Dynamic BH3 profiling method for rapid identification of active therapy in BH3 mimetics resistant xenograft mouse models

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Protocol
Dynamic BH3 profiling method for rapid identification of active therapy in BH3 mimetics resistant xenograft mouse models

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https://doi.org/10.1016/j.xpro.2021.100461

SUMMARY
The clinical effectiveness of BH3 mimetics therapy is limited by the inevitable emergence of acquired resistance. We present a protocol to model in vivo acquired resistance to BH3 mimetics in patient-derived xenograft (PDX) mouse models of acute myeloid leukemia. Using resistant PDXs as a valuable model, we next introduce a protocol for dynamic BH3 profiling (DBP) method. DBP allows functional identification of effective drug therapies based on measurements of drug-induced apoptosis signaling to overcome in vivo BH3 mimetics resistance. For complete details on the use and execution of this protocol, please refer to Bhatt et al. (2020).

BEFORE YOU BEGIN

 отметить Timing: must be prepared at least 1 week before experiment date

1. Prepare buffers and BH3 peptides.
2. Acquire female NSG mice, 6–8 weeks old, from Jackson Labs. Allow the mice to acclimate to their environments for 5–7 days before AML transplantation.

Prepare multiwell high-throughput BH3 peptide plates for DBP

смотреть Timing: 2–8 h

Pause point: BH3 peptide plates (96W or 384W) can be made at the time of the experiment or in advance and stored at −80°C for 6 months. We utilized pre-prepared 384W plates for our studies.

3. Prepare 2 × desired concentration of each BH3 peptide in MEB buffer supplemented with 0.002% Digitonin for a final volume of 15 μL per well.

Note: The digitonin has to be added fresh at this step because it is not stable in MEB for long-term unless kept frozen. The concentration of digitonin will be 0.002% at this time, but it will be diluted to a final concentration of 0.001% when cells are added to the MEB.

⚠ CRITICAL: When creating plates for DBP, we recommend using a titration of BH3 peptides from 100 μM to 0.001 μM.
4. Using an electronic multichannel pipette, add 15 µL of BH3 peptides resuspended in MEB buffer to each well. Mix the plate by gently tapping all 4 sides. Visually inspect the plate to ensure that the BH3 peptides and MEB buffer are not trapped on the sides of the wells.

**Note:** Manual plate preparation is possible, but we recommend using an automated liquid dispensing system for plate preparation to help reduce human-introduced error and disparities between plates.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Human FcR block (1:100 dilution) | Miltenyi Biotec | Cat# 130-059-901 |
| Mouse Anti-Human CD33 | BD Biosciences | Cat# 555450; RRID: AB_395843 |
| Mouse Anti-Human CD45 | BD Biosciences | Cat# 63879; RRID: AB_2744402 |
| Mouse Anti-Human Alexa Fluor 488 anti-human CD38 (1:100 dilution) | BioLegend | Cat# 303512 |
| Mouse Anti-Human CD34 581 APC/Cytochrome c Alexa Fluor 647 6H2.B4 (1:400 working dilution; 1:2400 final dilution) | BioLegend | Cat# 612310; RRID: AB_2565241 |
| Biological samples |        |            |
| Patient-derived xenograft models | cBioportal | N/A |
| Chemicals, peptides, and recombinant proteins |        |            |
| ABT-199 (venetoclax) | MedChem Express LLC | Cat# HY-15531 |
| Alamethicin | Enzo | Cat# BML-A150-0005 |
| Bovine serum albumin (BSA) | Gemini Bio-Products | Cat# 700-101P |
| D-Mannitol | Sigma-Aldrich | Cat# M9647-1KG |
| D-α-Tocopherol polyethylene glycol 1000 succinate (vitamin E/TPGS) | Sigma-Aldrich | Cat# 57668-5G |
| Digitonin 5% | Fisher Scientific | Cat# BN2006 |
| EGTA pH 7.4 0.5 M | Fisher Scientific | Cat# 50255956 |
| EDTA pH 7.4 0.5 M | Westnet Inc. | Cat# BM-711-K |
| Ficoll-Paque Plus | Fisher Scientific | Cat# 45001749 |
| Glycine | Fisher Scientific | Cat# BP3815 |
| Heat-inactivated fetal bovine serum (FBS) | Life Technologies | Cat# 10082147 |
| HEPES buffer | Sigma-Aldrich | Cat# H4034-500G |
| Paraformaldehyde* | Electron Microscopy Science Nm | Cat# 15714 |
| PBS 1x, pH7.4 10 x 500 mL | DFCI Invitrogen Supply Center | Cat# 7020 |
| Penicillin-streptomycin solution | Life Technologies | Cat# 15140163 |
| Phosal 50 PG | Fisher Scientific | Cat# NC0130871 |
| Poly (ethylene glycol) (PEG) | Sigma-Aldrich | Cat# 202398-500G |
| Potassium chloride (KCl) | Sigma-Aldrich | Cat# P4505-1KG |
| Potassium hydroxide (KOH) | Sigma-Aldrich | Cat# P4494-50ML |
| RBC lysis buffer | QIAGEN | Cat# 158904 |
| RPMI 1640 10 x 500 mL | Life Technologies | Cat# 11835055 |
| Succinate Acid-BioXtra | Sigma-Aldrich | Cat# S3674-250G |
| Tris Base | Research Products International | Cat# T60043-4000.0 |
| Tween-20 | Fisher Scientific | Cat# BP337100 |

(Continued on next page)
### MATERIALS AND EQUIPMENT

#### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| FS-1 Acetyl-QWREIAAGLRL AADNVNAQLER-Amide | New England Peptide | Custom |
| hBid Y Acetyl-EDIIRNIARHLAQVGDSMDRY-Amide | New England Peptide | Custom |
| hBim Acetyl-MRPEIWIAQRHRLGDEINAYYAR-Amide | New England Peptide | Custom |
| HRK-Y Acetyl-SSAAQTAAARKALGDEIHQY-Amide | New England Peptide | Custom |
| mBAd Acetyl-LWAAQRYGRELRRMSDEFEGSKGL-Amide | New England Peptide | Custom |
| mNOXa Acetyl-AELPPEFAAQLRKIGDKVY-Amide | New England Peptide | Custom |
| MS-1 Acetyl-RPEIWMTQGLRRLGDEINAYYAR-Amide | New England Peptide | Custom |
| PUMA Acetyl-EQWAREIGAQLRRMADDLNA-Amide | New England Peptide | Custom |

#### Critical commercial assays

- **Zombie Yellow Fixable Viability Kit** BioLegend Cat# 423104

#### Experimental models: Organisms/strains

- **Mouse model**: NOD-scid IL2R gamma null female mice (6-8 weeks old) The Jackson Laboratory 005557

#### Software and algorithms

- **BD FACS DIVA** BD Biosciences https://wwwbdbiosciences.com
- **BioRender** BioRender https://biorender.com/
- **FlowJo** FlowJo https://www.flowjo.com
- **Prism 8** GraphPad https://www.graphpad.com/

#### Other

- **24-well cell culture plate with lid** Thomas Scientific Cat# 1156F00
- **384 W Fluorotrac 200 Plate, PS, Medium Binding, Flat Bottom, Black** MedSupply Partners Cat# GR-781076
- **4MM Lancet** Fisher Scientific Cat# NC9922361
- **Alcohol wipes** Fisher Scientific Cat# 17730602
- **BD 309628 Syringe for 1cc LL** AmerisourceBergen Cat# 2757037
- **BD LSRFortessa flow cytometry** BD Biosciences N/A
- **CellStar EASYstrainer 70 μm** Fisher Scientific Cat# 07000223
- **DWP96/2000 Plate** Fisher Scientific Cat# E951033502
- **Electronic multichannel pipettes/tips** Eppendorf N/A
- **Micropipette blood collection tubes** Westnet Inc. Cat# 365974
- **Mortar and pestle** N/A N/A
- **Plastic feeding tubes for rats and mice** Instech Cat# FTP-20-38
- **Storage film** Fisher Scientific Cat# E0030127870
- **Tabletop centrifuge for 50 mL tubes** Eppendorf N/A

*Paraformaldehyde is toxic through contact and inhalation. Prepare and use this solution wearing gloves, a lab coat, and eye protection in a chemical hood.

### MEB buffer

| Reagent | Final concentration | Amount for 0.50 L |
|---------|---------------------|------------------|
| Mannitol | 150 mM              | 13.65 g          |
| HEPES-KOH pH 7.5 | 10 mM              | 1.19 g           |
| KCl     | 150 mM              | 5.59 g           |

(Continued on next page)
Solution preparations

**MEB buffer**

- Dissolve mannitol, HEPES, succinic acid, BSA, and KCl together. When mixed well, add EDTA and EGTA to the solution.
- Titrate the pH to 7.5 ± 0.1 with KOH.
- Add ddH₂O to bring the final volume to 0.5 L.
- Filter and store at 4°C for up to 6 months.

**Venetoclax**

- Add ethanol (final concentration 10%) to required quantity of venetoclax powder for the desired final volume and vortex very well to mix.
- Sequentially add PEG 400 (final concentration 30%) and Phosal 50 PG (final concentration 60%) for the desired final volume and vortex to mix very well.
- Rest the solution for 30 min.
- Store in a brown or amber bottle to protect from light at 20°C–22°C for up to 2 weeks.

*Note:* Our typical dosing volume for venetoclax is 0.2 mL with the drug given at 100 mg/kg/day, once a day. Hence, we prepare venetoclax suspensions at 5 g/10 mL.

**S63845**

- Formulate 2% Vitamin E/TPGS in 0.9% NaCl.
  
  - Weigh out 1 g of Vitamin E/TPGS and add 50 mL of NaCl 0.9%.
  
  - Mix solution with a stir bar until complete dilution (approximately 1 h).
- Formulate S63845.
  
  - Weigh out S63845 for the desired final volume (ex. 4 mg for 4 mg/mL).
  
  - Add the appropriate amount of Vitamin E/TPGS for the desired final volume (ex. 1 mL for 4 mg/mL).
  
  - Mix solution with a stir bar until complete dilution.
Pass through a 0.2 μm filter to sterilize the solution.

- Always make fresh and administer within 30 min of preparation.

    **Note:** S63845 is light-sensitive and should be kept in the dark at all times. The maximum solubility is 4 mg/mL.

**N2 buffer**

- Combine tris base and glycine in 90 mL of ddH₂O.
- Titrate the pH to 9.1 ± 0.1 if needed.
- Dilute with 100 mL ddH₂O to final volume.
- Filter and store at 20°C–22°C for up to 6 months.

**CytoC staining buffer**

- In 50 mL of PBS, combine Tween20 (2% final concentration) and BSA (10% final concentration).
- Filter and store at 4°C for up to 6 months.

    **Note:** Add the 1:400 dilution at of anti-Cytochrome c-Alexa fluor 647 at the time of experiment, not when preparing the buffer.

**STEP-BY-STEP METHOD DETAILS**

This protocol is organized into three main sections (see below). Section 1 and 2 both take several weeks to complete, and Section 3 can be carried out in 2 days after the conclusion of Section 2. Buffers and BH3 peptides described above may be prepared prior to starting the experiment.

**Section 1: Leukemia transplantation to create AML PDX models**

**Section 2: Generating acquired resistance to BH3 mimetics in AML PDX models**

**Section 3: Dynamic BH3 profiling on BH3 mimetics resistant myeloblasts**

**Section 1: Leukemia transplantation to create AML PDX models**

© Timing: 4 Weeks

Mice were injected with human AML cells to create PDX models for experimentation and analysis.

    **Note:** If not using a primary patient sample, a large repository of viably frozen PDX cells can be obtained from cBioportal (Townsend et al., 2016) (https://www.cbiportal.org). The time between tumor transplantation and leukemia development varies depending on PDX model. We were generally able to detect disease burden as circulating myeloblasts after 3 weeks of engraftment using FACS analysis.

1. Obtain human leukemia cells from a bone marrow (BM) biopsy.
2. Subject BM aspirate (BMA) of human patient to Ficoll-Paque gradient to isolate mononuclear cells.
   a. Dilute the BMA 1:5 (1 mL BMA: 5 mL 20°C–22°C PBS), filter through a 70 μm cell strainer on top of a 50 mL Falcon tube, and layer the BMA onto the Ficoll.
   b. Centrifuge at 400 × g for 30 min at 20°C–22°C without the brake. Collect the white blood cells from the interphase layer and transfer to a new 50 mL Falcon tube.
   c. Add 4°C PBS to a final volume of 50 mL and mix by inversion. Centrifuge at 600 × g for 5 min at 20°C–22°C. Look for pellet; aspirate the supernatant.
d. Resuspend the cells in 5 mL of 4°C PBS to a final volume of 30 mL. Count the cells.
e. Centrifuge at 600 × g for 5 min at 20°C–22°C. Look for pellet; aspirate the supernatant.

3. Resuspend mononuclear cells in 200 μL of 20°C–22°C PBS.

**Pause point:** We used immunocompromised NSG mice without prior pre-conditioning. We acclimated mice for 5-7 days before serial transplantation.

4. Place the mice under a lamp light to briefly warm them so that tail veins become more visible. Sterilize the mouse’s tail with an alcohol wipe.

5. Inject 0.6–1.0 × 10^6 human leukemia cells in 200 μL of PBS intravenously (IV) (Passage 1).

6. Bleed mice weekly to evaluate circulating peripheral blast counts and the engraftment progress via flow cytometry.
   a. Using a surgical lancet, puncture the facial submandibular vein and collect 20–50 μL of blood in a microtainer blood collection tube. **Troubleshooting 1**
   b. Lyse the cells by adding a 1:10 ratio of blood:RBC lysis buffer (Qiagen). Incubate for 10 min in a 5 mL FACS tube at 20°C–22°C.
   c. Wash the cells with 4 mL of PBS.

   △ CRITICAL: RBC lysis is a critical step of this protocol, but it is important to limit the incubation period to no longer than 10 min with lysis buffer. Extended exposure to RBC lysis buffer may cause the lysis of desired (non-RBC) cell populations too.

   d. Centrifuge at 600 × g for 5 min at 20°C–22°C. Look for pellet; aspirate the supernatant.
   e. Resuspend the cell pellet in FACS staining buffer and proceed for hCD45 antibody staining at 1:100 dilution.

7. Using FACS analysis, determine the percentage of human myeloblasts circulating in the blood by gating on SSC<sub>low</sub>hCD45<sub>dim</sub> cells (Figure 1).

8. Sacrifice the mice and harvest key organs once FACS analysis reveals >70% circulating human myeloblasts.
   a. Euthanize mice in CO₂ chamber equipped with a CO₂ delivery rate of 10%–30% of the chamber volume per min.
   b. Expose peritoneum to remove spleen and collect the pelvis, femur, and tibia bones from both legs. Remove excessive fat and muscle.
   c. Proceed with tissue processing to prepare the spleen and bone marrow for serial passaging of the leukemia cells.
      i. **For the spleen:** Place spleen onto 70 μm cell strainer on top of a 50 mL Falcon tube. Add 5 mL PBS, smash the spleen using the plunger from a 5 mL syringe, and add 20 mL PBS to prepare a single cell suspension.
      ii. **For the bone marrow:** Crush all bones in 1 mL of PBS using a mortar and pestle. Filter the bones through a 70 μm cell strainer on top of a 50 mL Falcon tube to prepare a single cell suspension.
      iii. **Perform RBC lysis:** Resuspend the cells in RBC lysis buffer (Qiagen) for 5 min at 20°C–22°C. Wash the cells with 4 mL of PBS.

9. To prepare cells for leukemia transplantation, resuspend 0.6–1.0 × 10^6 spleen myeloblasts in 200 μL of PBS. Cells can also be viability frozen using freezing media at this stage for future transplantations.

**Section 2: Generating acquired resistance to BH3 mimetics in AML PDX models**

© Timing: variable by PDX model and drug

Begin continuous treatment of leukemia-bearing mice with BH3 mimetics until leukemia blasts relapse, indicating acquired drug resistance.
Pause point: Time to develop acquired resistance depends on PDX model and individual drug. For BH3 mimetics, we typically observed resistance developing between 45–120 days on treatment, denoted by >70% circulating peripheral blast counts or signs of disease progression in the mice.

10. Repeat section 1 step 5 to perform leukemia transplantation with murine spleen cells (Passage 2).
Note: We utilized splenic cells to perform serial transplantation because the spleens typically yielded a higher number of cells compared to the bone marrow.

11. Bleed the mice weekly with section 1 steps 6 and 7 to assess for circulating peripheral blasts.
12. When FACS analysis reveals >5% circulating peripheral blasts, begin treatment with BH3 mimetics.
   a. To administer venetoclax: Use an oral gavage feeding tube to administer 100 mg/kg, PO, 5 days per week.
   b. To administer S6383845: Use a 28 1/2g needle to inject 40 mg/kg, IV, 2 days per week.
13. Continue to bleed the mice weekly to assess for circulating peripheral blasts. Troubleshooting 2
14. When FACS analysis reveals >70% circulating peripheral blasts, sacrifice the mice and harvest bones and spleen following section 1 steps 8a-c.
15. Proceed to dynamic BH3 profiling. Troubleshooting 3

Section 3: Dynamic BH3 profiling (DBP) on BH3 mimetics resistant myeloblasts

© Timing: 2 Days

Perform dynamic BH3 profiling (Montero et al., 2015) on cells with acquired resistance to BH3 mimetics to identify new or persistent drug vulnerabilities based on drug-induced mitochondrial priming (threshold to apoptosis).

16. Seed myeloblasts harvested from the spleen and bone marrow samples in section 2 separately in a 24-well plate at a density of 1.0 × 10⁶ cells per 1 mL of RPMI per well. Troubleshooting 4

Note: While we harvested both splenic and bone marrow cells for downstream molecular studies, we primarily utilized splenic cells for DBP. Bone marrow samples often did not yield enough cells to evaluate larger drug panels, though we performed DBP on them when possible.

17. Treat with desired drug panel for 14–16 h at 37°C.

18. Collect the cells for DBP in 1.5 mL Eppendorf tubes and prepare for cell surface staining.
   a. Centrifuge at 600 × g for 5 min at 20°C–22°C. Look for pellet; aspirate the supernatant.
   b. Resuspend 1.0 × 10⁶ cells in 100 μL of FACS staining buffer.
      i. Incubate with 1 μL Fc blocker in the dark at 4°C for 15 min.
      ii. Wash the cells with 1 mL of PBS.
      iii. In the meantime, prepare a cocktail of antibodies and Zombie Yellow Fixable Viability dye in 100 μL of FACS staining buffer, each at 1:100 dilution. Do this for anti-hCD45, anti-hCD33, anti-hCD34, and anti-hCD38.
      iv. Incubate the stained cells in the dark 4°C for 30 min.

△ CRITICAL: Cell surface staining steps are time-sensitive. For this reason, always prepare a little more of the antibody cocktail than is required to avoid coming up short.

   c. Wash the cells with 1 mL of PBS.

19. Proceed with DBP.
   a. Obtain pre-made peptide plates from the “Before you begin: Prepare multiwell high-throughput BH3 peptide plates for DBP” step, or prepare peptide plates in real-time as directed above.
      i. If using pre-made plates stored at −80°C, thaw the plates in a 26°C incubator for at least 30 min before use.
   b. Centrifuge the PBS-washed cells at 600 × g for 5 min at 20°C–22°C. Look for pellet; aspirate the supernatant.
c. Resuspend cells at $5 \times 10^4$ cells per 15 μL MEB buffer. Transfer the 15 μL cell suspension to a 384W BH3 peptide plate.

d. Mix the cells with the BH3 peptides by gently tapping all 4 sides of the plate. Visually inspect the plate to ensure that the cells are not stuck to the sides of the wells.

e. Incubate the cells with BH3 peptides in a 26°C incubator for 60 min.

△ CRITICAL: Mitochondrial outer membrane permeabilization (MOMP) processes are time- and temperature-sensitive. Make sure to incubate cells at a constant temperature no longer than the time described.

f. In a chemical hood, add 10 μL of 4% paraformaldehyde to each well to fix the cells and terminate BH3 peptide exposure. Repeat mixing, and incubate for 15 min at 20°C–22°C.

g. Add 10 μL of N2 buffer to each well to neutralize the 4% paraformaldehyde and terminate cell fixation. Repeat mixing, and incubate for 10 min at 20°C–22°C.

h. Add 10 μL of pre-made CytoC staining buffer and antibody mixture to the wells. Repeat mixing, and incubate at 4°C overnight (14–16 h) in the dark.

△ CRITICAL: Mixing well is very important to ensure homogenous staining across each well. For the last step, thoroughly mix the cells and stain by covering the plate with an adhesive sealing film and shaking the plate from side to side.

20. Proceed with acquisition and FACS analysis. Troubleshooting 5

EXPECTED OUTCOMES

Using the dynamic BH3 profiling methodology described above, we determined cytochrome c release in response to drug or DMSO treated cells after treatment with the BIM BH3 peptide (Figure 2). Response to the promiscuously interacting BIM BH3 peptide, which interacts with all anti-apoptotic molecules and which can directly activate BAX and BAK, can be used to determine differential drug response (Certo et al., 2006, Garcia et al., 2019). If treatment with a drug showed enhanced mitochondrial priming response, indicated by a >15% cytochrome c release compared to DMSO, then we would predict that drug to ultimately induce apoptosis of target cells and overcome resistance to BH3 mimetics in vivo (Bhatt et al., 2020).

QUANTIFICATION AND STATISTICAL ANALYSIS

We performed various statistical analyses to determine the individual efficacy for drugs of our interest. To calculate the percent change (delta) in mitochondrial priming, we determined percentage cytochrome c release for cells exposed to DMSO and drug (Table 1). Then, we calculated delta priming with the following formula:

$$\text{Delta Priming} = \text{percentage cytochrome c Loss}_{\text{Drug}} - \text{percentage cytochrome c Loss}_{\text{DMSO}}$$

Delta Priming for BIM 0.03 μM = 59.8 – 10.3 = 49.5

To understand whether ex vivo DBP can predict for in vivo response to drug treatments, we compared vehicle-treated and drug-treated responders using a one-sided Wilcoxon rank-sum test. We then determined overall survival advantages in animals using the log-rank test by the Kaplan-Meier method (Figure 3).

LIMITATIONS

A few limitations of this protocol hinge on the restraints of PDX models themselves. During engraftment of human leukemia cells into NSG mice, cells undergo clonal selection, due to which resulting leukemia may not retain complete intratumor heterogeneity of the original tumor (Ferrando and Lopez-Otin, 2017, Sanden et al., 2020). Since the prediction of drug vulnerability by DBP assay relies on drug response
against a population of tumor cells, DBP analysis may not truly encompass all of the drug vulnerabilities present in the original leukemia, due to selection pressure exerted during \textit{in vivo} engraftment.

Logistically, there is variability in the onset of tumor development and durability of response to drug treatment between different animals within the same cohort. Often, we saw instances where one mouse in a cohort developed measurable leukemia burden more rapidly than others. Further, mice responded at different rates, and for variable durations, to the BH3 mimetics, which emphasized the previously observed phenomenon of intratumor heterogeneity in AML and in different mouse strains.

While deriving models of acquired resistance, we had to prioritize tolerability to animals over the maximum effective drug doses of BH3 mimetics to allow prolonged drug treatment until emergence of resistance. Through our study design, we identified the optimal routes and dosages of venetoclax and S63845, but toxicity resulting from extended treatment periods limited us from using those exact doses, and we instead selected suboptimal doses. Therefore, it is possible that the resulting baseline priming of BH3 mimetics resistant myeloblasts may be different had we been able to use the optimized dosages for our long-term experiments.

**TROUBLESHOOTING**

**Problem 1**  
Bleeding-associated animal mortality (section 1, step 6a)

**Potential solution**  
In this protocol, we bled mice from the facial submandibular vein to avoid excessive pain or possibility of infection. However, we observed mouse mortality on one occasion. After collecting 20–50 $\mu$L of blood, it is important to check the mouse to ensure bleeding has stopped. If bleeding is severe, hold mouse in an upright position and apply direct pressure.

**Problem 2**  
Spontaneous death due to acquisition of BH3 mimetics resistance despite low circulating blast counts in the peripheral blood (section 2, step 13)

**Table 1. Condensed example of raw data for dynamic BH3 profiling from FACS output**

| Sample                        | Well          | % Cyto c Parent (DMSO) | % Cyto c Parent (Drug) |
|-------------------------------|---------------|------------------------|------------------------|
| DFAM-61786 Venetoclax Resistant | A1 (DMSO)    | 3.1                    | 2.5                    |
| DFAM-61786 Venetoclax Resistant | A2 (Alamethicin) | 96.9               | 94.7                   |
| DFAM-61786 Venetoclax Resistant | A3 (BIM 3 $\mu$M) | 81.6                   | 90.1                   |
| DFAM-61786 Venetoclax Resistant | A4 (BIM 0.3 $\mu$M) | 43.6                   | 70.2                   |
| DFAM-61786 Venetoclax Resistant | A5 (BIM 0.03 $\mu$M) | 10.3                   | 59.8                   |
Potential solution
We observed spatial heterogeneity within mice during acquisition of resistance to BH3 mimetics. We found that the myeloblasts were highly congregated in the bone marrow or spleen, as compared to non-resistant models, and witnessed spontaneous death in mice with low peripheral blood blast counts. Therefore, it is important to monitor overall mouse health and look for signs of disease progression, such as ungroomed fur, change in fur color, weight loss, lethargy, and/or hemolytic paralysis.

Problem 3
Lower cell recovery due to acquisition of BH3 mimetics resistance (section 2, step 15)

Potential solution
Because the cells at this step are BH3 mimetics resistant and on treatment, we anticipate obtaining cell numbers that are lower compared to if we performed DBP analysis on cells without any drug treatment. We planned to use cohort sizes of 5 mice per treatment arm. When we received cell numbers less than 5 million per mouse, we planned to enroll 10 mice per treatment arm at section 2 step 10 to ensure that we would obtain enough cells to perform subsequent DBP analyses and serial transplants.

Problem 4
Poor cell recovery from viably frozen PDX samples due to cell clumping (section 3, step 16)

Potential solution
Viably frozen splenic cells can be utilized for serial transplants. Since viable cells can become entangled with the DNA of dead cells during thawing, it is recommended to pre-treat with DNase to improve live cell recovery.

Problem 5
Too-high mitochondrial permeabilization (>25%) in DMSO treated wells due to leaky cells (section 3, step 20)

Potential solution
If baseline cytochrome c release (without peptide or drug exposure) appears >30%, it is important to reevaluate data from DMSO wells. We recommend re-gating the cell populations to ensure tight gating for viable cells and to exclude leaky cells.
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Shruti Bhatt (Shruti_bhatt@nus.edu.sg).

Materials availability
PDX models generated in this study have been deposited to cBioportal. Requests can be made https://www.cbioportal.org.

Data and code availability
This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS
S.B. acknowledges support from the LLS Career Development Fellow Award, AACR Basic Cancer Research Fellowship, and Claudia-Adams Barr Foundation. We thank Anthony Letai for his assistance conceptualizing the study and Marissa S. Pioso and Binyam Yilma for their assistance performing experiments and data analysis. We also thank David Weinstock for sharing valuable PDX models.

AUTHOR CONTRIBUTIONS
S.B. conceptualized study, designed the experiments, analyzed data, and wrote the manuscript. E.A.O. performed the experiments, analyzed data, and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests. Dana-Farber holds a patent for the dynamic BH3 profiling assay. Authors participating in this study are not part of the original invention.

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