Research article

Interaction between goniothalamin and peroxisomal multifunctional enzyme type 2 triggering endoplasmic reticulum stress

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ABSTRACT

Endoplasmic reticulum stress is one of the pathways involved in cell cytotoxicity. In this study, goniothalamin, one of styryllactone compounds found in plant *Goniothalamus spp.*, was observed to trigger ER stress in HeLa cell line. In addition, we demonstrated that peroxisomal multifunctional enzyme type2 (MFE2) was a specific goniothalamin-binding protein using an *in vitro* goniothalamin-linked bead pull-down assay. Since MFE2 has been reported to be an important mediator enzyme for peroxisomal β-oxidation of a very long chain fatty acid metabolism, therefore computational molecular docking analysis was performed to confirm the binding of goniothalamin and MFE2. The results indicated that goniothalamin structure binds to scp-2 domain, enoyl-CoA hydratase 2 domain and (3R)-hydroxyacyl-CoA dehydrogenase domain of MFE2. To further determine the effect of MFE2 on ER stress induction, MFE2 knockdown by siRNA in HeLa cell was conducted. The results implied that MFE2 triggered CHOP, a key mediator of ER stress-induced apoptosis, expression. Therefore, these data inferred that goniothalamin may interrupt the MFE2 function resulting in lipid metabolism perturbation associated with ER stress-independent activation of unfolded protein response. This is the first report to show that goniothalamin binds directly to MFE2 triggering ER stress activation probably through the lipid metabolism perturbation.

1. Introduction

Endoplasmic reticulum (ER) stress was reported to trigger cell death associated with mitochondria-mediated apoptosis pathway [1, 2]. In general, the main cause of ER stress induction is accumulation and aggregation of unfolded proteins in the ER and then activates an unfolded protein response (UPR) [3]. However, lipid perturbation also contributes to UPR activation [4]. Lipid perturbation was reported to have a cascade effect on peroxisomal lipid β-oxidation suppression via interrupting the peroxisomal lipid β-oxidation enzymes, including peroxisomal multifunctional enzyme type2 (MFE2). The roles of MFE2 is to catalyze the second and the third reactions of the peroxisomal lipid β-oxidation pathway [5]. The disturbance of MFE2 causes a very-long chain fatty acid (VLCFA) accumulation which related to the previous report that VLCFA accumulation could trigger ER stress [6]. The function of MFE2 is not only important for fatty acid degradation through β-oxidation, but also

for lipid metabolism in general too [7]. Thus, the disturbance of MFE2 is correlated with the ER stress induction via lipid perturbation.

Goniothalamin is one of styryllactone compounds which is mainly found in plant of the genus *Goniothalamus* (Annonaceae). It was reported to possess cytotoxic activities including antimicrobial [8], anticandidal [9], larvicidal [10] and anticancer activity [11, 12, 13, 14]. In the context of cytotoxic induction mechanism, goniothalamin has been reported to selectively trigger caspase induced apoptosis in cancer cells, with little activity on normal cell lines [13]. Moreover, goniothalamin was demonstrated to inhibit nucleocytoplasmic transport via targeting the chromosomal maintenance 1 (CRM1), also known as nuclear receptor Exportin 1, with a similar mechanism to a lactone compound leptomycin B and may have antiproliferative property [15]. Our previous study reported that goniothalamin induced apoptosis through mitochondria-mediated pathway associated with ER stress-induced JNK activation in cervical cancer cells [16]. Interestingly, although previous

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publication of Wach and co-worker [15] suggested that goniothalamin inhibited CRM1 with a similar mechanism to leptomycin B, however, our studies showed that only goniothalamin triggered ER stress, but not leptomycin B. Thus, the mechanism and target of goniothalamin associated ER stress remained uninvestigated.

Due to the previous publication, goniothalamin and leptomycin B could bind to CRM1 at the similar area [15]. However, there was no report indicated that suppression of CRM1 by both compounds related to ER stress induction. Thus, molecular docking analysis was carried out to determine other goniothalamin-binding proteins which may cause ER stress induction. The results demonstrated the effect of goniothalamin interaction with its binding proteins on apoptosis induction associated with ER stress.

2. Materials and methods

2.1. Cell culture, reagents and antibodies

Human cervical cancer, HeLa cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). It was maintained in Dulbecco's Modified Eagle Medium (DMEM: Gibco®, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Science, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (PAA Laboratories, AT) at 37 °C in a humidified 5% CO_2 atmosphere. The cells in the exponential growth phase were then used for subsequent assays. Goniothalamin was obtained from Assoc. Prof. Wilawan Mahabusarakam, Faculty of Science, Prince of Songkla University, Thailand in a purified powder form. It was purified from the stems of *Goniothalamus macrophyllus* as previously described [16]. Leptomycin B and tunicamycin were obtained from Sigma-Aldrich® (St. Louis, MO, USA).

Goniothalamin linked-bead and control (unlinked)-bead were provided from Prof. Makoto Muroi and Prof. Yasumitsu Kondoh, Chemical Library Validation Team, Chemical Biology Core Facility, Chemical Biology Department, RIKEN Advanced Science Institute, Japan. The beads were prepared as previously described [17]. Reagents for mRNA extraction and cDNA synthesis were purchased from QIAGEN® (Venlo, LI, NL) and Thermo Scientific™ (Carlsbad, CA, USA). Chemicals for quantitative PCR was obtained from Thermo Scientific™ (Carlsbad, CA, USA). Chemicals for immunoblotting analysis were purchased from the following distributors: mouse anti-CHOP (MA1-250) (Thermo Fisher Scientific, USA); mouse anti-KDEL (GRP94, GRP78; ADI-SPA-827) (Enzo Life Sciences, DE); rabbit anti-MFE2 (NBP1-85297) (Novus Biologicals, USA); rabbit anti-phospho-eIF2α (#9721), rabbit anti-Ero1-Lα (#3264), rabbit anti-PDI (#3501), rabbit anti-β-Actin (#4967), goat anti-rabbit IgG, HRP-linked antibody (#7074) and horse anti-mouse IgG, HRP-linked antibody (#7076) (Cell signaling Technology, USA); polyvinylidene fluoride (PVDF) membrane (ISEQ10100) and chemiluminescent HRP substrate (11536345) (Merck Millipore, DE). Chemical for RNA interference was obtained from Invitrogen™ (Carlsbad, CA, USA).

2.2. Detection of specific binding proteins for goniothalamin by pull-down assay

HeLa cells were used as a model for specific protein binding of goniothalamin. Cells were harvested and washed with PBS buffer then resuspended in detergent-free binding buffer [50mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1 mM DTT, 0.1 mM PMSF and protease inhibitor cocktail tablets (Roche Holding AG, Basel, CH); pH 7.5]. The collected cells were lysed by sonication; soluble fraction was separated by centrifugation and collected as cell lysate. Three milligram of protein in cell lysate was incubated with goniothalamin-beads or the control (unlinked)-beads for 3 h at 4 °C. The beads were washed with detergent-free binding buffer, and the binding proteins were eluted with SDS-PAGE sample buffer, and separated by SDS-PAGE, and protein pattern visualized by Coomassie Brilliant Blue R-250 staining. Protein identification was performed using MALDI-TOF-MS and LC-MS/MS as previously

described [18] to identify the specific goniothalamin binding protein. Human MFE2 was shown to be the the specific goniothalamin binding protein which then was confirmed by immunoblotting with anti-MFE2 antibody.

2.3. Molecular docking of human MFE2 and goniothalamin, compared with leptomycin B

Molecular docking was used to show the binding ability and binding site between goniothalamin and MFE2 compared with the leptomycin B. The molecular docking with Genetic Algorithm [19] was performed using AutoDock 4 Software [20] to find the most favorable binding interaction. Two ligands, goniothalamin and leptomycin B were docked into three domains of human MFE2 which are scp-2, enoyl-CoA hydratase and (3R)-hydroxyacyl-CoA dehydrogenase domains. The enzyme structures were obtained from X-ray crystallography (PDB ID: 1IKT [21], 1S9C [22] and 1ZBQ [23]). The 3D-structures of the ligands used in docking were obtained from PubChem compound database [24] (PubChem CID: 6440856 and 6917907). Docking of these ligands to all domains of the enzyme were evaluated by genetic algorithm with a standard docking protocol with a population size of 150 randomly placed individuals, crossover rate of 0.80, an elitism value of 1, a mutation rate of 0.02 and a maximum number of 2.5×10^7 energy evaluations. The grid maps representing the proteins were calculated using autogrid and size of the grid box is 126Å x 126Å x 126Å. Finally, the complex structures were constructed.

### 2.4. Detection of CRM1-dependent nuclear export of MEK1 inhibition

In general, CRM1 transports MEK1 from nucleus to cytoplasm, thus MEK1 accumulation in nucleus was observed upon CRM1 inhibition. The accumulated MEK1 in nucleus was evaluated by immunohistochemistry. Briefly, the treated cells were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS, and blocked in 3% BSA in PBS. The cells were stained with primary antibody against MEK1 (#8727, Cell signaling Technology, USA) and incubated with secondary antibody against rabbit IgG Alexa Fluor 546 (A-11010, Thermo Fisher Scientific, USA). For nuclear localization, the nucleus were stained with Hoechst 33258 (Sigma-Aldrich®: St. Louis, MO, USA) and observed under a laser-scanning confocal microscope system (Fluoview FV1200 model: Olympus, Tokyo, Japan). The MEK1 accumulation in nucleus was calculated by fluorescent intensity analysis using ImageJ software (National Institutes of Health, MD, USA).

2.5. Knockdown MFE2 expression using RNA interference

For the MFE2 knock down experiment, siRNA double-stranded oligonucleotides designed to interfere with MFE2 expression (Gene symbol: HSD17B4; Invitrogen™: Carlsbad, CA, USA) and the non-coding siRNA (Invitrogen™: Carlsbad, CA, USA) as a negative control were used. Lipofectamine® RNAiMAX™ (Invitrogen™: Carlsbad, CA, USA) was used for siRNA transfection according to the manufacturer's instructions.

2.6. Protein expression analysis by immunoblotting analysis

After treatment, the cells were lysed with a RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% $C_{24}H_{39}NaO_4$, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF). The extracted proteins were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Darmstad, DE) by blotting technique. Then, the membranes were incubated with primary then secondary antibody and incubated with Immobilon™ Western chemiluminescent HRP substrate (Merck Millipore, Darmstad, DE) and detected under Chemiluminescent Imaging System: GeneGnome gel documentation (Synoptics Ltd., MD, USA).
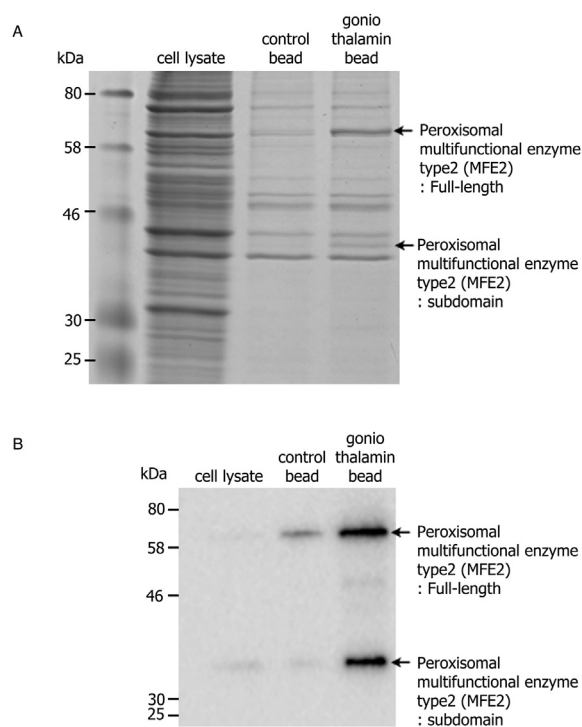
Figure 1. Identification of specific protein binding with goniothalamin-beads. After incubation of HeLa cell lysate with control (unlinked)-beads or goniothalamin-beads and eluted with SDS-PAGE sample buffer, the samples include whole cell lysate proteins to compare bead-unbound proteins, were separated on polyacrylamide gel and visualized by Coomassie brilliant blue R-250 staining. Different protein bands between control (unlinked)-beads and goniothalamin-beads were identified using MALDI-TOF-MS and LC-MS/MS, MFE2 was identified (A), and confirmed by immunoblotting with the anti-MFE2 (B). These results indicate that goniothalamin bind to human MFE2.

2.7. XBP1 splicing analysis by reverse transcription (RT)-PCR

The spliced *XBP1* mRNA was detected by RT-PCR with specific primers corresponding to the spliced site of *XBP1* gene. After treatment, the whole cells were collected and RNA was extracted by using QIAzol™ lysis reagent (QIAGEN®: Venlo, LI, NL) and cDNA synthesis by reverse transcription according RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific™: Carlsbad, CA, USA) using 2 μg of total RNA of each sample. PCR was carried out with *Taq* polymerase (Vivantis Technologies Sdn. Bhd., Selangor Darul Ehsan, MY), a pair of primers corresponding to the *XBP1* spliced site gene; forward 5′-AATGAAGTGAGGCCAGTGGCC-3′ and reverse 5′-AATACCGCCAGAATCCATGGG-3′. In details, the PCR was carried out in a 20 μl reaction volume, using 1 μl of cDNA with a PCR mixture containing 0.3 μM forward primer, 0.3 μM reverse primer, 0.25 mM deoxynucleotide triphosphate, 10 mM Tris-HCl, 50 mM KCl, 0.01% Triton X-100, 1.5 mM MgCl$_2$ and 2 units of *Taq* polymerase. The PCR thermal cycler condition: 94 °C for 2 min; 35 cycles at 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. The PCR products were separated by 8% polyacrylamide gel electrophoresis and visualized under UV transilluminator with ethidium bromide staining. Unspliced- and spliced-*XBP1* gene were indicated at 125 and 99 base pairs, respectively.

2.8. Statistical analysis

All data presented were obtained from at least three independent experiments and were presented as mean ± standard deviation (SD). Statistical comparisons were calculated based on student's *t*-test. Microsoft Excel version 2010 software (Microsoft Corporation, Redmond, WA, USA) was used to analyze the data. Statistical significance was set at *p*-value < 0.05.

3. Results

3.1. Identification of goniothalamin-binding protein

The results from goniothalamin beads and MALDI-TOF-MS and LC-MS/MS techniques indicated that MFE2 in HeLa cell lysate bound to goniothalamin specifically (Figure 1A), the details of mas spectrometry analysis is under the Supplementary data section. This binding was
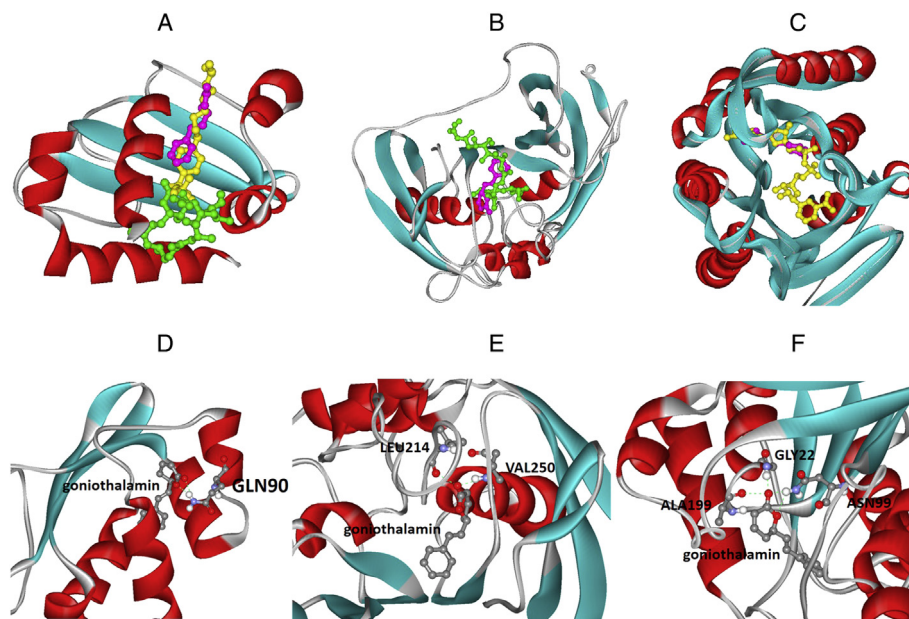
Figure 2. Comparison of the docking conformations of goniothalamin (pink) and leptomycin B (green) bound to scp-2 (A), enoyl-CoA hydratase (B) and (3R)-hydroxyacyl-CoA dehydrogenase (C) domains. Predicted binding poses are compared to the original ligand position from the X-ray structure (yellow). Schematic depiction of hydrogen bonding pattern between goniothalamin and scp-2 (D), enoyl-CoA hydratase (E) and (3R)-hydroxyacyl-CoA dehydrogenase (F).
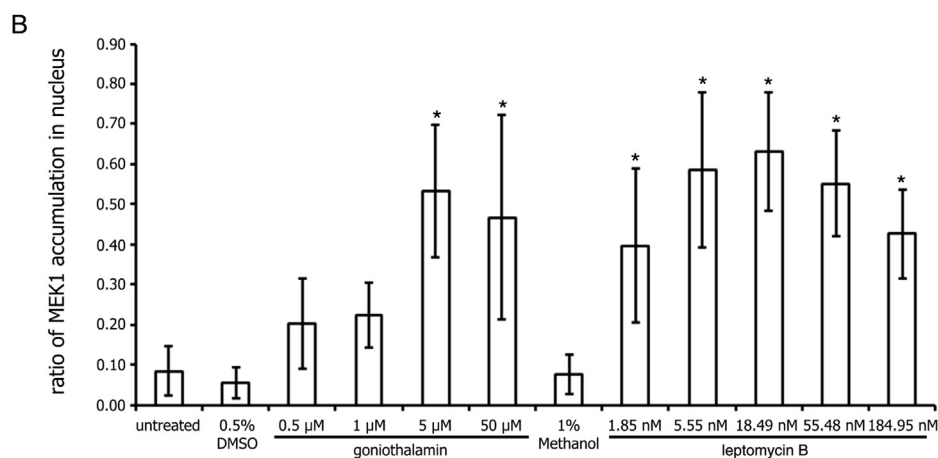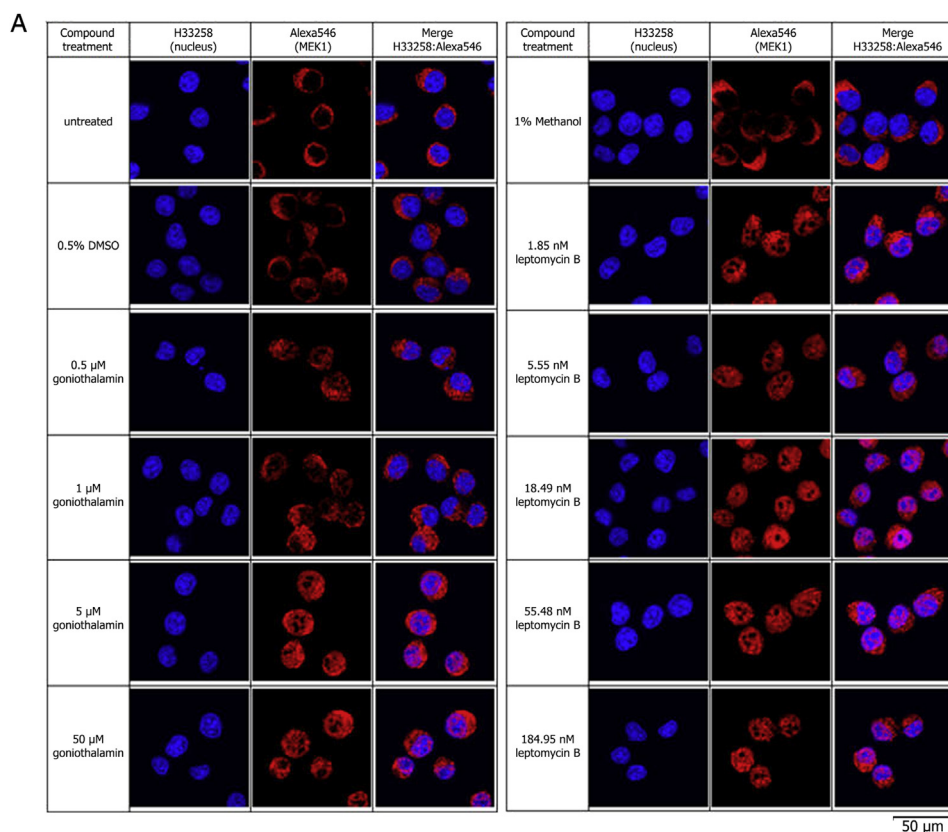
Figure 3. Immunocytochemistry staining with Hoechst33258 (H33258) and anti-MEK1against rabbit IgG Alexa Fluor 546 (Alexa546) was referred to nucleus- and MEK1- localization, respectively, in treatment of various concentration of goniothalamin and leptomycin B by using untreated cells as control condition (A). The fluorescent signal analysis of Alexa546 compared to H33258 indicated that MEK1 accumulation in nucleus (B). Data are mean ± SD. *p-value < 0.05.

confirmed by immunoblotting with MFE2 antibody (Figure 1B). Moreover, the binding ability and binding site between goniothalamin and MFE2 were evaluated by molecular docking method in the next step.

3.2. Molecular docking of goniothalamin on human MFE2

Goniothalamin and leptomycin B compounds were docked with three domains (scp-2, enoyl-CoA hydratase and (3R)-hydroxyacyl-CoA dehydrogenase domains) of human MFE2. In the scp-2 domains, the results showed that goniothalamin compound had better binding energy compared to leptomycin B compound. All of the top 10 docking conformations of goniothalamin compound were placed in the same position as the original ligand position from the X-ray structure, whereas docking conformations of leptomycin B compound could collide with different parts of protein structure and were usually found on the surface of the protein. In addition, the leptomycin B position from docking method was not the same as binding position in the X-ray structure (Figure 2A).

In the enoyl-CoA hydratase domains, all the docking processes were performed without a ligand in the active site of the X-ray structure. The results showed that docking conformations of goniothalamin compound were found in two positions of protein structure and demonstrated a better binding with enoyl-CoA hydratase domains. On the other hand, leptomycin B compound was found in varied position on the surface of the protein. In comparison to the binding pattern of two ligands with enoyl-CoA hydratase domains, most of leptomycin B docking structure protruded beyond the protein structure leading to the reduction of leptomycin B complex stability (Figure 2B).

Goniothalamin and leptomycin B were also docked to (3R)-hydrox-yacyl-CoA dehydrogenase domains. This docking analysis revealed that the poses of goniothalamin docked compound placing into two positions of the protein. One of those positions is the same position as the original ligand position from the X-ray structure. On the other hand, the docked conformations of leptomycin B compound were found in different a position from the original ligand binding position in the X-ray structure (Figure 2C). It should be noted that our docked poses of goniothalamin would agree with the X-ray structures. Base on the crystal structure binding mode, goniothalamin allowed binding to dehydrogenase domains.

The result of molecular docking analysis suggested that goniothalamin was able to bind human MFE2 specifically, while leptomycin B was not. However, goniothalamin was reported to bind to CRM1 at a similar region with leptomycin B. So, the effect of CRM1 on inhibition and executive mediator of ER-stress induced apoptosis by goniothalamin and leptomycin B were compared in the next step.

3.3. Goniothalamin and leptomycin B inhibit CRM1 in HeLa cell line

An *in vivo* nuclear export assay by immunostaining of MEK1 in HeLa cells showed that goniothalamin was an inhibitor of nucleocytoplasmic transport via inhibiting CRM1-dependent nuclear export of MEK1 in HeLa cell line, so as leptomycin B (Figure 3A). The ratio of Hoechst33258: Alexa546 signal in Hoechst33258 stained area referred that MEK1 accumulation in nucleus was increased by goniothalamin treatment similar to the leptomycin B treatment (Figure 3B). These results indicated that both goniothalamin and leptomycin B inhibited nuclear export of MEK1 via a similar mechanism targeting the CRM1.

3.4. Goniothalamin and leptomycin B having different effect on triggering ER stress in HeLa cell line

The expression of CHOP, an executive mediator of ER stress-induced apoptosis, was induced within 8 h after 50 μM goniothalamin treatments, interestingly leptomycin B treatment did not induce ER stress (Figure 4A). As mention above, both goniothalamin and leptomycin B interacted with CRM1 by a similar mechanism but only goniothalamin treatment triggered ER stress induction indicated by a slightly increased chaperone GRP78 and GRP94 expression and dramatically increased CHOP expression level in a time-dependent manner (Figure 4B). These results led us to focus on MFE2 as a molecular target of goniothalamin which causes ER stress induction and subsequent cell death induction.

3.5. Goniothalamin may trigger MFE2 inhibition and ER stress induction

We next investigated the effect of MFE2 knockdown on CHOP expression. CHOP expression was increased whenwhile MFE2 expression was decreased after three days of siMFE2-tranfection (Figure 5A). This result suggested interruption of MFE2 activity led to ER stress induction. So, we examined whether ER stress was induced by goniothalamin treatment in other cell lines. The results showed that goniothalamin induced CHOP expression but not MFE2 expression in other cell lines (Figures 5B and 5C). Moreover, goniothalamin induced XBP1 mRNA splicing in HeLa cells indicating that IRE1α, ER stress sensor transmembrane protein, was activated (Figure 5D). An activation of PERK, another ER stress sensor transmembrane protein, was known to phosphorylate eukaryotic initiation factor 2 alpha (eIF2α). The results showed that the phosphorylated eIF2α was increased by goniothalamin treatment and suggested that goniothalamin induced PERK activation (Figure 5E). Moreover, the mediators in the protein folding machinery of the ER, Ero1-Lα and PDI, were also observed but with no altered expression level.
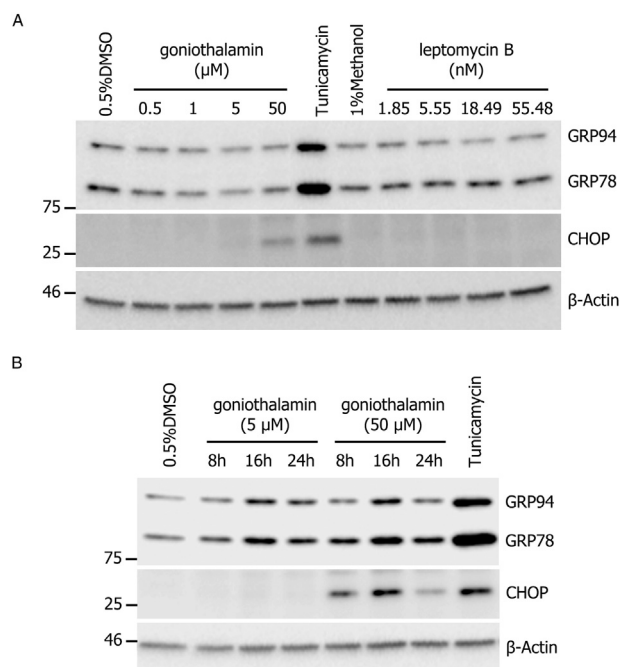

Figure 4. Comparison of goniothalamin and leptomycin B in the ability of ER stress induction. Goniothalamin showed CHOP, a mediator of ER stress-induced apoptosis, induction after 24 h treatment with 50 μM goniothalamin, while leptomycin B could not. (A). That corresponding with goniothalamin triggered CHOP induction after 8 h treatment with 50 μM goniothalamin while slightly increased level of chaperone GRP78 and GRP94 (B). Only goniothalamin triggered ER stress in HeLa cell line. 0.5% DMSO and 1% methanol were used as solvent control for goniothalamin and leptomycin B, respectively, while 12 μM tunicamycin, known ER stress inducer, was used as positive control.

4. Discussion and conclusion

Although goniothalamin was reported to have cytotoxic activity associated with ER stress induction in the previous study [16], but the mechanism has not been fully understood. In this study, the molecular target of goniothalamin which causes ER stress induction was investigated. As goniothalamin was reported to bind to nucleocytoplasmic transporter CRM1 with a similar mechanism to leptomycin B [15]. Our results confirmed this mechanism, but we found that only goniothalamin was able to induce CHOP, a mediator of ER stress-induced apoptosis, expression while leptomycin B was not. These results indicated goniothalamin may bind to other molecular targets which trigger ER stress induction and its binding was different from the leptomycin B. Thus, by using goniothalamin-beads binding assay and computational molecular docking analysis, we investigated the molecular target of goniothalamin which induced ER stress-associated apoptosis.

The goniothalamin-beads binding analysis revealed that MFE2 bound to goniothalamin directly and specifically. The computational molecular docking analysis indicated that goniothalamin can bind to scp-2 domain, enoyl-CoA hydratase domain and (3R)-hydroxyacyl-CoA dehydrogenase domain of MEF2 through the formation of hydrogen bonds. For peroxisomal β-oxidation, acyl-CoA oxidase catalyses the conversion of very long chain fatty acid (VLCFA) sequentially to 2E-enoyl-CoA which then was reversibly converted to 3R-hydroxyacyl-CoA by enoyl-CoA hydratase [5]. Thus, the perturbation of enoyl-CoA hydratase domain by goniothalamin may result in VLCFA accumulation, then triggers VLCFA-induced ER stress in a chain length dependent fashion [6]. So, MFE2 plays an important role in lipid metabolism and its deficiency leads to the very long chain fatty acid (VLCFA) accumulation which may cause ER stress
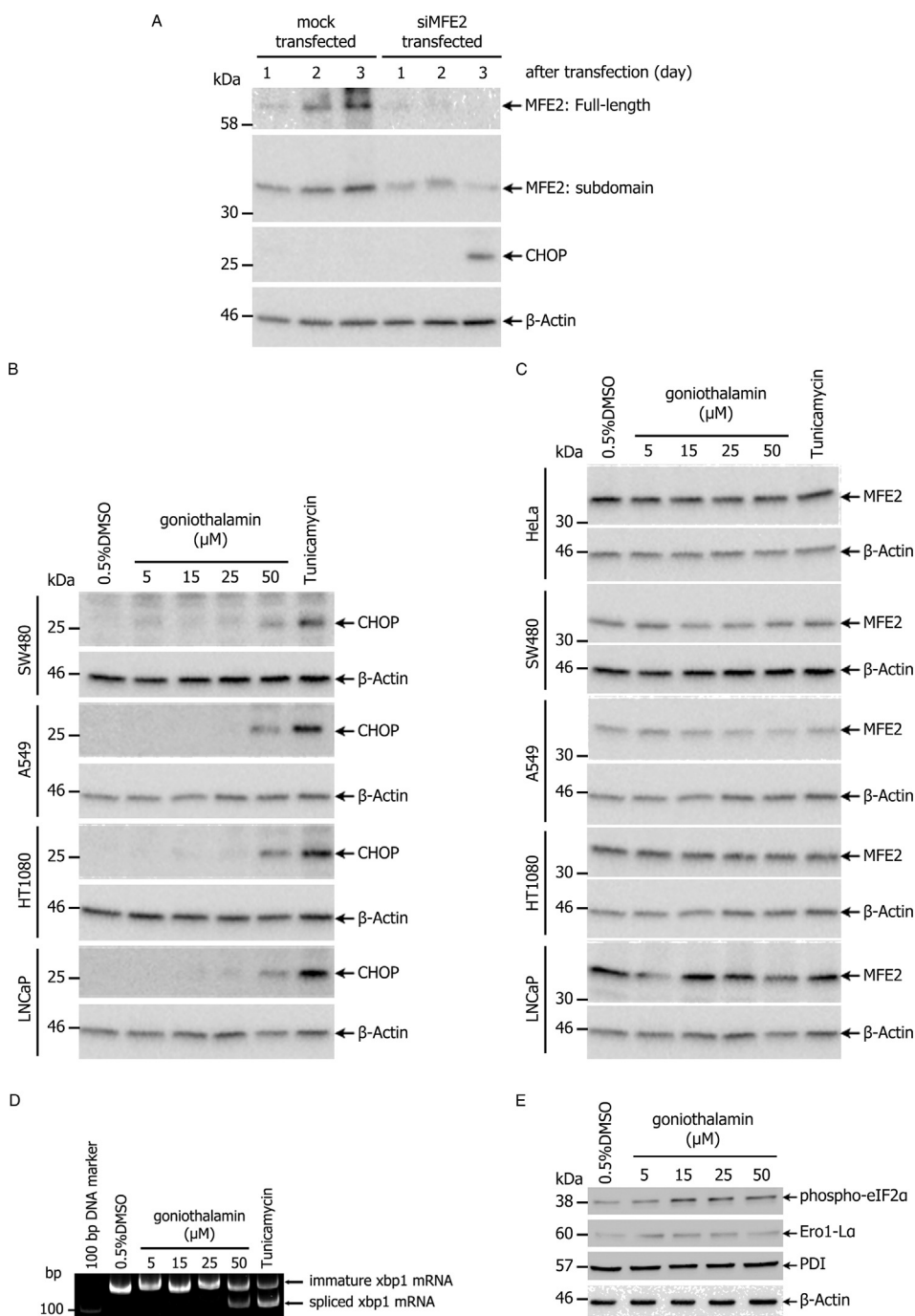
Figure 5. Effect of goniothalamin-MFE2 interaction. Knockdown MFE2 expression using siMFE2 transfection into HeLa cell line resulting in decreased MFE2 expression and CHOP induction after three days, mock-transfection into HeLa cell line was used as control (A). Goniothalamin treatment in other cell line showed similar results in CHOP induction (B), but no effect on MFE2 expression (C). The ER stress sensor transmembrane proteins activation by goniothalamin was observed via detection of XBP1 splicing, for IRE1α activation (D), and detection of phospho-eIF2α, for PERK activation (E). The mediators of protein folding process, Ero1-Lα and PDI were observed, but not altered in the expression. These results suggest goniothalamin - MFE2 interaction may cause indirect ER stress induction. The results of immunoblotting analysis (B,C,E) and reverse transcription-PCR (D) use 0.5% DMSO treatment as control.

induction by lipid perturbation [4, 5, 25, 26]. This study investigated the effect of MFE2 knockdown on ER stress induction, our results revealed that the decreased MFE2 expression was able to induce CHOP expression. In other words, interaction between goniothalamin and MFE2 may interrupt MFE2 activity and cause ER stress induction. Moreover, goniothalamin treatment in other cell lines showed CHOP induction in a cell line-independent manner. However, the activation of ER stress sensor transmembrane proteins, including PERK and IRE1α, were observed via cascade-activated intermediated molecules upon goniothalamin treatment. All of these results corresponded with the previous work [16] that expression of ER stress-mediated apoptosis molecules

were increased, indicating that the later stage of ER stress upon ER stress sensor transmembrane protein activation were triggered [27, 28, 29, 30]. These results suggested that goniothalamin triggers ER stress by lipid metabolism perturbation via the interrupted MFE2 activity, as illustrated in Figure 6. Interestingly, this study is the first investigation reporting that MFE2 is a novel molecular target of goniothalamin associated with ER stress induction.

In conclusion, our studies reveal that goniothalamin can bind to MFE2 and interrupts its activity, which then indirectly induced ER stress. However, the mechanism of ER stress-regulated MFE2 interruption should be further investigated.
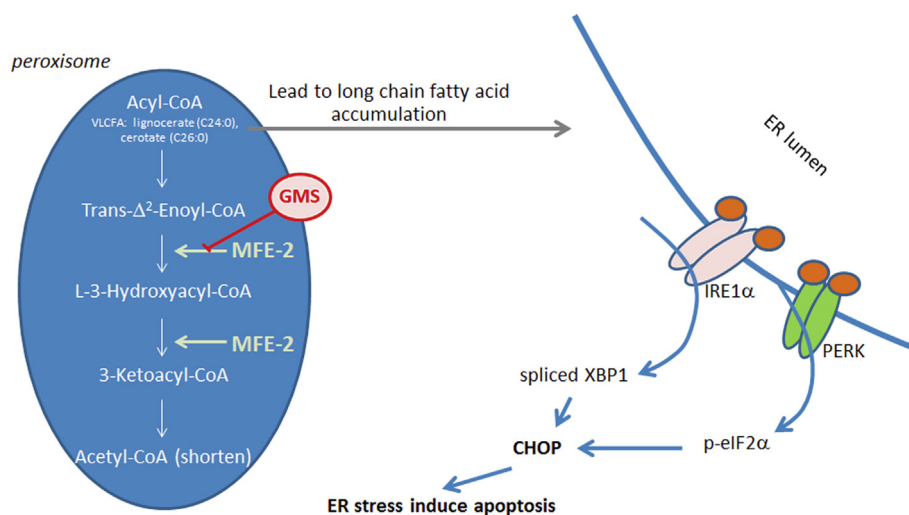
Figure 6. Illustration of MFE2 inhibition mechanism by goniothalamin (GMS) and subsequently triggers ER stress via lipid perturbation. Goniothalamin triggered ER stress induction indirectly, unrelated to unfolded protein accumulation in ER lumen.

Declarations

Author contribution statement

T. Sophonnithiprasert: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

O. Aruksakunwong: Performed the experiments; Analyzed and interpreted the data.

E. Tashiro, Y. Kondoh, M Muroi and H. Osada: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

M. Imoto: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

R. Watanapokasin: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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