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Tian Mi

Determining the mechanism behind imipramine blue's selectivity towards FLT3-ITD^{+} acute myeloid leukemia

By

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B.S., Emory University, 2018

Advisor:

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An abstract of a thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Science in the Graduate Division of Biological and Biomedical Sciences, Cancer Biology, 2019.

Abstract

Determining the mechanism behind imipramine blue's selectivity towards FLT3-ITD$^+$ acute myeloid leukemia

By Tian Mi

FLT3-ITD$^+$ acute myeloid leukemia (AML) accounts for a quarter of AML cases and is associated with very poor prognosis and high relapse rate. Although in recent years many FLT3 inhibitors have been extensively studied, they only produced transient responses. Imipramine blue (IB) and honokiol (HK) has emerged as promising new drugs to treat FLT3-ITD$^+$ AML targeting oncogenic reactive oxygen species (ROS) and signal transducer and activator of transcription 5 (STAT5). IB is found to cause cytosolic calcium release and more potent at inducing cell death in FLT3-ITD$^+$ AML cell lines than FLT3-WT. This thesis primarily aims to figure out why IB exhibits greater efficacy towards FLT3-ITD$^+$ AML. Contrary to our hypothesis, we found that IB did not induce ER stress in AML cell lines. We also discovered that dynamin related protein1 (Drp1), a key component of mitochondrial fission, had higher expression in FLT3-ITD$^+$ cell lines and IB treatment activated Drp1. Therefore, we proposed that more Drp1 had been activated by IB in FLT3-ITD$^+$ AML, leading to extensive mitochondrial fission and subsequently more robust apoptosis. However, inhibiting IB-induced activation of Drp1 did not eliminate the difference in sensitivity towards IB between FLT3-ITD$^+$ cell lines and FLT3-WT cell line. On the other hand, HK formed potent synergy with pimozide, an FDA-approved anti-psychotic drug.

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Introduction

Acute myeloid leukemia (AML) overview

AML is a cancer of blood and bone marrow. It is characterized by abnormal proliferation and differentiation of a clonal population of myeloid stem cells [1]. AML is a complicated disease with high relapse rate. Although 80% of adult AML patients achieve complete remission after initial chemotherapy, there are approximately 50% of them who experience relapse [2]. However, the general approach for AML treatment has not changed profoundly over three decades, with intensive chemotherapy and allogenic hematopoietic stem cell transplantation still being the two major strategies [3]. Therefore, there is a need for improved therapy for AML patients.

FLT3-ITD$^+$ AML

AML is an enormously heterogeneous disease with various subtypes [1]. Among all AML patients, around 20-30% carry the FLT3-internal tandem duplication (ITD) mutation, which is an activating mutation for the FLT3 receptor tyrosine kinase [4]. Such mutation not only overactivates FLT3, but also cause FLT3 mislocalization to the ER membrane, resulting in aberrant activation of downstream signaling pathway, promoting cell proliferation and survival, and leading to malignant phenotypes [5, 6]. STAT5 is an important target in such aberrant FLT3 signaling pathway. It is constitutively activated by ER-localized FLT3 through tyrosine phosphorylation and acts as a critical transcription factor regulating the proliferation and differentiation of hematopoietic cells [7]. AML patients carrying the FLT3 mutation are often associated with poorer prognostic outcomes. However, recent developments for targeted therapy to treat FLT3-ITD$^+$ AML have limited effect to prevent relapse, including FLT3 inhibitors such as quizartinib (Ac220) [4, 8], calling for more research on such topic.

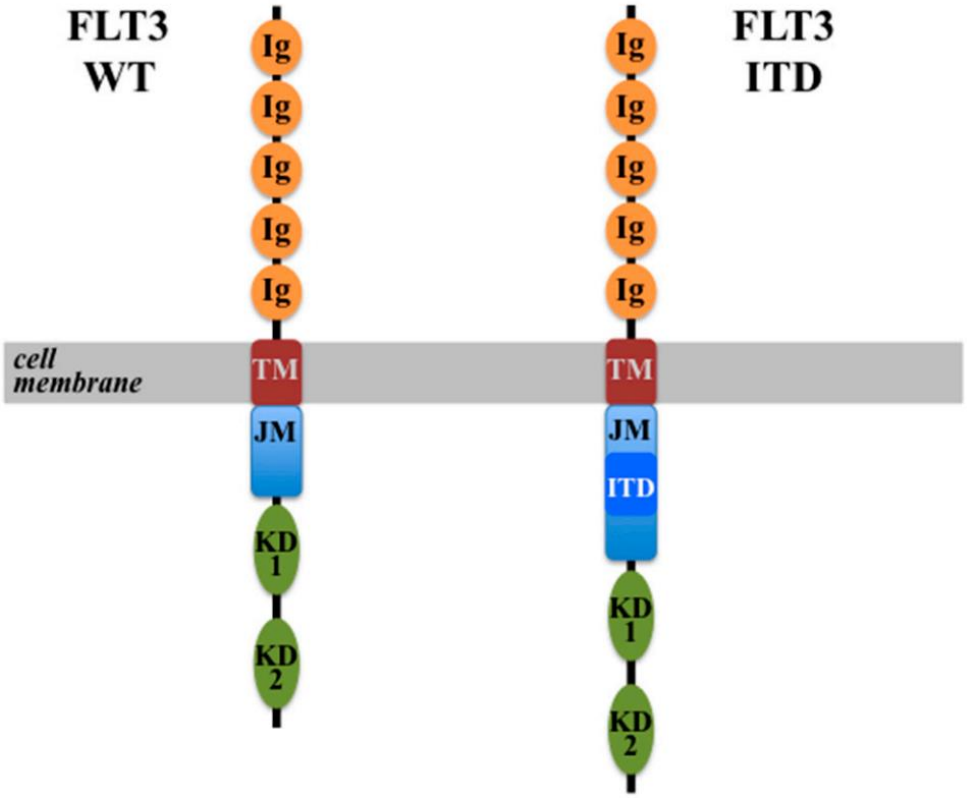

Figure 1 Structural domains of FLT3-WT and FLT3-ITD. Figure was from [9]

Positive feedback loop between STAT5 and ROS

Reactive oxygen species (ROS) are generated primarily as a cellular by-product. They are associated with cancer as increased ROS production causes DNA damage and performs a signaling function to promote cell proliferation and migration [4]. Interestingly, persistent activation of STAT5 signaling pathway in FLT3-ITD^{+} AML is associated with increased production of ROS through a positive feedback loop. STAT5 mediates ROS production by enhancing the transcription of NOX4, which is a protein that directly contributes to ROS formation [10, 11]. STAT5 also represses the expression of antioxidant enzymes to further promote the accumulation of ROS [12]. At the meantime, ROS promotes STAT5 activation by repressing the enzymatic activities of protein tyrosine phosphatase (PTP). While PTP opposes STAT5 activation by removing a phosphate group, ROS increase activation-specific

phosphorylation of STAT5 by inhibiting PTP [4]. Such correlation between STAT5 and ROS together contributes to the malignant phenotype of FLT3-ITD$^+$ AML.

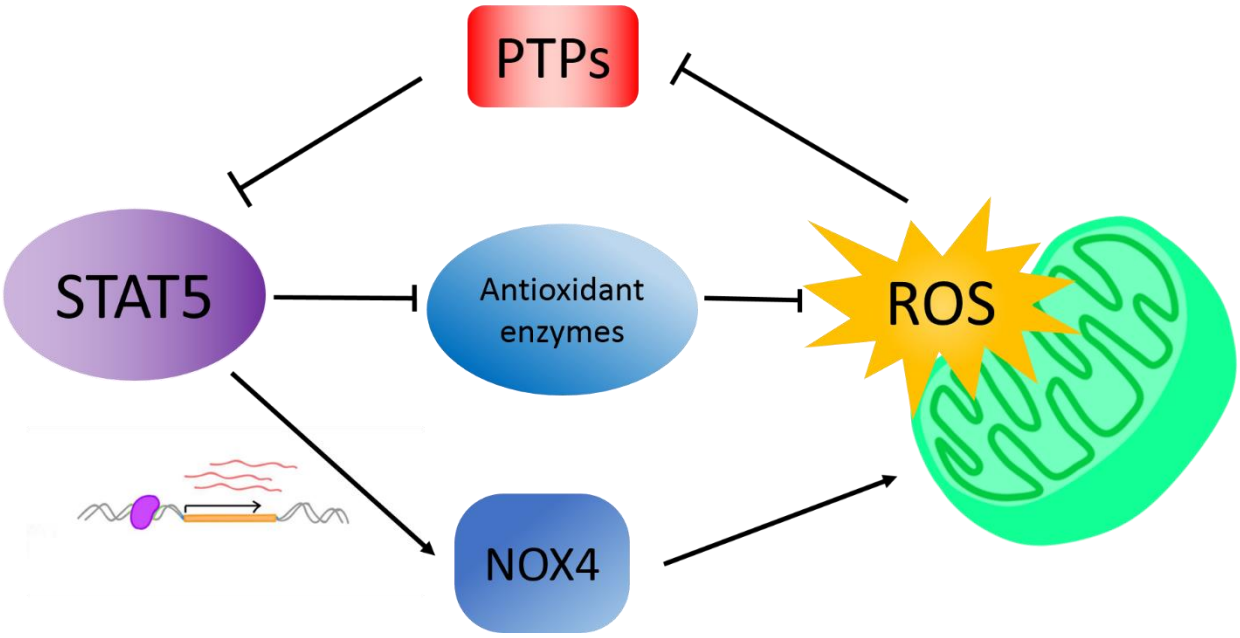

Figure 2 The cooperative relationship between STAT5 and ROS.

IB as potential therapy targeting FLT3-ITD$^+$ AML

Given the fact that FLT3-ITD$^+$ AML is a reactive-oxygen-driven type of cancer, imipramine blue (IB) arises as a promising new candidate for treating this disease. It is a new chimeric drug with the ability to quench ROS production in cells by binding a triphenylmethane dye to the FDA-approved tricyclic antidepressant imipramine. It was invented by Emory professor Dr. Jack Arbiser to treat glioblastoma initially due to its ability to cross the blood brain barrier [13]. Previous work has found that IB selectively targets FLT-ITD+ AML with a higher potency in

killing primary human FLT3-ITD$^+$ AML cells compared to FLT3-WT cells. It has also been found that IB has a dual mechanism of killing: inhibiting STAT5 activation though NOX inhibition and increasing cytosolic calcium level [14]. However, it remains to be explored why IB shows a selectivity towards FLT3-ITD$^+$ AML.

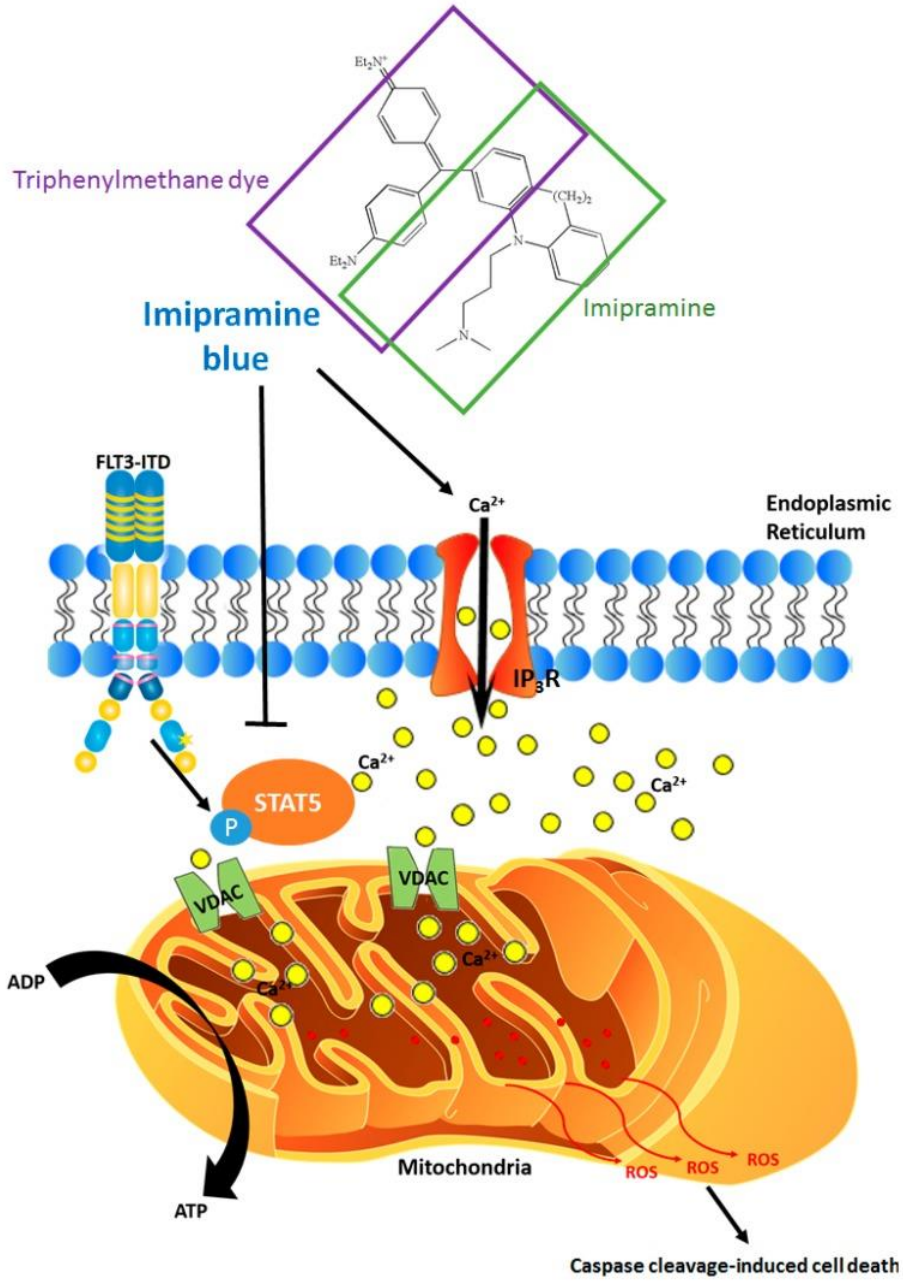

Figure 3 IB inhibits STAT5 phosphorylation and induces calcium release from ER to cause mitochondrial cell death.

Mitochondria-ER crosstalk

Direct interactions between ER and mitochondria have been implicated with many important cellular processes including calcium transfer [15]. Mitochondria-associated membranes (MAM) are ER regions that are in contact with mitochondria [16]. Several proteins are localized to MAM and are known to be involved in the inter-organelle communication between ER and mitochondria. The mitochondrial fission protein Fission 1 homologue (Fis1) interacts with Bap31 to form the Fis1-Bap31 complex, which connect ER and mitochondria for apoptotic signaling [17]. The voltage-dependent anion channel (VDAC) links with the ER calcium-release channel inositol 1,4,5-trisphosphate receptor (IP_3R) through the chaperone glucose-regulated protein 75(GRP75) to mediate calcium and cell death signaling between ER and mitochondria [18]. Mitofusin1 (Mfn1) connects ER to mitochondria for efficient mitochondrial calcium uptake [19]. Dynamin related protein (Drp1) is a cytosolic GTPase that induces mitochondrial fission. Upon sustained cytosolic calcium rise, cytosolic phosphatase calcineurin is activated and interacts with Drp1 to dephosphorylate it on serine 637. Upon dephosphorylation, Drp1 is activated and recruited to potential fission sites on the mitochondrial surface and promotes mitochondrial fission, a process that is actively involved in apoptosis [20-22]. Mammalian TOR complex 2 (mTORC2) controls MAM integrity through Akt signaling [23].

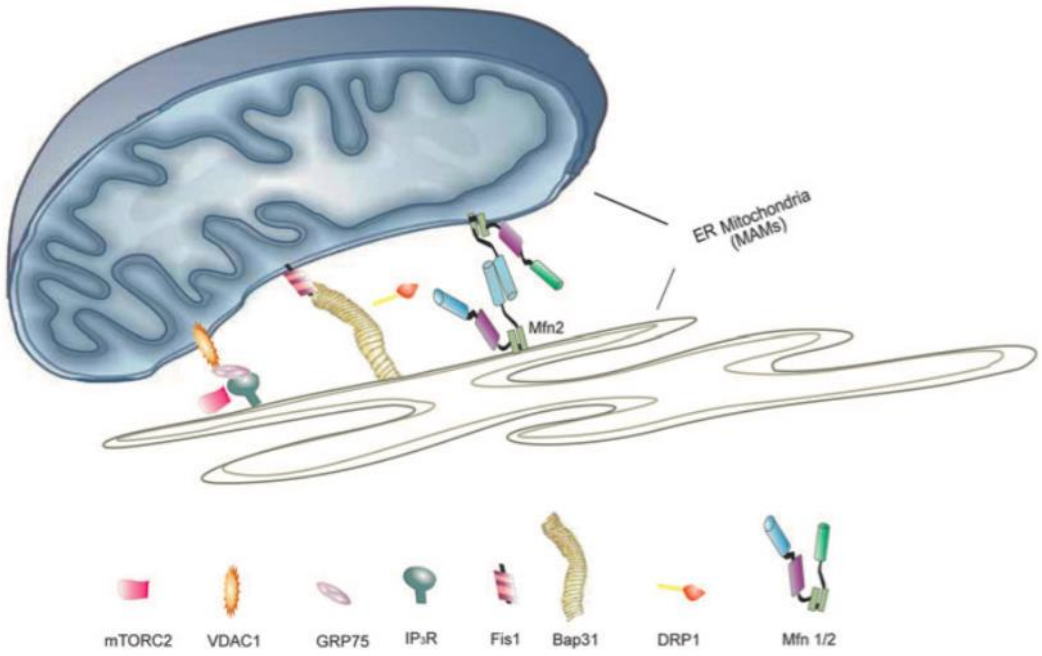

Figure 4 Proteins that are involved in the Mito-ER crosstalk. Figure was from [24]

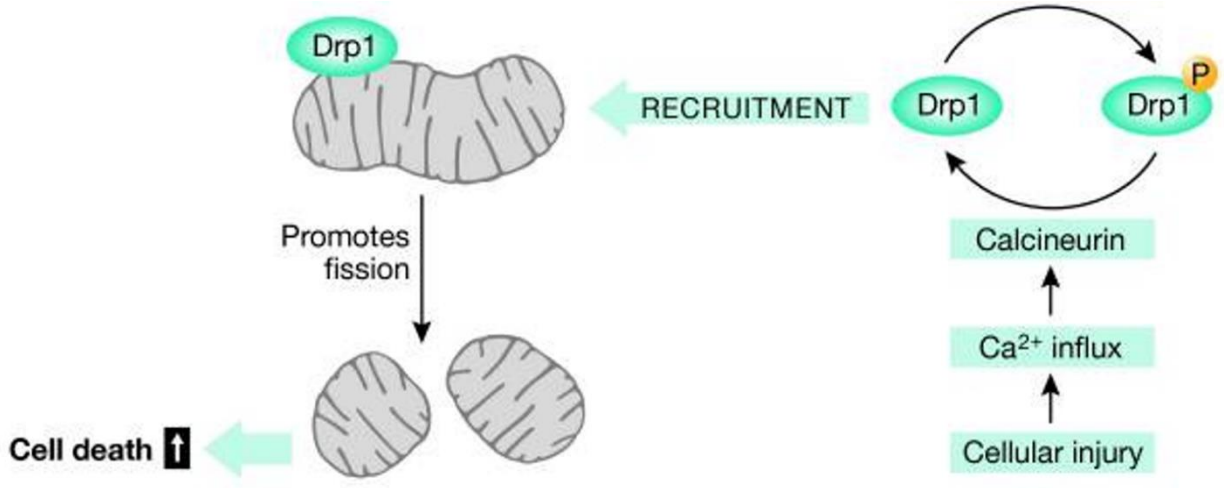

Figure 5 Drp1 promotes mitochondrial fission and subsequent cell death upon dephosphorylation. Figure was modified from [25]

ER stress and unfolded protein response (UPR)

A number of cellular stresses including the disruption of calcium homeostasis activate UPR, which is a part of the global ER stress responses [26]. Several proteins work together to constitute the UPR pathway. The glucose-regulated protein-78 (GRP78) is induced upon ER stress and activates three ER transmembrane sensors inositol-requiring enzyme 1 (IRE1), protein kinase R-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) [27]. Upon activation, IRE1 initiates downstream signaling through splicing of the X-box binding protein 1 (XBP1); PERK activates the stress-regulated protein p8, the activating transcription factor 3 and 4 (ATF3 & ATF4), which promotes the transcription of tribbles pseudokinase 3 (TRB3) [28-30].

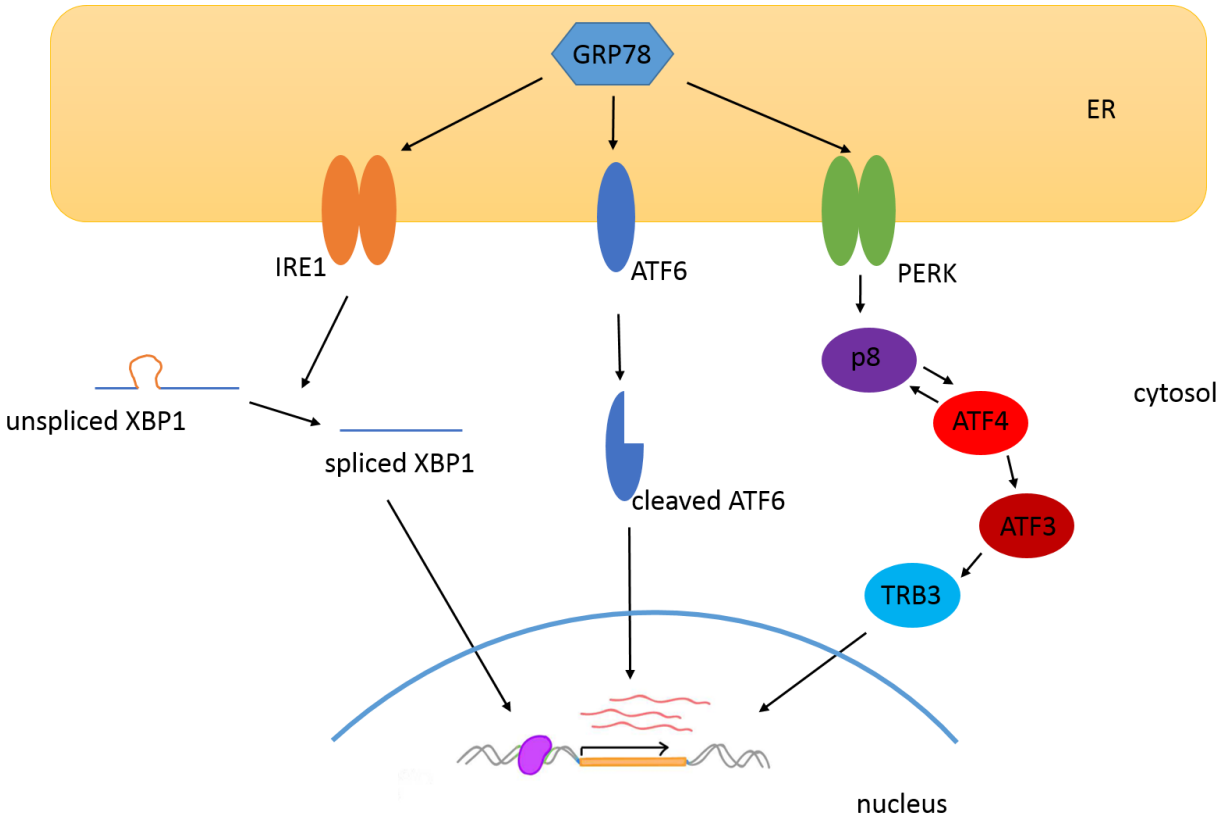

Figure 6 Proteins that are involved in the UPR pathway.

Drp1 in cancer

Drp1 expression is found to be associated with many different tumor models such as melanoma and lung cancer [31]. Since it is a key component in the mitochondrial fission machinery [32], Drp1 is involved in two important cellular processes: mitosis and apoptosis. Therefore, Drp1 has a contradictory role in tumorigenesis. On one hand, increased Drp1 activation promotes cell cycle progression and cell proliferation [33]. For instance, in glioblastoma, Drp1 is aberrantly activated in brain tumor initiating cells and promotes tumor growth [34]. on the other hand, under diverse stress conditions, Drp1 is intensively activated, leading to excessive mitochondrial fragmentation and consequent apoptosis [35]. Taken together, although tumor cells may utilize Drp1 to promote proliferation, it may sensitize them to cellular stress induced by various cancer therapies.

Honokiol (HK) as an alternative

HK is a biphenolic compound isolated from magnolia bark, which is widely used in Traditional Chinese medicine to eliminate "humidity". Previous research has shown that HK possesses anticancer property by quenching ROS and thereby suppressing the activation of important pro-survival transcription factors including NF-κβ and STAT5 [36-38]. It was therefore hypothesized that HK and IB have overlapping mechanism of actions. However, while IB may induce too much calcium release and hence introduce side effects, HK, on the other hand, may be on the low side of calcium release and may better compromise between calcium release and ROS inhibition. Therefore, HK may serve as an alternative for IB to reduce side effects and improve efficacy in vivo.

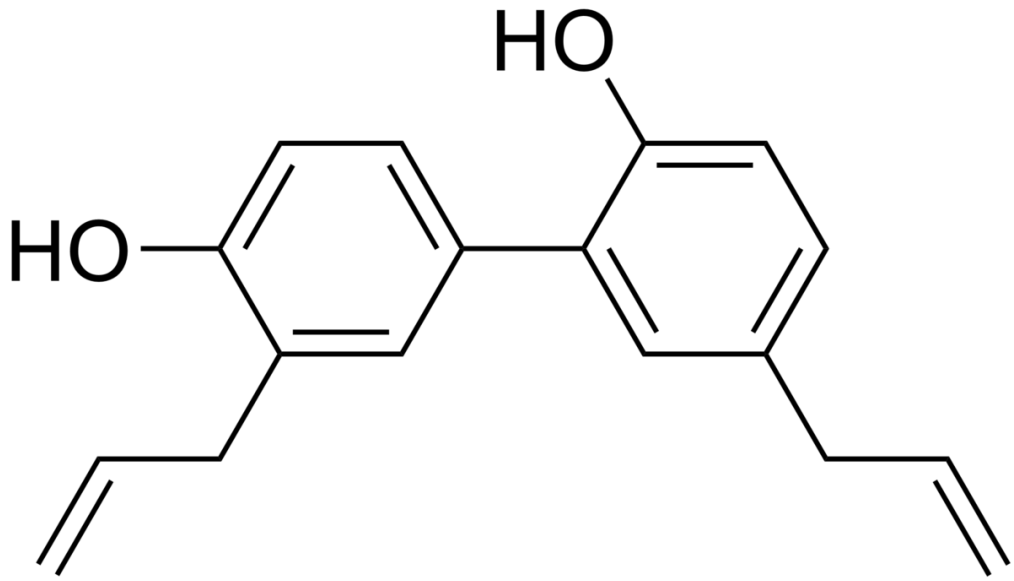

Figure 7 HK is a biphenolic compound.

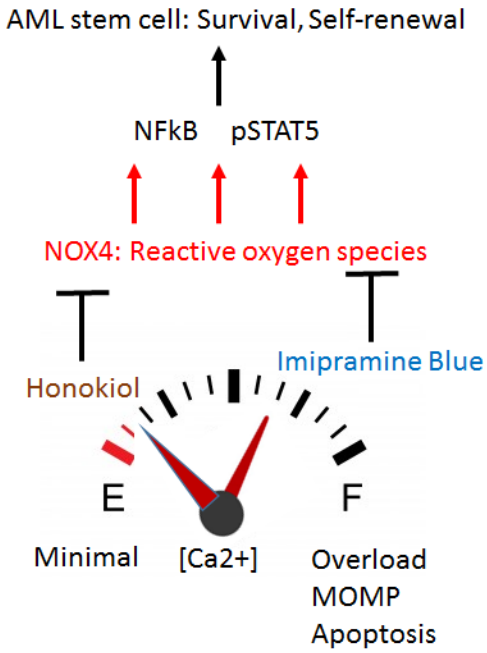

Figure 8 HK and IB have some overlapping mechanisms of action regarding ROS inhibition while HK may induce less calcium flux than IB.

Scope of the thesis

This thesis focuses on investigating why IB exhibits selectivity towards FLT-ITD+ cells. Since IB induces intracellular calcium release, it was hypothesized that 1) the mitochondria-ER crosstalk is upregulated in FLT3-ITD$^+$ cells so that more calcium ions that are released from the ER enter the mitochondria and induce apoptosis; 2) IB induces ER stress and the FLT3-ITD$^+$ cells are primed for ER stress responses so that they are more susceptible to IB-induced cell death.

This work also explores HK, a promising anticancer compound, as an alternative AML drug therapy targeting ROS and STAT5.

Materials and Methods

Cell culture

All cells were cultured in media with 1% antibiotic-antimycotic. The FLT3-ITD$^+$ MV4-11 and MOLM-13 cell lines were gifted by Doug Graham (Emory University). MV4-11 cells were cultured in IMDM supplemented with 10% FBS; MOLM-13 cells were cultured in RPMI supplemented with 10% FBS. The FLT3-WT OCI-AML3 cells were purchased from DSMZ and were cultured in α-MEM supplemented with 20% FBS.

Treatment reagents

Imipramine blue and honokiol was gifted by Jack Arbiser (Emory University). Pimozide, thapsigargin, mdivi-1 and cyclosporin A were purchased from Sigma. All reagents were dissolved in DMSO for 1mM stock solution preparation.

Trypan blue exclusion assay

10μL of cell suspension was placed in Eppendorf tube. 10μL trypan blue dye was added to the tube and mixed by pipetting up and down. One side of the hemacytometer was filled with 10μL of the cell suspension and dye mixture. All cells in the grid were counted under microscope. Clear cells were counted as viable cells; blue cells unviable.

Mitochondrial mass measurement

Cells were incubated with 50nM of MitoTracker Deep Red (Thermo Fisher, M22426) for 45min before they were washed with PBS. Flow cytometry was performed at 670/30 bandpass filter.

RT-PCR

Cells were lysed by Trizol reagent and total RNA was extracted using the RNeasy Mini® kit (QIAGEN, 74104). Reverse transcription for cDNA synthesis was conducted using SuperScript™ III First-Strand Synthesis System (Invitrogen, 18080051) according to

manufacturer's instructions. Primer design for selected genes was listed in Appendix I. The cDNA products were amplified by real-time PCR using FastStart Universal SYBR Green Master (Rox) (04913914001), 1 cycle at 50^{o}C for 2 min, 95^{o}C for 10 min, 40 cycles at 95^{o}C for 15 sec, 60^{o}C for 1 min, followed by dissociation stage of 1 cycle at 95^{o}C for 15 sec, 60^{o}C for 1 min, 95^{o}C for

15 sec. GAPDH was used as internal control and comparative cycle threshold method ($\Delta\Delta C_t$) for data analysis.

XBP1 splicing assay

Primers for XBP1 were designed to span the 26 base intron spliced by IRE1, listed in Appendix I. PCR was programmed at 95^{o}C for 3 min, 25 cycles of 95^{o}C for 30 sec, 52^{o}C for 30 sec, 72^{o}C for 30 sec, followed by 72^{o}C for 5 min. PCR products were electrophoresed on 3% gel. GAPDH was used as loading control.

Western blotting

Cells were lysed in RIPA buffer with protease inhibitor (Roche) and then centrifuged for 10 min at 10000 rpm. Protein concentrations were measured using the Bio-RAD protein assay (Bio-RAD, 500-0006). Equal amounts of proteins were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose (Fisher, 1215471) membrane, which was blocked in 5% BSA for one hour. Then, membrane was incubated with primary antibodies overnight at appropriate concentrations: Drp1 (Novus Biologicals, NB110-55288) at 1:500; phospho-Drp1 (Ser637) (Cell Signaling, 6319S) at 1:500; β-actin (Sigma, A2228) at 1:10000, followed by membrane wash and one-hour secondary antibodies (Licor, 925-32211, 925-68070) incubation. Membranes were detected by the Odyssey CLx imaging system (LI-COR Biosciences) and densitometry quantification was done using Image Studio v4.0 software.

Data analysis

Results are expressed as mean ± SD. Data were analyzed using student *t* test and p-value < 0.05 was considered significant. Prism 8.0 (GraphPad Software, San Diego, CA) and Excel 2016 (Microsoft) were used for statistical analysis.

Results

IB exhibited greater potency in FLT3-ITD$^+$ cell lines

Previous research has shown that IB at 48hrs demonstrated selective cell death for FLT3-ITD$^+$ cell lines while having relatively little effect on the FLT3-WT lines [14]. Consistent with this finding, the IC_{50} value for the FLT3-ITD$^+$ cell line is lower around 300nM while for the FLT3-WT cell line higher around 500nM. However, at higher concentrations (≥500nM), IB induced high percentage of cell death in both FLT-ITD+ and FLT3-WT cell lines (Fig9A). Moreover, IB started to reduce the number of viable cells at 100-200nM for the FLT3-ITD$^+$ lines and at 300nM for the FLT3-WT line, prior to cell-death induction (Fig9B). This result suggests that IB may be affecting cell proliferation before it starts to cause apoptosis.

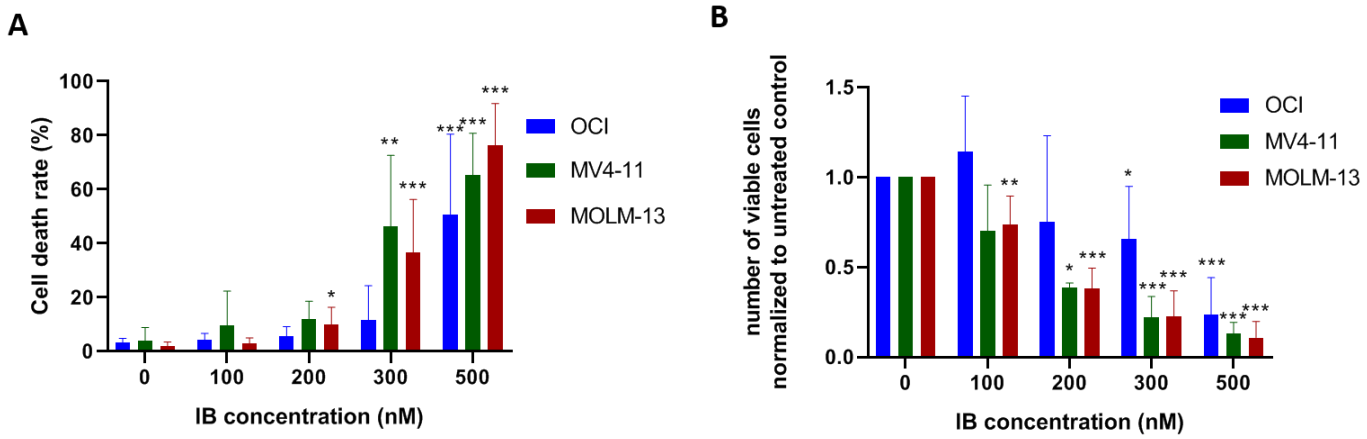

Figure 9 **IB induces cell death and growth inhibition at lower concentrations in FLT3-ITD$^+$ cell lines.** Trypan blue exclusion test on OCI-AML3 (FLT3-WT), MV4-11 (FLT3-ITD$^+$) and MOLM-13 (FLT3-ITD$^+$) after 48 hours of IB treatment with increasing dosage from 0nM to 500nM. (**A)** Cell death rate was calculated from the number of dead cell divided by the total number of cells. IB induced significant cell death at 300nM for the FLT3-ITD$^+$ cell lines but not for the FLT3-WT line. (**B**) Relative number of viable cells was calculated from the number of live cells after 48hrs IB treatment divided by the number of live cells untreated with IB. IB induced significant growth inhibition at 100-200nM for the FLT3-ITD$^+$ cell lines but not for the FLT3-WT line. Error bars represent standard deviation (SD), n=3-8. (*p<0.05, **p<0.01, ***p<0.001)

Drp1 mRNA expression was higher in FLT3-ITD$^+$ cell lines

In order to explain IB's selectivity towards the FLT3-ITD$^+$ cell lines, it was hypothesized that the crosstalk between ER and mitochondria was upregulated in the FLT3-ITD$^+$ cell lines so that when IB induced calcium release from ER, more calcium ions would enter mitochondria and cause mitochondria outer membrane permeabilization (MOMP) and mitochondrial apoptosis [14]. A panel of genes that are known to be involved in the contact between ER and mitochondria were chosen for analysis. In Fig.10A, mRNA expression levels of these mitochondria-ER crosstalk genes were measured in OCI (FLT3-WT) and MOLM-13 (FLT3-ITD$^+$) for comparison. Drp1 and mTORC2 were significantly up-regulated in MOLM-13 and emerged as two promising candidate genes for explaining the priming of FLT3-ITD$^+$ cells for IB-induced cell death. The mRNA expression levels of Drp1 and mTORC2 in FLT3-WT and FLT3-ITD$^+$ AML cell lines with or without IB treatment were then compared to confirm up-regulation in the FLT3-ITD$^+$ cell lines and to see if IB treatment would alter the expressions. Although for mTORC2 expression there was no significant increase for MV4-11 (FLT3-ITD$^+$) compared with OCI, Drp1 was found to be significantly up-regulated in both of the FLT3-ITD$^+$ cell lines (Fig.8B, C). However, for neither Drp1 nor mTORC2 was there alteration of gene expressions after IB treatment (Fig.10B, C).

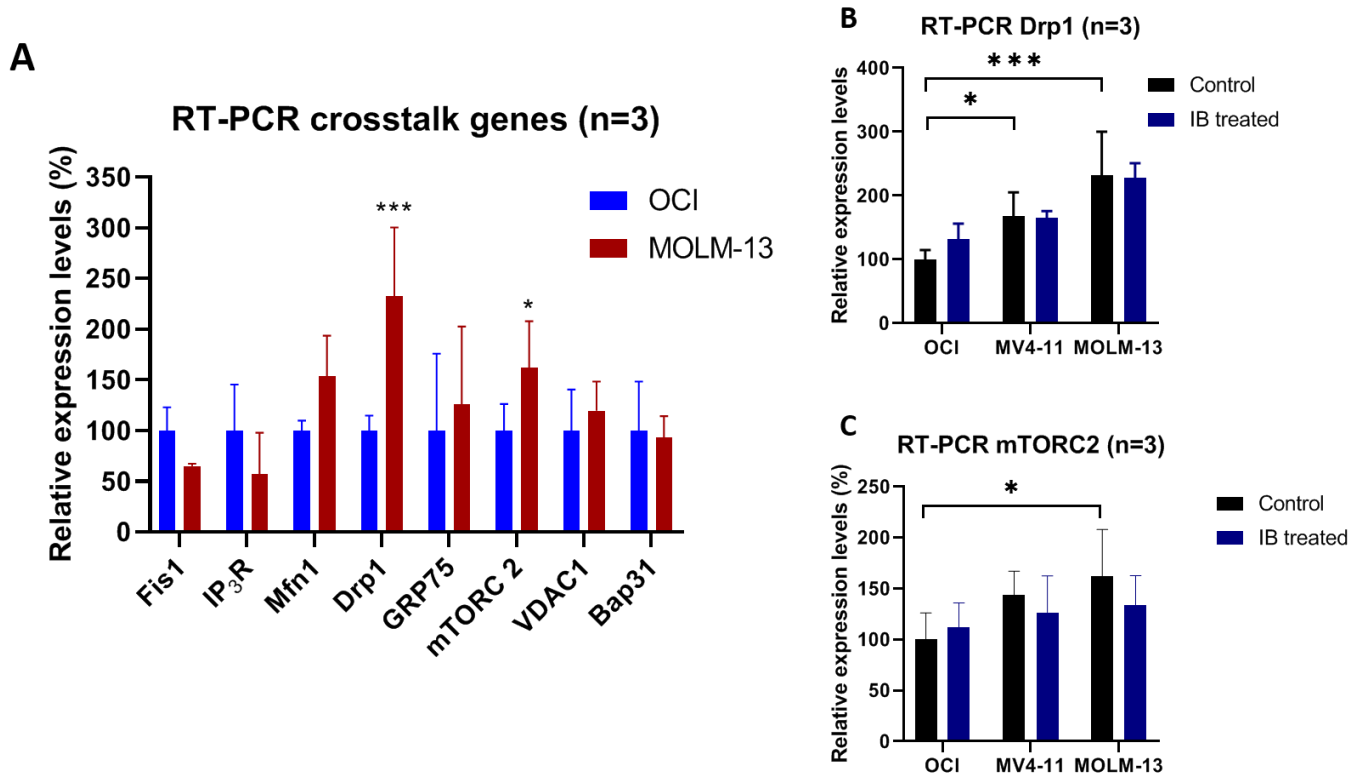

Figure 10 **Drp1 mRNA expression level was up-regulated in the FLT3-ITD$^+$ cell lines**. (**A**) Among Mito-ER crosstalk genes (Fis1, IP_3R, Mfn1, Drp1, GRP75, mTorc2, VDAC1, Bap31), Drp1 and mTORC2 had higher mRNA expression in MOLM-13 (FLT3-ITD$^+$) than OCI (FLT3-WT). (**B** and **C**) Drp1 expression was up-regulated in both MV4-11 and MOLM-13 (FLT3-ITD$^+$); Drp1 and mTORC2 expression remained unaltered after IB treatment at 400nM for four hours. GAPDH was used as loading control. Data were normalized to untreated OCI. Error bars indicate SD. P-values were calculated from unnormalized data. (*p<0.05, ***p<0.001)

Drp1 protein expression was higher in FLT3-ITD$^+$ cell lines and IB increased Drp1 activation

It was next determined whether Drp1 was up-regulated in the FLT3-ITD$^+$ cell lines on the protein level by conducting Western blotting on total and phospho-Drp1. In Fig11, the blots demonstrated that FLT3-ITD$^+$ cell lines had higher total Drp1 protein levels, which is consistent

with the RT-PCR results. Interestingly, phospho-Drp1 levels were also higher in the FLT3-ITD$^+$ cell lines, indicating that more Drp1 was inactivated in those lines. This might be a protective mechanism for the FLT3-ITD$^+$ cells to avoid excess mitochondrial fission due to the more abundant Drp1 in the cells. After IB treatment at 300nM overnight, there were decreased levels of phospho-Drp1 compared with untreated controls (Fig11C), suggesting that IB treatment increased Drp1 dephosphorylation, thus activating Drp1 to induce mitochondrial fission and perhaps subsequent apoptosis. Since IB induces cytosolic calcium release [14], it was proposed that such increase of cytosolic calcium concentration activates calcineurin, which in turn dephosphorylates and activates Drp1. By having more Drp1 to begin with, the FLT3-ITD$^+$ cells may thus be primed to such calcium-induced cell death of IB.

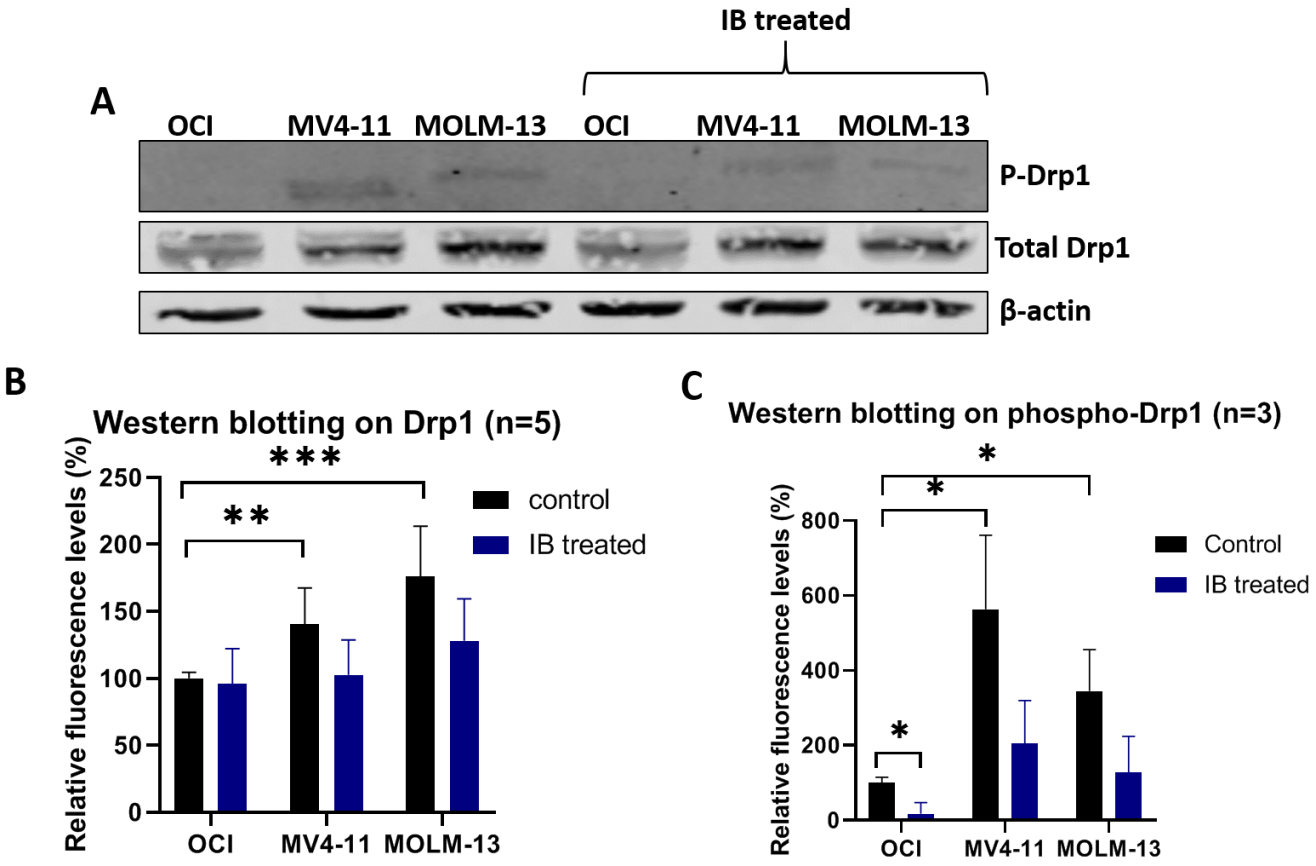

Figure 11 **Increased protein expression of Drp1 in the FLT3-ITD$^+$ cell lines and increased Drp1 activation upon IB treatment.** (A) Representative Western blot on total and phospho-Drp1 (Ser637) in FLT3 WT cell line (OCI) and FLT3-ITD$^+$ cell lines (MV4-11 and MOLM-13), either untreated or treated with 300nM IB overnight. Actin was used as loading control. (B and C) Densitometry analysis shows increased Drp1 in the two FLT3-ITD$^+$ cell lines and inhibition of phospho-Drp1 after IB treatment. Data were normalized to untreated OCI. Error bars indicate SD. P-values were calculated from unnormalized data. (*p<0.05, **p<0.01, ***p<0.001)

Calcineurin inhibition did not ablate the difference in IB sensitivity between FLT3-ITD$^+$ and FLT3-WT cell lines

In order to find out whether Drp1 contributes to the difference in IB sensitivity between FLT3-ITD$^+$ and FLT3-WT cell lines, mdivi-1 was used to inhibit Drp1 activity [39] to determine if that would increase cell survival under IB treatment and eliminate IB's selectivity towards the FLT3-ITD$^+$ cell lines. However, although mdivi-1 did inhibit Drp1 activity and increased mitochondrial mass, it was cytotoxic and caused cell death by itself (FigS1). Next, focus was shifted upstream. Cyclosporin A (CsA), an immunosuppressive agent that binds to cyclophilin to inhibit the phosphatase activity of calcineurin [40], was used to block the IB-induced activation of Drp1. In Fig12A, CsA's inhibition of calcineurin's phosphatase activity was confirmed by a rescue of Drp1 phosphorylation after 10nM CsA treatment. In Fig12B and C, AML cell lines were pre-treated with 10nM CsA 24 hours prior to the 48-hour IB treatment and calculated the cell death rate and the number of viable cells. However, CsA treatment did not have an effect on IB-induced cell death on AML cell lines. This result may suggest that Drp1 expression difference between FLT3-ITD$^+$ and FLT3-WT AML cell lines does not account for their difference in sensitivity towards IB. Nevertheless, shRNA knockout of Drp1 still needs to be conducted for a definite conclusion.

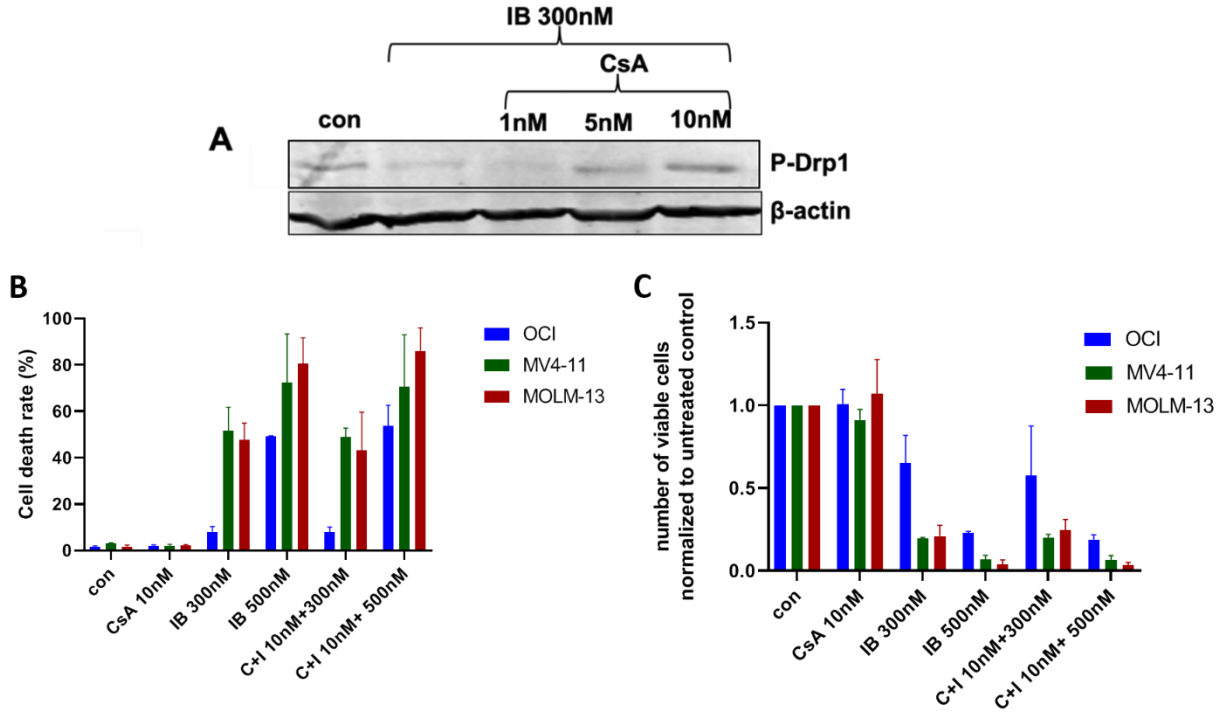

Figure 12 **CsA pretreatment did not affect IB-induced cell death.** (A) MOLM-13 cells were treated with 300nM of IB and increasing concentrations of CsA overnight before measuring the phospho-Drp1 level by Western blot. (B-C) FLT3-WT (OCI) and FLT3-ITD^{+} (MV4-11 & MOLM-13) AML cell lines were treated with CsA at 10nM 24 hours before the addition of 300nM or 500nM IB. 48 hours after IB addition, cells were counted in trypan blue dye. Cell death rate (B) and relative number of viable cells (C) were calculated. Error bars indicate SD, n=2.

Unlike thapsigargin (TG), IB may not induce ER stress in AML cell lines

Disruption of calcium signaling and homeostasis in the ER and the cytosol leads to activation of ER stress responses, including the unfolded protein response (UPR) [41]. Under sustained and unsolvable ER stress, apoptotic cell death will be initiated [42]. Since IB causes a surge in cytoplasmic calcium level, it was hypothesized that IB could induce ER stress and activate the UPR signaling pathway. On such note, it was further hypothesized that IB exhibited higher potency at killing the FLT3-ITD$^+$ cells because FLT3-ITD$^+$ cells were primed for ER stress response by up-regulating genes that are involved in the ER stress and UPR pathways, so that when IB induced ER stress, the FLT-ITD+ cells would become prone to cell death. RT-PCR was performed to compare a panel of genes that had been implicated with ER stress responses between the FLT3-ITD$^+$ and the FLT3-WT cell lines. However, amongst these UPR-related genes, none of them had shown significant up-regulation in both FLT3-ITD$^+$ cell lines (Fig13A). Therefore, XBP1 splicing assay was conducted to determine whether IB truly induce ER stress and activate the UPR pathways. Different from thapsigargin (TG), which induced evident XBP1 splicing in cells after 4 hours of treatment at 300nM, which indicates the presence of ER stress response, IB did not induce such splicing event even after overnight treatment at 300nM (Fig13B).However, XBP1 splicing is only one marker of ER stress response. Other markers need to be checked to confirm that IB does not induce ER stress in AML cell lines.

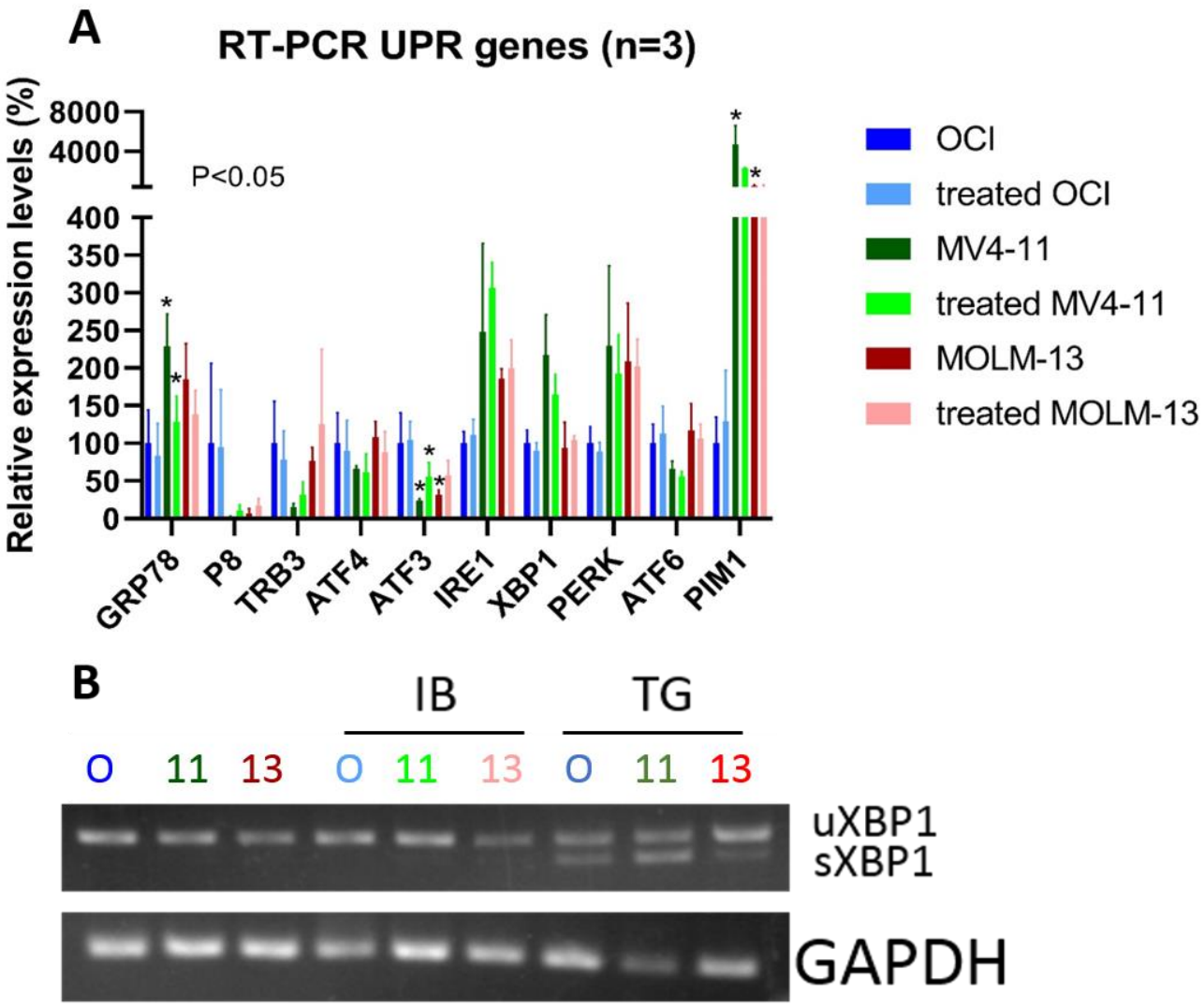

Figure13. IB may not induce ER stress in AML cell lines. (A) None of the UPR genes (GRP78, P8, TRB3, ATF4, ATF3, IRE1, XBP1, PERK, ATF6) was up-regulated in both MV4-11 and MOLM-13. PIM1, a STAT5 target gene, was used as positive control for up-regulation in MV4-11 and MOLM-13. GAPDH was used as loading control. Data were normalized to untreated OCI. Error bars represent SD. P-values were calculated from unnormalized data, comparing untreated MV4-11 and MOLM-13 to untreated OCI; IB-treated cell lines to untreated cell lines. (B) XBP1 mRNA was detected by semi-quantitative RT-PCR. Unspliced XBP1 (uXBP1) was observed at 152bp; spliced XBP1 was observed at 126bp. OCI (O), MV4-11 (11) and MOLM-13 (13) were untreated or treated with IB at 300nM overnight, or treated with thapsigargin (TG) at 300nM for four hours. TG-treated cells showed expression of spliced XBP1 whereas IB-treated cells did not. GAPDH was used as loading control.

HK synergized with pimozide

HK induced dose-dependent cell death on MOLM-13 cells. Although it only caused relatively low level of cell death at a concentration of 25μM or lower, a higher death rate was achieved at 50μM (Fig.14A). Interestingly, it formed a very potent synergy with pimozide, an FDA-approved antipsychotic drug that also inhibits STAT5 phosphorylation [43]. While HK at 25μM or pimozide at 2.5-10μM treatment alone only caused less than 20% of cell death, combined treatment had higher efficacy, inducing more than 40% of cell death (Fig14B-D).

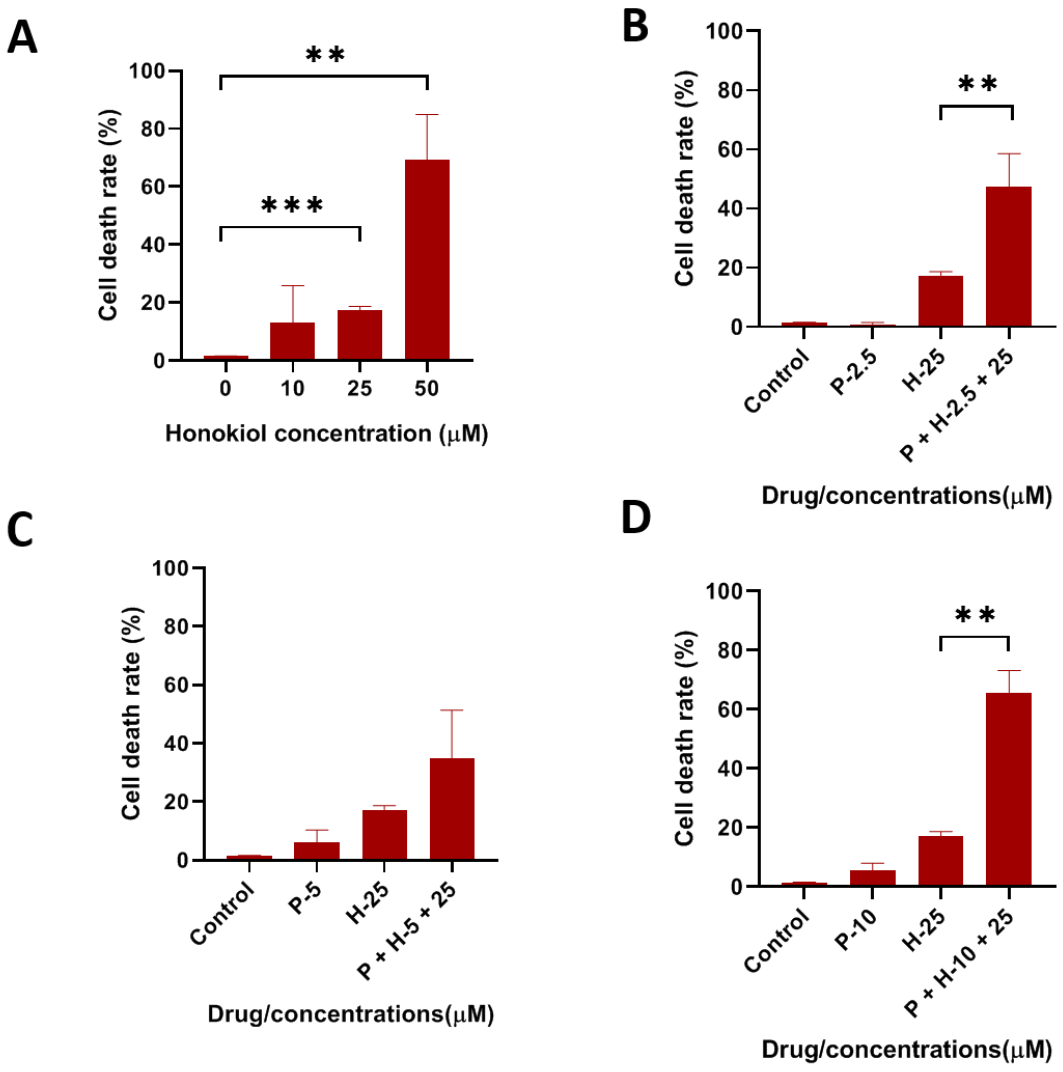

Figure 14 **HK synergized with pimozide**. Trypan blue exclusion assay after 48hrs of drug treatment. (A) HK induced cell death on MOLM-13 cells at μM concentration. (B-D) HK (H) and pimozide (P) combination treatments yielded higher cell death rate than HK or pimozide treatment alone on MOLM-13 cells. Error bars indicate SD. n=3 (**p<0.01, ***p<0.001)

Discussion

FLT3-ITD$^+$ AML portends poor prognosis and high relapse rate. Although several tyrosine kinase inhibitors (TKIs) have been tested in clinical trials, responses are transient and resistance invariably occurs [44]. The NOX inhibitor IB has emerged as a promising drug targeting FLT3-ITD$^+$ AML. It has been previously found that IB localized to the lysosomes and suggested that it caused apoptotic cell death via two mechanisms: 1) raising the intracellular calcium concentration; 2) inhibiting STAT5 activation and quenching ROS production [14]. Nonetheless, some control experiments are missing here and should be conducted in the future in order to better establish these two mechanisms as IB's way of killing. For instance, calcium channel antagonists such as nifedipine could be used to suppress ER calcium release [45] and therefore assess the role of calcium in IB-induced apoptosis. In addition, the aberrant activating signal for STAT5 comes from FLT3's mislocalization to the ER in FLT3-ITD$^+$ AML. Hence, a STAT5a^{S711F} mutant-expressing cell line, which has persistently active STAT5a by lacking the N-domain [46], could be tested to determine whether the cells still exhibit high sensitivity to IB treatment even when the activating signal for STAT5 is not coming from the ER.

The data presented here confirmed that IB possesses higher potency at killing AML cells carrying the FLT3-ITD mutation, with IC_{50} for FLT3-ITD$^+$ cell lines at around 300nM while for FLT3-WT cell line 500nM. This work had found that different expression level of Drp1 in the FLT3-ITD$^+$ and the FLT3-WT cell lines might be able to account for this phenomenon. There were higher levels of both Drp1 mRNA and protein in the FLT3-ITD$^+$ cell lines comparing to the FLT3-WT cell line. Furthermore, Drp1 was activated upon IB-induced calcium release through dephosphorylation by calcineurin, evidenced by decreased level of phospho-Drp1 after IB treatment. Upon activation, Drp1 induces mitochondrial fission, an important step in apoptosis [47] and thus it had been proposed that more Drp1 would be activated after IB treatment in the

FLT3-ITD$^+$ cells, leading to more mitochondrial fission and more apoptosis, accounting for IB's selectivity towards the FLT3-ITD$^+$ cell lines. However, pharmacological inhibition of calcineurin and subsequently inhibition of IB-induced Drp1 activation did not eliminate the difference in sensitivity of the FLT3-ITD$^+$ cell lines and the FLT3-WT cell line towards IB. Therefore, the difference in Drp1 expression in the FLT3-ITD$^+$ cells and the FLT3-WT cells may not be responsible for IB's selectivity towards the former cells. However, shRNA knockout of Drp1 on the AML cell lines still needs to be done in order to confirm this result.

Previous work has compared IB with TG and found that both of them increases cytosolic calcium concentration, suggesting that the two drugs may have similar working mechanisms [14]. However, this work has demonstrated that although TG induced ER stress in cells, IB might not produce the same effect and the two drugs may function differently to induce cell death. Nevertheless, only XBP1 splicing as one of the ER stress response markers has been looked at in this work and more markers need to be measured in the future to confirm that IB does not induce ER stress. Additionally, the underlying mechanism of how IB causes robust calcium release still remains to be explored.

Alternatively, HK can be used to target AML. This data has demonstrated that HK formed a potent synergy with pimozide, which can also inhibit STAT5 phosphorylation [43], and there is a big potential for combination drug therapy. However, more work needs to be done to compare IB and HK, both in regard to their induction of calcium release and their performance in vivo.

References

1. De Kouchkovsky, I. and M. Abdul-Hay, *'Acute myeloid leukemia: a comprehensive review and 2016 update'.* Blood Cancer J, 2016. **6**(7): p. e441.
2. Klco, J.M., et al., *Association Between Mutation Clearance After Induction Therapy and Outcomes in Acute Myeloid Leukemia.* Jama, 2015. **314**(8): p. 811-22.
3. Dohner, H., D.J. Weisdorf, and C.D. Bloomfield, *Acute Myeloid Leukemia.* N Engl J Med, 2015. **373**(12): p. 1136-52.
4. Mi, T., Z. Wang, and K.D. Bunting, *The Cooperative Relationship between STAT5 and Reactive Oxygen Species in Leukemia: Mechanism and Therapeutic Potential.* Cancers (Basel), 2018. **10**(10).
5. Hospital, M.A., et al., *FLT3 inhibitors: clinical potential in acute myeloid leukemia.* Onco Targets Ther, 2017. **10**: p. 607-615.
6. Choudhary, C., et al., *Mislocalized activation of oncogenic RTKs switches downstream signaling outcomes.* Mol Cell, 2009. **36**(2): p. 326-39.
7. Spiekermann, K., et al., *Overexpression and constitutive activation of FLT3 induces STAT5 activation in primary acute myeloid leukemia blast cells.* Clin Cancer Res, 2003. **9**(6): p. 2140-50.
8. Kayser, S. and M.J. Levis, *FLT3 tyrosine kinase inhibitors in acute myeloid leukemia: clinical implications and limitations.* Leuk Lymphoma, 2014. **55**(2): p. 243-55.
9. Tsapogas, P., et al., *The Cytokine Flt3-Ligand in Normal and Malignant Hematopoiesis.* Int J Mol Sci, 2017. **18**(6).
10. Weyemi, U. and C. Dupuy, *The emerging role of ROS-generating NADPH oxidase NOX4 in DNA-damage responses.* Mutat Res, 2012. **751**(2): p. 77-81.
11. Jayavelu, A.K., et al., *NOX4-driven ROS formation mediates PTP inactivation and cell transformation in FLT3ITD-positive AML cells.* Leukemia, 2016. **30**(2): p. 473-83.
12. Bourgeais, J., et al., *Oncogenic STAT5 signaling promotes oxidative stress in chronic myeloid leukemia cells by repressing antioxidant defenses.* Oncotarget, 2017. **8**(26): p. 41876-41889.
13. Maley, A.M. and J.L. Arbiser, *Gentian violet: a 19th century drug re-emerges in the 21st century.* Exp Dermatol, 2013. **22**(12): p. 775-80.
14. Metts, J., et al., *Imipramine blue sensitively and selectively targets FLT3-ITD positive acute myeloid leukemia cells.* Sci Rep, 2017. **7**(1): p. 4447.
15. Lombardi, A.A. and J.W. Elrod, *Mediating ER-mitochondrial cross-talk.* Science (New York, N.Y.), 2017. **358**(6363): p. 591-592.
16. Doghman-Bouguerra, M. and E. Lalli, *ER-mitochondria interactions: Both strength and weakness within cancer cells.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2019. **1866**(4): p. 650-662.
17. Iwasawa, R., et al., *Fis1 and Bap31 bridge the mitochondria-ER interface to establish a platform for apoptosis induction.* Embo j, 2011. **30**(3): p. 556-68.
18. Szabadkai, G., et al., *Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca2+ channels.* J Cell Biol, 2006. **175**(6): p. 901-11.
19. de Brito, O.M. and L. Scorrano, *Mitofusin 2 tethers endoplasmic reticulum to mitochondria.* Nature, 2008. **456**: p. 605.
20. Youle, R.J. and M. Karbowski, *Mitochondrial fission in apoptosis.* Nature Reviews Molecular Cell Biology, 2005. **6**: p. 657.
21. Cereghetti, G.M., et al., *Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(41): p. 15803-15808.
22. Smirnova, E., et al., *Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells.* Mol Biol Cell, 2001. **12**(8): p. 2245-56.
23. Betz, C., et al., *mTOR complex 2-Akt signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology.* Proceedings of the National Academy of Sciences, 2013. **110**(31): p. 12526-12534.

24. Zorzano, A., et al., *Mitofusin 2 as a Driver That Controls Energy Metabolism and Insulin Signaling.* Antioxidants & Redox Signaling, 2015. **22**(12): p. 1020-1031.
25. Jahani-Asl, A. and R.S. Slack, *The phosphorylation state of Drp1 determines cell fate.* EMBO reports, 2007. **8**(10): p. 912-913.
26. Senft, D. and Z.e.A. Ronai, *UPR, autophagy, and mitochondria crosstalk underlies the ER stress response.* Trends in Biochemical Sciences, 2015. **40**(3): p. 141-148.
27. Lee, A.S., *The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress.* Methods, 2005. **35**(4): p. 373-81.
28. Chen, Y. and F. Brandizzi, *IRE1: ER stress sensor and cell fate executor.* Trends in cell biology, 2013. **23**(11): p. 547-555.
29. Verfaillie, T., et al., *Linking ER Stress to Autophagy: Potential Implications for Cancer Therapy.* Int J Cell Biol, 2010. **2010**: p. 930509.
30. Jiang, H.Y., et al., *Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response.* Mol Cell Biol, 2004. **24**(3): p. 1365-77.
31. Lima, A.R., et al., *Dynamin-Related Protein 1 at the Crossroads of Cancer.* Genes (Basel), 2018. **9**(2).
32. Kageyama, Y., Z. Zhang, and H. Sesaki, *Mitochondrial division: molecular machinery and physiological functions.* Curr Opin Cell Biol, 2011. **23**(4): p. 427-34.
33. Zhan, L., et al., *Drp1-mediated mitochondrial fission promotes cell proliferation through crosstalk of p53 and NF-kappaB pathways in hepatocellular carcinoma.* Oncotarget, 2016. **7**(40): p. 65001-65011.
34. Xie, Q., et al., *Mitochondrial control by DRP1 in brain tumor initiating cells.* Nature Neuroscience, 2015. **18**: p. 501.
35. Pradeep, H., B. Sharma, and G.K. Rajanikant, *Drp1 in ischemic neuronal death: an unusual suspect.* Curr Med Chem, 2014. **21**(19): p. 2183-9.
36. Rauf, A., et al., *Honokiol: An anticancer lignan.* Biomedicine & Pharmacotherapy, 2018. **107**: p. 555-562.
37. Ishikawa, C., J.L. Arbiser, and N. Mori, *Honokiol induces cell cycle arrest and apoptosis via inhibition of survival signals in adult T-cell leukemia.* Biochim Biophys Acta, 2012. **1820**(7): p. 879-87.
38. Prasad, R. and S.K. Katiyar, *Honokiol, an Active Compound of Magnolia Plant, Inhibits Growth, and Progression of Cancers of Different Organs.* Adv Exp Med Biol, 2016. **928**: p. 245-265.
39. Peiris-Pagès, M., et al., *Mitochondrial fission as a driver of stemness in tumor cells: mDIVI1 inhibits mitochondrial function, cell migration and cancer stem cell (CSC) signalling.* Oncotarget, 2018. **9**(17): p. 13254-13275.
40. Fruman, D.A., et al., *Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A.* Proceedings of the National Academy of Sciences, 1992. **89**(9): p. 3686.
41. Krebs, J., L.B. Agellon, and M. Michalak, *Ca2+ homeostasis and endoplasmic reticulum (ER) stress: An integrated view of calcium signaling.* Biochemical and Biophysical Research Communications, 2015. **460**(1): p. 114-121.
42. Szegezdi, E., et al., *Mediators of endoplasmic reticulum stress-induced apoptosis.* EMBO reports, 2006. **7**(9): p. 880-885.
43. Nelson, E.A., et al., *The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors.* Blood, 2011. **117**(12): p. 3421-3429.
44. Leung, A.Y.H., C.H. Man, and Y.L. Kwong, *FLT3 inhibition: a moving and evolving target in acute myeloid leukaemia.* Leukemia, 2012. **27**: p. 260.
45. Fermini, B., et al., *L-type calcium channel antagonism – Translation from in vitro to in vivo.* Journal of Pharmacological and Toxicological Methods, 2017. **84**: p. 86-92.
46. Li, G., et al., *STAT5 requires the N-domain for suppression of miR15/16, induction of bcl-2, and survival signaling in myeloproliferative disease.* Blood, 2010. **115**(7): p. 1416-24.
47. Yu, X., et al., *Dephosphorylation by calcineurin regulates translocation of dynamin-related protein 1 to mitochondria in hepatic ischemia reperfusion induced hippocampus injury in young mice.* Brain Research, 2019. **1711**: p. 68-76.

Appendix I: primer list

gene	forward primer	reverse primer
IRE1	GCCGAAGTTCAGATGGAATC	ATCTGCAAAGGCCGATGA
XBP1	GGTCTGCTGAGTCCGCAGCAGG	GGGCTTGGTATATATGTGG
Drp1	TGCTTCCCAGAGGTACTGGA	TCTGCTTCCACCCCATTTTCT
VDAC1	CCAAGGGCTATGGATTTGGC	TGTAAACGTCAGGCCGTACT
IP_3R	GCGGCAGAGATTGACACATT	GAGACACAGAGGTCGGAGAG
Mfn1	TGAATGAGCGGCTTTCCAAG	TGCAGGCATCTTTCCATGTG
mTORC2	CTTCAGGTAGGTGAGCTCGT	TCTCACACATTCCCGTGTCA
GRP75	AGGCGGGATTATGCATCAGA	CCAGCACCTTTGCTCGTTTA
Fis1	TGGAGGACCTGCTGAAGTTT	ACTCAAACTGCGTGCTCTTG
Bap31	GCGGAGGTCTTTGTTGTGTT	CCACAAAGAAGGTGTTGCCA
GAPDH	CCTGCACCACCAACTGCTTA	GGCCATCCACAGTCTTCTGAG
PERK	GTCCGGAACCAGACGATGAG	GGCTGGATGACACCAAGGAA
ATF6	CCGCAGAAGGGGAGACACA	TCGGAGGTAAGGAGGAACTGACG
GRP78	GTTCTTGCCGTTCAAGGTGG	TGGTACAGTAACAACTGCATG
ATF3	CTGCAGAAAGAGTCGGAG	TGAGCCCGGACAATACAC
P8	GAAGAGAGGCAGGGAAGACA	CTGCCGTGCGTGTCTATTTA
TRB3	GCCACTGCCTCCCGTCTTG	GCTGCCTTGCCCGAGTATGA
ATF4	AGTCGGGTTTGGGGGCTGAAG	TGGGGAAAGGGGAAGAGGTTGTAA

Appendix II: Supplementary Figure

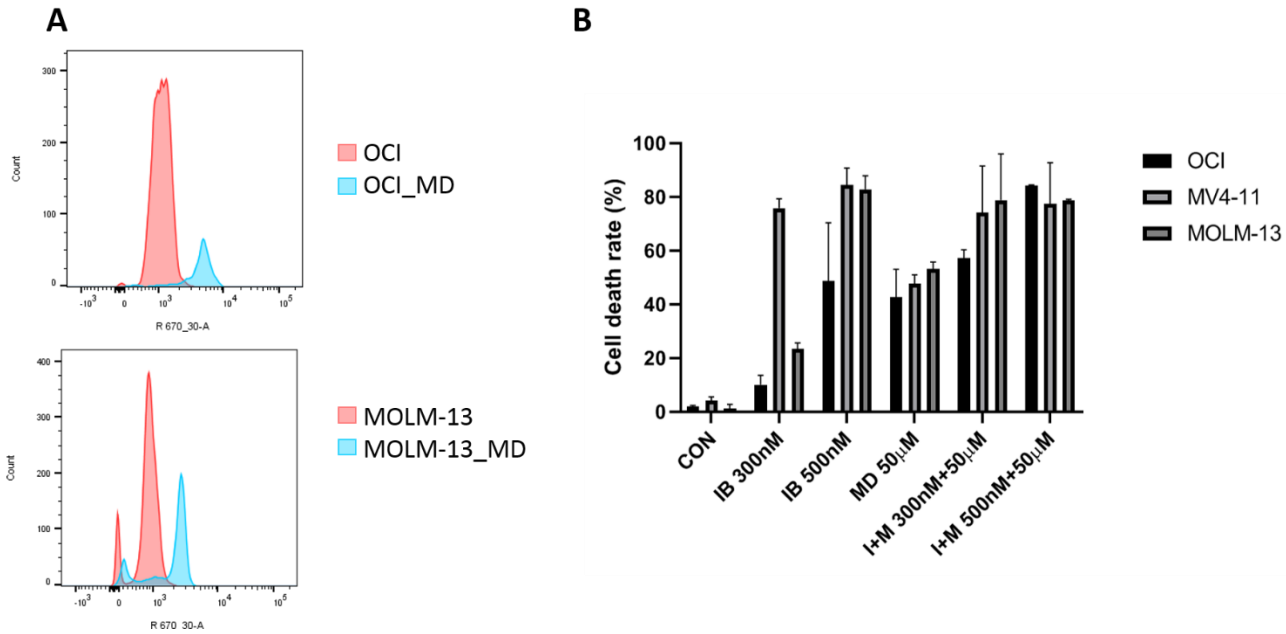

Figure S1 **Mdivi-1 is cytotoxic by itself.** (A) OCI and MOLM-13 cells were treated with 50μM of mdivi-1 (MD) for five days before they were stained with MitoTracker dye for flow cytometric assessment of mitochondrial mass. In both cell lines, mitochondrial mass increased with MD treatment. (B) Trypan blue exclusion assay on AML cell lines treated with IB and/or MD for 48 hours. MD at 50μM induced approximately 50% of cell death.