Polyphyllin I promotes cell death via suppressing UPR-mediated CHOP ubiquitination and degradation in non-small cell lung cancer

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[ABSTRACT] Polyphyllin I (PPI) purified from Polyphylla rhizomes displays puissant cytotoxicity in many kinds of cancers. Several investigations studied its anti-cancer activity. But novel mechanisms are still worth investigation. This study aimed to explore PPI-induced endoplasmic reticulum (ER) stress as well as the underlying mechanism in non-small cell lung cancer (NSCLC). Cell viability or colony-forming was determined by MTT or crystal violet respectively. Cell cycle, apoptosis, reactive oxygen species (ROS) and mitochondrial membrane potential were assessed by flow cytometry. Gene and protein levels were evaluated by qRT-PCR and immunoblotting respectively. Protein interaction was determined by immunoprecipitation or immunofluorescence assay. Gene overexpression or silencing was carried out by transient transfection with plasmids or small interfering RNAs. The Cancer Genome Atlas (TCGA) database was used for Gene Set Enrichment Analysis (GSEA), survival analysis, gene expression statistics or pathway enrichment analysis of gene or protein alterations including oncogenes (EGFR, ALK, MET, FGFR1, DDR2, ERBB2/3 \(^{[3]}\), KRAS, HRAS \(^{[6]}\), BRAF \(^{[9]}\) and so on) overexpression, fusion or mutation drive NSCLC development, subsequently lead to metastasis, tumor invasion and chemoresistance \(^{[1, 6, 7]}\). Additionally, the silence in tumor suppressor genes is also observed in the formation and progress of NSCLC \(^{[9]}\).

Chemotherapy regimens, which have been used over a decade in NSCLC, include 2 lines \(^{[9]}\): first-line chemotherapy consists of a platinum doublet \(^{[10]}\), and second-line chemotherapy usually covers either docetaxel or pemetrexed \(^{[11]}\). Patients who received both first-line and second-line chemotherapy demonstrated significant prolonged median survival compared with those who were not treated with chemotherapy or received first-line chemotherapy only, even in the cohort of patients aged 70 years and older. But better supportive care for untreated patients did not seem to extend their median survival \(^{[9]}\). It is worth mentioning that targeted therapies or immunotherapies show improved tumor response and progression-free survival outcome compared with cyto-
toxic chemotherapies[12]. But these benefits from new paradigms are interrupted inevitably by the development of drug resistance usually mediated by mutational oncogene, as it did in the case of epidermal growth factor receptor (EGFR) mutation[13]. Hence, more therapeutic strategies should be investigated.

Natural products isolated from herbal medicines represent a great many candidates for drug discovery[14]. Among them, saponins exert multidimensional pharmacological activities, for instance polyphyllin I (PPI, also called polyphyllin I or (+)-polyphyllin D), which is one of main active compounds purified from Polyphya rhizomes (Chonglou in Chinese), demonstrates potent anti-cancer effect on human NSCLC, breast cancer, glioblastoma, leukemia, hepatocellular carcinoma, glioma and cervix epithelioid carcinoma cell lines[14,15]. Previous studies demonstrated that PPI upregulates p21 and Bax, downregulates cyclin B1, CDK1 and Bcl-2, induces reactive oxygen species (ROS) generation, dissipates the mitochondrial membrane potential[16-18].

In this study, we found PPI inhibited glucose-regulated protein 78 (GRP78) levels, which is contrary to former studies[19]. Thus, more details were explored in NSCLC cell lines. We detected its anti-cancer effect and found PPI selectively regulated key protein levels of endoplasmic reticulum (ER) stress or unfolded protein response (UPR). Finally, we discovered that the GRP78-mediated C/EBP homologous protein (CHOP) ubiquitylation was suppressed by PPI.

Materials and Methods

Cell culture

All four human NSCLC cell lines were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). NCI-H1299, NCI-H661 and NCI-H460 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum containing 1.5 g L⁻¹ NaHCO₃, 2.5 g L⁻¹ glucose, 0.11 g L⁻¹ Sodium Pyruvate. A549 cells were maintained in F12K medium supplemented with 10% fetal bovine serum containing 2.5 g L⁻¹ NaHCO₃.

Reagents

PPI (purity ≥ 98%, Fig. 1A) was purchased from Shanghai Yuanye Biological Science and Technology Company (Shanghai, China). The compound was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) as a stock solution of 50 mmol L⁻¹ and then added to extracellular solutions to obtain the desired concentration. The final concentration of DMSO was lower than 0.1%, which did not affect the test. F12K, RPMI1640 medium were respectively purchased from Sigma-Aldrich (Cat#N3520) and Gibco (Cat#31800022).

Bortezomib (BTZ) was commercially available from Millennium Pharmaceuticals Inc. (Cambridge, MA). MG132 was obtained from Selleck (Nanjing, China). Thapsigargin (Tg) was purchased from MedChemExpress (Monmouth Junction, NJ). Annexin V-FITC/PI Kit (Cat#FMSAV-100) was purchased from FeMACS (Nanjing, China). Mitochondrial membrane potential assay kit with JC-1 (Cat#C2006), Cell Cycle and Apoptosis Analysis Kit (Cat#1052) and Reactive Oxygen Species Assay Kit (Cat#S0033M) were purchased from Beyotime (Nantong, Jiangsu, China). Primary antibodies of GAPDH, IRE1α, GRP78, phospho-eIF2α (Ser51), PERK, phospho-PERK (Thr980), pan AKT, phospho-AKT (Ser473), JNK, phospho-SARK/JNK (Thr183/Tyr185), p38, phospho-p38 MAPK (Thr180/Tyr182), ATF4, ATF6, TRAF2, ERK, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies of CHOP and phospho-IRE1α (S724) were purchased from Abcam (Cambridge, UK).

Cell viability and proliferation assay

NCI-H1299 (5 × 10³ cells/well), NCI-H460 (4 × 10³ cells/well) cells, NCI-H661 (5 × 10³ cells/well) and A549 (4 × 10³ cells/well) were seeded onto 96-well microplates, cultured for 24, 48 and 72 h. The cellular viability was assessed using MTT. For colony-forming assay, appreciable amounts of cells (1 × 10⁵/well) were incubated in 6-well plates and treated with incremental concentrations of drugs or 0.1% DMSO for two weeks. The cells were then fixed, stained with a crystal violet solution (Sigma-Aldrich, St. Louis, MO) for 30 min, and images of colonies were taken manually.

Annexin V-FITC staining assay for cell apoptosis

NSCLC cells that underwent apoptosis were evaluated via flow cytometry. Cells were plated in six-well culture plates (5 × 10⁵ cells per well) and treated with various concentrations of PPI or 0.1% DMSO for 24 h. Flow cytometry assay was measured according to the supplier’s protocol.

Propidium iodide (PI) staining assay for cell cycle

NSCLC cells were treated with PPI for 24 h and then stained with propidium iodide (PI). Flow cytometry assay was measured according to the given protocol.

Assay of intracellular ROS and Mitochondrial membrane potential

ROS were measured with the non-fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Mitochondrial membrane potential was assayed by JC-1. And flow cytometry assay was measured according to the commercial protocol.

Real-time quantitative PCR

Total RNA was extracted as previously described[20]. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using TaqMan polymerase with SYBR Green fluorescence (Nippon Gene, Tokyo, Japan) on a Light Cycler 480 Detector (Roche, Mannheim, Germany). The analysis was performed using specific primers (Supplementary Table S1).

Immunoblotting

Cells were lysed in western blotting lysis buffer and centrifuged at 14 000 g for 10 min. The proteins were extracted for western blotting as previously described[21]. Bound immuno-complexes were detected with a ChemiDOC™
Fig. 1  PPI markedly inhibited cell proliferation. (A) The structure, molecular formula and molecular weight of PPI. (B) NSCLC cells with $4 \times 10^3$–$5 \times 10^3$ cells/well were treated with PPI for 0–72 h. Cell viability was detected by MTT. The standard deviation or standard error was too small to display. The original data can be obtained from the authors. (C) NSCLC cells were planted in 6-well plates ($1 \times 10^3$ cells/well) and incubated with 0–1.6 μmol·L$^{-1}$ PPI for 14 days. Then colony-forming assay was performed. (D) Cells were plated in six-well culture plates ($5 \times 10^5$ cells per well) and treated with various concentrations of PPI or 0.1% DMSO for 24 h. Apoptosis were evaluated via flow cytometry. Data from three independent experiments are presented as mean ± SEM.
Rad Laboratories, Hercules, CA). The band intensity was measured with Image Lab 4.0 (Bio-Rad Laboratories).

Construction of expression plasmids

HA-GRP78, FLAG-CHOP and FLAG-ubiquitin were respectively sub-cloned into pcDNA3.1 vector by General Biosystems, Inc. (Anhui, China). All constructs were verified by sequencing.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and rinsed three times in 1 × PBS for 5 min each. Next, the cells were incubated in 5% bovine serum albumin (BSA) with 0.1% Triton X-100 for 60 min to permeabilize the cells and block nonspecific protein-protein interactions. The cells were then incubated with the indicated antibodies (dilution ratio selected were dependent on the product datasheets) overnight at 4 °C and then rinse three times in 1 × PBS for 5 min each. An Alexa Fluor® 488-conjugated goat anti-rabbit IgG polyclonal (1 : 200 dilution, Cell Signaling Technology) was used as the secondary antibody. Immunofluorescence experiments were visualized using an ImageXpress Micro® system (Molecular Devices, Sunnyvale, CA). A laser scanning confocal microscope LSM 700 (Carl Zeiss, Oberkochen, Germany) was used for colocalization analysis.

Small interfering RNA-mediated gene silencing

CHOP siRNAs (siCHOP#1: GCCUCGUAGAGGAC-CUGC; siCHOP#2: AGAACACCAGGAGGCACCAA), GRP78 siRNAs (siGRP78#1: GCGCAUUGAUACUAGAUAGA AUGA; siGRP78#2: AGACCGUGAACUAUUGCUUU), IRE1α siRNA (siRNA: GGACGUGACGGACGACAAATT) and non-targeting (NC) siRNA were synthesized by Biomics Biotechnologies (Nantong, China). The cells were transfected with 150 nmol·L⁻¹ each siRNA or siRNA hybrid using Lipofectamine 3000 reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer’s protocol. NC siRNA was used as a negative control under similar conditions. The operating methods were performed as previously described [29].

Immunoprecipitation assay

Immunoprecipitation assay was performed as described [23]. Briefly, the NSCLC cells were transiently transfected with specific plasmids and were treated as described before harvesting. The cells were lysed in cold lysis buffer supplemented with protease inhibitors, and then cell lysates were precleared with protein A/G beads and subsequently incubated for 6 h with a mixture of protein A/G beads covalently coupled with specific antibody and IgG. Immunoprecipitation (IP) samples were collected by centrifugation and washed more than three times with cold lysis or PBS buffer. The lysates and IP samples were subjected to SDS-PAGE followed by western blotting using the indicated antibodies.

Statistical analysis

All experiments were performed at least three times in a parallel manner. Statistics were calculated with GraphPad Prism version 6.0. One-way ANOVA was used to compare each parameter. The results were analyzed using one-way analysis of variance with Tukey’s multiple comparison test. The data are given as the mean ± standard error of the mean and P < 0.05 was considered to indicate a significant difference.

Results

PPI showed powerful anti-cancer effect on NSCLC cells

It was demonstrated that PPI displays extensive inhibitory activity on the proliferation of multiple cancer cells [14]. We also testified that PPI blocked the propagation of NSCLC cells in a concentration- and time-dependent manner (Fig. 1B and 1C). We are curious about the specific ways concerning its anti-cancer effect. Hence, more detection was performed. PPI significantly induced late apoptosis in NCI-H1299, NCI-H460, A549 and NCI-H661 cells (Fig. 1D, Supplementary Fig. 1). Cell cycle was also arrested by PPI at G2/M phase (Fig. 2A), which were coincided with its suppressive effect on the mRNA or protein levels of Ki-67, PCNA, cyclin A2, cyclin B1, cyclin B2 or cyclin D1, and its promoting effect on the cyclin-dependent kinase (CDK) inhibitors P15 and P21 (Fig. 2B and 2C). Besides, ROS levels were significantly increased (Fig. 2D), but the mitochondrial membrane potential was not disturbed (Fig. 2E) in 0–4 μmol·L⁻¹ PPI-treated NSCLC cells. These results certified that PPI exerted anti-cancer activity by blocking cell cycle, impeding cell proliferation, and inducing cytotoxicity through elevation of ROS levels on of ROS levels.

PPI selectively decreased GRP78 levels

Several studies have illuminated the mechanisms regarding the regulative effects of PPI on cell cycle [17] and cellular proliferation [19]. We focus on its ability leading to cell death since PPI markedly induced late apoptosis (Fig. 1D). In Gene Set Enrichment Analysis (GSEA) on The Cancer Genome Atlas (TCGA) database, we found that gene expression of NSCLC was highly enriched in genes involved in the sets of protein processing in ER (NES = −1.6641, NOM p-val = 0.0334 and FDR q-val = 0.0759) and unfolded protein binding: GO 0051082 (NES = 1.7774, NOM p-val = 0.0056 and FDR q-val = 0.1406) (Fig. 3A). Pathway enrichment assay also revealed the top twenty significant pathways including protein processing (Fig. 3B) [19]. Hence, we will focus on the levels of gene sets concerning PPI-mediated ER stress.

Consequences of ER stress-responsive genes by qRT-PCR detection revealed that DNA-damage-inducible transcript 3 (DDIT3, also known as CHOP) and EIF2AK3 (PERK alias) were two of the highest inducible genes after PPI stimulation (Fig. 3C) in NSCLC cells. Western blotting indicated that PPI selectively restrained levels of chaperone protein GRP78 (Fig. 3D, 3F and 3G, Supplementary Figs. 2, 3), the main UPR regulator which is preferentially overexpressed in aggressive, metastatic, and chemo-resistant cancers [30], but not IRE1α, PERK, ATF6 or JNK in NCI-H1299 and NCI-H460 cells. In addition, it seems that PPI forced CHOP expression in a concentration-dependent manner (Fig. 3E–3G). The speculation was subsequently confirmed by the upregula-
Fig. 2  PPI arrested cell cycle at G2/M phase and increased ROS levels. (A) NSCLC cells were incubated with 0−2.5 μmol·L\(^{-1}\) PPI for 24 h and cell cycle was determined by flow cytometry. (B) NCI-H1299 cells were treated with 0−3 μmol·L\(^{-1}\) PPI for 24 h and indicated gene levels were tested by qRT-PCR. (C) NCI-H1299 cells were cultured with 0−3 μmol·L\(^{-1}\) PPI for 24 h and CyclinD1 levels were detected by western blotting. NSCLC cells were treated by PPI, and (D) ROS levels and (E) mitochondrial membrane potential were monitored by flow cytometry. Data from three independent experiments are presented as mean ± SEM.
Fig. 3  PPI induced ER stress and selectively suppressed UPR chaperone GRP78. (A) GSEA and (B) gene enrichment assay on TCGA database. (C) Twelve ER-related genes were explored by qRT-PCR in the present of 0−3 μmol·L$^{-1}$ PPI for 24 h in NSCLC cells. (D−F) Cells were stimulated by 0−3 μmol·L$^{-1}$ PPI for 24 h, and (G) NCI-H1299 cells were transfected transiently with 150 nmol·L$^{-1}$ IRE1α or NC siRNA for 24 h. After that cells were washed twice with warm PBS and treated with 2.5 μmol·L$^{-1}$ PPI for 24 h, followed by immunoblotting analysis for the detection of indicated proteins with GAPDH (GA) as loading control. Thirty micrograms protein lysate of each sample was used for immunoblotting analysis. For the results of GSEA, |NES| > 1, NOM p-val < 0.05 and FDR q-val < 0.25 are considered as high enrichment.
To verify the results of bioinformatic analysis, we constructed GRP78 plasmid or synthesized two GRP78 small interfering RNAs. In order to avoid the off-target effect, we mixed the GRP78 siRNAs which aimed at two independent sites and used them for subsequent experiments. GRP78 overexpression or knockdown by using GRP78 construct or siGRP78 hybrid (siGRP78s) did not interrupt cell growth curve within 72 h (data not shown), but siGRP78s enhanced PPI-caused cell death compared with siNC and/or PPI treatment (data not shown). Flow cytometry assay confirmed this augmentation. In the presence of siGRP78s, PPI-treated groups showed noteworthy increase in the number of PI-positive cells (Fig. 4B and 4C). These results indicated that PPI may inhibit UPR by decreasing GRP78 levels and then actuated cell death.

**PPI attenuated GRP78-mediated CHOP degradation**

Under ER stress, p38 MAP kinase family activates CHOP transcriptional activation and induces the pro-apoptotic effect of CHOP (Fig. 3E−3G) [24]. In our previous study, we demonstrated that GRP78 interacts with CHOP and promotes CHOP multiubiquitination under ER stress condition [21]. Thus, CHOP levels were tested after treating with PPI. Overexpression of GRP78 reduced CHOP levels in NCI-H1299 cells (Fig. 5A). However, the basal level of CHOP...
was too low to be detected by immunoblotting (Fig. 5A), even in Tg-stimulated group or CHOP vector-transfected group. But it can be captured via confocal microscopy (Fig. 5B). The results showed fluorescents of exogenetic CHOP, conjugated with Cy5, were augmented concentration-dependently by 0−1 μmol·L⁻¹ PPI. Besides, with PPI treatment, not only were GRP78 levels decreased but its colocalization with CHOP was suppressed (Fig. 5B). Furthermore, immunoprecipitation assay demonstrated that interaction between GRP78 and CHOP in the absent or present of transient cotransfection with exogenetic HA-GRP78 and FLAG-CHOP existed in NSCLC cells (Fig. 5C and 5D). It seems that PPI at 2.5 μmol·L⁻¹ failed to impede the binding between FLAG-CHOP and HA-GRP78, or at least the impediment is

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**Fig. 5**  PPI abated GRP78-mediated CHOP degradation. (A) NCI-H1299 cells were transfected transiently with 5 μg GRP78 plasmid or 3 μg CHOP plasmid for 24 h. Then cells were treated with designative chemicals for 24 h. Immunoblotting was executed to determine each protein levels. (B) NCI-H1299 cells were planted into 96-well plates and transfected with FLAG-CHOP (0.5 μg) and HA-GRP78 (0.5 μg) for 24 h, and then treated with 0−1 μmol·L⁻¹ PPI for 24 h. Immunofluorescence analysis of Cy5-conjugated CHOP, conjugated with Cy5, were augmented concentration-dependently by 0−1 μmol·L⁻¹ PPI. Besides, with PPI treatment, not only were GRP78 levels decreased but its colocalization with CHOP was suppressed (Fig. 5B). Furthermore, immunoprecipitation assay demonstrated that interaction between GRP78 and CHOP in the absent or present of transient cotransfection with exogenetic HA-GRP78 and FLAG-CHOP existed in NSCLC cells (Fig. 5C and 5D). It seems that PPI at 2.5 μmol·L⁻¹ failed to impede the binding between FLAG-CHOP and HA-GRP78, or at least the impediment is...
hard to be detected by western blotting (Fig. 5C and 5D). In addition, when protein synthesis was blocked by cycloheximide (CHX), a prolonged CHOP half-life was observed after treating siGRP78s (data not shown), and GRP78 degradation was accelerated by PPI (Fig. 5E). In flow cytometry detection, NSCLC cells transfected with CHOP plasmid showed more sensitive to PPI, while siCHOPs-treated cells showed more resistant to PPI (Fig. 5F, Supplementary Fig. 4). These results indicated that PPI accelerated GRP78 degradation, and then stabilized CHOP.

**PPI inhibits GRP78-driven CHOP ubiquitination**

Bioactive assay *in vitro* illustrated that GRP78 plasmid attenuated PPI-induced cytotoxic effect or cell death, and GRP78 siRNAs heightened the activity of PPI (Fig. 4C). We speculated GRP78 scarcity contributed to CHOP stability (Fig. 5E) and thus enhanced CHOP-mediated cell death (Fig. 5F). Previous study proofed that GRP78 drives CHOP ubiquitination and subsequently propels its proteasomal degradation [13]. In NSCLC cells, PPI reduced levels of CHOP ubiquitination whether or not GRP78 construct existed (Fig. 6A, 6B, D and E). Overexpressed GRP78 fortified CHOP ubiquitination (Fig. 6C and 6F). GSEA also showed gene expression in NSCLC significantly enriched in the gene sets of ubiquitin mediated proteolysis (NES = −1.561, NOM p-val = 0.0442 and FDR q-val = 0.1064) and KEGG proteasome (NES = 1.7867, NOM p-val = 0.0062 and FDR q-val = 0.0463).
0.0463 (Fig. 6G). Two proteasome inhibitors MG132 and BTZ were recruited to confirm this deduction. Cell viability assay revealed that PPI combined with MG132 or BTZ notably reduced cell viability in NSCLC cells (Fig. 6H). These results demonstrated that CHOP, whose ubiquitination and degradation is mediated by GRP78, promoted PPI-induced cell death.

**Discussion**

CHOP, also known as DDIT3, growth arrest- and DNA damage-inducible gene 153 (GADD153) or C/EBPz, is a 29 kDa protein with 169 amino-acid residues in human. Previous studies proved that gene levels of CHOP were fortified in the condition of ER stress. p38 MAP kinase phosphorylates two serine residues (79 and 82) in the transactivation domain of CHOP. This effect promotes CHOP to exert its proapoptotic ability [24]. In this study, PPI markedly increased phosphorylated levels of p38 but the expression of total p38 were not augmented significantly (Fig. 3E–3G). The decrease of p38 at 2.5 μmol·L⁻¹ PPI may be due to the decrease of p38 gene mRNA level or the acceleration of p38 protein degradation. In addition, stimulated by PPI, NSCLC cells will launch the unfolded protein response (UPR) to maintain a homeostatic balance between demand and capacity for endoplasmic reticum (ER)-mediated protein folding. p38 probably be removed by ER-associated degradation (ERAD) or regulated-IRE1 dependent decay (RIDD).

We identified that PPI showed potent cytotoxicity, inhibited cell proliferation and promoted cell death in NSCLC cell lines. The ability of PPI to accelerate cell death may not refer to apoptosis induction, for the apoptotic cells induced by PPI mainly focus on non-viable apoptotic cluster (Fig. 1D). We deduced PPI directly forced ER stress-induced cell death or stimulated cell autophagy, subsequently led to cell death, for the reason that the proteasome inhibitors enhanced the anticancer effect of PPI (Fig. 6H). The apoptosis detected by flow cytometry (Figs. 1D, 4B and 5F) may be one of phenomena along with PPI-mediated poor cell fate. It means that in the process of cell death, PPI-induced apoptosis is sufficient but not necessary. However, more evidences would be needed to prove this point of view.

GRP78, also called as BIP or HSPA5, is a HSP70 family member. Its overexpression in cancer cells hinders cell apoptosis and is beneficial for cell survival. TCGA data confirmed that the high levels of HSPA45 in NSCLC tissues (P < 0.01) portended a poor prognosis (Fig. 4A). After treating with PPI, protein levels of GRP78 were restrained (Fig. 3D, 3F and 3G), regardless of its gene expression was increased or decreased (Fig. 3C), revealing a good therapeutic outcome.

Cells need their ER to maintain basic physiological activities including protein folding, modification, lipid biosynthesis and calcium homeostasis. These procedures require high concentrations of dedicated chaperones (such as GRP78) and enzymes thus could not occur in the cytosol [20]. If mistake happens during the above processes, unfolded or misfolded proteins will accumulate in the ER and then lead to ER stress. The UPR is activated after ER stress to eliminate the negative factors and promote cell survival. If these protections fail to resolve the stress, death programs are activated [23]. In GSEA and pathway enrichment assay, we screened out gene sets concerning protein or unfolded protein process in ER (Fig. 3A and 3B) and found the selective inhibition of PPI on GRP78 expression (Fig. 3D and 3F), which revealed a possible treatment option involving ER-related targets.

When ER stress occurs in NSCLC cells, CHOP gene is forced to be transcribed and translated, subsequently enhances downstream genes expression, and then promotes cell death. Molecular chaperone GRP78 blocks these courses by binding CHOP (Fig. 5C and 5D), assisting CHOP ubiquitination and facilitating the latter proteasomal degradation (Fig. 6A–6F). Thus, cellular fate may be reversed. Our results showed PPI protected CHOP levels from GRP78 inhibition.

Taken together, we reveal a novel molecular mechanism...
concerning the inhibitory effect of PPI on NSCLC cells (Fig. 7). PPI suppresses UPR, increases levels of CHOP via expediting CHOP gene expression and stabilizing CHOP protein. The UPR chaperone GRP78 restrained by PPI is, at least partly, the reason for CHOP stabilization. In consideration of the potent anti-cancer activity, the natural product PPI deserves further development.

**Fig. 7** Model indicating that PPI induces ROS and ER stress, and inhibits concomitant UPR in NSCLC cells. Under PPI treatment, suppressed UPR chaperone GRP78 fails to facilitate CHOP ubiquitination and degradation. Then stable CHOP accelerates downstream gene expression, subsequently promotes cell death, which generates a positive feedback loop.

### Supplementary Materials

Supplementary materials are available as Supporting Information, and can be requested by sending E-mail to the corresponding author.

### References


