



# A Multiplex Assay Approach to Screening Cytoactive Compounds for Cell Cycle Disruption and Apoptotic Activity on the Guava EasyCyte™ Plus System

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## Abstract

Control of cell cycle progression and initiation of the apoptotic cascade are tightly regulated processes complicated by the myriad of signaling pathways contributing to their regulation. Functional assays capable of interrogating both processes in live cells are critical for target validation and compound optimization. In order to improve screening efficiency, we have developed the microcapillary-based Guava EasyCyte Plus system, which, when used in conjunction with Guava's suite of functional assays, provides an ideal platform for cellular analyses. For over 200 test compounds analyzed, the following parameters were measured: (1) enzyme activity of caspases 3/7 and 8, (2) changes in mitochondrial membrane potential, (3) translocation of phosphatidylserine residues to the cell surface, (4) membrane permeability, and (5) cell cycle distribution. The simultaneous interrogation of caspase activity and cell cycle progression, along with the information regarding the relative frequencies of treated cells in the early, middle or late stages of apoptosis, offers a powerful tool for both "hit" compound identification and endpoint analysis. In addition, this strategy provides a more detailed understanding of the mechanism of action both at the level of the individual cell and the overall cell population.

## Introduction

The ability to induce growth arrest and apoptosis in tumor cells is critical for eliciting a positive response to chemotherapy. The importance of proper cell cycle regulation is underscored by the fact that unrestricted growth following loss of checkpoint control at the G1/S boundary is a hallmark of many solid tumors. Under appropriate cues, resting (G0/G1 phase) cells are directed to replicate their entire chromosomal complement (S phase). With DNA content doubled, G2/M phase cells are now poised for mitotic partitioning into two equivalent daughter cells. The Guava® Cell Cycle assay provides a simple screening method for detecting changes in cell cycle kinetics and DNA content through staining fixed/permeabilized samples with propidium iodide (PI), a fluorescent DNA intercalating agent<sup>1</sup>.

Apoptosis, or programmed cell death, is characterized by a progressive series of morphological and biochemical changes ultimately resulting in disassembly of the cell proper. These changes include the externalization of phosphatidylserine (PS) residues to the cell surface, loss of mitochondrial membrane potential coincident with release of cytochrome *c*, fragmentation of nuclear chromatin, and final loss of membrane integrity<sup>2-3</sup>. Caspases (cysteinyll directed

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aspartate-specific proteases) are a family of proteolytic enzymes that mediate key aspects of this process, from initiating early signaling events (caspase-2, -8, -9) to directing cellular breakdown through cleavage of structural proteins (caspase-3, -6, -7). In response to proper stimuli, site-directed cleavage results in conversion of functionally inert pro-caspase molecules to their active forms that then act to propagate the apoptotic cascade<sup>4-5</sup>.

The ability to obtain multi-parameter information on compound behavior in the context of a living cell quickly and easily is invaluable to the drug discovery process. Furthermore, with the use of Guava Technologies® on-demand cytometry platform, data is collected on individual cells in an automated fashion, enabling users to detect sub-population effects and analyze different cell types or lineages in the same assay. Multiple parameters of cell function can be interrogated in the same assay, allowing researchers to detect changes in cell viability, apoptosis, and other cellular processes that can reveal aspects of a compound's specificity, toxicity, and mechanism of action.

In the example illustrated here, 200 cytoactive compounds were evaluated on the Guava EasyCyte Plus microplate screening system for their ability to disrupt cell cycle progression by assessing cellular DNA content. Each compound was further profiled through an additional series of assays to assess cell viability, distinct apoptosis indicators (caspase-3/7, caspase-8, annexin-V), and changes in mitochondrial membrane potential. Taken together, this multiplex data set provides a more detailed view on the behavior of each of the test compounds with respect to both apoptotic induction and cell cycle progression.

## Methods CELLS

The non-adherent T-cell derived Jurkat (ATCC, #TIB-152) cell line was used for these studies. Prior to compound treatment, cells were then maintained in log phase using medium designed for optimal growth. Cultures were then synchronized for 24 hours in complete medium minus serum. For screening experiments, Jurkat cells were seeded at 50,000 cells/well (150 µL total volume) in 96-well round bottom polystyrene microplates (BD-Falcon, #353070) just prior to induction. Following induction, cells were harvested by centrifugation and assayed as described below.

## COMPOUNDS

A panel of 200 cytotoxic, immunosuppressive, anti-proliferative, and anti-inflammatory compounds was obtained from MicroSource Discovery Systems, Inc. For initial screening, a 10 mM stock solution of each compound was diluted in

2 steps to 10 mM in cell culture medium just prior to use. Negative controls (0.1% DMSO) and positive controls for apoptosis (1 mM staurosporine) were also included on each plate assayed. Treated plates were incubated for 4 and 20 hours. For hit confirmation and potency determination, selected compounds were titrated out to the indicated concentrations.

## GUAVA CASPASE-3/7 & CASPASE-8 ASSAYS

Both assays were performed according to manufacturer's instructions. Following harvest, cells were resuspended in 100 µL media. To this 10 mL of 10X FLICA reagent, caspase-3/7 specific (#4500-0290) or caspase-8 specific (#4500-0300), was added and cells were incubated at 37 °C for 1 hour in a 5% CO<sub>2</sub> incubator. Plates were removed, washed, and then labeled with propidium iodide for an additional 5 minutes. Samples (2000 cells/well) were acquired on a Guava EasyCyte system using ExpressPlus software.

## GUAVA MITOCHONDRIAL DEPOLARIZATION ASSAY

The Guava Mitochondrial Depolarization Assay (#4500-0250) was conducted according to manufacturer's instructions. Following harvest, cells were resuspended in 200 µL media. A volume of 2 µL 100X JC-1 solution and 2 µL 7-AAD was then added to each well. Plates were incubated for 30 minutes at 37 °C in a 5% CO<sub>2</sub> incubator. Samples (2000 cells/well) were acquired on a Guava EasyCyte system.

## GUAVA NEXIN® ASSAY

The Guava Nexin assay (#4500-0161) was conducted per the manufacturer's instructions. Following harvest, cells were resuspended in staining solution containing annexin V-PE and 7-AAD, then incubated in the dark at RT for 20 minutes. Samples (2000 cells/well) were acquired on the Guava EasyCyte system.

## GUAVA CELL CYCLE

Following induction, samples were harvested, washed in 1X PBS, and fixed overnight at -20 °C in 70% ethanol. Cells were washed, stained for DNA content with propidium iodide, and acquired (3000 cells/well) according to the Guava Cell Cycle protocol. A compound was considered to have induced cell cycle arrest if the percentages of cells in one of the phases of the cell cycle were three standard deviations (SD) above the average of the negative controls.

## Results

The Guava Cell Cycle Assay was used in combination with Guava Technologies' suite of apoptosis assays to screen a panel of small molecules for their ability to arrest cell division in human Jurkat cells. In addition, the 200 cytoactive compounds plus control compounds were assessed at two time points for the induction of the following markers of apoptotic progression: (1) externalization of phosphatidylserine (PS) (Nexin Assay), (2) elevations in active caspase enzymes (caspase-3/7 and 8 assays), and (3) loss of mitochondrial membrane potential (MitoPotential Assay). Each of these assays also contains a cell-impermeant DNA stain (either PI or 7-AAD) permitting distinction of late-stage apoptotic/dying cells.

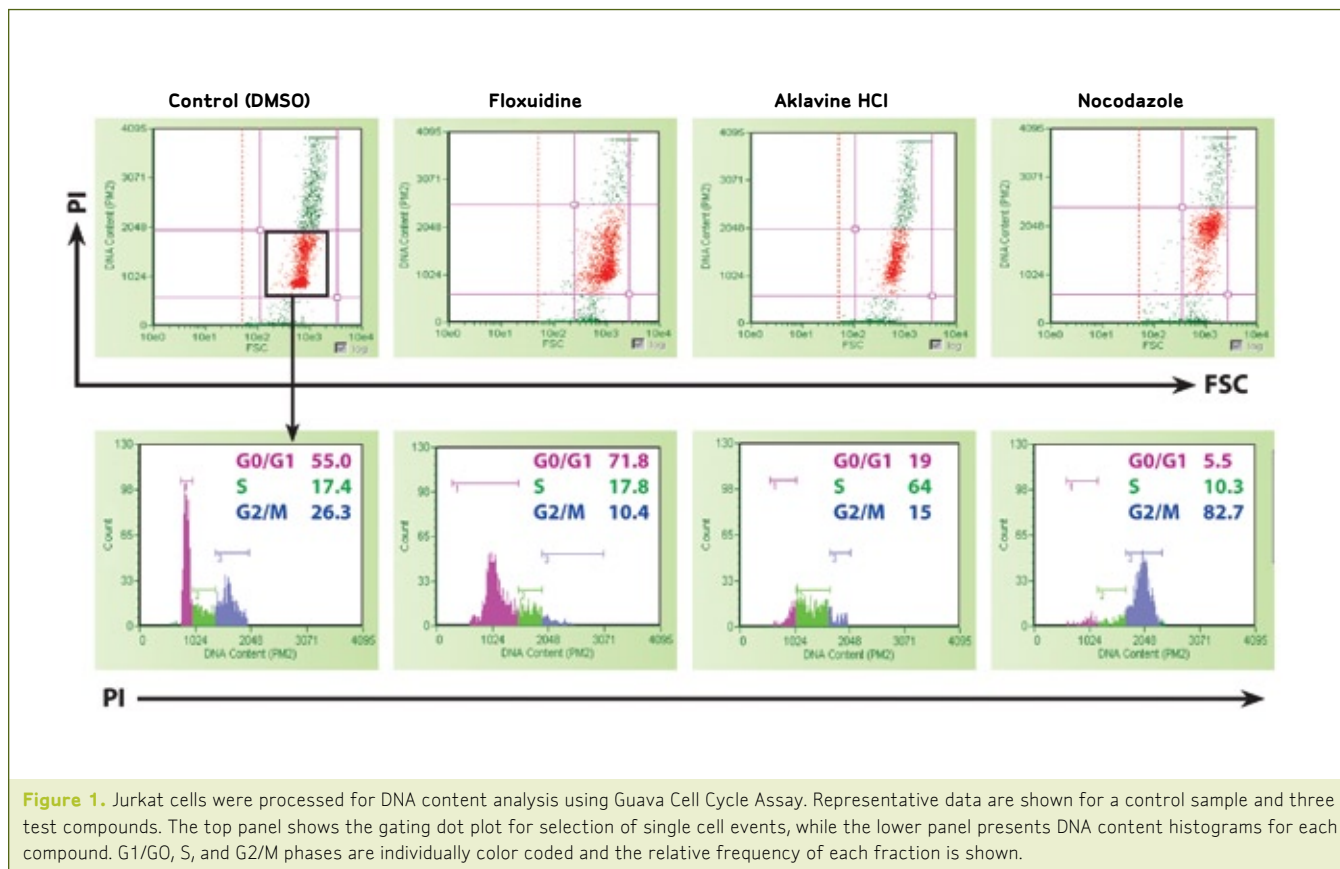
## GUAVA CELL CYCLE ASSAY

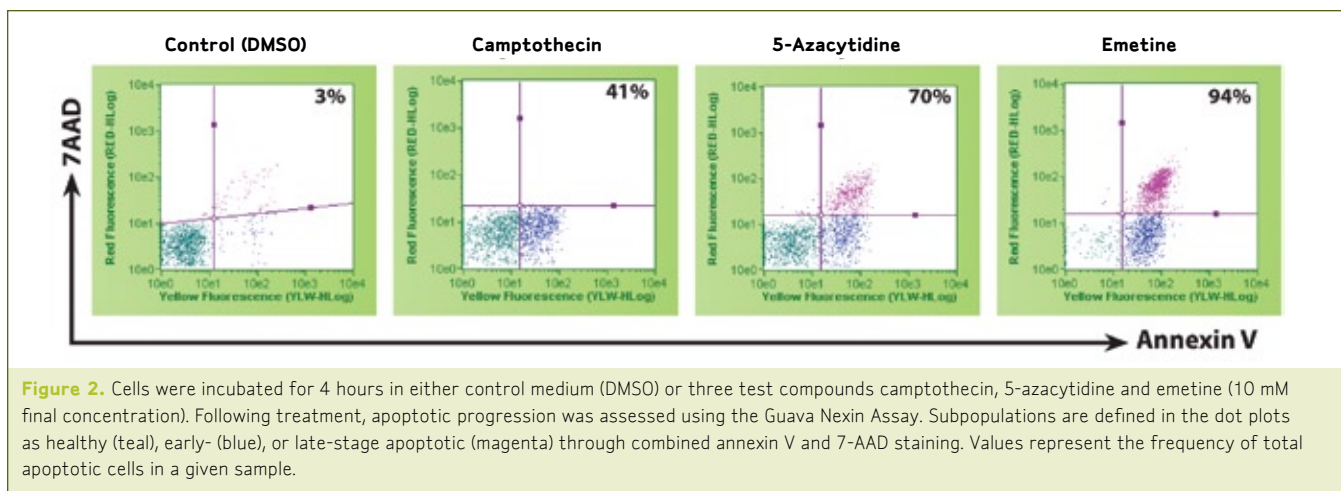
Once released from serum starvation and allowed to grow in complete medium, the cells were found primarily in the G0/G1 and G2/M phases. Treatment with drugs that block cell division led to changes in the frequency of cells in each phase relative to untreated cultures. Figure 1 shows DNA content histogram plots (PI staining) for untreated Jurkat cells as well as following exposure to three representative compounds. In each culture where cells have been arrested, the relative frequency of the arrested phase was increased over that seen in untreated samples. Treatment with floxuidine resulted in

an increase in the number of cells present in G0/G1 over that found in untreated samples. Aklavine HCl promoted S-phase arrest with 64% of cells in the S phase as compared to 17% in control wells. By contrast, exposure to nocodazole caused arrest of cultures in G2/M with 83% of cells in this phase as compared to 26% in control cells.

## GUAVA NEXIN ASSAY

Annexin V is a  $\text{Ca}^{2+}$ -dependent phospholipid binding protein with high affinity for phosphatidylserine (PS), a membrane bound component localized to the inner face of the cell membrane. An indicator of early-stage apoptosis is the detection of exposed PS residues that have translocated to the cell surface<sup>6-8</sup>. The Guava Nexin Assay permits simultaneous detection of early apoptotic events based on annexin V binding to exposed PS and late apoptotic/dead events through uptake of 7-AAD. From Figure 2, the vast majority of Jurkat cells in the untreated control were healthy and thus unstained for annexin V and 7-AAD. By contrast, exposure to any of the three compounds resulted in marked apoptotic induction, with emetine eliciting the strongest response (50% of apoptotic cells in late stages or dying). From the plots shown, it is evident that differences in the potency and mechanism of action contribute to variations in apoptotic progression induced by each test compound.





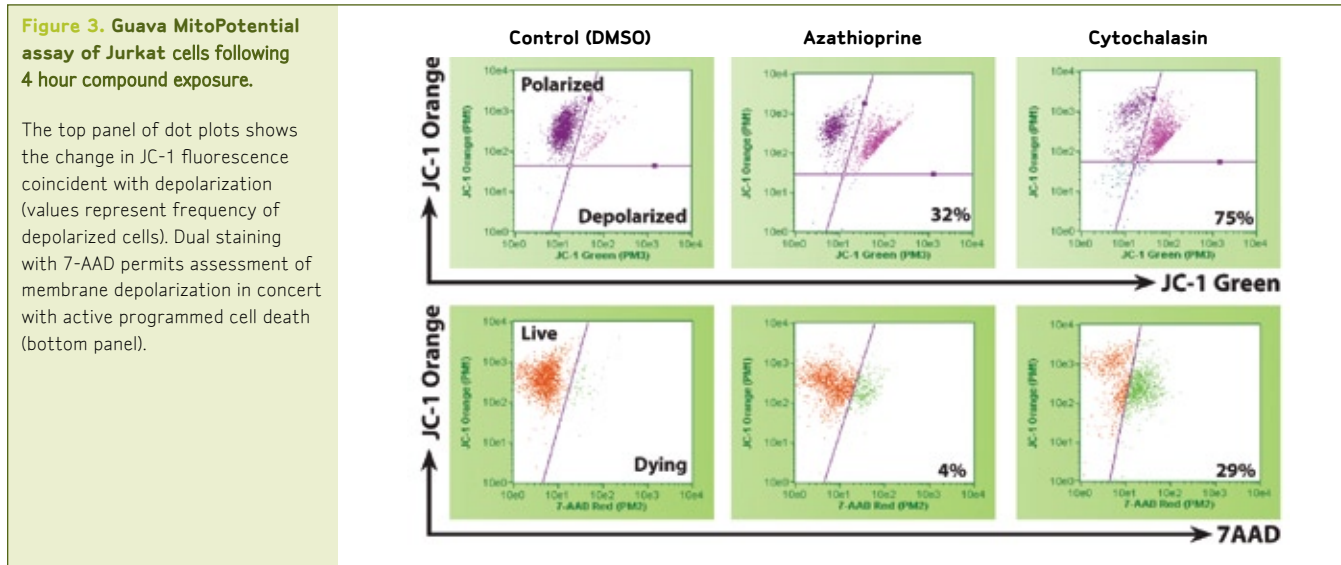
### GUAVA CASPASE ASSAYS

Caspase proteins are intracellular proteases actively involved in the degradation of a wide range of cellular components during the apoptotic cell death. The Guava caspase-8 and Caspase-3/7 Assay kits each employ a fluorescently-tagged cell permeable caspase-specific FLICA (Fluorescent Inhibitor of Caspases) reagent which binds specifically to active caspase molecules. The FLICA reagent is used in concert with the cell impermeant dye PI to permit simultaneous evaluation of apoptosis as a function of caspase activity and generalized cell death (membrane integrity).

### GUAVA MITOCHONDRIAL DEPOLARIZATION ASSAY

Collapse of the mitochondrial inner transmembrane potential is believed to be coincident with release of cytochrome c into the surrounding cytosol, ultimately resulting in the activation of downstream events in the apoptotic cascade<sup>9,10</sup>. The Guava Mitochondrial Depolarization Assay combines JC-1, a ratiometric dye that fluoresces either orange (polarized) or

green (depolarized) depending upon membrane potential,<sup>11</sup> with 7-AAD to assess membrane permeability changes commonly observed in late-stage apoptosis. This two-dye assay not only enables users to monitor the changes in mitochondrial membrane potential as a function of apoptotic state, but also allows for the identification of those physiological conditions where either cellular event may occur independently. Untreated Jurkat cells are predominantly healthy and, with respect to JC-1 fluorescence, in a polarized state (Figure 3). By contrast, exposure to either azathioprine or cytochalasin A for 4 hours resulted in a significant shift of mitochondrial membrane potential, with cultures being 32% and 75% depolarized, respectively. While each compound was effective at inducing depolarization, only wells treated with cytochalasin A showed high levels of cell death. While this finding suggests that azathioprine possesses no apoptotic capability, significant numbers of 7-AAD positive staining cells were found in cultures treated for 20 hours. From these findings, it is apparent that the kinetics of azathioprine action are slower than that of cytochalasin A.



## MULTIPARAMETER ANALYSIS OF "HIT" COMPOUNDS

In all, 200 cytoactive compounds were tested. By the second induction point, 35% of the compound panel (70 of 200) exhibited some biological effect, although the action and/or magnitude of this influence varied greatly from compound to compound. For compounds causing arrest in G0/G1, 7 of 12 had little or no pro-apoptotic activity. Two compounds, dichlorophene and suramin, however, did show a change in JC-1 fluorescence. Similarly, among the 11 small molecules that promoted a G2/M arrest, virtually no signs of apoptotic induction were detected after 4 hours. However, 55% (6 of 11) showed significant apoptotic progression following 24-hour treatment. For those compounds that blocked cell division at the G2/M boundary, 64% (7 of 11) also induced expression of apoptotic markers at the early time point. Some compounds elicited no effect on cell cycle regulation but showed significant capacity to alter membrane potential and/or apoptotic induction<sup>12</sup>.

## "HIT" COMPOUND TITRATION FOR EC50 DETERMINATION

Using Jurkat cells, six "hit" compounds were retested to confirm their activity and determine their EC50 values for the various stages of apoptosis<sup>12</sup>. Cells were cultured for 20 hours in the presence of varying concentrations of each test compound then assessed for apoptotic progression using the Guava Nexin and Guava Caspase-3/7 assays. In Figure 4, each curve represents the mean  $\pm$  SD for three independent trials. Results replicated what was observed in

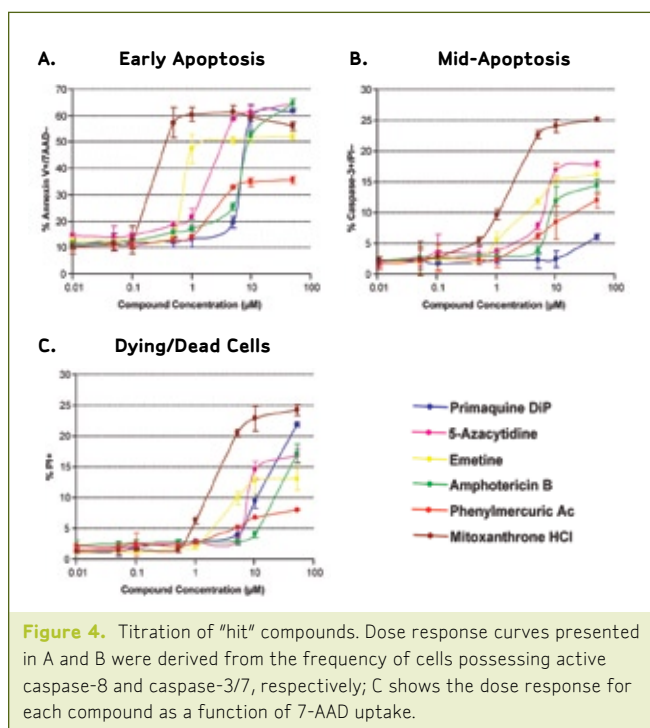
the previous screening experiment. Of the six, mitoxanthrone HCl demonstrated the strongest induction capacity at all stages, while all other compounds showed a much slower accumulation of dead and late-stage apoptotic cells. Interestingly, all six compounds tested induced apoptosis, whether directly or indirectly, through activation of the caspase-8 pathway.

## Summary

The microplate-based Guava EasyCyte Plus System, when used in conjunction with Guava Technologies' suite of functional assays, provides the ideal platform for the multiplex approach to cellular analysis required during both initial target validation and compound assessment for drug discovery. As shown for the 200 compounds tested, this combination of assays can easily identify both cell cycle inhibitors and apoptotic inducers, as well as characterize their EC50 values and quantify the percentage of cells affected. The detection of sub-population effects for these compounds highlights the benefit of a platform capable of analyzing populations at the single cell level. Because of the difference in the types of apoptotic cells detected (early vs. mid vs. late for Guava Nexin, Guava Caspase-8, and Caspase-3/7, respectively), initial screening of compounds for cytoactive capacity may only require the use of a single reliable apoptosis assay. However, to better understand the mechanism of action for a given compound and to gain more information concerning potential side effects (potency, toxicity, and specificity), the multi-assay approach can be applied.

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**Figure 4.** Titration of "hit" compounds. Dose response curves presented in A and B were derived from the frequency of cells possessing active caspase-8 and caspase-3/7, respectively; C shows the dose response for each compound as a function of 7-AAD uptake.



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