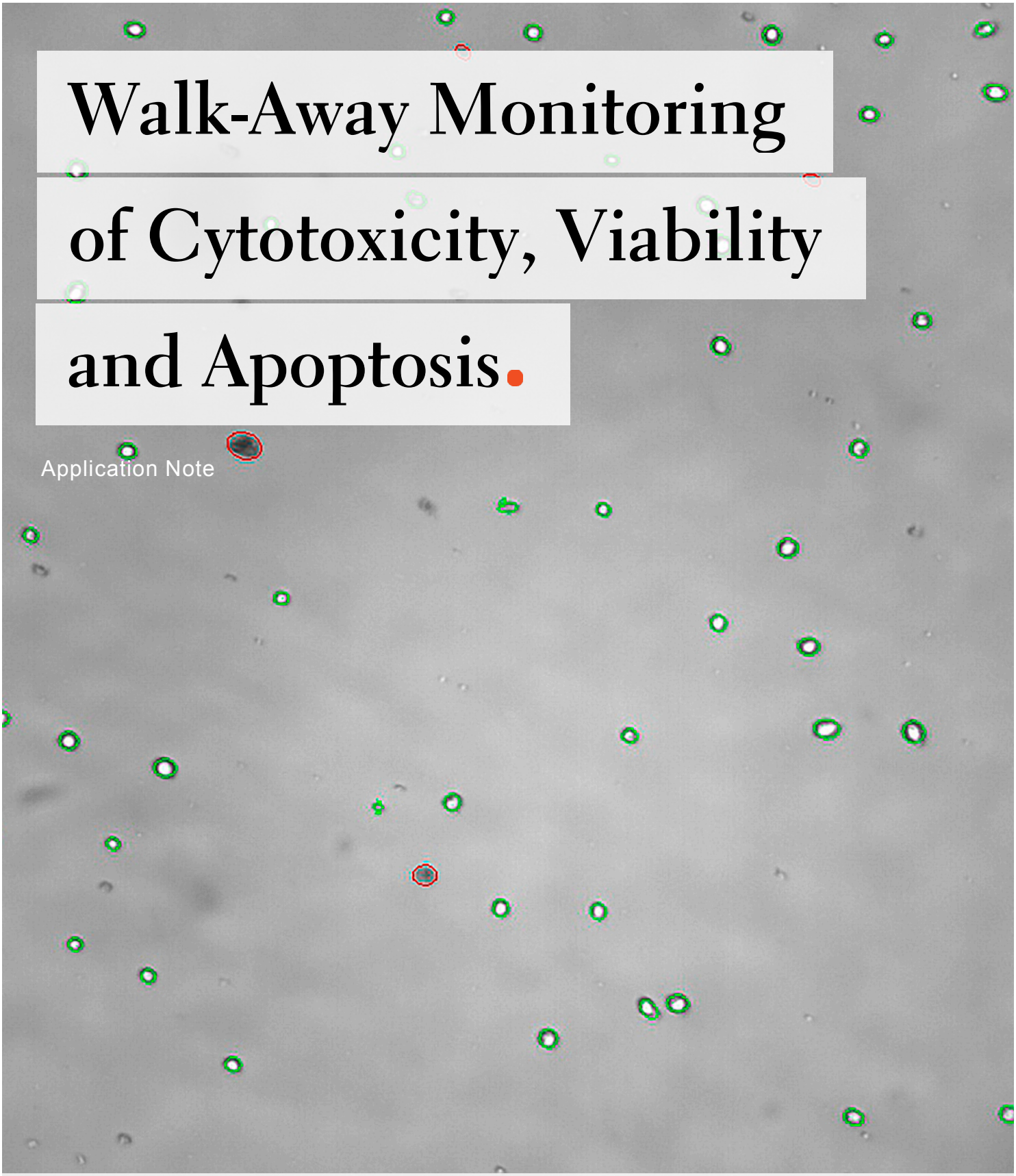
Walk-Away Monitoring of Cytotoxicity, Viability and Apoptosis.

Application Note



SIMPLIFYING CELL ANALYSIS WORKFLOWS WITH THE SPARK® MULTIMODE READER PLATFORM.

TECAN.

INTRODUCTION

Assaying cells in an appropriate window of time after a compound-mediated cytotoxic event can be extremely challenging. Missing this window may mean the difference between defining an effect as apoptotic or necrotic and can affect interpretation of a compound's mode of action. Monitoring membrane integrity changes and cell viability can reveal an exact point in time when primary or secondary necrotic events occur. Further multiplexing with a caspase-3/7 assay can help differentiate membrane breakdown due to apoptosis and secondary necrosis from an overt cytotoxic event.

This application note describes a method for kinetic monitoring of cytotoxicity and viability, using three Promega assays for viability, cytotoxicity and apoptosis assessment and Tecan's Spark® multimode reader (equipped with an enhanced fluorescence module) platform. Further studies investigate the ability of the instrument to automatically inject caspase-3/7 reagent using a cytotoxicity threshold set within the reader software. The effects of compounds, including six kinase inhibitors, on K562 cell health were evaluated over time.

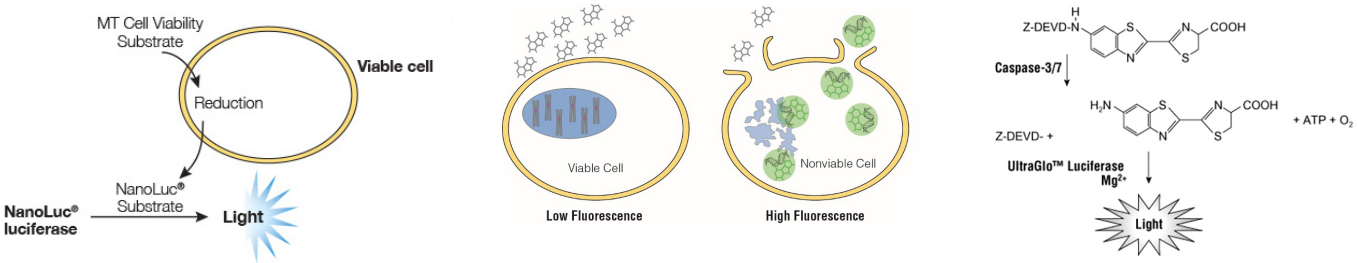
MATERIALS AND METHODS

Drug compounds and cells

A small panel of drugs was assembled, representing known cytotoxic agents and and apoptosis-inducing kinase inhibitors. Ionomycin (Sigma #I0634), Staurosporine (LC Laboratories #S9300), Bosutinib (LC Laboratories #B1788), Dasatinib (LC Laboratories #D3307), Imatinib (LC Laboratories #I5577), and Ponatinib (LC Laboratories #P7022) were prepared as 10mM DMSO stocks. K562 cells, a human model of Chronic Myelogenous Leukemia with aberrant bcr/abl kinase activity, was propagated in RPMI 1640 + 10% FBS (cell medium).

Assays

Three Promega assays were used in the experiments described here (Table 1).



RealTime-Glo™ MT Cell Viability Assay	CellTox™ Green Cytotoxicity Assay	Caspase-Glo® 3/7 Assay
• Non-lytic, kinetic	• Non-lytic, kinetic	• Lytic, endpoint
• Viable cells reduce pro-NanoLuc® substrate	• Asymmetric cyanine dye binds DNA released from dead cells	• Caspase-3/7 cleaves pro-luminescent DEVD-luciferin substrate.
• NanoLuc® luciferase uses NanoLuc® substrate to produce light	• Dye becomes fluorescent when bound to DNA	• Firefly luciferase uses luciferin substrate to produce light
• Luminescence is proportional to number of viable cells	• Fluorescence is proportional to number of dead cells	• Luminescence is proportional to caspase-3/7 activity (apoptosis)

Table 1: Assays used for viability, cytotoxicity and apoptosis detection. RealTime-Glo™ and CellTox™ Green Reagents were added at the time of cell plating. Caspase-Glo® 3/7 reagent was injected with the Spark.

Plate setup

K562 cells were pelleted and adjusted to 5x10^4 cells/mL in cell medium. RealTime-Glo™ (Promega #G9713) and CellTox Green™ (Promega #G8731) reagents were added at 2X concentration to the cell suspension. 20µl of cell suspension was dispensed into the wells of a white 384 well plate with clear bottom (Corning #3707). Wells containing only 2X RealTime-Glo™ and 2X CellTox Green™ in medium were included as assay background controls.

Compounds were diluted to 2X starting concentration in cell medium, then serially titrated 1:5 in cell culture medium containing 0.2% DMSO. 20 µl of 2X compound titration was added to the plated cells. No compound control wells containing 0.2% DMSO, 2X RealTime-Glo™ and 2X CellTox Green™ were also included.

The final assembled assays included: 1,000 K562 cells, 1X RealTime-Glo™ and CellTox Green™ reagents, 1X compound in 0.1% DMSO, 0.1% DMSO vehicle control, and a no cell control with 1X reagents.

The Spark multimode reader was pre-equilibrated to 37°C and 5% CO_2 prior to performing the experiments. The small humidity cassette was prepared by filling the moat region with deionized water. The assay plate, without lid, was placed into the humidity cassette. The covered cassette with plate was then placed into the equilibrated instrument.

The Spark multimode reader platform

The Spark has a number of features that are beneficial for cell-based experimentation. Table 2 highlights the assay requirements for the experiments described here and shows the corresponding Spark feature used during each step of the process.

Assay Requirement	Spark Feature Used
Incubate 384 well assay plate	Temperature control (37 °C)
Maintain 5% CO_2 and ambient O_2	Gas control
Prevent medium evaporation	Humidity cassette with liquid reservoir
Quantify cell viability	Lid lifter, top-read luminescence, 500 ms integration time
Quantify cytotoxicity	Monochromator, bottom-read fluorescence (EX 485/20, EM 520/20)
Add caspase-3/7 reagent	Lid lifter, reagent injector
Quantify apoptosis	Lid lifter, top-read luminescence, 500 ms integration time

Table 2: Benefits of the Spark multimode reader for cell-based applications.

Walk-away data generation was achieved with a simple workflow. Viability and cytotoxicity were monitored in real-time. Caspase-Glo® 3/7 (Promega #G8091) reagent addition was dictated by the user and added at specific intervals, or if certain experimental conditions were met.

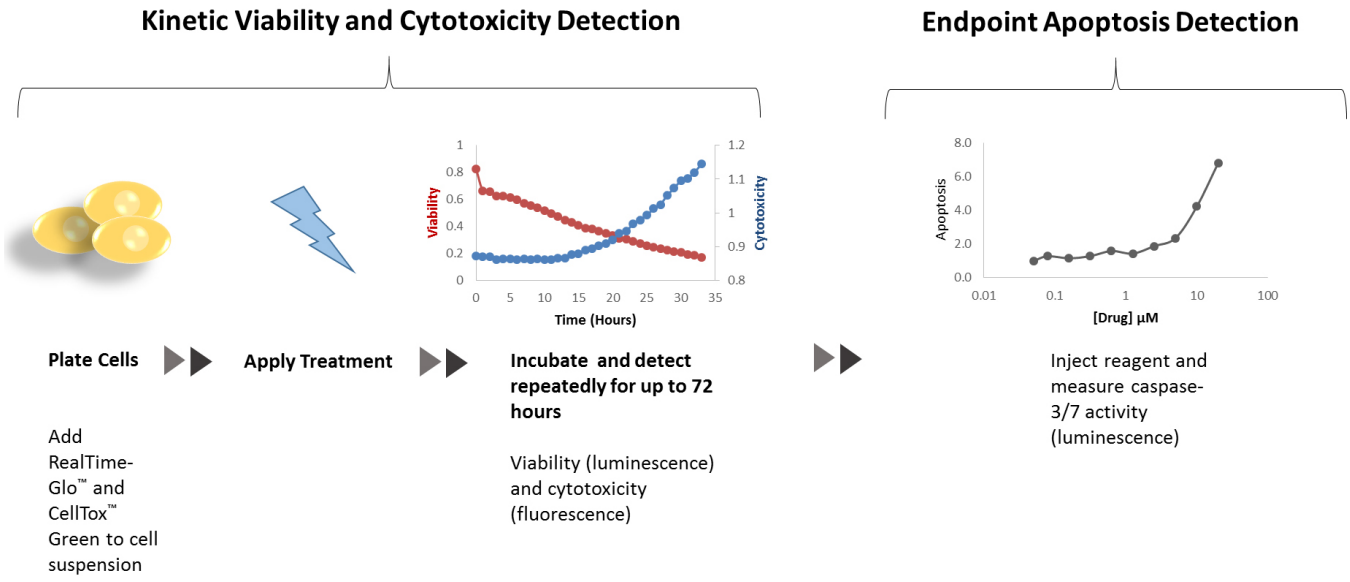


Figure 1: Plate setup for walkaway data generation.

Experiment 1: Assessing apoptosis at defined treatment interval time course

A first experiment was performed to assess the time- and dose-dependent effects on K562 cell health following treatment with the panel of test compounds. We were interested in evaluating if changes in cytotoxicity were any indication of cells undergoing apoptosis. The RealTime-Glo™ assay was included as a means to evaluate anti-proliferative effects due to compound treatment. A serial titration of test compound, starting at 100 µM concentration, was applied to the cells. Replicate titrations of each compound were set up on the plate to facilitate the time course study. Viability and cytotoxicity were monitored every hour. 40µl of Caspase-Glo® 3/7 reagent was injected into a subset of the wells at time 0 and pre-determined time points every 8-12 hours out to 72 hours. Caspase-3/7 reagent was injected to all wells in the dose series at each time point, the plate was incubated for 30 minutes, and luminescence from the apoptosis assay was quantified. The stock of Caspase-Glo® 3/7 Reagent supplied by the injectors was refreshed on the instrument every 24 hours.

Results from Experiment 1

Real-time detection of cell viability and cytotoxicity changes revealed a variety of results based on compound concentration and length of exposure. Immediate cytotoxicity was detected in some cases, particularly at high concentration, but anti-proliferative and cytotoxic effects emerged with time at lower doses. Figure 2A and 2B are examples of data acquired from Ponatinib treatment of K562 cells. Figure 2C shows corresponding endpoint apoptosis assay results across the dose range at the various time points in the time course. Immediate cytotoxicity with moderate caspase induction was noted at high concentration of Ponatinib (100 µM). Anti-proliferative effects with moderate caspase induction were noted at mid-range concentration of Ponatonib (20 µM). Longer term exposure to lower concentrations (≤ 4 µM) past 40 hours revealed the most potent caspase-3/7 induction with decreases in cell viability and increases in cytotoxicity beginning to emerge.

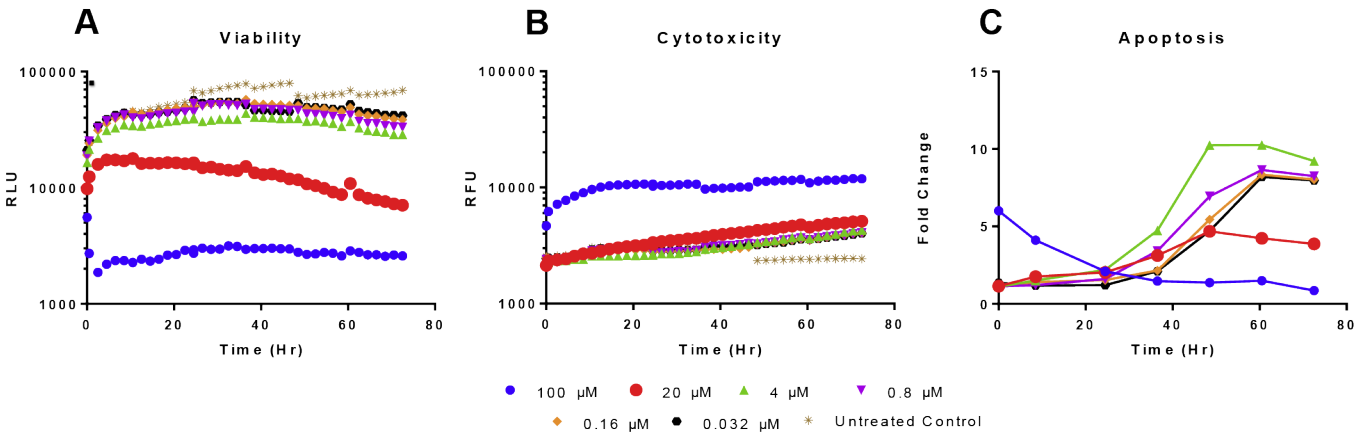


Figure 2A-C: Time and dose-dependent effects on K562 cell health following Ponatinib treatment.

Time course treatment with the other test compounds showed that detection of caspase-3/7 activity was correlated with K562 cytotoxicity and transient in nature. Evaluating the highest concentration tested, 100µM, and shown in Figure 3, the kinase inhibitors induced an increase in cytotoxicity over time due to treatment with the various drugs. Apoptosis (caspase-3/7 activity) was generally least detectable in cases of early cytotoxicity (figure 3A) as exemplified by treatment with Bosutinib.

Other drug treatments (Dasatinib, Imatinib, Staurosporine) displayed increasing apoptosis detection with time that correlated with an increase in cytotoxicity (figure 3B-D). The transient nature of caspase-3/7 detection was demonstrated in the case of Bosutinib (A) and Staurosporine (D) where the level of caspase-3/7 detection peaked early and then decreased with time.

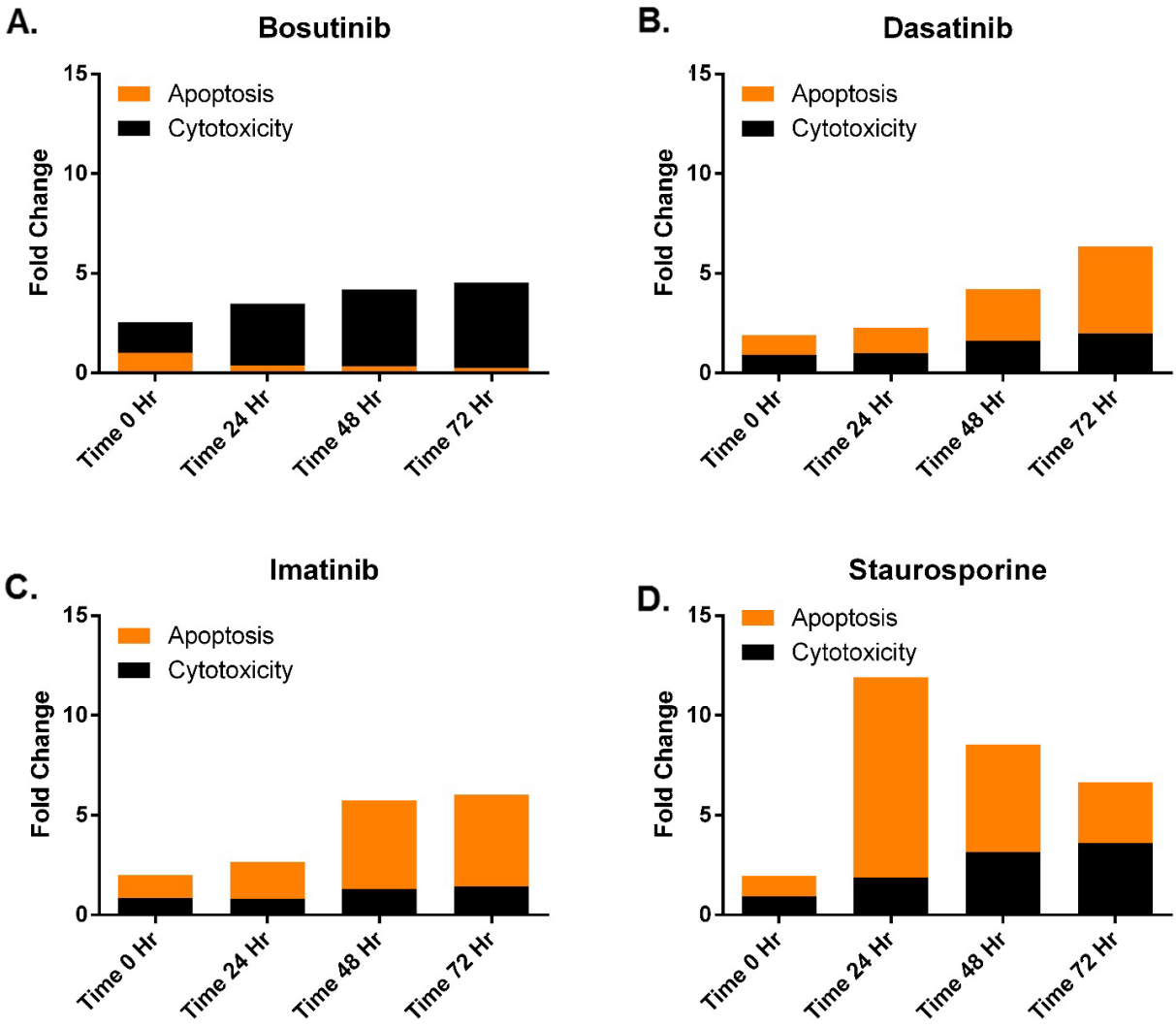


Figure 3: Fold changes in cytotoxicity and apoptosis as compared to an untreated control reveal varied cell health profiles in response to kinase inhibitor treatment.

Closer evaluation of the cytotoxicity data from the apoptosis-inducing compounds showed that, in general, when relative fluorescence cytotoxicity assay readings with any dose of compound increased to approximately 1.4 times the untreated cell control, caspase-3/7 activity was detectable within those wells. We were then interested in knowing whether or not a change in cytotoxicity, or cytotoxic threshold, could be set within the Spark software and used as a condition for automated injection of caspase-3/7 reagent.

Experiment 2: Using cytotoxicity readout to initiate automated injection of caspase-3/7 reagent

A follow-up experiment was conducted to assess the ability of the Spark to automatically inject caspase-3/7 reagent using a fluorescence cytotoxicity threshold set within the reader software. A fluorescence reading of the untreated cell control was taken at the time of compound treatment, and a numerical value approximately 1.4 times the RFU of the untreated control was used as the cytotoxic threshold for reagent injection for the 72 hour experiment. The assay plate was assembled in a similar manner as experiment 1, but this time the starting concentration of test compound within the dose range was decreased to 20 µM in order to prevent immediate automated injection of reagent due to an overt cytotoxic event. When the fluorescence threshold value at the 20 µM concentration was achieved, caspase-3/7 reagent was injected to all wells in the dose series, the plate was incubated for 30 minutes, and luminescence was quantified.

Results from Experiment 2

Using Bosutinib as an example, caspase-3/7 activity was detectable at 39 hours across the entire dose series of compound indicating potent induction of apoptosis compared to the control (figure 4). The viability assay showed that the highest concentration of Bosutinib exhibited anti-proliferative effects within 10 hours of treatment. As a result, a decrease in caspase-3/7 activity at 39 hours is likely due to the decreased number of K562 cells present in the well at the time of reagent injection. Normalizing apoptosis to cell number (viability) accounts for this anti-proliferative effect.

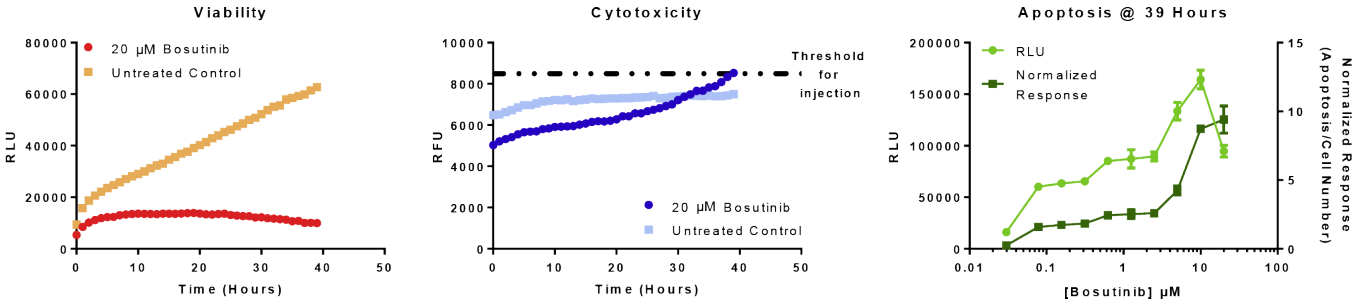


Figure 4: Cytotoxicity-dependent injection of caspase reagent and corresponding viability, cytotoxicity and apoptosis assessment

Table 3 summarizes the mechanism of action for all tested compounds. Staurosporine and bcr/abl-targeted inhibitors, with the exception of Ponatinib, showed detectable apoptosis at the time of reagent injection. Ponatinib results were in accordance with those obtained in figure 2; at 14 hours incubation only cytotoxicity was detectable and triggered injection of capase-3/7 reagent in the absence of apoptosis. With increased incubation apoptosis was likely to be measured. Ionomycin, known to cause cytotoxicity and necrosis, showed no apoptosis at 5 hours.

Compound [20 µM]	Mechanism of Action	Time of Injection	Apoptosis (Fold Change over untreated control)	Viability (Fold Change over untreated control)
Ionomycin	Calcium ionophore	5 Hours	1.1	0.61
Ponatinib	Bcr/abl kinase inhibitor	14 Hours	1.2	0.14
Staurosporine	Pan-kinase inhibitor	33 Hours	6.8	0.17
Imatinib	Bcr/abl kinase inhibitor	35 Hours	12.0	0.32
Bosutinib	Bcr/abl kinase inhibitor	39 Hours	5.8	0.16
Dasatinib	Bcr/abl kinase inhibitor	53 Hours	7.2	0.36

Table 3: Confirmation of mechanism of action for all tested compounds.

CONCLUSIONS

The data summarized in this note show that Promega Cell Health Assays and the Spark multimode reader support mechanistic toxicity determination with data acquisition for multiple days.

Viability and cytotoxicity could be monitored in real-time up to 72 hours during which the lid lifter provided access to the assay plate for data acquisition and timed or automatic reagent injection. The window during which apoptosis is measured can drastically affect the interpretation of mode of action. By using the onset of cytotoxicity as a trigger for apoptosis detection, it is more likely that a researcher will not only be able to find the window of apoptosis induction specific to their drug and/or dosage, but also that the mode of action of the drug will be correctly assigned. Our preliminary studies indicated that membrane integrity changes correlated with the level of apoptosis detected, and early detection of cytotoxicity correlated with fast-acting apoptosis inducers or overtly cytotoxic compounds (1° necrosis). Delayed detection of cytotoxicity correlated with more potent induction of apoptosis (2° necrosis). The cytotoxicity measurement could be used to dictate the time of caspase-3/7 reagent injection. Caspase-3/7 activity at cytotoxic threshold values correlated with compounds known to induce apoptosis.

ACKNOWLEDGEMENTS

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1) RealTime-Glo™ MT Cell Viability Assay Kit Protocol (TM431, 9/14)
2) CellTox™ Green Cytotoxicity Assay Kit Protocol (TM375, 5/15)
3) Caspase-Glo® 3/7 Assay Kit Protocol (TB323, 3/15)

About the author

Dr Katrin Flatscher is an application scientist at Tecan Austria. She studied molecular biology at the University of Salzburg and focused on cell biology and immunology research during her PhD.

She joined Tecan in 2007 and has been involved in the development of the Infinite as well as the Spark reader series.

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