BH3 profiling as pharmacodynamic biomarker for the activity of BH3 mimetics

Recent years witnessed remarkable advances in developing BH3 mimetic drugs that target anti-apoptotic BCL-2, BCL-XL, and MCL-1 proteins.[1,2] With BCL-2 inhibitor venetoclax (ABT-199) approval and its remarkable clinical results, the development of BH3 mimetics at most pharmaceutical companies has been accelerated and the number of new BH3 mimetic compounds has been dramatically increased. Nonetheless, with a huge number of preclinical studies and hundreds of clinical trials ongoing in cancers, there is no convenient pharmacodynamic (PD) biomarkers for the activity of BH3 mimetics. PD biomarker provide information about the pharmacologic effects of a drug on its target, important for successful development of candidate drugs. When a BCL-2 inhibitor engages its target BCL-2, the pro-apoptotic proteins sequestered by BCL-2 would be replaced and the freed pro-apoptotic proteins could subsequently be bound by other anti-apoptotic proteins such as MCL-1, assuming that the studied cells express both proteins. As a result, the treated cells would become less dependent on BCL-2 and more dependent on MCL-1 for survival. The opposite would happen when an MCL-1 inhibitor binds MCL-1. An assay that can determine these dynamic changes can thus be used in PD studies of BH3 mimetics. BH3 profiling is a functional assay which can determine cell's different dependence on BCL-2 anti-apoptotic proteins.[2-7] In the current study, we tested whether BH3 profiling can serve as a reliable peripheral blood-based PD biomarker for the activity of BH3 mimetics.

Our previous work established that BH3 profiling can identify cell dependence on different anti-apoptotic proteins for survival.[2,3,8] In the current study, we first used two cell lines with defined dependency to validate the robustness of the technique. O-BCL2 and O-MCL1 are leukemia cell lines overexpressing BCL-2 and MCL-1, respectively (*Online Supplementary Figure S1A*), where the overexpression overrides the cells' dependency on other anti-apoptotic proteins for survival.[9,10] In a blinded fashion, we performed BH3 profiling on the two cell lines (Methods as previously described[3,11]) and found that among the selective peptides and BH3 mimetics (*Online Supplementary Figure S1B*), only the BCL-2 binding ABT-199 and the BCL-2/BCL-XL binding BAD peptide induced cytochrome *c* release from mitochondria of O-BCL2 (*Online Supplementary Figure S1C)* and only the MCL-1 binding MS-1 peptide or S63845 induced cytochrome *c* release from O-MCL1. This result validated that BH3 profiling can reliably identify anti-apoptotic dependence of tested cells. We reasoned that BH3 profiling should be able to detect changes of anti-apoptotic dependence induced by BH3 mimetics and the changes may serve as a PD biomarker for the activity of BH3 mimetics (i.e., the engagement of a given BH3 mimetic on its target protein).

We next validated that BCL-2 inhibitor BCL201, and MCL-1 inhibitor S63845 are *bona fide* BH3 mimetics and then chose them as representative BH3 mimetics to test our hypothesis. As shown in *Online Supplementary Figure S1D*, BCL201 and S63845 respectively induced cytochrome c release from O-BCL2 and O-MCL1 cells, but not from cell lines with other dependency, suggesting that BCL201 and S63845 are highly selective for BCL-2 and MCL-1 respectively, thus being ideal compounds for this study.

A PD biomarker is used to evaluate whether a compound engages its target in an expected fashion. We did not choose to use primary cancer cells in this study because they vary from sample to sample and different samples may have high, low, or even zero expression of the targeted proteins (in this case, BCL-2 and MCL-1), making it difficult to evaluate whether a BH3 mimetic engages its target protein. Instead, we chose to use primary human lymphocytes based on the following reasons: i) both normal T and B cells express significant levels of BCL-2 and MCL-1;[12] ii) they can be easily isolated from a simple blood draw from patients; iii) T or B cells readily respond to BH3 peptides-the probes for BH3 profiling (Figure 1A), suggesting they would respond to BH3 mimetics as well. The changes of anti-apoptotic dependence in normal peripheral T/B cells taken from a treated tumor patient, should be detectable by BH3 profiling. The observed changes in T/B cells (e.g., a shift of dependence from BCL-2 to MCL-1) can serve as a PD biomarker to evaluate whether a given BH3 mimetic at used doses is able to act upon its target protein.

Human peripheral blood mononuclear cells (PBMC) were isolated using CPT tubes (BD, cat#362753) via a quick centrifugation (*Online Supplementary Figure S1E* for the workflow). Use of human samples was approved by the Dana-Farber Cancer Institute and Harvard University human studies review boards. Next, three million of PBMC cells were treated with BCL-201 or S63844 at concentrations that are clinically achievable in blood plasma as revealed by our pharmacodynamic studies. After treatment, BH3 profiling was performed on PBMC cells as previously described.[3,11] Using BH3 peptides as probes, we analyzed the treatment-induced changes of mitochondrial priming (delta priming) of viable $CD3^+CD19/20^-$ T cells and $CD3^-CD19/20^+$ B cells (*Online Supplementary Figure S1F* for gating strategy). Delta priming is defined as the values of mitochondrial priming of treated cells from which the values of baseline priming (priming of vehicle treated cells) are subtracted.

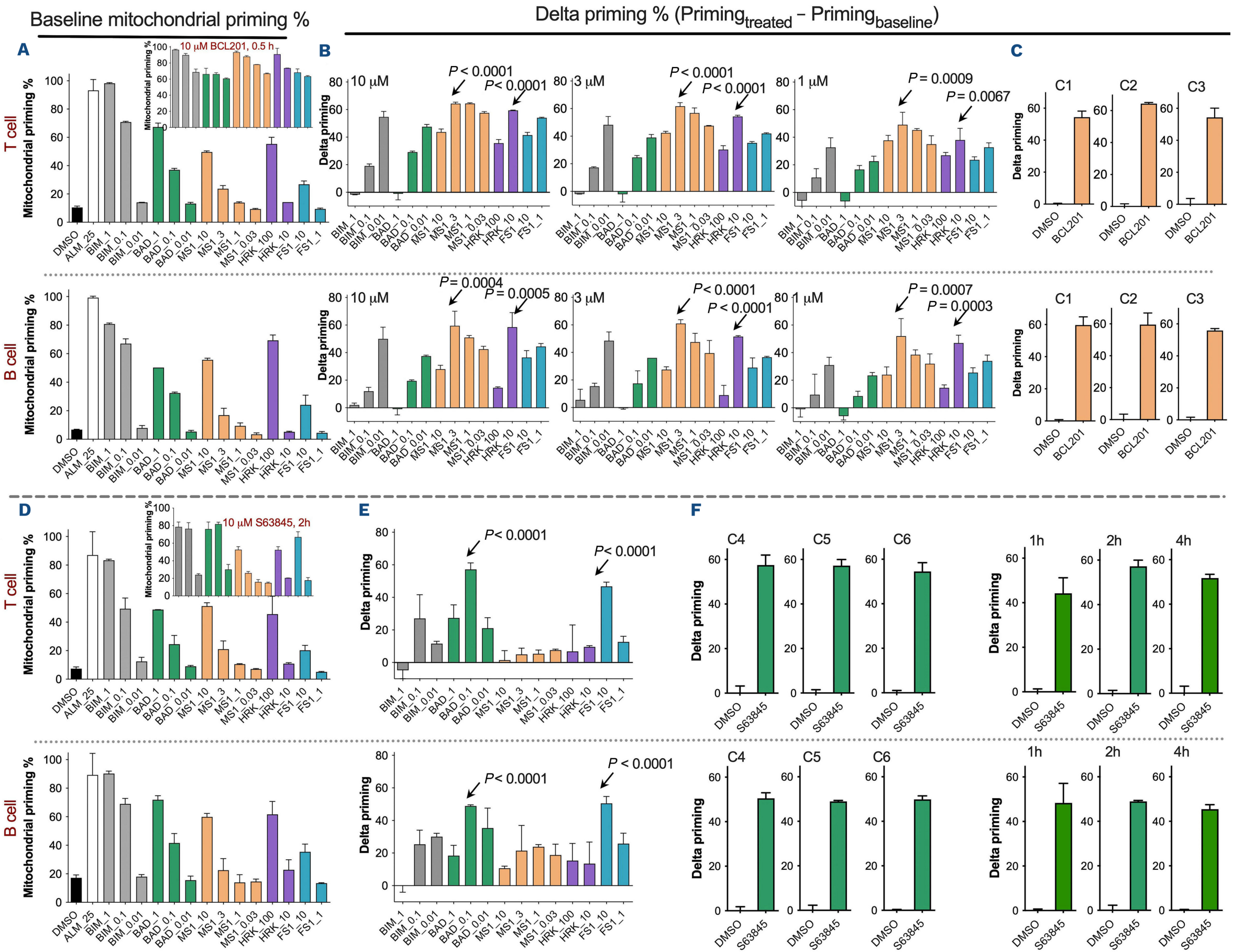

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Figure 1. BH3 profiling can serve as a pharmacodynamic biomarker for the activity of BH3 mimetics *in vitro*. (A) Baseline priming of human T and B cells in peripheral blood mononuclear cell (PBMC) samples (i.e., untreated control). The inserted bar graph in the upper panel shows mitochondrial priming of T cells after treatment with 10 μM BCL201 for 0.5 hours (h), serving as an example of 'Priming$_{treated}$'. (B) Delta priming of human T and B cells after treatment with indicated concentrations of BCL201 (0.5 h treatment). Arrows indicate bars with largest values in each graph. *P* value for the arrowed bar was calculated using Student's *t* test to compare the values of Priming$_{treated}$ and Priming$_{baseline}$. (C) Delta priming of T and B cells from 3 PBMC samples as determined using MS1 peptide at 3 μM (BCL201 treatment for 0.5 h, 3 μM). (D) Baseline priming of T and B cells (i.e., untreated control). The inserted bar graph in the upper panel shows overall mitochondrial priming of T cells after treatment with 10 μM S63845 for 2 h, serving as an example of 'Priming$_{treated}$'. (E) Delta priming of T and B cells after treatment with 10 μM S63845 (2-h treatment). (F) Δ priming of T and B cells from 3 different samples after 2-h treatment with 10 μM S63845 (left panels). Delta priming of T and B cells from 1 representative donor after 1-, 2-, or 4-h treatment with 10 μM S63845. BH3 profiling was performed using BAD peptide at 0.1 μM. Data were presented as mean ± standard error of the mean of triplicate experiments. *P* value was calculated using Student's *t* test to compare the values of Priming$_{treated}$ and Priming$_{baseline}$.

Increased priming indicates increased dependence on corresponding anti-apoptotic proteins. In order to explore which BH3 peptide is the most sensitive probe in detecting delta priming, we included BIM, BAD, MS-1, HRK, and FS-1 peptide (*Online Supplementary Figure S1B*) in our panel.

After treatment with 1 μM BCL201 for 30 minutes, we conducted BH3 profiling on viable T cells and found that among all the tested peptides, MS-1 peptide performs the best, especially when used at 3 μM (Figure 1B). We further tested other concentrations of BCL201 like 3 μM and 10 μM (achievable levels *in vivo*). MS-1 peptide is again the most robust probe in detecting delta priming caused by BCL201 treatment (Figure 1B). Importantly, the induced delta priming by BCL201 were consistently and readily detected when we used blood samples from three different donors (Figure 1C). Similarly, MS-1 peptide at 3 μM also worked the best at detecting delta priming in B cells after BCL201 treatment (Figure 1C, lower panels). Since MS-1 peptide is selective for MCL-1, these results suggest that BCL201 replaced pro-apoptotic proteins that are sequestered by BCL-2, and freed pro-apoptotic proteins could be subsequently bound by MCL-1 in T/B cells. Therefore, BCL201 treatment augments T/B-cell dependency on MCL-1 and BH3 profiling of T/B cells with the MCL-1 selective MS-1 peptide can serve as a PD marker for the activity of a BCL-2 inhibitor.

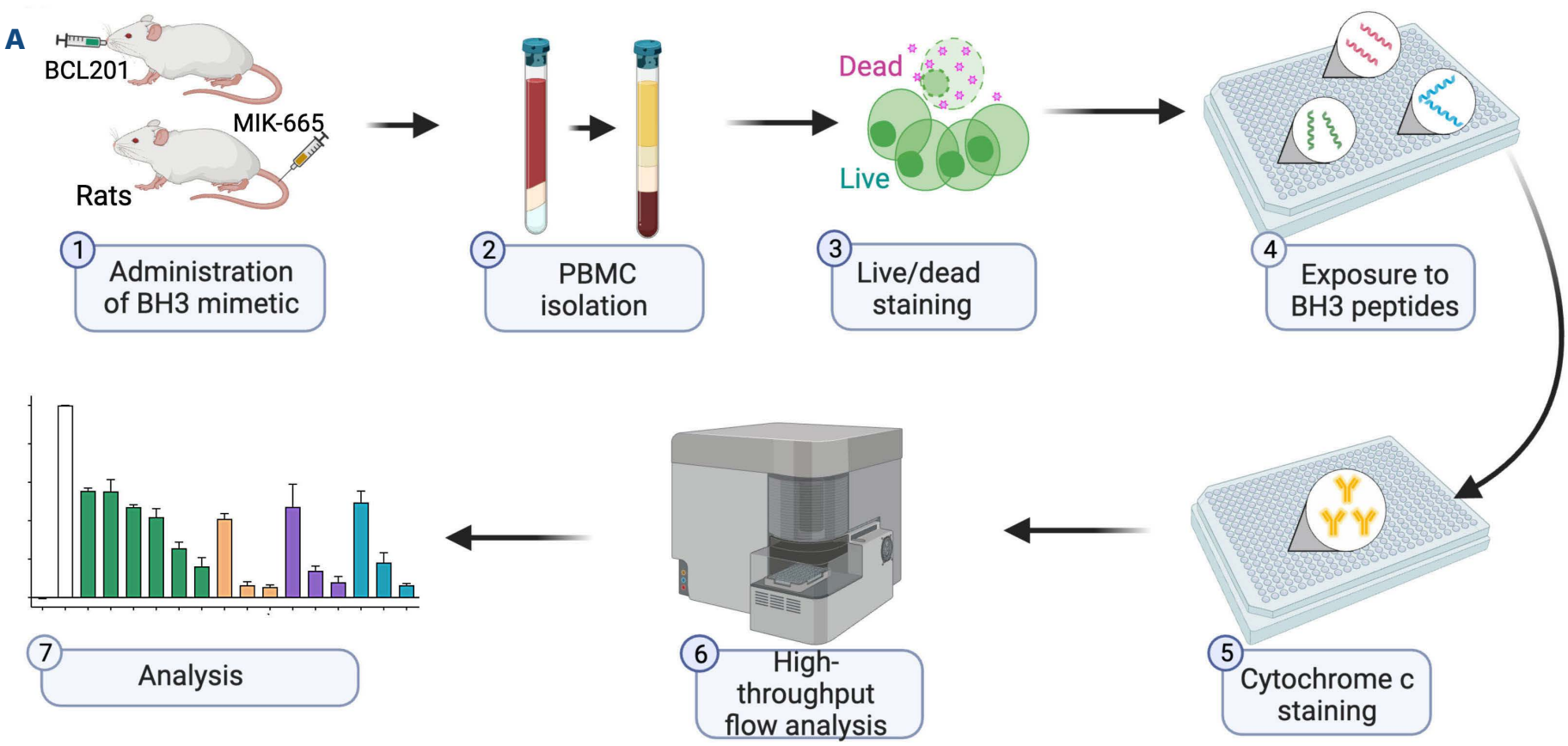
We next studied whether BH3 profiling can serve as a PD marker for the activity of an MCL-1 inhibitor. We found that in both T and B cells, MCL-1 inhibitor S63845 significantly increased mitochondrial sensitivity to BAD and FS1 peptides (Figure 1E). BAD peptide also reliably detected the increased delta priming of samples from multiple donors (N=3) after treatment with S63845 (Figure 1F), or at different time points such as 1, 2, or 4 hours (h) (Figure 1F, right panels), suggesting BH3 profiling of T/B cells with BAD peptide may serve as a robust PD marker for the activity of an MCL-1 inhibitor.

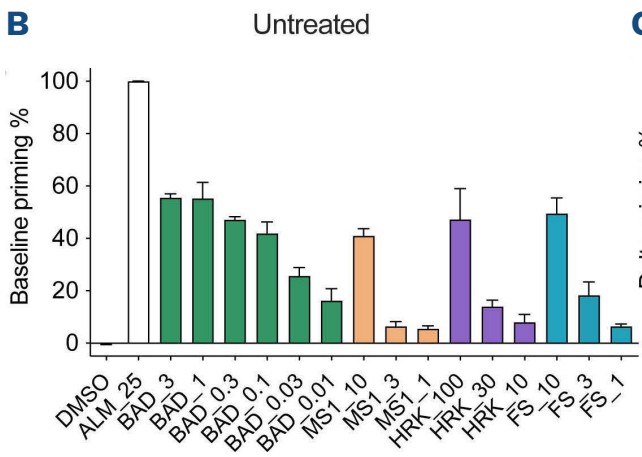
We next asked if we could use BH3 profiling as a PD marker for *in vivo* treatment with BH3 mimetics. For *in vivo* studies, we chose rat models because 3 mL of blood can be readily obtained via retro-orbital bleeding (see Figure 2A for workflow). The animal experiments were performed in accordance with the approved Institutional Animal Care and Use Committee (IACUC) guidelines at Novartis animal facility. Animal experiments were performed on 3-4 months old, body weight 200-300 g, female WISTAR HAN rats from Charles River Laboratories (catalog #273). BCL201 was administered via oral gavage at 100 mg/kg. Blood was collected 2 h later and PBMC were isolated using CPT tubes. Since T cells and B cells performed quite similarly in the *in vitro* studies, we further simplified the process and performed BH3 profiling on isolated lymphocytes without staining for T/B cells. We found that again MS-1 peptide is the most sensitive probe in detecting the delta priming caused by BCL201 (Figure 2C), consistent with our *in vitro* results. Taken together, BH3 profiling with MS-1 peptide can provide a PD biomarker for the activity of a BCL-2 inhibitor.

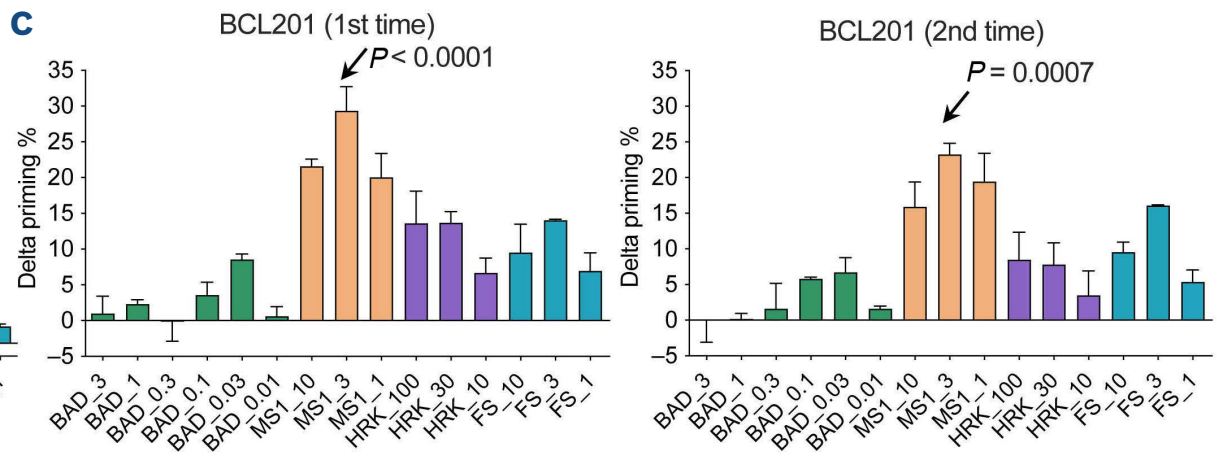
MCL-1 inhibitor MIK665 (S64315) is the clinical counterpart of the above-used S63845 with better *in vivo* pharmacokinetics.[13] It was thus chosen for rat *in vivo* study. MIK665 was formulated in clinical liposomal formulation, administered via intravenous (tail lateral vein) injection at 5 mL/kg. Blood was collected 30 minutes post dosing with MIK665. Rat PBMC were isolated and profiled with BH3 peptides. Consistent with the *in vitro* results, BAD peptide is the most robust probe in detecting delta priming caused by the MCL-1 inhibitor (Figures 2D, E). This stays true for both tested doses of 15 mg/kg or 30 mg/kg, the regularly used MIK665 doses (intravenously) for rat studies. Our results suggest that BH3 profiling with BAD peptide can work as a PD biomarker for the activity of an MCL-1 inhibitor, both *in vitro* and *in vivo*.

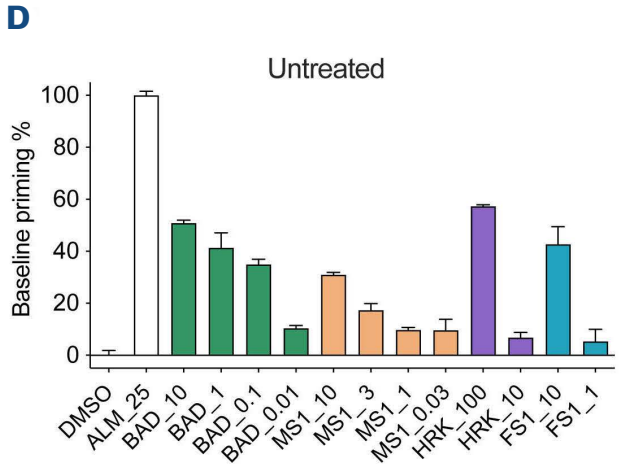
Finally, we conducted additional tests on paired human cancer cells and surrogate lymphocytes to evaluate the proposed strategy. PBMC were isolated from untreated acute myeloid leukemia (AML) patients according to protocols approved by the institutional human studies review boards. After treating PBMC *in vitro* with venetoclax for 1 h, BH3 profiling was performed with 3 μM MS-1, the identified peptide and dose sensitive in detecting delta priming caused by BCL-2 inhibitors (Figures 1B and 2C). Consistent with our *in vitro* and *in vivo* findings, MS-1 peptide reliably detected the increased delta priming of CD3^{+}/CD19^{+}CD45^{hi} lymphocytes in all four AML patient samples (Figures 3A-C). Furthermore, a significant increase in mitochondrial prim-

ing was observed in $CD3^{-}19^{-}33^{+}64^{-}11b^{-}16^{-}$ myeloblast cells from all four AML samples (Figure 3D). It is worth noting that myeloblasts from two AML samples became significantly more primed than the other two (Figure 3E), which could ensue from different expression profiles of BCL-2 proteins in different samples. Nevertheless, the observed increased priming in all four tested AML samples aligns with our earlier report that AML generally have dependence on BCL-2 for survival.[8] Altogether, BH3 profiling with MS-1 peptide readily detected the PD effects of venetoclax on AML blasts and lymphocytes. Non-cancerous T/B cells are reliable and arguably more sensitive and consistent surrogates than AML blasts for pharmacodynamic data. Baseline priming results suggests that both T and B cells









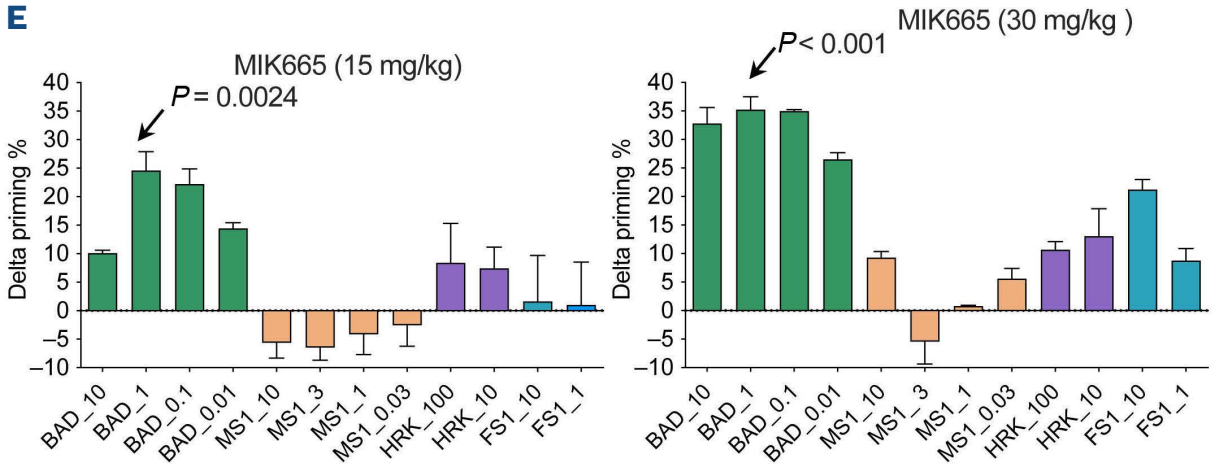


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Figure 2 BH3 profiling can serve as a pharmacodynamic biomarker for the activity of BH3 mimetics *in vivo*. (A) Workflow of BH3 profiling of rat peripheral blood mononuclear cells (PBMC) after *in vivo* treatment with BH3 mimetics. BCL201 was administered by oral gavage and MIK665 was given by tail vein injection. (B) Baseline priming of lymphocytes from untreated rats (untreated control). (C) Delta priming of rat lymphocytes after *in vivo* treatment with BCL201. The drug was administered via oral gavage at 100 mg/kg and peripheral blood was collected 2 hours (h) after a single dose of BCL201. The rat experiments were repeated twice. (D) Baseline priming of lymphocytes from untreated rats (untreated control for panel E). (E) Delta priming of rat lymphocytes after treatment with MIK665. MIK665 was given by intravenous injection at 15 or 30 mg/kg and peripheral blood was collected 0.5 h after a single dose of MIK665. Data were presented as mean ± standard error of the mean of triplicate experiments. Arrows indicate bars with largest values in each graph. *P* value for the arrowed bar was calculated using Student's *t* test to compare the values of Priming$_{treated}$ and Priming$_{baseline}$.

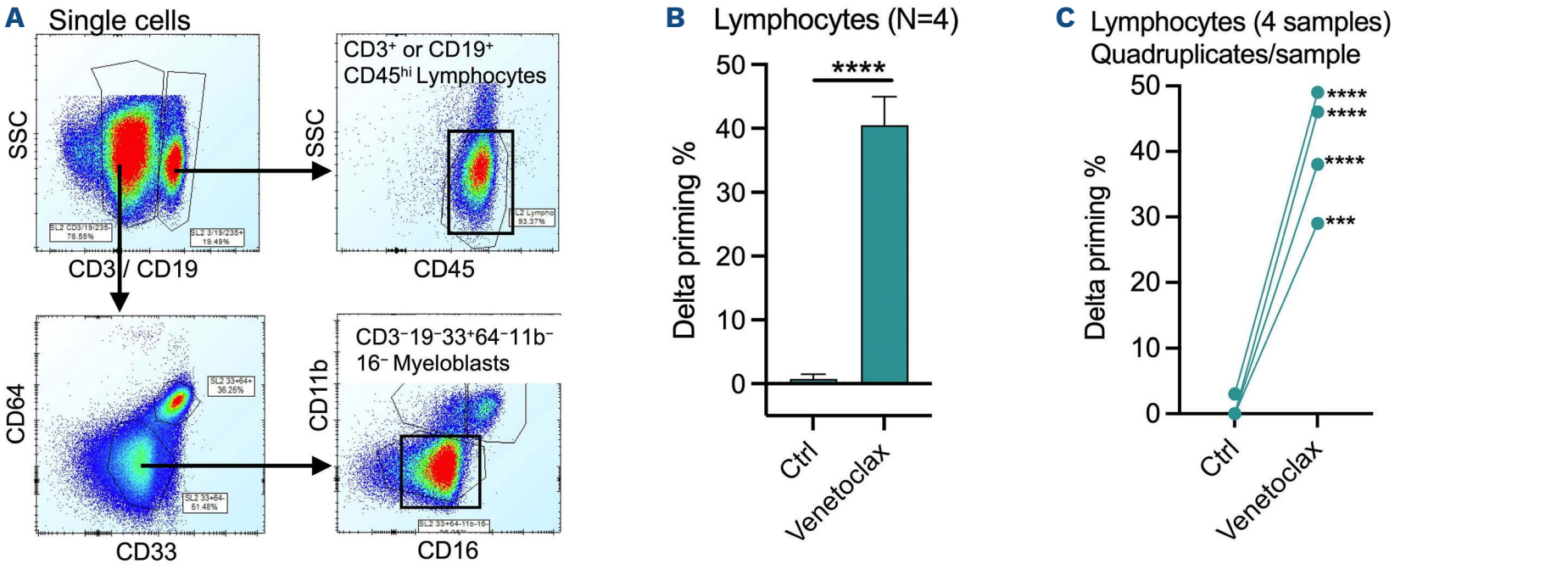

Figure 3. BH3 profiling manifested the pharmacodynamic effects of venetoclax in paired lymphocytes and myeloblasts from acute myeloid leukemia patients. (A) Gating strategy for human primary acute myeloid leukemia (AML) myeloblasts and lymphocytes. (B, C) Delta priming of CD45⁺CD3/19⁺ lymphocytes from four AML patients after treatment with 0.1 μM venetoclax for 1 hour (h). Frozen AML peripheral blood mononuclear cells (PBMC) were thawed, treated, and then probed with 3 μM MS-1 peptide using BH3 profiling. (D, E) Delta priming of CD3⁻19⁻33⁺64⁻11b⁻16⁻ myeloblast from four AML patients after treatment with 0.1 μM venetoclax for 1 h. Frozen AML PBMC were thawed, treated, and then probed with 3 μM MS-1 peptide. Data were presented as mean ± standard error of the mean of quadruplicate experiments. *P* value was calculated using Student's *t* test to compare the values of control and venetoclax-treated samples.

respond well to BAD, MS1, and HRK peptides (Figure 1A, D), indicating that they have dependence on BCL-2, MCL-1,and BCL-XL for survival. When treated with BCL-2 inhibitor, the anti-apoptotic capability of BCL-2 protein would be significantly neutralized and the released pro-apoptotic proteins from BCL-2 sequestration could then be significantly buffered by MCL-1. The mitochondria are thus becoming more dependent on MCL-1, which can be readily determined by the MCL-1-selective MS-1 peptide. Similarly, a significant portion of pro-apoptotic proteins freed from MCL-1 sequestration by an MCL-1 inhibitor can be sequestered by BCL-2 and BCL-XL in lymphocytes. The mitochondria thus become more dependent on BCL-2 and BCL-XL and this change can be readily detected by BH3 profiling using BAD peptide. This likely explains why BH3 profiling works well as a PD biomarker for BH3 mimetics.

In conclusion, we found that BH3 profiling, with lymphocytes as surrogate cells, provides a robust and convenient PD biomarker for the activity of BH3 mimetics. The current study indicates that BH3 profiling could be used as a PD

biomarker for the activity of BH3 mimetics in cellular assays and preclinical animal studies. These findings provide the preclinical basis for testing of BH3 profiling as a PD marker in clinical trials of BH3 mimetics.

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Disclosures

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Contributions

RP, EH and AL designed the research. RP, JR, YW and SQ performed the research. MV and JR contributed vital reagents. RP, YW, EH, EM and AL analyzed the data. RP wrote the original draft. RP, EM, EH and AL reviewed and edited the manuscript.

Data-sharing statement

Interested people can obtain data through contacting the corresponding author.

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