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

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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 due to the fact that 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 [1,2,3]. Insufficient blood or oxygen supply is a leading causal factor, and can lead to chronic, non-healing ulcers [4,5,6]. 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 additional studies [8] which have examined the potential protective ability of antioxidants against hypoxia and its downstream effects.

Here we demonstrate 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 wide-field fluorescence microscopy. The latter provides quantitative ratios of affected cells relative to the total cell population using the cellular analysis feature of Gen5™ 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 shown 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 in order 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/Oxidative Stress assay. All measurements were performed on the Cytation™3 Cell Imaging Multi-Mode Reader.

BioTek Instrumentation

Figure 1 – Cytation3 Cell Imaging Multi-Mode Reader. Cytation3 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 BioTek's patented Hybrid Technology™ for microplate detection, Cytation3 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.

Fluorescence microscopy is a powerful technique for visualizing cellular responses to understand cell proliferation, protein expression, cytotoxicity and other cellular processes. The ability to perform both conventional quantitative fluorescence measurements and cell imaging provides unique capabilities such as screening microplate wells for a fluorescence intensity threshold that triggers the reader to follow-up on the screen with imaging of those wells that passed the intensity threshold. This serves to reduce analysis time and data storage requirements by imaging only those wells of interest which pass the intensity threshold.

Cytation3's design places special emphasis on live-cell assays: features include temperature control to 45 °C, CO_2/O_2 gas control, orbital shaking and full support for kinetic studies with BioTek's Gen5 software, specifically designed to make plate reading and image capture easy. Other technology advances are found throughout Cytation3's design including high-intensity LED light sources, matched filter cubes, hard coated optical filters, Olympus objectives, and superior autofocus for totally software controlled digital microscopy.

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 luminescent detection system was used to detect the luminescent signal from the CellTiter-Glo® Assay using the following settings: Delay after plate movement: 100 msec; Integration Time: 0.3 sec; Read height: 4.5 mm. 20x imaging was then performed with the Hypoxia/Oxidative Stress multiplexed assay and Hoechst 33342 fluorescent probe using the microscopy capabilities. Gen5 software was used for initial data analysis.

Cyto-ID® 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 non-fluorescent 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 hydroxylamino (NHOH) and amino (NH_2) group. The original reduction that degrades releasing the fluorescent probe. Oxidative Stress Detection Reagent is a non-fluorescent, 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.

Materials and Methods

Assay Method

The assay protocol described here was created following initial optimization. Keratinocytes, at a concentration of 1.0×10^5 cells/mL, were added to the 96-well plate 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 2 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/Oxidative Stress Detection and 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 Cytation3 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 5 hours. In the second, six different concentrations of $CoCl_2$ were added to the cells, ranging from 0-1000 µM (1x), and incubated at 37 °C/5% CO_2 for 2 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.
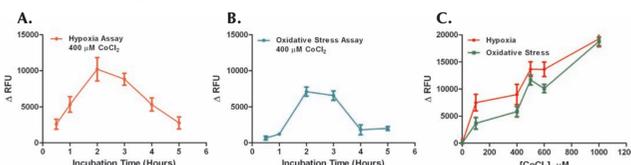

Figure 2 – Evaluation of incubation time effect using 400 µM $CoCl_2$ for (A) Hypoxia and (B) Oxidative Stress assays. $CoCl_2$ dose response also performed with both assays (C) using optimal 2 hour incubation time.

The results generated from the first experiment using 400 µM $CoCl_2$ concentrations and multiple incubation times (Figure 2A) 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 2B) confirms that the overproduction of ROS plays in the creation of a hypoxic condition within the cells. It can also be seen that a 2 hour incubation time creates the largest change in fluorescence from either assay.

When variable concentrations of $CoCl_2$ are added to the keratinocytes and incubated for 2 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.
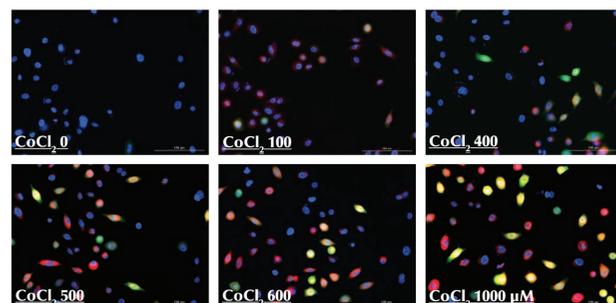

Figure 3 – 20x Cytation3 combined images of Hoechst 33342 (blue), Hypoxia (red), and Oxidative Stress (green) probes following 2 hour $CoCl_2$ incubation.
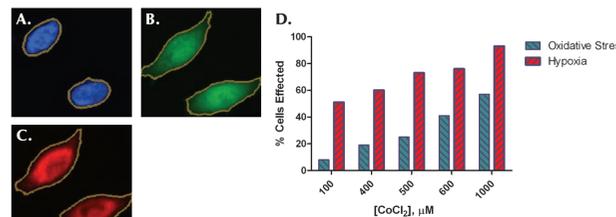

Figure 4 – Cellular Analysis images of (A) Hoechst 33342; (B) Oxidative Stress; and (C) Hypoxia stained cells. Analysis parameters included Threshold: 10,000 RFU; Minimum Object Size: 10 µm; and Maximum Object Size: 150 µm. (D) Ratio of effected to total cell number for increasing $CoCl_2$ concentrations.

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.

From the microplate read and 20x imaging results it was decided that a 500 µM $CoCl_2$ concentration and 2 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.

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.

Gen5 Microplate Reader/Imager Hypoxia Inhibition Hit Pick Protocol

A single protocol was created with the Gen5 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 5).

Figure 5 – Gen5 plate layout for Enzo REDOX Compound Library Screen. Yellow wells: NAC ROS scavenger; Red wells: Uninhibited 500 µM $CoCl_2$ positive control; Green wells: 0 µM $CoCl_2$ negative control.

The Hit Pick procedure calls for all wells of the plate to be read using the Hypoxia assay read parameters (Figure 6). 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.
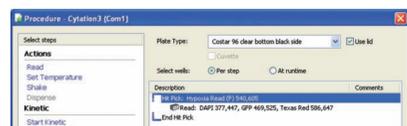

Figure 6 – Hit Pick microplate read and imaging procedure.

Enzo Screen-Well REDOX Library Screen

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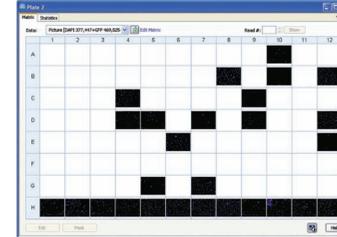

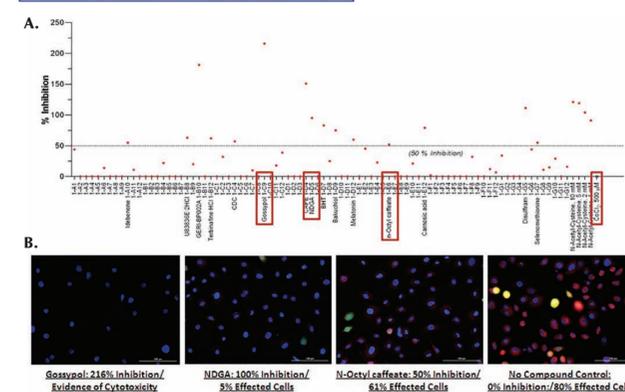*Figure 7 – Wells imaged using Hit Pick procedure criteria.*

*Figure 8 – (A) Screen-Well REDOX Library screen percent inhibition results calculated from whole well microplate reads using the following formula: (1-((RFU Value(Test Well) – RFU Value(Neg Ctl))/(RFU Value(Pos Ctl) – RFU Value(Neg Ctl))))*100. (B) 20x images from select wells exhibiting ≥50% inhibition, and no compound control, using Hit Picking feature in Gen5.*

Gossypol: 216% Inhibition/ Evidence of Cytotoxicity   NDGA: 100% Inhibition/ 5% Effected Cells   N-Octyl caffeate: 50% Inhibition/ 61% Effected Cells   No Compound Control: 0% Inhibition/80% Effected Cells

A number of antioxidant compounds were identified as being able to inhibit $CoCl_2$ induced hypoxia by ≥50% (Figure 8A). As previously mentioned, the Hit Pick feature in the Gen5 software allowed for imaging of wells containing only "Hit" compounds plus identified control wells (Figure 7), 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.

Select Compound Inhibition/Cytotoxicity Analysis

Ten concentrations of each compound was tested, ranging from 0-100 µM for library compounds and 0-10 mM for NAC, plus uninhibited positive control and uninduced negative control wells as included in the library screen. A final 1x concentration of 500 µM $CoCl_2$ was added to test and positive control wells. Following reading of the Hypoxia/Oxidative Stress assays and 20x imaging, an equal volume of CellTiter-Glo reagent was added to the wells. The luminescent signal was then detected from each well to assess cell's viability. Dose response curves for the seven library compounds chosen, plus NAC, were plotted using the percent inhibition values calculated for each concentration tested (Figure 9).
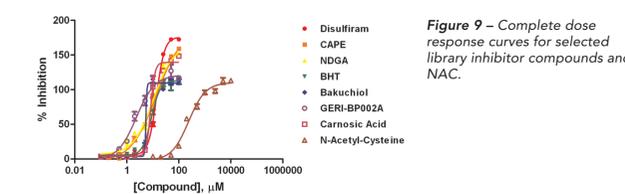

Figure 9 – Complete dose response curves for selected library inhibitor compounds and NAC.

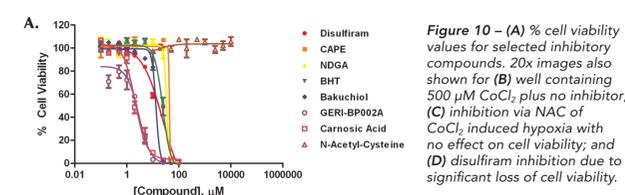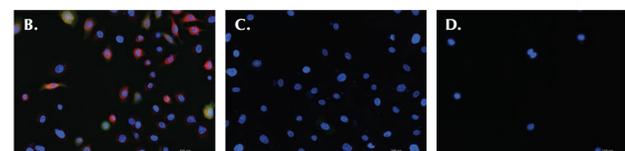Effect on keratinocyte cell viability was also assessed across the full compound dose range.

Figure 10 – (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.

From the cell viability data generated using the CellTiter-Glo assay, and 20x images captured (Figure 10), 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].

Conclusions

1. The Hypoxia/Oxidative Stress Detection kit provides an easy-to-use, multiplexed cell-based approach for the assessment of hypoxia induction and ROS creation.

2. Optical paths can be used to collect whole well fluorescent and luminescent intensities for each incorporated assay chemistry.

3. The two probes can also be used with fluorescence microscopy. The automated digital widefield fluorescence module of Cytation3, in addition to the cell segmentation capabilities of Gen5 software, provide 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.

4. For screening of inhibition of phenotypes, whole well intensities can be used for rapid screening of plates and only those wells which display reduction of signal below a chosen threshold need be imaged for hit confirmation and quantification of phenotype.

5. The combination of the multiplexed assay chemistry and process, combined with an easy-to-use instrument for microplate reading and imaging, provides an ideal method to assess the phenotypic effects caused by the two cellular phenomenon.

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