DEVELOPMENT OF A ROBUST BH3 PROFILING TOOLKIT FOR PRECISION MEDICINE IN HEMATOLOGIC MALIGNANCIES.

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INTRODUCTION

Hematologic malignancies (HM) are a type of cancer that affect the bone marrow, the blood and the lymph node of the patient and include Chronic Lymphocytic Leukemia (CLL), Non-Hodgkin Lymphoma (B and T), multiple myeloma, as well as Acute Myeloid Leukemia. Recently, a novel class of pro-apoptotic anti-HM drugs, called **BCL2 homology 3 (BH3) mimetics,** has been discovered. But each disease remains heterogeneous which makes it hard to provide standardized treatment for each patient and highlight the need for new diagnosis tools to deliver precision medicine. To answer this, a technique called **BH3 profiling** has been developed, allowing to measure the apoptosis priming of the cells as well as their dependencies on specific anti-apoptotic proteins. This technique uses specific BH3 sensitizer peptides to measure the percentage of cytochrome c released for each anti-apoptotic protein.

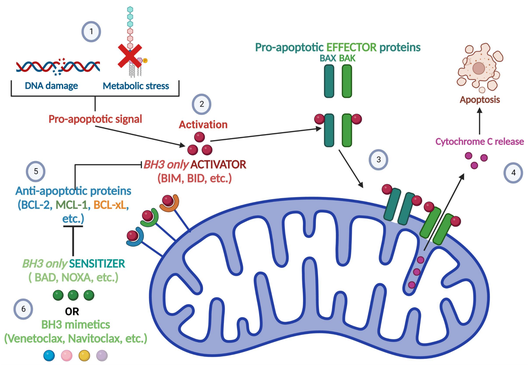
BH3 toolkit, a variation of BH3 profiling, use BH3 mimetics instead of BH3 peptides. Those molecules mimic the effect of BH3 sensitizer proteins without the two major sources of variability present in the original BH3 profiling: cell permeabilization and peptide instability.

Another variation, called **dynamic BH3 profiling (DBP)**, allows the measurement of early drug-induced changes in net pro-apoptotic signaling, to identify modification of anti-apoptotic dependencies.

AIM

Our aim is to develop a standardized and reproducible BH3 toolkit (baseline and dynamic), with internal control, to allow an absolute measurement of specific anti-apoptotic dependencies as well as identifying optimal therapeutic combination in HM.

APOPTOSIS INTRINSIC PATHWAY



1. Intrinsic signal such as DNA damage or metabolism stress will activate a pro-apoptotic signal cascade.
2. Pro-apoptotic cascade activates *BH3 only* ACTIVATOR proteins wih in turn activate EFFECTOR proteins such as BAX or BAK.
3. BAX or BAK homodimerise, translocate to mitochondrial membrane and create pores in it, allowing the release of Cytochrome C .
4. The release of Cytochrome C will allow the formation of the apoptosome and thus the caspase cascade and cell apoptosis.
5. Anti-apoptotic proteins will negatively regulate apoptosis by sequestrating *BH3 only* ACTIVATOR proteins.
6. *BH3 only* SENSITIZER or BH3 mimetics indirectly favor apoptosis by inhibiting anti-apoptotic proteins threw catalytic pocket competition.

BASELINE BH3 PROFILING

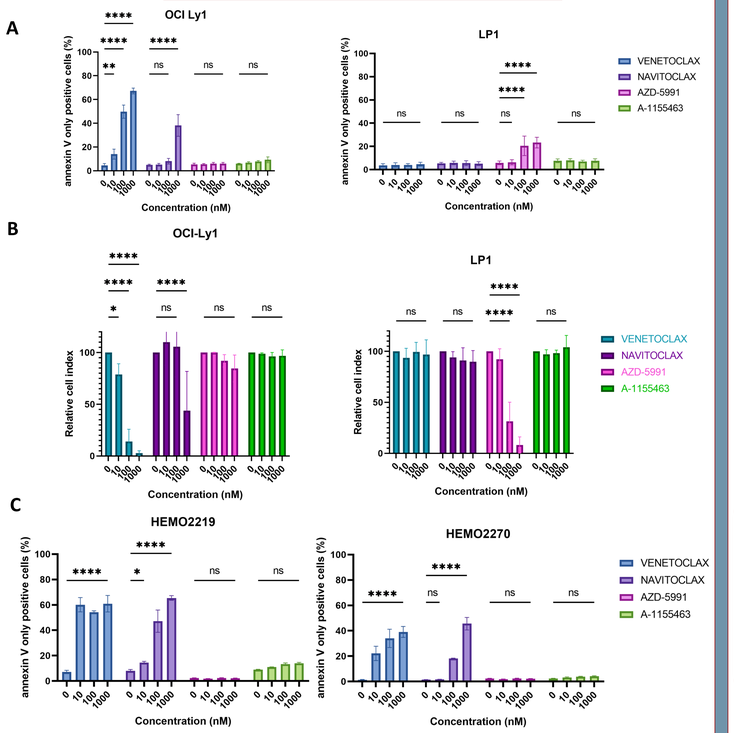


FIGURE 1 : Dynamic BH3 toolkit discriminate anti-apoptotic dependecies in cell lines and patient samples

A) OCI-Ly1 and LP1 cell lines treated with BH3 mimetics for 4h and stained with Annexin V-APC and 7aad. Cells were analysed by flow cytometry. Results represent the mean percentage and SD of three independent experiments. Statistical analysis was done with a two way ANOVA test *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; NS, nonsignificant. x**B)** Relative cell number measured by Cell-Titer Glo assay after 24h of BH3 mimetics treatment. **C)** Primary cells treated with BH3 mimetics for 4h, stained with Annexin V-APC and 7aad and analyse by flow cytometry.

DYNAMIC AND PATIENT BH3 PROFILING

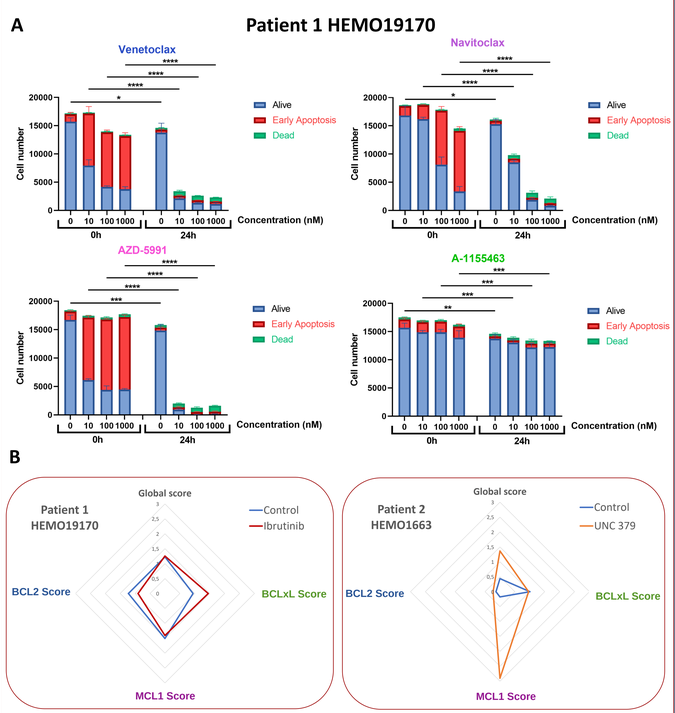


FIGURE 2 : Dynamic BH3 toolkit is able to predict patient response

A) Primary samples treated with BH3 mimetics for 4h and then stained with Annexin V-APC and 7aad. Counting beads were added prior to flow cytometry analysis. Cell number was calculated using cell count for each subpopulation and Counting beads count. The same experiment was conducted 24h later to compare cell number. Statistical analysis was done with a two way ANOVA test on the live cells (Alive + Early Apoptosis cells) *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; NS, nonsignificant. **B)** Two patients primary samples treated with Ibrutinib (left panel) or UNC 379 (right panel) or DMSO for 24h and BH3 mimetics for 4h. Global score represent the mean of all individual anti-apoptotic score.

METHOD

For primary cells testing, 96 well plates are seeded with Resto6 stromal cells and incubated for 24h. Then, primary patient samples are added upong thawing using robotic liquid handler and cultured for 20h. For DBP, the drug tested is added simultaneoulsly with primary cells. Next, we treat them with 4 BH3 mimetics (or vehicle) at concentration ranging from 10nM to 1µM for 4h :

- **Venetoclax (VEN, Bcl2 inhibitor)**
- **Navitoclax (NAV, Bcl2 and Bcl-xl inhibitor)**
- **AZD-5991 (MCL1i, MCL1 inhibitor)**
- **A-1155463 (BCLXLi, Bcl-xl inhibitor)**

Finally, cells were stained using Annexin V and 7AAD and analyzed by flow cytometry. Our gating strategy focuses on live cells to measure annexin V flipflop as an apoptotic mark. We also added precision counting beads to this assay in order to calculate cell number in our samples.

For cell line testing, we use the same protocol without the stromal cells.

BCL2, MCL1 and BCLxL scores are established as followed : Annexin V percentage is normalized on the control value. Then we divided the primary cells annexin V value by the control cell line value, OCI-Ly1 for Venetoclax and A-1155463 and LP1 for AZD-5991. Global score is the mean of the BCL2, MCL1 and BCLXL scores.

CONCLUSION

Our preliminary results show the robustness of our protocol and its ability to detect potential new therapeutic strategies. We hope that it will be an easy to use tool for both researchers and clinicians in the future.

CONTACT INFORMATION

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