Flow Cytometry Multiplexing and its Use in Detailed Characterization of Cell Health

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272 B112

ABSTRACT

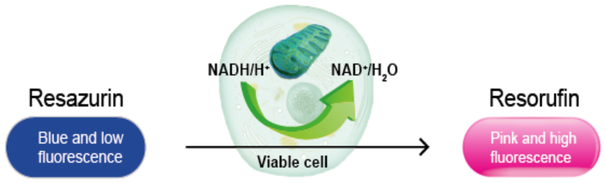
High throughput screening (HTS) is an extremely effective method for allowing researchers to identify putative compounds of interest which play a role in their area of cellular research. Recently, flow cytometry has emerged as a powerful HTS tool with the added benefit of cell-by-cell analysis. Flow cytometry not only allows a researcher to study drug effectiveness towards different cell types but also can be used to analyze protein-protein interactions, metabolic activities, as well as DNA/mRNA content in a single or multiplexed assay format.

In this study, we screened the MicroSource Discovery System Killer Plate compound library and compared HTS using a plate reader with flow cytometer multiplexing. Jurkat and Ramos cells, T and B lymphocyte cell types respectively, were used as cell models and cultured under standard conditions and screened either at hypoxic (1%) or hyperoxic (19%) oxygen levels at varied lengths of time (24, 48, or 72 hrs). To assess compound veracity, post-screening analysis was implemented to establish EC50s of "hits" from the compound library. Membrane integrity and metabolic activity were measured as an initial screening output for evaluating cellular viability. Using the multiplexed capabilities of flow cytometry, we performed secondary and tertiary assays to further characterize the possible mechanism of action by analysis of cell cycle, specific RNA expression levels, oxidative stress, and caspase activation across differing concentrations of the compounds. This method and analysis highlights the complicated nature of assessing toxicity in cellular screening assays and the advantages of Flow Cytometry in particular for characterizing cell health.

PrestoBlue® Cell Viability Reagent

Cell viability as indicated by the fluorescence of reduction product.

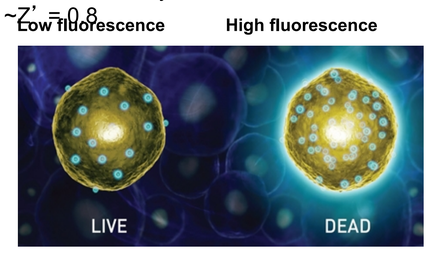
- Resazurin (PrestoBlue reagent) is reduced by the intracellular environment of living cells. ~Z' = 0.7
- Cell viability was determined by measuring the fluorescence intensity of total cells per well using a Varioskan™ Flash plate reader.



LIVE/DEAD® Fixable Aqua Dead Cell Stain

Cell viability evaluated based on cell membrane integrity.

- For cells with compromised plasma membranes, amine reactive dye labels both the cell surface and the cell interior. For intact cells, dye labels only the cell surface.
- Cell viability was determined by the mean fluorescent intensity (MFI) of total cell population. ~Z' = 0.8



RESULTS

Screening

Figure 1a. Varioskan™ High Throughput Screening of Ramos Cells with PrestoBlue Cell Viability Reagent

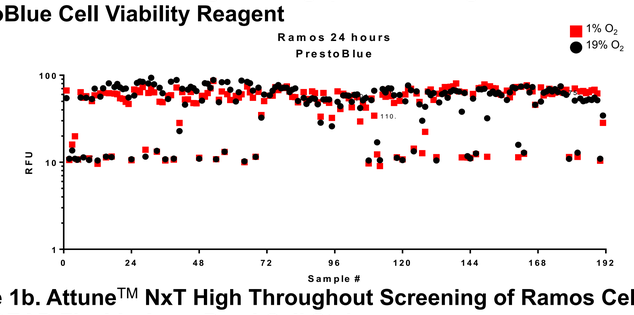
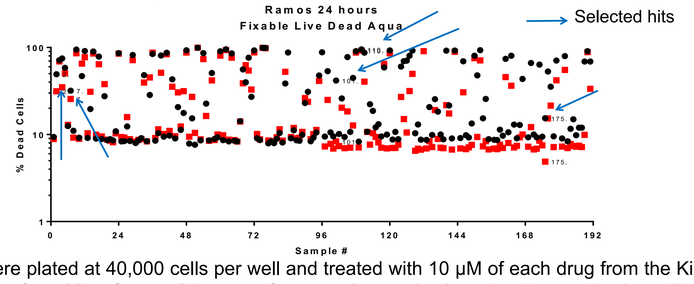


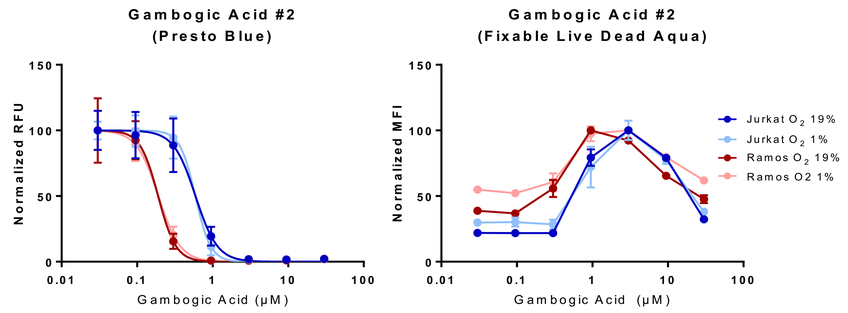
Figure 1b. Attune™ NxT High Throughout Screening of Ramos Cells with LIVE/DEAD Fixable Aqua Dead Cell Stain



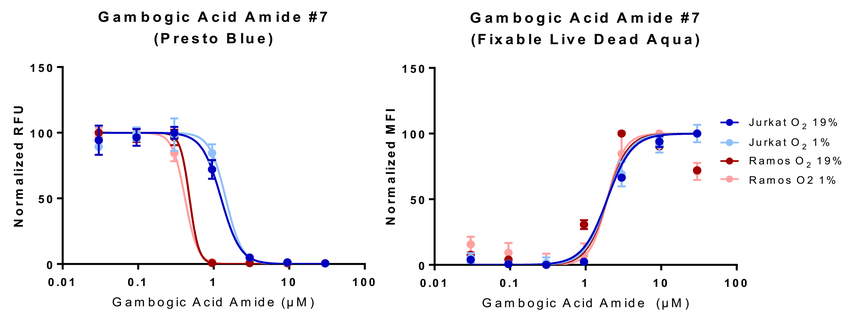
Cells were plated at 40,000 cells per well and treated with 10 µM of each drug from the Killer Collection from MicroSource Discovery Systems, Inc. and cultured under standard conditions and either at hypoxic (1% O_2) or hyperoxic (19% O_2) levels using a Heracell™ VIOS 160i Tri-Gas Incubator. Cells were incubated at three time periods with the compound library. At 24, 48, and 72 hours post compound treatment, cells were assayed with either PrestoBlue Cell Viability Reagent or LIVE/DEAD Fixable Aqua Dead Cell Stain. These were analyzed with either the Varioskan™ Flash Multimode Reader or the Attune™ NxT Acoustic Focusing Cytometer.

Attune™ NxT Autosampler HTS: "Cherry Picking": Different Readouts Confirm Potency of Drug

Figure 2. Gambogic acid is more potent than the amide form



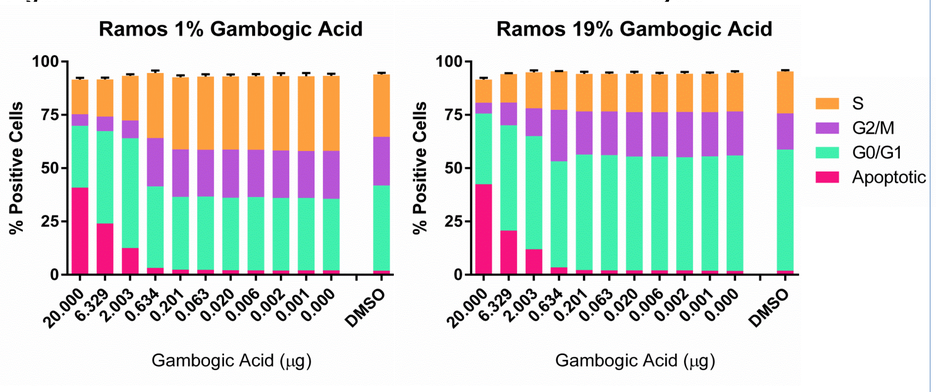
Gambogic Acid Response EC_{50}				
PrestoBlue			LIVE/DEAD	
19% O_2	1% O_2		19% O_2	1% O_2
0.6 µM	0.6 µM	Jurkat	~0.6 µM	~0.5 µM
0.2 µM	0.2 µM	Ramos	~0.3 µM	~0.3 µM

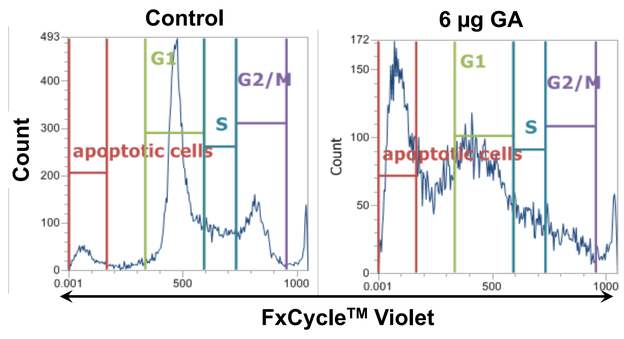


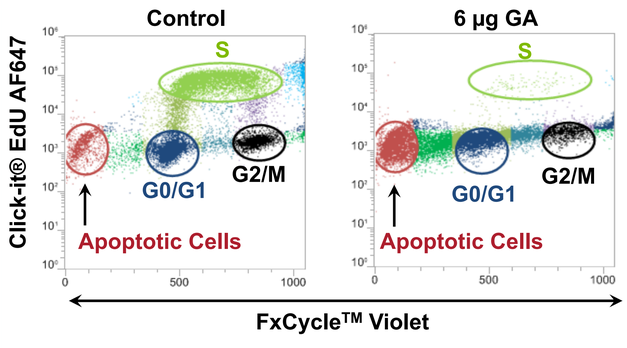
Gambogic Acid Amide Response EC_{50}				
PrestoBlue			LIVE/DEAD	
19% O_2	1% O_2		19% O_2	1% O_2
1.3 µM	1.5 µM	Jurkat	2.5 µM	2.8 µM
0.5 µM	0.4 µM	Ramos	1.0 µM	2.3 µM

Secondary Assays: Test mechanism of Action

Figure 3. Flow allows for detailed characterization of the cell cycle

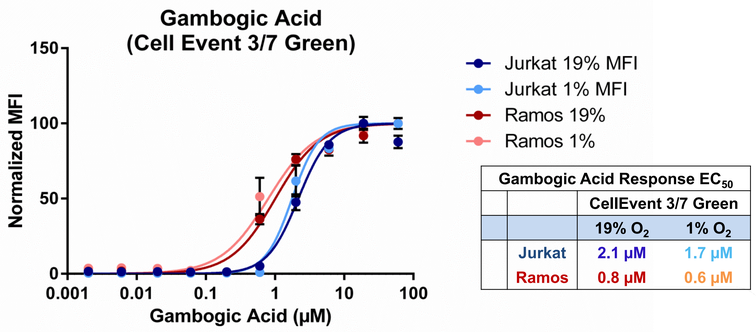




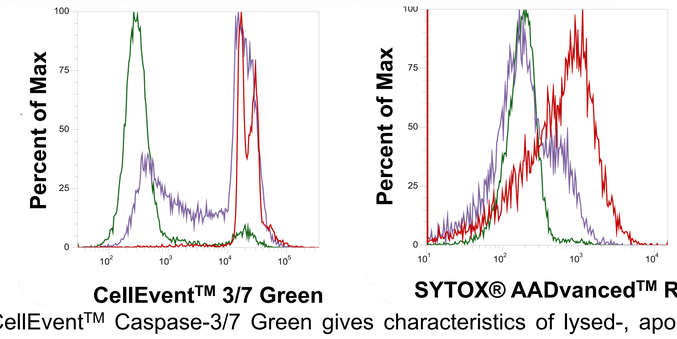


- Click-it® EdU allows for full cell cycle analysis on the Attune NxT flow cytometer.
- A greater number of proliferating cells is observed at 1% vs 19% oxygen levels.

Figure 4. Caspase Assay Confirms Differential Potency of Gambogic Acid

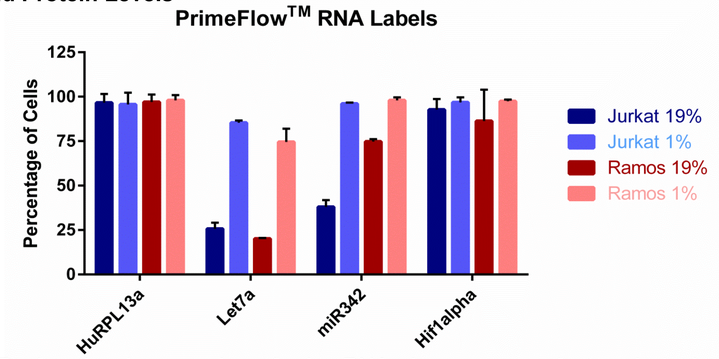


Gambogic Acid Response EC_{50}		
	CellEvent 3/7 Green	
	19% O_2	1% O_2
Jurkat	2.1 µM	1.7 µM
Ramos	0.8 µM	0.6 µM

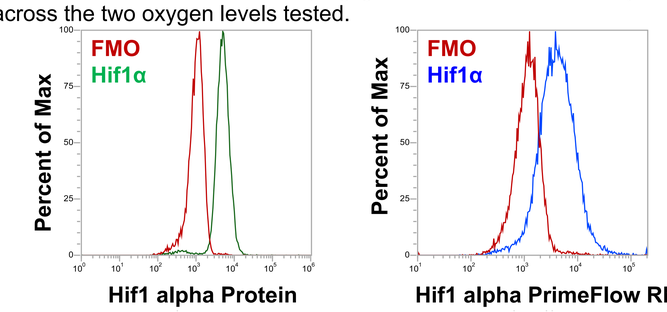


- CellEvent™ Caspase-3/7 Green gives characteristics of lysed-, apoptotic-, and normal cells.

Figure 5. PrimeFlow™ RNA allows for detailed characterization of RNA and Protein Levels



- Dysfunctional expression of microRNAs is a common feature in leukemia diseases, comparing the expression profiles of Burkitt's lymphoma (B-cells) and T-cell acute lymphoblastic leukemia (T-cells) cells , microRNA 342-3p shows higher expression levels in B-cells (Ramos-) than in T-cells (Jurkat).
- Introducing hypoxic conditions to T- and B-cells demonstrates microRNA levels are diverse. We show that common mciroRNAs such as let-7a are up-regulated in hypoxic conditions (1% O_2).
- Human RPL13a is a housekeeping gene that is shown to stay consistent across the two oxygen levels tested.



- Hif1α is a tumor suppressor gene and is up-regulated in hypoxic conditions. The duration of hypoxia incubation may promote or inhibit leukemia progression and maintenance, thus explaining the oncogenic or tumor suppressor activity.

SUMMARY

- Jurkat and Ramos cells displayed differential responses to compound treatment when studied across cell health, cell cycle and RNA expression assays.
- Cell health determination based on reduction potential of the cell using an HTS plate reader format and membrane integrity assay using flow cytometry showed higher potency of Gambogic Acid than it's derivative compound Gambogic Acid Amide.
- The statistics of flow cytometry allow for more detailed characterization of "hits"
- Biological applications on flow cytometry (Click-it EdU, Fixable LIVE/DEAD, PrimeFlow RNA, etc…) allow for a more specific analysis of a compound's possible mechanism of action.
- Future directions- Further analysis of "hits" or lead compounds to include assays shown here and an expanded miRNA panel.