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Mitochondrial Unfolded Protein Response Regulator ATF5 in Mitochondrial Targeted Therapies in AML

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Mitochondrial Unfolded Protein Response Regulator ATF5 in Mitochondrial Targeted
Therapies in AML

by

Ran Zhao, B.S.

APPROVED:

Michael Andreeff, M.D., Ph.D.
Advisory Professor

Ishizawa Jo, M.D., Ph.D.

Marina Konopleva, M.D., Ph.D.

Richard E. Davis, M.D.

Gautam Borthakur, M.D.

APPROVED:

Dean, The University of Texas
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

Mitochondrial Unfolded Protein Response Regulator ATF5 in Mitochondrial Targeted
Therapies in AML

A

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Master of Science

by

Ran Zhao, B.S.
Houston, Texas

December, 2021

Abstract

Mitochondrial unfolded protein response (UPR^{mt}) is an adaptive transcriptional response induced by damaged proteins accumulated in mitochondria. UPR^{mt} signaling involves induction of mitochondrial specific chaperones and proteases such as HSP60, LonP1 and ClpP, aiding in the restoration of mitochondrial protein pool homeostasis. However, the cell-protective roles of UPR^{mt} in the context of mitochondrial stress-induced cell death in AML has not been well explored. We demonstrate that AML cells are susceptible to mitochondrial targeted agents such as ONC201, an agonist of the mitochondrial protease ClpP, and gamitrinib, an inhibitor of mitochondrial chaperone TRAP1, however, these agents also induce activating transcription factor 5 (ATF5), a primary mediator of UPR^{mt} in mammals. Thus, we hypothesized that inactivating the cell-protective UPR^{mt} pathway, by inhibiting ATF5, could potentially sensitize AML cells to mitochondrial stress. Consistently, flow cytometry-based apoptosis assays demonstrated that stable knockdown of ATF5 by short-hairpin RNA (shRNA) sensitized AML cells to single treatments of ONC201 and gamitrinib. In contrast, ATF5 knockdown did not further enhance AML cell killing when these agents were combined with the BCL2 inhibitor venetoclax, suggesting that venetoclax combination may overcome the cell-protective response of UPR^{mt}. However, tetracycline-inducible shRNA against ATF5 did not sensitize AML cells to any of the above treatments. Similarly, pharmacological inhibition of ATF5 function by cell penetrating peptide Dpep was also unable to sensitize AML cells to mitochondrial targeted agents, suggesting that short-term inactivation of ATF5 is not sufficient to abrogate its cell-protective functions. Of note, while knockdown of ATF5 gene expression was confirmed in both stable and transient short hairpin models, canonical downstream genes of UPR^{mt} (e.g.,

LONP1 and *HSPD1*) were not significantly affected in neither model, suggesting a disconnect between ATF5 expression and regulation of UPR^{mt} gene expression, perhaps uniquely in AML cells.

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Mitochondrial Unfolded Protein Response Regulator ATF5 in Mitochondrial Targeted Therapies in AML

Ran Zhao, B.S

Advisory Professor: Michael Andreeff, M.D., Ph.D.

Introduction

Mitochondria are responsible for cellular bioenergetics and metabolite production necessary for cell growth and proliferation (1). Another important role of mitochondria is the regulation of apoptosis, a programmed cell death pathway mediated by proapoptotic Bcl-2 family members and pharmacologically inducible by BH3 mimetics. One such BH3 mimetic is venetoclax, a specific inhibitor of the anti-apoptotic protein Bcl-2. Venetoclax is FDA approved for AML, in combination with hypomethylating agents or cytarabine (2). Additionally, AML cells have greater copy numbers of mitochondrial DNA and higher rates of oxygen consumption in comparison to normal hematopoietic stem cells (HSCs), suggesting a greater reliance on mitochondria (3). Mitochondrial metabolites involved in glucose metabolism are also reported to have prognostic value in AML (4). Thus, mitochondria are a potential therapeutic target in AML. Indeed, AML cells have been shown to be vulnerable to mitochondrial targeted agents such as inhibitors of mitochondrial respiratory chain complex I (e.g., IACS-010759), potent agonists of mitochondrial caseinolytic protease P (ClpP) (ONC201 and ONC212), an inhibitor of mitochondrial-

specific heat shock protein-75 (HSP75) (gamitrinib), and inhibitor of mitochondrial importer mia40 (MitoBlock6) (Figure 1B) (2, 5, 6).

While AML cells are sensitive to mitochondrial targeted agents, mitochondrial dysfunction induced by mitochondrial targeted agents also triggers mitochondrial unfolded protein response (UPR^{mt}), a protective and adaptive transcriptional response induced upon cellular crisis by damaged proteins when mitochondrial protein compositions or folding conditions are dysregulated (8, 9). UPR^{mt} signaling involves transcriptional induction of mitochondrial specific chaperones and proteases including HSP60 (HSPD1), HSP70 (HSPA9), LonP1 (LONP1) and ClpP, some of which are highly expressed in various cancers including AML, promoting mitochondrial protein pool homeostasis and repair (9). Previous studies have shown that expression of these UPR^{mt} induced chaperones and proteases contributes to tumor growth and survival, whereas their inhibition enhances apoptosis and reduced tumor cell proliferation (10,11).

Activating transcription factor 5 (ATF5), a bZip family transcription factor, has recently been proposed as a main regulator of UPR^{mt} in mammals (8, 9). ATF5 protein is reported to contain both mitochondrial localization and nuclear localization sequences. Previous studies propose that under normal physiological conditions, ATF5 accumulates in mitochondria, while under mitochondrial stress conditions ATF5 instead accumulates in the nucleus, activating transcription of downstream UPR^{mt} genes and aiding in restoration of mitochondrial protein pool homeostasis (Figure 1A) (9, 12). ATF5 is considered oncogenic in several cancers, including glioblastoma, breast carcinoma, leukemia and lung adenocarcinoma. Targeting and depletion of ATF5 in glioblastoma results in tumor regression and growth inhibition (12). We have previously reported that mitochondrial stress

inducers such as ONC201, Tigecycline (inhibitor of mitochondrial ribosomal EF-Tu), and MitoBlock6 induce high levels of ATF5 gene expression in AML cell lines (7). Thus, ATF5-mediated UPR^{mt} signaling could be protective against mitochondrial targeted agents in AML cells, and inactivation of ATF5 possibly sensitize AML cells to mitochondrial targeted agents. The cell-protective roles of ATF5-regulated UPR^{mt} against mitochondrial targeted agents in AML cells have not been well explored. Here we hypothesize that ATF5 regulates UPR^{mt} which protects AML cells from mitochondrial targeted therapies.

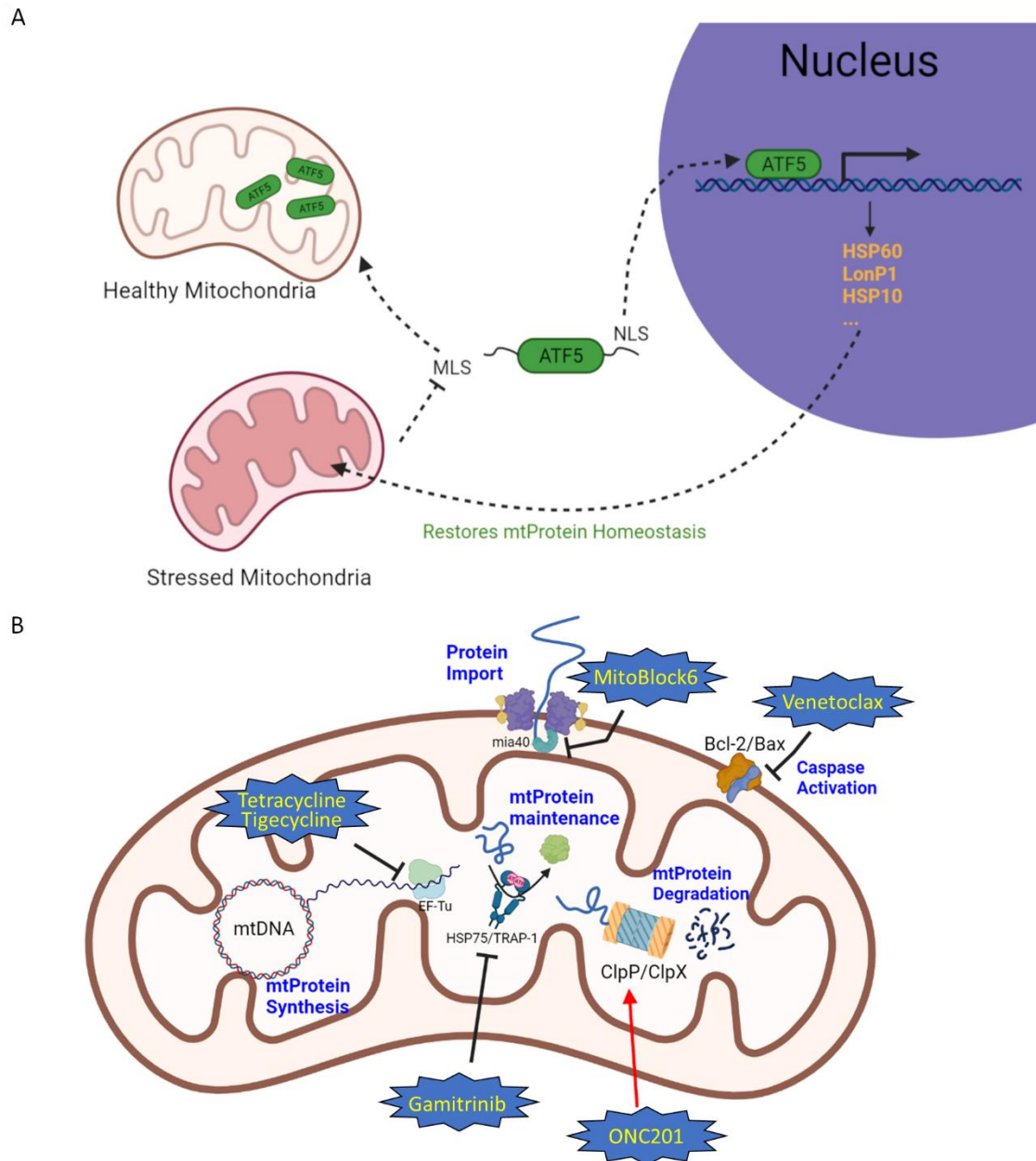


Figure 1. Overview of ATF5 regulation of UPR^{mt}.

(A) Proposed cellular mechanism of UPR^{mt} mediation by ATF5. (B) mitochondrial targeted agents explored in this study and their molecular targets.

Material and Methods:

Cells and culture conditions:

OCI-AML3, MOLM13 and MV4;11 cells were grown and maintained in RPMI-1640 with 10% fetal bovine serum (FBS). HEK-293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS. Cell lines were previously confirmed to be mycoplasma negative by DNA fingerprinting (5).

Cell viability and apoptosis assays:

AML cell lines OCI-AML3, MOLM13 and MV4;11 were seeded at 1.5×10^5 per mL in either 24-well or 96-well plates for 72-hour timepoint apoptosis assays with venetoclax (chemgood), ONC201 (MedChemExpress), gamitrinib (obtained from Dr. Altieri at the Wistar Institute) and Dpep (details below). For tetracycline-inducible shRNA models targeting ATF5, AML cell lines were first pre-treated with tetracycline for 48 hours before treatment with ONC201, gamitrinib and venetoclax along with continuous tetracycline induction. Cells were seeded at 1.5×10^5 / mL initial concentration and passaged at a ratio of 1:4 after 48 hours to avoid over-confluency and spontaneous cell death due to extended experimental timepoints.

In all apoptosis experiments, final cell concentration in untreated wells were adjusted to be $< 1.6 \times 10^6$ per mL to avoid over-growth and spontaneous cell death. For apoptosis analysis, Annexin V and DAPI binding assays were performed using flow cytometry. Annexin V-negative and/or DAPI-negative populations were considered live cells, while

Annexin V-positive and Annexin V/DAPI-double-positive populations were categorized as apoptotic cells.

Dpep was purchased from AlanScientific in acetate salt form with the following sequence RQIKIWFQNRRMKWKKLVELSAENEKLHQRVEQLTRDLAQLRQFFK (14). Peptide was dissolved in sterile 10% glycerol dH₂O and aliquoted for storage at -80 Celsius.

Immunoblotting

Cell line lysates were extracted using 2% SDS protein lysis buffer (2% SDS, 10% glycerol, 0.02% BPB, 4% 2-ME in 0.25M pH 6.8 Tris-HCL) with added phosphatase protease inhibitor (Cell Signaling) at 2 x10⁶ cells / 100uL. SDS-Page was performed using Bio-Rad 10% or 12% pre-cast gels and quantified with Odyssey imaging system. Antibody used: ATF5 (Invitrogen), β -Actin (sigma)

Real-time PCR

mRNA was extracted from 1-2 x10⁶ OCI-AML3, MOLM13 and MV4;11 cells with Qiagen RNeasy kit. Single-strand cDNA were generated using SuperScript™ III First-Strand Synthesis System Super Mix from extracted mRNA. RT-PCR was performed on 96-well plates with TaqMan fluorophore probes and TaqMan™ Fast Advanced Master Mix using Thermofisher QuantStudio 3 instrument.

Lentiviral infection and ATF5 knock down models.

Mature shRNA sequences targeting ATF5 (“38” and “42”) are as follows:
TRCN0000017638: AAGTCTTCCATCTGTTCCAGC, TRCN0000017642:
AAGTCCAGAGTATCCAAGACA. Vectors of stable-expression ATF5 shRNA clone 38 and 42 in PLKO.1 backbone was directly purchased from vendor (Dharmacon). Tetracycline-

inducible model of shATF5 clone 38 vector was generated by removing the stuffer between restriction sites AgeI and EcoRI then ligating the ATF5 shRNA sequence between the restriction sites on tet-on PLKO.1 backbone, which contains an upstream Tetracycline response element (TRE) followed by puromycin-resistance element for selection.

Lentivirus of both stable and tet-on shATF5 models were generated by transfecting pMD2.G and psPAX2 together with shATF5 vector in HEK-293T cells using JetPrime transfection reagent according to manufacturer's protocol. After 5 hours, culture media of transfected HEK-293T cells were replaced with 10% FBS Iscove's DMEM as virus producing media. Lentivirus containing supernatant was collected after 48 hours of incubation. OCI-AML3, MOLM13 and MV4;11 cells were infected for 24 hours using viral supernatant along with polybrene (OCI-AML3- 8ug/mL, MOLM13 6ug/mL, MV4;11 6ug/mL). After viral incubation, polybrene containing virus media were washed out by centrifugation at 200xg for 10 minutes. Infected cells were then incubated in 10% FBS RPMI-1640 media for approximately two doubling times before beginning puromycin selection at initial concentration of 0.5ug/mL. Selection of transformed cells was concluded when cells reached 95% viability under 1ug/mL puromycin treatment.

RESULTS

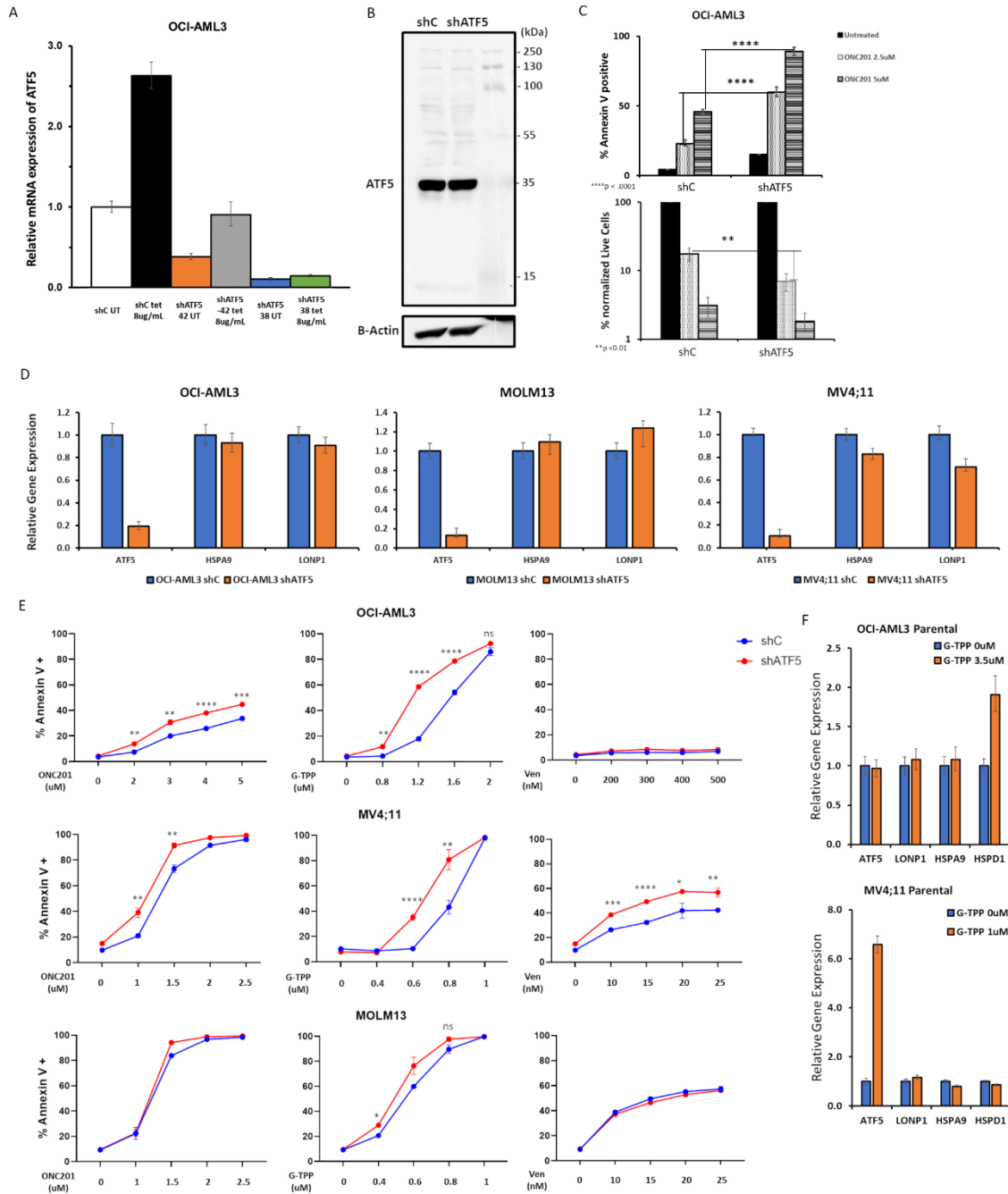


Figure 2. Stable Knockdown of ATF5 Sensitizes AML Cells to Mitochondrial targeted agents.

(A) RT-PCR confirmation of ATF5 knockdown in OCI-AML3 cells transduced with short-hairpin ATF5 scramble control, clone 38 and clone 42 PLKO.1 vector. Cells were treated with tetracycline 8ug/mL for 48 hours to induce ATF5 expression.

(B). Western blot of stable expression shATF5 clone 38 transduced OCI-AML3 cells. ATF5 is expected at 31kDa, but

detected band is at 35 kDa. (C). Apoptosis and Live cell % of OCI-AML3 shATF5 clone 38 cells after 72 hours of ONC201 treatment. (D) RT-PCR of ATF5 and UPR^{mt} genes using newly generated stable expression shATF5 clone 38 OCI-AML3, MOLM13 and MV4;11 cell lines. (E) Apoptosis effect of mitochondrial targeted agents (ONC201, venetoclax, gamitrinib(G-TPP)) in shATF5 MOLM13, OCI-AML3 and MV4;11 cells compared to scramble control after 72 hours. False positive adjusted p-value (q-value) * <0.01 , ** <0.001 , *** <0.0001 , **** <0.00001 . (F) RT-PCR verification of ATF5 and UPR^{mt} gene expression induced by classical UPR^{mt} inducer gamitrinib(G-TPP) in OCI-AML3 cells after 6hr treatment.

Stable knockdown of ATF5 sensitizes AML cells to mitochondrial targeted agents.

To explore the effects of genetic knockdown of ATF5 in AML cell lines and their sensitivity to mitochondrial targeted agents, we designed a stable expression model of shRNA targeting ATF5. For initial assessment, OCI-AML3 cells were transformed with two shRNAs, shATF5 38 and shATF5 42 by lentiviral infection. Stable expression of shATF5 38 significantly downregulated *ATF5* mRNA expression in OCI-AML3 cells and inhibited high-dose tetracycline-induced ATF5 upregulation (Figure 2A). Confirmation of ATF5 reduction at the protein level was unsuccessful, possibly due to the unreliable anti-ATF5 antibody as the molecular weight of detected bands did not match the expected 31 kDa molecular weight (Figure 2B). (There is no established anti-ATF5 antibody commercially available, per personal conversation with Dr. Cole M. Haynes, an expert of UPR^{mt} in University of Massachusetts Medical School.)

We have previously reported ONC201 as an inducer of ATF5 and UPR^{mt} (7). OCI-AML3 with stable expression of shATF5 (shATF5 OCI-AML3 cells) and control shRNA (shC) were treated with ONC201 for cell death assay. shATF5 OCI-AML3 cells showed significantly greater apoptosis and less proliferation with ONC201 treatment, compared to those in shC OCI-AML3 cells (Figure 2C). Of note, in untreated conditions, shATF5 OCI-AML3 cells showed higher spontaneous apoptosis and lower proliferation compared to

scramble control, suggesting that ATF5 knockdown alone exerts anti-AML effects (Figure 2C).

To verify the sensitization of AML to mitochondrial targeted agents by ATF5 knockdown, we applied the shATF5 model also in MOLM13 and MV4;11 cells and generated another clone of OCI-AML3 shATF5 cells (Figure 2D), and cells were treated with ONC201, gamitrinib and venetoclax. While varying among the three AML cell lines, data show that stable expression of ATF5 knockdown sensitizes AML cells to mitochondrial targeted agents. Specifically, shATF5 OCI-AML3 cells were more sensitive to ONC201 and gamitrinib but not to venetoclax, compared to control cells; shATF5 MV4;11 were sensitive to all three treatments, compared to control cells; and shATF5 MOLM13 was only slightly more sensitive to gamitrinib treatment compared to control cells, and showed no significant difference to the other two compounds (Figure 2E). Of note, although gamitrinib is an established UPR^{mt} inducer, changes in UPR^{mt} genes observed in AML cells after gamitrinib treatment were variable (Figure 2F). In OCI-AML3 cells, *HSPD1* mRNA was upregulated by gamitrinib while *ATF5*, *LONP1* and *HSPA9* mRNA were not. In MV4;11 cells, only *ATF5* mRNA was upregulated while the other three genes are not affected.

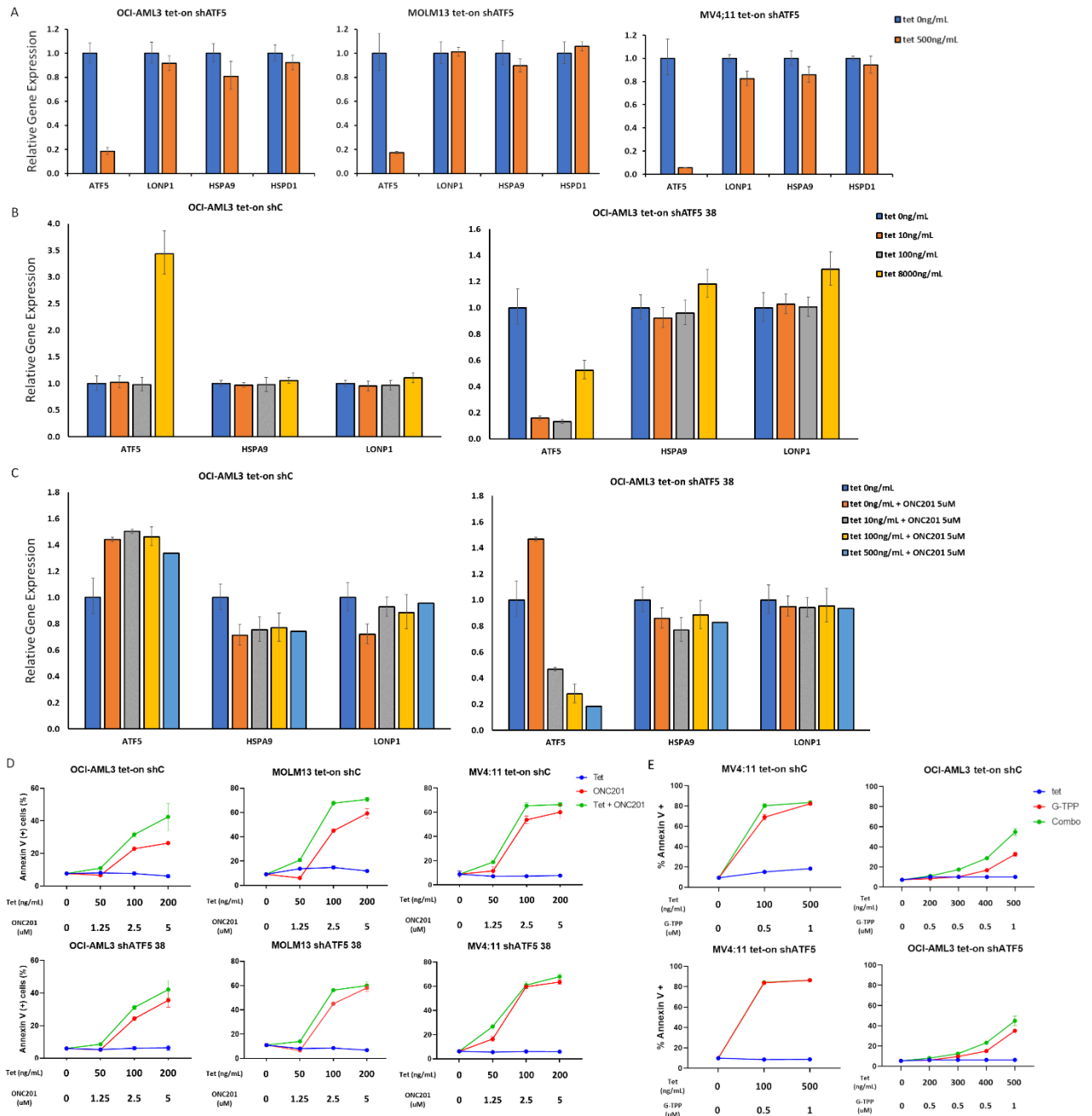


Figure 3. Transient tetracycline inducible ATF5 knockdown does not sensitize AML cells to mitochondria targeted therapeutics.

(A) RT-PCR verification of tet-on ATF5 knockdown and UPRmt genes panel. OCI-AML3, MOLM13 and MV4;11 cells were treated with tetracycline at indicated concentrations for 48 hours prior to lysate extraction. (B) RT-PCR of ATF5 knockdown efficacy and effects on UPRmt genes by dose-dependent and high-dose tetracycline treatment at 48hr timepoint. (C) RT-PCR of ATF5 knockdown efficacy and effects on UPRmt genes by 24hr ONC201 treatment after 48 hours of

tetracycline induction. (D) 72-hour apoptosis assay comparison between control and tet-on shATF5 OCI-AML3, MV4;11 and MOLM13 cells after treatment with ONC201. (E) 48-hour apoptosis assay comparison between control and tet-on shATF5 OCI-AML3 and MV4;11 cells after treatment with gamitrinib(G-TPP).

Tetracycline-inducible ATF5 knockdown does not sensitize AML cells to mitochondria targeted agents.

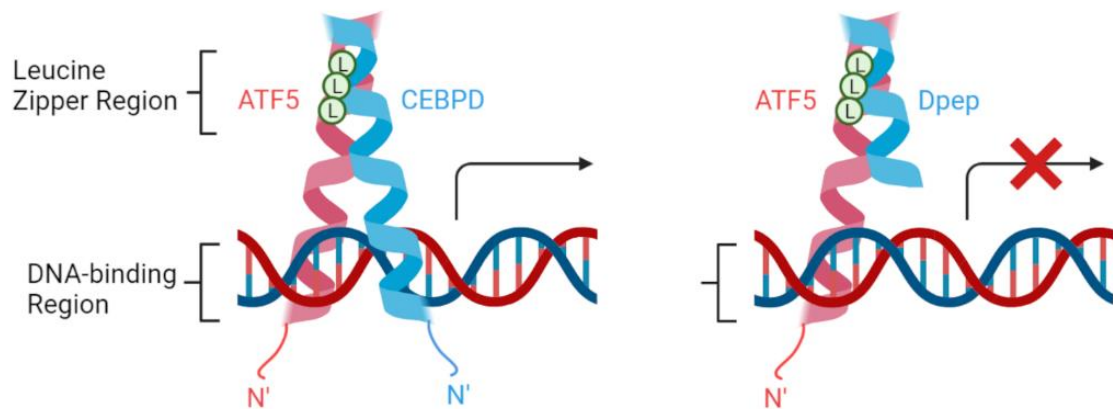
To test whether acute inactivation of ATF5 sensitizes AML to mitochondrial targeted agents, we generated a tetracycline-inducible model of ATF5 knockdown by shRNA (tet-on shATF5) which could potentially mimic pharmacological inhibition of ATF5. OCI-AML3, MOLM13 and MV4;11 cells were transfected with tet-on shATF5 vector by lentiviral infection. The cells were then treated with tetracycline to induce ATF5 knockdown, at a low dose that is sufficient to induce expression of the shRNA, but not at the high dose used previously to induce mtUPR. (Figure 3A). The tet-on shATF5 model in OCI-AML3 cells was able to successfully knock down both basal and high-dose tet-induced *ATF5* expression (Figure 3B). Tet-on ATF5 was also able to knock down ONC201-induced ATF5 by tetracycline treatment in a dose dependent manner (Figure 3C). Of note, increase or decrease ATF5 gene expression by either knockdown or mitochondrial targeted agents did not change expressions of downstream UPR^{mt} genes significantly (Figure 3A-C). This suggest a possible disconnection of ATF5 regulation and UPR^{mt} genes in AML cells.

To evaluate the effect of transient ATF5 knockdown on killing of AML cells by mitochondrial targeted agents, we pre-treated tet-on shATF5 OCI-AML3, MOLM13 and MV4;11 cells with tetracycline to knock down ATF5, then treated with the cells with ONC201 or gamitrinib. Contrary to results observed in the stable shATF5-expressing model (Figure 2E), transient ATF5 knockdown by this inducible model did not sensitize OCI-AML3, MOLM13 and MV4;11 cells to ONC201 treatment (Figure 3D). Similarly, transient

tet-on ATF5 knockdown did not sensitize OCI-AML3 and MV4;11 against gamitrinib treatment compared to controls (Figure 3E), as opposed to the sensitization observed in stable knockdown of ATF5 (Figure 2E). This finding suggests that stable knockdown of ATF5 may have different biological phenotypes and UPR^{mt} regulation compared to transient tet-on ATF5 knockdown, possibly due to chronic depletion of ATF5.

Of note, a drawback of tet-on shATF5 model in our context of UPR^{mt} is that tetracycline at higher doses also acts as a UPR^{mt}/ATF5 inducer, as tetracycline disrupts mitochondrial protein translation. Indeed, even in shC cells, tetracycline caused synergistic cell death in combination with ONC201 or gamitrinib, likely reflecting its inhibitory effects on mitochondrial protein translation. However, even at the highest dose of 500ug/mL, we confirmed that ATF5 knockdown is still successful compared to shC (Figure 3B-C), indicating that the inducible knockdown system is functional and useful for the comparison between shC vs shATF5 cells.

A



B

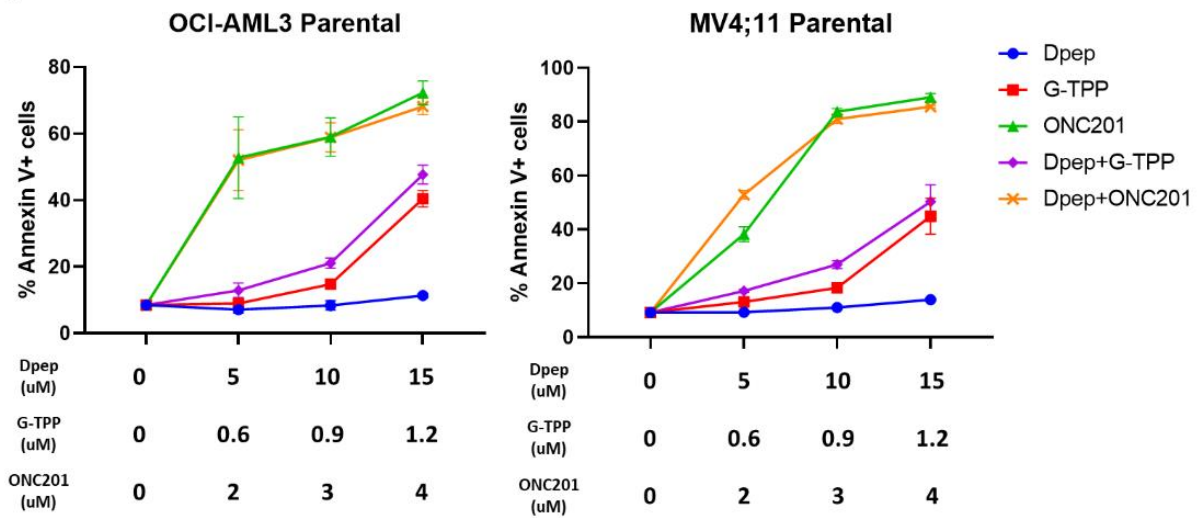


Figure 4. Pharmacological inhibition of ATF5 by Dominant-negative Peptide of CEBPD Does Not Sensitize AML Cells to Mitochondrial Targeted Agents

(A) Principle of Dpep's functional inactivation of ATF5. (B) Apoptosis results of OCI-AML3 and MV4;11 parental cells after 72 hours of Dpep + gamitrinib (G-TPP) /ONC201 combination treatment. Similar range of Dpep concentration was used in a previous report on solid tumors such as breast cancers and glioblastoma which induced cell death (14).

Pharmacological inhibition of ATF5 by dominant-negative peptide CEBPD does not sensitize AML cells to mitochondrial targeted agents.

We then tested if pharmacological inhibition of ATF5 would sensitize AML cells to mitochondrial targeted agents. ATF5 must form dimers with another bZip transcription factor to function. ATF5 does not form homodimers by itself, and instead forms heterodimers with its binding partners CEBPD and CEBPB. Dpep, a cell penetrating, dominative-negative peptide of ATF5's binding partner CEBPD without a DNA binding domain can be used to functionally inactivate ATF5 (Figure 4A) (14). To test cell killing effects of pharmacological inhibition of ATF5 combined with mitochondrial targeted agents, we co-treated OCI-AML3 cells using Dpep with ONC201 or gamitrinib. Similar to transient genetic knockdown of ATF5, Dpep co-treatment did not enhance apoptosis in OCI-AML3 and MV4;11 cells by ONC201 or gamitrinib (Figure 4B). These data suggest that pharmacological inactivation of ATF5 does not sensitize AML cells to mitochondrial targeted agents, consistent with the findings with transient tet-on shATF5 knockdown.

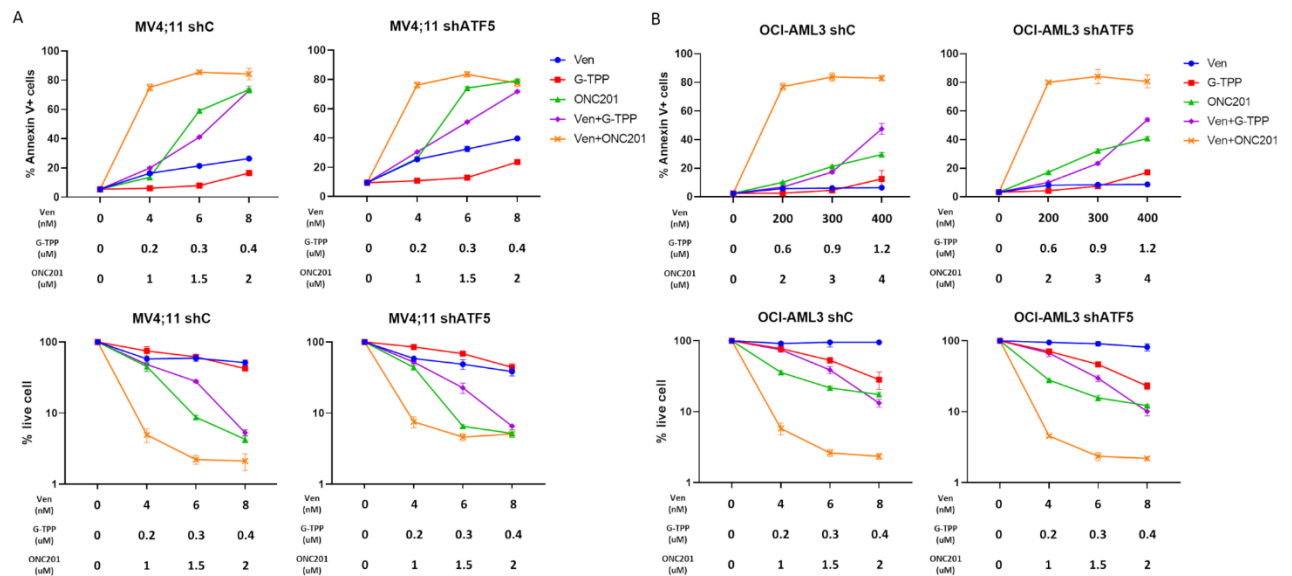


Figure 5. Stable knockdown of ATF5 Did not Sensitize AML Cells to Venetoclax Combination Treatment with Mitochondria Targeted Therapeutics.

(A) Apoptosis and live cell data of MV4;11 with or without stable ATF5 knockdown when treated with venetoclax and G-TPP/ONC201 for 72 hours. (B) Apoptosis and live cell data of OCI-AML3 with or without stable ATF5 knockdown when treated with venetoclax and gamitrinib (G-TPP) for 72 hours.

Stable knockdown of ATF5 did not sensitize AML cells to venetoclax combination treatment with mitochondria targeted agents

Venetoclax is often used in the clinic in combination therapies with other therapeutics such as azacitidine or cytarabine, since single agent venetoclax treatment only exhibits modest activity in AML (15). We have previously reported that venetoclax synergizes with various mitochondrial targeted agents such as ONC201, tigecycline and MitoBlock6 in AML cell lines (7). Venetoclax is also synergistic *in vivo* and *in vitro* in combination with ONC212, a more potent analogue of ONC201 (16). While transient genetic and pharmacological inactivation of ATF5 did not sensitize AML cells to mitochondrial targeted agents, we further examined if stable ATF5 knockdown would sensitize AML cells to venetoclax in combination with mitochondria targeted agents. OCI-AML3 and MV4;11 were treated with venetoclax in combination with ONC201 or gamitrinib. While venetoclax exhibited strong apoptogenic synergism in combination with both ONC201 and gamitrinib as expected, stable ATF5 knockdown did not further enhance the efficacy of combination therapies (Figure 5A, B), suggesting that the potent synergy induced by these combinations can overcome ATF5-mediated cell-protective effects.

Discussion:

In this study, we have shown that stable shATF5 knockdown sensitized AML cells to individual mitochondrial targeted agents but not when combined with venetoclax *in vitro*.

Thus, ATF5 gene expression potentially protects AML cells against mitochondrial targeted agents, while the potent synergy induced by the combination with venetoclax could overcome the ATF5-mediated cell-protective response.

Also, an unexpected finding was that upregulated ATF5 expression by mitochondrial targeted agents in AML did not significantly enhance the expressions of UPR^{mt} downstream genes (e.g., 3F, 3B and 3C), making it challenging for us to validate the functional inactivation of ATF5 in our knockdown models in the present study. Both stable and transient knockdown of ATF5 also did not significantly decrease UPR^{mt} gene expression, suggesting a disconnect between ATF5 and UPR^{mt}, and that ATF5 gene expression level does not directly regulate proposed downstream UPR^{mt} gene expression. A potential explanation of the disconnect between ATF5 upregulation and downstream effects is that AML cells have a unique ATF5-independent regulation of UPR^{mt} genes. Previous studies on ATF5-mediated UPR^{mt} used HeLa and HEK-293T cells, thus the discrepancy we observed in this study may be tissue- or tumor-type-dependent. Further investigation of downstream genes by more comprehensive screening such as microarray or RNA-sequencing using our ATF5 knockdown cells would be useful to address this hypothesis. As a future direction, rather than targeting ATF5, knockdown or knockout of specific UPR^{mt} downstream genes such as LONP1 or HSP70 could be used to study UPR^{mt} mediated protection against mitochondrial targeted agents in the context of AML.

There was a difference in phenotype between transient and stable ATF5 knockdown in terms of AML cell sensitivity to cell death induction by mitochondrial targeted agents. Tet-on ATF5 knockdown, while successfully suppressing ATF5 gene expression, did not sensitize AML cells to the mitochondrial targeted agents while stable ATF5 knockdown did,

using the same agents in the same AML cell lines. This suggests that chronic depletion of ATF5 could be the key factor to mitochondrial targeted agents' sensitization of AML, by inducing unexplored genetic or proteomic changes. Under steady state conditions, ATF5 protein is reported to have a very short half-life due to protease-dependent and caspase-dependent mechanisms, but its stability can be greatly enhanced by chaperone HSP70 (17). This suggests the possibility that a short, transient knockdown of ATF5 may not be sufficient to deplete remaining ATF5 protein bound to stabilizing chaperones. A CRISPR-cas9 ATF5 knockout model could provide more reliable ATF5 depletion for future investigations. Results from pharmacological inhibition of ATF5, however, do not support this possibility as Dpep functionally inhibits ATF5, yet still did not enhance AML cell sensitivity to mitochondrial targeted agents. Dpep concentration used in AML testing was able to induce cell death in solid tumor models such as melanoma and glioblastoma (14).

The proof of concept in this study relies on transcript levels of ATF5 alone, as there is no validated, published antibody to ATF5. Previous studies of ATF5 and UPR^{mt} used antibodies generated in individual laboratory and are unavailable commercially (8). Future studies on this topic should be augmented with working ATF5 specific antibodies, or by genetically inserting FLAG epitopes into the *ATF5* locus for protein level assessment

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Vita:

Ran Zhao graduated from Bellaire Senior High School in 2012 and entered the University of Houston in Houston, Texas. He received Bachelor of Science with a major in Biology with university honors in winter 2015. Afterwards, he worked for 3 years as a research assistant under Dr. Michael Andreeff and Dr. Ishizawa Jo in the Department of Leukemia in University of Texas MD Anderson Cancer Center. In August 2019, he joined The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Science in pursuit of a Masters in Biomedical Sciences with a focus on cancer biology.