Application Note

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Induction and Inhibition Studies of Hypoxia and Oxidative Stress in Immortalized Keratinocytes

Using traditional microplate detection and automated digital widefield fluorescence microscopy

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Abstract

This application note demonstrates a multiplexed assay that can monitor the induction of hypoxia and oxidative stress phenotypes using fluorogenic probes. Data readout can be achieved using whole-well fluorescence intensities measured using either monochromators or spectral filters for rapid measurements depicting relative phenotype changes. Absolute ratios of effected cells for each phenotype can also be obtained through cellular analysis using automated digital widefield fluorescence microscopy. The ability of a library of antioxidants to inhibit hypoxia was then assessed in a separate assay. The library was screened rapidly using whole-well intensities. A hit picking feature in the instrument software was activated to image only those wells meeting the predetermined criteria. Imaging then provided qualitative and quantitative assessments of the extent of hypoxia phenotype inhibition. Excessive inhibition relative to controls was found to be caused by compound toxicity using a luminescent cell viability assay. All cell-based assays were performed using the Agilent BioTek Cytation 3 cell imaging multimode reader.

Introduction

Hypoxia is a pathological condition where the entire body, or a portion of the body, is deprived of adequate oxygen supply. Variations in oxygen concentration can be part of normal physiology; such as during strenuous exercise, high-altitude climbs, or deep-sea dives. However, hypoxia can also be a serious condition. Many children born prematurely experience generalized hypoxia because their lungs have not fully developed, and oxygenated blood is not adequately distributed throughout the body.

Hypoxic skin injuries are also an important pathological process in many diseases, including multiple types of ulcer such as pressure, diabetic, and varicose ulcers.[1–3] Insufficient blood or oxygen supply is a leading causal factor, and can lead to chronic, nonhealing ulcers.[4–6] It has been shown that oxidative stress, the overproduction of reactive oxygen species (ROS), is intimately associated with hypoxic injury of skin.[7] This has led to more studies[8] which have examined the potential protective ability of antioxidants against hypoxia and its downstream effects.

This application note demonstrates an *in vitro* multiplexed microplate assay that can monitor induction of hypoxia and oxidative stress through ROS production. Cobalt chloride ($CoCl_2$), a well-known mimetic agent of hypoxia[9], was used to chemically induce these phenotypes in immortalized keratinocytes. Relative whole-well fluorescence intensity data were acquired for both assays using PMT-based fluorescence microplate detection, in addition to using automated digital widefield fluorescence microscopy. The latter provides quantitative ratios of affected cells relative to the total cell population using the cellular analysis feature of Agilent BioTek Gen5 microplate reader and imager software in addition to qualitative visual confirmation of oxidative stress and hypoxia.

A screen of documented antioxidant compounds was performed to determine whether induction of the hypoxic condition could be inhibited using the cell model included here. A hit picking feature of the instrument's software allowed for rapid screening of compounds using whole-well intensities followed by imaging of only hit wells to collect phenotypic data.

Dose-response tests were then performed to confirm the effects seen from select inhibitor compounds. Finally, a luminescent cell viability assay was incorporated where cell viability measurements were performed in the same well following detection of the fluorescent signals from the hypoxia and oxidative stress assay. All measurements were performed on the Cytation 3 cell imaging multimode reader.

Materials and methods

Materials

Cells

Immortalized transformed keratinocytes (part number CRL-2309) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were propagated in keratinocyte serum-free medium (part number 17005-042) supplemented with bovine pituitary extract (part number 13028-014) and EGF recombinant human protein (part number PHG0311) from Life Technologies (Carlsbad, CA).

Assay chemistries

Hypoxia/Oxidative stress detection kit (part number ENZ-51042-K100) was donated by Enzo Life Sciences (Farmingdale, NY). CellTiter-Glo Luminescent Cell Viability Assay (part number G7572) was purchased from Promega Corporation (Madison, WI). BisBenzimide H33342 trihydrochloride (Hoechst 33342) (part number 14533) was purchased from Sigma-Aldrich (St. Louis, MO).

Hypoxia inducer

Cobalt(II) chloride, hexahydrate (part number C2911) from Sigma-Aldrich (St. Louis, MO) was used to chemically induce hypoxia in the keratinocytes.

Inhibitors

N-acetyl-L-cysteine (NAC) (part number A7250), was purchased from Sigma-Aldrich (St. Louis, MO). The Screen-Well REDOX Library, V.1.1 (part number BML-2835-0100) was donated by Enzo Life Sciences (Farmingdale, NY).

Cell plates

96-Well flat clear bottom, black PS, TC-treated microplates (part number 3904) were purchased from Corning Life Sciences (Corning, NY).

Instrumentation

Agilent BioTek Cytation 3 cell imaging multimode reader

Agilent BioTek Cytation 3 combines automated digital widefield microscopy and conventional microplate detection. This patent-pending design provides rich phenotypic cellular information with well-based quantitative data. Equipped with patented Agilent BioTek Hybrid Technology for microplate detection, Cytation 3 includes both high sensitivity filter-based detection and a flexible monochromator-based system for unmatched versatility and performance. The upgradable automated digital fluorescence microscopy module provides researchers rich cellular visualization analysis without the complexity and expense of standard microplate-based imagers.

The filter-based system was used to detect the green fluorescent signal from the Oxidative Stress Detection Reagent with the following settings: 485/20 nm excitation filter; 528/20 nm emission filter; 510 nm cutoff mirror; delay after plate movement: 100 msec; read height: 4.5 mm. The monochromator-based system was used to detect the red fluorescent signal from the Hypoxia Detection Reagent with the following settings: 540 nm excitation; 605 nm emission; delay after plate movement: 100 msec; read height: 4.5 mm.

The luminescence detection system was used to detect the luminescent signal from the CellTiter-Glo assay using the following settings: Delay after plate movement: 100 ms; Integration time: 0.3 seconds; Read height: 4.5 mm. 20x imaging was then performed with the Hypoxia/Oxidative Stress multiplexed assay and Hoescht 33342 fluorescent probe using the microscopy capabilities. Agilent BioTek Gen5 software was used for initial data analysis.

Assay chemistries

Hypoxia/Oxidative stress detection kit:

The Hypoxia/Oxidative stress detection kit from Enzo Life Sciences (Farmingdale, NY) is designed for functional detection of hypoxia and oxidative stress levels in live cells (both suspension and adherent). The kit includes fluorogenic probes for hypoxia (red) and for oxidative stress levels (green) as two major components. Red hypoxia detection probe is a nonfluorescent or weakly fluorescent aromatic compound containing a nitro (NO_2) moiety. Due to increased nitroreductase activity in hypoxic cells, the nitro group is converted in a series of chemical steps to hydroxamino (NHOH) and amino (NH_2) group. The original molecule then degrades releasing the fluorescent probe. Oxidative stress detection reagent is a nonfluorescent, cell-permeable total ROS detection dye, which reacts directly with a wide range of reactive species yielding a green fluorescent product indicative of cellular production of different ROS types.

Methods

Assay protocol

The assay protocol was created following initial optimization. Keratinocytes, at a concentration of 1.0×10^5 cells/mL, were added to the 96-well cell plates in a volume of 100 µL and incubated overnight. For the agonist protocol, 50 µL of 3x cobalt chloride ($CoCl_2$) was then added to the well and incubated at 37 °C/5% CO_2 for the appropriate time. For the inhibitor protocol, 25 µL of 6x inhibitor was added to the well and incubated for 60 minutes at 37 °C/5% CO_2. 25 µL of 6x $CoCl_2$ was then added to the well and incubated for two hours using the same conditions. Following incubation the medium was removed, and the plate was washed once with 100 µL of 1x PBS. 50 µL of PBS containing the hypoxia, oxidative stress, and Hoechst 33342 fluorescent probes was then added to the wells and incubated at 37 °C/5% CO_2 for 30 minutes. Upon completion the plate was washed three times with 100 µL PBS, and a final volume of 50 µL PBS was added to the wells before the plate reads and imaging were performed.

Hypoxia/ROS stimulation optimization

Initial experiments were performed to determine whether $CoCl_2$ could induce hypoxia in the cell model being used, as well as the ability of the Agilent BioTek Cytation 3 to detect the fluorescent signals from the fluorogenic probes for both hypoxia (red) and oxidative stress (green) using conventional microplate detection. In the first experiment, 400 µM $CoCl_2$ (1x) was added to the cells and incubated using the same conditions between 30 minutes and five hours. In the second, six different concentrations of $CoCl_2$ were added to the cells, ranging from 0 to 1,000 µM (1x), and incubated at 37 °C/5% CO_2 for two hours. Microplate reads were performed on all wells for each experiment as well as 20x imaging of the various [$CoCl_2$]s of the dose response.

REDOX compound library screen

The 84 member REDOX compound library was then evaluated to determine if inhibitors of chemically induced hypoxia could be identified. A single well of each compound was tested at a final 1x concentration of 10 µM. Four individual concentrations of the known ROS scavenger, NAC were also included along with uninhibited positive control and uninduced negative control wells. A final 1x concentration of 500 µM $CoCl_2$ was added to test and positive control wells.

Agilent BioTek Gen5 microplate reader/imager hypoxia inhibition hit pick protocol

A single protocol was created with the Agilent BioTek Gen5 microplate reader and imager software for use during the compound library screen. The protocol eliminates the need to image the entire cell plate, therefore obviating unnecessary data generation and storage. The plate layout created in Gen5 identifies the location of control and test wells (Figure 1).

The hit pick procedure calls for all wells of the plate to be read using the Hypoxia assay read parameters (Figure 2A). Those wells with an RFU value greater than one standard deviation lower than the average from the four positive control wells, which corresponds to greater than or equal to 50% inhibition, are then imaged (Figure 2B).

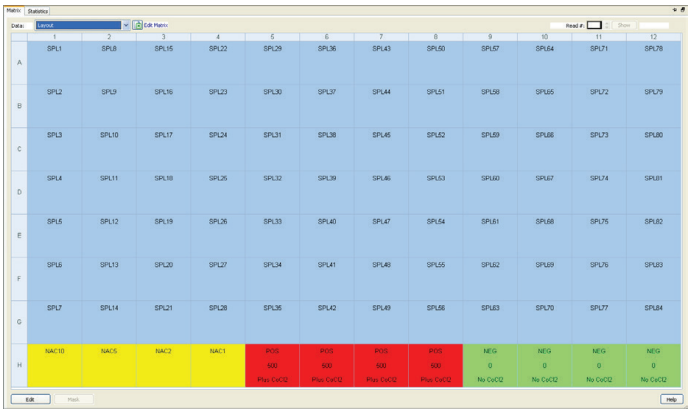
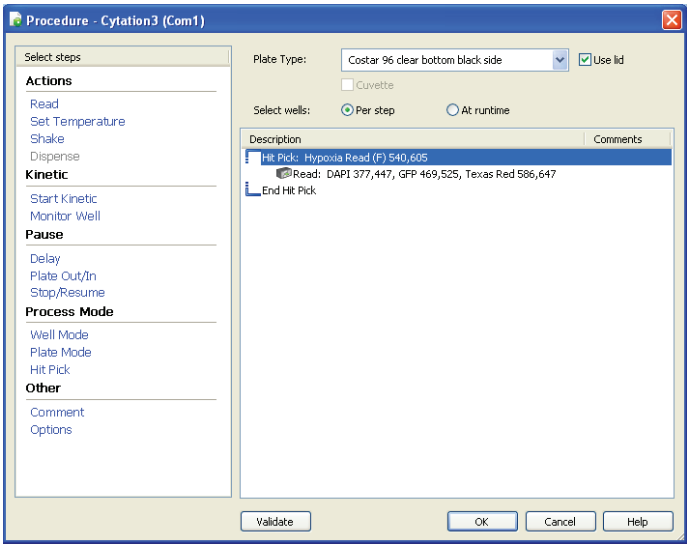


Figure 1. Agilent BioTek Gen5 plate layout for Enzo REDOX compound library screen.

A



B

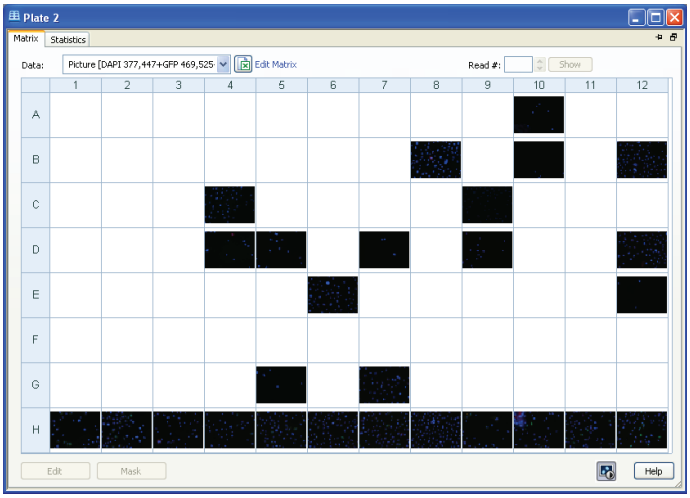


Figure 2. (A) Hit pick microplate read and imaging procedure. (B) Wells imaged using hit pick procedure criteria.

Select compound inhibition/cytotoxicity analysis

Seven compounds plus NAC were selected for further analysis to determine the complete inhibitory profile and cytotoxic potential of each. Ten concentrations of inhibitor were tested, ranging from 0 to 100 µM for library compounds and 0 to 10 mM for NAC, plus uninhibited positive control and uninduced negative control wells as included with the library screen. A final 1x concentration of 500 µM $CoCl_2$ was once again added to test and positive control wells. The cell wash and probe addition process were performed as previously described. Following reading of the Hypoxia/Oxidative Stress assays and 20x imaging, an equal volume (50 µL) of CellTiter-Glo reagent was added to the wells. The plate was shaken for 30 seconds and incubated for 10 minutes at room temperature. The luminescent signal was then detected from each well to assess cell's viability.

Results and discussion

Hypoxia/Oxidative stress detection and optimization

The results generated from the first experiment using 400 µM $CoCl_2$ concentrations and multiple incubation times (Figure 3A) demonstrate that $CoCl_2$ induces hypoxia in the immortalized keratinocytes used here, similar to what has been shown with other keratinocyte cell models.[9] Furthermore, the similarity in the tracking of the delta RFU values seen from the Oxidative Stress assay (Figure 3B) confirms the role that overproduction of ROS plays in the creation of a hypoxic condition within the cells. It can also be seen that a two-hour incubation time creates the largest change in fluorescence from either assay.

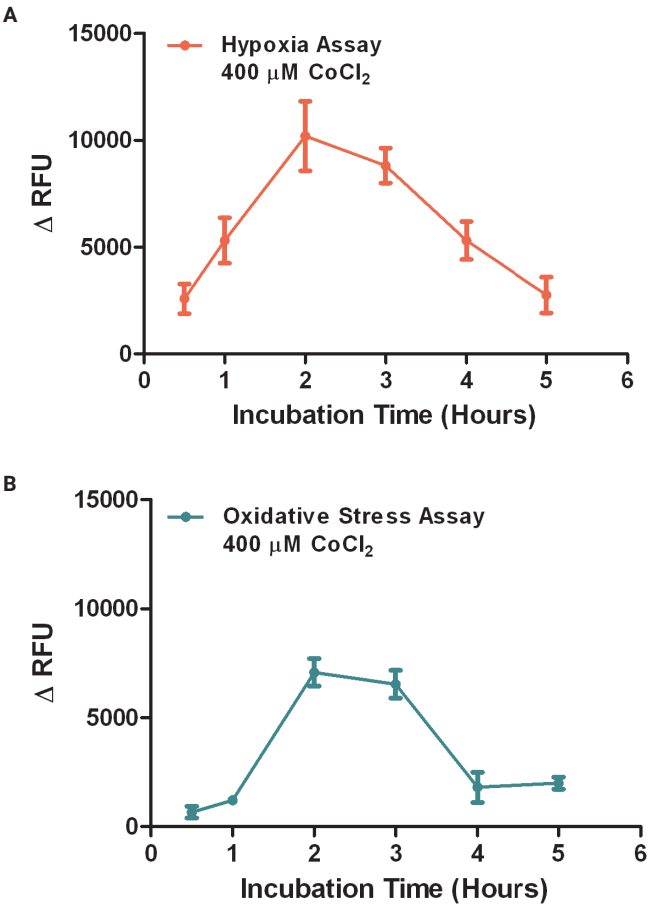


Figure 3. Evaluation of incubation time effect using 400 µM $CoCl_2$ for (A) Hypoxia and (B) Oxidative Stress assays.

When variable concentrations of $CoCl_2$ are added to the keratinocytes and incubated for two hours (Figure 4), it is apparent that the 500 µM concentration, while not causing the greatest change in RFU values, still causes a significant hypoxic effect in the cells. This is also verified qualitatively when examining the 20x images captured in the second experiment (Figure 5).

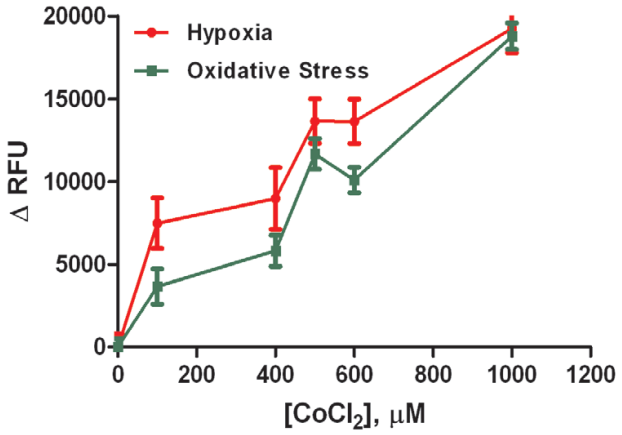


Figure 4. Evaluation of $CoCl_2$ dose response using two-hour incubation for (A) Hypoxia and (B) Oxidative Stress assays.

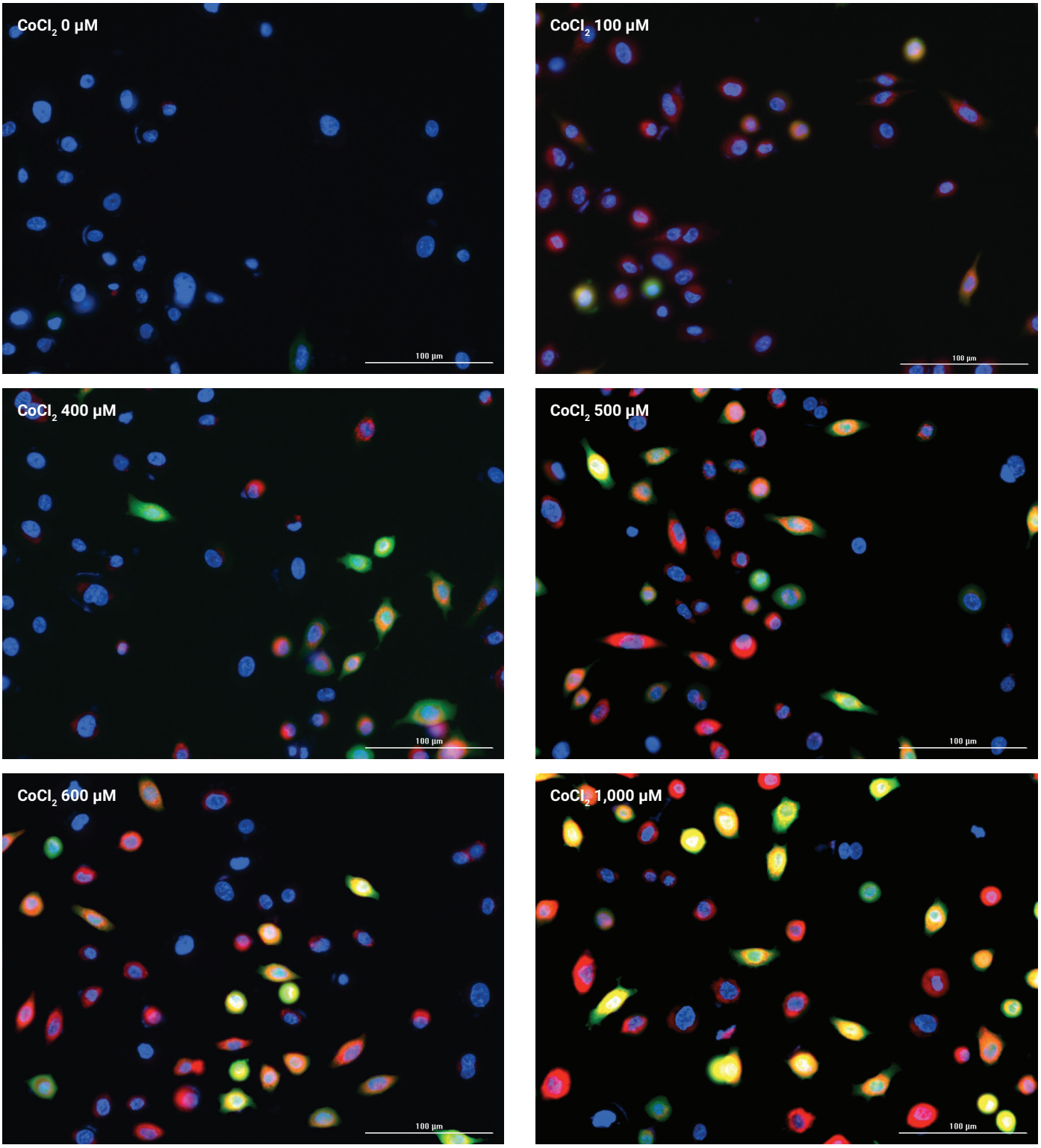


Figure 5. 20x Agilent BioTek Cytation 3 cell imaging multimode reader combined images of Hoechst 33342 (blue), hypoxia (red), and oxidative stress (green) probes following two-hour $CoCl_2$ incubation.

Quantitative results from the images expressed as the ratio of affected to total cells in each concentration of $CoCl_2$ can also be obtained using the Cellular Analysis function in the Gen5 software to further understand the effect of $CoCl_2$ on the cells. This function segments cells in the blue (Hoechst 33342), green (oxidative stress) and red (hypoxia) channels and allows for the calculation of % cells affected by oxidative stress by taking the ratio of green to blue channel; and % cells effected by hypoxia, the ratio of red to blue channel. This provides quantification of the number of cells affected by the phenotypes instead of the relative effects seen using whole-well intensities.

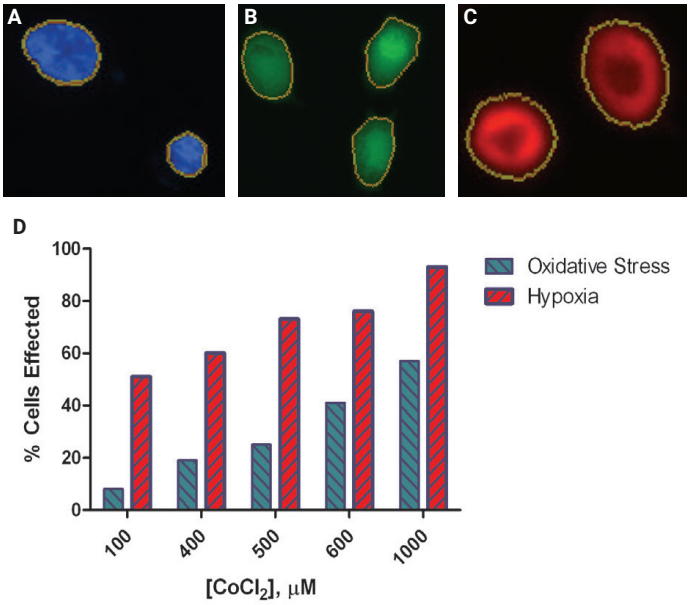


Figure 6. Cellular Analysis images of (A) Hoechst 33342; (B) oxidative stress; and (C) hypoxia stained cells. Analysis parameters included threshold: 10,000 RFU; minimum object size: 10 µm; and maximum object size: 150 µm. (D) Ratio of effected to total cell number for increasing $CoCl_2$ concentrations.

From the microplate read and 20x imaging results it was decided that a 500 µM $CoCl_2$ concentration and two-hour incubation time would be used for subsequent inhibitor experiments of hypoxia as this concentration yielded a change in well fluorescence intensity of ~15,000 RFU and ~75% of cells affected.

Enzo Screen-Well REDOX library screen

The Screen-Well REDOX library, which contains various known antioxidant compounds, was screened for inhibition of $CoCl_2$ induced hypoxia. Since the two phenotypes of oxidative stress and hypoxia were linked, only hypoxia was assessed in the screen.

Some antioxidant compounds were identified as being able to inhibit $CoCl_2$ induced hypoxia by ≥50% (Figure 7). As previously mentioned, the Hit Pick feature in the Agilent BioTek Gen5 software allowed for imaging of wells containing only Hit compounds plus identified control wells (Figure 2B), eliminating needless data creation and storage. Several of these compounds, along with the positive control compound, NAC, were carried forward for dose-response tests to determine their exact inhibitory characteristics and potential cytotoxic effects.

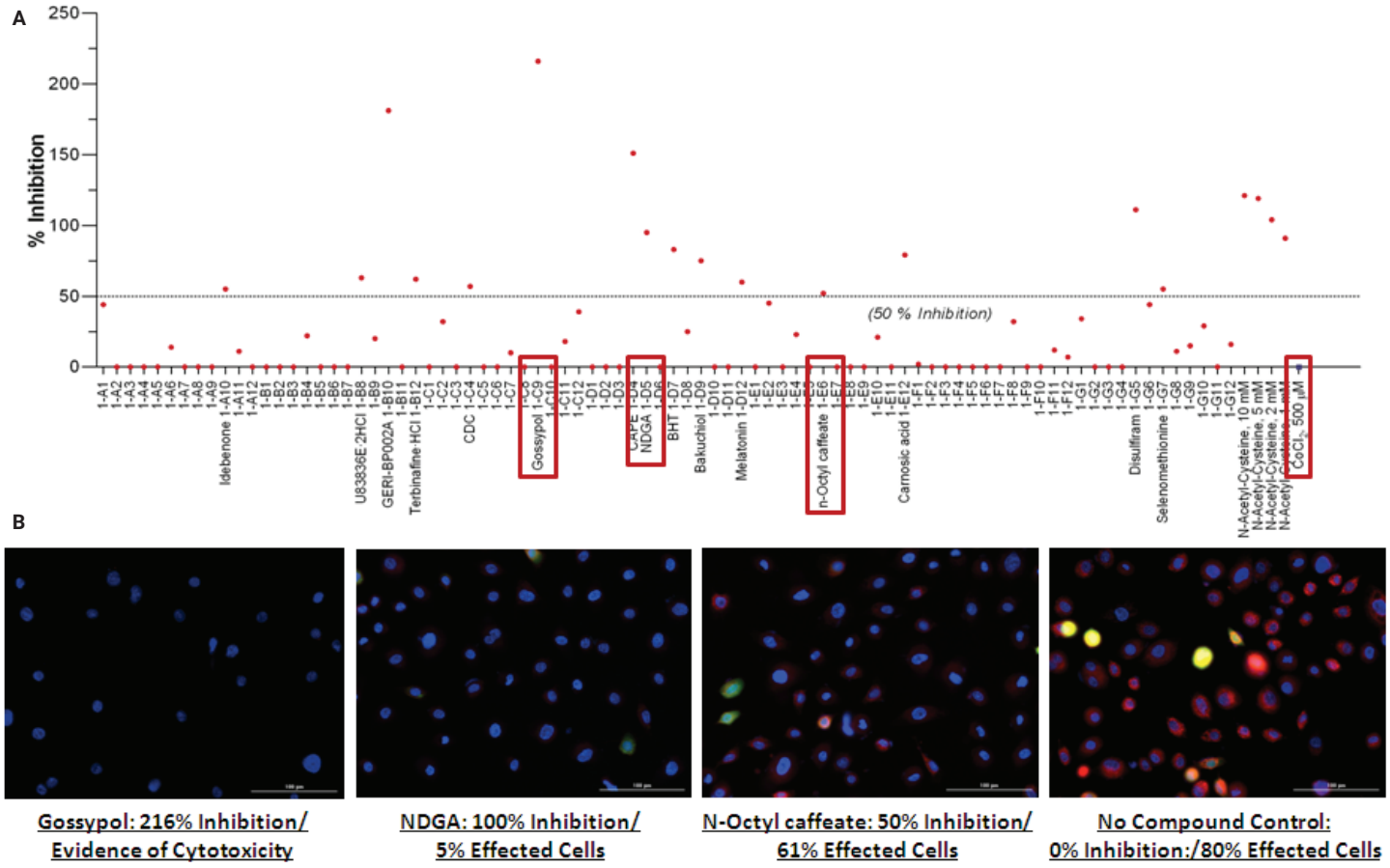


Figure 7. (A) Screen-Well REDOX library screen percent inhibition results calculated from whole-well microplate reads using the following formula: $(1 - ((\text{RFU Value}_{(\text{Test Well})} - \text{RFU Value}_{(\text{Neg Ctl})})/(\text{RFU Value}_{(\text{Pos Ctl})} - \text{RFU Value}_{(\text{Neg Ctl})}))) \times 100$. (B) 20x images from select wells exhibiting ≥50% inhibition, and no compound control, using the Hit Picking feature in Agilent BioTek Gen5 microplate reader and imager software.

Select compound inhibition/cytotoxicity confirmation

Dose-response curves for the seven library compounds chosen, plus NAC, were plotted using the percent inhibition values calculated for each concentration tested (Figure 8). The results demonstrate that each compound fully inhibits hypoxia at the highest concentrations. Excessive inhibition is observed for some the compounds tested, as evidenced by Gossypol (Figure 7B).

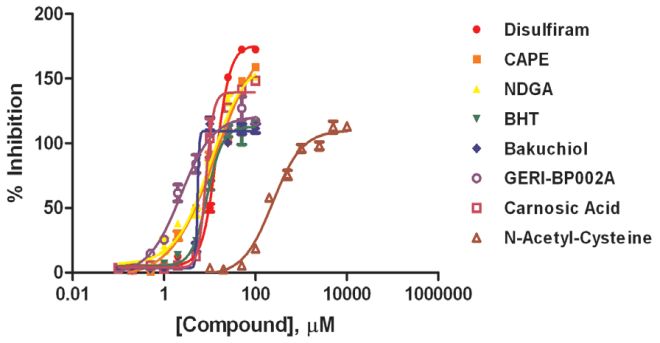


Figure 8. Complete dose-response curves for selected library inhibitor compounds and NAC.

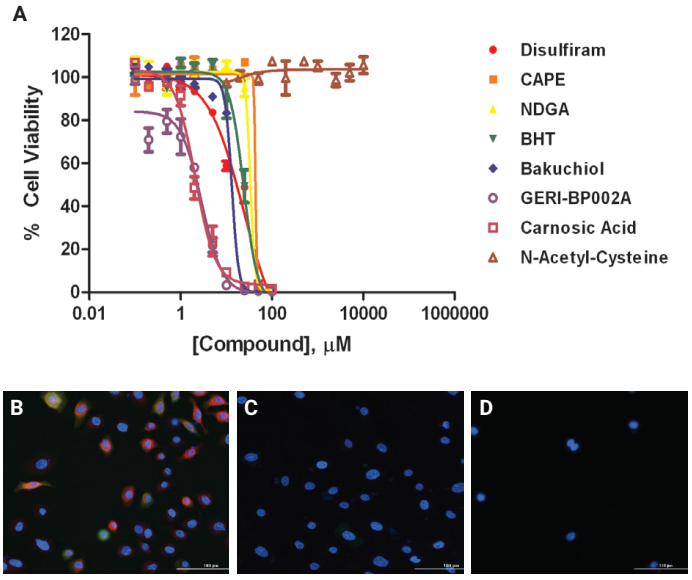


Figure 9. (A) % cell viability values for selected inhibitory compounds. 20x images also shown for (B) well containing 500 µM $CoCl_2$ plus no inhibitor; (C) inhibition via NAC of $CoCl_2$ induced hypoxia with no effect on cell viability; and (D) disulfiram inhibition due to significant loss of cell viability.

Effect on keratinocyte cell viability was also assessed across the full compound dose range.

From the cell viability data generated using the CellTiter-Glo assay, and 20x images captured (Figure 9), it is apparent that high concentrations of most compounds tested demonstrate significant cytotoxic effects on the keratinocyte cells. Therefore a decreased fluorescent signal from the Hypoxia assay cannot always be attributed to true inhibition of the $CoCl_2$ induced effects.

These results are in keeping with previously published findings illustrating the fact that exogenous antioxidants are beneficial at physiological concentrations, but can have detrimental effects at high concentrations.[10]

Conclusion

The Hypoxia/Oxidative stress detection kit provides an easy-to-use, multiplexed cell-based approach for the assessment of hypoxia induction and ROS creation. Upon the addition of an end point luminescent cell viability reagent, a triplexed method is created which also allows for the detection of potential cytotoxicity. Optical paths can be used to collect whole-well fluorescence and luminescence intensities. In this work, spectral filters were used for the assay quantifying the oxidative stress phenotype as the spectral characteristics of the probe are equivalent to fluorescein, which is a filter set that comes standard with all multimode readers. The spectral characteristics of the probe for the hypoxia phenotype is less common. Therefore, the inherent wavelength flexibility and fast reading speed of monochromators were used for this dye. Finally, the dedicated luminescence detection system is used to quantify the signal from the cell viability reagent.

The two probes can also be used with fluorescence microscopy. The automated digital widefield fluorescence module of Agilent BioTek Cytation 3 cell imaging multimode reader, in addition to the cell segmentation capabilities of Agilent BioTek Gen5 microplate reader and imager software, provides rich phenotypic data including qualitative visual confirmation of cells affected by the phenotypes and absolute quantification of the ratio of cells affected by the phenotype relative to total cell populations.

For screening of inhibition of phenotypes, whole-well intensities can be used for rapid screening of plates and only those wells that display reduction of signal below a chosen threshold need be imaged for hit confirmation and quantification of phenotype. This serves to dramatically speed up screening compared to using the digital widefield microscopy alone common to some of the less expensive HCS instruments and relieve issues with data storage. Compared to typical multimode readers, e.g., those without automated digital fluorescence microscopy, Cytation 3 provides much richer phenotypic data and confirmatory results.

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