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High-throughput flow cytometric screening of a kinase inhibitor library for apoptosis induction

Cayman CHEMICAL

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Key Information

- Cayman's multiparametric Early Apoptosis Detection Assay Kit used with Miltenyi Biotec's MACSQuant® X Flow Cytometer creates a powerful, efficient, and exquisitely detailed screening tool.
- Using this approach to screen a library of kinase inhibitors for cytotoxicity, apoptosis inducers were distinguished from necrosis-causing or mitochondrial-uncoupling compounds.
- This simple approach can be adapted to many other probes for cellular function to help determine the effects of potential therapeutic compounds during the drug development process.

Introduction

Apoptosis, or programmed cell death, is one way that cells can react to an external stimulus. Several different apoptotic pathways are well described, including both extrinsic and intrinsic drug-induced pathways. A non-inflammatory apoptotic response differs from necrosis (a more pro-inflammatory type of cell death), which could be highly damaging if induced by a drug. For this reason, cytotoxicity screening is a very important component of pre-clinical drug testing.

Several probes have been used to detect different modes and stages of cell death. The protein Annexin V binds to externalized phosphatidylserine in the plasma membrane, one of the processes downstream of all apoptotic pathways. More recently, the nucleic acid dye TO-PRO®-3 has been described to be selectively transported by pannexin channels in the plasma membrane, which open early in apoptosis to allow the release of 'find me' and 'eat me' signals. Therefore, TO-PRO-3 fluorescence can detect cells early in apoptosis, before the externalization of phosphatidylserine. In addition, TMRE, a fluorescent mitochondrial membrane potential probe, accumulates in healthy mitochondria. Since the opening of the mitochondrial permeability transition pore is a key event in apoptosis, loss of TMRE fluorescence can serve as another indicator of apoptotic processes. Notably, mitochondria can lose membrane potential without triggering cell death, making the addition of TMRE important for measuring gross mitochondrial health (figure 1).

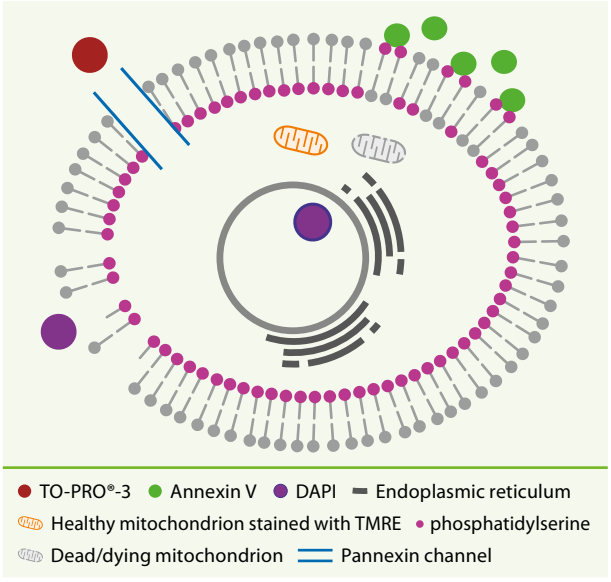

Figure 1: Detection of different modes and stages of apoptosis.

Traditional methods for detecting cytotoxicity of potential therapeutic compounds have relied on less sensitive techniques, which are amenable to high throughput. These include the detection of LDH release, tetrazolium salt reduction to formazan, and ATP. These methods can be useful, but finer understanding of the mechanism of cell death may be more helpful in developing effective therapeutics. Flow cytometry has not traditionally been used as a screening tool due to low throughput. That is, samples are analyzed individually, and automation is frequently unreliable, resulting in a requirement for high user input. The MACSQuant® X Flow Cytometer was developed to counter these issues and make flow cytometry possible at significantly higher throughput levels. This flow cytometer can analyze as little as 5 µL of sample reliably, can automatically sample 384-well plates, and maintains the multiplex-ability that makes flow cytometry an attractive tool for obtaining maximal information from each sample.

Methods

Jurkat cells were plated at 4×10^5/well in two 96-well plates in complete medium and treated overnight with 1 µM compound from Cayman's Kinase Screening Library (Cayman Item No. 10505). Up to 160 compounds are in this library, and the remainder of the wells were used as controls. DMSO was used as a vehicle control, and staurosporine was used as a positive control. Cells were stained as described in the kit booklet for the Early Apoptosis Detection Assay Kit (Cayman Item No. 601360), using Annexin V FITC, TMRE, and TO-PRO®-3. After staining, cells were washed, resuspended in PBS with DAPI, and transferred to 384-well plates in quadruplicate. Cells were analyzed in the 384-well plates using the MACSQuant X Flow Cytometer with the following settings: Collect 15 µL sample, high speed, fast wash, shake the plate every six wells; collect data in channels V1, B1, B2, and R1. Using these settings, each 384-well plate was collected in about 2.5 hours. An overview of the experimental setup is displayed in figure 2.

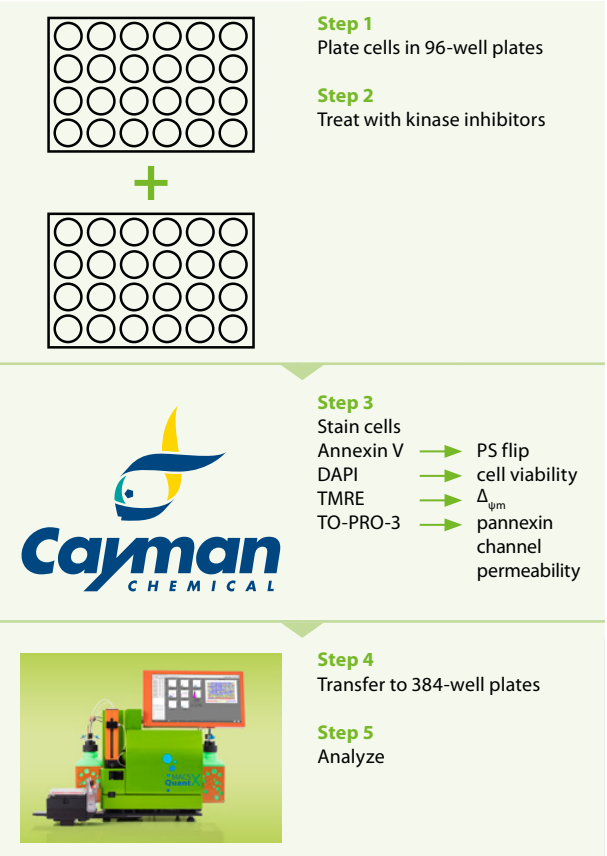

Figure 2: Experimental overview of high-throughput multiparameter apoptosis screening.

Data analysis

Flow cytometric data were analyzed in FlowLogic™ Software as shown in figure 3. An initial gate was drawn to enumerate "necrotic" cells (fig. 3A, $DAPI^+$). DAPI-negative cells were further gated on Annexin V-positive and -negative cells (fig. 3B). The Annexin V-negative population was then gated using forward scatter to differentiate "apoptotic" cells from apoptotic bodies and debris (fig. 3C). The Annexin V-negative cells were visualized in a histogram of TO-PRO-3 (fig. 3D), in which positive cells were described as "early apoptotic" and negative cells were "live".

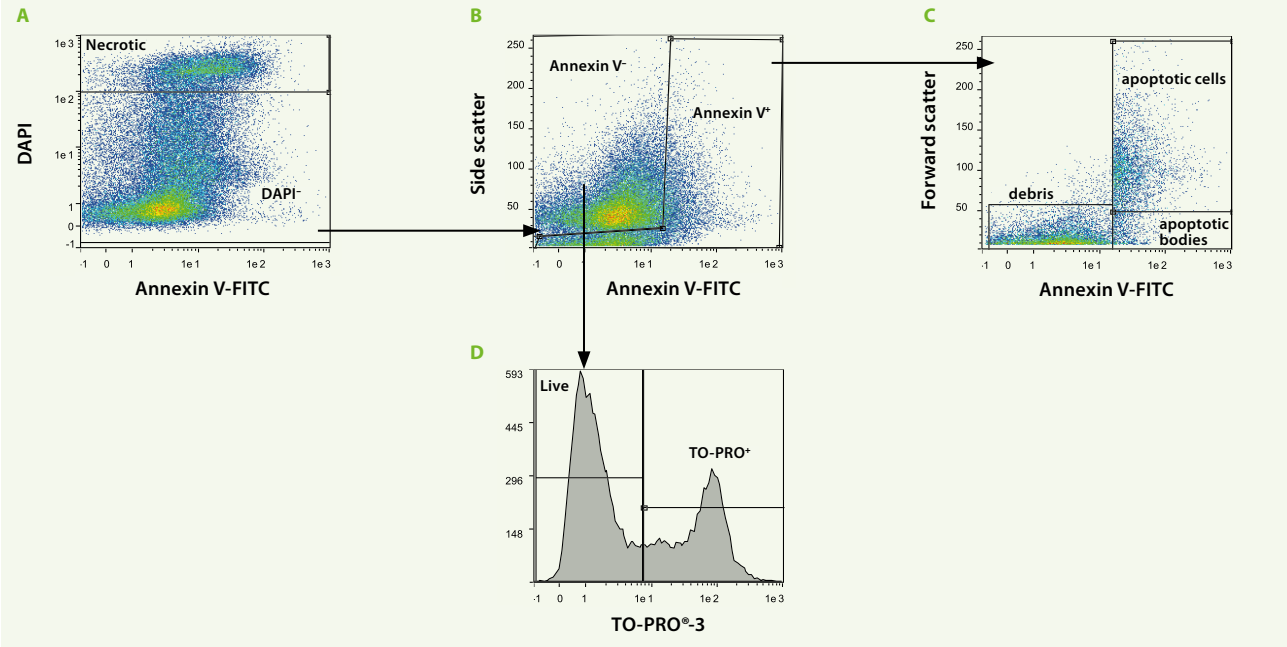

Figure 3: Gating scheme for analysis of flow cytometric data.

Results

The percentage of the total population falling into the "live" (DAPI⁻, Annexin V⁻, TO-PRO-3⁻) category was used to flag cytotoxic compounds, as shown in the figure 4 dot plot of all compounds. The average of the DMSO and staurosporine-treated controls was 83% and 1.6%, respectively. Compounds that fell more than three standard deviations below the mean vehicle-treated live percentage (fig. 4 asterisk and dotted line) were flagged as potentially cytotoxic and are shown in the heat map in figure 5. Among the eleven flagged compounds, only staurosporine and PIK-75 (hydrochloride) were nearly completely cytotoxic, with most of the cell death being necrotic (>50% DAPI⁺).

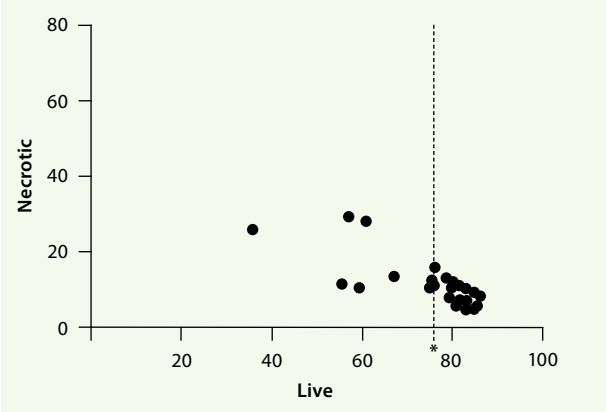

Figure 4: Flagging compounds that induce cell death. Each point represents the average of quadruplicate values for a single compound. The percentage of live cells at three standard deviations from the mean less than vehicle-treated samples is indicated with an asterisk and dotted line. Any compound with fewer live cells was flagged for further analysis.

The remainder of the cytotoxic compounds, as shown in figure 5 induced highly mixed and variable populations, suggesting different mechanisms of cytotoxicity. For example, paclitaxel, JNJ-10198409, and 17β-hydroxy wortmannin were equally cytotoxic overall (around 60% live), but paclitaxel and JNJ-10198409 tended to induce more early and late apoptotic responses, while 17β-hydroxy wortmannin induced much more necrosis in these cells. It may be worth noting that, generally, the TO-PRO-3 early apoptosis marker seemed to be more sensitive than Annexin V in this experiment. In most cases, when TO-PRO-3 was higher than average, Annexin V was also higher than average, providing support to the theory that these independent markers measure related processes in apoptosis. However, in a few cases (e.g., NVP-BEZ235), the percentage of cells that were TO-PRO-3-positive was above average while the Annexin V percentage was below average. This may be due to the cells being primarily early in the apoptotic process, or it may indicate some biologic effect of the compound on the pannexin channel itself. The multiparametric nature of this screen can help to elucidate the activity of the compounds in question in finer detail than commonly used screens like LDH release.

Furthermore, the addition of TMRE staining to the apoptotic stains in this screen allows the concomitant analysis of the health of the mitochondria, indicating potential mitochondrial toxicity. TMRE staining only occurs in healthy mitochondria with an intact membrane potential, while loss of staining indicates depolarization of the mitochondria. In general, TMRE tracked with apoptotic markers, and treatments that induced apoptosis also decreased TMRE positivity (figure 6). Notably, however, there were compounds that seemed to induce a loss of TMRE staining in the absence of an increase in apoptosis. For example, AG-17 showed relatively low induction of apoptosis and modest necrosis, but almost no TMRE staining, suggesting profound depolarization of the mitochondria without direct induction of the apoptotic pathway. This conclusion is supported by literature describing mitochondrial disruption by AG-17 in the low micromolar range.[1]

Cayman item no.	Name	Live	Early apoptotic	Necrotic	Apoptotic
10461	Paclitaxel	59.48	15.075	10.975	3.91
81590	Staurosporine	0.90	12.425	68.575	3.47
10565	NVP-BEZ235	74.73	8.295	11.1	1.08
11445	Tunicamycin	67.13	8.47	13.825	4.01
11491	AZD 7762	35.73	18.625	26.575	4.96
13812	17β-hydroxy wortmannin	57.13	7.3525	29.975	0.69
11569	GSK1059615	76.18	6.4875	11.825	1.55
10009210	PIK-75 (hydrochloride)	2.29	20.425	55.775	10.83
10010248	AG-17	76.13	3.4775	16.4	0.47
10008131	JNJ-10198409	55.63	15.325	12.125	3.89
10009557	SC-1	75.60	5.545	12.675	1.14

Figure 5: Further analysis of flagged compounds. The mean of quadruplicate percentages of total cells falling in each of the gates described in figure 3 are shown. Values near the mean of vehicle-treated cells are shown as the green end of the heat map, while values near the mean of staurosporine-treated cells are shown as the orange end of the heat map.

Cayman item no.	Name	Live	Early apoptotic	Necrotic	Apoptotic	TMRE (% control)
10461	Paclitaxel	59.48	15.075	10.975	3.91	80.36
81590	Staurosporine	0.90	12.425	68.575	3.47	-0.62
10565	NVP-BEZ235	74.73	8.295	11.1	1.08	155.19
11445	Tunicamycin	67.13	8.47	13.825	4.01	124.89
11491	AZD 7762	35.73	18.625	26.575	4.96	28.93
13812	17β-hydroxy wortmannin	57.13	7.3525	29.975	0.69	125.94
11569	GSK1059615	76.18	6.4875	11.825	1.55	169.73
10009210	PIK-75 (hydrochloride)	2.29	20.425	55.775	10.83	0.86
10010248	AG-17	76.13	3.4775	16.4	0.47	0.62
10008131	JNJ-10198409	55.63	15.325	12.125	3.89	72.03
10009557	SC-1	75.60	5.545	12.675	1.14	108.32

Figure 6: Addition of TMRE to assess mitochondrial health. TMRE staining is represented as a percentage of control (vehicle-treated cells). 100% indicates similar numbers of TMRE-positive cells as vehicle-treated samples (green), while 0% indicates similar numbers of TMRE-positive cells to staurosporine-treated samples (orange, loss of TMRE staining).

Conclusion

The multiplexed nature of this multiparameter cytotoxicity assay means that the effects of compounds can be finely dissected with a single experiment. We have shown, using the high-throughput MACSQuant® X Flow Cytometer, that a library of compounds can be screened efficiently and accurately by flow cytometry, yielding more complete results than commonly used screening methods. Several different cellular parameters can be analyzed in a single experiment in a highly automated manner, increasing the reproducibility of results.

Ref. no.	Product name	Provider
601360	Early Apoptosis Detection Assay Kit	Cayman Chemical
10505	Kinase Screening Library (96-well)	Cayman Chemical
81590	Staurosporine	Cayman Chemical
130-105-100	MACSQuant X Flow Cytometer	Miltenyi Biotec

Figure 7: Products used in this application.

Reference

1. Burger, A.M. *et al.* Tyrphostin AG17, [(3,5-Di-tert-butyl-4-hydroxybenzylidene)-malononitrile], inhibits cell growth by disrupting mitochondria (1995) Cancer Res. 55(13), 2794-2799.

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