Ectopic ATP Synthase Blockade Suppresses Lung Adenocarcinoma Growth by Activating the Unfolded Protein Response

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Abstract

Ectopic expression of the mitochondrial F₁F₀-ATP synthase on the plasma membrane has been reported to occur in cancer, but whether it exerts a functional role in this setting remains unclear. Here we show that ectopic ATP synthase and the electron transfer chain exist on the plasma membrane in a punctuated distribution of lung adenocarcinoma cells, where it is critical to support cancer cell proliferation. Applying ATP synthase inhibitor citreoviridin induced cell cycle arrest and inhibited proliferation and anchorage-independent growth of lung cancer cells. Analysis of protein expression profiles after citreoviridin treatment suggested this compound induced the unfolded protein response (UPR) associated with phosphorylation the translation initiation factor 2α (eIF2α), triggering cell growth inhibition. Citreoviridin-enhanced eIF2α phosphorylation could be reversed by siRNA-mediated attenuation of the UPR kinase PKR-like endoplasmic reticulum kinase (PERK) combined with treatment with the antioxidant N-acetylcysteine, establishing that reactive oxygen species (ROS) boost UPR after citreoviridin treatment. Thus, a coordinate elevation of UPR and ROS initiates a positive feedback loop that convergently blocks cell proliferation. Our findings define a molecular function for ectopic ATP synthase at the plasma membrane in lung cancer cells and they prompt further study of its inhibition as a potential therapeutic approach.

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Introduction

F₁F₀-ATP synthase catalyzes the phosphorylation of ADP to ATP by exploiting a transmembrane proton gradient (1). Although F₁F₀-ATP synthase was initially thought to be located exclusively in the mitochondrial inner membrane, its presence has now been described on the outside of the plasma membrane of highly proliferated cells both normal cells and tumor cells (2–9). However, these studies reveal ectopic ATP synthase with a variety functions depending on cell types and relatively little is known about ectopic ATP synthase in tumor cells.

Ectopic ATP synthase has been shown to have several roles in normal cells. In endothelial cells, ectopic ATP synthase is the receptor of angiotatin, an endogenous angiogenesis inhibitor that blocks neovascularization (2, 10). Angiotatin and the ATP synthase F₁ inhibitor protein IF₁ can block ATP synthesis and hydrolysis by the enzyme and inhibit the proliferation and migration of cultured endothelial cells (2, 11). In hepatocytes, ectopic ATP synthase was identified as the receptor for high-density lipoprotein (HDL) endocytosis, and IF₁ significantly decreases HDL internalization in HepG2 cells, showing the participation of ectopic ATP synthase in the regulation of cholesterol homeostasis (5). In keratinocytes, ectopic ATP synthase mediates the release secretion of ATP into the culture medium, which plays a crucial role in normal epidermal homeostasis and wound healing (8). In neural cells, ectopic ATP synthase has been found to bind to amyloid precursor protein and amyloid β-peptide, which are involved in the pathogenesis of Alzheimer’s disease (12). During adipogenesis, ectopic ATP synthase is markedly increased, and may be a potential target for anti-obesity drugs (4, 7, 13).

Although ectopic ATP synthase has been found on the extracellular surface of several different cancer cell types (6, 14–16), unlike in normal cells, its function in tumor cells...
is unknown. Expression of the ectopic ATP synthase β subunit has been reported on Daudi, K562, and RPMI 8226 tumor cells, and this subunit can be recognized by γ/δ T lymphocytes through interaction with the T-cell receptor via apoA-1 (9). Thus, ectopic ATP synthase may be an antigen of tumor cells and involved in the immune response to tumor cells. Treating these cancer cells with biological (i.e., antibody or inhibitor of F₁ of ATP synthase, IF1) or chemical synthetic inhibitors of ectopic ATP synthase markedly inhibits cell growth (15, 17), thereby highlighting ectopic ATP synthase as a potential and novel therapeutic target for cancer.

By understanding how inhibitors of ectopic ATP synthase induce cytotoxicity and by elucidating the molecular mechanisms underlying this process, we aim to improve our understanding of their potential anticancer activity and lay the foundations for therapeutic application. Specifically, we used a proteomics approach and constructed a protein–protein interaction (PPI) network, to investigate the role of tumor ectopic ATP synthase and the effects of its inhibitor citreoviridin, which exhibits specific inhibition on F₁F₀ ATP synthase (18, 19).

Materials and Methods

Cell culture

Human A549 lung carcinoma cells and IMR-90 lung fibroblasts were obtained from the American Type Culture Collection. Human lung carcinoma cells CL1-0 were cultured as previously described (20). Cells were cultured at 37°C and 5% CO₂ in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS and routinely passaged when 90% to 95% confluent. All the cells were free of mycoplasma as determined by a PCR-based mycoplasma detection method (MBI Fermentas).

Drug treatment

The ATP synthase inhibitor citreoviridin (Fermentek Biotechnology) was solubilized in dimethyl sulfoxide (DMSO) at 20 mmol/L and diluted in medium at the concentrations indicated. The control samples were treated with the same volume of DMSO only (Sigma-Aldrich). All the procedures including drug preparation and treatment were carried out in the dark.

Immunofluorescence staining and flow cytometry

Cells were plated onto poly-l-lysine–coated glass coverslips for 24 hours and fixed in 4% formaldehyde. Immunofluorescence was carried out as described previously (15). Primary antibodies used to probing NDUFB4, SDHA, UQCRCC2, COX5A, and ATP5B were purchased from Abcam and anti-ATP synthase complex mouse monoclonal antibody was obtained from MitoSciences. The secondary antibody used was Alexa Fluor 488–conjugated goat anti-mouse IgG (Molecular Probes). All antibody incubations were carried out at room temperature for 1 hour, after which the samples were washed three times with PBS. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes, the mitochondria were stained with Mito-ID Red Detection Kit (Enzo Life Sciences Inc.) and coverslips were mounted with AntiFade Prolong solution (Molecular Probes). Cells were analyzed with a fluorescence microscope or a Leica TCS SP5 spectral scanning confocal microscope with a Leica HCX PL APO CS 100×1.40 OIL. objective (Leica Lasertechnik). A stack of consecutive image planes with vertical distances was taken for each sample.

For flow cytometric analysis, cells were labeled with ATP5B and ATP complex antibodies. Cells were washed with PBS twice, suspended in PBS and then analyzed by FACSCanto instrument (Becton Dickinson). The fluorescence data were further analyzed with WinMDI 2.9 software (Scripps Research Institute, Jupiter, FL).

Extracellular ATP generation assay

The levels of extracellular ATP (eATP) secreted by A549, CL1-0, and IMR-90 cells were assayed by a bioluminescence assay kit (Sigma-Aldrich) according to the manual. A total of 2 × 10⁶ cells were seeded in 24-well plate and allowed to attach for 16 hours. The cells were refreshed with medium containing 5 μmol/L citreoviridin or DMSO for 30 minutes. Then, after adding 200 μmol/L ADP for 1 minute, the samples were centrifuged to eliminate the cells, and the concentration of ATP in the aliquots was determined according to the user manual by the bioluminescence assay kit using FlexStation III (Molecular Devices). Data are expressed in micromoles of ATP per 1 × 10⁶ cells on the basis of standards determined for each independent experiment.

Flow cytometric detection of mitochondrial membrane potential

To assess the mitochondrial membrane potential (MMP), after incubation with 5 μmol/L citreoviridin for 48 hours, cells were incubated with 100 nmol/L DiOC₆ for 15 minutes at 37°C. Then, the cells were washed with PBS twice, suspended in PBS and then analyzed by FACSCanto instrument (Becton Dickinson). The fluorescence data were further analyzed with WinMDI 2.9.

Flow cytometric detection of reactive oxygen species

For reactive oxygen species (ROS) detection, cells were treated with citreoviridin at the IC₅₀ for 6, 12, and 24 hours. Cells were then washed, trypsinized, and incubated with 1 μmol/L 2′,7′-dichlorofluorescein diacetate (H₂DCFDA, Molecular Probes) in the dark at 37°C for 30 minutes. Cells were then washed twice with PBS and analyzed by FACSCanto instrument (Becton Dickinson) and FlowJo 7.1 (Treestar, Inc.).

Proliferation assay using xCELLigence system and MTS assays

The xCELLigence System (Roche), an electronic analyzer with sensor electrodes coated on the tissue culture plate, provides growth information in real time, which can reflect the cell behavior immediately once recording after drug treatment. xCELLigence cell index impedance measurements were done according to the manufacturer’s instructions. In brief, after 30 minutes equilibration in the medium, 5,000 cells were seeded in 100-μL culture medium to each well of the E-plate 16, and the attachment, spreading, and proliferation of the cells were monitored every hour by the xCELLigence
system. Approximately 24 hours after seeding, when the cells were in log phase growth, the cells were exposed to 50 μL of medium containing the ATP synthase inhibitor citreoviridin or DMSO only as the control. The final concentration of citreoviridin was 0, 2, 4, and 6 μmol/L. The concentration of DMSO was 0.1%. All experiments were repeated three times and examined every hour for 48 hours. The average IC_{50} was calculated throughout the 48 hours for each cell lines by xCELLigence system.

Five thousand cells were plated in 96-well plates and allowed to adhere overnight. The medium then was discarded, and the cells were pretreated with chemical chaperones or antioxidants. Chemical chaperones 10 μmol/L tauroursodeoxycholic acid (TUDCA, Sigma-Aldrich) and 1 mmol/L 4-phenyl butyric acid (4-PBA, Sigma-Aldrich) were pretreated for 2 hours, and 10 mmol/L antioxidant N-acetylcysteine (NAC, Sigma-Aldrich) was pretreated for 30 minutes. Citreoviridin was treated for further 24 hours after chemical chaperones and antioxidant removed. For extracellular nucleotide and calcium signaling involvements, 10 μmol/L ATP (Sigma-Aldrich) and 0.1 mmol/L EGTA (Sigma-Aldrich) were added 5 minutes after citreoviridin treatment. The concentration of citreoviridin for each cell lines was used as their IC_{50}. Growth inhibition was measured by using MTS (Promega Corporation) assay. One hundred percent viability refers to the MTS value for 0.1% DMSO-treated cells.

**Colony formation assay**

For anchorage-dependent growth assays, 200 A549 or CL1-0 cells/well were seeded in 6-well plates, incubated with citreoviridin at their IC_{50} or 0.1% DMSO control for 10 days, fixed with methanol, and stained with crystal violet. For anchorage-independent growth assays, 500 cells were mixed with 2 mL low melting point agar (0.35% in DMEM with citreoviridin or DMSO mentioned above) and overlaid on 0.7% agar (2 mL) in each well of 6-well plates. The plates were incubated for 14 days, fixed, and stained. Colonies with a diameter greater than 100 μm were counted.

**DNA content analysis**

To determine cell cycle distributions, 1.5 × 10^6 A549 or CL1-0 cells were exposed to citreoviridin at their IC_{50} or 0.1% DMSO control in DMEM with 10% FBS for 12 hours or 24 hours. Cells were washed, trypsinized, collected and fixed in 70% cold ethanol (−20°C) overnight. Cells were then washed twice with PBS and resuspended in PBS containing 1 mg/mL RNase A and incubated at 37°C for 30 minutes and followed by propidium iodide (PI, 10 μg/mL) staining for 15 minutes. The DNA content of cells was then analyzed with a FACSCanto instrument (Becton Dickinson). The percentage of cells in different phases of the cell cycle was calculated by MultiCycle (DeNovo software).

**Protein extraction**

Total protein was extracted from 1 × 10^7 cells by 0.5 mL lysis solution containing 7 M urea (Boehringer), 2 M thiourea (J. T. Baker), 4% CHAPS (J. T. Baker), and 0.002% bromophenol blue (Amersco). The mixture was discontinuously sonicated for 2 minutes on ice. The lysates were centrifuged for 30 minutes at 4°C at 15,000 × g. The supernatant was collected, and the protein concentration was measured by a protein assay kit (Bio-Rad) according the manual.

**Transfection of siRNA**

The siRNAs directed against human PKR-like endoplasmic reticulum kinase (PERK siRNA, pools of three target specific 19 to 25 nt siRNAs) and the nontargeting negative control siRNA (Control siRNA) were purchased from Santa Cruz Biotechnology Inc. Another independent siRNA targeting PERK and scrambled control siRNA were obtained from OriGene (SR306267-3, OriGene). Cancer cells were transfected with the PERK siRNA or control siRNA with Lipofectamine 2000 (Invitrogen) for 48 hours according to the manufacturer’s protocol and treated with citreoviridin or DMSO for a further 12 hours. The final concentration of the siRNAs was 10 nmol/L.

**Western blotting**

The proteins were extracted using lysis buffer as described previously. Total proteins (20 μg) were separated by PAGE and blotted onto polyvinyl-difluoride membranes (Millipore). After blocking with 5% nonfat milk in PBST at room temperature for 30 minutes, membranes were probed with antibodies. Antibodies against BiP, PERK, EroLα, PDI, IRE1α, eIF2α, phospho-eIF2α, and phospho-PERK were purchased from Cell Signaling Technology Inc. Actin antibody was purchased from Millipore. All secondary antibodies were obtained from Sigma-Aldrich. After incubation with primary and secondary antibodies, immunoblots were visualized with the ECL detection kit (Pierce Biotechnology Inc.) and exposed to Fuji medical X-ray film.

**Statistical analysis**

All experiments were carried out at least 3 times. Data are expressed as mean ± SD. Unpaired 2-tailed t tests were used for the comparison of two groups. P values < 0.05 were considered significant.

**Results**

**ATP synthase and the electron transport chain are expressed on the surface of lung cancer cells**

ATP synthase consists of 2 regions, the transmembrane F_{0} portion and the F_{1} portion with the ATPase activity. The F_{1} sector is composed of α_{5}, β_{5}, γ, δ, e subunit, where β subunit provides enzyme activity to convert ADP to ATP as well as hydrolysis of ATP. To measure the expression levels of ATP synthase on lung cells, antibodies probed for ATP synthase β subunit and the ATP synthase complex were applied to quantitative and qualitative measurements by flow cytometry (Fig. 1A) and confocal microscopy (Fig. 1B). ATP synthase β subunit and the whole complex were found to be expressed on the cell surface of A549 and CL1-0 lung cancer cells but not on normal fibroblast IMR-90. Without permeabilization of the cell, the antibodies were restricted outside the cell and can only recognize the structures projecting from the cell. Both the expression of ATP synthase complex and β subunit are localized on the cell surface not colocalized with mitochondria staining in A549 and CL1-0 cells (Fig. 1B). With
not only the β subunit but the whole catalytic complex located on the lung cancer cell surface, we suggested that the complete ectopic ATP synthase may exhibit enzymatic activities. To further confirm this implication, bioluminescence assay for eATP detection was carried out after adding inhibitor and DMSO control. Relative to control, eATP concentration significantly decreased following treatment with 5 μmol/L citreoviridin for 30 minutes in A549 and CL1-0 cells, but not the normal IMR-90 fibroblasts. The expression of ATP synthase β (anti-ATP5B) and the ATP synthase complex (anti-ATP synthase complex) were analyzed by flow cytometry. Expression of ectopic ATP synthase was observed by confocal microscopy in A549 and CL1-0 lung cancer cells. DAPI was used to stain nuclei. eATP concentration was determined after treatment with citreoviridin (Citreo) or DMSO (vehicle control) for 30 minutes in three cell lines. Asterisks indicate significant differences between the control and the treated group from three independent experiments (P < 0.01).
ectopic ATP synthase negative cells IMR-90 (Fig. 1C). The re-
remaining level of eATP suggest that there are other ATPase or 
ATP-permeable release channels contributing to the homeo-
stasis of eATP, and the inhibitory efficacy of citreoviridin may 
be specific for F1F0 ATP synthase.

As the generation of proton gradient is required for ATP 
production by ATP synthase, we examined the existence of the 
electron transport chain (ETC) on plasma membrane by 
immunocytochemistry and purification of plasma membrane 
proteins. The distribution pattern of the examined proteins in 
nonpermeable cells was punctuated (Fig. 1D), and was quite 
different from that in Triton X-100 permeable ones showing 
typical mitochondrial pattern (Fig. S1). In addition, the ETC 
proteins and the ATP synthase \( \beta \) were presented in the 
biotinylated purification of plasma membrane proteins (Fig. 
1E and Supplementary Fig. S2). We revealed that not only ATP 
synthase but ETC proteins were located on the plasma mem-
brane with a punctuated distribution. Other proteomics stud-
ies have also been reviewed for the respiratory chain on cell 
surfaces (21), which supports our findings. Taken together, 
these results indicate that ectopic ATP synthase and ETC are 
localized on the plasma membrane of lung cancer cells and can 
generate ATP.

Citreoviridin inhibits the proliferation of lung cancer 
cells by inducing Go–G1 phase arrest

To further explore the role of the ectopic ATP synthase on 
cell survival of lung cancer cells, we treated both lung cancer 
cells and normal lung cells with the ATP synthase inhibitor 
citreoviridin and observed their real-time cell growth curves. 
Citreoviridin inhibited lung cancer cell proliferation in a dose-
dependent manner but did not have an effect on the growth of 
normal human IMR-90 fibroblasts (Fig. 2A). According to the 
results, cell proliferation and attachment was inhibited 4 to 6 
hours after treatment with citreoviridin. The average half 
maximal inhibitory concentrations (IC\(_{50}\)) for 48 hours treatment 
were 1.5, 4.65, and more than 6 \( \mu \)mol/L for A549, CL1-0, 
and IMR-90 cells, respectively. The variation of IC\(_{50}\) may be 
due to the difference of the amount of ectopic ATP synthase in 
each cell line. We assumed that the more expression of ectopic 
ATP synthase, the higher concentration of citreoviridin was 
needed to reach the inhibitory threshold. To distinguish the 
effects of citreoviridin on cell proliferation, whether the mito-
chondria function is inhibited may need further to be explored. 
To determine the effects of the inhibitor treatment on the 
mitochondrial electron transfer chain, we measured the MMP 
by DiOC\(_6\) in both CL1-0 and A549 cells (Fig. 2B and C). 
Comparing to paraformaldehyde caused depletion of MMP 
(Gate M1), cells treated with citreoviridin for 48 hours were 
maintained in the population of M2 where retained the MMP 
as the DMSO treated control. The results suggest that citreo-
viridin only inhibit the activity of ectopic, and not mitochon-
drial, ATP synthase.

Next, we examined the means by which the inhibition of 
ectopic ATP synthase inhibited lung cancer cell proliferation. 
Cell-cycle analysis by flow cytometry indicated that
citreoviridin increased the percentage of cells in the G₀–G₁ phase in both CL1-0 and A549 lung cells within 12 hours and markedly at 24 hours after treatment (Fig. 3A and B). This suggests that citreoviridin may inhibit cell proliferation through the inhibition of cell cycle progression at a specific phase.

**Citreoviridin reduces anchorage-independent growth of lung cancer cells**

To determine whether citreoviridin may inhibit anchorage-dependent or anchorage-independent growth by blocking oncogenes that are required for cell survival and/or growth signals normally provided to adherent nontransformed cells by the extracellular matrix via integrins, we investigated anchorage-dependent growth by colony formation on tissue culture plates and anchorage-independent growth by soft agar assay at their IC₅₀ to both cell lines. We found that citreoviridin inhibited anchorage-dependent growth of both CL1-0 and A549 cells after treatment of 2 weeks (Fig. 3C). Regarding anchorage-independent growth, cells treated with IC₅₀ citreoviridin formed significantly fewer and smaller colonies in the soft agar than cells treated with DMSO alone (Fig. 3D). These results suggest that the inhibition of ectopic ATP synthase is involved in the attenuation of anchorage-independent growth, a feature of malignant transformation of lung cancer cells.

**Proteomic analysis identifies changes in CL1-0 cells with citreoviridin treatment**

To investigate the effects of citreoviridin on protein expression, comprehensive time-course protein expression profiles were analyzed by proteomic analysis (Supplementary Fig. S3). Performing 2DE, the amounts of protein spots were quantified using ImageMaster and proteins were identified using mass spectrometry (see Table S1 for the differentially expressed proteins). We also analyzed the PPI network of the identified differentially expressed proteins. In total, 30 of 49 MS-identified proteins were mapped to the PPI network ($P < 0.005$). Gene ontology (GO) functional enrichment analysis of the dataset indicated that protein folding (8 proteins), negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle...
(7 proteins), and mRNA processing (5 proteins) were the top three cellular functions that are altered by citreoviridin treatment (Fig. 4).

**Citreoviridin induces the unfolded protein response**

Further analysis of the GO terms revealed that protein folding was the process most affected by the citreoviridin treatment (Fig. 4). Disruption of ER homeostasis leads to the accumulation of unfolded proteins. The ER has developed an adaptive mechanism known as the unfolded protein response (UPR) to cope with altered protein folding. Accumulation of unfolded proteins in the ER induces the dissociation of the chaperone protein BiP from an ER transmembrane sensor known as PKR-like ER-localized eIF2α kinase (PERK) and inositol-requiring 1α (IRE1α). The dimerization and phosphorylation of PERK and IRE1α subsequently activate the UPR. Activated PERK phosphorylates eIF2α, which in turn inhibits the assembly of translational machinery and thereby represses protein synthesis, which reduces the workload of the ER and can reduce protein accumulation in the ER. Expression of the ER stress markers in CL1-0 and A549 lung cancer cells was investigated by western blotting (Fig. 5A and B). Citreoviridin treatment increased the expression of chaperone proteins, including BiP, and protein disulfide isomerase (PDI). Oxidizing proteins, such as IRE1α, were also increased. The most immediate response to ER stress is transient attenuation of mRNA translation by increased phosphorylation of eIF2α. Accordingly, although the protein levels of eIF2α remain constant following citreoviridin treatment, the data showed that eIF2α was phosphorylated following treatment with the inhibitor, in both cell lines tested (Fig. 5C). We observed a corresponding increase in the expression of PERK following citreoviridin treatment (Fig. 5B). These results suggested that the growth attenuation of citreoviridin-treated cells may be due to the induction of the UPR and the inhibition of protein synthesis.

To confirm that PERK was involved in eIF2α phosphorylation in citreoviridin treated cells, we carried out PERK knockdown in both cell lines using RNA interference for 48 hours, and then treated them with citreoviridin for 12 hours. The phosphorylation and expression levels of eIF2α were analyzed by western blotting and normalized to the expression of actin (Fig. 5D). The phosphorylation of eIF2α was abolished after citreoviridin treatment in the PERK siRNA cells, whereas the total eIF2α levels were unaffected. These data suggest that the phosphorylation of eIF2α is mediated by PERK.

**Citreoviridin induces ROS dependent UPR**

Studies have indicated cross-talk between ER stress and oxidative stress (22, 23). Our proteomics data also showed that glutathione S-transferase Mu 3 and glutathione S-transferase P, enzymes participating in detoxification by conjugating reduced glutathione to electrophilic substrates were upregulated upon citreoviridin treatment. To verify whether citreoviridin caused ROS accumulation, H$_2$DCFDA was used to measure the level of endogenous ROS. The geometric mean of fluorescence intensity was measured by flow cytometric analysis (Fig. 6A). The results indicated that ROS levels were elevated by citreoviridin in a time-dependent manner. To see whether the citreoviridin induced UPR was ROS dependent, free radical scavenger NAC was used. Treating with citreoviridin, the phosphorylation level of eIF2α was reduced (Fig. 6B) and the cell viability was recovered (Fig. 6C) upon NAC pretreatment, indicating citreoviridin induced UPR was ROS dependent.
Citreoviridin-induced UPR does not activate the apoptotic cascade

Misfolded or unassembled proteins retained in the ER are degraded by ER-associated degradation (ERAD) through the ubiquitin/26S proteosome-dependent pathway (24) or autophagy (25). If stressed cells fail to cope with the UPR, cells undergo cell death, mainly via apoptosis (26). However, we did not observe apoptotic cell death by annexin V/PI staining in citreoviridin-treated CL1-0 and A549 cells (Supplementary Fig. S4), indicating the citreoviridin-induced inhibition on cell proliferation was caused by restriction of cell cycle progression but not cell death. Recent studies have highlighted that p53 selectively transactivate quite different responses, ranging from cell cycle arrest to cell death and senescence (27–29).

Although the detailed regulating mechanism remains unclear, we postulate that the p53 regulated cell cycle/apoptosis decision may be involved in the citreoviridin-induced pathway. To further confirm this possibility, we treated the ectopic ATP synthase expressed p53 null cell line H1299 with citreoviridin ranged from 0 to 8 μmol/L (Supplementary Fig. S5). The results showed citreoviridin only inhibited 12.5% of cell proliferation at the highest concentration, indicating citreoviridin induced inhibition on cell growth is p53 mediated.

Discussion

In the past decade, ectopic ATP synthase has been shown to involve a variety of functions in lipid metabolism, immune recognition, and invasiveness of tumors (3, 6, 9, 16), regulation of intracellular pH (14, 30), differentiation (13), control of proliferation and cell death (3, 10, 15). Ectopic ATP synthase has been shown to localize on the membrane of different cancer cell types. Here, we show that the ATP synthase complex and ETC are localized on the membrane of lung cancer cells. In an attempt to shed light on the cellular processes affected by the action of this complex, and to provide further insights into the mechanistic action of the ATP synthase inhibitor citreoviridin, we show that the inhibition of ectopic ATP synthase is associated with the inhibition of lung cancer cell growth and the activation of UPR. By disrupting the homeostasis of the ER, citreoviridin could specifically target ectopic ATP synthase-expressing cancer cells and effectively inhibit growth with limited side effects on normal cells.

Cancer cells can pose numerous microenvironmental challenges to surrounding tissues, such as through hypoxia, nutrient limitation, oxidative stress, metabolic dysregulation, or low pH. In turn, these stresses can promote the activation of specific signaling pathways, sometimes from the ER via the accumulation of misfolded proteins in the lumen (31). To account for this severe microenvironment and support proliferation, tumor cells have a higher capacity for rapid protein synthesis and degradation than normal cells (32–35). Inhibition of the ERAD pathway by proteasomal inhibitors (36) or protein folding by PDI inhibitors (37) induces the UPR and cytotoxicity in tumor cells. This implies that the homeostasis of ER capacity is critical in tumor progression and recurrence (38, 39).

But how could citreoviridin activate UPR? There are several possible mechanisms. First, it may be due to the inhibition of eATP formation by citreoviridin. Because we have now shown that lung cancer ectopic ATP synthase generates ATP (Fig. 1C), it is reasonable to speculate that citreoviridin disturbs the homeostasis of extracellular nucleotides, which may have further effects on cell signaling. For example, eATP can activate plasma membrane-localized ATP-gated ion channel (P2X) receptors and G protein-coupled (P2Y) receptors in an...
autocrine or paracrine manner (40). Furthermore, ATP is degraded rapidly to ADP and 5′-AMP, the latter of which is subsequently converted by ectopic 5′-nucleotidases into adenosine, which acts as an agonist of the P1 receptor (41). Although extracellular nucleotides and nucleosides are important for growth and death signal transduction (40, 42), whether the disruption of P1 and P2 receptor signaling by the inhibition of ectopic ATP synthase activity participates in the regulation of growth or other effects remains to be seen. Yang and colleagues showed a delicate proteomics study of the human ABCC1 interacting proteome and revealed ATP synthase binds to ABCC1 in plasma membranes and may cooperate to regulate eATP level and purinergic signaling cascade (43), supporting the regulation of eATP by ectopic ATP synthase.

Second, citreoviridin-induced acidosis may activate the UPR. Treatment with citreoviridin for 48 hours decreased the pH of the culture medium from 7.0 to 6.7 and 7.1 to 6.8 for CL1-0 and A549 cells, respectively. The acidosis of culture medium may induce ER stress and cause cytotoxicity (44). Ectopic ATP synthase may act as an intracellular pH regulator because of its role in proton transport (12, 14, 45), and the inhibition of ectopic ATP synthase is enhanced under acidic conditions (14). The inhibition of ectopic ATP synthase by citreoviridin may disrupt homeostasis of intracellular pH and cause extracellular acidosis, thus triggering the UPR by increasing inhibitory efficacy of citreoviridin.

Third, the induction of ROS forms a positive feedback loop to enhance ER stress. Our results showed that citreoviridin induces ROS production (Fig. 6A). Raj and colleagues reported that piperlongumine selectively kills cancer cells by increasing the level of ROS, but this was dependent on the cancer genotype (46). Furthermore, a mitochondrial mutation in MTATP6, one of the subunit of ATP synthase, which results in an elevated level of cytosolic ROS and MMP has been shown (47), suggesting the dysfunction of ATP synthase may contribute to impaired oxidative phosphorylation and ROS production. As there is the whole respiratory chain in the plasma membrane that generates partial eATP in lung cancer cells and the MMP was not affected by citreoviridin, we assumed that the citreoviridin-induced production of ROS may be due to the impairment of oxidative phosphorylation in the plasma membrane but not mitochondria. The active excessive ROS oxidize proteins and ultimately results in protein damage. Generation of ROS directly or indirectly affects ER homeostasis and protein folding by calcium signaling, thus results in ER stress and vise versa (48). Our results therefore suggest that citreoviridin-induced ROS elevation may contribute to the selective inhibition of growth in cancer cells.
extensive modifications, including glycosylation and disulfide bond formation. The ER contains a pool of calcium-dependent molecular chaperone proteins (49), such as Grp94, calnexin, and calreticulin, which assist in protein folding, disulfide bond formation, and N-linked glycosylation. The depletion of ER luminal calcium and increased cytosolic calcium cause dysfunction of the molecular chaperones and leads to the UPR (50).

The addition of ATP, the antioxidant NAC, chemical chaperones, or the calcium chelator EGTA rescued citreoviridin-induced growth inhibition (Fig. 6C), suggesting that regulation of the citreoviridin-induced inhibition of cell proliferation is complex, and many mechanisms in addition to UPR activation are involved (Supplementary Fig. 56). It would therefore be interesting to investigate whether pathways enriched in the PPI network could have a synergic/additive effect with UPR in citreoviridin-induced growth inhibition. Here, we provided evidence that in combination of only dose of 1 μmol/L citreoviridin with 10 μmol/L 26S proteasome inhibitor bortezomib caused significantly decreasing in cell viability when comparing to single agent treatment (Fig. S7), implying the possibility of synergic/additive therapy for citreoviridin and proteasome inhibitors.

This study provides the first evidence that the inhibition of ectopic ATP synthase induces the UPR, which disrupts the balance between life and death in lung cancer cells and highlights the therapeutic potential of ectopic ATP synthase inhibition in cancer cells. Further investigations of the ectopic ATP synthase PPI network, including the active and nonactive state downstream signaling, will be crucial for a more comprehensive understanding of its function in the cell membrane of tumor cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H.-Y. Chang, H.-C. Huang, T.-C. Huang, P.-C. Yang, H.-F. Juan
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