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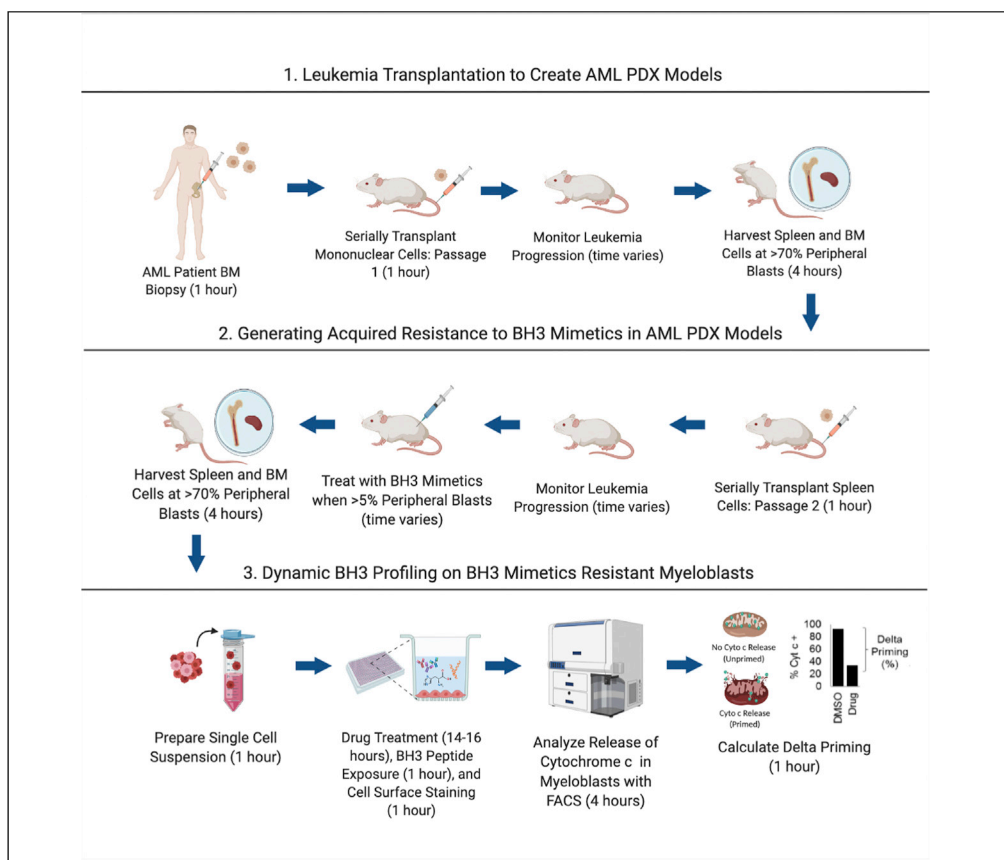
Protocol

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



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.

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HIGHLIGHTS

Generating *in vivo* PDX mouse models with acquired resistance to BH3 mimetics

Isolating human myeloblasts from the bone marrow and spleen of leukemia-engrafted mice

Identifying drug sensitivities of resistant myeloblasts using dynamic BH3 profiling

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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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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

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

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.

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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 WM53 PE (1:100 dilution)	BD Biosciences	Cat# 555450; RRID: AB_395843
Mouse Anti-Human CD45 HI30 BV421 (1:100 dilution)	BD Biosciences	Cat# 563879; RRID: AB_2744402
Mouse Anti-Human Alexa Fluor 488 anti-human CD38 (1:100 dilution)	BioLegend	Cat# 303512
Mouse Anti-Human CD34 581 APC/Cyanine7 (1:100 dilution)	BioLegend	Cat# 343514; RRID: AB_1877168
Mouse Anti-Human 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 ACS	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[a]	Electron Microscopy Science Nm	Cat# 15714
PBS 1×, PH7.4 10 × 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 × 500 mL	Life Technologies	Cat# 11835055
S63845	Servier/Novartis	Provided
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*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
FS-1 Acetyl-QWVREIAAGLRL AADNVNAQLER-Amide	New England Peptide	Custom
hBID-Y Acetyl-EDIIRNIARHLA QVGDSMDRY-Amide	New England Peptide	Custom
hBIM Acetyl-MRPEIWIAQEL RRIGDEFNA-Amide	New England Peptide	Custom
HRK-Y Acetyl-SSAAQLTAAR LKALGDELHQY-Amide	New England Peptide	Custom
mBAD Acetyl-LWAAQRYGRELRRMSDE FEGSFKGL-Amide	New England Peptide	Custom
mNOXA Acetyl-AELPPEFAA QLRKIGDKVYC-Amide	New England Peptide	Custom
MS-1 Acetyl-RPEIWMTQGLR RLGDEINAYYAR-Amide	New England Peptide	Custom
PUMA Acetyl-EQWAREIGAQ LRRMADDLNA-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		
BDFACS DIVA	BD Biosciences	https://www.bdbiosciences.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 Fluotrac 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
Microtainer 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

[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.

MATERIALS AND EQUIPMENT

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*)

Continued		
Reagent	Final concentration	Amount for 0.50 L
EGTA 0.5M	0.02 mM	20 μL
EDTA 0.5M	0.02 mM	20 μL
BSA	0.1%	0.500 g
Succinate	5 mM	0.295 g
ddH_2O	N/A	Up to 0.50 L
Once prepared, store at 4°C for up to 6 months.		

Other Solutions	Reagents
Venetoclax	Formulated in 60% phosal 50 PG, 30% PEG 400, 10% EtOH
S63845	Provided by Servier/Novartis. Formulated in 2% Vitamin E/TPGS in 0.9% NaCl
RPMI Media	Supplemented with 10% heat-inactivated FBS, 1% Penicillin-Streptomycin
FACS Staining Buffer	2% FBS in PBS
N2 Buffer	1.7 M Tris, 1.25 M Glycine pH 9.1
CytoC Staining Buffer	10% BSA, 2% Tween20, PBS, 1:400 dilution of anti-Cytochrome c-Alexa fluor 647
Freezing Media	5% DMSO in FBS
Once prepared, store at RPMI, FACS Staining Buffer, and Freezing Media at 4°C for up to 6 months.	

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 ddH_2O 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 4mg/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 ddH_2O.
- Titrate the pH to 9.1 ± 0.1 if needed.
- Dilute with 100 mL ddH_2O 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.cbioportal.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 $SSC^{low}hCD45^{dim}$ cells (Figure 1).
8. Sacrifice the mice and harvest key organs once FACS analysis reveals >70% circulating human myeloblasts.
   a. Euthanize mice in CO_2 chamber equipped with a CO_2 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.

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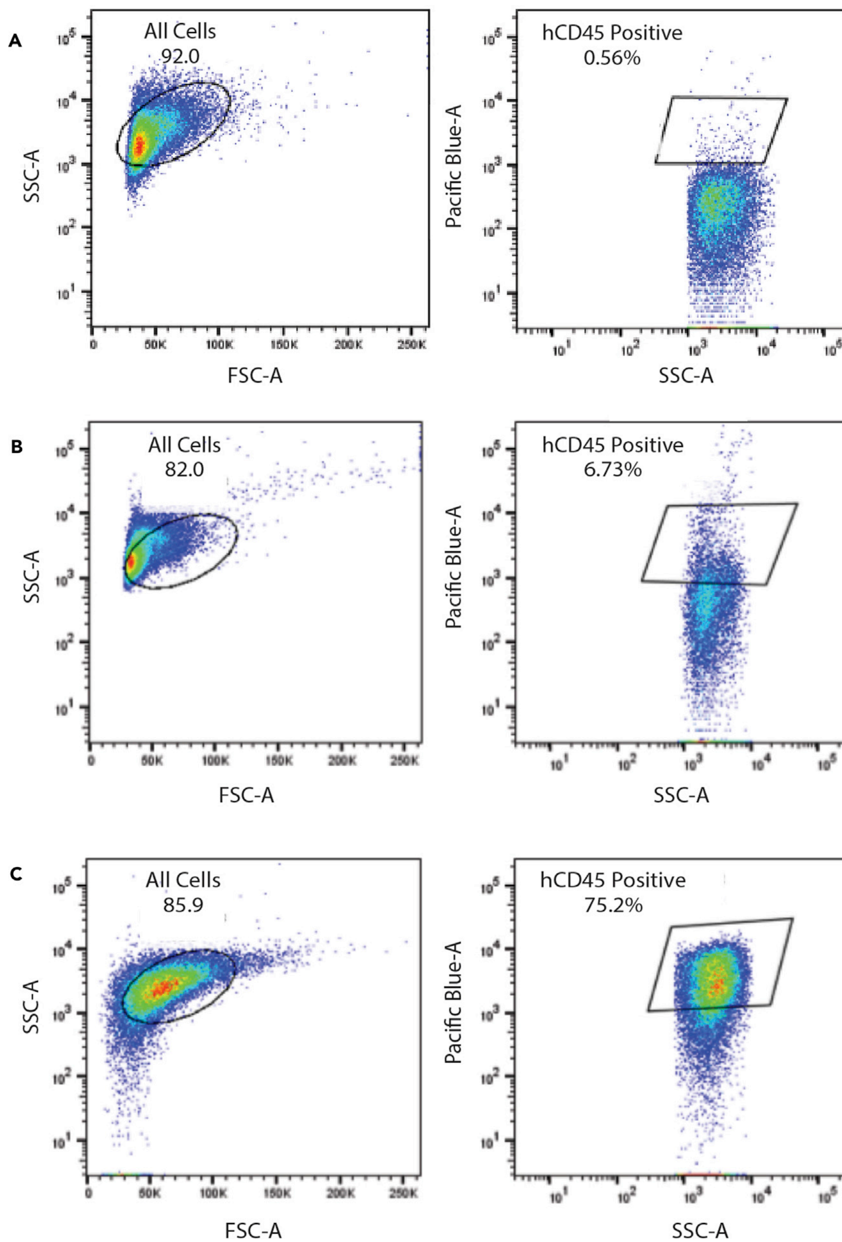

Figure 1. Gating strategy for identification of circulating human myeloblasts in AML PDXs

Representative flow cytometry plots for circulating human myeloblasts in the peripheral blood of NSG mice.

(A) Healthy NSG mice (non-transplanted).

(B) NSG mice at 3 weeks post leukemia engraftment.

(C) NSG mice after leukemia progression.

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 $28^{1/2}$g 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 1.0×10^6 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 1.0×10^6 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 × 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:

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

$$\text{Delta Priming for BIM } 0.03\ \mu\text{M} = 59.8 - 10.3 = 49.5$$

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

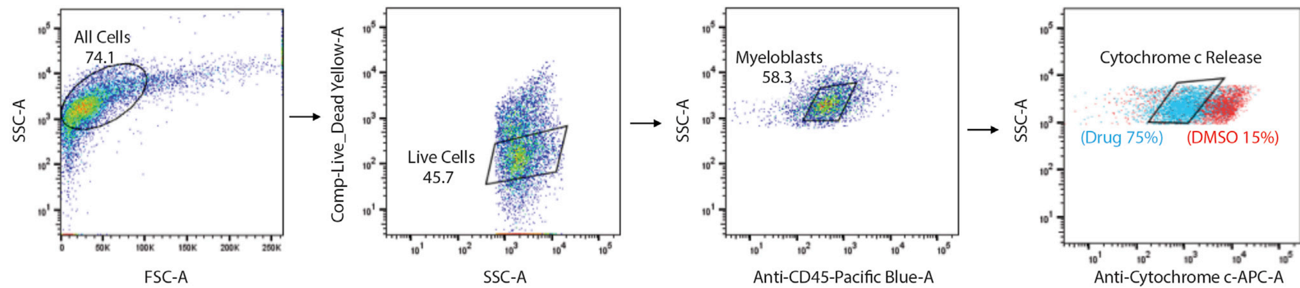

Figure 2. Example of identification of active drug using dynamic BH3 profiling in venetoclax resistant AML PDXs.
Myeloblasts harvested from the spleen of a venetoclax resistant AML PDX mouse was subjected to dynamic BH3 profiling using the BIM peptide. Increased cytochrome c release caused by drug treatment for 16 h is seen by the shift left. Representative plots before each arrow include cells that are selected and gated on in subsequent plots.

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 *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 μL 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 μM)	81.6	90.1
DFAM-61786 Venetoclax Resistant	A4 (BIM 0.3 μM)	43.6	70.2
DFAM-61786 Venetoclax Resistant	A5 (BIM 0.03 μM)	10.3	59.8

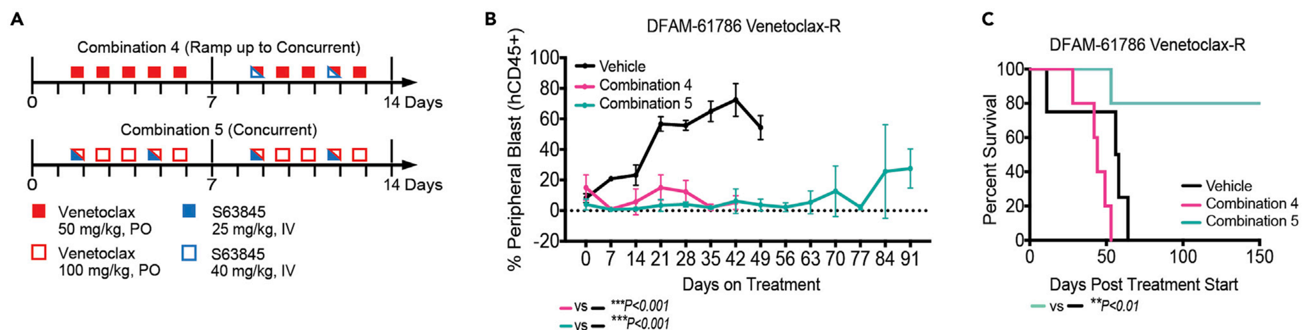

Figure 3. Example of *in vivo* activity of drugs identified using dynamic BH3 profiling

(A) Schematic of treatment combination regiments for venetoclax and S63845.

(B) Percentage of hCD45+ leukemia cells after treatment with combination treat of venetoclax and S63845 in venetoclax resistant DFAM-61786.

(C) Kaplan-Meier curves showing comparison of survival analysis between different treatment groups.

*p<0.05, **p<0.01, ***p<0.001; log rank test. Figure modified and reprinted with permission from Bhatt et al., 2020.

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.

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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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