cells (%) = (number of blue cells / number of total cells) x 100

2.7 Data analysis and statistics

Statistical comparisons were calculated using individual unpaired t tests to compare means between the presence and absence of 4μ8c within treatment groups. Analysis of variance (ANOVA) was used to analyze the group means among a range of concentrations of 4μ8c. Statistical significance was set at P < 0.05. All data are reported as means ± SD.

3 Results

Recently published data have demonstrated that the IRE1α inhibitor, 4μ8c, directly targeted IRE1α endoribonuclease activity and reduced XBP1 splicing (XBP1s) in mouse embryonic fibroblasts (MEFs) [3] and multiple myeloma cells (MM) [1]. To confirm the efficacy of this inhibitor in H4IIE cells, we first examined the ability of 60 µM 4μ8c to inhibit IRE1α-mediated XBP1s. As shown in Figure 1, incubation with 4μ8c significantly decreased XBP1s at 2, 4, and 6 hours in control cells (LG) and in cells treated with the ER stress inducer, thapsigargin (Thap). These time points were chosen because they characterize a period of time in which active cell proliferation and minimal cell death occurs in Thap-treated cells.

Given 4μ8c’s ability to inhibit cell proliferation in MM cells during ER stress [3], we next investigated whether 60 µM 4μ8c also inhibited cell proliferation in H4IIE cells. The presence of the inhibitor at 60 µM significantly reduced cell proliferation in both LG and Thap conditions (Figure 2). These data suggest that IRE1α regulates cell proliferation in both unstressed and stressed H4IIE cells. We next examined a group of genes markers of cell
proliferation: Topoisomerase 2A (TOP2A), ki-67 (MKI67), and yes-associated protein 1 (YAP1). The presence of 4μ8c did not induce changes in any of these markers in LG or Thap (Supplementary Table 1).

Although previous studies used a range of inhibitor concentrations up to 120 μM, the concentration of 4μ8c that produced a 50% reduction in IRE1α RNase activity in other cell types was ~7 μM (1, 3, 4). We therefore performed additional experiments across a range of inhibitor concentrations in H4IIE cells. Equivalent inhibition of XBP1s was observed with concentrations of 4μ8c ranging from 10-90 µM (Figure 3). We also examined 14 putative RIDD-targeted mRNAs (Supplementary Table 2) of which 3 displayed changes consistent with RIDD targets. Carboxylesterase 1 (CES1c) and Biogenesis of Lysosome-Related Organelles Complex 1 Subunit 1a and Subunit 1c (BLOC1s1a, BLOC1s1c) were reduced in the presence of thapsigargin and normalized by 10 µM 4μ8c (Figure 4).

No change was observed in the phosphorylation state of IRE1α with low (10µM) and high (60µM) concentrations of 4μ8c (Figure 5). In contrast to the consistent reduction of IRE1α RNase activity observed across all concentrations of 4μ8c, a dose-dependent inhibition of cell proliferation was observed in LG and Thap (Figure 6). Importantly, cell proliferation was not significantly reduced at concentrations of 4μ8c (10-30 μM) that produced maximal inhibition of IRE1 RNase activity in LG (Figure 6). Consistent with past publications [1, 3], all concentrations of 4μ8c significantly decreased cell proliferation in the presence of Thap. In addition, trypan blue positive cells were not significantly increased at any of the concentrations of 4μ8c in LG or Thap (Supplementary Figure 1).

### Table 1: Primers used in qRT-PCR.

| Gene            | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| XBP1s           | 5'-GTCTGCTGAGCGACGACGG-3'                                                |
|                 | 5'-GATTAGCAGACTTGGGGAAG-3'                                               |
| YAP1            | 5'-TCATGCCAGCAAGCAAAATG-3'                                              |
|                 | 5'-CATCTGAGTCCCTCCATCC-3'                                               |
| MKI-67          | 5'-CTGCAGAGAAGGTTGGGATAA-3'                                              |
|                 | 5'-CTGACCTTGGCCAGAGATGAA-3'                                              |
| TOP2A           | 5'-GAACAGCCAGTAGGAAATAC-3'                                              |
|                 | 5'-GTGAAATCTCCTCGCGTAAGA-3'                                              |
| CHOP            | 5'-CCAGCAGAGGTCACAAAGCAC-3'                                              |
|                 | 5'-CGACCTGACACTCTGTTC-3'                                                |
| GADD34          | 5'-CTTCTCTCTCGTTCTCTGTC-3'                                              |
|                 | 5'-CCGGCTCTCCTCCCAATGTC-3'                                              |
| GRP-78          | 5'-AACCAGATGAGGTCTGAGCA-3'                                              |
|                 | 5'-ACATCAAGCAGAACCAGGTCA-3'                                              |

**Fig 2** Effects of the IRE1α inhibitor 4μ8c on cell proliferation. H4IIE liver cells were incubated for 4 hours in low glucose (LG) control media or LG supplemented with thapsigargin (Thap; 780 nM), in the absence (-) or presence (+; 60 µM) of 4μ8c. Data represent mean ± SD for 3 independent experiments. *= P < 0.05 from condition minus 4μ8c. The lack of a SD for BLOC1s1a and BLOC1s1c in the 10 uM condition was do to the fact that the values from the 2 experiments were the same.

**Fig 3** Effects of 10-90 µM 4μ8c on XBP1s. H4IIE liver cells were incubated for 6 hours in low glucose (LG) control media or LG supplemented with thapsigargin (Thap; 780 nM) in the absence (-) or presence (+; 10 µM-90 µM) of 4μ8c. Data represent mean ± SD for 3 independent experiments. *= P < 0.05 from condition minus 4μ8c.
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Our data suggests that 4µ8c exerts effects on cell proliferation that are independent of IRE1α-mediated RNase activity. Therefore, we next examined whether 4µ8c influenced other components of the unfolded protein response. Activation of the PERK branch of the UPR results in a global but transient reduction in protein synthesis mediated by the phosphorylation of eIF2α (p-eIF2α) [26]. The presence of 60µM 4µ8c increased p-eIF2α in LG and Thap conditions (Figure 7b). In contrast, 10µM 4µ8c did not result in significant changes of p-eIF2α (Figure 7a).

Additionally, we examined genes that are typically upregulated in the presence of ER stress. C/EBP homologous protein (CHOP) is a transcription factor involved in the regulation of metabolism and apoptosis and is typically upregulated during ER stress [27]. CHOP mRNA increased ~5 fold with 60µM 4µ8c and ~18 fold with 90µM 4µ8c in LG (Figure 8). In the presence of Thap, all concentrations of 4µ8c resulted in a significant decrease in CHOP mRNA expression (Figure 8b). CHOP protein expression was also measured and although it was not significant, a similar trend was observed (Figure 8c). Similar trends were observed for growth arrest and DNA damage-inducible protein (GADD34), a protein that plays a role in ER-mediated cell death via reversal of translation inhibition by dephosphorylation of eIF2α [13], and 78 kDa glucose-regulated protein (GRP-78), a molecular chaperone that is induced upon ER stress [13] (Supplementary Table 3).
Fig 7 Effects of 10 μM (a) and 60 μM (b) 4μ8c on eIF2α phosphorylation (p-eIF2α). H4IIE liver cells were incubated for 4 hours in low glucose (LG) control media or LG supplemented with thapsigargin (Thap; 780 nM). All conditions represent the fold change of the ratio of p-eIF2α to total eIF2α in the absence (-) or presence (+; 60 μM) of 4μ8c. The representative gel was a single gel that was split, as other samples were run in between LG and Thap samples. Data represent mean ± SD for 3 independent experiments. *= P < 0.05 from condition minus 4μ8c.

Fig 8 Effects of 10-90 μM 4μ8c on gene and protein expression of CHOP. H4IIE liver cells were incubated for 6 hours in low glucose (LG) control media (a) or LG supplemented with thapsigargin (Thap; 780 nM) (b) in the absence (-) or presence (+; 10 μM-90 μM) of 4μ8c. A representative blot of protein expression is shown in c. Data represent mean ± SD for 2 independent experiments. *= P < 0.05 from condition minus 4μ8c.
4 Discussion

In the present study we investigated the role of IRE1α RNase activity in the regulation of cell proliferation under control and ER stress conditions in H4IIE cells using a small-molecule inhibitor, 4μ8c. Three independent experiments were performed in the absence and presence of chemically-induced ER stress. The results demonstrate that 4μ8c successfully inhibited IRE1α RNase activity in H4IIE cells under control conditions as well as during ER stress. Further, we demonstrate that while IRE1α RNase activity is involved in the regulation of cell proliferation during Thap-induced ER stress it does not play a significant role in the regulation of cell proliferation in unstressed cells. Lastly, we demonstrate that higher concentrations of 4μ8c result in effects on both cell proliferation and the UPR that appear to be independent of IRE1α RNase activity.

Cross et al., characterized 4μ8c as an effective inhibitor of IRE1α RNase activity base on reduced splicing of the downstream effector XBPI and reduced RIDD in MM and/or MEF cell lines [1]. Our first goal was to determine the effectiveness of this inhibitor in H4IIE cells. Our results indicate that 4μ8c reduced XBPIs across a wide range of concentrations (10-90 µM) and time points (2-6 hours) in both unstressed and ER stressed H4IIE cells. In addition, 4µ8c prevented the thapsigargin-induced reduction of three putative RIDD-targeted mRNAs [20, 28]. This extends previous reports by demonstrating that 4µ8c is a potent inhibitor of IRE1α RNase activity in a hepatoma cell line [1, 3, 4]. Previous studies have observed a Kₐ for 4µ8c of ~7µM, which approximates the lowest concentration used in the present study (1).

The regulation of IRE1α RNase activity and the downstream outputs that arise as a result of IRE1α RNase activity involve complex protein-protein interactions, as well as post-translational modifications to IRE1α [29-31]. IRE1α has been linked to the regulation of cell proliferation and ER membrane expansion in both unstressed and ER stressed cells [12, 32]. However, whereas IRE1α-mediated XBPI splicing has been linked to most prosurvival outputs, RIDD appears to be associated with apoptosis [20]. Although the present study was not designed to examine the relative roles of XBPIs and RIDD in cell fate decisions, our results provide new evidence for the regulation of cell proliferation in H4IIE cells by the small molecule inhibitor 4μ8c. In the present study, 4μ8c inhibited cell proliferation in H4IIE cells treated with thapsigargin, a finding consistent with previous work in MM and MEF cells [1]. In contrast, the concentration of 4μ8c that resulted in inhibition IRE1α RNase activity (10µM) had no effect on cell proliferation in unstressed (control) H4IIE cells. Higher concentrations of 4μ8c (60µM) reduced cell proliferation in unstressed (control) H4IIE cells, suggesting that this inhibition is likely independent of IRE1α RNase activity, and perhaps the result of off-target effects.

Given that we observed effects of 4μ8c on cell proliferation that appeared to be independent of IRE1α RNase activity, we also assessed the effects of 4μ8c on additional markers of UPR activation: p-eIF2α, CHOP, GADD34, and GRP-78 [13, 33]. Under control conditions, we observed a significant increase in CHOP mRNA and protein expression and GADD34 mRNA with 90 µM 4µ8c in H4IIE cells. Since IRE1α-mediated XBPIs and RIDD were equivalently reduced with 10 µM and 90 µM 4µ8c, these data suggest that changes in CHOP and GADD34 involve off-target effects of 4µ8c. In contrast to control conditions, these same UPR markers were reduced by 10 µM 4µ8c in cells treated with thapsigargin. It is possible that IRE1α RNase activity contributes to the upregulation of CHOP and GADD34 in response to chemical induction of ER stress. However, genetic deletion of IRE1α in the liver has typically resulted in increased expression levels of CHOP in response to chemical induction of ER stress [14]. It is also possible that the restoration of RIDD targets following exposure to 4µ8c result in suppression of CHOP and GADD34.

In summary, 4μ8c is an effective inhibitor of IRE1α RNase activity in H4IIE cells. Inhibition of IRE1α RNase activity by 4μ8c confirms that this UPR pathway regulates cell proliferation during ER stress. However, regulation of cell proliferation in unstressed cells appears to not involve IRE1α RNase activity. Importantly, higher concentrations of 4μ8c appear to have effects on both cell proliferation and UPR-related genes that are independent of IRE1α RNase activity. These effects do not involve cytotoxicity. Therefore, to understand IRE1α regulation in cellular processes using 4μ8c requires the use of multiple concentrations and assessment of a broad range of UPR markers.

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Conflict of Interest: The authors state no conflict of interest.
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Supplemental Data:

**Supplementary Figure 1:** Effects of 10-90 µM 4μ8c on cytotoxicity. H4IIE liver cells were incubated for 6 hours in low glucose (LG) control media or LG supplemented with thapsigargin (Thap; 780 nM) in the absence (-) or presence (+; 10 µM-90 µM) of 4μ8c. Data represent mean ± SD for 3 independent experiments. *= P < 0.05 from condition minus 4μ8c.

**Supplementary Table 1:** Effects of the IRE1α inhibitor 4μ8c on gene markers of cell proliferation. H4IIE liver cells were incubated for 4 hours in low glucose (LG) control media or LG supplemented with thapsigargin (Thap; 780 nM) in the absence (-) or presence (+; 60 µM) of 4μ8c. All conditions represent the fold change of the ratio of β2-microglobulin to each gene. Data represent mean ± SD for 3 independent experiments. *= P < 0.05 from condition minus 4μ8c.
**Supplementary Table 2:** RIDD substrate primers used in qRT-PCR: GPC3, Glypican 3; PDK2, Pyruvate Dehydrogenase Kinase 2; YWHAQ, Tyrosine 3-Monoxygenase/Tryptophan 5-Monoxygenase Activation Protein Theta; Geminin; PEPD, Peptidase D; RUVBL1, RuvB Like AAA ATPase 1; BLOC1s1, Biogenesis of lysosome-related organelles complex 1 subunit 1; CD59, Protectin; CES1, Carboxylesterase 1; IRE1, inositol-requiring enzyme 1; RTN4, Reticulon.

| Gene       | Sequence                  |
|------------|---------------------------|
| GPC3       | 5'- CGAATGTGGTACTGCTCTTAC - 3' |
|            | 5'- CTACCACACCTGCCACACAG - 3' |
| PDK2       | 5'- GCTGGTTTCTCGTACAGTAG - 3' |
|            | 5'- TGGTGTCAGAGTGAGTGAGG - 3' |
| YWHAQ      | 5'- CTGGCTGAACTGCTGCTTGG - 3' |
|            | 5'- GGATGTGTAAGTGCTGATCTC - 3' |
| Geminin    | 5'- CAGAGGCGACACAGAGGATAG - 3' |
|            | 5'- CGATGGAAGTAAAGACGAGAG - 3' |
| PEPD       | 5'- GGATGGAAGTAAAGACGAGAG - 3' |
|            | 5'- TGGCAGCAGAGTGAGGAGG - 3' |
| RUVBL1     | 5'- GATCGATAACACGCTCAAGACT - 3' |
|            | 5'- GCCTCCTACTGCTCTCTTCG - 3' |
| BLOC1s1a   | 5'- GGCTCATGACACAGGAGGAGG - 3' |
|            | 5'- TCCAAACTGCAACATCCTG - 3' |
| BLOC1s1c   | 5'- CAACCGCCACCTAAGGAGGATC - 3' |
|            | 5'- GCTGCCCTTGTAGAAGCTACT - 3' |
| CD59a      | 5'- CCTGTGCCACAGTGTTAG - 3' |
|            | 5'- GCAAGAGTACGAGTGGAGG - 3' |
| CD59b      | 5'- CTTGCTCCAAAGTACCCCTAA - 3' |
|            | 5'- CTACCTCAAGTGCTGTTAT - 3' |
| CD59c      | 5'- TT GGTAAGCAGCAGGAAAGG - 3' |
|            | 5'- CTCCGAAATGCAAAAGGAGG - 3' |
| CES1c      | 5'- GAAATAGCTAGCTGTTAGAGG - 3' |
|            | 5'- GTGAAGAGTGGTGGCTTCTTAC - 3' |
| IRE1       | 5'- ACCACACCGGAGACCTTAAA - 3' |
|            | 5'- ACTGGTCGACCGCTGAGGAGG - 3' |
| RTN4       | 5'- GGAGGATAGTGGAAGGGGAGG - 3' |
|            | 5'- GAGTAAAGGAGGCCAGGATGAGG - 3' |

**Supplementary Table 3:** Effects of the IRE1α inhibitor 4μ8c on gene markers of the UPR: H411E liver cells were incubated for 6 hours in low glucose (LG) control media or LG supplemented with thapsigargin (Thap; 780 nM) in the absence (-) or presence (+; 10 μM-90 μM) of 4μ8c. All conditions represent the fold change of the ratio of β2-microglobulin to each gene. Data represent mean ± SD for 3 independent experiments. *= P < 0.05 from condition minus 4μ8c.

| 4μ8c Concentration | Gene ; Treatment |
|--------------------|------------------|
|                    | - 4μ8c | 10μM | 30μM | 60μM | 90μM |
| 1 ± 0 | 0.8 ± 0.3 | 0.95 ± 0.3 | 1.84 ± 0.2 | 2.13 ± 0.1* | GRP78 ; LG |
| 10.8 ± 5.0 | 6.0 ± 3.5 | 3.5 ± 2.0 | 2.6 ± 2.2 | 1.74 ± 1.2 | GRP78 ; Thap |
| 1 ± 0 | 1.1 ± 0.2 | 1.2 ± 0.03 | 2.6 ± 0.8 | 4.8 ± 3.2 | GADD34 ; LG |
| 50.6 ± 2.0 | 22.0 ± 1.0* | 12.8 ± 3.1* | 9.3 ± 1.7* | 6.7 ± 2.95* | GADD34 ; Thap |