Application Note

Cell Analysis

Agilent

Trusted Answers

Long-Term High-Throughput Cytotoxicity Profiling

xCELLigence RTCA HT instrument

Author

Agilent Technologies, Inc.

Introduction

The current portfolio of Agilent xCELLigence real-time cell analysis (RTCA) instruments has brought label-free cell-based assays to the forefront of drug discovery research. Applications include cell proliferation, cytotoxicity, cell adhesion, cell invasion and migration, and receptor responses. These cover a wide range of disease research areas including cancer, inflammation, diabetes, and infection. In addition to label-free assays, RTCA instruments also offer real-time monitoring which provides important advantages over traditional endpoint assays. For the first time, a comprehensive representation of the entire time course of the assay is possible, allowing the user to make informed decisions about the timing of manipulations and cell treatments. Quantitative data from cellular kinetic responses provide important information about biological status, such as cell growth, morphological changes, and apoptosis. Precise time-dependent cell response profiles (TCRPs) offer important information about the mechanisms of drug action and the underlying toxicity of off-target interactions.[1]

The xCELLigence RTCA high throughput (HT) instrument makes all the features and benefits of the xCELLigence system technology available to high-throughput compound screening and profiling. The RTCA HT instrument consists of the RTCA HT control unit with the RTCA HT software, analyzer, and up to four HT stations. Each HT station holds a 384-well plate (Agilent E-Plate 384) for measuring cellular response to treatments/agents using electrical impedance. Cells are seeded one day before treatment for attachment to the biosensors located in the bottom of the E-Plate 384 wells. After the E-Plate 384 is placed on the RTCA HT station, cellular interactions are detected by biosensors in the RTCA HT analyzer. Changes in electrical impedance are produced after a small current is run through the microelectrode sensors in the bottom of each well of the E-Plate 384.

Impedance measurements are converted to Cell Index (CI) values by the RTCA HT software. CI values reflect the number of cells, as well as morphological parameters, such as size, shape, and degree of cell attachment to the substrate. Sampling time can be set as short as 15 seconds for real-time readout of rapid responses, such as those mediated by GPCR activation and long-term cytotoxic effects. A heating element on the RTCA HT station ensures that all assays are performed at physiological temperature, ensuring biological relevance under screening conditions. The compact footprint of the RTCA station is easily integrated into existing liquid handling and automation workflows (see Figure 1).

This application note illustrates how the RTCA HT instrument reveals distinct profiles of different cell lines.The findings demonstrate that this system is useful for high-throughput screening of cell phenotype that alters its cell attachment, morphology, and growth characteristics. Also shown are antiproliferative agents, through different mechanisms of action, exhibit distinctive kinetic profiles using the RTCA HT instrument.

Compound potencies measured on the RTCA HT instrument are also correlated with those obtained in standard endpoint assays. Comparing the kinetic profile of one of these compounds to the kinetics of apoptosis induction revealed a high correlation between the two assays. This illustrates the applicability of kinetic readout in pinpointing the optimal time points for performing endpoint assays. In summary, the RTCA HT instrument is user-friendly for developing assays that screen long-term cellular responses in high-throughput workflows using 384-well culture plates.

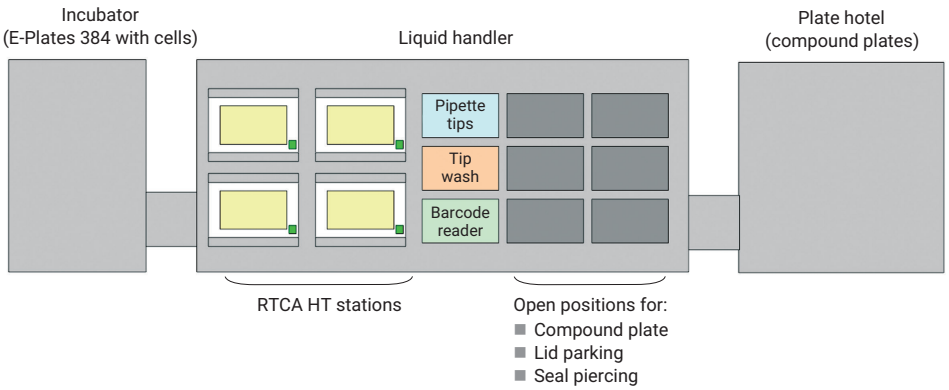

Figure 1. Schematic showing the Agilent xCELLigence RTCA HT instrument incorporated into a typical automation platform configuration. Up to four RTCA HT stations may be incorporated directly onto the deck of the automated liquid handling platform. These stations are connected by cables to the adjacent RTCA HT analyzer (not shown). In a typical assay, the Agilent E-Plate 384 containing growth media (dispensed online or offline) are loaded into the incubator and background impedance readings are taken on the RTCA HT stations after barcode reading. Cells are then plated in the E-Plate 384 plates (dispensed online or offline), which are again loaded into the incubator. The E-Plate 384 may be monitored for cell growth over time by cycling in and out of the RTCA HT stations at regular intervals. Up to four plates may be read simultaneously for a user-defined number of independent impedance readings. For compound addition, the user's standard automation workflow may be carried out. Compounds may be added to the E-Plate 384 at an open position or, in cases where detection of rapid response kinetics is required, directly on the RTCA HT station while the cells are being monitored for impedance changes.

Results and discussion

Dynamic monitoring of cell proliferation on the RTCA HT instrument

To characterize growth profiles of different cell lines using the RTCA HT instrument, four human cell lines were chosen: the HeLa cervical cancer cell line, A549 and H460 lung carcinoma cell lines, and the HT1080 fibrosarcoma cell line. Different numbers of cells (125 to 4,000 per well) were seeded and growth was monitored every hour for 80 hours (see Figure 2). Each cell line produced distinct profiles with the RTCA HT instrument, corresponding to differences in growth rate, cell morphology, and attachment quality. For example, at a density of 4,000 cells per well, HT1080 and HeLa cells reached the maximum Cell Index value at approximately 30 hours, while A549 and H460 cells reached maximum at 80 to 90 hours. Each cell line exhibited a unique kinetic profile, depending on cell size and degree of attachment to the surface of the plate. These characteristic profiles may be used to verify homogeneity and monitor contamination in each cell line, as well as for accurate calculation of attachment kinetics, doubling time, and different growth phases.[2] Such parameters may be significant in screens for modulators of differentiation or other long-term cellular responses, which may be conducted over a span of days (or even weeks) using the integrated RTCA HT instrument.

Dynamic monitoring of antiproliferative agents on the RTCA HT instrument

xCELLigence technology has proven useful in screening for antiproliferative agents. The kinetic profiles generated can indicate a mechanism of action and identify potential off-target or toxic effects.[1] To investigate the utility of the RTCA HT instrument in assessing the effects of antiproliferative agents on target cells, four well-characterized compounds were tested. These were: two DNA-damaging agents (camptothecin and doxorubicin), a proteasome inhibitor (MG132), and a PI3K kinase/mTOR inhibitor (PI-103). A549 lung carcinoma cells were seeded on E-Plate 384 plates, grown overnight, and treated with the indicated concentrations of compound while still in logarithmic growth phase, and cell responses were monitored by taking readings every hour for 72 hours (see Figure 3).

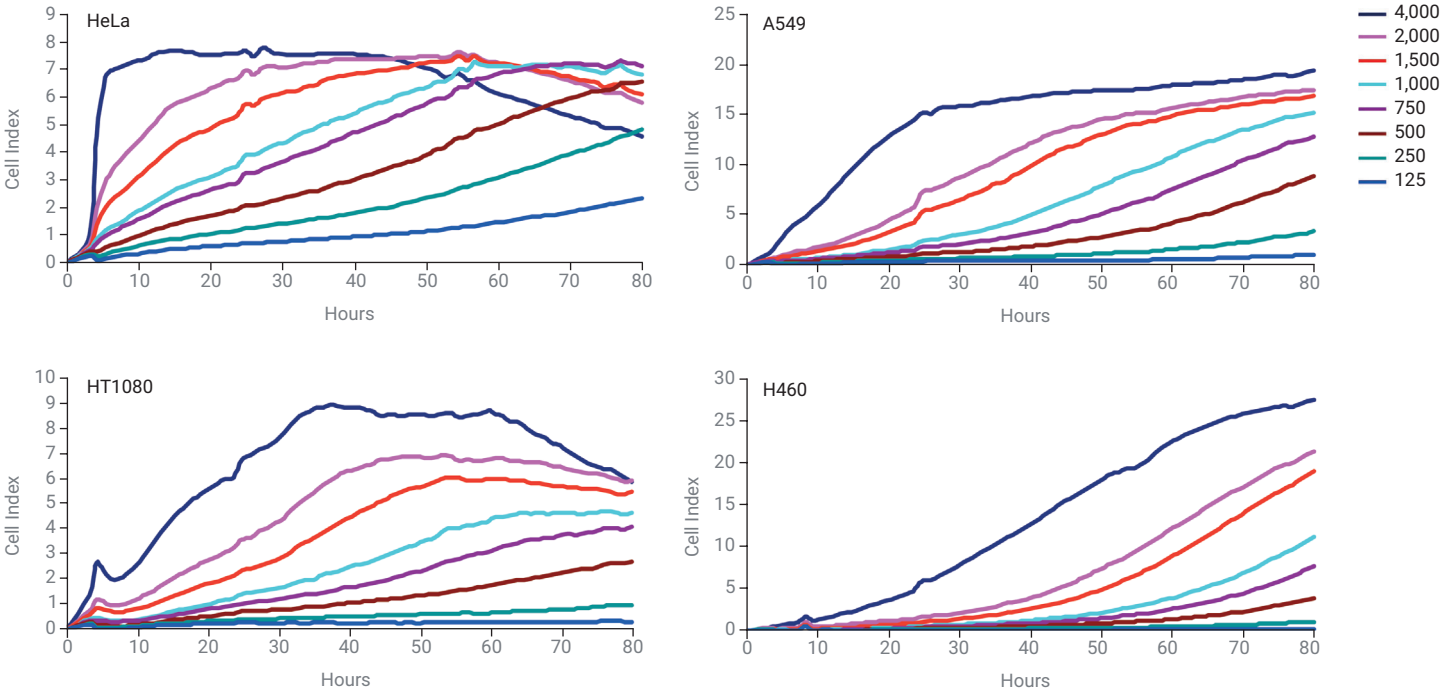

Figure 2. Cell adhesion and proliferation profiling on the Agilent xCELLigence RTCA HT instrument. The indicated cell lines were seeded on the Agilent E-Plate 384 devices at the indicated cell densities and monitored by automated cycling between the RTCA HT station and the incubator every hour for 80 hours. Mean values of 12 replicate wells are shown.

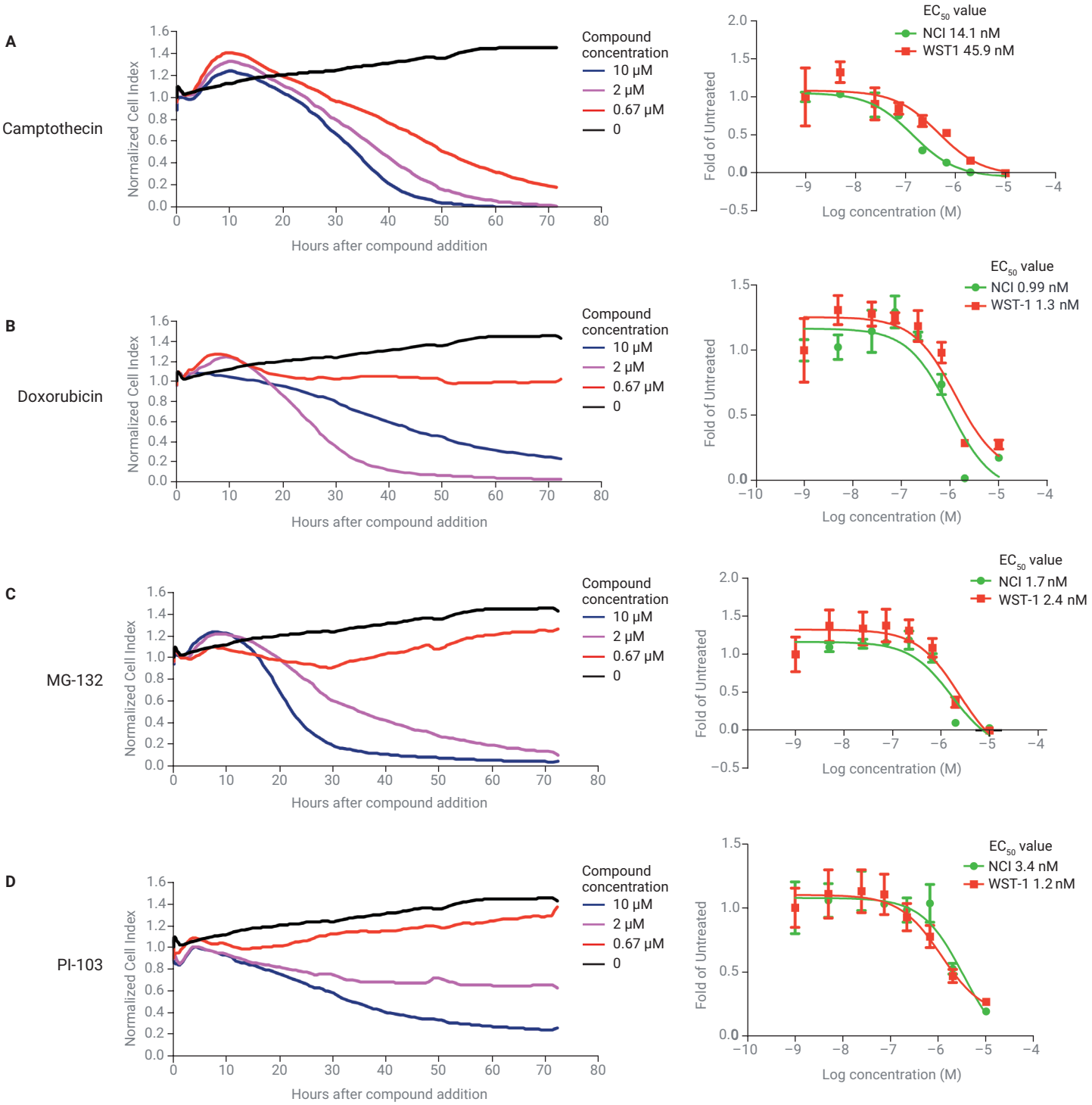

Figure 3. Profiling of cytotoxic agents on the Agilent xCELLigence RTCA HT instrument. A549 lung carcinoma cells were seeded on the Agilent E-Plate 384 devices at 4,000 cells per well and grown overnight. The compounds shown in each image were added at indicated concentrations. Responses were monitored hourly by automated cycling between the RTCA HT station and the incubator. Left panels show cellular responses measured using the RTCA HT instrument. Normalized Cell Index (NCI) values are plotted as the mean value from four replicates. Only selected concentrations are shown for clarity. Right panels show the dose-response curves at the 72-hour time point from both the xCELLigence system and WST-1 assays. Values are plotted as the mean value from four replicates, with error bars representing one standard deviation. The EC_{50} values derived from each assay are shown.

All compounds mediated a decrease in Cell Index, as expected for any antiproliferative agent. But the kinetic profile of the cellular response differed in a dose-dependent manner with each compound's mechanism of action. The DNA-damaging agents generated a transient increase in CI immediately after compound addition, possibly reflecting a transient morphological change, followed by a gradual, dose-dependent decrease in CI (see the left panels of Figures 3A and 3B). The proteasome inhibitor MG-132 produced a similar profile with slightly more rapid onset (see the left panel of Figure 3C). In contrast, the PI3 kinase/mTOR inhibitor PI-103 produced a more gradual decrease in Cell Index over the entire course of the experiment (see the left panel of Figure 3D).

To verify whether the CI values measured by the RTCA HT instrument correlate with the antiproliferative effects of these compounds, an independent, colorimetric-based cell proliferation assay (WST-1) was performed in a parallel experiment. A549 cells were cultured in an E-Plate 384 and treated as previously described. At 72 hours after compound addition, the WST-1 reagent was added, the plate was incubated for 1 hour, and the lysate was transferred to a regular 384-well plate for absorbance measurements.

The resulting dose-response curves and calculated EC_{50} values obtained using both the RTCA HT instrument and the WST-1 assay are shown for each compound in the right panels of Figures 3A through 3D. These curves were very similar between the WST-1 and xCELLigence assays, indicating that quantitative changes in CI accurately reflect changes in cellular viability.

The DNA-damaging agents and the proteasome inhibitor tested here ultimately induce apoptosis in A549 cells, albeit with different kinetics. To investigate the relationship between the kinetic CI response profile generated using the RTCA HT and the kinetics of apoptosis induction, the Agilent Cell Death Detection ELISA kit was used. A549 cells were treated with the DNA-damaging agent camptothecin, as described above, and monitored using the xCELLigence system. Identical experiments were conducted in parallel using the Agilent E-Plate 96, and subjected to the Cell Death Detection assay at 24, 48, or 72 hours after compound addition.

Representative results for CI monitoring and the apoptosis assay of one dose of camptothecin are shown in Figures 4A and 4B. Robust apoptosis induction was observed at the 24 and 48-hour time points (see Figure 4B). This corresponds with a time shortly after the Cell Index value begins a rapid decrease (approximately 18 hours), and the time at which it reaches the minimal value, respectively (approximately 50 hours; see Figure 4A). At 72 hours, the apoptotic signal is greatly reduced, suggesting that most of the cells are dead. At the 48-hour time point, the dose-response profiles from two assays are nearly perfect mirror images, as apoptosis induction is detected with increasing camptothecin concentration, while the CI value is correspondingly decreased (see Figure 4C). In addition, the calculated EC_{50} values are comparable in the two assays (see Figure 4C). The RTCA HT instrument has provided a quantitatively accurate measure of apoptosis induction. Taken together, these findings illustrate the utility of the real-time kinetic readout in determining the optimal time points for conducting follow-up assays.

The RTCA HT instrument is ideal for screening compounds that elicit long-term cellular responses. The integration of up to four RTCA HT stations with an automated plate-handling system permits the continuous cycling of a large number of plates between incubator and station. This feature, combined with rapid plate-reading times (as little as 15 seconds), enables a high-throughput rate. The built-in heating element of the RTCA HT ensures that all readings obtained are at physiological temperatures, minimizing the disruption of cellular processes during readings and over the course of the screen. In this way, long-term biological responses over days or weeks may reliably be used for small molecule screening by the RTCA HT instrument.

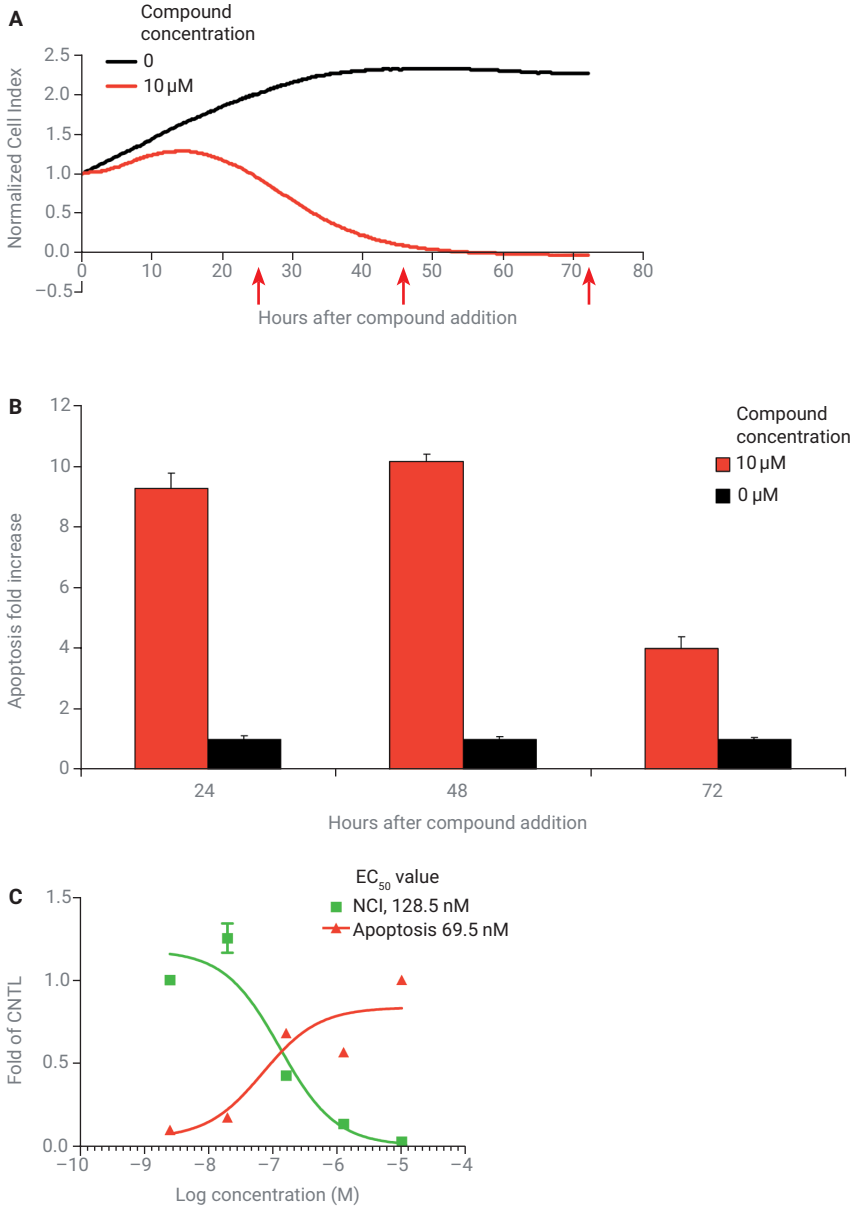

Figure 4. Apoptotic response comparison. (A) A549 lung carcinoma cells were seeded on Agilent E-Plate 384 devices at 6,000 cells per well and grown overnight. Camptothecin was added at the indicated concentrations and cellular responses continually monitored. Red arrows indicate time points used for apoptosis assays. (B) In a parallel experiment, A549 lung carcinoma cells were seeded on regular 96-well plates, treated as in A, and apoptotic induction at the indicated time points was determined, according to the instructions from the Cell Death Detection ELISA kit. The ratio of the resulting apoptosis signal over untreated wells for each dose is plotted as the mean value from four replicates, with error bars representing one standard deviation. (C) Dose-response curves derived from data obtained at the 48-hour time point in (A) and (B). Cell Index values were normalized (NCI) as in B, and the EC_{50} values derived from each assay are indicated. CNTL = untreated samples.

Findings show that cells with distinctive morphology, attachment, and growth characteristics produce biologically distinctive profiles when assayed by the xCELLigence RTCA HT instrument (see Figure 2). Disease-relevant changes in these cellular characteristics, such as cell death and more subtle cell morphological changes, such as those resulting from differentiation processes, may be effectively screened using the RTCA HT instrument. For the antiproliferative agent tests conducted here, the RTCA HT instrument exhibited high sensitivity, with calculated EC_{50} values obtained in high correlation with the WST-1 and apoptosis assays. The major advantage of the xCELLigence assay compared to standard assays stems from the kinetic nature of the readout. Previous work has established that the time-dependent cell response profile (TCRP) obtained from small molecule or siRNA treatments using xCELLigence instruments may be indicative of the mechanism of action, including the identification of undesirable off-target effects.[1,3] For the four antiproliferative agents tested here, three distinct mechanisms of action were involved, as indicated by the different TCRP profiles.

These kinetic patterns can also be used to determine the mechanism of action of novel compounds interacting with unknown targets by comparison to compounds with known mechanisms. In this case, the TCRP profiles can be seen as a reflection of the inherent global cellular response to a specific treatment, all detectable as measurable effects on cell adhesion, morphology, and proliferation, which may be revealed in the primary screen, including undesirable cytotoxic effects. The RTCA HT instrument expands the biological spatial and temporal resolution in drug screening. It provides unique profiles that can be used in a high-throughput manner to categorize compounds with important biological activities, based on their mechanism of action, all from primary screening data.

Conclusion

- The RTCA HT instrument is well suited for high-throughput screening of cellular responses over long experiment periods.
- Cells with different morphologies, attachment, and growth rates produce distinct kinetic profiles on the RTCA HT.
- Antiproliferative agents produce distinct profiles on the RTCA HT instrument, corresponding to their different mechanisms of action.
- Compound potencies measured using the RTCA HT correlate with widely used and more time-consuming functional endpoint assays.

References

1. Abassi, Y. A. *et al.* Kinetic Cell-Based Morphological Screening: Prediction of Mechanism of Compound Action and Off-Target Effects. *Chem. Biol.* **2009**, *16*, 712–23.
2. Kirstein, S. L. *et al.* Live Cell Quality Control and Utility of Real-Time Cell Electronic Sensing for Assay Development. *Assay Drug Dev. Technol.* **2006**, *4(5)*, 545–53. https://doi.org/10.1089/adt.2006.4.545
3. Ke, N. *et al.* Screening and Identification of Small Molecule Compounds Perturbing Mitosis Using Time-Dependent Cellular Response Profiles. *Anal Chem.* **2010**, *82*, 6495–503.

www.agilent.com/chem

RA.6860069444

Printed in the USA, August 27, 2020
5994-2001EN

Agilent

Trusted Answers