Modulation of Navitoclax Sensitivity by Dihydroartemisinin-Mediated MCL-1 Repression in BCR-ABL⁺ B-Lineage Acute Lymphoblastic Leukemia

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Abstract

Purpose: BCR-ABL⁺ B-ALL leukemic cells are highly dependent on the expression of endogenous antiapoptotic MCL-1 to promote viability and are resistant to BH3-mimetic agents such as navitoclax (ABT-263) that target BCL-2, BCL-XL, and BCL-W. However, the survival of most normal blood cells and other cell types is also dependent on Mcl-1. Despite the requirement for MCL-1 in these cell types, initial reports of MCL-1–specific BH3-mimetics have not described any overt toxicities associated with single-agent use, but these agents are still early in clinical development. Therefore, we sought to identify approved drugs that could sensitize leukemic cells to ABT-263.

Experimental Design: A screen identified dihydroartemisinin (DHA), a water-soluble metabolite of the antimalarial artemisinin, as a potent inhibitor of MCL-1 expression by cancer cell lines, and primary patient-derived xenografts, the effect of DHA on survival was tested, and mechanistic studies were carried out to discover how DHA functions. We further tested in vitro and in vivo whether combining DHA with ABT-263 could enhance the response of leukemic cells to combination therapy.

Results: DHA causes the downmodulation of MCL-1 expression by triggering a cellular stress response that represses translation. The repression of MCL-1 renders leukemic cells highly sensitive to synergistic cell death induced by ABT-263 in a mouse model of BCR-ABL⁺ B-ALL both in vitro and in vivo. Furthermore, DHA synergizes with ABT-263 in human Ph⁺ ALL cell lines, and primary patient-derived xenografts of Ph⁺ ALL in culture.

Conclusions: Our findings suggest that combining DHA with ABT-263 can improve therapeutic response in BCR-ABL⁺ B-ALL. Clin Cancer Res 23(24): 7558–68. ©2017 AACR.

Introduction

One in 5 pediatric patients with acute lymphoblastic leukemia (ALL) are diagnosed with poor prognosis disease (including BCR-ABL⁺ ALL; ref. 1). Adults with ALL have even poorer survival rates (<40%) in part due to the dominant role of the BCR-ABL oncogene (2). In Philadelphia chromosome (Ph⁺) leukemia, BCR-ABL fusion proteins encode constitutively active tyrosine kinases [e.g., p185 in B-cell acute lymphoblastic leukemia (B-ALL) and p210 in chronic myelogenous leukemia (CML)] essential for cell transformation (3, 4). CML therapy has been revolutionized by the BCR-ABL tyrosine kinase inhibitors (TKI), which induce and maintain remission without serious side effects (5). In contrast, TKI treatment of Ph⁺ B-ALL results in short remissions and rapid outgrowth of TKI-resistant clones (6, 7). As a result, Ph⁺ ALL patients require intensive chemotherapy and stem cell transplants (1). Thus, new therapies are needed to improve patient outcomes.

Leukemic cells often feature the elevated expression of antiapoptotic molecules that inhibit cell death by preventing BAX and BAK-dependent mitochondrial outer membrane permeabilization and caspase activation. Small-molecule inhibitors, known as BH3-mimetics, including navitoclax (ABT-263) and venetoclax (ABT-199), have been developed to inhibit the antiapoptotic molecules BCL-2, BCL-XL, BCL-W, or only BCL-2, respectively (8, 9). These agents are effective because malignant cells often become addicted to the function of antiapoptotic BCL-2 members to permit their survival, thus making them more sensitive to inhibitors of this pathway than normal cells (10). ABT-199 is approved for treatment of some chronic lymphocytic leukemia with a 17p deletion. In contrast, ABT-263 is still in development with the caveat that it provokes a dose-limiting thrombocytopenia due to the on-target effect of inhibiting BCL-XL in platelets (11). Although these BH3-mimetics show promise in treating malignancies, elevated antiapoptotic MCL-1 expression represents a common resistance mechanism (12–15). MCL-1 levels are commonly elevated in cancer, but potent MCL-1 inhibitors are still in development.
Translational Relevance

The BH3-mimetic agents venetoclax (ABT-199) and ABT-263 are promising drug candidates, particularly for hematologic malignancies that are dependent on the BCL-2 and BCL-X<sub>L</sub> survival genes, respectively. Despite their promise, experimental evidence has indicated that elevated expression of other prosurvival molecules, such as MCL-1, represents a common resistance mechanism to these BH3-mimetics. Furthermore, in MCL-1-dependent malignancies, ABT-199 and ABT-263 resistance remains a significant barrier. We sought to identify agents that could render ABT-263-resistant BCR-ABL<sup>+</sup> cells sensitive to ABT-263. Our efforts identified dihydroartemisinin, an antimalarial drug, as a repressor of MCL-1 protein expression. We demonstrate in mouse and human BCR-ABL<sup>+</sup> B-lineage leukemia that combining DHA treatment with ABT-263 induces significant synergistic responses in cell culture, patient-derived xenografts, and in animal models. These findings suggest that combinatorial therapy of DHA with BH3-mimetic agents can significantly improve leukemic response.

Materials and Methods

Plasmids, expression constructs, and generation of mutants

BCR-ABL<sup>(p185)</sup> was generated from Dr. Witte (University of California, Los Angeles, Los Angeles, CA) and BCR-ABL<sup>T315I</sup> mutant was generated by site-directed mutagenesis. Murine embryonic fibroblasts (MEF) were stably expressed in leukemia cells by retroviral transduction.

Ecotropic retroviral production and cell transduction

Retroviruses were produced as described previously (19). Stable cells were generated by retroviral transduction and drug selection or by FACS.

Cells and cell culture

Genetically engineered mouse (GEM) p185<sup>+</sup> or p185<sup>T315I</sup> Arp<sup>-/-</sup> or Tp53<sup>+/+</sup> hereafter referred to as p53-deficient B-ALL were grown in RPMI with 10% FBS, 55 μmol/L 2-mercaptoethanol, 2 mmol/L glutamine, penicillin, and streptomycin. SV40-transformed wild-type, Pmaip1-deficient and Ddit3-deficient murine embryonic fibroblasts (MEF) were cultured in DMEM with 10% FBS, 55 μmol/L 2-mercaptoethanol, 2 mmol/L glutamine, and gentamycin (20, 21). ABT-263 was provided by AbbVie. DHA was from AvaChem Scientific. Human Ph<sup>+</sup> leukemia cell lines, OP-1, TOM-1, SUP-B15, and BV-173 were grown in RPMI with 20% FBS, 55 μmol/L 2-mercaptoethanol, 2 mmol/L glutamine, penicillin, and streptomycin.

Immunoblotting and antibodies

Protein expression was assessed as described previously (19). Antibodies used were: anti-MCL-1 (Rockland Immunochemical), anti-human MCL-1, anti-PARP, anti-CHOP, and anti-BCL-X<sub>L</sub>. (Cell Signaling Technology), anti–BCL-2 (BD Biosciences), anti-NOXA and anti-PUMA (Sigma Aldrich), and anti-actin (Millipore). Anti-rabbit or anti-mouse horseradish peroxidase–conjugated secondary antibodies were from Jackson Immunochemical. Immunoblots were acquired on a Li-COR Odyssey (Li-COR) and densitometry assessed for all blots in replicate for statistical analysis (Supplementary Fig. S7).

Microarray analysis and GSEA

Affymetrix Mouse Gene 2.0 ST arrays were used to assay RNA samples. Data were submitted to NIH GEO accession number: GSE95809. The 4-hour DHA-treated samples were statistically tested by unequal variance t test to the DMSO control (3 replicates), and the volcano plot was generated by converting the resultant p value to a score (−log<sub>10</sub>(P value)) using Spotfire (TIBCO). Gene set enrichment analysis (GSEA; version 2.2.3) was performed using 4-hour DHA samples versus DMSO control samples and the c5.all.v5.2.symbol.gmt Gene Ontology gene sets. Gene set permutation was performed 1,000 times using the weighted enrichment statistic and genes ranked by signal to noise metric.

Cell death experiments

Cells (6 × 10<sup>4</sup>) were seeded in 96-well plates and DHA and ABT-263 (solubilized in DMSO or DMSO vehicle controls) added at indicated concentrations. After 24 hours, apoptotic cells were determined by staining with Annexin-V-APC and propidium iodide (BD Biosciences) and measured by flow cytometry.

Real-time PCR

RNA extracted using Ambion RNA Extraction Kit (Life Technologies) was reverse transcribed with SuperScript III (Life Technologies). Real-time PCR was performed using primers and Fast SYBR Green (Thermo Fisher Scientific). Data were analyzed by the ΔΔC<sub>T</sub> method in a Quantstudio 7 Flex real-time PCR machine (Thermo Fisher Scientific) with housekeeping gene (ubiquitin) and compared with unstimulated cells. Primer sequences are available by request.

Response surface modeling

Response surface modeling, implemented in MATLAB version R2016a (MathWorks), was used to determine changes in the response on viable cells (Annexin-V<sup>-</sup> and PI<sup>-</sup>) of two drugs given alone and in combination (22–24). A drug combination was considered either synergistic or antagonistic if the interaction term (c) describing the change in response relative to the additive model was either positive or negative, respectively.
CRISPR/Cas9 mutagenesis of mouse BCR-ABL+ B-ALL cells

Ddis+/– murine BCR-ABL+ B-ALL cells were generated using CRISPR-Cas9 technology. Briefly, 4 × 10⁶ B-ALL cells were transiently cotransfected with 1 μg of gRNA (5′ GACACCGTCTC-CAAGGTGAA 3′) and 1 μg Cas9 expression plasmid via nucleofection (Lonza, 4D-Nucleofector X-unit) using solution SF, program CA-137 in small cuvettes according to the manufacturer-recommended protocol. Cells were single-cell sorted by flow cytometry, clonally selected, and verified for disruption of the endogenous locus via targeted deep sequencing to identify frameshift mutations.

Generation of patient-derived xenograft mice

Leukemia from adult patients with BCR-ABL+ ALL obtained from the Eastern Cooperative Oncology Group E2993 study (ClinicalTrials.gov identifier NCT00002514) and from the University Health Network was transplanted into unirradiated immunodeficient NOD.Cg-Pkdcsid1H2gpmo1NSjSgy (NSG) mice (Jackson Laboratories) for 8 to 10 weeks prior to reisolation (25–27). Mice were bled and utilized in accordance with St. Jude Children’s Research Hospital Animal Care and Use Committee (SJCRHACUC).

Treatment of murine leukemia in recipient mice

Mouse BCR-ABL+ Arf−/− Luciferase+ B-ALL cells were injected (2 × 10⁶) into nonconditioned, 6- to 8-week-old, female C57BL/6 recipients (The Jackson Laboratory). Five days after the transfer, recipients were treated with DHA and ABT-263 by oral gavage. ABT-263 was formulated in a mixture of 60% Phosal 50 PG, 30% PEG 400, and 10% EtOH and dosed at 100 mg/kg/day as described previously (28). DHA was formulated in 0.5% carboxymethylcellulose, 0.5% Tween-80, and 0.5% benzyl alcohol and dosed at 200 mg/kg/day. Treatment was given daily for 15 days (days 5–20) during and after which the mice were monitored.

Bioluminescence imaging was assessed by Xenogen IVIS (PerkinElmer) after mice were injected with o-luciferin (PerkinElmer) at 150 mg/kg. Images (photons/second) were quantified through application of a contour drawn around the target region and normalized to maximum luminescence activity. For ex vivo analysis of MCL-1 expression, recipient mice 10 days after transplant of 2 × 10⁶ mouse BCR-ABL+ B-ALL cells were treated with vehicle or DHA (200 mg/kg) by gavage. Four or 8 hours after treatment, splenic blast cells were isolated and subjected to immunoblotting.

Results

DHA induces apoptosis in BCR-ABL+ B-ALL cells

Using a GEM model for BCR-ABL+ B+, we previously demonstrated that endogenous MCL-1 is required to maintain leukemic cell survival (15). This is a powerful model to interrogate the biology of human, poor-prognosis BCR-ABL+ B-ALL (15, 29). Although MCL-1 is clearly an important therapeutic target, potent and selective MCL-1 inhibitors are still in development and have only recently entered human phase 1 trials (17). Therefore, we sought to identify alternative strategies by which MCL-1 function or expression can be attenuated to render BCR-ABL+ B-ALL cells susceptible to apoptosis induced by currently available BH3-mimetic small molecules.

A library of approved drugs was screened to identify compounds that killed mouse BCR-ABL+ B-ALL leukemic cells (30). This screen identified members of the artesminin class of antimalarial agents including DHA, a widely used, orally delivered drug for malaria with favorable pharmacokinetics and bioavailability in humans (31). DHA possesses anticancer properties; however, the mechanism(s) by which DHA functions to kill cancer cells is unclear (32–36). Treatment of mouse BCR-ABL+ B-ALL cells with DHA induced apoptosis (Fig. 1A; Supplementary Fig. S1A). Consistent with the induction of apoptosis, the leukemic cells responded to DHA treatment by cleaving PARP (Fig. 1B; Supplementary Fig. S1B). Caspase inhibitors (e.g., Q-VD) or treatment of Bax and Bak-deficient (hereafter DKO) BCR-ABL+ B-ALL mouse cells with DHA repressed the induction of leukemic apoptosis and cleavage of PARP (Fig. 1A and C; Supplementary Fig. S1C).

DHA represses MCL-1 expression in murine BCR-ABL+ B-ALL cells

Treatment of mouse BCR-ABL+ B-ALL cells with DHA, at significantly lower doses than those required for cytotoxicity, produced a loss of MCL-1 expression, but the expression levels of BCL-XL, BCL-2 were only marginally affected (Fig. 1B; Supplementary Fig. S1B). The loss of MCL-1 expression was still observed in DHA-treated cultures when cell death was blocked by caspase inhibitors (Q-VD) or in mouse DKO BCR-ABL+ B-ALL cells (Fig. 1C; Supplementary Fig. S1C). Therefore, the decline of MCL-1 expression is independent of caspase activation and BAX/BAK-dependent mitochondrial permeabilization. Diminished MCL-1 expression was detectable as early as 8 hours after DHA treatment, preceding evidence of apoptosis (Supplementary Fig. S1B).

Although DHA treatment may affect a variety of cellular pathways at high concentration to induce single-agent killing, overexpression of antiapoptotic MCL-1 rendered mouse BCR-ABL+ B-ALL cells more resistant to DHA treatment as expected (Supplementary Fig. S1D and S1E).

DHA results in posttranscriptional repression of MCL-1 expression

MCL-1 is a labile protein regulated at many levels including transcription, translation, and protein degradation by the proteasome (37). To determine mechanistically how MCL-1 expression is repressed by DHA, RNA expression of antiapoptotic BCL-2 family members was assessed by quantitative PCR in mouse BCR-ABL+ B-ALL cells treated with DHA in vitro. In response to DHA treatment, prosurvival BCL-2 family member expression was either unchanged or induced and notably MCL-1 mRNA expression was unaffected (Fig. 1D). When protein translation was assayed by pulsing mouse BCR-ABL+ B-ALL cells with 35S-containing media, the treatment of DHA substantially repressed new protein synthesis of many cellular proteins, including MCL-1 (Supplementary Fig. S2A). MCL-1 is a well-recognized labile protein and cellular signaling has been reported to modulate its turnover by the proteasome (19, 38). However, when mouse BCR-ABL+ B-ALL cells were pretreated with DHA and then pulsed with cycloheximide to attenuate new MCL-1 protein synthesis, MCL-1 protein was eliminated at the same rate whether cultured with DHA or DMSO (Supplementary Fig. S2B). Unsurprisingly, the loss of MCL-1 protein expression triggered by DHA treatment could be rescued by treatment with the proteasome inhibitor bortezomib (Supplementary Fig. S2C). These data indicate that DHA treatment does not attenuate MCL-1’s transcription, but rather inhibits protein translation leading to
MCL-1’s elimination by the proteasome in mouse BCR-ABL+ B-ALL cells.

DHA induces a cellular stress program that represses MCL-1 expression

DHA has been proposed to induce killing of cancer cells through the induction of reactive oxygen species (ROS; refs. 33, 34). Indeed, DHA treatment does induce ROS generation; however, multiple ROS scavengers that repressed the induced ROS were unable to block the attenuation of MCL-1 expression triggered by DHA (Supplementary Fig. S2D and S2E). Therefore, we focused on identifying other mechanisms by which DHA represses MCL-1 expression. To decipher the cellular response to DHA, microarray analysis was performed to identify pathways of gene expression triggered by DHA. When compared with control treated mouse BCR-ABL+ B-ALL cells, cells treated with DHA for 4 hours revealed 18 genes that were significantly induced by DHA treatment (top induced genes were Ddit3, Chac1, Trib3, Atf3, Sess2, Egr1, and Nupr1; Supplementary Fig. S3A). GSEA indicated that the gene set induced by DHA repression correlates significantly with known endoplasmic reticulum (ER) stress pathway signatures (Fig. 2A). The induction of these genes was confirmed by quantitative PCR and immunoblot analysis for CHOP, which is encoded by the Ddit3 gene (Supplementary Fig. S3B; Fig. 2B).

To mechanistically address how MCL-1 expression is attenuated by DHA treatment, we established that DHA could repress MCL-1 expression in MEF cell lines without inducing apoptosis (Fig. 2C). As in BCR-ABL+ B-ALL cells, MEF lines treated with DHA also induced the expression of CHOP, a key regulator of the ER stress pathway, as well as proapoptotic NOXA (Fig. 2C). Importantly, NOXA has been previously implicated in repressing MCL-1 protein expression by promoting its proteasome-mediated degradation, making it an attractive candidate (39, 40).

Despite the induction of NOXA in wild-type MEFs, we still observed NOXA in wild-type MEFs, we still were unable to block the attenuation of MCL-1 expression triggered by DHA (Supplementary Fig. S2D and S2E). Furthermore, the induction of the BH3-only gene Pmaip1, which encodes the proapoptotic protein NOXA, was detected in BCR-ABL+ B-ALL cells in response to DHA treatment (Supplementary Fig. S2; Fig. 2C). Importantly, NOXA has been previously implicated in repressing MCL-1 protein expression by promoting its proteasome-mediated degradation, making it an attractive candidate (39, 40).

Despite the induction of NOXA in wild-type MEFs, we still...
Figure 2.
DHA treatment induces an ER stress response that represses MCL-1 expression. A, GSEA (version 2.2.3) was performed using microarray data from mouse BCR-ABL 
+ B-ALL cells treated for 4 hours with vehicle (control) versus 312 nmol/L DHA and the c5.all.v5.2 symbol.gmt Gene Ontology gene sets. B, Mouse BCR-ABL 
+ B-ALL cells were treated with 1,250 nmol/L DHA or 5 nmol/L thapsigargin (Thapsi, positive control for ER stress). Cell lysates were analyzed by immunoblot to detect indicated proteins. This experiment is representative of three independently performed assays. C, SV40-transformed wild-type MEFs were treated with 10 ng/mL DHA and 5 nmol/L thapsigargin (Thapsi). This experiment is representative of three independently performed assays. D, Mouse BCR-ABL 
+ B-ALL cells were treated with 10 ng/mL DHA or 20 nmol/L thapsigargin. After 24 hours, the MEFs were harvested and cell lysates analyzed by immunoblot analysis. This experiment is representative of three independently performed assays. E, SV40-transformed wild-type MEFs were treated with 10 ng/mL DHA and 5 nmol/L thapsigargin (Thapsi). This experiment is representative of three independently performed assays.
observed the repression of MCL-1 expression when Pmapi-1-deficient MEF lines were treated with DHA, suggesting that NOXA induction is not required to repress MCL-1 expression (Supplementary Fig. S3C). In support of this conclusion, when BCR-ABL+S53-deficient B-ALL cells are treated with DHA, neither NOXA nor PUMA BH3-only proteins are induced (Supplementary Fig. S3D). Although the BCR-ABL−p53-deficient B-ALL cells exhibit substantial resistance to cell death induced by DHA, MCL-1 expression is still diminished by DHA treatment (Supplementary Fig. S5D and S5E). These data suggest that although NOXA and PUMA are clearly important in the induction of apoptosis in response to DHA, they are not essential for the repression of MCL-1 expression. In contrast, Ddit3-deficient MEFs and BCR-ABL− B-ALL cells, which lack CHOP protein, exhibit much less repression of MCL-1 protein in response to DHA treatment (Fig. 2D and E), suggesting that CHOP induction contributes to repression of MCL-1 by DHA. Therefore, in both MEF lines and BCR-ABL− B-ALL cells, a CHOP-dependent cellular stress pathway is triggered by DHA treatment and leads to MCL-1 repression.

DHA synergizes with ABT-263 in killing murine BCR-ABL+ B-ALL cells

Resistance to TKI is a significant barrier to treatment of both Ph+ CML and Ph+ ALL (6, 7, 41, 42). Therefore, TKI-resistant mouse leukemic cell lines were generated using a common "gatekeeper" mutation (BCR-ABL+T315I) that is observed in TKI-resistant, adult Ph+ B-ALL patients. As expected, the BCR-ABL+T315I B-ALL cells are resistant to TKI treatment (Supplementary Fig. S4A). In contrast, treatment with DHA triggered apoptosis in both wild-type BCR-ABL+ and BCR-ABL+T315I B-ALL cells to a similar extent at high concentrations (Supplementary Fig. S4B). At lower concentrations of DHA, the expression of MCL-1 was similarly repressed in both wild-type BCR-ABL+ and BCR-ABL+T315I B-ALL cells (Supplementary Fig. S4C). These data indicate that DHA can repress MCL-1 expression in both BCR-ABL+ and TKI-resistant BCR-ABL+T315I B-ALL cells, unlike first-generation TKIs.

Mouse BCR-ABL+ B-ALL cells depend upon the antiapoptotic activity of MCL-1 to mediate their survival and, accordingly, are relatively insensitive to BH3-mimetic small molecules, such as ABT-263 (15, 43). Therefore, we posited that the DHA-mediated MCL-1 repression would lead to increased response of the mouse BCR-ABL+ B-ALL cells to ABT-263. To test this hypothesis, both BCR-ABL+ and BCR-ABL+T315I B-ALL cells were treated in vitro with suboptimal doses of DHA and/or ABT-263 after which cell killing was assessed. Although single agents induced modest cell death, when DHA treatment was combined with ABT-263, significantly more cell death was observed in both BCR-ABL+ and BCR-ABL+T315I B-ALL cells (Fig. 3A and B, respectively). Response surface modeling indicated synergy for both BCR-ABL+ and BCR-ABL+T315I B-ALL cells when DHA and ABT-263 are combined (Fig. 3C and D, respectively). These data support the hypothesis that DHA can repress MCL-1 expression to sensitize BCR-ABL+ B-ALL cells to BH3-mimetic drugs.

DHA represses MCL-1 expression in human leukemia cells

To establish whether we could potentiate the response of human Ph+ leukemia cell lines to ABT-263 treatment by cotreating with DHA, we assessed the response of TOM-1 (Ph+ B-ALL), SUP-B15 (Ph+ B-ALL), and OP-1 (Ph+ CML blast crisis cell line), and OP-1 (Ph+ B-ALL) cells to treatment with DHA. Similar to our observations in murine BCR-ABL+ B-ALL cells, the treatment of these human Ph+ leukemia cell lines with DHA repressed MCL-1 expression, but did not affect the expression levels of BCL-X, or BCL-2 (Fig. 4A; Supplementary Fig. S5A–S5C). In these human Ph+ cell lines, the repression of MCL-1 occurred even when cell death was inhibited by caspase inhibitors.
However, two Ph− sensitive to apoptosis induced by ABT-263 treatment alone; apoptotic cells (Annexin-V+) were assessed. Each experiment was performed from at least 3 separate recipient mice, and each assay was carried out in triplicate. The average is plotted with SEM. Following 24 hours of incubation, apoptotic cells were treated with indicated doses of DHA. After 24 hours, the cells were lysed and immunoblotted for expression of indicated proteins.

As DHA repressed MCL-1 protein levels in human Ph− leukemia cell lines, we tested whether we could potentiate the apoptosis induced by ABT-263 by combining with DHA in culture. Although both PDX samples were sensitive to single-agent ABT-263, they were quite resistant to cell death induced by DHA alone. However, the killing by low-dose ABT-263 synergized with DHA in both PDX models (Fig. 4C and D). Response surface modeling indicated synergy of both primary patient xenografts PDX10940 (α = 118; P = 2.64 × 10−13) and PDX10380 (α = 13667; P = 2.67 × 10−15) by response surface modeling.

DHA synergizes with ABT-263 to repress mouse BCR-ABL+ B-ALL leukemic progression in vivo

The ability of DHA to sensitize cultured BCR-ABL+ B-ALL cells to apoptosis induced by ABT-263 suggests that combining the two

Figure 4.
Synergistic effect of Ph− human cell lines and primary patient samples to combined treatment with DHA and ABT-263. A, Human Ph− ALL cell line TOM-1 was treated with indicated doses of DHA. After 24 hours, the cells were lysed and immunoblotted for expression of indicated proteins. B, TOM-1 human Ph− cells were cultured with ABT-263 (0, 20, or 40 nmol/L concentrations) and combined with DHA at indicated doses. Following 24 hours of incubation, apoptotic cells (Annexin-V+) were assessed by flow cytometry. Each experiment was performed 3 times in triplicate and the average plotted with SEM. One-way ANOVA with Bonferroni multiple comparison indicates significance P < 0.001*** between the DHA alone (0 nmol/L ABT-263) and both combined treated arms (20 and 40 nmol/L) at indicated doses. Human primary patient xenograft (PDX) Ph− B-ALL cells from PDX10940 (C) and PDX10380 (D) were cultured with ABT-263 (0, 10, or 40 nmol/L concentrations) and combined with DHA at indicated doses in culture. Following 24 hours of incubation, apoptotic cells were assessed. Each experiment was performed from at least 3 separate recipient mice, and each assay was carried out in triplicate. The average is plotted with SEM. One-way ANOVA with Bonferroni multiple comparison indicates significance P < 0.001*** between the DHA alone (0 nmol/L ABT-263) and both combined treated arms (10 and 40 nmol/L) at indicated doses. The combination of DHA and ABT-263 showed a synergistic interaction in TOM-1 Ph− cells (B, α = 9.43; P = 0.004) primary patient xenograft PDX10940 (C, α = 118; P = 2.64 × 10−13) and PDX10380 (D, α = 13667; P = 2.67 × 10−15) by response surface modeling.

As DHA repressed MCL-1 protein levels in human Ph− leukemia cell lines, we tested whether we could potentiate the apoptosis induced by ABT-263 by combining with DHA in culture. Although DHA alone did not provoke substantial apoptosis in these human cell lines, when DHA was combined with ABT-263, significant synergy was observed by response surface modeling (TOM-1: α = 9.43; P = 0.004; BV-173: α = 75; P = 7.11 × 10−4; OP-1: α = 902; P = 2.82 × 10−13; and SUP-B15: α = 23602; P = 1.91 × 10−4; Fig. 4B; Supplementary Fig. SSD–SSF, respectively). These data demonstrate that, in all of the human Ph− leukemia cell lines tested, apoptosis is potentiated when ABT-263 is combined with DHA.

To extend these studies beyond established human Ph− leukemia cell lines, we took advantage of primary patient-derived xenografts (PDX) that were established by direct injection of patient leukemia into immunodeficient (NSG) recipients. Of the 5 Ph− B-ALL PDXs leukemia assessed, most were extremely sensitive to apoptosis induced by ABT-263 treatment alone; however, two Ph− leukemia (PDX 10940 and 10380) were further tested for in vitro synergy. Leukemic cells were isolated from the recipient mice and subjected to treatment with DHA and/or ABT-263 in culture. Although both PDX samples were sensitive to single-agent ABT-263, they were quite resistant to cell death induced by DHA alone. However, the killing by low-dose ABT-263 synergized with DHA in both PDX models (Fig. 4C and D). Response surface modeling indicated synergy of both primary patient xenografts PDX10940 (α = 118; P = 2.64 × 10−13) and PDX10380 (α = 13667; P = 2.67 × 10−15). These data indicate that combining DHA with ABT-263 can potentiate the response of primary patient leukemia when treated in culture. Future studies will be necessary to determine whether the combination therapy can be similarly efficacious when delivered in vivo to NSG mice bearing luciferase-labeled human PDX Ph− leukemia. DHA synergizes with ABT-263 to repress mouse BCR-ABL+ B-ALL leukemic progression in vivo

The ability of DHA to sensitize cultured BCR-ABL+ B-ALL cells to apoptosis induced by ABT-263 suggests that combining the two
agents together would lead to improved responses of BCR-ABL

B-ALL in animals. To test this concept, murine luciferase-expressing BCR-ABL

B-ALL cells were transplanted into nonconditioned C57BL/6 recipient mice. Recipient mice received either vehicle controls, DHA (200 mg/kg), ABT-263 (100 mg/kg), or DHA (200 mg/kg) and ABT-263 (100 mg/kg) daily from day 5 after transplantation to day 20 by oral gavage. Mice receiving either vehicle or ABT-263 alone rapidly succumbed to a fatal leukemia, indicating no survival extension from mice treated daily with the BH3-mimetic agent (Fig. 5A). Treatment with DHA alone marginally extended survival, indicating a small, but significant, effect. In contrast, combining DHA and ABT-263 together for 15 days extended survival when compared with the other treatment groups (Fig. 5A). Similar to overall survival data, mice receiving the combination treatment had reduced bioluminescence when compared with the mice treated with single agents or control (Fig. 5B; Supplementary Fig. S6A). Complete blood counts from mice also showed a repression in circulating leukemic cells in combination-treated animals when compared with mice only receiving single agents (Supplementary Fig. S6B). Ex vivo analysis of mouse BCR-ABL

B-ALL cells isolated from syngeneic recipient mice after in vivo DHA treatment revealed that MCL-1 protein expression was decreased when compared with vehicle-treated animals at 8, but not 4, hours after DHA treatment (Fig. 5C). It remains to be established whether human leukemia cells will similarly repress MCL-1 expression when treated in vivo. The mice receiving combined treatment with both DHA and ABT-263 harvested on day 13 did not exhibit any overt toxicities (data not shown). The leukemia resulting from the transplanted cells was highly aggressive, replacing normal hematopoiesis in the bone marrow. Furthermore, the leukemic cells invaded the central nervous system and lymphatics and often resulted in hind-limb paralysis (Supplementary Fig. S6C). The resultant leukemic cells expressed B220

cell surface marker and were Pax5

positive (Supplementary Fig. S6D). Despite the extension of survival over the 15 days of treatment, all mice, including those in the combination-treated group, eventually succumbed to leukemia (Fig. 5A). To assess whether the leukemia from the combination-treated mice became resistant, leukemic cells were isolated from the bone marrow of moribund mice (sacrificed on day 42) that received combined DHA and ABT-263 in vivo. The ex vivo leukemia cells were treated in

Figure 5.

Potentiated response of BCR-ABL

B-ALL cells treated in vivo with DHA and ABT-263. Mouse BCR-ABL

B-ALL cells were adoptively transferred into syngeneic, nonirradiated C57BL/6 recipients. Five days after the transfer, the recipients were divided into four treatment arms: vehicle controls, ABT-263 alone, DHA alone, and ABT-263 and DHA. Mice received the treatment daily (indicated by "Treatment") for 15 days. A, Kaplan-Meier survival curve of the cohorts of mice (n = 10/group). Log-rank test shows P < 0.001 for combined treatment group. B, Quantification of bioluminescence measurements for the indicated mouse cohorts from analysis on day 13 after leukemia transplant. Each symbol represents one animal, the lines indicate the average radiance, and the error bars are the SEM. One-way ANOVA followed by t test with post hoc correction for multiple comparisons indicate significance between indicated groups. P < 0.05 and P < 0.01. C, Splenic blast cells harvested from mouse BCR-ABL

B-ALL recipient mice, 4 or 8 hours after treatment with vehicle or DHA (200 mg/kg), were lysed and immunoblotted for indicated proteins. Significant repression was observed only at 8-hour time point. Each lane represents a recipient mouse. D, Mouse BCR-ABL

B-ALL cells from moribund recipients that received the combined treatment with ABT-263 and DHA (on day 42) were recultured with ABT-263 (0, 39, or 78 nmol/L concentrations) and then combined with DHA at indicated doses in culture. Following 24 hours of incubation, cell viability was assessed. Each experiment was performed 3 times in triplicate, and the average apoptotic cells (Annexin-V

positive) are plotted with SEM. Two-way ANOVA indicates significance P < 0.001 between the DHA alone (0 nmol/L ABT-263) and both combined treated arms at all doses. The combination of DHA and ABT-263 still showed a synergistic interaction in ex vivo mouse BCR-ABL

B-ALL cells (α = 1.87; P = 3.24 × 10^{-5}) by response surface modeling.
We have identified that the antimalarial drug DHA can repress MCL-1 protein expression in both mouse and human BCR-ABL\(^+\) B-ALL cells. Although treatment with DHA alone can trigger mouse BCR-ABL\(^+\) B-ALL killing by a variety of mechanisms, including induction of NOXA and PUMA, DHA treatment represses MCL-1 protein expression by triggering a cellular stress response that blocks protein translation. When MCL-1 is repressed in mouse and human BCR-ABL\(^+\) B-ALL cells by DHA treatment, they are significantly more sensitive to ABT-263-induced cell death. Importantly, sensitization was observed not only in wild-type BCR-ABL\(^+\) B-ALL cells, but also in TKI-resistant BCR-ABL\(^{T315I}\) mouse leukemia, indicating that DHA can repress MCL-1 in a mechanism distinct from the action of TKIs. These data suggest that combining DHA and ABT-263 together may lead to improved therapeutic responses of poor prognosis BCR-ABL\(^+\) B-ALL.

MCL-1 is a prosurvival molecule that is frequently amplified in human cancer (16). Furthermore, elevated MCL-1 expression in malignant cells is a chief mediator of resistance to both ABT-263 and the currently FDA-approved ABT-199 (12–14). As a result, laboratories in industry and academia have expended significant efforts to identify and develop specific and potent MCL-1 inhibitors (18). Many of these inhibitors suffer from a lack of specificity and potency for MCL-1 inhibition (44). Recently, the first highly potent MCL-1 inhibitor, S63845, was reported to be well tolerated in mice and efficacious in a model of mouse and human cancer (17). The lack of toxicity associated with S63845 treatment stands in stark contrast to mouse Mcl-1 deletion experiments, indicating that transient inhibition of MCL-1 may be better tolerated than complete and permanent genetic deletion. Although promising, specific MCL-1 inhibitors like S63845 will require substantial development and testing before impacting human disease. Furthermore, it is unclear whether MCL-1 inhibition will exacerbate toxicities to normal cell types, especially when delivered with conventional chemotherapy in humans. Therefore, we focused on identifying available drugs that can attenuate MCL-1 expression and enhance the sensitivity of cancer cells to BH3-mimetic drugs that are currently being tested in humans. The dose-limiting thrombocytopenia associated with ABT-263 represents a barrier to the combined use with DHA; however, this proof-of-principle study suggests that DHA can synergize with BH3-mimetic agents.

DHA has previously been reported to have anticancer properties; however, the specificity of activity and the concentrations needed for efficacy have remained unclear (32–35, 45). Furthermore, how DHA kills cancer cells has been linked to a number of mechanisms (32–36, 45). The treatment of mouse BCR-ABL\(^+\) B-ALL cells and MEF cells with DHA treatment induces the activation of a genetic program that is similar to the ER stress pathway as well as the proapoptotic protein NOXA. Although NOXA has been reported to destabilize MCL-1 protein levels, we were unable to abrogate MCL-1 repression by DHA in MEF cells lacking the NOXA-encoding Puma gene. Importantly, the loss of p53 function in mouse BCR-ABL\(^+\) B-ALL cells rendered the cells quite resistant to DHA-induced apoptosis, suggesting that NOXA and PUMA induction are critical apoptotic regulators.

We observed that DHA treatment of mouse BCR-ABL\(^+\) B-ALL cells triggered the potent induction of a genetic program consistent with an ER stress response. One of the key features of an ER stress response is the inhibition of protein translation (46). As MCL-1 is a very labile protein, blocking protein synthesis rapidly induces its clearance by the proteasome (47). Consistent with this hypothesis, it has been observed in other systems that the induction of ER stress can rapidly eliminate MCL-1 expression (48–51). When we genetically ablated the CHOP-encoding cellular stress gene Ddit3 in MEFs or mouse BCR-ABL\(^+\) B-ALL cells, we observed a significant decrease in MCL-1 repression triggered by DHA. These data suggest that the induction of cellular stress by DHA induces CHOP expression and represses MCL-1 protein levels. The fact that DHA treatment does not inhibit MCL-1 mRNA expression or induce enhanced degradation suggests that MCL-1 protein translation is repressed. How MCL-1 protein translation is being inhibited in a CHOP-dependent manner is still unclear, but these data suggest that CHOP mediates the expression of additional target genes that regulate MCL-1 translation. Indeed, a myriad of gene targets are CHOP dependent and involved in many cellular pathways, including transcription and translation (52). In addition, some noncoding miRNAs have been found to be CHOP dependent and regulate translation of protein targets (53). Studies are ongoing to identify how DHA triggers the cellular stress pathway as identifying these cellular pathway(s) may help to identify additional therapeutic targets by which MCL-1 expression can be even more specifically antagonized.

The labile nature of MCL-1 and its response to cellular signaling pathways makes it a good candidate for combinatorial therapy. Indeed, other strategies have been reported for repressing MCL-1 expression and leading to improved responses in cancer cells (14, 15, 54–60). Our identification of DHA represents an additional therapeutic strategy in which the induction of a cellular stress pathway triggers the repression of MCL-1 protein expression in mouse BCR-ABL\(^+\) B-ALL cells. We look forward to conducting future experiments testing whether combined DHA and ABT-263 treatment of NSG recipient mice bearing Ph\(^+\) primary patient leukemia will reveal similarly synergistic responses and MCL-1 repression. Furthermore, we are also investigating whether this mechanism might be common to other tumor and leukemia types. Importantly, in leukemic cells, DHA may have the added benefit that it bypasses resistance to kinase inhibitors that would be expected to occur in the clinic. Therefore, our data indicate that DHA represents an additional strategic pathway by which MCL-1 expression can be repressed, leading to enhanced response in poor prognosis BCR-ABL\(^+\) B-ALL.

**Disclosure of Potential Conflicts of Interest**

J.T. Opferman reports receiving speakers bureau honoraria from Mt. Sinai Tisch Cancer Institute, Texas A&M, University of Chicago, University of Michigan, and University of Pittsburgh and is a consultant/advisory board member for AbbVie. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

J.T. Opferman is supported by the NIH R01HL102175 and R01CA201069; the American Cancer Society 119130-RSG-10-255-01-LIB, P30CA021765, and the American Lebanese Syrian Associated Charities.

We thank the Opferman laboratory, Drs. Hendershot, Singh, and Rehg for helpful discussions. The Ddit3-deficient MEFs were from Dr. Ron [Cambridge Institute for Medical Research] and Pmaip1-deficient MEFs were from Dr. Li [University of Louisville]. We thank S.T. Peters and Dr. S.M. Pruett-Miller of St. Jude's Center for Advanced Genome Engineering for generating the mouse Ddit3-deficient BCR-ABL1+ B-ALL cells.

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Received April 27, 2017; revised August 31, 2017; accepted September 28, 2017, published OnlineFirst October 3, 2017.

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Modulation of Navitoclax Sensitivity by Dihydroartemisinin-Mediated MCL-1 Repression in BCR-ABL + B-Lineage Acute Lymphoblastic Leukemia

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