blood

American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
editorial@hematology.org

Ceramide-induced integrated stress response overcomes Bcl-2 inhibitor resistance in acute myeloid leukemia

Tracking no: BLD-2021-013277R2

Alexander Lewis (University of South Australia and SA Pathology, Australia) Victoria Pope (University of South Australia and SA Pathology, Australia) Melinda Tea (University of South Australia and SA Pathology, Australia) Manjun Li (University of South Australia and SA Pathology, Australia) Gus Nwosu (University of South Australia and SA Pathology, Australia) Thao Nguyen (University of Adelaide, Australia) Craig Wallington-Beddoe (University of South Australia and SA Pathology, Australia) Paul Moretti (University of South Australia and SA Pathology, Australia) Dovile Anderson (Monash University, Australia) Darren Creek (Monash University, Australia) Maurizio Costabile (University of South Australia, Australia) Saira Ali (University of South Australia and SA Pathology, Australia) Chloe Thompson-Peach (South Australian Health and Medical Research Institute (SAHMRI), Australia) B Dredge (University of South Australia and SA Pathology, Australia) Andrew Bert (University of South Australia and SA Pathology, Australia) Gregory Goodall (Department of Medicine, University of Adelaide, Australia) Paul Ekert (Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia, Australia) Anna Brown (University of Adelaide, Australia) Richard D'Andrea (University of South Australia, Australia) Nirmal Robinson (University of South Australia and SA Pathology, Australia) Melissa Pitman (University of South Australia and SA Pathology, Australia) Daniel Thomas (Stanford University, United States) David Ross (University of South Australia and SA Pathology, Australia) Briony Gliddon (University of South Australia and SA Pathology, Australia) Jason Powell (University of South Australia and SA Pathology, Australia) Stuart Pitson (University of Adelaide, Australia)

Abstract:
Inducing cell death by the sphingolipid ceramide is a potential anti-cancer strategy, but the underlying mechanisms remain poorly defined. Here, we show that triggering accumulation of ceramide in acute myeloid leukaemia (AML) cells by inhibition of sphingosine kinase induces an apoptotic integrated stress response (ISR) through protein kinase R-mediated activation of the master transcription factor ATF4. This leads to transcription of the BH3-only protein, Noxa, and degradation of the pro-survival Mcl-1 protein on which AML cells are highly dependent on for survival. Targeting this novel ISR pathway in combination with the Bcl-2 inhibitor venetoclax synergistically killed primary AML blasts, including those with venetoclax-resistant mutations, as well as immunophenotypic leukemic stem cells, and reduced leukemic engraftment in patient-derived AML xenografts. Collectively, these findings provide mechanistic insight into the anti-cancer effects of ceramide and pre-clinical evidence for new approaches to augment Bcl-2 inhibition in the therapy of AML and other cancers with high Mcl-1 dependency.

Conflict of interest: No COI declared

COI notes:

Preprint server: No;

Author contributions and disclosures: ACL, MNT, GON, VSP, TMN, CWB, PABM, DA, DJC, MRP, BLG and JAP performed experiments. PGE provided intellectual input and reagents. DMR, ALB and RJD provided patient material and clinical notes. ACL, JAP, DT and SMP designed the studies and analysed the data. ACL, JAP and SMP wrote the manuscript, which all authors critically reviewed and edited.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: emails to the corresponding author

Clinical trial registration information (if any):

Ceramide-induced integrated stress response overcomes Bcl-2 inhibitor resistance in acute myeloid leukemia

Running Title: Ceramide and the integrated stress response in AML

Alexander C. Lewis[1,#], Victoria S. Pope[1], Melinda N. Tea[1], Manjun Li[1], Gus O. Nwosu[1], Thao M. Nguyen[1,2], Craig T. Wallington-Beddoe[1,2,3,4], Paul A. B. Moretti[1], Dovile Anderson[5], Darren J. Creek[5], Maurizio Costabile[1,6], Saira R. Ali[1], Chloe A. L. Thompson-Peach[2,7], B. Kate Dredge[1], Andrew G. Bert[1], Gregory J. Goodall[1], Paul G. Ekert[8,9,10], Anna L. Brown[1,2,11], Richard D'Andrea[1], Nirmal Robinson[1], Melissa R. Pitman[1,12], Daniel Thomas[2,7,13], David M. Ross[1,2,3,4,7,14], Briony L. Gliddon[1], Jason A. Powell[1,2,15,*], Stuart M. Pitson[1,2,12,15,*]

[1] Centre for Cancer Biology, University of South Australia and SA Pathology, Bradley Building, North Terrace, Adelaide, SA 5001, Australia

[2] Adelaide Medical School, Faculty of Health Sciences, University of Adelaide, Adelaide SA, 5000, Australia

[3] College of Medicine and Public Health, Flinders University, Bedford Park SA, 5042, Australia

[4] Flinders Medical Centre, Bedford Park SA, 5042, Australia

[5] Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia

[6] Clinical and Health Sciences, University of South Australia, Adelaide, SA 5001, Australia

[7] Precision Medicine Theme, South Australian Health and Medical Research Institute, Adelaide, Australia

[8] Children's Cancer Institute, Lowy Cancer Research Centre, University of New South Wales, Sydney, NSW, Australia.

[9] Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

[10] Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria, Australia

[11] Department of Genetics and Molecular Pathology, SA pathology, Adelaide, Australia

[12] School of Biological Sciences, University of Adelaide, Adelaide SA, 5000, Australia

[13] Institute for Stem Cell Biology and Regenerative Medicine, Stanford School of Medicine, Stanford University, Stanford, CA

[14] Department of Haematology and Bone Marrow Transplantation, Royal Adelaide Hospital, Adelaide, Australia.

[15] Equal senior author

[#]Present Address: The Peter MacCallum Cancer Centre, Melbourne, 3000 VIC, Australia.

[*] **Authors for correspondence**: Stuart M Pitson, Bradley Building, North Tce, Adelaide, SA 5001, Australia, E-mail: stuart.pitson@unisa.edu.au, Tel: +61 8 8302 7832. Jason A Powell, Bradley Building, North Tce, Adelaide, SA 5001, Australia, E-mail: jason.powell@sa.gov.au, Tel: +61 8 8302 7898.

Text word count 3,990, abstract word count 151, 7 figures, 68 references

Scientific category: Myeloid Neoplasia

Key points

- Enhancing cellular ceramide levels in AML activates protein kinase R to induce the integrated stress response (ISR).
- This ISR induces the BH3-only protein Noxa, resulting in degradation of Mcl-1 and sensitization of AML to Bcl-2 inhibition.

Abstract

Inducing cell death by the sphingolipid ceramide is a potential anti-cancer strategy, but the underlying mechanisms remain poorly defined. Here, we show that triggering accumulation of ceramide in acute myeloid leukaemia (AML) cells by inhibition of sphingosine kinase induces an apoptotic integrated stress response (ISR) through protein kinase R-mediated activation of the master transcription factor ATF4. This leads to transcription of the BH3-only protein, Noxa, and degradation of the pro-survival Mcl-1 protein on which AML cells are highly dependent on for survival. Targeting this novel ISR pathway in combination with the Bcl-2 inhibitor venetoclax synergistically killed primary AML blasts, including those with venetoclax-resistant mutations, as well as immunophenotypic leukemic stem cells, and reduced leukemic engraftment in patient-derived AML xenografts. Collectively, these findings provide mechanistic insight into the anti-cancer effects of ceramide and pre-clinical evidence for new approaches to augment Bcl-2 inhibition in the therapy of AML and other cancers with high Mcl-1 dependency.

Introduction

The discovery of novel signaling mechanisms that enable induction of pro-apoptotic BH3-only proteins independent of TP53 has immense therapeutic potential for both TP53 mutant cancer and tumors resistant to Bcl-2 inhibitors. Pro-apoptotic Noxa is a Bcl-2 family protein that belongs to a subclass of BH3-only proteins and can induce apoptosis via both TP53-dependent and TP53-independent processes, depending on cellular context. Certain cytotoxic drugs have been shown to upregulate Noxa protein, principally through upregulating mRNA transcription,[1] but the upstream signals and physiological stimuli are not well defined nor are they optimised for therapy. Through its ability to bind and neutralise both A1 and Mcl-1 pro-survival proteins, upregulation of Noxa[2] has therapeutic potential in multiple cancers that show intrinsic or acquired resistance to Bcl-2 inhibitor therapies such as venetoclax.

The BH3 mimetic, venetoclax is a highly selective oral inhibitor of the pro-survival protein Bcl-2 approved for treatment of 17p(del) chronic lymphocytic leukemia.[3] Venetoclax shows modest activity as a single agent in AML (overall response rate 19%)[4] but promising results when combined with chemotherapy (complete response CR, 62%) or hypomethylating agents (CR 67%),[5,6] and has been recently approved by the FDA in adults 75 years or older, or who have comorbidities precluding intensive induction chemotherapy (NCT02993523 and NCT03069352). Unlike lymphoid leukemias, AML cells rely on the pro-survival protein Mcl-1 for disease maintenance,[7] suggesting that its inhibition may prove beneficial in achieving deep molecular remission. Pre-clinical studies revealed Mcl-1 as a biomarker for venetoclax resistance due to the inability of this drug to sequester Mcl-1, highlighting the importance of concurrent inhibition of multiple proteins in the Bcl-2 family.[4,8]

An alternative mechanism for induction of programmed cell death can occur through the ceramide/sphingosine-1-phosphate (S1P) rheostat.[9] Ceramide accumulation is thought to contribute to the effects of many anti-cancer therapies including ionizing radiation, daunorubicin, etoposide and gemcitabine as well as some targeted therapies such as tyrosine kinase inhibitors.[10] Ceramides are lipids present in high concentrations in cell membranes, but accumulate following blockade of downstream conversion of sphingosine to S1P, that occurs principally through the activity of sphingosine kinase 1 (SPHK1). SPHK1 can promote tumorigenic pathways such as survival and

proliferation in multiple solid and blood cancers, and is a key player of the sphingolipid rheostat in maintaining the balance between pro-apoptotic ceramide and sphingosine and pro-survival S1P.[11] But, how sphingolipid signaling is integrated with intrinsic BAX/BAK-dependent apoptosis is not well understood.

We previously demonstrated that targeting SPHK1 in AML depletes the pro-survival protein, Mcl-1 and can synergise with Bcl-2/Bcl-X_L inhibitor, navitoclax.[12] However, the mechanistic basis of synergy between SPHK1 inhibition and navitoclax remained poorly defined. Here, we show that triggering accumulation of ceramide in AML cells by inhibition of SPHK1 induces upregulation of the BH3-only protein Noxa via ceramide-mediated activation of protein kinase R (PKR), as part of the integrated stress response (ISR) pathway, and subsequent activation of the transcription factor ATF4. Targeting this novel pathway synergizes with the clinically relevant Bcl-2 inhibitor, venetoclax to exert anti-leukemic activity against AML patient blasts, including those harboring mutations associated with venetoclax resistance and immunophenotypic $CD34^+CD38^-CD123^+$ leukemic stem cells (iLSCs), both in vitro and in vivo. Collectively this advocates the use of ceramide modulating agents as ISR activators to augment Bcl-2 inhibiting strategies for the treatment of AML and other cancers with high dependency on Mcl-1.

Methods

Study approval

Animal studies were approved by the SA Pathology/CALHN and UniSA Animal Ethics Committees. Human samples were obtained from the South Australian Cancer Research Biobank from AML patients after informed consent, and studies were approved by the Royal Adelaide Hospital Human Ethics Committee (Protocol # 041009).

Mutational analysis of primary AML biopsies

Mutations in primary AML biopsies were identified using either whole exome sequencing or targeted gene sequencing as described previously.[12]

Cell lines & primary AML samples

AML cell lines MV411, THP-1, MOLM13 and UT7 were cultured as previously described.[12] OCI-AML3 were cultured in RPMI with 10% fetal calf serum (FCS; HyClone Thermo Scientific), HL-60 in IMDM with 20% FCS, and HEK293T cells in DMEM with 10% FCS. Cell line authentication was confirmed by STR profiling. Mononuclear cells (MNC) from diagnostic bone marrow or apheresis product samples were isolated by Ficoll-Hypaque density-gradient centrifugation and resuspended in IMDM containing 10% FCS. Factor dependent myeloid (FDM) wild type and $Bax^{-/-}/Bak^{-/-}$ cells were cultured in DMEM (Low glucose) supplemented with 10% FCS and 0.25ng/ml mIL-3. Parental and $PERK^{-/-}$ HAP1 cells (Horizon Discovery) were cultured in IMDM containing 10% FCS.

In vivo primary AML xenograft model

6 week old female NOD/SCID/IL-2R$\gamma^{-/-}$ (NSG) mice were IV injected with $5x10^6$ human primary AML cells. Mice were tail bled weekly to confirm human cell engraftment by flow cytometry (>1% hCD45+). MP-A08 (100mg/kg i.p {PEG 400}) and venetoclax (75mg/kg p.o. {60% Phosal 50PG, 30% PEG 400 and 10% ethanol}) were administered daily for two weeks. Mice were sacrificed following treatment cessation to collect bone marrow to measure hCD45+ cells by flow cytometry. Immunohistochemistry on mouse sternum was performed as previously described using the human specific mitochondrial antibody (Thermo Fisher Scientific Cat# MA1-21891).[12]

Data Sharing Statement: For original data please contact stuart.pitson@unisa.edu.au

Additional details are provided in supplemental methods

Results

BH3-only protein Noxa is essential for AML cell death induced by SPHK1 inhibition

Previous studies have shown that SPHK1 inhibitors, including MP-A08, induce AML cell death.[12-16] We also showed this occurs in an Mcl-1-dependent manner,[12] however, the exact mechanism remained unclear. The BH3-only protein Noxa is a selective binding partner of Mcl-1, with this interaction known to promote Mcl-1 degradation and induction of apoptosis.[17] Treatment of MV411 AML cells with the SPHK1 inhibitor MP-A08 increased Noxa expression and augmented the association of Noxa with Mcl-1 (Figure 1a). This increase in Noxa protein expression coincided with

144 increased Noxa mRNA levels following MP-A08 treatment (Figure 1b; Supp Figure 1) and could be
145 blocked through inhibition of protein synthesis by cycloheximide (Figure 1c), suggesting that
146 increased Noxa expression occurs via transcriptional upregulation. Similar increases in Noxa
147 expression were also observed in both MV411 cells and primary AML patient samples (Supp. Table
148 1) in response to another structurally different SPHK1 inhibitor, SK1-I (Supp. Figure 2). A broader
149 analysis of Bcl-2 and BH3-only proteins in response to MP-A08 prior to apoptosis induction (6 h) in
150 multiple AML cell lines revealed dose-dependent increases in Noxa and to a lesser extent other BH3
151 only proteins Bim and cleaved Bid, but not other Bcl-2 family proteins nor TP53 (Figure 1d,e; Supp
152 Figure 3a,b). Doxycycline-inducible shRNA knockdown of Noxa partially reversed the degradation of
153 Mcl-1 and rescued the effects of MP-A08 on cell viability (Figure 1f; Supp Figure 3c). In contrast,
154 Bim knockdown had only a minor effect on cell viability (Figure 1g) but could not reverse loss of
155 Mcl-1, whereas Bid knockdown had no effect (Figure 1h). This implicates Noxa as an important
156 determinant of the apoptotic effects of SPHK1 inhibition in AML cells.
157
158 ***SPHK1 inhibition induces ATF4-dependent Noxa transcription***
159 Tumour suppressor TP53 has been previously shown to directly induce Noxa transcription in response
160 to chemotherapeutics.[18] Unlike daunorubicin and cytarabine, MP-A08 treatment of multiple AML cell
161 lines was associated with a lack of increased TP53 expression, suggesting that the increase in Noxa is
162 independent of TP53 (Figure 2a; Supp Figure 4). Previous work investigating the mechanism of MP-
163 A08-induced AML cell death using Ingenuity Pathways Analysis (IPA) of RNA-Seq data revealed
164 enrichment of genes associated with the unfolded protein response (UPR).[12] In response to cellular
165 stresses that cause misfolded proteins to accumulate within the endoplasmic reticulum (ER), cells
166 activate the three UPR transmembrane proteins, ATF6 (activating transcription factor-6), IRE1
167 (inositol-requiring kinase 1) and protein kinase R-like ER kinase (PERK) to re-establish proteostasis
168 (Figure 2b). Further examination of this RNA-Seq data revealed that MP-A08 treatment of MV411
169 cells induced almost exclusively activation of the PERK arm of the UPR, typified by upregulation of
170 ATF4 and downstream effectors, including CHOP (Supp. Figure 5a).[12] Direct protein analysis of this
171 pathway demonstrated that MP-A08 induced clear activation/phosphorylation of eIF2a, a central

component of the pathway, and induction of ATF4 in AML cell lines (Figure 2c; Supp Figure 5b). Similar effects were also observed with CRISPR/Cas9-mediated knockout of SPHK1 (Figure 2d; Supp. Figure 5c). Intriguingly, consistent with the RNA-Seq data, no changes in XBP1 splicing were observed in response to MP-A08 treatment (Supp. Figure 5d), further demonstrating that the effects induced by SPHK1 inhibition are limited to the PERK arm of the UPR. Notably, prolonged activation of the PERK pathway can culminate in upregulation of BH3-only proteins, Noxa and Bim through the transcription factors ATF4 and CHOP, respectively.[19,20] To confirm that ATF4 was necessary to mediate Noxa transcription in response to MP-A08, we utilised the eIF2b agonist, ISRIB to render cells insensitive to eIF2a phosphorylation and block ATF4 production[21] which nullified Noxa transcription observed with MP-A08 treatment (Figure 2e; Supp Figure 1). An ATF4 shRNA recapitulated the effects of ISRIB (Figure 2f; Supp Figure 6). Chromatin immuno-precipitation (ChIP) analysis of the Noxa promoter confirmed the involvement of ATF4 in Noxa transcription with significant enrichment of ATF4 following MP-A08 treatment (Figure 2g). Like observed in AML cell lines, MP-A08 treatment resulted in dose-dependent increases in eIF2a phosphorylation, Noxa expression and ATF4 expression in a series of primary AML patient blasts (Figure 2h).

Ceramide accumulation activates an apoptotic integrated stress response

Ceramides have been shown to evoke UPR activation and contribute to disease pathogenesis.[22] Furthermore, saturated lipids, of which the ceramides and other sphingolipids are a major class, have been shown to induce IRE1 and PERK activation independent of unfolded proteins via direct sensing of the lipid composition within the ER membrane.[23-26] As the ER is the main location of de novo sphingolipid biosynthesis, we hypothesized that the accumulation of sphingolipids, such as ceramides, at this site in response to SPHK1 inhibition may facilitate the PERK activation observed in response to MP-A08. Indeed, mass spectrometric lipidomic analysis of MV411 cells treated with MP-A08 for 6 h revealed a broad increase in the cellular levels of various ceramides and dihydroceramides, as well as sphingosine and dihydrosphingosine (Figure 3a-c). Similar MP-A08-induced increases in ceramides, but not dihydroceramides, were also observed in MOLM13 and OCI-AML3 cells, with an apparent bias towards increases in long chain ceramides over very long chain ceramides (Supp Figure

7). In MV411 cells increases in ceramides and dihydroceramides were observed as early as 2 h (Supp. Figure 8), in alignment with the upregulation of ATF4 and Noxa observed at this time point after SPHK1 inhibition by MP-A08 (Figure 2c). Consistent with a role for ceramides/dihydroceramides in these effects, use of PF-543, a potent SPHK1 inhibitor that blocks S1P generation but inexplicably does not increase ceramide levels,[27] did not induce eIF2a phosphorylation, nor increase ATF4 and Noxa levels, nor reduce Mcl-1 levels (Figure 3d). In contrast, the ceramidase inhibitor, ceranib-2, known to elevate cellular ceramide levels,[28] caused induction of Noxa and loss of Mcl-1, as well as effective cell death of AML cell lines (Figure 3e; Supp Figure 9). Together, this data suggests that accumulation of ceramides mediates the anti-AML effects of SPHK1 inhibition, rather than loss of S1P signaling. Indeed, addition of exogenous S1P failed to rescue AML cell death induced by MP-A08 (Supp. Figure 10). To more directly examine the effects of ceramide we added exogenous C2- or C6-ceramide to MV411 cells which, unlike the respective dihydroceramides, induced ATF4 and Noxa expression and loss of Mcl-1, consistent with a role for ceramides in evoking activation of the ATF4 pathway (Figure 3f).

Since our data demonstrated a clear involvement of eIF2a and ATF4 in mediating the effects of SPHK1 inhibition on Noxa accumulation, we next examined the dependency on PERK through the use of both CRISPR/Cas9 knockout of PERK in HAP1 chronic myeloid leukemia cells and doxycycline-inducible shRNA knockdown of PERK in MV411 cells. Unexpectedly, loss of PERK had no effect on MP-A08-induced ATF4 and Noxa accumulation (Figure 3g,h,i) suggesting this may be driven by an alternative mechanism leading to activation/phosphorylation of eIF2a.

Ceramides drive an integrated stress response via direct activation of PKR

In addition to PERK, eIF2a can be phosphorylated by three other protein kinases that are activated under varying stress conditions as part of the ISR. These kinases, PKR, GCN2 (general control non-derepressible 2) and HRI (heme-regulated inhibitor) can all phosphorylate eIF2a to increase translation of the master ISR transcription factor, ATF4[29] (Figure 4a). Pharmacological interrogation of the role of these kinases in ATF4 and Noxa induction revealed a clear role for PKR with two different PKR inhibitors C16[30] and 2-aminopurine (2-AP),[31] both blocking the effects of MP-A08

(Figure 4b,c). In contrast, inhibition of GCN2 (with A-92) or PERK (with AMG-44) had little to no effect (Figure 4b). Similar effects were observed in all five AML cell lines examined (Figure 4b,c). Furthermore, the anti-AML effects of MP-A08 on THP-1 and HL-60 cells were mitigated by either pharmacological targeting of PKR with 2-AP (Figure 4d; Supp Figure 11), or CRISPR/Cas9 knockout of PKR in MV411 cells (Figure 4e). Collectively, this indicates a critical role for PKR in sensing SPHK1-inhibitor-induced ceramide accumulation, culminating in activation of eIF2a, leading to activation of ATF4 and Noxa, loss of Mcl-1 and consequent AML cell death.

Previous findings that ceramide can directly modulate the function of various proteins,[32-37] suggested the potential for PKR to be directly activated by ceramide. To examine this we assessed the effect of ceramide on the activity of isolated PKR in vitro. Strikingly, our results demonstrated enhanced PKR activity in the presence of ceramide suggesting that ceramide directly binds and enhances PKR kinase activity (Figure 4f). To further assess the interaction of ceramide with PKR we employed ceramide conjugated agarose beads to probe cell lysates for PKR. The data (Figure 4g) demonstrates a clear pull-down of PKR with ceramide beads compared to control beads, indicative of an interaction of PKR with ceramide.

SPHK1 inhibition synergises with venetoclax in AML cell lines

Mcl-1 is known to mediate resistance to Bcl-2 inhibition in AML,[8] and approaches to target Mcl-1 have been shown to enhance the efficacy of Bcl-2 inhibition in inducing AML cell death.[12,38-42] Thus, we next investigated the therapeutic potential for modulating sphingolipid signaling to impact Bcl-2 antagonism. Anti-AML synergy of MP-A08 and venetoclax could be observed as early as two hours (Figure 5a) and coupled with enhanced association of Noxa with Mcl-1 (Supp. Figure 12a,b).

Viability studies on factor-dependent myeloid cell lines from wild type and $Bax^{-/-}/Bak^{-/-}$ mice[43] demonstrated that wild type cells underwent synergistic cell death in response to MP-A08 and venetoclax, but $Bax^{-/-}/Bak^{-/-}$ cells were completely resistant (Figure 5b), verifying mitochondrial mediated apoptosis via the necessity of executioner proteins, Bax and Bak.

Combining MP-A08 with sub-cytotoxic doses of venetoclax strongly enhanced cell death in venetoclax-sensitive MV411, HL-60 and MOLM13 cell lines (Figure 5c-f), as well as in the

venetoclax-resistant OCI-AML3 cell line (Figure 5g). Statistical analysis using the Chou-Talalay method[44] confirmed combinational treatment induced synergistic cell death (synergy = combination index (CI) <1). Treatment of MV411 cells with another SPHK1 inhibitor, SK1-I, recapitulated the effects observed with MP-A08, causing synergistic loss of cell viability with venetoclax treatment (Supp. Figure 12c). Synergy between SPHK1 and Bcl-2 inhibition was further confirmed by doxycycline-inducible shRNA targeting of Bcl-2 in MV411 cells (Figure 5h). Interestingly, at doses capable of achieving drug synergy in other cells, MP-A08 and venetoclax treatment exhibited little effect in UT7 cells (Figure 5i). This lack of response to either drug, however, may be explained either by the strong expression of Bcl-X_L (Supp. Figure 12d)[45] or by the lack of Mcl-1 degradation in response to MP-A08 (Figure 5j). Combined treatment was associated with synergistic activation of caspase 3 and loss of Mcl-1 across several AML cell lines (Figure 5k,l, Supp. Figure 12e).

MP-A08 and venetoclax treatment exhibits anti-leukemic activity in primary AML cells in vitro and in vivo

MP-A08 treatment of primary AML samples induced changes in levels of Mcl-1, Noxa and Bid (Figure 6a). Combining both MP-A08 and venetoclax also resulted in synergistically enhanced cell death of primary AML blasts (Figure 6b-c) and chemo-refractory and relapse driving iLSCs (Figure 6d) even when co-cultured with human bone marrow derived mesenchymal stem cells (MSC) (Figure 6e; Supp. Figure 13a). This suggests this dual targeting approach is sufficient to induce cell death across different AML subtypes even in the presence of extrinsic factor support from MSCs. Further analysis also showed that combining MP-A08 and venetoclax reduced colony forming units in primary AML patient cells (Supp Figure 13b). Notably, however, even high concentrations of MP-A08 and venetoclax had minimal effects on CD34+ hematopoietic stem/progenitor cells isolated from healthy volunteers (Figure 6f; Supp Figure 13c).

We next assessed the efficacy of MP-A08 and venetoclax in vivo using primary AML patient xenografts in NSG mice. Immunohistochemical staining for human leukemic cells in the sternum confirmed systemic orthotopic engraftment in two separate sets of primary AML patient xenografts (Figure 6g). Mice with established disease were treated daily with MP-A08 (100mg/kg), venetoclax

(75mg/kg) or the combination for two weeks. This combinational approach significantly reduced the leukemic engraftment compared with the respective monotherapies, as assessed by flow cytometric analysis of $hCD45^+$ cells in the bone marrow of these mice (Figure 6h-i). Notably, similar dual therapy with MP-A08 and venetoclax daily for two weeks in control C57Bl/6 mice showed no obvious toxicity, with no deleterious effects on body weight, major bone marrow lineages or blood cell counts (Supp Figure 14).

Since MP-A08 enhanced venetoclax-induced killing of AML cells, we sought to expand this finding by identifying venetoclax-resistant patients using the Beat AML Master Trial data (ex vivo drug sensitivity analysis with 122 drugs on 409 patient samples) to assess whether these patients could be resensitised to venetoclax[46]. In addition to the $TP53^{mut}$ cohort identified in the phase II studies of venetoclax with cytarabine or hypomethylating agents[6,47] we identified patients with *KRAS*, *PTPN11* and *SF3B1* mutations as resistant to venetoclax treatment (Figure 6j), consistent with previous findings[40]. Combined MP-A08 and venetoclax treatment exhibited synergistic anti-AML activity in diagnostic patient samples containing either *PTPN11* or *TP53* mutations (Supp. Table 1, Figure 6k-l) with little effect in patient cells containing a *KRAS* mutation (Figure 6m). Although only from single patients with each mutation, collectively, this data begins to provide pre-clinical evidence for combination therapy with SPHK1 inhibitors to augment the efficacy of venetoclax in a variety of AML subtypes.

Discussion

The success of the Bcl-2 inhibitor venetoclax in CLL has revolutionised the therapeutic landscape. Yet, in AML which relies on Mcl-1 for survival,[7] the modest single agent activity of venetoclax has fuelled the search for new therapies that can be combined with this drug.[4] In this study, we found that enhancing ceramide accumulation, through targeting SPHK1, may be such an approach with ceramide directly activating PKR, inducing ATF4-mediated transcriptional upregulation of Noxa, a known inducer of Mcl-1 degradation, and acts synergistically with venetoclax to induce apoptosis in AML cells. This synergy extended to both primary AML blasts and iLSCs, suggesting this combinational approach may reduce relapse rates by depleting leukemia-initiating cells. Importantly, this approach

also reduced disease engraftment in orthotopic AML patient xenograft models, providing pre-clinical evidence of the targeting of SPHK1 and Bcl-2 as a valid combinational therapy approach in AML.

The Beat AML master trial is a multi-centre clinical trial integrating genomics data with in vitro responses to both clinical and pre-clinical agents.[46] Using this data, we identified patients with a number of mutations including those in *KRAS*, *PTPN11* and *SF3B1* to confer venetoclax resistance in addition to the $TP53^{Mut}$ cohort observed in a phase II study examining cytarabine and venetoclax.[6] Excitingly, SPHK1 inhibition combined with venetoclax was effective in patient samples containing *PTPN11* or *TP53* mutations (Figure 5j,k). The absence of any effect observed in a *KRAS* mutant case may be related to the protective effects of *KRAS* in activating the IRE1 pathway to promote survival, as observed in HSCs.[48] Follow up analysis with cells from further *KRAS* mutant AML patients is clearly required to confirm if these apparent protective effects of mutant *KRAS* against venetoclax/SPHK1 inhibition holds true. Notably, our analysis suggests AML with *NRAS* mutations, which are more common,[49] remain sensitive to combination therapy with venetoclax/SPHK1 inhibition, as demonstrated by data from HL-60 and OCI-AML3 cell lines (Figure 5e,g) and patient sample AML11 (Figure 6i). Collectively this suggests that SPHK1 inhibition may be an effective combination therapy approach with venetoclax in cases that are venetoclax insensitive. Since SPHK1 inhibition increases Noxa in a manner independent of TP53, its combination with drugs like cytarabine that are ineffective in cohorts such as $TP53^{Mut}$ patients, may also be warranted.

Approaches to enhance cellular ceramide as a potential anti-AML therapy have been investigated over the last two decades,[10] including with inhibitors of SPHK1[12,13,50] and acid ceramidase,[51,52] and more recently with direct nanoliposomal ceramide formulations.[53,54] The mechanisms whereby ceramide elicits its anti-AML effects, however, have not been clear. In this study we showed that accumulation of ceramides through SPHK1 inhibition activates an apoptotic ISR that is dependent on PKR. Although ISR activation has been reported previously in response to addition of exogenous ceramides,[55] this is the first time to our knowledge that endogenous ceramides have been associated with ISR activation, and the first report of direct activation of PKR by ceramides. Furthermore, we have provided evidence that Noxa is a significant effector of the apoptotic effects of ceramide accumulation through the ISR, expanding the role of sphingolipids in

dictating cell survival. This builds on previous studies suggesting ceramide may act both prior to apoptosis through Bcl-2 dephosphorylation[56] and Bad activation,[57] as well as during apoptosis through the formation of ceramide-induced channels in the mitochondrial outer membrane to facilitate release of cytochrome c and other pro-apoptotic mediators.[58]

Notably, an oncogenic role for PKR in AML has been previously reported, with high PKR expression in AML associated with poor patient prognosis. This appears to be due to the suppressive effects of PKR on the DNA damage response (DDR), an important feature of efficacy to chemotherapeutics such as cytarabine and daunorubicin.[59] Thus, these observations, combined with the findings of the current study that show that the anti-AML effects of SPHK1 inhibition appear to be independent of TP53 and DDR activation and dependent on PKR activation, make it tempting to speculate that high PKR expressing patients may respond better to SPHK1 inhibition than to chemotherapy.

Our findings support a prominent role for the PKR/ATF4/Noxa/Mcl-1 axis identified in this study in mediating ceramide-induced AML cell death. However, efficient Noxa knockdown does not completely rescue AML cell killing by MP-A08 (Figure 1f), supporting the notion that other pathways may play minor roles in the anti-AML effects of MP-A08. Our analysis of gene expression changes in AML cells in response to MP-A08[12] suggest a range of altered pathways that could potentially be involved, including oxidative stress and autophagy, as well as other target genes downstream of ATF4 and one of its effectors, CHOP (Supp Table 2). This warrants further investigation.

Recent work has revealed an importance for ATF4, the master regulator of the ISR, in leukemic survival.[60,61] In particular, ATF4 has been shown to promote cell survival in FLT3-ITD$^+$ AML by enhancing autophagy.[62] Others have shown that primitive leukemic stem cells (LSCs) exhibit higher basal ISR activity and ATF4 levels than leukemic progenitors/blasts and this may protect LSCs against amino acid deprivation.[60] Yet, we and others have found that ATF4 can prime cancer cells for apoptosis via transcriptional upregulation of Noxa.[20,63] How LSCs control the dichotomous signaling of ATF4 to favour survival signaling and how ceramide disrupts this requires further investigation. As ATF4 is regulated by phosphorylation,[64] we cannot discount the premise that ceramide, a known activator of protein phosphatases, including PP2A,[65] may also modulate ATF4 at the post-translational

level. Indeed, a recent study demonstrated that PP2A activation caused upregulation of ATF4 and Noxa, strengthening the potential link between ceramide and the ISR.[66] Furthermore, ceramides induce loss of oxidative phosphorylation, a metabolic vulnerability of quiescent LSPCs.[67,68] Collectively this suggests that ceramide inducing agents together with venetoclax may represent a LSC specific therapy by activating the ISR, reducing the likelihood of relapse and chemotherapy resistant disease (Figure 7).

Acknowledgements

This work was supported by a Research Training Program Scholarship and Royal Adelaide Hospital Dawes Top-up scholarship (to ACL), the Fay Fuller Foundation, the Royal Adelaide Hospital Research Fund, The Hospital Research Foundation, a Senior Research Fellowship (GNT1156693 to SMP), an Early Career Fellowship (PG101400 to TMN) and project grants (GNT1145139 and GNT1184485) from the National Health and Medical Research Council of Australia. DT is supported by Leukemia & Lymphoma Translation Research Program and CSL Centenary Fellowship. The authors are grateful to the South Australian Cancer Research Biobank (SACRB) and the patients who donated samples. We thank Sarah Tamang for technical assistance.

Author contributions

ACL, MNT, VSP, ML, GON, TMN, CWB, PABM, DA, DJC, MC, SRA, CT-P, BKD, AGB, MRP, BLG and JAP performed experiments. PGE, GJG and NR provided intellectual input and/or reagents. DMR, ALB and RJD provided patient material and clinical notes. ACL, JAP, DT and SMP designed the studies and analysed the data. ACL, JAP and SMP wrote the manuscript, which all authors critically reviewed and edited.

Conflict of interest

The authors declare no relevant conflicts of interest.

References

1. Albert MC, Brinkmann K, Kashkar H. Noxa and cancer therapy: Tuning up the mitochondrial death machinery in response to chemotherapy. *Mol Cell Oncol*. 2014;1(1):e29906.
2. Morsi RZ, Hage-Sleiman R, Kobeissy H, Dbaibo G. Noxa: Role in Cancer Pathogenesis and Treatment. *Curr Cancer Drug Targets*. 2018;18(10):914-928.
3. Roberts AW, Davids MS, Pagel JM, et al. Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. *N Engl J Med*. 2016;374(4):311-322.
4. Konopleva M, Pollyea DA, Potluri J, et al. Efficacy and Biological Correlates of Response in a Phase II Study of Venetoclax Monotherapy in Patients with Acute Myelogenous Leukemia. *Cancer Discovery*. 2016.
5. Wei AH, Montesinos P, Ivanov V, et al. Venetoclax plus LDAC for newly diagnosed AML ineligible for intensive chemotherapy: a phase 3 randomized placebo-controlled trial. *Blood*. 2020;135(24):2137-2145.
6. DiNardo CD, Tiong IS, Quaglieri A, et al. Molecular patterns of response and treatment failure after frontline venetoclax combinations in older patients with AML. *Blood*. 2020;135(11):791-803.
7. Glaser SP, Lee EF, Trounson E, et al. Anti-apoptotic Mcl-1 is essential for the development and sustained growth of acute myeloid leukemia. *Genes Dev*. 2012;26(2):120-125.
8. van Delft MF, Wei AH, Mason KD, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell*. 2006;10(5):389-399.
9. Newton J, Lima S, Maceyka M, Spiegel S. Revisiting the sphingolipid rheostat: Evolving concepts in cancer therapy. *Exp Cell Res*. 2015;333(2):195-200.
10. Lewis AC, Wallington-Beddoe CT, Powell JA, Pitson SM. Targeting sphingolipid metabolism as an approach for combination therapies in haematological malignancies. *Cell Death Discovery*. 2018;4(1):72.
11. Pitson SM. Regulation of sphingosine kinase and sphingolipid signaling. *Trends Biochem Sci*. 2011;36(2):97-107.
12. Powell JA, Lewis AC, Zhu W, et al. Targeting sphingosine kinase 1 induces MCL1-dependent cell death in acute myeloid leukemia. *Blood*. 2017;129(6):771-782.
13. Paugh SW, Paugh BS, Rahmani M, et al. A selective sphingosine kinase 1 inhibitor integrates multiple molecular therapeutic targets in human leukemia. *Blood*. 2008;112(4):1382-1391.
14. Dick TE, Hengst JA, Fox TE, et al. The apoptotic mechanism of action of the sphingosine kinase 1 selective inhibitor SKI-178 in human acute myeloid leukemia cell lines. *J Pharmacol Exp Ther*. 2015;352(3):494-508.
15. Hengst JA, Dick TE, Sharma A, et al. SKI-178: A Multitargeted Inhibitor of Sphingosine Kinase and Microtubule Dynamics Demonstrating Therapeutic Efficacy in Acute Myeloid Leukemia Models. *Cancer Transl Med*. 2017;3(4):109-121.
16. Hengst JA, Hegde S, Paulson RF, Yun JK. Development of SKI-349, a dual-targeted inhibitor of sphingosine kinase and microtubule polymerization. *Bioorg Med Chem Lett*. 2020;30(20):127453.
17. Czabotar PE, Lee EF, van Delft MF, et al. Structural insights into the degradation of Mcl-1 induced by BH3 domains. *Proc Natl Acad Sci U S A*. 2007;104(15):6217-6222.
18. Oda K, Arakawa H, Tanaka T, et al. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell*. 2000;102(6):849-862.
19. Puthalakath H, O'Reilly LA, Gunn P, et al. ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell*. 2007;129(7):1337-1349.
20. Wang Q, Mora-Jensen H, Weniger MA, et al. ERAD inhibitors integrate ER stress with an epigenetic mechanism to activate BH3-only protein NOXA in cancer cells. *Proceedings of the National Academy of Sciences*. 2009;106(7):2200.
21. Zyryanova AF, Weis F, Faille A, et al. Binding of ISRIB reveals a regulatory site in the nucleotide exchange factor eIF2B. *Science*. 2018;359(6383):1533.
22. Bennett MK, Wallington-Beddoe CT, Pitson SM. Sphingolipids and the unfolded protein response. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2019;1864(10):1483-1494.

23. Bennett MK, Li M, Tea MN, et al. Resensitising proteasome inhibitor-resistant myeloma with sphingosine kinase 2 inhibition. *Neoplasia*. 2022;24(1):1-11.
24. Halbleib K, Pesek K, Covino R, et al. Activation of the Unfolded Protein Response by Lipid Bilayer Stress. *Molecular Cell*. 2017;67(4):673-684.e678.
25. Volmer R, Ron D. Lipid-dependent regulation of the unfolded protein response. *Curr Opin Cell Biol*. 2015;33:67-73.
26. Volmer R, van der Ploeg K, Ron D. Membrane lipid saturation activates endoplasmic reticulum unfolded protein response transducers through their transmembrane domains. *Proc Natl Acad Sci U S A*. 2013;110(12):4628-4633.
27. Schnute ME, McReynolds MD, Kasten T, et al. Modulation of cellular S1P levels with a novel, potent and specific inhibitor of sphingosine kinase-1. *Biochem J*. 2012;444(1):79-88.
28. Draper JM, Xia Z, Smith RA, Zhuang Y, Wang W, Smith CD. Discovery and evaluation of inhibitors of human ceramidase. *Mol Cancer Ther*. 2011;10(11):2052-2061.
29. Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. The integrated stress response. 2016;17(10):1374-1395.
30. Jammi NV, Whitby LR, Beal PA. Small molecule inhibitors of the RNA-dependent protein kinase. *Biochem Biophys Res Commun*. 2003;308(1):50-57.
31. Kaufman RJ. Double-stranded RNA-activated protein kinase mediates virus-induced apoptosis: a new role for an old actor. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96(21):11693-11695.
32. Mukhopadhyay A, Saddoughi SA, Song P, et al. Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the regulation of protein phosphatase 2A activity and signaling. *FASEB J*. 2009;23(3):751-763.
33. Heinrich M, Wickel M, Schneider-Brachert W, et al. Cathepsin D targeted by acid sphingomyelinase-derived ceramide. *EMBO J*. 1999;18(19):5252-5263.
34. Huwiler A, Brunner J, Hummel R, et al. Ceramide-binding and activation defines protein kinase c-Raf as a ceramide-activated protein kinase. *Proc Natl Acad Sci U S A*. 1996;93(14):6959-6963.
35. Zhang Y, Yao B, Delikat S, et al. Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell*. 1997;89(1):63-72.
36. Dadsena S, Bockelmann S, Mina JGM, et al. Ceramides bind VDAC2 to trigger mitochondrial apoptosis. *Nat Commun*. 2019;10(1):1832.
37. Fekry B, Jeffries KA, Esmaeilniakooshkghazi A, et al. C16-ceramide is a natural regulatory ligand of p53 in cellular stress response. *Nat Commun*. 2018;9(1):4149.
38. Ewald L, Dittmann J, Vogler M, Fulda S. Side-by-side comparison of BH3-mimetics identifies MCL-1 as a key therapeutic target in AML. *Cell Death Dis*. 2019;10(12):917.
39. Niu X, Zhao J, Ma J, et al. Binding of Released Bim to Mcl-1 is a Mechanism of Intrinsic Resistance to ABT-199 which can be Overcome by Combination with Daunorubicin or Cytarabine in AML Cells. *Clin Cancer Res*. 2016;22(17):4440-4451.
40. Phillips DC, Jin S, Gregory GP, et al. A novel CDK9 inhibitor increases the efficacy of venetoclax (ABT-199) in multiple models of hematologic malignancies. *Leukemia*. 2020;34(6):1646-1657.
41. Ramsey HE, Fischer MA, Lee T, et al. A Novel MCL1 Inhibitor Combined with Venetoclax Rescues Venetoclax-Resistant Acute Myelogenous Leukemia. *Cancer Discov*. 2018;8(12):1566-1581.
42. Zhang H, Nakauchi Y, Kohnke T, et al. Integrated analysis of patient samples identifies biomarkers for venetoclax efficacy and combination strategies in acute myeloid leukemia. *Nat Cancer*. 2020;1(8):826-839.
43. Ekert PG, Jabbour AM, Manoharan A, et al. Cell death provoked by loss of interleukin-3 signaling is independent of Bad, Bim, and PI3 kinase, but depends in part on Puma. *Blood*. 2006;108(5):1461-1468.
44. Chou T-C. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. *Pharmacological reviews*. 2006;58:621-681.

45. Soderquist RS, Crawford L, Liu E, et al. Systematic mapping of BCL-2 gene dependencies in cancer reveals molecular determinants of BH3 mimetic sensitivity. *Nature Communications*. 2018;9(1):3513.
46. Tyner JW, Tognon CE, Bottomly D, et al. Functional genomic landscape of acute myeloid leukaemia. *Nature*. 2018;562(7728):526-531.
47. Nechiporuk T, Kurtz SE, Nikolova O, et al. The TP53 Apoptotic Network is a Primary Mediator of Resistance to BCL2 inhibition in AML Cells. *Cancer Discovery*. 2019:CD-19-0125.
48. Liu L, Zhao M, Jin X, et al. Adaptive endoplasmic reticulum stress signalling via IRE1α-XBP1 preserves self-renewal of haematopoietic and pre-leukaemic stem cells. *Nature cell biology*. 2019;21(3):328-337.
49. Yu J, Li Y, Li T, et al. Gene mutational analysis by NGS and its clinical significance in patients with myelodysplastic syndrome and acute myeloid leukemia. *Exp Hematol Oncol*. 2020;9:2.
50. Bonhoure E, Pchejetski D, Aouali N, et al. Overcoming MDR-associated chemoresistance in HL-60 acute myeloid leukemia cells by targeting sphingosine kinase-1. *Leukemia*. 2006;20(1):95-102.
51. Tan SF, Liu X, Fox TE, et al. Acid ceramidase is upregulated in AML and represents a novel therapeutic target. *Oncotarget*. 2016;7(50):83208-83222.
52. Pearson JM, Tan SF, Sharma A, et al. Ceramide Analogue SACLAC Modulates Sphingolipid Levels and MCL-1 Splicing to Induce Apoptosis in Acute Myeloid Leukemia. *Mol Cancer Res*. 2020;18(3):352-363.
53. McGill CM, Brown TJ, Fisher LN, et al. Combinatorial Efficacy of Quercitin and Nanoliposomal Ceramide for Acute Myeloid Leukemia. *Int J Biopharm Sci*. 2018;1(1).
54. Barth BM, Wang W, Toran PT, et al. Sphingolipid metabolism determines the therapeutic efficacy of nanoliposomal ceramide in acute myeloid leukemia. *Blood Adv*. 2019;3(17):2598-2603.
55. Ruvolo PP, Gao F, Blalock WL, Deng X, May WS. Ceramide regulates protein synthesis by a novel mechanism involving the cellular PKR activator RAX. *J Biol Chem*. 2001;276(15):11754-11758.
56. Ruvolo PP, Deng X, Ito T, Carr BK, May WS. Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A. *J Biol Chem*. 1999;274(29):20296-20300.
57. Basu S, Bayoumy S, Zhang Y, Lozano J, Kolesnick R. BAD enables ceramide to signal apoptosis via Ras and Raf-1. *J Biol Chem*. 1998;273(46):30419-30426.
58. Chang KT, Anishkin A, Patwardhan GA, Beverly LJ, Siskind LJ, Colombini M. Ceramide channels: destabilization by Bcl-xL and role in apoptosis. *Biochim Biophys Acta*. 2015;1848(10 Pt A):2374-2384.
59. Cheng X, Byrne M, Brown KD, et al. PKR inhibits the DNA damage response, and is associated with poor survival in AML and accelerated leukemia in NHD13 mice. *Blood*. 2015;126(13):1585-1594.
60. van Galen P, Mbong N, Kreso A, et al. Integrated Stress Response Activity Marks Stem Cells in Normal Hematopoiesis and Leukemia. *Cell Rep*. 2018;25(5):1109-1117.e1105.
61. Zhou C, Martinez E, Di Marcantonio D, et al. JUN is a key transcriptional regulator of the unfolded protein response in acute myeloid leukemia. *Leukemia*. 2017;31(5):1196-1205.
62. Heydt Q, Larrue C, Saland E, et al. Oncogenic FLT3-ITD supports autophagy via ATF4 in acute myeloid leukemia. *Oncogene*. 2018;37(6):787-797.
63. Armstrong JL, Flockhart R, Veal GJ, Lovat PE, Redfern CP. Regulation of endoplasmic reticulum stress-induced cell death by ATF4 in neuroectodermal tumor cells. *J Biol Chem*. 2010;285(9):6091-6100.
64. Sharma K, D'Souza RC, Tyanova S, et al. Ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr-based signaling. *Cell Rep*. 2014;8(5):1583-1594.
65. Chalfant CE, Kishikawa K, Mumby MC, Kamibayashi C, Bielawska A, Hannun YA. Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid. *J Biol Chem*. 1999;274(29):20313-20317.
66. Ryder CB, Narla G. Direct-Acting Small Molecule Activators of PP2A (SMAPs) in Myeloid Malignancies: Understanding Mechanisms of Cytotoxicity to Inform Rational Combinatorial Therapeutic Strategies. *Blood*. 2017;130(Supplement 1):2517-2517.
67. Morad SAF, Ryan TE, Neufer PD, et al. Ceramide-tamoxifen regimen targets bioenergetic elements in acute myelogenous leukemia. *Journal of lipid research*. 2016;57(7):1231-1242.

68. Lagadinou ED, Sach A, Callahan K, et al. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell stem cell*. 2013;12(3):329-341.

Figure Legends

Figure 1: ***BH3 only proteins Noxa and Bim are essential for MP-A08 induced cell death***

A) Mcl-1 immunoprecipitation of MV411 cells treated with MP-A08 for 6 h and subjected to immunoblot analysis with the indicated antibodies. **B)** Quantitative PCR analysis of MV411 cells treated with MP-A08 (20μM) over 6 h. **C)** Immunoblot analysis of MV411 cells treated with MP-A08 in the presence of cycloheximide for 6 h. **D)** MV411 or **E)** OCI-AML3 were treated with increasing concentrations of MP-A08 for 6 h and subject to immunoblot analysis with the indicated antibodies. MV411 cells were lentivirally transduced with shRNAs targeting **F)** Noxa, **G)** Bim or **H)** Bid, treated with doxycycline for 48 h and MP-A08 (15μM) for either 6 h for immunoblot analysis or 24 h for cell viability using Annexin V staining. All qualitative data is representative of at least three independent experiments, and all quantitative data shown represents mean ±SEM from three independent experiments Statistical significance was assessed by Student's t test.*** $p<0.0001$, ** $p<0.005$, * $p<0.05$.

Figure 2: ***MP-A08 induces ATF4 dependent Noxa transcription***

A) MV411 cells were treated with MP-A08 (20μM), daunorubicin (DNR) (1μM) or cytarabine (Ara-C) (1μM) for 6 h, lysed and subject to immunoblot analysis with indicated antibodies. **B)** Schematic of the unfolded protein response. **C)** MV411 cells were treated with MP-A08 (20μM) over a 6 h period, lysed and subject to immunoblot analysis with the indicated antibodies. **D)** MV411 cells were stably transduced with a two different CRISPR guide sequences targeting SPHK1 (g1 and g2) and lysed and subject to immunoblot analysis with indicated antibodies. SPHK1 knockout efficiency was confirmed via SPHK1 activity assays of lysates from these cells using assay conditions largely selective for SPHK1 over SPHK2 (Supp. Figure 5c). **E)** MV411 cells were treated with MP-A08 (20μM) alone or in combination with eIF2b agonist, ISRIB (200nM) over 6 h for quantitative PCR analysis of Noxa mRNA levels and immunoblot analysis with the indicated antibodies. Statistical significance was assessed by Student's t test. * $p< 0.05$ (n=3). **F)** MV411 cells were stably transduced

with a doxycycline inducible shRNA targeting ATF4. Cells were treated with 1μg/ml doxycycline for 48 h and MP-A08 (20μM) for 6 h prior to cell lysis for immunoblot analysis. ns denotes non-specific band. **G)** Chromatin immunoprecipitation analysis of the Noxa promoter in response to MP-A08 treatment (20μM) of MV411 cells for 6 h. Statistical significance was assessed by Student's t test. * $p<0.05$ (n=4). **H)** Primary AML samples were treated with increasing concentrations of MP-A08 and subject to immunoblot analysis with the indicated antibodies. All qualitative data is representative of at least three independent experiments, and all quantitative data shown represents mean ±SEM from at least three independent experiments.

Figure 3: ***Ceramides drive a PERK-independent ISR***

A-C) MV411 cells were treated with vehicle control (0.1% DMSO; black bars) or 20μM MP-A08 (red bars) for 6 h and analysed by LC-MS. **A)** Quantitation of individual ceramide (Cer) and dihydroceramide (dhCer) species. **B)** Quantitation of sphingosine (Sph) and dihydrosphingosine (dhSph) species. **C)** Quantitation of sphingosine 1-phosphate (S1P) and dihydrosphingosine 1-phosphate (dhS1P). All data are presented as pmol per million cells, mean ±SD from four independent experiments. Statistical significance was assessed by Student's t test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **D)** MV411 cells were treated with MP-A08 (20μM), PF-543 (1μM) and SK1-I (10μM) for 6 h, lysed and subject to immunoblot analysis. **E)** MV411 cells were treated with Ceranib-2 for 16 h and assessed for cell viability by Annexin V/propidium iodide staining. Data represents mean ±SD from two independent experiments. MV411 cells were treated with Ceranib-2 (10μM) for 6 h and subject to immunoblot analysis with the indicated antibodies. **F)** MV411 cells were treated with MP-A08 (20μM) C2-Ceramide (10μM), C6-Ceramide (10μM), C2-dhCeramide (10μM), C6-dhCeramide (10μM) (all introduced from 2.5mM stock solutions in DMSO), Bortezomib (10nM) for 6 h, lysed and subject to immunoblot analysis with the indicated antibodies. **G)** HAP1 wild type and *PERK*$^{-/-}$ cells were treated with MP-A08 (20μM) for 6 h, lysed and subject to immunoblot analysis with the indicated antibodies. **H,I)** MV411 cells were stably transduced with a doxycycline inducible shRNA targeting PERK . Cells were treated with 1μg/ml doxycycline for 48 h and MP-A08 (20μM) for 6 h

prior to (H) quantitative PCR analysis of PERK mRNA levels and (I) immunoblot analysis with the indicated antibodies. Data shown represents mean ±SEM from three independent experiments. Statistical significance was assessed by Student's t test. ** $p< 0.01$.

Figure 4: ***Ceramides drive a PKR-dependent integrated stress response***

A) Schematic of the integrated stress response (ISR). **B,C)** MV411 cells, OCI-AML3 cells, MOLM13 cells, HL-60 cells and THP-1 cells were treated with MP-A08 alone (20 μM) or in combination with GCN2 inhibitor A-92 (5μM), PKR inhibitors C16 (5μM) or 2-AP (0.1-10mM) or PERK inhibitor AMG-44 (5μM) for 6 h prior to immunoblot analysis with indicated antibodies. **D)** MV411 cells were treated with MP-A08 (10μM), and 2-AP (5mM) for 16 h and assessed for cell viability by Annexin V/propidium iodide staining. Data shown represents mean ± SEM from three independent experiments. Statistical significance was assessed by Student's t test. **E)** WT or PKR knockout MV411 cells were treated with MP-A08 (15μM) for 16 h and assessed for cell viability by Annexin V/ propidium iodide staining. Data shown represents mean ± SEM from four independent experiments. Statistical significance was assessed by Student's t test. Immunoblot analysis of WT or PKR knockout MV411 cells with the indicated antibodies. **F)** PKR-HA was immunoprecipitated from transiently transfected HEK 293T cells, incubated with exogenous C6-ceramide (10μM) for 30 mins and subjected to a PKR activity assay, using auto-phosphorylation as the read-out. Data shown represents mean ± SEM from four independent experiments. **G)** Lysates from HEK293T cells transfected with pcDNA3/PKR-HA was incubated with ceramide conjugated to agarose beads or control beads overnight at 4°C, washed and resolved by SDS-PAGE and the associated PKR detected by immunoblotting with anti-HA antibodies using an Odyssey imaging system.

Figure 5: ***MP-A08 and venetoclax induces potent synergistic activity in AML cell lines***

A) MV411 cells were treated with 20μM MP-A08 (black bars), 10nM venetoclax (red bars) alone or in combination (white bars) for up to 6 h. Cell viability was analysed every 2 h by Annexin V/propidium iodide staining. Data is representative of mean ±SEM (n=4). Statistical significance was

assessed by Student's t test. (**** $p < 0.0001$). Drug synergy was assessed using the Chou-Talay combination Index (CI) method whereby CI values less than 1 are classified as synergy. **B)** FDM wild type and Bax/Bak$^{-/-}$ cells treated with MP-A08 (20μM) and/or venetoclax (10nM). Data is representative of mean ±SEM (n=4). Statistical significance was assessed by Student's t test. (**** $p < 0.0001$). **C,D)** MV411, **E)** HL-60 **F)** MOLM13 **G)** OCI-AML3. **H)** MV411 cells were stably transduced with a doxycycline inducible shRNA targeting Bcl-2 and treated with doxycycline (1μg/ml) for 48 h and MP-A08 (10μM) for 24 h. Cell viability was assessed by Annexin V staining. All data represents mean ±SEM from three independent experiments. Statistical significance was assessed by Student's t test. *** $p<0.0001$. **I)** UT-7 cells were treated with MP-A08 and venetoclax for 24 h and assessed for cell viability by Annexin V/propidium iodide staining. Drug synergy was assessed using the Chou-Talay combination Index (CI) method whereby CI values less than 1 are classified as synergy. **J)** UT-7 were treated with increasing concentrations of MP-A08 for 6 h and subject to immunoblot analysis. **K)** MV411 and **L)** OCI-AML3 cells were treated with MP-A08, venetoclax or in combination for 6 h and subject to immunoblot analysis with the indicated antibodies. All qualitative data is representative of at least three independent experiments, and all quantitative data shown represents mean ±SEM from at least three independent experiments.

Figure 6: ***MP-A08 and venetoclax treatment exhibits anti-leukemic activity in primary AML samples***

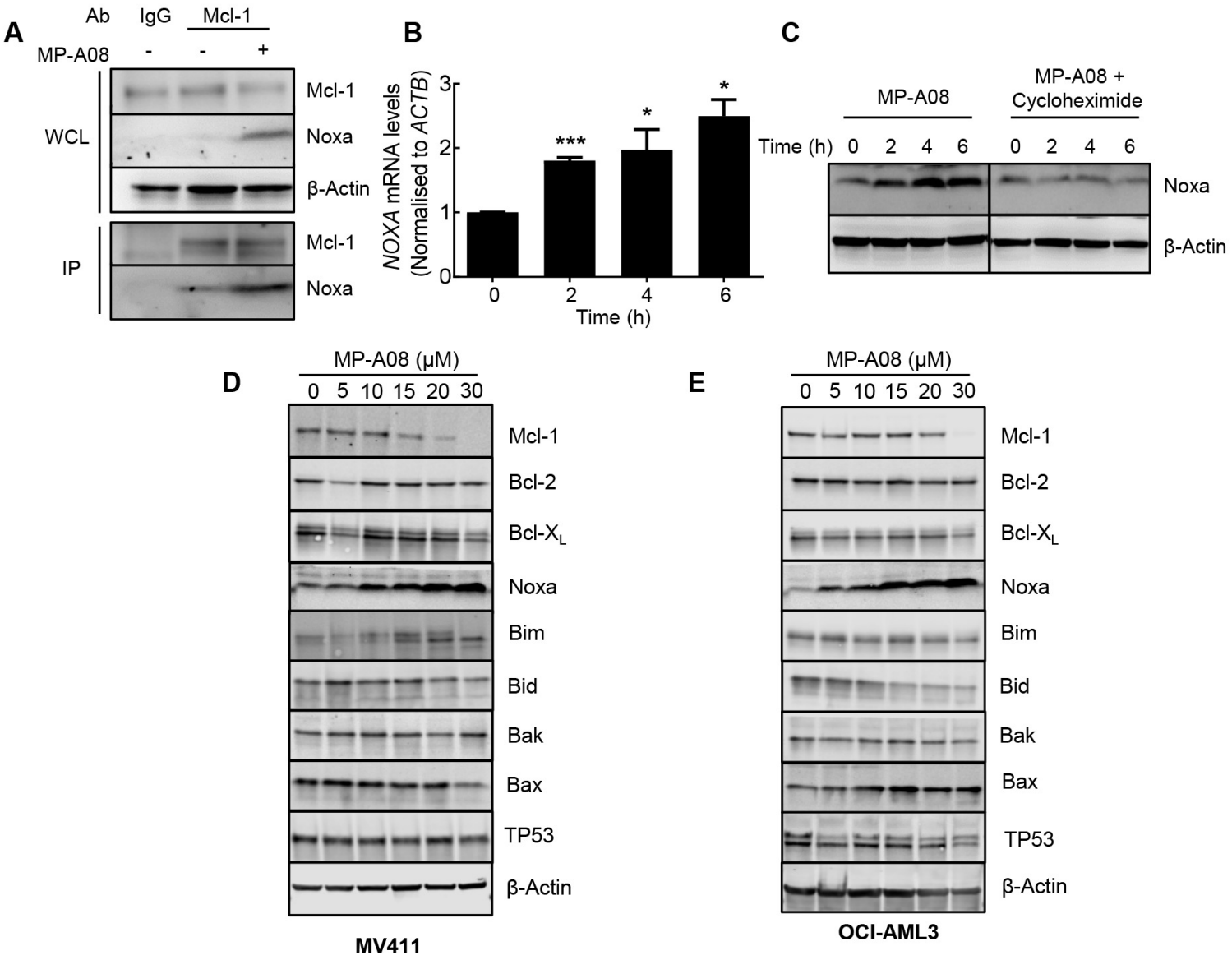
A) Primary AML cells were treated with increasing concentrations of MP-A08 for 6 h, lysed and subject to immunoblot analysis with indicated antibodies. **B,C)** Primary AML samples were treated with MP-A08 and venetoclax for 6 h and assessed for cell viability by Annexin V staining. Data is displayed as duplicate technical replicates mean ± range Statistical significance was assessed by Student's t test. (* $p < 0.05$, *** $p < 0.0005$). Synergy was determined by the CI method. **D)** FACS purified iLSCs were seeded alone or **E)** on a MSC co-culture layer, treated with MP-A08 and venetoclax for 24 h and assessed for cell viability by Annexin V staining. Data is displayed as

duplicate technical replicates mean ± range. Statistical significance was assessed by Student's t test. (* $p < 0.05$). Synergy was determined by the Webb fractional product (FP) method. **F)** Normal bone marrow derived $CD34^+$ cells were treated with MP-A08 and venetoclax for 6 h and assessed for cell viability by Annexin V staining. Data is displayed as duplicate technical replicates mean ± range. **G)** Representative immunohistochemistry staining using human specific mitochondrial antibody (MTC02) of a NSG mouse sternum engrafted with primary AML cells. Scale bar equals 100μM. **H,I)** NSG mice were engrafted with primary AML blasts, and bled weekly to confirm disease engraftment (>1% $hCD45^+$ in peripheral blood). Mice were administered with either vehicle, MP-A08 (i.p. 100 mg/kg), venetoclax (p.o. 75mg/kg) or both daily for two weeks. Engraftment was quantified by assessing the percentage of human $CD45^+$ cells in the bone marrow of recipient mice. Each symbol represents the percentage of $CD45^+$ cells observed in a separate mouse. Significance was assessed by Student's t test. **J)** Mutational analysis of AML patient samples treated with venetoclax from the Beat AML Project.[46] Average area-under-the curve (AUC) is a measure of drug sensitivity (higher the AUC = more resistant) derived from an ex vivo drug sensitivity assays. Statistical significance was assessed by Student's t test with Welch's correction . (** $p < 0.01$, *** $p < 0.0001$). **K)** Primary AML samples identified by whole exome sequencing containing PTPN11, **L)** TP53 or **M)** K-Ras mutations were treated with MP-A08 and venetoclax for 6 h and assessed for cell viability by Annexin V staining. Data is displayed as duplicate technical replicates, mean ± range. Statistical significance was assessed by Student's t test. (** $p < 0.01$).

Figure 7: ***Ceramides induce ISR activation and sensitises cells to Bcl-2 inhibition***

The accumulation of pro-apoptotic sphingolipids such as ceramide in response to SPHK inhibition is sensed by eIF2a kinase PKR. PKR activation culminates in an apoptotic ISR mediated by master transcription factor ATF4. ATF4 promotes Bcl-2 dependency by the transcriptional upregulation of Noxa and the subsequent binding to and inactivation of Mcl-1.

Figure 1: BH3 only proteins Bim and Noxa are essential for MP-A08 induced cell death.



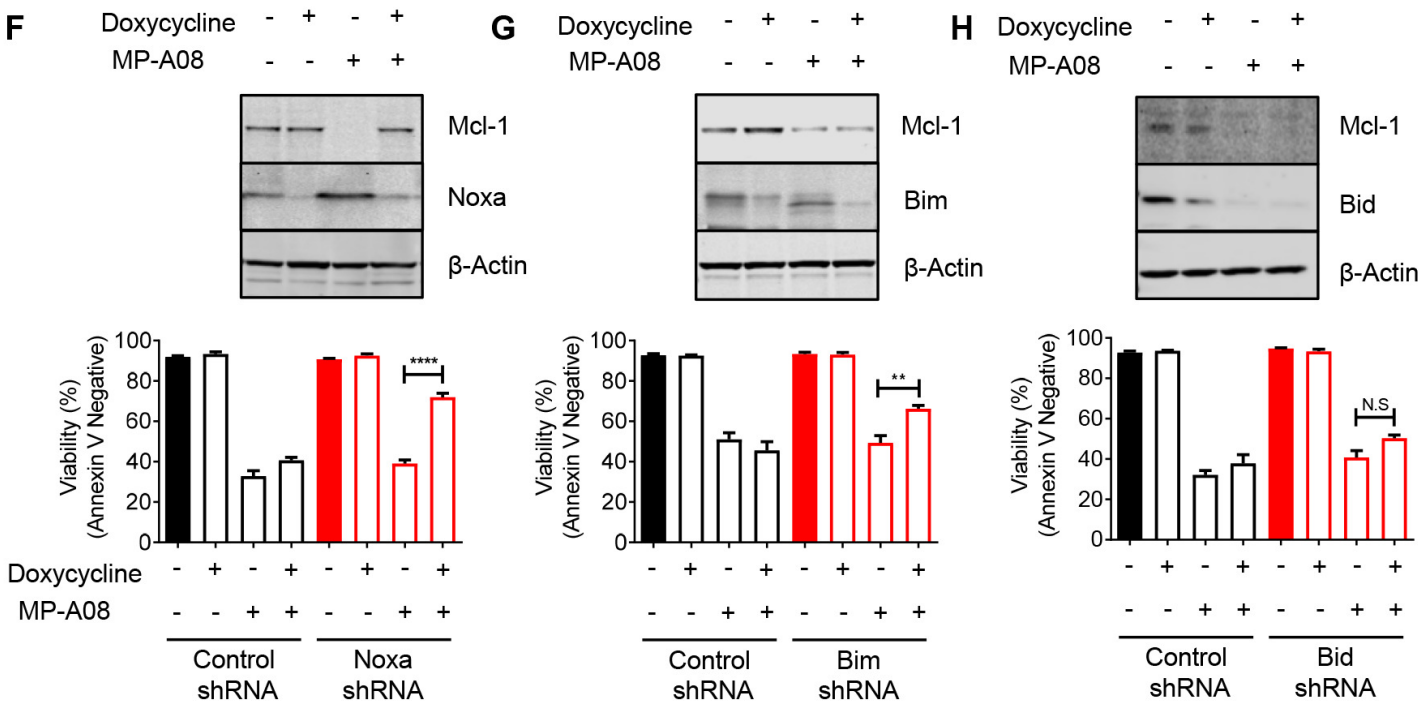


Figure 2: MP-A08 induces ATF4 dependent Noxa transcription

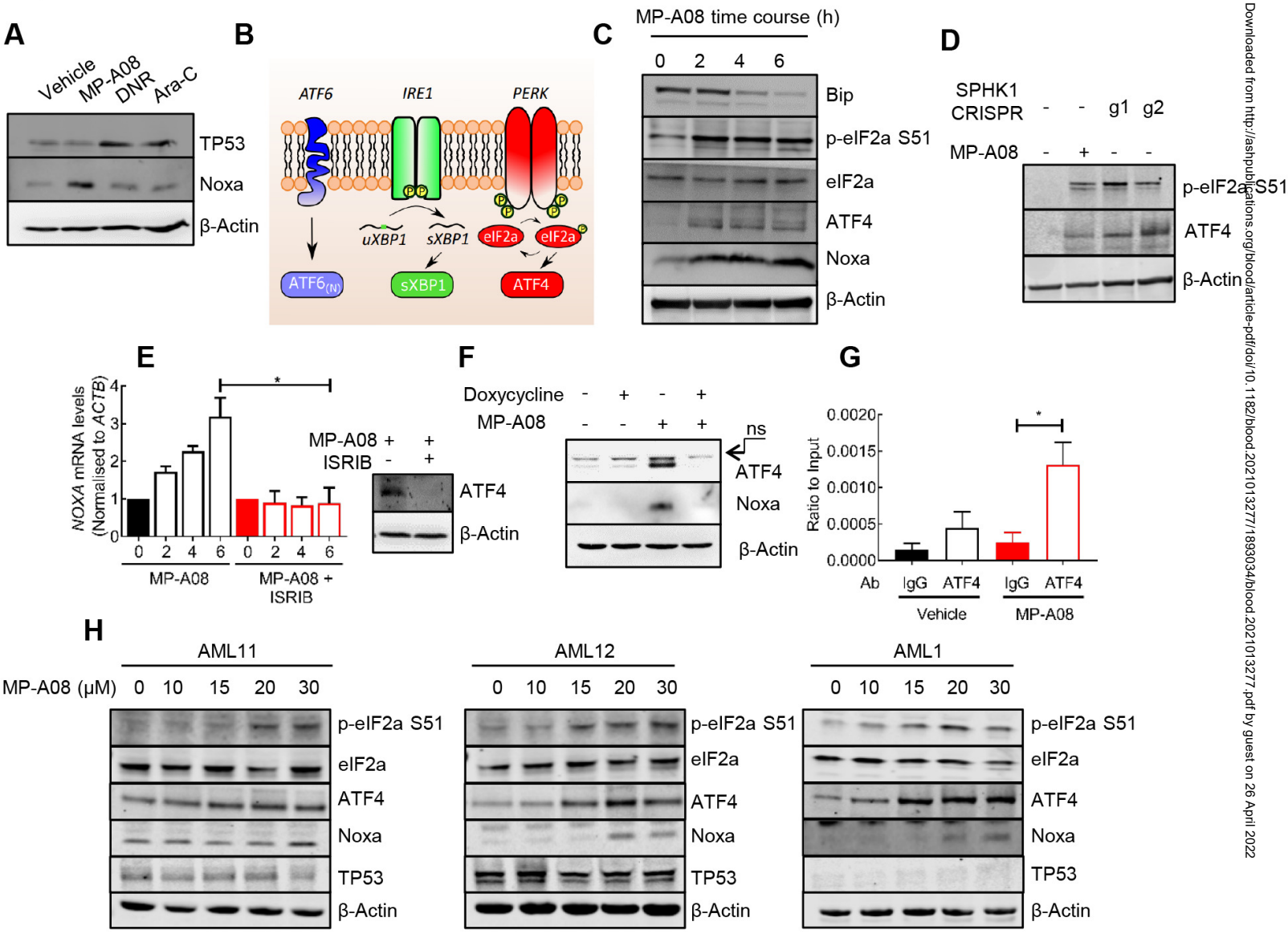


Figure 3: Ceramides drive a PERK-independent ISR

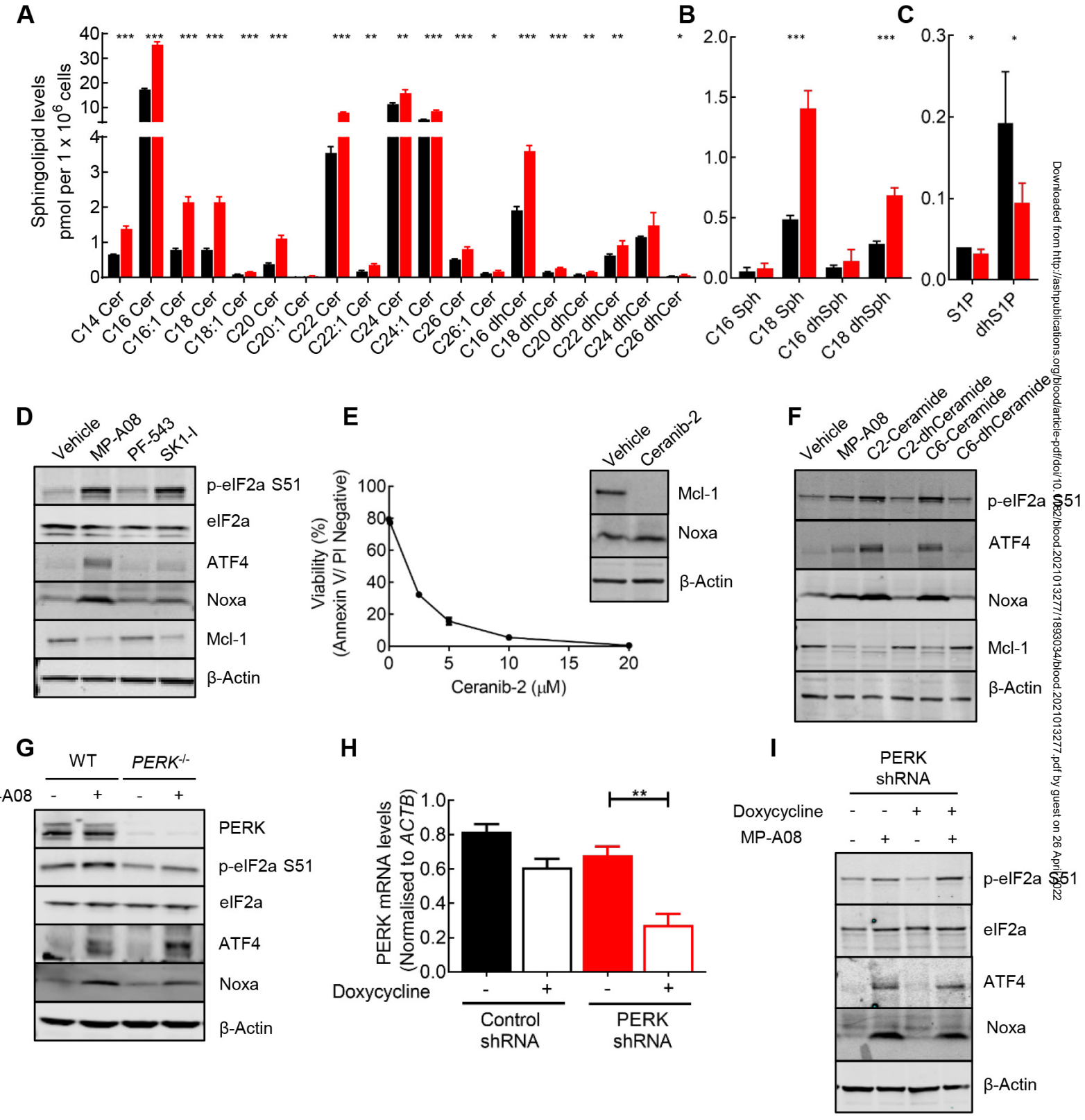


Figure 4: Ceramides drive a PKR-dependant integrated stress response (ISR)

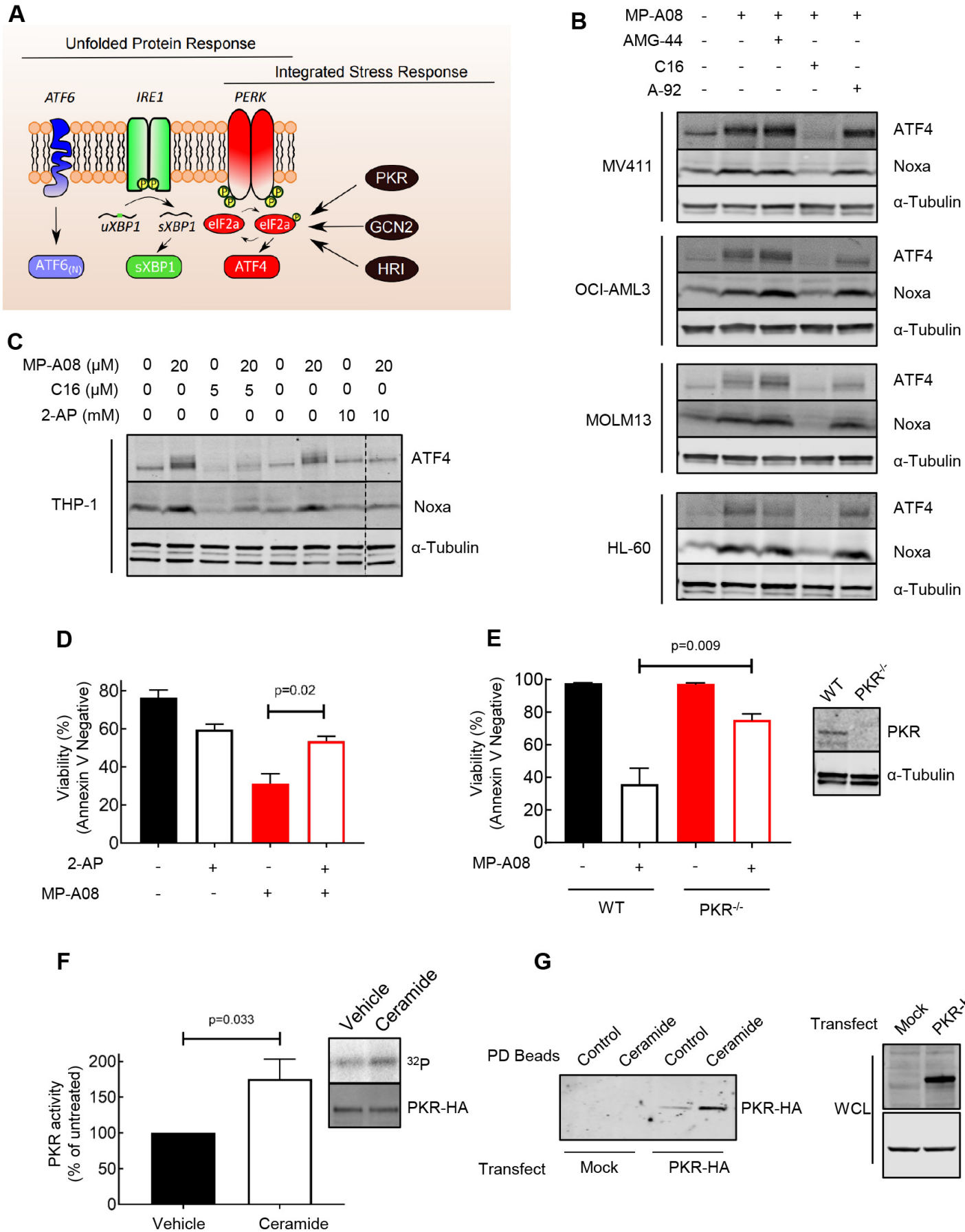


Figure 5: MP-A08 and venetoclax induces potent synergistic activity in AML cell lines

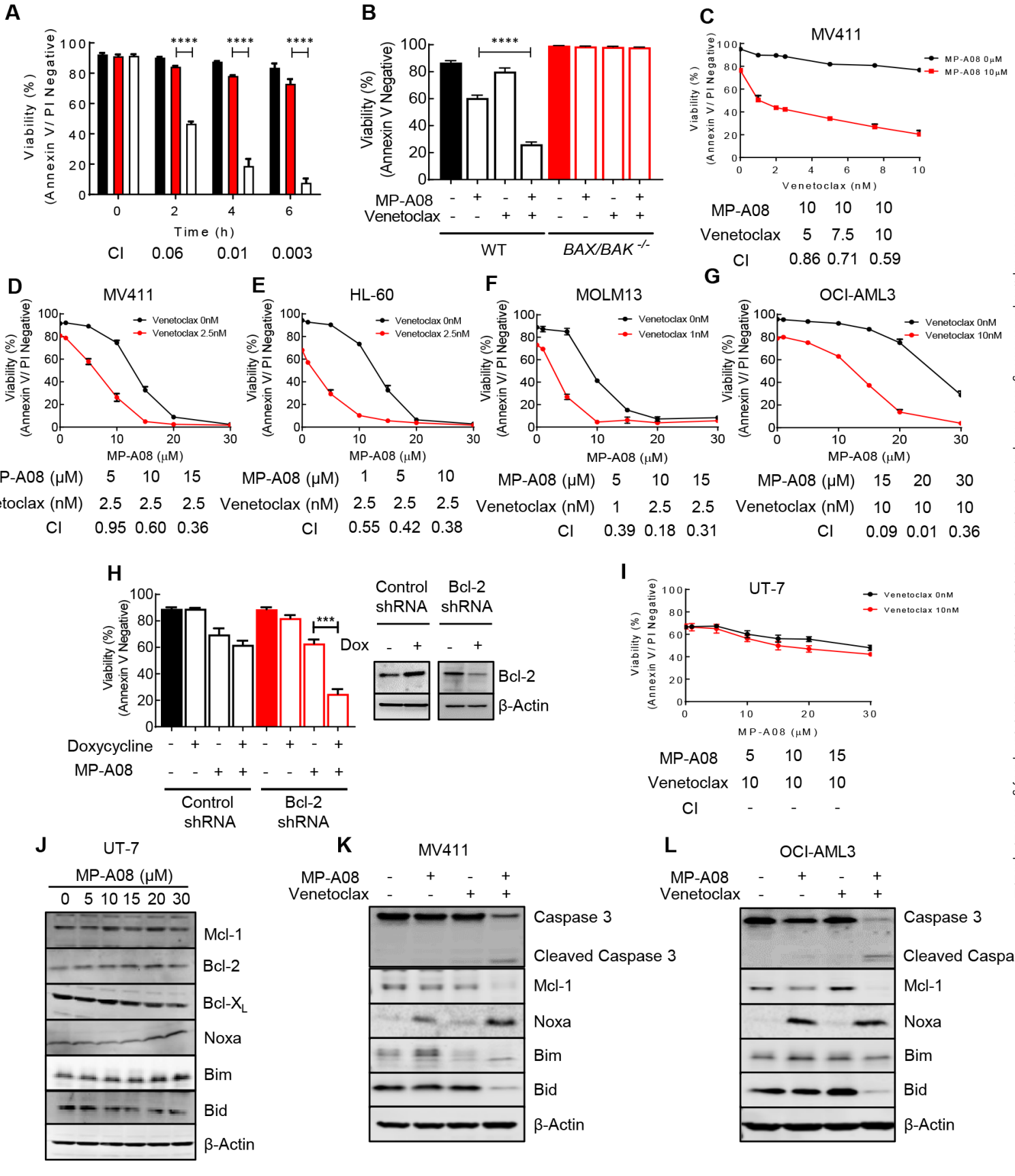


Figure 6: MP-A08 & Venetoclax treatment exhibits anti-leukemic activity in primary AML samples

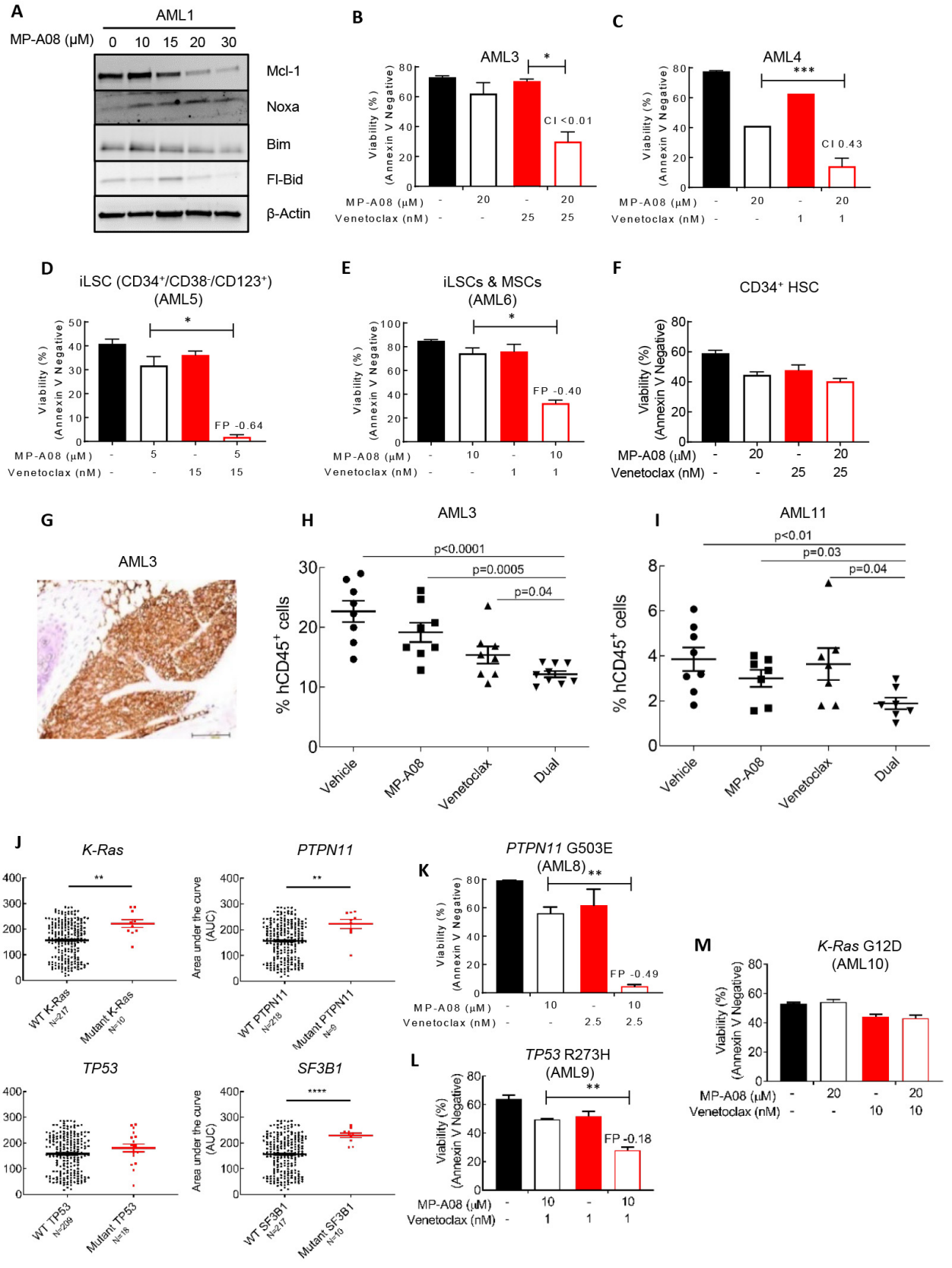


Figure 7: ***Ceramides induce ISR activation and sensitises cells to Bcl-2 inhibition***