Flow Cytometry Multiplexing Used to Analyze Metabolic Activity and Assess Pharmaceutical Compound Toxicity as A High Throughput Screening Tool

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ABSTRACT

High throughput screening (HTS) is an extremely effective method for allowing researchers to identify putative active compounds for therapeutics. Recently, flow cytometry has emerged as a powerful HTS tool with the added benefit of cell-by-cell analysis. Flow cytometry allows a researcher to study protein-protein interactions, metabolic activities, as well as DNA content in a single or multiplexed assay format. For this study, we screened the MicroSource Discovery System Killer Plates compound library and compared HTS using a plate reader with a flow cytometry live/dead assay.

Jurkat (T lymphocyte cell type) and Ramos (B lymphocyte cell type) cells were used as cell models. Cells were cultured under standard conditions and either at hypoxic (1% using a Heracell™ VIOS 160i incubator) or hyperoxic (19%) oxygen levels. We also varied length of time the cells were treated with compound library (24, 48, or 72 hrs.). Membrane integrity and metabolic activity were measured as an output for evaluating cellular viability. To further assess cellular activity, post-screening analysis was implemented to establish EC50s of "hits" from compound library.

Screening analysis identified target drugs that were further analyzed using cell health readouts with different concentrations of "hits" to assess compound toxicity. Results indicate that compound "hits" and potency differed in the screen depending on cell type, the mechanistic readout, and mode of readout used to perform the HTS experiments.

INTRODUCTION

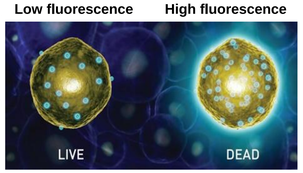
High-throughput screening has become the industry standard for pharmaceutical drug screening and drug analysis. The ideal tool for investigating drug functions on cells is flow cytometry. Within this study we compared an acoustic flow cytometry platform with a fluorescent plate reader platform, and analyzed cell viability with different mechanisms of read-out. In combination with the different platforms used, we investigated the difference in oxygen levels and compound potency. The selection of promising compound leads is often poorly characterized due to the oxygen level difference in clusters of abnormal cell types within the human body.

❖Jurkat (T lymphocyte cell type) and Ramos (B lymphocyte cell type) cells were used to study the affect of drugs on different types of white blood cells. White blood cells protect the body against foreign intruders; using these cells allows us to evaluate the effect of drugs tested and their involvement in cell-mediated immunity (T cells) and humoral immunity (B cells).

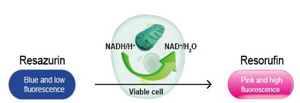
❖Methods/Analysis – At 24, 48, and 72 hours post compound treatment cells were treated with either LIVE/DEAD™ Fixable Aqua Dead Cell Stain for 40 minutes or PrestoBlue™ Cell Viability Reagent for 30 minutes and analyzed on either the Attune™ NxT flow cytometer or the Varioskan™ Flash.

MATERIALS AND METHODS

❖LIVE/DEAD Fixable Aqua Dead Cell Stain – Used to evaluate cell viability by flow cytometry. Cell viability was determined by the mean fluorescent intensity (MFI) of total cell population. Reactive dye permeates compromised cell membranes and reacts with free amines on the cell surface and the interior on the cell (Cell Membrane Integrity Read-out). ~Z' = 0.8



❖PrestoBlue Cell Viability Reagent – Used to measure cytotoxicity and the proliferation of cells by a spectrometer. Cell viability was determined by measuring the mean fluorescent intensity of total cells per well and obtaining the relative fluorescent units (RFU) per well. Resazurin (PrestoBlue reagent) is reduced by the environment of living cells (Metabolic Read-out). ~Z' = 0.7



❖Attune NxT Flow Cytometer – Designed to use acoustic-assisted hydrodynamic focusing, allowing for fast sample throughput rates.

❖Varioskan Flash – Spectral scanning reader for fluorescence, absorbance, time resolved fluorescence, and luminescence.

Screening

Figure 1. Screening of Ramos and Jurkat Cells Using PrestoBlue Cell Viability Reagent as the Read-out.

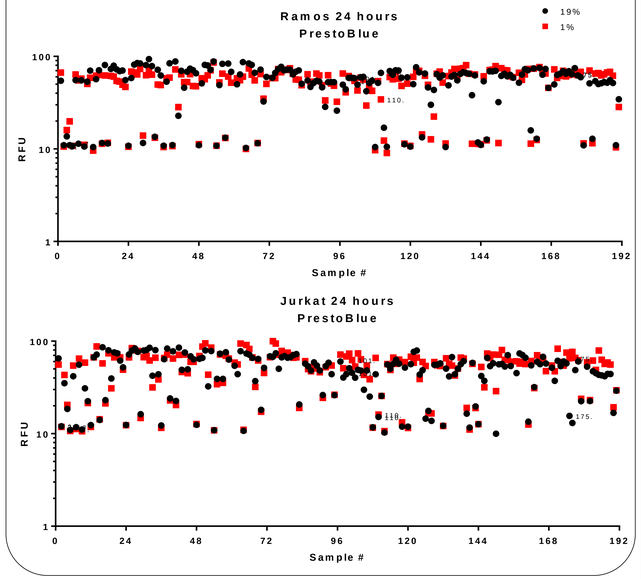
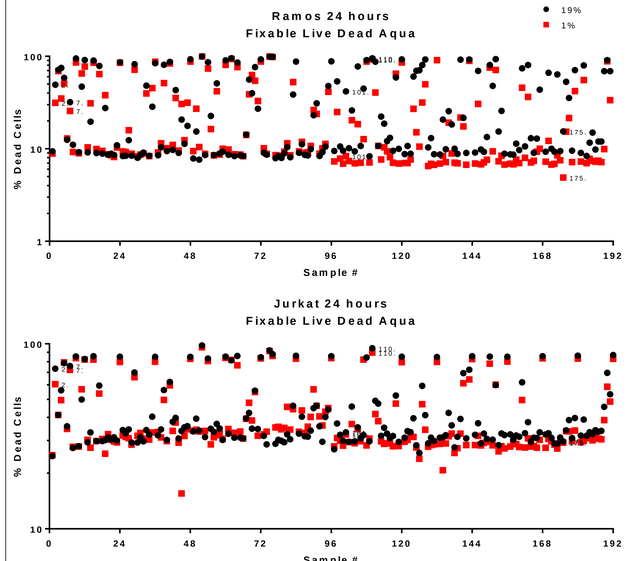


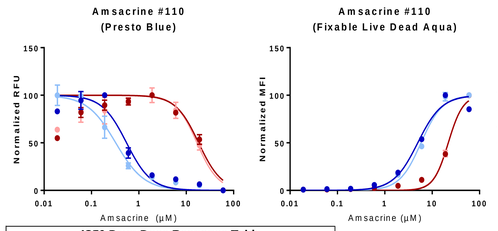
Figure 2. Screening of Ramos and Jurkat Cells Using LIVE/DEAD Fixable Aqua Dead Cell Stain as the Read-out.



Cells plated at 40,000 cells per well, then treated with 10 µM of each drug from the Killer Collection from MicroSource Discovery Systems, Inc., and stored at either 19% Oxygen or 1% Oxygen in 5% CO_2 and at 37 ºC for 24, 48, and 72 hours. At for 24, 48, and 72 hours cells were treated with either LIVE/DEAD Fixable Aqua Dead Cell Stain or PrestoBlue Cell Viability Reagent and analyzed on either the Attune NxT or the Varioskan Flash.

Cherry Picking

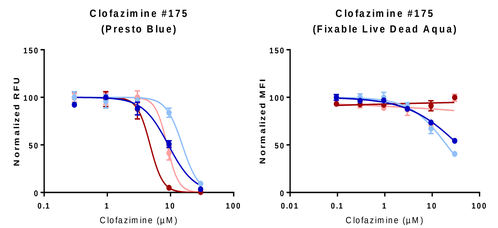
Figure 3. Jurkat and Ramos Cells React Differently to the Same Drug



IC50 Drug Dose Response Table				
Amsacrine	PrestoBlue Cell Viability Reagent		LIVE/DEAD Fixable Aqua Dead Cell Stain	
Cell Type	**19% Oxygen**	**1% Oxygen**	**19% Oxygen**	**1% Oxygen**
Jurkat	~ 0.57 µM	0.26 µM	4.96 µM	6.60 µM
Ramos	~ 23.30 µM	~ 20.17 µM	~ 71.57 µM	~ 64.95 µM

Amsacrine (known chemotherapeutic for Leukemia) is a more potent drug for Jurkat cells than Ramos cells.

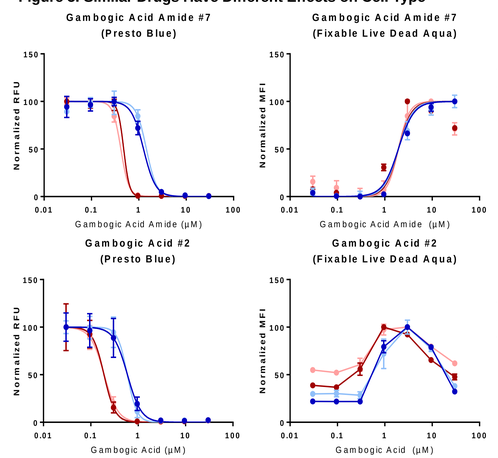
Figure 4. Oxygen Levels have an Effect on Drug Potency



IC50 Drug Dose Response Table				
Clofazimine	PrestoBlue Cell Viability Reagent		LIVE/DEAD Fixable Aqua Dead Cell Stain	
Cell Type	**19% Oxygen**	**1% Oxygen**	**19% Oxygen**	**1% Oxygen**
Jurkat	10.69 µM	~ 30.86 µM	NA	NA
Ramos	4.77 µM	~ 9.21 µM	NA	NA

Clofazimine (antibiotic used in multi-drug therapy for leprosy) is a more potent drug at 19% Oxygen for both Jurkat and Ramos cells when analyzed using cellular reduction potential as a cell health readout.

Figure 5. Similar Drugs Have Different Effects on Cell Type



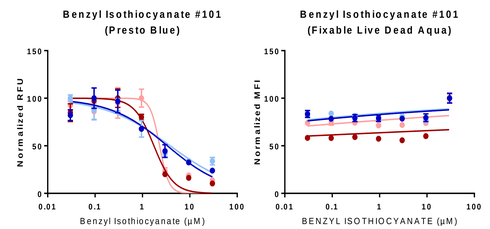
IC50 Drug Dose Response Table				
Gambogic Acid Amide	PrestoBlue Cell Viability Reagent		LIVE/DEAD Fixable Aqua Dead Cell Stain	
Cell Type	**19% Oxygen**	**1% Oxygen**	**19% Oxygen**	**1% Oxygen**
Jurkat	0.60 µM	0.58 µM	2.50 µM	2.78 µM
Ramos	0.19 µM	0.19 µM	1.01 µM	2.25 µM

IC50 Drug Dose Response Table				
Gambogic Acid	PrestoBlue Cell Viability Reagent		LIVE/DEAD Fixable Aqua Dead Cell Stain	
Cell Type	**19% Oxygen**	**1% Oxygen**	**19% Oxygen**	**1% Oxygen**
Jurkat	0.60 µM	0.58 µM	~ 0.57 µM	~ 0.53 µM
Ramos	0.19 µM	0.19 µM	~ 0.30 µM	~ 0.33 µM

Gambogic Acid (natural product from ***Garcinia hanburyi*** shown to activate caspases and have anti-cancer activity) is a more potent drug than the amide version of same compound.

Flow Analysis

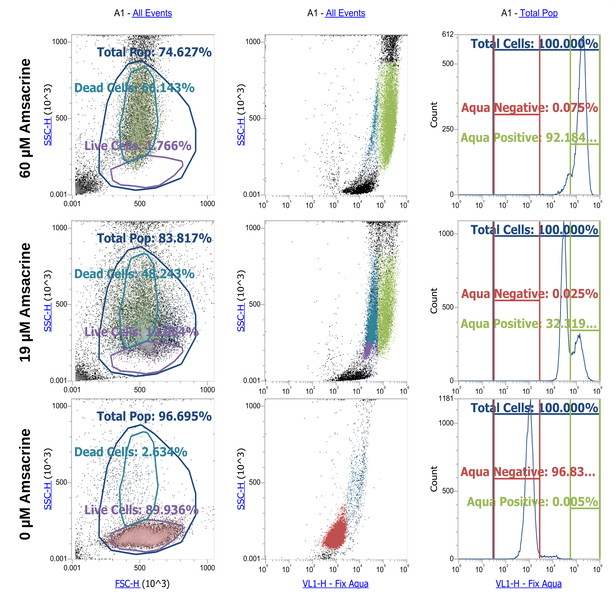
Figure 6. Mechanistic Read-outs Give Two Different results



IC50 Drug Dose Response Table				
Benzyl Isothiocyanate	PrestoBlue Cell Viability Reagent		LIVE/DEAD Fixable Aqua Dead Cell Stain	
Cell Type	**19% Oxygen**	**1% Oxygen**	**19% Oxygen**	**1% Oxygen**
Jurkat	1.48 µM	1.06 µM	NA	NA
Ramos	1.50 µM	~2.63 µM	NA	NA

Analyzing the redox potential of a cell gave a difference in compound potency compared to analyzing for membrane integrity.

Figure 7. Flow Analysis of Ramos cells Treated with Amsacrine



Three distinct populations are seen with flow cytometry; live, dead, and unhealthy cells. Further analysis with a different readout method(s) are planned for the near future.

CONCLUSIONS

❖Analysis of cell health by screening can benefit from a multi-parametric approach, including different mechanistic and platform readouts.

❖Flow cytometry and the use of multi-well plate auto samplers is an excellent tool for high throughput screening, and should be considered as a viable option for primary, secondary or tertiary compound screening.

❖Oxygen concentrations need to be considered when performing screens and determining compound potency.

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