Flow Cytometric Screening of a Kinase Inhibitor Library for Apoptosis Induction

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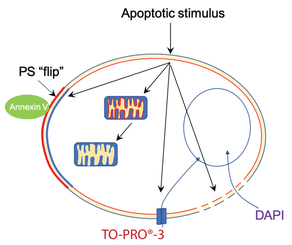
KEY FINDING

Multiparametric screening of kinase inhibitor cytotoxicity distinguishes multiple modes of cell death.

Introduction

Traditional methods of detecting cytotoxicity of potential therapeutic compounds have relied on less sensitive methods, which are amenable to high throughput. These include detecting 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.

Fluorescent probes can detect many different components of cell death and have the advantage of being able to be multiplexed, given the proper analysis platform. Annexin V binds to externalized phosphatidylserine, a marker of apoptosis. DAPI is a nucleic acid dye, which is only internalized after the plasma membrane is compromised in necrotic cells. TO-PRO®-3 is trafficked into the cell early in apoptosis through pannexin channels. TMRE is fluorescent only in healthy mitochondria.

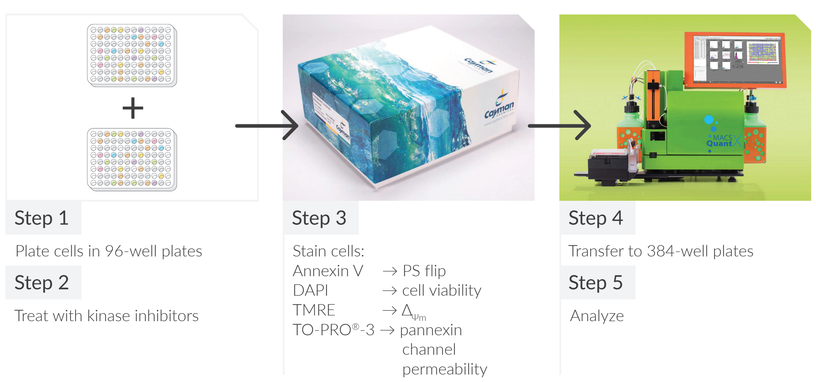


Flow cytometry has not traditionally been used as a screening tool due to low throughput. That is, samples are analyzed individually, and automation is frequently unreliable, thus demanding high user input. The MACSQuant® X has been developed to counter these issues and make flow cytometry significantly higher throughput. This flow cytometer can analyze as little as 5 µl of sample reliably, can automatically sample 384-well plates, and maintains the mutliplexability that makes flow cytometry an attractive tool for obtaining maximal information from each sample.

Methods

Jurkat cells were plated at 4 x 10^5/well in two 96-well plates in complete medium and treated overnight with 1 µM of compound from the Kinase Screening Library (Cayman Item No. 10505). Approximately 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 (Cayman Item No. 81590) 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 6 wells; collect data in channels V1, B1, B2, and R1. Using these settings, each 384-well plate was sampled in about 2.5 hours.

Cell Staining and MACSQuant® X Flow Cytometry Workflow



Results

Analysis of Flow Cytometric Data

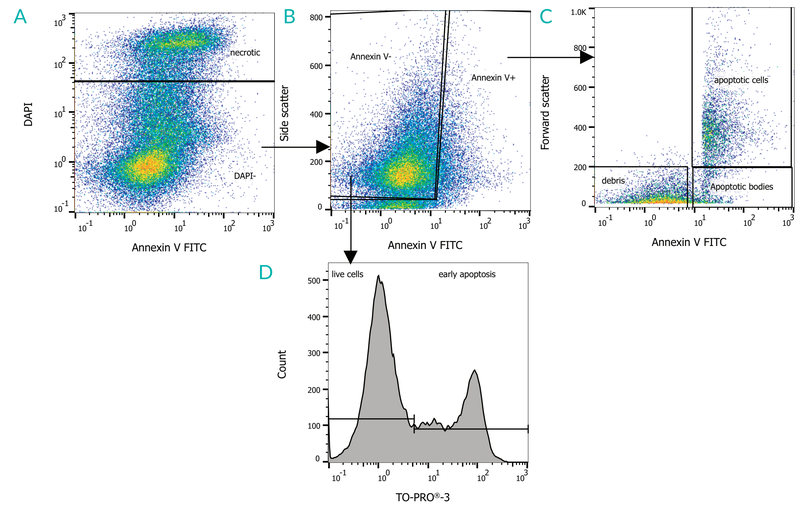


Figure 1. A. An initial gate was drawn to enumerate necrotic cells (DAPI+). B. DAPI-negative cells were further gated on annexin V-positive and -negative cells. C. The annexin V-positive population was then gated using forward scatter to differentiate cells from apoptotic bodies and debris. D. The annexin V-negative cells were visualized in a histogram of TO-PRO®-3, in which positive cells were described as early apoptotic and negative cells were live.

Heat Map Reveals Potentially Cytotoxic Compounds

Item No.	Item Name	Live
10398	TG003	84.88
11022	BIBF 1120	85.25
11793	SB-505124	86.25
13122	CHIR99021	85.78
13303	NSC 663284	85.23
13325	Iso-Olomoucine	84.28
13601	ZM 447439	81.83
62575	N,N-Dimethylsphingosine (d18:1)	82.90
10459	PKC 412	81.05
11029	SMI-4a	84.88
11811	INK128	79.85
13123	BIO	83.88
13305	D 4476	85.65
13332	(S)-Glycyl-H-1152 (hydrochloride)	84.60
13622	AS-041164	84.43
70920	LY294002	82.88
10460	Doramapimod	83.95
11140	CAY10657	81.63
12076	Canertinib (hydrochloride)	84.45
13139	Imatinib (mesylate)	84.83
13308	NU 7026	84.75
13333	Bisindolylmaleimide VIII (acetate)	86.68
13641	NVP-AEW541 (hydrochloride)	84.38
70970	U-0126	83.58
10461	Paclitaxel	59.48
	Control	84.85
13031	SB-431542	84.98
13159	Sunitinib (malate)	83.50
13311	Gö 6983	85.30
13334	Bisindolylmaleimide IX (mesylate)	86.80
13643	PP242	81.60
81590	Staurosporine	0.90
10483	Erlotinib	83.80
11314	Chelerythrine (chloride)	85.38
13032	PD 173074	85.53
13166	Gefitinib	84.98
13312	H-9 (hydrochloride)	85.15
13337	ST638	85.30
13653	ABT-869	84.45
	Control	83.55
10565	NVP-BEZ235	74.73
11445	Tunicamycin	67.13
13033	Valproic Acid (sodium salt)	83.83
13198	PP2	84.98
13314	Indirubin-3'-monoxime	84.68
13338	SU6656	84.85
13687	CAY10622	84.33
9000980	AS-605240 (potassium salt)	82.98
10735	Phthalazinone pyrazole	83.88
11491	AZD 7762	35.73
13034	PD 0325901	84.08
13242	3-Methyladenine	85.50
13317	NU 6102	84.08
13341	LY364947	85.13
13812	17β-hydroxy Wortmannin	57.13
9000988	PD 166326	78.85
10793	AG-879	79.43
11569	GSK1059615	76.18
13067	SB 203580	84.23
13298	Bisindolylmaleimide I	85.23
13318	KN-62	85.78
13344	SB 203580 (hydrochloride)	84.60
13838	CAY10626	84.10
10004914	O-1918	79.88
10954	1-NA-PP1	83.40
11609	Ruxolitinib	84.20
13108	VX-702	83.83
13299	Bisindolylmaleimide IV	84.93
13319	KN-93	84.38
13371	CAY10621	83.93
13873	SU 6668	83.68
10005583	Y-27632 (hydrochloride)	82.95
10997	Torin 1	81.55
11658	Necrostatin-1	84.00
13109	Emodin	84.63
13300	Bisindolylmaleimide V	85.23
13322	CGP 57380	83.25
13576	YM-201636	80.28
18218	PHA-767491	84.28
10006148	Leelamine	82.95
10006726	PD 98059	84.63
10009052	AS-252424	85.60
10010175	AS-604850	85.10
10010244	AG-1478	85.28
10010309	RG-13022	84.98
10010399	SB 202190	83.95
10011246	WHI-P131	82.58
10012591	CCT018159	79.98
10006727	PD 169316	84.85
10009078	CAY10505	84.73
10010177	PI3-Kinase α Inhibitor 2	85.55
10010246	SB-216763	86.68
10010310	RG-14620	85.45
10010400	CAY10571	85.30
10011247	CAY10574	82.03
10012600	Myricetin	78.50
10007349	TGX-221	85.10
10009209	PI-103	85.45
10010233	CAY10567	85.43
10010247	SB-415286	86.93
10010311	AG-490	86.28
10010422	Nilotinib	85.33
	Control	81.60
10527	Necrostatin-5	78.90
10007653	(S)-H-1152 (hydrochloride)	83.10
10009210	PIK-75 (hydrochloride)	2.29
10010236	ML-9	85.68
10010248	AG-17	76.13
10010312	AG-82	85.38
10010466	SP 600125	86.05
10011249	CAY10576	85.00
10008005	OSU03012	80.93
10007707	AS-605240	84.93
10009222	Sphingosine Kinase Inhibitor 2	85.20
10010237	Triciribine	85.18
10010249	H-8 (hydrochloride)	86.58
10010313	AG-99	86.30
10010541	L-*threo*-Sphingosine (d18:1)	85.73
	Control	85.15
10010301	CAY10554	82.50
10007907	Sphingosine (d18:1)	85.00
10009366	Piceatannol	85.18
10010238	Erbstatin analog	85.45
10010265	LFM-A13	86.25
10010314	AG-213	85.90
10010556	H-89 (hydrochloride)	85.83
10011251	TWS119	81.93
	Control	80.15
10008131	JNJ-10198409	55.63
10009557	SC-1	75.60
10010239	Kenpaullone	86.63
10010267	SC-514	86.60
10010315	AG-183	86.35
10010559	HA-1077 (hydrochloride)	85.98
10011255	NSC 210902	85.25
	Control	80.50
10008614	Leelamine (hydrochloride)	85.10
10009569	(R)-Roscovitine	85.60
10010240	Olomoucine	86.20
10010275	Apigenin	86.15
10010329	Lavendustin C	85.95
10010568	AG-370	85.63
10011256	CAY10577	80.03
	Control	80.20
	Control	84.75
10009644	Sorafenib	85.40
10010242	AG-494	85.80
10010300	AG-18	86.20
	Control	61.03
10010591	Wortmannin	85.45
10011264	CAY10578	83.60
	Control	79.20
10008618	Lauric Acid Leelamide	84.45
10010043	CAY10561	85.05
10010243	AG-825	85.10
10010302	DRB	84.70
10010375	5-Iodotubercidin	84.78
10010592	AG-1296	82.83
10012431	PD 184161	82.73
	Control	80.93

Figure 2. The percentage of the total population falling into the "live" (DAPI-, annexin V-, TO-PRO®-3-) category was used to flag cytotoxic compounds (blue: live, red: dead). The average of the DMSO and staurosporine-treated controls was 83% and 1.6%, respectively.

Further Analysis of Potentially Cytotoxic Compounds

Item No.	Item 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 3. This heatmap shows the percentage of the total population within each of the indicated gates. Colors represent the deviation from the vehicle-treated cells (blue: within 3 SD of vehicle-treated cells; red: value of staurosporine-treated cells). 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+). The remainder of the cytotoxic compounds 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. In most cases, when TO-PRO®-3 was higher than average, annexin V was also higher than average, providing support that these independent markers measure related processes in apoptosis. However, in a few cases (*e.g.*, **NVP-BEZ235**), the percentage of cells which were TO-PRO®-3-positive was above average while the annexin V percentage was below average.

TMRE Staining of Treated Cells

Item No.	Item 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	154.19
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 4. The addition of TMRE staining (last column, percent of control-treated cells, blue: percentage of cells TMRE+ in vehicle, red: percentage of cells TMRE+ in staurosporine) to the apoptotic stains in this screen allows for the concomitant analysis of the health of the mitochondria, elucidating potential mitochondrial toxicity. While several compounds shown to induce apoptosis predictably had low levels of TMRE positivity, one stood out. Notably, **AG-17** showed lower than average apoptosis induction, average necrosis levels, and almost no TMRE staining, suggesting a profound uncoupling 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]

Conclusions

- Eleven compounds in the kinase screening library induced significant cell death.
- By analyzing the mode of cell death using the Early Apoptosis Detection Assay Kit (Cayman Item No. 601360), we were able to determine that there are differences in the way that each of these compounds kills, which may affect their therapeutic efficacy.
- We have shown, using the high-throughput MACSQuant® X Flow Cytometer by Miltenyi, that a library of compounds can be screened efficiently and accurately by flow cytometry.

Reference

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

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